Aerobic and Anaerobic Oxidation of Methane in Sediments of Lake Constance

Dissertation

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Abbreviations

ANME	anaerobic methanotrophic archaea
AOM	anaerobic oxidation of methane
FISH	fluorescence *in-situ* hybridization
MCR	methyl-coenzyme M reductase
MMO	methane monooxygenase
mmoX	gene encoding the α-subunit of the soluble methane monooxygenase
MOB	methane-oxidizing bacteria (aerobic)
NC10 bacteria	bacteria belonging phylogenetically to candidate division Nullarbor caves 10
nifH	gene encoding nitrogenase reductase
mxaF	gene coding for the α-subunit of methanol dehydrogenase
PCR	polymerase chain reaction
pmoA	gene encoding the α-subunit of the particulate methane monooxygenase
pMMO	particulate (= membrane bound) methane monooxygenase
ppb	parts per billion
qPCR	quantitative (real time) PCR
RuMP	ribulose monophosphate
sMMO	soluble methane monooxygenase
SRB	sulfate reducing bacteria
T-RFLP	terminal restriction fragment length polymorphism
T-RF	terminal restriction fragment

Chapter 1

General Introduction

Methane and the Global Methane Cycle

In 1776, Alessandro Volta discovered methane when he stirred in anoxic sediments of Laggo Maggiore. Methane is the most reduced carbon compound and its tetrahedral structure and the strong C-H bonds cause the low reactivity of methane (Crabtree, 1995; Shilov and Shul'pin, 1997). Methane is known to be a potent greenhouse gas with a global warming potential 25 times higher than that of CO_2 on a 100-year period. It has an even 72 times higher global warming potential on a 20 year period due to its short lifetime in the atmosphere of about 10 years (Lelieveld et al., 1998; IPCC, 2007). Atmospheric methane concentrations were stable for the last 650 thousand years (Spahni et al., 2005) and increased from about 700 ppb in the pre-industrial age to nearly 1800 ppb in 2008 (Rigby et al., 2008). A further raise of atmospheric methane concentrations in the future is discussed to date as the increase seemed to level off since 1999 (IPCC, 2007), but recent insights indicate a renewed increase of atmospheric methane concentrations (Bousquet et al., 2006; Rigby et al., 2008). The abiotic processes of mining and burning of fossil fuels or biomass burning account for 25% of the global methane production. However, the main source is the biological formation of methane by methanogenic archaea which constitutes 69% of the global methane production (Conrad, 2009). Minor methane sources are chemical decomposition of plant material (Conrad, 2009) and geothermic methane (Etiope et al., 2004). The main sink of atmospheric methane is the chemical reaction with hydroxyl radicals in the troposphere, which accounts for about 90% of the total atmospheric methane oxidation (Crutzen, 1995). Minor sinks are losses of methane to the stratosphere, reaction with chlorine (Allan et al., 2005) and microbial oxidation in upland soils (Conrad, 1996). Even though microorganisms play only a minor role in the consumption of atmospheric methane they play a crucial role in mitigating methane emissions. Methane-oxidizing microorganisms act as biofilters and oxidize a large proportion of the methane formed by methanogenic archaea in all kinds of anoxic habitats (Fig. 1.1) (Frenzel et al., 1990; Frenzel, 2000; Reeburgh, 2003; Reeburgh, 2007).

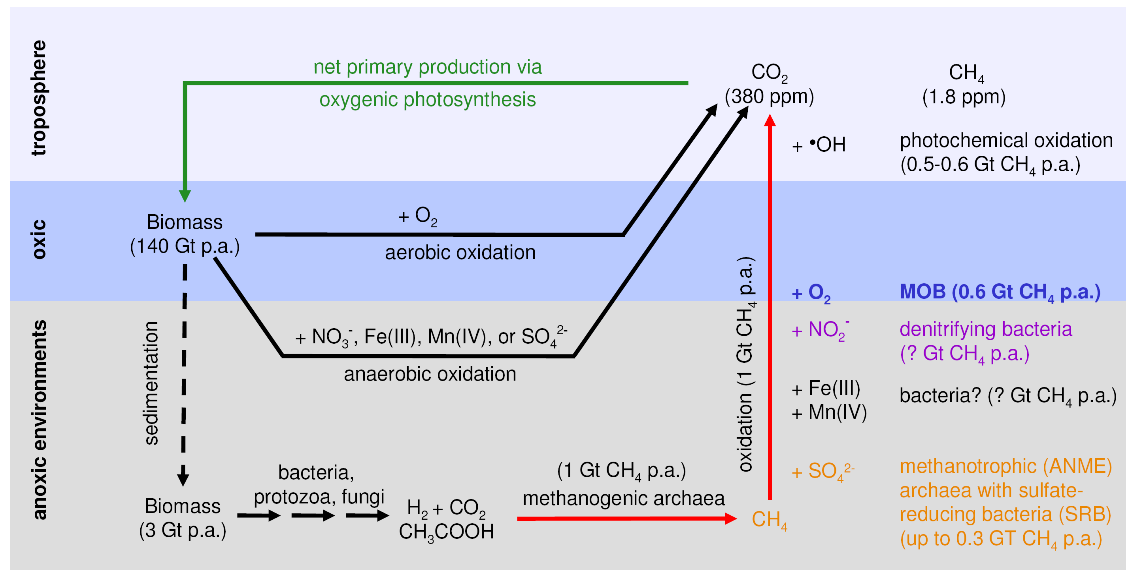

Fig. 1.1. The global methane cycle (modified after Thauer, 2010). Microorganisms that catalyze aerobic methane oxidation (MOB, blue) or anaerobic oxidation of methane with nitrite (magenta) or sulfate (orange) are highlighted. p.a. = per year.

Biological Methanogenesis

Biological methane production takes place wherever organic matter is decomposed in the absence of external electron acceptors like oxygen, nitrate, nitrite, sulfate or oxidized metal species (Schink, 1997; Thauer, 2010). Methane is formed by methane producing archaea, called methanogens, which are a phylogenetically diverse group of obligately anaerobic *Euryarcheota.* They share a limited substrate spectrum and use only $H_2 + CO_2$, acetate, formate, methanol, and methylamines as carbon source (Boone et al., 1993; Zinder, 1993; Liu and Whitman, 2008). The main substrates, acetic acid and $CO_2 + H_2$, are provided by bacteria or fungi degrading complex organic matter to monomers and syntrophic bacteria subsequently fermenting those to acetic acid and $CO_2 + H_2$ (Fig. 1.1) (Zinder, 1993; Schink and Stams, 2006). Acetogenic bacteria provide an additional link between acetic acid and $CO_2 + H_2$ when the environmental parameters are suitable (Kotsyurbenko et al., 2001). Although the different groups of methanogens vary in parts of their energy metabolism (Thauer et al., 2008), the basic reaction steps and the final release of methane by methyl-coenzyme M reductase (MCR) are shared by all methanogens (Thauer, 1998). MCR with its nickel porphinoid coenzyme F_{430} (Ermler et al., 1997) is therefore the key enzyme in methanogenesis and is widely used as phylogenetic marker gene and to study methanogenic communities in the environment (Ritchie et al., 1997; Friedrich and Jared, 2005; Juottonen et al., 2006; Steinberg and Regan, 2009).

Aerobic Methane-Oxidizing Bacteria

Aerobic oxidation of methane by microorganisms is known for more than 100 years (Kaserer, 1906; Söhngen, 1906) but intense research on methanotrophic bacteria started in 1970 when Whittenbury and colleagues isolated more than 100 strains of methanotrophic bacteria belonging to 5 groups later assigned to different genera (Whittenbury et al., 1970). Methane-oxidizing bacteria (MOB) are defined by their ability to use methane as sole source of carbon and energy. The "classical" proteobacterial MOB were classified in two groups, type I and type II MOB, based on morphological, physiological and phylogenetical characteristics by Whittenbury and colleagues (1970). These two groups differ in some physiological properties like their ultra structure, phospholipid fatty acid profiles, and their carbon assimilation pathway. Type I MOB use the ribulose monophosphate pathway (RuMP) to assimilate one carbon compounds, while type II methanotrophs use the serine pathway (Hanson and Hanson, 1996). To date, 17 genera containing isolated strains of "classical" methane-oxidizing bacteria (MOB) have been described (Tab. 1.1). Cultured MOB of the α-proteobacterial clade (type II MOB) comprise the genera *Methylosinus* and *Methylocystis* of the family Methylocystaceae and the family Beijerinckiaceae including the genera *Methylocella, Methyloferula* and *Methylocapsa*. Classical cultured methanotrophic γ-proteobacteria belong to the family Methylococcaceae (type I MOB), which includes 12 described genera. Type I MOB were further divided into two groups, type Ia and type Ib MOB, the latter (called type X in earlier publications) including the genera *Methylococcus, Methylocaldum* (and probably the recently described *Methylogaea*), which possess characteristics of type II as well as of type I MOB (Trotsenko and Murrell, 2008). However, as more and more exceptions to this classification are described and 16S rRNA gene sequences are widely used as main phylogenetic marker, this classification got more and more outdated. For example, *Methylocystis heyeri*, a type II MOB, possesses signature fatty acids of type I methanotrophs (Dedysh et al., 2007), *Methylocella* and *Methyloferula* species possess no particulate methane monooxygenase, which was previously thought to be present in all MOB (Dedysh et al., 2000; Vorob'ev et al., in press), and *Methylocella* spp. and *Methylocapsa aurea* are not even obligate methanotrophs, but prefer acetate as carbon source (Dedysh et al., 2005; Dunfield et al., 2010). Additionally, it is not possible to assign environmental DNA sequences to type I or type II MOB in the strict sense, because the physiological and morphological data defining these groups are lacking. However, this classification is still widely applied in a phylogenetic sense to distinguish the traditional groups of MOB and will also be used in this sense in this thesis.

