Anaerobic oxidation of methane in lake environments: rates, pathways, environmental controls and microorganisms

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Statement of personal contribution

This PhD project was supported by the China Scholarship Council (CSC), and carried out under the supervisions of Prof. Moritz Lehmann, Dr. Jakob Zopfi and Dr. Helge Niemann. Additional fundings came from the Department of Environmental Sciences of the University Basel and the Swiss National Science Foundation (Grant No. 153055). Personal contributions to the thesis chapters are detailed below.

Chapter 3: Fabio Lepori and Stefano Beatrizotti (SUPSI, University of Applied Sciences and Arts of Southern Switzerland) operated the research boat for all sampling campaigns on Lake Lugano. Fabio Lepori and Andreas Bruder provided the oxygen profile data for the two basins of Lake Lugano. DNA samples of 2009 and 2014 in the North Basin were collected and extracted by Christine Wenk and Yuki Weber/Jakob Zopfi, respectively. Water samples in the North Basin were collected together with Jana Tischer. Jana Tischer measured sulfide and nitrite concentrations in the North Basin. Judith Kobler Waldis analysed dissolved iron and manganese concentrations. Helge Niemann helped with the sample preparation the measurements of water column methan oxidation rates. Jean-Claude Walser (GDC, Genetic Diversity Centre, ETHZ) performed the initial treatment of raw read sequencing data. The remaining chemical analyses, DNA extraction, qPCR, and data analyses were performed by myself with the help from Jakob Zopfi.

Chapter 4: The personnel of the Piora Centro Biologia Alpina provided access to the sampling platform. Maciej Bartosiewicz and Jana Tischer helped with the retrieval of the sediment cores. Martin Ley, Haoyi Yao, and Lei Yang helped with subsample preparation in the lab. Judith Kobler Waldis operated the ICP for the measurements of dissolved iron and manganese concentrations. Samples for DIC concentrations were prepared by myself and measured by Ruth Strunk. DIC samples for carbon isotope analyses were prepared by myself and measured by Thomas Kuhn. Methane isotope measurements were performed by myself with the assistance of Serge Robert at the Eawag. All remaining analyses of the chemical parameters and AOM rate measurements were done by myself. I also performed the slurry incubation experiments, extracted DNA and the lipid biomarkers. Helge Niemann performed the GC-IRMS analyses of the lipids at the NIOZ (The Netherlands). Jean-Claude Walser provided bioinformatic support for the raw sequence data treatment. Microbial data

were analyzed by myself with the help from Jakob Zopfi. Lipid biomarker analysis and interpretation was performed by myself with the help from Haoyi Yao and Lea Steinle.

Chapter 5: Robert Lovas and Stefano Beatrizotti were operaterating the boats during the sampling campaigns on Lakes Sempach and Lugano. Moritz Lehmann, Jakob Zopfi, Helge Niemann, Jana Tischer, Adeline Cojean and Yuki Weber helped with the collection of sediment cores. Adeline Cojean, Yuki Weber and Jana Tischer helped with sample preparation in the lab. Judith Kobler Waldis performed the measurements of dissolved iron and manganese concentrations. All other sediment and pore water geochemical analyses, AOM rate measurements and DNA extractions were performed by myself. Microbial data were analyzed by myself with help from Jakob Zopfi, except for the treatment of raw sequence data, which was done by Jean-Claude Walser. Alexandre Bagnoud helped with the phylogenetic tree construction using iTOL.

Chapter 6: The personnel of the Piora Centro Biologia Alpina provided access to the sampling platform on Lake Cadagno. Lea Steinle and Maciej Bartosiewicz helped with the retrieval of the sediment cores. Martin Ley, Haoyi Yao and Lei Yang helped with subsample preparation in the lab. I measured methane concentrations and AOM rates. Stable carbon isotope ratios in methane were determined with the assistance of Serge Robert. Judith Kobler Waldis measured dissolved iron and manganese concentrations by ICP. All other sediment and pore water geochemistry analyses were performed by myself.

I wrote the remaining chapters (i.e., Chapter 1, 2 and 7) with inputs, comments and corrections form Jakob Zopfi and Moritz Lehmann.

Summary

Freshwater lakes represent an important source of methane to the atmosphere, and large amounts of methane are produced in anoxic sediments by anaerobic methanogens. The produced methane dissolves and accumulates in the anoxic sediment pore water, from where it may escape, by ebullition or diffusion, into the near-bottom waters of the lake. Microbial methane oxidation, including aerobic methane oxidation, catalyzed by methane-oxidizing bacteria in oxic environments, and anaerobic oxidation of methane (AOM) by anaerobic methanotrophs under anoxic condition, is the only biological process mitigating methane emissions from natural systems. So far, most studies in freshwater lakes focussed on aerobic methane oxidation at the sediment surface or in oxic water layers. Methane oxidation within anoxic sediments, or within waters of, e.g. eutrophic or permanently stratified freshwater bodies, remains largely unexplored. Recently, however, AOM has been shown to also occur in freshwater environments, raising questions regarding its potential to reduce methane emission from sediments. In addition to sulfate (typically inveolved in AOM in marine settings), oxidants such as nitrite, nitrate, iron and manganese oxides may serve as terminal electron acceptors in AOM. However, the knowledge about the exact pathways and the microorganisms mediating AOM in anoxic freshwater habitats, as well as the environmental controls are still rudimentary. Understanding these controls is crucial for predicting future changes in lacustrine methane emissions under changed climatic/environmental conditions.

In this PhD project, I evaluated different methods to quantify AOM rates in lake sediments and scrutinized different freshwater systems for the presence of AOM. I then selected two representative lake environments, where I could verify the presence of AOM, for greater-detail investigations into the exact modes of, and controls on, AOM: I studied AOM in the anoxic waters in Lake Lugano and within sediments of Lake Cadagno, both lakes in southern Switzerland.

In Lake Lugano, I investigated methane oxidation in the anoxic waters of the two hydrologically connected basins of this lake, which display very different mixing regimes. In both basins, I measured maximum methane oxidation rates below the redoxcline. In the seasonally stratified South Basin, putatively aerobic methane oxidizing (MOx) bacteria belonging to the *Methylococcaceae* family (Type I MOB)

dominated the methanotrophic community, and were mostly responsible for the methane oxidation in the water column. In the permanently stratified North Basin, methane consumption at the oxic-anoxic interfaces can be attributed to both *Methylococcaceae* and *Candidatus* Methylomirabilis, i.e., bacteria that were previously reported to perform nitrite-depedent AOM (i.e. AOM coupled to nitrite reduction/denitrification). A secondary methane turnover maximum was observed well within the anoxic water column, where both *Methylococcaceae* and *Candidatus* Methylomirabilis were present and ammonium oxidation was apparently indicated. Hence, both (micro-) aerobic methane oxidation and nitrite-dependent, true, AOM appear to be important methane-consuming processes in the water column of the North Basin. Most intriguingly, I could demonstrate that water column stability is the prime environmental factor that controls the growth and abundance of the denitrifying methanotrophs. The stably anoxic conditions in the North Basin are particularly conducive to the proliferation of denitrifying AOM bacteria, whereas the seasonal mixing and shorter-term fluctuations in the redox regime in the South Basin seem to prevent the thriving of nitrite dependent AOM bacteria.

In anoxic lake sediments, maximum AOM rates of ~15 nmol/cm^3/d were observed in the deep sediments of sulfate-rich Lake Cadagno, as well as in the surface sediments of Lake Sempach and Lake Lugano. For Lake Cadagno, I present a conclusive data set (radiolabel-based AOM rate measurements, stable isotope probing of lipid biomarkers, 16S rRNA gene-sequencing) which highlights that AOM is coupled to sulfate reduction, and carried out by uncultured archaea of the candidate genus Methanoperedens. Depth distributions of *Candidatus* Methanoperedens and potential sulfate reducing ANME partners in the AOM zones suggests that methane oxidation is most likely performed in archaea-bacteria association. Furthermore, I could demonstrated that this process is indirectly supported by the continuous sulfate production via oxidation of reduced sulfur compounds with other oxidants (e.g., manganese oxide). In this way, sulfate-dependent AOM (involving a cryptic sulfur cycle) may be "disguised" as AOM coupled to manganese reduction. Our study suggest that methanotrophic archaea in syntrophy with sulfate-reducing bacterial partners play an important role in mitigating methane emissions from terrestrial freshwater environments to the atmosphere, analogous to ANME-1, -2, and -3 in marine settings.

In both Lake Sempach and Lake Lugano, in situ AOM rate measurements combined with the molecular data and slurry incubation experiments suggest that putative anaerobic methane oxidation is likely performed by the Genus *Methylobacter*. I hypothesized that these tentative "aerobic" methanotrophs may switch to an anaerobic respiration mode, and are able to utilize electron acceptors other than O_2, such as humic substances in the surface lake sediments.

The presented research significantly expands the range of freshwater habitats where AOM activity could be verified, highlighting its ecological importance of anaerobic methane oxidizing microorganisms (bacteria and archaea, benthic and pelagic) as sentinels of methane emission in freshwater environments. I speculate that both sulfate and nitrite-dependent AOM may act as an important biological methane filter both in the water column of permanently stratified lakes worldwide, as well as in anoxic lacustrine sediments.

Chapter 1: Introduction

Methane (CH_4) is the simplest and most abundant organic compound in the atmosphere. It is a potent greenhouse gas with a global warming potential that is more than 25 folds higher than that of carbon dioxide (Intergovernmental Panel on Climate Change [IPCC], 2006). CH_4 can stem from both anthropogenic and natural sources, each contributing approximately equally to the atmospheric methane budget (Kirschke et al. 2013). Anthropogenic sources of methane include fossil fuels, biomass burning, agriculture and waste. Figure 1 shows the relative importance of natural methane sources to the atmosphere during the last decade (2000-2009). Of these natural sources, wetlands represent the most important source with 62.5% of total emissions (Fig. 1). Freshwater lakes and rivers account for only a small portion of the global area (Downing et al. 2006), however, they contribute up to 40 Tg CH_4 annually, or 11.5%, to total natural methane emissions. In contrast, geological sources (including the oceans) only account for 15.6% of the total emissions (approximately 54 Tg CH_4 each year) to the atmosphere. Other natural sources (8.4%) comprise methane production associated with wild animals, wildfires and termites. On the other hand, CH_4 can be broadly grouped into three categories: pyrogenic, thermogenic and microbial.

Generally, microbial methane from wetlands, soils, marine or freshwater environments is produced under anoxic conditions during the final step in the degradation of organic matter. Methane accumulation is mostly observed in environments/sediments characterized by high sedimentation rate and/or organic matter concentrations (Reeburgh 2007). Microbial methane production (methanogenesis) is catalyzed by methanogenic archaea, which are strictly anaerobic microorganisms and generate methane via the disproportionation of acetate (CH_3COOH) or by utilizing CO_2 as the terminal electron acceptor (Berg et al. 2010). Most of the methane produced in anoxic environments, regardless of its origin, is consumed by methane-oxidizing microorganisms before it reaches to the atmosphere (e.g., Knittel & Boetius 2009).

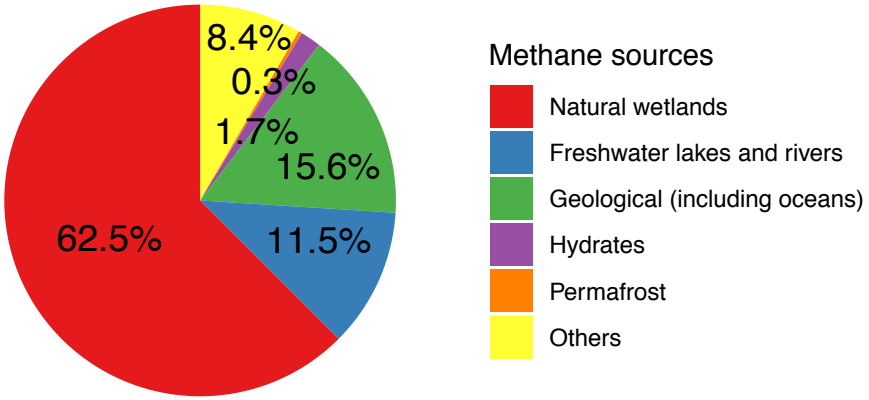

Figure 1. Relative importance of natural methane sources (total of 347 Tg CH_4/yr) to the atmosphere. Values are taken from bottom-up estimates for the time-period 2000-2009 (Kirschke et al. 2013).

Biological oxidation of methane

Microbially-mediated methane oxidation is the counter process to methanogenesis, and it is carried out by specialized microorganisms, so-called methanotrophs, which convert CH_4 to CO_2. These microorganisms are ubiquitous in many oxic and anoxic environments, and have traditionally been considered to consist of aerobic methane oxidizing bacteria and anaerobic methanotrophic archaea. Methane oxidation represents a major sink of methane and efficiently controls the input of methane produced in freshwater and marine sediments, terrestrial and subsurface environments to the atmosphere (Reeburgh 2007).

Aerobic methane oxidation.

Many studies have provided evidence for aerobic methane oxidation in both marine and freshwater systems. Aerobic methane oxidation is usually mediated by methane oxidizing bacteria (MOB), which are generally members of Alpha- or Gammaproteobacteria and utilize molecular oxygen (O_2) as the terminal electron acceptor. But based on physiological differences related to their carbon assimilation and nitrogen fixation capacity (Hanson and Hanson 1996), these aerobic methanotrophs are divided into gammaproteobacterial type I or alphaproteobacterial type II MOB. Type I MOB use the ribulose monophosphate (RuMP) pathway,

whereas Type II MOB fix carbon via the serine pathway. The initial step of this pathway is catalyzed by the enzyme methane monooxygenases (MMO), which transform methane to methanol, and involve the particulate MMO (pMMO) and/or the soluble MMO (sMMO) (Fig. 2). The gene encoding for pMMO (*pmoA*) is expressed by most methanotrophs and often used as a marker gene (Costello and Lidstrom 1999). However, the gene for sMMO (mmoX) is only found in some methanotrophs (Semrau et al. 2010). Methanol is further oxidized to formaldehyde by methanol dehydrogenase (MDH) and formaldehyde is oxidized to formate by formaldehyde dehydrogenase (FADH). Finally, formate is converted to CO_2 by formate dehydrogenases (FDH). Interestingly, it has been reported that some aerobic methanotrophs are able to catalyze methane oxidation also under anoxic conditions, seemingly coupled to nitrate/nitrite reduction (Kits et al. 2015a; b). Indeed, several other studies from stratified lake environments also showed that under oxygen-deficient conditions, aerobic MOB are abundant and active (Blees et al. 2014; Oswald et al. 2016).

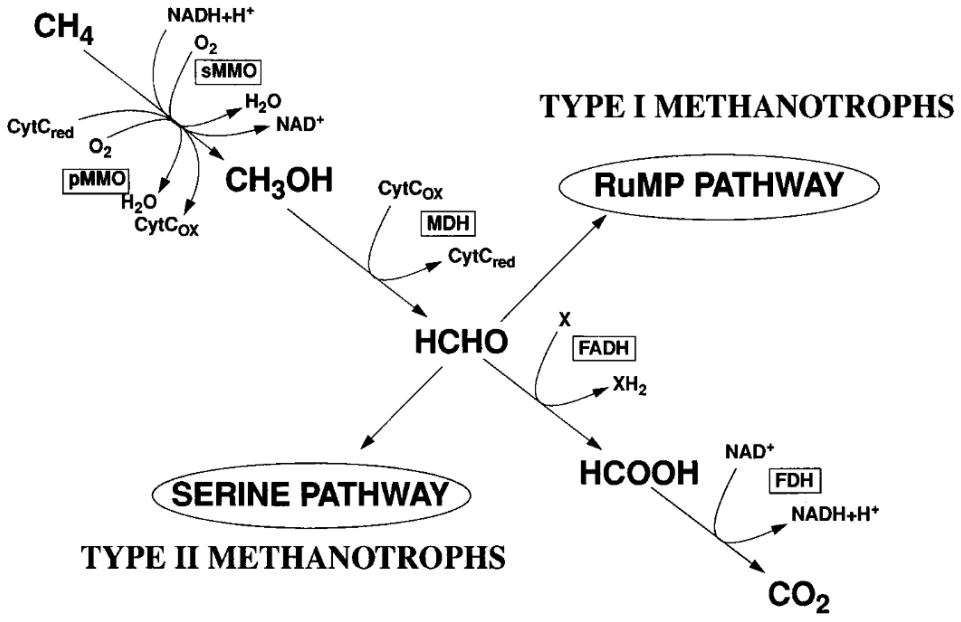

Figure 2. Enzymatic pathways for the oxidation of methane and assimilation of formaldehyde (Hanson and Hanson 1996). Abbreviations: CytC, cytochrome c; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

Anaerobic oxidation of methane.

Anaerobic oxidation of methane (AOM) is a microbially-mediated process carried out by anaerobic methanotrophic archaea (ANME), oxidizing methane in the absence of oxygen. This process is one of the most scientifically intriguing subjects of environmental microbiology. In addition to aerobic methane oxidation, AOM represents another important methane sink in natural environments by reducing the efflux of this potent greenhouse gas into the atmosphere. Furthermore, our knowledge about the AOM and AOM-related organisms in natural environments, particularly in freshwater habitats such as lakes, is still rudimentary. During the last decades, AOM has been intensively studied but almost exclusively in marine systems. Here, it is generally assumed that AOM proceeds in association with sulfate reduction (Hoehler et al. 1994; Hinrichs et al. 1999; Knittel and Boetius 2009), and sulfate-dependent AOM is almost certainly the most important methane consuming process in the ocean. It is typically mediated by a microbial consortium of of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRBs) (Boetius et al. 2000; Orphan et al. 2001; Niemann et al. 2006; Knittel and Boetius 2009). It has been recently shown that ANME-2 archaea are able to perform AOM coupled to dissimilatory sulfate reduction independent of SRBs, producing elemental sulfur (S^0) as metabolic product (Milucka et al. 2012).

Although the exact metabolic mechanism of sulfate-coupled AOM remains unresolved (Valentine and Reeburgh 2000; Caldwell et al. 2008), the occurrence of AOM has been recently demonstrated in freshwater systems (Sivan et al. 2011; Norði et al. 2013; Norði and Thamdrup 2014; Martinez-cruz et al. 2017; Weber et al. 2017; Bar-Or et al. 2017). AOM in lakes, for example, may thus represent an important methane sink that was previously overlooked (Hu et al. 2014; Deutzmann et al. 2014; Segarra et al. 2015). In marine environments, sulfate concentrations are relatively high and sulfate-coupled-AOM can be expected. However, in freshwater environments, this process is likely to be limited by relatively low sulfate concentrations, and alternative electron acceptors are likely to be involved. Both in marine and freshwater environments, organic matter mineralization can be coupled to a variety of electron acceptors (Fig. 3). Similarly, where methane co-occurs with oxidants other than O_2 or sulfate (e.g., nitrate, nitrite, iron and manganese oxides, among others), these alternative substrates may serve as electron acceptors for AOM. NOx-, Fe-, or Mn-dependent AOM may in fact be thermodynamically and biochemically more feasible than sulfate-coupled AOM.

Indeed, several novel modes of AOM with different terminal electron acceptors have been reported, including inorganic electron acceptors such as nitrite (Ettwig et al. 2010), nitrate (Haroon et al. 2013), metal oxides (Ettwig et al. 2016; Cai et al. 2018) and organic electron acceptors (i.e., humic substances) (Scheller et al. 2016). Compared with marine systems, the role of AOM is largely unknown in freshwater environments (e.g., lakes), and evidence of these newly discovered AOM pathways is still sparse. In addition, knowledge about microbial activities, environmental controls, and identity of microbial players involved in AOM in lakes is still limited. Hence, one of the most important rationales in methane biogeochemical research to date is to taxonomically identify key microorganisms in freshwater AOM, and to investigate the potential for metabolic versatility of lacustrine methanotrophic microorganisms. Given the fact that lakes represent one of the most significant methane sources on earth, such information will be vital for predicting future methane emissions from lakes to the atmosphere, and at the same time, would enable us to better understand the interaction between the biogeochemical cycles of methane and probably other important elements in the system.

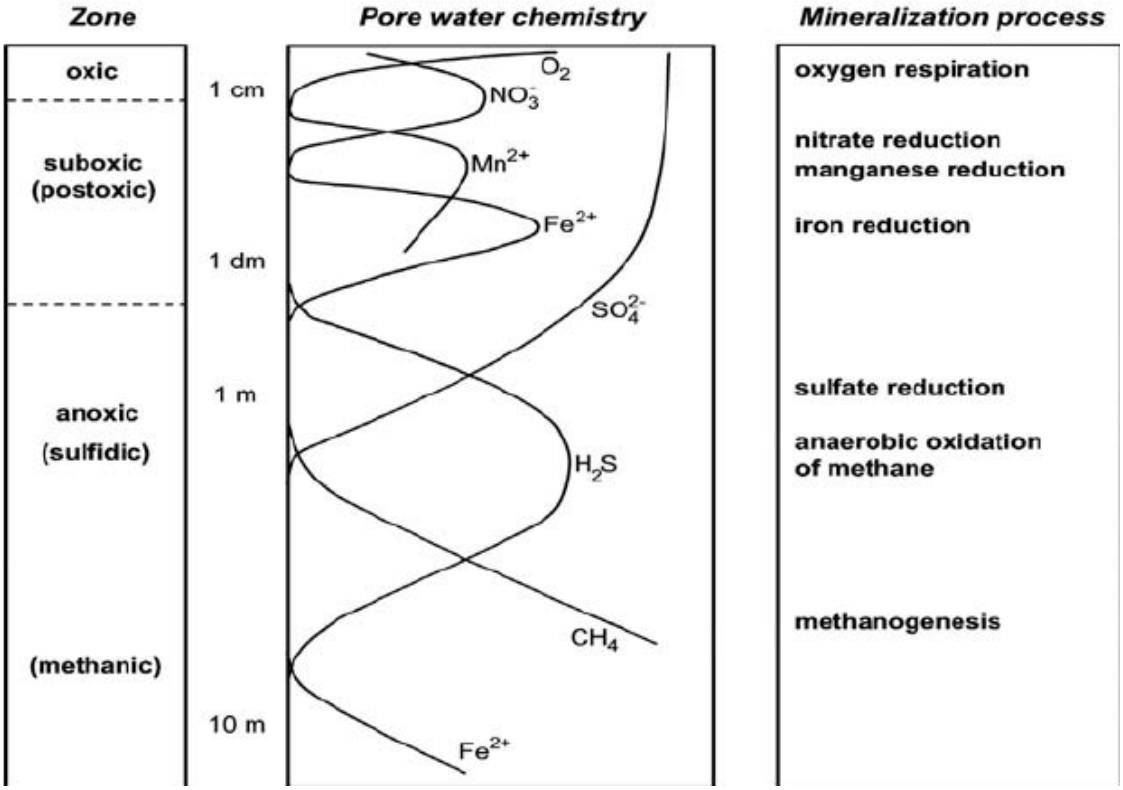

Figure 3. Schematic representation of the biogeochemical zonation in marine sediments (Jøgensen and Kasten 2006). The chemical profiles reflect the depth sequence of electron acceptor usage for the dominant mineralization processes, through which organic matter is oxidized to CO_2.

Study sites

In this PhD work, I investigated AOM in the anoxic water column and sediments of various Swiss lakes, including Lake Lugano, Lake Cadagno and Lake Sempach. Lake Lugano is a eutrophic lake located on the Swiss-Italian border, and consists of two hydrodynamically contrasting basins. The South Basin (45°57′N, 8°54′E) is seasonally stratified with the development of a benthic nepheloid layer and anoxia during summer, while the North Basin (46°01′N, 9°02′E) is permanently stratified below the chemocline. Lake Cadagno (46°33′N, 8°43′E) is a meromictic lake located in the catchment in the Piora valley of the Canton Ticino. The water column and surface sediments of Lake Cadagno contain relatively high concentrations of sulfate, which make it an excellent model environment to study freshwater sulfate-dependent AOM. Lake Sempach (47°09′N, 8°09′E) is located in the central part of Switzerland and is artificially aerated with air in the hypolimnion, which maintains oxic conditions throughout the year in the bottom waters of the lake.

Objective, research questions and outline of the thesis

Objective of the thesis

The main objectives and research goals of this PhD project were to study AOM in anoxic lake waters and sediments, including the quantification of AOM rates, investigation of possible electron acceptors, environmental controls and other biogeochemical processes involved in AOM, and finally the identification of the responsible microorganisms. Towards these goals, I tested and used radiotracers approaches involving $^{14}CH_4$ and $^{3}H\text{-}CH_4$ to determine in situ AOM rates in the anoxic water column or sediments. I also performed slurry incubation experiments using $^{14}CH_4$ or $^{13}CH_4$, supplemented with different electron acceptors, and measured methane oxidation potentials to distinguish between possible modes of AOM processes and gain constraints on direct or indirect links to other element cycles. Finally, I performed lipid biomarker and phylogenetic analyses, in order to identify and/or verify the microorganisms responsible for AOM in the investigated lacustrine environments.

Specifically, I was interested in the following research questions:

- What is the best approach to quantify AOM in lacustrine sediment and how may different AOM rate measurement techniques bias results?
- Is AOM taking place in the anoxic water columns of the two Lake Lugano basins? What is the magnitude of methane oxidation rates?
- What is the relative importance of water column AOM with respect to aerobic methane oxidation at the redoxcline?
- What are the microbial players involved and how abundant are they? What are the environmental factors controlling methane oxidation in the water column?
- What are the dominant pathways of methane consumption in freshwater sediments where metal oxides (e.g., iron and manganese) and/or sulfate are available?
- If electron acceptors other than O_2 and sulfate are involved, is AOM directly coupled to the reduction of these oxidants, or may sulfate-coupled AOM simply be supported by the reduction of metal oxides via a cryptic sulfur cycle producing sulfate?
- Regardless of the electron acceptor involved, which microorganisms are responsible for AOM, and do synthrophic interactions play such an important role as in marine AOM?
- Is AOM in anoxic sediments possibly catalyzed by tentatively aerobic methanotrophs? What are the possible electron acceptors in the absence of oxygen, and what is the role of humic substances during AOM?

Outline of the thesis

This thesis consists of five main chapters that focus on different aspects of AOM in lakes, including one review and four research articles.

Chapter 2: Anaerobic oxidation of methane in freshwater environments: new perspectives on mechanisms, pathways and microorganisms. This chapter is written as a review article, summarizing the current knowledge of the different modes of AOM in freshwater systems, it includes recent reports on discoveries of the pathways and associated microorganisms involved in AOM. Methods used for the investigation of AOM, pathways and identification of methanotrophic communities are presented and critically discussed.

Chapter 3: Water-column stability as prime environmental factor fostering nitrite-dependent anaerobic methane oxidation in stratified lake basins. In this study, I investigated AOM in the water columns of the two hydrodynamically contrasting basins of Lake Lugano. In the stable water column of the permanently stratified northern basin, nitrite-dependent AOM was demonstrated to be an important process. In contrast, I provide conclusive evidence that the dynamic mixing regime in the southern basin is inhibitive to the growth of nitrite dependent AOM bacteria (*Candidatus* Methylomirabilis), even when the chemical conditions see most favorable. Time series data on the evolution of the abundance of *Candidatus* Methylomirabilis in the the north Basin further confirm the requirement of lasting, stably low redox-conditions to establish bacterial AOM.

Chapter 4: Manganese/iron-supported sulfate-dependent anaerobic oxidation of methane by methanotrophic archaea in lacustrine sediments. In marine systems, sulfate-dependent AOM is usually catalyzed by microbial consortia of anaerobic methanotrophs and sulfate-reducing bacteria. In the sulfate-rich Lake Cadagno sediments, however, this process was carried out by a novel ANME strain belonging to *Candidatus Methanoperedens* (i.e., ANME-2d), probably in synthopy with a new group of SRB, representatives of the family Desulfobulbaceae. In addition, I demonstrate that under sulfate-starved conditions, sulfate dependent AOM may be supported by metal (Mn, Fe) oxides through the re-oxidation of reduced sulfur species to sulfate.

Chapter 5: Anaerobic oxidation of methane by aerobic methanotrophic bacteria in lake sediments. True anaerobic oxidation of methane (AOM) in marine systems is primarily catalyzed by ANMEs. However, aerobic methanotrophs were also reported capable of performing AOM in anoxic lake sediments. In this chapter, I confirm a high degree of metabolic versatility by aerobic methanotrophic bacteria. I observed high rates of methane oxidation in the anoxic sediments of Lake Sempach, Lake Lugano, and Lake Cadagno. Type-I aerobic methanotrophs dominated the methanotrophic community at all sites, suggesting that they are able to perform AOM in the absence of O_2. I demonstrate that methane oxidation is not stimulated by any of the common inorganic electron acceptors, suggesting that organic oxidants (i.e., humic substances) may be involved.

Chapter 6: Evaluating radioisotope-based approaches to measure anaerobic methane oxidation rates in lacustrine sediments. Four different $^{14}CH_4$-based

approaches to quantify absolute AOM rates in lake sediments were compared , using samples/sediment cores from Lake Cadagno: The method comparison included sub-core, glass syringe, whole-core and slurry incubations. Different aspects that can bias rate-measurement results (e.g., affecting sample yield, reproducibility and accuracy) were evaluated, and recommendations for further work are provided. I make a convincing case that whole-core incubation is the method-of-choice in future AOM rate measurement studies in sediments, to be preferred over subcore, glass syringe and slurry incubations.

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Chapter 2: Anaerobic oxidation of methane: new perspectives on the processes, pathways and microorganisms

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Abstract

Anaerobic oxidation of methane (AOM) represents one of the dominant biological methane sinks in natural environments and substantially reduces the emission of this potent greenhouse gas to the atmosphere. AOM coupled to sulfate reduction has been most extensively studied in marine systems, where a diversity of anaerobic methane oxidizing archaea (ANMEs) are able to catalyze methane oxidation in syntrophic interaction with their partner bacteria, sulfate-reducing bacteria (SRB). In marine environments, the ANMEs may gain energy exclusively from AOM with sulfate as the terminal electron acceptor. However, recent discoveries of new pathways that microorganisms perform AOM coupled to alternative electron acceptors, including nitrite, nitrate and metal oxides (i.e., iron and manganese), have greatly extended our understanding of these versatile methaneotrophs under anoxic conditions. Increasing evidence has showed that these processes occurred in freshwater environments and has the potential to substantially reduce methane emissions from sediments to the atmosphere. In addition to sulfate-coupled ANMEs in marine environments, microorganisms that are able to perform AOM in freshwater systems have also been identified, with methanotrophic bacteria (NC10 phylum) performing nitrite-dependent AOM and methanotrophic archaea (ANME-2d) catalyzing methane oxidation coupled to nitrate reduction. By applying new tools and techniques, methane oxidation under anoxic conditions have been investigated during recent decades. These studies have provided increasing evidence for AOM in both marine and freshwater habitats and enabled us a better understanding of this microbial process. However, some underlying aspects including physiology of the methanotrophs and the metabolisms of this process as well as the ecological importance particularly in freshwater systems remain insufficiently understood.

Introduction

Methane is a potent greenhouse gas with a global warming potential that is more than 25 folds that of carbon dioxide (ICPP 2006). Microbial methane, produced by anaerobic methanogens (methanogenesis) in anoxic environments, is an important source of this gas and represents more than 50% of the total emissions (Neef et al. 2010). As a major sink of methane, microbially mediated anaerobic oxidation of methane (AOM) plays an important role in maintaining the balance of atmospheric

methane concentrations because this process efficiently controls the input of methane produced in marine sediments, terrestrial and subsurface environments to the atmosphere (Reeburgh 2007).

Since the first discovery of AOM (Barnes and Goldberg 1976; Reeburgh 1976), increasing evidence has supported the existence of AOM based on the geochemical studies and laboratory experiments, including radioactive tracer measurements with $^{14}C/^{3}H$-labeled methane and/or ^{35}S-labeled sulfate (Reeburgh 1980; Iversen and Blackburn 1981; Devol and Ahmed 1981; Devol 1983; Iversen and Jørgensen 1985; Ward et al. 1987; Iversen et al. 1987; Alperin 1989; Reeburgh et al. 1991; Joye et al. 1999, 2004; Treude et al. 2003; Hensen et al. 2003; Orcutt et al. 2005; Durisch-Kaiser et al. 2005; Schubert et al. 2006) and stable isotope investigations (Whiticar and Faber 1986; Reeburgh et al. 1991; Blair and Aller 1995; Martens et al. 1999; Whiticar 1999; Borowski et al. 2000; Valentine and Reeburgh 2000; Nauhaus et al. 2002; Orphan et al. 2002; Wegener et al. 2008; Kellermann et al. 2012). This process has been observed in a variety of different environments (Knittel and Boetius 2009), ranging from marine systems, including methane seep sediments (Hinrichs et al. 1999; Boetius et al. 2000; Michaelis et al. 2002; Orphan et al. 2002), hydrothermal sediments (Teske et al. 2002; Wankel et al. 2012), sulfate-methane transition zones (SMTZs) (Niemann et al. 2005; Treude et al. 2005), marine water column (Daffonchio et al. 2006; Schubert et al. 2006) and deep biosphere (Roussel et al. 2008), to non-marine systems such as terrestrial mud volcanoes (Alain et al. 2006), landfills (Grossman et al. 2002) and freshwater systems, for example, lake water column (Eller et al. 2005). AOM can be carried out by a diversity of both methanotrophic archaea and bacteria with different respirations involved (Orphan et al. 2002; Knittel et al. 2005; Ettwig et al. 2010, 2016; Haroon et al. 2013) and versatile electron transfer pathways in anoxic environments (Wang et al. 2014; Wegener et al. 2015; Scheller et al. 2016; Shi et al. 2016).

Based on our current understanding, AOM has been mostly studied in marine environments and is generally coupled to sulfate reduction, which has been considered as the dominant methane-consuming process in anoxic marine environments (Reeburgh 2007; Knittel & Boetius 2009; Egger et al. 2018). However, none of the organisms that perform AOM in marine systems have been isolated as pure cultures yet. In freshwater systems, methane oxidation under anoxic conditions is much less explored and knowledge about this process is still limited. Recently,

increasing evidence for AOM has been shown to occur in freshwater environments such as lakes and wetlands (Sivan et al. 2011; Norði et al. 2013; Segarra et al. 2015). Indeed, both methanotrophic bacteria of NC10 phylum (Deutzmann et al. 2014; Graf et al. 2018) and methanotrophic archaea (Weber et al. 2017) could be involved and are likely to play a role in the mitigation of methane emission in lacustrine environments.

So far, several AOM processes depending on different terminal electron acceptors have been discovered, including sulfate-dependent AOM, methane oxidation coupled to denitrification (nitrate/nitrite-dependent AOM) and metal oxide reduction linked to AOM (e.g., iron- and manganese-dependent AOM). This review summarizes current knowledge of different AOM processes, including the mechanisms of these processes and associated microorganisms. Finally, methods used for the investigation of AOM pathways and identification of responsible microbes are also summarized and discussed.

Microbial Processes of AOM

Sulfate-dependent AOM. For the first time, the evidence of AOM was shown within anoxic marine sediments and waters where methane was consumed and sulfate was reduced (Barnes and Goldberg 1976; Reeburgh 1976; Martens and Berner 1977). Field and laboratory studies suggested that methane oxidation in anoxic sediments was probably mediated by a consortium of methanogenic archaea and sulfate-reducing bacteria (SRB) through reversal of the methanogenesis pathway (Hoehler et al. 1994). That is, the methanogens oxidized methane and produced hydrogen via a reversal of CO_2 reduction. Hydrogen is known to be a competitive substrate in anaerobic environments and sulfate reducers can utilize hydrogen as electron donor efficiently. Simultaneously, low concentrations of hydrogen was maintained to allow for syntrophic oxidation of methane through the process of interspecies hydrogen transfer and create conditions that thermodynamically favor this biological process (Hoehler and Alperin 1996; Schink 1997).

Investigation of archaeal 16S rRNA gene sequences revealed that a new group was dominant in the methane seep samples. This cluster was phylogenetically distinct from known methanogens and proposed as anaerobic methanotrophic archaea (Hinrichs et al. 1999). The result of this study also found a mixture of archaea and

bacteria, and the bacterial 16S rRNA sequences retrieved were mainly related to known anaerobes (e.g., SRB), suggesting a coupling of methane oxidation and sulfate reduction. Subsequent studies of microbial cell identification confirmed that microbial aggregates of methanotrophic archaea and SRBs mediated anaerobic oxidation of methane in anoxic marine environments (Boetius et al. 2000; Michaelis et al. 2002; Knittel et al. 2003). The anabolic activities were further shown in the microbial consortia using the combination of fluorescence in situ hybridization and secondary ion mass spectrometry (FISH-SIMS) (Orphan et al. 2001a, 2002, 2009; House et al. 2011; Dekas et al. 2016). In addition, in vitro experiment using sediments that were naturally enriched in microbial consortia consisting of methanotrophic archaea and SRB successfully demonstrated AOM coupled to sulfate reduction (Nauhaus et al. 2002). These findings supported that methane was oxidized via the cooperation of anaerobic methanotrophic archaea and sulfate-reducing bacteria under anoxic conditions (Hoehler et al. 1994). During sulfate-coupled AOM, electrons are transferred from ANME to the bacterial partner. ANMEs usually form close associations with SRB in dense aggregates (Hinrichs et al. 1999; Boetius et al. 2000; Michaelis et al. 2002; Vigneron et al. 2013), which could support the mechanism of direct interspecies electron transfer (DIET) (Orcutt and Meile 2008; Alperin and Hoehler 2009, 2010). DIET is usually achieved using multi-heme cytochrome c proteins (MHCs) (Kletzin et al. 2015; Wegener et al. 2015; McGlynn et al. 2015) and conductive pili (i.e., nanowires) (Wegener et al. 2015; Krukenberg et al. 2016). However, further investigations for more direct evidences are required to better understand the coupling mechanisms for cell-to-cell electron transfer (Lovley 2017) and specific microbial communities (Timmers et al. 2017).

Recently, many proteomic and genomic studies supporting the previously proposed pathway of AOM (i.e., reverse methanogenesis) (Hoehler et al. 1994) have been well documented and discussed (Widdel and Rabus 2001; Hallam et al. 2003, 2004; Krüger et al. 2003; Meyerdierks et al. 2010; Scheller et al. 2010; Thauer 2011; Timmers et al. 2017). All known methanogens express the methyl coenzymeM reductase (mcr), an enzyme composes of three subunits (alpha, beta and gamma) and two tightly but noncovalently bound molecules of a nickel porphinoid, cofactor F_{430} (ELLERMANN et al. 1988), and catalyzes the terminal step in biogenic methane production (Reeve et al. 1997; K.Thaue 1998; Ferry 1999). That is, methyl-coenzyme M ($CoM\text{-}S\text{-}CH_3$) is reduced with coenzyme B (H-S-CoB) in the final step of

methanogenesis, yielding methane and the heterodisulfide of coenzyme B and coenzymeM (CoM-S-S-CoB). Hallam et al. studied Mcr genes in ANME archaea isolated from various marine environments and identified four types of novel *mcr*A genes (coding for the alpha subunit of MCR), providing a basis for identifying methanotrophic archaea (Hallam et al. 2003). The detection of the *mcr*A gene is currently still used as an indication of microbial communities catalyzing AOM (Inagaki et al. 2004; Nercessian et al. 2005; Nunoura et al. 2006; Lösekann et al. 2007; Heller et al. 2008; Biderre-Petit et al. 2011; Takeuchi et al. 2011; Biddle et al. 2012; Rissanen et al. 2018; Winkel et al. 2018). Furthermore, Krüger et al. extracted a nickel protein (Ni-protein I) from AOM-mediating microbial mats. The new protein probably played a catalytic role in AOM similar to the authentic cofactor F_{430} of the MCR but was not found in any methanogenic archaea (Krüger et al. 2003). Recently, Scheller et al. reported that purified MCR from a methanogen could cleave the particularly strong C-H bond of methane without the involvement of highly reactive oxygen-derived intermediates into methyl coenzyme M (Scheller et al. 2010). However, there were also studies showing that pure cultures of methanogens were not able to oxidize methane under low hydrogen and high methane conditions (Valentine & Reeburgh 2000) and the low thermodynamic yield of reverse methanogenesis, which would have to be shared by both archaea and SRB, rendered this mechanism doubtful and alternative mechanisms were proposed, including acetogenesis (Zehnder and Thomas 1980; Hoehler et al. 1994; Valentine and Reeburgh 2000) and methylogenesis (Moran et al. 2008). Until now, the mechanistic details accounting for reverse methanogenesis during sulfate-dependent AOM in various environments remain unresolved, which may be due to the differences in the environmental conditions and the physiological characteristics of ANME consortia members.

