PhD thesis

Ecophysiology of Anaerobic Oxidation of Methane

Olivia Rasigraf
2016

Olivia Rasigraf (2016): Ecophysiology of Anaerobic Oxidation of Methane. PhD thesis, Radboud University, Nijmegen

This PhD project was financially supported by ERC AG 232937 & 339880. The PhD thesis was made possible by Gravitation Grant OCW/NWO SIAM 024 002 002 and NESSC 024 002 001.

Cover: Element Cycling and Phylogenetic Tree of Life. Design by O. Rasigraf.

Print: Gildeprint Drukkerijen

ISBN: 978-94-6233-186-0

Ecophysiology of Anaerobic Oxidation of Methane

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus, volgens
besluit van het college van decanen
in het openbaar te verdedigen
op woensdag 27 januari 2016
om 16:30 uur precies

door
Olivia Rasigraf
geboren op 23 Juli 1985 te Ewpatorija, Oekraïne

Promotor:

Prof. dr. ir. Mike Jetten

Copromotoren:

Dr. Claudia Lüke

Dr. Katharina Ettwig

Manuscriptcommissie:

Prof. dr. Leon Lamers

Prof. dr. ir. Fons Stams
Wageningen Universiteit

Prof. dr. Antje Boetius
Max Planck Instituut Bremen

"*There is no use trying...one can't believe impossible things.*"
"*I dare say you haven't had much practice...When I was your age, I always did it for half an hour a day. Why, sometimes I've believed as many as six impossible things before breakfast.*"

Lewis Carroll, *"Alice in Wonderland"*

To my parents.

(Посвящается моим родителям)

Contents

Prologue

The driving force of all life on Earth is the supply of energy from the Sun and Earth's internal heat, which creates chemical disequilibria and forms the basis for biogeochemical element cycling. Thus, solar and geochemical energy are eventually converted into chemical energy stored in energy-rich bonds of ATP, acetyl-CoA or acetyl phosphate. The history of Earth's surface chemistry is inevitably connected to the biosphere as already after approximately 600 My after the accretion, the first evidence for life was found in the oldest preserved rocks (Arndt & Nisbet, 2012). Despite still ongoing speculations on what types of respiration those early organisms might have had, they were most likely devoid of oxygen. Geochemical data show that for a period of almost 2 billion years, the Earth's atmosphere was mostly anoxic driving the evolution towards a plethora of anaerobic biochemical diversity. However, it was the invention of photosystem II – a reaction complex capable to split water into reducing equivalents and molecular oxygen – which was among the most significant events for the further course of life's evolution and diversification. Due to its strong oxidizing properties, oxygen was not only highly toxic to most then existing organisms, but it also started a cascade of abiotic reactions changing Earth's surface chemistry towards a more oxidized state (Kump *et al.*, 2011). During this time, oceans started to accumulate sulfate, oxidized forms of reactive nitrogen and metals. It was speculated that the availability of these new electron sinks was a turning point for invention of several respiratory processes considered today to be essential for biogeochemical element cycles (Falkowski & Godfrey, 2008, Godfrey & Falkowski, 2009). Eventually, adaptions to detoxify oxygen and its usage as terminal electron acceptor for respiration of energy-rich carbon-based substrates enabled organisms to exploit this strong source of metabolic energy and evolve towards multicellularity.

All Earth's biogeochemical element cycles are interconnected and a cascade of feedbacks controls the overall balance, which is primarily driven by the activity of microorganisms. The goal of this thesis was to shed more light on such interconnections at oxic/anoxic sedimentary interfaces and to understand the physiology of some microbial key players.

chapter 1

General introduction

Methane in the biological Carbon cycle

Electron flow along physicochemical disequilibria is the basis for energy generating processes related to living systems and reactions driving biogeochemical element cycles. The carbon cycle (Figure 1) forms the basis and connects all other element cycles, not only because of the property of carbon to form the backbone of all macromolecules related to life, but also because of the vast reservoir of energy stored in reduced carbon compounds. The most abundant form of carbon – fully oxidized in carbonate minerals or carbon dioxide gas – enters the biosphere via fixation by photo- and chemolitho-/organoautotrophic organisms. During respiration and fermentation, the reduced carbon is oxidized back to carbon dioxide (CO_2), however, its fate differs profoundly depending on available electron acceptors. Under aerobic conditions, even the most thermodynamically stable substrates can be rapidly oxidized directly to CO_2, whereas in the absence of oxygen the degradation often occurs stepwise in metabolic co-operation of several organisms. The initial hydrolysis of organic matter yields a variety of oligo- and monomers which are then further converted into carboxylic acids, hydrogen and CO_2. Generally, methanogenesis is the terminal reaction in anaerobic degradation processes when simple organic C-compounds, CO_2 and hydrogen are converted to methane by methanogenic archaea. Thus, carbon dioxide and methane – the most oxidized and most reduced gaseous forms of carbon – are the end products of degradation. While CO_2 can be then immobilized in minerals, released to the atmosphere and be fixed again, most of the produced methane is oxidized by microorganisms to CO_2 before reaching the atmosphere. Thermodynamically, methane is among the most energy-rich organic substrates while at same time being among the most stable ones, with a H-C bond activation energy of 439 kJ/mol (Thauer & Shima, 2008). For decades, this high thermodynamic stability was the reason for assuming that methane oxidation was only possible under aerobic conditions. During the aerobic process, the methane monooxygenase enzyme is used by oxygen-dependent methanotrophic bacteria to split oxygen into radicals one of which breaks a H-C bond in methane to form methanol and the other is reduced to form water (Hakemian & Rosenzweig, 2007). The ability of bacteria to utilize methane as a single source of energy and cell carbon was discovered as early as 1906 (Söhngen, 1906) and the dogma on the exclusive role of oxygen in methane oxidation persisted until the late 1970s.

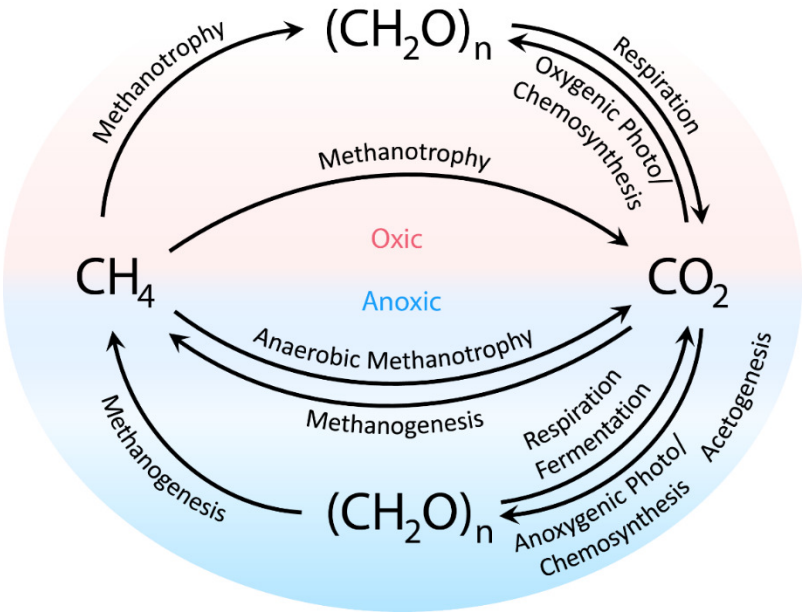

Figure 1: Biological carbon cycle. Transformations between the most oxidized (CO_2), the most reduced (CH_4) and intermediate ((CH_2O)n) carbon compounds driven by particular metabolic processes are shown with arrows. Both, anabolic and catabolic processes are shown.

Anaerobic methane oxidation

First indications on the possibility of methane oxidation under anoxic conditions came from geochemical observations of methane disappearance in sulfate-rich waters and sediments (Barnes & Goldberg, 1976, Reeburgh, 1976, Panganiban *et al.*, 1979). Since then numerous biogeochemical and molecular studies have provided evidence for sulfate-dependent anaerobic methane oxidation (S-AOM) (Knittel & Boetius, 2009). The discovery of this anaerobic way of methane degradation fundamentally changed our understanding on environmental methane fluxes and the carbon cycle as a whole. As the majority of biogenic methane is produced in deep anoxic sediments, it diffuses through the sediment column towards the oxic/anoxic interface (Figure 2). Thus, the anoxic sediment column acts as 'filter' and not all methane reaches the oxic layer where the aerobic methane oxidizing organisms are active. It has been estimated that in marine sediments up to 90% of methane is oxidized via the S-AOM process in the sulfate/methane transition zone (SMTZ)

where both compounds co-occur (Hinrichs & Boetius, 2003). S-AOM was shown to be performed by consortia of sulfate-reducing bacteria (SRB) and methanotrophic archaea (ANME) which use the reverse methanogenesis pathway for methane activation (Knittel & Boetius, 2009). This metabolic co-operation was hypothesized to be based on a transfer of reducing equivalents from archaea to SRB which would make the overall reaction more exergonic (Hoehler *et al.*, 1994). More recently, it was shown that some ANME might reduce sulfate on their own and shuttle intermediate oxidized sulfur species to SRB which in turn would perform a dismutation reaction to sulfide and sulfate (Milucka *et al.*, 2012). Moreover, some environmental ANME populations were found not to be associated with SRB, raising the possibility of a non-syntrophic S-AOM process (Orphan *et al.*, 2002, Treude *et al.*, 2007). All these findings underline that mechanisms driving S-AOM still need further investigation. While sulfate is of great importance in fully marine sediments due to its constant supply from the overlaying water, nitrogen oxides and oxidized metals are often more abundant electron acceptors in freshwater and brackish environments. Furthermore, according to calculations S-AOM yields only between -22 and -35 kJ/mol CH_4 (Valentine & Reeburgh, 2000) – energy which must be shared between both metabolic partners. These values are close to thermodynamic limits of life as the minimum quantum of energy usable for phosphorylation of ADP must be at least between one third and one fifth of the ATP unit (Schink, 1997). Based on physical parameters and partial pressures of hydrogen, calculations on marine sediments revealed that the minimum free energy needed to sustain microbial sulfate reduction is around -19 kJ/mol SO_4^{2-} and -11 kJ/mol CH_4 to sustain methanogenesis (Hoehler *et al.*, 2001). In context of anaerobic methane oxidation, the use of nitrogen oxides and oxidized metals as terminal electron acceptors would yield significantly greater amounts of energy for microbial metabolism. The free energy gain from nitrite/nitrate reduction coupled to methane oxidation would yield -928/-503 kJ/mol CH_4 and from Fe^{3+}/Mn^{4+} reduction -454/-502 kJ/mol CH_4 at standard conditions. First evidence for nitrogen oxide-dependent methane oxidation (N-AOM) came from an enrichment culture of ANME-related archaea and uncharacterized bacteria originating from a highly eutrophic freshwater sediment of Twentekanaal in the Netherlands (Raghoebarsing *et al.*, 2006). The enrichment culture oxidized methane to CO_2 while performing full denitrification to N_2. Based on combined observations of this study and previous knowledge about S-AOM, it was hypothesized that a metabolic co-operation with interspecies reducing-

equivalent transfer between methanotrophic archaea and denitrifying bacteria was responsible for N-AOM.

However, this hypothesis was revised later by the observation that the partner bacteria could perform nitrite-dependent AOM in the absence of archaea (Ettwig *et al.*, 2008). Subsequent metagenome sequencing and physiological experiments provided evidence for bacterial nitrite-dependent intra-aerobic methane oxidation metabolism in which nitrite is first reduced to nitric oxide (NO) which is then dismutated to molecular nitrogen and oxygen (Ettwig *et al.*, 2010).

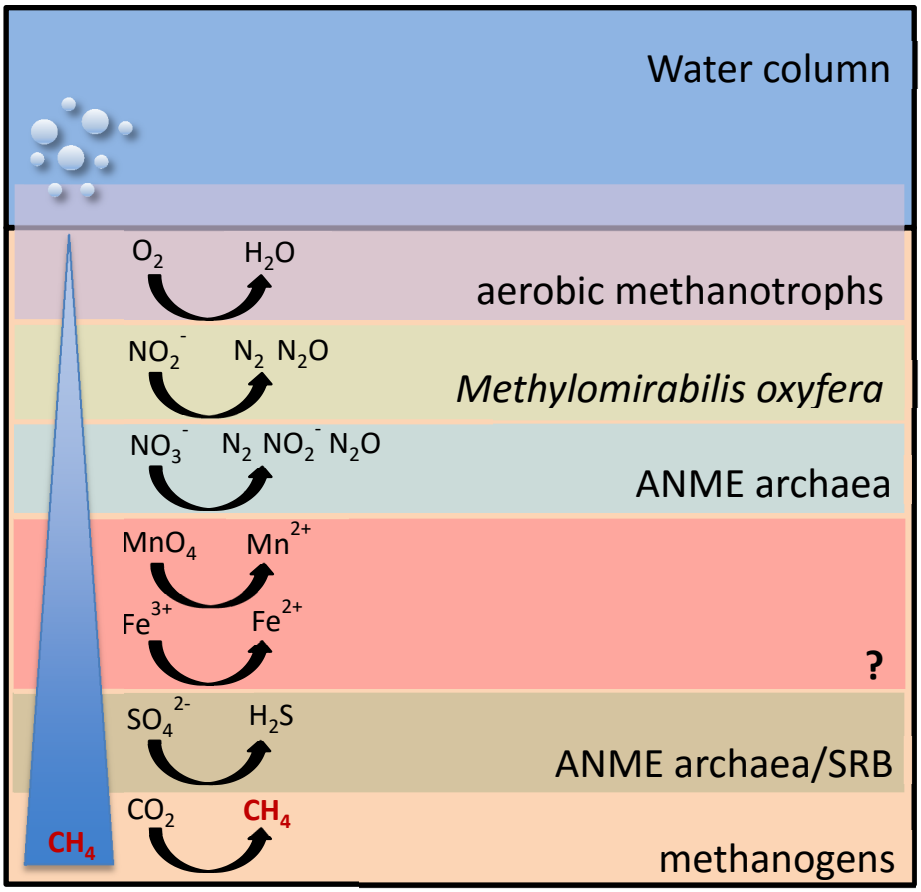

Figure 2: Fate of methane within a sediment column with theoretical distribution of available electron acceptors based on their electron potential. The free energy gain from reduction decreases with the increasing sediment depth. Known organisms respiring each oxidant with methane as substrate are shown at the right. Abbreviations: ANME, anaerobic methanotrophs; SRB, sulfate reducing bacteria

The thus internally produced oxygen is assumed to be used by the methane monooxygenase for methane oxidation. The bacterium responsible for this process, M*ethylomirabilis oxyfera*, was shown to belong to a new phylum, provisionally named NC10, which does not have any other cultured members.
Recently, the archaeal member of an N-AOM consortium, *Methanoperedens nitroreducens*, was shown to perform methane oxidation via reverse methanogenesis in a similar fashion as other ANME archaea, but by coupling it directly to nitrate reduction (Haroon *et al.*, 2013).
Besides sulfate and nitrogen oxides, oxidized metal species were hypothesized to be suitable electron acceptors for methane oxidation and several studies have confirmed the occurrence of metal-dependent AOM (M-AOM) in various ecosystems (Crowe *et al.*, 2011, Sivan *et al.*, 2011, Amos *et al.*, 2012, Norði *et al.*, 2013, Egger *et al.*, 2015). However, so far the responsible pathway remains to be elucidated and several organisms have been speculated to be involved in observed activities. Iron- and manganese oxides are abundant constituents of various freshwater and marine sediments and directly control associated element cycles (e.g. phosphorous). Thus, metal reduction in methane rich sediments could be of particular importance for sedimentary nutrient dynamics.

Ecology of N-AOM organisms

Nitrogen oxides are common constituents at oxic/anoxic interfaces where ammonium, diffusing from deeper anoxic sediments, is oxidized with oxygen diffusing from overlaying oxic column. Here, methane, when not fully removed in deeper sediment, can be used as electron donor and sustain a population of N-AOM organisms. Moreover, in view of ever expanding eutrophication around the globe (Galloway *et al.*, 2008), increasing hypoxic zones with elevated concentrations of reactive nitrogen and methane create more and more potential habitats for N-AOM organisms.
Since their discovery, numerous molecular surveys based on 16S rRNA and methane monooxygenase (encoded by *pmoA*) genomic biomarkers have provided evidence for the widespread occurrence of *M. oxyfera* bacteria in various freshwater, estuarine and even marine environments. Besides their original enrichment sources in eutrophic Dutch channels and ditches (Raghoebarsing *et al.*, 2006, Ettwig *et al.*, 2009), *M. oxyfera*-like bacteria have also been detected in the Lake Biwa sediment (Kojima *et al.*, 2012), various waste water treatment plants (Luesken *et al.*, 2011,

Kampman *et al.*, 2014), a minerotrophic peatland (Zhu *et al.*, 2012), oligotrophic sediments of Lake Constance (Deutzmann & Schink, 2011, Deutzmann *et al.*, 2014), Jiaojiang Estuary of the East Sea (Li-dong *et al.*, 2014), various Chinese wetlands (Zhu *et al.*, 2015), freshwater lakes on the Yunnan Plateau (Liu *et al.*, 2015), surface and subsurface sediments of the South China Sea (Chen *et al.*, 2014), coastal sediment of Xiaogan Island (He *et al.*, 2015), Japanese paddy field soils (Hatamoto *et al.*, 2014), sediments of Qiantang River (Shen *et al.*, 2014), coal tar contaminated aquifer in South Glens Falls (Hanson & Madsen, 2015), Banisveld aquifer in the Netherlands (Luesken *et al.*, 2011) and sewage contaminated aquifer in Cape Cod (Olivia Rasigraf, unpublished results).

Since the recent characterization of the N-AOM performing archaeon, the occurrence of *M. nitroreducens* has been investigated in rice field soils (Lee *et al.*, 2015, Vaksmaa *et al.*, 2015) and freshwater wetlands (Adrienne Narrowe, unpublished results). Moreover, before physiological and genomic characterization of *M. nitroreducens*, related 16S rRNA gene sequences have been detected in diverse freshwater environments. So far, it is not known, whether these archaea are also thriving in brackish or marine sediments.

Based on physiological studies, nitrite was shown to be the main product of nitrate reduction by N-AOM archaea (Baoli Zhu, PhD thesis, Haroon *et al.,* 2013). In high concentrations, nitrite becomes toxic and must be removed. This creates a basis for metabolic co-operation with nitrite scavenging organisms. The first described culture of *M. nitroreducens* contained anaerobic ammonium oxidizing (anammox) bacteria which use nitrite for respiration (Haroon *et al.*, 2013). The original N-AOM culture described in 2006 contained archaea closely related to *M. nitroreducens* which were enriched together with *M. oxyfera* bacteria, the latter being known by now to use nitrite as electron acceptor (Raghoebarsing *et al.*, 2006). Thus, anammox and *M. oxyfera*-like bacteria are most likely common metabolic partners of N-AOM archaea as both methane and ammonium are often present at oxic/anoxic interfaces.

Physiological aspects of *Methylomirabilis oxyfera* bacteria

M. oxyfera is a methanotrophic bacterium which oxidizes methane under anoxic conditions via the aerobic pathways similar to aerobic methane oxidizers with methane monooxygenase as the key enzyme (Fig. 3). This discrepancy between the aerobic lifestyle and the lack of external oxygen in the habitat of *M. oxyfera* was solved with a hypothesis of nitric oxide (NO) dismutation to molecular nitrogen and

oxygen, with the latter being used by the methane monooxygenase (Ettwig *et al.*, 2010). The hypothesis was based on physiological experiments showing molecular oxygen production after acetylene-mediated blockage of methane monooxygenase and genomic analysis which revealed absence of genes encoding for known nitrous oxide reductase enzymes (Ettwig *et al.*, 2010). Hitherto, the only two enzymes known to produce molecular nitrogen were nitrous oxide reductase in denitrifiers and hydrazine dehydrogenase in anaerobic ammonium oxidizers (anammox). In *M. oxyfera*, dinitrogen was hypothesized to be produced directly from NO by nitric oxide dismutases (Nod) – enzymes related to quinol-dependent nitric oxide reductases (qNor), but lacking essential features of the latter for quinol binding and pointing to their electron-neutral overall reaction (Ettwig *et al.*, 2012). The putative Nod-encoding genes were shown to be among the highest transcribed and expressed in *M. oxyfera* enrichment cultures further pointing to their crucial role in metabolism (Luesken *et al.*, 2012). However, it remains to be shown whether these proteins catalyze their hypothesized reaction.

Besides two genes encoding putative Nod enzymes, *M. oxyfera* also encodes a canonical qNor which is also constitutively expressed at low levels (Luesken *et al.*, 2012). The role of this qNor is currently unclear as nitrous oxide is not an intermediate of the proposed main metabolism of *M. oxyfera*. Furthermore, the genome encodes and expresses a membrane-bound nitrate reductase pointing to the ability to use nitrate. However, previous physiological studies and observed reaction stoichiometry could not confirm usage of nitrate by *M. oxyfera* cultures, thus leaving also this aspect of its metabolism for future investigation.

M. oxyfera is a methanotrophic bacterium which utilizes the aerobic pathways for methane oxidation with CO_2 being the terminal product of the reaction (Ettwig *et al.*, 2010). Oxygen – generated from the dismutation of NO – is primarily used for the activation of methane during the first step of the pathway, however, according to proposed reaction stoichiometry not all of it can be used by methane monooxygenase. The genome of *M. oxyfera* was shown to encode several types of terminal respiratory oxidases. A complimentary array of experiments showed that *M. oxyfera* functionally produced a *bo*-type ubiquinol oxidase for oxygen respiration, further supporting its intra-aerobic metabolism (Wu *et al.*, 2011). However, additions of oxygen (2 and 8%) to enrichment cultures showed a clear stress response on transcriptional level (Luesken *et al.*, 2012). It remains to be investigated how the cultures would respond to lower O_2 concentrations.

Another aspect of the unusual metabolism of *M. oxyfera* is its way of carbon assimilation. Typically, proteobacterial methanotrophs derive at least half of their cellular carbon from methane via serine or all via the ribulose-monophosphate pathway (Hanson & Hanson, 1996). However, the genome lacked key genes for some essential enzymes for both pathways, but encoded and transcribed all genes of the Calvin-Benson-Bassham (CBB) cycle. Thus, the pathway of carbon assimilation by *M. oxyfera* bacteria was unclear.

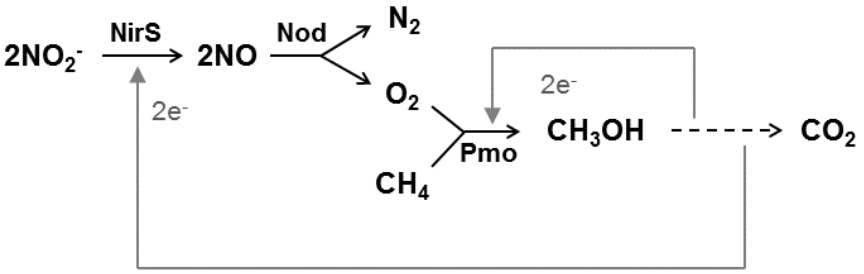

Figure 3: Proposed metabolic pathway of *Methylomirabilis oxyfera* bacterium (modified after Ettwig et al., 2010). Key enzymes (at reaction arrows) and the overall reaction stoichiometry are shown. Abbreviations: NirS, cytochrome cd1-dependent nitrite reductase; Nod, nitric oxide dismutase; Pmo, particulate methane monooxygenase.

Thesis outline

A complementary array of methods and experiments performed within this PhD project aimed to elucidate some aspects *of M. oxyfera* physiology and to investigate the genomic basis for sedimentary element cycling with regard to nitrogen, iron and methane transformations.

In **chapter 2**, the carbon and hydrogen isotope fractionation during the methane oxidation metabolism by *M. oxyfera* was investigated. The fractionation is based on reaction kinetics of lighter isotopes to react faster, thus leaving the remaining pool of substrates enriched with slightly heavier molecules. This leads to a specific isotopic signature under no substrate limitation which can be used for environmental studies to quantify methane oxidation. Our research revealed that the *M. oxyfera* isotopic signature during methane oxidation was not significantly different to that of other methanotrophs.

In **chapter 3**, the carbon assimilation route of *M. oxyfera* bacteria was investigated. As the *M. oxyfera* genome lacks essential genes necessary for carbon assimilation from methane via the serine or ribulose monophosphate pathways, but contains all genes of the CBB cycle for CO_2 fixation, we investigated whether CO_2 was indeed the main carbon source for *M. oxyfera* cultures. Lipid labeling and RubisCO enzyme activity assays showed that CO_2 was the main source of cellular carbon in *M. oxyfera* enrichment cultures.

In **chapter 4**, the response of an *M. oxyfera* enrichment culture to additions of NO was investigated. In a batch reactor set-up, NO was added as a saturated solution and concentrations of N-metabolites and methane was monitored. Combined physiological and transcriptomic data pointed to severe nitrosative stress at micromolar concentration of free NO.

In **chapter 5**, the capacity of the Bothnian Sea sediments for M-AOM was investigated. Batch incubations of slurries from the original sediment showed iron-dependent methane oxidation activity. Subsequent metagenomic sequencing of the original sediment and active incubation slurries pointed to the involvement of iron-reducing bacteria, methanotrophic/-genic archaea, putatively fermentative *Clostridia* and sulfate/thiosulfate reducing bacteria.

In **chapter 6**, the genomic potential for various metabolic transformations of nitrogen compounds in the Bothnian Sea sediment was investigated. Results showed that nitrogen fixation, anammox and dissimilatory nitrite reduction to ammonium played only a minor role, but denitrification to N_2 was probably the dominant pathway in the Bothnian Sea nitrogen cycle.

In **chapter 7**, all findings are summarized and a general outlook for future research directions is discussed.

chapter 2

Carbon and hydrogen isotope fractionation during nitrite-dependent anaerobic methane oxidation by *Methylomirabilis oxyfera*

Published previously as:

Olivia Rasigraf, Carsten Vogt, Hans-Hermann Richnow, Mike S. M. Jetten, Katharina F. Ettwig (2011). Carbon and hydrogen isotope fractionation during nitrite-dependent anaerobic methane oxidation by *Methylomirabilis oxyfera.* Geochimica et Cosmochimica Acta, **89**: 256-264.

ABSTRACT

Anaerobic oxidation of methane coupled to nitrite reduction is a recently discovered methane sink of as yet unknown global significance. The bacteria that have been identified to carry out this process, Candidatus *Methylomirabilis oxyfera*, oxidize methane via the known aerobic pathway involving the monooxygenase reaction. In contrast to aerobic methanotrophs, oxygen is produced intracellularly and used for the activation of methane by a phylogenetically distinct particulate methane monooxygenase (pMMO). Here we report the fractionation factors for carbon and hydrogen during methane degradation by an enrichment culture of *M. oxyfera* bacteria. In two separate batch incubation experiments with different absolute biomass and methane contents, the specific methanotrophic activity was similar and the progressive isotope enrichment identical. Headspace methane was consumed up to 98% with rates showing typical first order reaction kinetics. The enrichment factors determined by Rayleigh approach were -29.2 ± 2.6‰ for $\delta^{13}C$ (ε_C) and -227.6 ± 13.5‰ for $\delta^{2}H$ (ε_H), respectively. These enrichment factors were in the upper range of values reported so far for aerobic methanotrophs. In addition, two-dimensional specific isotope analysis ($\Lambda = (\alpha_H^{-1}-1)/(\alpha_C^{-1}-1)$) was performed and also the determined Λ value of 9.8 was within the range determined for other aerobic and anaerobic methanotrophs. The results showed that in contrast to abiotic processes biological methane oxidation exhibits a narrow range of fractionation factors for carbon and hydrogen irrespective of the underlying biochemical mechanisms. This work will therefore facilitate the correct interpretation of isotopic composition of atmospheric methane with implications for modeling of global carbon fluxes.

INTRODUCTION

Methane is the most abundant hydrocarbon in the earth's atmosphere and is a potent greenhouse gas with an approximately 25 times higher global warming potential than carbon dioxide (IPCC, 2007). About 69% of all methane is produced by the catabolic activity of methanogenic archaea (Conrad, 2009), thriving in anoxic environments (e.g. rice paddy fields, swamps, continental margins) rich in organic carbon and limited in oxidants stronger than carbon dioxide.

Most of the produced methane is oxidized back to carbon dioxide by two major sinks – abiotic oxidation by hydroxyl radicals in the upper atmosphere, and microbial oxidation under both oxic and anoxic conditions (Wuebbles & Hayhoe, 2002,

Conrad, 2009, Montzka *et al.*, 2011). During biological methane oxidation, the initial activation to methanol is mechanistically the most difficult step due to the exceptional thermodynamic stability of methane with an activation barrier of +439 kJ/mol (Thauer & Shima, 2008). For decades, oxygen was believed to be the only possible electron acceptor for methane oxidation (Strous & Jetten, 2004). Aerobic methane oxidation is exclusively performed by Bacteria, belonging to α- or γ-Proteobacteria and recently discovered *Verrucomicrobia* (Semrau *et al.*, 2008, Op den Camp *et al.*, 2009). However, anaerobic oxidation with sulfate as terminal electron acceptor in consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria was shown to exist and to be the dominant methane-oxidizing process in marine sediments (Knittel & Boetius, 2009). In terms of thermodynamics, all electron acceptors in the range between sulfate and oxygen (~ -220 mV – +818 mV) could potentially be used for the oxidation of methane. In 2006, it was for the first time described that oxidized nitrogen species (i.e., nitrate, nitrite) could be used for the anaerobic oxidation of methane (AOM) (Raghoebarsing *et al.*, 2006). The process of nitrite-dependent anaerobic methane oxidation (N-DAMO) is performed by *Candidatus* 'Methylomirabilis oxyfera' bacteria which belong to the newly described NC10 phylum (Ettwig *et al.*, 2008, Ettwig *et al.*, 2009, Ettwig *et al.*, 2010). Though living anaerobically, *M. oxyfera* activates methane via the known pathway of aerobic methanotrophs, involving the monooxygenase reaction as the initial step of the process (Ettwig *et al.*, 2010, Wu *et al.*, 2011). Notably, the molecular oxygen used for methane oxidation is generated intracellularly by the reduction of nitrite to nitric oxide and probably dismutation of the latter to molecular nitrogen and oxygen (Ettwig *et al.*, 2010). Methane monooxygenase is the key enzyme of oxygen-dependent methane oxidation and occurs in nature in two different forms: membrane-bound particulate (pMMO) and cytoplasmic soluble methane monooxygenase (sMMO) (Hakemian & Rosenzweig, 2007). Both enzyme forms differ in structure, active site composition and catalytic mechanism, with sMMO being expressed under copper-limited conditions and exhibiting a broader substrate range than pMMO (Elliott *et al.*, 1997, Murrell *et al.*, 2000, Hakemian & Rosenzweig, 2007). Most known methanotrophs preferentially express pMMO instead of sMMO with only one genus (*Methylocella*) exclusively using the latter, and only a small number can expresses both simultaneously (Murrell *et al.*, 2000, Hakemian & Rosenzweig, 2007). In its genome, *M. oxyfera* possesses a single copy gene encoding particulate methane monooxygenase, whose full length amino acid

sequence of the alpha-subunit (*pmoB*) shares at most only 41% identity to the *pmoB* sequence of *Methylococcus capsulatus*.

So far, little is known about the occurrence and significance of *M. oxyfera* in the environment. Besides the eutrophic freshwater canal in the Netherlands (Raghoebarsing *et al.*, 2006), these bacteria have also been enriched from other freshwater sediments and a waste water treatment plant (Ettwig *et al.*, 2009, Hu *et al.*, 2009, Luesken *et al.*, 2011). 16S rRNA sequences of NC10 phylum bacteria were retrieved from several ecosystems worldwide, including contaminated aquifers, soils, lake and river sediments (Ettwig *et al.*, 2009), thereby providing evidence for their ubiquity. Moreover, with the development of primer sets specifically targeting the *pmoA* sequence of *M. oxyfera* bacteria (Deutzmann & Schink, 2011, Luesken *et al.*, 2011), it was possible to approach their detection on the functional gene level. The *pmoA* sequences were detected in several anoxic aquifers, anaerobic waste water treatment plants, peat lands and an oligotrophic lake (Deutzmann & Schink, 2011, Luesken *et al.*, 2011, Luesken *et al.*, 2011), showing the potential for nitrite-dependent anaerobic methane oxidation in these ecosystems.

The experimental evidence for active AOM coupled to nitrite reduction *in situ* is scarce. So far, the concomitant disappearance of methane and nitrate has only been shown to occur in a sewage-contaminated aquifer and the sediment of an oligotrophic lake (Smith *et al.*, 1991, Deutzmann & Schink, 2011). However, several studies have reported AOM in anoxic freshwater peat sediments and lake water columns, without identifying the responsible electron acceptor and organisms (Lehmann *et al.*, 2004, Smemo & Yavitt, 2007, Schubert *et al.*, 2010). It was hypothesized that nitrate and nitrite might be of particular importance for the observed methane oxidation in these ecosystems (Smemo & Yavitt, 2007).

As methane is the second most important greenhouse gas in the atmosphere, the quantification and evaluation of its fluxes on a global scale has been the subject of extensive research during the last decades (Petit *et al.*, 1999, Robertson *et al.*, 2000, Wuebbles & Hayhoe, 2002, Dlugokencky *et al.*, 2009, Ettwig *et al.*, 2009, Montzka *et al.*, 2011). The unusually strong depletion in the heavy ^{13}C isotope of biogenic methane makes it possible to distinguish between thermogenic and biotic sources (Whiticar, 1999), but the picture is often complicated by the effect of biological oxidation. In this respect, determination of the global C1 budget based on mixing ratios and isotopic composition of methane has become an essential tool in biogeochemical studies (Whiticar, 1999, Dlugokencky *et al.*, 2009, Kai *et al.*, 2011).

Usually, (bio)chemical bond cleavages are associated with isotope discrimination of the substrate. The atomic mass differences between isotopes lead to different bond strengths inside the molecules, with heavier nuclei possessing lower zero-point energies and thus stronger bonds (Urey, 1947). The molecules with weaker bonds react faster and the remaining substrate pool becomes enriched with the heavier isotope. However, the preference of different processes, enzymes or even different enzyme isomers for lighter substrates is not uniform. Thereby, different pathways of substrate conversion can often be distinguished by the specific isotopic signature of the remaining pool or the product formed (Meckenstock *et al.*, 2004, Mahieu *et al.*, 2006, Fischer *et al.*, 2008, Vogt *et al.*, 2008).

The weighted average $\delta^{13}C$ of methane produced by all biotic and abiotic sources is about -53 ± 5‰, but the average $\delta^{13}C$ of atmospheric methane is only -47‰ (Quay *et al.*, 1999), indicating that methane consumption processes lead to its enrichment with the heavy isotope. During aerobic methane oxidation, the remaining methane pool becomes enriched with ^{13}C and ^{2}H under non-limiting substrate conditions, which can be attributed to the kinetic effect of the initial and irreversible step of the pathway, the monooxygenase reaction (Nesheim & Lipscomb, 1996, Templeton *et al.*, 2006). Fractionation occurs for both hydrogen and carbon, but its extent is different for both elements. In comparison to the $^{2}H/^{1}H$ couple, where the relative mass difference amounts to 100%, the difference for $^{13}C/^{12}C$ is only 8.3%. Thus, the fractionation effect is always higher for hydrogen than for carbon.

The isotope enrichment of the residual substrate pool is expressed in enrichment factors (ε). So far, several enrichment factors for carbon and hydrogen during aerobic methane oxidation were determined (Coleman *et al.*, 1981, Tyler *et al.*, 1994, Bergmaschi & Harris, 1995, Reeburgh *et al.*, 1997, Snover & Quay, 2000, Kinnaman *et al.*, 2007, Feisthauer *et al.*, 2011). The factors showed a broad range and could not be linked to a distinct type of methane monooxygenase, phylogenetic affiliation or cultivation condition. Furthermore, also enrichment cultures from marine sources exhibiting sulfate-dependent anaerobic methane oxidation produced enrichment factors for carbon and hydrogen similar to those of aerobic methanotrophs (Alperin *et al.*, 1988, Kessler *et al.*, 2006, Holler *et al.*, 2009), despite the profoundly different underlying biochemical methane activation mechanisms.

As the observed bulk stable isotope effect can be influenced by masking effects, e. g. diffusion or transport limitation, a two-dimensional analysis on the basis of hydrogen and carbon has been proposed (Feisthauer *et al.*, 2011). The masking effects are considered to influence the bulk isotope fractionation of each element to

the same extent, thus the ratio of both fractionation factors could potentially provide a better picture about a particular biotransformation process in the environment (Feisthauer *et al.*, 2011).
This study aimed to investigate the isotope enrichment factors for carbon and hydrogen and to determine the two-dimensional fractionation factor during nitrite-dependent anaerobic methane oxidation by an *M. oxyfera* enrichment culture.

MATERIALS & METHODS

Enrichment culture and methane degradation

The culture of *M. oxyfera* bacteria (corresponding to strain "Twente" in Ettwig et al. (2010)) was enriched anoxically in a sequencing batch reactor under continuous supply of methane and nitrite as described by Wu et al. (2011). The incubation for isotope analyses was performed in batch incubations using 60 ml glass serum bottles with two different headspace to volume ratios: 0.35 (further referred to as R0.35) and 2.8 (R2.8). Biomass was concentrated two times in nitrate-free mineral salt medium (Ettwig *et al.*, 2009) buffered with 5 mM 3-(N-morpholino) propanesulfonic acid (MOPS) under oxic conditions. After aerobically dispensing the biomass, serum bottles were sealed with red butyl rubber stoppers (Rubber BV, Hilversum, Netherlands), crimped with aluminium caps and made anaerobic by 5 cycles of successive vacuuming and gassing with helium, and a final flushing with helium for 5 min. In each serum bottle, an overpressure of 0.4 bar was applied. Thereafter, methane (Air Liquide, Eindhoven, Netherlands) was added to a concentration of 3-5% (v/v) of headspace gas. Culture bottles were incubated horizontally under shaking (170 rpm, Innova® 40, New Brunswick Scientific, United States) at 30°C. Each serum bottle was sacrificed at a certain level of methane biodegradation (0-98% of initial concentration) by injection of 1 mL of 4 M sodium hydroxide and stored at 4°C until analyses. In addition to biotic culture incubation, abiotic control serum bottles with medium instead of biomass were prepared.

Analysis of nitrite, methane and protein content

Methane concentrations were analyzed by gas chromatography as described by Ettwig et al. (2008). Each sample was measured in duplicate by manual injection of 100 µL headspace gas with a gas-tight syringe (Hamilton, Switzerland). Protein

content from 3 representative batch incubations per experiment was analyzed by bicinchoninic acid assay according to the manufacturer's instructions (Ettwig *et al.*, 2008). Nitrite content was monitored throughout the incubation with Merckoquant test strips (Merck, Darmstadt, Germany) in order to prevent nitrite limitation. In experiment R2.8, additional nitrite was supplied by a 100 mM anaerobic stock solution.

Isotope-ratio mass spectrometry

The isotopic composition of the headspace methane was analyzed with an isotope-ratio mass spectrometer (Finnigan MAT 253, Thermo Finnigan Bremen, Germany) coupled to a gas chromatograph (GC, HP 7890A Series, Agilent Technology, Santa Clara, United States for H and HP 6890 Series, Agilent Technology, Santa Clara, United States for C isotopes) via a combustion device. Dependent on the concentration of methane in the headspace, 50 to 1000 µL gas were injected into the GC by a sample-lock syringe (Hamilton, Switzerland). Helium was used as a carrier gas with a constant flow of 2 mL min^{-1} at 40°C. Each sample was measured at least 3 times. The standard deviation was always lower than 0.6 ‰ for $\delta^{13}C$ and 7.8 ‰ for $\delta^{2}H$, respectively.

Determination of isotope enrichment factors, fractionation factors and Λ

For calculations of the isotope enrichment factors (Eq. 1), the isotopic abundance ($\delta^{13}C$, $\delta^{2}H$) was expressed in per mill (‰) relative to Vienna PeeDee Belemnite (VPDB) and standard mean ocean water (SMOW) as international standards, respectively.

$$\boldsymbol{\delta^{13}C \text{ or } \delta^{2}H\ [‰]} = \frac{(R_{sample} - R_{standard})}{R_{standard}} \bullet 1000 \qquad \text{Eq. 1}$$

in which R_{sample} and $R_{standard}$ represent the $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios in sample and international standard, respectively.

Because batch incubation experiments represent closed systems, a simplified Rayleigh approach as described by Coleman et al. (1981) can be applied for determination of enrichment factors during methane degradation. According to Coleman et al. (1981), the initial concentration of light methane isotopes can be approximated by the total methane concentration as the natural abundance of ^{13}C and

^{2}H is small (1.1% and 0.015%, respectively). Furthermore, the simplified Rayleigh approach applies for first order reaction kinetics, where concentration of methane is the rate limiting factor (Coleman *et al.*, 1981).
In the current study, the isotope enrichment factors (ε) for carbon and hydrogen were calculated by a simplified Rayleigh approach as previously described (Elsner *et al.*, 2005) (Eq. 2).

$$\frac{R_t}{R_0} = \frac{C_t^{\frac{\varepsilon}{1000}}}{C_0} \quad \text{Eq. 2}$$

in which R_t, C_t, R_0 and C_0 represent stable isotope ratios (R) and concentrations (C) of headspace methane at the beginning (time point 0) and after a certain time of the experiment (time point *t*).
By combining the equations 1 and 2, the isotope enrichment factor can be expressed according to equation 3.

$$\frac{\delta_t + 1000}{\delta_0 + 1000} = \frac{C_t^{\frac{\varepsilon}{1000}}}{C_0} \quad \text{Eq. 3}$$

The isotope enrichment factor was determined from the slope of the linear regression after plotting of $\ln((\delta_t + 1000)/(\delta_0 + 1000))$ versus $\ln(C_t/C_0)$. δ_t and δ_0 represent the isotope values at the beginning and after a certain time of the experiment, respectively. The standard error originating from the slope was calculated with 95% confidence interval as previously described according (Elsner *et al.*, 2007).
The isotope fractionation factor (α) can then be calculated according to equation 4.

$$\boldsymbol{\alpha} = \frac{\varepsilon}{1000} + 1 \quad \text{Eq. 4}$$

In order to account for potential masking effects, a two-dimensional specific isotope analysis based on isotope fractionation of two elements was performed according to Elsner et al. (2007) and is presented in equation 5.

$$\boldsymbol{\Lambda} = \frac{(\alpha_H^{-1} - 1)}{(\alpha_C^{-1} - 1)} \quad \text{Eq. 5}$$

RESULTS & DISCUSSION

Methane degradation kinetics

In R0.35, methane was consumed up to 98% within 20 h of incubation, in R2.8 the degradation did not exceed 84% within 136 h (Figure 1). The different degradation rates in R0.35 and R2.8 were consistent with the absolute protein content: the specific methane consumption activity was comparable for both incubation experiments, 3 ± 0.3 in R0.35 and 3.2 ± 0.2 μmol g^{-1} protein min^{-1} in R2.8, respectively. However, the overall consumption patterns differed between R0.35 and R2.8, with an observable *lag*-phase during the first 10 h in R2.8. In both R0.35 and R2.8, methane degradation followed first order reaction kinetics; most culture bottles of each experiment exhibited similar methane oxidation rates
(Fig. 1). During both incubation experiments a significant increase in biomass could be excluded, as the doubling time of *M. oxyfera*-like bacteria lies in the range of one to two weeks (Ettwig *et al.*, 2009). In a previously conducted activity test, the stoichiometry of methane to nitrite consumption was determined to be close to the theoretical ratio of 3:8, indicating that N-DAMO was the predominant denitrifying pathway.

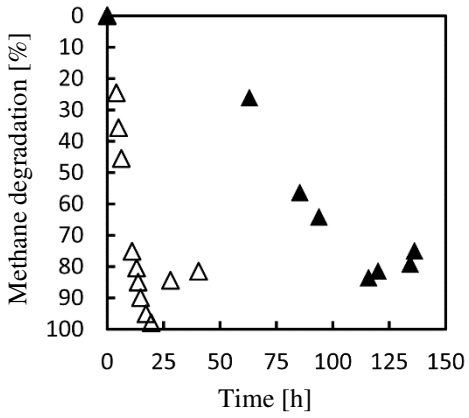

Figure 1: Methane degradation kinetics of *Methylomirabilis oxyfera* enrichment culture during both incubation experiments with different headspace to liquid ratios (R0.35 and R2.8). Headspace methane contents at the time of sacrification are plotted. R0.35 is shown with open triangles, R2.8 with filled triangles.

Isotope fractionation of carbon and hydrogen

Both R0.35 and R2.8 exhibited an enrichment in heavy isotopes of carbon and hydrogen during the course of incubation. The isotopic signature of ^{13}C-methane was -37.8 ± 0.6‰ at the start of incubation and increased to +84.6 ± 0.4‰ after 98% of

headspace methane was consumed (Fig. 2A). The enrichment in deuterium proceeded in the same manner as ^{13}C and increased from -130 ± 0.5‰ to +434 ± 6.4‰ after 90% of headspace methane was consumed (Fig. 2B).
The isotope enrichment trend did not differ between both R0.35 and R2.8, indicating

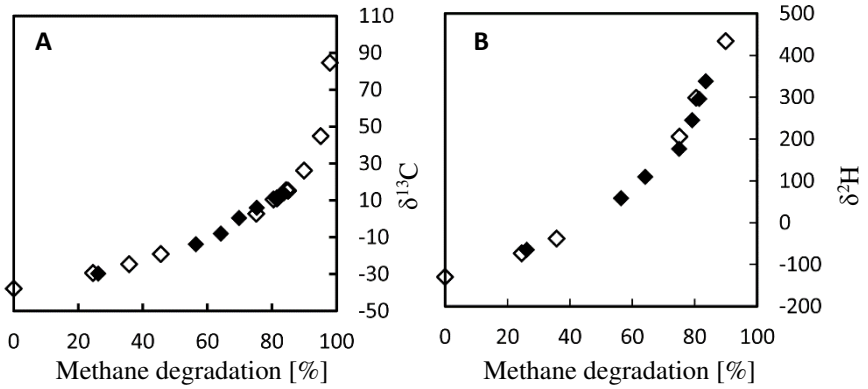

Figure 2: Enrichment in heavy isotopes (A, carbon; B, hydrogen) with progressive methane degradation by a *Methylomirabilis oxyfera* enrichment culture during both incubation experiments. Values of R0.35 are shown as open rhombs, values of R2.8 as filled rhombs.

that no diffusion limitation occurred in R0.35 and that slow consumption rates and *lag*-time at the beginning of the incubation in R2.8 did not affect isotope fractionation. The isotope enrichment correlated very well with the decreasing concentrations of headspace methane typical for closed incubation systems. The determined isotope enrichment factors from the slopes of simplified Rayleigh plots were -29.2 ± 2.6‰ for carbon (ε_C) and -227.6 ± 13.5‰ for hydrogen (ε_H) (Fig. 3). Both regression lines came close to a correlation factor (R^2) of 1 (Figure 3).
The results showed that *M. oxyfera*-like methanotrophs discriminate against the heavier isotopes of carbon and hydrogen with values in the upper range of what has been reported so far for other methanotrophs and methane-oxidizing environmental samples (compiled in Feisthauer et al. 2011). Neither the peculiar methane oxidation mechanism nor the distinct sequence of *M. oxyfera*-specific pMMO were reflected in its specific enrichment factors. Similar observations were made by Feisthauer et al. (2011), where type I and type II methanotrophs produced similar enrichment factors for methane regardless of the type of expressed MMO. Although Nesheim and Lipscomb (1996) determined that isotope fractionation during biological

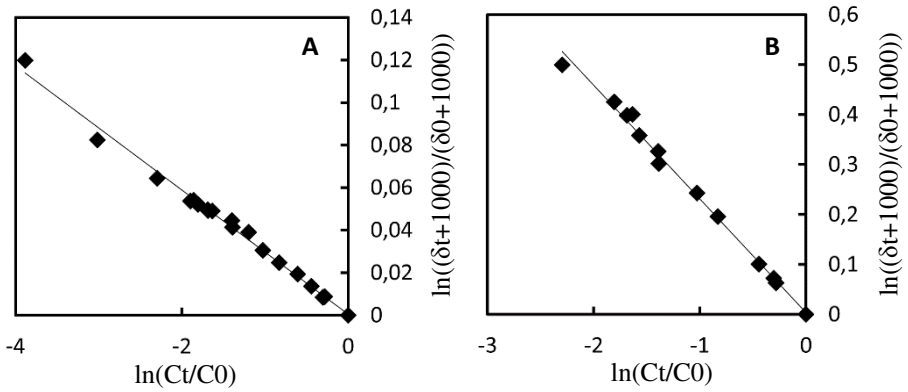

Figure 3: Rayleigh plots for stable isotope fractionation (A, carbon; B, hydrogen). Data from both incubation experiments are included.

methane oxidation is primary due to catalysis by MMO, the experiments with whole cells showed that other processes might play a significant role for observed bulk isotope effect as well.

Previous studies on microbial aerobic degradation of phenol and benzoate provided evidence that growth rates and physiological features were major parameters for the variation of isotopic discrimination of carbon (Hall *et al.*, 1999). These factors correlated, directly or indirectly, with kinetics of substrate transport into the cell and thus its availability for the enzyme. As concluded by Kinnaman et al. (2007), substrate limitation and transport rates during aerobic oxidation of C1-C4 alkanes were the main determinants of fractionation control in methane seep enrichment cultures.

As the *M. oxyfera* enrichment culture primary consisted of aggregated cells, substrate diffusion limitation to inner cells could be one of the parameters, which would affect the extent of bulk isotope fractionation and possibly mask the true fractionation in case of non-limiting substrate condition for each individual cell. Substrate availability is controlled by two factors, the bulk substrate concentration on one hand, and cell biomass content and its activity on the other hand. A previous study showed that cell density had a significant impact on fractionation of carbon during aerobic toluene degradation, with lower cell numbers of single-cell cultures leading to highest isotope fractionation factors (Kampara *et al.*, 2009). In case of biofilms or cell clusters the effect could be even higher since the ratio of biovolume to surface area is much higher, restricting the access of substrate even more. Templeton et al. (2006) found that methane fractionation by whole cells of

proteobacteria was mostly regulated by the total amount of substrate oxidized per unit time, which is dependent on the cell numbers and finally the number of active MMOs, and was regardless of the type of organism or type of expressed MMO. In general, lower cell densities might lead to determination of the true biological fractionation effect of methane, as the amount of catalyst (MMO) and not substrate is limiting. During the current study, the ratio of available catalyst (proportional to the total protein content) to total methane differed by a factor of 8 between both incubation experiments, however it did not have an effect on the isotope fractionation by *M. oxyfera*. One of the factors leading to the high enrichment factor of $\delta^{13}C$ could be the availability of oxygen for pMMO (Templeton *et al.*, 2006). The effect of oxygen would be inverse to that of methane concentrations. Theoretically, under low oxygen concentrations the process of methane oxidation would be slowed down favoring fractionation. Thus, even at low methane availability, the kinetic isotope effect could be offset by low oxygen. As *M. oxyfera* produces its oxygen intracellularly from nitrite via nitric oxide, this step is likely to be rate-limiting, thus restricting oxygen availability for pMMO (Templeton *et al.*, 2006).