Recently, *Crenothrix polyspora* and *Clonothrix fusca* have been characterized as facultative methanotrophs which are phylogenetically related to the γ-proteobacterial type I MOB (Stoecker et al., 2006; Vigliotta et al., 2007). Together with the finding that also the phylum Verrucomicrobia includes methanotrophs (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008; op den Camp et al., 2009) this implies that methanotrophy might be far more widespread in nature than previously thought.

Despite the growing number of isolates that are available for physiological characterization, the physiological adaptations determining the distribution of different MOB strains in the environment or, the other way round, the environmental factors shaping MOB communities *in-situ* are largely unknown. Furthermore, many clusters of environmental marker gene sequences lack cultured representatives, thus, no detailed physiological information is available. Experiments showed that type II MOB might be generalists, as they outcompete type I MOB under nutrient limitation (Graham et al., 1993; Baani and Liesack, 2008; Steenbergh et al., 2010, Amaral, 1995), have more resistant resting stages (Whittenbury et al., 1970) and are less susceptible to grazing (Murase and Frenzel, 2008), whereas within the type I MOB more specialized strains have been described. Many known heat-tolerant (Tsubota et al., 2005), halo-tolerant (Kalyuzhnaya et al., 2008a) or psychrophilic methanotrophs (Trotsenko and Khmelenina, 2005) belong to the type I MOB. A recent meta-analysis of *pmoA* gene sequences revealed distinct distribution patterns of several clusters of methanotrophs (Lüke, 2010). Uncultured type Ia MOB seem to dominate freshwater and marine habitats, type Ib dominate wet rice paddies and some freshwater habitats. Landfills are dominated by type II MOB, *Methylocaldum*, and *Methylosarcina*, whereas *Methylococcus* is found mainly in grassland. A large group of uncultivated MOB, represented by 7 sequence clusters, are upland soil methanotrophs which likely oxidize methane at atmospheric concentrations.

Tab. 1.1. Characteristics of different genera of methanotrophs. Data were adopted from previous publications (Dunfield et al., 2010; Murrell, 2010; Geymonat et al., in press; Iguchi et al., 2011; Vorob'ev et al., in press). C1 assimilation: Pathway used to assimilate one carbon compounds; ND not determined; NA not applicable; pMMO & sMMO particulate & soluble methane monooxygenase, respectively; ICM: intracytoplasmic membranes; PLFA: phospholipid fatty acid. Genera including facultative methanotrophs are shown in bold. The classification after Whittenbury is shown in parenthesis.

Genus name	Phylogeny	MMO type	C_1 assimilation	ICM type	N_2 fixation	G+C content*	Major PLFA	Growth capability
Methylovulum	γ Proteobacteria (type Ia)	pMMO ± sMMO	RuMP	Type I	No	49.3	16:0	Mesophilic
Methylosoma	γ Proteobacteria (type Ia)	pMMO	ND	Type I	Yes	49.9	16:1	Not extreme
Methylosarcina	γ Proteobacteria (type Ia)	pMMO	RuMP	Type I	No	54	16:1	Not extreme
Methylobacter	γ Proteobacteria (type Ia)	pMMO	RuMP	Type I	No	49–54	16:1	Some psychrophilic
Methylomicrobium	γ Proteobacteria (type Ia)	pMMO ± sMMO	RuMP	Type I	No	49–60	16:1	(Halo)alkaliphilic
Methylomonas	γ Proteobacteria (type Ia)	pMMO ± sMMO	RuMP	Type I	Some	51–59	16:1	Some psychrophilic
Methylosphaera	γ Proteobacteria (type Ia)	pMMO	RuMP	ND	Yes	43–46	16:1	Psychrophilic
Methylococcus	γ Proteobacteria (type Ib)	pMMO ± sMMO	RuMP/Serine	Type I	Yes	59–66	16:0	Thermophilic
Methylogaea	γ Proteobacteria (type Ib)	pMMO	ND	Type I	No?	63.1	16:0	Mesophilic
Methylocaldum	γ Proteobacteria (type Ib)	pMMO	RuMP/Serine	Type I	No	57	16:0	Thermophilic
Methylohalobius	γ Proteobacteria (type I)	pMMO	RuMP	Type I	No	58.7	18:1	Halophilic
Methylothermus	γ Proteobacteria (type I)	pMMO	RuMP	Type I	No	62.5	18:1/16:0	Thermophilic
Methylocapsa	α Proteobacteria (type II)	pMMO	Serine	Type III?	Yes	63.1	18:1	Acidophilic
Methyloferula	α Proteobacteria (type II)	sMMO	Serine/RuMP	NA	Yes	56-58	18:1	Acidophilic
Methylocella	α Proteobacteria (type II)	sMMO	Serine	NA	Yes	60–61	18:1	Acidophilic
Methylocystis	α Proteobacteria (type II)	pMMO ± sMMO	Serine	Type II	Yes	62–67	18:1/(16:1)	Some acidophilic
Methylosinus	α Proteobacteria (type II)	pMMO ± sMMO	Serine	Type II	Yes	63–67	18:1	Not extreme
Crenothrix	γ Proteobacteria	pMMO	ND	Type I	ND	ND	ND	Not extreme
Clonothrix	γ Proteobacteria	pMMO	ND	Type I	ND	ND	ND	Not extreme
"Methylokorus"	*Verrucomicrobia*	pMMO	Serine	Type IV?	No	ND	ND	Thermoacidophilic
"Methyloacida"	*Verrucomicrobia*	ND	ND	Type IV?	Yes	ND	ND	Thermoacidophilic
"Acidimethylosilex"	*Verrucomicrobia*	pMMO	RuMP?	Type IV?	No	ND	18:0	Thermoacidophilic

* in mol %

The reaction mechanism of aerobic oxidation of methane has been well investigated. Although aerobic oxidation of methane is highly exergonic (Eqn. 1.1), aerobic methanotrophs have to invest two redox equivalents into a monooxygenase reaction to activate the strong C-H bond of methane (Higgins and Quayle, 1970; King, 1992). Two different isoforms of this enzyme are known to date, which differ significantly in their properties. Both enzymes differ substantially in their primary structure (Murrell et al., 1998) as well as their substrate spectra (Burrows et al., 1984). The structure and function of soluble methane monooxygenase (sMMO), which contains a non-heme di-iron center as active site, is well known (Lipscomb, 1994; Rosenzweig et al., 1995). Its low substrate specificity allows the oxidation of e.g., n-alkanes, n-alkenes, mono- and di-aromatics (Colby et al., 1977) and can be used to detoxify halogenated hydrocarbons (Fox et al., 1990). The membrane-bound (particulate) methane monooxygenase (pMMO) has a much narrower substrate spectrum and enzyme preparations contained copper, iron and nickel, whose presence and function in pMMO are still under discussion (Hakemian and Rosenzweig, 2007; Himes and Karlin, 2009), but a copper-catalyzed reaction seemed convincing (Balasubramanian et al., 2010). However, almost all known methanotrophs posses pMMO, some can express both isoforms and only *Methylocella* and *Methyloferula* harbor only sMMO (Dedysh et al., 2000; Vorob'ev et al., in press). In bacteria possessing both genes, sMMO is not expressed when copper concentrations are sufficient to express pMMO (Murrell et al., 2000).