In recent two decades, the diversity of anaerobic methanotrophic populations mediating sulfate-dependent AOM has been intensively investigated in marine environment (Orphan et al. 2002; Knittel et al. 2005; Lösekann et al. 2007; Orcutt et al. 2008; Lever & Teske 2015; Timmers et al. 2015; Ruff et al. 2016; Wegener et al. 2016; Winkel et al. 2018). Based on the 16S rRNA and archaeal *mcr*A gene analyses, three distinct phylogenic clusters of Euryarchaeota, namely ANME-1, -2 and -3 were identified as AOM mediators (Knittel and Boetius 2009). ANME-1 (including ANME-1a and ANME-1b) are distantly related to the orders *Methanosarcinales* and *Methanomicrobiales*, which comprise a major part of the cultivated methanogens

(Hinrichs et al. 1999; Michaelis et al. 2002; Orphan et al. 2002; Knittel et al. 2005). ANME-2 (including four distinct subgroups) are affiliated with the order *Methanosarcinales* (Orphan et al. 2001b; Knittel et al. 2005), and ANME-3 are related to the genera *Methanococcoides* (Niemann et al. 2006; Lösekann et al. 2007; Lazar et al. 2011). ANMEs often form consortia with SRB to catalyze anaerobic oxidation of methane. Investigations have showed that archaea of ANME-1 and ANME-2 (ANME-2a, -2b and -2c) are usually associated with sulfate-reducing bacteria (SRB) within *Desulfosarcina/Desulfococcus* (DSS) branch of the Deltaproteobacteria (Orphan et al. 2001b, 2002; Michaelis et al. 2002; Knittel et al. 2003) while ANME-3 archaea identified from submarine mud volcanoes were reported to form aggregates with SRB of the *Desulfobulbus* (Niemann et al. 2006; Lösekann et al. 2007), but were also detected together with DSS in shallow subsurface gas-hydrate-bearing sediments (Knittel and Boetius 2009). In Eel River Basin sediments, however, both consortia of ANME-2c/*Desulfobulbus* and ANME-2c/DSS were identified (Pernthaler et al. 2008), indicating a versatility in bacterial partnership and AOM syntrophy. A physical association with SRB is not obligatory for all three clades of ANME archaea. Most ANME-1 archaea exist as single cells or form monospecific chains without any attached partner (Orphan et al. 2002; Maignien et al. 2013). ANME-2 (Treude et al. 2005) and ANME-3 (Lösekann et al. 2007) have also been shown not directly associated with sulfate-reducing partners and could probably perform AOM independently. In addition, SRB are not the only bacterial partners of ANMEs. For example, *Sphingomonas* of Alphaproteobacteria and betaproteobacterial *Burkholderia* have been identified microscopically as the dominant or sole bacterial partner associated with ANME-2c (Knittel and Boetius 2009). ANME-3 cells have also been shwoed to occur with yet unidentified bacteria, forming mixed-type aggregates Very few consortia were detected with a yet-unidentified bacterial partner. (Lösekann et al. 2007). These findings suggest that ANMEs do not necessarily depend on SRB and might be able to live syntrophically with other bacterial partners as well. Sulfate-dependent AOM has been well studied in marine environments but in freshwater systems, this process remains poorly understood. Very recently, a novel ANME strain belonging to *Candidatue* Methanoperedens and closely affiliated with nitrate-dependent ANME, has been demonstrated to perform sulfate-dependent AOM probably associated with an uncultivated Desulfobulbaceae bacterium (Su et al , unpublished data).

Methane oxidation coupled to denitrification. Methane oxidation with nitrate or nitrite as electron acceptor is thermodynamically more favorable than sulfate (Shima and Thauer 2005; Thauer and Shima 2008). There were experimental evidences showing that methane oxidation was associated with denitrification in different systems (Sollo et al. 1976; Smith et al. 1991; Mason 1997; Islas-Lima et al. 2004; Knowles 2005), but microorganisms capable of coupling methane oxidation to denitrification were first identified in an enrichment culture with inocula from anoxic sediment of a canal (Raghoebarsing et al. 2006). AOM in the enrichment culture was mediated by a microbial consortium of bacteria of the NC10 phylum and archaea of the order *Methanosarcinales* distantly related to marine ANME archaea. Prolonged incubation of the same culture with methane and nitrite resulted in the disappearance of the archaea (Ettwig et al. 2008) and the dominant NC10 bacteria were able to couple AOM to denitrification without the involvement of archaea (Ettwig et al. 2008, 2009). The anaerobic denitrifying bacteria, subsequently named *Candidatus Methylomirabilis oxyfera* (*M. oxyfera*), were shown to perform the well-established aerobic pathway for methane oxidation based on the complete genome analysis (Ettwig et al. 2010). That is, *M. oxyfera* utilized an intra-aerobic pathway through the disproportionation of two molecules of nitric oxide (NO) to dinitrogen (N_2) and oxygen (O_2). The O_2 produced during this step was used for aerobic methane oxidation with methane monooxygenase (Ettwig et al. 2010, 2012). The second step of canonical aerobic methane oxidation is the conversion of methanol to formaldehyde, catalyzed by either a calcium-dependent MxaFI-type or a lanthanide-dependent XoxF-type methanol dehydrogenase (MDH) (Chistoserdova and Kalyuzhnaya 2018; Versantvoort et al. 2018). The proposed pathway of 'intra-aerobic denitrification' did not involved some known genes for complete denitrification (e.g., genes for dinitrogen production), but suggested a novel enzyme as yet unknown functional analogue for N_2 production (NO dismutase). However, the genome of *M. oxyfera* includes genes encoding the complete pathway for aerobic methane oxidation, indicating that this microorganism might exclusively and independently mediate the whole process (i.e., coupling of nitrite reduction to the oxidation of methane) (Ettwig et al. 2010; Versantvoort et al. 2018).

So far, our knowledge regarding NC10 phylum is largely limited to *M. oxyfera*. By using molecular techniques, recent studies have demonstrated that representatives

of the NC10 phylum are present in diverse environments, ranging from peatlands (Zhu et al. 2012), paddy soils (Zhou et al. 2014; He et al. 2016) and wastewater treatment plants (Luesken et al. 2011) to marine environments (Chen et al. 2015; He et al. 2015; Padilla et al. 2016), estuary (Yan et al. 2015; Zhang et al. 2018), reservoir (Kojima et al. 2014) and lacustrine environments (Kojima et al. 2012; Yang et al. 2012; Deutzmann et al. 2014; Graf et al. 2018). Three different names have been proposed for distinct strains belonging to *Candidatus* Methylomirabilis genus. *Candidatus* Methylomirabilis sinica (*M. sinica*), distantly related to *M. oxyfera* (85.3% similarity of *pmoA* gene sequence) were found to be widely distributed in various habitats (He et al. 2016). *Candidatus* Methylomirabilis limnetica (*M. sinica*) were detected in lacustrine environments and they were more distantly related to *M. oxyfera* than *M. sinica* (Graf et al. 2018). *Candidatus* Methylomirabilis lanthanidiphila (*M. lanthanidiphila*) were originally present in the culture of *M. oxyfera*, but they used a lanthanide-dependent XoxF-type MDH as the sole methanol-oxidizing enzyme, which was different from *M. oxyfera* employing XoxF-MxaI heterodimer (Versantvoort et al. 2018). It has also been shown that *M. limnetica* possesses only a single copy of the lanthanide-dependent xoxF-type MDH to produce an active MDH (Graf et al. 2018).

More recently, *Candidatus* Methanoperedens nitroreducens or *M. nitroreducens*, that belongs to a novel ANME lineage (ANME-2d) and was enriched from laboratorial bioreactors (Hu et al. 2009), has been demonstrated to participate in AOM independently by using nitrate as the terminal electron acceptor (Haroon et al. 2013). In contrast to sulfate-dependent AOM, ANME-2d do not need a bacterial partner and transfer electrons directly to an unusual Nar-like protein complex in the membrane-bound electron transport chain that also includes $F_{420}H_2$ dehydrogenase and an unusual Rieske/cytochrome b complex (Arshad et al. 2015). During nitrate-dependent AOM, methane was probably activated by methyl-CoM reductase and subsequently oxidized to CO_2 via reverse methanogenesis, all the enzymes of which were present and expressed in the investigated culture (Haroon et al. 2013; Arshad et al. 2015). However, genes for the complete denitrification process were lacking in *M. nitroreducens*, therefore explaining why this methanotrophic archaea often occurs in aggregates with denitrifying methanotrophic bacteria, *M. oxyfera* (Raghoebarsing et al. 2006; Hu et al. 2009, 2011) or in the presence of excess ammonium, with anaerobic ammonia-oxidizing (ANAMMOX) bacteria (i.e., *Candidatus* Kuenenia stuttgartiensis)

(Haroon et al. 2013) to remove toxic nitrite (Oni and Friedrich 2017). So far, few evidences of ANME-2d lineage (*Methanoperedenaceae*) performing nitrate-dependent AOM have been reported in natural environments (Shen et al. 2017; Vaksmaa et al. 2017; Xu et al. 2018). Interestingly, *M.nitroreducens* was affiliated with ANME-2d, the fourth subgroup of ANME-2 for sulfate-dependent AOM, suggesting a certain relationship between the two processes of nitrate-dependent and sulfate-dependent AOM. In a low-sulfate, iron-rich freshwater lake sediment, anaerobic methanotrophic archaec of the ANME-2d cluster were reported to perform AOM probably coupled to sulfate and/or iron redcution (Norði et al. 2013; Weber et al. 2017) and a novel ANME strain within *Candidatus* Methanoperedens retrieved from sulfate-rich lake sediments has also been demonstrated to perform sulfate-dependent AOM (G. Su, J. Zopfi, H. Niemann & M. F. Lehmann, unpublished data). In addition to NC10 and ANME-2d, crenothrix of gamma-proteobacteria was experimentally and genomically shown to conduct anaerobic respiration under nitrate-reducing conditions, but the proposed pathway of methane oxidation coupled to nitrate reduction by crenothrix remains to be confirmed in future studies (Oswald et al. 2017).

Metal oxide reduction linked to AOM. Although AOM coupled to metal oxide (e.g., Fe^{III} and Mn^{IV}) reduction is thought to be both thermodynamically favorable and biochemically feasible (Table 1), conclusive field evidence for the occurrence of metal-dependent AOM in natural systems is still scarce. Anaerobic oxidation of methane coupled to metal reduction has been demonstrated or suggested in freshwater environments (Crowe et al. 2011; Sivan et al. 2011; Segarra et al. 2013; Norði et al. 2013; Torres et al. 2014), a mud volcano (Chang et al. 2012), a contaminated aquifer (Amos et al. 2012), and marine sediments (Beal et al. 2009; Wankel et al. 2012; Riedinger et al. 2014; Egger et al. 2014; Treude et al. 2014; Rooze et al. 2016). However, microorganisms responsible for iron/manganese-dependent methane oxidation were not identified in these studies. It was speculated that JS1 bacteria, methanogens and archaea related to Methanohalobium/ANME-3 could be responsible for iron-dependent AOM (Oni et al. 2015) whereas ANME-1 and/or Methanococcoides/ANME-3 would probably explain manganese-dependent AOM together with a bacterial partner (Beal et al. 2009). Furthermore, ANME-2d was also shown to be involved in AOM coupled to chromium(VI) reduction (Lu et al. 2016). In

a recent study, Ettwig et al. used highly enriched cultures from freshwater sediments to show that archaea of the order Methanosarcinales, related to *M. nitroreducens*, can independently couple methane oxidation to the reduction of insoluble Fe^{III} and Mn^{IV} minerals (Ettwig et al. 2016). AOM coupled to iron/manganese reduction might be more widespread and has the potential to be a large methane sink than previously thought, as both metal oxides are distributed throughout the sediments and can be oxidized and reduced 100 to 300 times before burial (Canfield et al. 1993). Using isotope labeling experiments and metagenomic analysis, a novel methanotrophic archaeon named *Candidatus* Methanoperedens ferrireducens was demonstrated to perform AOM couple to Fe(III) reduction with genes encoding the reverse methanogenesis pathway (Cai et al. 2018). In addition, MHCs were also identified in the genome of these organisms, suggesting that they could independently mediate AOM using Fe(III) as the terminal electron acceptors. These MHCs proteins have been previously reported in bacteria such as *Geobacter* and *Shewanella* species and are important as biological nanowires to connect between cell and mineral for extracellular electron transfer (Reguera et al. 2005; El-Naggar et al. 2010; Summers et al. 2010).

Table 1. The free energy yields of AOM with environmentally relevant electron acceptors under standard conditions discussed in this review.

Reaction	**$\Delta G^{\circ\prime}$ (kJ/mol CH_4)**
AOM processes with identified electron acceptors	
$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	–16.6
$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O$	–928
$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O$	–765
AOM processes with possible or hypothetic electron acceptors	
$CH_4 + 8Fe(OH)_3 + 15H^+ \rightarrow HCO_3^- + 8Fe^{2+} + 21H_2O$	–270.3
$CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O$	–556
$CH_4 + 4AQDS + 3H_2O \rightarrow HCO_3^- + 4AQH_2DS + H^+$	−41
$CH_4 + ClO_4^- + 7H^+ \rightarrow CO_2 + Cl^- + 2H_2O$	–932.2
$CH_4 + 4HAsO_4^{2-} + 4H^+ \rightarrow CO_2 + 4H_2AsO_3^{2-} + 2H_2O$	–263.3

Methanogen has been documented to be capable of methanotrophic growth

dependent on reduction of Fe(III) (Soo et al. 2016; Yan et al. 2018), which is very similar to the pathway proposed for anaerobic methanotrophic archaea (ANME-2a) based on metagenomic and transcriptomic analyses (Wang et al. 2014). During iron-dependent reverse methanogenesis, methane is oxidized to methyl-coenzymeM (CH_3-SCoM), which is transferred to tetrahydrosarcinapterin (H_4SPT) by Mtr representing the reversal of methanogenic pathway. The exergonic oxidation of coenzyme M (HSCoM) and coenzyme B (HSCoB) coupled to Fe(III) reduction drives the endergonic methane oxidation and the transfer reactions of CH_3-H_4SPT is driven with the Na^+ gradient generated by Rnf/cytochrome c complex catalyzing the highly exergonic oxidation of ferredoxin (Fdx^{2-}) and reduction of iron (Yan et al. 2018). The homologs of heterodisulfide reductases (HdrA2B2C2) is also proposed to play a role in iron-dependent reverse methanogenesis (Yan and Ferry 2018).

Other potential electron acceptors during AOM. Sulfate-coupled AOM has been considered as the dominant process in anoxic marine environments (Reeburgh 2007). Despite the decoupling from the syntrophic SRB partners using artificial oxidants (e.g., AQDS and humic acids), ANME from marine sediments was still shown to sustain high rates of methane oxidation in the absence of sulfate, suggesting the possibility of these humic substance analogues as electron acceptors for AOM (Scheller et al. 2016). Humic substance as electron shuttles could enhance the microbial capacity to reduce less accessible electron acceptors (e.g., insoluble Fe (III) oxides) (Lovley et al. 1996) and humic acids was even shown as electron acceptors for anaerobic microbial oxidation of organic compounds (Bradley et al. 1998). Humic substance constitutes a significant fraction of natural organic matter in terrestrial and aquatic environments (Stevenson 1994), and under recurrent anoxic conditions, the electron accepting capacity of humic substances can be fully regenerable (Klüpfel et al. 2014), which could provide potential electron acceptors for AOM in environments such as redox boundaries of water column or surface sediments of a seasonal stratified lake (Blees et al. 2014). In addition to the known and well-tested electron acceptors (i.e., sulfate and nitrite/nitrate), it might also be possible that other environmentally relevant oxidants (Table 1, e.g., arsenate, perchlorate) could be coupled to anaerobic oxidation of methane (Thauer and Shima 2008; Caldwell et al. 2008). Recently, anaerobic oxidation of methane was observed by aerobic methanotrophs (i.e., type I MOB related to *Methylobacter*) in the anoxic water column or lake sediments (Blees

et al. 2014; Martinez-cruz et al. 2017). A new proposed pathway of aerobic methane oxidation coupled to oxygenic photosynthesis (Oswald et al. 2015; Milucka et al. 2015; Rissanen et al. 2018) or cryptic oxygen cycling (Garcia-Robledo et al. 2017) could explain the methane oxidation in anoxic waters of shallow lakes by gamma-proteobacterial MOB. In freshwater lake sediments, we also observed methane oxidation under anoxic conditions and but only aerobic methane-oxidizing bacteria were detected (Figure 1), further supporting that aerobic methanotrophs may catalyze AOM. However, the responsible electron acceptors for methane oxidation in the anoxic lake sediments and also in deep anoxic water column (Blees et al. 2014; Roland et al. 2018) remained unclear. Further investigations in this regard are required to reveal the possible pathways and the electron acceptors involved.

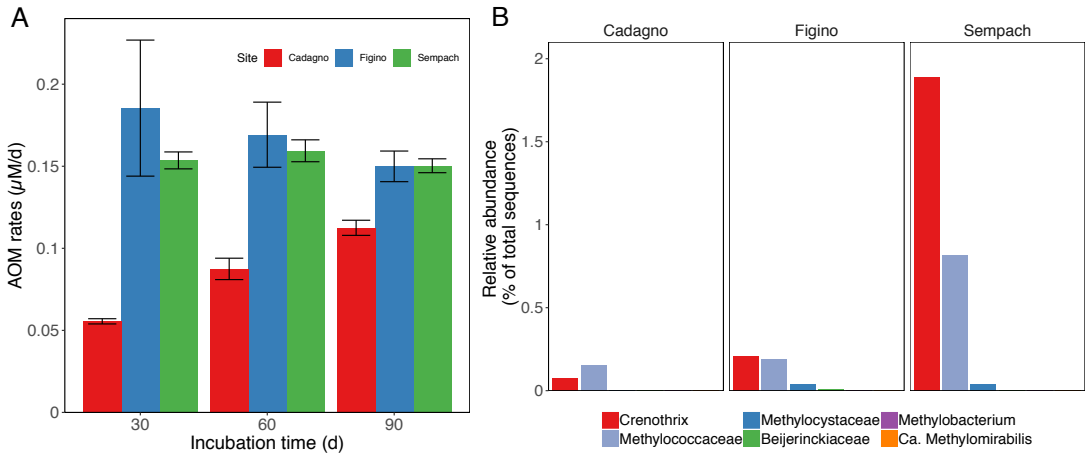

Figure 1. (A) Rates of anaerobic oxidation of methane (AOM) over incubation time using slurry with surface sediments (top 10 cm) amended with ^{14}C-labeled methane. No methane oxidation rates were detected in the killed controls (data not shown here) and (B) composition and relative abundance of methanotrophic bacteria detected in the surface sediments (0-2 cm) of the investigated lakes: Lake Sempach (March 2015), Lake Lugano (Figino, February 2015) and Lake Cadagno (July 2016).

Tools and Approaches to Identifying AOM

AOM rate measurements and geochemical observations. Despite that AOM has been intensively studied, the process of anaerobic methanotrophy in natural environments have not been sufficiently quantified. Direct rate measurements using radiotracers could provide compelling evidence of AOM and are also essential for evaluating the efficacy of this important methane sink and to better understand the

modulating controls on the balance between methane production and consumption. The first attempts using ^{14}C-CH_4 (Reeburgh 1980; Iversen and Blackburn 1981) for rate measurements provided direct evidences that AOM occurred in the anoxic sediments. Indeed, depth-specific AOM rate profiles are able to provide information of where, and to what extent, methane is oxidized in natural environments, which is conducive to subsequent sample selection and analysis. In addition to marine systems (Treude et al. 2003; Niemann et al. 2006; Steinle et al. 2016), recent studies using ^{14}C-CH_4 to actually quantify methane oxidation rates have also been reported in freshwater sediments (Segarra et al. 2013, 2015; Norði et al. 2013). On the other hand, profiles of geochemical parameters could suggest potential AOM processes in natural environments, for example, sulfate-dependent AOM within or below SMTZs (Hoehler et al. 1994; Reeburgh 2007; Knab et al. 2008; Dale et al. 2008; Treude et al. 2014), and laboratory incubation experiments which were amended with potential electron acceptors could also facilitate the identification of AOM process within a specific environmental context (Beal et al. 2009; Egger et al. 2014).

Fluorescent in situ hybridization (FISH). FISH is a cultivation-independent method that has been widely used for the identification of microbial communities (Amann & Fuchs 2008). When combined with other techniques, FISH can be used for better characterization of phylogenetically defined microbial populations and inferring their functional traits in complex environments (Wagner et al. 2003). Boetius et al. first identified a marine microbial consortium of archaea and SRB with FISH by using specific fluorescently labeled 16S rRNA-targeted oligonucleotide probes (Boetius et al. 2000). The combination of FISH and secondary ion mass spectrometry (SIMS), which is a sensitive method for the detection of stable and radioactive isotopes, could also be used for the identification of naturally occurring microorganisms and the detection of their metabolic activities simultaneously (Orphan et al. 2001a, 2002). Single microbial cells can also be analyzed for the identity and function by a combination of stable-isotope Raman spectroscopy and FISH (Huang et al. 2007). FISH-NanoSIMS with a much higher resolution has recently been even used to investigate the potential role of nitrogen substrates in ANME consortia (Green-Saxena et al. 2014) and to measure the anabolic activity of single cell and AOM aggregates (Dekas et al. 2016). The immunomagnetic capture Magneto-FISH was also combined with highthroughput sequencing and isotope labeling to characterize

potentially hidden microbial groups and simplifies metagenomic sequencing by reducing community complexity (Pernthaler et al. 2008). By combining bioorthogonal noncanonical amino acid tagging (BONCAT) with rRNA-targeted FISH, microbial interactions were shown to be occurring between ANME and members of the poorly understood phylum *Verrucomicrobia* within the slow-growing archaeal-bacterial consortia (Hatzenpichler et al. 2016). Other combinations such as CARD-FISH that combines catalysed reported deposition (CARD) of fluorescently labelled tyramides with single-cell identification by FISH (Schönhuber et al. 1997; Pernthaler et al. 2002), and MAR-FISH that combines the detection of radioactively labelled substrates by microautoradiography and with single-cell identification by FISH (Lee et al. 1999; Ouverney et al. 1999) could improve the sensitivity of FISH. CARD-FISH have recently been widely used for the identification of both methanotrophic archaea and bacteria, including AOM consotia (Halm et al. 2009; Robert Hamersley et al. 2009; Schubert et al. 2011; Dekas et al. 2014; van Kessel et al. 2015; Zigah et al. 2015; Cai et al. 2016; Oswald et al. 2016a, 2017; Trembath-Reichert et al. 2016). However, to our knowledge, the MAR-FISH has not yet been used to characterize AOM communities.

Culture-dependent/independent 16S rRNA gene and metagenomic analyses. Identification of the whole-community genomes in the enrichment cultures (or environmental samples) has provided us a significant insight into the phylogeny and metabolism of AOM communities. By using culture-independent molecular phylogenetic surveys, Hinrichs et al. first identified ANME-1 from methane seep sediments in the Eel River basin and the group was phylogenetically distinct from known methanogens but peripherally related to the methanogenic orders *Methanomicrobiales* and *Methanosarcinales* (Hinrichs et al. 1999). Hallam et al. used genomic analyses on an enrichment of marine AOM consortia and the identified most of the genes associated with methanogenesis in the ANME groups (Hallam et al. 2004), supporting the previous hypothesis of reverse methanogenesis for AOM (Hoehler et al. 1994). Anaerobic methane oxidizers are notoriously slow-growing, with generation times from months to years due to the extremely low energy yield and biosynthesis (Knittel and Boetius 2009). A continuous-flow bioreactor that simulated in situ conditions was developed and demonstrated to support the growth and metabolism of anaerobic methanotrophic archaea (Girguis et al. 2005). Long-term in

vitro incubations with a continuous supply of methane and sulfate resulted in growth of ANME-2/DSS clusters with the same morphology as those present in the original sediment inoculum (Nauhaus et al. 2007). By using laboratory enrichment and metagenomics, two AOM processes have by now been identified, and the corresponding microbial players and the pathways were also proposed: nitrite-dependent AOM by *M. oxyfera* (Raghoebarsing et al. 2006; Ettwig et al. 2009, 2010) and nitrate-dependent AOM by *M. nitroreducens* (Hu et al. 2009; Haroon et al. 2013).

Biomarker analysis combined with stable isotope probing (SIP). Specific lipid biomarkers and their stable carbon isotope signatures could attain AOM population identification independently. This approach provides the primary evidence for the presence of AOM and has been widely used particularly in natural environments where direct measurements of methane distribution and AOM rates were not difficult (Hinrichs et al. 1999; Orphan et al. 2001a, 2002; Orcutt et al. 2005). A recent review summarizes significant lipid biomarker signatures for a chemotaxonomic identification of known AOM communities (Niemann and Elvert 2008) and isotopically light lipid biomarkers are important signals of the presence of AOM consortia responsible for methane oxidation (Elvert et al. 1999; Hinrichs et al. 1999, 2000; Thiel et al. 1999; Pancost et al. 2000; Orphan et al. 2001b; Kellermann et al. 2012). Furthermore, in vitro $^{13}CH_4$ labeling studies (Blumenberg et al. 2005; Nauhaus et al. 2007), which can be combined with substrate-amended incubation experiments, could provide another direct approach to investigating the potential pathways and identifying AOM communities by tracking the incorporation of $^{13}CH_4$ into methanotrophic biomass (i.e., specific lipids). In addition to lipid-SIP, strategy that combines genomics with SIP (e.g., DNA-SIP) has been shown as a powerful technique for identifying microbial populations that were actively involved in specific metabolic activities (Radajewski et al. 2000). However, most studies using DNA/RNA-based SIP were restricted to aerobic methanotrophs (Morris et al. 2002; Hutchens et al. 2004; Lin et al. 2004; Dumont et al. 2011; Graef et al. 2011; Cai et al. 2016; Martinez-cruz et al. 2017; Weber et al. 2017), but this technique should also be considered in future AOM investigations. Finally, a DNA-SIP-based metagenome analysis increases the feasibility of linking genetic information to distinct microbial species, thus exploiting isotopically labeled genomes and revealing potential functions of uncultivated microorganisms in different environments (Friedrich 2006).

Conclusions and Perspectives

AOM is a microbially mediated process catalyzed by anaerobic methanotrophs in the absence of oxygen. This process occurs widely in anoxic environments of both marine and freshwater habitats and serves as an important methane sink. Currently, several processes are considered to be responsible for AOM, including sulfate-coupled AOM, nitrite/nitrate-dependent AOM, methane oxidation coupled to the reduction of metal oxide (e.g., Fe^{III}) and potentially humic substances. However, some of these processes have not been sufficiently documented and require further investigation. The different AOM processes by a diversity of methanotrophic archaea and bacteria have greatly expanded our understanding of these biogeochemically significant microorganisms and their potential for metabolic versatility in anoxic environments. So far, metagenomic and metatranscriptomic studies have provided evidence that methanotrophic archaea use the reverse methanogenesis pathway during AOM whereas NC10 bacteria use canonical aerobic methane oxidation pathway with oxygen derived from intra-aerobic denitrification. However, the exact mechanism of AOM is still not well understood and future studies are required to further shed light on the molecular basis of the reverse methanogenesis pathway in different ANME types.

Despite the slow-growing characteristic, the successful cultivation of individual ANME will enable a targeted investigation of AOM process with more complete physiological and molecular details. These knowledge would greatly enhance our understanding of the microorganism and the mechanism behind anaerobic methane oxidation in anoxic environments. Finally, studies that integrate work of different aspects (e.g., geochemical characterizations, enrichment experiments, rate measurements, biomarker, microscopy, isotope and phylogenetic analyses) and combine recent tools and techniques will simultaneously demonstrate metabolic activity, microbial processes, pathways and responsible microorganisms of AOM in the environments.

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Chapter 3: Water column dynamics controls nitrite-dependent anaerobic oxidation of methane in stratified lakes

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Abstract

The anaerobic oxidation of methane (AOM) with nitrate/nitrite as the terminal electron acceptor may play an important role in mitigating methane emissions from lacustrine environments to the atmosphere. We investigated AOM in the water column of two hydrodynamically contrasting basins of a south-alpine lake in Switzerland (Lake Lugano). The North Basin is permanently stratified with year-round anoxic conditions below 95 m water depth while the South Basin undergoes seasonal stratification with the development of a benthic bacterial nepheloid layer and anoxia during summer. Here we show a substantial fraction of methane was oxidized coupled to nitrite reduction by *Candidatus* Methylomirabilis below the redoxcline of the North Basin and $^{14}CH_4$-labeled incubation with concentrated biomass from this basin showed at least 43-52%-enhanced AOM rates with nitrate/nitrite added as electron acceptor. Time series data on the evolution and recovery of *Methylomirabiliaceae* in the North Basin since the last exceptional mixing events in 2005/2006 reveals their requirement for lasting, stably low redox-conditions to establish. In contrast, the absence of *Candidatus* Methylomirabilis in the South Basin despite a hydrochemical framework during the stratified season that seems even more favorable for nitrite-dependent AOM further confirms the importance of water column stability for the growth of denitrifying anaerobic methananotrophs. Based on our findings, we predict that the process of nitrite-dependent AOM has the potential to be a globally important methane sink due to the increasing stratified lakes around the world resulting from global warming.

Introduction

Freshwater habitats such as lakes are important sources of methane (CH_4), a potent greenhouse gas in the atmosphere (Bastviken et al. 2011). A large fraction of methane is produced in lake sediments by anaerobic methanogenic archaea, from where it may escape by ebullition or diffusion into bottom waters. Several studies have evidenced aerobic methane oxidation at the sediment surface or in the water column of lakes (He et al. 2012; Blees et al. 2014a; b; Milucka et al. 2015; Oswald et al. 2016). However, under anoxic conditions, methane can also be oxidized anaerobically. For a long time, sulfate was considered to be the only suitable terminal electron acceptor for the anaerobic oxidation of methane (AOM), which is an

important process to reduce methane emissions to the atmosphere (Knittel and Boetius 2009). This process is mainly performed by microbial consortia of anaerobic methanotrophic archaea (ANME-1, -2 and -3) and sulfate-reducing bacteria (SRB) (Boetius et al. 2000; Michaelis et al. 2002; Orphan et al. 2002; Niemann et al. 2006). Recent studies have reported other potential electron acceptors for AOM, including nitrogenous compounds (Raghoebarsing et al. 2006; Ettwig et al. 2010; Haroon et al. 2013), iron and/or manganese (Beal et al. 2009; Sivan et al. 2011; Ettwig et al. 2016; Cai et al. 2018), and possibly humic substances (Scheller et al. 2016; Valenzuela et al. 2019).

Indeed, recent evidence suggests that under anoxic conditions not only in marine but also freshwater environments, AOM with electron acceptors other than sulfate may represent a significant methane sink (Sivan et al. 2011; Norði et al. 2013; Segarra et al. 2015; Weber et al. 2017; Su et al. 2020). For nitrogen-dependent anaerobic oxidation of methane (N-AOM), two microbial processes and the corresponding microbial players have been identified. One is the oxidation of methane with nitrite as terminal electron acceptor by the bacterium *Candidatus* Methylomirabilis oxyfera (Ettwig et al. 2009; He et al. 2016; Versantvoort et al. 2018). These methanotrophs are capable of producing oxygen intracellularly (intra-aerobic pathway) by the disproportionation of nitric oxide to N_2 and O_2, which is then used for canonical aerobic methane oxidation (Ettwig et al. 2010). The other process is methane oxidation coupled to nitrate reduction, catalyzed by the methanotrophic archaeon *Candidatus* Methanoperedens nitroreducens (Haroon et al. 2013). Although the exact metabolic mechanisms of nitrate/nitrite-dependent AOM are not entirely elucidated, evidence for this process has been recently reported in freshwater environments (Ettwig et al. 2009; Hu et al. 2009, 2014; Deutzmann and Schink 2011; Wang et al. 2012; Norði and Thamdrup 2014; Graf et al. 2018; Mayr et al. 2020) and marine oxygen minimum zones (Padilla et al. 2016).

Given the prevalence of nitrate in freshwater lakes, N-AOM may play an important role in the mitigation of methane emissions from lake sediments. In lacustrine environments, highest methane oxidation rates were often observed near oxic/anoxic transition zones at the sediment-water interface (Lidstrom and Somers 1984; Kuivila et al. 1988; Frenzel et al. 1990; Bender and Conrad 1994; He et al. 2012) or in the water column of stratified lakes (Rudd et al. 1974; Blees et al. 2014a, b). However, methane consumption at these boundaries was usually thought to be

carried out by aerobic methanotrophs, fueled by oxygen supplied by diffusion, cryptic production, or intrusion events (Hanson and Hanson 1996; Bastviken et al. 2002; Pasche et al. 2011; He et al. 2012; Milucka et al. 2015; Oswald et al. 2016). Indeed, redox transition zones may also represent sites where nitrate/nitrite is typically produced/regenerated through the oxidation of ammonium and reduction of nitrate by nitrogen-transforming microorganisms (Kuypers et al. 2018). Hence, here the N-AOM might be masked by, or misinterpreted as, aerobic methane oxidation (Deutzmann et al. 2014). As a result, methane oxidation with nitrate/nitrite as terminal electron acceptor may play a greatly underappreciated role in lakes. While nitrate-dependent *Ca.* Methanoperedens has not been observed in freshwater lake environments (Su et al. 2020), high abundance as well as transcriptional activity of nitrite-dependent *Ca.* Methylomirabilis limnetica has been recently reported in two permanently stratified lakes (Graf et al. 2018; Mayr et al. 2020). However, the ecology, and more importantly, the role of these denitrifying methane oxidizers may play in lacustrine methane and nitrogen cycles still remain largely unknown.

In this study, we investigated methanotrophy in the anoxic waters of two main basins of Lake Lugano. Previous studies in this lake were mostly concerned with aerobic methane oxidation, and highlighted the prominent role of Type I methanotrophs near the redoxcline in the North Basin (Blees et al., 2014a), but also in the seasonal formation of a benthic nepheloid layer in the South Basin (Blees et al. 2014b). The data on the potential of AOM in the water column particularly of the North Basin remained ambiguous because potential methane oxidation rate maxima were found below the oxycline (Blees et al. 2014a). Here, we aimed at further elucidating the mode of methane oxidation (in particular the scope for true AOM), and more importantly, the environmental factors that control the occurrence, growth and activity of methanotrophs in the two contrasting lake basins. Lake Lugano is an excellent setting where to investigate the physico-chemical controls on AOM as a function of ecosystem dynamics, because the two hydrologically connected lake basins differ significantly in their mixing, water column-stability, and hence redox regimes (see below). We quantified methane oxidation rates in the water column of the two basins and used incubation experiments with concentrated lake-water biomass to determine the effectiveness of different electron acceptors (nitrate, nitrite and sulfate) for AOM. Moreover, using 16S rRNA amplicon sequencing, we identified the key taxa of the aerobic and anaerobic methanotrophic guilds, and demonstrate that *Ca.*

Methylomirabilis is an important microbial player in anaerobic oxidation of methane in the North Basin. DNA samples collected during previous studies at the same site allowed us to track the population dynamics of *Ca.* Methylomirabilis in the years following the exceptional mixing event in 2005 and 2006.

Materials and methods

Site Description and Sampling. Lake Lugano is located at the Swiss-Italian border and consists of two hydrodynamically contrasting basins that are separated from each other by a shallow sill. The eutrophic 95m-deep South Basin undergoes seasonal stratification with the development of a benthic "bacterial" nepheloid layer and anoxia during summer and fall (Lehmann et al. 2004). The 288m-deep north basin is permanently stratified, and a chemocline at about 130 m separates the oxic mixolimnion from the anoxic monimolimnion (Blees et al 2014a). The permanent stratification since the 1960's was interrupted by the exceptional mixing of the whole water column occured in 2005 and 2006 due to cold and windy winters, and led to the transient oxygenation of the monimolimnion (Holzner et al. 2009; Lehmann et al. 2015).

Water samples were collected in late November 2016, in the center of the southern basin off Figino (45°57′N, 8°54′E), and off Gandria in the northern basin (46°06′N, 9°12′E). Oxygen concentrations were measured using a conductivity, temperature and depth (CTD) probe (Idronaut Ocean Seven 316 Plus). Water samples from distinct depths were collected using 5L-Niskin bottles, and subsamples were taken directly from the Niskin bottle and filtered (0.45 μm) and/or processed as outlined below. Water samples for methane oxidation potential measurements were collected in 20 mL glass vials, which were filled carefully through the tubing, allowing water to overflow for about 2-3 volumes. The bottles were filled completely and care was taken not to introduce any air bubbles. The vials where crimp-sealed with Br-butyl rubber stoppers (Niemann et al. 2015). Samples for methane concentration measurements were collected in 120 mL serum bottles, crimp sealed with thick butyl rubber stoppers (Niemann et al. 2015) and a 20 mL headspace was created before fixing the sample by adding 5 mL of 20% NaOH.

Analytical Methods. Methane concentrations in the headspace of NaOH-fixed water samples were measured using a gas chromatograph (GC, Agilent 6890N) with a flame ionization detector and He as a carrier gas (Blees et al 2014b). Ammonium (NH_4^+) concentrations were determined colorimetrically using indophenol reaction, and nitrite (NO_2^-) using Griess reagent (Hansen and Koroleff (1999). NO_x (nitrate plus nitrite) was determined using a NO_x-Analyzer (Antek Model 745). Nitrate (NO_3^-) concentrations were calculated from the difference between NO_x and NO_2^-. Filtered samples for sulfide concentrations were stabilized immediately after sampling with zinc acetate and analyzed in the laboratory photometrically (Cline 1969). Sulfate was analyzed by ion chromatography (881 IC compact plus, Metrohm, Switzerland). Water samples for dissolved iron (Fe^{2+}) and manganese (Mn^{2+}) were fixed with HCl (6 M) after filtration through a 0.45 µm membrane filter, and total Fe and Mn concentrations (unfiltered) were measured using inductively coupled plasma optical emission spectrometry (ICP-OES). Concentrations of iron species were additionally determined photometrically using the ferrozine assay (Stookey 1970). Particulate iron was calculated from the difference between the total Fe^{2+} concentrations after reduction, and the dissolved Fe^{2+} in the filtered sample.

Methane oxidation rate measurements. In situ methane oxidation rates were determined with trace amounts of tritium-labeled methane (^3H-CH_4) (Steinle et al. 2015). Upon the retrieval of water samples, 5 µL anoxic ^3H-CH_4 solution (~1.8 kBq) was injected into the 20 mL bubble-free glass vials and samples were incubated in the dark at 4 °C for 42 h. To terminate incubations, 2 mL water samples were directly transferred into 6 mL scintillation vials, mixed with 2 mL of a scintillation cocktail (Ultima Gold, PerkinElmer) and immediately measured for total radioactivity of ^3H. 10 mL samples were then taken and transferred into 20 mL scintillation vials containing 1 mL saturated NaCl solution. After stripping the remaining radio-labeled methane from the vials for 30 min, samples were mixed with 8 mL of the scintillation cocktail prior to ^3H_2O radioactivity measurement via liquid scintillation counting (2200CA Tri-Carb Liquid Scintillation Analyzer). Methane oxidation rates (*MOR*) were calculated according to Eq. 1.

$$MOR = [CH_4] \times \frac{A_{H_2O}}{A_H} \times t^{-1} \qquad \text{(Eq. 1)}$$

Where A_H and A_{H_2O} represent the radioactivity of total ^3H and ^3H_2O from methane oxidation, respectively, [CH_4] is the methane concentration in the water column sample, and *t* the incubation time.

Incubation experiments with ^{14}C-labelled methane. We used microbial biomass from anoxic water layers of both basins to test different electron acceptors for their potential to stimulate anaerobic methane oxidation. Briefly, the biomass of a 500 mL water sample was collected on a glass fiber filter, which was then transferred to a 120 mL serum bottle containing 100 mL anoxic artificial lake water. The bottles were subsequently purged with nitrogen until the oxygen concentrations in the control bottles, equipped with trace oxygen sensor spots (TROXSP5, pyroscience), were below the detection limit (0.1 µM). Under an N_2-atmosphere in an anaerobic chamber, potential electron acceptors, i.e., nitrate, nitrite, and sulfate, were added from anoxic stock solutions to a final concentration of 4 mM, 4 mM, and 2 mM, respectively. Molybdate, a specific inhibitor of dissimilatory sulfate reduction, was added to some incubations (4 mM final concentration) to test for sulfate-dependent anaerobic methane oxidation. After these additions, the bottles were filled headspace-free with anoxic artificial medium, and closed with grey stoppers. The stoppers had been heated in boiled water, and were stored in a Schott bottle with Helium to remove dissolved oxygen in the elastomer. Finally, 10 µL of $^{14}CH_4$ tracer were injected, and samples were incubated at 25 °C. To exclude potential oxygen contamination during the long incubation time, the closed incubation bottles were kept permanently under N_2-atmosphere in an anaerobic chamber. Both live controls (without added electron acceptors) and base-killed controls (pH>13) were treated in the same way and incubated in parallel for the two basins. At the end of the incubation, 20 mL of headspace was created by exchanging the medium with N_2 gas. Biological activity was stopped by adding 5 mL saturated NaOH solution (50% w/w). The radioactivity of residual $^{14}CH_4$ (combusted to produce $^{14}CO_2$), $^{14}CO_2$ produced by methane oxidation, and radioactivity in the remaining samples was determined by liquid scintillation counting (e.g., Blees et al. 2014b). The first order rate constants (*k*) were calculated according to Eq. 2.

$$k = \frac{A_{CO_2} + A_R}{A_{CH_4} + A_{CO_2} + A_R} \times t^{-1} \qquad \text{(Eq. 2)}$$

Where A_{CH_4}, A_{CO_2}, and A_R represent the radioactivity of methane, carbon dioxide, and the remaining radioactivity, respectively. t represents the incubation time. Methane oxidation rates (MOR) were calculated using the value for k and the methane concentration at the start of the incubation (Eq. 3).

$$\text{MOR} = k \times [CH_4] \qquad \text{(Eq. 3)}$$

DNA extraction, PCR amplification, Illumina sequencing and data analysis. Water samples from different depths of the two basins were collected and sterile-filtered for biomass using 0.2 µm polycarbonate membrane filters (Cyclopore, Whatman). Biomass DNA was then extracted using FastDNA SPIN Kit (MP Biomedicals) following the manufacturer's instructions. A two-step PCR approach (Monchamp et al. 2016) was applied in order to prepare the library for Illumina sequencing at the Genomics Facility Basel (https://www.bsse.ethz.ch/genomicsbasel/service-support/illumina-sequencing.html). Briefly, 10 ng of extracted DNA were used and the PCR was performed using universal primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3') targeting the V4 and V5 regions of the 16S rRNA gene (Parada et al. 2016). The primers of the first PCR were composed of the target region and an Illumina Nextera XT specific adapter sequence. Four sets of forward and reverse primers, which contained 0-3 additional and ambiguous bases after adapter sequence, were used in order to introduce frame shifts to increase complexity (see Table S1 in supporting information). Sample indices and Illumina adaptors were added in a second PCR of 8 cycles. Purified, indexed amplicons were finally pooled at equimolar concentration, denatured, spiked with 10% PhiX, and sequenced on an Illumina MiSeq platform using the 2×300 bp paired-end protocol (V3-Kit). After sequencing, quality of the raw reads was checked using FastQC (v 1.2.11; Babraham Bioinformatics). FLASH (Magoč and Salzberg 2011) was used to merge forward and reverse reads into amplicons of about 374 bp length with an average merging rate of 96%, allowing a minimum overlap of 15 nucleotides and a mismatch density of 0.25. Full-length primer region was trimmed using USEARCH (v10.0.240), allowing a maximum of one mismatch. In a next step, the merged and primer-trimmed amplicons were quality-filtered (size range: 250-550, no ambiguous nucleotides, minimum average quality score of 20) using PRINSEQ (Schmieder and

Edwards 2011). OTU (operational taxonomic unit) clustering with a 97% identity threshold was performed using the UPARSE-OTU algorithm in USEARCH v10.0.240 (Edgar 2010, 2013). Taxonomic assignment of OTUs was done using SINTAX (Edgar, 2016) and the SILVA 16S rRNA reference database v128 (Quast et al. 2013). Downstream sequence analysis was done in R v3.5.1 using Phyloseq v1.25.2 (McMurdie and Holmes 2013) as detailed in the supporting information (see supplementary method). Phylogenetic analysis of specific partial 16S rRNA gene sequences was performed in Mega 7 (Kumar et al. 2016) using the neighbor-joining method (Tamura et al. 2004), and the robustness of tree topology was tested by bootstrap analysis (1000 replicates).