Two-dimensional specific isotope analysis

The two-dimensional specific isotope analysis based on carbon and hydrogen during methane degradation by *M. oxyfera* resulted in a lambda (Λ) value of 9.8. This approach was recently introduced in order to identify specific biodegradation processes (Elsner *et al.*, 2005, Elsner *et al.*, 2007). However, the study of Elsner et al. (2007) focused on biodegradation of methyl *tert*-butyl ether, a compound more complex and containing several non-reactive atom positions in contrast to methane. In a recent study, Feisthauer et al. (2011) determined several Λ values for sMMO and pMMO of phylogenetically distinct *Proteobacteria*, but could not observe significantly different lambda values dependent on the type of enzyme expressed. The authors compared the determination of Λ by two approaches, by plotting $\delta^{13}C$ versus $\delta^{2}H$ and calculation according to equation 5. The first approach was shown to be applicable only for enrichment factors of hydrogen not exceeding -100‰, which is out of the range during the current study. In general, there is a broad range of variation in Λ known from previous reports on methane oxidizing environmental gas samples and enrichment cultures. The values range between 3.2 and 19 without an obvious correlation with phylogeny or environmental conditions. Thus, neither the phylogenetic affiliation nor the specific catalytic mechanism can be inferred from

the two-dimensional specific isotope analysis during oxygen-dependent methane oxidation.

Environmental implications

It is evident from all studies on stable isotope fractionation during aerobic methane oxidation conducted so far that the determined enrichment and Λ factors do not reflect neither the underlying enzymatic pathway nor the phylogenetic affiliation of active methanotrophs. Moreover, a study of Holler et al. (2009) on isotope fractionation by enrichment cultures of AOM consortia (ANME II clade) from various marine environments revealed ε_C values of -11.9‰ – -37.5‰ and ε_H of -100.7‰ – -229.6‰. Thus, despite the profoundly different biochemical mechanisms underlying both processes, they resemble isotope enrichment factors as those of aerobic methanotrophs. Similar values were also reported for gas samples from anoxic marine environments (Martens *et al.*, 1999, Kessler *et al.*, 2006). These findings indicate a narrow range of fractionation specific for biological methane oxidation. This, however, can be valuable for the quantitative distinction between biotic and abiotic methane oxidation. The hydroxyl-driven abiotic methane oxidation in the atmosphere is responsible for almost one third of all methane removal (Thauer, 2011) and exhibits an Λ value of 75, a value of one magnitude larger than that of biologically mediated oxidation (Saueressig *et al.*, 1996, Bergamaschi *et al.*, 2000, Saueressig *et al.*, 2001, Feilberg *et al.*, 2005, Feisthauer *et al.*, 2011), making both processes well distinguishable.

An important factor which must be taken into consideration when interpreting isotope data from anoxic environments is methanogenesis. Methanogenic activity may overlap with anaerobic methanotrophy, leading to a partial recycling of produced carbon dioxide and complicating the interpretation of isotope data. However, under nitrate/nitrite rich conditions in the habitat of *M. oxyfera* methanogenesis might be restricted due to the high redox potential and unfavorable kinetics in comparison with denitrifiers.

The enrichment factors determined within the current study have a potential to be applied in evaluation of methane fluxes in anoxic freshwater environments, and to contribute to source determination and modeling of global methane fluxes, which is important in context of global warming.

ACKNOWLEDGEMENTS

We would like to thank M. L. Wu for providing biomass and U. Günther for technical assistance. O. Rasigraf and M. S. M. Jetten were supported by the ERC (grant no. 2322937), and K. F. Ettwig by the Darwin Center for Biogeology (project 3071). C. Vogt and H.-H. Richnow acknowledge funding from the German Research Foundation (priority Program 1319).

chapter 3

Autotrophic carbon dioxide fixation by the denitrifying methanotrophic bacterium *Methylomirabilis oxyfera*

Published previously as:

Olivia Rasigraf, Dorien M. Kool, Mike S. M. Jetten, Jaap S. Sinninghe Damsté, Katharina F. Ettwig (2014). Autotrophic carbon dioxide fixation via the Calvin-Benson-Bassham cycle by the denitrifying methanotroph *Methylomirabilis oxyfera*. Applied and Environmental Microbiology, **80**(8): 2451-2460.

ABSTRACT

Methane is an important greenhouse gas and the most abundant hydrocarbon in the earth's atmosphere. Methanotrophic microorganisms can use methane as their sole energy source and play a crucial role in mitigation of methane emissions in the environment. *Methylomirabilis oxyfera* is a recently described "intra-aerobic" methanotroph that is assumed to use NO to generate internal oxygen to oxidize methane via the 'conventional' aerobic pathway including the monooxygenase reaction. Previous genome analysis has shown that, in contrast to most known methanotrophs, *M. oxyfera* lacks the complete common pathways for carbon assimilation from methane (i.e. serine and ribulose monophosphate pathways). Instead, the complete pathway of the Calvin-Benson-Bassham (CBB) cycle was encoded and transcribed by *M. oxyfera*. Here we provide multiple independent lines of evidence for autotrophic carbon dioxide fixation by *M. oxyfera* via the CBB cycle. Activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), a key enzyme of the CBB cycle, in cell-free extracts from the *M. oxyfera* enrichment culture was shown to account for up to 10% of the total methane oxidation activity. Labeling studies with whole cells in batch incubations supplied with either ^{13}C-CH_4 or ^{13}C-bicarbonate revealed that *M. oxyfera* biomass and lipids became significantly more enriched in ^{13}C after incubation with ^{13}C-labeled bicarbonate (and unlabeled methane) than after incubation with ^{13}C-labeled methane (and unlabeled bicarbonate), providing the conclusive evidence of autotrophic carbon dioxide fixation. Besides this experimental evidence, detailed genomic and transcriptomic analysis demonstrated an operational CBB cycle in *M. oxyfera*. Altogether, these results showed that the CBB cycle is active and plays a major role in carbon assimilation by *M. oxyfera* bacteria. Our results suggest that autotrophy might be more widespread among methanotrophs than previously assumed and implies that a methanotrophic community in the environment is not necessarily revealed by ^{13}C-depleted lipids.

INTRODUCTION

Methane is an important volatile product of the anaerobic degradation of organic matter and is the most abundant hydrocarbon in the earth's atmosphere (Cicerone & Oremland, 1988). It is the most reduced form of carbon, but while bearing a vast amount of energy it is thermodynamically one of the most difficult organic

compounds to activate. The biological oxidation of methane occurs under both oxic and anoxic conditions, performed by specialized groups of Bacteria or Archaea. Aerobic methanotrophs belong to the Bacteria and all oxidize methane in a similar manner, using oxygen for the first oxidation step of methane to methanol by a monooxygenase. In the following reactions, catalyzed by dehydrogenases, methanol is oxidized to carbon dioxide with formaldehyde and formate as intermediates (Hanson & Hanson, 1996).

The aerobic methanotrophs belonging to the Proteobacteria were divided into two types based on their morphology and physiological properties, including the route of C1 assimilation (Whittenbury *et al.*, 1970). Type I methanotrophs utilize mostly the ribulose monophosphate (RuMP) pathway in which all cellular carbon is derived from methane and enters the pathway at the level of formaldehyde (Whittenbury *et al.*, 1970, Anthony, 1986, Hanson & Hanson, 1996). Type II methanotrophs use the serine pathway in which half of the cellular carbon is derived from methane via formaldehyde, and the other half originates from carbon dioxide which enters the pathway via the phosphoenolpyruvate carboxylation reaction (Anthony, 1986). Both pathways represent chemoorganoheterotrophic modes of metabolism and were considered to be universal among aerobic methanotrophs. However, some proteobacterial methanotrophs do possess complete gene sets for autotrophic CO_2 fixation (Taylor *et al.*, 1981, Baxter *et al.*, 2002). The genomes of type I methanotrophs *Methylococcus capsulatus*, *Methylocapsa acidiphila* B2, *Methylocaldum szegediense* O-12, and of type II methanotrophs *Methyloferula stellata* AR4 and *Methylocella silvestris* BL2 possess complete sets of the reductive pentose phosphate cycle, commonly known as the Calvin-Benson-Bassham (Dedysh *et al.*, 2002, Ward *et al.*, 2004, Chen *et al.*, 2010, Vorobev *et al.*, 2011). It still remains to be experimentally validated which role the CBB cycle plays in these organisms.

As methanotrophic bacteria thus seemed to derive at least half of their cellular carbon from CH_4, stable carbon isotopic analysis has been commonly used to identify methanotrophy in culture-independent environmental studies (Hutchens *et al.*, 2004, Cébron *et al.*, 2007, Qiu *et al.*, 2008, Dumont *et al.*, 2011). It is in those cases assumed that the distinct $\delta^{13}C$ signature of biogenic methane (i.e. strongly depleted in ^{13}C) is also reflected in lipids and biomass of methanotrophic communities. Extremely depleted $\delta^{13}C$ values were indeed observed in microbial biomass and lipids (Freeman *et al.*, 1990, Hinrichs *et al.*, 2000, Orphan *et al.*, 2001, Blumenberg *et al.*, 2004, Coolen *et al.*, 2004, Deines *et al.*, 2007). Based on these observations

autotrophy was generally dismissed as a dominant mode of carbon fixation in methanotrophic organisms.

For a century after their first discovery in 1906, methanotrophic bacteria were believed to be restricted to α- and γ-Proteobacteria (Trotsenko & Murrell, 2008). However, since 2007 several independent studies showed that bacterial methanotrophs are phylogenetically much more diverse and are also found within the verrucomicrobial and NC10 phyla (Dunfield *et al.*, 2007, Pol *et al.*, 2007, Islam *et al.*, 2008, Ettwig *et al.*, 2010). The discovery of the (acidophilic) verrucomicrobial methanotrophs revealed not only a wider environmental and phylogenetic spectrum for aerobic methanotrophy, but also demonstrated that these methanotrophs lack essential genes of both the RuMP and the serine pathways (Hou *et al.*, 2008). Instead, they were shown to utilize the CBB cycle for carbon dioxide fixation, challenging the paradigm that methanotrophs are organotrophs deriving a large part of their biomass from CH_4 (Khadem *et al.*, 2011). Similarly, genome analysis suggested that also the first described member of the NC10 phylum – the nitrite-dependent methane oxidizer *Methylomirabilis oxyfera* – may employ the CBB cycle for carbon assimilation (Ettwig *et al.*, 2010, Wu *et al.*, 2011). *M. oxyfera* oxidizes methane via a similar sequence of reactions as employed by aerobic methanotrophs, however, it does so in the complete absence of external oxygen. Instead, nitrite is reduced to nitric oxide and the latter is hypothesized to be dismutated to molecular nitrogen and oxygen (Ettwig *et al.*, 2010, Wu *et al.*, 2011, Ettwig *et al.*, 2012). The internally produced oxygen can then be used for methane oxidation by a methane monooxygenase.

The current study aimed to investigate the mode of C1 assimilation in *M. oxyfera* enrichment cultures through a multidisciplinary approach. We carried out detailed genome and transcriptome analysis focusing on the potential for autotrophic CO_2 fixation via the CBB cycle and employed enzyme activity assays to detect the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) in cell free extracts. In (whole cell) batch incubations we performed ^{13}C-labeling experiments using labeled methane and/or bicarbonate to identify the effective source of C assimilation in *M. oxyfera*-specific lipid biomarkers and total enrichment culture biomass.

MATERIALS & METHODS

M. oxyfera enrichment culture

The culture of *M. oxyfera* bacteria strain "Ooij" was enriched in an anoxic sequencing batch bioreactor as previously described (Ettwig *et al.*, 2008). The enrichment culture was dominated by *M. oxyfera*, making up approximately 80% of the total community as estimated from previous fluorescence *in-situ* hybridization (FISH) analysis.

Transcriptomic and genomic analysis

The phylogenetic sequence analysis of the large chain RubisCO genes was performed by comparison of *cbbL* gene sequences obtained from GenBank using the ClustalW algorithm within the MEGA 5.0 software (Thompson *et al.*, 1994, Tamura *et al.*, 2011). The resulting alignment was manually checked. The phylogenetic tree was calculated using the neighbor-joining method (Saitou & Nei, 1987) and a bootstrap test of 1,000 replicates. The Dayhoff matrix was used for reconstruction of evolutionary distances. The transcriptome data used in this study were obtained from (Luesken *et al.*, 2012). Mapping of the total RubisCO reads was performed with CLC Bio Genomics Workbench 5.0 software using a sequence dataset downloaded from GenBank as mapping reference. The genome analysis was performed on the basis of published data with additional BLAST analysis.

Cell extract preparations

Biomass was obtained from the enrichment culture and centrifuged (under oxic conditions) for 30 min at 16,500 x *g* (Sorvall® RC5B Plus, DuPont, Bad Homburg, Germany) at 4°C. The supernatant was discarded and the pellet resuspended in 20 mM Tris-HCl (pH 8.0) containing 50 mM sodium pyrophosphate (PPi). The final volume of resuspended pellet was 7 mL to which 1 tablet of a protease inhibitor cocktail (Boehringer, Mannheim, Germany) and 1 mg DNAse (Roche Diagnostics, Mannheim, Germany) were added. The disruption of cells was performed on ice by ultrasonication at 110 MPa 15 times for 10 s each with time pause intervals of 10 s. The sonicated biomass was centrifuged for 10 min at 12,000 x *g* at 4°C. The pellet was discarded and the supernatant was further centrifuged for 10 min under identical

conditions. Finally, the dark reddish-brownish supernatant was stored on ice for enzyme assays.

RubisCO activity assays

The method for the RubisCO activity assay was adapted from (Khadem *et al.*, 2011). It is based on the determination of ^{13}C-CO_2 liberation after the destruction of labeled 3-phosphoglycerate which is formed after the RubisCO-specific carboxylation of ribulose-1,5-bisphosphate (RuBP) by CO_2 originating from labeled bicarbonate. The RubisCO activity can then be deduced from the increase in ^{13}C-CO_2 during a time-series of samples.
The assay was performed in 2 mL septum vials (Labco, UK) with a liquid volume of 250 µL. Depending on the amount of cell-free extract used, either 175 or 200 µL of 20 mM potassium phosphate buffer (pH 6.9) containing 10 mM $MgCl_2$ were used. Cell-free extract (50 or 25 µL) and 20 µl of 100 mM ^{13}C-$NaHCO_3$ were added with a syringe to closed vials containing buffer, vortexed for 2 s and incubated for 10 min at 30°C. Then, 5 µL of 25 mMRuBP were added and vortexed for 2 s. Further incubation was performed at 30°C and samples of 50 µL were withdrawn by a syringe and injected into closed 3 mL exetainer vials (Labco Limited, High Wycombe, UK) in intervals of 5 or 10 min over a total period of 20 min. Each exetainer was amended with 20 µL of 0.5 M HCl and dried under vacuum at 50°C over night. The dried samples were amended with 0.5 mL ice-cold 0.1% $KMnO_4$ in 0.1 M H_3PO_4 in closed exetainers, vortexed for 5 s and incubated for 25 min at 50°C. After incubation, samples were kept at room temperature for 1 h for equilibration. The final measurement of headspace CO_2 was performed by gas chromatography coupled to a mass spectrometer (GC-MS, Agilent 5975C, Santa Clara, USA).Each measurement was performed in duplicate by injection of 100 µL of headspace gas with a gastight syringe.

Carbon isotope tracing experiments

The effective assimilation of carbon from CH_4 or bicarbonate/CO_2 into biomass and lipids of *M. oxyfera* was investigated with stable isotope probing (SIP) experiments. Various treatments were performed in which *M. oxyfera* biomass was incubated with either ^{13}C-CH_4 or ^{13}C-bicarbonate. The ^{13}C-CH_4 treatment also contained (unlabeled) bicarbonate, and the treatment with ^{13}C-bicarbonate also received CH_4 as energy

source. A control was included with ^{13}C-bicarbonate without CH_4, and as a reference biomass was also incubated with unlabeled CH_4 and unlabeled bicarbonate. Additional treatments were included to test the use of H_2 and/or formate as alternative electron donors. All treatments, summarized in Tab. 1, received nitrite as electron acceptor.

Incubation set-up

The reactor biomass was washed in 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.4) and resuspended in nitrate-free mineral salt medium described in (29). MOPS was used as a buffering agent (pH 7.4) with a final concentration of 5 mM. Duplicate incubations were performed in 60 mL glass serum bottles amended with different combinations of ^{13}C-labeled or unlabeled sodium bicarbonate (2.5 mM), methane (~4% in headspace) or formate (2.5 mM). All incubations were amended with sodium nitrite (0.3 mM) at the start of incubation and spiked with additional nitrite when the concentration in the bottles was close to zero, as estimated by Merckoquant test strips. The bottles were sealed with red butyl rubber stoppers and crimped with aluminum rings. Immediately after sealing, the batch cultures were made anoxic by 5 cycles of successive vacuuming and gassing with argon, and a final flushing with argon for at least 10 min. In each bottle, an overpressure of 0.4 bar was applied. Thereafter, the incubations were amended with ^{13}C-labeled (99-atom%, IsotecInc, Matheson Tri, USA) or -unlabeled methane (Air Liquide, Eindhoven, Netherlands) and some treatments with hydrogen to final concentrations of approximately 4% and 16% of headspace gas, respectively. Additionally, 2 mL unlabeled CO_2 was added to each bottle for a bicarbonate/CO_2 equilibrium. The cultures were incubated horizontally on a shaker (170 rpm, Innova®40, New Brunswick Scientific, United States) at 30°C. The pH was measured with a pH meter (Metrohm 691, Herisau, Switzerland) at the start and the end of incubation. The headspace pressure was monitored several times during the experiment with a needle pressure meter (GMH 3111, Greisinger, Germany).

Table 1: Summary of the labeling experiment treatments of the *Methylomirabilis oxyfera* enrichment culture.

$NaHCO_3$ Treatments	Electron donor
^{13}C	^{12}C-CH_4
^{12}C	^{13}C-CH_4
^{12}C	^{12}C-CH_4
^{13}C	-
-	^{12}C-CH_4
^{12}C	^{13}C-CH_4+H_2
^{13}C	H_2
^{13}C	^{12}C-CH_4+H_2
^{13}C	^{12}C-CH_4
^{12}C	^{13}C-$HCOO^-$
^{13}C	^{12}C-$HCOO^-$
-	^{13}C-CH_4

Analysis of methane, nitrite, formate and protein concentrations

Methane concentration in the headspace was measured by gas chromatography with a flame ionization detector (HP 5890 Series II, Agilent Technologies, SantaClara, United States). Each measurement was performed in duplicate by injection of 100 µL headspace gas with a gastight syringe. Measurements of carbon dioxide, oxygen and hydrogen were performed by GC-MS (see above). At the same time, liquid samples of 200 µL each were taken for nitrite and formate determination. After each activity experiment, 0.5 mL of biomass was taken from each incubation bottle for determination of the total protein content. The remaining biomass was centrifuged in 50 mL tubes at 4,000 x *g* for 20 min, supernatant was discarded and pellets were kept at -80°C until subsequent freeze-drying and following lipid and isotope analysis. Nitrite and protein concentrations were determined colorimetrically as described in (Ettwig *et al.*, 2008). Formate determination was performed with a

method slightly modified from (Sleat & Mah, 1984). 0.1 mL sample was mixed with freshly prepared 0.2 mL working solution (0.5 g citric acid and 10 g acetamide in 100 mL 2-propanol), 0.01 mL sodium acetate (30% (w/v) in MilliQ) and 0.7 mL acetic anhydride. All samples (triplicate) were incubated for two hours at room temperature in the dark and the extinction was measured at 510 nm with a spectrophotometer (Ultrospec K, LKB Biochrom Ltd., Cambridge, United Kingdom).

During the experiment the enrichment culture exhibited a methanotrophic activity between 1.3 and 1.6 nmol CH_4 min^{-1} mg^{-1} protein. Associated NO_2^- consumption closely followed the theoretical stoichiometry of 3:8 (CH_4:NO_2^-). In the treatments where we checked for the use of alternative electron donors, we confirmed that neither formate nor hydrogen were used as electron donors by the *M. oxyfera* enrichment culture (data not shown). The presence of hydrogen did not have any effect on the methanotrophic activity. Interestingly, the nitrite- and methane-oxidizing activity was considerably lower in incubations without added bicarbonate although the pH difference to cultures with added bicarbonate was not significant.

Bulk- and compound-specific isotope analysis

The freeze-dried biomass was separated into subsamples for bulk and compound specific isotope analysis. For bulk isotope analysis of the biomass, the sub-samples were acidified in silver foil cups (2 N HCl; dried at 60°C overnight) to remove all inorganic carbon. Isotope values were determined by elemental analysis followed by isotope ratio mass spectrometry (Thermo Flash 2000 Elemental Analyzer coupled to a Thermo DeltaV IRMS system). Lipid extraction of the sub-sample for compound-specific analysis was carried out as described previously (Kool *et al.*, 2012). In short, after saponification the obtained extracts were methylated with boron trifluoride (BF_3) in MeOH and subsequently separated by column chromatography over activated alumina (Al_2O_3) into apolar and polar fractions. Previous analysis had revealed that the apolar fraction comprised the majority of the total lipids (Kool *et al.*, 2012). The apolar fractions were subsequently analyzed by gas chromatography (GC; Agilent 6890) and gas chromatography-mass spectrometry (GC-MS; Thermo Trace GC Ultra coupled to a Thermo Trace DSQ). The GC was equipped with a fused silica column (25 m x 0.32 mm) coated with CP Sil-5 (film thickness = 0.12 μm) with He as carrier gas. Relative abundance of the fatty acid methyl esters was derived from the (integrated) GC profile. Compounds were identified with GC-MS

following methods described previously (Kool *et al.*, 2012). For stable carbon isotope analysis, fractions were analyzed by GC coupled to isotope ratio mass spectrometry as described previously (Schouten *et al.*, 1998). Raw data were corrected for the $\delta^{13}C$ of the carbon derived from BF_3-methanol that was added during preparation of the methyl esters of the fatty acids.

RESULTS

Genome- and transcriptome analysis of C1 assimilation potential by *M. oxyfera*

Detailed analysis of the previously published genome data (Ettwig *et al.*, 2010) showed that neither serine nor the RuMP pathways were complete in *M. oxyfera*. For both pathways, important key genes could not be annotated. In the RuMP pathway 3-hexulose-6-phosphate synthase (HPS) and 6-phospho-3-hexuloisomerase (PHI) were missing. Malyl-CoA lyase, malate thiokinase and glycerate 2-kinase were absent in the serine pathway.

When we consider the six known autotrophic pathways, only the CBB cycle gene set was complete. Analysis of the previously published transcriptomic and proteomic data (Ettwig *et al.*, 2010, Luesken *et al.*, 2012) revealed that all CBB cycle genes were also expressed in *M. oxyfera* bacteria (Tab. 2). The CBB genes are arranged in four separate clusters (Fig. 1) with additional copies of a phosphoribulokinase (PRK) and a fructose-1,6-bisphosphatase (FBPase) not being located within a *cbb*-operon.

Table 2: Relative expression of CBB cycle-associated genes and (for comparison) key catabolic genes in *Methylomirabilis oxyfera*[a]

orf identifier (damo_)	**Gene name**	**Encoded protein**	**EC[b] no.**	**Relative expression[c]**
0174	*cbbF1*	Fructose-1,6-bisphosphatase II	3.1.3.11	8.3
0175	*cbbA*	Fructose-bisphosphate aldolase II, CBB cycle subtype	4.1.2.13	9.8
0176	*cbbE*	Ribulose-phosphate 3-epimerase	5.1.3.1	8.8
0339	*cbbG1*	Glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12	9.4

0340	*cbbK*	Phosphoglycerate kinase, 5' end	2.7.2.3	9.1
0341	*cbbK*	Phosphoglycerate kinase, 3' end	2.7.2.3	8.3
0342	*tpiA*	Triosephosphate isomerase	5.3.1.1	9.0
0804	*oxyR*	LysR-type transcriptional activator	-	7.5
0861	*oxyR*	LysR-type transcriptional activator	-	8.7
2116	*cbbP1*	Phosphoribulokinase	2.7.1.19	3.9
2163	*cbbF2*	Fructose-1,6-bisphosphatase II	3.1.3.11	9.6
2164	*rpiA*	Ribulose-5-phosphate isomerase A	5.3.1.6	8.1
2165	*cbbL*	Ribulose-1,6-bisphosphate carboxylase/oxygenase, large chain	4.1.1.39	9.3
2166	*cbbS*	Ribulose-1,6-bisphosphate carboxylase/oxygenase, small chain	4.1.1.39	10.1
2167	*cbbX*	Ribulose-1,6-bisphosphate carboxylase/oxygenase activase, AAA ATPase	-	9.9
2168	*ppcA*	Phosphoenolpyruvate carboxylase	4.1.1.31	9.3
2170	*cynT*	Carbonic anhydrase	4.2.1.1	10.3
2650	*cbbF3*	Fructose-1,6-bisphosphatase II	3.1.3.11	9.6
2651	*cbbT*	Transketolase	2.2.1.1	7.9
2652	*cbbG2*	Glyceraldehyde-3-phosphate-dehydrogenase	1.2.1.12	7.9
2653	*cbbP2*	Phosphoribulokinase	2.7.1.19	9.9
2986	*cbbF4*	Fructose-1,6-bisphosphatase II	3.1.3.11	9.1

[a] The transcriptome analysis was performed on the basis of data published previously (Luesken *et al.*, 2012). The presented data refer to the anoxic period.
[b] EC, enzyme nomenclature designation.
[c] Relative expression was determined from $\log_2$(RPKM+1), where RPKM is reads per kilobase per million mapped reads.

Two enzymes are exclusive to the CBB cycle: RubisCO and PRK (Tabita, 1988). The large subunit of the *M. oxyfera* RubisCO belongs to the red-like type 1c (Fig. 2) sharing 83% sequence similarity on the amino acid level with *Rhodospirillum centenum* SW and *Beggiatoa alba* as closest homologues. Also the small RubisCO subunit encoded by *cbbS* and a putative RubisCO activase encoded by *cbbX* share 73% and 78% sequence similarity, respectively, with *B. alba* as the closest relative. Additionally, two isoforms of a LysR-type transcriptional activator were identified in the genome of *M. oxyfera*. Many autotrophs employ a regulation of the CBB cycle gene expression via the LysR-type transcriptional regulator, designated as CbbR (Gibson & Tabita, 1993). It was shown previously that CbbR is essential for the induction of autotrophy in *Xanthobacter flavus* (van den Bergh *et al.*, 1993, Meijer *et al.*, 1996, van Keulen *et al.*, 2003). However, the *cbbR*-like genes of *M. oxyfera* are not located in the vicinity of any *cbb* cluster and it remains to be shown whether the expressed LysR-type proteins are specifically involved in the regulation of the CBB cycle or other metabolic processes.

Two accessory functions are often associated with an active CBB cycle: the presence of CO_2-concentrating micro-compartments, commonly known as carboxysomes, and a photorespiration pathway employed to detoxify 2-phosphoglycolate formed due to the oxygenation activity of RubisCO (Bowes *et al.*, 1971, Yeates *et al.*, 2008). However, neither carboxysome-encoding genes nor carboxysome-like structures could be observed in *M. oxyfera* cells (Wu *et al.*, 2012), indicating a cytoplasmic CO_2 fixation. Besides CO_2, O_2 is a competing substrate of RubisCO, which leads to a reduced CO_2 fixation efficiency and the production of 2-phosphoglycolate (Bowes *et al.*, 1971), a toxic compound which needs to be detoxified. The detoxification of phosphoglycolate eventually leads to the formation of 3-phosphoglycerate which can re-enter the CBB cycle. So far, three routes of the photo-respiratory pathway are known: C2 cycle, glycerate pathway and decarboxylation (Eisenhut *et al.*, 2008, Zarzycki *et al.*, 2013). In each pathway, glycerate is the key intermediate and steps leading to its formation are common for all. Glycerate is formed via glycolate from 2-phosphoglycolate catalyzed by a phosphoglycolate phosphatase and glycolate oxidase, respectively. No genes encoding a phosphoglycolate phosphatase were identified in *M. oxyfera*, however, the glycolate oxidase encoding gene *glcD* is present and transcribed. Furthermore, except for a glycerate kinase catalyzing the last step, all other proteins involved in C2 cycle are present in *M. oxyfera*.

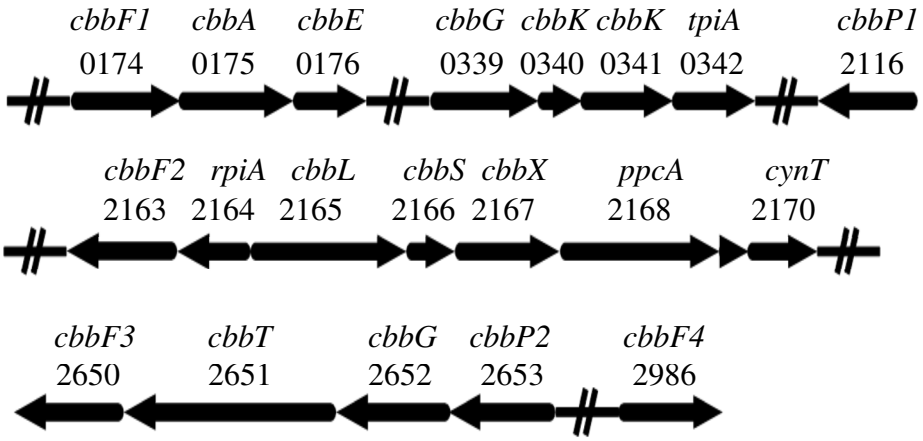

Figure 1: Gene clusters and single genes involved in or associated with the CBB cycle in *Methylomirabilis oxyfera*. The direction of transcription is indicated with arrows and ORF identifiers are shown. Encoding genes: *cbbF*, fructose-1,6-bisphosphatase II; *cbbA*, fructose-bisphosphate aldolase II; *cbbE*, ribulose-phosphate 3-epimerase; *cbbG*, glyceraldehyde 3-phosphate dehydrogenase; *cbbK*, phosphoglycerate kinase; *tpiA*, triosephosphate isomerase; *cbbP*, phosphoribulokinase; *rpiA*, ribulose-5-phosphate isomerase A; *cbbL*, ribulose-1,6-bisphosphate carboxylase/oxygenase, large chain; *cbbS*, ribulose-1,6-bisphosphate carboxylase/oxygenase, small chain; *cbbX*, putative ribulose-1,6-bisphosphate carboxylase/oxygenase activase; *ppcA*, phosphoenolpyruvate carboxylase; *cynT*, carbonic anhydrase; *cbbT*, transketolase

RubisCO activity

Cell-free extract preparations of *M. oxyfera* enrichment culture exhibited an average RubisCO-specific activity of 0.2 nmol $^{13}C\text{-}CO_2*min^{-1}*mg\ protein^{-1}$ (Fig. 3). This activity corresponded to approximately 10% of the methanotrophic activity of the culture at the time of the experiments. No activity was observed in incubations without addition of either RuBP or cell-free extract (data not shown).

Interestingly, only freshly prepared cell-free extract exhibited the CO_2-fixing activity, freezing or storing on ice for longer than one day resulted in a complete loss of activity. Since the cell-free extract was prepared from an enrichment culture with approximately 20% other community members, we also investigated the transcriptome for reads mapping to a comprehensive RubisCO data set obtained from GenBank. Over 90% percent of the total RubisCO reads were specific to *M. oxyfera*,

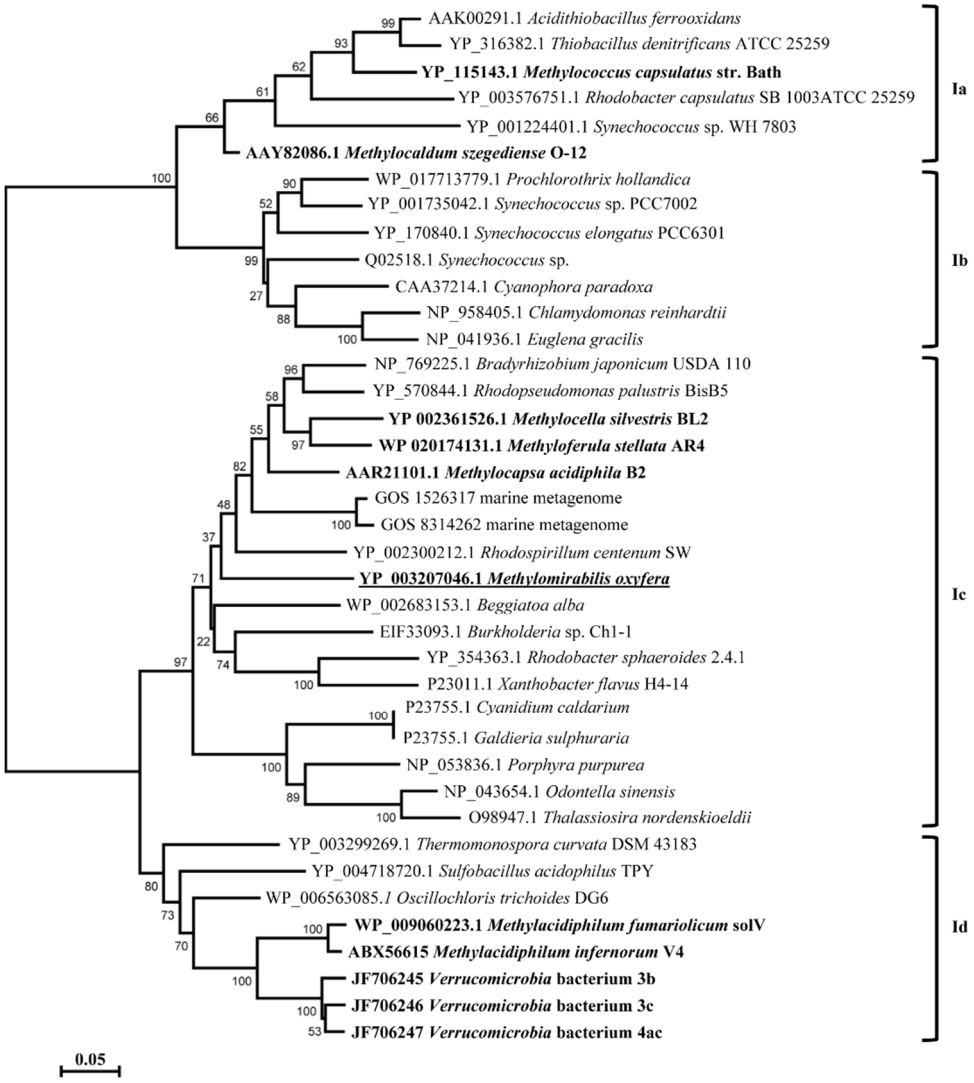

Figure 2: The bootstrap consensus tree of selected RubisCO form I sequences calculated from 1,000 replicates. The evolutionary history was inferred using the neighbor-joining method and the evolutionary distances using the Dayhoff matrix. Scale bar represents the number of amino acid changes per site. The alignment gaps were eliminated in a pairwise comparison. RubisCO sequences of methanotrophs are highlighted and the sequence of *Methylomirabilis oxyfera* is underlined. The classification is based on (Tabita, 1995), with types Ia and Ib belonging to the green-like, and types Ic, Id and Ie belonging to the red-like RubisCOs.

suggesting that the measured specific RubisCO activity could be attributed mainly to *M. oxyfera.*

^{13}C-labeling of bulk biomass and specific lipids

Bulk stable carbon isotope data showed that more ^{13}C was incorporated into the total biomass in the treatment with ^{13}C-bicarbonate than with ^{13}C-CH_4 (Fig. 4a). In the absence of CH_4, also when substituted with formate or H_2 as electron donor, the incorporation from ^{13}C-bicarbonate was minimal. The absolute amount of ^{13}C incorporation into biomass was estimated from the bulk isotope enrichment and the total organic carbon data, together with the overall amount of CH_4 oxidation (1.3 to 1.6 nmol CH_4 min^{-1} mg^{-1} protein amounts to an average 422 µg C oxidized per total batch incubated). By the end of the incubation, the ^{13}C-bicarbonate treatment contained approximately 27 µg more ^{13}C than the control without any label (absolute amount per batch incubated). In the ^{13}C-methane treatment, about 11 µg ^{13}C had been incorporated, which approximates the amount of C indirectly assimilated from CH_4 via CO_2. In total, approximately 38 µg of ^{13}C was thus assimilated into the biomass of each batch over the course of the experiment, which amounts to about 9% of the amount of CH_4-carbon oxidized. This correlates well with the observed specific RubisCO activity.

The compound-specific stable carbon isotopic analysis of fatty acids showed the same trend as the bulk carbon isotope enrichment, with highest ^{13}C enrichment when bicarbonate was ^{13}C-labeled in the presence of (unlabeled) CH_4 (Fig. 4b). The three fatty acids $C_{16:0}$, $10MeC_{16:0}$, and $10MeC_{16:1\Delta7}$, which are of main interest because of their relative abundance and specificity (Kool *et al.*, 2012), all followed the same trend as the total ^{13}C uptake. Some minor fatty acids showed a different response to the labeling: the fatty acids $isoC_{15}$ and aiC_{15} became similarly enriched from ^{13}C-bicarbonate regardless of the presence of CH_4. For the fatty acids $C_{18:1}$, $C_{18:0}$ and C_{19cyc} a higher degree of labeling was obtained from ^{13}C-CH_4 than from ^{13}C-CO_2.

DISCUSSION

Based on the genome information of *M. oxyfera*, we followed the hypothesis that the CBB cycle would be the major route of C assimilation in *M. oxyfera*. Our labeling

experiments (Fig. 4) now provide conclusive proof that *M. oxyfera,* in contrast to most known methanotrophic bacteria, is an autotrophic organism. In the absence of

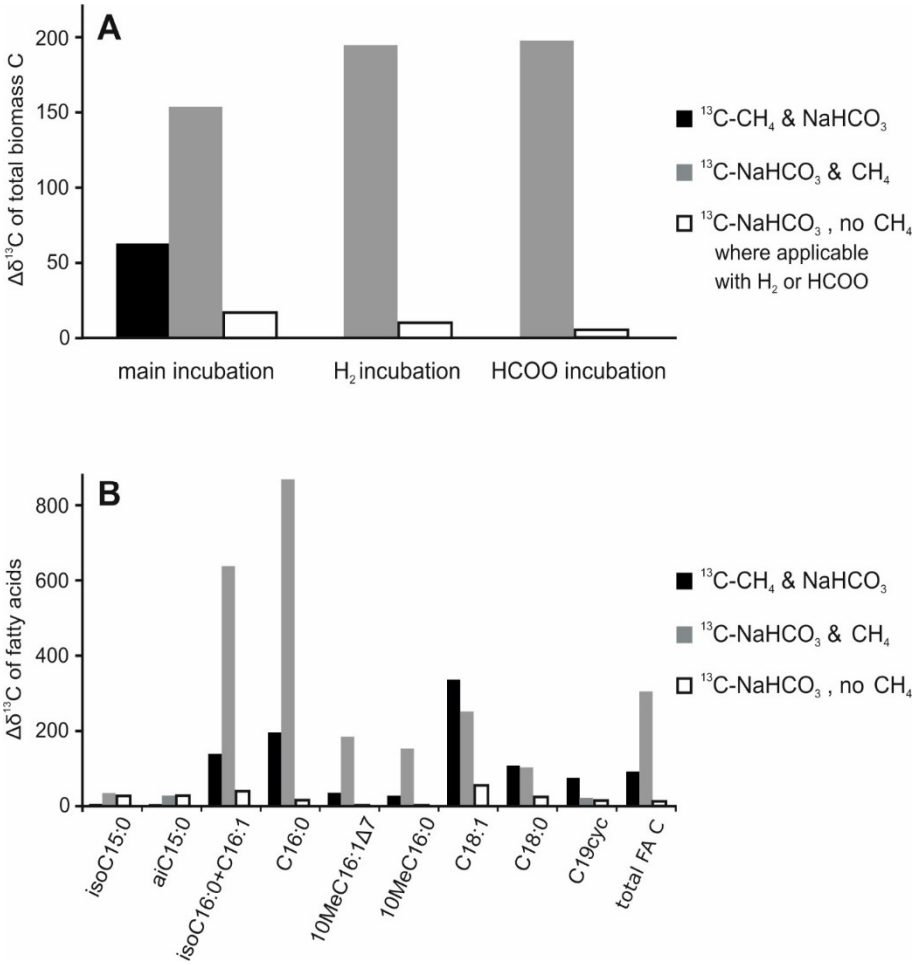

Figure 4: Changes in $\delta^{13}C$ in fatty acids and total biomass carbon of *Methylomirabilis oxyfera* after incubation with ^{13}C-labeled substrates, relative to the control incubation without ^{13}C labeling. The 'cyc' denotes a cyclopropyl moiety; 'total fatty acid carbon' is the weighted average of the $\Delta\delta^{13}C$ of all fatty acids.

CH_4 the cultures did not incorporate significant amounts of ^{13}C label, showing that CH_4 was essential as energy source, but the bulk stable carbon isotope analysis suggests that bicarbonate/CO_2 was actually the main carbon source for *M. oxyfera* cells (Fig. 4a). However, as the *M. oxyfera* biomass is only available in enrichment culture, the risk always remains that the other community members may have

contributed to the observed ^{13}C incorporation. The compound-specific stable carbon isotope analysis provides the decisive data that bicarbonate/CO_2 was the carbon source for cell material production by *M. oxyfera* because a similar type of enrichment pattern was observed for the *M. oxyfera*-specific fatty acids $10MeC_{16:0}$, and $10MeC_{16:1\Delta7}$ (Kool *et al.*, 2012). Although some labeling was observed in the incubations with ^{13}C-CH_4 and unlabeled bicarbonate/CO_2, its extent was clearly lower than in the incubations with ^{13}C-bicarbonate and unlabeled methane. This labeling signal was most probably caused by an indirect ^{13}C-CO_2 incorporation originating from ^{13}C-CH_4 oxidation (i.e. scrambling). Additionally, the labeling results of a minor fatty acid fraction also indicated the presence of other CO_2-fixing community members and chemoorganoheterotrophic methanotrophs.

At the time of the earliest nitrite-dependent methane oxidizing enrichment culture, a labeling experiment with ^{13}C-CH_4 showed that after 3 to 6 days of incubation the ^{13}C label was indeed incorporated into bacterial lipids (Raghoebarsing *et al.*, 2006). However, the anticipated biomarker lipid $10MeC_{16:0}$ fatty acid did not become significantly enriched and the ^{13}C content of $10MeC_{16:1\Delta7}$ fatty acid could not be determined due to its low abundance and co-elution (Raghoebarsing *et al.*, 2006). This could, at least in part, be caused by the slow growth of *M. oxyfera*. However, a carbon source other than CH_4 can also explain this observation. This was not tested at that time; our data now provide strong indications that this alternative carbon source could be CO_2.

The test for the specific activity of RubisCO with cell-free extract of *M. oxyfera* confirmed that *M. oxyfera* indeed exhibited a CO_2-fixing activity. The measured activity rate may seem low compared to the two weeks doubling time of *M. oxyfera* reported previously (Ettwig *et al.*, 2009). However, this doubling time was observed during the initial enrichment period and represents exponential growth of *M. oxyfera*. In the current study the culture exhibited a constant steady-state activity, probably close to stationary phase, during which enzyme expression and specific activity are expected to be lower than during exponential growth phase. Moreover, the observed specific RubisCO activity compared well with the estimated C assimilation activity based on our ^{13}C labeling experiments.

The genomic information and the absence of carboxysome-like structures suggest the CO_2 fixation in *M. oxyfera* to be non-carboxysomal, but rather cytoplasmic. Carboxysomal CO_2 fixation probably evolved in order to increase the rates at low ambient CO_2 concentrations and to minimize photorespiration (Zarzycki *et al.*, 2013). As in the environmental niche of *M. oxyfera* the CO_2 concentrations are

unlikely to be limiting and external oxygen is not present the carboxysomal CO_2 fixation might not offer an advantage in comparison with cytoplasmic CO_2 fixation. The incomplete photorespiration pathway also implies that photorespiration might not be relevant in *M. oxyfera*, however, the mechanism of the hypothesized internal oxygen metabolism is not known yet and the possibility of internal RubisCO oxygen exposure cannot be ruled out.

The rapid increase in genomic information has revealed that several known proteobacterial methanotrophs, which are considered to be chemoorganoheterotrophs, do possess the genomic potential for an autotrophic lifestyle. Moreover, it was recently shown that autotrophy is widespread among the newly discovered verrucomicrobial methanotrophs (Khadem *et al.*, 2011, Sharp *et al.*, 2012). The finding that autotrophy might be a more common mode of C1 metabolism among methanotrophs has implications for the detection of methanotrophy, and assessment of its significance, in the environment. It implies that the stable carbon isotopic signature of these bacteria would not identify them as part of the methanotrophic community *in situ* and in conventional stable isotope studies. A recent report on methanotrophy in a geothermal soil demonstrated that labeling with ^{13}C-CH_4, as commonly used in SIP studies targeting methanotrophs, failed in detecting the active methanotrophic community. Instead a modified SIP method including ^{13}C-bicarbonate/CO_2 was necessary and successful to detect autotrophic methanotrophs. The same would apply for the detection of *M. oxyfera*-like methanotrophs in mesophilic, anoxic and suboxic environments. Thus, *M. oxyfera* might have escaped detection by means of stable isotope analysis and, more generally, the contribution of autotrophic methanotrophic bacteria to methane cycling might have been hitherto overlooked.

ACKNOWLEDGEMENTS

We would like to thank Mingliang Wu for help with cell-free extract and SDS-PAGE techniques, Huub Op den Camp for providing a *cbbL* core alignment, Claudia Lüke for access to methanotroph genome data and Margaret Butler for initial genome annotation. O.R. and M.S.M.J. were supported by the ERC (grant no. 2322937), D.K., J.S.S.D. and K.F.E. by the Darwin Center for Biogeosciences (projects 142.16.3071 and 142.16.3072).

chapter 4

Physiological response of *Methylomirabilis oxyfera* enrichment cultures to nitric oxide (NO)

ABSTRACT

The methanotrophic bacterium *Methylomirabilis oxyfera* employs a novel "intra-aerobic" denitrification pathway in which nitrite is converted to dinitrogen gas as the end-product. Nitrite is presumably reduced to nitric oxide by a cytochrome *cd1*-dependent nitrite reductase NirS. The following step, the conversion of nitric oxide, is hypothesized to proceed via a reaction which was so far unknown to biology - dismutation of nitric oxide to molecular nitrogen and oxygen. In this pathway, nitrous oxide is bypassed as an intermediate. While nitrogen is released, the produced oxygen is presumed to be mainly used for the oxidation of methane to carbon dioxide. However, a small fraction of generated oxygen can also be reduced to water by a terminal oxidase.

The mechanism of nitric oxide dismutation still remains elusive. It is hypothesized to be catalyzed by a divergent nitric oxide reductase-like enzyme. The *M. oxyfera* genome encodes two homologues of the putative nitric oxide dismutase (Nod), both encoding genes are highly transcribed under all conditions tested so far. Besides the two putative nitric oxide dismutases, *M. oxyfera* genome also encodes one copy of a "conventional" quinol-dependent nitric oxide reductase (qNor). Thus, in *M. oxyfera* nitric oxide is a critical metabolite which conversions are far from being understood. Here, we exposed an enrichment culture of *M. oxyfera* bacteria to elevated concentration of free nitric oxide and analyzed its metabolic response from metabolite measurements and sequenced mRNA. Different experimental stages revealed a severe nitrosative stress. After NO had decreased under 1 µM, the methanotrophic and nitrite reducing activities resumed. Transcriptomic results, however, indicated an ongoing downregulation of central cellular processes including genes involved in both nitrogen and methane metabolism. Upregulation was observed for protein synthesis and DNA repairing processes. Following experiments will focus on NO concentration under 1 µM in chemostat cultures in order to investigate the roles of two putative Nod proteins and conventional qNor.