$CH_4 + 2\ O_2 \rightarrow CO_2 + 2\ H_2O \qquad \Delta G^{\circ\prime} = -819\ kJ/mol\ CH_4 \qquad$ Eqn. 1.1

Oxidation of Methane Coupled to Denitrification

Recently, anaerobic oxidation of methane coupled to denitrification (Eqn. 1.2) was discovered (Raghoebarsing et al., 2006). The first assumption of a syntrophic association of archaea and nitrate-reducing bacteria was proven false after prolonged incubation, because the archaea disappeared from the denitrifying enrichment. It turned out that bacteria belonging to the candidate division NC10 carry out the process of denitrifying methane oxidation on their own (Ettwig et al., 2008). These bacteria most likely produce oxygen from nitrite via NO (Ettwig et al., 2010). Thus, this anaerobic oxidation of methane takes places in an anoxic environment, but methane is activated by molecular oxygen through a methane monooxygenase reaction as in aerobic methanotrophs. Thus, the oxidation of the methane molecule is not an anaerobic process. A *pmo* gene cluster encoding particulate methane monooxygenase has been identified in the metagenome and is actively transcribed and translated (Ettwig et al., 2010). However, the enzyme responsible for the disproportionation

of 2 NO to N_2 and O_2 has not been identified, and it cannot be ruled out that the "pMMO" uses NO directly to activate methane (Thauer, 2010). The NC10 phylum has no isolated representatives so far, but few highly enriched cultures are available (Raghoebarsing et al., 2006; Ettwig et al., 2009; Hu et al., 2009). Environmental 16S rRNA gene sequences of NC10 bacteria have been obtained from different freshwater habitats. However, it is not known how widespread this kind of methane oxidation is within the NC10 phylum or even in other bacterial clades and detailed studies on the distribution of NC10 bacteria are lacking.

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O \qquad \Delta G^{\circ\prime} = -928\ kJ/mol\ CH_4 \qquad \text{Eqn. 1.2}$$

Anaerobic Oxidation of Methane

Anaerobic oxidation of methane (AOM) with sulfate as electron acceptor was postulated first on the basis of methane and sulfate profiles measured in marine sediments because sulfate and methane disappear in the same depth of the sediment, the sulfate-methane transition zone (Barnes and Goldberg, 1976; Reeburgh and Heggie, 1977). Soon, rate measurements of sulfate reduction and methane oxidation followed, using radioactive tracers (Reeburgh, 1980; Iversen and Jørgensen, 1985). Tracer experiments with freshwater sediment, digested sludge, and marine sediment slurries, as well as pure culture experiments, led to the hypothesis that sulfate-dependent AOM is carried out by an association of sulfate reducers and methanogens (Zehnder and Brock, 1980). However, methane oxidation was always accompanied by net methane formation in these experiments (Zehnder and Brock, 1980). The hypothesis of a methanogen-sulfate reducer consortium was later verified by laboratory experiments and *in-situ* measurements (Hoehler et al., 1994). A few years later, this hypothesis was slightly modified by demonstrating that different groups of archaea are involved in AOM. These archaea (later called anaerobic methanotrophic [ANME] archaea) are in fact related to methanogens, but form distinct phylogenetic groups. This has been shown first by analysis of lipid biomarkers (Hinrichs et al., 1999), but the tight syntrophic association of these ANME archaea with sulfate-reducing bacteria (SRB) was finally demonstrated by fluorescence *in-situ* hybridization (Boetius et al., 2000). Meanwhile, sulfate-dependent AOM and even growth of the ANME consortia has been reported *in vitro* (Nauhaus et al., 2002; Nauhaus et al., 2007), but no defined co-culture is available to date. Sulfate dependent AOM is mediated by three distinct clusters of archaea, namely ANME-1, ANME-2, and ANME-3, all of which are related to methanogens of the orders Methanosarcinales and Methanomicrobiales. The physiology, ecology, preferred habitats, and bacterial partners of the different ANME clusters have been reviewed recently (Knittel and Boetius, 2009).

$$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + HS^- + 2\,H_2O \qquad \Delta G°' = -21\ kJ/mol \qquad \text{Eqn. 1.3}$$

The energy gain in sulfate-dependent AOM (Eqn. 1.3) is close to the theoretical minimum for ATP synthesis of $\Delta G°' = -20\ kJ\ mol^{-1}$ (Schink, 1997). Thus, sulfate-dependent AOM could hardly feed two organisms in a syntrophic cooperation under standard conditions. However, sulfate-rich (marine) habitats experiencing high hydrostatic pressures allow methane concentrations to rise far above the solubility of 1.4 mM at atmospheric pressure (Yamamoto et al., 1976) and can provide conditions where sulfate dependent AOM is energetically favorable even for a syntrophic cooperation. The high methane concentration needed for AOM with sulfate to be energetically favorable corresponds well with the observed K_m value of 10 mM CH_4 for sulfate dependent AOM *in vitro* (Nauhaus et al., 2002).

The activation of methane under anoxic conditions is a chemically challenging task. As more and more evidence pointed to an involvement of methanogen-like archaea in AOM with sulfate, the potential of reversed methanogenesis as possible reaction pathway was investigated. A "conspicuous nickel protein" very similar to methyl-coenzyme M reductase (MCR) of methanogens was found in microbial mats mediating AOM (Krüger et al., 2003). Environmental genomics of enriched ANME-SRB consortia also provided evidence for the reversed methanogen hypothesis, because nearly all genes typically associated with methane production were present in one group of archaea (Hallam et al., 2004). Recently, it was shown that even MCR of the methanogen *Methanothermobacter marburgensis* catalyses the activation of methane to methyl-CoM at rates comparable to those of AOM *in-situ* (Scheller et al., 2010).

Besides the well investigated sulfate-dependent methane oxidation, AOM coupled to iron(III) and manganese(IV) reduction (Beal et al., 2009) or humic compound reduction (Smemo and Yavitt, 2007) has been proposed. However, direct coupling of these electron acceptors to AOM has not been shown and the organisms responsible for these processes are unknown. The energy yield of AOM coupled to iron(III) or manganese(IV) reduction would be high enough to permit AOM at moderate substrate concentrations (Thauer and Shima, 2008) and might enable AOM to take place even at lower methane partial pressures.

Marker Genes for Studying MOB

The by far most frequently used phylogenetic marker for studying microbial diversity is the 16S rRNA gene. Due to its conserved physiological function at the nucleotide level and its occurrence in all prokaryotes this gene provides an ideal target for phylogenetic studies (Ludwig and Schleifer, 1994). However, in many cases phylogeny and physiological

properties do not coincide (Achenbach and Coates, 2000). Thus, investigations on certain groups of microorganisms target functional genes that code for key enzymes of the respective metabolic pathways. The key enzymes unique to MOB are the particulate and the soluble methane monooxygenase. Consequently, the genes used for phylogenetic studies are the *pmoA* and *mmoX* gene, respectively (McDonald et al., 2008). As the *pmoA* gene is present in almost all MOB, this gene is widely used in MOB diversity research (McDonald et al., 2008). Phylogenetic relations calculated on the basis of *pmoA* sequence diversity are largely congruent with those based on 16S rRNA gene phylogeny (Kolb et al., 2003; Lüke, 2010), which renders *pmoA* a suitable phylogenetic marker gene widely used for detection and characterization of methanotrophic communities. The frequency of amino acid exchanges among PmoA sequences of different classical MOB is about 1.9 times higher than the nucleotide substitution rate among the 16S rRNA gene of the respective MOB (Lüke, 2010).

Methane Oxidation in Lakes

The contribution of freshwater lakes to the global methane emissions is a matter of dispute (IPCC, 2007; EPA, 2010). Estimates are up to 1.6-9.6% of the total or 6-16% of the natural methane emissions (Bastviken et al., 2004), but actual emissions might be even higher because small lakes were underestimated (EPA, 2010; Bastviken et al., 2011).

In mesotrophic or oligotrophic lakes, where oxygen is available throughout the water column, aerobic methane oxidation takes place at the sediment-water interface (Lidstrom and Somers, 1984; Frenzel et al., 1990), whereas in eutrophic or other stratified lakes aerobic methane oxidation often occurs within the water column at the chemocline between anoxic bottom water (hypolimnion) and the oxic epilimnion (Harrits and Hanson, 1980; Hanson and Hanson, 1996; Carini et al., 2005; Sundh et al., 2005). At this narrow zone, oxygen concentrations are low and methane diffuses upwards from the anoxic hypolimnion. Two famous examples of these different lake types have been studied extensively besides Lake Constance. Aerobic methane oxidation at the sediment-water interface has been investigated in Lake Washington (Lidstrom and Somers, 1984; Costello and Lidstrom, 1999; Costello et al., 2002; Kalyuzhnaya et al., 2008b), a mesotrophic freshwater lake with similar properties as Lake Constance, and research on pelagic methane oxidation was performed in Mono Lake (California) an alkaline salt lake (Oremland et al., 1987; Carini et al., 2005; Lin et al., 2005). However, numerous reports are also available on various other lakes, which help not only to access the importance of freshwater lakes for the global methane budget (Bastviken et al., 2004), but also the

importance of MOB in mitigating emissions and their distribution and adaptation to their habitats (Lüke, 2010).