Quantitative PCR (qPCR). The abundance of *Ca.* Methylomirabilis was quantified using the primers qP1F (5′-GGGCTTGACATCCCACGAACCTG-3′) and qP1R (5′-CGCCTTCCTCCAGCTTGACGC-3′) amplifying positions 1001 to 1201 of 16S rRNA gene (Ettwig et al. 2009). qPCR reactions of all DNA samples were performed using the SensiFAST SYBR No-ROX Kit (Bioline) on a Mic (Magnetic Induction Cycler) real time PCR machine (Bio Molecular Systems, Inc). An initial denaturing step of 95 °C for 3 min was followed by 40 cycles of 5 s at 95 °C, 10 s at 65 °C, and 15 s at 72 °C. The specificity of the amplification was assessed by examining the melting curves from 60 °C to 95 °C, and by agarose gel electrophoresis. The calibration curves were generated using serial dilutions of pGEM-T Easy plasmid DNA (Promega, USA) carrying a single copy of the target gene fragment (qp1F/qp1R). Standard curves with these clones had a slope of -3.44, an R^2 of 0.994, and an amplification efficiency of 95%. The number of gene copies in plasmid DNA was calculated using the equation reported previously (Ritalahti et al. 2006).

Results and discussion

Hydrochemistry and methane oxidation in the water column of Lake Lugano.

The water column of the deep North Basin (NB) was permantly stratified during the sampling year 2016 and O_2 concentrations decreased with depth and fell below the detection limit (1 μM) at 95 m depth (Fig. 1A), as was defined as the redoxcline. Methane concentrations increased linearly from redoxcline to 20 μM at 155 m, at depths where nitrite was below the limit of detection (0.02 μM) and nitrate were in

the low micromolar range (< 1 µM, Fig. 1B). Below the redoxcline or in the anoxic water, concentrations of other reduced compounds such as sulfide, ammonium and dissolved Fe^{2+} rose above their background concentrations and increased continuously with water depth (Fig. S3). In the seasonally stratified South Basin (SB), the benthic nepheloid layer (NBL) stareted during summer and was fully developed in October. This turbid oxygen-depleted layer extends from the lake ground up to the chemocline, consists of microbial biomass, produced locally in large parts by methanotrophs (Blees et al. 2014b). During the sampling time in the southern basin, concentrations of O_2 decreased with depth towards the redoxcline at 63 m and methane concentrations increased remarkably to 28 µM in the bottom water (Fig. 2A). The anoxic hypolimnia below redoxcline were characterised by considerable amounts of nitrate (38-70 µM), with low conccentrations of nitrite (1.2-3.9 µM) (Fig. 2B). Similar to the northern basin, ammonium accumulated in anoxic water column and increased with depth. Particular Fe reached up to 9.1 µM close to surface sediment and Mn species were present below the redoxcline (Fig. S4). Sulfate was relatively abundant and remained mostly above 100 µM below the redoxclines of both basins.

To quantify methane oxidation in the water column of Lake Lugano, we performed incubation experiments with ^3H-CH_4 to determine in situ rates of methane oxidation across the redoxclines particularly in the anoxic waters of both basins. In the NB, methane oxidation was occurring across and mostly below the redoxcline with two peaks observed (Fig. 1C). The first peak of methane oxidation (0.06 ± 0.01 µmol L^{-1} d^{-1}) was at 100 m, right below the redoxcline, and aerobic methane oxidation was the most likely cause for this observed peak (Blees et al. 2014a). However, methane oxidation continued into the lower, anoxic parts of chemocline, and a secondary rate maximum of 0.08 ± 0.07 µmol L^{-1} d^{-1} was detected at 125 m. A similar bimodal pattern has also been observed before, albeit the two separate peaks and the oxycline were located at greater depths (Blees et al. 2014a). In contrast, we found a similar increase in methane oxidation rates across the redoxcline in the SB, with the highest rates of 0.18 ± 0.1 µmol L^{-1} d^{-1} observed at 70 m (Fig. 2C). Although Type I methane-oxidizing bacteria (MOB) were shown to dominate the biomass in the BNL of SB, where the highest methanotrophic activity was observed (Blees et al. 2014a), it remained unclear whether the observed activity was soley due to these aerobic methanotrophs. The presence of both nitrate/nitrite and sulfate in the BNL bears the

potential that methane could also be oxidized anaerobically with either of these oxidants.

Methane oxidation within oxic-anoxic transition zones of other meromictic lakes was often attributed to aerobic methanotrophs (Biderre-Petit et al. 2011; Oswald et al. 2016). In lakes with shallow redox transition zones (RTZs), cryptic oxygen production by phototrophs could sustain aerobic methane oxidation even in seemingly anoxic waters (Oswald et al. 2015; Milucka et al. 2015). At the depths of the RTZs in Lake Lugano, particularlay in the NB, O_2 production by phototrophs is an unlikely mechanism. Alternatively, Blees et al. (2014b) suggested that aerobic methane oxidizers can survive prolonged periods of O_2 starvation, and can resume high MOx activity upon episodic downwelling of O_2, for example during cooling events. Yet potential mechanisms that inject O_2 to the deep hypolimnion were not investigated and it remains speculative if and how deep such events occur. Thus, methane oxidation far below the redoxcline in Lake Lugano North Basin may indeed be anaerobic, and nitrate/nitrite and sulfate may serve as potential oxidants for anaerobic methane oxidation.

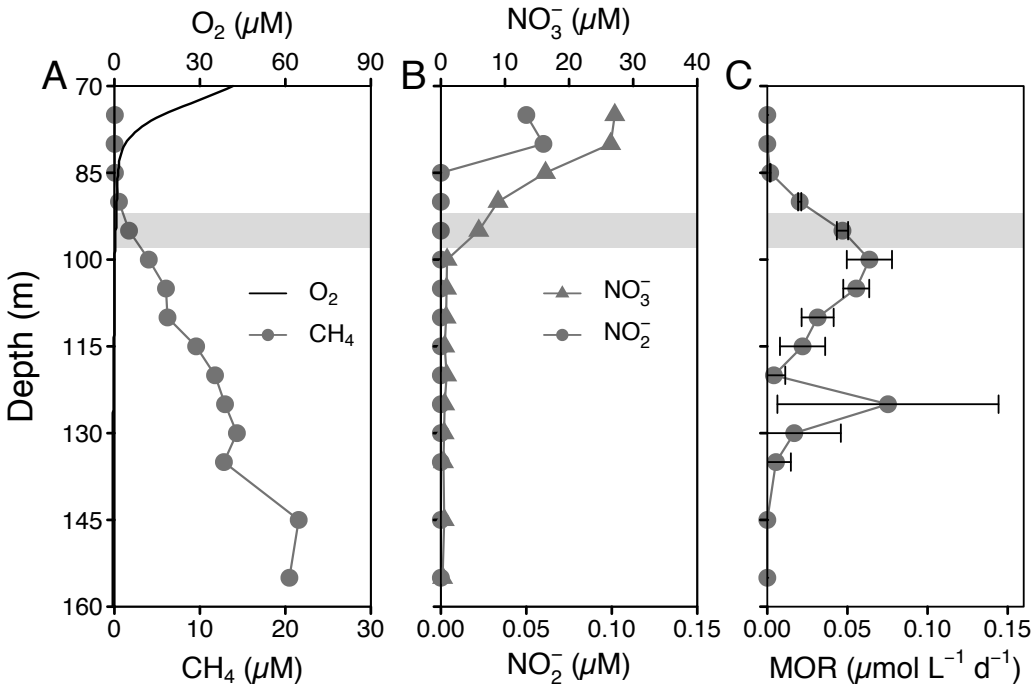

Figure 1. Water column profiles of (A) oxygen (O_2) and methane (CH_4) concentrations, (B) nitrate and nitrite concentrations and (C) methane oxidation rates (MOR) in the North Basin of Lake Lugano in November 2016. Redoxcline, defined as the depth where oxygen drops below detection limit (1 µM) is located at ~95 m and indicated with grey bar. Error bars of MOR represent standard deviation (n = 3).

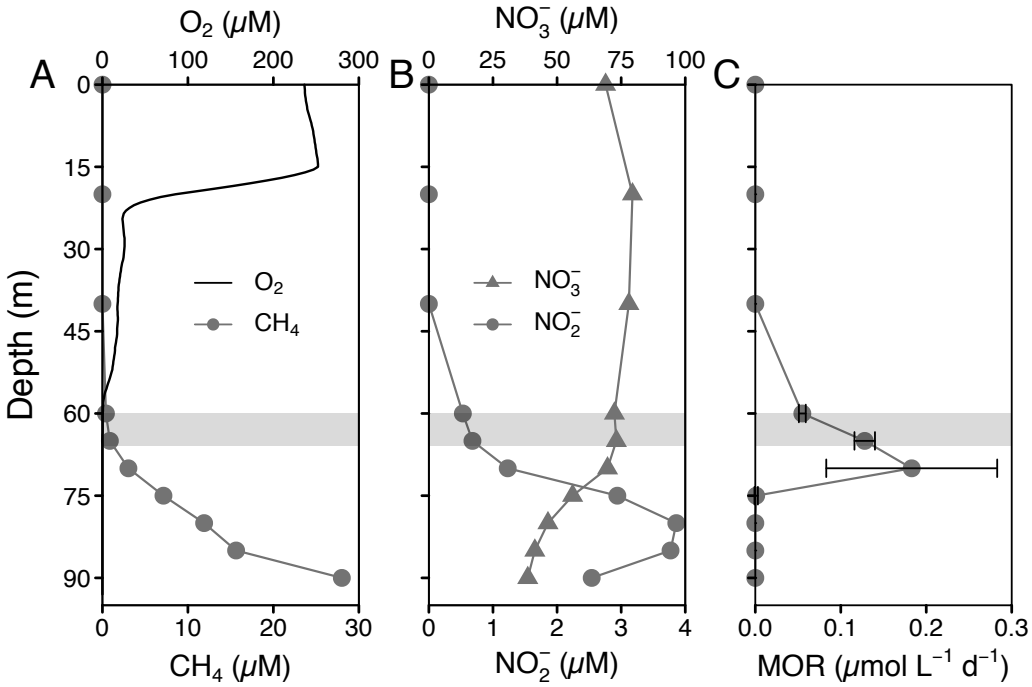

Figure 2. Water column profiles of (A) oxygen (O_2) and methane (CH_4) concentrations, (B) nitrate and nitrite concentrations and (C) methane oxidation rates (MOR) in the South Basin of Lake Lugano in November 2016. Redoxcline (located at ~63 m) is indicated with grey bar. Error bars of MOR represent standard deviation (n = 3).

Evidence for nitrate/nitrite-dependent AOM

To test for the presence of active anaerobic methanotrophs, and to indentify the potential oxidants for methane oxidation, we set up anoxic incubation experiments with $^{14}CH_4$ as substrate, different electron acceptors (i.e., nitrate, nitrite and sulfate), and concentrated biomass. The biomass was collected from 85-90 m in the South Basin, a depth well below the RTZ, but where nitrate, nitrite, and sulfate were present. Biomass from the North Basin was collected at 105-110 m, below the RTZ where nitrite was undetectable but nitrate (low) and sulfate were still available.

With biomass from water column right below the redoxcline of the meromictic NB, we found that both nitrate and nitrite stimulated AOM rates considerably (Fig. 3). Compared to the control experiments (i.e., no electron acceptor added), AOM rates increased by 52% (16 days, 54.8 ± 15.2 μmol L^{-1} d^{-1}) and 72% (32 days, 60.8 ± 5.9 μmol L^{-1} d^{-1}) in the presence of nitrate, and by 43% after 16 days (51.5 ± 6.3 μmol L^{-1} d^{-1}) and 44% after 32 days (50.4 ± 15.8 μmol L^{-1} d^{-1}) when nitrite was added. However, methane oxidation was not enhanced by the addition of sulfate compared to

controls and there were no significant differences between the controls and amendments with sulfate of both the 16-day and 32-day incubations (Table S3). In addition, no such stimulation occurred in the presence of molybdate and AOM rates were not significantly different between incubation bottles with sulfate and molybdate (Table S3). With respct to incubations with biomass from the anoxic water of the SB, no significant stimulation of methane oxidation was observed for all the treatments with added electron acceptors relative to the live controls after 16 days (Fig. 3 and Table S3). These results suggest that the oxidation of methane was not driven by any of the electron acceptors, and in turn, that AOM was likely not a major mode of methane removal in the South Basin, in spite of the presence of nitrate, nitrite and sulfate in the water column. Interestingly, after 32 days of incubation, the methane oxidation rates in all incubations were higher than after 16 days, independently of the added compounds, including molybdate, a known inhibitor of sulfate-reduction and thus of sulfate-dependent AOM (Wilson and Bandurski 1958).

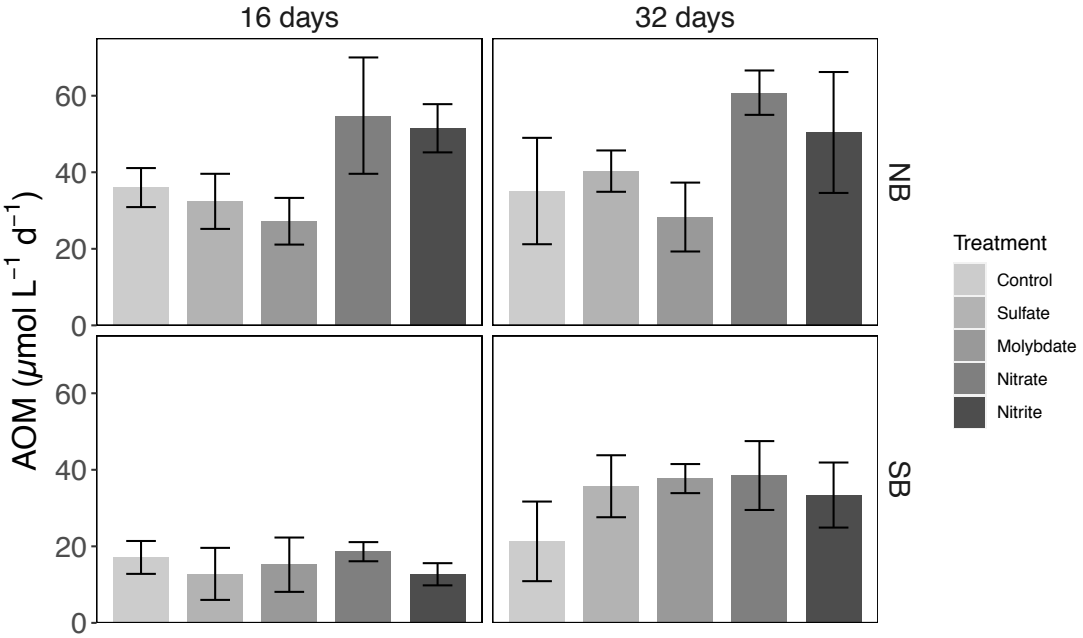

Figure 3. Effect of different electron acceptors on anaerobic methane oxidation (AOM) rates (n=3) in comparison to the control experiments (without addition of electron acceptors). The incubation was conducted with concentrated biomass collected in November 2016 from anoxic water layers in both NB and SB, and amended with $^{14}CH_4$ and different oxidants (nitrate, nitrite, sulfate and molybdate). In the killed controls (n = 3), no tracer conversion was observed after 32 days.

Puzzling at first was the observation that we measured significant AOM rates in all live controls (both NB and SB), in the absence of any additional electron acceptors.

Ambient concentrations of nitrate and nitrite were below the detection limit, but ambient sulfate was likely still present, which could explain the anaerobic oxidation of methane to some extent. However, the fact that no significant difference between sulfate- and molybdate-amended incubations was observed in all cases (Table S3) allows us to exclude any significant role of sulfate-dependent AOM in the live controls. One possiblity inferred from the incubation experiments particularly in the SB was that the methanotrophs in the concentrated biomass oxidized methane with the particulate Fe and/or Mn oxides (Beal et al. 2009; Ettwig et al. 2016; Cai et al. 2018), which were present in the water column and were concentrated on the filters together with the biomass (Fig. S3 and S4). In addition, the presence of traces of oxygen cannot be excluded. Greatest care was taken to avoid any oxygen contamination during preparation of the incubations and sampling. We did not, however, add a reducing agent to remove chemically any traces of oxygen. Thus, if trace amounts of O_2 (<100 nM) were still present in the incubations, they might be sufficient to serve as substrate for the enzyme methane monooxygenase. After the oxygen-dependent initial attack of methane, its further transformation could proceed anaerobically by fermentation, whereby hydrogen, formate acetate, and other compounds are produced (Kalyuzhnaya et al. 2013). Because short chain fatty acids are volatile under acidic conditions, radiolabeled formate and acetate could have been purged from the samples together with CO_2, and contribute to the measured methane oxidation rate. This fermentative conversion of methane to excreted organic compounds by gammaproteobacterial methanotrophs, could represent an important methane elimination pathway under severe oxygen limitation (Kalyuzhnaya et al. 2013) in both the nepheloid layer of the SB and water column below the RTZ of the NB of Lake Lugano.

Although the electron acceptors used for methane oxidation in all live controls remained to be explored, incubation experiments with biomass from the NB demonstrated that both nitrate and nitrite played a significant role and enhanced methane oxidation under anoxic condition, providing evidence for methane oxidation coupled to nitrate and/or nitrite reduction. Assuming that methane consumption in the live controls was an effect due to micro-aerobic methane oxidation or metal-dependent AOM, rates in the nitrite/nitrate amendments beyond the controls (net consumption) can be attributed to true NO_x-dependent AOM (N-AOM). Indeed, nitrate/nitrite-dependent AOM contributed 52-73% and 43% to the overall methane

consumption in nitrate/nitrite-added incubations with water from below the chemocline in Lake Lugano North Basin, hightlighting the potentially large role N-AOM plays in lacustrine methane cycling.

Abundance of diversity of methanotrophic bacteria

Based on 16S rRNA amplicon sequencing using an optimized PCR cycle (Supplementary method), a total of 4518 OTU's were obtained in the combined datasets of the South and the North Basin, with a total of 7247 OTU's before rarefaction (Weiss et al. 2017). Among them, we identified 32 OTU's of potential methanotrophic bacteria, 23 of which were related to gamma-proteobacterial (Type I) methanotrophs. One OTU was related to Methylomirabilia, the former NC10 class (Fig. S5). No evidence of typical anaerobic methane-oxidizing archaea such as *Ca.* Methanoperedens or representatives of the ANME-1, -2 or -3 groups were detected in any of the samples from the water column of both basins.

In the permanently stratified NB, we detected 16S rRNA gene sequences that were affiliated with *Ca.* Methylomirabilis, which was capable of mediating AOM with nitrite as terminal electron acceptor (Ettwig et al. 2010). With a relative sequence abundance of up to 6.7% at the redoxcline (95 m), this single OTU was equally abundant as all *Methylococcaceae* sequences combined, which accounted for ~5% of total sequences below the redoxcline (Fig. 4, Nov. 2016). Both *Ca.* Methylomirabilis and *Methylococcaceae* coexisted in the micro-oxic water column (95-105 m), where methane consumption rates were high. These observations together with incubation experiment from the NB suggested that both aerobic and anaerobic methanotrophs were important members of the methanotrophic guild and contributed together to the efficiency of the pelagic methane filter close to the redoxcline (Fig. 1). However, the factors facilitating the secondary peak of methane oxidation activity at 125 m (i.e., in anoxic water) remain uncertain. The lack of evidence for both sulfate or iron-utilizing anaerobic methanotrophs suggested that this peak was most probably attributed to *Ca.* Methylomirabilis (Fig. S6). Although the abundance of this denitrifying methane oxidizer did not vary in tandem with AOM rate at that depth (1.5% rel. abundance) and nitrite concentrations were below the detection limit throughout the water column, *Ca.* Methylomirabilis may still depend directly on the in-situ production of nitrite through microaerobic ammonium oxidation or nitrate reduction. Indeed, relatively high concentrations of ammonium were observed at these depths, and the

abundance of ammonium-oxidizing bacteria (AOB) was peaking at 130 m (0.42% relative abundance), closed to the depth where the secondary methane oxidation maximum was observed (Fig. S7B). O_2 was not detected at this depth, but these microorganisms could utilize minimal O_2 concentrations (at low nanomolar levels), which occurred at redoxcline boundaries or through sporadic O_2 intrusions oxygen and sustained aerobic ammonium oxidation (Thamdrup et al. 2012; Bristow et al. 2016). Likewise, ANAMMOX bacteria that use nitirte as electron acceptor (Strous et al. 1999), were also found to have the maximum abundance of 0.86% at this depth (Fig. S7D). This further corroborated the in-situ production of nitrite, which sustained the growth of the denitrifying anaerobic methanotroph. Thus, the virtual absence of nitrite was a reflection of the very efficient consuption rather than a lack of production, which implies that well below the chemocline of the NB, these anaerobic processes, at least for nitrite-dependent AOM and ANAMMOX were likely limited by the ammonium oxidation, and likely in turn by the oxygen flux supporting it.

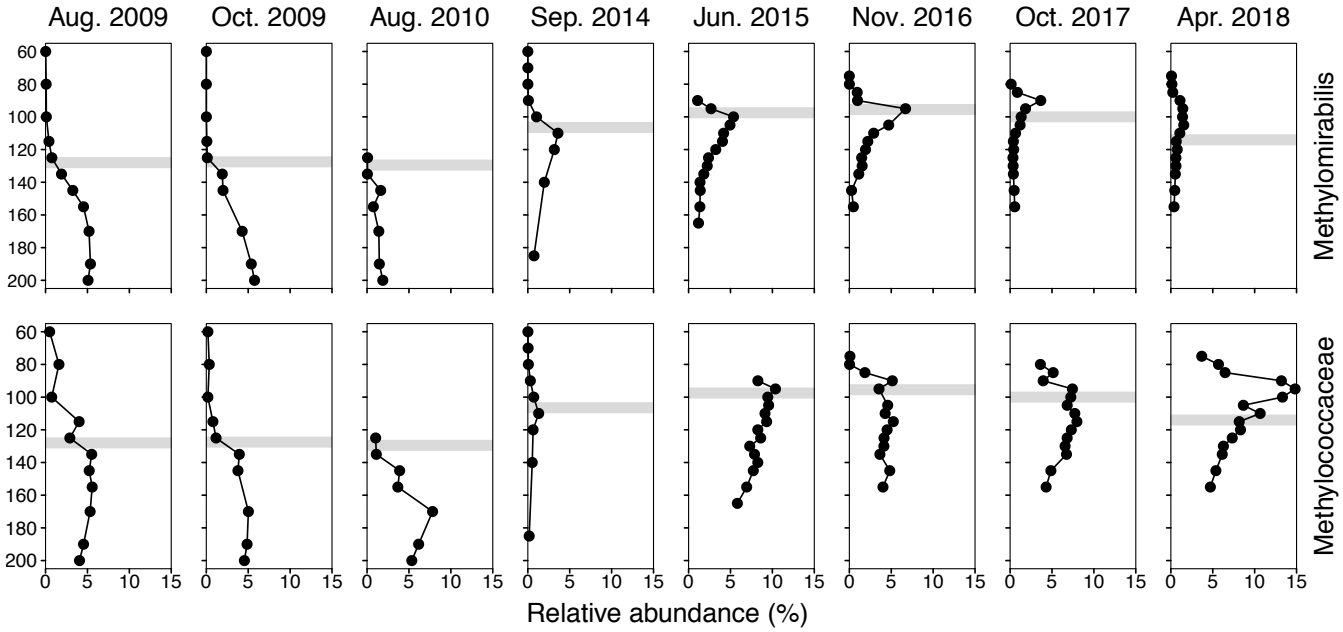

Figure 4. Abundance profiles of *Ca.* Methylomirabilis and *Methylococcaceae* and their dynamics in the water column of Lake Lugano North Basin with year after mixing events in 2005/2006. Data are based on relative read abundances of 16S rRNA gene sequences. Redoxclines are indicated with grey bars in different sampling dates.

By comparison, we were not able to detect any 16S rRNA gene sequences that belonged to *Ca.* Methylomirabilis in the water column of the seanonally stratified SB,

but only in the surface sediment with a very low relative abundance of 0.0014% of total sequences (Fig. 5C). The apparent absence of true anaerobic methane oxidizers was consistent with the lack of any significant stimulation of AOM with nitrate/nitrite in the incubations with biomass from this basin. However, Type I methane-oxidizing bacteria or *Methylococcaceae* were dominating the aerobic methanotrophic community, which was in accordance with the previous finding (Blees et al. 2014b). Among the members of *Methylococcaceae* (including *Methylobacter*, *Methylomonas*, *Methyloglobulus*, *Methylosarcina, Methyloparacoccus, Methylocaldum* and *Ca. Methylospira*), *Methylobacter* represented the most abundant genera, followed by *Crenothrix* (Fig. 5B), which has recently been shown to contribute to methane oxidation in two other stratified lakes in Switzerland (Oswald et al. 2017). Despite their occurrence in anoxic waters, these gamma-protebacterial methanotrophs are considered aerobic methanotrophs, as they use molecular oxygen for the particulate methane monooxygenase and the initial activation of methane. Nevertheless, genomes of several aerobic methanotrophs, including *Crenothrix*, encode putative nitrate (*narG*, *napA*), nitrite (*nirS*, *nirK*), and/or nitrogen oxide reductases (*norB*) (e.g. Kits et al. 2015; Oswald et al. 2017). *Methylomonas denitrificans*, for example, can couple the oxidation of methane (and methanol) to the reduction of nitrate to N_2O, under severe oxygen limitation (Kits et al. 2015). But oxidation of methane under completely anoxic conditions has neither been shown for *Crenothrix* nor any other of the gamma-proteobacterial methanotrophs to date. When comparing the diversity of *Methylococcaceae* in the water column of the two basins, six out of the total 23 OTU's were shared by both basins, 4 OTU's were observed in the North Basin only, and 13 in the South Basin only (Fig. S5). Thus, the methanotrophic guild was more diverse (19 OTUs) in the dynamic water column of the SB, where oxic and anoxic conditions alternate seasonally.

Taken together, the peak activity of methane oxidation at 70 m in the SB was essentially due to aerobic *Methylococcaceae* (22% rel. abundance) thriving under hypoxic conditions in the BNL. More importantly, in the NB of Lake Lugano, methane oxidation below the redoxcline was mediated by both type I MOB and nitrite-dependent *Ca.* Methylomirabilis. Furthermore, we propose that the observed methane oxidation in the seemingly anoxic water column was largely attributed to denitrifying methanotrophs using cryptic nitrite from "nano-aerobic" bacterial nitrification, but we could not exclude the cryptic aerobic methane oxidation.

Independent of the mode of methane oxidation (aerobic or anaerobic), O_2 appears as the ultimate and key limiting factor that controls methane oxidation directly (MOx) or indirectly (nitrite-dependent AOM) in the North Basin. Yet, it remains enigmatic as to why we see the accumulation of aerobic and nitrite-dependent methanotrophs, and even more intriguingly a methane turnover rate peak, at a very specific water depth well below the redoxcline (Fig. 1C).

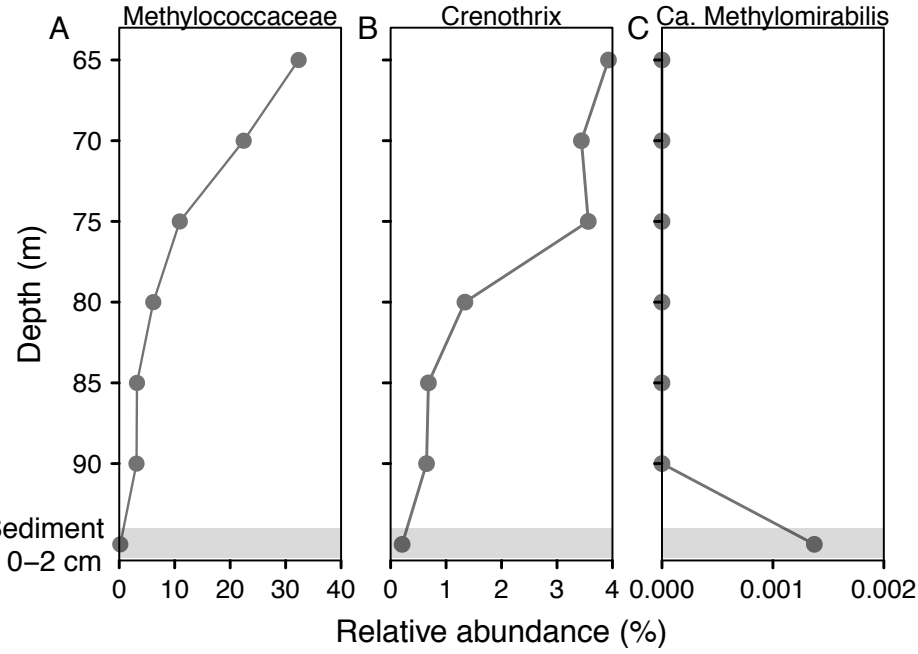

Figure 5. Relative abundance (% of total sequences) of (A) *Methylococcaceae*, (B) *Crenothrix* and (C) *Ca.* Methylomirabilis in the water column of Lake Lugano South Basin in November 2016. Surface sediment (0-2 cm) was taken in June 2017. Data are based on relative read abundances of 16S rRNA gene sequences.

Water column stability as an ecological factor fostering nitrite-dependent anaerobic methane oxidation

After ~40 years of permanent meromixis in Lake Lugano North Basin, two exceptionally strong mixing events in 2005 and 2006 led to complete oxygenation of the whole water column (Holzner et al. 2009; Lehmann et al. 2015). Thereafter, the water column re-stabilized rapidly again and remained stratified with anoxia below 125 m depth (e.g., Wenk et al. 2013). Interestingly, the abundance of *Ca.* Methylomirabilis was low around the redoxcline but increased with depth in 2009, reaching ~5% of total sequences at 200 m. The vertical mixing brought considerable amounts of O_2 to the deep water column and resulted in oxidation of ammonia, which

provided nitrite for the growth of *Ca.* Methylomirabilis. Once nitrite was used up and was not produced again, the abundance of this methanotrophic bacteria started to decreased and increased towards the redoxclines (Fig. 4). Notably, an upward migration of the redoxcline occurred after 2010 but the peak abundances of *Ca.* Methylomirabilis were consistently observed around the redoxclines at 110 m, 100 m and 95 m, respectively, representing up to 3.6% of total sequences in September 2014, 5.4 % in June 2015 and 6.7% in November 2016. The steadily increased relative abundances of *Ca.* Methylomirabilis based on read numbers were further confirmed by qPCR data (Fig. S8), demonstrating that water column stability was an important environmental factor for the growth of nitrite-dependent anaerobic methanotrophs. Further putative evidence is provided when comparing the two lake basins. The chemical conditions in the deep water column of the SB were conducive to nitrate/nitrite-dependent AOM (e.g., nitrate and nitrite concentrations up to 73 and 3.9 μmol/L, respectively, Fig. S2), but *Ca.* Methylomirabilis was not detected at all where nitrite-dependent AOM should be operative and only found in the anoxic surface sediment. These observations suggest that the seasonal mixing regime with a stratified anoxic period of ~5 months (shorter than our estimated doubling time of ~6 months, Fig. S9) did not support the development of stable populations of the slow-growing anaerobic methanotrophs and stable water column condition was a more critical factor than previously believed. In addition, ventilation of hypolimnetic waters in 2017 and 2018 resulted in a decline of the redoxcline and the strikingly decrease of the abundance of *Ca.* Methylomirabilis (Fig. 4). However, this oxygenation did not affect but appeared to stimulate the growth of *Methylococcaceae* above the redoxcline, indicating that they were most likely outcompeting the denitrify anaerobic methanotrophs that migh be intolerant of high O_2 concentrations.

Based on the existing time-series data demonstrating the increasing relative importance of *Ca.* Methylomirabilis in the North Basin, we speculate that the observed evolution reflects the slow dynamic recovery of the nitrite-dependent AOM community after the mixing events in 2005/2006. Episodic mixing and ventilation of hypolimnetic waters marks a significant ecological perturbation, which likely has detrimental effects particularly on this slow-growing anaerobic methanotrophic bacteria that require stable and low-redox environmental conditions (Luesken et al. 2012). Once quasi-permanent anoxia under stably stratified conditions was restored, the population *Ca.* Methylomirabilis seemed to grow back in the deeper hypolimnion,

and remained a permanent and important component of the water-column methane filter in the North Basin.

Conclusions

In this study, we have shown that nitrite-dependent AOM was an important methane sink in the permanently stratified North Basin of Lake Lugano and that this process was mediated by *Candidatus* Methylomirabilis below the redoxcline and in the anoxic water column. in the North Basin since then. Time series data demonstrate that stable and low redox conditions in the meromictic North Basin are particularly conducive to the development of AOM-performing bacterial populations since the mixing. In the more dynamic South Basin, the duration of seasonal stratification and anoxia is likely too short, relative to the slow growth rate of *Ca.* Methylomirabilis, to allow the establishement of anaerobic methanotrophs, in spite of the favorable hydrochemical conditions. Our research on methanotrophy in the two connected but hydrodynamically differing lake basins highlights that the chemical conditions alone cannot fully determine which microorganims will thrive and prevail in a system. Instead, physical processes such as water column dynamics/stability may be equally important, yet often neglected, factors determining microbial community structures, and with that the modes and activity of biogeochemical processes. Our findings link water column stability and nitrite-dependent AOM within the stratified lake and have important implications for both anaerobic processes and the prediction of future methane emission under the scenarios of future climate change.

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Supporting Information

Water column dynamics controls nitrite-dependent anaerobic oxidation of methane in stratified lakes

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Supplementary method

Library preparation

The 16S rRNA gene sequencing has become an increasingly important approach to help discover and understand the bacterial and archaeal world around them. However, different amplification conditions may result in biases during polymerase chain reaction (PCR), and thus have a severe effect on the accuracy of the sequence result. One of the most important parameters that could lead to biases is the PCR cycle number. The cycle numbers may vary upon the amount of DNA input and high cycle number could produce a sufficient yield of PCR product, but could also lead to biased amplification and reduce the accuracy of the replication of target DNA, particularly for the rare species in the environmental samples. Here, we tested the effect of two different cycle numbers (18 vs. 25 cycles) during the first PCR step using the primers in Table S1. 16S rRNA sequencing data were analyzed using R (v3.5.1) (R Core Team, 2014, http://www.r-project.org/). Rarefaction (using function "rarefy_even_depth"), alpha diversity estimates (Chao1, Shannon and InvSimpson) and principal coordinate analyses (PCoA) were done using the R package Phyloseq (v1.25.2) (McMurdie and Holmes 2013).

Rarefaction curves show that 25 cycles yielded better estimates of the species richness than 18 cycles, although, saturation was not reached with neither cycle number (Figure S1). Read numbers and singletons were presented using the raw data per sample. We performed an ANOVA in R using the function “aov” to test whether different alpha diversity meassures/indices (i.e., Observed, Chao1, Shannon and InvSimpson) were affected by either the sequencing depth or the amplification cycle number. Some indices (e.g., Shannon and InvSimpson) are significantly affacted by cycle numbers, while others (e.g., Observed richness and Chao1) depend on sampling depths (Table S2). In order to eliminate the effect of sequencing depth (sample size), samples were rarefied to the sample with the least reads to compare alpha diversity estimates (Chao1, Shannon and InvSimpson) of the two cycles, the numbers of operational taxonomical units (OTU) and zero-level operational taxonomical units (ZOTU) (Table S3) (Weiss et al. 2017). Principal coordinate analysis (PCoA) was performed after data were rarefied to the same depth. Although PCoA plots show that both 18 or 25 PCR amplification cycles yield similar microbial community structure (Figure S3), the 25 amplification cycle data were used for the microbial community analysis in this study and relative abundances of taxa were calculated using the function “transform_sample_counts” in the Phyloseq package. However, the sample “Ga_100” in the 25 cycles was excluded due to a sequencing failure (Table S3 and Figure S2),

Supplementary tables

Table S1. Design of the four different sets of forward and reverse primers used for library preparation. The primers contained 0-3 additional ambiguous bases (indicated in red) between the adapter sequence and the amplicon PCR primer 515F-Y/926R (Parada et al. 2016) to increase the nucleotide diversity and improve template generation during Illumina sequencing. Increasing the nucleotide diversity at this stage allowed reducing the amount of PhiX to 10%.

Primer		Sequence
515F-Y		GTGYCAGCMGCCGCGGTAA
926R		CCGYCAATTYMTTTRAGTTT
Forward primer sets	16S_Par0_fw	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GA-GTGYCAGCMGCCGCGGTAA-3'
	16S_Par1_fw	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-**N**-GA-GTGYCAGCMGCCGCGGTAA -3'
	16S_Par2_fw	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-**NN**-GA-GTGYCAGCMGCCGCGGTAA -3'
	16S_Par3_fw	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-**NNN**-GA-GTGYCAGCMGCCGCGGTAA -3'
Reverse primer sets	16S_Par0_rev	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-CA-CCGYCAATTYMTTTRAGTTT -3
	16S_Par1_rev	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-**N**-CA-CCGYCAATTYMTTTRAGTTT -3'
	16S_Par2_rev	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-**NN**-CA-CCGYCAATTYMTTTRAGTTT-3'
	16S_Par3_rev	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-**NNN**-CA-CCGYCAATTYMTTTRAGTTT-3'

Table S2. ANOVA test showing potential relationship between experimental factors (sequencing depth and PCR cycle number) and the alpha diversity measures (Observed, Chao1, Shannon and Inversed Simpson). A significant effect (p value *) of sequencing depth on Observed and Chao1 can be observed, as well as of cycle number on Shannon and InvSimpson.

Indices / Factors	Observed	Chao1	Shannon	InvSimpson
Sequencing depth	F=11.68, p=0.001 *	F=6.26, p=0.017 *	F=0.04, p=0.844	F=0.463, p=0.500
Cycle number	F=1.36, p=0.251	F=1.79, p=0.188	F=8.46, p=0.006 *	F=10.71, p=0.002 *

Table S3. Effect of PCR amplification cycle number on (Z)OTU numbers and alpha diversity measures. Overview of the Lake Lugano 16S rRNA gene sequence. Two libraries were generated, one with 18 and one with 25 PCR amplification in the first PCR, and sequenced together in the same Illumina sequencing run. Total reads and numbers of singletons (merged sequences that only occur once in the sample) are based on raw reads. Total numbers of OTUs (97% sequence similarity), ZOTUs (zero-radius OTUs, i.e. amplicon sequence variants) and some selected alpha diversity measures (Chao1, Shannon and InvSimpson) were determined after rarefying to even sequencing depths.

Cycles / Samples	Reads		Singletons		OTU number		ZOTU number		Alpha diversity					
									Chao1		Shannon		InvSimpson	
	18	25	18	25	18	25	18	25	18	25	18	25	18	25
Fi_65m	38657	98914	359	351	370	367	473	489	519	594	3.6	3.5	11.5	10
Fi_70m	34948	33400	328	343	357	401	461	538	539	563	3.5	4	9.6	16.5
Fi_75m	29039	30049	340	410	369	404	483	513	526	705	4	4.1	21.6	27.8
Fi_80m	27890	31947	383	479	407	442	530	589	617	739	4	4.2	18.6	23.3
Fi_85m	52758	38723	478	484	400	442	532	591	667	816	3.6	4	10.9	15.6
Fi_90m	56930	67764	580	724	470	538	630	733	834	838	4	4.3	16.6	23.9
Ga_75m	31370	34607	225	292	329	384	465	547	464	548	3.2	3.9	5.1	11.7
Ga_80m	39557	111195	250	303	330	336	459	486	447	483	3.1	3	4.9	4.5
Ga_85m	32943	106091	322	467	416	420	567	540	598	617	4.1	3.8	24	14
Ga_90m	31961	45567	345	406	435	464	566	571	670	707	3.9	4.1	15.3	19.6
Ga_95m	23937	44800	295	426	432	474	568	629	622	696	3.8	4	16.3	20.5
Ga_100m	15070	**7160**	289	**10**	382	**62**	489	**72**	525	**67**	3.4	**3.3**	7.4	**20.2**
Ga_105m	16946	56802	296	517	410	491	506	621	593	751	3.6	4	10.2	17.2
Ga_110m	16465	43192	334	535	472	515	596	656	688	846	3.8	4	12.8	16.6
Ga_115m	15078	40146	312	531	422	560	558	730	600	1044	3.7	3.9	9.5	12.3
Ga_120m	27477	62134	440	713	501	574	628	721	802	882	3.8	4.2	10.3	16.9
Ga_125m	37707	39503	570	627	582	658	747	796	1043	1076	4	4.3	11.7	17.8
Ga_130m	60645	35240	616	546	565	621	738	770	894	1036	3.9	4.3	9.7	16.9
Ga_135m	37500	36390	611	609	620	645	782	799	1016	986	3.9	4.3	9	17
Ga_145m	29141	38604	480	622	527	587	673	738	860	1001	3.5	3.8	6	9.1
Ga_155m	25232	40902	499	668	572	609	716	811	779	942	3.7	4	7.2	9.6
Total	681251	1043130	4518	5627	2284	2531	3267	3650	–	–	–	–	–	–

Table S4. Statistical differences of AOM rates (n=3) in incubations (both 16 and 32 days) of the two basins with different treatments in comparison to the corresponding live control or incubation with sulfate addition. Statistical differences were determined with a t-test and statistical parameter is indicated in parentheses (p-value) with the significance level α set at 0.05. **NS** indicates the difference between the two treatments is not significant.