INTRODUCTION

Nitric oxide (NO) is central metabolite in the biological Nitrogen cycle (N-cycle) as all so far known organisms and pathways involved in nitrogen transformations can form and utilize NO (Schreiber *et al.*, 2012). At the same time it is one of most important signaling molecules in prokaryotic and eukaryotic cells. In microbes,

various cellular processes have been implicated to be regulated by NO including denitrification (Van Spanning *et al.*, 1995, Kwiatkowski & Shapleigh, 1996, Van Spanning *et al.*, 1999), biofilm swarming (Barraud *et al.*, 2006, Liu *et al.*, 2012), symbiosis (Wang & Ruby, 2011) and defense mechanisms (Gusarov *et al.*, 2009).
Production of nitric oxide in denitrifiers is mediated by dissimilatory nitrite reductase (Nir), an enzyme which was shown to occur in two structurally dissimilar but functionally identical forms: copper-dependent NirK and cytochrome *cd1*-dependent NirS (Zumft, 1997). Besides dissimilatory nitrite reductases, several other enzymatic systems were shown to produce NO including bacterial nitric oxide synthases (NOS) (Adak *et al.*, 2002), hydroxylamine oxidoreductases (Hao) (Campbell *et al.*, 2011, Maalcke *et al.*, 2013) and pentaheme nitrite reductases (Corker & Poole, 2003).
Besides its important role in cellular processes, NO is at the same time a highly reactive and toxic intermediate when its transient concentrations rise above physiological limits. Thus, a variety of mechanisms have evolved to further transform NO to less toxic intermediates (e.g. nitrite, nitrous oxide). The abiotic chemistry of nitric oxide is complex and involves several reactive nitrogen species (RNS) when reacting with oxygen and superoxide (Bowman *et al.*, 2011). These RNS include peroxinitrite, nitroxyl and nitrosonium, with the oxidation state of N atom varying between I and III (Hughes, 1999, Poole, 2005). Each of these highly reactive and transient intermediates induces partly distinct physiological responses (Bowman *et al.*, 2011). Known microbial detoxification mechanisms involve flavohemoglobins (Hmp) (Kim *et al.*, 1999, Stevanin *et al.*, 2002), cytochrome *c* nitrite reductases (NrfA) (Costa *et al.*, 1990, Stach *et al.*, 2000, Mills *et al.*, 2008) and flavorubredoxins (NorV) (Gardner *et al.*, 2002).
In most denitrifiers, NO is metabolized to nitrous oxide (N_2O) by respiratory nitric oxide reductases (Nor) which occur in two forms: quinol- (qNor) and cytochrome *bc1*-dependent (cNor) forms (Shiro, 2012). Both are transmembrane proteins which catalyze the following reaction (Shiro, 2012):

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$$

Until recently, the only pathways for NO conversion to dinitrogen gas (N_2) were believed to proceed either via N_2O during denitrification or N_2H_4 during anaerobic ammonium oxidation (anammox). In denitrifiers, the reaction is catalyzed by the activity of Nor and nitrous oxide reductase (Nos) (Zumft, 1997), and in anammox bacteria by hydrazine synthase and hydrazine dehydrogenase (Kartal *et al.*, 2011). However, recent description of a novel methane-oxidizing denitrifier

Methylomirabilis oxyfera challenged this paradigm with a proposed hypothesis of NO dismutation to molecular oxygen (O_2) and N_2, thereby bypassing any free reaction intermediates (Ettwig *et al.*, 2010). The genome of *M. oxyfera* encodes and expresses two paralogues of qNor-like proteins, which have been proposed to catalyze NO dismutation and have been provisionally named nitric oxide dismutases (Nod) (Ettwig *et al.*, 2012). Amino acid sequence analysis and comparison to conventional qNor proteins revealed important structural modifications at the active and quinol-binding site, which imply an electron-neutral reaction in accordance with a proposed dismutation reaction (Ettwig *et al.*, 2012). However, mechanisms of NO dismutation are currently unknown and need further investigation including isolation and biochemical characterization of putative proteins. Besides two structurally related Nod proteins, *M. oxyfera* genome also encodes and expresses a conventional qNor. The role of *M. oxyfera* qNor is currently unknown as N_2O was hypothesized to be bypassed as an intermediate. However, enrichments of *M. oxyfera* cultures produce low levels of N_2O and the proportion of produced N_2O from nitrite rises with elevated concentrations of nitrite (unpublished). Thus, its role might be in detoxification of excessive NO. However, this hypothesis needs further validation since the reaction mechanism and speed of putative Nod proteins are not known. Its potential involvement in NO detoxification would imply the dismutation to be the limiting step during the denitrification pathway of *M. oxyfera.* Besides the unclear role of a conventional qNor, the *M. oxyfera* genome also encodes and expresses other enzymes involved in dissimilatory part of the N-cycle, namely membrane-bound/periplasmic nitrate reductases (Nar/Nas) and hydroxylamine oxidoreductase (Hao). Former enzymes perform reduction of nitrate to nitrite, however previous physiological studies and the proposed pathway suggest that nitrate is not used by *M. oxyfera*. The role of Hao is also not clear since this enzyme is believed to be essential in aerobic ammonia oxidation where it converts hydroxylamine to nitrite. Besides aerobic ammonia oxidizers, many methanotrophic bacteria were shown to harbor Hao (Stein & Klotz, 2011). In methanotrophs, Hao was hypothesized to be involved in detoxification of hydroxylamine which is formed by methane monooxygenase when ammonium is present (Nyerges & Stein, 2009, Stein & Klotz, 2011). Thus, *M. oxyfera* Hao might be involved in detoxification of ammonium-derived hydroxylamine since ammonium is a common compound at oxic/anoxic interfaces. In aerobic ammonia oxidizers, hydroxylamine is oxidized to nitrite with O_2 (Hooper *et al.*, 1983). However, since *M. oxyfera* occurs in habitats where external oxygen is not present or very low, the main product of hydroxylamine

oxidation might be NO. It has been shown previously that Hao-like proteins from anaerobic ammonium oxidizing bacteria (anammox) form NO (Maalcke *et al.*, 2013). However, it cannot be excluded that some of the internally produced oxygen could be used for the oxidation of hydroxylamine to nitrite. All N-cycle and in methane oxidation pathway involved enzymes harbored by *M. oxyfera* are summarized in Figure 1.

The objective of this research was to investigate the physiological response of an *M. oxyfera* enrichment culture to controlled additions of NO in a bioreactor system by measuring concentrations of nitrogenous compounds and methane. Furthermore,

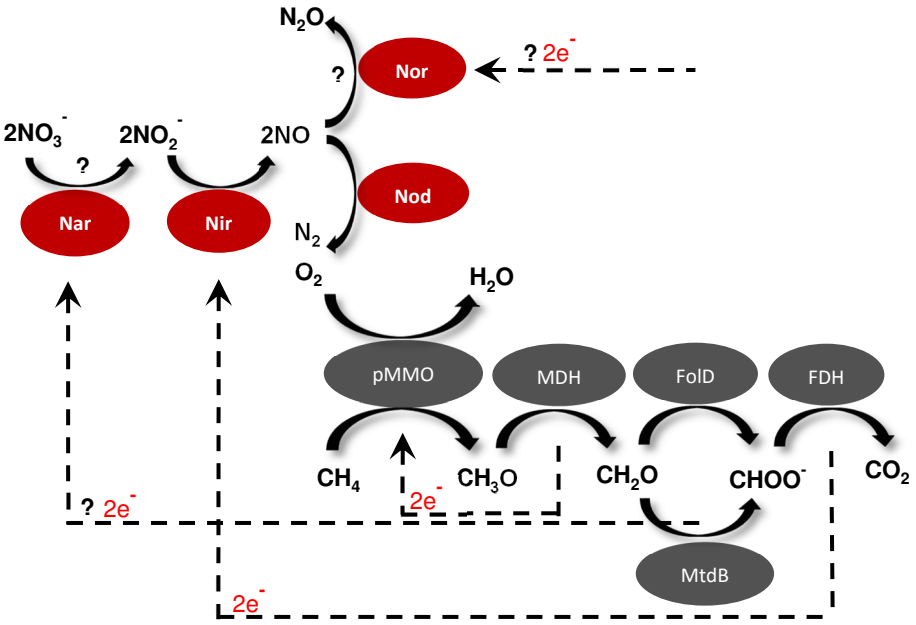

Figure 1: Genomic metabolic potential of *Methylomirabilis oxyfera* in respect to catabolic nitrogen- and methane conversions. Steps designated with questions marks are so far not investigated and their role/function unclear. Abbreviations: Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nod, nitric oxide dismutase; pMMO, particulate methane monooxygenase, MDH, methanol dehydrogenase; FolD, methylene-H_4F dehydrogenase/methenyl-H_4F cyclohydrolase; FDH, formate dehydrogenase; MtdB, methylene-H_4MPT dehydrogenase (Modified after Ettwig et al., 2010 and Wu et al., 2011).

total RNA extracted from the enrichment culture during the exposure experiment was sequenced with Ion Torrent technology and analyzed with the CLC Genomics Workbench software. Exposure to micromolar concentrations of NO induced a stress response which resulted in inhibited methane oxidation, nitrite reduction activity and

downregulation of central metabolic genes. Only after the NO concentration decreased below 1 µM in liquid, activities resumes while the downregulation for central catabolic genes was still ongoing.

MATERIALS & METHODS

Experimental set-up

The *M. oxyfera* enrichment culture used in this study was enriched in an anoxic sequencing batch reactor as described previously (Ettwig *et al.*, 2008). Prior to NO exposure experiments, the culture was transferred to a 3 L continuous membrane reactor under anoxic conditions. The culture was fed with nitrate-free mineral medium (composition described previously in Ettwig et al., 2008) and bubbled with CH_4/CO_2 (95/5%) gas mixture. The pH of the culture was maintained around 7.2. The denitrifying activity of the culture prior to experiments was approximately 6 nmol NO_2^-*min^{-1}*mg $protein^{-1}$.
NO was supplied to the reactor from an anoxic saturated mineral medium (nitrate-, nitrite- and trace element-free) solution bubbled with a 50% NO (in He) gas.

NO exposure experiment: closed headspace

A modified set up with a closed headspace was employed for simultaneous quantification of headspace gas concentrations including methane. Prior to the experiment, the influent was stopped in order to deplete the culture in all nitrogenous electron acceptors. At the same time, the culture was flushed for 2 h with an Ar/CO_2 (95/5%) gas mixture to lower the headspace methane concentrations. At the time point when the NO solution started to be supplied to the reactor, the effluent speed was adjusted to the same speed as the NO solution influent so that the headspace volume and pressure remained approximately constant. Headspace NO, N_2, O_2 and N_2O concentrations were measured throughout the experiment with gas chromatography coupled to mass spectrometry (GC-MS, Agilent 5975C, Santa Clara, USA), CH_4 was measured with gas chromatography (GC, HP 5890 Series II, Agilent Technologies, SantaClara, United States). Liquid samples of 0.5 mL were taken throughout the experiment for nitrogenous ion determination. Biomass samples of 10 ml each were taken in duplicate at different stages of the experiment

for RNA extraction. The samples were immediately frozen at -80°C until the subsequent RNA isolation. Before each biomass sampling, the same volume of Ar gas was injected into the headspace to avoid under pressure.

NO exposure experiment: open headspace

Prior to NO exposure experiments with the open headspace, the medium influent to the reactor was stopped in order deplete the culture of nitrogenous electron acceptors. During the experiment, the reactor was supplied with CH_4/CO_2 gas at the rate of 32 ml/min. Simultaneously, the headspace effluent gas was connected to NOx analyzer (CLD 700EL, EcoPhysics, Michigan) for on-line determination of free NO. Further measurements, sampling and operation were performed as described above.

Analysis of nitrite and protein concentrations

Nitrite and protein concentrations were measured colorimetrically as described previously (Ettwig *et al.*, 2008).

RNA isolation

The total RNA was isolated from the enrichment biomass with the TRIzol reagent (Ambion, Life technologies) following the manufacturer's instructions. The concentration and quality of isolated RNA was assessed with NanoDrop 1000 (Thermo Scientific). After isolation and quantification, the RNA was treated with DNAse I (Sigma Aldrich) following the manufacturer's instructions. After the initial isolation and DNAse I treatment, the RNA was checked with gel electrophoresis. The removal of 16S and 23S ribosomal RNA was performed with the RiboPure™ kit (Ambion, Life technologies). The subsequent removal of 5S ribosomal and transfer RNA was performed with the MEGAClear™ kit (Ambion, Life technologies). After each step, the RNA quality was checked with the Bioanalyzer 2100 (Agilent technologies) following the manufacturer's instructions.

mRNA sequencing and analysis

The rRNA-depleted mRNA was used for sequencing by Ion PGM™ system (Ion Torrent™ platform, Life technologies). In short, the cDNA libraries were prepared by using the Ion Total RNA-Seq Kit v2 (Life technologies) following the manufacturer's instructions. The quality and quantity of cDNA was assessed with the Bioanalyzer 2100. The sequencing of cDNA was performed with the Ion 318™ Chip kit (Life technologies) following the manufacturer's instructions.
Following mRNA samples were sequenced: T0, before NO exposure (reference transcriptome), T4 (315 min after the start of NO exposure), T5 (1578 min after the start of NO exposure) and T8 (2923 min after the start of NO exposure). T5 and T8 transcriptomes were sequenced from two biological replicated. All sequence data were cross-assembled with CLC Genomics Workbench 6.5 and analyzed with crAss v1.3 online tool (http://edwards.sdsu.edu/crass/) for identification of assembled reads in contigs. Blast 2.2.27+ was used for annotation of assembled contigs against non-redundant protein database (version from 07/2014) with an e-value cutoff of 1E-5. Analysis revealed no significant difference between T5 and T8 transcriptomes. Thus, final data interpretation was performed for T0, T4 and T5 data points.

RESULTS & DISCUSSION

Analysis of metabolite concentrations

After the start of continuous supply of NO solution to the reactor, an immediate non-linear increase of free NO in reactor headspace was observed (Fig. 2, experiment with closed headspace). Simultaneously with NO, also concentrations of N_2O and NO_2^- increased. Both compounds must have originated from abiotic reactions occurring while saturation of N-compound-free mineral medium with NO. This saturated solution was eventually pumped into the reactor and it cannot be excluded that parallel to biotic processes some abiotic reactions of NO with mineral medium compounds were still ongoing inside the reactor. The non-linear increase of free NO concentrations was probably caused by reactions of NO with biomass and medium, where after the initial saturation the increase began to be linear. In the batch mode (317 min after the start of the NO exposure), net nitrite and NO consumption by the culture were observed. However, the methane oxidizing activity was inhibited during the entire phase when free NO in the liquid phase was higher than 0.7 µM. Below

0.7µM NO, methane oxidizing and nitrite reducing activities started to recover. During this "active" phase of the exposure experiment, approximately between 1250 and 2628 min, calculated methane consumption activity was 2 nmol CH_4*min^{-1}*mg $protein^{-1}$. The nitrite reducing activity was approximately 2.8 nmol NO_2^-*min^{-1}*mg $protein^{-1}$, which was 52% of the theoretical activity based on calculated methane consumption activity and assumed theoretical stoichiometry of 8:3 (nitrite:methane). The net nitrous oxide gas production activity was 0.26 nmol N_2O*min^{-1}*mg $protein^{-1}$ and the N_2 production activity 2.6 nmol N_2*min^{-1}*mg $protein^{-1}$. Overall, the N_2 production activity matched well with measured CH_4 consumption activity calculated from the 4:3 stoichiometry. During this phase, free NO was still observed in headspace of the culture and was consumed simultaneously with nitrite.

At the point when the nitrite and NO were not measurable anymore, after 2427 min, methane oxidation activity declined and net nitrous oxide consumption was observed at a rate of 0.27 nmol N_2O*min^{-1}*mg $protein^{-1}$. This indicated that methane oxidation in the culture was coupled to availability of nitrite and/or nitric oxide, but not to nitrous oxide reduction. This observation would confirm the hypothesized metabolism of *M. oxyfera* in which nitrous oxide is not a reaction intermediate. Nitrous oxide was probably consumed by other denitrifying community members. In order to investigate whether the observed activity trends were caused by biological or abiotic reactions, an abiotic experiment (with open headspace) was performed with the same set up but without biomass. From the beginning of the NO addition an immediate increase of free NO was observed (data not shown). During the NO supply phase, also an increase in N_2O (approximately 50% of the NO increase slope) and NO_2^- was observed, indicating an abiotic source for both compounds.

During batch phase, concentrations of all N-compounds except unlabeled N_2 remained constant indicating the purely biotic origin of all activities in the biotic experiments. Slow increase in N_2 concentrations was most likely due to air diffusion into the reactor headspace. Abiotic nitrous oxide production has been reported previously and occurs during the process of chemodenitrification when reduced metals act as electron donors for NO reduction to nitrous oxide or dinitrogen (Chalk & Smith, 1983, Parkes *et al.*, 2007). These abiotic reactions are not well investigated and their extent is not known, but would be dependent on the availability of reduced metals and pH. Since culture media contain various trace metals, this reactions are likely to take place. Thus, in culture experiments, the origin of N_2O might be difficult to trace since several biotic and abiotic processes take place simultaneously.

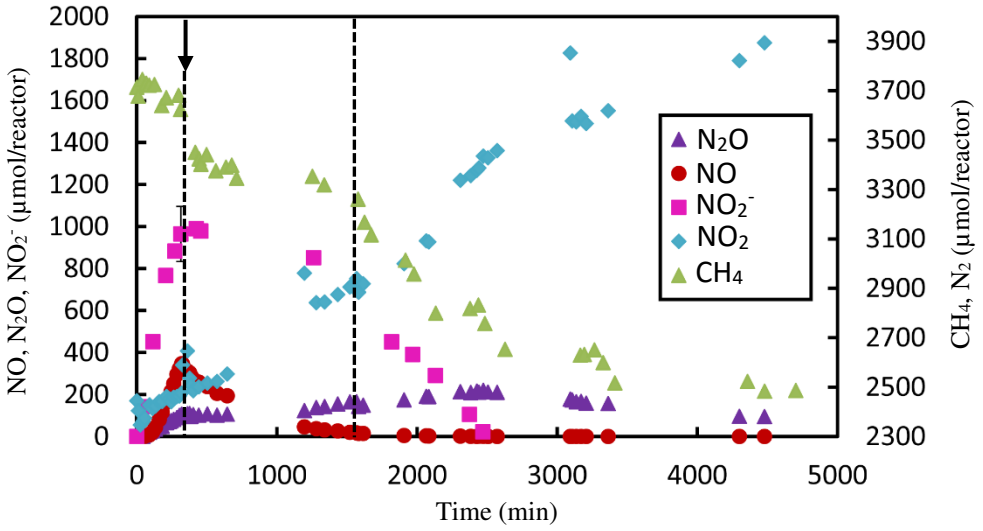

Figure 2: Absolute amounts (µmol) of N-compounds throughout the nitric oxide (NO) exposure experiment of *Methylomirabilis oxyfera* enrichment culture. The data from an experiment with closed headspace are shown. After the supply of nitric oxide was stopped (indicated with arrow), the experiment was continued in batch mode. Dotted lines indicate time points at which culture transcriptome was analyzed.

Transcriptome analysis

The transcriptomic analysis of the culture revealed the presence of a microdiverse population of *M. oxyfera* bacteria in the enrichment culture (data not shown). Thus, mapping to the previously published genome appeared to be a not optimal approach for transcriptome analysis. Instead, an alternative approach was applied. Here, all transcriptome data was combined in a single dataset which was then used for a *de novo* assembly. Assembled and assigned contigs were then used as mapping template for individual transcriptomes.

It also should be noted that the enrichment culture consisted of flocks which create concentration gradients. Thus, depending on the location of individual cells and reaction kinetics of nitric oxide, they might have been exposed to different concentrations of nitric oxide, which in turn could have caused different metabolic responses. Therefore, obtained data represent an “average” metabolic response of different strains of *M. oxyfera* present in the reactor and cells located at different locations inside the flocks.

Metabolite measurements revealed a severe inhibition of methane oxidizing activity of enrichment culture by added NO (batch phase between 317 min and 1579 min). Transcriptome analysis was performed for the time points T4 (315 min after the start of NO exposure, NO concentration at sampling time was 14 µM in liquid) and T5 (1578 min after the start of NO exposure, NO concentration at sampling time was 0.6 µM in liquid). Results revealed an overall trend towards downregulation of most enzymes involved in central metabolic processes, however, for some a clear trend could not be observed since some parts of the same gene assembled in separate contigs showed partial up- and downregulation (Tab. 1). At T4, when free NO concentration was highest, among the genes involved in nitrogen transformations downregulation was observed for *nirJ*, encoding for heme *d1* biosynthesis protein involved in nitrite reductase synthesis and *nirS*, encoding cytochrome *cd1* nitrite reductase. Among two putative Nod enzymes, most contigs with decreased expression values affected one of the paralogues, Nod2. Though not being involved in central energy metabolism, also the expression of hydroxylamine oxidoreductase (Hao) was diminished. At time point T5, the expression results were partially not consistent for the same gene. Here, contig_14512 covering the 100-154 AA region on Nap showed an over 7 fold upregulation and contig_15191 covering the 748-788 AA region an over 3 fold downregulation of the same gene. Similar observation was made for the Nar-encoding genes. However, contig_530 covering the full NarG encoding gene showed slight overexpression pointing to an increased nitrate-utilizing activity of *M. oxyfera*. At T5, most Nir encoding genes were downregulated and only *lrp* gene, encoding transcriptional regulator of AsnC family, showed an over 7 fold overexpression for the AA region of 249-280. Overall expression of nitrite reductase encoding genes, in particular NirS, was also downregulated. As nitrite reductase encoding genes are located on the same operon as NapA, this might point to an overall reduced expression of both enzyme systems. Both Nod encoding genes showed an overall trend towards downregulation, except contig_9017 covering the Nod1 AA region of 741-803 with an over 7 fold overexpression. Thus, a clear trend towards a differential expression of the two Nod paralogues could not be concluded. At T5, the conventional qNor showed an upregulation and Hao was downregulated.

For particulate methane monooxygenase encoding genes (*pmoCAB*), it was possible to assemble contigs covering full genes, with contig_1681 covering both *pmoA* and *pmoB*, and contig_655 covering *pmoC*. Also, most *M. oxyfera*-specific *pmo* coding reads could be mapped to fully assembled contigs. At T4, no significant expression

changes could be observed, however, clear downregulation of all *pmo* genes occurred at T5 pointing to a diminished methane oxidizing capacity of the enrichment culture.

Methanol dehydrogenase (MDH) is present in the *M. oxyfera* genome in two different forms: pyrroloquinolone (PQQ)-dependent MxaF1 and XoxF form. The latter is present in two forms, XoxF1 and XoxF2 (Wu *et al.*, 2015). Protein purification and enzyme activity studies have shown that under standard culturing conditions *M. oxyfera* functionally expresses the XoxF1 form (Wu *et al.*, 2015). Interestingly, at T4, an upregulation was observed for MxaF1 and XoxF2 encoding genes, while no significant transcription change occurred for XoxF1 encoding genes. At T5, however, the only transcribed MDH form was XoxF2, and no transcription was observed for either MxaF1 or XoxF1. This points to differential expression of different MDH homologues at different stages of physiological changes induced by nitrosative stress.

The MxaF1 MDH has been well characterizes in the past, it contains PQQ as prosthetic group and Ca^{2+} as a cofactor (Anthony & Williams, 2003), and is present in a variety of methano- and methylotrophic bacteria (McDonald & Murrell, 1997). XoxF proteins, however, share less than 50% sequence similarity to known MxaF (Kalyuzhnaya *et al.*, 2008, Keltjens *et al.*, 2014, Wu *et al.*, 2015). The genes encoding XoxF proteins were shown to be widespread in natural systems and even to be present in organisms not affiliated with methylotrophic lifestyle (Kalyuzhnaya *et al.*, 2008, Taubert *et al.*, 2015). Phylogenetic analysis revealed a division of XoxF in 5 families many of which are dependent on rare earth elements of the lanthanide-group instead of Ca^{2+} as a cofactor (Keltjens *et al.*, 2014, Pol *et al.*, 2014). Interestingly, so far group XoxF2 has only been detected in acidophilic methanotrophic *Verrucomicrobia* and *M. oxyfera* (Pol *et al.*, 2014, Wu *et al.*, 2015). Regarding the catalytic mechanism of methanol oxidation, some evidence suggests the higher substrate affinity and reaction speed of XoxF in comparison to MxaF (Keltjens *et al.*, 2014). Also, XoxF2 from *Methylacidiphilum fumariolocum* SolV was shown to oxidize methanol directly to formate bypassing formaldehyde as intermediate (Pol *et al.*, 2014). This feature has been speculated to be related to the use of Calvin Cycle for CO_2 fixation by this organism, while both ribulose-monophosphate and serine cycle require formaldehyde derived from methane (Keltjens *et al.*, 2014).

As discussed earlier, downregulation was observed for Pmo and also for formaldehyde activating enzyme (Fae) encoding genes at the time point T5. Here,

the overregulation of XoxF2 could potentially serve for oxidation of lower concentrations of methanol directly to formate and bypassing formaldehyde as reaction intermediate. Thus, *M. oxyfera* might switch between the expression of different types of MDH depending on substrate availability and stress conditions. However, this hypothesis needs further validation.

At T4, most contigs showing overexpression, besides central nitrogen and methane metabolism, included gene parts encoding histidine kinases, peptidase M50, endonuclease, thioredoxin reductase, ribosomal protein L35, guanylate cyclase, response regulator in two-component system, NADH-quinone oxidoreductase and menaquinol-cytochrome *c* reductase (data not shown). At T5, most overexpression was observed for genes encoding proteins involved in DNA processing including DNA gyrase, DNA mismatch repair protein, DNA polymerase, DNA methyltransferase, several proteins involved in tRNA synthesis, NADH-quinone oxidoreductase, chaperones, enoyl-CoA hydratase, acetylglutamate kinase and two proteins involved in CBB cycle, CbbX and transketolase (data not shown). This indicated that NO mediated stress caused cells to overexpress genes encoding proteins mainly involved in signal transduction, DNA repair and protein synthesis. Remaining annotated genes genes either did not show significant expression changes or were downregulated pointing to an overall nitrosative stress response. Notably, numerous contigs with significant over- and downregulation could not be annotated and manual blast analysis showed significant similarity to non-annotated regions of published *M. oxyfera* genome.

NO is a highly reactive small radical molecule which can diffuse freely into the cell and cause damage by reacting with metals, lipids, proteins and nucleic acids. Proteins with metals in active sites and rich in thiol groups are considered to be damaged the most; this would especially affect the cytochrome proteins (Cooper, 1999). Besides transition metals, also sulfhydryl groups in proteins were shown to be binding targets for NO (Clementi *et al.*, 1998, Cooper, 1999). It has been shown previously that NO mediated inhibition of NADH dehydrogenase in mitochondria is not due to NO binding to iron in the enzyme, but due to reaction with sulfhydryl groups (Clementi *et al.*, 1998).

Global downregulation of genes involved in central metabolic processes indicated severe nitrosative stress of *M. oxyfera* bacteria at micromolar concentration of free NO. Although methane oxidizing and nitrite reducing activity increased after 1579 min, approximately the time point from which the T5 transcriptome originated, more downregulation of central metabolic genes was observed. This might indicate that

the culture responded with a delay to nitrosative stress by ongoing downregulation. Thus, activity recovery could not be deduced for the transcriptomic response of the culture. It should be noted however, that transcriptome does not reflect activity changes which are occurring at the protein level. Moreover, it been shown that elevated NO concentrations (lower micromolar range) can diminish nitric oxide reductase activity in *Paracoccus denitrifcans* (Girsch & de Vries, 1997, Koutný & Kučera, 1999). This was assumed to occur due to NO binding to oxidized enzyme which is considered to be inactive (Girsch & de Vries, 1997). Although there are structural differences between Nor and Nod, similar mechanisms might apply for Nod as well. As reactions of NO with enzyme-bound iron are mostly reversible (Cooper, 1999), drop in inhibitory concentrations of NO can lead to recovery of activity which would be uncoupled to transcriptomic or translational response for NO utilizing proteins. However, this needs to be tested for *M. oxyfera* cells with lower NO concentrations. Furthermore, lower concentrations of free NO might give insight into differential expression of two Nod paralogues in case of different enzyme affinities to NO. Genome analysis of *M. oxyfera* revealed no known detoxification systems such as flavohemoglobin, flavorubredoxin or pentaheme nitrite reductase. Also the kinetics of the putative nitric oxide dismutation are so far unknown, but if this reaction is indeed a limiting step during the nitrogen metabolism of *M. oxyfera*, cells would benefit from highly regulated NO detoxification mechanisms. This function could be taken over by the conventional qNor (CBE68939) which is constitutively expressed. Our previous batch activity studies with elevated partly inhibitory concentrations of nitrite revealed increased production of nitrous oxide, which was up to 50% of $N\text{-}NO_2^-$ (data not shown). This points to N_2O production as a putative detoxification mechanism. However, further validation is needed. Also the trend towards overexpression of qNor at T5 indicated a possible involvement in detoxification. The role of Hao in respect to detoxification still needs further investigation.

Interestingly, physiological results obtained within this study further confirmed observations made by Ettwig et al. 2010 (Supplementary Fig. 5). Experiments performed with ^{15}N-labeled NO pointed to an inhibitory effect of free NO on methane oxidizing activity of *M. oxyfera* enrichment culture, which only recovered after the NO concentration dropped to approximately 1 µM. At the moment when labeled NO was added labeled N_2 and N_2O were produced simultaneously (Ettwig *et al.*, 2010). However, it should be noted that due to chemodenitrification the fraction of N_2O produced by biological activity is not known.

PERSPECTIVES

Our combined results pointed to an overall inhibitory effects of NO at tested micromolar concentrations. Both methane oxidizing and nitrite reducing activity only recovered after free NO concentrations decreased lower than 1 μM. Thus, it would be feasible to perform future NO exposure experiments just below and above the inhibitory threshold in order to investigate associated physiological changes. Moreover, a continuous chemostat set up would provide an advantage to investigate physiological and transcriptomic changes caused exclusively by nitric oxide. As there is still a possibility of a delayed transcriptomic response of the culture to changing external parameters, an experiment of longer duration could potentially provide a clear picture of different transcriptomic regulation patterns. Moreover, the role of hydroxylamine oxidoreductase and nitrate reductase still need to be investigated. Also, differential expression of different methanol dehydrogenases might point to metabolic flexibility of *M. oxyfera* at changing methanol availability. Thus, the physiological response analysis to other N-compounds such as ammonium, hydroxylamine, elevated concentrations of nitrate and nitrite and methanol would provide a better understanding of *M. oxyfera* physiology and thus its role in different environments.

ACKNOWLEDGMENTS

We would like to thanks Arjan Pol and Boran Kartal for technical advice and support, and Theo van Alen for help with sequencing. O.R. and M.S.M.J. were supported by the ERC (grant no. 2322937), K.F.E. by the Darwin Center for Biogeosciences (projects 142.16.3071 and 142.16.3072)

Table 1: Transcription values for central nitrogen and methane metabolic genes cross-assembled in contigs from *M. oxyfera* enrichment culture exposed to free nitric oxide. Comparison of T4 and T5 transcriptoms relative to T0 is shown as fold change values. Overexpression was defined for values higher than 1 (marked in green) and downregulation for values lower than -1 (marked in red).

Gene description	**Gene name**	**Contig identifier**	**GenBank Accession (CBE)**	**Protein length (AA)**	**Protein sequence coverage range[a]**	**% identity[b]**	**Fraction[c]**			**Fold change[d]**	
							T0	**T4**	**T5**	**T4/T0**	**T5/T0**
Periplasmic nitrate reductase	*napA*	contig_14512	69459	788	100-154	73	7,84E-06	5,28E-06	7,55E-05	-0,10	7,70
	napA	contig_8007	69459	788	200-625	85-91	2,65E-04	2,44E-04	8,30E-04	0,15	-0,61
	napA	contig_15191	69459	788	748-788	95	2,49E-05	2,66E-05	4,39E-05	1,15	-3,21
Membrane-bound nitrate reductase	*narI*	contig_1955	67839	227	45-95	94	2,44E-05	1,32E-05	4,34E-05	-0,56	2,48
	narG	contig_530	67843	1216	1-1216	77-98	4,93E-06	3,36E-06	6,31E-06	-0,12	1,15
	narJ	contig_2833	67840	299	1-295	66-80	8,96E-07	9,60E-07	6,12E-07	0,10	0,40
	narI	contig_676	67839	227	102-227	89-91	2,51E-05	1,49E-05	1,05E-05	-0,88	0,28
	narH	contig_8004	67841	509	295-371	84	1,48E-05	7,44E-06	4,12E-06	-0,54	-0,34
	narH	contig_8782	67841	509	254-301	83-96	1,34E-06	1,44E-06	0,00E+00	0,09	-1,29
	narH	contig_3365	67841	509	409-491	77	2,24E-06	1,68E-06	0,00E+00	-0,75	-1,69
	narG	contig_12321	67843	1216	547-576	100	7,84E-06	5,28E-06	7,55E-05	-0,98	-2,47

	narG	contig_7894	67843	1216	574-618	77	2,65E-04	2,44E-04	8,30E-04	0,09	-3,85
	narJ	contig_10847	67840	299	144-178	97	2,49E-05	2,66E-05	4,39E-05	-0,39	-4,55
Dissimila-tory nitrite reductase	*lrp*	contig_8819	69457	384	249-280	94	2,24E-07	2,40E-07	7,77E-05	0,07	7,91
	lrp	contig_4659	69457	384	3-251	83	8,56E-05	7,20E-05	8,42E-05	-0,25	-0,02
	nirJ	contig_6034	69461	406	14-116	77	4,03E-06	1,68E-06	3,16E-06	-1,21	-0,34
	nirS	contig_4747	69462	546	120-477	85-99	6,34E-05	2,88E-05	4,15E-05	-1,13	-0,61
	nirF	contig_2787	69460	409	159-405	88	1,48E-05	7,44E-06	6,81E-06	-0,98	-1,11
	nirS	contig_6641	69462	546	51-121	99	9,85E-06	3,84E-06	3,65E-06	-1,34	-1,41
	nirS	contig_16564	69462	546	483-535	91	1,70E-05	1,46E-05	4,83E-06	-0,22	-1,80
	nirS	contig_12241	69462	546	501-546	87-94	1,57E-06	3,36E-06	3,06E-07	1,05	-2,04
	nirF	contig_5491	69460	409	31-158	91-92	4,03E-06	1,92E-06	6,12E-07	-1,03	-2,54
	nirJ	contig_12484	69461	406	213-378	84	6,94E-06	2,40E-06	9,18E-07	-1,49	-2,79
Putative nitric oxide dismutase	*nod1*	contig_9017	69496	808	741-803	53	6,05E-06	1,15E-05	2,07E-04	0,92	5,07
	nod1	contig_3194	69496	808	11-319	93	4,10E-05	2,40E-05	3,61E-05	-0,77	-0,18
	nod1	contig_18979	69496	808	499-530	75	8,96E-07	2,16E-06	6,59E-07	1,18	-0,39
	nod2	contig_79	69502	810	1-810	92-98	2,57E-03	1,53E-03	1,74E-03	-0,75	-0,56

nod2	contig_15007	69502	810	438-509	99	2,46E-06	2,16E-06	1,62E-06	-0,18	-0,57
nod1	contig_11813	69496	808	322-357	81	1,57E-06	1,44E-06	9,18E-07	-0,11	-0,71
nod2	contig_9390	69502	810	268-307	80	6,94E-06	8,88E-06	3,77E-06	0,35	-0,86
nod2	contig_492	69502	810	701-753	98	2,02E-06	2,40E-07	1,01E-06	-2,64	-0,93
nod2	contig_11728	69502	810	502-545	95	2,24E-06	2,16E-06	9,65E-07	-0,05	-1,13
nod2	contig_11713	69502	810	225-281	97-100	8,96E-07	0,00E+00	3,53E-07	-3,32	-1,14
nod2	contig_493	69502	810	723-777	96	1,79E-06	7,20E-07	7,07E-07	-1,21	-1,23
nod1	contig_717	69496	808	593-808	91-96	3,15E-04	2,06E-04	1,22E-04	-0,61	-1,37
nod2	contig_748	69502	810	112-137	92	2,75E-05	1,39E-05	1,00E-05	-0,98	-1,45
nod1	contig_8693	69496	808	371-420	96	2,46E-06	4,80E-07	6,12E-07	-2,14	-1,85
nod2	contig_9890	69502	810	13-214	77	6,94E-06	5,52E-06	1,27E-06	-0,33	-2,36
nod2	contig_6703	69502	810	30-82	92	4,48E-07	0,00E+00	0,00E+00	-2,45	-2,45
nod2	contig_14326	69502	810	239-278	95	8,96E-07	0,00E+00	0,00E+00	-3,32	-3,32
nod1	contig_12211	69496	808	364-445	89	8,96E-07	4,80E-07	0,00E+00	-0,78	-3,32
nod1	contig_11676	69496	808	741-784	84	8,96E-07	7,20E-07	0,00E+00	-0,28	-3,32
nod2	contig_10780	69502	810	1-31	81	1,55E-05	6,96E-06	1,37E-06	-1,14	-3,41

	nod2	contig_11312	69502	810	70-216	95	5,15E-06	1,20E-06	3,53E-07	-2,01	-3,53
	nod1	contig_20579	69496	808	72-122	69	1,12E-06	2,40E-07	0,00E+00	-1,84	-3,61
	nod2	contig_16015	69502	810	321-358	100	1,12E-06	7,20E-07	0,00E+00	-0,57	-3,61
	nod2	contig_19168	69502	810	616-661	96	1,79E-06	2,40E-07	0,00E+00	-2,48	-4,24
Nitric oxide reductase	*qnor*	contig_17166	68939	730	584-661	90-100	2,89E-05	2,57E-05	2,75E-04	-0,17	3,24
	qnor	contig_10582	68939	730	661-725	94	1,34E-06	1,20E-06	1,32E-06	-0,15	-0,03
	qnor	contig_1849	68939	730	186-302	80-83	8,96E-07	0,00E+00	6,12E-07	-3,32	-0,48
	qnor	contig_16072	68939	730	81-131	76	1,12E-06	7,20E-07	6,59E-07	-0,57	-0,68
Hydroxyl-amine oxido-reductase	*hao*	contig_9987	69546	462	46-125	89	2,02E-06	1,20E-06	1,27E-06	-0,70	-0,63
	hao	contig_2004	69546	462	299-455	91	8,49E-05	4,87E-05	3,81E-05	-0,80	-1,15
	hao	contig_10028	69546	462	120-231	92	6,72E-06	3,12E-06	0,00E+00	-1,08	-6,09
	hao	contig_11741	69546	462	199-297	91	8,96E-07	7,20E-07	1,62E-06	-0,28	0,79
Methane metabolism											
Methane monooxy-genase	*pmoC*	contig_10135	69521	258	1-24	100	8,96E-07	0,00E+00	0,00E+00	-3,32	-3,32
	pmoC	contig_10803	69521	258	1-122	86	3,14E-06	1,44E-06	6,59E-07	-1,07	-2,09
	pmoC	contig_13085	69521	258	212-252	98	2,24E-07	7,20E-07	7,07E-07	1,34	1,32

	pmoA	contig_14662	69517	422	312-349	87-100	8,96E-07	7,20E-07	0,00E+00	-0,28	-3,32
	pmoC	contig_15629	69521	258	147-194	94	4,70E-06	5,04E-06	0,00E+00	0,10	-5,59
	pmoA *pmoB*	contig_1681	69517 69519	422 243	*pmoA:* 1-422 *pmoB*: 1-243	93 93-95	2,30E-04	3,04E-04	7,66E-05	0,40	-1,58
	pmoA	contig_18155	69517	422	99-138	98	0,00E+00	2,40E-07	0,00E+00	1,77	0,00
	pmoB	contig_20036	69519	243	194-240	85	1,12E-06	7,20E-07	0,00E+00	-0,57	-3,61
	pmoA	contig_20740	69517	422	36-66	91	4,48E-07	4,80E-07	0,00E+00	0,08	-2,45
	pmoA	contig_5442	69517	422	260-334	95	7,39E-06	6,96E-06	1,30E-05	-0,09	0,81
	pmoC	contig_655	69521	258	1-265	78-93	5,68E-04	4,19E-04	3,10E-04	-0,44	-0,87
	pmoA	contig_7562	69517	422	375-422	98	1,57E-06	1,68E-06	3,06E-07	0,09	-2,04
Methanol dehydro-genase	*xoxF1*	contig_17727	67239	634	534-593	98	4,48E-07	2,40E-07	0,00E+00	-0,69	-2,45
	mxaF	contig_18750	67228	601	344-401	82-100	1,34E-06	4,80E-06	0,00E+00	1,76	-3,85
	mxaF	contig_19291	67228	601	576-601	96	2,24E-07	9,60E-07	0,00E+00	1,71	-1,70
	xoxF2	contig_19484	67248	613	588-613	92	4,48E-07	4,80E-07	3,06E-07	0,08	-0,43
	mxaF	contig_19977	67228	601	278-339	97	1,57E-06	9,60E-07	0,00E+00	-0,65	-4,06
	xoxF1	contig_20618	67239	634	329-391	94-100	6,72E-07	4,80E-07	0,00E+00	-0,41	-2,95

	xoxF2	contig_6156	67248	613	513-545	94	4,48E-07	3,60E-06	8,55E-06	2,76	3,98
	xoxF2	contig_7400	67248	613	546-606	93	7,17E-06	9,36E-06	6,88E-06	0,38	-0,06
	xoxF2	contig_7537	67248	613	438-473	81	2,24E-07	1,44E-06	1,62E-06	2,25	2,41
5,10-methenyl-tetrahydro-folate cyclo-hydrolase; Methylene tetrahydro-methano-pterin dehydro-genase; Formal-dehyde activating enzyme	*folD*	contig_14471	68902	288	189-244	82	2,24E-07	4,80E-07	6,12E-07	0,84	1,14
	fae *mtdB*	contig_1682	67531 67532	166 294	*Fae*: 1-166 *MtdB*: 1-288	98 92	9,68E-05	8,64E-05	1,51E-04	-0,16	0,64
	folD	contig_4820	68902	288	95-196	90	4,26E-06	2,16E-06	4,74E-05	-0,95	3,45
	folD	contig_6595	68902	288	18-104	84-92	2,46E-06	1,20E-06	2,28E-06	-0,98	-0,10
	folD	contig_8720	68902	288	246-282	89	2,46E-06	5,04E-06	4,17E-06	1,00	0,74
	fae	contig_15307	67531	166	12-74	95	4,48E-07	0,00E+00	3,53E-07	-2,45	-0,27
	fae	contig_18178	67531	166	82-129	100	5,53E-05	3,82E-05	2,26E-05	-0,53	-1,29
	fae	contig_19314	67531	166	132-166	91	1,12E-06	7,20E-07	0,00E+00	-0,57	-3,61
Formyl-transferase/ hydrolase; Methenyl-tetrahydro-methano-pterin cyclo-hydrolase	*fhcD*	contig_10086	67536	304	27-81	78	0,00E+00	2,40E-07	3,53E-07	1,77	2,18
	fhcB	contig_10888	67534	451	254-330	62	6,72E-07	4,80E-07	6,59E-07	-0,41	-0,02
	fhcA	contig_11321	67535	548	382-471	91	1,34E-06	7,20E-07	3,06E-07	-0,82	-1,83
	fhcB	contig_11887	67534	451	105-157	68	2,69E-06	7,20E-07	3,06E-07	-1,77	-2,78

	fhcB	contig_16477	67534	451	17-105	70	1,05E-05	1,20E-05	2,03E-06	0,19	-2,32
	fhcC	contig_17513	67537	269	163-222	80-100	2,24E-07	4,80E-07	0,00E+00	0,84	-1,70
	fhcB	contig_18161	67534	451	375-432	72	1,57E-06	1,92E-06	0,00E+00	0,28	-4,06
	fhcA	contig_314	67535	548	1-548	92	1,61E-04	1,11E-04	1,28E-04	-0,53	-0,33
	fhcB		67534	451	1-439	94-96					
	mch	contig_2501	67538	312	1-304	81-93	5,58E-05	5,57E-05	5,96E-05	0,00	0,10
	fhcC		67537	269	1-269	86					
Formyl-tetrahydro-folate deformy-lase	*purU*	contig_12220	69659	286	96-150	76-83	4,48E-07	0,00E+00	3,06E-07	-2,45	-0,43
	purU	contig_13724	69659	286	195-274	91	1,41E-05	1,20E-05	1,51E-05	-0,23	0,10
	purU	contig_3881	69659	286	41-84	82-92	4,48E-07	1,20E-06	1,22E-06	1,25	1,27
Formate dehydro-genase	*fdhI*	contig_13529	67886	715	389-446	81	2,24E-07	0,00E+00	3,53E-07	-1,70	0,48
	fdhA2	contig_13903	67914	899	74-173	82	3,14E-06	3,60E-06	7,01E-05	0,19	4,44
	fdhI	contig_13910	67886	715	659-714	70	1,34E-06	2,40E-07	0,00E+00	-2,09	-3,85
	fdhA2	contig_14646	67914	899	188-243	83-88	1,34E-06	4,80E-07	0,00E+00	-1,32	-3,85
	fdhA2	contig_1845	67914	899	262-570	60-81	6,94E-06	4,80E-06	2,64E-06	-0,52	-1,36
	fdhI	contig_2576	67886	715	25-123	56	1,79E-06	9,60E-07	2,59E-06	-0,84	0,51
	fdhI	contig_3337	67886	715	148-257	79	2,69E-06	1,92E-06	2,19E-06	-0,46	-0,28
	fdhI	contig_3383	67886	715	542-657	88	4,26E-06	5,52E-06	4,73E-06	0,37	0,15

fdhA2	contig_4983	67914	899	1-40	80	2,36E-03	3,09E-03	2,20E-04	0,39	-3,43
fdhD	contig_7207	68661	269	61-176	46	4,70E-06	2,64E-06	3,56E-06	-0,81	-0,39
fdhA2	contig_8959	67914	899	849-899	82	2,44E-05	3,12E-05	1,49E-05	0,35	-0,71
fdhA2	contig_9316	67914	899	596-847	83-85	9,18E-06	5,76E-06	2,03E-06	-0,66	-2,13
fdhD	contig_9455	68661	269	160-220	97	3,14E-06	1,92E-06	1,62E-06	-0,68	-0,91
fdhD	contig_9851	68661	269	184-217	91	3,14E-06	2,64E-06	3,06E-07	-0,24	-2,99

[a] protein coverage range of translated amino acid sequence from gene assembled in respective contig
[b] % identity on amino acid level to protein sequence of *M. oxyfera* in GenBank
[c] mapped read fraction normalized to total transcriptome size
[d] logarithmic fold change in T4 and T5 transcriptomes in relation to T0 reference point

chapter 5

Iron-dependent anaerobic oxidation of methane in Bothnian Sea sediments

Previously published as:

Matthias Egger, Olivia Rasigraf, Célia J. Sapart, Tom Jilbert, Mike S. M. Jetten, Thomas Röckmann, Carina van der Veen, Narcisa Bândă, Boran Kartal, Katharina F. Ettwig, and Caroline P. Slomp (2014) Iron-Mediated Anaerobic Oxidation of Methane in Brackish Coastal Sediments. Environmental Science & Technology **49**(1): 277-283.

Submitted:

Olivia Rasigraf, Matthias Egger, Katharina F. Ettwig, Mike S.M Jetten, Caroline P. Slomp, Claudia Lüke (2015) Metagenomic analysis of iron-dependent anaerobic oxidation of methane in Bothnian Sea sediments: a playground for various microbial guilds.

ABSTRACT

The Bothnian Sea is an oligotrophic, low-salinity brackish basin located in the northern part of the Baltic Sea. Its sediments are characterized by high amounts of reactive iron below a shallow sulfate-methane transition zone (SMTZ). Previous geochemical studies suggested that methane produced in these sediments is consumed by sulfate-dependent anaerobic oxidation of methane (S-AOM) within, and iron-dependent AOM (Fe-AOM) below the SMTZ. In this study, we analyzed the potential of the *in situ* sediment microbial community from Bothnian Sea for iron-dependent anaerobic methane oxidation with ferrihydrite and ^{13}C-labeled methane additions and sequenced total DNA from 3 subsequent depths and active incubation slurries. The microbial community was analyzed in relation to the methane cycle. Based on 16S rRNA and functional key genes (*mcrA* and *pmoA/mmoX)* involved in methane transformations, we identified an abundant aerobic type I methanotrophic community at the oxic/anoxic interface (0-2.5 cm below seafloor, cmbsf) with *Methylobacter* as the dominant member. In this surface layer, obvious anaerobic methane cycling processes were absent. In the S-AOM zone (5-12.5 cmbsf), we were able to retrieve anaerobic methanotrophic archaea (ANME) sequences which belonged to the ANME-2a cluster accompanied by abundant sulfate reducing bacteria from the order *Desulfobacterales*. The ANME archaea were also present in the methanic/Fe-AOM zone below the SMTZ (30-35 cmbsf), and became even more abundant in Fe-AOM incubation slurries. Methanogens were present in both S-AOM and methanic/Fe-AOM zones with *Methanosarcina* and *Methanoregula* as dominant genera. Putatively fermentative *Clostridia* and *δ*-Proteobacteria from the order *Desulfuromonadales (Geobacteraceae)* also increased in numbers possibly indicating an indirect mechanism of iron and methane transformation processes in the slurries. Moreover, genes encoding for oxidized sulfur species reduction processes (thiosulfate and sulfite reductase) were also enriched in incubation slurries pointing to an operational cryptic sulfur cycle. Altogether, our results suggest that Fe-AOM in Bothnian Sea sediments is most likely driven by an interplay of several metabolic processes involving methanotrophic/-genic archaea, iron reducers, sulfate reducers and putatively fermentative *Clostridia*.

INTRODUCTION

Biological methane oxidation has been discovered more than a century ago (Söhngen, 1906) and since then extensive research has advanced our knowledge about mechanisms of methane oxidation in the presence of oxygen. The reaction is initiated by a methane monooxygenase (Mmo) which utilizes the potent chemistry of oxygen radicals for the transformation of the highly stable methane molecule into methanol (Hakemian & Rosenzweig, 2007). Further reactions involving various dehydrogenases ultimately lead to carbon dioxide (CO_2) as the end product (Hanson & Hanson, 1996). So far, all known organisms capable of this process belonged exclusively to bacteria within the phyla of *Proteobacteria* (α- and γ-) and *Verrucomicrobia* (Hanson & Hanson, 1996, Op den Camp *et al.*, 2009, van Teeseling *et al.*, 2014)

In contrast, biological methane production is a strictly anaerobic process typically occurring in deep sediments where organic matter is degraded to acetate and hydrogen/CO_2, which in the absence of electron acceptors other than CO_2 are converted to methane by acetoclastic and hydrogenotrophic methanogenic archaea. Via diffusion towards the sediment surface, methane passes through zones of alternative electron acceptors, ultimately reaching the oxic layers or the overlying water column. Thus, anaerobic methane oxidation with alternative electron acceptors has been subject of research in the past decades. Among these, sulfate was shown to play a crucial role as electron acceptor for methane oxidation in marine sediments (Knittel & Boetius, 2009) where its constant supply from overlying water leads to the formation of a sulfate methane transition zone (SMTZ) due to sulfate-dependent methane oxidation (S-AOM).

S-AOM is performed by specialized groups of anaerobic methanotrophic archaea (ANME) and associated sulfate-reducing bacteria (SRB) (Knittel & Boetius, 2009) and has been recognized as a major sink for methane in marine environments where it can attenuate up to 90% of methane diffusing from the underlying methanic zone (Reeburgh, 2007). Based on 16S rRNA gene phylogeny, known ANME archaea were classified into 3 non-monophyletic clusters related either to *Methanosarcinales* (ANME 2 and 3) or *Methanomicrobiales* (ANME 1) (Knittel & Boetius, 2009). Associated SRB partners of ANME-1/ANME-2 clades were shown to belong to *Desulfosarcina/Desulfococcus* and those of ANME-3 clade to *Desulfobulbus* clusters (Knittel & Boetius, 2009).