Besides aerobic methane oxidation, a few studies reported also anaerobic oxidation of methane in freshwater lakes. In Lake Plußsee, a small oligotrophic kettle lake (Eller et al., 2005b), and Lago di Cadagno, a sulfate-rich alpine lake (Schubert et al., 2011), anaerobic oxidation of methane has been described based on isotopic signatures and concentration profiles.

Methane Oxidation in Lake Constance

Lake Constance is an oligotrophic, monomictic, pre-alpine lake and the second largest water body in Europe. Long-term data on various aspects of limnology are available and provide a helpful background for interpreting recent data. Lake Constance is a deep lake (max. 254 m) which harbors a stable and undisturbed environment in high water depths below the thermocline. Thus, profundal sediments constantly experience no light, water temperatures of about 4°C, and almost no disturbances of the sediment whereas littoral sediments are exposed to wind and wave action, seasonal fluctuations of water level and temperature, and day-night cycles of light and oxygen availability.

Methane formation and oxidation as well as the respective microorganisms have been studied in detail over the past years (Frenzel et al., 1990; Bosse et al., 1993; Thebrath et al., 1993; Schulz and Conrad, 1995; Bussmann et al., 2004; Pester et al., 2004; Bussmann, 2005; Bussmann et al., 2006). Cultivation-dependent methods were used to explore methanotrophic diversity in Lake Constance (Bussmann et al., 2004; Bussmann et al., 2006), finally leading to the isolation of a novel MOB representing a new genus (Rahalkar et al., 2007). Other studies focused on the environmental importance and distribution of MOB in the sediments. In profundal sediments of Lake Constance, 93% of the total methane diffusing upwards was consumed under aerobic conditions (Frenzel et al., 1990), whereas in the littoral only 79% was consumed (Bosse et al., 1993) when ebullition caused by disturbances *in-situ* was neglected (Bussmann, 2005). In undisturbed sediments, stable gradients of methane and oxygen are formed causing MOB to experience different substrate concentrations on a very fine spatial scale. These gradients can be measured with high spatial resolution with a recently developed diffusion based methane sensor (Bussmann and Schink, 2006). This provides the opportunity to characterize with high accuracy the environmental conditions that MOB experience in sediments of Lake Constance. Methanotrophic bacteria and the differences in their community structure regarding profundal and littoral sediments have been investigated

by culture-independent methods targeting *pmoA* and 16S rRNA gene diversity (Pester et al., 2004; Rahalkar and Schink, 2007), but the depth distribution of MOB along the substrate gradients remained unresolved.

Another important aspect for understanding the methane cycle in Lake Constance was the discovery of hundreds of pockmark-like depressions in its Eastern part (Wessels et al., 2010). Pockmarks are concave depressions of the sediment surface which are known to occur in marine sediments (Hovland and Judd, 1988; Hovland et al., 2002; Judd and Hovland, 2007), but are rarely reported in freshwater habitats. The pockmarks found in Lake Constance are circular depressions with diameters of up to 8 meters and 0.5 to 1.5 m depth (Wessels et al., 2010) in which biogenic methane bubbles out of the sediment. Those pockmarks provide a habitat for methanotrophic bacteria that is presumably distinct from the situation in usual sediments. Methane from gas bubbles can dissolve into the well-oxygenated water and diffuse into the sediment providing environmental niches with high methane and oxygen concentrations, which might harbor a distinct community of methanotrophs.

Despite some reports on AOM in freshwater habitats (Eller et al., 2005b; Smemo and Yavitt, 2007; Schubert et al., 2011) and the intense research on methane oxidation in Lake Constance, AOM had not been reported for Lake Constance. However, the prerequisites for AOM coupled to iron reduction (Beal et al., 2009), reduction of humic compounds (Smemo and Yavitt, 2010) or denitrification (Raghoebarsing et al., 2006) are met because the respective electron acceptors are present in anoxic sediment layers in Lake Constance (Kappler et al., 2004; Gerhardt et al., 2005; C. Schmidt pers. comm.).

Aims of this Thesis

Methane oxidation and methanotrophic bacteria have been well studied in Lake Constance, but still there is no complete picture of the complex methanotrophic communities, the environmental niches of different methanotrophic strains, and their role in mitigating methane emissions. The focus of this thesis was to refine our current knowledge on methane oxidation in Lake Constance and to provide a detailed view on the methanotrophic communities that carry out this process in littoral and profundal sediments as well as recently discovered methane seeps.

To characterize the active zone of methane oxidation in the sediments, the depth distribution and abundance of MOB in littoral and profundal sediments was investigated and compared to the distribution of their major substrates, methane and oxygen (chapter 2).

Furthermore, the methanotrophic activity as well as MOB abundance and community structure at recently discovered methane seeps was investigated, because the environmental conditions at these sites likely differ substantially from common diffusion-controlled littoral and profundal sediments (chapter 3).

As the factors determining the methanotrophic community composition *in-situ* are still largely unknown, another goal of this study was to isolate novel MOB from Lake Constance to gain access to the physiological characteristics of yet uncultivated organisms (chapter 4).

Anaerobic oxidation of methane is still a cryptic process in freshwater habitats, thus, we checked for anaerobic methane oxidation with different electron acceptors (sulfate, nitrate, nitrite) in littoral and profundal sediments, and aimed to identify the responsible organisms (chapter 5).

Chapter 2

Abundance and Activity of Methanotrophic Bacteria in Littoral and Profundal Sediments of Lake Constance (Germany)

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Abstract

The abundances and activities of aerobic methane-oxidizing bacteria (MOB) were compared in depth profiles of littoral and profundal sediments of Lake Constance, Germany. Abundances were determined by quantitative PCR (qPCR) targeting the *pmoA* gene and by fluorescence *in-situ* hybridization (FISH), and data were compared to methane oxidation rates calculated from high-resolution concentration profiles. qPCR using type I MOB-specific *pmoA* primers indicated that type I MOB represented a major proportion in both sediments at all depths. FISH indicated that in both sediments, type I MOB outnumbered type II MOB at least fourfold. Results obtained with both techniques indicated that in the littoral sediment, the highest numbers of methanotrophs were found at a depth of 2 to 3 cm, corresponding to the zone of highest methane oxidation activity, although no oxygen could be detected in this zone. In the profundal sediment, highest methane oxidation activities were found at a depth of 1 to 2 cm, while MOB abundance decreased gradually with sediment depth. In both sediments, MOB were also present at high numbers in deeper sediment layers where no methane oxidation activity could be observed.

Introduction

Methane is formed by natural processes and by anthropogenic processes and is 25 times more effective as a greenhouse gas than carbon dioxide (Dalton, 2005). Methane emissions from lakes contribute about 6 to 16% of the total non-anthropogenic global methane emission (Bastviken et al., 2004). Microbial methane oxidation is an important process for prevention of the escape of the methane produced in anoxic sediment layers (Thebrath et al., 1993; Bastviken et al., 2004) to the atmosphere and ultimately controls global warming.

In mesotrophic or oligotrophic lakes that are oxic down to the sediment surface, aerobic oxidation of methane occurs at the sediment-water interface (Lidstrom and Somers, 1984). Methane oxidation in freshwater lakes was considered to be an exclusively aerobic process

(Kuivila et al., 1988; Frenzel et al., 1990), but recently, anaerobic oxidation of methane in Lake Plußsee has been reported (Eller et al., 2005b). In Lake Constance, a large, deep, oligotrophic lake, methane production and oxidation processes have been studied intensively in the past (Bosse et al., 1993; Schulz and Conrad, 1995). Recently, a diffusion methane sensor was used to measure methane profiles at millimeter-level resolution both in littoral sediments and in profundal sediments of Lake Constance (Bussmann and Schink, 2006). The sensor consists of a steel cannula with small openings, which are covered by thin silicone tubing. Methane diffuses into the cannula and is flushed directly to a flame ionization detector for quantification.