		16 days		32 days	
Sites	Treatments	Difference to control	Difference to sulfate	Difference to control	Difference to sulfate
NB	Nitrate	**NS**	**> (0.023)**	**NS**	**> (0.014)**
	Nitrite	**NS**	**> (0.044)**	**> (0.049)**	**NS**
	Molybdate	**< (0.002)**	**NS**	**NS**	**NS**
	Sulfate	**NS**	–	**NS**	–
	Control	–	–	–	–
SB	Nitrate	**NS**	**NS**	**NS**	**NS**
	Nitrite	**NS**	**NS**	**NS**	**NS**
	Molybdate	**NS**	**NS**	**> (0.026)**	**NS**
	Sulfate	**NS**	–	**> (0.047)**	–
	Control	–	–	–	–

Supplementary figures

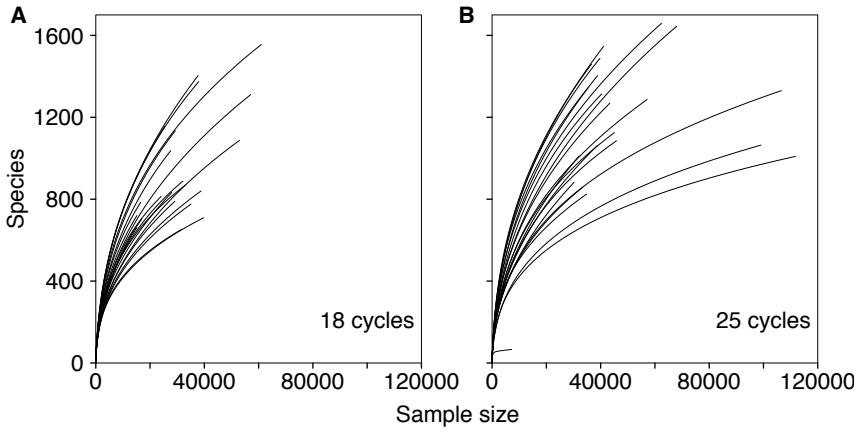

Figure S1. Rarefaction curves of samples from libraries generated with different PCR cycle numbers (A) 18 cycles and (B) 25 cycles. Each curve represents one sample, and shows the number of different OTUs ("Species") found in a given number of sampled sequences ("Sample size").

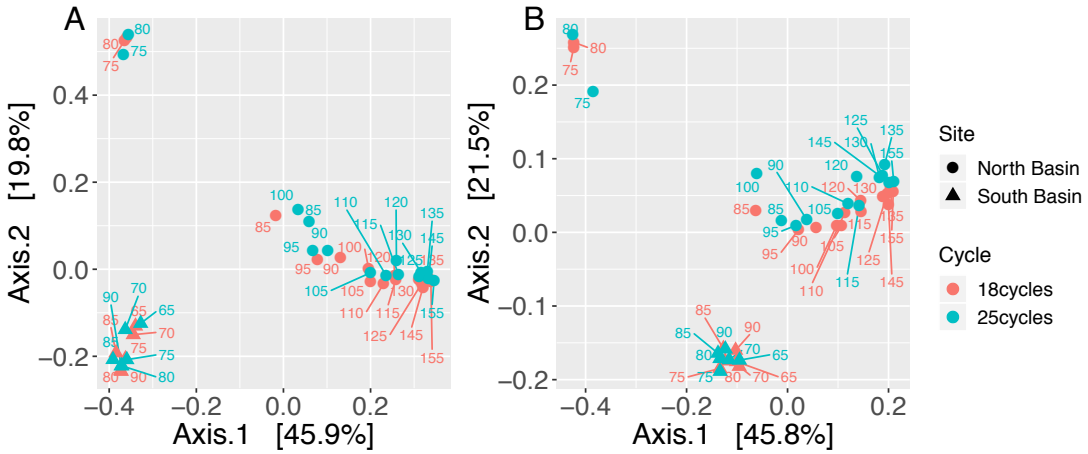

Figure S2. Principal coordinate analysis (PCoA) of microbial community structures in samples of the two basins (North Basin and South Basin) in Lake Lugano with two different PCR amplification cycle numbers (18 cycles and 25 cycles). Plots of PCoA on (A) Bray-Curtis and (B) weighted UniFrac distances both show that one of the samples (Ga_100 m of 25 cycles) is most likely affected by a PCR amplification artifact. However, both 18 and 25 cycles yield highly similar results and therefore we opted to use sequence data of 18 cycles in the manuscript. The x- and y-axes are indicated by the first and second coordinates, respectively, and the values in square brackets show the percentages of the community variation explained.

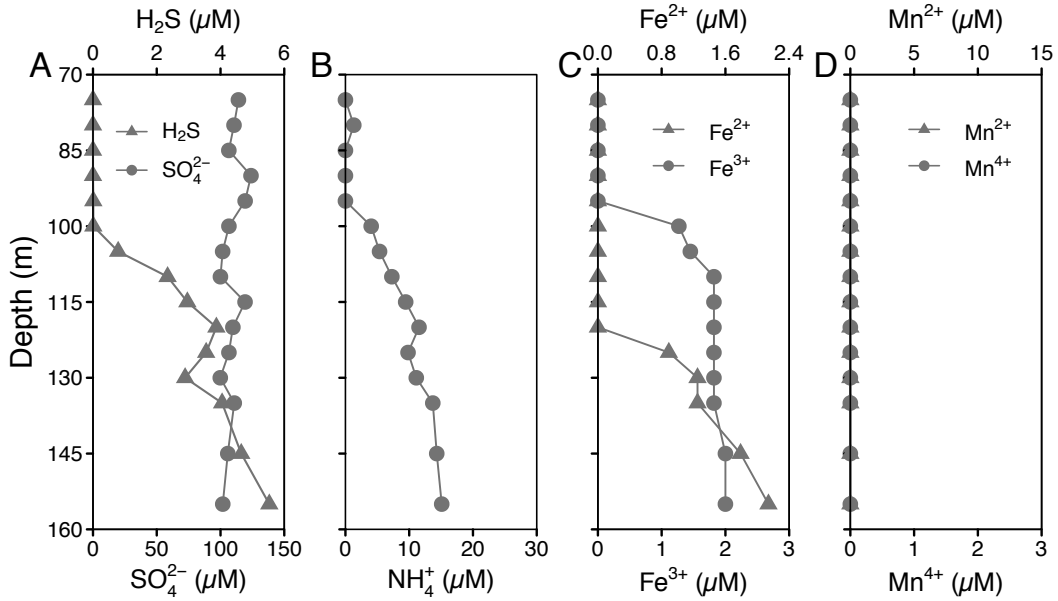

Figure S3. Concentration profiles of (A) sulfur species (sulfate and sulfide), (B) ammonium, (C) iron species and (D) manganese species in the water column of the permanently stratified North Basin of Lake Lugano in November 2016. Manganese species (both Mn^{2+} and Mn^{4+} were below detection limit throughout the investigated depths).

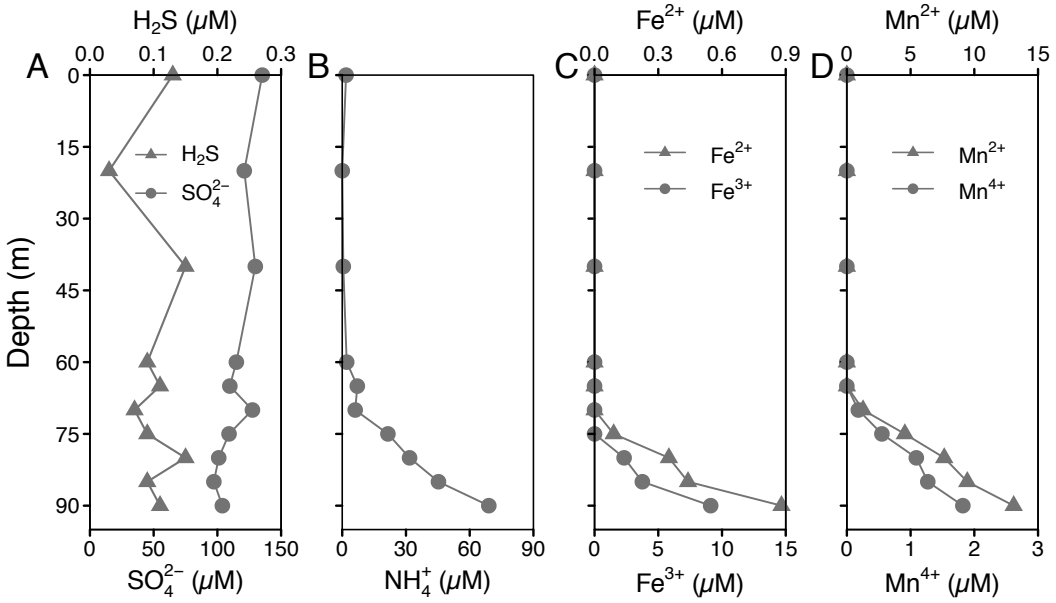

Figure S4. Concentration profiles of (A) sulfur species (sulfate and sulfide), (B) ammonium, (C) iron species and (D) manganese species in the water column of the seasonally stratified South Basin of Lake Lugano in November 2016.

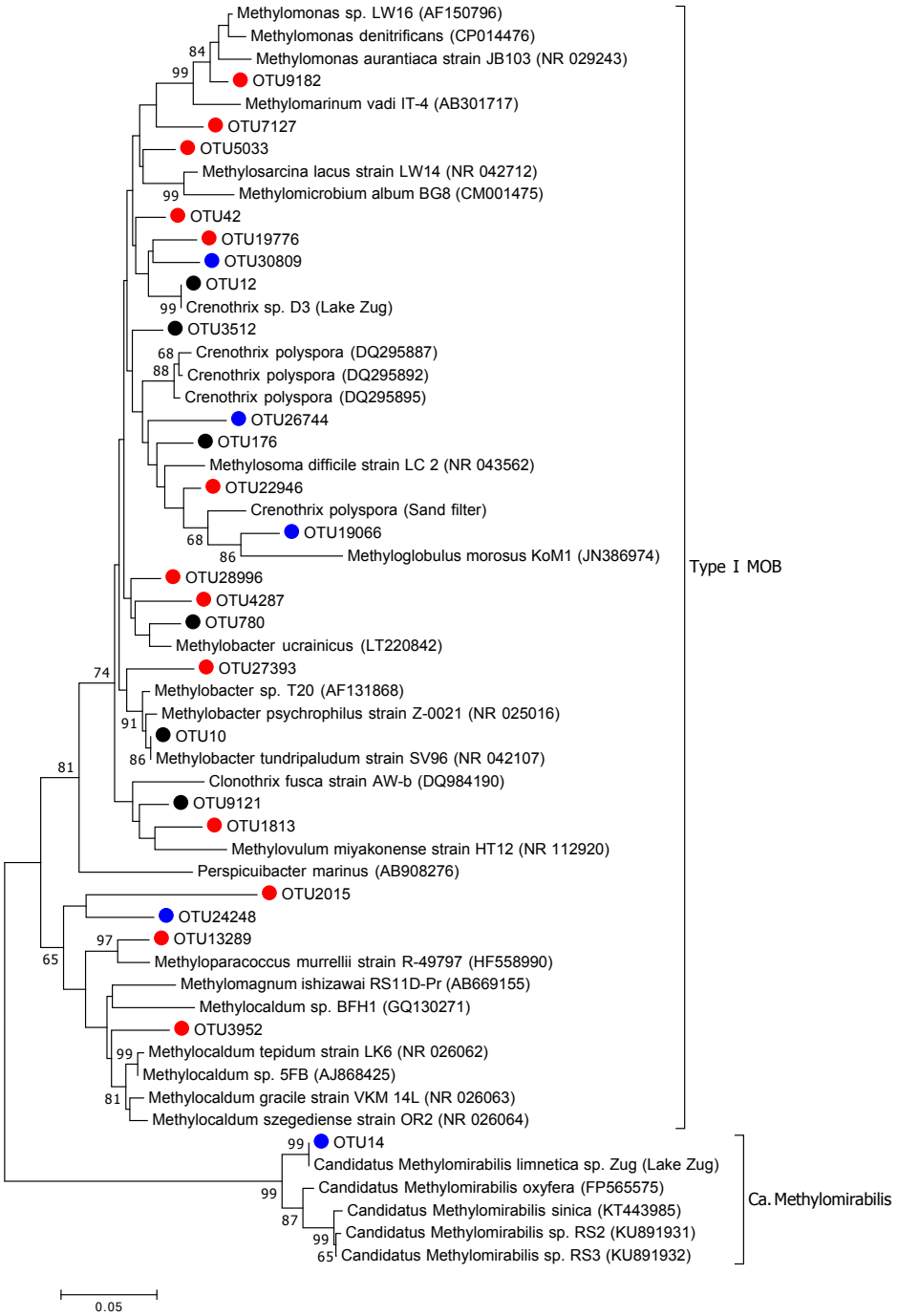

Figure S5. Neighbor-joining phylogenetic tree of 16S rRNA genes of both *Methylococcaceae* sp. and *Candidatus Methylomirabilis* detected only in the South Basin (red bullets), only in the North Basin (blue) and in both basins (black), where methane oxidation occurred. The tree was constructed using Maximum Composite Likelihood correction and complete gap deletion (Kumar et al. 2016). Bootstrap values (> 65%) based on 1000 resamplings are indicated at each node. Scale bar represents 5% of the sequence divergence.

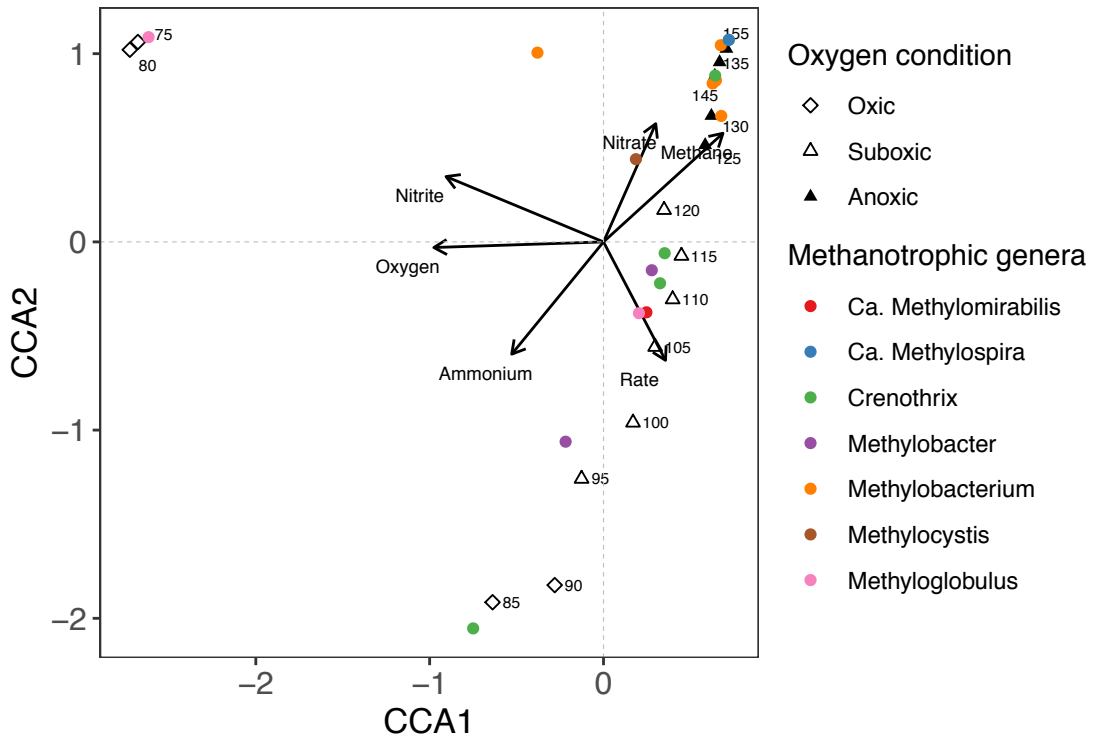

Figure S6. Canonical correspondence analysis (CCA) based on nutrient concentrations, rate measurements and potential methanotrophs detected in the North Basin of Lake Lugano. Triangles and diamonds represent samples in this basin under different oxygen conditions (with numbers indicating the water depths). Ordination was performed on the sequence data using Bray-Curtis distance. Taxa abundance and environmental variables (concentrations and rates) were Hellinger transformed prior to ordination. The CCA triplot shows that *Ca.* Methylomirabilis (red filled circle) is found in the suboxic water column of the permanently stratified North Basin (open triangles), and is positively correlated to the methane oxidation rate. Interestingly, the plot also shows that *Ca.* Methylomirabilis is anti-correlated to nitrite, suggesting that this taxon may be responsible for the depletion of the nitrite/nitrate pool in the habitat. Arrows represent solute concentrations, with arrowheads indicating their direction of increase.

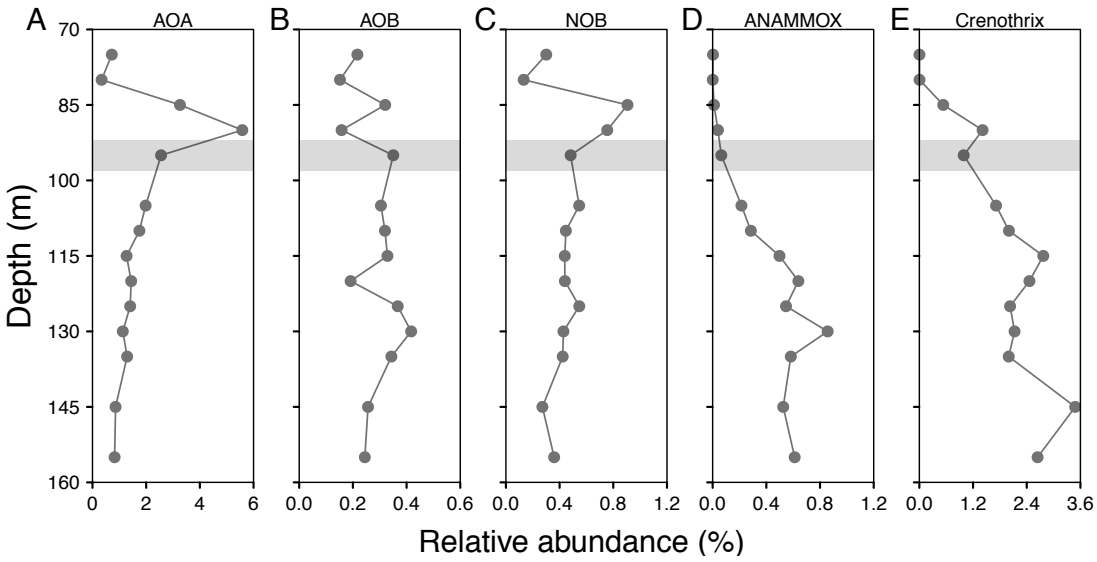

Figure S7. Abundance profiles of nitrogen-transforming microorganisms and *Crenothrix* across and below the redoxcline (indicated with grey) of the Lake Lugano North Basin in November 2016. (A) ammonium-oxidizing archaea (AOA, *Ca.* Nitrosopumilus and *Ca.* Nitrosoarchaeum), (B) ammonium-oxidizing bacteria (AOB, *Nitrosomonas* and *Nitrosospira*), (C) nitrite-oxidizing bacteria (NOB, *Nitrobacter* and *Nitrospira*), (D) ANAMMOX bacteria including *Ca.* Anammoximicrobium and *Ca.* Brocadia and (E) *Crenothrix*, potential methane oxidizers. Data are based on relative read abundances of 16S rRNA gene sequences.

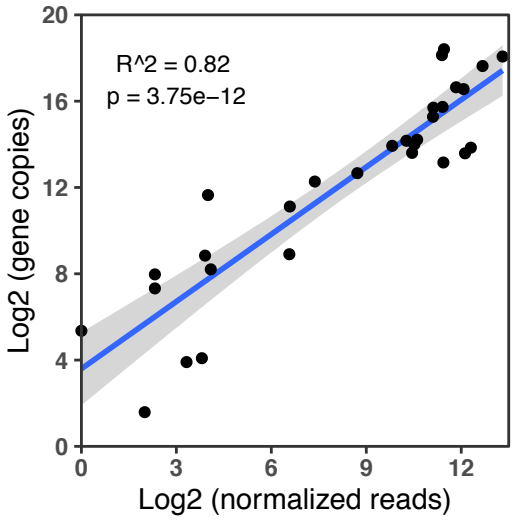

Figure S8. Relationship between 16S rRNA gene copy numbers and the normalized read numbers of 16S rRNA gene sequences of *Ca.* Methylomirabilis in the North Basin water column. Samples are from October 2010, September 2014 and November 2016.

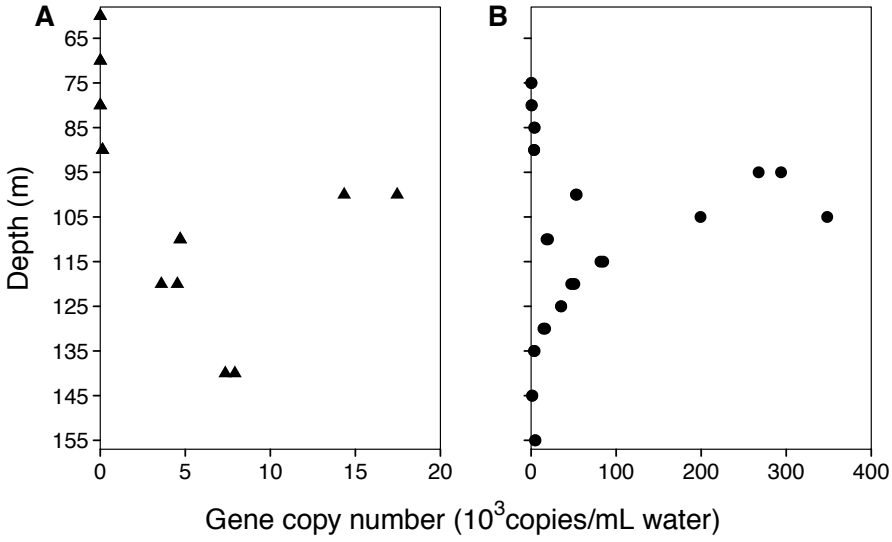

Figure S9. Depth profiles of 16S rRNA gene copy numbers of *Ca.* Methylomirabilis in the water column of the permanently stratified North Basin of Lake Lugano in (A) September 2014 and (B) November 2016. The redoxcline was located at 106 m in 2014, and at 95 m in 2016. Based on qPCR data (i.e., the local maximum gene copy numbers between the two years), the estimated apparent doubling time of *Ca.* Methylomirabilis was approximately 6 months, longer than the anoxic stratification period of ~ 5 months in the South Basin.

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Chapter 4: Manganese/iron-supported sulfate-dependent anaerobic oxidation of methane by archaea in lake sediments

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Abstract

Anaerobic oxidation of methane (AOM) by methanotrophic archaea is an important sink of this greenhouse gas in marine sediments. However, evidence for AOM in freshwater habitats is rare, and little is known about the pathways, electron acceptors and microbes involved. Here, we show that AOM occurs in anoxic sediments of a sulfate-rich lake in southern Switzerland (Lake Cadagno). Combined AOM-rate and 16S rRNA gene-sequencing data suggest that *Candidatus* Methanoperedens archaea are responsible for the observed methane oxidation. Members of the *Methanoperedenaceae* family were previously reported to conduct nitrate- or iron/manganese-dependent AOM. However, we demonstrate for the first time that the methanotrophic archaea do not necessarily rely upon these oxidants as terminal electron acceptors directly, but mainly perform canonical sulfate-dependent AOM, which under sulfate-starved conditions can be supported by metal (Mn, Fe) oxides through oxidation of reduced sulfur species to sulfate. The correspondence of high abundances of *Desulfobulbaceae* and *Candidatus* Methanoperedens at the same sediment depth confirm the interdependence of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria. The relatively high abundance and widespread distribution of *Candidatus* Methanoperedens in lake sediments highlight their potentially important role in mitigating methane emissions from terrestrial freshwater environments to the atmosphere, analogous to ANME-1, -2 and -3 in marine settings.

Introduction

A major fraction of the methane (CH_4) produced in lakes is oxidized by methanotrophic bacteria right at the redox transition zone within sediments or in the water column (Bastviken et al. 2002; Blees et al. 2014). Yet, more recent reports indicate that methane in lakes is also oxidized in the absence of oxygen (Schubert et al. 2011; Sivan et al. 2011; Deutzmann et al. 2014; Martinez-Cruz et al. 2018). Information on the controls on lacustrine methane oxidation in general, and on the alternative electron acceptors involved in anaerobic oxidation of methane (AOM) in particular, is important for understanding the regulation of the biological methane filter in lakes (Sivan et al. 2011; Norði et al. 2013; Deutzmann et al. 2014; Weber et al. 2017).

AOM coupled to sulfate reduction has been recognized as the most important

sink in marine environments, where sulfate concentrations are high (Reeburgh 2007; Knittel and Boetius 2009). This microbial process is primarily mediated by consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) (Boetius et al. 2000; Orphan et al. 2002; Niemann et al. 2006; Wegener et al. 2015). AOM, putatively coupled to sulfate reduction, has also been observed recently in freshwater ecosystems, for example wetlands (Segarra et al. 2015), iron-rich lake sediments (Norði et al. 2013; Weber et al. 2017), and ditch sediments (Timmers et al. 2016). However, in most lacustrine environments (e.g., in anoxic lake sediments), sulfate-dependent AOM is likely limited by relatively low sulfate concentrations.

Possible alternative terminal electron acceptors for AOM include nitrate and nitrite. Indeed, both nitrate- and nitrite-dependent AOM have recently been documented in laboratory enrichment cultures or freshwater systems (Raghoebarsing et al. 2006; Ettwig et al. 2010; Haroon et al. 2013; Deutzmann et al. 2014). Moreover, AOM coupled to the reduction of metal oxides (i.e., ferrihydrite and birnessite) has been demonstrated in anoxic marine sediments (Beal et al. 2009), a freshwater enrichment culture (Ettwig et al. 2016), and in lake sediments (Sivan et al. 2011; Norði et al. 2013). Despite the potential for iron- and manganese-coupled AOM as major methane sink in many Fe/Mn-rich sedimentary environments, the electron transport mechanisms that couple AOM with metal oxides (as well as sulfate) are still not fully understood (Milucka et al. 2012; Wegener et al. 2015; McGlynn et al. 2015). Moreover, it remains unclear whether microorganisms in environments where both metal oxides and sulfate are present (Egger et al. 2015; Weber et al. 2017) can independently mediate AOM using iron or manganese oxides (i.e., Fe(III)/Mn(IV)) as the terminal electron acceptors (Ettwig et al. 2016; Cai et al. 2018), or whether canonical sulfate-dependent AOM is indirectly stimulated by metal oxides that drive sulfide/sulfur oxidation via a cryptic sulfur cycle (Holmkvist et al. 2011a; Hansel et al. 2015).

Despite growing evidence that anaerobic methanotrophs play an important role in removing methane from lake ecosystems and reducing fluxes to the atmosphere, our knowledge about the microorganisms that perform AOM, particular within lake sediments, is still rudimentary. So far, only a few studies have identified freshwater AOM-mediating microorganisms (Ettwig et al. 2010, 2016; Schubert et al. 2011; Haroon et al. 2013; Weber et al. 2017; Graf et al. 2018; Versantvoort et al. 2018). In freshwater lake sediments, methanotrophic bacteria related to *Candidatus*

Methylomirabilis oxyfera have been reported to perform methane oxidation coupled to denitrification (Deutzmann et al. 2014), and archaea within the phylum Euryarchaeota possibly carried out sulfate- and/or iron-dependent AOM (Schubert et al. 2011; Weber et al. 2017). Although anaerobic methanotrophic archaea (e.g., ANME-2a) are potentially versatile with regards to the mode of AOM (Wang et al. 2014), the biogeochemical controls on possible metabolic adaptions in lacustrine environments are still poorly understood.

In the present study, we investigated methane oxidation in the anoxic sediments of Lake Cadagno. Using a complementary approach combining radio-tracer techniques ($^{14}CH_4$) for rate determination, incubation experiments with ^{13}C-labeled methane and different electron acceptors and stable isotope probing (SIP) of lipid biomarkers, as well as 16S rRNA gene sequencing, we aimed at 1) revealing the microbial processes and mechanisms involved in AOM with particular focus on the potential role of metal oxides in stimulating sulfate-dependent AOM, and 2) identifying the AOM-mediating microorganisms responsible for methane oxidation within the Lake Cadagno sediments. We demonstrate that methane oxidation is primarily coupled to sulfate reduction (even at sediment depths where sulfate is depleted), yet AOM cannot be attributed to the typical ANME lineages that were found to perform AOM with sulfate (Knittel and Boetius 2009), but is mediated by thus far uncultured archaea (Schubert et al. 2011), related to *Candidatus* Methanoperedens (formerly named ANME-2d or AAA).

Materials and Methods

Study Site. Lake Cadagno is an alpine meromictic lake located in the southern Alps of Switzerland (46°33′N, 8°43′E). The permanent chemocline in the water column of this lake separates the oxic mixolimnion from the anaerobic and sulfidic monimolimnion. Due to water infiltration from high-ionic strength subaquatic springs, Lake Cadagno displays relatively high concentrations of sulfate (>1 mmol/L).

Sampling. A total of six undisturbed sediment cores (inner diameter 62 mm) were recovered with a gravity corer from the deepest site (21 m water depth) in Lake Cadagno in October 2016, and subsampled in the home laboratory for geochemical analyses and AOM rate measurements. Samples for dissolved methane concentrations

were collected onsite with cut-off syringes through holes in one of the core tubes that were covered with tape during coring. 3 mL of sediment samples were fixed with 7.0 mL 10% NaOH in 20 mL glass vials, which were then immediately sealed with thick butyl rubber stoppers (Niemann et al. 2015). A second sediment core was sacrificed for the quantification of sulfur species, dissolved and particulate iron/manganese, as well as for DNA extraction. The sediment core was sectioned into 1 or 2 cm segments, and DNA samples were collected and stored frozen at -20 °C until further processing. Pore water was extracted by centrifuging the sediment samples under anoxic condition, and filtering the supernatant through 0.45 µm filters. Porewater samples (200 µL) for sulfide concentration measurements were fixed with Zn acetate (5% w/v) immediately after filtration. For the analysis of dissolved iron and manganese concentrations, 1 mL of the filtered sample was amended with 200 µL 6 M HCl. For the analysis of dissolved inorganic carbon (DIC), sulfate and nitrogen species concentrations, the remaining samples were stored at 4 °C, respectively.

Porewater and sediment geochemical analyses. Methane concentrations in the headspace of NaOH-fixed samples were measured using a gas chromatography (GC, Agilent 6890N) with a flame ionization detector, and helium as a carrier gas. The C isotopic composition ($^{13}C/^{12}C$) of methane from the headspace was determined using a pre-concentration unit (Precon, Finnigan) connected to an isotope ratio mass spectrometer (IRMS; Delta XL, Finnigan). Stable C-isotope values are reported in the conventional δ notation (in ‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB). $\delta^{13}CH_4$ values have an analytical error of ± 1‰. A total carbon analyzer (Shimadzu, Corp., Kyoto, Japan) was used to quantify dissolved inorganic carbon (DIC) concentrations in the porewater. Hereby, DIC was quantified as the difference between the total dissolved carbon concentration and the dissolved organic carbon concentration, which was analyzed separately after acidification of the sample with HCl. Porewater concentrations of ammonium, sulfate (detection limit of 2 µmol/L) and nitrate were analyzed by ion chromatography (Metrohm, Switzerland). Sulfide concentrations were determined spectrophotometrically using the Cline method (Cline 1969). Dissolved iron (Fe(II)), manganese (Mn(II)), and total manganese concentrations were measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Reactive Fe(III) (FeOx) in the solid phase was extracted with 0.5 M HCl and then reduced to Fe(II) with 1.5 M hydroxylamine. Concentrations

of Fe(II) were then determined photometrically using the ferrozine assay (Stookey 1970). Particulate reactive iron was calculated from the difference between the total Fe(II) concentrations after reduction, and the dissolved Fe(II) in the filtered sample.

Flux calculations. Diffusive fluxes J (in mmol/m^2/s) of methane and sulfate in the sediment porewater were calculated according to Fick's first law of diffusion, assuming steady-state conditions (Eq. 1):

$$J = -D_{sed}\frac{\partial C}{\partial X} \qquad \text{(Eq. 1)}$$

where D_{sed} is the molecular diffusion coefficient D_0 (in m^2/s) for methane and sulfate, respectively, corrected for sediment porosity (0.93) and the corresponding tortuosity. $\frac{\partial C}{\partial X}$ is the solute concentration gradient (in mmol/m^4), which was estimated based on the linear portions of concentration profiles within the investigated depth intervals. Molecular diffusion coefficients D_0 were adopted from Boudreau (1997), under consideration of the in situ temperature in Lake Cadagno sediments (9.48×10^{-11} and 5.08×10^{-10} m^2/s for methane and sulfate, respectively).

AOM Rate Measurements. A radiotracer $^{14}CH_4$ technique (Iversen and Jørgensen 1985) was chosen to obtain depth-specific ex-situ AOM rate profiles in the sediments. A 20 μL gas bubble of $^{14}CH_4/N_2$ (>2.5 kBq, American Radiolabeled Chemicals) was applied and directly injected to the whole core through pre-drilled side-holes at a depth interval of 2 cm (Su et al. 2019). Subsequent incubations were performed at in situ temperature (4 °C) in the dark. After incubation, the core was extruded and triplicate samples (~4 mL) were collected from 2-cm sediment slabs using 20-mL cut-off syringes, and transferred into vials with aqueous NaOH (5% wt:wt) to stop bacterial activity (e.g., Su et al. 2019). $^{14}CH_4$ activity was measured in the residual methane (as CO_2 after combustion), the CO_2 produced by AOM, and the remaining biomass via liquid scintillation counting (Blees et al. 2014; Steinle et al. 2016). AOM first order rate constants (k) were calculated according to Eq. 2.

$$k = \frac{A_{CO_2} + A_R}{A_{CH_4} + A_{CO_2} + A_R} \times t^{-1} \qquad \text{(Eq. 2)}$$

where A_{CH_4}, A_{CO_2} and A_R represent the radioactivity of methane, CO_2, and the remaining radioactivity. t represents the incubation time. Methane oxidation rates

(MOR) were then calculated using the value for k and the methane concentration at the start of the incubation (Eq. 3).

$$MOR = k \times [CH_4] \qquad \text{(Eq. 3)}$$

$^{13}CH_4$ Incubation Experiments. Sediment cores retrieved from the lake were stored at in situ temperature (4 °C) in the dark. Sediments from three sediment zones: 14-19 cm (where maximum AOM rates were observed; Fig. 1A), 19-24 cm and 24-29 cm (where sulfate concentrations were low and close to the detection limit) from four replicate cores were combined, each section with approximately 600 cm^3 of fresh sediment, and mixed with 2.5 L anoxic artificial mineral medium (Ettwig et al. 2009) to yield homogenized mixtures for the slurry incubation experiments (see Table S1 for components of medium). The slurries were degassed with He to remove any traces of O_2 and background methane. All slurries (from three different zones) were pre-incubated under anoxic condition for at least two weeks to allow the microbial community to recover from any potential perturbation during sample handling, and the supernatant was replaced with anoxic sulfate-free water (this step was repeated until sulfate concentrations were below detection limit) (Segarra et al. 2013). In a first set of experiments, we prepared incubations with sediments only from 14-19 cm and from 19-24 cm. For each sediment zone, a total of ten 240-mL serum vials were subsequently filled with ~ 200 mL of the homogeneous sediment slurry. All bottles except for the controls were amended either with nitrate, sulfate, amorphous manganese or iron oxide, with final concentrations of 4.8 mM, 2.4 mM, 10 mM and 10 mM, respectively (Table 1). Control experiments include live controls (slurries without additional electron acceptors), killed controls (autoclaved unamended slurries), and incubations with sulfate-reduction inhibition (with 20 mM molybdate). To further differentiate whether sulfate or manganese oxide served as direct electron acceptor, we performed a second set of experiments (Table 1). Here, we split the slurries from the first set of incubation experiments (Cadagno sediments 19-24 cm) at the end of incubation period (after 96 days), re-amended them with MnO_2 or sulfate, and in addition added 20 mM molybdate to some of them, respectively. Similarly, we divided the live control and added 0.5 mM nitrate to one of the splits. All incubation bottles were supplemented with 20 mL pure $^{13}CH_4$ (99.8 atom %, Campro Scientific) to the He headspace, and were incubated in the dark in an anoxic chamber with N_2

atmosphere at 25 °C. At different time points (0, 4, 8, 16, 32, 48, 64, 96 days), the sample was homogenized and 5 mL of the supernatant was collected and filtered with a 0.45 μm membrane filter for subsequent sulfate, dissolved iron/manganese, DIC concentration and stable carbon isotope ratio analyses. To determine the carbon isotope composition of DIC, 1 mL of water sample was transferred into a 12 mL He-purged exetainer (Labco Ltd) containing 200 μL zinc chloride (50 % w/v), and then acidified with ~100 μL concentrated H_3PO_4. The liberated CO_2 was analyzed in the headspace using a purge-and-trap purification system (Gas Bench) coupled to a GC-IRMS (Thermo Scientific, Delta V Advantage). Absolute ^{13}C-DIC abundances were determined from the DIC concentrations and the $^{13}C/^{12}C$ ratios converted from δ^{13}C-DIC values (Oswald et al. 2015). The temporal change in ^{13}C-DIC with incubation time was then used to calculate slurry-incubation-based potential methane oxidation rates, and to compare rates between the different treatments (Table 1).

Microbial Lipid Extraction and Sample Analysis. Lipids were extracted from incubation slurries and further treated according to previously described methods (Elvert et al. 2003; Niemann et al. 2005). Briefly, total lipid extracts (TLEs) were obtained by ultrasonication of the slurry samples in four steps with solvent mixtures of decreasing polarity: (1) dichloromethane (DCM):methanol (MeOH) 1:2; (2) DCM:MeOH 2:1; (3) and (4) DCM. TLEs were then saponified with methanolic KOH-solution (12%) at 80 °C for 3 h. After extracting the neutral fraction, fatty acids (FAs) were methylated using BF_3 in methanol, and analyzed later as FA methyl esters. The double-bond positions of monounsaturated fatty acids were determined by analyzing their dimethyl disulfide (DMDS) adducts (Nichols et al. 1986; Moss and Lambert-Fair 1989). Neutral compounds were further separated into hydrocarbon, ketone and alcohol fractions using silica glass cartridges, followed by derivatization of alcohol fractions into trimethylsilyl ethers prior to analysis. Individual lipid compounds were quantified and identified by gas chromatography with flame ionization detection (GC-FID) and GC-mass spectrometry (GC-MS, Thermo Scientific DSQ II Dual Stage Quadrupole), respectively. Compound-specific stable carbon isotope ratios were determined using a GC-isotope ratio mass spectrometer (GC-Isolink Delta V Advantage, Thermo Scientific). Both concentrations and δ^{13}C values of lipids were corrected for the introduction of carbon atoms during derivatization. Accuracy and reproducibility of lipid concentrations and δ^{13}C were

monitored by repeated analysis of internal standards (n-C19:0-FA, n-C19:0-OC, α-Cholestane and n-C36:0). Reported $\delta^{13}C$ values have an analytical error of ± 1‰.

DNA extraction, PCR amplification, Illumina sequencing and data analysis. DNA was extracted from both, samples of Lake Cadagno core sediments as well as from slurry sediments at the end of incubations, using a FastDNA SPIN Kit (MP Biomedicals) following the manufacturer's instructions. A two-step PCR approach was applied in order to prepare the library (cf. Illumina support document 16S Metagenomic Sequencing Library Preparation (15044223 B)) for sequencing at the Genomics Facility Basel (https://www.bsse.ethz.ch/genomicsbasel). Briefly, a first PCR (25 cycles) was performed using primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3') targeting the V4 and V5 regions of the 16S rRNA gene (Parada et al. 2016). Sample indices and Illumina adaptors were added in a second PCR of eight cycles. Purified indexed amplicons were finally pooled at equimolar concentration into one library and sequenced on an Illumina MiSeq platform using the 2×300 bp paired-end protocol (V3 kit). After sequencing, quality of the raw reads was checked using FastQC (v 0.11.8) (Andrews 2010). FLASH (Magoč and Salzberg 2011) was used to merge forward and reverse reads into amplicons of about 374 bp length, allowing a minimum overlap of 15 nucleotides and a mismatch density of 0.25. Quality filtering (min Q20, no Ns allowed) was carried out using PRINSEQ (Schmieder and Edwards 2011). Classical OTU (operational taxonomic unit) clustering with a 97 % cutoff was performed using the UPARSE-OTU algorithm in USEARCH v10.0.240 (Edgar 2013). Amplicon sequence variants were determined by denoising using the UNOISE algorithm (unoise3 command) and are herein referred to as ZOTU (zero-radius OTU). Taxonomic assignment was done using SINTAX v10.0.240_i86linux64 (Edgar 2016) and the SILVA 16S rRNA reference database v128 (Quast et al. 2013). Subsequent data analyses were carried out with Phyloseq (McMurdie and Holmes 2013) in the R environment (R Core Team, 2014) (http://www.r-project.org/).

Data availability. Raw reads are deposited at the NCBI Sequence Read Archive (SRA) in the BioProject PRJNA497531, and can be accessed under the accession numbers SRR8130729-SRR8130745. Additionally, amplified sequence variants of *Candidatus* Methanoperedens and *Desulfobulbaceae* (ZOTU307) used to construct

phylogenetic trees have been deposited in the GenBank, under the accession numbers MK087688-MK087694.

Results and Discussion

Anaerobic oxidation of methane in Lake Cadagno sediments. Methane oxidation has been investigated in Lake Cadagno sediments previously. Schubert et al. (2011) observed a strong ^{13}C enrichment within the porewater methane pool close to the sediment-water interface, and attributed the elevated $\delta^{13}CH_4$ values to the high C isotope fractionation associated with AOM. The apparent restriction of AOM hotspots to the uppermost 2-4 centimeters of the sediment column, where sulfate concentrations were up to 2 mM, led them to the conclusion that methane was most likely oxidized with sulfate as electron acceptor, and that AOM was constrained by the availability of sulfate in the sediment porewater. Here, we confirm the biogeochemical evidence for AOM in the sulfate-rich Lake Cadagno sediments by providing, for the first time, downcore AOM rate measurements for these lake sediments. Yet, our rate measurements clearly reveal that AOM is not restricted to the uppermost sediment layers and that AOM rates peaked at ~17 cm depth, with highest rates of 15 nmol/cm^3/d (i.e., two orders of magnitude higher than at the sediment surface) (Fig. 1A). The AOM rate maximum lines up nicely with a significant drop in CH_4. The inability to detect any ^{13}C-CH_4 isotope enrichment at this depth, however, may be attributed to the balancing effects of co-occurring oxidation and production of methane and/or to the microbially mediated carbon isotope equilibration between methane and carbon dioxide at low sulfate concentrations (which can even lead to ^{13}C-CH_4 depletion) (Yoshinaga et al. 2014). The maximum AOM rates observed are comparable to those reported for other freshwater environments (Norði et al. 2013; Segarra et al. 2013, 2015).