ANME archaea are related to methanogens and employ the methyl coenzyme M reductase (Mcr) enzyme in reverse for methane activation (Krüger *et al.*, 2003). Early research on S-AOM hypothesized a syntrophic partnership between ANME and SRB, in which the SRB would scavenge reducing equivalents from ANME, thus shifting the thermodynamics of the overall process towards a more exergonic state (Hoehler *et al.*, 1994). The nature of exchanged reducing equivalents remains elusive to date. Moreover, ANME archaea have also been reported to thrive in the absence of SRB pointing to the ability of certain ANME groups to perform S-AOM on their own (Orphan *et al.*, 2002, Treude *et al.*, 2007). A recent study investigating the nature of exchanged metabolites between an ANME-2 clade and associated SRB suggested the ability of archaea to reduce sulfate to sulfur, which after a reaction with sulfide to polysulfide was disproportionated by SRB to sulfide and sulfate (Milucka *et al.*, 2012). However, it remains to be investigated whether all ANME archaea are able to perform sulfate reduction and how ubiquitous this particular interaction is in nature.

Distinct clades of ANME archaea were shown to possess different environmental niches mainly defined by temperature and availability of methane (Nauhaus *et al.*, 2005). The same study investigated the potential for anaerobic oxidation of methane with alternative electron acceptors including metal oxides, nitrate and sulfur. All were reduced (at lower rates than sulfate), but the processes could not directly be linked to methane oxidation (Nauhaus *et al.*, 2005). Another study, however, found that ANME-2 archaea/SRB consortia originating from the same environment – Hydrate Ridge sediments in the Pacific Ocean– were stimulated in their sulfate-reducing activity by iron oxides (Sivan *et al.*, 2014). Based on isotope fractionation signatures of sulfate, it was proposed that the natural sulfate reduction was enhanced, but not altered in its mechanism (Sivan *et al.*, 2014). The involvement of iron oxides in biological S reduction has been shown previously. *Sulfurospirillum deleyianum*, a sulfur- and thiosulfate-reducing bacterium, was shown to grow in the presence of ferrihydrite, where sulfide was re-oxidized by ferric iron to mixed forms of oxidized sulfur species used by the organism (Straub & Schink, 2004, Lohmayer *et al.*, 2014). A similar mechanism was proposed to take place in iron-rich marine sediments in which S-AOM was shown to occur (Holmkvist *et al.*, 2011). In this process, a cryptic sulfur cycle is generated below the SMTZ where sulfide diffuses downward and is re-oxidized to intermediate oxidized sulfur species by *in-situ* iron oxides. Further transformation to sulfate via disproportionation reactions was also speculated to

occur, thus sustaining an *in-situ* population of active sulfate- and sulfur-reducing organisms (Lohmayer *et al.*, 2014).

The early research on AOM focused on sulfate as an electron acceptor due to its high concentration in seawater and marine sediment. However, AOM with electron acceptors other than sulfate would in theory provide sufficient energy to sustain microbial metabolism. The first described enrichment culture capable of AOM in the presence of nitrate and nitrite (N-AOM) was hypothesized to utilize a mechanism similar to that of S-AOM – a syntrophic consortium of archaea and bacteria in which bacteria would scavenge reducing equivalents for reduction of nitrogen oxides (Raghoebarsing *et al.*, 2006). Recent studies have shown that bacterial and archaeal members of the consortium can oxidize methane without each other, both possessing their own independent pathways for the process. The bacterial member, *Methylomirabilis oxyfera*, utilizes nitrite which after the reduction to NO was hypothesized to generate intra-cellular oxygen via a dismutation reaction (Ettwig *et al.*, 2010, Ettwig *et al.*, 2012). *M. oxyfera* possesses the full pathway for aerobic methane oxidation, in which methane monooxygenase would use the internally produced oxygen to oxidize methane to methanol (Wu *et al.*, 2011). In contrast, the archaeal member, *Methanoperedens nitroreducens*, reduces nitrate to nitrite by oxidizing methane via reverse methanogenesis with Mcr as the key enzyme (Haroon *et al.*, 2013).

The first indication for the use of oxidized metal species as electron acceptors for AOM (M-AOM) came from marine sediment incubations (Beal *et al.*, 2009). Since then, several studies of various terrestrial and marine ecosystems reported metal-dependent AOM activity (Crowe *et al.*, 2011, Sivan *et al.*, 2011, Amos *et al.*, 2012, Norði *et al.*, 2013, Segarra *et al.*, 2013, Egger *et al.*, 2015), however, so far no organisms or possible pathways could be deduced. Thus, the significance and mechanism of this process in the environment remains largely unknown. In view of the ubiquitous occurrence of iron and manganese oxides in both freshwater and marine sediments, a better understanding of the underlying mechanisms of observed M-AOM activity would greatly enhance our knowledge of sedimentary metal- and methane cycles.

Previous work indicated the potential of iron-rich Bothnian Sea sediments for M-AOM (Slomp *et al.*, 2013). Though being part of the Baltic Sea – a sea suffering from high nutrient loading and widespread bottom water anoxia – the Bothnian Sea has been impacted by eutrophication to a far lesser degree (Lundberg *et al.*, 2009). However, mild eutrophication during the last decade has led to a shift of a narrow

SMTZ towards the sediment surface below which abundant iron oxides coincide with high methane concentrations (Slomp *et al.*, 2013, Egger *et al.*, 2015). Here, we investigated the potential of iron- and methane-rich Bothnian Sea sediment for iron-dependent anaerobic methane oxidation. The slurried original sediments was incubated with nanoparticulate ferric oxides and labeled methane, and the total DNA extracted from different layers from original and incubated sediment material was sequenced with the Ion Torrent technology and analyzed using various metagenomic pipelines.

MATERIALS & METHODS

Sampling, geochemical analysis and diffusion model simulations

Replicate sediment cores were taken in the Bothnian Sea at sampling site US5B during the R/V *Aranda* cruise in August 2012 (Fig. 1, location, sampling procedure and core storage are described in Egger et al. 2015). Biogeochemical parameters were measured either onboard or later in the lab and diffusion model calculation performed as described previously (Egger *et al.*, 2015).

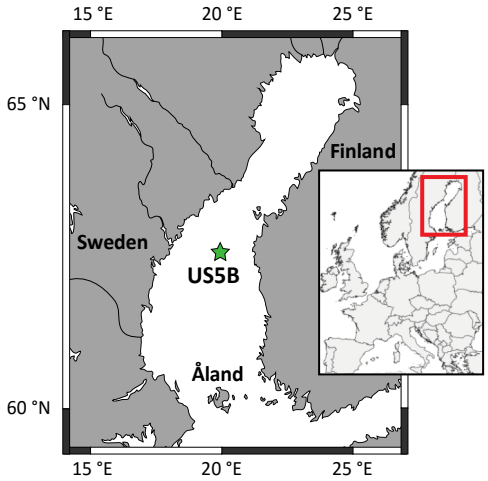

Figure 1: Location map of the study site. Site US5B (62°35.17' N, 19°58.13' E) is located in the deepest part of the Bothnian Sea at 214 m depth with an average bottom water salinity of around 6 g/L.

Incubation experiments

Replicate sediment cores taken in 2012 were sealed immediately and stored onboard at 4°C. Back in the lab, they were sliced in sections of 5 cm under strictly anaerobic conditions and stored anaerobically in the dark at 4 °C. The incubation experiments started within half a year after sampling. Per culture bottle, 30 g of wet sediment (~25 mL) was homogenized in 75 mL of SO_4^{2-}-depleted medium mimicking *in-situ* bottom water conditions within a helium-filled glovebox (containing ~2% hydrogen gas, H_2). An 85-mL aliquot of this sediment/medium slurry was distributed over 150-mL culture bottles and an additional 11.4 mL of medium was added to all bottles with the exception of the Fe treatments, for which 11.4 mL of a Fe-nanoparticle solution (Bosch *et al.*, 2010) (20 mmol/L (Fe^{3+}), resulting in 2 mmol Fe^{3+} per bottle) was added instead. The approximate ratio of 1 part sediment to 3 parts medium was chosen in agreement to reported incubation studies (Beal *et al.*, 2009, Segarra *et al.*, 2013). After the contents were mixed, the culture bottles were sealed with airtight red butyl rubber stoppers and secured with open-top Al screw caps. After being sealed, 5mL of CO_2 and either 45 mL of nitrogen ("cntl") or 45mL of $^{13}CH_4$ ("$^{13}CH_4$" and "$^{13}CH_4$ & Fe^{3+}") were injected into the headspace of duplicate incubations to yield 1 bar overpressure (volume headspace = 50 mL) and incubated in the dark at 20°C under gentle shaking. Dissolved sulfide and SO_4^{2-} were sampled within the glovebox by allowing the sediment to settle out of suspension and taking a subsample (1.5 mL) of the supernatant water via a needle syringe. Analysis of sulfide was performed as described for sediment porewater in Egger et al. 2014 (detection limit of <1 µmol/L). Samples for total dissolved S were measured by ICP-OES after acidification with 10 µL of 35% suprapur HCl and assumed to represent only SO_4^{2-} due to the release of sulfide to the gas phase during acidification (Jilbert & Slomp, 2013) (detection limit of <82 µmol/L). Headspace samples (30 µL) were analyzed by gas chromatography (GC, Agilent 6890 series, USA) using a Porapak Q column at 80°C (5 min) with helium as the carrier gas (flow rate 24 mL/min). The GC was coupled to a mass spectrometer (Agilent 5975C inert MSD, Agilent, USA) to quantify the masses 44 and 45 (CO_2). To account for the medium loss due to subsampling of the solution and because of an observed leveling-off of measured headspace $^{13}CO_2$ concentrations, an additional 11.4 mL of medium ("cntl" and "$^{13}CH_4$") and Fe-nanoparticle solution ("$^{13}CH_4$ & Fe^{3+}") was added to the culture bottles after 55 days. Fe-AOM rates were determined from the linear slope of $^{13}CO_2$ production in duplicate incubations from 20-30 cm and 30-35 cm depths, before and

after second addition of Fe^{3+} taking into account the CO_2 dissolved in the liquid and removal thereof during sampling. Thus, the statistical mean is based on a total number of eight rate estimates.

DNA isolation and sequencing

A separate core dedicated to molecular analysis was sliced under aerobic conditions in intervals of 2.5 cm, immediately frozen in liquid nitrogen and stored at -80°C until DNA extraction. Total DNA was extracted from each homogenized sediment core slice with the PowerSoil Total RNA Isolation Kit with DNA Elution Accessory kit (MoBio, USA). For each extraction, 2 g of sediment material was used according to manufacturer's instructions. The quality and quantity of isolated DNA was assessed with NanoDrop 1000 (Thermo Scientific). For each analyzed depth (0-2.5, 5-12.5 and 30-35 cmbsf), the isolated DNA was pooled in equimolar concentrations, if necessary. DNA samples were stored at -20°C until further metagenomic library preparation.
Metagenomic library preparation was performed with IonXpress™ Plus gDNA Fragment Library kit (Ion Torrent™ platform, Life technologies) following the manufacturer's instructions. The initial shearing of DNA was performed by ultrasonication (Bioruptor®, Diagenode). The quality and quantity of DNA was assessed with the Bioanalyzer 2100 during the library preparation procedure. Sequencing was performed with the Ion PGM™ system (Ion Torrent™ platform, Life technologies).

Molecular data analysis

Raw sequence data were trimmed to >100nt length with CLC Bio Genomics Workbench 7.0.3 (CLC Bio, Qiagen, USA) resulting in following read numbers: 3,966,999 (average length 289 nt) for 0-2.5 cmbsf, 3,615,605 (average length 296 nt) for 5-12.5 cmbsf, 3,225,702 (average length 291 nt) for 30-35 cmbsf and 2,180,088 (average length 303 nt) for incubation slurry. The phylogenetic characterization of *in-situ* microbial community was performed based on 16S rRNA gene diversity. The raw metagenomic sequence reads were mapped to a reference SSU rRNA gene dataset obtained from the SILVA database (Quast *et al.*, 2013) (RefNR99 dataset, release 115) using CLC Genomics Workbench with the following mapping parameters: mismatch cost 2, insertion cost 3, deletion cost 3, length

fraction 0.5, similarity fraction 0.8. The reads that mapped to SSU rRNA reference gene set were extracted and used for subsequent nucleotide blastn analysis against the same SSU reference dataset (e-value cut off 10E-6). Significant hits were extracted and aligned using the SINA Aligner (Pruesse *et al.*, 2012). The aligned SSU rRNA reads were then imported and analyzed using the ARB software version 5.5 (Ludwig *et al.*, 2004) and the SILVA SSU RefNR99 database (release 115) as a reference.

Functional gene analysis of *pmoA* and *mcrA* gene reads was performed as described previously (Lüke *et al.*, 2015). In short, metagenomes were blasted against manually curated *pmoA* (Dumont *et al.*, 2014) and *mcrA* gene databases (e-value cut off 10E-6). For the *mcrA* gene database, *mcrA* gene sequences were obtained from NCBI and manually curated to cover the known methanogen diversity (Supplementary material). Then, significant hits were extracted and blasted against non-redundant protein database (version 11/2014). Bit scores from both blast outputs were plotted against each other with the RStudio software (R version 3.1.2) and bit score ratio was used to filter out non-significant matches. All *pmoA* and *mcrA* sequences from the Bothnian Sea sediment metagenomes were loaded into ARB, corrected for sequencing errors and aligned. Afterwards, all sequences were exported and analyzed with MEGAN 5.10.3 (Huson *et al.*, 2011) using previously published database for *pmoA* (Dumont *et al.*, 2014) and an *mcrA* gene database of manually curated full and partial length sequences of known methanogens and methanotrophs for taxonomic assignment (Supplementary material). The *mcrA* database was created by importing and characterizing gene sequences in ARB. Accessory genes encoding for thiosulfate reductase (*mopB*), sulfite reductase (*dsrA*) and multi-heme cytochrome *c* proteins (*cytC*) of *Geobacter*/*Shewanella*/*M. nitroreducens* were analyzed by blasting metagenomes against non-curated gene datasets obtained from NCBI and Kletzin et al. 2015 (Supplementary material). After a first blast run, significant reads were extracted and blasted against non-redundant protein database. After plotting both bit score outputs and extracting significant reads, all were imported into MEGAN and analyzed based on standard NCBI taxonomy. For quantitative comparison, all analyzed gene reads except for *cytC* were normalized to metagenome size and average gene length according to the following formula: normalized read count (*nrc*) = (gene read count*1.000.000.000)/(total metagenome read count*average gene length [nt]). The obtained *nrc* values (representing reads per million read per 1000 bp) were used to generate gene heat maps with the RStudio software. Gene assemblies were done with the CLC Genomics workbench 8.0 with

the following parameters: bubble size 5,000, minimum contig length 400, word size 30, mismatch cost 3, insertion cost 3, deletion cost 3, length fraction 0.5 and similarity fraction 0.8. MEGAN analysis was performed with the following settings: min score 50, max expected 0.01, top percent 5, max support percent 0.0, min support 1, LCA percent 50, min complexity 0.

RESULTS & DISCUSSION

Geochemical profiles

Vertical pore water profiles for station US5B (Fig. 2) revealed a shallow SMTZ at a depth of ca. 4 to 9 cm, where SO_4-dependent AOM results in the depletion of pore water SO_4^{2-} and CH_4. Reductive dissolution of Fe-oxides driven by sulfide production during SO_4-AOM induces a distinct minimum in sedimentary Fe-oxides and precipitation of Fe-sulfides (mostly FeS). Abundant reducible Fe-oxides below the SMTZ were accompanied by very high dissolved ferrous Fe (Fe^{2+}).concentrations (> 1.8 mmol/l). The depth trend in total (Fe/Al) indicated that the Fe is not only repartitioned between oxide and sulfide phases within the SMTZ, but that Fe-oxide reduction below the SMTZ triggered upward migration of dissolved Fe^{2+} with an enrichment of total Fe in the sulfidic zone. However, reductive Fe-oxide dissolution by dissolved sulfide is unlikely in the deeper sediments since the sulfide generated by SO_4^{2-} reduction is sequestered in the form of authigenic Fe-sulfides in the SMTZ (Fig. 2). Because no sulfide remains to diffuse into the zone where Fe reduction is occurring, a cryptic sulfur cycle as observed in Baltic Sea (Holmkvist *et al.*, 2011) and Black Sea (Holmkvist *et al.*, 2011) sediments, where SO_4^{2-} is generated by sulfide reacting with deeply buried ferric Fe (Fe^{3+}) species (Holmkvist *et al.*, 2011, Holmkvist *et al.*, 2011), is unlikely to occur. High dissolved Fe^{2+} concentrations further preclude Fe-oxide reduction via sulfide released during disproportionation of elemental sulfur (Bak & Pfennig, 1987, Thamdrup *et al.*, 1993), as any sulfide produced locally would be immediately scavenged to form Fe-sulfides. Thus, two alternative mechanisms may explain the high dissolved Fe^{2+} concentration in the porewater below the SMTZ. The first mechanism is organoclastic Fe reduction, i.e. Fe reduction coupled to organic matter degradation. The second is Fe-AOM, i.e. Fe reduction coupled to AOM dissolved Fe^{2+} in these sediments.

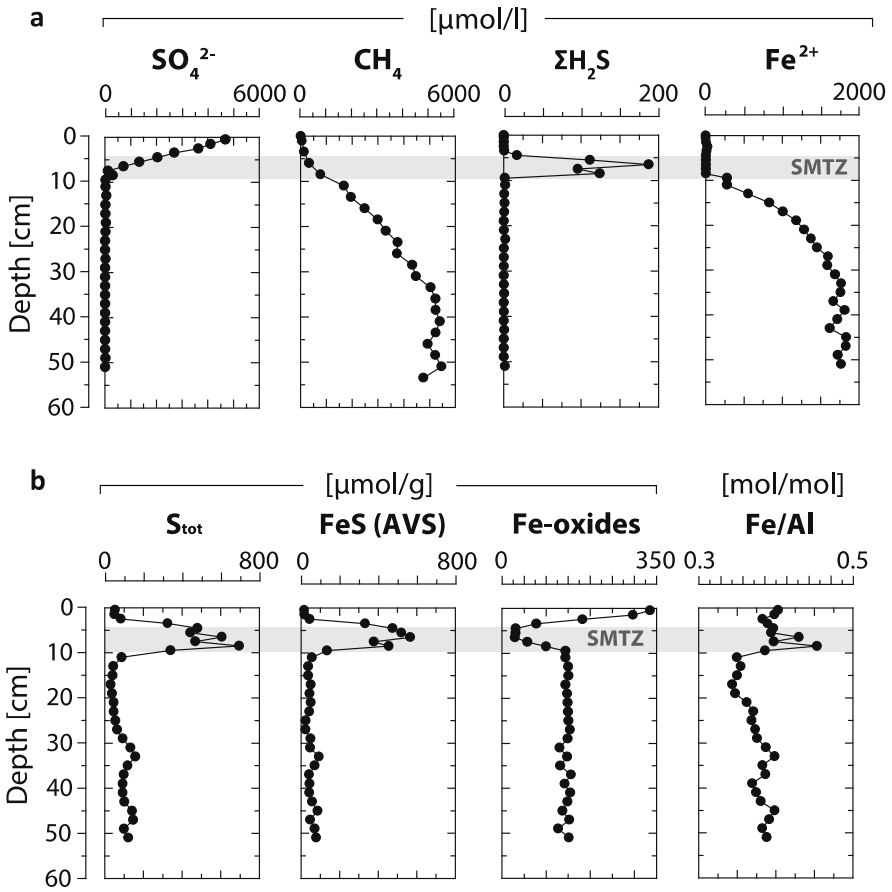

Figure 2: Geochemical profiles for site US5B. a, Porewater profiles of SO_4^{2-}, CH_4, sulfide ($\sum H_2S$ = $H_2S + HS^- + S^{2-}$) and Fe^{2+}. Grey bar indicates the sulfate/methane transition zone (SMTZ) b, Sediment profiles of total sulfur (S_{tot}), FeS (acid volatile sulfide, AVS), Fe-oxides (see Supplementary Fig. 1 for the calculation of the Fe-oxide fraction) and total (Fe/Al).

Fe-AOM activity in the Bothnian Sea sediment

The potential of the microbial community present in the sediments below the SMTZ to perform Fe-AOM was experimentally tested with slurry incubation studies. Fresh sediment samples from several depth layers were incubated with ^{13}C-labeled CH_4 ($^{13}CH_4$), CO_2, and a SO_4^{2-}-depleted medium mimicking Bothnian Sea bottom water conditions. Duplicate incubations were amended with either only $^{13}CH_4$ or $^{13}CH_4$ and 20 mmol/L Fe-hydroxide nanoparticles.

In the control slurries, nitrogen was used instead of $^{13}CH_4$. AOM rates were then determined by measuring production of $^{13}CO_2$, i.e. the end product of $^{13}CH_4$ oxidation. The addition of Fe hydroxide nanoparticles almost doubled AOM activity (1.7 fold increase) in the sediment samples between 20 and 35 cm depth compared to the slurries where no additional Fe^{3+} was added (Fig. 3). The increase in $\delta^{13}C\text{-}CO_2$ as a response to Fe^{3+} addition thus suggests that the microbial community present in the sediment below the SMTZ is capable of coupling AOM to Fe reduction. Throughout the whole experiment, sulfide stayed below detection limit (<1 µmol/L) and SO_4^{2-} concentrations stayed below 350 µmol/L. A slight decrease in background SO_4^{2-} during the incubation period might indicate low
levels of SO_4^{2-} reduction of ~1.9 pmol SO_4^{2-} cm^{-3} day^{-1}. Similar rates of SO_4^{2-} reduction (~1 pmol SO_4^{2-} cm^{-3} day^{-1}) were reported for methanogenic Baltic Sea

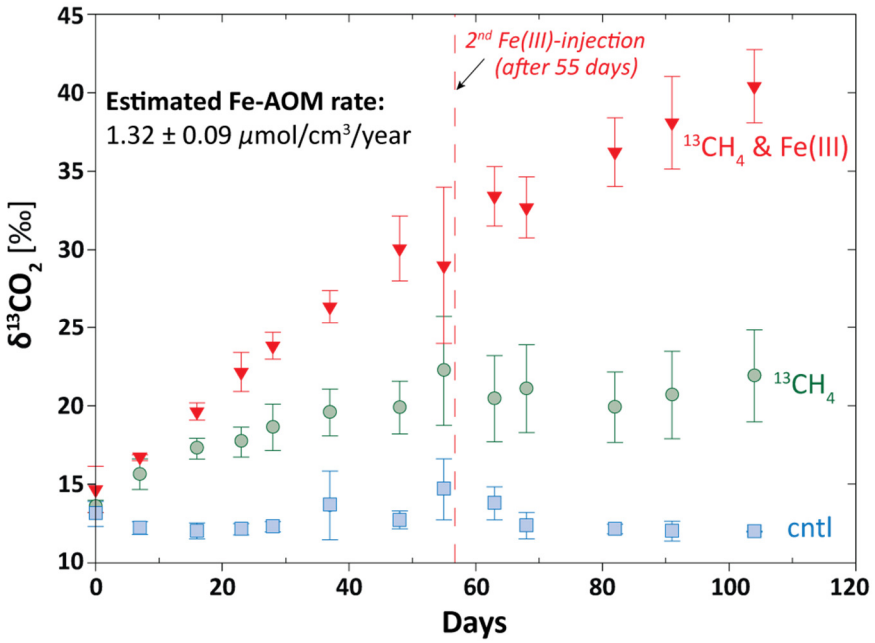

Figure 3: Incubation experiment with ^{13}C-labeled CH_4 conducted on sediments from 20-35 cm depth. SO_4^{2-}-depleted slurry incubations showed an increasing enrichment of headspace CO_2 in ^{13}C after addition of Fe(III) ("$^{13}CH_4$ & Fe(III)", red triangles) compared to treatments where no additional Fe(III) was added ("$^{13}CH_4$", green circles), suggesting stimulation of Fe-AOM. Elevated $\delta^{13}C\text{-}CO_2$ values ($[^{13}CO_2]$ / ($[^{12}CO_2]+[^{13}CO_2]$) of the $^{13}CH_4$-treatment without additional Fe(III) compared to the control ("cntl", blue squares) indicate Fe-AOM with remaining Fe-oxides present in the sediment. 20 mmol/l Fe(III) were added at t_0 (0 days) and after 55 days (dashed red line). Error bars are based on duplicates for 20-30 cm and 30-35 cm (i.e. n = 4).

sediments (Holmkvist *et al.*, 2011). Taking into account indirect Fe-stimulated S-AOM through a cryptic sulfur cycle (Holmkvist *et al.*, 2011, Holmkvist *et al.*, 2011), where redox reactions between sulfide and Fe-oxides result in the reoxidation of sulfide to SO_4^{2-} in a 17:1 stoichiometric ratio, we estimate a gross rate of SO_4^{2-} reduction of ~2 pmol SO_4^{2-} cm^{-3} day^{-1}. S-AOM is thus unlikely to contribute more than 0.1% to the total $^{13}CO_2$ production in the investigated Bothnian Sea sediment layers. These findings support our hypothesis that the accumulation of dissolved Fe^{2+} in the porewater below the SMTZ is, at least partly, a result of Fe-AOM. The potential rate of Fe-AOM in our incubations is 1.32 ± 0.09 μmol cm^{-3} $year^{-1}$, which compares well to recent estimates of potential Fe-AOM rates in slurry incubations of brackish wetland (1.42 ± 0.11 μmol cm^{-3} $year^{-1}$ (Segarra *et al.*, 2013)) and Fe^{3+}-amended mesocosm studies of intact deep lake sediment cores (1.26 ± 0.63 μmol cm^{-3} $year^{-1}$(Sivan *et al.*, 2011)). It should be noted, however, that these rates are all derived from stimulated microbial communities and thus could be lower under *in-situ* conditions.

16S rRNA gene-based phylogenetic community composition

The microbial community composition over the sediment core from the site USB5 in the Bothnian Sea was analyzed with respect to 16S rRNA diversity and CH_4 cycling by metagenomics from 3 different depths: the oxic/anoxic interface, active S-AOM zone and potential Fe-AOM zone. Previous geochemical analysis and activity measurements have indicated the active ferric iron-dependent AOM in the sediment below SMTZ. However, responsible organisms and thus possible metabolic processes behind the observed activity are still unknown. The current study used the approach of metagenomics in order to investigate the microbial community changes associated with Fe-AOM incubation and to deduce possible routes for the production of labeled CO_2 from methane.

Bacterial in-situ sediment composition

Total 16S rRNA gene reads from each Bothnian Sea sediment sample comprised approximately 5 to 6% of total raw reads in respective metagenomes. The majority was assigned to bacteria (80% in 0-2.5 cmbsf, 95% in 5-12.5 cmbsf and 80% in 30-35 cmbsf). The most abundant bacterial phylum in 0-2.5 cmbsf and 30-35 cmbsf was *Proteobacteria* with 55% and 32%, respectively. Other abundant phyla were

comprised by *Bacteroidetes* (7% in both samples), *Planctomycetes* (5% in 0-2.5 cmbsf and 6% in 30-35 cmbsf) and *Chloroflexi* (3% in 0-2.5 cmbsf and 9% in 30-35 cmbsf). In 5-12.5 cmbsf, *Proteobacteria* were as abundant as *Planctomycetes* (28% each), followed by *Bacteroidetes* (7%) and *Chloroflexi* (6%). The proportional distribution of the most abundant archaeal, bacterial and proteobacterial groups in all samples is shown in Fig. 4.

The distribution within the proteobacterial population was significantly different between all depth samples. Whereas the most dominant groups in 0-2.5 cmbsf were comprised by *Methylococcales* (14%), *Campylobacterales* (9%) and unclassified group Sh765B-TzT-29 (7%), the 5-12.5 cmbsf zone was dominated by *Desulfobacterales* (18%), group Sh765B-TzT-29 (10%), group 43F-1404R (8%) and Order Insertae Sedis/Family Insertae Sedis/Marine (7%). The 30-35 cmbsf zone was dominated by group Sh765B-TzT-29 (13%), group 43F-1404R (8%), *Syntrophobacterales* (7%) and *Xanthomonadales* (7%). The occurrence of *Methylococcales* in the upper depth corresponded with availability of both methane and oxygen in this depth which is essential for the methanotrophic lifestyle of this group. Moreover, we found abundant *pmoA* gene reads encoding the particulate methane monooxygenase enzyme. This finding points to the importance of aerobic methane oxidation at the oxic/anoxic sediment interface, which also shows that methane was not effectively removed in the deeper anoxic layers. *Campylobacterales*, the second most abundant proteobacterial group in 0-2.5 cmbsf, comprised the genera *Sulfurimonas* and *Sulfurovum* belonging to the family *Helicobacteraceae*. Both *Sulfurovum* and *Sulfurimonas* spp. are commonly found in marine environments, they respire oxygen or nitrate with reduced sulfur species as electron donors (Inagaki *et al.*, 2003, Inagaki *et al.*, 2004, Zhang *et al.*, 2009). These findings are corroborated by profiles of nitrate and oxygen which were available only within the uppermost cmbsf, but were not measurable below. The coupling between biogeochemistry and community structure was also apparent in the S-AOM zone. Here, the *Desulfobacterales* were the most dominant. This was also observed in previous studies investigating S-AOM (Michaelis *et al.*, 2002, Siegert *et al.*, 2011). Members of the *Desulfobacterales*, such as the genus *Desulfococcus*, are commonly found in association with ANME archaea, with whom they perform syntrophic S-AOM process (Knittel *et al.*, 2005). Interestingly, the uncharacterized group Sh765B-TzT-29 was found to be abundant in all depths, however, no information is available about metabolic capacities of this group. Sh765B-TzT-29 sequences were found in various environments and it was speculated that its members might be

involved in Fe-AOM in anoxic environments (Siegert *et al.*, 2011). Also no information is currently available about metabolic capabilities for the group 43F-1404R. Sequences belonging to 43F-1404R have been detected previously in marine sediments with active sulfur cycling (Asami *et al.*, 2005), marine hydrothermal field (Kato *et al.*, 2009) or paddy soils (Itoh *et al.*, 2013). In depth 30-35 cmbsf, members of *Syntrophobacterales* were more abundant than in upper analyzed sediment layers. Some of the members of this order are capable of sulfate reduction (McInerney *et al.*, 2008). They were shown to be metabolically flexible and, depending on environmental conditions and metabolic partners, able to perform both sulfate respiration and fermentation (Plugge *et al.*, 2011).

Syntrophobacterales are often found in syntrophic partnerships with hydrogen-consuming organisms in anoxic methanogenic environments (Lueders *et al.*, 2004, Stams & Plugge, 2009). An abundant methanogen population would potentially act as a hydrogen sink in this depth.

The *Planctomycete* population differed substantially within the sediment transect, with a remarkable abundance of *Brocadiales*-related sequences (65% of all *Planctomycete* reads) in 5-12.5 cmbsf versus 5% in 0-2.5 cmbsf and non in 30-35 cmbsf (more detailed discussion in Chapter 6).

Archaeal *in-situ* sediment composition

The most dominant groups within the archaeal population at all depths were the thaumarcheal Marine Group 1 (MG-1), the euryarchaeal Deep Sea Hydrothermal Vent Group 6 (DSHVG-6), the euryarchaeal order *Thermoplasmatales* and anaerobic methanotrophic archaea (ANME) from group 2a.

Group MG-I comprised 65% of all archaeal 16S rRNA gene reads in 0-2.5 cmbsf, while in 5-12.5 and 30-35 cmbsf its abundance was with 24% and 28% significantly lower, respectively. Previous studies have shown that at least some representatives of this group are capable of aerobic ammonia oxidation (Hu *et al.*, 2011, Stahl & de la Torre, 2012). Furthermore, MG-1 seems to be dominant among archaea in various marine water and sediment habitats underlining its importance for biogeochemical element cycling (DeLong, 1992, Galand *et al.*, 2009). Although most gene reads belonging to MG-1 were detected in 0-2.5 cmbsf where both oxygen and nitrate co-occurred, their relatively high abundance in deeper anoxic layers was puzzling. Similar observations were reported previously from deep oligotrophic sediment subsurface (Sørensen *et al.*, 2004, Inagaki *et al.*, 2006, Teske, 2006). However, the metabolic function of those deep-sediment MG-I group archaea remains unclear, and

a metabolism other than oxygen-dependent ammonia oxidation might be more likely.

Based on 16S rRNA gene results, group DSHVG-6 was the second most abundant in the Bothnian Sea sediment and its relative abundance increased with depth (17% in 0-2.5 cmbsf, 27% in 5-12.5 cmbsf and 38% in 30-35 cmbsf).

Metabolic capacities of members from this group are unknown, however sequences have been often found in marine anoxic sediments. Investigations of methane seep sediments from Nankai Through have revealed similar trends in archaeal population with groups MG-1 and DSHVG-6 being the most dominant and similar trend showing a decrease in MG-1 and increase in DSHVG-6 abundance with depth (Nunoura *et al.*, 2012). The first habitats in which DSHVG-6 sequences were detected were hydrothermal vents (Takai & Horikoshi, 1999).

Thermoplasmatales archaea were found in relatively constant abundance (8-10%) at all depths over the sediment transect. The sequences clustered mainly within 5 groups: ASC21, AMOS1A-4113-D04, Marine Group II, Terrestrial Miscellaneous Group (TMEG) and 2B5. Their metabolism is not known, but related 16S rRNA sequences have been detected in methane seeps of the North Sea (Wegener *et al.*, 2008), subseafloor sediments in a gas hydrate area (unpublished), oxygen minimum zone in the Pacific Ocean (unpublished) and methanogenic estuarine sediments in Orikasa River (Kaku *et al.*, 2005). Moreover, during *mcrA* gene analysis we detected methanogens of the genus *Methanomassiliicoccus* which is related to *Thermoplasmatales* and might have contributed to observed 16S rRNA inventories.

ANME archaea were only found in 5-12.5 (24%) and 30-35 (8%) cmbsf which is in agreement with the presence of corresponding *mcrA* genes in both depths. These archaea are involved in reverse methanogenesis in cooperation with SRB from the order *Desulfobacterales* (Knittel *et al.*, 2005). This corresponds to biogeochemical data pointing to active S-AOM zone in 5-12.5 cmbsf. The presence of ANME in deeper sediment with no measurable sulfate might be a relict of their previous activity in this depth as the sulfate-methane transition zone in the Bothnian Sea had moved towards the sediment surface in previous decades (Slomp *et al.*, 2013). An alternative possibility would be a methanotrophy metabolism coupled to electron acceptors other than sulfate.

.

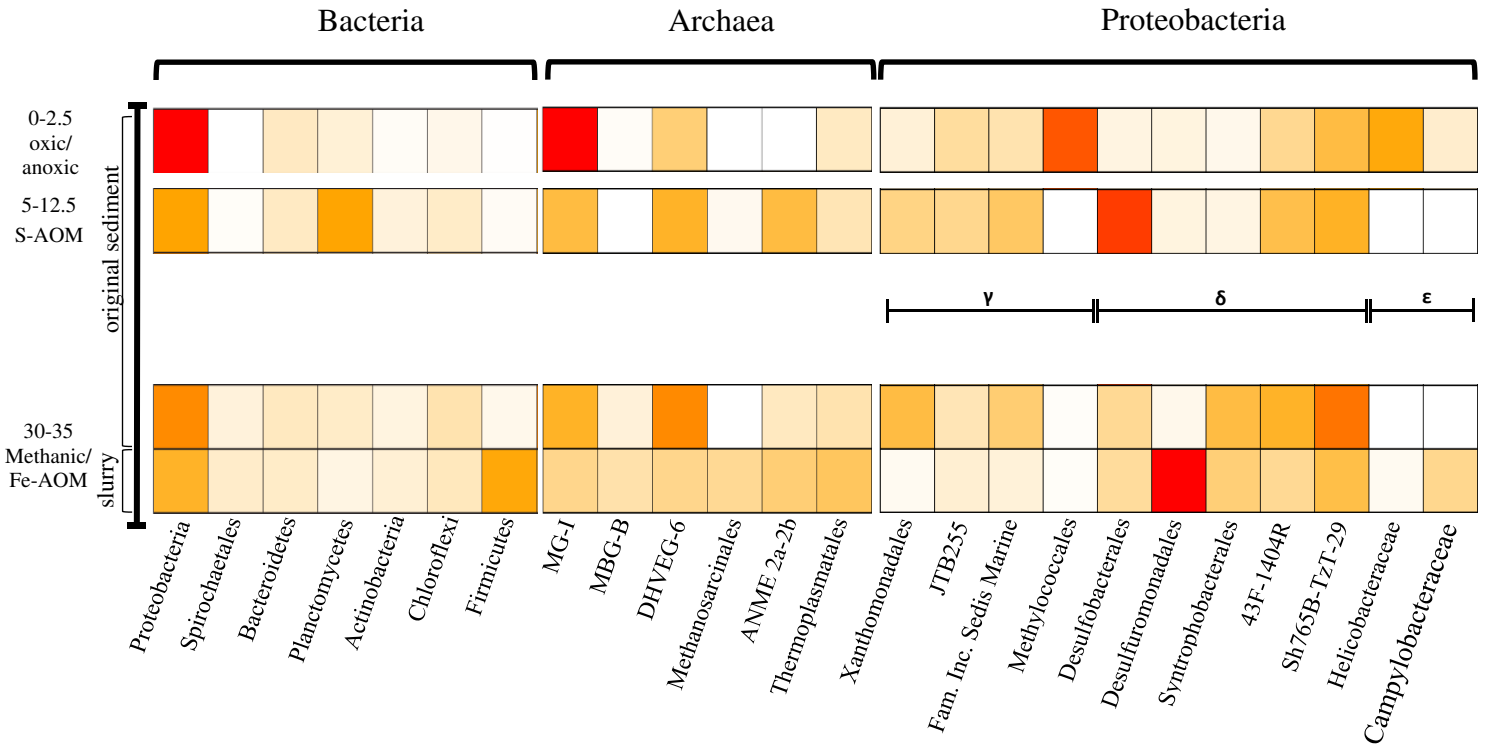

Figure 4: 16S rRNA gene analysis heat maps from the Bothnian Sea metagenomes. Heatmaps (Red: high abundance, white: low abundance) showing most abundant archaeal, bacterial and proteobacterial groups (>5%) in original sediment and incubation slurry. Abbreviations: MG-I, Marine Group I *Thaumarchaeota*; MBG-B, Marine Benthic Group B; DHVEG-6, Deep Sea Hydrothermal Vent Group 6; ANME, Anaerobic Methanotrophic Archaea; JTB255, Xanthomonadales JTB255 Marine Benthic Group; Fam. Inc. Sedis Marine, Family Incertae Sedis Marine.

Community change associated with Fe^{3+} and CH_4 incubation

Metagenome analysis revealed that the abundances of most major bacterial and archaeal clades decreased after the incubation period of 3 months in Fe-AOM slurry incubations. Among bacteria only members of *Firmicutes* (putatively fermentative *Clostridia*), *δ*-Proteobacteria (*Desulfuromonadales*) and *ε*-Proteobacteria (*Campylobacterales*) increased in numbers after the incubation period (Fig. 4): *Clostridia* (from 3 to 24%), *Desulfuromonadales* (from 1 to 23% within all *Proteobacteria* (equivalent to <0.5% to 5.5% within all bacteria)) and *Campylobacterales* (from below detection limit in original sediment to 5% within all *Proteobacteria* (0 to 1.3% within all bacteria)).

Within archaea, several taxa also increased in abundance, mostly belonging to euryarchaeal clades of *Methanosarcinales* (from below detection limit to 14%), ANME-2a (from 8 to 18%), *Thermoplasmatales* (from 10 to 21%) and uncharacterized Marine Benthic Group B (MBG-B) (from 5 to 11%).

The archaeal lineage MBG-B was first detected in deep-sea sediments of the Atlantic Ocean (Vetriani *et al.*, 1999). Since then, 16S rRNA gene signatures of MBG-B have been detected in a wide range of coastal, marine, intertidal subsurface and sediment habitats (Inagaki *et al.*, 2003, Sørensen *et al.*, 2004, Kim *et al.*, 2005, Knittel *et al.*, 2005, Lloyd *et al.*, 2006). Notably, many of those habitats were characterized by active methane cycling such as methane seeps, gas hydrates and methane-consuming mats. It has been hypothesized that MBG-B archaea might be involved in anaerobic methane oxidation, either directly or indirectly (Sørensen & Teske, 2006, Teske & Sorensen, 2007). Moreover, a study comparing microbial subsurface communities detected significant dominance of MBG-B in methane-bearing but not in methane-free sediment (Inagaki *et al.*, 2006). This shows that MBG-B archaea are associated with methane cycling in anoxic sediment, however, neither possible metabolic pathways nor associated genes are known to date. Investigation of total archaeal lipids from methane-hydrate bearing sediments revealed assimilation of non-methane organic carbon derived from sediments, despite the fact that most carbon cycling in those sediment was driven by AOM (Biddle *et al.*, 2006). It was argued that MBG-B might still be involved in oxidation, but not assimilation of methane (Biddle *et al.*, 2006).

A significant increase in *Methanosarcinaceae* 16S rRNA gene reads was accompanied with an increase in *mcrA* gene read numbers related to *Methanosarcina* spp. Methanogens of the order *Methanosarcinales* have been well characterized, both genetically and physiologically. They are among the most metabolically

versatile methanogens, being able to utilize all methanogenic substrates except formate (Thauer *et al.*, 2008). Their increase in numbers indicated favorable conditions for methanogenesis during incubation which could have been stimulated by several factors. Addition of ferric iron provided electron acceptor in excess thus stimulating favorable conditions for respiration of organic matter present in the sediment slurry. This could have led to mobilization of simple organic compounds which would benefit methanogens. Also, a stark increase in putatively fermentative *Clostridia* we observed in the incubation slurry could have caused a decrease in redox potential which in turn would stimulate both methanogens and iron reducers. The effect of lower redox potential on increased ferrihydrite reduction by *Geobacter* spp. has been shown previously (Straub & Schink, 2004). A previous study showed that *Methanosarcina* and ANME archaea are stimulated with addition of ferrihydrite in hydrocarbon-dependent methanogenesis/-trophy (Siegert *et al.*, 2011). An increase in methanogen population in our incubation slurries could then at least partly explain the increase in observed methane oxidation activity as it is known that *Methanosarcina*-related methanogens are able to oxidize some methane during the regular methanogenesis activity (Zehnder & Brock, 1979). Moreover, methanogens of the order *Methanosarcinales* have been previously shown to be able to reduce ferric iron with hydrogen as the electron donor during methanogenesis (Liu *et al.*, 2011, Liu *et al.*, 2011). Inhibitory effects of ferric iron on methanogenesis activity of *Methanosarcina* were also reported (van Bodegom *et al.*, 2004).

Besides methanogenic *Methanosarcina* spp., known methanotrophic archaea from the ANME-2a clade also increased in both 16S rRNA and *mcrA* (group 2e) gene reads. ANME archaea are known to be involved in S-AOM (Knittel & Boetius, 2009). More recently, an archaeon closely related to ANME group 2a was shown to be capable to couple nitrate reduction to methane oxidation (Haroon *et al.*, 2013). It has been speculated that ANME archaea could also be potentially involved in AOM linked to ferrihydrite reduction (Wankel *et al.*, 2012, Kletzin *et al.*, 2015), however, any evidence for this is still lacking or is indirect.

In comparison to soluble electron acceptors (e.g. sulfate, nitrite, nitrate), iron reduction is more challenging due to the insoluble nature of iron oxides at physiological conditions. Although siderophore-mediated assimilative uptake of chelated ferric iron has been well described in bacteria (Andrews *et al.*, 2003), this mechanism is unlikely to play a role for dissimilative processes due to relatively high metabolic investment. Microbial iron reduction is a widespread process and several mechanisms have been described for its mediation. Microorganisms can employ

direct contact either via conductive pilin-structures and cell surface contact to iron oxides, or indirect iron reduction via secondary redox shuttles such as humic substances (Weber *et al.*, 2006). In any case, electrons from methane oxidation must be directed into the periplasm and ultimately outside the cell.

Theoretically, an ability to shuttle electrons onto an external electron acceptor (ferrihydrite particle) such as known from bacterial iron reducers like *Geobacter* or *Shewanella* could enable ANME archaea to link methane oxidation to iron reduction without a metabolic partner. The ability to reduce iron is generally associated with high numbers of multi-heme cytochrome *c* protein encoding genes in respective genomes (Shi *et al.*, 2007). Notably, the genome of ANME archaeon *M. nitroreducens* encodes up to 38 multi-heme cytochrome *c* proteins (Haroon *et al.*, 2013, Kletzin *et al.*, 2015), and this feature was speculated to be linked to possibility for iron metabolism (Kletzin *et al.*, 2015).

All 16S rRNA gene reads of ANME archaea found in original Bothnian Sea sediment as well as in incubation slurry were assigned to clade 2a which corresponds to group 2e in *mcrA* phylogeny (reviewed in Knittel and Boetius, 2009). Both archaeal clades are phylogenetically closely related (Fig. 7). So far, one metagenome of ANME-2a enrichment culture has been sequenced from an aggregate and revealed that this lineage of ANME harbors abundant multi-heme cytochrome *c* protein encoding genes (Wang *et al.*, 2014), a feature very similar to ANME-2d. Based on genome information, it was speculated that ANME-2a possess high metabolic flexibility in substrate utilization and energy-converting mechanisms (Wang *et al.*, 2014). However, so far there is no evidence for dissimilatory iron reduction by these methanotrophs. Even if not performed by one organisms alone, iron reduction coupled to methane oxidation still could occur in a metabolic co-operation with iron reducers similar to that described previously for S-AOM. In this scenario, archaea would oxidize methane and shuttle reducing equivalents to known bacterial iron reducer. In fact, the observed increase in 16S rRNA genes assigned to *Desulfuromonadales* (*Geobacteracaea*) indicated that this scenario might have been at least partially responsible for the observed AOM activity in our incubations. The majority of reads (64%) assigned to *Desulfuromonadales* could be assembled to a single full length 16S rRNA contig sharing 97% identity to *Desulfuromonas michiganensis* strain BB1 (NR_114607). This organism was shown to utilize a wide range of electron acceptors including ferric iron with several electron donors (Sung *et al.*, 2003).

Members of *Desulfuromonadales* order, *Geobacter* spp., have been observed previously in the zone of iron-dependent AOM in an anoxic aquifer (Amos *et al.*, 2012). Also Beal et al. (2009) reported a significant increase in known metal reducing organisms in their incubations with iron.

A parallel increase in 16S rRNA gene reads belonging to *Campylobacteraceae* most closely related to *Sulfurospirillum* spp. might point to an alternative route to iron reduction in incubation slurry. These organisms have been shown to use a variety of electron acceptors including oxygen, nitrate, sulfur and thiosulfate (Eisenmann *et al.*, 1995, Finster *et al.*, 1997, Sikorski *et al.*, 2010). As our incubations were anoxic without added nitrate, growth of those bacteria was probably associated with intermediate oxidized sulfur species. These would have been generated internally from the oxidation of sulfide, probably abiotically by added iron hydroxide – via a cryptic sulfur cycle previously described for incubations of marine sediment (Holmkvist *et al.*, 2011). In order to further investigate this hypothesis we analyzed both metagenomes for the presence of poly-/thiosulfate reductase encoding genes related to *Sulfurospirillum* spp. The read numbers increased from below detection limit in the original sediment to 4.3 normalized read counts in the incubation slurry, which would fit to an operational cryptic sulfur cycle. However, it is not known to what extent the generated sulfur species contributed to iron reduction and whether there was a link to methane oxidation activity. We did not observe an increase in 16S rRNA gene reads belonging to known sulfate reducers. Also, some species of *Sulfurospirillum* were shown to be capable of iron reduction (Stolz *et al.*, 1999). Our batch incubation medium used for slurry preparation contained low concentration of sulfate (200 µM) to mimic *in-situ* conditions. The importance of low sulfate concentrations for a cryptic sulfur cycle in ferrihydrite-rich systems has been pointed out by a recent study (Hansel *et al.*, 2015). In flow experiments with ferrihydrite-amended columns and low medium sulfate concentrations, the *in situ* microbial community was enriched for known sulfate- and sulfur-reducing organisms and iron-reducing organisms increased in numbers only after all sulfate was depleted within the column (Hansel *et al.*, 2015). Mass balance calculations and molecular data pointed to preferential usage of sulfate over highly reactive iron oxides, despite the better theoretical reduction potential of ferric iron (Hansel *et al.*, 2015).