Aerobic methane-oxidizing bacteria (MOB) have been classified as type I and type II methanotrophs based on their phylogenetic position, carbon assimilation pathways, and the arrangement of intracellular membranes, and they belong to the classes Gammaproteobacteria and Alphaproteobacteria, respectively (Bowman, 2000). Very recently, Verrucomicrobium-like bacteria have also been reported to oxidize methane in acidic environments (Pol et al., 2007; Islam et al., 2008). MOB from natural ecosystems such as soil, rice paddies, freshwater marshes, and lakes have been quantified by cultivation methods like the most-probable-number method (MPN) (Eller, 2001; Bussmann et al., 2004) and gradient cultivation (Bussmann et al., 2006) and by culture-independent techniques such as Southern hybridization with probes for the *pmoA* or the *mmoX* gene (Auman and Lidstrom, 2002; Costello et al., 2002), phospholipid fatty acid profiles (Sundh et al., 2005), methane oxidation rates (Lin et al., 2005), fluorescence *in-situ* hybridization (FISH) (Eller et al., 2001; Carini et al., 2005), and quantitative real-time PCR (qPCR) (Kolb et al., 2003). Using quantitative cultivation of methanotrophs in the littoral sediment of Lake Constance, we determined 10^4 cells per ml by the MPN method in micro titer plates (Bussmann et al., 2004) and up to 10^5 cells per ml by gradient cultivation (Bussmann et al., 2006).

Littoral sediments differ from profundal sediments by their exposure to daily light/dark cycles (Gerhardt et al., 2005), their higher content of organics, and the frequency of disturbances by either bioturbation or sediment resuspension (Wetzel, 2001). We therefore expected major differences in community structures (Rahalkar and Schink, 2007) and abundances of methanotrophs in these two different compartments of the lake.

In the present study, we determined methanotrophic abundance at high spatial resolution by using two independent molecular methods, qPCR targeting the *pmoA* gene and FISH targeting 16S rRNA. The abundance of MOB was correlated with the rates of methane oxidation

calculated from high-resolution profiles of methane concentrations in the littoral and profundal sediments of Lake Constance.

Materials and Methods

Sediment sampling. Littoral sediment samples were collected by scuba diving from the lower infralittoral zone ("Litoralgarten") of Lake Constance at a water depth of 2 to 5 m. Profundal sediment was collected with a ship-borne multicorer from a depth of 80 m in the "Überlinger See." Littoral and profundal sediment cores were collected in late winter (February and April 2007) and were taken to the laboratory within 0.5 to 3 h. To simulate *in-situ* conditions, cores were kept in a water bath at *in-situ* temperature and their surface was flushed continuously with aerated lake water. Water temperatures were 5°C for the profundal core in February 2007 and 8°C for the littoral core in April 2007. The methane and oxygen sensors were calibrated before sediment sampling, and measurements started within 30 min after arrival.

Density and porosity of the sediment samples were determined by drying 0.5-cm slices of sediment for 2 days at 70°C (data determined as wet weight and dry weight), followed by volume determination of the dried sediment in 50-ml volumetric flasks (according to the method described at http://www.ifm-geomar.de/index.php?id = mg_dichtebestimmung). The littoral sediment, with a porosity of 0.54 to 0.62, consisted of fine sand. The profundal sediment, with a porosity of 0.85 to 0.89, consisted of fine-grained material and clay.

Microsensor profiles and methane oxidation activities. Upon retrieval of the sediment cores, three to five oxygen profiles were measured with a Clark-type microelectrode (Ox-50; Unisense, Denmark). The oxygen sensor was two-point calibrated in air-saturated water and in anoxic sediment. The detection limit was 0.3 µmol liter^{-1}. Molar concentrations of oxygen were calculated according to the methods used previously (Garcia and Gordon, 1992). The sensor was mounted on a micromanipulator and was moved into the sediment at 0.5-mm steps.

Methane profiles of high spatial resolution were determined with a diffusion-based microsensor (Bussmann and Schink, 2006). For calibration, three methane standard solutions were prepared with glass beads to mimic sediment diffusivity (Bussmann and Schink, 2006). Standard solutions were incubated in a water bath at the respective *in-situ* temperatures. The aerated water bath was taken as a zero-methane standard. Additional standards contained 3, 44, and 93 µM of methane. The detection limit was 2 µmol liter^{-1}. The relative accuracy of

the sensor was ±15% with a precision of ±7.5%. Methane in sediment cores was measured in three parallel profiles at 2-mm intervals to a depth of 6 cm.

Depth profiles of methane oxidation and production were calculated by two methods. One method used was a computer-implemented diffusion-reaction model (Berg et al., 1998). In a first step, the best-fitting concentration profile was calculated. In the next step, the simplest production-consumption profile that reproduces the concentration profile was chosen. The other method was an application of Fick's second law of diffusion to the best-fitting concentration profile. The calculated activities were then smoothed by a running average. Sediment diffusivity (D_s) was determined by the equation $D_s = \Phi^2 \times D$, where Φ is porosity and D is the diffusivity of methane in free water. The methane diffusion coefficient ranged from 1.13 x 10^{-5} cm^2 s^{-1} at 5°C to 1.25 10^{-5} cm^2 s^{-1} at 8°C, for profundal and littoral sediments, respectively (gas tables from Unisense, Denmark). The *in-situ* biodiffusivities for profundal and littoral sediments were calculated by multiplying D_b (biodiffusion coefficient for each organism group) by the average *in-situ* density of the respective group (Matisoff and Wang, 2000).

Sample preservation and DNA isolation. After the methane and oxygen profiles were measured, the uppermost 5-cm parts of the sediment cores were cut into 0.5-cm slices and stored at –20°C. DNA was extracted from 300 to 400 mg wet sediment by using the Fast DNA spin kit for soil (MP Biomedicals Germany). The final concentration of the diluted DNA was determined by the Sybr green quantification method (with Sybr green I; Cambrex Bioscience, Maine) (Zipper et al., 2003).

PCR and qPCR. Trial qPCR assays, namely, MTOT, MBAC and TYPEII, as described by Kolb et al. (2003), were run using DNA from littoral and profundal sediments as templates. The MTOT assay (with the A189f-mb661r primer set [Auman et al., 2000]) has been designed to quantify the *pmoA* gene as a target for all methanotrophs (Kolb et al., 2003). For this assay, a plasmid carrying the *pmoA* clone (littoral site 1, clone 12, NCBI accession number DQ235460) from the *pmoA* clone library of DNA found from the Lake Constance littoral sediment was used as a standard (Rahalkar and Schink, 2007). For exact quantification, the concentration of the plasmid was determined by the Sybr green quantification method. *pmoA* target molecules per ng of DNA were calculated assuming a molecular mass of 660 Da per DNA base pair (Fierer et al., 2005). A dilution series with 10-fold dilution steps resulting in 10^1 to 10^7 target molecules of DNA μl^{-1} was used as standards.

Usually a small amount (i.e., 1 to 2 ng) of sediment DNA was used in a 20-µl PCR mixture, to avoid any possible effects of PCR inhibitors in the sediment DNA. Power Sybr green qPCR kit (Applied Biosystems) was used, and the qPCR was performed in an ABI-7500 instrument (Applied Biosystems). The reaction mixture consisted of 10 µl of the master mix and 10 pmol of each primer in a final 20-µl reaction mixture. Melting curve analyses were performed with samples and standard assays from which the data acquisition temperature was calculated. The data acquisition temperature is the temperature above the melting temperature of the primer dimers and was determined to be 77.5°C for the MTOT assay. The qPCR program for the MTOT assay was modified as follows: 94°C for 15 s, 56°C for 30 s, and 60°C for 30 s, and data acquisition at 77.5°C for 34 s for 40 cycles followed by denaturation. All standards and samples were used in triplicate. Standard graphs of threshold cycle were plotted against the logarithm of the copy number. The copy numbers of the samples were calculated with the help of 7500 system SDS software (Applied Biosystems) or with Microsoft Excel.

The MBAC assay targeting the *Methylobacter/Methylosarcina* group was found not to amplify all the *pmoA* sequences retrieved from Lake Constance (Bussmann et al., 2006). Therefore, a new qPCR assay was developed to detect the abundance of type I MOB in Lake Constance (LC type I assay) by designing a reverse degenerate primer, LC Type I r (5'TTCTDACRTAGTGGTAACC3'), to cover the detected *pmoA* diversity of type I MOB from Lake Constance (Bussmann et al., 2006). The specificity of the reverse primers was checked by performing a BLAST search at the NCBI site (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990), using the MegAlign program in the DNASTAR software and using the ARB software package (version 2.5b; http://www.arb-home.de) (Ludwig et al., 2004). Annealing temperatures were determined by amplifying this particular region of the *pmoA* gene from a *pmoA* clone (littoral site 1, clone 12, accession number DQ235460) (Rahalkar and Schink, 2007), for which only a single band of correct size was obtained and annealing and data acquisition temperatures of 54°C and 78°C, respectively, were determined. The LC type I assay was additionally validated by creating clone libraries from the sediment, using this primer set, and it was found that all clones belonged to type I methanotrophs.