Interestingly, the highest AOM activity was observed within sediment layers where our measurements from a parallel core show that sulfate was still available, but at relatively low concentrations (~ 0.1 mmol/L) (Fig. 1B). While this can be taken as indication for AOM coupled to sulfate reduction (Reeburgh 2007), the vertical methane flux and downward diffusion of sulfate suggest an imbalance between the electron donor and its potential electron acceptor in the sediments (-110.5 and 190.9 μmol/m^2/d for methane and sulfate, respectively). Moreover, a clearly defined sulfate-

methane transition zone (SMTZ), as has been commonly described in most diffusive marine settings (Reeburgh 1980, 2007; Iversen and Jørgensen 1985, Jørgensen et al., 2001), was not observed at the depth of maximum AOM rates. In fact, relatively high concentrations of both methane and sulfate were found in the surface sediments, where AOM activity was very low, or not detected at all. Furthermore, anaerobic methane turnover continued well below the AOM rate maximum, at depths where sulfate was almost depleted (~ 0.04 mmol/L). These observations together indicate that AOM was not necessarily limited by the availability of free sulfate within the sediment, and potentially suggest other environmental controls on benthic AOM rates. For example, iron-dependent AOM was recently proposed in the methanogenic zone of lake sediments, well below the SMTZ (Bar-Or et al. 2017).

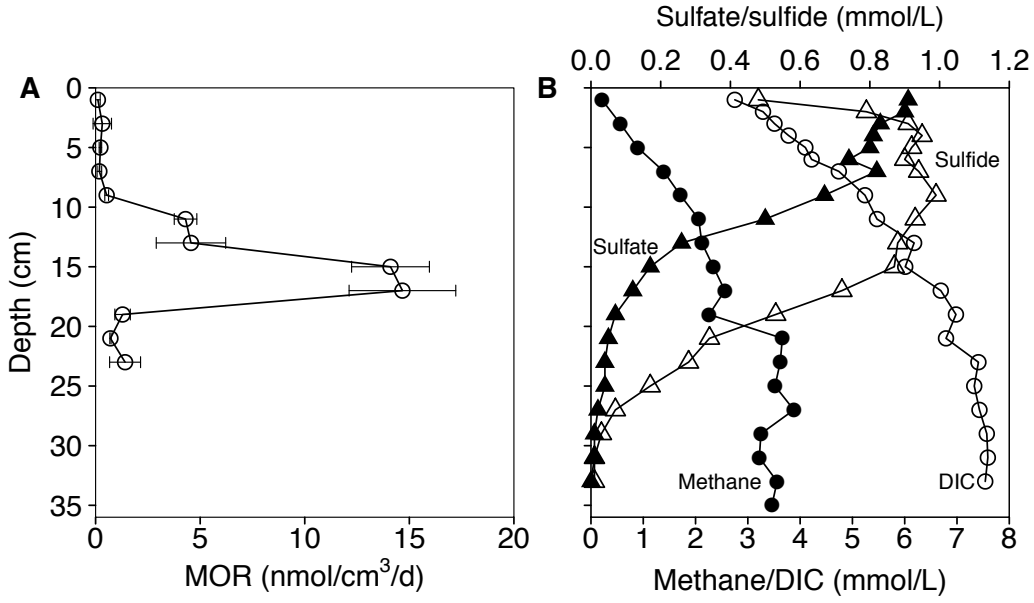

Figure 1. (A) Depth-specific in situ AOM rates determined by radioisotope-based approaches using whole-core incubations, (B) profiles of dissolved methane, DIC, sulfate and sulfide concentrations in the sediments of Lake Cadagno.

The combined geochemical and radiotracer-based rate data imply that AOM in the deeper Lake Cadagno sediments may not depend on free sulfate alone. Anaerobic oxidation of methane coupled to nitrate/nitrite reduction, which has recently been reported for other lake sediments (Deutzmann et al. 2014), seems implausible for Lake Cadagno, as nitrate and nitrite concentrations were below the detection limit

(<0.3 μmol/L), both in the euxinic water column and the sediment porewater. While we cannot fully exclude cryptic NOx production by the anaerobic oxidation of ammonium with oxidized metal species (Luther et al. 1997), NO_x as an important electron acceptor for AOM in the Lake Cadagno setting seems unlikely. Porewater profiles suggest that the reduction of iron and manganese at, and below, the AOM zone may be involved (Fig. S1B). Though fermentative/respiratory metal reduction by organotrophs could play a role too in the organic-rich (~15% organic carbon) sediments (Schubert et al. 2011), AOM may be coupled to iron or manganese reduction, as has been suggested for other lakes (Crowe et al. 2011; Sivan et al. 2011; Norði et al. 2013) or methane-seep marine sediments (Beal et al. 2009; Sivan et al. 2014).

Modes of sedimentary AOM. To further investigate the pathway of AOM in Lake Cadagno sediments, we performed slurry incubation experiments using sediments from three sediment depth segments (Table 1). Segments were chosen based on the methane oxidation rate profiles and the corresponding concentrations of potential electron acceptors (as determined in a separate core). They cover the zone of maximum AOM activity (14-19 cm and 19-24 cm) and the zone below (24-29 cm), where AOM rates were still significant, sulfate concentrations very low, and metal oxide concentrations relatively high (Fig. S1). Moreover, all three segments show the presence of *Candidatus* Methanoperedens, a proven microbial player in AOM (see below). In the first set of experiments (sediments from 14-19 cm and 19-24 cm), we detected only a slight methane turnover in killed controls, which must have been abiotic. In the un-amended live controls (i.e., slurries without additional electron acceptors) and in incubations with 20 mM molybdate, a competitive inhibitor for microbial sulfate reduction (Wilson and Bandurski 1958), AOM was slightly elevated relative to killed controls at both depths, as indicated by the small amount of excess $^{13}CO_2$ that was produced at the end of the incubation period. The low-level AOM might be attributed to the ambient substrates remaining in the slurries after their preparation and conditioning (e.g., sulfate). Most strikingly, at both sediment depths, methane oxidation was considerably enhanced upon the addition of either sulfate or MnO_2 (Fig. 2A and 2B). Excess $^{13}CO_2$ production (i.e. methane turnover) was immediately detectable in incubations with added sulfate, whereas a delay of approximately two weeks was observed in our MnO_2-amended experiments. Though

mostly in the incubations with sediments from the 19-24 cm, FeOx-supplemented slurries, similar to the MnO_2 amendments, also showed a stimulation of AOM with a ~2 week delay.

Table 1. $^{13}CH_4$ incubation experiments with slurries from different sediment depths in Lake Cadagno.

Cadagno sediment slurries Sampling interval*	Treatment		Number of incubations
14-19 cm or 19-24 cm (first set of experiments)	Killed control	Autoclaved	1
	Live control	-	2
	FeOx†	+10 mM	2
	MnO_2†	+10 mM	2
	Sulfate	+2.4 mM	2
	Nitrate	+4.8 mM	1
	Molybdate	+20 mM	1
24-29 cm (second set of experiments)	Killed control	Autoclaved	1
	MnO_2	+10 mM	1
	MnO_2 + Molybdate	+10 mM + 20 mM	1
	Sulfate	+4.8 mM	1
	Sulfate + Molybdate	+4.8 mM + 20 mM	1
19-24 cm (second set of experiments)	Live control	-	1
	Nitrate	+0.5 mM	1
	MnO_2	+10 mM	1
	MnO_2 + Molybdate	+10 mM + 20 mM	1
	Sulfate	+4.8 mM	1
	Sulfate + Molybdate	+4.8 mM + 20 mM	1

*Experiments were performed in two subsequent sets (see also Fig. 2). In the second set of experiments, based on results from the first set, the effect of sulfate-reduction inhibition by molybdate was specifically tested (see text). For the incubations with 19-24 cm sediments, we used the same material as in the first set of incubation experiments; i.e., slurries were split at the end of the first incubation (after 96 days) and re-amended with nitrate, MnO_2, sulfate and molybdate, respectively. See Supplementary Information for details on how FeOx and MnO_2 assays were prepared.

To further differentiate whether sulfate or manganese oxide served as direct electron acceptor, we performed a second set of experiments, in which we (re-)amended sediments from 19-24 cm from the first set of experiments, as well as

"fresh" sediments from 24-29 cm, with sulfate/MnO_2/nitrate and/or molybdate (Table 1). This way, we wanted to test whether 1.) AOM was solely and directly driven by sulfate reduction (sulfate-dependent AOM), 2.) Mn(IV) reduction was directly coupled to AOM, or 3.) whether the added MnO_2 fueled a cryptic sulfur or nitrogen cycle, in which alternative electron acceptors (i.e., sulfur intermediates, sulfate or nitrate) were produced through the oxidation of sulfide or ammonium, respectively (Luther et al. 1997; Zopfi et al. 2004; Holmkvist et al. 2011a). During the first 48 days of incubation, no AOM activity was detected in the live control or in incubations with added molybdate. Also thereafter, $^{13}CH_4$ oxidation was insignificant in these incubations. In contrast, AOM rates in the nitrate-amended treatment (during the second half of the incubation experiment) were higher compared to the untreated live control, suggesting that, at least under sulfate-depleted conditions, AOM can be coupled to denitrification, or that AOM is stimulated by nitrate indirectly. Most obviously, and consistent with the first set of experiments, both sulfate and MnO_2 boosted $^{13}CO_2$ production by AOM compared to the control and the molybdate-addition experiments (Fig. 2C and 2D).

Molybdate has been shown to have no effect on anaerobic methanotrophs or manganese-reducing microorganisms (Oremland and Taylor 1978; Nealson and Saffarini 1994). In the case of true manganese-dependent AOM, we would have expected $^{13}CO_2$ production independent of any molybdate amendment. Yet, because AOM was not promoted in incubations with MnO_2 plus molybdate, in contrast to the MnO_2-only treatments, we suggest that MnO_2 was not directly used as electron acceptor for AOM. In the presence of MnO_2, sulfate can be continuously produced by the chemical oxidation of dissolved sulfide or particulate FeS/FeS_2 (Yao and Millero 1996; Schippers and Jørgensen 2001), thus fostering sulfate-dependent AOM. Indeed, we observed a net increase in the sulfate concentrations during the incubations with MnO_2, providing substrate for sulfate-dependent AOM (Fig. S2). In the molybdate-amended experiments, we did not measure sulfate concentrations but a similar increase in sulfate concentration can be assumed. Again, the sulfate was produced by the oxidation of reduced sulfur species with MnO_2, however, AOM was now inhibited by molybdate at the sulfate-reduction step. In the second set of experiments, the enhancing effect of the MnO_2 addition was approximately three times greater than when sulfate was added to sediments from 19-24 cm (Fig. 2C), while the opposite was observed for sediments from 24-29cm. At this point, we lack an obvious

explanation for the observed discrepancy between the MnO_2 and sulfate-only experiments other than that our observation may leave some scope for true metal-driven AOM in the shallower sediments.

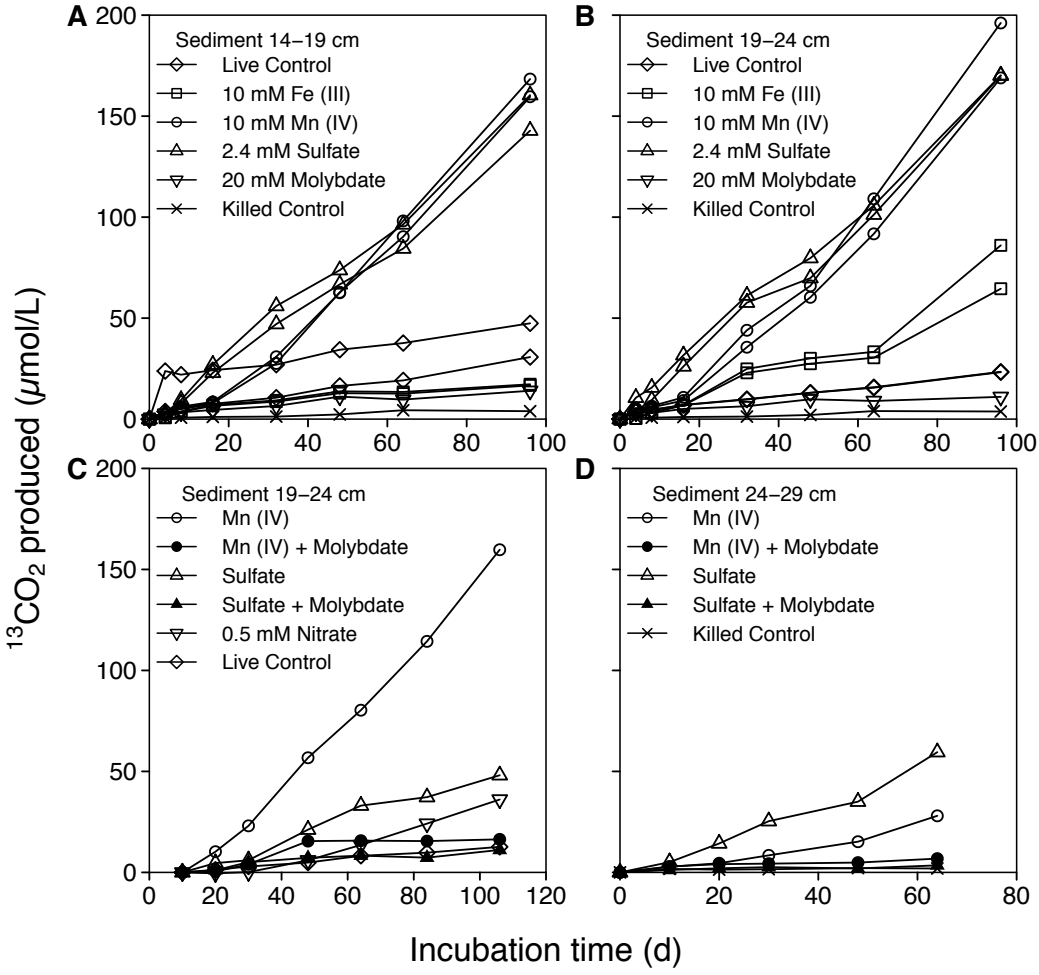

Figure 2. Concentrations of produced $^{13}CO_2$ in incubation experiments with ^{13}C labeled methane. First set of experiments (A-B): slurry incubations with Cadagno sediments from the depths of (A) 14-19 cm and (B) 19-24 cm, amended with different potential electron acceptors (iron, manganese and sulfate) and sulfate-reduction inhibitor (molybdate). Second set of experiments (C-D): molybdate inhibition tests in incubations with sulfate and MnO_2 using sediments from 19-24 cm (C) and 24-29 cm (D), respectively. The effect of nitrate was only tested in slurries from 19-24 cm. In killed-control incubations, autoclaved slurries were used. In live controls no electron acceptors were added.

Increasing sulfate concentrations in the incubations with nitrate (Fig. S2) suggests that nitrate might have had a similar effect (i.e., the oxidation of reduced sulfur with nitrate was promoted), but it seemed to stimulate AOM much less than MnO_2. We conclude that nitrate can, at least in our experiments, like MnO_2, serve as oxidant for the oxidation of reduced sulfur, producing sulfate that is then available for sulfate-dependent AOM. It is difficult, to explain the weaker effect of nitrate on AOM (i.e., less stimulation compared to the MnO_2 amendment), if AOM was strictly coupled to sulfate only. More precisely, both the nitrate and MnO_2 addition resulted in the production of almost equivalent concentrations of sulfate (Fig. S2). This may be taken as additional evidence that MnO_2 not only affects AOM indirectly through its role in generating sulfate, but also directly by serving as oxidant for true Mn-dependent AOM.

With regards to the effect of FeOx, we expected a stimulation of AOM analogous to that by MnO_2. However, the overall AOM activity was lower than in the sulfate and MnO_2 treatments (Fig. 2A and 2B). The weaker $^{13}CO_2$ production in the FeOx versus the MnO_2 treatments is best explained by the fact that sulfate is not a major product of the reaction of FeOx with sulfide (Yao and Millero 1996; Zopfi et al. 2004).

Our incubation results clearly demonstrate that sulfate, added MnOx and FeOx (and potentially nitrate) promoted AOM in the anoxic sediments of Lake Cadagno. In all instances, sulfate appears to be the key electron acceptor used by microorganisms to oxidize methane. We are aware of the limitations with regards to the applicability of high-concentration experimental results to natural environments, and we acknowledge that our combined incubation data leave some scope for true metal-dependent AOM. Yet, we propose that canonical sulfate-dependent AOM is the dominant methane oxidation pathway in the studied sediments. In the upper AOM zone (14-19 cm), where sulfate is replete, AOM is directly coupled to sulfate reduction. In the lower parts of the sediment column (19-29 cm), where free sulfate concentrations are low, sulfate-driven AOM still happens, and is likely maintained by the continuous supply of sulfate produced by the oxidation of reduced sulfur species with metal-oxide phases buried in the sediment (Holmkvist et al. 2011b; Weber et al. 2016).

Lipid biomarker constraints on methane oxidizing microbes.

At the end of the slurry incubation period, we did not find any ^{13}C-labelled lipids typical for the known archaeal methanotrophs, for example, archaeol and hydroxyarchaeol, or phytane and biphytane (e.g. Niemann and Elvert 2008). We cannot completely rule out that undetectable ^{13}C-label incorporation into archaeal lipids during the AOM-incubation experiments may simply be explained by the slow growth of ANME-archaea, with doubling times of several months under laboratory conditions (Nauhaus et al. 2007; Wegener et al. 2008). Similarly, an enrichment culture of *Candidatus* Methanopredens nitroreducens in a bioreactor showed a lag-phase of ~300 days before substantial growth was detected (Vaksmaa et al., 2017).

In contrast to archaeal lipids, we found (in the first set of experiments with sulfate and manganese oxide) some microbial fatty acids that were highly enriched in ^{13}C, including monounsaturated C16:1 fatty acids (i.e., C16:1ω7c, C16:1ω7t, C16:1ω5c) and iC17:0 (Fig. 3). In the live controls no enrichment of ^{13}C was detected in these specific bacterial fatty acids. The extent of ^{13}C fatty acid biosynthesis (and thus $^{13}CH_4$ uptake) differed both between individual compounds, as well as treatments. The most strongly ^{13}C-enriched fatty acid was C16:1ω5c in the 14-19 cm incubation with sulfate (161‰), and in the 19-24 cm incubation with MnO_2 (307‰). This fatty acid was previously found in SRB associated to ANME -2 and -3, and to a lesser degree in SRB associated with ANME-1 (Niemann and Elvert 2008). The AOM-associated SRB are known to assimilate the end product of AOM, inorganic carbon (DIC) (Wegener et al. 2008; Kellermann et al. 2012). The observed incorporation of ^{13}C can thus be attributed to $^{13}CH_4$ oxidation and assimilative uptake of the produced ^{13}DIC. Other ^{13}C-enriched FA in the treatments with sulfate and/or MnO_2 (e.g., C16:1ω7c and C16:1ω7t; Fig. 3) were also found in AOM sediments elsewhere, however, they are less useful as chemotaxonomic markers (Niemann and Elvert 2008). C16:1ω7c is often associated with SRB, but is also present in many other bacteria and eukaryotes. Similarly, iC17:0 has been found in several SRBs (Rütters et al. 2001; Bühring et al. 2006). Strikingly, while we found C16:1ω5c, we did not observe any other fatty acids synthesized by SRBs typically associated with ANMEs, for example the methyl-branched FAs iC15:0 and aiC15:0, or cy-C17:0ω5 and C16:1ω6 (Elvert et al. 2003; Niemann et al. 2006; Niemann and Elvert 2008). The observed mismatch in the FAs pattern may thus indicate that AOM in Lake Cadagno is not associated to any of the known SRB partners. High ^{13}C-uptake into some of these lipids, most likely by methanotrophic bacteria, has been recently observed in sediments of Lake Kinneret

(Bar-Or et al. 2017). In our sediments, typical gamma or alphaproteobacterial methanotrophs were not detectable or only at very low relative abundances (See next section and Fig. S3), suggesting that different organisms and/or modes of AOM are operating in the sediments of Lake Cadagno and Lake Kinneret, respectively. The lipid pattern found in Lake Cadagno sediments does also not fit to *Methylomirablis oxyfera* (Kool et al. 2012), currently the only known bacterium mediating AOM with nitrite (Ettwig et al. 2010). Thus, along with gamma- and alphaproteobacterial methanotrophs, also *Methylomirablis oxyfera* seems an unlikely candidate contributing to the observed methane oxidation in our experiments.

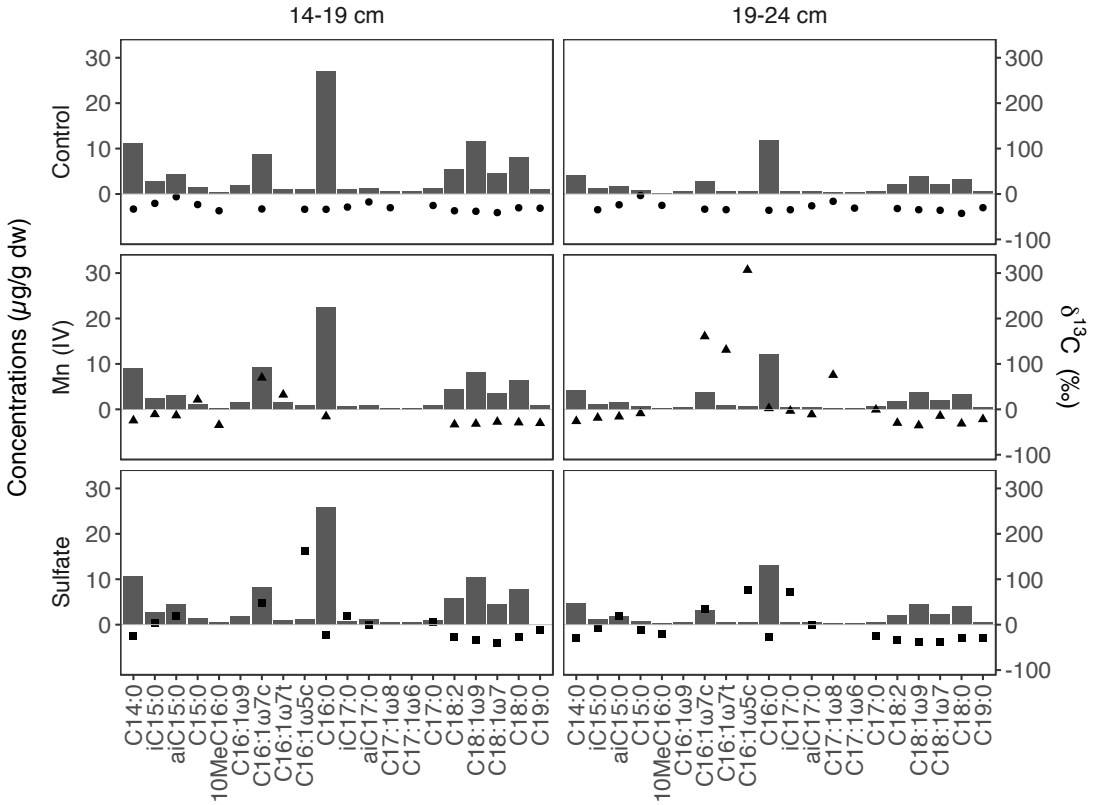

Figure 3. Concentrations (black bars) and compound-specific $\delta^{13}C$-values of fatty acids from control experiments (filled circles), samples with MnO_2 addition (filled triangles) and sulfate (filled squares) of two parallel incubations (sediments from 14-19 cm and 19-24 cm) after 96 days.

Microorganisms potentially performing AOM. Given the clear evidence of AOM coupled to sulfate reduction in our incubation experiments, we analyzed the sediments

for the presence of anaerobic methanotrophic archaea (i.e., ANME-1, -2 and -3) that are typically found in marine sediments in syntrophy with sulfate-reducing bacteria (i.e., Seep-SRB1 and *Desulfobulbus* sp.) (Hinrichs et al. 1999; Boetius et al. 2000; Niemann et al. 2006; Knittel and Boetius 2009). We used primers that match with a large fraction of the deposited sequences of anaerobic methanotrophic archaeal clades (see Supplementary Information, Table S2), but, consistent with our results of the biomarker and gene sequence analyses from the incubation experiments (Fig. 3 and Fig. S3), and with previous 16S rRNA gene analyses in Lake Cadagno sediments (Schubert et al. 2011), we were not able to detect any of the typical ANME-archaea found in marine systems (Knittel and Boetius 2009).

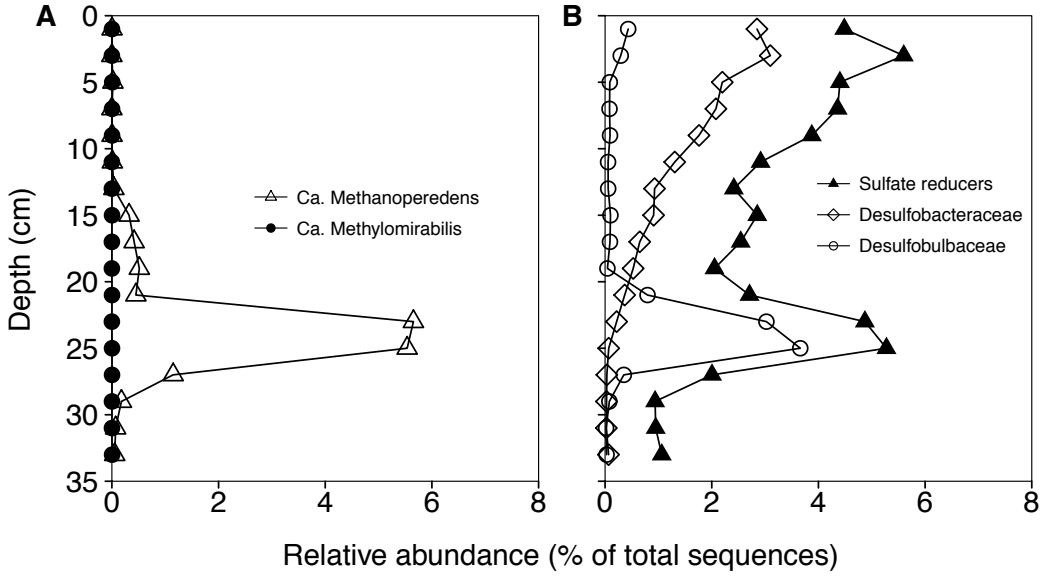

Figure 4. Depth profiles of relative abundances of (A) *Candidatus* Methanoperedens and *Candidatus* Methylomirabilis and (B) sulfate-reducing bacteria (SRBs) in the sediments of Lake Cadagno. Among the SRBs, representatives of *Desulfobulbaceae* and *Desulfobacteraceae* have been shown to form consortia with anaerobic methane oxidizing archaea. Data are based on read abundances of 16S rRNA gene sequences.

A significant number of 16S rRNA gene sequences that were retrieved at/below the maximum AOM zone (0.3-5.7% of total sequences at >10 cm sediment depth; Fig. 4A), belonged to *Candidatus* Methanoperedens. There is some discrepancy between the vertical distribution of the relative abundance of *Candidatus* Methanoperedens

and the AOM rate profile; i.e., the abundance peak was offset by several cm with respect to the maximum AOM rate determined in a separate core. We attribute the apparent offset between the peaks to the heterogeneity of different sediment cores and to lesser degree to artifacts during subsample manipulation. Given that the general shape/quality of the two profiles is essentially equivalent, however, it is reasonable to assume synchronicity, and to link the high methane oxidation rates primarily to this phylotype. The latter was dominated by four amplified sequence variants (ASVs) that showed similar vertical distribution patterns (Fig. S4), and share high sequence similarities within the V4-V5 region of the 16S rRNA gene with recently described *Candidatus* Methanoperedens strains. These are, for example: *Candidatus* Methanoperedens sp. BLZ-1 (Arshad et al. 2015) (98%-100% similarity) and *Candidatus* Methanoperedens nitroreducens ANME-2d (Haroon et al. 2013) (97%-99% similarity) in bioreactors, and *Candidatus* Methanoperedens nitroreducens Vercelli in paddy field soils (Vaksmaa et al. 2017) (97%-98% similarity), which are capable of coupling methane oxidation to nitrate reduction (Haroon et al. 2013). However, recent RNA stable isotope probing results suggest that archaea of this clade may also perform AOM coupled to iron and/or sulfate reduction in the iron-rich but sulfate-poor sediments of Lake Ørn (Denmark) (Weber et al. 2017). The ANME-2d sequences in these sediments share >98% sequence similarity with the *Methanoperedenaceae* phylotypes found in Lake Cadagno (Fig. S5). Moreover, there is genomic evidence for the presence of numerous multiheme c-type cytochromes in *Methanoperedens*-like archaea, suggesting that they can transfer electrons to a broad range of electron acceptors (Arshad et al. 2015; McGlynn et al. 2015), as shown experimentally for Fe(III)- and Mn(IV)-dependent AOM (Ettwig et al. 2016). Multiheme c-type cytochromes in ANME-1 archaea have also been shown to facilitate the electron transfer to syntrophic partner organisms such as SRB (Wegener et al. 2015).

In Lake Cadagno sediments, we also found sequences of sulfate reducing bacteria, including the SEEP-SRB1 cluster and members of the *Desulfobulbus* group, which have been shown to be associated to ANME-1, -2 and -3, respectively, as bacterial partners in marine settings (Knittel and Boetius 2009). The relative abundances of SRBs decreased with sediment depth, in parallel with decreasing sulfate concentrations, but then increased again at about 24 cm depth (Fig. 4B). This secondary maximum was due to a local enrichment of *Desulfobulbaceae*, which were

dominated by a single uncultured representative of this family (Figs. S4, S5, S6). The correspondence of high abundances of *Desulfobulbaceae* and the *Candidatus* Methanoperedens peak at the same sediment depth (Fig. 4B and Fig. S7), together with the lipid-SIP results, suggests that the anaerobic methane-oxidizing archaea and sulfate-reducing bacteria detected in Lake Cadagno sediments are interdependent. The exact nature of the syntrophic interaction (e.g., formation of consortia or an indirect association of SRB and AOM) awaits further investigation. We note, however, that this interdependence is most likely facultative, as one of the amplified sequence variants of *Candidatus* Methanoperedens (ZOTU202) showed a second abundance peak at a depth (13-19 cm), where no *Desulfobulbaceae* partner sequences were detected (Fig. S4A; Fig. 4B). This is consistent with our incubation experiments, and suggests that at least this strain of *Candidatus* Methanoperedens can perform AOM independently, provided a suitable electron acceptor is present.

Concluding remarks

In the present study, patterns of AOM activity, pathways and microbial diversity were investigated in the sediments of euxinic Lake Cadagno. We present clear evidence that microorganisms performed anaerobic oxidation of methane coupled to sulfate reduction below 10 cm sediment depth, with relatively high AOM rates even at depths where sulfate concentrations are relatively low. Incubation experiments show that the addition of sulfate, manganese, iron, and/or nitrate promotes AOM. While there is some evidence for metal oxide-dependent AOM, we argue that the stimulation of AOM by the non-sulfate oxidants was mostly indirect. Sulfate-dependent methane oxidation was fueled by continuous (and at greater depths, cryptic) sulfate production by the oxidation of reduced sulfur compounds with metal oxides. Our microbial community analysis revealed that AOM was driven by uncultured archaea of the candidate genus Methanoperedens. The parallel depth distribution of the abundances of *Candidatus* Methanoperedens and potential sulfate-reducing ANME partners in the sediment zone where high AOM rates were observed suggests that methane oxidation is performed in archaeal-bacterial association. The coupling of AOM to sulfate reduction by novel *Methanoperedenaceae* (and the possible disguise as Mn-/Fe-dependent methanotrophs) not only expands our understanding of this biogeochemically significant group and their potential for

metabolic versatility, but also has broad implications for future AOM investigations in freshwater environments, where sulfate concentrations are low and metal (Mn, Fe) concentrations are often high. Here, *Candidatus* Methanoperedens may represent important sentinels of methane emission to the atmosphere, taking over a similar ecological role as ANME-1, -2 and -3 in marine sediments.

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Author contributions

MFL, JZ and HN conceived the research. GS performed all the experiments with support from JZ. GS, JZ, MFL, and HN performed data analyses and interpretation. HY and LS assisted in the interpretation of lipid biomarker data. GS prepared the manuscript with support from MFL, JZ, and HN.

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Supporting Information

Manganese/iron-supported sulfate-dependent anaerobic oxidation of methane by archaea in lake sediments

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Supplementary information on methods

Preparation of Fe(III)- and Mn(IV)-oxides

Two-line Ferrihydrite ($Fe_5O_8H \cdot H_2O$, hereafter "FeOx") for $^{13}CH_4$ incubation experiments was produced according to Schwertmann and Cornell (Schwertmann and Cornell 2000).

Reaction: $5FeCl_3 + 15NaOH \rightarrow Fe_5O_8H \cdot H_2O + 15NaCl + 6H_2O$

1. In a large beaker, 27 g $FeCl_3$ was dissolved in about 300 mL distilled water, and NaOH solution (20% w/w) was added until pH 7 was reached.
2. The suspension was stirred for ~30 minutes, then the FeOx was washed (by decanting/resuspending) 8-10 times with distilled water until most of NaCl was removed. FeOx was kept as an aqueous slurry under N_2 atmosphere in glass bottles (Schott) with thick butyl rubber septa. The concentration of Fe in suspension was determined gravimetrically by drying a known volume, and dehydrating the ferrihydrite to Fe_2O_3 at 105 °C for 24 h.

Preparation of MnO_2 (Vernadite) according to Kostka and Nealson (1998).

Reaction: $2KMnO_4 + 3MnCl_2 + 2H_2O \rightarrow 5MnO_2 + 4H^+ + 2K^+ + 6Cl^-$

1. 5.92 g $KMnO_4$ was dissolved in 148 mL distilled water, heated to 90 °C, then 7.5 ml 5N NaOH was added.
2. 13 g $MnCl_2 \cdot 4H_2O$ was dissolved in 60 ml distilled water and then slowly added to the alkaline $KMnO_4$ solution.
3. Heating was stopped, and after 30 minutes of continued stirring at room temperature, the suspension was washed several times to remove excess salts and acidity. MnO_2 was kept as an aqueous slurry under N_2 atmosphere in glass bottles (Schott) with thick butyl rubber septa. For determining the Mn content of the suspension, a known volume was centrifuged and dried at 105 °C for 24 h. The molar concentration was calculated from the weight of the dry MnO_2 precipitate.

Supplementary Figures

Geochemical data

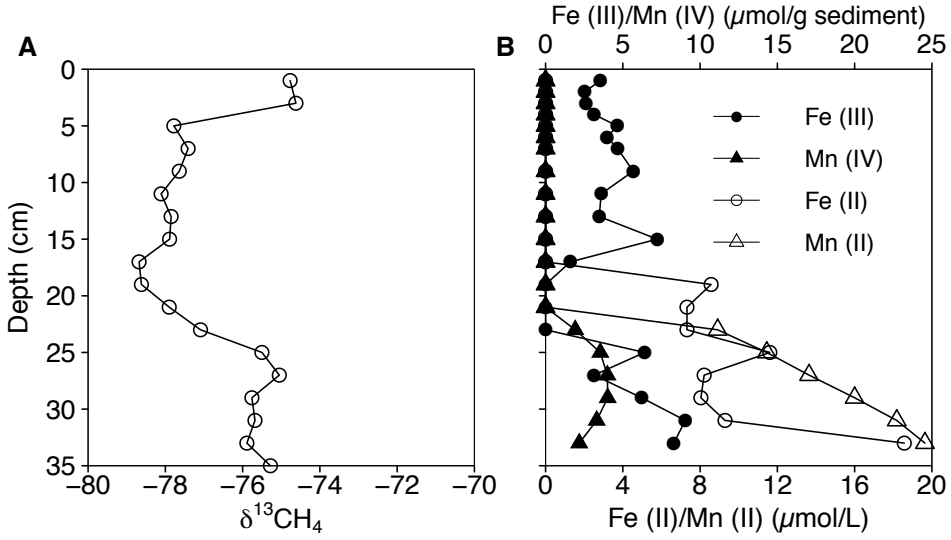

Figure S1. Depth profiles of (A) $\delta^{13}CH_4$ (in ‰ vs. V-PDB) in the sediments of Lake Cadagno and (B) concentrations of dissolved Fe(II) and Mn(II) in the sediment porewater, HCl-extractable Fe(III)) and Mn (IV) in the solid phase. Fe(II)/Mn(II) concentrations in the porewater likely included metal complexes or colloids that passed through the 0.45 μm filter pores, as they should not coexist freely with sulfide at the depth interval of 19-25 cm.

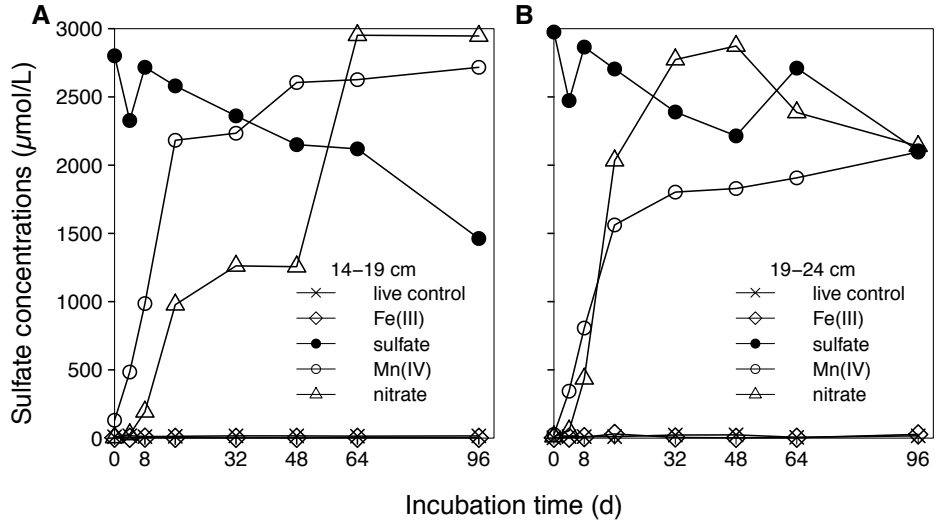

Figure S2. Sulfate concentrations with incubation time in slurry incubations (first set of experiments) using sediments from (A) 14-19 cm and (B) 19-24 cm depth in Lake Cadagno. Different symbols represent the different electron acceptors added.

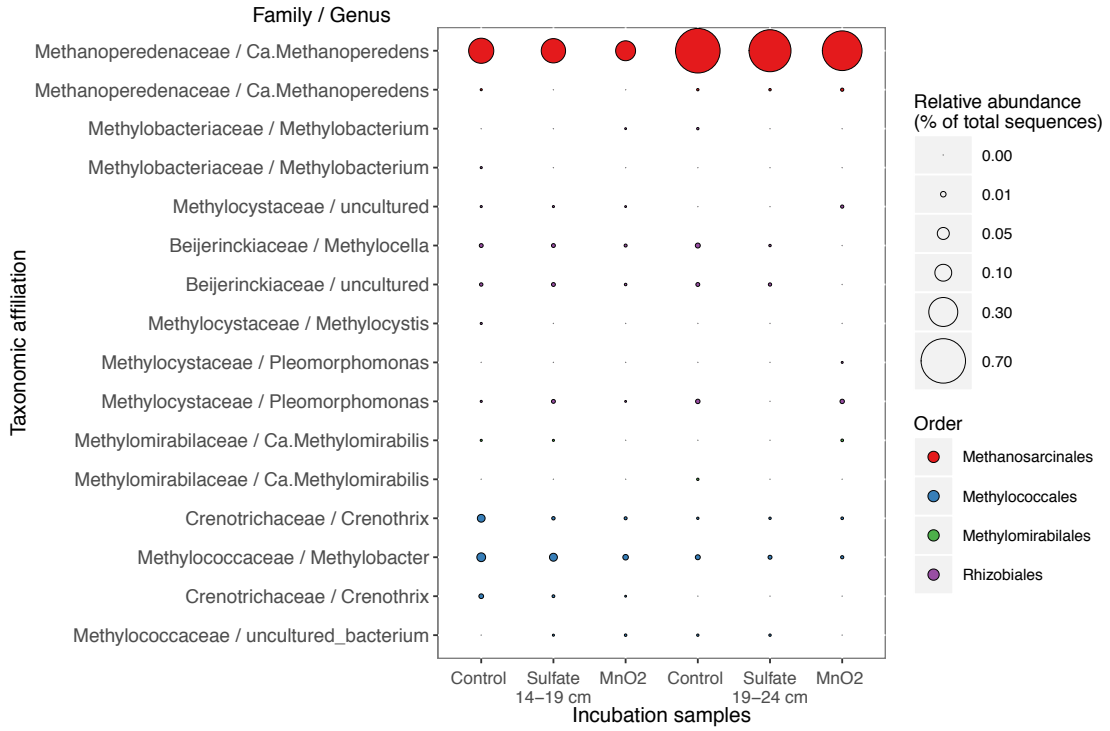

Figure S3. Relative abundances of aerobic and anaerobic methanotrophs detected in incubation samples with sulfate and manganese oxide, and in control experiments after 96 days. Consistent with the down-core molecular data, incubations were dominated by 16S rRNA gene sequences affiliated to *Candidatus* Methanoperedens, implying that AOM in the Lake Cadagno sediments is primarily driven by this phylotype. The relative abundance of *Ca.* Methanoperedens appeared to decrease during the incubation, which was most likely due to growth of other organisms (e.g., SRBs upon sulfate addition).