Aerobic methane cycle as inferred from *pmoA* gene inventory

Our metagenome survey revealed that up to 14% of all proteobacterial 16S rRNA gene reads in the 0-2.5 cmbsf sample belonged to the γ-proteobacterial order of *Methylococcales* – aerobic methanotrophs commonly found in various terrestrial and marine environments. Previous comparative analysis of methane cycle associated communities has shown that *Methylococcales* are also common community members in various marine methane seep ecosystems (Tavormina *et al.*, 2010, Ruff *et al.*, 2015). The analysis of particulate methane monooxygenase-encoding sequence reads from the sediment transect revealed that most methanotrophs in 0-2.5 cmbsf belonged to the type Ia with *Methylobacter* being the most abundant top blast hit (Fig. 2). Not a single monooxygenase sequence could be retrieved from 5-12.5 cmbsf – hot spot of S-AOM in the sediment transect. In 30-35 cmbsf, no typical

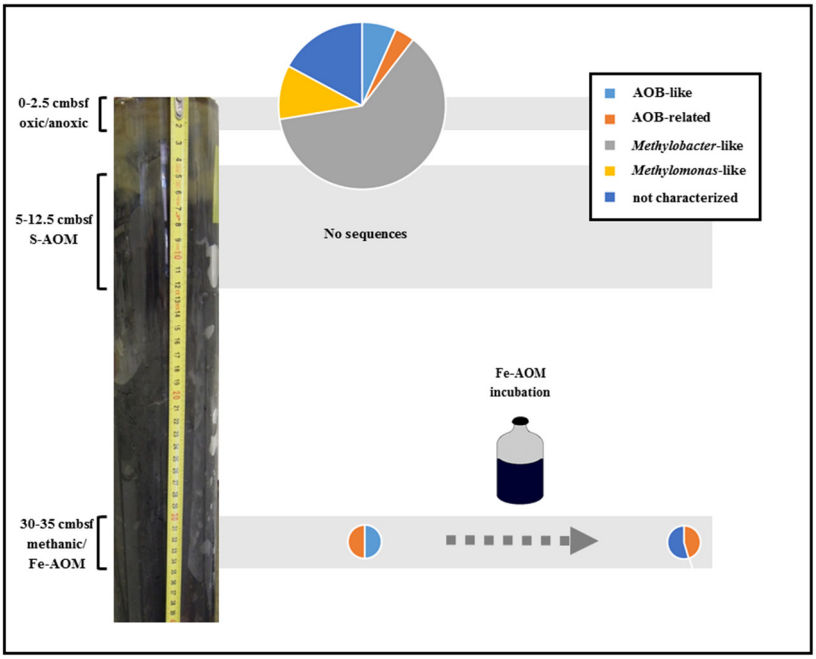

Figure 5: Methane monooxygenase (*pmoA*) gene read analysis in the Bothnian Sea sediment. Normalized taxonomically assigned gene read distribution is shown. Normalized and summarized gene read counts: 10.5 in 0-2.5 cmbsf, 0 in 5-12.5 cmbsf, 1 in 30-35 cmbsf and 0.6 in incubation slurry. Pie chart area is categorized as following: 10-12 nrc: 100%, 7-10 nrc: 80%, 5-7 nrc: 60%, 2-5 nrc: 40%, 0.1-2 nrc: 20%. Abbreviations: AOB, ammonia oxidizing bacteria.

type Ia methanotrophic *pmoA* sequences could be detected, but instead some divergent monooxygenase sequences related to both methane and ammonia monooxygenase clades from various freshwater environments. Some sequences could not be classified with the used reference database (1.8 nrc from 0-2.5 cmbsf and 0.3 nrc from 30-35 cmbsf, designated as 'not characterized' in Fig. 2), as the database was developed for classification of amplicon pyrosequences of partial sequence length (Dumont *et al.*, 2014). Failed classification was either being out of range of the alignment with database sequences and/or low sequence similarity to known monooxygenases. The sediment was also analyzed for the presence of soluble methane encoding genes (*mmoX*). Only one *mmoX* gene read could be retrieved from the 0-2.5 cmbsf zone, and none from two deeper layers indicating that this enzyme did not play an important role in the Bothnian Sea methane cycle. Functional gene data were congruent with 16S rRNA gene data, which confirmed the dominance of *Methylococcaceae* family in 0-2.5 cmbsf. In conclusion, the methane cycle at the oxic/anoxic interface was exclusively driven by aerobic methanotrophy, as neither a single 16S rRNA belonging to methanogens nor *mcrA* gene sequences could be retrieved from the sequenced metagenome. This finding also indicated that methane was not effectively removed in the deeper sediment transect via AOM, and that some methane reached the oxic zone to fuel aerobic methanotrophs. This finding is congruent with results reported by Egger *et al.* 2015, showing an isotopic signature of methane above the SMTZ which is indicative of the activity of methanotrophic organisms.

Anaerobic methane cycle as inferred from *mcrA* gene inventory

In stark contrast to absence of methanogen related sequences in 0-2.5 cmbsf, *mcrA* gene reads were detected in deeper layers and increased in abundance with depth (Fig. 6; 2.3 nrc in 5-12.5 cmbsf, 2.7 ncr in 30-35 cmbsf). Also, normalized *mcrA* read numbers increased nearly 4 times in incubation slurry from 30-35 cmbsf (11.3 nrc) in comparison to the original sediment indicating a possible stimulation by added Fe^{3+}.

Taxonomic analysis revealed that the majority of *mcrA* reads in 5-12.5 cmbsf belonged to ANME archaea related to group 2e, followed by methanogens related to *Methanosarcina* and *Methanoregula*. These findings further support the hypothesis that ANME drive the S-AOM process in this zone. ANME-2e *mcrA* genes were also detected in 30-35 cmbsf, although in slightly lower percentage (60% vs 77%). Here,

in contrast to 5-12.5 cmbsf, *Methanosarcina*-related *mcrA* were not detected, but *Methanoregula* slightly increased in abundance (13% vs 8%) and also *Methanomassiliicoccus*-like *mcrA* genes were detected (20%). *Methanomassiliicoccus*-like methanogens were only recently described from a human gut microbiome (Dridi *et al.*, 2012, Borrel *et al.*, 2013). Phylogenetic analysis suggests their common ancestry with Marine Group B, Marine Benthic Group D and *Thermoplasmatales* (Borrel *et al.*, 2013). These methanogens were shown to be restricted to methylotrophic H_2-dependent metabolism lacking most genes involved in H_2/CO_2 methanogenesis and the oxidative part of methylotrophic methanogenesis (Dridi *et al.*, 2012, Borrel *et al.*, 2013). Recent data suggest their widespread occurrence in various environments (Kemnitz *et al.*, 2005, Biderre-Petit *et al.*, 2011, Iino *et al.*, 2013, Zhou *et al.*, 2015). As these methanogens are related to *Thermoplasmatales* (Dridi *et al.*, 2012), their ribosomal genes might have contributed to observed increases of 16S rRNA reads assigned to *Thermoplasmatales*. The presence of methanogen-related *mcrA* and 16S rRNA gene sequences is congruent with our previous isotope fraction modeling studies, which suggested a concurrent hydrogenotrophic methanogenesis and Fe-AOM below the SMTZ (Egger *et al.*, 2015).

In comparison to the original sediment, we observed increases in all detected methanogen- and methanotroph-related *mcrA* gene reads in active incubation slurry. In particular, the most pronounced changes were in *Methanosarcina*-like and ANME-2e-like *mcrA* read abundances which pointed to increased methane utilization capacity in the incubation slurries. Assemblies of corresponding 16S rRNA gene reads revealed that the ANME archaea at our sampling site belonged to the same genotype irrespective of depth in which it was detected, resembling 99% identity to an environmental sequence OT-A17.11 (GenBank: AB2524242) originating from the marine sediment of the Yonaguni Knoll IV hydrothermal field and which was assigned to ANME-2a cluster (Inagaki *et al.*, 2006). Assemblies of *mcrA* gene reads pointed to microdiversity within the detected groups, but all ANME-like sequences detected at our sampling site formed a separate cluster in an amino acid-based phylogenetic tree (Fig. 7) pointing to a new ANME-2e-related species.

Also, in view of abundant multi-heme cytochrome *c* (CytC) encoding genes found in both ANME-2a and group AAA (16S rRNA phylogeny) genomes and known iron reducers from the order *Desulfuromonadales*, we compared metagenomes from the original sediment and slurry incubation for *Geobacter*/*Shewanella*/*M.*

nitroreducens-like CytC protein encoding genes (Tab. 1). This analysis showed a clear increase in *cytC* gene reads in incubation slurries in comparison to the original sediment. Notably, blastx results indicated an increase in *cytC* gene reads with *M. nitroreducens* as closest match, however, with protein identities between 32 and 82% on the amino acid level. The low identities could partially be explained by frame shifts due to sequencing errors (analyzed *cytC* genes encoding reads were not corrected for sequencing errors) in raw reads, but also due a lack of protein information of ANME-2e-related archaea in NCBI blast database.

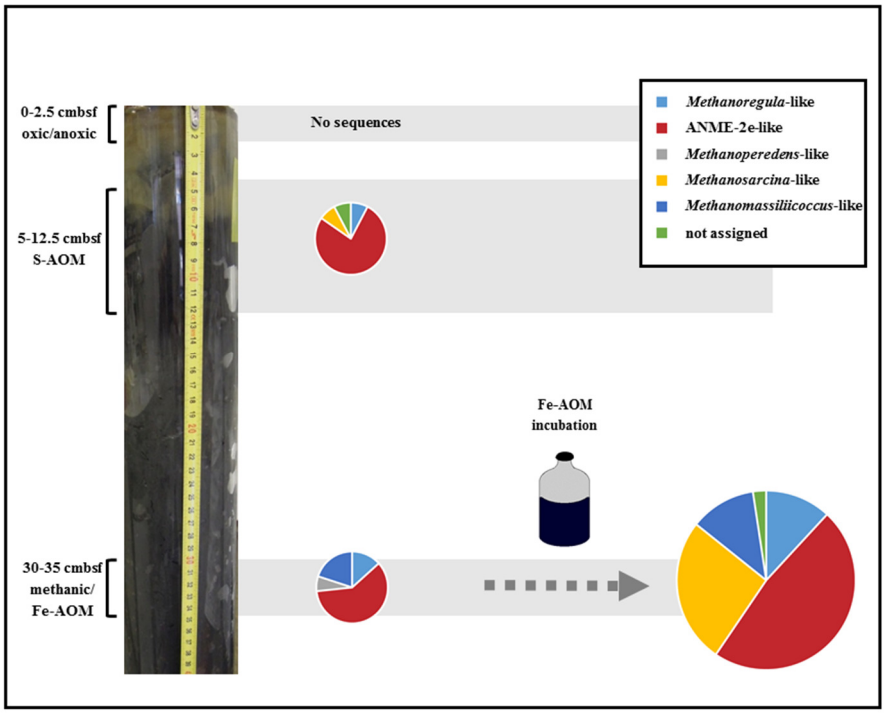

Figure 6: Methyl-coenzyme M reductase (*mcrA*) gene read analysis in the Bothnian Sea sediment. Normalized taxonomically assigned gene read distribution is shown. Normalized and summarized gene read counts: 0 in 0-2.5 cmbsf, 2.1 in 5-12.5 cmbsf, 2.7 in 30-35 cmbsf and 11.3 in incubation slurry. Pie chart area is categorized as following: 10-12 nrc: 100%, 7-10 nrc: 80%, 5-7 nrc: 60%, 2-5 nrc: 40%, 0.1-2 nrc: 20%.

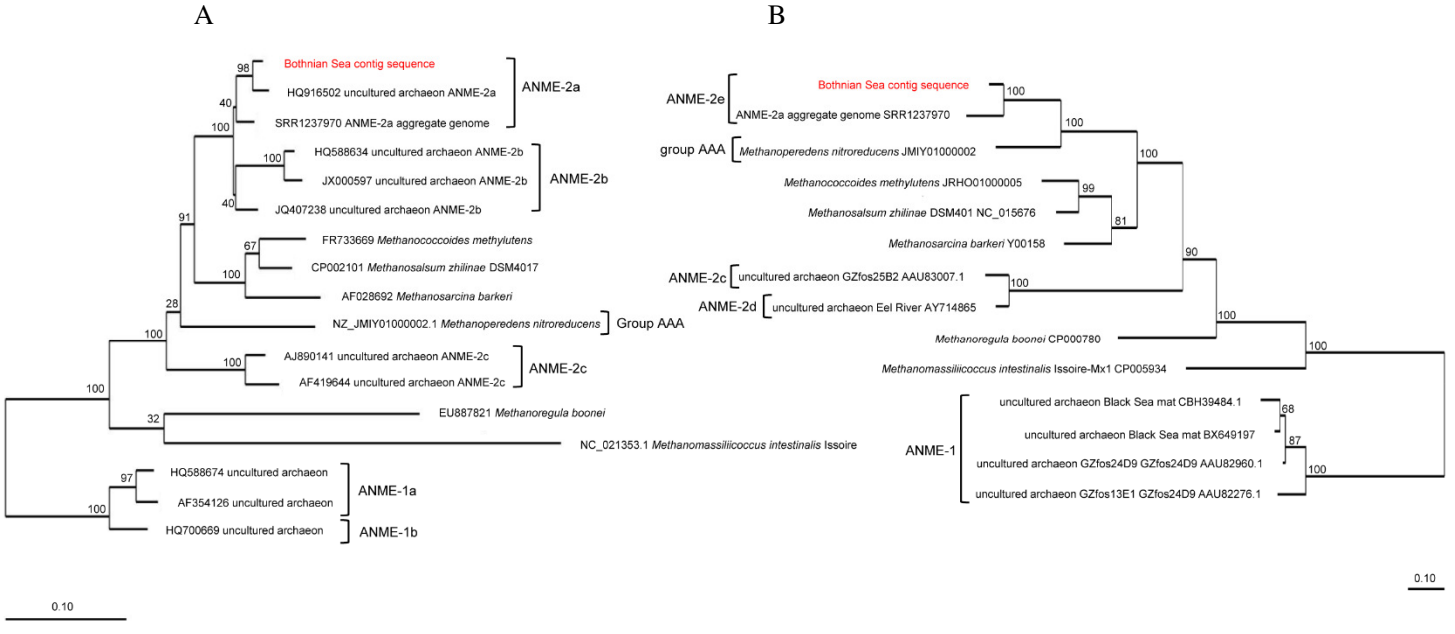

Figure 7: Maximum likelihood phylogenetic trees of selected methanogenic and methanotrophic archaea. Designated groups of methanotrophic archaea (ANME) are shown. A: 16S rRNA-based tree. B: *mcrA*-based amino acid tree. Abbreviations: ANME, anaerobic methanotrophic archaea; AAA, AOM-associated archaea. Group AAA designation is synonymous to ANME-2d lineage in 16S rRNA gene phylogeny.

Table 1: Multi-heme cytochrome *c* (cytC) protein encoding gene blastx output after analysis for *Geobacter* (*omcE/omcS*), *Shewanella* (*mtrC/omcA*) and *M. nitroreducens*-like (all multi-heme cytC protein encoding genes) specific genes. Absolute read numbers are shown. The total metagenome size of original sediment metagenome was 3225702 and of incubation slurry 2180088 reads.

		30-35 (cm below sediment surface, cmbsf)	
		original sediment	**incubation slurry**
Total		134	493
Archaea (all)		58	205
Archaeoglobi	*Ferroglobus*-like	17	72
Methanomicrobia	*Methanoperedens*-like	35	116
Bacteria (all)		68	248
Deltaproteobacteria	*Desulfuromonadales*	7	44
	Desulfobacterales	2	12
Gammaproteobacteria	*Alteromonadales*	4	4
Acidobacteria		7	24
Clostridia		1	24

Scenarios for Fe-AOM in Bothnian Sea sediment

Based on our observations of population changes in the incubation slurry in comparison to the original sediment, several scenarios can possibly explain the observed methane oxidation activity (Fig. 8), which are not mutually exclusive.
First, ANME archaea could perform Fe-AOM alone without a metabolic partner by shuttling electrons directly onto Fe^{3+} particles. Abundant archaeal *cytC* encoding gene reads detected in the incubation slurry metagenome supported this scenario. Secondly, they also could shuttle reducing equivalents directly or indirectly to iron reducers (*Desulfuromonadales*) for ferric iron reduction or to sulfate reducers for sulfate/sulfur reduction. *Clostridia*, probably stimulated by ferric iron, could contribute to both enzymatic iron reduction and release of fatty acids and

hydrogen. Both, fatty acids and hydrogen, are potential substrates for methanogens, iron reducers, sulfate and sulfur reducers. The majority of *Clostridia*-assigned 16S rRNA gene reads could be assembled to a single contig sequence sharing 98% identity with *Acidaminobacter hydrogenoformans*, an organism which was shown to ferment various amino acids while producing acetate, propionate, hydrogen and CO_2 (Stams & Hansen, 1984). Moreover, its growth was shown to be stimulated in co-cultures with sulfate reducers or methanogens, which would scavenge H_2 and stimulate fermentation (Stams & Hansen, 1984). Some *Clostridia* have been shown previously to be able to reduce ferric iron (Ottow, 1971, Dobbin *et al.*, 1999, Park *et al.*, 2001, Lovley *et al.*, 2004, Scala *et al.*, 2006). Furthermore, as discussed earlier, *Clostridia* could lower the overall redox potential inside the incubation slurry which would stimulate methanogens, ANME archaea and iron reducers even further. Based on genomic potential, there was a possibility for a cryptic sulfur cycle in the incubation slurry supported by an increase in 16S rRNA and thiosulfate reductase encoding gene reads of *Sulfurospirillum*-like organisms. Also, despite no obvious increases in 16S rRNA gene reads of known sulfate reducers, we analyzed both original sediment and incubation slurry metagenomes for sulfite reductase encoding genes (*dsrA*) and observed a clear enrichment in incubation slurry samples (17.8 nrc vs 5.4 ncr). Hence, ANME archaea mediating anaerobic methane oxidation may also shuttle reducing equivalents to sulfate reducers, together mediating S-AOM.

CONCLUSIONS & OUTLOOK

Our investigations of the Bothnian Sea sediment for iron-dependent anaerobic methane oxidation leave room for several possible mechanisms. Incubation slurries which exhibited methanotrophic activity harbored increased populations of known iron reducing bacteria of the order *Desulfuromonadales*, methanogenic archaea belonging to *Methanosarcina*, *Methanomassilicoccus* and *Methanoregula*, and ANME-2a-like methanotrophic archaea. The strongest population increase was observed for putatively fermentative *Clostridia*. A concomitant increase in *Desulfuromonadales*-specific and archaeal multi-heme cytochrome *c* and *mcrA*-encoding genes further indicated crucial roles for Fe-AOM activity. At the same time, functional genes involved in reduction of oxidized sulfur methanotrophic archaea. The strongest population increase was observed for putatively fermentative *Clostridia*. A concomitant increase in *Desulfuromonadales*-specific and archaeal multi-heme cytochrome *c* and *mcrA*-encoding genes further indicated crucial roles

for Fe-AOM activity. At the same time, functional genes involved in reduction of oxidized sulfur species also increased in abundance indicating an active sulfur cycle as well. Altogether, our data support that Fe-AOM is active in the Bothnian Sea sediment with ANME archaea, iron reducing bacteria, fermentative *Clostridia* and sulfur/sulfate reducing bacteria being the most likely organisms involved. Enrichment cultures are ultimately necessary to unravel the responsible mechanisms.

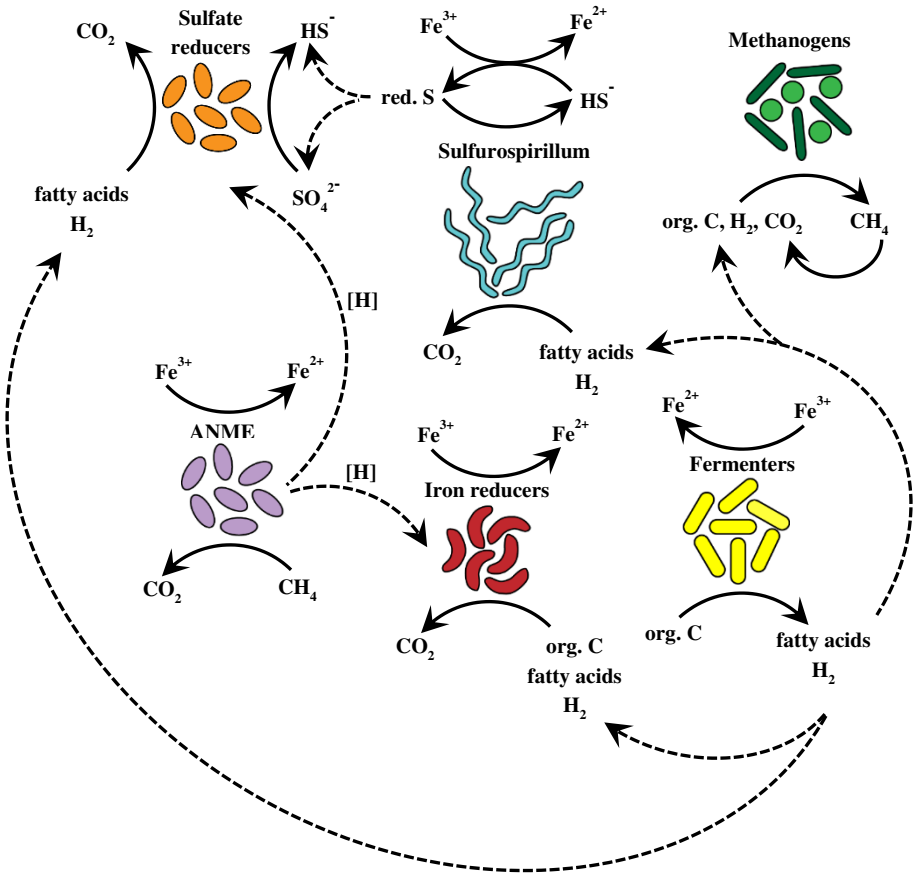

Figure 8: Possible scenarios for iron and methane transformations in the Bothnian Sea Fe-AOM incubation slurry as deduced from 16S rRNA and relevant functional gene changes assigned to major functional organism groups in compared metagenomes from the original sediment and incubation slurry from the Bothnian Sea. Possible processes are shown in parallel.

ACKNOWLEDGEMENTS

We would like to thank Theo van Alen and Geert Cremers for technical assistance with sequencing and B. Kartal for discussions. O. Rasigraf and M. Jetten were supported by the ERC (grant no. 232937) M. Jetten and C. Lüke were supported by ERC AG (339880), SIAM (024002002) and NESSC (024001001). C. Slomp and M. Egger were supported by ERC SG (278364) and VICI (865.13.005). K. Ettwig was supported by VENI 863.13.007.

chapter 6

Genomic potential for nitrogen cycling in the Bothnian Sea sediment

Submitted:

Olivia Rasigraf, Julia Schmitt, Mike S.M. Jetten, Claudia Lüke (2015) Metagenomic potential for and diversity of N-cycle driving microorganisms in the Bothnian Sea sediment.

ABSTRACT

The biological nitrogen cycle is driven by a plethora of reactions transforming nitrogen compounds between various redox states, which can be performed by a variety of different microorganisms. Whereas some of these processes are phylogenetically fairly widespread (e.g. denitrification), some others are only restricted to narrow groups (e.g. anammox). Here we investigated the metagenomic potential for nitrogen cycle processes of the *in-situ* microbial community in an oligotrophic, low salinity environment of the Bothnian Sea sediment. Total DNA from three depths below the sediment surface was isolated and sequenced with the Ion Torrent technology. The diversity of diagnostic functional genes coding for nitrate reductases (*napA*;*narG*), nitrite reductases (*nirK*;*nirS*;*nrfA*), nitric oxide reductase (*nor*), nitrous oxide reductase (*nosZ*), hydrazine synthase (*hzsA*), ammonia monooxygenase (*amoA*), hydroxylamine oxidoreductase (*hao*) and nitrogenase (*nifH*) was analyzed by blastx analysis against curated reference databases. In addition, PCR-based amplification was performed on the *hzsA* gene of anammox bacteria. Our results reveal high genomic potential for full denitrification to N_2, but minor importance of anaerobic ammonium oxidation (anammox), dissimilatory nitrite reduction to ammonium (DNRA) and nitrogen fixation. Genomic potential for aerobic ammonia oxidation was dominated by *Thaumarchaeota* while bacterial *amoA* genes were scarce at all sediment depths. In general, phylogenetic composition of core microbial communities correlated well with biogeochemical characteristics of particular depths. Moreover, despite their lower abundance in Bothnian Sea sediment, we detected a higher diversity of anammox bacteria in metagenomes than with PCR-based technique. Our metagenome results reveal the quantitative importance of various N-cycle driving processes and highlight the advantage in detection of novel microbial key players which might be overlooked by using traditional PCR-based methods.

INTRODUCTION

The biogeochemical nitrogen cycle (N-cycle) is among the key element cycles in nature. N is a major component in building blocks for life (amino acids, nucleic acids and metabolites) and N-oxides are used as alternate electron acceptors in anaerobic respiration. The biggest pool of nitrogen is air, which comprises the most stable and inert form – N_2 (Robertson & Vitousek, 2009). It enters the reactive

nitrogen pool as ammonia via biological nitrogen fixation by nitrogenase (Nif). This phylogenetically deeply rooted and highly regulated protein is widespread among both bacteria and archaea (Postgate, 1998) and the *nifH* gene is widely used as biomarker to track this process in the environment (Gaby & Buckley, 2012). Ammonium can then either be directly assimilated into biomass or nitrified to nitrite by ammonia oxidizing bacteria (AOB) and archaea (AOA). Aerobic ammonia oxidation is catalyzed by ammonia monooxygenase (Amo) (Norton *et al.*, 2002, Klotz *et al.*, 2006), an enzyme only distantly related between AOB and AOA (Walker *et al.*, 2010), with the *amoA* gene – coding for the beta-subunit of the enzyme – being used as the biomarker for ecological studies. Although being structurally different, both AOB- and AOA-specific Amo oxidizes ammonium to hydroxylamine (Arp *et al.*, 2002, Vajrala *et al.*, 2013), the fate of which has been well investigated in AOB but is still elusive in AOA. AOB use hydroxylamine oxidoreductase (Hao) – a multi-heme cytochrome *c* protein – to oxidize hydroxylamine further to nitrite (Arp *et al.*, 2002). The use of *hao* gene as biomarker has been explored previously (Schmid *et al.*, 2008), but is complicated by divergent multiple gene copies in some genomes. AOA seem to utilize a novel so far unknown mechanism for hydroxylamine oxidation. Their genomes lack multi-heme cytochrome *c* proteins, but instead harbor a great variety of multi-copper oxidase (Mco) encoding genes. These Mco have been implicated in hydroxylamine oxidation (Hallam *et al.*, 2006, Walker *et al.*, 2010). In the presence of oxygen, nitrite can be further oxidized to nitrate by nitrite oxidizing bacteria (NOB). NOB are mostly aerobic organisms that convert nitrite to nitrate by a molybptopterin iron-sulfur containing nitrite:nitrate oxidoreductase (Nxr), an enzyme phylogenetically and structurally related to membrane-bound nitrate reductase (Nar). Nitrate is the most oxidized and more stable form of reactive nitrogen in the environment. When oxygen becomes limiting, nitrogen oxides can be respired during denitrification – a sequential reduction process which proceeds via NO and N_2O to molecular nitrogen, catalyzed by respiratory membrane-bound or periplasmic molybdenum-containing nitrate reductases (Nar, Nap), copper- or iron-containing nitrite reductases (NirK, NirS), quinol- or cytochrome-dependent nitric oxide reductases (Nor) and copper-containing nitrous oxide reductase (Nos), respectively. A recent discovery of nitrite-dependent anaerobic methane oxidation (N-AOM) has indicated a presence of an alternative mechanism of denitrification in which N_2O as an intermediate is bypassed and NO is directly converted to N_2 by simultaneously producing molecular oxygen which is used for methane oxidation (Ettwig *et al.*, 2010). The genome of the

responsible bacterium was shown to encode alternative Nor-like proteins, designated as nitric oxide dismutases (Nod) (Ettwig *et al.*, 2012). In the environment, denitrifying organisms compete with anaerobic ammonia oxidizers (anammox) and dissimilatory nitrite/nitrate reducers to ammonium (DNRA with *nrfA* gene as diagnostic marker) for the electron acceptor. Thus, the fate of nitrite/nitrate depends on various environmental parameters which favor a particular process (Kraft *et al.*, 2014). While denitrification is a phylogenetically fairly widespread ability and usually requires organic carbon, reduced sulfur compounds or hydrogen as electron donors (Zumft, 1997), anammox is a unique process which combines nitrite and ammonium to N_2 as the end product (Strous *et al.*, 2006). The anammox reaction proceeds via nitric oxide (NO) and hydrazine (N_2H_4) as intermediates with hydrazine synthase (Hzs) being the key enzyme and *hzsA* gene being used as biomarker for environmental studies (Kartal *et al.*, 2011, Harhangi *et al.*, 2012). Anammox seems to have evolved once and is restricted to a monophyletic order of *Brocadiales* within the *Planctomycete* phylum. Extensive research in the past decade has revealed the key role of anammox bacteria for nitrogen cycling in a wide range of habitats. It has been estimated that up to half of reactive nitrogen in anoxic basins and marine oxygen minimum zones – hot spots of nature's biogeochemical nitrogen cycling – is lost due to anammox activity (Devol, 2003, Kuypers *et al.*, 2003, Kuypers *et al.*, 2005, Lam *et al.*, 2009). Environmental studies investigating the diversity and activity of anammox bacteria have shown that marine environments are inhabited by representatives of the *Scalindua* genus whereas freshwater and brackish ecosystems can harbor a wider diversity including the known genera *Jettenia*, *Brocadia*, and *Kuenenia*, (Kuypers *et al.*, 2003, Schmid *et al.*, 2007, Dale *et al.*, 2009, Humbert *et al.*, 2009, Hirsch *et al.*, 2011, Hu *et al.*, 2011). Marine coastal sediments are of particular interest for biogeochemical element cycling as they represent hot-spots of microbial activity due to excess availability of organic matter. The solid matrix of sediments limits the diffusion of substrates and so, through biotic and abiotic reactions, redox gradients establish and spatially separate aerobic and anaerobic metabolisms (Joye & Anderson, 2008). The Bothnian Sea is located in the northern part on the Baltic Sea – one of the world's biggest anoxic basins which has been heavily impacted by eutrophication in the previous decades (Lundberg *et al.*, 2009). However, due to physical factors such as topography, water exchange dynamics and weak stratification, the Bothnian Sea has been influenced to a far lesser degree of eutrophication and is generally considered as an oligotrophic ecosystem (Lundberg *et al.*, 2009). The main input sources for organic matter in the Bothnian Sea were

calculated to be riverine and from intrusions from highly eutrophic Baltic Proper (Algesten *et al.*, 2006). Nitrate penetration depth is restricted to the upper centimeter (Slomp *et al.*, 2013). Studies about nitrogen cycling in Bothnian Sea sediments are scarce and previous activity measurements and flux calculations have indicated that several N-cycle processes might be involved in reactive nitrogen loss from the ecosystem (Stockenberg & Johnstone, 1997). Knowledge about the genomic potential of the microbial community of the sediment involved in N-cycle is lacking. In this study we investigated the phylogenetic composition and metagenomic potential of the *in-situ* microbial community with respect to the biological N-cycle in the Bothnian Sea sediment at three specific depths. DNA was extracted and sequenced by Ion Torrent technology. Curated data sets of the diagnostic N-cycle proteins were used to estimate the abundance and diversity of the various reactions with specific emphasis on the anammox process.

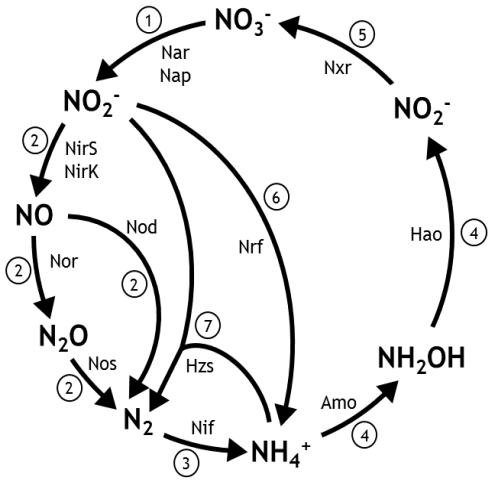

Figure 1: Biological N-cycle illustrating major metabolic processes (number-coded) with associated known key enzymes. Abbreviations: Nar/Nap, dissimilatory nitrate reductase; NirK/NirS, dissimilatory NO-forming nitrite reductase; Nor, nitric oxide reductase; Nod, nitric oxide dismutase; Nos, nitrous oxide reductase; Nif, nitrogenase; Amo, ammonia monooxygenase; Hao, hydroxylamine oxidoreductase; Nxr, nitrite oxidoreductase; Nrf, dissimilatory ammonia-forming nitrite reductase; Hzs, hydrazine synthase. Number-coded metabolic processes: 1, nitrate reduction; 2, denitrification; 3, nitrogen fixation; 4, aerobic ammonia oxidation; 5, aerobic nitrite oxidation; 6, dissimilatory nitrite reduction to ammonium (DNRA); 7, anaerobic ammonia oxidation.

MATERIALS & METHODS

Sampling site and core processing

Sediment cores were taken in the Bothnian Sea at sampling site USB5 during the R/V *Aranda* cruise in August 2012 (location, sampling procedure and core storage

are described in Egger et al. 2014). Processing of the core dedicated to molecular analysis is described in (Rasigraf et al., in preparation, Chapter 5).

DNA isolation and sequencing

DNA isolation and sequencing were performed as previously described (Rasigraf et al., in preparation, Chapter 5).

Polymerase chain reaction (PCR)

PCR reactions were performed to amplify the *hzsA* gene specific to anammox bacteria in samples from 0 to 25 cmbsf on each 2.5 cm interval sample individually. PCR reactions were composed as previously described (Harhangi *et al.*, 2012). Following primer pairs were used: hzsA_757F and hzsA_1829R to cover the diversity of known freshwater anammox bacteria, and hzsA_757F Scalindua and hzsA_1829R Scalindua to cover known marine anammox bacteria (Harhangi *et al.*, 2012). PCR was performed in a thermocycler (Professional thermal cycler, Biometra, Jena) with following parameters: initial denaturation for 4 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 54° to 66°C (parallel PCR reactions were performed at different annealing temperatures), elongation for 2 min at 72°C, and final elongation for 10 min at 72°C. PCR products were checked with gel electrophoresis. Due to low final concentration of PCR products, a semi-nested PCR was performed with hzsA_1600F Scalindua and hzsA_1829R Scalindua primers (Harhangi *et al.*, 2012). PCR reaction products for each depth sample were pooled and subjected to gel electrophoresis. Bands of correct size were cut and purified from gels with the Gene Jet Gel extraction kit (Thermo Scientific, Waltham, USA) following manufacturer's instructions. A semi-nested PCR was performed with purified PCR products as template under following conditions: initial denaturation for 4 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 56°, elongation for 2 min at 72°C, and final elongation for 10 min at 72°C. Ligation, transformation and cloning of PCR products were performed as previously described (Harhangi *et al.*, 2012).

Metagenome analysis

The 16S rRNA gene-based phylogenetic characterization of *in-situ* microbial community at USB5 sampling site was described previously (Rasigraf et al., in preparation, Chapter 5). Functional gene analysis was performed as previously described (Lüke *et al.*, 2015). The following functional genes were analyzed: *narG/napA* (dissimilatory nitrate reductases), *hzsA* (hydrazine synthase), *hao* (hydroxylamine oxidoreductase), *amoA* (ammonia monooxygenase), *nor* (nitric oxide reductase), *nirS/nirK* (dissimilatory NO-forming nitrite reductases), *nifH* (nitrogenase), *nosZ* (nitrous oxide reductase), *nrfA* (dissimilatory ammonia-forming nitrite reductase). The gene databases used for blast analysis were described previously (Lüke *et al.*, 2015). Data analysis and normalization was performed as previously described (Rasigraf et al., in preparation, Chapter 5).

RESULTS & DISCUSSION

N-cycle metabolism related gene analysis

Detailed 16S rRNA gene analysis of the Bothnian Sea sediment at USB5 sampling site was described previously (Rasigraf et al., in preparation, Chapter 5). Therefore, we focused here on combined functional biomarker and selective 16S rRNA gene analysis relevant to particular metabolic processes involved in N-cycle. General overview of normalized functional gene counts is shown in Tab. 1.

Table 1: Overview over blastx-based functional marker gene analysis of the Bothnian Sea sediment transect. Most abundant phylogenetic groups with assigned normalized gene read counts (nrc, counts per 1 Mio reads per 1 kb) and corresponding metabolic processes are shown.

Phylogenetic assignment		**Depth (cmbsf, cm below sediment surface)**		
Phylum	**Order/group**	**0-2.5**	**5-12.5**	**30-35**
Total prokaryotic 16S rRNA		337	338	313
N_2 fixation				
nifH (total)		10.1	6.1	1.7
Proteobacteria	*Methylococcales*	7.6	0	0
	Chromatiales	1.1	0	0
Euryarchaeota	*Methanomicrobia*	0	3.1	1.4

Nitrate reduction		55.1	33.9	3.2
narG (total)				
Proteobacteria	*Rhodocyclales*	3.9	4.7	0
	Methylococcales	2.6	0.2	0
	Desulfobacterales	0.8	2.3	0.1
	Desulfuromonadales	3.0	1.1	0.1
Deinococci	-	1.0	1.8	0.1
Candidate division OP3	*Omnitrophicaceae*	9.1	3.8	0.9
Euryarchaeota	*Haloarchaea*	2.8	2.9	0.3
napA (total)		29.6	12.4	0.7
Bacteroidetes	*Flavobacteriia*	1.6	0.3	0
Planctomycetes	-	0.9	0.8	0
Proteobacteria	*Burkholderiales*	0.4	0.7	0
	Desulfuromonadales	0.6	0.9	0.1
	Desulfobacterales	1.3	1.2	0
	Campylobacterales	3.0	0.9	0
	Alteromonadales	2.8	0.1	0.2
Nitrite reduction				
nirS (total)		73.8	52.9	4.6
Proteobacteria	*Rhodobacterales*	2.4	2.8	0.2
	Burkholderiales	3.3	3.2	0
	Hydrogenophilales	2.9	4.3	0
	Rhodoyclales	3.2	1.7	0.4
	Alteromonadales	3.2	1.1	0
	Chromatiales	2.4	1.3	0.4
	Methylococcales	8.1	0.3	0
	Oceanospirillales	4.9	4.7	0.2
nirK (total)		91.5	68.2	23.9
Actinobacteria	-	3.4	7.0	0
Chloroflexi	-	1.1	2.9	0
Proteobacteria	*Rhizobiales*	3.5	2.7	0.5
	Methylococcales	4.3	0	0
Crenarchaea	*Thaumarchaeota*	39.4	11.3	14.5
Nitric oxide reduction				
nor (total)		37.7	28.9	5.0
Bacteriodetes	*Flavobacteriia*	3.1	3.7	0.9
Planctomycetes	-	2.0	0.9	0
Proteobacteria	*Burkholderiales*	2.1	0	0
	Desulfuromonadales	2.0	3.4	0.5
	Myxococcales	2.1	1.1	0.2
	Methylococcales	2.0	0.6	0
	Strain HdN1-like	2.9	2.2	0
Spirochaetes	-	2.0	0	0

Nitrous oxide reduction				
nosZ (total)		52.5	40.4	7.5
Bacteriodetes	*Flavobacteriia*	12.2	5.4	1.4
	Order II Insertae Sedis	2.7	2.4	0.3
	Cytophagia	3.1	1.4	0.3
Chlorobi	*Ignavibacteriae*	1.6	2.1	0.8
Chloroflexi	-	2.1	1.0	0.2
Proteobacteria	*Myxococcales*	1.2	2.1	0.2
Hydroxylamine oxidation				
hao (total)		33.4	52.0	6.4
Bacteriodetes/Chlorobi	-	1.3	2.8	0.5
Deinococci	-	1.3	1.4	0.4
Planctomycetes	-	3.9	8.9	0.7
Proteobacteria	*Desulfobacterales*	1.4	8.4	0.7
	Desulfovibrionales	1.0	2.8	0.2
	Desulfuromonadales	1.2	1.7	0.2
	Myxococcales	2.9	4.0	0.2
	Syntrophobacterales	1.9	1.4	0.2
	Alteromonadales	1.4	2.5	0
	Vibrionales	2.2	1.4	0
Candidate division OP3	*Omnitrophicaceae*	1.4	1.3	0.5
Anammox				
hzsA (total)		0.6	2.4	0
Planctomycetes	*Brocadiales*	0.6	2.4	0
Aerobic ammonia oxidation				
amoA (total)		22.9	8.9	6.2
Crenarchaea	*Thaumarchaeota*	21.7	8.9	5.2
Proteobacteria	-	1.0	0	1.0

Dissimilatory nitrate reduction/anammox: nitrate reductase (*narG/napA*)

The ability to perform nitrate reduction is a widespread trait among facultative and strictly anaerobic bacteria, archaea and some eukarya. Nitrate is a fairly common electron acceptor in many ecosystems at the oxic/anoxic interface and is readily available when oxygen becomes scarce. Nitrate is reduced to nitrite by a dissimilatory nitrate reductase which occurs in two forms: membrane-bound (encoded by Nar gene cluster) and soluble periplasmic (encoded by Nap gene cluster) (Zumft, 1997), with genes encoding subunits NarG and NapA being used as phylogenetic markers for environmental studies. The product of nitrate reduction – nitrite – is a central intermediate within the biogeochemical nitrogen cycle. It can be reduced to dinitrogen gas or any intermediate nitrogen oxide during denitrification,

to ammonium via dissimilatory nitrite reduction to ammonium (DNRA) or to dinitrogen gas via anammox. Furthermore, it can be oxidized back to nitrate during the last step of nitrification by nitrite oxidoreductase (Nxr) – a homologue enzyme to Nar.

The nitrate reducing community harboring *narG* showed a clear stratification within the sediment transect. Most reads (55 nrc) were found in the upper 0-2.5 cmbsf zone, where oxygen and nitrogen oxides were still available for respiration. Roughly half of all reads were assigned to phylum *Proteobacteria* with the most dominant groups belonging to *Methylococcales* (5%), *Desulfuromonadales* (5%) and *Rhodocyclales* (7%). Other dominant *narG*-containing groups were assigned to candidate division OP3 (*Omnitrophicae*, 17%) and *Haloarchaea* (5%). In the 5-12.5 cmbsf zone (34 nrc), the major groups containing *narG* were comprised by *Rhodocyclales* (14%), *Desulfobacterales* (7%), *Deinococcales* (5%), candidate division OP3 (*Omnitrophicae*, 11%) and *Haloarchaea* (9%). Notably, the change in biogeochemical parameters towards the absence of oxygen and dominance of sulfur cycle in this zone was accompanied with the shift in *narG*-harboring community towards sulfate reducing bacteria (*Desulfobacterales*) and decline in methane oxidizing bacteria (*Methylococcales*) and iron reducing bacteria (*Desulfuromonadales*). The deeper layer of 30-35 cmbsf was characterized by a stark decrease in overall *narG* read numbers (3 nrc) most of which were assigned to candidate division OP3 (*Omnitrophicae*, 30%) with the remaining reads being distributed among *Proteobacteria*. These results were congruent with all other observations and indicated the low potential and need for nitrate reduction in this depth.

Conspicuous was the dominance of candidate division OP3 within the *narG*-harboring community in all depths. There are so far no cultured representatives from this group and their metabolic potential remains elusive. 16S rRNA gene information revealed that candidate division OP3 belongs to the *Planctomycetes/Verrucomicrobia/Clamydiae* (PVC) superphylum and it was suggested that members of this group are most likely anaerobes thriving in marine sediments, lakes and aquifers (Glöckner *et al.*, 2010, Ragon *et al.*, 2013). The currently available single-cell genome of one of the members of this group, *Omnitrophus fodinae* SCGC AAA011-A17, revealed the presence of genes coding for respiratory nitrate reductase and heme/copper-type cytochrome/quinol oxidase. Other genes of the denitrification pathway could not be detected. This points to a facultative anaerobic lifestyle of this organism.

The *napA* gene reads were less abundant than *narG*. The distribution followed the pattern of the *narG* with most reads detected in 0-2.5 cmbsf (30 nrc), where most abundant groups belonged to *Flavobacteriia* (5%), *Campylobacterales* (10%) and *Alteromonadales* (10%). The distribution of *napA* in 5-12.5 cmbsf (12 nrc) was more evenly spread with most abundant groups belonging to *Planctomycetes* (6%), *Burkholderiales* (5%), *Desulfobacterales* (10%), *Desulfuromonadales* (7%) and *Campylobacterales* (7%). In 30-35 cmbsf, *napA* was with 1 nrc only of minor importance consistent with the finding on *narG* and other N-cycle genes.

Denitrification/anammox: dissimilatory NO-forming nitrite reductase *(nirS;nirK)*

Nitrite reductase is the key enzyme within the denitrification process in which it catalyzes the conversion of nitrite to nitric oxide – the defining step of the process. There are two forms of nitrite reductase described in denitrification so far: iron-containing cytochrome *cd1* (NirS) and copper-dependent (NirK) forms. Both enzymes are structurally and phylogenetically dissimilar, but serve the same physiological function (Zumft, 1997). It has been believed that both genes were mutually exclusive (Averill, 1996), however, a recent study has found both genes in the genome of *Methylomonas denitrificans* strain FJG1 (Kits *et al.*, 2015).
Numerous studies have used *nirS/nirK* genes for environmental studies for evaluation of diverse habitats for their denitrification potential and phylogeny of associated denitrifiers (Braker *et al.*, 2000, Oakley *et al.*, 2007).
The distribution of both *nirK* and *nirS* gene reads followed the same trend as for other N-cycle related genes with decreasing abundance with increasing depth (Tab.1). The vast majority (43%) of detected *nirK* reads in 0-2.5 cmbsf were assigned to *Thaumarchaeota*. Other abundant groups were assigned to *Methylococcales* (5%), *Rhizobiales* (4%) and *Actinobacteria* (4%). The most abundant *nirK* groups in 5-12.5 cmbsf depth belonged to *Thaumarchaeota* (17%), *Actinobacteria* (10%), *Chloroflexi* (4%) and *Rhizobiales* (4%), indicating a clear community shift with the increasing depth. Interestingly, deeper in the sediment transect (30-35 cmbsf), the thaumarchaeal *nirK* seemed to increase in abundance again (61%) and the total normalized number was even higher than in 5-12.5 cmbsf. The available genome information of AOA has confirmed the presence of multiple gene copies of multicopper oxidoreductase type nitrite reductases (Mco), a trait which seems to be highly conserved among AOA (Lund *et al.*, 2012). This pointed

to the ability of AOA to nitrifier denitrification which is probably responsible for the archaeal N_2O production in the ocean (Santoro *et al.*, 2011). The physiological role of AOA NirK is still unclear, it might be involved in detoxification of nitrite or use of nitrite as the alternative electron acceptor to oxygen under hypoxia (Walker *et al.*, 2010). Environmental studies have revealed wide distribution of thaumarchaeal *nirK* genes in marine water columns and sediments (Venter *et al.*, 2004, Yakimov *et al.*, 2011, Lund *et al.*, 2012), also indicating distinct communities between water columns and sediments (Lund *et al.*, 2012). The calculated ratios of *nirK* to group MG-1 16S rRNA showed a decrease with increasing depth (3.5 in 0-2.5 cmbsf, 2.7 in 5-12.5 cmbsf and 0.9 in 30-35 cmbsf). This again pointed to distinct populations of MG-1 archaea with potentially different modes of metabolism. Normalized *nirK/amoA* gene ratios were calculated to be 1.8 for 0-2.5 cmbsf, 1.3 for 5-12.5 cmbsf and 2.8 for 30-35 cmbsf.

Proteobacteria was the most dominant bacterial phylum harboring *nirS*-like genes in 0-2.5 cmbsf (48%), 5-12.5 cmbsf (41%) and 30-35 cmbsf (50%). *Methylococcales* comprised with 11% of all reads the most abundant group in 0-2.5 cmbsf. This finding corresponded with widespread occurrence of *Methylococcales* bacteria in this depth as inferred from 16S rRNA and findings of other genes of the denitrification pathway (e.g. *narG*, *nor, nirK*). Available genome data of MOB confirm widespread presence of genes involved in denitrification and their contribution to N_2O production (Stein & Klotz, 2011). Recent work has shown that *Methylomonas* species encode and express denitrification genes when exposed to nitrate and are able to link the reduction of nitrate to the oxidation of methane under hypoxia in a bioenergetically favorable manner (Kits *et al.*, 2015).

Denitrification/anammox: dissimilatory nitric oxide reductase (*nor*)

Nitric oxide reductases are membrane-bound heme-copper oxidase proteins catalyzing reduction of NO to N_2O. The step of NO reduction is of high biological significance as a highly toxic and reactive gas is reduced to a non-toxic form. Thus, NO-reductases are not exclusively restricted to denitrifying organisms but are widely spread among organisms for detoxification reasons (Shiro, 2012).

So far, two forms of respiratory nitric oxide reductases (Nor) have been characterized: a cytochrome *c*-dependent (NorBC) and a quinol-dependent (qNor) form (Shiro, 2012). However, with the advance in metagenomics sequencing, many novel genes encoding alternative Nor enzymes have been identified (Pace et al.,

unpublished). In particular, a recent discovery of nitrite-dependent AOM (N-AOM) has revealed that the crucial oxygen-generating step from NO is most likely to be catalyzed by a NO-dismutase (Nod), an enzyme structurally related to qNor (Ettwig *et al.*, 2012). Genes with similar sequence modifications have since been identified in several other organisms including γ-proteobacterial strain HdN1 (Zedelius *et al.*, 2011) and phylum *Bacteroidetes* belonging *Mariniradius saccharolyticus* (Bhumika *et al.*, 2013), *Cecembia lonarensis* (Kumar *et al.*, 2011) and *Muricauda ruestringensis* (Bruns *et al.*, 2001).

nor-like genes were detected in all depths over the sediment transect with decreasing abundance with increasing depth (38 nrc in 0-2.5 cmbsf, 29 nrc in 5-12.5 cmbsf and 5 nrc in 30-35 cmbsf). In 0-2.5 cmbsf, no clear dominance of any phylogenetic group could be observed. Here, 5-6% of all *nor*-like reads were assigned to *Spirochaetales*, *Methylococcales*, *Desulfuromonadales*, *Myxococcales*, *Burkholderiales* and *Planctomycetes*. Notably, two groups which possessed the most *nor*-like reads showed nearest identity to *Flavobacteriia* and strain HdN1. Several *nor*-like reads from the *Flavobacteriia* order revealed highest identity to *Muricauda ruestringensis* and other organisms containing alternative *nor*-like genes with sequence features found in Nod proteins. Also, sequences resembling nearest identity to strain HdN1 *nod* sequence pointed to an abundant population of bacteria containing Nod-like proteins. However, no 16S rRNA genes affiliated with either *M. oxyfera* or strain HdN1 could be detected pointing to novel *nod*-like gene harboring bacterial groups. The observation of abundant *nod*-like gene reads would be congruent with biogeochemical data indicating the presence of methane and nitrogen oxides in 0-2.5 cmbsf for N-AOM. However, an alternative Nod-catalyzed metabolism might be possible. Also, similar *nor*-containing phylogenetic groups were identified in 5-12.5 cmsbf, however, the abundances differed to 0-2.5 cmbsf. In particular, *nor*-containing *Desulfuromonadales* and *Flavobacteriia* populations increased to 12 and 13%, respectively. The abundance of *nod*-like gene containing strain HdN1-like population remained similar (7%). This finding showed that despite the absence of nitrogen oxides in 5-12.5 cmbsf, *nod*-like genes were still relatively abundant. This pointed to either a non-active population of bacteria containing Nod proteins or an active population using Nod-like proteins to perform an alternative metabolism.