With qPCR, we detected positive products of correct size with the MTOT assay (total methanotrophs), the MBAC assay (for *Methylobacter/Methylosarcina* type I methanotrophs) (Kolb et al., 2003), and the LC type I assay (this study) but did not get any amplification for the type II MOB-specific assay at the annealing temperature mentioned by Kolb et al. (2003). Thus, only the MTOT assay and the LC type I assay were used further for quantifying the total *pmoA* genes and *pmoA* genes of type I methanotrophs, respectively.

For estimation of the bacterial 16S rRNA gene copy numbers, bacterial primers Eub338 and Eub518r (Fierer et al., 2005) were used. The same program as described for the two PCRs described above was used except that the annealing temperature was 53°C, and a plasmid containing a 16S rRNA gene fragment was used as a standard after appropriate dilutions. Finally, to compare the cell numbers obtained by FISH with those obtained by qPCR, *pmoA* copy numbers were divided by 2 (average copy number of *pmoA* in methanotrophs) (Kolb et al., 2003), and for total bacteria, the copy numbers were divided by 4 (Case et al., 2007). Standard errors for qPCR were on average 0.1 x 10^7 bacteria per g of sediment (wet weight) for the MOB from the littoral sediment (in both the MTOT assay and the LC type I assay), and on average 0.7 x 10^7 bacteria per g of sediment (wet weight) for the MOB from the profundal sediment (in both the MTOT assay and the LC type I assay). The standard errors for the total bacterial assay for the littoral and profundal sediments were 0.1 x 10^9 and 0.2 x 10^9 per g of sediment (wet weight), respectively.

Extraction of cells from the sediment and FISH. FISH was performed on samples collected in February and April 2007. Immediately after the sediment was sliced, samples of 240 to 820 mg (fresh weight) were fixed by the addition of formalin to a 4% final formaldehyde concentration and incubation at room temperature for at least 1 h or overnight at 4°C. Formaldehyde was removed by centrifugation at 10,000 x g for 2 min. The supernatant was removed, and 1 ml of 1x phosphate-buffered saline (PBS), 160 µl of Na-pyrophosphate (0.1 M), and 1 drop of Tween 80 was added to the pellet. The suspension was mixed vigorously for one minute and then incubated at room temperature for 30 min. After a further brief mixing, the samples were centrifuged for 2 min at 720 x g and the pellet was washed twice with 1 ml PBS in a similar way. All three supernatants were pooled and centrifuged for 10 min at 14,000 x g. The obtained cell pellet was resuspended in 100 µl of PBS-ethanol mix (1:1). After sonication for eight short intervals (in total, 10 s) at cycle 0.5/amplitude 50 (instrument settings), samples were stored at –20°C. Extraction efficiency was checked with three littoral sediment samples, three profundal sediment samples, and three stored fixed sediment samples. For an estimate of the extraction efficiency of our protocol, we counted the bacterial numbers after each extraction step. The third extraction step yielded less than 20% of the total number of extracted cells. Therefore a fourth extraction step was omitted. Sonication of the samples (15 intervals at cycle 0.5/amplitude 50) increased the total bacterial count by 20%, but the background fluorescence of the sediment increased dramatically to render counting, especially of profundal sediment samples, barely possible.

FISH was performed in 10-well microscopic slides (Roth, Germany) with 10 µl of sample that had been sonicated briefly once more, as described by Eller et al. (2001), and stained with DAPI (4',6-diamidino-2-phenylindole; 1 µg/ml). Hybridizations with probes for type I and type II methanotrophs were performed separately but combined with the Eub338 probe linked to fluorescein. Probes used for type I methanotrophs were Cy3-linked Mγ84 and Mγ705 (Eller et al., 2001) and Cy3-linked Mα450 for type II methanotrophs (Eller et al., 2001). All probes were purchased from ThermoHybaid (Germany).

Slides were dried, Citifluor antifading agent (Citifluor Ltd., United Kingdom) was added, and the slides were stored at –20°C until counting. Slides were observed with an Axiophot fluorescence microscope (Zeiss) with the filter sets suitable for observing DAPI, Cy3, and fluorescein and photographed with a cooled charge-coupled-device camera (Magnafire; INTAS). Only cells showing clear signals with all three excitation filters and fluorescing in the proper colors were counted as methanotrophs. For calculation of final numbers, we used a calculation similar to that used previously (Dedysh et al., 2001) except that at minimum 35 fields of view were counted, and this number would correspond to 11,000 to 140,000 DAPI counts.

For DAPI, only five squares were counted. Cells were found evenly distributed on the slides. Because of the high background fluorescence of the sediment, only brightly fluorescing cells were counted. Bacterial cells hybridized with the Eub338 probe were not counted, because background fluorescence of sediment particles was too high for reliable counts with the corresponding filter to be obtained. However, differentiation between bacteria and inorganic particles was facilitated by comparison of three pictures with different filters to determine the MOB-specific counts.

Results

Microsensor profiles and activities. The distributions of oxygen and methane in littoral and profundal sediments were analyzed with microsensors at high vertical and horizontal resolutions. In the littoral sediment, the oxic zone was narrow, with oxygen penetrating down to a depth of 0.35 cm (Fig. 2.1A). In the profundal sediment, oxygen penetrated to a depth of 0.65 cm (Fig. 2.1B). The highest methane concentrations in the littoral sediment (140 µM) were measured at a depth of 5.5 cm. A steep decrease of methane was found at a depth of 2 to 3 cm, and concentrations were close to zero in the uppermost 1 cm. Methane determinations in different months during 2005 and 2006 showed similar profiles (data not shown). In the profundal sediment, a maximum of 113 µM of methane was measured at a depth of 6 cm

(Fig. 2.1B), and there was a linear decrease toward zero in the top 1 cm. By performing additional measurements in the profundal sediment during 2005 and 2006, we observed comparable profiles as well (data not shown).

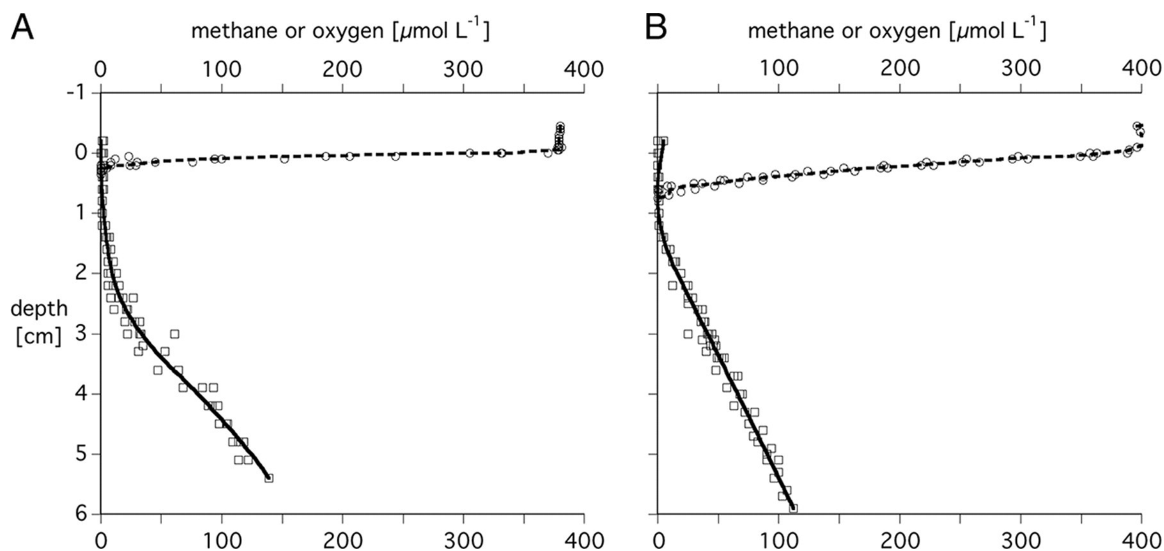

Fig. 2.1. Concentration profiles of oxygen (circles) and methane (squares) in littoral (A) and profundal (B) sediments. Lines indicate the means of three oxygen measurements and the calculated best-fitting profile of methane concentration.

In order to localize and quantify the zones of methane consumption or methane production, we assumed steady-state conditions. We calculated the activities directly from the concentration gradients and additionally applied a model to the methane profiles (Berg et al., 1998).