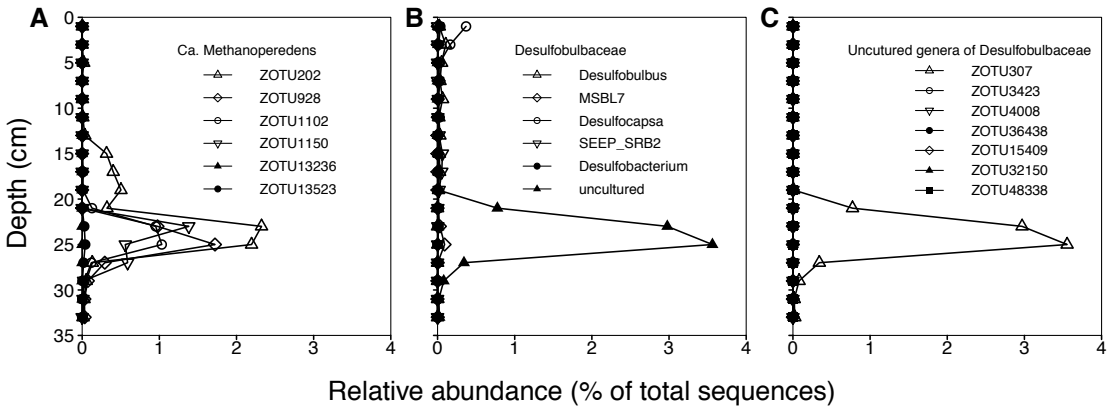

Figure S4. (A) Relative abundances of the amplicon sequence variants (i.e. zero-radius OTU, ZOTU) forming the *Ca*. Methanoperedens phylotype in the sediments of Lake Cadagno. The four dominant ZOTUs peaked at the same depth as uncultured *Desulfobulbaceae* (B), a family previously shown to be associated with anaerobic methanotrophs in marine environments. (C) The depth distribution of all detected *Desulfobulbaceae* ZOTUs reveals that this family was dominated by a single amplified sequence variant (ZOTU307). Interestingly, *Ca.* Methanoperedens ZOTU202 showed a second abundance peak at a depth (13-19 cm) where ZOTU307 was not detected, suggesting that this strain may also be able to perform AOM independently in presence of a suitable electron acceptor. Data are based on read abundances of 16S rRNA gene sequences.

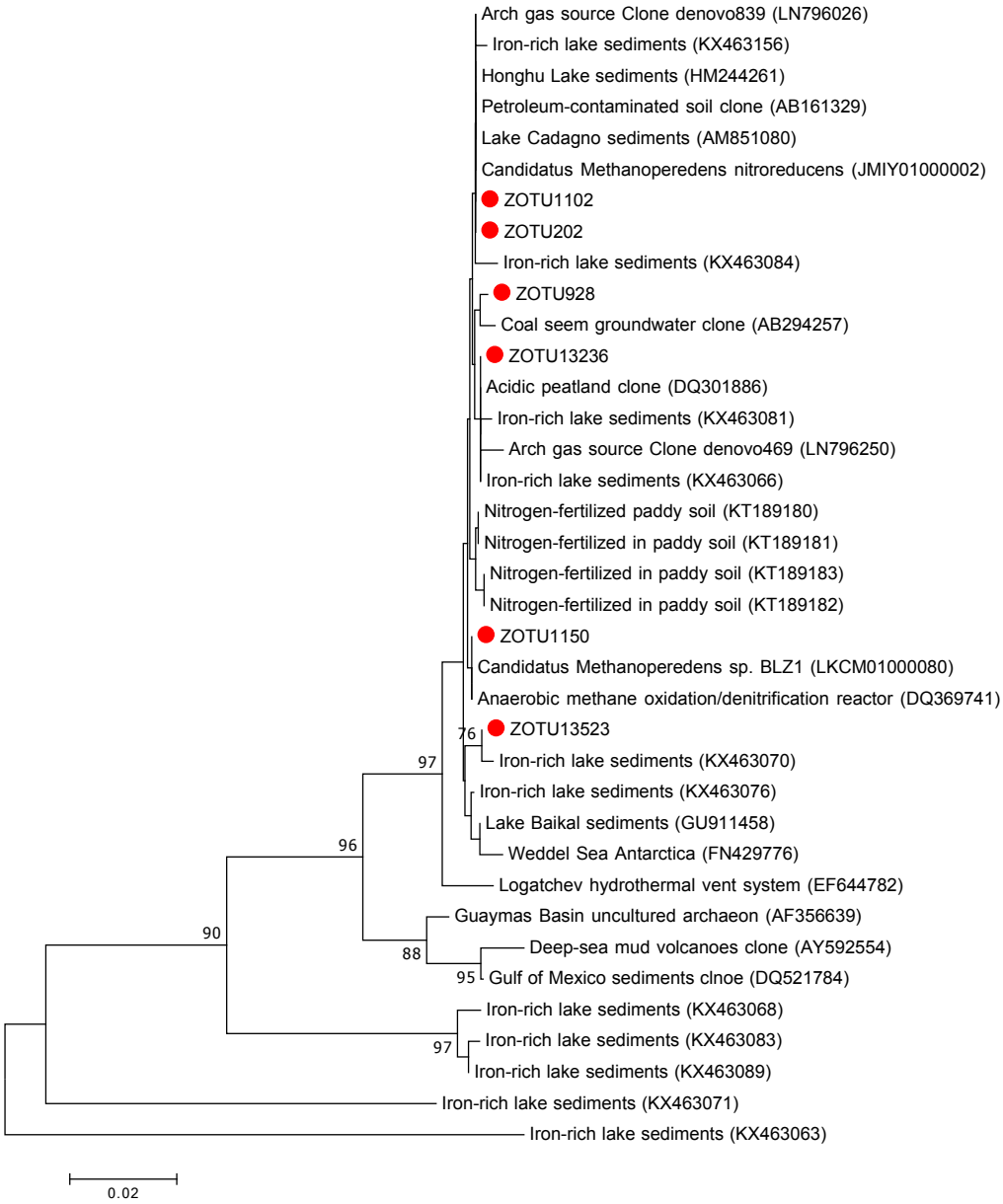

Figure S5. Neighbor-joining phylogenetic tree showing representative 16S rRNA gene sequences of *Methanoperedenaceae* including the amplified sequence variants (ZOTU) retrieved form Lake Cadagno sediments (red circles) and closely related sequences from different environments. The tree was constructed using Maximum Composite Likelihood correction and complete gap deletion (Kumar et al. 2016). Bootstrap values >65% for 1000 re-samplings are shown and the scale bar represents 2% estimated sequence divergence.

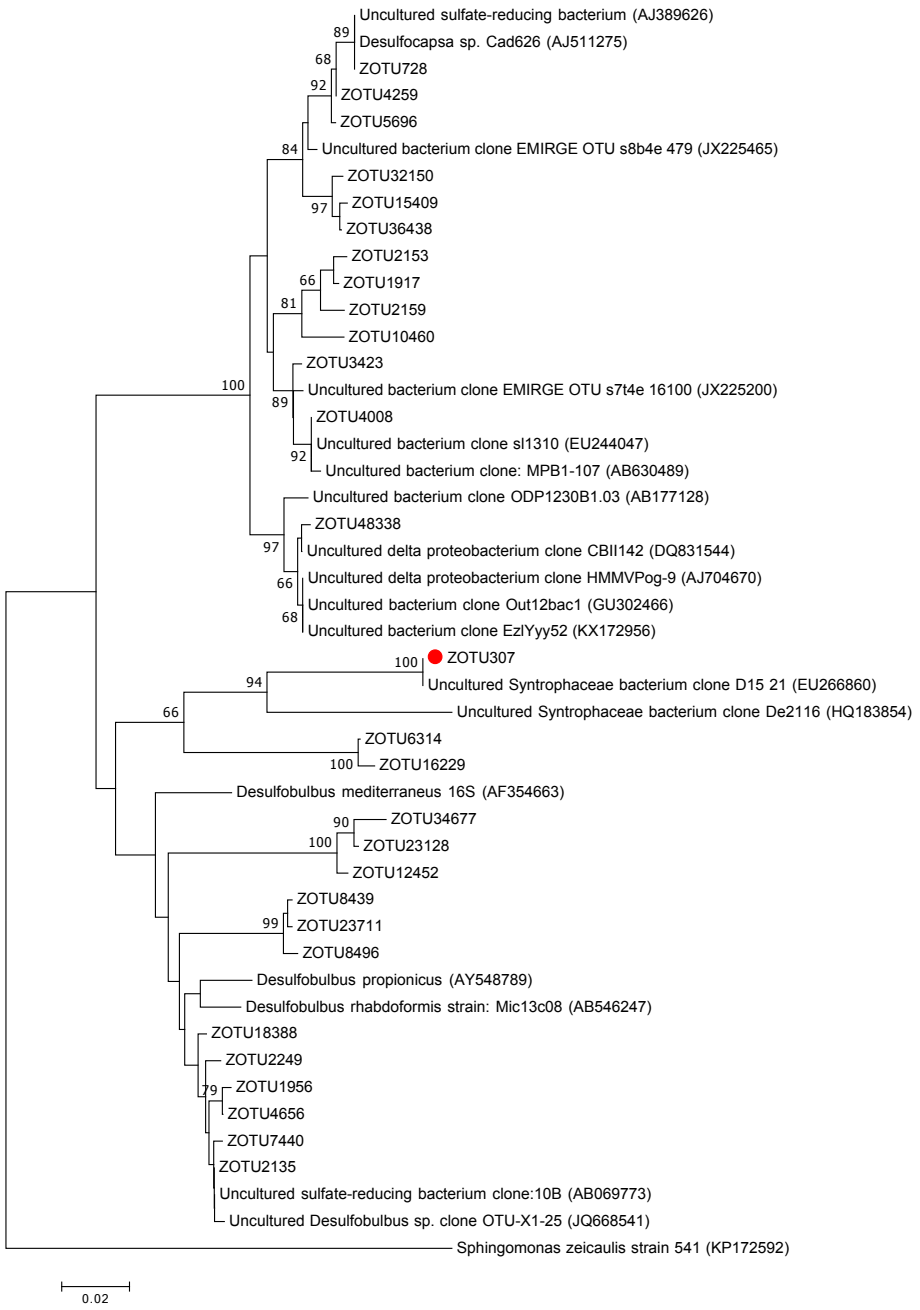

Figure S6. Neighbor-joining phylogenetic tree of representative *Desulfobulbaceae* 16S rRNA gene sequences including the amplified sequence variants (ZOTU) retrieved form Lake Cadagno sediments. ZOTU307 (red circle) was by far the most abundant sequence and exhibited a vertical distribution pattern very similar to that of *Ca.* Methanoperedens. The tree was constructed using Maximum Composite Likelihood correction and partial gap deletion. Bootstrap values >65% for 1000 re-samplings are shown and the scale bar represents 2% estimated sequence divergence.

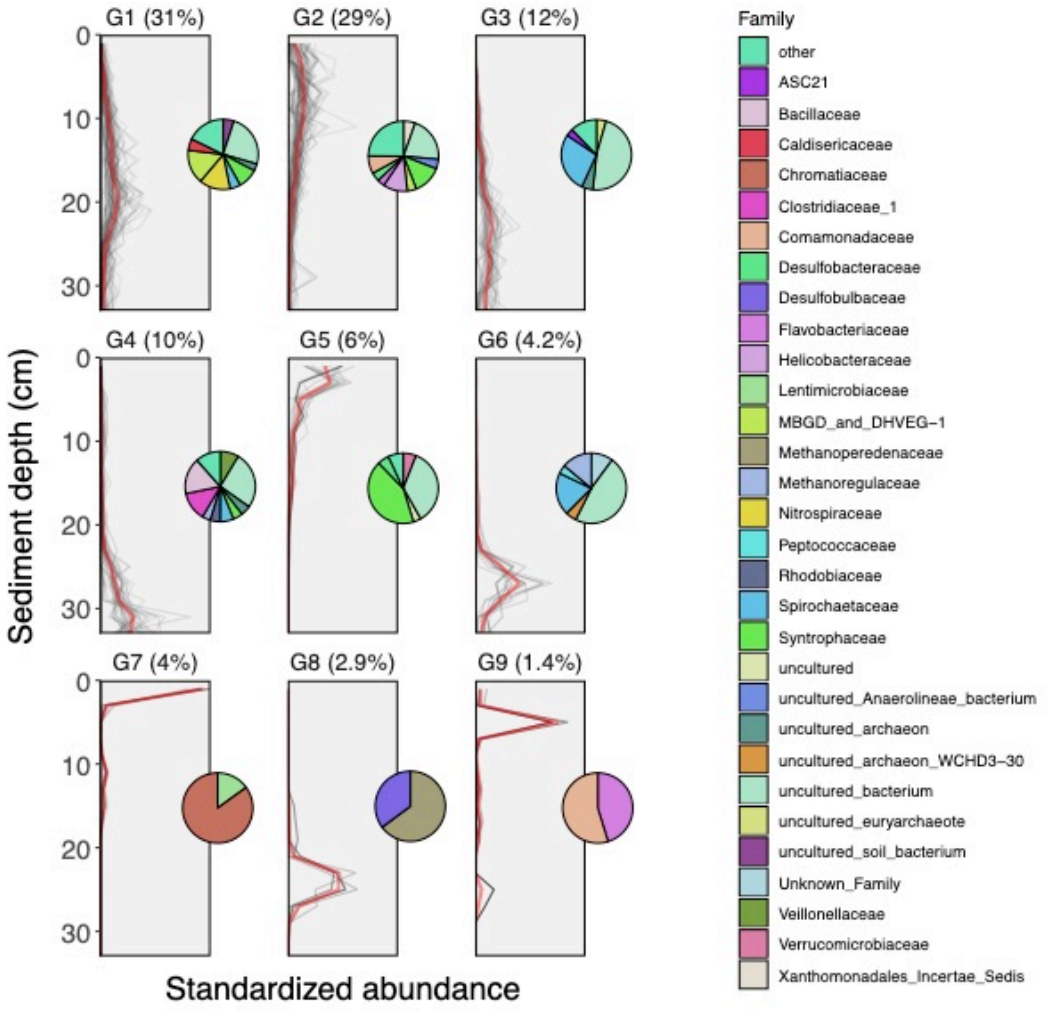

Figure S7. In order to test whether other microbial taxa than *Ca.* Methanoperedens and *Desulfobulbaceae* show a depth distribution pattern matching the subsurface maximum of anaerobic methane oxidation (AOM), we clustered the vertical abundance profiles of 265 ZOTUs, representing 99% of all sequences, into 9 distribution patterns (G1-9). Percentages in parentheses indicate the portion of all sequences falling into a specific grouping. The grey lines represent the profiles of normalized abundances of individual ZOTUs, and the red lines indicates the mean of all ZOTUs in a group. The cluster analysis revealed an AOM-like pattern only for *Desulfobulbaceae*, and *Methanoperedenaceae* (G8). Clustering was done using "ward.D", based on Euclidian distances of normalized vertical abundance profiles. Calculations, clustering and graphs were done using the "plot_groups" function in the R library "phylo.lipids" (Weber et al. 2018) (https://github.com/yukiweber/phylo.lipids).

Supplementary Tables

Table S1. Components of medium used for $^{13}CH_4$ incubation experiments. All medium components were autoclaved separately and mixed aseptically after cooling. The medium was sulfate-free and modified concentrations of basic salts were used (Ettwig et al. 2009).

Components	In 10 L medium	Final concentration (mM)
KH_2PO_4	0.68 g	0.5
$MgCl_2.6H_2O$	1.015 g	0.5
$CaCl_2.2H_2O$	0.735 g	0.5
$NaHCO_3$ (200 mM)	100 ml	2.0
Acidic trace element sol. [a]	5 ml	-
Alkaline trace element sol. [b]	2 ml	-

[a] 1L 100 mM HCl, containing: 2.085 g $FeSO_4 \cdot 7H_2O$, 0.068 g $ZnSO_4 \cdot 7H_2O$, 0.12 g $CoCl_2 \cdot 6H_2O$, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.32 g $CuSO_4$, 0.095 g $NiCl_2 \cdot 6H_2O$, 0.014 g H_3BO_4

[b] 1L 10 mM NaOH, containing: 0.067g SeO_2, 0.050g $Na_2WO_4 \cdot 2H_2O$, 0.242g Na_2MoO_4

Table S2. Coverage of the primer pair 515F-Y / 926R for ANME sequences in the SILVA database (SSU-128, RefNr) assessed by the TestPrime tool (Klindworth et al. 2013) (https://www.arb-silva.de/search/testprime/).

Taxa		Coverage (%)	Accessions	Match	Mismatch
Phylum	Family/Genus				
Euryarchaeota	ANME-1a	85.4	43	35	6
Euryarchaeota	ANME-1b	91.3	104	94	9
Euryarchaeota	ANME-2a/b	91.2	126	114	11
Euryarchaeota	ANME-2b	91.7	12	11	1
Euryarchaeota	ANME-2c	90.6	86	77	8
Euryarchaeota	ANME-3	88.9	63	56	7
Euryarchaeota	Methanoperedenaceae (ANME-2d)	96.2	106	102	4

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Chapter 5: Anaerobic oxidation of methane by aerobic methanotrophic bacteria in the lake sediments

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Abstract

Freshwater lakes represent an important source of the potent greenhouse gas methane to the atmosphere. Methane emissions are regulated to large parts by aerobic (MOx) and anaerobic (AOM) oxidation of methane,,important sinks of this gas within lacustrine sediments. In contrast to marine benthic environments, our knowledge about the modes of AOM, the related methanotrophic microorganisms, and the environmental controls on AOM rates in anoxic lake sediments is still rudimentary. Here, we investigated AOM in the surface sediments of different lakes in Switzerland. Radioisotope incubations with $^{14}CH_4$ were used for both in situ quantification of AOM rates in the sediments, and potential rate measurements in experiments amended with different potential electron acceptors (NO3, Fe3+, SO4). High AOM rates were measured in the surface sediments of both Lake Sempach and Lake Lugano while in the sediments of meromictic Lake Cadagno, AOM rates right at the surface were comparatively low, and maximum rates were observed in the subsurface. Our molecular data indicate that *Methylobacter*, belonging to the Type I aerobic methanotrophs, dominated the methanotrophic community within the surface sediments. Under oxic or micro-oxic conditions, *Methylobacter* clearly use oxygen to oxidize methane. Under anoxic condition, however, the "aerobic" microorganisms seem to be capable of switching to anaerobic respiration and perform AOM. Since methane oxidation was not stimulated by any of the inorganic electron acceptors tested, we speculate that other oxidants such as humic substances may serve as methane oxidizing agent in the lake sediments. If true, and given the widespread distribution of humic substances in lacustrine environments, such mode of anaerobic methane oxidation may play an underappreciated role in reducing methane emissions from anoxic lake sediments.

Introduction

Methane (CH_4) is a powerful greenhouse gas in the atmosphere and a major portion of this gas in aquatic and terrestrial ecosystems is produced biologically in anoxic environments (e.g., anoxic waters and sediments) by anaerobic methanogens. In contrast to oceans, freshwater habitats such as lakes cover only a small portion of the earth's surface (0.9%; Downing et al. 2006), yet, they contribute a significant part to the global emissions of methane to the atmosphere (6-16%; Bastviken et al. 2004).

The comparatively low methane concentrations in both surface and bottom waters of lakes (Blees et al. 2015; Donis et al. 2017) compared to those (mM ranges) in the anoxic sediments (Blees et al. 2014) indicate that methane from methanogenesis is largely consumed by methanotrophy before it is released to the water column. However, the primary sink for biogenic methane in anoxic lake sediments is still not well understood, and many aspects of the process including the physiological mechanisms and modes of benthic methane oxidation have not yet been unraveled.

In freshwater lakes, methane oxidation is thought to be most active at sediment-water interfaces between oxic and anoxic zones (Lidstrom and Somers 1984; Kuivila et al. 1988; Frenzel et al. 1990; Bender and Conrad 1994; He et al. 2012). It is generally assumed that the mitigation of methane emissions in lakes is carried out by methane-oxidizing bacteria (MOB) via aerobic methane oxidation, and fueled by oxygen diffusion or intrusion events from the oxic water column (Hanson and Hanson 1996; Bastviken et al. 2002; Pasche et al. 2011; He et al. 2012; Oswald et al. 2016b). These aerobic methanotrophs performing methane oxidation under oxic conditions usually belong to type I and type X? (gammaproteobacteria) or type II (alphaproteobacteria) MOB, which are classified based on physiological differences related to their carbon assimilation and nitrogen fixation capacity (Hanson and Hanson 1996). In the anoxic water column of some stratified, shallow lakes, oxygenic photosynthesis could potentially provide the O2 for methane oxidation by methanotrophic bacteria (Oswald et al. 2015; Milucka et al. 2015; Rissanen et al. 2018). ,Moreover, a recently identified bacteria of the NC10 phylum (i.e., Candidatus Methylomirabilis) can couple aerobic methane oxidation to nitrite reduction (Raghoebarsing et al. 2006; Ettwig et al. 2008) and has the capacity to produce oxygen intracellularly via the dismutation of nitric oxide (Ettwig et al. 2010). Such denitrifying methanotrophic bacteria have been recently reported for both freshwater lake sediment (Kojima et al. 2012; Deutzmann et al. 2014) and water column (Graf et al. 2018).

True anaerobic oxidation of methane (AOM) is usually performed by anaerobic methanotrophic archaea (ANME) that couple methane oxidation to the reduction of sulfate (Boetius et al. 2000; Orphan et al. 2002; Knittel et al. 2005; Milucka et al. 2012), nitrate (Haroon et al. 2013) or metal oxides (Beal et al. 2009; Ettwig et al. 2016; Cai et al. 2018). However, evidence of the occurrence of any of these pathways in freshwater habitats, particularly in lakes, is still sparse (Crowe et al. 2011; Schubert

et al. 2011; Sivan et al. 2011; Bar-Or et al. 2017; Norði et al. 2013; Weber et al. 2017; Su et al. submitted). In addition to the inorganic electron acceptors mentioned above, humic substances could serve as electron shuttles (Lovley et al. 1996; Scott et al. 1999) and have recently been shown to support methane oxidation under anoxic conditions (Scheller et al. 2016). Some aerobic methanotrophs were reported capable to catalyze methane oxidation coupled to nitrate/nitrite reduction under oxygen limitation (Kits et al. 2015a; b). In addition, it was shown that Crenothrix (i.e., type I methanotrophs) in stratified lakes have the potential to catalyze methane oxidation under nitrate-reducing conditions (Oswald et al. 2017). In another water column study on a permanently stratified lake in Switzerland, maximum activities of methane oxidation were consistently observed in the deeper anoxic hypolimnion, which was ascribed to the activity of Methylobacter, yet the exact mechanism of this apparent AOM by putatively aerobic bacteria and the responsible electron acceptor remained enigmatic (Blees et al. 2014). Similarly, for some sulfate-rich or ferruginous lake environments, it was demonstrated that the microbial community well below the permanent redox clines or within the anoxic sediments was composed predominantly of aerobic methanotrophs (Biderre-Petit et al. 2011; Milucka et al. 2015; Oswald et al. 2016). Most recently, it was shown that aerobic methanotrophs can perform anaerobic oxidation of methane in anoxic lake sediments (Martinez-cruz et al. 2017), where light penetration into the sediments seemed unlikely for oxygenic photosynthesis (Oswald et al. 2015; Milucka et al. 2015). It appears that aerobic methanotrophs are versatile in their oxygen requirements and are capable of conducting alternative metabolisms in oxygen-deficient or anoxic environments.

In this study, we investigated methane oxidation in the anoxic surface sediments of different lakes in Switzerland, with the particular goal to elucidate the microbial players involved and to verify whether the apparent anoxic methane sink in the studied lakes represents true AOM. Towards this goal, we combined sediment pore water hydrochemical data with in situ AOM rate measurements using radio-labeled methane, as well as slurry incubation experiments to study the impact of alternative oxidants (i.e., sulfate, nitrate, iron and manganese) on AOM. In addition we characterized the microorganisms that are involved in methane oxidation using 16S rRNA gene sequencing.

Materials and Methods

Study sites.

We investigated three eutrophic lakes with different stratification regimes and sediment biogeochemistry in Switzerland. Lake Sempach is located in central Switzerland and is artificially destratified by hypolimnetic aeration, maintaining the bottom waters permanently oxic. The Lake Lugano South Basin undergoes seasonal stratification with the development of a bacterial benthic nepheloid layer (Blees et al. 2014) and anoxia during summer, while Lake Cadagno is a meromictic lake with permanent stratification, and relatively high concentrations of sulfate.

Sampling and sample processing.

Sampling campaigns were carried out independently in Lake Sempach (March 2015, April 2016), Lake Lugano (February 2015, September 2016) and Lake Cadagno (July 2016, October 2016) either for in situ AOM rate measurements or slurry incubation experiments to investigate the potential role of different electron acceptors. For each sampling, undisturbed sediment cores (inner diameter 62 mm) were recovered with a gravity corer from the deepest site of the respective lake. Samples for dissolved CH_4 concentrations were collected on site with cut-off syringes through pre-drilled holes in the sediment core tube, which were covered with tape. 3 ml of sediment samples were fixed with 7.0 ml 10% NaOH in 20 ml glass vials, which were then immediately sealed with butyl rubber stoppers (Niemann et al. 2015). Sediment porewater was extracted by sectioning a second sediment core and centrifuging the sediment samples under anoxic condition. Supernatant was then filtered through 0.45 μm filters. Porewater samples for sulfide concentration measurements were fixed with Zn acetate (5%) immediately after filtration. Samples (xxml) for dissolved iron and manganese concentration determination were added to 200 μl 6 M HCl. Particulate iron and manganese in the sediment solid phase were extracted with 0.5 M HCl and filtered through 0.45 μm filters. Porewater samples for sulfate and nitrogen species (i.e., NH_4^+, NO_2^- and NO_3^-) were stored at 4°C for further analysis.

Porewater and sediment geochemical analyses.

Methane concentrations in the headspace of NaOH-fixed samples were measured using a gas chromatograph (GC, Agilent 6890N) with a flame ionization detector, and

helium as a carrier gas. Photometric analysis was used for the determination of nitrite concentrations using the Griess reaction (Berichte 1879). Porewater concentrations of NH_4^+, SO_4^{2-} and NO_3^- were analyzed by ion chromatography (Metrohm, Switzerland). Sulfide concentrations were determined spectrophotometrically using the Cline method (Cline 1969). Dissolved iron (Fe^{2+}), manganese (Mn^{2+}) and total manganese concentrations were measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Iron oxides in the solid phase were extracted with 0.5 M HCl and then reduced to Fe^{2+} with 1.5M hydroxylamine. Concentrations of Fe^{2+} were determined photometrically using the ferrozine assay (Stookey 1970). Particulate iron was then calculated from the difference between the total Fe^{2+} concentrations after reduction and the dissolved Fe^{2+} in the filtered sample.

AOM Rate Measurements. To obtain depth-specific in situ AOM rate profiles in the sediments, three small plexiglass tubes (inner diameter 16 mm) were inserted into the fresh sediment core in order to to extract sub-cores. Subsequently, $^{14}CH_4$ radiotracer injection was applied to the triplicate sub-cores according to Treude et al. (2003)) and incubations were performed at in situ temperature (4 °C) in the dark. At the end of the incubation, 1 or 2 cm segments were pushed out from the subcore liner, directly transfered into 100 ml vials containing 20 ml of the aqueous NaOH (5% w:w) to terminate microbial activity. Radioactivities of residual $^{14}CH_4$ in the incubation samples (as CO_2 after combustion), the $^{14}CO_2$ produced by AOM, and the remaining biomass were measured via liquid scintillation counting (Mau et al. 2013; Blees et al. 2014; Steinle et al. 2016). AOM first order rate constants (k) were calculated according to Eq. 1,

$$k = \frac{A_{CO_2} + A_R}{A_{CH_4} + A_{CO_2} + A_R} \times t^{-1} \qquad \text{(Eq. 1)}$$

where A_{CH_4}, A_{CO_2} and A_R represent the radioactivity of CH_4, CO_2, and the remaining radioactivity. t represents the incubation time. Methane oxidation rates (MOR) were then calculated using the value for k and the methane concentration at the start of the incubation (Eq. 2).

$$\text{MOR} = k \times [CH_4] \qquad \text{(Eq. 2)}$$

Substrate-amended Slurry Incubation Experiments. The top 10 cm of four replicate sediment cores were combined (~1200 cm^3) and mixed with anoxic artificial

mineral medium to yield approximately 8 L homogenized mixtures for slurry incubations. The slurries were purged with N_2 to remove any traces of O_2 and background CH_4. 120 mL serum vials were filled with ~100 mL of the homogeneous sediment slurries, which were purged again to maintain anoxic conditions. 1.5 ml pure CH_4 were injected into the 20 ml headspace, and the bottles were gently shaken for 24 h, resulting in a final concentration of 100 µmol/L in the slurries. Incubation slurries, except for controls, were amended with either nitrate, sulfate, amorphous manganese or iron oxide, with final concentrations of 4 mM, 2 mM, 5 mM and 5 mM, respectively. Control experiments included live controls (slurries without additional electron acceptors), killed controls (slurries with 5 ml saturated NaOH) and incubations with sulfate reduction inhibition (with 4 mM molybdate). Finally, all incubation bottles were supplemented with $^{14}CH_4$ gas tracer before filling the bottles bubble-free with anoxic water (100 µmol/L CH_4), and were incubated in the dark in an anoxic chamber with N_2 atmosphere at 25 ºC. At different time points (0, 30, 60 and 90 days), the incubation samples were stopped by adding 5 ml saturated NaOH after creating 20 ml headspace. Supernatant water in the bottles was collected and filtered with a 0.45 µm membrane filter for subsequent sulfate, nitrate, ammonium and dissolved iron/manganese analyses. AOM rates were analyzed as mentioned before and calculated according to Eq. 1 and Eq. 2.

DNA extraction, PCR amplification, Illumina sequencing and data analysis. Surface sediments (0-2 cm) from three different lakes were collected for DNA extraction using FastDNA SPIN Kit (MP Biomedicals) following the manufacturer's instructions. A two-step PCR approach was applied in order to prepare the library for Illumina sequencing at the Genomics Facility Basel. Briefly, a first PCR (25 cycles) was performed using universal primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3') targeting the V4 and V5 regions of the 16S rRNA gene (Parada et al. 2016). Sample indices and Illumina adaptors were added in a second PCR of 8 cycles. Purified indexed amplicons were finally pooled at equimolar concentration into one library and sequenced on an Illumina MiSeq V2 platform using the 2×300 bp paired-end protocol. After sequencing, FastQC (v 1.2.11; Babraham Bioinformatics) was used to check the quality of the raw reads. Forward and reverse reads was merged into amplicons of about 374 bp length using FLASH (Magoč and Salzberg 2011), allowing a minimum overlap of 15

nucleotides and a mismatch density of 0.25. Quality filtering (min Q20, no Ns allowed) was performed using PRINSEQ (Schmieder and Edwards 2011). OTU (operational taxonomic unit) clustering with a 97% cutoff was carried out using the UPARSE-OTU algorithm in USEARCH v10.0.240 (Edgar 2010, 2013). Taxonomic assignment prediction of OTUs was done using SINTAX (Edgar 2016) and the SILVA 16S rRNA reference database v128 (Quast et al. 2013). Subsequent data analyses were carried out with Phyloseq package (McMurdie and Holmes 2013) in the R environment (R Core Team, 2014) (http://www.r-project.org/).

Results

Porewater Chemistry and benthic AOM rates. Oxygen penetrated less than 3 mm depth into the sediments of permanently oxic Lake Sempach (March 2015) and the monomictic south basin of Lake Lugano (February 2015) (Figure S1). Porewater nitrate was only detected in the uppermost 3 cm sediments of Lake Sempach and Lugano, and nitrite was below detection limit at all three sites (Figure S2). Porewater sulfate concentrations decreased with sediment depth and maximum concentrations at the sediment water interface ranged from 78 µmol/L (Sempach), 86 µmol/L (Lugano) to 910 µmol/L (Cadagno) in the surface sediments. Considerable amounts of porewater methane were present in the sediments of the three studied lakes. In all cases, methane concentrations increased with depth, ranging from a few hundred µmol/L to 1-2 mmol/L (Lakes Sempach and Cadagno) or from ~1.6 mmol/L to 4.8 mmol/L (Lake Lugano) (Figure 1). AOM rates measurements revealed active methane oxidation in the surface sediments of both Lake Sempach and Lake Lugano. AOM activity decreased with depth, with the maximum rates (68.0 ± 19.0 and 217.7 ± 154.8 nmol/cm^3/d) observed in the upper 0-2 cm of the sediments. Depth-integrated rates in the anoxic sediments (0-10 cm) of Lake Lugano (Melide) were 409.8 ± 204.4 nmol/cm^2/d, higher than those in Lake Sempach (284.8 ± 32.9 nmol/cm^2/d). In contrast, AOM was not detected in the surface sediment layers of Lake Cadagno, where the rates increased with depth (Figure 1) to 4.0 ± 1.7 nmol/cm^3/d. Here, the integrated AOM rates were much lower (15.7 ± 7.9 nmol/cm^2/d), i.e., one to two orders of magnitude lower than those in Lake Sempach and Lake Lugano. These measurements represent first direct evidence of AOM in the anoxic sediments of three lakes.

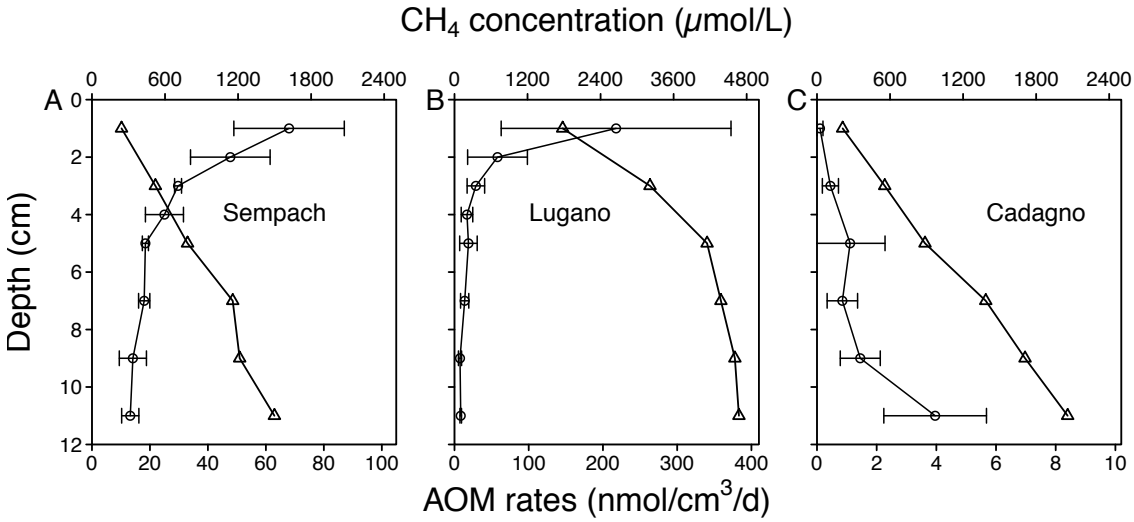

Figure 1. Methane concentrations (open triangles) and depth-specific in situ AOM rates (open circles) determined in the sediments of three lakes in Switzerland. (A) Lake Sempach (March 2015): permanently oxic bottom water; (B) Lake Lugano (Melide): seasonal stratified, anoxic bottom water (February 2015); (C) Lake Cadagno (October 2016): permanently stratified, anoxic bottom waters and sulfate-rich. Error bars represent standard deviations of triplicate rate measurements.

Microbial community in surface lake sediments

The microbial community of the surface lake sediments was analyzed by amplicon sequencing of 16S rRNA gene sequences. A very large diversity of both bacterial and archaeal phylogenetic groups was observed in the sediments of all three lakes (Table 1). Among the bacterial community, Proteobacteria comprising potential aerobic methanotrophs represented the most abundant phylum in all cases (34.81% in Lake Sempach, 23.93% in Melide, Lake Lugano, 21.84% in Figino, Lake Lugano) and 50.48% (Lake Cadagno) of total sequences. A close look at the composition of this phylum revealed that type I methane-oxidizing bacteria (Gamma-Proteobacteria) dominated the methanotrophic groups, accounting for 2.70% (Lake Sempach), 0.35% or 0.37% (Lake Lugano) and 0.35% (Lake Cadagno) of total microbial community (Figure 2A). Type II aerobic methanotrophs (Alpha-Proteobacteria) were also detected in Lake Sempach and Lake Lugano with relatively low abundances of less than 0.1% of total reads. In all the samples, the archaeal groups constitute only a minor fraction of the total microbial community, with most of the sequences assigned to Euryarchaeota in Lake Sempach (0.97%) and Lake Lugano (Melide, 0.98%), and Woesearchaeota in Lake Lugano (Figino, 1.62%) and Lake Cadagno (1.35%). Within

the Euryarchaeota, we did not detect any sequence of anaerobic methanotrophs (i.e., ANME-1, -2 and -3) that are known to perform sulfate-dependent AOM. However, we found very low abundances of Candidatus Methanoperedens in Lake Lugano (Melide) and Lake Cadagno (< 0.003% of total sequences), bacteria that can use nitrate as electron acceptor to oxidize methane. Likewise, sequences related to the methane-oxidizing NC10 phylum (i.e., Candidatus Methylomirabilis) were not detected in Figino, and made up less than 0.003% of total sequences for all the other sites. Verrucomicrobia could also be detected in all the lake sediments, but sequences related to Methylacidiphilum, known to perform aerobic methane oxidation in acidic environments, were only present in Lake Sempach and Lugano (Melide) and made up less than 0.03% of the community (Figure 2A).

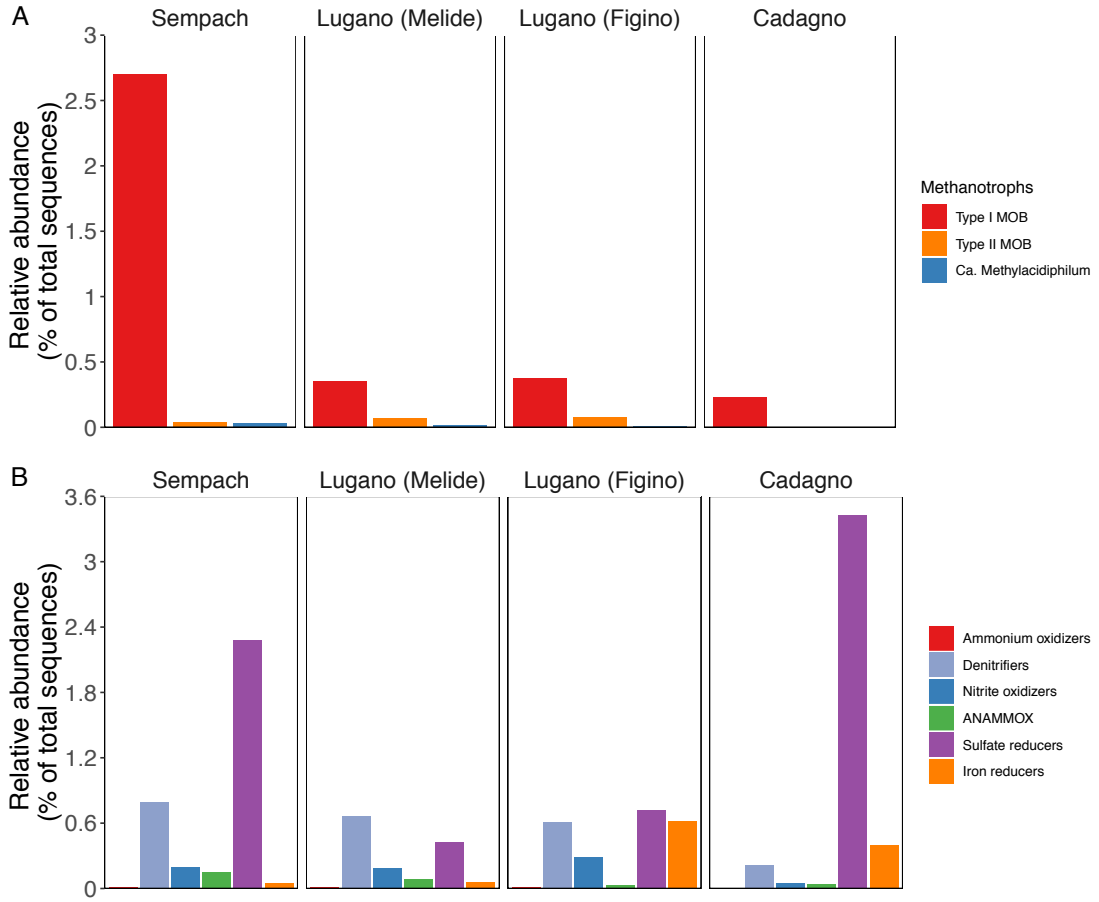

Figure 2. Composition and relative abundance of (A) aerobic methanotrophs and (B) nitrogen-transforming microorganisms, iron and sulfate reducers detected in the surface sediments (0-2 cm) of the three investigated lakes with different geochemical conditions.

In addition to potential methanotrophs, diverse groups of microorganisms that could carry out different biogeochemical processes were also detected in the

sediments, including nitrogen-transforming microorganisms, iron and sulfate reducers (Figure 2B). Of the nitrogen cycling groups in the lake sediments, denitrifiers were the most abundant (0.21-0.80%), followed by nitrite oxidizers (0.05-0.20%), ANAMMOX (0.04-0.15%) and ammonium oxidizers (<0.02%). The relative abundances of sulfate-reducing bacteria were found considerably higher in both Lake Sempach (2.28%) and the sulfate-rich Lake Cadagno (3.43%) than Lake Lugano (0.42%). Furthermore, we retrieved sequences of known iron reducers at all the three sites, including Deferrisoma, Thermincola, Geobacter and Geothrix and the relative abundances accounted for 0.06-0.40% of all reads.

Table 1. Composition and relative abundance of top bacterial and archaeal phyla in the surface sediments of different lakes. Only bacterial phylogenetic groups that make up ≥1% of total sequences and top 5 arhcaeal phyla are shown. Phyla comprising potential methanotrophs are highlighted in bold.