Denitrification: nitrous oxide reductase (*nosZ*)

Nitrous oxide reductase is a soluble homodimeric heme-copper oxidase which catalyzes the final step in denitrification sequence, the conversion of N_2O to molecular nitrogen (Zumft, 1997). First characterized nitrous oxide reductases originated from denitrifying cultures (Zumft, 1997), but it became evident that the capacity to reduce N_2O to N_2 was not a defining step of denitrification. In fact, many denitrifiers lack the necessary genes and stop the process at the level of N_2O, thereby contributing to its emissions to the atmosphere (Beaulieu *et al.*, 2011). Previous studies have shown that the capacity of denitrifying communities to reduce N_2O might be regulated by environmental parameters such as availability of nitrate, organic carbon or pH (Weier *et al.*, 1993, Van Den Heuvel *et al.*, 2011). Recent genomic surveys have pointed to a much wider distribution of atypical *nos* genes which are not restricted to denitrifiers, but are also present in non-denitrifiers and DNRA-performing organisms (Sanford *et al.*, 2012). Moreover, these abundant atypical nitrous oxide reductases were shown to be functional (Sanford *et al.*, 2012). Our metagenome analysis revealed high abundance of *nosZ*-encoding gene reads (53 in 0-2.5, 40 in 5-12.5 and 8 in 30-35 cmbsf nrc, respectively) which was approximately within the same range as the abundance of *narG*- and and *nirS*-encoding gene reads. Most dominant phylum harboring *nosZ* gene in 0-2.5 cmbsf was assigned to *Bacteroidetes* with *Flavobacteriia* (23%), *Cytophagia* (6%) and Bacteroidetes Order Insertae II Sedis (5%) groups being the most dominant. Similar bacterial groups were dominating the 5-12.5 cmbsf depth, however the abundances changed. The most prominent change was observed in *nosZ* abundance of *Flavobacteriia* which decreased to 13% and *Myxococcales* which increased to 5%. *Flavobacteriia* was with 11% of all assigned reads also the most dominant *nosZ*-harboring group in 30-35 cmbsf.
These results pointed to widespread capacity for nitrous oxide reduction in the Bothnian Sea sediment along the whole sediment transect with molecular nitrogen being the most likely product of denitrification.

Nitrogen fixation: nitrogenase (*nifH*)

Nitrogenase is the only known enzyme system capable of reduction of molecular nitrogen to biologically available form as ammonia. It consists of two components, a MoFe, VFe or FeFe metalloprotein and a second Fe metalloprotein (Eady, 1996,

Rees & Howard, 2000). Nitrogenase (encoded by *nif* gene cluster) has a fairly wide distribution within bacteria, but within archaea was only shown to be present in methanogenic/-trophic *Euryarchaeota* (Dos Santos *et al.*, 2012, Boyd & Peters, 2013).

Analysis for nitrogen fixation potential in the sediment transect revealed low abundance of *nifH* gene reads in all sediment depths (10 nrc in 0-2.5 cmbsf, 6 nrc in 5-12.5 cmbsf and 2 ncr in 30-35 cmbsf) as well as clear differences in microbial populations responsible for the process. The majority (75%) of detected *nifH* gene reads in 0-2.5 cmbsf belonged to the order *Methylococcales* with *Methylobacter* spp. as the most dominant genus (up to 100% identity on protein level). This dominance of *Methylococcales* was congruent with 16S rRNA gene data. Moreover, the majority of gene reads encoding the particulate methane monooxygenase in this sediment layer were assigned to *Methylobacter* (Rasigraf et al., in preparation, Chapter 5), further confirming its dominance at the oxic/anoxic interface. Only one read was assigned to *δ-Proteobacteria* (95% protein identity with *Desulfocapsa sulfexigens*). The remaining 14% were assigned to orders *Chromatiales* and *Aeromonadales* within *γ*-Proteobacteria.

The *nifH* inventory in 5-12.5 cmbsf revealed a shift in nitrogen fixing population towards *Methanomicrobia* (>55% of all reads). Two reads shared an over 95% protein identity to *Methanosarcina acetivorans*, the phylogenetic identity of remaining reads within *Methanomicrobia* could not be clearly resolved due to lower identity to known species. The remaining nitrogen fixing population (25%) was represented by sulfate reducing bacteria (SRB) from *δ-Proteobacteria*, *Nitrospirae* and *Firmicutes*.

The phylogenetic affiliation of *nifH* reads within the sediment transect reflected the dominance of major functional microorganism groups in each particular depth observed from the 16S rRNA analysis (Rasigraf et al., in preparation, Chapter 5).

Aerobic ammonium oxidation: ammonia monooxygenase (*amoA*)

Ammonia monooxygenase catalyzes oxygen-dependent oxidation of ammonia to hydroxylamine. Structurally, ammonia monooxygenase (Amo) is related to methane monooxygenase (MMO), both exhibit *vice versa* activity, although at lower rates (Bédard & Knowles, 1989). For decades, the ability to oxidize ammonia was considered to be restricted to two monophyletic groups within *Proteobacteria*: *β*-Proteobacteria including genera *Nitrosomonas*, *Nitrosospira* and *Nitrosovibrio*, and

γ-Proteobacteria including genus *Nitrosococcus* (Teske *et al.*, 1994). Beginning in 2006, studies reported that a distinct mesophilic group within *Crenarchaeaa* (*Thaumarchaeaota*) was able to oxidize ammonium to nitrite as end product. These archaea contained a homolog of Amo only distantly related to its bacterial counterpart (Könneke *et al.*, 2005). Since then, numerous studies have shown the ubiquity of ammonia oxidizing archaea (AOA) in both terrestrial and marine environments, and their tremendous importance for the N-cycle (Francis *et al.*, 2005, Beman *et al.*, 2008).

At our sampling site, *amoA* gene sequences were found in all three analyzed depths, with decreasing abundance with increasing sediment depth (Tab.1). The highest abundance was observed in 0-2.5 cmbsf, sediment zone where oxygen and ammonium still co-occured, thus providing substrates for ammonia oxidizers. Taxonomic assignment revealed that the majority of *amoA* reads was assigned to *Thaumarchaeota* (Tab. 1), which strongly pointed to their dominance in aerobic ammonia oxidation process in the Bothnian Sea sediment. All archaeal sequences fell within the Marine Group 1.1a. Reads revealed high similarity to sequences found in ecosystems ranging from fully marine over brackish to terrestrial. The closest cultured representatives were *Nitrosopumilus* and *Nitrosoarchaeum* spp. *AmoA* reads were also detected in the S-AOM zone of 5-12.5 cmbsf and methanogenic zone of 30-35 cmbsf, where electron acceptors other than sulfate or CO_2 were not measurable. Most sequences detected in the deeper sediment revealed high similarity to sequences found in marine, estuarine and freshwater habitats.

Based on these observations, it was evident that AOA were not restricted to sediment zones where oxygen was still present, but rather occurred in all analyzed sediment depths. This corresponded with 16S rRNA gene results of marine group I (MG-I) Thaumarchaeota which were detected in all depths and decreased in abundance with increasing sediment depth. The ratio of *amoA* to 16S rRNA of MG-I was approximately 2 for the upper two depths and 0.3 for 30-35 cmbsf. Currently available genomic information of archaeal ammonia oxidizers shows *amoA* and 16S rRNA being single copy genes in sequenced genomes of thaumarchaeal ammonium oxidizers. However, several previous studies have reported *amoA*/16S rRNA ratios to be higher than 1 and speculated on several *amoA* copies in AOA genomes (Beman *et al.*, 2008, Santoro *et al.*, 2010, Lund *et al.*, 2012), which might be the case for novel sedimentary AOA. The occurrence of group MG-I *Thaumarchaeota* in anoxic sediment layers has been reported previously (Sørensen *et al.*, 2004, Roussel *et al.*, 2009, Jorgensen *et al.*, 2012). It has been speculated that electron acceptors other

than oxygen might be used for ammonia oxidation in these organisms, or that ammonia monooxygenase might serve a different function than ammonia oxidation (Mußmann *et al.*, 2011, Jorgensen *et al.*, 2012). It has also been shown recently that not all *Thaumarhaeota* are capable of ammonia oxidation, but are metabolically more flexible and can grow with organic nitrogen substrates (Weber *et al.*, 2015).

Aerobic ammonium oxidation/anammox: hydroxylamine oxidoreductase (*hao*)

Hydroxylamine oxidoreductase is the second key enzyme during aerobic ammonia oxidation where it catalyzes the oxidation of hydroxylamine to nitrite in AOB (Igarashi *et al.*, 1997). Hao from *Nitrosomonas europaea* is a homotrimeric protein with each monomer containing 7 *c*-type hemes and one P460 heme, the latter being involved in catalysis and covalent binding of the adjacent monomer (Igarashi *et al.*, 1997, Cedervall *et al.*, 2009). In AOB, Hao forms a functional complex with cytochrome C_{554} and cytochrome Cm_{552} where two electrons are shuttled to AMO via C_{554} and other two electrons to cytochrome *c* oxidase via Cm_{552}. This functional unit is conserved in all AOB on the genomic level where HAO, C_{554}, Cm_{552} encoding genes are located in a single operon (Arp *et al.*, 2007).
Besides AOB, several organisms with diverse phylogenetic backgrounds were shown to harbor octa-heme Hao-like proteins. It has been hypothesized that those proteins are capable of both reductive and oxidative N-compound transformation in the N-redox state between -3 and +3 (Kartal *et al.*, 2011, Maalcke *et al.*, 2014). Previous studies have shown octa-heme cytochrome *c* protein mediated reduction of nitrite and hydroxylamine to ammonium in *Shewanella oneidesis* MR-1 (Atkinson *et al.*, 2007), reduction of nitrite to ammonium in *Thioalkalivibrio nitratireducens* (Polyakov *et al.*, 2009), oxidation of hydrazine to molecular nitrogen (Kartal *et al.*, 2011) and reduction of hydroxylamine to nitric oxide in anammox bacteria (Maalcke *et al.*, 2014).
hao-like gene reads and its multi-heme cytochrome *c* homologs were detected in all depths of the sediment transect, however, unlike other N-cycle genes, more reads were detected in 5-12.5 cmbsf than in the 0-2.5 cmbsf (52 vs. 33 nrc). This higher abundance was mostly attributable to the dominance of anammox (order *Brocadiales*) and sulfate reducing bacteria (orders *Desulfobacterales* and *Desulfovibrionales*). Based on available genome information, anammox bacteria contain up to 10 divergent *hao*-like gene paralogs (Strous *et al.*, 2006, Kartal *et al.*, 2012, van de Vossenberg *et al.*, 2013), which would explain the relative high

abundance of *hao*-like genes affiliated to anammox bacteria in 5-12.5 cmbsf. This observation was in congruence with 16S rRNA and *hzsA* gene data which all indicated higher abundance of anammox bacteria in this depth interval. The relative abundance of anammox related *hao*-like reads within 0-2.5 cmbsf sample was 12% and increased to 18% in 5-12.5 cmbsf. Blast-based phylogenetic analysis of the *hao*-like reads assigned to anammox bacteria revealed that *Kuenenia stuttgartiensis* was the most common next relative in 5-12.5 cmbsf (75%) with identities between 50 and 100%, the remaining reads revealed highest identity to *Scalindua* spp. (14%, identities between 54 and 100%), *Brocadia* spp. (9%, identities between 52 and 70%) and strain KSU-1 (2%, 58% identity). In contrast, the majority of reads assigned to anammox in 0-2.5 cmbsf resembled highest identity to *Scalindua* spp. (65%, accession nr. WP_034410018) with identities ranging between 54 and 100%, the rest was assigned to *Kuenenia* spp. (15%, identities between 47 and 65%), *Brocadia* spp. (7%, identities between 53 and 64%) and strain KSU-1 (11%, identities between 76 and 78%). These results demonstrated diversity differences in both depths and pointed to potentially novel anammox species, an observation which was supported by data derived from 16S rRNA and *hzsA* gene phylogeny. However, since the phylogenetic resolution was limited due to short read length the interpretation of data should be treated with care and needs further investigation.

Previous surveys revealed marine ecosystems to be dominated by anammox bacteria of the *Scalindua* genus and freshwater terrestrial habitats by the genera *Kuenenia*, *Brocadia*, *Jettenia*, *Anammoxoglobus* and strain KSU-1 (Kuypers *et al.*, 2003, Penton *et al.*, 2006, Galán *et al.*, 2009, Humbert *et al.*, 2009). Molecular studies based on amplification of anammox-specific 16S rRNA and functional genes have reinforced a hypothesis salinity being the major environmental factor shaping the community shifts between the dominance of either *Scalindua* or other anammox genera (Dale *et al.*, 2009, Hirsch *et al.*, 2011), the latter designated as “freshwater” anammox genera. However, physiological studies have shown that members of the genus *Kuenenia* can gradually be adopted to high salt concentrations in a bioreactor system (Kartal *et al.*, 2006), thus indicating that environmental parameters other than salinity might play a role in anammox distribution.

In general, highest proportion of *hao*-like gene reads in all depths was comprised by δ-Proteobacteria: 25% in 0-2.5 cmbsf, 35% in 5-12.5 cmbsf and 22% in 30-35 cmbsf, with *Desulfobacterales*, *Desulfovibrionales* and *Myxococcales* being the most abundant orders. Due to limited gene sequence information, accurate function predictions of detected *hao*-like genes fragments were not possible. However,

previous studies have shown several sulfate reducing bacteria (SRB), in particular within the δ-subdivision, to possess multi-heme cytochrome *c* proteins of the C_{554} and other families (Pereira *et al.*, 2011). In SRB, these proteins were speculated to be involved in respiration as in storage of electrons derived from periplasmic hydrogen oxidation (Heidelberg *et al.*, 2004), enzymatic metal reduction (Lovley *et al.*, 1993, Lovley & Phillips, 1994, Michel *et al.*, 2001), regulation (Pereira *et al.*, 2011), or detoxification (Greene *et al.*, 2003). To our knowledge, there is no evidence for *bona fide* hydroxylamine oxidoreductase proteins in these organisms.
Interestingly, *hao*-like gene reads affiliated with AOB were detected in low abundance in all analyzed depths with no difference between 0-2.5 and 5-12.5 cmbsf (3% and 2% within all *hao*-like reads, respectively). All AOB *hao*-like reads belonged to the order *Nitrosomonadales* within β-Proteobacteria. In addition to 16S rRNA and *amoA* gene data, this was another indication of AOB being not of high importance in ammonium oxidation at the USB5 sampling site.
In contrast to the two upper depths, *hao*-like genes were of much less importance in 30-35 cmbsf depth (6 nrc). This trend followed other N-cycle genes, which pointed to a much lower importance of nitrogen cycling in this depth.

Dissimilatory ammonia-forming nitrite reductase (*nrfA*)

Dissimilatory ammonia-forming nitrite reductase (Nrf) is a pentaheme cytochrome *c* protein catalyzing a single-step $6e^-$ reduction of nitrite to ammonia (Einsle, 2001). Nrf is the key enzyme during ammonification – a "shortcut" process in the N-cycle which directly links pools of nitrite and ammonium. This process is of particular importance for investigations of reactive nitrogen balance in anoxic ecosystems as it competes for nitrite with denitrification and anammox but unlike them does not contribute to net reactive nitrogen loss as N_2.
nrfA-like gene reads were detected in all depths of the sediment transect, however their abundance was considerably lower than of those involved in denitrification. Also, more *nrfA*-like gene fragments were detected in 5-12.5 cmbsf (16 ncr) than in 0-2.5 cmbsf (12 nrc). The most abundant groups possessing *nrfA* in 0-2.5 cmbsf belonged to *Desulfuromonadales* (15%), *Bacteroidetes*/*Chlorobi* (25%) and *Verrucomicrobia* (13%). In 5-12.5 cmbsf, verrucomicrobial *nrfA* could not be detected anymore, but *Bacteroidetes*/*Chlorobi* (24%) and *Desulfuromonadales* (13%) still comprised the most abundant *nrfA*-possessing groups. Here, *nrfA* fragments assigned to *Myxococcales* increased to 8%. Also in depth 30-35 cmbsf

most of the *nrfA*-like reads were assigned to *Bacteroidetes*/*Chlorobi* (70%). Thus, the *nrfA*-bearing *Verrucomicrobia* detected in 0-2.5 cmbsf might be adapted to higher sediment redox state and probably able to tolerate some oxygen.
Previous studies investigating DNRA in estuarine environments reported its relative importance in comparison to denitrification in organic carbon- and sulfide-rich sediments, speculating on inhibitory role of sulfide on denitrification (An & Gardner, 2002). Moreover, it has been speculated that salinity might play a crucial role for fate of nitrate reduction pathway, where denitrification was inhibited at higher salinities while DNRA was not affected (Giblin *et al.*, 2010). Based on those previous observations, the combination of low-sulfide, oligotrophic and hyposaline conditions in the Bothnian Sea sediment would likely favor denitrification over DNRA for nitrate reduction. In fact, the low overall abundance of *nrfA* in comparison to denitrification-related gene reads supported this hypothesis.
Reports on *nrfA*-bearing communities in sediments are scarce. So far, these have been analyzed in 3 estuary ecosystems exhibiting gradients in organic carbon, sulfide and salinity parameters (Takeuchi, 2006, Smith *et al.*, 2007, Song *et al.*, 2014). The majority of *nrfA* sequences detected in the Colne estuary, United Kingdom, was comprised by representatives of δ-Proteobacteria most closely related to order *Desulfuromonadales*. Despite the very different biogeochemical properties of our sampling site in the Bothnian Sea with the hypernutrified sediment of the Colne estuary, we observed a similar trend in DNRA community towards the dominance of those particular *δ*-proteobacterial groups.

Anammox: hydrazine synthase (*hzsA*)

Hydrazine synthase is a unique enzyme complex catalyzing the condensation reaction of NO and ammonium to hydrazine (N_2H_4) during the anammox pathway (Kartal *et al.*, 2011). The enzyme is characteristic for anammox bacteria and based on the available genome information, primers were developed for HzsA subunit encoding gene to detect anammox bacterial diversity in the environment (Harhangi *et al.*, 2012).
Metagenome analysis revealed higher *hzsA* gene read abundance in 5-12.5 cmbsf sample (2.4 nrc) than in 0-2.5 cmbsf (0.6 nrc). This results contradicted the assumption of anammox bacteria being more abundant in layers where they would have access to oxidized nitrogen oxides for respiration. Comparing the identity of *hzsA* reads between both depths, a clear difference was observed. Whereas all reads

found in 0-2.5 cmbsf were assigned to *Scalindua* spp., all reads but one in 5-12.5 cmbsf were assigned to *Kuenenia* spp. Only one read in 5-12.5 cmbsf was assigned to *Scalindua*. No *hzsA* gene reads could be identified in the 30-35 cmbsf. Also the majority of 16S rRNA reads assigned to *Brocadiales* from 5-12.5 cmbsf were affiliated with *Kuenenia* and other freshwater anammox genera supporting the findings at the *hzsA* gene level. Despite low read numbers assigned to *hzsA* gene, these results pointed to a vertical stratification of the anammox community within the Bothnian Sea sediment transect. Findings of brackish sediments inhabited by anammox bacteria belonging to different genera including *Scalindua* have been reported before (Dale *et al.*, 2009, Fu *et al.*, 2015). However, shifts in community structure between freshwater and marine genera have been investigated in horizontal gradients and attributed to changing environmental parameters like salinity, pH or C/N ratio (Dale *et al.*, 2009, Fu *et al.*, 2015). Slight changes in pH, nitrate/nitrite availability, C/N ratio or interactions with different metabolic partners between 0-2.5 and 5-12.5 cmbsf might be responsible for observed vertical anammox community structure in the Bothnian Sea sediment. Also, due to its geographical location the Bothnian Sea is strongly influenced by riverine input from mainland and occasional intrusions of saltier waters from the North Sea. This might have contributed to introduction and preservation of microbial communities from other locations including freshwater habitats of the mainland.

PCR on extracted total DNA with primer pair combinations targeting either the *Scalindua* genus or other five known genera of anammox bacteria resulted in positive amplification only for *Scalindua*-specific *hzsA* gene. Positive amplification was observed for 3 samples between 0 and 7.5 cmbsf indicating significant presence of *Scalindua*-specific *hzsA* genes only in this upper layer. The absence on *hzsA* below 7.5 cmbsf is congruent with metagenome results for 30-35 cmbsf where no reads could be assigned to *hzsA* and *Brocadiales*-specific 16S rRNA genes. However, as PCR product concentration was too low for ligation, a semi-nested PCR reaction was performed with *Scalindua*-specific primers for greater yield and further cloning procedure resulting in a final amplicon sequence length of 229nt. In total, 60 amplicon sequences could be retrieved: 21 for 0-2.5 cmbsf, 18 for 2.5-5 cmbsf and 21 for 5-7.5 cmbsf. All sequences shared 97-100% amino acid identity with uncultured *Scalindua* spp. originating from the marine sediments in Guyamas Basin (AGV76990) (Russ *et al.*, 2013). However, due to short sequence length the uncertainty in correct phylogenetic annotation is high and solid information can only be deduced at the genus level.

Biogeochemical parameters of the sediment transect showed measurable nitrate only within the upper 0.5 cmbsf, below this depth the sediment was anoxic. Thus, it is not clear where an abundant anammox bacterial population would derive nitrogen oxides for respiration below 2.5 cmbsf. The presence of other genes involved in aerobic processes (*amoA*) below 2.5 cmbsf might point to occasional fluxes of both oxygen and nitrogen oxides which would be rapidly consumed thus keeping concentrations below detection limits. An alternative explanation could be the presence of dormant not active community which was preserved during the sedimentation process. To our knowledge, deep bioturbation (below 2 cmbsf) which would introduce oxygen in deeper layers was not occurring at the USB5 sampling site (Matthias Egger, personal communication).

CONCLUSIONS & OUTLOOK

The results of our study indicate the importance of nitrogen cycling in the upper more oxidized Bothnian Sea sediment layers, where, based on genomic potential, full denitrification to N_2 seemed to dominate the N-cycle driving processes. Aerobic ammonia oxidation was almost exclusively attributed to *Thaumarchaeota* which presence was detected throughout the whole sediment transect with decreasing numbers towards deeper transect. Unexpectedly, the peak of anammox bacterial community was detected below the oxidized layer, a zone where no oxygen or nitrogen oxides were detectable and sulfate reduction with methane was the dominant metabolic process. Moreover, the anammox community composition seemed to be stratified between different layers with potentially novel species which were not detectable by PCR with specific primers. These findings show that gene amplification-based techniques might lead to underestimation or lack of detection of microbial key players of particular metabolic processes. This also shows that our understanding of microbial metabolic networks in coastal sediments is still far from being complete.

chapter 7

Discussion and Outlook

Physiological aspects of intra-aerobic methanotrophy: current knowledge and future directions

Since the discovery of nitrite-dependent methane oxidizer *Methylomirabilis oxyfera*, numerous molecular studies have provided evidence for its ubiquity in diverse oxygen limited habitats pointing to the important role in methane cycle. While molecular data can give a valuable insight in their distribution and phylogenetic diversity, it is physiological characterization which can eventually help us to understand why they occur in certain environmental niches, what role they play in the microbial community and what interactions they undergo with other community members.

The isotopic composition of methane has been shown to give an accurate information about its sources and sinks, and make predictions about its cycling in many habitats (Whiticar, 1999). Methanogens produce isotopically light methane, which is well distinguishable from abiotic sources. Moreover, based on both hydrogen and carbon isotopic signature it is even possible to distinguish between hydrogenotrophic and acetoclastic methanogenesis (Whiticar, 1999). On the "sink-side", under non-limiting substrate conditions methanotrophic organisms consume methane with a preference for lighter isotopes of both carbon and hydrogen, thus leaving the residual pool of methane enriched in heavier isotopes. The fractionation usually occurs at the first non-reversible enzymatic conversion step when the back reaction is not significant. For methanotrophic bacteria, this would apply for the first step in the methane oxidation pathway – catalyzed by methane monooxygenase – which has also been experimentally shown (Nesheim & Lipscomb, 1996). The unusual methanotrophic lifestyle of *M. oxyfera* and its ubiquitous occurrence in the environment led to the motivation to investigate its specific isotopic signature was different to other methanotrophs using methane monooxygenase for methane oxidation. However, the results showed that despite its unusual metabolism, the fractionation factors for both carbon and hydrogen were in same range as of other methanotrophs. *M. oxyfera*-specific methane monooxygenase clusters separately from other methanotrophic bacteria in phylogenetic trees, indicating some characteristic features which are only present in these methanotrophs. Although unlikely according to the calculated and observed reaction stoichiometry, it still cannot be excluded that nitric oxide could be used directly as a substrate for the enzyme. Nevertheless, even with its most likely substrate – molecular oxygen – the kinetic isotope fractionation mechanism is still elusive, since oxygen is hypothesized

to be supplied by putative Nod enzymes. The reaction mechanism and kinetics of this dismutation reaction are so far unknown and remain a subject for future research. Here, one among the still many questions is whether there is a close physical proximity between Nod and Pmo to shuttle oxygen. In case of a slow and limiting oxygen supply, the isotopic fractionation of methane could be offset due to slower reaction rates. The mechanism of the *M. oxyfera*-specific Pmo still needs further investigation.

The methane isotope fractionation factors obtained for *M. oxyfera* can be used in future studies for a more precise modelling of methane fluxes in anoxic environments. It has been shown is several previous studies that *M. oxyfera* bacteria are highly abundant in certain habitats (Deutzmann *et al.*, 2014, Hanson & Madsen, 2015). These habitats would provide ideal grounds for methane isotope measurements and application of fractionation factors determined for *M. oxyfera* bacteria. Thus, the abundance, phylogeny, classical activity methane oxidation activity measurements and modelling of overall methane cycling in those habitats could provide a holistic picture on the role of *M. oxyfera* in the environment.

In the context of environmental detection of active methanotrophy in the environment, many studies were based on the knowledge of methanotrophs assimilating at least half of their cellular carbon from methane, which would be reflected in the C isotope composition of lipids and DNA of methanotrophic bacteria. Thus, heavy ^{13}C-labeled methane was used for incubations of environmental samples in order to detect active methanotrophs by extraction and amplification of ^{13}C-labeled DNA (DNA-SIP). Although the Calvin Cycle encoding genes for CO_2 fixation were known for a long time to be present and even expressed in some proteobacterial methanotrophs (Kao *et al.*, 2004), their function remained elusive since it was shown that those methanotrophs were still mainly using carbon assimilation pathways with formaldehyde as the main substrate. The use of intermediate formaldehyde for assimilation into cell carbon seemed logical, as in comparison to the reduction CO_2 to the redox level of cellular carbon significantly less reducing equivalents are needed. However, recent characterization of verrucomicrobial methanotrophs and *M. oxyfera*-like bacteria challenged this dogma, since both were shown to use the Calvin Cycle for CO_2 fixation and lack essential genes involved either in serine or ribulose monophosphate pathways (Khadem *et al.*, 2011, Rasigraf *et al.*, 2014). This trait seemed peculiar at first, but growing genomic and experimental evidence suggests that methanotrophic bacteria are metabolically more flexible than previously thought (Chistoserdova, 2011,

Kalyuzhnaya *et al.*, 2013). Here, recent characterization of novel methanol dehydrogenases of the XoxF type adds another puzzle piece to the above mentioned metabolic plasticity. XoxF from *Methylobacterium extorquens* AM1 was shown to be more efficient in the catalysis of methanol oxidation and even able to oxidize methanol to formate in one step (Schmidt *et al.*, 2010). This has been speculated to be a possible solution for the CO_2 fixation via the Calvin Cycle in *M. fumariolicum* SolV since this methanotroph was shown to only possess the XoxF type methanol dehydrogenase (Keltjens *et al.*, 2014). The genomes of all so far known methylotrophs were shown to possess XoxF (Taubert *et al.*, 2015), thus providing a great potential for physiological investigations of their function in different methano- and methylotrophs. The genome of *M. oxyfera* encodes MxaF1 and two types of XoxF methanol dehydrogenases, with XoxF1 being functionally expressed under standard culturing conditions (Wu *et al.*, 2015). Our nitric oxide exposure experiments indicated a differential expression of all three MDH types, with only XoxF2 type being transcribed at the time point when Pmo, Fae and other methane oxidation pathway encoding genes were downregulated and NO concentrations dropped to under 1 µM. At this time point, the methane oxidation activity was still inhibited and resumed shortly after. The kinetics and catalytic mechanism of XoxF2 from *M. oxyfera* are not known. It might be adapted to lower methanol supply from methane monooxygenase, which was likely to be inhibited at that time point. These results point to metabolic flexibility of *M. oxyfera* under different stages of metabolic stress and open room for future physiological studies to investigate gene transcription and expression switches at varying physiological conditions. Furthermore, knowing the physiological conditions at which XoxF2 is expressed would allow its expression and possibly further purification and biochemical characterization.

Testing the physiological response of *M. oxyfera* cultures with lower concentrations of NO (below 1 µM) could potentially resolve the roles of two Nod and conventional qNor proteins. The slightly higher expression of the conventional qNor at the time point when NO dropped under 1 µM but the activity was still inhibited (T5) and our previous experiments with elevated semi-toxic concentrations of nitrite (unpublished results) have indicated an elevated production of nitrous oxide as a potential detoxification mechanism. As the reduction of NO to N_2O requires two electrons, the question remains whether they are derived from the central methane oxidation metabolism or are coupled to another electron donor.

The dismutation mechanism still remains hypothetical, the knowledge of the conditions regulating a potential switch between the expression of different Nod encoding genes could provide a tool for physiological studies and protein purification for biochemical characterization. However, slow growth in flocks with doubling times in a matter of weeks still remains a challenge. Here, the exposure to nanomolar concentrations of nitric oxide might offer an effective trigger for swarming (Barraud *et al.*, 2006) and physiological changes associated with enhanced growth rates. NO concentration seems to be a crucial factor, since it has been shown that both biofilm formation and dissociation can be induced at different NO concentrations (Schmidt *et al.*, 2004). Thus, at lower concentrations (in nanomolar range) *M. oxyfera*'s potential for motility could be investigated in a continuous chemostat reactor system.

The ability to reduce nitrate still remains a question since nitrate is not used under standard culturing conditions, but the genes encoding for both periplasmic and membrane-bound nitrate reductases are transcribed under all tested conditions. Furthermore, if nitrate would be used as the only substrate for O_2 formation, 4 molecules of O_2 would not be enough for the oxidation of 5 molecules of methane. Here, a possibility remains that *M. oxyfera* NarG, which is closely related to Nxr, is involved in nitrite oxidation instead of nitrate reduction. In fact, *M. oxyfera* NarG was shown to be closely related to Nxr proteins of nitrite oxidizing bacteria *Nitrobacter*, *Nitrococcus* and *Nitrolancetus* (Sorokin *et al.*, 2012). However, nitrate production was so far not observed in *M. oxyfera* cultures and it is well possible that nitrate reductase encoding genes were obtained via the horizontal gene transfer and are not essential for *M. oxyfera*.

M. oxyfera is a methanotrophic bacterium which employs methane monooxygenase for methane oxidation. Structurally, methane is very similar to ammonium and it is known that methane monooxygenase can oxidize ammonium to hydroxylamine as a side reaction. Hydroxylamine is a highly toxic intermediate and many methanotrophic bacteria possess hydroxylamine oxidoreductase for its detoxification. Also the genome of *M. oxyfera* encodes and expresses a hydroxylamine oxidoreductase. However, the fate of oxidized hydroxylamine remains unknown since external oxygen is not available as a substrate for the enzyme. In its environmental niche at the oxic/anoxic interface, *M. oxyfera* would often encounter elevated concentrations of ammonium. It remains to be investigated at which concentrations ammonium would become toxic and how the reaction is integrated in the overall metabolism of *M. oxyfera* in terms of the electron flow

mechanisms. Hao mediated oxidation of hydroxylamine to NO instead of nitrite might provide a physiological advantage since it could directly be used by dedicated NO utilizing enzymes, either Nod or qNor. Experiments involving ^{15}N-labeled ammonium and mass spectrometric detection of labeled metabolites such as NO and hydroxylamine might provide an insight into the role of Hao in *M. oxyfera*.

Another great under-explored research field concerns interactions of *M. oxyfera* bacteria with other community members, which might either benefit or compete with them for substrates. Oxic/anoxic interfaces harbor a great variety of organisms which depend on the supply of electron acceptors from the oxic zone such as nitrate and nitrite, and availability of electron donors such as various fermentation products, ammonium and methane produced in the deeper anoxic layers. *M. oxyfera* is a denitrifying bacterium, which depends on nitrite as the electron donor. Thus, possible competition for electron acceptor could occur with denitrifiers, anammox bacteria and DNRA performing organisms. Several previous studies have focused on investigations of factors determining the dominance of either denitrification or DNRA, and several factors such as organic carbon content, sulfide concentrations, salinity and availability of reactive nitrogen have been implicated as crucial factors for the dominance of either process (Giblin *et al.*, 2010, An & Gardner, 2002, Kraft *et al*., 2014). Both denitrification and DNRA compete for organic carbon or reduced sulfur species as electron donors, *M. oxyfera* however does not rely on these electron donors but uses methane instead. Thus, the affinity to nitrite would be among the main factors determining its competitiveness with denitrifiers and DNRA organisms. But other factors such as salinity or sulfide exposure would play important roles as well. All these aspects of *M. oxyfera* physiology are currently mostly unknown and studies based on both environmental sequencing of total commnity DNA and laboratory-based controlled physiological tests in bioreactor systems would give some insights into possible environmetal interactions. In case of interactions with anammox bacteria, a previous study showed that both can be enriched together and co-exist in a bioreactor system (Luesken et al., 2011b). Another study, however, showed that *M. oxyfera* could be effectivelly outcompeted by anmmox bacteria in bioreactor systems (Hu *et al*., 2015). Environmetal molecular studies have shown that both co-occur in anoxic environments (Wang *et al*., 2012). It is likely that different substrate affinities and reaction kinetics of different *M. oxyfera* and anammox strains determine individual competition advantages and outcomes of competition interactions.

In the environment, *M. oxyfera* bacteria depend on some community members for the supply of nitrite. Thus, ammonia oxidizers and nitrate reducing organisms are essential partners, however, little is known about possible interactions with these organisms. The first described N-AOM culture contained both nitrate-reducing archaea (group AAA) and *M. oxyfera* bacteria (Raghoebarsing *et al*., 2006). By now it is known that group AAA archaea reduce nitrate to nitrite (Baoli Zhu, PhD thesis, Haroon *et al*., 2013), which can be effectively scavenged by *M. oxyfera*. Since both use methane as the electron donor, a natural co-occurance of both is a very likely scenario, since also group AAA archaea would benefit from the removal of toxic nitrite. In contrast, no studies have yet investigated possible interactions with aerobic ammonia oxidizers. Both would benefit from each other where *M. oxyfera* would revome toxic nitrite and ammonia oxidizers would make create anoxic environment for *M. oxyfera* by respiring oxygen. It would be particularly interesting to investigate whether ammonia oxidizing bacteria or *Thaumarchaeota* would be the preferred metabolic partners and what environmental factors would determine possible metabolic partner preference. Also here, total community DNA sequencing and culturing could provide some insights.
Moreover, in view of previously discussed metabolic plasticity of methanotrophic organisms, and in particular of *M. oxyfera* bacteria, it cannot be excluded that also substrates other than methane could be used depending on varying environmetal conditions. This would inevitably add another level of complexity to investigations of metabolic interactions with other organisms.

Iron-dependent anaerobic oxidation of methane: in quest for microbial key players

Within a sediment column, methane produced in deeper anoxic layers diffuses through zones of alternative electron acceptors before it reaches the oxic/anoxic interface where nitrite-, nitrate- and oxygen-dependent methanotrophs are active. During the past few decades a combination of geochemical, biogeochemical and molecular studies provided solid evidence for the importance of sulfate for methane oxidation in marine sediments which is performed by consortia of ANME archaea and sulfate-reducing bacteria at the sulfate-methane transition zone (SMTZ). The majority of methane is oxidized in this zone before ever reaching the upper sediment layers. Here, methane is oxidized by methanogen-related archaea via the methyl-coenzyme M reductase (Mcr) and the reducing equivalents are shuttled to partner

sulfate-reducing bacteria. However, in freshwater and brackish environments where overall salinity and sulfate concentrations are low, other electron acceptors like oxidized metal species (e.g., birnessite, ferrihydrite, goethite, magnetite etc.) play a more important role for anaerobic respiration. Several environmental studies have provided evidence for metal-dependent methane oxidation activity in various environments, but underlying processes still remain elusive. Thus, solving the physiological and biochemical enigma responsible for observed activities could provide an important insight into the methane cycling processes in iron-rich sediments. Here, based on its biogeochemical characteristics of abundant reactive iron oxides below a narrow SMTZ where sulfate concentrations stay below the detection limit, the Bothnian Sea sediment provides a suitable ecosystem to study the iron-dependent anaerobic methane oxidation (Fe-AOM). Our batch incubations with reactive iron oxides and added methane showed that Fe-AOM was indeed active the Bothnian Sea sediment and subsequent metagenomic sequencing revealed several groups of organisms being stimulated by added iron and methane. Thus, a specific group of organisms responsible for observed activities could not be identified, which rather pointed to a possibility of a metabolic network of different groups involved either directly in iron reduction and methane oxidation or indirectly in stimulation of the former by produced metabolites. Furthermore, there was evidence for a functional cryptic sulfur cycle which was indicated by enrichments of thiosulfate and sulfite reductase encoding genes in active incubation slurries. However, the phylogenetic affiliation of the detected sulfite reductase genes revealed that they belonged to various sulfate reducing bacteria without a clear dominance of *Desulfobacterales* which have been implicated as metabolic partners for sulfate-dependent anaerobic methane oxidation (S-AOM). Thus, the increase of these genes might have been decoupled to AOM since the putatively fermentative *Clostridia* – strongly enriched in our incubation slurries – could have provided substrates for microbial sulfate reduction in form of various fermentation products. In order to investigate whether the observed increase in sulfite reductase genes was indeed coupled to AOM via a cryptic sulfur cycle, a modified experimental set up is needed and will be perfumed in follow up studies. Sulfate could be provided in different concentrations to Fe^{3+}-amended and Fe^{3+}-free incubations with methane, and the associated methane oxidation activities and microbial community changes could be investigated. Furthermore, an application of microbial sulfate reduction inhibitors might proof to be feasible, however, it cannot be excluded that also other metabolic processes can be co-inhibited, in particular the Fe-AOM.

The community analysis of active Fe-AOM batch incubations also showed increases in both 16S rRNA and McrA encoding genes assigned to ANME-2a archaea. It is tempting to conclude that these archaea were directly involved in the observed Fe-AOM activity, however, as mentioned above, a possibility for an active S-AOM via a cryptic sulfur cycle at least to some degree cannot be excluded. An interesting genomic feature of this particular type of ANME archaea is the high abundance of cytochrome *c* encoding genes which has also been shown for another closely related nitrate-reducing ANME-like archaeon, *Methanoperedens nitroreducens* (Haroon *et al.*, 2013, Wang *et al.*, 2014, Kletzin *et al.*, 2015). Based on this feature, the latter has been speculated to be able to reduce reactive iron (Kletzin *et al.*, 2015). In case ANME-2a archaea can use their abundant cytochrome-containing proteins for electron transfer to the outer membrane, two scenarios for their involvement in Fe-AOM would be possible: direct reduction by the transfer of electrons onto the iron particle and indirect reduction either by reduction of some kind of redox shuttles or shuttling of electrons to an iron-reducing partner. The dominance of the same ANME genotype in the SMTZ of the Bothnian Sea sediment transect points to its ability to perform S-AOM. However, it has been discussed previously that based on their genomic features ANME-2a archaea might be metabolically flexible (Wang *et al.*, 2014). Thus, depending on environmental conditions, the usage of both sulfate and iron as electron acceptors could still be possible. However, this questions could only be answered with an enrichment and subsequent physiological characterization of this particular Bothnian Sea specific ANME-2a lineage. Additionally, co-cultures of related archaea with *Desulfuromonas*-like iron reducers could be performed in order to investigate possibilities for interspecies reducing equivalent transfer.

Besides the ANME-2a, also the McrA encoding genes of several methanogens (*Methanosarcina*, *Methanoregula* and *Methanomassiliicoccus*) increased in abundance in our Fe-AOM incubations. Although involved in methanogenesis, it has been shown in the past that members of *Methanosarcina* can oxidize some methane to carbon dioxide (Zehnder & Brock, 1979). This could have contributed to the observed labeled CO_2 production. It is likely that the increase in methanogens was stimulated by fermentative *Clostridia* which would have provided substrates for methanogenesis in form of carboxylic acids and hydrogen. In this case, their activity would contribute to the "dilution" of ^{13}C-labeled methane with unlabeled methane originating from the above mentioned clostridial substrates.

Genomic potential for nitrogen cycle in the Bothnian Sea sediment

In addition to investigations of methane cycling processes in the Bothnian Sea, also the genomic potential for nitrogen cycle was analyzed. It appeared that full denitrification to N_2 was the most important process based on the abundance of genes involved in each enzymatic step. Other anaerobic processes such as dissimilatory nitrite reduction to ammonium (DNRA), anaerobic ammonium oxidation (anammox) and nitrogen fixation seemed to be of only minor importance. The aerobic part was dominated by ammonia oxidizing *Thaumarchaeota* and their genomic biomarkers based on 16S rRNA, AmoA and NirK encoding genes were detected in all analyzed depths including deeper anoxic layers. This observation seems obscure since oxygen is absent in the deeper layers. Interestingly, similar trends were observed in marine sediments (Jorgensen *et al.*, 2012). This points to the ubiquitous occurrence of these deep ammonium oxidizer-like organisms which most likely employ a metabolism other than aerobic ammonium oxidation. It cannot be excluded, however, that these genomic markers are remnants from the past when this sediment layers still contained oxygen. Since an alternative metabolism is another likely possibility, the potential substrates and electron acceptors are unknown. Nevertheless, ammonium might still play a role as the possible substrate with an implication for anaerobic ammonium oxidation with electron acceptors other than oxygen or nitrite. This novel metabolism could for example be coupled to iron oxide or sulfate reduction which would have major implications for sedimentary nitrogen cycling and add another puzzle piece to theoretical microbial metabolisms "yet to be discovered". Here, a genomic approach with full genome assembly might be a suitable approach since the genomic data might provide some cues about its metabolic features and subsequently lead to successful enrichments and further physiological characterization. Surprising observations were also made regarding the community composition and distribution of anammox bacteria within the sediment column. Here, the majority of anammox genomic biomarkers including 16S rRNA, Hzs and Hao encoding genes were detected in the S-AOM sediment sample and not at the oxic/anoxic interface where biogeochemical conditions would imply a higher availability of nitrite – the electron acceptor for anammox metabolism. PCR amplification of *hzsA* genes indicated that this peak of anammox biomarkers was located at the top of sulfate-methane transition zone. Furthermore, metagenomics revealed potentially novel anammox species closer related to *Kuenenia*, while the anammox biomarkers at the oxic/anoxic interface were mostly

related to *Scalindua*. This vertical stratification is intriguing and was so far never reported before. It remains to be shown whether these deep located anammox bacteria are active or dormant, and in case of their activity where they would derive electron acceptor for respiration. Enrichment cultures of these potentially novel anammox bacteria from the original sediment would enable to perform their physiological characterization which in turn would possibly shed more light on this peculiar distribution pattern. Moreover, genome assemblies and environmental transcriptomics could be used for comparative genomics and transcriptomics with the so far sequenced and characterized anammox species.

References

Adak S, Bilwes AM, Panda K, Hosfield D, Aulak KS, McDonald JF, Tainer JA, Getzoff ED, Crane BR & Stuehr DJ (2002) Cloning, expression, and characterization of a nitric oxide synthase protein from *Deinococcus radiodurans*. *Proceedings of the National Academy of Sciences* **99**: 107-112.

Algesten G, Brydsten L, Jonsson P*, et al.* (2006) Organic carbon budget for the Gulf of Bothnia. *Journal of Marine Systems* **63**: 155-161.

Alperin MJ, Reeburgh WS & Whiticar MJ (1988) Carbon and hydrogen isotope fractionation resulting from anaerobic methane oxidation. *Journal Name: Global Biogeochemical Cycles; (USA); Journal Volume: 2:3* Medium: X; Size: Pages: 279-288.

Amos RT, Bekins BA, Cozzarelli IM, Voytek MA, Kirshtein JD, Jones EJP & Blowes DW (2012) Evidence for iron-mediated anaerobic methane oxidation in a crude oil-contaminated aquifer. *Geobiology* **10**: 506-517.

An S & Gardner WS (2002) Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). *Marine Ecology Progress Series* **237**: 41-50.

Andrews SC, Robinson AK & Rodríguez-Quiñones F (2003) *Bacterial iron homeostasis*.
Anthony C (1986) Bacterial oxidation of methane and methanol. *Advances in Microbial Physiology* **27**: 113-210.

Anthony C & Williams P (2003) The structure and mechanism of methanol dehydrogenase. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1647**: 18-23.

Arndt NT & Nisbet EG (2012) Processes on the Young Earth and the Habitats of Early Life. *Annual Review of Earth and Planetary Sciences* **40**: 521-549.

Arp DJ, Sayavedra-Soto LA & Hommes NG (2002) Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch Microbiol* **178**: 250-255.

Arp DJ, Chain PSG & Klotz MG (2007) The Impact of Genome Analyses on Our Understanding of Ammonia-Oxidizing Bacteria. *Annual Review of Microbiology* **61**: 503-528.

Asami H, Aida M & Watanabe K (2005) Accelerated Sulfur Cycle in Coastal Marine Sediment beneath Areas of Intensive Shellfish Aquaculture. *Applied and Environmental Microbiology* **71**: 2925-2933.

Atkinson SJ, Mowat CG, Reid GA & Chapman SK (2007) An octaheme *c*-type cytochrome from *Shewanella oneidensis* can reduce nitrite and hydroxylamine. *FEBS Letters* **581**: 3805-3808.

Averill BA (1996) Dissimilatory Nitrite and Nitric Oxide Reductases. *Chemical Reviews* **96**: 2951-2964.

Bak F & Pfennig N (1987) Chemolithotrophic growth of *Desulfovibrio sulfodismutans* sp. nov. by disproportionation of inorganic sulfur compounds. *Archives of Microbiology* **147**: 184-189.

Barnes RO & Goldberg ED (1976) Methane production and consumption in anoxic marine sediments. *Geology* **4**: 297-300.

Barraud N, Hassett DJ, Hwang S-H, Rice SA, Kjelleberg S & Webb JS (2006) Involvement of Nitric Oxide in Biofilm Dispersal of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **188**: 7344-7353.

Baxter NJ, Hirt RP, Bodrossy L, Kovacs KL, Embley MT, Prosser JI & Murrell CJ (2002) The ribulose-1,5-bisphosphate carboxylase/oxygenase gene cluster of *Methylococcus capsulatus* (Bath). *Archives of Microbiology* **177**: 279-289.

Beal EJ, House CH & Orphan VJ (2009) Manganese- and Iron-Dependent Marine Methane Oxidation. *Science* **325**: 184-187.

Beaulieu JJ, Tank JL, Hamilton SK*, et al.* (2011) Nitrous oxide emission from denitrification in stream and river networks. *Proceedings of the National Academy of Sciences* **108**: 214-219.

Bédard C & Knowles R (1989) Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+, and CO oxidation by methanotrophs and nitrifiers. *Microbiological Reviews* **53**: 68-84.

Beman JM, Popp BN & Francis CA (2008) Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California. *ISME Journal* **2**: 429-441.

Bergamaschi P, Bräunlich M, Marik T & Brenninkmeijer CAM (2000) Measurements of the carbon and hydrogen isotopes of atmospheric methane at Izaña, Tenerife: Seasonal cycles and synoptic-scale variations. *Journal of Geophysical Research: Atmospheres* **105**: 14531-14546.

Bergmaschi P & Harris GW (1995) Measurements of stable isotope ratios ($^{13}CH_4/^{12}CH_4$; $^{12}CH_3D/^{12}CH_4$) in landfill methane using a Tunable Diode Laser Absorption Spectrometer. *Global Biogeochemical Cycles* **9**: 439-447.

Bhumika V, Srinivas TNR, Ravinder K & Anil Kumar P (2013) *Mariniradius saccharolyticus* gen. nov., sp. nov., a member of the family *Cyclobacteriaceae* isolated from marine aquaculture pond water, and emended descriptions of the genus *Aquiflexum* and *Aquiflexum balticum*. *International Journal of Systematic and Evolutionary Microbiology* **63**: 2088-2094.

Biddle JF, Lipp JS, Lever MA*, et al.* (2006) Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 3846-3851.

Biderre-Petit C, Jézéquel D, Dugat-Bony E, Lopes F, Kuever J, Borrel G, Viollier E, Fonty G & Peyret P (2011) Identification of microbial communities involved in the methane cycle of a freshwater meromictic lake. *FEMS Microbiology Ecology* **77**: 533-545.

Blumenberg M, Seifert R, Reitner J, Pape T & Michaelis W (2004) Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. *Proceedings of the National Academy of Sciences* **101**: 11111-11116.

Borrel G, O'Toole PW, Harris HMB, Peyret P, Brugère J-F & Gribaldo S (2013) Phylogenomic Data Support a Seventh Order of Methylotrophic Methanogens and Provide Insights into the Evolution of Methanogenesis. *Genome Biology and Evolution* **5**: 1769-1780.

Borrel G, Harris HMB, Parisot N*, et al.* (2013) Genome Sequence of "Candidatus *Methanomassiliicoccus intestinalis*" Issoire-Mx1, a Third *Thermoplasmatales*-Related Methanogenic Archaeon from Human Feces. *Genome Announcements* **1**.

Bosch J, Heister K, Hofmann T & Meckenstock RU (2010) Nanosized Iron Oxide Colloids Strongly Enhance Microbial Iron Reduction. *Applied and Environmental Microbiology* **76**: 184-189.

Bowes G, Ogren WL & Hageman RH (1971) Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. *Biochemical and Biophysical Research Communications* **45**: 716-722.

Bowman LAH, McLean S, Poole RK & Fukuto JM (2011) The Diversity of Microbial Responses to Nitric Oxide and Agents of Nitrosative Stress: Close Cousins but Not Identical Twins. *Advances in Microbial Physiology,* Vol. Volume 59 (Robert KP, ed.) pp. 135-219. Academic Press.

Boyd ES & Peters JW (2013) New insights into the evolutionary history of biological nitrogen fixation. *Frontiers in Microbiology* **4**: 201.

Braker G, Zhou J, Wu L, Devol AH & Tiedje JM (2000) Nitrite Reductase Genes (*nirK* and *nirS*) as Functional Markers To Investigate Diversity of Denitrifying Bacteria in Pacific Northwest Marine Sediment Communities. *Applied and Environmental Microbiology* **66**: 2096-2104.