In the littoral sediment, methane was oxidized in the top 3.5 cm, with a zone of low activity (average, 0.04 µmol liter^{-1} h^{-1}) in the top 1.6 cm (Fig. 2.2A) followed by a zone of higher activity (average, 0.18 µmol liter^{-1} h^{-1}) from 1.6 to 3.5 cm. Below 3.5-cm sediment depth, methane production started. In total, 4.01 µmol methane m^{-2} h^{-1} was oxidized. Use of the model of Berg et al. (1998) revealed the same depth zonation and similar activities (total oxidation rate, 3.4 µmol m^{-2} h^{-1}).

In the profundal sediment, methane oxidation was restricted to the top 0.5 to 2.3 cm, with an average rate of 0.28 µmol liter^{-1} h^{-1} (Fig. 2.2B). The total methane oxidation rate was 5.74 µmol m^{-2} h^{-1}. Within a depth of 2.3 to 5 cm, no notable activity of methane oxidation or production was observed. The methane production zone presumably started below the investigated depth. Use of the model of Berg et al. (1998) revealed a much broader zone of methane oxidation, reaching from 0 cm to almost 3 cm. However, the overall methane oxidation rate was almost the same (5.76 µmol m^{-2} h^{-1}).

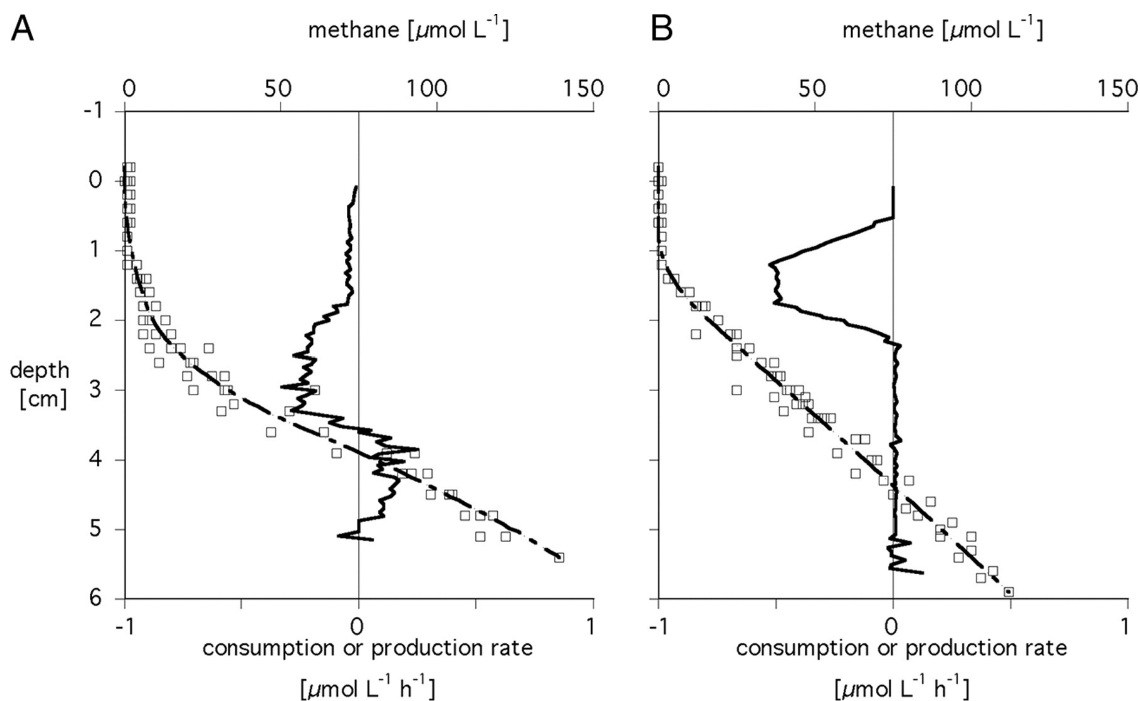

Fig. 2.2. Methane consumption and production rates (bold continuous line) calculated via Fick's second law of diffusion from the concentration profiles (dashed line) measured for littoral (A) and profundal (B) sediments. The individual data are plotted as squares.

For both the profundal sediment and the littoral sediment, we found the zone of methane oxidation extending 2 to 3.5 cm into the sediment, which is significantly deeper than the respective oxygen penetration depths (0.35 and 0.65 cm, respectively).

Abundance of methanotrophs. Quantification of MOB by qPCR of *pmoA* genes (MTOT assay) revealed that in the littoral sediment (Fig. 2.3A), the numbers of methanotrophs as determined by quantification of the *pmoA* copies in the littoral sediment were slightly lower in the top 0.5 cm (9.6 x 10^6 MOB per g of sediment [wet weight]) than in the sediment below. Their numbers increased with depth and were almost constant down to a depth of 3.5 cm. The highest numbers of MOB were found at a depth of 2 to 3 cm (average of 2.7 x 10^7 MOB per g of sediment [wet weight]). Below 3.5 cm, the *pmoA* copy number decreased. Type I MOB (in the LC type I assay) constituted a large proportion of total MOB in all layers. They contributed to 60 to 100%, and on average 70%, of the total methanotrophs in terms of copy numbers. In the zone of highest abundance, i.e., 2 to 3 cm, they contributed 70% of the total *pmoA* copies.

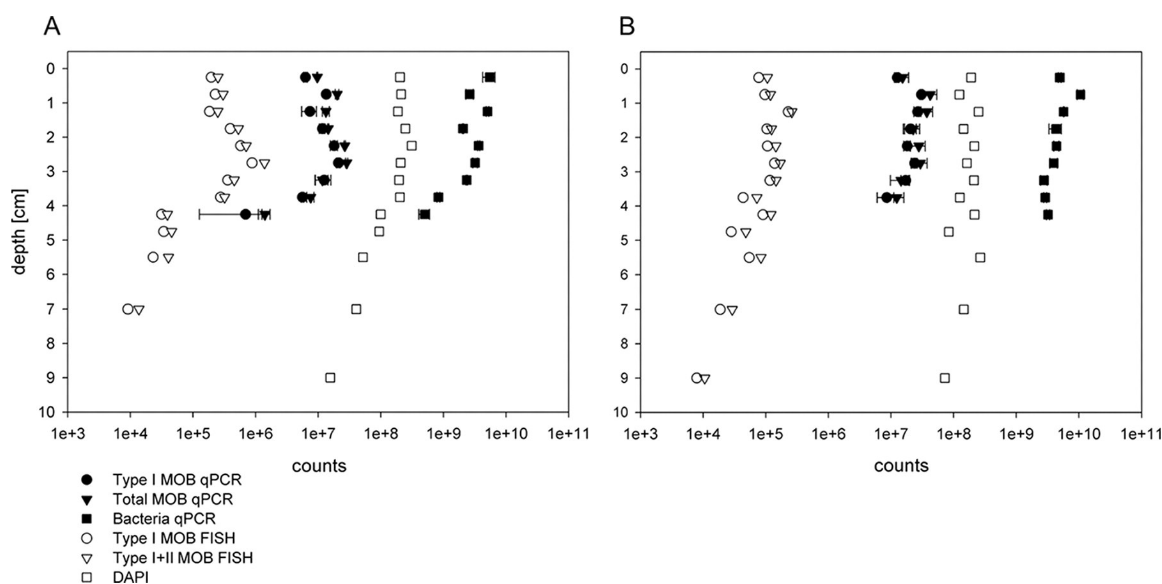

Fig. 2.3. Abundance of methanotrophs in littoral (A) and profundal (B) sediments. MOB as quantified by FISH (cells per g of sediment [wet weight]) and by qPCR (cells per g of sediment [wet weight]). Type I MOB (determined with Mγ84 and Mγ705 for FISH and the LC type I assay for qPCR), total numbers of MOB (determined with Mγ84, Mγ705, and Mα450 for FISH and the MTOT assay for qPCR), and total bacterial numbers (determined by DAPI counts and 16S rRNA gene qPCR) are shown.

The detection of MOB in the littoral sediment with FISH also showed a clear maximum of MOB abundance at a depth of 2 to 3.5 cm (6 x 10^5 cells per g of sediment [wet weight]). Type II MOB were found in lower numbers (2 x 10^5 per g of sediment [wet weight]). In the upper 4 cm of the sediment, type I MOB were about four times more abundant than type II MOB. Both types were detected down to a depth of 8 cm. Type I methanotrophs were sometimes visible as pairs of cylindrical or elliptical cells. These cells were quite large (2 to 4 µm) compared to the other bacteria. The type II methanotrophs were mainly single coccoid cells.