Relative abundance of top bacterial phyla (making up ≥ 1% of total sequences)							
Lake Sempach		Lake Lugano (Melide)		Lake Lugano (Figino)		Lake Cadagno	
Proteobacteria	34.81	**Proteobacteria**	23.93	**Proteobacteria**	21.84	**Proteobacteria**	50.48
Acidobacteria	11.14	Firmicutes	21.96	Bacteroidetes	17.37	Bacteroidetes	13.01
Firmicutes	11.10	Planctomycetes	8.36	Cyanobacteria	8.07	Chloroflexi	4.32
Bacteroidetes	7.60	Bacteroidetes	8.19	Firmicutes	6.82	Firmicutes	4.08
Chloroflexi	6.42	Acidobacteria	6.5	Planctomycetes	5.84	Aminicenantes	3.33
Planctomycetes	6.24	Cyanobacteria	4.81	Chloroflexi	3.78	Cyanobacteria	3.04
Chloroflexi	6.42	Chloroflexi	3.20	Acidobacteria	3.72	Planctomycetes	2.61
Actinobacteria	5.45	Aminicenantes	3.18	Modulibacteria	3.24	Acidobacteria	1.92
Cyanobacteria	4.66	Actinobacteria	3.07	**Verrucomicrobia**	3.04	**Verrucomicrobia**	1.87
Verrucomicrobia	3.20	**Verrucomicrobia**	2.67	Spirochaetae	2.68	Actinobacteria	1.60
Ignavibacteriae	1.69	Caldiserica	2.26	Ignavibacteriae	2.16	Latescibacteria	1.21
Nitrospirae	1.09	Ignavibacteriae	1.40	Omnitrophica	2.03	Omnitrophica	1.08
-	-	Armatimonadetes	1.16	Aminicenantes	1.93	-	-
-	-	Latescibacteria	1.04	Actinobacteria	1.60	-	-
Relative abundance of top 5 archaeal phyla (% of total sequences)							
Lake Sempach		Lake Lugano (Melide)		Lake Lugano (Figino)		Lake Cadagno	
Euryarchaeota	0.97	**Euryarchaeota**	0.98	Woesearchaeota	1.62	Woesearchaeota	1.35
Woesearchaeota	0.14	Woesearchaeota	0.38	**Euryarchaeota**	1.18	**Euryarchaeota**	0.48
Bathyarchaeota	0.10	Thaumarchaeota	0.30	MEG	0.25	MEG	0.12
Thaumarchaeota	0.06	Bathyarchaeota	0.25	Lokiarchaeota	0.13	Lokiarchaeota	0.07
WSA2	0.008	MEG	0.03	Bathyarchaeota	0.10	Thaumarchaeota	0.04

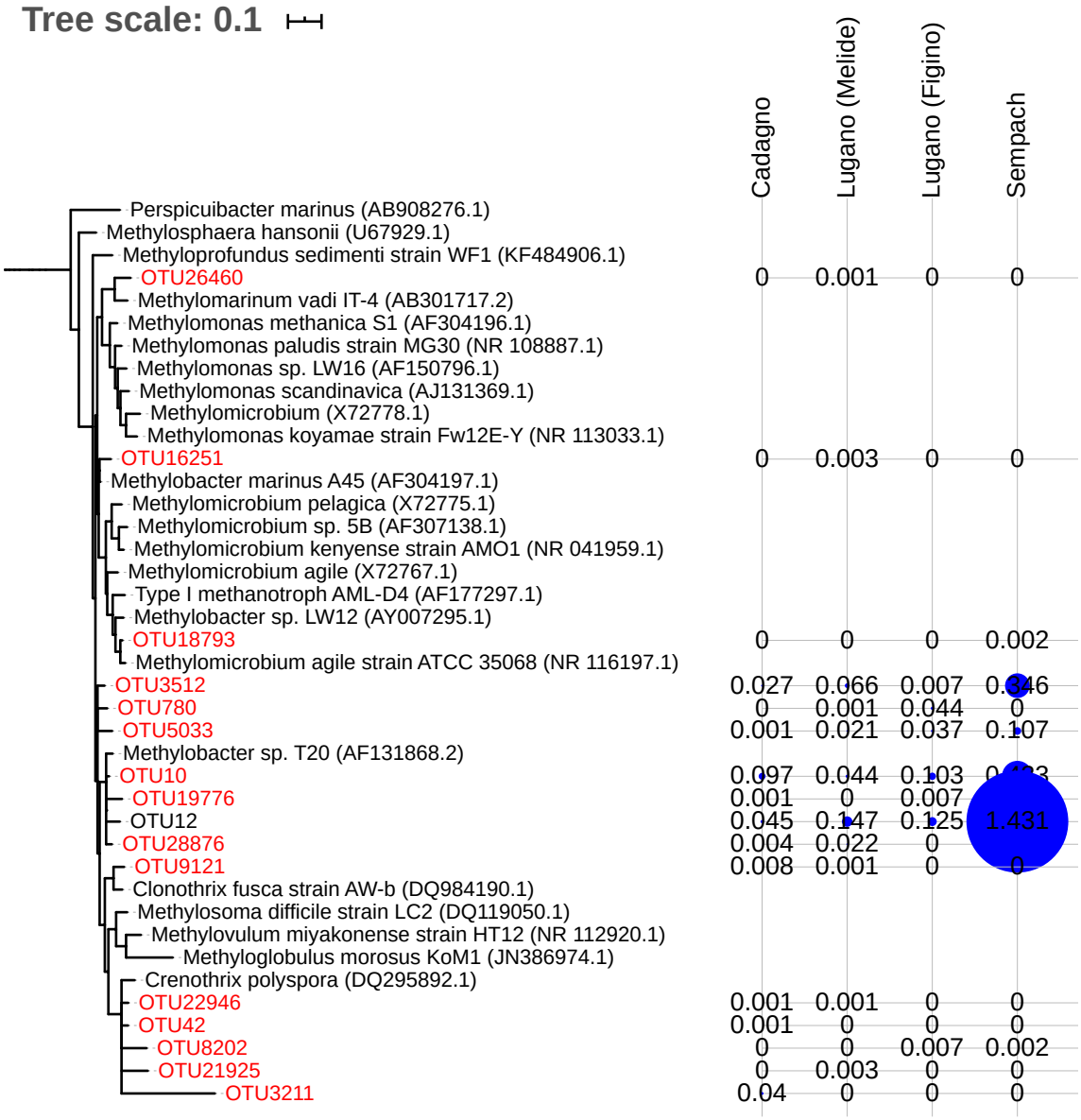

Figure 3. RAxML evolutionary placements of 16S rRNA gene OTUs (97% similarity) of type I methanotroph retrieved from different lake sediments, on a maximum-likelihood reference tree generated by IQ-TREE. Nucleotide accession numbers of the reference sequences are listed in brackets. The numbers (bubble plot) indicate relative abundances for each OTU within type I MOB in three different lakes.

Possible electron acceptors for AOM. To find out AOM processes observed in the surface sediments of lakes, we tested several oxidants, which have been previously reported as electron acceptors for AOM, including sulfate (Boetius et al. 2000; Orphan et al. 2001), nitrate (Haroon et al. 2013) and metal oxides (Beal et al. 2009). AOM was detected in all the controls and incubations of some treatments (Figure 4). In the anoxic slurries with sediments from Lake Sempach, methane oxidation was

completely inhibited in the incubations with both nitrate and manganese oxides. The addition of iron oxide lowered AOM activities relative to the control experiments, but the rates increased with incubation time, from 0.056 ± 0.037 µmol/L/d (30 days) to 0.103 ± 0.036 µmol/L/d (90 days). In the incubations with sediments from Lake Lugano, AOM rates were considerably lower when nitrate, sulfate or manganese oxides were added. Interestingly, sulfate additions to the slurries from Lake Sempach or iron additions to those from Lake Lugano generated similar rates compared to the controls throughout the incubation experiments. Similarly, the amendments of slurries from Lake Cadagno with all potential electron acceptors for AOM (i.e., nitrate, sulfate, iron and manganese) lowered the rates relative to the controls. However, AOM rates of both controls and incubations with sulfate and iron/manganese increased with time. Overall, in all of the incubations, we did not observe any stimulation of AOM with either nitrate, sulfate or Fe^{III}/Mn^{IV} compared to the controls without any external electron acceptors.

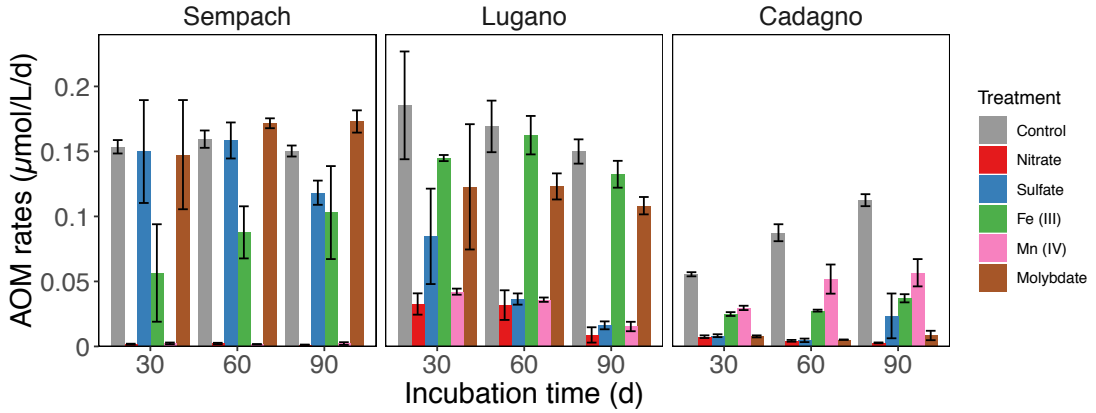

Figure 4. Rates of anaerobic oxidation of methane (AOM) over incubation time using surface sediments (top 10 cm) from Lake Sempach, Lake Lugano (Figino) and Lake Cadagno. Samples were amended with 14C-labeled methane and different electron acceptors including amorphous manganese and iron oxides, nitrate and sulfate. Control incubations were conducted without electron acceptors (grey bars). No methane oxidation rates were detected in the killed controls.

Discussions

The anaerobic oxidation of methane has the potential to be an important methane sink in freshwater lakes and may efficiently control the emissions of this greenhouse gas from sediments to the atmosphere since methane concentrations decreased

remarkably from bottom to top sediment layers. AOM was only recently shown in freshwater lake environments and direct rate measurements are rare (Norði et al. 2013). Our direct rate measurements provide clear evidence for AOM in the anoxic sediments of all the studied lakes. Of the three lakes, Lake Sempach and Lake Lugano exhibited the same pattern of AOM rate distribution in the sediments, which was consistent with previous results in freshwater wetlands (Segarra et al. 2013, 2015). However, in the sulfate-rich Lake Cadagno, the depth-specific AOM rate profile was different with the highest activity observed in the bottom sediments. Eutrophic lakes receive large inputs of nutrients and are therefore potential settings for diverse metabolic activities The geochemical and rate profiles in the sediments suggest the potential roles of sulfate, nitrate and/or metal oxides for the observed methane oxidation (Figure 1, Figure S2 and S3). In Lake Cadagno, sulfate (~ 0.9 mM) most likely served as the electron acceptor (Boetius et al. 2000; Orphan et al. 2002; Milucka et al. 2012), despite the fact that sulfate concentrations are still much lower in this lake than in marine systems where sulfate-dependent AOM is the dominant methane consuming process (Reeburgh 2007). Likewise, in the sediment porewater of both Lake Sempach and Lake Lugano, sulfate was the only known electron acceptor available at high enough in situ concentrations (~ 80 μmol/L) to possibly account for the measured AOM rates. However, sulfate-coupled AOM seems to be very unlikely especially for Lake Semapch and Lugano, as there was no visible consumption of SO_4^{2-} and H_2S was below detection limits in the sediment porewater. The absence of targeted groups of known ANMEs performing sulfate-dependent methane oxidation could further confirm that classical AOM was not occurring in the surface lake sediments.

The methanotrophic community was dominated by type I MOB in the surface sediments of all investigated lakes. Phylogenetic analysis revealed that type I methanotrophs mainly comprised the Genus Methylobacter, representing 2.67% (Lake Sempach), 0.31% (Lake Lugano) and 0.22% (Lake Cadagno) of total community (Figure 3). Sequences related to Candidatus Methanoperedens (Haroon et al. 2013; Arshad et al. 2015; Vaksmaa et al. 2017) that catalyze nitrate-dependent AOM, Candidatus Methylomirabilis (Raghoebarsing et al. 2006; Ettwig et al. 2010; Graf et al. 2018) that perform AOM with nitrite as electron acceptors and Methylacidiphilum known to perform aerobic methane oxidation in acidic environments (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; van Teeseling

et al. 2014), were either not detected or too low in abundance to account for the observed methane oxidation in the anoxic sediments. Our molecular data indicate that Methylobacter were central to the methane turnover in the anoxic lake sediments.

Active Methylobacter in the surface sediments may be sustained by transient supply of O_2 diffusion in the bottom oxic waters during incubation. Yet, O_2 penetration to less than 3 mm depth in the sediments of both Lake Sempach and Lugano suggested that aerobic respiration was not the only process for methane assimilation. This conclusion is further supported by the results of slurry incubation experiments where methane consumption was active under anoxic condition. However, results of amended slurries in all cases remained elusive. Stimulation of AOM was not discernible with added sulfate and iron/manganese, similar to previous observations in the anoxic water column incubation (Oswald et al. 2016). Compared with the control experiments, addition of some oxidants (e.g., nitrate and manganese oxide) appeared to even suppress AOM activity or caused complete inhibition. The decreased AOM rates or suppression of AOM with the same electron acceptors relative to the control were also observed in the slurry incubations with freshwater wetland sediments (Segarra et al. 2013) and in the anoxic lake water incubations (Rissanen et al. 2018). In all experiments with nitrate, complete denitrification was observed within first 30 days. On the other hand, the elevated ammonium concentrations in the slurries with both nitrate and manganese relative to other amendments indicated active organic matter mineralization with the oxidants (Canfield et al. 1993) or potential microbial processes such as dissimilatory reduction of nitrate to ammonium (DNRA) (Burgin and Hamilton 2007). While the addition of metal oxides did not enhance methane oxidation, dissolved Fe and Mn concentrations did increase over incubation time (Figure S4), indicating reduction of iron and manganese oxides. So far, aerobic methanotrophs have not been shown to be capable of direct metal reduction in anoxic sediments, but have been demonstrated to switch to anaerobic dormancy with endogenous metabolism for long-term survival in anoxic environments (Roslev and King 1994, 1995). Recently, genomic studies have revealed that Methylobacter had the capacity to encode respiratory nitrate and nitrite reductases (Svenning et al. 2011; Hamilton et al. 2015) and methane oxidation coupled to nitrate/nitrite reduction have been experimentally shown under oxygen limitation by *Methylomonas* and *Methylomicrobium* (Kits et al. 2015a; b). The inability to detect AOM or much lower rates in the incubations with nitrate, however,

excludes the possibility that aerobic methanotrophs in the sediments used nitrate as electron acceptors to oxidize methane.

At this point, we are unable to constrain the electron acceptors involved in the anoxic lake sediments based on the performed slurry incubation experiments, as none of the electron acceptors used resulted in the stimulation of AOM. Thus, alternative electron acceptors that were already present in the lake sediments (e.g., humic substances) could be involved. Indeed, humic substances have been recognized as a viable electron acceptor through the reduction of their quinone moieties (Lovley et al. 1996; Scott et al. 1999) and organic electron acceptors in the wetlands such as humic acids were suggested to play a role in AOM (Smemo and Yavitt 2011; Blodau and Deppe 2012). In addition, ANMEs from marine sediments were recently demonstrated to use humic substance analogues (i.e., AQDS and humic acids) as electron acceptors in the absence of sulfate (Scheller et al. 2016). Furthermore, humic substance as electron shuttles could even enhance the microbial capacity to reduce less accessible electron acceptors (e.g., insoluble Fe (III) oxides) (Lovley et al. 1996). All the lakes investigated here are rich in humic substances, which has the potential to be the electron acceptors for methane oxidation in anoxic lake sediments. External addition of oxidants in the slurries may change the redox condition of the systems and favor the growth of specific microorganisms, which outcompeted potential methanotrophs. This could to some extent explain the suppressed AOM rates or complete inhibition of methane oxidation in the incubations with added substrates. However, further investigations are apparently required to explore the role of humic substances in the methane oxidation and the specific biochemical mechanism mainly by Methylobacter in anoxic lake sediments.

Conclusions

Our results of in situ AOM rate measurements combined with the molecular data indicate that methane oxidation in the surface lake sediments could be mainly due to type I aerobic methanotrophs belonging to the Genus *Methylobacter*. Our slurry incubations indicated that none of the added oxidants served as the electron acceptor responsible for the observed methane oxidation. However, we proposed that humic substances might be an alternative electron acceptor in the anoxic sediments that were rich in organic matter. Under oxic or micro-oxic conditions, *Methylobacter* are able to

use oxygen as the oxidant and oxidize methane, however, under anoxic condition, these "aerobic" microorganisms might switch to anaerobic respiration and likely utilized humic substances as the terminal electron acceptor. While the exact mechanism remained elusive, we could not rule out the possibility that these aerobic methanotrophs were associated with heterotrophic anaerobic microorganisms such as denitrifiers or iron-reducers detected in the lake sediments for extracellular electron transfers. Compared with other inorganic electron acceptors, the proposed mode of AOM involving humic substances as potential electron acceptors has not been proposed for freshwater lake sediments but certainly deserves further investigation. Given the widespread distribution of humic substances in lacustrine environments, hmuics-dependent AOM may play an unrecognized role in reducing methane emissions from anoxic lake sediments.

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Supporting Information

Supplementary Figures

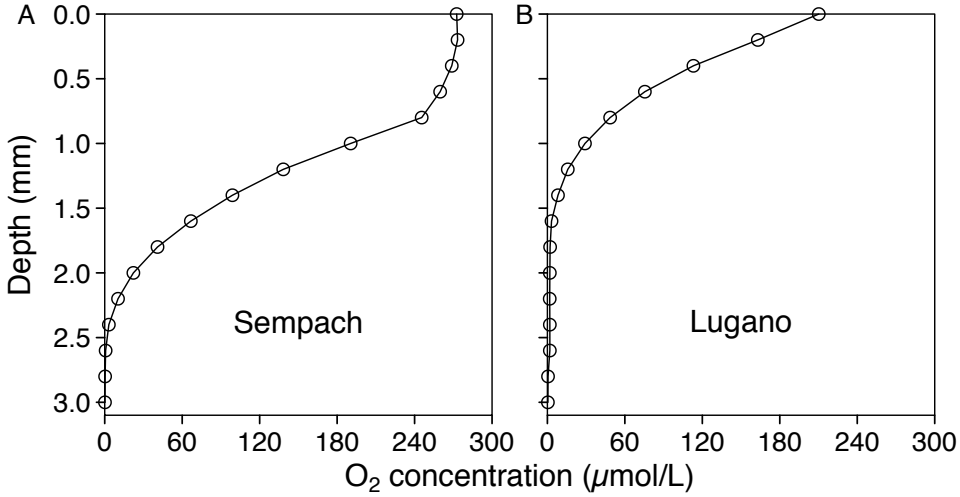


Figure S1. Oxygen profiles of sediment cores incubated under oxic conditions from (A) Lake Sempach (March 2015) and (B) Lake Lugano (Melide, February 2015).

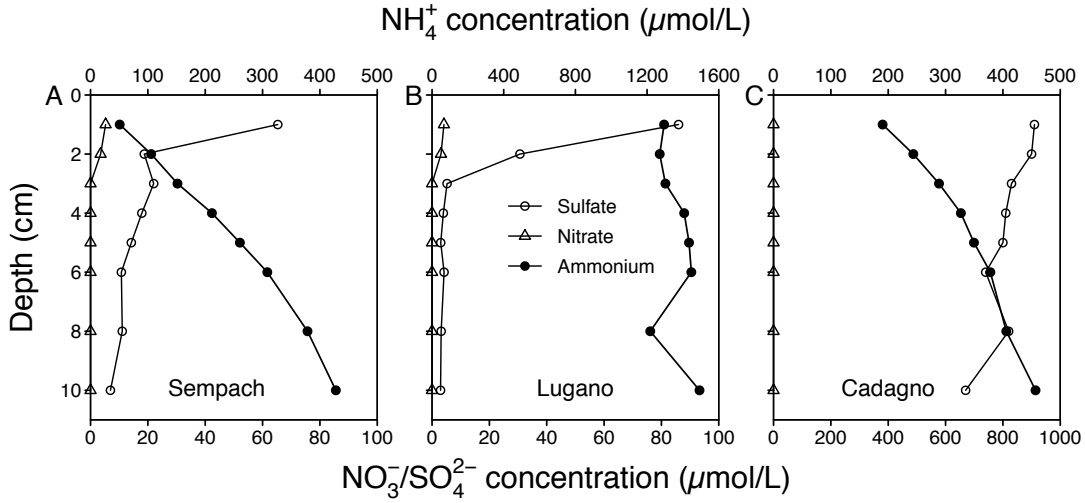


Figure S2. Porewater nutrient concentrations in the sediments of (A) Lake Sempach (March 2015) (B) Lake Lugano (Melide, February 2015) and (C) Lake Cadagno (October 2016).

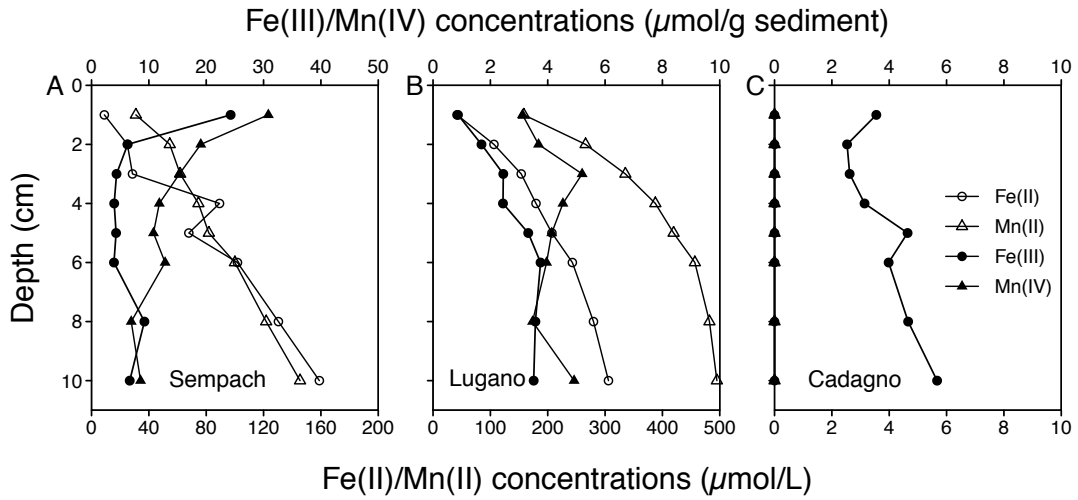
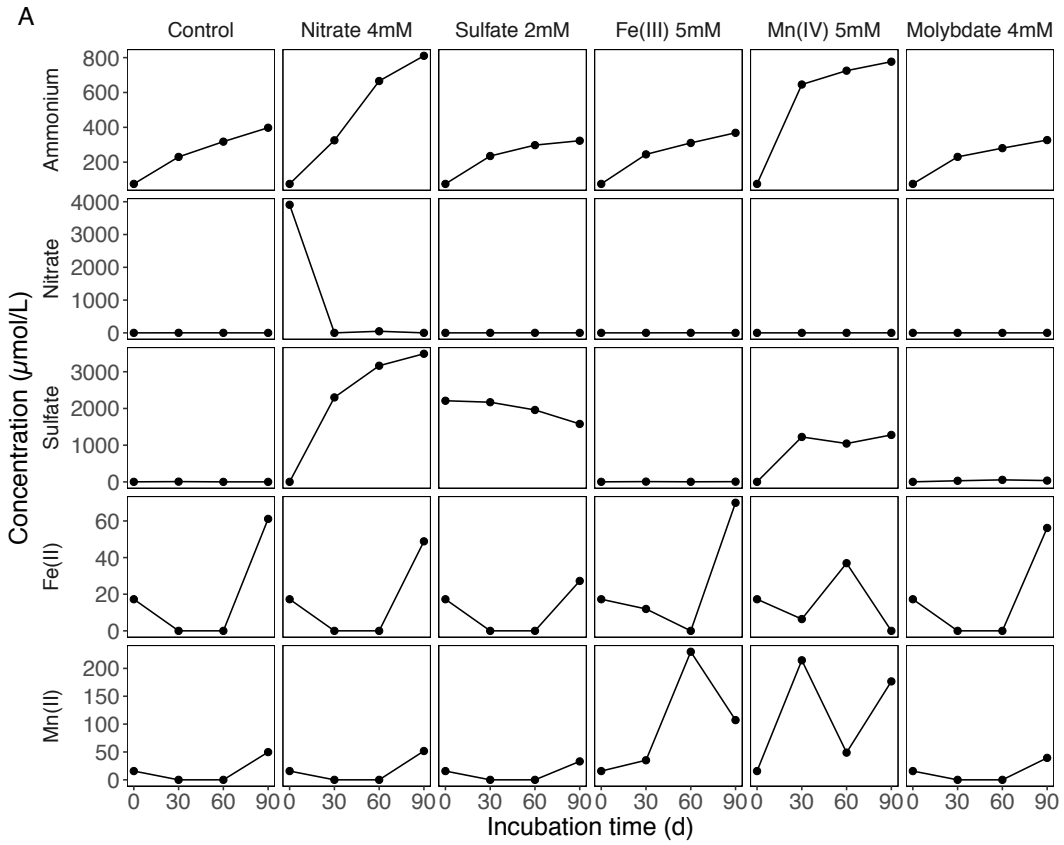


Figure S3. Concentration profiles of dissolved Fe^{II}, Mn^{II}, HCl-extractable Fe^{III} and solid Mn-phases in the sediments of (A) Lake Sempach (March 2015) (B) Lake Lugano (Melide, February 2015) and (C) Lake Cadagno (October 2016).



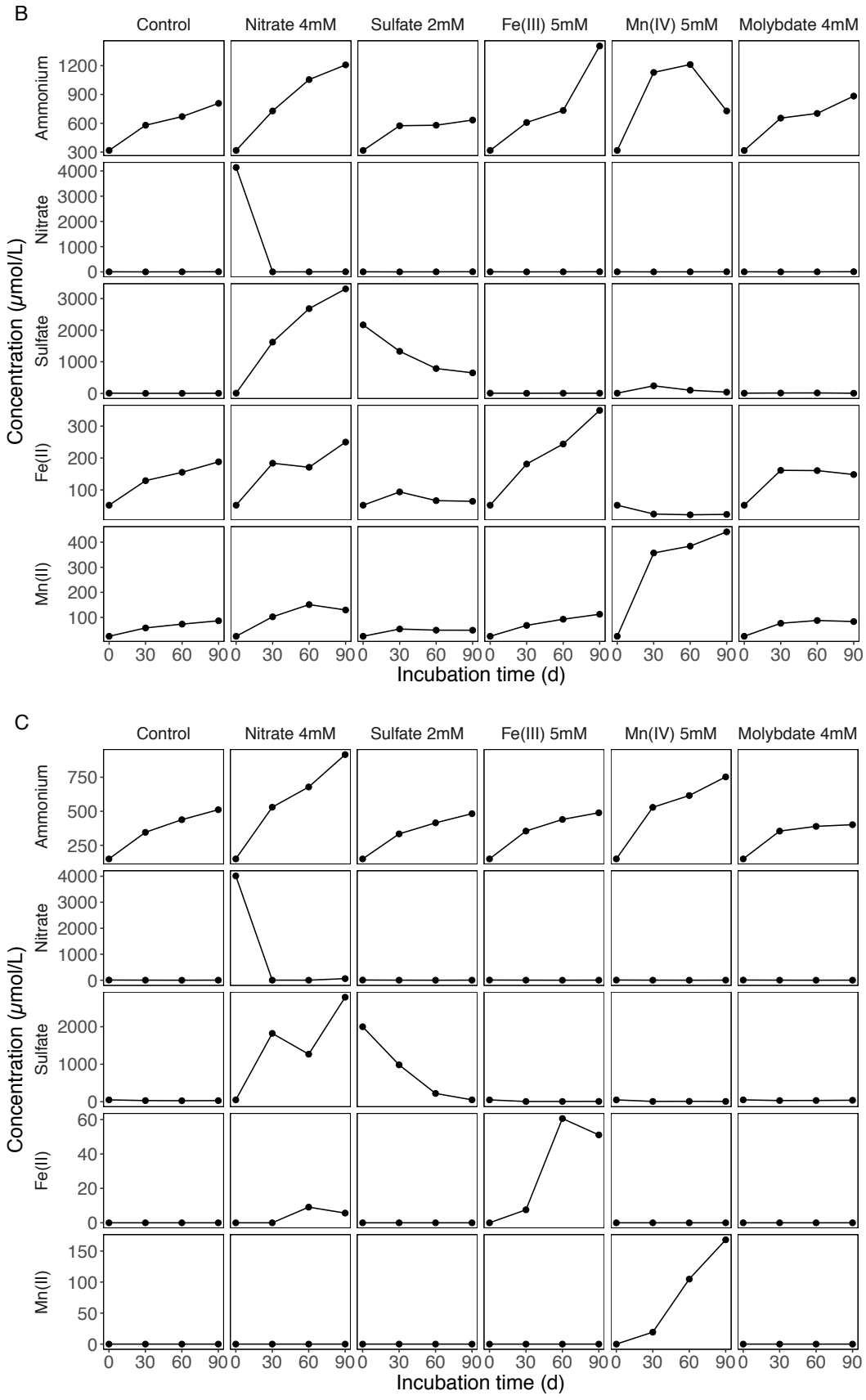


Figure S4. Time course concentration of ammonium, nitrate, sulfate, dissolved Fe(II) and Mn(II) in the supernatant of incubation slurries using surface sediments from (A) Lake Sempach, (B) Lake Lugano (Figino) and (C) Lake Cadagno and amended with different substrates.

Chapter 6: Evaluating radioisotope-based approaches to measure anaerobic methane oxidation rates in lacustrine sediments

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Abstract

The microbial anaerobic oxidation of methane (AOM) is the dominant sink for methane in anoxic sediments. AOM rate measurements are essential for assessing the efficacy of the benthic methane filter to mitigate the evasion of this potent greenhouse gas to the atmosphere. Incubation techniques with trace amounts of radiolabelled substrate (typically $^{14}CH_4$) represent the most sensitive approach for methane oxidation rate measurements. Yet, radiotracer application can be performed in different ways, rendering the comparability of AOM rate measurements in field and laboratory investigations problematic. We compared four different $^{14}CH_4$-based short-term incubation approaches to quantify methane turnover rates in lake sediments. Three of the applied methods (i.e., sub-core, glass syringe and whole-core incubations) yielded similar and reliable downcore rate profiles. They provided clear evidence for AOM with maximum rates of 15 nmol/cm^3/d at ~17 cm sediment depth. Using the short-term slurry incubation method, however, we were unable to detect the AOM activity maximum that we observed with the other approaches. We hypothesize that changes in the microbial structure and disruption of physical interactions due to mixing of sediments negatively affected the activity of AOM communities and longer incubation times are necessary to enhance the sensitivity of this approach. Minor variabilities in rate measurement that we found in the non-slurry incubation may be related to small-scale sediment heterogeneity, differential partial methane loss during sample handling, and/or an uneven application of the radio-tracer. Whole-core incubations interfere the least with the in-situ conditions, but the ultimate choice of the AOM rate measurement method will depend on the individual sampling requirements.

Introduction

Methane (CH_4) is an important contributor to greenhouse forcing (Lelieveld et al. 1998), providing a strong rationale for characterizing global fluxes of CH_4. Lakes represent an important source of CH_4 to the atmosphere (Bastviken et al. 2004; Borrel et al. 2011) and most of the lacustrine methane is produced by methanogenic microorganisms within anoxic sediments. Yet, a large fraction (57%-100%) of the biogenic CH_4 produced is oxidized by methanotrophic microorganisms within anoxic or oxic sediments, or in the water column (Bastviken et al. 2002; Schubert et al. 2011;

Blees et al. 2014a, b). The anaerobic oxidation of methane (AOM) has mostly been studied in benthic marine environments and it is typically coupled to sulfate reduction (Boetius et al. 2000; Knittel and Boetius 2009), but other electron acceptors are possible (Ettwig et al. 2010, 2016; Sivan et al. 2011; Haroon et al. 2013; Deutzmann et al. 2014; Cai et al. 2018). Evidence for AOM in freshwater environments is rare and not always conclusive (Schubert et al. 2011; Crowe et al. 2011; Bray et al. 2017). In contrast, there is multiple evidence for aerobic methane oxidation (MOx) in lakes, and a great diversity of bacteria and niches of the different aerobic methanotrophs were described (Hanson and Hanson 1996; Blees et al. 2014a, b; Oswald et al. 2016). Independent of the mode of methane oxidation, microbes play a pivotal role in modulating lacustrine methane fluxes and mitigating CH_4 emissions to the atmosphere, with important implications for the global CH_4 budget (Reeburgh 2007).

AOM and MOx rate measurements are essential for evaluating the efficacy of the sequential biological methane filter described above, and to better understand the controls on the balance between CH_4 production and consumption. Environmentally realistic methane oxidation rates also allow us to better understand the observed methane concentrations and the discrepancy between high production of methane in the sediments and relatively low flux of methane to the atmosphere (Bastviken et al. 2004; Reeburgh 2007). Despite a plethora of methane dynamics-related studies in lakes focusing on net fluxes of methane in the water column or to the atmosphere (e.g. Bogard et al. 2014; Blees et al. 2015; Wik et al. 2016), we still only know little about absolute rates and their controls on anaerobic methane oxidation in lakes, particularly in anoxic lake sediments. Similarly, our knowledge about the electron acceptors involved in AOM in freshwater environments, where sulfate-based AOM is likely to be less important than that in marine environments, is still rudimentary. Ongoing and future work to address this knowledge gap relies on the capacity to provide accurate and reliable quantitative assessments of the location and magnitude of methane oxidation in lacustrine sediments.

Multiple geochemical and microbiological approaches exist to elucidate methane oxidation pathways in marine and freshwater environments. Potential sites of methane oxidation, both in the water column and within sediments, may be pinpointed from CH_4 concentration profiles, characteristic stable carbon isotope signatures of CH_4 or through phylogenetic analyses of the microbial community and specific microbial biomarkers. Approaches to actually quantify methane oxidation rates in sediments

include the application of one-dimensional numerical reaction-diffusion models (Jørgensen et al. 2001), flux measurements combined with analysis of methane concentration gradients (Reeburgh 1976), monitoring of CH_4 concentration changes in sediment slurry incubations (Nauhaus et al. 2002; Hershey et al. 2015), as well as incubations with $^{13}CH_4$ (Moran et al. 2008; Beal et al. 2009; Egger et al. 2015), or with a radioactive tracer such as ^{14}C-CH_4 (Reeburgh 1980; Iversen and Blackburn 1981; Boetius et al. 2000; Segarra et al. 2013; Norði et al. 2013; Steinle et al. 2016).

For $^{13}CH_4$ and $^{14}CH_4$ assays, the incorporation of labeled methane into metabolic products is quantified by stable isotope ratio mass spectrometry (IRMS) and liquid scintillation counting, or less common, by accelerator mass spectrometry (Pack et al. 2011). Tracer-based approaches are generally most direct, leaving little doubt as to which process is quantified. Yet, stable isotope (i.e., $^{13}CH_4$) derived rate measurements can be problematic in carbonate-rich sediments, where the dissolved inorganic carbon (DIC) concentration in porewater (i.e., the natural ^{13}C background) is very high, so that excess ^{13}C-DIC from methane turnover is difficult to quantify. The radiolabel $^{14}CH_4$ incubation approaches are more sensitive than $^{13}CH_4$ based methods, and the measured rates tend to be more precise because high background levels are not an issue (Reeburgh 1980; Iversen and Blackburn 1981; Iversen and Jørgensen 1985; Treude et al. 2003). Thus, despite the strict safety regulations for handling radio-tracer materials in the field and in the laboratory, the $^{14}CH_4$ incubation (with standard decay counting techniques) is the most commonly used method to measure rates of anaerobic methane oxidation in marine settings. In contrast, radiolabel AOM rate measurements in lake sediments are still rather sparse (Deutzmann and Schink 2011; Norði et al. 2013).

To obtain depth-specific rate profiles in marine or lacustrine sediments, radiotracer application can be performed in different ways. $^{14}CH_4$ can be applied by the whole-core-injection method (Jørgensen 1978), where the dissolved tracer is injected at various sediment depths, either into the sediment cores directly (Iversen and Blackburn 1981), or in sub-sampled push-cores (Jøgensen 1977; Treude et al. 2003; Niemann et al. 2006; Steinle et al. 2016). Radiotracer can also be applied to discrete samples after down-core subsampling with cut-off glass syringes, either directly to the syringe (e.g., Treude et al. 2003; Niemann et al. 2005; Norði et al. 2013) or after transfer of the sediment slurries to separate incubation vials. Moreover, depending on the approach, the $^{14}CH_4$ can be applied as gas bubble or injected in

dissolved form. The different approaches are likely to have different strengths and limitations. The best choice of application for a given environment will represent a compromise between minimizing the use of radioactive material/costs and maximizing the sensitivity of rate measurements, with minimum interference with the natural conditions. To the best of our knowledge, there has been no comprehensive study to compare different $^{14}CH_4$ incubation methods for measuring depth-specific methane oxidation rates, neither in marine nor in lacustrine sediments.

In this study, our objective was to compare and evaluate different radioactive $^{14}CH_4$ tracer incubation methods to quantify anaerobic methane oxidation in lake sediments. We demonstrate that despite minor variations in the absolute rates measured, which may be related to the differences in subsample handling, all but one of the methods tested (i.e., sub-core, glass syringe and whole-core incubations) allowed us to detect AOM in the studied lake sediments, providing a coherent picture as to where in the sediment column AOM occurs.

Materials and Procedures

Study site

Lake Cadagno is an alpine meromictic lake located in the southern Alps of Switzerland (46°33'03"N 8°42'42"E). A chemocline located between 9 m and 14 m depth separates the oxic mixolimnion, the upper layer in a meromictic lake, from the sulfidic monimolimnion (the lower layer). Due to water infiltration from high-ionic strength subaquatic springs, Lake Cadagno features relatively high concentrations of sulfate (> 1 mmol/L) (Dahl et al. 2010). Previous work has provided geochemical evidence for AOM in the sediments of Lake Cadagno (Schubert et al. 2011), but no rate measurements were performed. Sediment samples were collected from a sampling platform in the deepest part of the lake, at 21 m depth.

Sample processing

Using a gravity corer, a total of six undisturbed sediment cores (inner diameter 62 mm, PVC) were recovered in October 2016. For the analysis of dissolved CH_4 concentrations and $\delta^{13}CH_4$ in the porewater, 3 ml of wet sediment was taken at different depths immediately after core recovery. Sediment samples were collected through pre-drilled holes at a 2-cm resolution using cut-off 3 mL syringes,

immediately transferred into a 20 ml glass serum vial containing 7 ml 10% NaOH, and sealed with black bromobutyl stoppers (Rubber B. V., The Netherlands, part nr.: 7395; Niemann et al. 2015). One sediment core for porewater extraction and four additional cores for rate measurement were taken back to the home laboratory and stored at 4 °C in the cold room until further processing on the next day.

For AOM rate measurements, we used four different approaches (for details see the supplementary file): 1) sub-core incubation (SC), 2) glass syringe incubation (GS), 3) whole-core incubation (WC) and 4) slurry incubation (SL). For the SC treatment, one of the intact gravity cores was subsampled vertically by inserting three smaller push-core liners (inner diameter of 16 mm, and 29 cm long, PVC) (Jøgensen 1977; Treude et al. 2003; Niemann et al. 2006; Steinle et al. 2016). Care was taken that the water-sediment interfaces in both the original core and the push-cores were approximately at the same level. Sub-cores were then closed with grey thick rubber stoppers without headspace. 20 µl $^{14}CH_4$ tracer dissolved in anoxic water (activity ~ 0.23 kBq) was injected into each sub-core through the side-holes sealed with silicon gel at a depth interval of 1.5 cm. For the GS treatment, triplicate samples were taken every 2 cm from a gravity core through pre-drilled side ports using cut-off glass syringes, which were then sealed with black butyl stoppers. The same $^{14}CH_4$ solution as for the SC incubations was injected (20 µl) directly into each of the glass syringes containing 4 ml sediment. For the WC treatment (Jørgensen 1978), similar to the SC treatment, the entire gravity core was spiked with $^{14}CH_4$ tracer through pre-drilled side-holes sealed with silicon gel at 2-cm intervals. Given the much larger sediment volume in the whole-core, we had to introduce a substantially higher amount of ^{14}C-CH_4 to yield consistent relative activities (^{14}C-CH_4 per volume of incubated sediment) across the different approaches applied here. To account for this, the radiotracer was always injected as a gas bubble (20 µl) for WC treatments. Finally, for the SL treatment, 4 ml wet sediment samples were taken from a fourth sediment core through pre-drilled side ports every 2 cm by using 5 ml cut-off syringes. Samples were then transferred into 20 ml glass serum vials containing autoclaved and anoxic sulfate-amended water, at sulfate levels that are representative for the sediment-water interface in Lake Cadagno (i.e., ~1 mM). Sediment samples were gently pushed into the vials and then closed headspace-free with polytetrafluoroethylene (PTFE) coated gray chlorobutyl stoppers (Wheaton, USA, part No.: 224100-175, Niemann et al. 2015). In order to eliminate potential O_2 contamination, slurry samples were pre-

incubated for approximately two hours (i.e., until no oxygen was detected in parallel control vials fitted with TRACE O_2 sensor spots (PyroScience, Germany) for O_2 concentration monitoring) before the tracer was added. Then, 20 µl of the same tracer used for both SC and GS was injected into the 20 ml vials containing the slurries. Immediately after the tracer injection, all samples were incubated for 48 hours, at in situ temperature (4 °C) and in the dark.

At the end of the incubations, bacterial activity was stopped by adding aqueous NaOH (5% w:w). For SC samples, 2 cm segments were pushed out of the sub-core liner, directly transferred into 100 ml glass bottles (DURAN GL45) containing 20 ml of the aqueous NaOH solution. The bottles were closed quickly with thick butyl rubber stoppers (Ochs Glasgerätebau, Germany, order nr.: 444704) and shaken thoroughly to equilibrate the porewater methane between the aqueous and the gas phase. Biological activity of sediments in glass syringes, and slurries in incubation vials, was stopped in the same way, i.e., samples were transferred into 100 ml bottles and fixed with 20 ml NaOH (5% w:w). For the termination of the whole-core incubation, the core was extruded, and triplicate samples (~ 4 ml) were collected from 2 cm slabs from the sediment, using 20 ml cut-off syringes, and quickly transferred into bottles with NaOH solution.