Bruns A, Rohde M & Berthe-Corti L (2001) *Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment. *International Journal of Systematic and Evolutionary Microbiology* **51**: 1997-2006.

Campbell MA, Nyerges G, Kozlowski JA, Poret-Peterson AT, Stein LY & Klotz MG (2011) Model of the molecular basis for hydroxylamine oxidation and nitrous oxide production in methanotrophic bacteria. *FEMS Microbiology Letters* **322**: 82-89.

Cébron A, Bodrossy L, Chen Y, Singer AC, Thompson IP, Prosser JI & Murrell JC (2007) Identity of active methanotrophs in landfill cover soil as revealed by DNA-stable isotope probing. *FEMS Microbiology Ecology* **62**: 12-23.

Cedervall PE, Hooper AB & Wilmot CM (2009) Crystallization and preliminary X-ray crystallographic analysis of a new crystal form of hydroxylamine oxidoreductase from *Nitrosomonas europaea*. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* **65**: 1296-1298.

Chalk PM & Smith CJ (1983) Chemodenitrification. *Gaseous Loss of Nitrogen from Plant-Soil Systems,* Vol. 9 (Freney JR & Simpson JR, eds.), pp. 65-89. Springer Netherlands.

Chen J, Zhou Z-C & Gu J-D (2014) Occurrence and diversity of nitrite-dependent anaerobic methane oxidation bacteria in the sediments of the South China Sea revealed by amplification of both 16S rRNA and *pmoA* genes. *Applied Microbiology and Biotechnology* **98**: 5685-5696.

Chen Y, Crombie A, Rahman MT, Dedysh SN, Liesack W, Stott MB, Alam M, Theisen AR, Murrell JC & Dunfield PF (2010) Complete genome sequence of the aerobic facultative methanotroph *Methylocella silvestris* BL2. *Journal of Bacteriology* **192**: 3840-3841.

Chistoserdova L (2011) Modularity of methylotrophy, revisited. *Environmental Microbiology* **13**: 2603-2622.

Cicerone RJ & Oremland RS (1988) Biogeochemical aspects of atmospheric methane. *Global Biogeochemical Cycles* **2**: 299-327.

Clementi E, Brown GC, Feelisch M & Moncada S (1998) Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proceedings of the National Academy of Sciences* **95**: 7631-7636.

Coleman DD, Risatti JB & Schoell M (1981) Fractionation of carbon and hydrogen isotopes by methane-oxidizing bacteria. *Geochimica et Cosmochimica Acta* **45**: 1033-1037.

Conrad R (2009) The global methane cycle: recent advances in understanding the microbial processes involved. *Environmental Microbiology Reports* **1**: 285-292.

Coolen MJL, Hopmans EC, Rijpstra WIC, Muyzer G, Schouten S, Volkman JK & Sinninghe Damsté JS (2004) Evolution of the methane cycle in Ace Lake (Antarctica) during the Holocene: response of methanogens and methanotrophs to environmental change. *Organic Geochemistry* **35**: 1151-1167.

Cooper CE (1999) Nitric oxide and iron proteins. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1411**: 290-309.

Corker H & Poole RK (2003) Nitric Oxide Formation by *Escherichia coli*: dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohemoglobin Hmp. *Journal of Biological Chemistry* **278**: 31584-31592.

Costa C, Macedo A, Moura I, Moura JJG, Le Gall J, Berlier Y, Liu MY & Payne WJ (1990) Regulation of the hexaheme nitrite/nitric oxide reductase of *Desulfovibrio desulfuricans*, *Wolinella succinogenes* and *Escherichia coli*: A mass spectrometric study. *FEBS Letters* **276**: 67-70.

Crowe SA, Katsev S, Leslie K*, et al.* (2011) The methane cycle in ferruginous Lake Matano. *Geobiology* **9**: 61-78.

Dale OR, Tobias CR & Song B (2009) Biogeographical distribution of diverse anaerobic ammonium oxidizing (anammox) bacteria in Cape Fear River Estuary. *Environmental Microbiology* **11**: 1194-1207.

Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W & Tiedje JM (2002) *Methylocapsa acidiphila* gen. nov., sp nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. *International Journal of Systematic and Evolutionary Microbiology* **52**: 251-261.

Deines P, Bodelier PL & Eller G (2007) Methane-derived carbon flows through methane-oxidizing bacteria to higher trophic levels in aquatic systems. *Environmental Microbiology* **9**: 1126-1134.

DeLong EF (1992) Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 5685-5689.

Deutzmann JS & Schink B (2011) Anaerobic Oxidation of Methane in Sediments of Lake Constance, an Oligotrophic Freshwater Lake. *Applied and Environmental Microbiology* **77**: 4429-4436.

Deutzmann JS, Stief P, Brandes J & Schink B (2014) Anaerobic methane oxidation coupled to denitrification is the dominant methane sink in a deep lake. *Proceedings of the National Academy of Sciences* **111**: 18273-18278.

Devol AH (2003) Nitrogen cycle: Solution to a marine mystery. *Nature* **422**: 575-576.

Dlugokencky EJ, Bruhwiler L, White JWC*, et al.* (2009) Observational constraints on recent increases in the atmospheric CH_4 burden. *Geophysical Research Letters* **36**: n/a-n/a.

Dobbin PS, Carter JP, García-Salamanca San Juan C, von Hobe M, Powell AK & Richardson DJ (1999) Dissimilatory Fe(III) reduction by *Clostridium beijerinckii* isolated from freshwater sediment using Fe(III) maltol enrichment. *FEMS Microbiology Letters* **176**: 131-138.

Dos Santos P, Fang Z, Mason S, Setubal J & Dixon R (2012) Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. *BMC Genomics* **13**: 162.

Dridi B, Fardeau M-L, Ollivier B, Raoult D & Drancourt M (2012) *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology* **62**: 1902-1907.

Dumont MG, Pommerenke B, Casper P & Conrad R (2011) DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. *Environmental Microbiology* **13**: 1153-1167.

Dumont MG, Lüke C, Deng Y & Frenzel P (2014) Classification of *pmoA* amplicon pyrosequences using BLAST and the lowest common ancestor method in MEGAN. *Frontiers in Microbiology* **5**: 34.

Dunfield PF, Yuryev A, Senin P*, et al.* (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. *Nature* **450**: 879-U818.

Eady RR (1996) Structure-function relationships of alternative nitrogenases. *Chemial Reviews* **96**: 3013-3030.

Egger M, Rasigraf O, Sapart CJ*, et al.* (2015) Iron-Mediated Anaerobic Oxidation of Methane in Brackish Coastal Sediments. *Environmental Science & Technology* **49**: 277-283.

Einsle O (2001) Cytochrome *c* nitrite reductase. *Handbook of metalloproteins,*(Messerschmidt A, Huber, R., Wieghardt, K. & Poulos, T., eds., ed.) pp. John Wiley & Sons, New York.

Eisenhut M, Ruth W, Haimovich M, Bauwe H, Kaplan A & Hagemann M (2008) The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiontically to plants. *Proceedings of the National Academy of Sciences* **105**: 17199-17204.

Eisenmann E, Beuerle J, Sulger K, Kroneck PH & Schumacher W (1995) Lithotrophic growth of *Sulfurospirillum deleyianum* with sulfide as electron donor coupled to respiratory reduction of nitrate to ammonia. *Archives of Microbiology* **164**: 180-185.

Elliott SJ, Zhu M, Tso L, Nguyen HHT, Yip JHK & Chan SI (1997) Regio- and Stereoselectivity of Particulate Methane Monooxygenase from *Methylococcus capsulatus* (Bath). *Journal of the American Chemical Society* **119**: 9949-9955.

Elsner M, Zwank L, Hunkeler D & Schwarzenbach RP (2005) A New Concept Linking Observable Stable Isotope Fractionation to Transformation Pathways of Organic Pollutants. *Environmental Science & Technology* **39**: 6896-6916.

Elsner M, McKelvie J, Lacrampe Couloume G & Sherwood Lollar B (2007) Insight into Methyl tert-Butyl Ether (MTBE) Stable Isotope Fractionation from Abiotic Reference Experiments. *Environmental Science & Technology* **41**: 5693-5700.

Ettwig KF, van Alen T, van de Pas-Schoonen KT, Jetten MSM & Strous M (2009) Enrichment and Molecular Detection of Denitrifying Methanotrophic Bacteria of the NC10 Phylum. *Applied and Environmental Microbiology* **75**: 3656-3662.

Ettwig KF, Speth DR, Reimann J, Wu ML, Jetten MSM & Keltjens JT (2012) Bacterial oxygen production in the dark. *Frontiers in Microbiology* **3**.

Ettwig KF, Shima S, Van De Pas-Schoonen KT, Kahnt J, Medema MH, Op Den Camp HJM, Jetten MSM & Strous M (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. *Environmental Microbiology* **10**: 3164-3173.

Ettwig KF, Butler MK, Le Paslier D*, et al.* (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543-548.

Falkowski PG & Godfrey LV (2008) Electrons, life and the evolution of Earth's oxygen cycle. *Philosophical Transactions of the Royal Society B* **363**: 2705-2716.

Feilberg KL, Griffith DWT, Johnson MS & Nielsen CJ (2005) The ^{13}C and D kinetic isotope effects in the reaction of CH_4 with Cl. *International Journal of Chemical Kinetics* **37**: 110-118.

Feisthauer S, Vogt C, Modrzynski J, Szlenkier M, Krüger M, Siegert M & Richnow H-H (2011) Different types of methane monooxygenases produce similar carbon and hydrogen isotope fractionation patterns during methane oxidation. *Geochimica et Cosmochimica Acta* **75**: 1173-1184.

Finster K, Liesack W & Tindall BJ (1997) *Sulfurospirillum arcachonense* sp. nov., a New Microaerophilic Sulfur-Reducing Bacterium. *International Journal of Systematic Bacteriology* **47**: 1212-1217.

Fischer A, Herklotz I, Herrmann S, Thullner M, Weelink SAB, Stams AJM, Schlömann M, Richnow H-H & Vogt C (2008) Combined Carbon and Hydrogen Isotope Fractionation Investigations for Elucidating Benzene Biodegradation Pathways. *Environmental Science & Technology* **42**: 4356-4363.

Francis CA, Roberts KJ, Beman JM, Santoro AE & Oakley BB (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 14683-14688.

Freeman KH, Hayes JM, Trendel J-M & Albrecht P (1990) Evidence from carbon isotope measurements for diverse origins of sedimentary hydrocarbons. *Nature* **343**: 254-256.

Fu B, Liu J, Yang H, Chang Hsu T, He B, Dai M, Kao SJ, Zhao M & Zhang X-H (2015) Shift of anammox bacterial community structure along the Pearl Estuary and the impact of environmental factors. *Journal of Geophysical Research: Oceans* n/a-n/a.

Gaby JC & Buckley DH (2012) A Comprehensive Evaluation of PCR Primers to Amplify the *nifH* Gene of Nitrogenase. *PLoS ONE* **7**: e42149.

Galán A, Molina V, Thamdrup B, Woebken D, Lavik G, Kuypers MMM & Ulloa O (2009) Anammox bacteria and the anaerobic oxidation of ammonium in the oxygen minimum zone off northern Chile. *Deep Sea Research Part II: Topical Studies in Oceanography* **56**: 1021-1031.

Galand PE, Casamayor EO, Kirchman DL, Potvin M & Lovejoy C (2009) Unique archaeal assemblages in the Arctic Ocean unveiled by massively parallel tag sequencing. *ISME Journal* **3**: 860-869.

Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP & Sutton MA (2008) Transformation of the Nitrogen Cycle: Recent Trends, Questions, and Potential Solutions. *Science* **320**: 889-892.

Gardner AM, Helmick RA & Gardner PR (2002) Flavorubredoxin, an Inducible Catalyst for Nitric Oxide Reduction and Detoxification in *Escherichia coli*. *Journal of Biological Chemistry* **277**: 8172-8177.

Giblin A, Weston N, Banta G, Tucker J & Hopkinson C (2010) The Effects of Salinity on Nitrogen Losses from an Oligohaline Estuarine Sediment. *Estuaries and Coasts* **33**: 1054-1068.

Gibson JL & Tabita FR (1993) Nucleotide sequence and functional analysis of cbbR, a positive regulator of the Calvin cycle operons of *Rhodobacter sphaeroides*. *Journal of Bacteriology* **175**: 5778-5784.

Girsch P & de Vries S (1997) Purification and initial kinetic and spectroscopic characterization of NO reductase from *Paracoccus denitrificans*. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1318**: 202-216.

Glöckner J, Kube M, Shrestha PM, Weber M, Glöckner FO, Reinhardt R & Liesack W (2010) Phylogenetic diversity and metagenomics of candidate division OP3. *Environmental Microbiology* **12**: 1218-1229.

Godfrey LV & Falkowski PG (2009) The cycling and redox state of nitrogen in the Archaean ocean. *Nature Geoscience* **2**: 725-729.

Greene EA, Hubert C, Nemati M, Jenneman GE & Voordouw G (2003) Nitrite reductase activity of sulphate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria. *Environmental Microbiology* **5**: 607-617.

Gusarov I, Shatalin K, Starodubtseva M & Nudler E (2009) Endogenous Nitric Oxide Protects Bacteria Against a Wide Spectrum of Antibiotics. *Science* **325**: 1380-1384.

Hakemian AS & Rosenzweig AC (2007) The Biochemistry of Methane Oxidation. *Annual Review of Biochemistry* **76**: 223-241.

Hall JA, Kalin RM, Larkin MJ, Allen CCR & Harper DB (1999) Variation in stable carbon isotope fractionation during aerobic degradation of phenol and benzoate by contaminant degrading bacteria. *Organic Geochemistry* **30**: 801-811.

Hallam SJ, Mincer TJ, Schleper C, Preston CM, Roberts K, Richardson PM & DeLong EF (2006) Pathways of Carbon Assimilation and Ammonia Oxidation Suggested by Environmental Genomic Analyses of Marine Crenarchaeota. *PLoS Biology* **4**: e95.

Hansel CM, Lentini CJ, Tang Y, Johnston DT, Wankel SD & Jardine PM (2015) Dominance of sulfur-fueled iron oxide reduction in low-sulfate freshwater sediments. *ISME J*ournal **9**: 2400-2412.

Hanson BT & Madsen EL (2015) *In situ* expression of nitrite-dependent anaerobic methane oxidation proteins by *Candidatus* Methylomirabilis oxyfera co-occurring with expressed anammox proteins in a contaminated aquifer. *Environmental Microbiology Reports* **7**: 252-264.

Hanson RS & Hanson TE (1996) Methanotrophic bacteria. *Microbiological Reviews* **60**: 439-471.

Harhangi HR, Le Roy M, van Alen T, Hu B-l, Groen J, Kartal B, Tringe SG, Quan Z-X, Jetten MSM & Op den Camp HJM (2012) Hydrazine Synthase, a Unique Phylomarker with Which To Study the Presence and Biodiversity of Anammox Bacteria. *Applied and Environmental Microbiology* **78**: 752-758.

Haroon MF, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan Z & Tyson GW (2013) Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* **500**: 567-570.

Hatamoto M, Kimura M, Sato T, Koizumi M, Takahashi M, Kawakami S, Araki N & Yamaguchi T (2014) Enrichment of Denitrifying Methane-Oxidizing Microorganisms Using Up-Flow Continuous Reactors and Batch Cultures. *PLoS ONE* **9**: e115823.

He Z, Geng S, Cai C, Liu S, Liu Y, Pan Y, Lou L, Zheng P, Xu X & Hu B (2015) Halophilic anaerobic oxidation of methane coupled to nitrite reduction by marine NC10 bacteria. *Applied and Environmental Microbiology* **81**: 5538-5545.

Heidelberg JF, Seshadri R, Haveman SA*, et al.* (2004) The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nature Biotechnology* **22**: 554-559.

Hinrichs K-U, Summons RE, Orphan V, Sylva SP & Hayes JM (2000) Molecular and isotopic analysis of anaerobic methane-oxidizing communities in marine sediments. *Organic Geochemistry* **31**: 1685-1701.

Hinrichs KU & Boetius A (2003) The Anaerobic Oxidation of Methane: New Insights in Microbial Ecology and Biogeochemistry. *Ocean Margin Systems,*(Wefer G, Billett D, Hebbeln D, Jørgensen B, Schlüter M & van Weering TE, eds.), pp. 457-477. Springer Berlin Heidelberg.

Hirsch M, Long Z & Song B (2011) Anammox Bacterial Diversity in Various Aquatic Ecosystems Based on the Detection of Hydrazine Oxidase Genes (*hzoA/hzoB*). *Microbial Ecology* **61**: 264-276.

Hoehler TM, Alperin MJ, Albert DB & Martens CS (1994) Field and laboratory studies of methane oxidation in an anoxic marine sediment: Evidence for a methanogen-sulfate reducer consortium. *Global Biogeochemical Cycles* **8**: 451-463.

Hoehler TM, Alperin MJ, Albert DB & Martens CS (2001) Apparent minimum free energy requirements for methanogenic Archaea and sulfate-reducing bacteria in an anoxic marine sediment. *FEMS Microbiology Ecology* **38**: 33-41.

Holler T, Wegener G, Knittel K, Boetius A, Brunner B, Kuypers MMM & Widdel F (2009) Substantial $^{13}C/^{12}C$ and D/H fractionation during anaerobic oxidation of methane by marine consortia enriched *in vitro*. *Environmental Microbiology Reports* **1**: 370-376.

Holmkvist L, Ferdelman TG & Jørgensen BB (2011) A cryptic sulfur cycle driven by iron in the methane zone of marine sediment (Aarhus Bay, Denmark). *Geochimica et Cosmochimica Acta* **75**: 3581-3599.

Holmkvist L, Kamyshny Jr A, Vogt C, Vamvakopoulos K, Ferdelman TG & Jørgensen BB (2011) Sulfate reduction below the sulfate–methane transition in Black Sea sediments. *Deep Sea Research Part I: Oceanographic Research Papers* **58**: 493-504.

Hooper AB, Debey P, Andersson KK & Balny C (1983) Heme P460 of Hydroxylamine Oxidoreductase of *Nitrosomonas*. *European Journal of Biochemistry* **134**: 83-87.

Hou S, Makarova K, Saw J*, et al.* (2008) Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum Verrucomicrobia. *Biology Direct* **3**: 26.

Hu A, Jiao N, Zhang R & Yang Z (2011) Niche Partitioning of Marine Group I *Crenarchaeota* in the Euphotic and Upper Mesopelagic Zones of the East China Sea. *Applied and Environmental Microbiology* **77**: 7469-7478.

Hu B-l, Rush D, van der Biezen E, Zheng P, van Mullekom M, Schouten S, Sinninghe Damsté JS, Smolders AJP, Jetten MSM & Kartal B (2011) New Anaerobic, Ammonium-Oxidizing Community Enriched from Peat Soil. *Applied and Environmental Microbiology* **77**: 966-971.

Hu S, Zeng RJ, Burow LC, Lant P, Keller J & Yuan Z (2009) Enrichment of denitrifying anaerobic methane oxidizing microorganisms. *Environmental Microbiology Reports* **1**: 377-384.

Hu S, Zeng RJ, Haroon MF, Keller J, Lant PA, Tyson GW & Yuan Z (2015) A laboratory investigation of interactions between denitrifying anaerobic methane oxidation (DAMO) and anammox processes in anoxic environments. *Scientific Reports* **5**: 8706.

Hughes MN (1999) Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1411**: 263-272.

Humbert S, Tarnawski S, Fromin N, Mallet M-P, Aragno M & Zopfi J (2009) Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. *ISME Journal* **4**: 450-454.

Huson DH, Mitra S, Ruscheweyh H-J, Weber N & Schuster SC (2011) Integrative analysis of environmental sequences using MEGAN4. *Genome Research* **21**: 1552-1560.

Hutchens E, Radajewski S, Dumont MG, McDonald IR & Murrell JC (2004) Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. *Environmental Microbiology* **6**: 111-120.

Igarashi N, Moriyama H, Fujiwara T, Fukumori Y & Tanaka N (1997) The 2.8 A structure of hydroxylamine oxidoreductase from a nitrifying chemoautotrophic bacterium, *Nitrosomonas europaea*. *Nature Structural & Molecular Biology* **4**: 276-284.

Iino T, Tamaki H, Tamazawa S, Ueno Y, Ohkuma M, Suzuki K-i, Igarashi Y & Haruta S (2013) Candidatus *Methanogranum caenicola*: a Novel Methanogen from the Anaerobic Digested Sludge, and Proposal of *Methanomassiliicoccaceae* fam. nov. and *Methanomassiliicoccales* ord. nov., for a Methanogenic Lineage of the Class Thermoplasmata. *Microbes and Environments* **28**: 244-250.

Inagaki F, Takai K, Nealson KH & Horikoshi K (2004) *Sulfurovum lithotrophicum* gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the ε-Proteobacteria isolated from Okinawa Trough hydrothermal sediments. *International Journal of Systematic and Evolutionary Microbiology* **54**: 1477-1482.

Inagaki F, Takai K, Kobayashi H, Nealson KH & Horikoshi K (2003) *Sulfurimonas autotrophica* gen. nov., sp. nov., a novel sulfur-oxidizing ε-proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough. *International Journal of Systematic and Evolutionary Microbiology* **53**: 1801-1805.

Inagaki F, Suzuki M, Takai K, Oida H, Sakamoto T, Aoki K, Nealson KH & Horikoshi K (2003) Microbial Communities Associated with Geological Horizons in Coastal Subseafloor Sediments from the Sea of Okhotsk. *Applied and Environmental Microbiology* **69**: 7224-7235.

Inagaki F, Nunoura T, Nakagawa S*, et al.* (2006) Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 2815-2820.

Inagaki F, Kuypers MMM, Tsunogai U*, et al.* (2006) Microbial community in a sediment-hosted CO_2 lake of the southern Okinawa Trough hydrothermal system. *Proceedings of the National Academy of Sciences* **103**: 14164-14169.

IPCC IPoCC (2007) Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. pp. Cambridge University Press, Cambridge.

Islam T, Jensen S, Reigstad LJ, Larsen O & Birkeland NK (2008) Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the *Verrucomicrobia* phylum. *Proceedings of the National Academy of Sciences* **105**: 300-304.

Itoh H, Ishii S, Shiratori Y, Oshima K, Otsuka S, Hattori M & Senoo K (2013) Seasonal Transition of Active Bacterial and Archaeal Communities in Relation to Water Management in Paddy Soils. *Microbes and Environments* **28**: 370-380.

Jilbert T & Slomp CP (2013) Iron and manganese shuttles control the formation of authigenic phosphorus minerals in the euxinic basins of the Baltic Sea. *Geochimica et Cosmochimica Acta* **107**: 155-169.

Jorgensen SL, Hannisdal B, Lanzén A*, et al.* (2012) Correlating microbial community profiles with geochemical data in highly stratified sediments from the Arctic Mid-Ocean Ridge. *Proceedings of the National Academy of Sciences* **109**: E2846–E2855.

Joye SB & Anderson IC (2008) Nitrogen cycling in coastal sediments. *Nitrogen in the marine environment,*(Capone DG, Bronk DA, Mulholland MR & Carpenter EJ, eds.), pp. 868–915. Academic Press, Amsterdam.

Kai FM, Tyler SC, Randerson JT & Blake DR (2011) Reduced methane growth rate explained by decreased Northern Hemisphere microbial sources. *Nature* **476**: 194-197.

Kaku N, Ueki A, Ueki K & Watanabe K (2005) Methanogenesis as an Important Terminal Electron Accepting Process in Estuarine Sediment at the Mouth of Orikasa River. *Microbes and Environments* **20**: 41-52.

Kalyuzhnaya MG, Hristova KR, Lidstrom ME & Chistoserdova L (2008) Characterization of a Novel Methanol Dehydrogenase in Representatives of *Burkholderiales*: Implications for Environmental Detection of Methylotrophy and Evidence for Convergent Evolution. *Journal of Bacteriology* **190**: 3817-3823.

Kalyuzhnaya MG, Yang S, Rozova ON*, et al.* (2013) Highly efficient methane biocatalysis revealed in a methanotrophic bacterium. *Nature Communications* **4**. doi:10.1038/ncomms3785

Kampara M, Thullner M, Harms H & Wick L (2009) Impact of cell density on microbially induced stable isotope fractionation. *Applied Microbiology and Biotechnology* **81**: 977-985.

Kampman C, Temmink H, Hendrickx TLG, Zeeman G & Buisman CJN (2014) Enrichment of denitrifying methanotrophic bacteria from municipal wastewater sludge in a membrane bioreactor at 20°C. *Journal of Hazardous Materials* **274**: 428-435.

Kao W-C, Chen Y-R, Yi EC, *et al.* (2004) Quantitative Proteomic Analysis of Metabolic Regulation by Copper Ions in *Methylococcus capsulatus* (Bath). *Journal of Biological Chemistry* **279**: 51554-51560.

Kartal B, van Niftrik L, Keltjens JT, Op den Camp HJM & Jetten MSM (2012) Chapter 3 - Anammox -Growth Physiology, Cell Biology, and Metabolism. *Advances in Microbial Physiology,* Vol. Volume 60 (Robert KP, ed.) pp. 211-262. Academic Press.

Kartal B, Koleva M, Arsov R, van der Star W, Jetten MSM & Strous M (2006) Adaptation of a freshwater anammox population to high salinity wastewater. *Journal of Biotechnology* **126**: 546-553.

Kartal B, Maalcke WJ, de Almeida NM, *et al.* (2011) Molecular mechanism of anaerobic ammonium oxidation. *Nature* **479**: 127-130.

Kato S, Yanagawa K, Sunamura M, *et al.* (2009) Abundance of *Zetaproteobacteria* within crustal fluids in back-arc hydrothermal fields of the Southern Mariana Trough. *Environmental Microbiology* **11**: 3210-3222.

Keltjens JT, Pol A, Reimann J & Op den Camp HJM (2014) PQQ-dependent methanol dehydrogenases: rare-earth elements make a difference. *Applied Microbiology and Biotechnology* **98**: 6163-6183.

Kemnitz D, Kolb S & Conrad R (2005) Phenotypic characterization of Rice Cluster III archaea without prior isolation by applying quantitative polymerase chain reaction to an enrichment culture. *Environmental Microbiology* **7**: 553-565.

Kessler JD, Reeburgh WS & Tyler SC (2006) Controls on methane concentration and stable isotope (δ^2H-CH_4 and δ^{13}C-CH_4) distributions in the water columns of the Black Sea and Cariaco Basin. *Global Biogeochemical Cycles* **20**: n/a-n/a.

Khadem AF, Pol A, Wieczorek A, Mohammadi SS, Francoijs KJ, Stunnenberg HG, Jetten MSM & Op den Camp HJM (2011) Autotrophic methanotrophy in Verrucomicrobia: *Methylacidiphilum fumariolicum* SolV uses the Calvin-Benson-Bassham cycle for carbon dioxide fixation. *Journal of Bacteriology* **193**: 4438-4446.

Kim BS, Oh HM, Kang H & Chun J (2005) Archaeal diversity in tidal flat sediment as revealed by 16S rDNA analysis. *The Journal of Microbiology* **43**: 144-151.

Kim SO, Orii Y, Lloyd D, Hughes MN & Poole RK (1999) Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. *FEBS Letters* **445**: 389-394.

Kinnaman FS, Valentine DL & Tyler SC (2007) Carbon and hydrogen isotope fractionation associated with the aerobic microbial oxidation of methane, ethane, propane and butane. *Geochimica et Cosmochimica Acta* **71**: 271-283.

Kits KD, Klotz MG & Stein LY (2015) Methane oxidation coupled to nitrate reduction under hypoxia by the Gammaproteobacterium *Methylomonas denitrificans*, sp. nov. type strain FJG1. *Environmental Microbiology* **17**: 3219-3232.

Kletzin A, Heimerl T, Flechsler J, Van Niftrik L, Rachel R & Klingl A (2015) Cytochromes *c* in Archaea: distribution, maturation, cell architecture and the special case of *Ignicoccus hospitalis*. *Frontiers in Microbiology* **6**.

Klotz MG, Arp DJ, Chain PSG*, et al.* (2006) Complete Genome Sequence of the Marine, Chemolithoautotrophic, Ammonia-Oxidizing Bacterium *Nitrosococcus oceani* ATCC 19707. *Applied and Environmental Microbiology* **72**: 6299-6315.

Knittel K & Boetius A (2009) Anaerobic Oxidation of Methane: Progress with an Unknown Process. *Annual Review of Microbiology* **63**: 311-334.

Knittel K, Lösekann T, Boetius A, Kort R & Amann R (2005) Diversity and Distribution of Methanotrophic Archaea at Cold Seeps. *Applied and Environmental Microbiology* **71**: 467-479.

Kojima H, Tsutsumi M, Ishikawa K, Iwata T, Mußmann M & Fukui M (2012) Distribution of putative denitrifying methane oxidizing bacteria in sediment of a freshwater lake, Lake Biwa. *Systematic and Applied Microbiology* **35**: 233-238.

Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB & Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543-546.

Kool DM, Zhu BL, Rijpstra WIC, Jetten MSM, Ettwig KF & Damsté JSS (2012) Rare branched fatty acids characterize the lipid composition of the intra-aerobic methane oxidizer "*Candidatus* Methylomirabilis oxyfera". *Applied and Environmental Microbiology* **78**: 8650-8656.

Koutný M & Kučera I (1999) Kinetic Analysis of Substrate Inhibition in Nitric Oxide Reductase of *Paracoccus denitrificans*. *Biochemical and Biophysical Research Communications* **262**: 562-564.

Kraft B, Tegetmeyer HE, Sharma R, Klotz MG, Ferdelman TG, Hettich RL, Geelhoed JS & Strous M (2014) The environmental controls that govern the end product of bacterial nitrate respiration. *Science* **345**: 676-679.

Krüger M, Meyerdierks A, Glockner FO*, et al.* (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. *Nature* **426**: 878-881.

Kumar PA, Srinivas TNR, Madhu S, Kumar RS, Singh S, Naqvi SWA, Mayilraj S & Shivaji S (2011) *Cecembia lonarensis* gen. nov., sp. nov., a novel haloalkalitolerant bacterium of the family *Cyclobacteriaceae,* isolated from a haloalkaline lake and emended descriptions of the genera *Indibacter*, *Nitritalea*, *Belliella* and *Aquiflexum*. *International Journal of Systematic and Evolutionary Microbiology* **61**: 1762.

Kump LR, Junium C, Arthur MA, Brasier A, Fallick A, Melezhik V, Lepland A, CČrne AE & Luo G (2011) Isotopic Evidence for Massive Oxidation of Organic Matter Following the Great Oxidation Event. *Science* **334**: 1694-1696.

Kuypers MMM, Lavik G, Woebken D, Schmid M, Fuchs BM, Amann R, Jørgensen BB & Jetten MSM (2005) Massive nitrogen loss from the Benguela upwelling system through anaerobic

ammonium oxidation. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 6478-6483.

Kuypers MMM, Sliekers AO, Lavik G, Schmid M, Jorgensen BB, Kuenen JG, Sinninghe Damste JS, Strous M & Jetten MSM (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422**: 608-611.

Kwiatkowski AV & Shapleigh JP (1996) Requirement of Nitric Oxide for Induction of Genes Whose Products Are Involved in Nitric Oxide Metabolism in *Rhodobacter sphaeroides* 2.4.3. *Journal of Biological Chemistry* **271**: 24382-24388.

Lam P, Lavik G, Jensen MM, van de Vossenberg J, Schmid M, Woebken D, Gutiérrez D, Amann R, Jetten MSM & Kuypers MMM (2009) Revising the nitrogen cycle in the Peruvian oxygen minimum zone. *Proceedings of the National Academy of Sciences* **106**: 4752-4757.

Lee HJ, Jeong SE, Kim PJ, Madsen EL & Jeon CO (2015) High resolution depth distribution of Bacteria, Archaea, methanotrophs, and methanogens in the bulk and rhizosphere soils of a flooded rice paddy. *Frontiers in Microbiology* **6**.

Lehmann MF, Bernasconi SM, McKenzie JA, Barbieri A, Simona M & Veronesi M (2004) Seasonal variation of the $d^{13}C$ and $d^{15}N$ of particulate and dissolved carbon and nitrogen in Lake Lugano: Constraints on biogeochemical cycling in a eutrophic lake. *Limnology and Oceanography* **49**: 415-429.

Li-dong S, Qun Z, Shuai L, Ping D, Jiang-ning Z, Dong-qing C, Xiang-yang X, Ping Z & Bao-lan H (2014) Molecular evidence for nitrite-dependent anaerobic methane-oxidising bacteria in the Jiaojiang Estuary of the East Sea (China). *Applied Microbiology and Biotechnology* **98**: 5029-5038.

Liu D, Wang H, Dong H, Qiu X, Dong X & Cravotta Iii CA (2011) Mineral transformations associated with goethite reduction by *Methanosarcina barkeri*. *Chemical Geology* **288**: 53-60.

Liu D, Dong H, Bishop ME, Wang H, Agrawal A, Tritschler S, Eberl DD & Xie S (2011) Reduction of structural Fe(III) in nontronite by methanogen *Methanosarcina barkeri*. *Geochimica et Cosmochimica Acta* **75**: 1057-1071.

Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA & Boon EM (2012) Nitric Oxide Regulation of Cyclic di-GMP Synthesis and Hydrolysis in *Shewanella woodyi*. *Biochemistry* **51**: 2087-2099.

Liu Y, Zhang J, Zhao L, Li Y, Yang Y & Xie S (2015) Aerobic and nitrite-dependent methane-oxidizing microorganisms in sediments of freshwater lakes on the Yunnan Plateau. *Applied Microbiology and Biotechnology* **99**: 2371-2381.

Lloyd KG, Lapham L & Teske A (2006) An Anaerobic Methane-Oxidizing Community of ANME-1b Archaea in Hypersaline Gulf of Mexico Sediments. *Applied and Environmental Microbiology* **72**: 7218-7230.

Lohmayer R, Kappler A, Lösekann-Behrens T & Planer-Friedrich B (2014) Sulfur Species as Redox Partners and Electron Shuttles for Ferrihydrite Reduction by *Sulfurospirillum deleyianum*. *Applied and Environmental Microbiology* **80**: 3141-3149.

Lovley DR & Phillips EJP (1994) Reduction of Chromate by *Desulfovibrio vulgaris* and Its *c3* Cytochrome. *Applied and Environmental Microbiology* **60**: 726-728.

Lovley DR, Holmes DE & Nevin KP (2004) Dissimilatory Fe(III) and Mn(IV) Reduction. *Advances in Microbial Physiology,* Vol. Volume 49 pp. 219-286. Academic Press.

Lovley DR, Roden EE, Phillips EJP & Woodward JC (1993) Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Marine Geology* **113**: 41-53.

Ludwig W, Strunk O, Westram R*, et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32**: 1363-1371.

Lueders T, Pommerenke B & Friedrich MW (2004) Stable-Isotope Probing of Microorganisms Thriving at Thermodynamic Limits: Syntrophic Propionate Oxidation in Flooded Soil. *Applied and Environmental Microbiology* **70**: 5778-5786.

Luesken F, van Alen T, van der Biezen E*, et al.* (2011) Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. *Applied Microbiology and Biotechnology* **92**: 845-854.

Luesken FA, Wu ML, Op den Camp HJM, Keltjens JT, Stunnenberg H, Francoijs K-J, Strous M & Jetten MSM (2012) Effect of oxygen on the anaerobic methanotroph '*Candidatus* Methylomirabilis oxyfera': kinetic and transcriptional analysis. *Environmental Microbiology* **14**: 1024-1034.

Luesken FA, Zhu B, van Alen TA, Butler MK, Diaz MR, Song B, Op den Camp HJM, Jetten MSM & Ettwig KF (2011) *pmoA* Primers for Detection of Anaerobic Methanotrophs. *Applied and Environmental Microbiology* **77**: 3877-3880.

Luesken FA, Sanchez J, van Alen TA, Sanabria J, Op den Camp HJM, Jetten MSM, Kartal B, (2011b) Simultaneous nitrite-dependent anaerobic methane and ammonium oxidation. *Applied and Environmental Microbiology* **77**: 6802-6807.

Lüke C, Speth DR, Kox M, Villanueva L & Jetten MSM (2015) Potential of microbially mediated nitrogen and carbon cycling in the Arabian Sea oxygen minimum zone. *In preparation*.

Lund MB, Smith JM & Francis CA (2012) Diversity, abundance and expression of nitrite reductase (*nirK*)-like genes in marine thaumarchaea. *ISME Journal* **6**: 1966-1977.

Lundberg C, Jakobsson B-M & Bonsdorff E (2009) The spreading of eutrophication in the eastern coast of the Gulf of Bothnia, northern Baltic Sea – An analysis in time and space. *Estuarine, Coastal and Shelf Science* **82**: 152-160.

Maalcke WJ, Dietl A, Marritt SJ, Butt JN, Jetten MSM, Keltjens JT, Barends TRM & Kartal B (2014) Structural Basis of Biological NO Generation by Octaheme Oxidoreductases. *Journal of Biological Chemistry* **289**: 1228-1242.

Mahieu K, Visscher AD, Vanrolleghem PA & Cleemput OV (2006) Carbon and hydrogen isotope fractionation by microbial methane oxidation: Improved determination. *Waste Management* **26**: 389-398.

Martens CS, Albert DB & Alperin MJ (1999) Stable isotope tracing of anaerobic methane oxidation in the gassy sediments of Eckernförder Bay, German Baltic Sea. *American Journal of Science* **299**: 589-610.

McDonald IR & Murrell JC (1997) The methanol dehydrogenase structural gene *mxaF* and its use as a functional gene probe for methanotrophs and methylotrophs. *Applied and Environmental Microbiology* **63**: 3218-3224.

McInerney MJ, Struchtemeyer CG, Sieber J, Mouttaki H, Stams AJM, Schink B, Rohlin L & Gunsalus RP (2008) Physiology, Ecology, Phylogeny, and Genomics of Microorganisms Capable of Syntrophic Metabolism. *Annals of the New York Academy of Sciences* **1125**: 58-72.

Meckenstock RU, Morasch B, Griebler C & Richnow HH (2004) Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated acquifers. *Journal of Contaminant Hydrology* **75**: 215-255.

Meijer WG, van den Bergh ER & Smith LM (1996) Induction of the gap-pgk operon encoding glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase of *Xanthobacter flavus* requires the LysR-type transcriptional activator CbbR. *Journal of Bacteriology* **178**: 881-887.

Michaelis W, Seifert R, Nauhaus K*, et al.* (2002) Microbial Reefs in the Black Sea Fueled by Anaerobic Oxidation of Methane. *Science* **297**: 1013-1015.

Michel C, Brugna M, Aubert C, Bernadac A & Bruschi M (2001) Enzymatic reduction of chromate: comparative studies using sulfate-reducing bacteria. *Applied Microbiology and Biotechnology* **55**: 95-100.

Mills PC, Rowley G, Spiro S, Hinton JCD & Richardson DJ (2008) A combination of cytochrome *c* nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. *Microbiology* **154**: 1218-1228.

Milucka J, Ferdelman TG, Polerecky L, Franzke D, Wegener G, Schmid M, Lieberwirth I, Wagner M, Widdel F & Kuypers MMM (2012) Zero-valent sulphur is a key intermediate in marine methane oxidation. *Nature* **491**: 541-546.

Montzka SA, Dlugokencky EJ & Butler JH (2011) Non-CO_2 greenhouse gases and climate change. *Nature* **476**: 43-50.

Murrell JC, McDonald IR & Gilbert B (2000) Regulation of expression of methane monooxygenases by copper ions. *Trends in Microbiology* **8**: 221-225.

Mußmann M, Brito I, Pitcher A*, et al.* (2011) Thaumarchaeotes abundant in refinery nitrifying sludges express *amoA* but are not obligate autotrophic ammonia oxidizers. *Proceedings of the National Academy of Sciences* **108**: 16771-16776.

Nauhaus K, Treude T, Boetius A & Krüger M (2005) Environmental regulation of the anaerobic oxidation of methane: a comparison of ANME-I and ANME-II communities. *Environmental Microbiology* **7**: 98-106.

Nesheim JC & Lipscomb JD (1996) Large Kinetic Isotope Effects in Methane Oxidation Catalyzed by Methane Monooxygenase: Evid□n□□f□r □–H B□nd □l□v□g□in □R□□ti□n □y□l□ Intermediate. *Biochemistry* **35**: 10240-10247.

Norði Kà, Thamdrup B & Schubert CJ (2013) Anaerobic oxidation of methane in an iron-rich Danish freshwater lake sediment. *Limnology and Oceanography* **58**: 546-554.

Norton J, Alzerreca J, Suwa Y & Klotz M (2002) Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Archives of Microbiology* **177**: 139-149.

Nunoura T, Takaki Y, Kazama H, Hirai M, Ashi J, Imachi H & Takai K (2012) Microbial Diversity in Deep-sea Methane Seep Sediments Presented by SSU rRNA Gene Tag Sequencing. *Microbes and Environments* **27**: 382-390.

Nyerges G & Stein LY (2009) Ammonia cometabolism and product inhibition vary considerably among species of methanotrophic bacteria. *FEMS Microbiology Letters* **297**: 131-136.

Oakley BB, Francis CA, Roberts KJ, Fuchsman CA, Srinivasan S & Staley JT (2007) Analysis of nitrite reductase (*nirK* and *nirS*) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone. *Environmental Microbiology* **9**: 118-130.

Op den Camp HJM, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S, Jetten MSM, Birkeland N-K, Pol A & Dunfield PF (2009) Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. *Environmental Microbiology Reports* **1**: 293-306.

Orphan VJ, House CH, Hinrichs K-U, McKeegan KD & DeLong EF (2001) Methane-Consuming Archaea Revealed by Directly Coupled Isotopic and Phylogenetic Analysis. *Science* **293**: 484-487.

Orphan VJ, House CH, Hinrichs K-U, McKeegan KD & DeLong EF (2002) Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 7663-7668.

Ottow JCG (1971) Iron reduction and gley formation by nitrogen-fixing clostridia. *Oecologia* **6**: 164-175.

Panganiban AT, Patt TE, Hart W & Hanson RS (1979) Oxidation of methane in the absence of oxygen in lake water samples. *Applied and Environmental Microbiology* **37**: 303-309.

Park HS, Kim BH, Kim HS, Kim HJ, Kim GT, Kim M, Chang IS, Park YK & Chang HI (2001) A Novel Electrochemically Active and Fe(III)-reducing Bacterium Phylogenetically Related to *Clostridium butyricum* Isolated from a Microbial Fuel Cell. *Anaerobe* **7**: 297-306.

Parkes S, Jolley D & Wilson S (2007) Inorganic nitrogen transformations in the treatment of landfill leachate with a high ammonium load: A case study. *Environmental Monitoring and Assessement* **124**: 51-61.

Penton CR, Devol AH & Tiedje JM (2006) Molecular Evidence for the Broad Distribution of Anaerobic Ammonium-Oxidizing Bacteria in Freshwater and Marine Sediments. *Applied and Environmental Microbiology* **72**: 6829-6832.

Pereira IAC, Ramos AR, Grein F, Marques MC, Da Silva SM & Venceslau SS (2011) A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea. *Frontiers in Microbiology* **2**.

Petit JR, Jouzel J, Raynaud D*, et al.* (1999) Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* **399**: 429-436.

Plugge CM, Zhang W, Scholten JCM & Stams AJM (2011) Metabolic Flexibility of Sulfate-Reducing Bacteria. *Frontiers in Microbiology* **2**: 81.

Pol A, Heijmans K, Harhangi HR, Tedesco D, Jetten MSM & den Camp H (2007) Methanotrophy below pH1 by a new Verrucomicrobia species. *Nature* **450**: 874-U817.

Pol A, Barends TRM, Dietl A, Khadem AF, Eygensteyn J, Jetten MSM & Op den Camp HJM (2014) Rare earth metals are essential for methanotrophic life in volcanic mudpots. *Environmental Microbiology* **16**: 255-264.

Polyakov KM, Boyko KM, Tikhonova TV, Slutsky A, Antipov AN, Zvyagilskaya RA, Popov AN, Bourenkov GP, Lamzin VS & Popov VO (2009) High-Resolution Structural Analysis of a Novel Octaheme Cytochrome *c* Nitrite Reductase from the Haloalkaliphilic Bacterium *Thioalkalivibrio nitratireducens*. *Journal of Molecular Biology* **389**: 846-862.

Poole RK (2005) Nitric oxide and nitrosative stress tolerance in bacteria. *Biochemical Society Transactions* **33**: 176-180.

Postgate JR (1998) *Nitrogen Fixation*. Cambridge University Press.

Pruesse E, Peplies J & Glöckner FO (2012) SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* **28**: 1823-1829.

Qiu Q, Noll M, Abraham W-R, Lu Y & Conrad R (2008) Applying stable isotope probing of phospholipid fatty acids and rRNA in a Chinese rice field to study activity and composition of the methanotrophic bacterial communities *in situ*. *ISME Journal* **2**: 602-614.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J & Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**: D590-D596.

Quay P, Stutsman J, Wilbur D, Snover A, Dlugokencky E & Brown T (1999) The isotopic composition of atmospheric methane. *Global Biogeochemical Cycles* **13**: 445-461.

Raghoebarsing AA, Pol A, van de Pas-Schoonen KT*, et al.* (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440**: 918-921.

Ragon M, Van Driessche AE, Garcia Ruiz JM, Moreira D & Lopez-Garcia P (2013) Microbial diversity in the deep-subsurface hydrothermal aquifer feeding the giant gypsum crystal-bearing Naica mine, Mexico. *Frontiers in Microbiology* **4**.

Rasigraf O, Kool DM, Jetten MSM, Sinninghe Damsté JS & Ettwig KF (2014) Autotrophic Carbon Dioxide Fixation via the Calvin-Benson-Bassham Cycle by the Denitrifying Methanotroph "*Candidatus* Methylomirabilis oxyfera". *Applied and Environmental Microbiology* **80**: 2451-2460.

Reeburgh WS (1976) Methane consumption in Cariaco Trench waters and sediments. *Earth and Planetary Science Letters* **28**: 337-344.

Reeburgh WS (2007) Oceanic Methane Biogeochemistry. *Chemical Reviews* **107**: 486-513.

Reeburgh WS, Hirsch AI, Sansone FJ, Popp BN & Rust TM (1997) Carbon kinetic isotope effect accompanying microbial oxidation of methane in boreal forest soils. *Geochimica et Cosmochimica Acta* **61**: 4761-4767.

Rees DC & Howard JB (2000) Nitrogenase: standing at the crossroads. *Current Opinion in Chemical Biology* **4**: 559-566.

Robertson GP & Vitousek PM (2009) Nitrogen in Agriculture: Balancing the Cost of an Essential Resource. *Annual Review of Environment and Resources* **34**: 97-125.

Robertson GP, Paul EA & Harwood RR (2000) Greenhouse Gases in Intensive Agriculture: Contributions of Individual Gases to the Radiative Forcing of the Atmosphere. *Science* **289**: 1922-1925.

Roussel EG, Sauvadet A-L, Chaduteau C, Fouquet Y, Charlou J-L, Prieur D & Cambon Bonavita M-A (2009) Archaeal communities associated with shallow to deep subseafloor sediments of the New Caledonia Basin. *Environmental Microbiology* **11**: 2446-2462.

Ruff SE, Biddle JF, Teske AP, Knittel K, Boetius A & Ramette A (2015) Global dispersion and local diversification of the methane seep microbiome. *Proceedings of the National Academy of Sciences* **112**: 4015-4020.

Russ L, Kartal B, Op Den Camp HJM, Sollai M, Le Bruchec J, Caprais J-C, Godfroy A, Sinninghe Damsté JS & Jetten MSM (2013) Presence and diversity of anammox bacteria in cold hydrocarbon-rich seeps and hydrothermal vent sediments of the Guaymas Basin. *Frontiers in Microbiology* **4**.

Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Sanford RA, Wagner DD, Wu Q*, et al.* (2012) Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proceedings of the National Academy of Sciences* **109**: 19709-19714.

Santoro AE, Casciotti KL & Francis CA (2010) Activity, abundance and diversity of nitrifying archaea and bacteria in the central California Current. *Environmental Microbiology* **12**: 1989-2006.

Santoro AE, Buchwald C, McIlvin MR & Casciotti KL (2011) Isotopic Signature of N_2O Produced by Marine Ammonia-Oxidizing Archaea. *Science* **333**: 1282-1285.

Saueressig G, Bergamaschi P, Crowley JN, Fischer H & Harris GW (1996) D/H kinetic isotope effect in the reaction CH_4+Cl. *Geophysical Research Letters* **23**: 3619-3622.

Saueressig G, Crowley JN, Bergamaschi P, Brühl C, Brenninkmeijer CAM & Fischer H (2001) Carbon 13 and D kinetic isotope effects in the reactions of CH_4 with O(1D) and OH: New laboratory measurements and their implications for the isotopic composition of stratospheric methane. *Journal of Geophysical Research: Atmospheres* **106**: 23127-23138.

Scala DJ, Hacherl EL, Cowan R, Young LY & Kosson DS (2006) Characterization of Fe(III)-reducing enrichment cultures and isolation of Fe(III)-reducing bacteria from the Savannah River site, South Carolina. *Research in Microbiology* **157**: 772-783.

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews* **61**: 262-280.

Schmid MC, Hooper AB, Klotz MG, Woebken D, Lam P, Kuypers MMM, Pommerening-Roeser A, Op Den Camp HJM & Jetten MSM (2008) Environmental detection of octahaem cytochrome *c* hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. *Environmental Microbiology* **10**: 3140-3149.

Schmid MC, Risgaard-Petersen N, Van De Vossenberg J*, et al.* (2007) Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. *Environmental Microbiology* **9**: 1476-1484.

Schmidt I, Steenbakkers PJM, op den Camp HJM, Schmidt K & Jetten MSM (2004) Physiologic and Proteomic Evidence for a Role of Nitric Oxide in Biofilm Formation by *Nitrosomonas europaea* and Other Ammonia Oxidizers. *Journal of Bacteriology* **186**: 2781-2788.

Schmidt S, Christen P, Kiefer P & Vorholt JA (2010) Functional investigation of methanol dehydrogenase-like protein XoxF in *Methylobacterium extorquens* AM1. *Microbiology* **156**: 2575-2586.

Schouten S, Klein Breteler WCM, Blokker P, Schogt N, Rijpstra WIC, Grice K, Baas M & Sinninghe Damsté JS (1998) Biosynthetic effects on the stable carbon isotopic compositions of algal lipids: implications for deciphering the carbon isotopic biomarker record. *Geochimica et Cosmochimica Acta* **62**: 1397-1406.

Schreiber F, Wunderlin P, Udert KM & Wells GF (2012) Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions and novel technologies. *Frontiers in Microbiology* **3**.

Schubert CJ, Lucas FS, Durisch-Kaiser E, Stierli R, Diem T, Scheidegger O, Vazquez F & Müller B (2010) Oxidation and emission of methane in a monomictic lake (Rotsee, Switzerland). *Aquatic Sciences* **72**: 455-466.

Segarra KEA, Comerford C, Slaughter J & Joye SB (2013) Impact of electron acceptor availability on the anaerobic oxidation of methane in coastal freshwater and brackish wetland sediments. *Geochimica et Cosmochimica Acta* **115**: 15-30.

Semrau JD, DiSpirito AA & Murrell JC (2008) Life in the extreme: thermoacidophilic methanotrophy. *Trends in Microbiology* **16**: 190-193.

Sharp C, Stott M & Dunfield P (2012) Detection of autotrophic verrucomicrobial methanotrophs in a geothermal environment using stable isotope probing. *Frontiers in Microbiology* **3**: 1-9.

Shen L-d, Liu S, Zhu Q, Li X-y, Cai C, Cheng D-q, Lou L-p, Xu X-y, Zheng P & Hu B-l (2014) Distribution and Diversity of Nitrite-Dependent Anaerobic Methane-Oxidising Bacteria in the Sediments of the Qiantang River. *Microbial Ecology* **67**: 341-349.

Shi L, Squier TC, Zachara JM & Fredrickson JK (2007) Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem *c*-type cytochromes. *Molecular Microbiology* **65**: 12-20.

Shiro Y (2012) Structure and function of bacterial nitric oxide reductases: Nitric oxide reductase, anaerobic enzymes. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1817**: 1907-1913.

Siegert M, Krüger M, Teichert B, Wiedicke M & Schippers A (2011) Anaerobic Oxidation of Methane at a Marine Methane Seep in a Forearc Sediment Basin off Sumatra, Indian Ocean. *Frontiers in Microbiology* **2**: 249.

Siegert M, Cichocka D, Herrmann S, Gründger F, Feisthauer S, Richnow H-H, Springael D & Krüger M (2011) Accelerated methanogenesis from aliphatic and aromatic hydrocarbons under iron- and sulfate-reducing conditions. *FEMS Microbiology Letters* 315: 6-16.

Sikorski J, Lapidus A, Copeland A*, et al.* (2010) Complete genome sequence of *Sulfurospirillum deleyianum* type strain (5175(T)). *Standards in Genomic Sciences* **2**: 149-157.

Sivan O, Antler G, Turchyn AV, Marlow JJ & Orphan VJ (2014) Iron oxides stimulate sulfate-driven anaerobic methane oxidation in seeps. *Proceedings of the National Academy of Sciences* **111**: E4139-E4147.

Sivan O, Adler M, Pearson A, Gelman F, Bar-Or I, John SG & Eckert W (2011) Geochemical evidence for iron-mediated anaerobic oxidation of methane. *Limnology and Oceanography* **56**: 1536-1544.

Sleat R & Mah RA (1984) Quantitative method for colorimetric determination of formate in fermentation media. *Applied and Environmental Microbiology* **47**: 884-885.

Slomp CP, Mort HP, Jilbert T, Reed DC, Gustafsson BG & Wolthers M (2013) Coupled Dynamics of Iron and Phosphorus in Sediments of an Oligotrophic Coastal Basin and the Impact of Anaerobic Oxidation of Methane. *PLoS ONE* **8**: e62386.

Smemo KA & Yavitt JB (2007) Evidence for Anaerobic CH_4 Oxidation in Freshwater Peatlands. *Geomicrobiology Journal* **24**: 583-597.

Smith CJ, Nedwell DB, Dong LF & Osborn AM (2007) Diversity and Abundance of Nitrate Reductase Genes (*narG* and *napA*), Nitrite Reductase Genes (*nirS* and *nrfA*), and Their Transcripts in Estuarine Sediments. *Applied and Environmental Microbiology* **73**: 3612-3622.

Smith RL, Howes BL & Garabedian SP (1991) *In situ* measurement of methane oxidation in groundwater by using natural-gradient tracer tests. *Applied and Environmental Microbiology* **57**: 1997-2004.

Snover AK & Quay PD (2000) Hydrogen and carbon kinetic isotope effects during soil uptake of atmospheric methane. *Global Biogeochemical Cycles* **14**: 25-39.

Söhngen NL (1906) Über Bakterien, welche Methan als Kohlenstoffnahrung und Energiequelle gebrauchen. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **15**: 513-517.

Song B, Lisa JA & Tobias CR (2014) Linking DNRA community structure and activity in a shallow lagoonal estuarine system. *Frontiers in Microbiology* **5**: 460.

Sørensen KB & Teske A (2006) Stratified Communities of Active Archaea in Deep Marine Subsurface Sediments. *Applied and Environmental Microbiology* **72**: 4596-4603.

Sørensen KB, Lauer A & Teske A (2004) Archaeal phylotypes in a metal-rich and low-activity deep subsurface sediment of the Peru Basin, ODP Leg 201, Site 1231. *Geobiology* **2**: 151-161.

Sorokin DY, Lücker S, Vejmelkova D*, et al.* (2012) Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum *Chloroflexi*. *ISME Journal* **6**: 2245-2256.

Stach P, Einsle O, Schumacher W, Kurun E & M.H. Kroneck P (2000) Bacterial cytochrome *c* nitrite reductase: new structural and functional aspects. *Journal of Inorganic Biochemistry* **79**: 381-385.

Stahl DA & de la Torre JR (2012) Physiology and Diversity of Ammonia-Oxidizing Archaea. *Annual Review of Microbiology* **66**: 83-101.

Stams AJM & Hansen TA (1984) Fermentation of glutamate and other compounds by *Acidaminobacter hydrogenoformans* gen. nov. sp. nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate-reducing and methanogenic bacteria. *Archives in Microbiology* **137**: 329-337.

Stams AJM & Plugge CM (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nature Reviews Microbiology* **7**: 568-577.

Stein LY & Klotz MG (2011) Nitrifying and denitrifying pathways of methanotrophic bacteria. *Biochemical Society Transactions* **39**: 1826–1831.

Stein LY & Klotz MG (2011) Nitrifying and denitrifying pathways of methanotrophic bacteria. *Biochemical Society Transactions* **39**: 1826-31.

Stevanin TM, Poole RK, Demoncheaux EAG & Read RC (2002) Flavohemoglobin Hmp Protects *Salmonella enterica* Serovar Typhimurium from Nitric Oxide-Related Killing by Human Macrophages. *Infection and Immunity* **70**: 4399-4405.

Stockenberg A & Johnstone RW (1997) Benthic Denitrification in the Gulf of Bothnia. *Estuarine, Coastal and Shelf Science* **45**: 835-843.

Stolz JF, Ellis DJ, Blum JS, Ahmann D, Lovley DR & Oremland RS (1999) Note: *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the *Sulfurospirillum* clade of the ε-Proteobacteria. *International Journal of Systematic Bacteriology* **49**: 1177-1180.

Straub K & Schink B (2004) Ferrihydrite reduction by *Geobacter* species is stimulated by secondary bacteria. *Archives of Microbiology* **182**: 175-181.

Straub KL & Schink B (2004) Ferrihydrite-Dependent Growth of *Sulfurospirillum deleyianum* through Electron Transfer via Sulfur Cycling. *Applied and Environmental Microbiology* **70**: 5744-5749.

Strous M & Jetten MSM (2004) Anaerobic Oxidation of Methane and Ammonium. *Annual Review of Microbiology* **58**: 99-117.

Strous M, Pelletier E, Mangenot S*, et al.* (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature* **440**: 790-794.

Sung Y, Ritalahti KM, Sanford RA, Urbance JW, Flynn SJ, Tiedje JM & Löffler FE (2003) Characterization of Two Tetrachloroethene-Reducing, Acetate-Oxidizing Anaerobic Bacteria and Their Description as *Desulfuromonas michiganensis* sp. nov. *Applied and Environmental Microbiology* **69**: 2964-2974.

Tabita FR (1995) The Biochemistry and Metabolic Regulation of Carbon Metabolism and CO_2 Fixation in Purple Bacteria. *Anoxygenic Photosynthetic Bacteria,* Vol. 2 (Blankenship RE, Madigan MT & Bauer CE, eds.), pp. 885-914. Springer Netherlands.

Takai K & Horikoshi K (1999) Genetic Diversity of Archaea in Deep-Sea Hydrothermal Vent Environments. *Genetics* **152**: 1285-1297.

Takeuchi J (2006) Habitat Segregation of a Functional Gene Encoding Nitrate Ammonification in Estuarine Sediments. *Geomicrobiology Journal* **23**: 75-87.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M & Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Taubert M, Grob C, Howat AM, Burns OJ, Dixon JL, Chen Y & Murrell JC (2015) XoxF encoding an alternative methanol dehydrogenase is widespread in coastal marine environments. *Environmental Microbiology* n/a-n/a.

Tavormina PL, Ussler W, Joye SB, Harrison BK & Orphan VJ (2010) Distributions of putative aerobic methanotrophs in diverse pelagic marine environments. *ISME Journal* **4**: 700-710.

Taylor SC, Dalton H & Dow CS (1981) Ribulose-1,5-bisphosphate carboxylase/oxygenase and carbon assimilation in *Methylococcus capsulatus* (Bath). *Journal of General Microbiology* **122**: 89-94.

Templeton AS, Chu K-H, Alvarez-Cohen L & Conrad ME (2006) Variable carbon isotope fractionation expressed by aerobic CH_4-oxidizing bacteria. *Geochimica et Cosmochimica Acta* **70**: 1739-1752.

Teske A & Sorensen KB (2007) Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME Journal* **2**: 3-18.

Teske A, Alm E, Regan JM, Toze S, Rittmann BE & Stahl DA (1994) Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. *Journal of Bacteriology* **176**: 6623-6630.

Teske AP (2006) Microbial Communities of Deep Marine Subsurface Sediments: Molecular and Cultivation Surveys. *Geomicrobiology Journal* **23**: 357-368.

Thamdrup B, Finster K, Hansen JW & Bak F (1993) Bacterial Disproportionation of Elemental Sulfur Coupled to Chemical Reduction of Iron or Manganese. *Applied and Environmental Microbiology* **59**: 101-108.

Thauer RK (2011) Anaerobic oxidation of methane with sulfate: on the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO_2. *Current Opinion in Microbiology* **14**: 292-299.

Thauer RK & Shima S (2008) Methane as Fuel for Anaerobic Microorganisms. *Annals of the New York Academy of Sciences* **1125**: 158-170.

Thauer RK, Kaster A-K, Seedorf H, Buckel W & Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. *Nature Reviews Microbiology* **6**: 579-591.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Treude T, Orphan V, Knittel K, Gieseke A, House CH & Boetius A (2007) Consumption of Methane and CO_2 by Methanotrophic Microbial Mats from Gas Seeps of the Anoxic Black Sea. *Applied and Environmental Microbiology* **73**: 2271-2283.

Trotsenko YA & Murrell JC (2008) Metabolic aspects of aerobic obligate methanotrophy. *Advances in Applied Microbiology,* Vol. Volume 63 (Allen I. Laskin SS & Geoffrey MG, eds.), pp. 183-229. Academic Press.

Tyler SC, Crill PM & Brailsford GW (1994) 13C12C Fractionation of methane during oxidation in a temperate forested soil. *Geochimica et Cosmochimica Acta* **58**: 1625-1633.

Urey HC (1947) The thermodynamic properties of isotopic substances. *Journal of the Chemical Society (Resumed)* 562-581.

Vajrala N, Martens-Habbena W, Sayavedra-Soto LA, Schauer A, Bottomley PJ, Stahl DA & Arp DJ (2013) Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 1006-1011.

Vaksmaa A, Lüke C, van Alen T, Vale G, Lupotto E, Jetten M & Ettwig KF (2015) Archaea dominate the anaerobic methanotrophic community in a nitrogen-loaded Italian paddy soil. *FEMS Microbiology Ecology*, submitted.

Valentine DL & Reeburgh WS (2000) New perspectives on anaerobic methane oxidation. *Environmental Microbiology* **2**: 477-484.

van Bodegom PM, Scholten JCM & Stams AJM (2004) Direct inhibition of methanogenesis by ferric iron. *FEMS Microbiology Ecology* **49**: 261-268.

van de Vossenberg J, Woebken D, Maalcke WJ*, et al.* (2013) The metagenome of the marine anammox bacterium '*Candidatus* Scalindua profunda' illustrates the versatility of this globally important nitrogen cycle bacterium. *Environmental Microbiology* **15**: 1275-1289.

van den Bergh ER, Dijkhuizen L & Meijer WG (1993) CbbR, a LysR-type transcriptional activator, is required for expression of the autotrophic CO_2 fixation enzymes of *Xanthobacter flavus*. *Journal of Bacteriology* **175**: 6097-6104.

Van Den Heuvel RN, Bakker SE, Jetten MSM & Hefting MM (2011) Decreased N_2O reduction by low soil pH causes high N_2O emissions in a riparian ecosystem. *Geobiology* **9**: 294-300.

van Keulen G, Ridder ANJA, Dijkhuizen L & Meijer WG (2003) Analysis of DNA binding and transcriptional activation by the LysR-type transcriptional regulator CbbR of *Xanthobacter flavus*. *Journal of Bacteriology* **185**: 1245-1252.

Van Spanning RJM, Houben E, Reijnders WNM, Spiro S, Westerhoff HV & Saunders N (1999) Nitric Oxide Is a Signal for NNR-Mediated Transcription Activation in *Paracoccus denitrificans*. *Journal of Bacteriology* **181**: 4129-4132.

Van Spanning RJM, De Boer APN, Reijnders WNM, Spiro S, Westerhoff HV, Stouthamer AH & Van der Oost J (1995) Nitrite and nitric oxide reduction in *Paracoccus denitrificans* is under the control of NNR, a regulatory protein that belongs to the FNR family of transcriptional activators. *FEBS Letters* **360**: 151-154.

van Teeseling MCF, Pol A, Harhangi HR, van der Zwart S, Jetten MSM, Op den Camp HJM & van Niftrik L (2014) Expanding the verrucomicrobial methanotrophic world: description of three novel species of *Methylacidimicrobium* gen. nov. *Applied and Environmental Microbiology* **80**: 6782-6791.

Venter JC, Remington K, Heidelberg JF*, et al.* (2004) Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science* **304**: 66-74.

Vetriani C, Jannasch HW, MacGregor BJ, Stahl DA & Reysenbach A-L (1999) Population Structure and Phylogenetic Characterization of Marine Benthic Archaea in Deep-Sea Sediments. *Applied and Environmental Microbiology* **65**: 4375-4384.

Vogt C, Cyrus E, Herklotz I, Schlosser D, Bahr A, Herrmann S, Richnow H-H & Fischer A (2008) Evaluation of Toluene Degradation Pathways by Two-Dimensional Stable Isotope Fractionation. *Environmental Science & Technology* **42**: 7793-7800.

Vorobev AV, Baani M, Doronina NV, Brady AL, Liesack W, Dunfield PF & Dedysh SN (2011) *Methyloferula stellata* gen. nov., sp nov., an acidophilic, obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase. *International Journal of Systematic and Evolutionary Microbiology* **61**: 2456-2463.

Walker CB, de la Torre JR, Klotz MG*, et al.* (2010) Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proceedings of the National Academy of Sciences* **107**: 8818-8823.

Wang F-P, Zhang Y, Chen Y, He Y, Qi J, Hinrichs K-U, Zhang X-X, Xiao X & Boon N (2014) Methanotrophic archaea possessing diverging methane-oxidizing and electron-transporting pathways. *ISME Journal* **8**: 1069-1078.

Wang Y & Ruby EG (2011) The roles of NO in microbial symbioses. *Cellular Microbiology* **13**: 518-526.

Wang Y, Zhu G, Harhangi HR, Zhu B, Jetten MSM, Yin C, Op den Camp HJM (2012) Co-occurrence and distribution of nitrite-dependent anaerobic ammonium and methane-oxidizing bacteria in a paddy soil. *Fems Microbiology Letters* **336**: 79-88.

Wankel SD, Adams MM, Johnston DT, Hansel CM, Joye SB & Girguis PR (2012) Anaerobic methane oxidation in metalliferous hydrothermal sediments: influence on carbon flux and decoupling from sulfate reduction. *Environmental Microbiology* **14**: 2726-2740.

Ward N, Larsen Ø, Sakwa J*, et al.* (2004) Genomic Insights into Methanotrophy: The Complete Genome Sequence of *Methylococcus capsulatus* (Bath). *PLoS Biology* **2**: e303.

Weber EB, Lehtovirta-Morley LE, Prosser JI & Gubry-Rangin C (2015) Ammonia oxidation is not required for growth of Group 1.1c soil *Thaumarchaeota*. *Fems Microbiology Ecology*: doi: 10.1093/femsec/fiv001

Weber KA, Achenbach LA & Coates JD (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nature Reviews Microbiology* **4**: 752-764.

Wegener G, Shovitri M, Knittel K, Niemann H, Hovland M & Boetius A (2008) Biogeochemical processes and microbial diversity of the Gullfaks and Tommeliten methane seeps (Northern North Sea). *Biogeosciences* **5**: 1127-1144.

Weier KL, Doran JW, Power JF & Walters DT (1993) Denitrification and the Dinitrogen/Nitrous Oxide Ratio as Affected by Soil Water, Available Carbon, and Nitrate. *Soil Science Society of America Journal* **57**: 66-72.

Whiticar MJ (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chemical Geology* **161**: 291-314.

Whittenbury R, Phillips KC & Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *Journal of General Microbiology* **61**: 205-218.

Wu ML, Ettwig KF, Jetten MSM, Strous M, Keltjens JT & van Niftrik L (2011) A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium *Candidatus* 'Methylomirabilis oxyfera'. *Biochemical Society Transactions* **39**: 243-248.

Wu ML, Wessels HJCT, Pol A, Op den Camp HJM, Jetten MSM, van Niftrik L & Keltjens JT (2015) XoxF-Type Methanol Dehydrogenase from the Anaerobic Methanotroph "*Candidatus* Methylomirabilis oxyfera". *Applied and Environmental Microbiology* **81**: 1442-1451.

Wu ML, de Vries S, van Alen TA, Butler MK, Op den Camp HJM, Keltjens JT, Jetten MSM & Strous M (2011) Physiological role of the respiratory quinol oxidase in the anaerobic nitrite-reducing methanotroph '*Candidatus* Methylomirabilis oxyfera'. *Microbiology* **157**: 890-898.

Wu ML, van Teeseling MCF, Willems MJR, van Donselaar EG, Klingl A, Rachel R, Geerts WJC, Jetten MSM, Strous M & van Niftrik L (2012) Ultrastructure of the denitrifying methanotroph "*Candidatus* Methylomirabilis oxyfera," a novel polygon-shaped bacterium. *Journal of Bacteriology* **194**: 284-291.

Wuebbles DJ & Hayhoe K (2002) Atmospheric methane and global change. *Earth-Science Reviews* **57**: 177-210.

Yakimov MM, Cono VL, Smedile F*, et al.* (2011) Contribution of crenarchaeal autotrophic ammonia oxidizers to the dark primary production in Tyrrhenian deep waters (Central Mediterranean Sea). *ISME Journal* **5**: 945-961.

Yeates TO, Kerfeld CA, Heinhorst S, Cannon GC & Shively JM (2008) Protein-based organelles in bacteria: carboxysomes and related microcompartments. *Nature Reviews Microbiology* **6**: 681-691.

Zarzycki J, Axen SD, Kinney JN & Kerfeld CA (2013) Cyanobacterial-based approaches to improving photosynthesis in plants. *Journal of Experimental Botany* **64**: 787-798.

Zedelius J, Rabus R, Grundmann O*, et al.* (2011) Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. *Environmental Microbiology Reports* **3**: 125-135.

Zehnder AJ & Brock TD (1979) Methane formation and methane oxidation by methanogenic bacteria. *Journal of Bacteriology* **137**: 420-432.

Zhang M, Zhang T, Shao MF & Fang HHP (2009) Autotrophic denitrification in nitrate-induced marine sediment remediation and Sulfurimonas denitrificans-like bacteria. *Chemosphere* **76**: 677-682.

Zhou Z, Cao H, Han P & Gu J-D (2015) Analysis of methane-producing and metabolizing archaeal and bacterial communities in sediments of the northern South China Sea and coastal Mai Po Nature Reserve revealed by PCR amplification of *mcrA* and *pmoA* genes. *Frontiers in Microbiology* **5**.

Zhu B, van Dijk G, Fritz C, Smolders AJP, Pol A, Jetten MSM & Ettwig KF (2012) Anaerobic Oxidization of Methane in a Minerotrophic Peatland: Enrichment of Nitrite-Dependent Methane-Oxidizing Bacteria. *Applied and Environmental Microbiology* **78**: 8657-8665.

Zhu B, (2015) Microbial and Environmental Aspects of Anaerobic Oxidation of Methane. PhD Thesis, Radboud University Nijmegen.

Zhu G, Zhou L, Wang Y*, et al.* (2015) Biogeographical distribution of denitrifying anaerobic methane oxidizing bacteria in Chinese wetland ecosystems. *Environmental Microbiology Reports* **7**: 128-138.

Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* **61**: 533-616.

Summary/Synthesis

Since the discovery in 1906 by Söhngen that methane can support growth of microorganisms, it was believed for a long time that methane could only be transformed in the presence of oxygen and remained inert in anoxic waters and sediments. It occurred as impossible that any other environmentally relevant oxidant could have a reduction potential high enough to be used for the oxidation of methane. Anaerobic oxidation of methane (AOM) seemed to be an "impossible" process until roughly 40 years ago when biogeochemical profiles and compound flux calculations in marine waters and sediments gave first hints that methane was not as stable under anoxic conditions as previously believed.

From that time onwards, AOM with sulfate as electron acceptor was shown to attenuate up to 90% of methane in anoxic marine sediments indicating its major importance for global carbon cycling. Moreover, we now also know that not only sulfate but also nitrogen oxides can be used for AOM and several biochemical mechanisms can be used by responsible organisms. *Methylomirabilis oxyfera* is one of those organisms which oxidizes methane under anoxic conditions, however it still uses the power of oxygen for C-H bond breakage in methane. This elegant biochemical solution to methane activation arises from the dismutation of nitric oxide to molecular nitrogen and oxygen, a novel reaction which was till then unknown to biology.

Since its discovery, numerous molecular studies have provided strong indications for the ubiquity of *M. oxyfera* bacteria in diverse habitats pointing to its important role in methane cycling. However, the knowledge about its physiology still remains to be fully explored thus making it difficult to draw conclusions about niche specialization, activity and potential interactions with other organisms.

In **chapter 2**, we addressed the specific isotopic signature which *M. oxyfera* bacteria impose during their methane oxidizing activity. As isotopically lighter substrates react faster, they are preferentially used by enzymes at non-limiting substrate conditions. During aerobic methane oxidation, the isotope fractionation is hypothesized to occur at the first and irreversible step of the pathway – at the methane monooxygenase. Various methanotrophs were shown to produce isotopic signatures which were not correlated to their phylogenetic affiliation or methane monooxygenase protein sequence. Our research showed that also the isotopic signature of *M. oxyfera* was no different to aerobic methanotrophs, and that all values

fell within a relatively narrow range. Previous molecular studies revealed habitats which contain considerable populations of *M. oxyfera*-like bacteria. Integration of methane isotope measurements in those habitats in combination with methane flux modelling, molecular approaches and classical batch activity measurements would provide a more complete picture on the role and importance of *M. oxyfera* in natural systems.

In context of environmental detection of active methanotrophy, numerous studies were based on the knowledge of methanotrophs assimilating at least half of their cellular carbon from methane, which would be reflected in the isotopic signature of the carbon atoms in lipids and DNA of methanotrophic bacteria. Thus, heavy ^{13}C-labeled methane was used for incubations of environmental samples in order to detect active methanotrophs by extraction and amplification of ^{13}C-labeled DNA (DNA-SIP). While this still applies to so far known proteobacterial aerobic methanotrophs, recently discovered verrucomicrobial aerobic methanotrophs were shown to use Calvin Cycle for CO_2 fixation. Thus, the incorporation of C-isotope label from methane would occur to a far lesser degree indirectly from methane-derived CO_2 and probably fail to detect these methanotrophs. In **chapter 3**, we showed that also *M. oxyfera*-like methanotrophs fix CO_2 via the Calvin Cycle. These recent findings point to the necessity for modified DNA-SIP methods for improved detection of methanotrophy in environmental samples.

Despite the available genome information and some physiological and biochemical studies on *M. oxyfera* bacteria, their metabolic pathway still needs further investigation. Here, nitric oxide is of particular interest as it was hypothesized to be dismutated to nitrogen and oxygen by a hypothesized Nod enzyme. However, the mechanism remains elusive and in view of the genomic potential of *M. oxyfera* to metabolize NO, further research is needed. Our nitric oxide exposure experiments described in **chapter 4** revealed severe stress response both at the transcriptomic and physiological levels at micromolar concentrations of free NO. This was evident from downregulation of central metabolic enzyme encoding genes involved in both nitrogen and methane metabolism. Also upregulation was observed, however, it mostly involved unannotated DNA regions on the *M. oxyfera* genome. Manual check and possible reannotation of those DNA regions could provide some insight into their function. This remains subject for future investigation. The activity started to recover after free NO concentration fell under 1 µM, however, the downregulation of central metabolic genes seemed to continue. Following studies should focus on concentrations of NO below 1 µM and include ^{15}N-labeled NO for more precise

tracing of its metabolites. Of particular interest is the role of the conventional qNor, but also possible differential expression of two paralogues of putative Nod enzymes.

Anoxic brackish sediments – hot-spots of element cycling

Within a sediment column, methane produced in deeper anoxic layers diffuses through zones of alternative electron acceptors before it reaches the oxic/anoxic interface where nitrite-, nitrate- and oxygen-dependent methanotrophs are active. During the past few decades a combination of geochemical, biogeochemical and molecular studies provided solid evidence for the importance of sulfate for methane oxidation in marine sediments which is performed in consortia of ANME archaea and sulfate-reducing bacteria at the sulfate-methane transition zone. The majority of methane is oxidized in this zone before reaching upper sediment layers. Here, methane is oxidized by methanogen-related archaea via the methyl-coenzyme M reductase and the reducing equivalents are shuttled to partner sulfate-reducing bacteria. However, in freshwater and brackish environments where overall salinity and sulfate concentrations are low, other electron acceptors like oxidized metal species (e.g., birnessite, ferrihydrite, goethite, magnetite etc.) play an important role in anaerobic respiration. Several environmental studies have provided evidence for metal-dependent methane oxidation activity in various environments, but underlying processes still remain elusive. In **chapter 5**, we investigated the potential of *in-situ* microbial community for iron-dependent anaerobic methane oxidation (Fe-AOM) in the Bothnian Sea sediments. Due to its non-steady state diagenetic history, abundant iron oxides have been preserved below a shallow sulfate-methane transition zone and based on previous geochemical analysis, Fe-AOM was hypothesized to take place in these sediments. Our batch incubations of original sediment from iron- and methane-rich anoxic layers showed significant iron-dependent methane oxidation activity. Analysis of total DNA could not identify one specific group of organisms responsible for the process, but rather pointed to an interplay of several potential metabolic partners each either involved in iron reduction, methane oxidation or stimulation of both with production of metabolites. We identified population increases in known methanogens related to *Methanosarcina*, *Methanoregula* and *Methanomassiliicoccus*, anaerobic methanotrophic archaea closely related to the ANME-2a clade, iron-reducing bacteria from the order *Desulfuromonadales*, putative fermentative *Clostridia* and thiosulfate/sulfate reducing bacteria. The role of methanotrophic archaea observed in our incubations is of particular interest since the genomes of most closely related organisms contain various cytochrome *c*

proteins in great abundance and the possibility of a direct electron transfer has been discussed as a potential mechanism for Fe-AOM. It remains unclear whether the Bothnian Sea specific ANME-2a subcluster can oxidize methane by directly coupling it to iron reduction, shuttling the reducing equivalents to iron reducers or even sulfate reducers which would derive sulfate from abiotic sulfide oxidation by reactive iron during a cryptic sulfur cycle. As we observed the same genotype of ANME archaea within the sulfate-methane transition zone, it shows that these archaea are involved in sulfate-dependent anaerobic methane oxidation. An enrichment of these archaea would enable further physiological characterization and show whether they also could be involved in iron transformations.

In **chapter 6**, we investigated the nitrogen cycle potential of the low salinity, oligotrophic sediment of Bothnian Sea. Full denitrification to N_2 seemed to dominate the anaerobic part of the N-cycle, while nitrogen fixation, DNRA and anammox seemed to be of only minor importance. The aerobic ammonia oxidation was dominated by ammonia oxidizing *Thaumarchaeota* which seemed to occur also in deeper anoxic layers. This observation remains enigmatic, since also other studies reported similar results at other marine locations. The metabolism of these deep sediment *Thaumarchaeota* could be different from aerobic ammonia oxidation and it remains elusive why ammonia monooxygenase still can be detected in those deep sediment layers.

Surprisingly, more anammox bacterial biomarkers were detected at the sulfate-methane transition zone instead of oxic/anoxic interface where oxidized nitrogen species would be available for anammox respiration. PCR results indicated that this peak of anammox biomarkers was located at the top of sulfate-methane transition zone. Furthermore, metagenomics revealed potentially novel anammox species closer related to freshwater genus *Kuenenia*, while the anammox biomarkers at the oxic/anoxic interface were mostly related to marine species of *Scalindua* genus. This vertical stratification is intriguing and was so far never reported before. It remains to be shown whether these deep located anammox bacteria are active or dormant, and in case of their activity where they would derive electron acceptor for respiration. Enrichment cultures of these potentially novel anammox bacteria from original sediment would enable to perform their physiological characterization.

Extensive AOM research from last decades has greatly advanced our understanding of global methane and carbon cycles. A process which was disregarded as “impossible” has been shown to be a major player in a wide range of ecosystems.

We also know now that there is no mechanistically “one fits all” solution, but rather a variety of biochemical inventions and metabolic co-operations. It seems that our knowledge of it is still relatively limited, and thus in the near future running deeper into the AOM “rabbit hole” will bring more exiting examples of “impossible things” performed by “impossible” microbes.

Samenvatting

Sinds de ontdekking in 1906 door Nicolas Söhngen, dat micro-organismen op methaan kunnen groeien, dacht men lang dat methaan alleen kon worden omgezet in aanwezigheid van zuurstof en inert was in anoxische milieus. Het werd "onmogelijk" gedacht dat andere electron acceptoren zoals nitraat, ijzer of sulfaat gebruikt konden worden voor de oxidatie van methaan. De mogelijkheid van anaërobe oxidatie van methaan (AOM) werd aan de kand geschoven tot ongeveer 40 jaar geleden, toen biogeochemische profielen en flux berekeningen in mariene ecosystemen de eerste aanwijzingen gaven dat methaan niet zo stabiel was onder zuurstofloze condities als voorheen werd gedacht.

Vanaf dat moment, bleek AOM met sulfaat als elektronenacceptor zo'n 90% van de methaan in zuurstofloze mariene sedimenten te om te zetten waarmee ook duidelijk werd dat AOM een grote rol kon spelen in de mondiale koolstofcyclus. Bovendien weten wij nu dat niet alleen sulfaat maar ook stikstofoxides kunnen worden gebruikt voor AOM en dat miscroorganismen verschillende biochemische mechanismen kunnen gebruiken. *Methylomirabilis oxyfera* is een van die organismen die methaan kan oxideren onder anoxische omstandigheden, maar toch gebruik maakt van de kracht van zuurstof voor het breken van de C-H binding in methaan. Deze elegante biochemische oplossing die *M. oxyfera* uitgevonden heeft is de dismutatie van 2 moleculen stikstofmonoxide tot moleculaire stikstof en zuurstof; diet is een nieuwe reactie die tot nu toe onbekend was in de biologie. Sinds deze ontdekking, hebben tal van moleculaire studies sterke aanwijzingen verschaft voor de anwezigheid van *M. oxyfera* bacteriën in diverse habitats, die mogelijk wijzen op een belangrijke rol in de methaan-cyclus. Echter, de kennis over de fysiologie van *M. oxyfera* is nog weining onderzocht, waardoor het moeilijk is om conclusies te trekken over niche specialisatie, activiteit en mogelijke interacties met andere organismen.

In hoofdstuk 2 hebben we de specifieke isotoopsignatuur van *M. oxyfera* bacteriën onderzocht die zij tijdens de oxidatie van methaan achterlaten. Isotopisch lichtere substraten (bijvoorbeeld $^{12}CH_4$) worden bij vorkeur gebruikt door enzymen wanner het substraat niet beperkended is. Tijdens aërobe methaanoxidatie, wordt verondersteld dat de isotopenfractionering optreedt tijdens de eerste en onomkeerbare activatie door methaan monooxygenase. Verschillende methanotrofen bleken isotope kenmerken te produceren die niet gecorreleerd waren met hun fylogenetische affiliatie op basis van methaan monooxygenase

eiwitsequenties. Ons onderzoek toonde aan dat ook de isotoopfractionering door *M. oxyfera* niet heel anders was dan die aërobe methanotrofen, en dat alle tot nu toe waargenomen waardes binnen een relatief smalle bandbreedte vielen. Moleculaire studies laten zien dat er aanzienlijke populaties van *M. oxyfera*-achtige bacteriën in ecosystemen aanwezig kunnen zijn. Integratie van methaan isotoop metingen in deze systemen, in combinatie met methaan-flux modellering, moleculaire studies, en klassieke activiteit metingen, zou een completer beeld over de rol en het belang van *M. oxyfera* in natuurlijke systemen kunnen geven.

In de context van milieu-detectie van actieve methanotrofie, werden er bij talrijke studies vanuit gegaan dat methanotrofen ten minste de helft van hun cellulaire koolstof uit methaan assimileren, wat weerspiegeld word in de isotopische waardes van de koolstofatomen in lipiden en DNA van methanotrofe bacteriën. Daarom wordt er vaak zwaar ^{13}C-gelabeld methaan gebruikt voor incubaties van milieumonsters teneinde de actieve methanotrofen te detecteren met behulp van ^{13}C-gelabeld DNA (DNA-SIP). Hoewel dit nog steeds geldt voor de tot nu toe bekende proteobacteriële aërobe methanotrofen, bleek dat de onlangs ontdekte verrucomicrobiële aërobe methanotrofen de Calvin Cyclus gebruiken voor CO_2-fixatie. In dit soort methanotrofen zou daarom de opname van ^{13}C-gelabeld methaan in veel mindere mate optreden en ze zullen waarschijnlijk niet eens gedetecteerd worden met deze methode. **In hoofdstuk 3** hebben we aangetoond dat ook *M. oxyfera*-achtige methanotrofen CO_2 opnemen middels de Calvin cyclus en het enzyme ribulose-bisfosfaat carboxylase. Deze bevinding wijst op de noodzaak om de DNA-SIP methoden te optimaliseren zodat ook autotrofe methanotrofen in milieumonsters gedecteerd kunnen worden.

Ondanks de beschikbare genoom informatie en een aantal fysiologische en biochemische studies over *M. oxyfera* bacteriën, is meer onderzoek nodig moet om hun metabole route beter te begrijpen en experiminteel te verifiëren. Stikstofmonoxide is hier van bijzonder belang vanwege de mogelijke omzwtting naar stikstof en zuurstof door een zogenaam NO dismutase eiwit (NOD). In onze experimenten waarin we M. oxyfera blootstelden aan micromolare concentraties stikstofmonoxide (**hoofdstuk 4**), bleek er in de cellen een stress respons op zowel transcriptoom als fysiologisch niveau op te treden.Dit bleek uit de repressie van genen die coderen voor de centrale metabole enzymen in zowel het stickstof als methaan metabolisme. Daarnast werd ook eent aantal zogenaamde gene die coderen voor hypothetische eiwitten sterker to expressie gebracht onder invloed van NO.

Handmatige analyse van deze gene kan mogelijk inzicht geven in hun mogelijke functie. Dit is een interessant onderwerp voor toekomstig onderzoek.
Nadat de vrije NO concentratie onder 1 µM kwam in ons experiment, kwan de activitiet van *M. oxyfera* cellen weer opgang, maar de expressie van genen voor het centrale metabolisme bleef laag. Verfolg studies moeten zich richten op exprerimenten waar de NO concentraties lager zijn dan 1 µM en waar ^{15}N-gelabeld NO word toegevoegd om intermediaire metabolieten nauwkeuriger in kaart te brengen. Van bijzonder belang is daarbij de rol van het conventionele qNor gen, maar ook de mogelijke differentiële expressie van twee paralogen van de vermeende NO dismutase enzymen.

Zuurstofloze brakke sedimenten - hot-spots van de element cycli

Methaan dat word geproduceerd in de diepere anoxische lagen zal in sedimenten omhoog diffunderen en daarbij zones van alternatieve electronenacceptoren doorkruisen voordat methaan het oxische/anoxische grensvlak bereikt. Gedurende de laatste decennia toonde een combinatie van geochemische, biogeochemische en moleculaire studies in marine sedimenten al aan hoe belangrijk sulfaat voor anaërobe methaanoxidatie is, die wordt uitgevoerd in consortia van ANME archaea en sulfaat-reducerende bacteriën in de sulfaat-methaan overgangszone (SMTZ). Het merendeel van het methaan wordt al geoxideerd in deze zone alvorens methaan de bovenste sedimentlagen bereikt. In de SMTZ wordt methaan geoxideerd via het methyl-coenzym M reductase van aan methanogenen verwandte archaea en worden de reductie equivalenten overgedragen naar de sulfaat-reducerende partner bacteriën mogelijk via dunne nanodraden en multiheme cytochromen.
Echter, in zoet water en brakke omgevingen waar totale zout en sulfaat concentraties laag zijn, spelen andere elektronen acceptoren zoals geoxideerde metalen (bv birnessiet, ferrihydriet, goethiet, magnetiet etc.) mogelijk een belangrijke rol in de anaërobe ademhaling en methaanoxidatie. Enkele studies in deze ecosystemen hebben al sterke aanwijzingen gegeven voor metaal afhankelijke methaanoxidatie, maar de onderliggende processen blijven tot nu toe nog steeds een raadsel. **In hoofdstuk 5** onderzochten we het potentieel van de in-situ microbiële gemeenschap voor ijzer-afhankelijke anaërobe methaanoxidatie (Fe-AOM) in de Botnische Zee sedimenten. Vanwege de *niet-steady state* diagenetische geschiedenis zijn behoorlijke hoeveelheden ijzeroxiden bewaard gebleven beneden de SMTZ, die mogelijk Fe-AOM zoden kunnen laten plaats vinden. Onze incubaties van sediment

uit de ijzer- en methaan-rijke anoxische lagen lieten een significante ijzer-afhankelijke methaanoxidatie activiteit zien van 1.1 µmol/g/jaar. Een metagenoom analyse van DNA geextraheerd uit deze incubaties toonde aan dat er moglijk een samenspel is van verschillende potentiële metabole partners die elk betrokken kunnen zijn bij ijzerreductie, methaanoxidatie of elkaar van metabolieten kunnen vorzien. De metagenoomanalyse liet zien dat de celaantallen van een aantal verschillende groepen microorganismen toenamen; dit waren bekende methanogene archaea gerelateerd aan *Methanosarcina*, *Methanoregula* en *Methanomassiliicoccus*, anaërobe methanotrofe archaea die nauw verwant zijn aan de ANME-2a clade, ijzerreducerende bacteriën uit de orde van *Desulfuromonadales*, fermentatieve *Clostridia* en bacteriën die betrokken zijn bij de zwavelcyclus. De rol van methanotrofe archaea in onze incubaties is van bijzonder belang omdat de genomen van de meest nauw verwante organismen grote aantallen van verschillende cytochroom c bevatten en daarmee de directe elektronenoverdracht als een potentieel mechanisme van Fe-AOM mogelijk maken zoals recent ook voor S-AOM is aangetoond. Het blijft onduidelijk de specifieke ANME-2a subcluster archaea in de Botnische Zee methaanoxidatie direct kunnen koppelen aan ijzerreductie, of dat er indirecte mechanismen bij betrokken zijn, dat er zelfs zogenaamde "geheime" abiotische zwavelcyclus bij betrokken is. Een actieve verrijkingscultuur van de gevonden AOM archaea zou hun fysiologische karakterisering verder mogelijk maken en ook kunnen laten zien of ze ook betrokken zijn bij de reductie van ijzer.

In hoofdstuk 6 onderzochten we de stikstofkringloop in sedimenten van de Botnische Zee. Denitrificatie naar N_2 leek de anaërobe N-cyclus te domineren, terwijl stikstoffixatie, DNRA en anammox van minder belang leken te zijn. De aërobe ammoniak-oxidatie wordt gedomineerd door *Thaumarchaeota* die zich ook in de diepere zuurstofloze bleken voor te komen. Wat de *Thaumarchaeota* daar precies doen blijft raadselachtig, maar ook andere studies hebben deze archaea in anoxische sedimentlagen aan getroffen.

Verrassenderwijs werden er ook meer anammox-bacteriële biomarkers ontdekt in de SMTZ in plaats in oxische/anoxische overgangsfase. De PCR resultaten gaven aan dat deze piek van anammox biomarkers aan de bovenkant van de SMTZ lag. Bovendien, onthulde de metagenoomanalyse ook potentieel nieuwe anammox-soorten die dichter verwant bleken te zijn aan de zoetwater *Kuenenia* soorten. De anammox biomarkers in oxische/anoxische overgangszone waren meer gerelateerd aan de mariene *Scalindua* anammox-soorten. Deze verticale stratificatie is intrigerend en is tot nu toe nog niet vaak eerder geobserveerd. Er moet nu nog

aangetoond worden of anammox-bacteriën in de SMTZ dawerkelijk actief zijn, en welke verbinding ze als electronenacceptor voor hun ademhaling zouden gebruiken. Ophopingsculturen van deze potentieel nieuwe anammox-bacteriën uit *in-situ* sedimenten zou ons in-staat stellen hun fysiologische eigenschappen te karakteriseren.

Het uitgebreide AOM onderzoek van de laatste decennia heeft ons begrip van de mondiale methaan en koolstof cycli sterk utgebreid. Het zogenaamde "onmogelijke" process van anaërobe methaanoxidatie is nu daadwerelijk aangetoond en het blijkt een belangrijke speler te zijn in een groot aantal ecosystemen. We weten nu ook dat er geen mechanistisch "*one fits all*" oplossing bestaat, maar dat er diverse biochemische mechanismen en verschillende metabolische samenwerkingsverbanden bij betrokken kunnen zijn. Er zijn due nog genug uitdagingen om in de nabije toekomst nieuwe ontdekkingen op het gebied van anaërobe methaanoxidatie te doen en daarbij nieuwe “onmogelijke” organismen te ontdekken.

Acknowledgments

A lot of people have influenced me and the process of this PhD research in many ways, for which I would like to thank all of them. First of all, of course, **Mike** for everything! From noticing that first e-mail from a German bachelor's student wanting to do an internship, then for the opportunity to continue with my Master's thesis, PhD thesis, great support to do a course in the USA, nomination for the Frye Stipendium and many more things. Through your mentorship, you have influenced my scientific development as no other person in these last 5 years! Also a big thanks to **Katharina** and **Claudia** for daily supervision. With the contribution of both of you I became a wiser and more knowledgeable person. Claudia, I really appreciate your positivity and patience, not only with ARB struggles but also at the gym where I could practice my personal trainer skills on you. I try to do my best to follow your legendary advice to "follow the iron!" anywhere, at the lab or at the gym :D **Theo**, you were my first internship supervisor at the department. Your great enthusiasm and dedication made every so little PCR result exiting. I couldn't have wished for a better supervisor back then! And of course now, your support with all the sequencing related issues is irreplaceable.

Ming, **Naomi** and **Xristina**, we've spent countless evenings with discussions, lots of wine, cigarettes, music and awesome food. Times have changes but memories will always stay. Either digging for oysters and clams at the beach, trip to Athens, tulip gardens, cocktails at Demain, it all were great times we shared.

Martine, the Viking mud run was an extreme experience which will never be forgotten! We both came close to our limits, but your optimism and faith that we can really do it made it possible to finish.

Ahmad, my Ecological Microbiology assistant buddy made every day of the course a pleasure. Having a lot of fun while doing science is the best thing anyways.

Katinka, **Guylaine**, **Boran** and **Arjan**, your help in the (reactor) lab was essential. I really value your advices on experiments, technical help with reactors, GC problems etc. Without your contribution some experiments would not have been possible.

Karin, I'm very grateful to you always having an open ear for me, either in happy or bad times! I really appreciate that we can talk just about anything! And, I still use your wrist protectors every time I train and they have accompanied me from the very

beginning to all recent victories! I had a great time in Groesbeek at the 4daagse and maybe we could even walk it ourselves one day ☺ Who knows.
Annika, my Eastern European "partner in crime", thanks for all the fun we had together, and I hope more will follow!
Judith, you are always there when any burocratic stuff needs to be clarified and I'm still amazed how fast things can be solved as there was no issue at all. And your passion for travelling around the world inspires me every time we talk about it. Up for the next trip!
The new gym connection, **Arslan**, **Mo** and **Lavinia**. So nice to see more sport fanatics in the department and very curious which way it will take for all of you.
Daan and **Muriel**, our joint PhD adventure is also ending for the two of you. I wish you both all the best with the new start in California and Sweden! And am very curious which turn our scientific journeys will take in future.
My office mates, **Maartje** and **Francisca**, always nice to have the after 5 talks and thanks for all the support.
I would like to thank my current and former colleagues, **Huub**, **Baoli**, **Dorien**, **Lina**, **Nardy, Mara, Marianne, Michela, Wang, Mamoru, Adam, Jaime, Kim, Jennifer, Bas, Sarah, Frauke, Wouter M., Wouter V., Harry, Wim**, **Rob G.**, **Rob M.**, **Baoli**, **Jan**, **Harry**, **Marjan, Suzanne, Geert, Sebastian, Sepehr, Simon, Dorien, Michiel, Ziye, Aniela, Dimitra, Jeroen, Eric, Tijs, Alvaro** for sharing all the experiences, help with experiments, discussions, tips, advices and all the fun shared either at the coffee corner or somewhere at the city during these last 5 years.
A special thanks to all the students I've guided during this time! **Robin**, **Caspar** and **Julia**, I think we all learned a lot from each other, in one way or the other.
I count myself very fortunate being a part of this group which gave me all those great opportunities to meet interesting people, learn, have fun, travel and develop further every single say.

Ke, my dear friend since the beginnings when I came to Nijmegen, with your influence I have become a wiser and more open person. I always enjoy our talks about life, politics, science, anything.. and of course the food! You are still the best cook I've ever known. If the expression "you are what you eat" is true, I wouldn't even know anymore who we are.
Chanthal, we know each other just for only a little more than a year but it feels like for many years! Last year was not an easy one, but you were always there no matter

if things did not go well for me or in the happy moments of triumph. Thanks for everything!
And after a few inactive years, the Radboud Sport Center also slowly became a very important part of my PhD life. There, I was fortunate to meet awesome people who shared my passion for physical culture and influenced my athletic journey. Therefore, special thanks to **Frency**, **Ferry**, **Louis**, **Robin**, **Kelly** and others. And I'm very much looking forward to all the future sportive endeavors with you.

Und natürlich ohne meine Familie wäre all das nicht möglich! Ob in guten oder schlechten Zeiten, ich weiss dass es immer jemanden gibt der mir beistehen wird. Und für meine Eltern: diese Arbeit ist euch gewidmet! Ihr hattet mich gefördert und an mich geglaubt, von Anfang an auch wenn niemand anders es tat. Der lange und steinige Weg; die Schule in der Ukraine, dann deutsche Schule nach der Immigration, dann die Uni.. all das hat sich jetzt an diesem Punkt kulminiert! Ich habe es tatsächlich geschafft.

Curriculum Vitae

Olivia Rasigraf was born on 23 July 1985 in Ewpatorija (Ukraine) where she also completed secondary school education. After immigration to Germany in 2002, she obtained German Abitur in 2005 and went for a short Au-Pair stay to the United Kingdom. She started her academic education in Mathematics at the Friedrich-Schiller-University in Jena, which she quickly changed for Biogeosciences. After obtaining Bachelor's degree in Biogeosciences, she continued her Master's studies in Microbiology at the same University and completed it with the distinction in form of Rector's Examination Award. During Master's education, Olivia came as an Erasmus exchange student to the Microbiology Department of Prof. Mike Jetten where she completed her Master's thesis and continued as PhD student. During her PhD research, Olivia focused on studying various ecosystems with regard to environmentally relevant greenhouse gases and their microbially driven conversions. The results of the PhD research are presented in the current thesis.

List of Publications

Olivia Rasigraf, Carsten Vogt, Hans-Hermann Richnow, Mike S. M. Jetten, and Katharina F. Ettwig, 2012. Carbon and hydrogen isotope fractionation during nitrite-dependent anaerobic methane oxidation by *Methylomirabilis oxyfera*. *Geochimica et Cosmochimica Acta* **89**, 256-264.

Olivia Rasigraf, Dorien M. Kool, Mike S. M. Jetten, Jaap S. Sinninghe Damsté, Katharina F. Ettwig, 2014. Autotrophic carbon dioxide fixation via the Calvin-Benson-Bassham cycle by the denitrifying methanotroph *Methylomirabilis oxyfera*. *Applied and Environmental Microbiology* **80(8)**, 2451-2460.

Matthias Egger, Olivia Rasigraf, Célia J. Sapart, Tom Jilbert, Mike S. M. Jetten, Thomas Röckmann, Boran Kartal, Katharina F. Ettwig, Carina van der Veen, Narcisa Banda, Caroline P. Slomp, 2014. Iron mediated anaerobic oxidation of methane in brackish coastal sediments. *Environmental Science and Technology* **49(1)**, 277-283.

Olivia Rasigraf, Matthias Egger, Katharina F. Ettwig, Mike S.M Jetten, Caroline P. Slomp, Claudia Lüke (2015) Metagenomic analysis of iron-dependent anaerobic oxidation of methane in Bothnian Sea sediments: a playground for various microbial guilds. *Submitted.*

Olivia Rasigraf, Julia Schmitt, Mike S.M. Jetten, Claudia Lüke (2015) Metagenomic potential for and diversity of N-cycle driving microorganisms in the Bothnian Sea sediment. *Submitted*.