Porewater and sediment geochemical analyses

Methane concentrations in the headspace were measured with a gas chromatograph (GC, Agilent 6890N) linked to a flame ionization detector, using hydrogen as a carrier gas (e.g., Blees et al. 2014b). The carbon isotope composition of CH_4 from the headspace was determined using a mass spectrometer (T/GAS PRE-CON, Micromass UK Ltd), connected to a preconcentration unit (e.g., Oswald et al. 2015). Stable carbon isotope ratios are reported in the conventional δ-notation (in ‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB). The $\delta^{13}C$ values of methane have an analytical error of ± 1‰.

Porewater was extracted by centrifugation of the sediment and the supernatant was filtered through 0.45 µm membrane filters (PES, Sarstedt, Germany). Porewater concentrations of sulfate was analyzed by ion chromatography (882 Compact IC plus, Metrohm, Switzerland) (Oswald et al., e.g., 2016a). Porewater samples for sulfide measurements were fixed with aqueous Zn acetate (5% w/w) immediately after filtration, and sulfide concentrations were determined spectrophotometrically using

the Cline method (Cline 1969). Dissolved inorganic carbon concentrations were quantified using a total carbon analyzer (TOC-5050A, Shimadzu, Kyoto, Japan; Sullivan et al. 2013).

Methane oxidation rate (MOR) measurements

$^{14}CH_4$ activity measurement: Following the procedure of Treude et al. (2003), samples from all the different incubation experiments were treated the same way after the incubations were stopped (Treude et al. 2003; Mau et al. 2013; Blees et al. 2014). In brief, the headspace of a fixed sample was purged with air (30 ml/min for 30 min) through a heated (850 °C) quartz tube filled with copper oxide, where the residual $^{14}CH_4$ was combusted to $^{14}CO_2$. The produced $^{14}CO_2$ was then captured in two scintillation vials (20 ml) containing 8 ml of CO_2-absorbant (a mixture of phenylethylamine and methoxyethanol with a volume ratio of 1/7). The cumulative sample radioactivity was then determined by liquid scintillation counting (2200CA Tri-Carb Liquid Scintillation Analyzer) after the addition of 8 ml of a scintillation cocktail (Ultima Gold, PerkinElmer) to each vial and thorough mixing using a vortex-mixer.

$^{14}CO_2$ analysis: For the quantification of microbially produced $^{14}CO_2$, we proceeded as previously described (Treude et al. 2003; Mau et al. 2013), with some modifications. In brief, product of the $^{14}CH_4$ oxidation was released from the alkaline liquid phase (i.e., from the carbonate pool of the NaOH-fixed sample) by adding 3 ml of concentrated HCl (37%), shifting the carbonate equilibrium towards CO_2. The $^{14}CO_2$ was subsequently purged and trapped as described above. Measured $^{14}CO_2$ radioactivities were blank-corrected by subtraction of the average amount of $^{14}CO_2$ recovered in killed controls (Treude et al. 2005). Excess $^{14}CO_2$ activity was considered to be zero if the corrected value was lower than three times the standard deviation of the control mean values.

Remaining activity: A minor fraction of the labeled methane may be assimilated into biomass (Blees et al. 2014a). Therefore, after the $^{14}CO_2$ activity measurement, we determined the remaining radioactivity in a 2 ml aliquot of the acidified mixture (amended with 4 ml Ultima Gold) by liquid scintillation counting (Blees et al. 2014a, b).

AOM rate calculation: First order rate constants (k) were calculated using the activity of the three different carbon pools (Eq. 1).

$$k = \frac{A_{CO_2} + A_R}{A_{CH_4} + A_{CO_2} + A_R} \times t^{-1} \qquad (1)$$

where A_{CH_4}, A_{CO_2}, and A_R represent the radioactivity of CH_4, CO_2, and the residual radioactivity (which includes biomass and metabolic intermediates), respectively, and *t* represents the incubation time in days. Methane oxidation rates (MOR) were then calculated using the values for k and the methane concentration at the start of the incubation (Eq. 2).

$$MOR = k \times [CH_4], \quad in\ \mu mol/cm^3/d \qquad (2)$$

For $[CH_4]$ we used the in situ methane concentration determined separately in the core dedicated to geochemical analyses. Given that the tracer addition did not increase the total methane concentration in a sample much over in situ levels, calculated MOR values can be considered to be absolute rates. As all incubations were conducted under anaerobic conditions, with sediments/slurries containing substantial amounts of sulfide as an oxygen buffer, MOR determined here represent rates of AOM rather than MOx.

Assessment and Discussion

Geochemical profiles

Methane concentrations in the anoxic Lake Cadagno sediments displayed maximum values between 3-4 mmol/L in the lower parts of the sediment core (Fig. 1a) and decreased towards the sediment-water interface to < 0.2 mmol/L. The methane depth distribution thus suggests that methane is oxidized within the sediments. The sulfate profile showed an inverse pattern with porewater concentrations decreasing from 0.9 mmol/L at the sediment surface to 0.01 mmol/L at 30 cm depth. Considerable amounts of sulfide (~ 1 mmol/L) were detected between 3 and 15 cm, providing clear evidence for sulfide production from microbial sulfate respiration. However, in contrast to most diffusive marine settings, a clear sulfate-methane transition zone that would allow us to pinpoint the location where in the sediments sulfate-dependent AOM rates are highest was not observed. Relatively high concentrations of both methane and sulfate coexisted in the surface sediments, indicating favorable conditions for AOM down to 30 cm sediment depth.

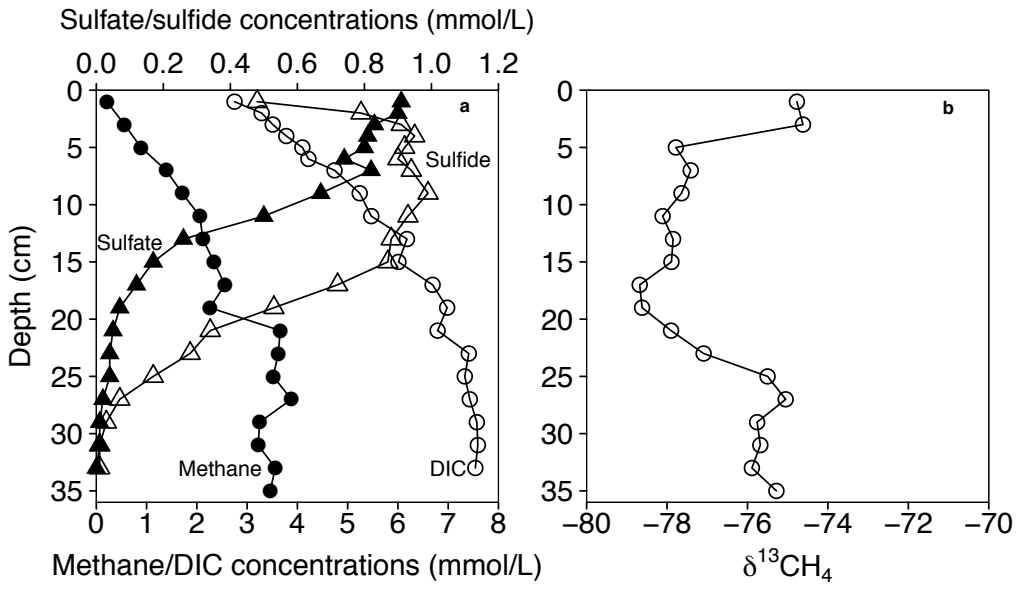

Fig. 1. (a) Porewater concentrations of methane (CH_4, filled circles) dissolved inorganic carbon (DIC, open circles), sulfate (SO_4^{2-}, filled triangles) and total sulfide (H_2S, open triangles) in Lake Cadagno sediments (October 2016). (b) Stable carbon isotope ratios ($\delta^{13}CH_4$) of dissolved methane in the porewater. The solubility of CH_4 in water at in-situ temperature (4°C) is 2.25 mmol/L.

Previous work has provided putative evidence that AOM is constrained to the uppermost sediment layers, with relatively strong positive shifts in the methane $\delta^{13}C$ towards the sediment water interface (Schubert et al. 2011). AOM tends to increase the porewater carbonate alkalinity through production of bicarbonate, yet a clear sediment horizon where AOM takes place was not evident from the DIC concentration profile (Fig. 1a). Moreover, a typical methane concentration-to-carbon-isotope relationship, with strongly increasing methane $\delta^{13}C$ values with decreasing methane concentrations right underneath the sediment surface, in contrast to previous work by Schubert et al. (2011), was not observed (Fig. 1b). In general, $\delta^{13}CH_4$ profiles alone may be misguiding with respect to assessing methane oxidation, because multiple microbial processes (including hydrogenotrophic and acetoclastic methanogenesis) can be involved, co-occurring at corresponding sediment depths and producing interfering isotope signatures. The methane $\delta^{13}C$ in this study showed only a relatively subtle increase from -78.7‰ to −74.8 ‰ below the sediment-water interface. Thus, both the porewater biogeochemical as well as the $\delta^{13}CH_4$ profiles in this study are clearly more ambiguous with respect to where exactly methane oxidation within the sediments may take place primarily, highlighting the necessity to measure AOM rates as a function of sediment depth.

AOM rates

The vertical AOM rate profiles obtained from three of the different incubation methods (SC, GS, WC) displayed very similar patterns (Fig. 2a, b and c). Generally, no or very low AOM activities were detected in the upper sediment layers. Methane oxidation rates started to increase below ~ 9 cm and highest rates were consistently observed at 17 ± 1 cm below the sediment-water interface (i.e. 15.9 ± 2.1, 15.2 ± 8.8 and 14.7 ± 2.6 nmol/cm^3/d when measured with the SC, GS, WC approach, respectively). Below the maximum, methanotrophic activity decreased rapidly, with AOM rates of 2.1 ± 1.2, 1.6 ± 0.8 and 1.4 ± 0.7 nmol/cm^3/d at 23 cm sediment depth (cmsd) respectively. Thus, all three approaches provided clear and consistent geochemical evidence for AOM in the sediments of Lake Cadagno, and the results agreed well with regards to the location of, and maximum rates within, the AOM zone. It is beyond the scope of this study to discuss in detail the biogeochemical and ecological context of the observed AOM rate profiles (i.e., potential electron acceptors and microbial players involved). But what becomes clear from the data set presented here is that all three approaches (SC, GS, WC) are useful to reliably estimate the biologic methane filtering capacity within the sediments (Fig. 2 and Fig. 3), and our results imply that ecosystem methane flux comparisons remain unbiased in the event that more than one of the three different AOM rate measurements techniques are involved. Generally, the AOM rates in Lake Cadagno sediments as obtained from the non-slurry incubations (SC, GS and WC) were comparable to those reported for other freshwater lake sediments (Norði et al. 2013) and wetlands (Segarra et al. 2013, 2015). Compared to marine sediments, they fell within the lower range of reported AOM rates (Iversen and Blackburn 1981; Iversen and Jørgensen 1985; Treude et al. 2003; Niemann et al. 2006, 2009; Steinle et al. 2016).

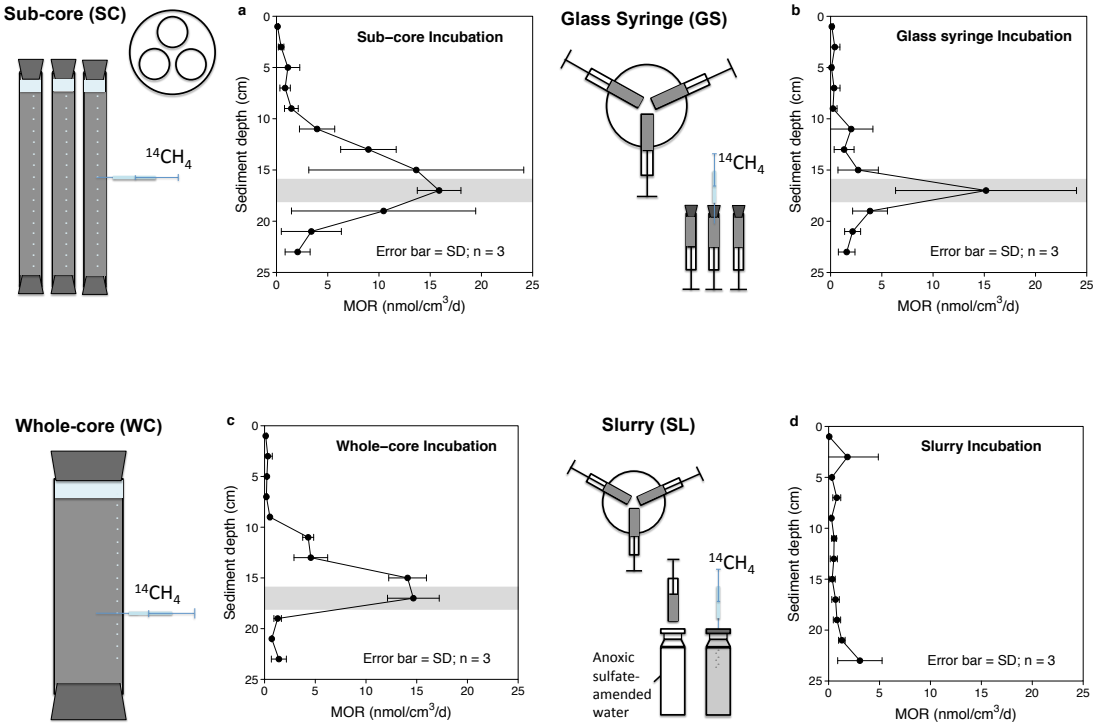

Fig. 2. Depth-specific in-situ methane oxidation rates (MOR) determined by different radioisotope based approaches: (a) sub-core incubation, (b) glass syringe incubation, (c) whole-core incubation, and (d) slurry incubation. Error bars represent standard deviations of triplicate measurements and the grey bars indicate the maximum rates at 17 cm depth.

Although the patterns of rate profiles in the sediments were similar among the non-slurry approaches, discrepancies were observed for single depths above and below the AOM rate peak, which resulted in considerable differences in the integrated rates within the top 23 cm of sediment (SC: 122.5 ± 19.5 nmol/cm^2/d; GS: 58.2 ± 19.9 nmol/cm^2/d; WC: 83.3 ± 8.6 nmol/cm^2/d). Moreover, between different approaches, the reproducibility was quite variable. For example, a significantly higher standard deviation was observed for the rate maximum determined by SC compared to WC (e.g., at 15 cm 13.65 ± 10.49 for SC and 14.10 ± 1.86 for SC). We cannot fully exclude that part of the data scatter is due to true natural variability. Yet, the relatively large error for the maximum AOM rates in the SC treatment can be easily explained, when considering the single replicate *k* profile (Fig. 3). While maximum k values for both GS and WC were found at 17 cm in all triplicates, depth-dependent k values for the three different sub-cores were located at 15, 17, and 19 cm depth, respectively, indicating a shift of the sediment horizons due to differential sediment

compression/displacement when pushing the sub-cores into the gravity core (this effect can be observed visually during subsampling). To account for this effect, we aligned the profiles of the replicate methane oxidation rate measurements (correction for the ±1-cm offset), yielding more consistent depth profiles, and the standard deviations for the peak-rates are much lower (Fig. 4). In addition to displacement, smearing of sediments during core introduction may result in a further shift of 'sediment depths' in the sub-cores. Finally, sediment heterogeneity or minor tilting of the sediment layers in the parent core may also contribute to the observed discrepancies (Treude et al. 2003). For future studies, we recommend that the degree of core compaction may be verified, for example through comparative measurements of porewater sulfate concentrations in both the parent and sub-cores.

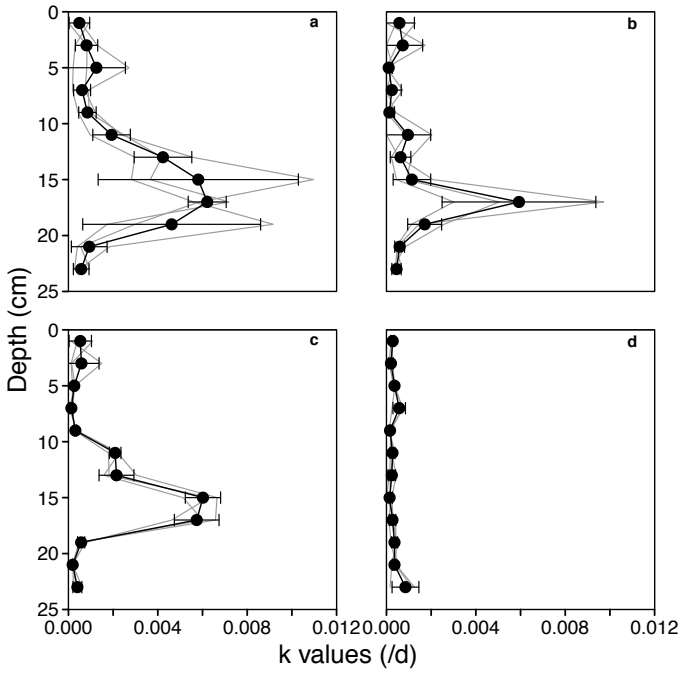

Fig. 3. Depth-dependent first order rate coefficients (k values) for AOM based on different incubation approaches: (a) SC, (b) GS, (c) WC and (d) SL. Mean (black filled circles) and standard deviation of the three replicate measurements (grey lines) are shown.

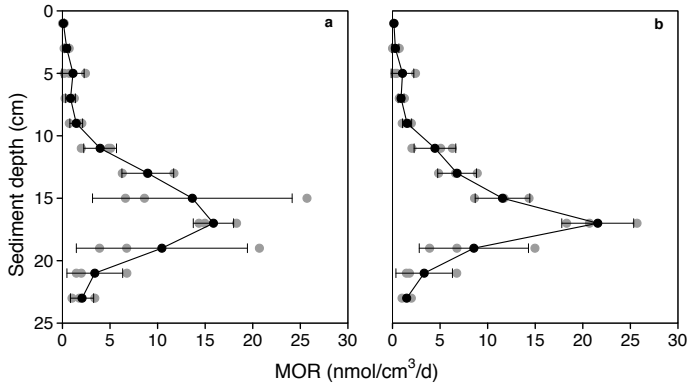

Fig. 4. AOM rate profile of SC incubation (a) before and (b) after depth correction (± 1-cm offset) for two sub-cores. Mean (black filled circles) and standard deviation of the three replicate measurements (grey filled circles) are shown. Lower standard deviations for the peak-rates were achieved after alignment.

To some extent, the rate differences observed between methods and replicates may also be attributed to differential methane loss, which will result in a lower total radioactivity and can have a biasing effect on the final AOM rates. Some approaches may be more susceptible to methane loss than others during handling. In order to assess whether parts of methane were lost, total radioactivity in the AOM samples can be compared with the radioactivity of injected tracer. It is important to note, however, that variation in the total radioactivity will not affect tracer turnover, and in turn the calculation of k values. Only when there is methane loss after the AOM incubation, the final absolute rate will be overestimated because the residual $^{14}CH_4$ radioactivity is underestimated, leading to increased k values (see Eq. 1).

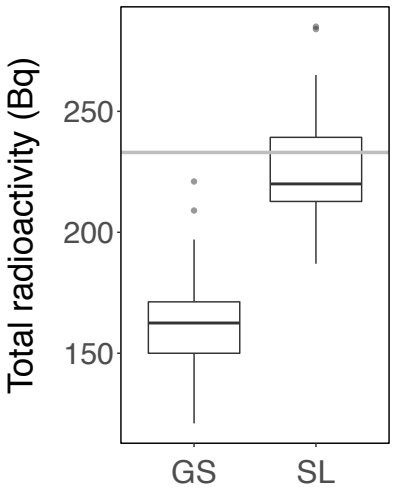

Fig. 5. Box-and-whisker plot showing total radioactivities of AOM samples at the end of glass syringe (GS, n=36) or slurry (SL, n=36) incubations. The grey line indicates the theoretical total radioactivity of 20 µl $^{14}CH_4$ tracer (~ 233 Bq). The horizontal black line represents the median total radioactivities of the respective datasets, the outer horizontal lines of each box represents the upper and lower quartiles, and the ends of the vertical lines represent minimum and maximum scores. The black dots represent the outliers and were excluded from the datasets. Total radioactivities can only be reliably assessed in the case where the ^{14}C tracer was added to a confined volume (not the case for SC and WC).

Radioactivity yields can best be assessed in the two approaches where the ^{14}C tracer is injected into a defined and closed volume (i.e., SL and GS). Total radioactivities for SL samples (mean ± sd, 227 ± 23 Bq) were very similar to the directly measured value (20 µl, ~ 233 Bq) (Fig. 5), indicating no significant methane loss. In contrast, total radioactivities in the GS incubation samples were much lower (mean ± sd, 162 ± 22 Bq), although the same amount of $^{14}CH_4$ was injected. The lower total radioactivity can possibly be attributed to diffusive methane loss at the plunger side of the glass syringe during the incubation. Theoretically, only $^{14}CH_4$ loss occurring at the end of incubation or during AOM sample transfer would result in overestimated rates (Treude et al. 2005; Knab et al. 2008). At least at some depths, GS-based rates were, if at all, lower when compared to rates determined using SC and WC. This suggests that the apparent radiogenic methane loss occurred right at the beginning of the incubation, when the tracer was applied or $^{14}CH_4$ was lost continuously during incubation. Overall, the different non-slurry approaches provided a reliable quantification of actual AOM rates in the studied sediment, and the relatively good agreement between these approaches suggests that the $^{14}CH_4$ loss was not significant enough to markedly affect the calculated k values. At least, our data do not provide any clear evidence for one approach being more, or less, susceptible to $^{14}CH_4$ loss-driven AOM rate overestimation than the other (except for SL).

Most strikingly, the AOM rate profiles obtained from the slurry incubations (SL) were completely different compared to the other approaches (Fig. 2d). They did not reveal the clear pattern of AOM rates that we observed wit the other methods within the subsurface sediments, as absolute rates in the methane oxidation zone were barely above zero (i.e., 1.8 ± 3.0 nmol/cm^3/d at 3 cm, 1.3 ± 0. 2 nmol/cm^3/d at 19 cm, and 3.1 ± 2.2 nmol/cm^3/d at 21 cm). Hence, information as to where the main AOM zone is located could not be gained from the slurry-incubation based rate profiles**.** In contrast to SC, GS and WC incubations, the samples used for SL-AOM incubations

were mixed with anoxic sulfate-containing water, disrupting the sediment texture and changing the microhabitat completely. Anaerobic methanotrophs in marine sediments usually depend on syntrophic partners, e.g. sulfate-reducing bacteria, and AOM is often carried out by microbial consortia (Boetius et al. 2000; Orphan et al. 2002). If this was the case also in the lake sediments studied here, the perturbation during slurry preparation may have affected the structure of AOM-hosting aggregates and thereby compromised the methanotrophic activity in the SL incubations. The very low methane turnover (i.e., k values) also at depths where the other approaches revealed maximum AOM rates (Fig. 3), support the idea that low/absent AOM may be due to changes of the microenvironmental conditions and/or disruption of the interactions between consorting organisms. The effect, however, seems to be transient as slurry incubations have been used successfully to detect AOM in marine sediments, albeit with much longer incubation times than used in the present study here (Beal et al. 2009; Egger et al. 2015). Thus, we recommend that subsamples for methane oxidation rate measurement are pre-incubated for anaerobic conditioning of the microbial communities (Segarra et al. 2015), and that the main slurry experiments are performed over longer periods of time (i.e. > 2 weeks).

Concluding remarks

Here we compared four different $^{14}CH_4$-tracer-based techniques to assess depth-specific in situ AOM rate measurement in lake sediments. Overall, our data demonstrate that those approaches, which involved incubations of the undiluted sediments (i.e., not mixed to slurries) yielded consistent and reliable results, allowing us to pinpoint the zone of AOM and to quantitatively assess methane oxidation rates. Obviously, whole-core incubations induce minimal interference with in situ conditions, which may be particularly important where metabolic or physical interaction between the methane oxidizing microorganisms and partner bacteria are expected. The whole-core incubation approach produced the best reproducibility among the tested techniques, yet it requires larger quantities of $^{14}CH_4$, which implies higher costs and more radioactive waste. Moreover, in some situations, whole-core incubations may not be feasible. For example, when using long piston/gravity cores, commonly only one core exists, which in turn cannot be sacrificed for AOM measurements alone. Ultimately, the choice of the right AOM measurement method

will depend on the individual sampling requirement and must factor in potential needs for complementary (e.g., hydrochemical or molecular) analyses. While slurry incubations do not appear to be a feasible approach for in situ AOM rate determination, they are viable in longer-term experimental studies seeking, for example, to investigate AOM modes by testing the involvement of different electron acceptors.

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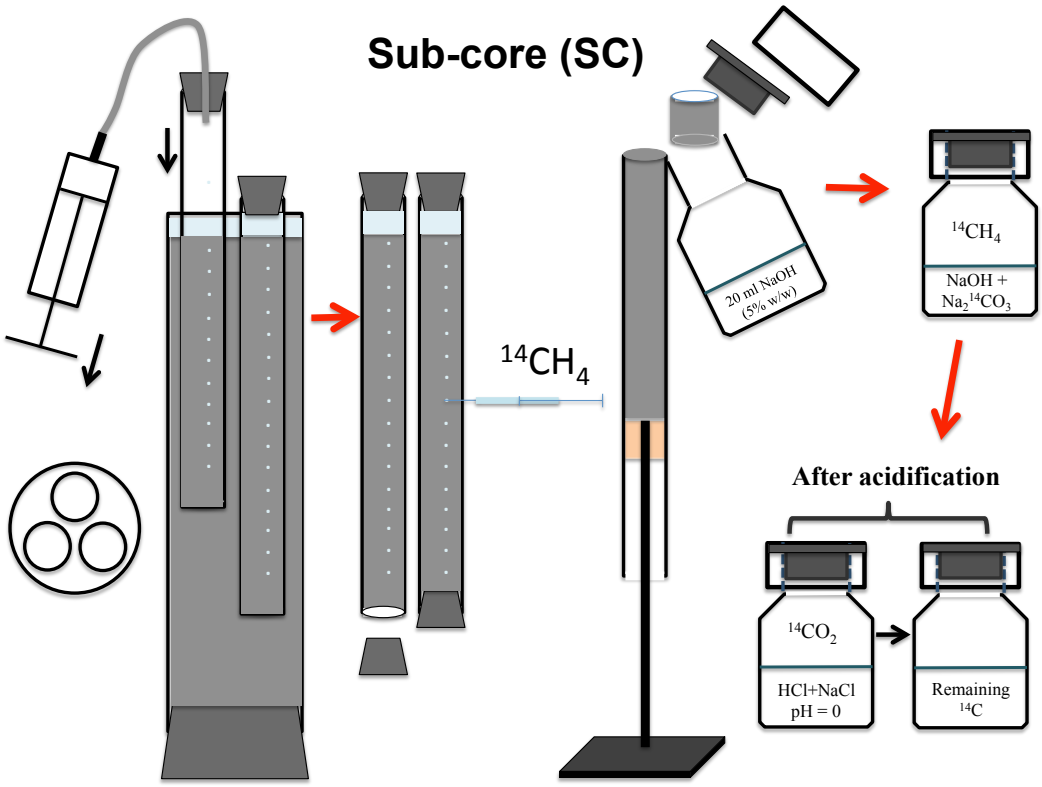
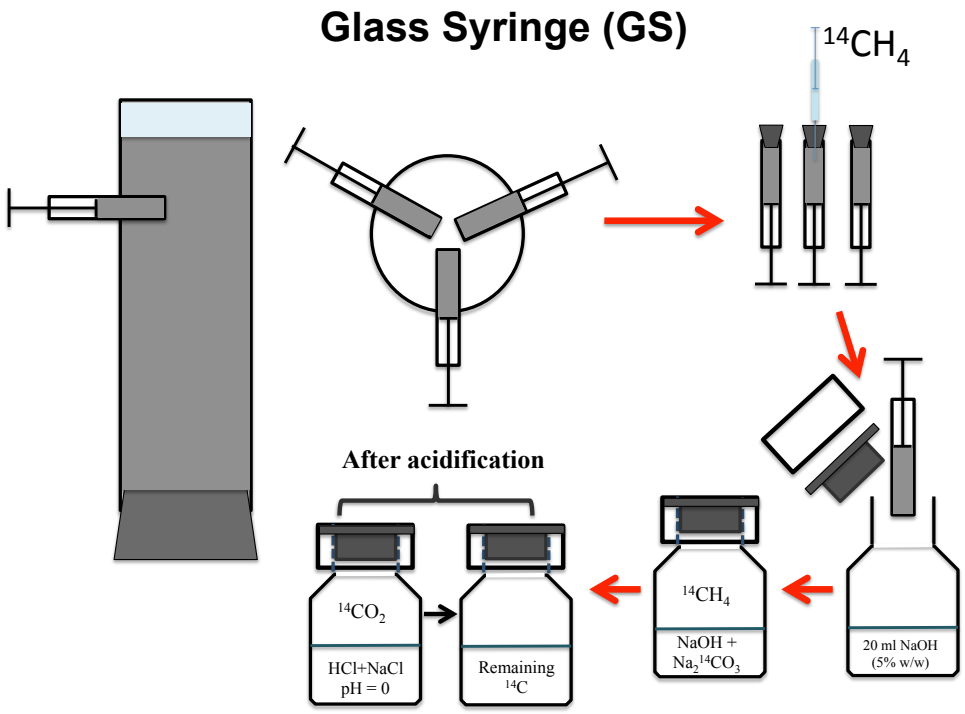
Supporting information

Sediment sample preparation

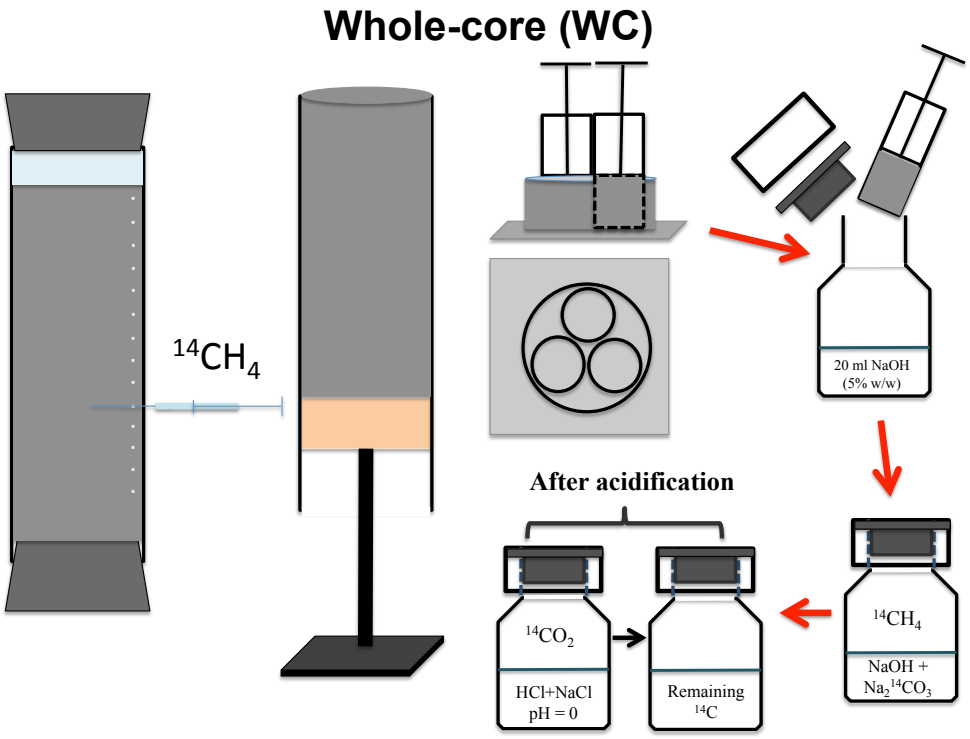
1. Sub-core incubation. One of the intact gravity cores (inner diameter 62 mm) was subsampled vertically by inserting three smaller push-core liners (inner diameter of 16 mm). Care was taken that the water-sediment interface in both the original core and the push-cores were approximately aligned. Sub-cores were then closed on both ends with grey thick rubber stoppers without headspace. The $^{14}CH_4$ working solution was injected in the replicate sub-cores through side-holes sealed with silicon gel at a depth interval of 1.5 cm. After incubation, 2 cm segments were pushed out of the sub-core liner, directly transferred into 100 ml glass bottles (DURAN GL45) containing 20 ml NaOH solution (5% w:w). The bottles were closed quickly with thick butyl rubber stoppers and shaken thoroughly to equilibrate the porewater methane between the aqueous and the gas phase. Sufficiently high pH and ion concentration are necessary to ensure that ΣCO_2 is bound as CO_3^{2-} and all CH_4 is driven into the headspace.



2. Glass syringe incubation. Triplicate samples were taken every 2 cm from a gravity core through pre-drilled side ports using cut-off glass syringes, which were then sealed with black butyl stoppers. The $^{14}CH_4$ working solution was injected directly into each of the glass syringes containing 4 ml sediment. After incubation, samples were transferred into 100 ml bottles and fixed with 20 ml NaOH (5% w:w). Sufficiently high pH and ion concentration are necessary to ensure that ΣCO_2 is bound as CO_3^{2-} and all CH_4 is driven into the headspace.



3. Whole-core incubation. An entire gravity core was spiked with $^{14}CH_4$ tracer through pre-drilled side-holes sealed with silicon gel at 2-cm intervals. After incubation, the whole core was extruded, and triplicate samples (~ 4 ml) were collected from 2-cm slabs from the sediment core, using 20 ml cut-off syringes, and quickly transferred into 100 ml bottles with NaOH solution (5% w:w). Sufficiently high pH and ion concentration are necessary to ensure that ΣCO_2 is bound as CO_3^{2-} and all CH_4 is driven into the headspace.



4. Slurry incubation. Triplicate samples were taken every 2 cm from a gravity core through pre-drilled side ports using cut-off syringes (4 ml sediment), which were then transferred into 20 ml glass vials containing anoxic sulfate-amended water. Vials were then closed bubble-free by using a needle. After pre-incubation for about 2 hours to remove oxygen in the slurry (by checking the control vials with oxygen sensor spots), the $^{14}CH_4$ working solution was injected into each of the glass vials. After incubation, samples were transferred into 100 ml bottles and fixed with 20 ml NaOH (5% w:w). (See above)

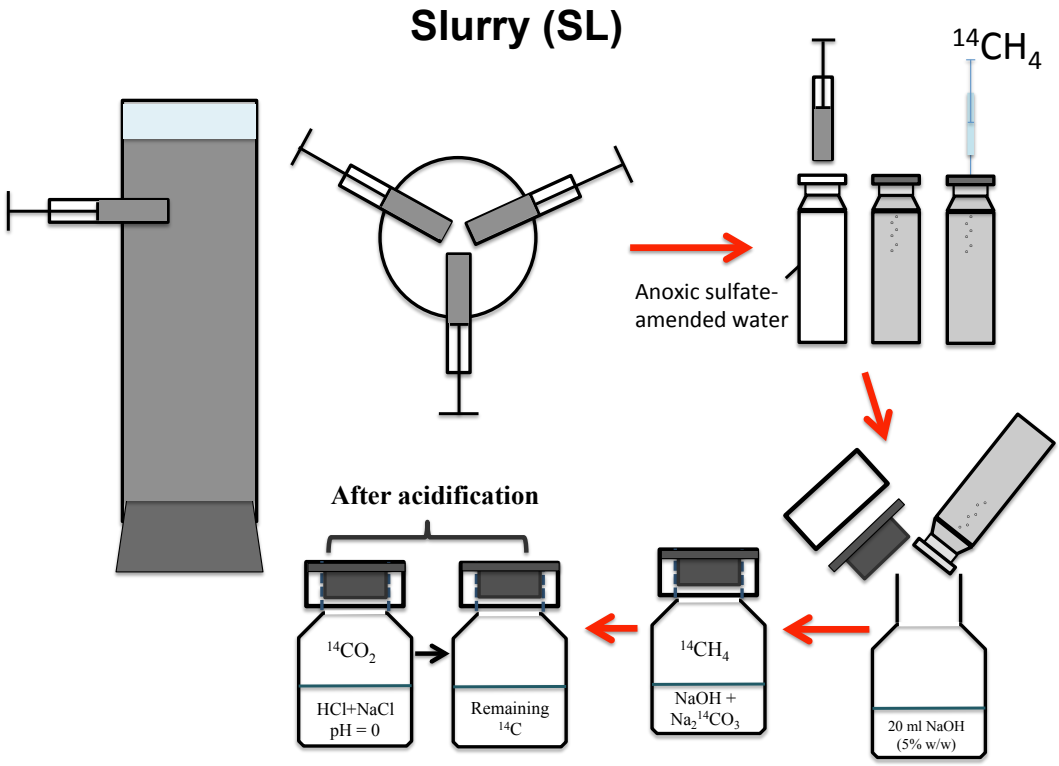


Table S1. Overview of four different set-ups (type of tracer injected, amount of tracer and sediment volume used for rate measurement).

Approach	Type of tracer application	Amount of tracer and activity per injection (µl, Bq)	Sediment used for rate measurement (cm^3)
SC	$^{14}CH_4$ solution	20, 233	4
GS	$^{14}CH_4$ solution	20, 233	4
WC	$^{14}CH_4$ bubble	20, na	4
SL	$^{14}CH_4$ solution	20, 233	4

na: not available.

Chapter 7: Conclusions and outlook

Lakes represent important sites of methane production, and contribute significantly to total natural methane emissions. Understanding the mechanisms that prevent the evasion of methane from lake environments to the atmosphere is of fundamental importance for constraining methane budgets and for understanding how emission rates will change in the future. In this thesis, anaerobic oxidation of methane (AOM) was investigated in the anoxic water columns and sediments of different Swiss lakes. By applying an interdisciplinary and complementary approach that combined radioactive tracer techniques ($^{14}CH_4$) for rate determination, incubation experiments ($^{13}CH_4$) with different electron acceptors, stable isotope probing (SIP) of lipid biomarkers and 16S rRNA gene sequencing, this thesis provides further understanding about AOM in lacustrine environments. The aspects addressed in this thesis are vital for assessing and understanding the environmental controls on the biological methane filter systems in freshwater environments. More specifically, this thesis represents an important step forward in our on-going efforts to taxonomically identify key microorganisms in freshwater AOM and to constrain the electron acceptors involved in AOM. Our study highlights the potential for metabolic versatility of lacustrine methanotrophic microorganisms, but also underscores the vulnerability particularly of bacterial communities that couple AOM with denitrification, to physical/redox perturbations in the open water column of lakes. Based on our results, we argue that nitrite-dependent AOM can be an important methane-consuming process in anoxic waters and has the potential to be a globally significant methane sink in stratified lakes, linking the biogeochmical cycles of methane and nitrogen. Finally, we provide an elegantly simple mechanism that links sulfate reduction and AOM in natural environments where these links may not be expected. Such a mechanism could very well explain previous observations where direct coupling of AOM and either manganese or iron has been invoked.

Directions for future research

This thesis has demonstrated the importance of AOM in lacustrine environments, and has highlighted the multi-faceted ways/modes it can act to mitigate CH_4

emissions. However, multiple questions remained to be answered. In the following, some research questions are suggested for future work.

In Chapter 3, we detected methane turnover in the anoxic water column of the South Basin where only aerobic methanotrophs were detected. The mechanism as to how those methanotrophic bacteria carry out methane oxidation in anoxic waters is still unknown, and where, if they use O_2, this O_2 originates is unclear. Unraveling the metabolic pathways of these apparently aerobic methanotrophs under anoxic conditions would yield important insights into the mechanism of how aerobic methanotrophs control the methane flux from the anoxic lake systems. Time-series analyses and seasonal comparisons of depth-specific methane oxidation rates and microbial abundances could provide additional perspectives with regards to the potential players in the water column. In the North Basin, nitrite-dependent AOM has been shown to be an important methane sink in the permanently stratified water column. The inability to detect nitrite suggests that nitrite is efficiently consumed by microorganisms such as *Candidatus* Methylomirabilis. However, the source of nitrite that sustained nitrite-dependent AOM in the anoxic waters and the other possible nitrite sinks remained to be discerned. Intensified investigations into the microbial community in the anoxic water column will be critical to the understanding of how *Candidatus* Methylomirabilis obtains nitrite for anaerobic respiration. Although *Candidatus* Methylomirabilis use intracellularly produced oxygen to catalyze canonical aerobic methane oxidation, oxygen exposure (both at high concentrations and under micro-oxic conditions) has been shown to inhibit their denitrifying activity. However, our observations suggest that *Candidatus* Methylomirabilis actively performed methane oxidation at the oxic-anoxic interface in Lake Lugano, where at least their peak abundance was observed. In turn the question remains whether these bacteria can use external oxygen to oxidize methane or not.

In Chapter 4, we have demonstrated that uncultured archaea of the Genus *Candidatus* Methanoperedens performed AOM in the sediments of Lake Cadagno. It still remains unclear whether this phylotype has the capacity to encode the pathway of sulfate reduction and is able to catalyze sulfate-coupled AOM independent of SRBs. While the metabolic activity of these archaea has been demonstrated within this PhD study, the environmental factors that control their distribution in the sediments still needs to be resolved. More precisely, it remained uncertain as to why *Candidatus* Methanoperedens was not observed at higher sediment depths, where elevated

concentrations of both methane and sulfate were encountered. In addition, although our incubation with nitrate stimulated AOM, it remains to be confirmed whether this new clade could directly use nitrate as terminal electron acceptor in the absence of sulfate, based on experimental support and genomic analyses.

In Chapter 5, we proposed that aerobic methanotrophs might use humic substances for anaerobic respiration, and thus oxidized methane in the anoxic lake sediments. However, the biochemical mechanism of this hypothetical process remains to be confirmed, and more evidence is required in this regard. Also, we cannot exclude the possibility that some unknown phylogenetic groups/different phyla are able to mediate methane oxidation. Using culture-dependent genomic and/or metagenomic analyses, combined with stable isotope probing may ultimately shed light on the key microbial players, as well as the possible mechanisms that could explain the observed “aerobic AOM” in anoxic lake sediments.

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