According to qPCR analysis, the total number of bacteria in the littoral sediment was maximal in the top 0.5 to 1.5 cm, with 5 x 10^9 bacteria per g of sediment (wet weight) (Fig. 2.3A) and on an average 2.8 x 10^9 bacteria per g of sediment (wet weight) were counted at a depth of 4.5 cm. Counting the cells after DAPI staining revealed on average a similar distribution of cells in the top 4.5 cm but numbers lower by 1 order of magnitude (2.1 x 10^8 cells per g of sediment [wet weight]) (Fig. 2.3A). With both methods, the abundance of total bacteria decreased by 1 order of magnitude toward deeper sediment layers.

In the profundal sediment, *pmoA* qPCR revealed comparable, high MOB numbers at a depth of 0.5 to 1.5 cm, which were around 4 x 10^7 per g (wet weight) (Fig. 2.3B). Numbers decreased only slightly below a depth of 1.5 cm. Average numbers of 2 x 10^7 copies per g fresh sediment were observed at a sediment depth of 2 to 4 cm. Type I MOB (LC type I

assay) represented a large proportion, i.e., 65 to 100%, and on average 81%, of the total methanotrophs.

Quantification of MOB with FISH probes revealed in profundal sediment maximal numbers between 1- and 1.5-cm depth. Both type I and type II MOB were detectable down to a depth of 10 cm, which was the deepest layer investigated. The ratio of type I MOB to type II MOB was similar to that observed in the littoral sediment. Total bacteria as detected by qPCR were almost constant from a depth of 0 to 4.0 cm, and on average, 4.7 x 10^9 cells per g of sediment (wet weight) were detected. Total MOB abundance in the uppermost 4 cm in the profundal sediment ([1.4 ± 0.6] x 10^5 cells per g of sediment [wet weight]) was lower by a factor of 4 to 5 than that in the littoral sediment ([5.3 ± 3.9] x 10^5 cells per g of sediment [wet weight]).

The total numbers of bacteria in the littoral and profundal sediments by qPCR were approximately (4.4 ± 1.1) x 10^9 and (5.0 ± 1.8) x 10^9 bacteria per g of sediment (wet weight), respectively. With DAPI staining, one-tenth as many bacteria were found in each of the two sediments, i.e., 2 x 10^8 cells per g of sediment (wet weight). For both sediments, plots of the ratios of total MOB (sum of type I and type II MOB) to DAPI counts (details not shown) corresponded well depthwise with the zones of methane oxidation. The highest ratios were found in the littoral sediment at a depth between 1.5 and 3.5 cm (zone of high methane oxidation activity) and a depth between 0.5 cm and 2.0 cm in the profundal sediment. According to qPCR results, MOB represented around 0.2 to 0.9% of the total bacteria in the littoral sediment and 0.3 to 0.7% in the profundal sediment. However, the ratios of total MOB to total bacteria when plotted against sediment depths showed a more or less unequal distribution (i.e., a zigzag line).

Discussion

Concentration and activity profiles. Methane consumption and production rates were calculated from high-resolution determinations of methane concentrations in sediment samples. In contrast to the case for previously used methods, the sediment cores were not destroyed and could be used for microbiological investigations afterwards. Additionally, the profile measurements lasted only approximately 3 h, and thus, incubation artifacts could be minimized.

We used two approaches to calculate the methane oxidation rate: direct application of Fick's second law of diffusion and model calculations (Berg et al., 1998). For littoral sediment, our calculated flux data (0.46 µmol m^{-2} h^{-1}) compared well with modeled flux data (0.56 µmol m^{-2} h^{-1}) and flux data calculated earlier on the basis of sediment core incubations

(0.61 µmol m^{-2} h^{-1}) (Bussmann, 2005). The results of the two approaches for the profundal sediment did not agree as well as the results for the littoral sediment. Although the overall methane oxidation rates were similar, the zonations were different. Direct calculation of the activity revealed a zone of methane oxidation activity which was much more consistent with the concentration profiles. From core incubations of profundal sediment of Lake Constance, a methane oxidation rate of 18.7 µmol m^{-2} h^{-1} has been calculated (Frenzel et al., 1990), and this rate is twice as high as the rate we calculated from our data (8.8 µmol m^{-2} h^{-1}). However, these data were obtained later in the year and at a time when the phosphate content of Lake Constance water was still about six times higher than it is presently. Thus, our estimates of methane oxidation activities appear to represent realistic values for the *in-situ* activity.

In the littoral sediment of Lake Constance, the group of *Chironomidae* is the most abundant macrofaunal group (Mörtl, 2003). In profundal sediments, the *Tubificidae* are the dominant infauna (Sauter and Güde, 1996). Including biodiffusivity in the model did not change the zonation of methane oxidation rates in littoral and profundal sediments. Chironomids dwelling in the top 1 cm of littoral sediment (Stief and de Beer, 2006) had only a minor influence on the overall methane oxidation rate. *Tubificidae* with deeper-reaching burrows in profundal sediment increased the overall methane oxidation rate by a factor of 1.7. However, more data on the distribution and activity pattern of the respective infauna would be necessary to assess their influence more precisely.

Methane production rates were calculated based on methane fluxes and oxidation rates. It turned out that in the profundal sediment, about 98% of the produced methane was oxidized, and in the littoral sediment, this value was about 90%. Earlier studies reported 93% methane oxidation in the profundal sediment and 79% in littoral sediment of Lake Constance (Frenzel et al., 1990; Bosse et al., 1993). It should be noted that these data (Frenzel et al., 1990; Bosse et al., 1993) were obtained when Lake Constance was still rather eutrophic. The overall oxygen consumption rates in littoral and profundal sediment were 250 and 228 µmol m^{-2} h^{-1}. Given a stoichiometry of 2 mol O_2 per mol CH_4 oxidized, methane oxidation contributed to about 3% and 5% of the total oxygen consumption in littoral and profundal sediments, respectively.

Quantification and abundance of methanotrophs. For quantification of methanotrophs, we used two independent molecular methods, namely, qPCR based on the abundance of a functional gene (*pmoA*) amplified from the DNA obtained from the sediment and FISH based on the hybridization of 16S rRNA with specific probes for type I and type II MOB, to

estimate the abundance of total methanotrophs relative to the active fraction. Although FISH and qPCR showed very similar profiles of distribution of MOB in the sediment, there was a considerable difference in the absolute numbers obtained. Under the assumption that every cell contains two copies of the *pmoA* gene (Kolb et al., 2003), we detected an average of 1.7×10^7 MOB cells per g fresh weight in the littoral sediment and 2.5×10^7 MOB cells per g fresh weight in the profundal sediment in the upper 4 cm. With FISH, we obtained much lower numbers, i.e., a total of 5.3×10^5 MOB per g of sediment (wet weight) in the littoral sediment and 1.4×10^5 MOB per g of sediment (wet weight) in the profundal sediment. To explain this discrepancy, we compared clone library data for type I and type II methanotrophs from the study site (Rahalkar and Schink, 2007) with the oligonucleotide sequence of the FISH probe and assumed that one mismatch resulted in no detectable signal (Eller, 2001). Thus, with the FISH probe set we employed (mγ705 and mγ84), we missed about 32% of the clones present in littoral sediment and about 63% of the profundal clones. When the FISH numbers were corrected for these mismatches, we obtained 7.1×10^5 and 3.3×10^5 cells per g of sediment (wet weight) for littoral and profundal sediments, respectively. In a comparison of these numbers with the qPCR data, the FISH numbers for the littoral and profundal sediments were still 23 and 75 times lower, respectively.

The high numbers determined by qPCR could be due to the fact that growing cells contain more than one genome copy per cell, and this could result in copy numbers being higher than the number of cells (Kolb et al., 2003). Another reason for this discrepancy could be that binding of the FISH probe to cells depends on the number of rRNA molecules, which in turn depends on the activity status of the cells (Bouvier and del Giorgio, 2003). Thus, inactive or slowly growing cells would not be detected with the FISH method, thus resulting in lower counts. In contrast, qPCR is based on DNA, and therefore, all cells would be counted, even if they are not active. In addition, the extraction of cells from the sediment for FISH could cause considerable losses. However, additional sonication used to estimate losses due to our extraction protocol yielded only 21% more cells. The true extraction efficiency is still unknown. Nevertheless, DAPI counts in littoral and profundal sediments of Lake Constance in the present study were comparable with those of previous studies of the littoral sediment; in these studies, 4×10^8 to 8×10^8 bacteria per g of sediment (wet weight) were detected, whereas in the profundal sediment, 1×10^9 to 4×10^9 bacteria per g of sediment (wet weight) were detected (Sala and Guede, 2006). Bacterial numbers detected by qPCR were a bit higher than those detected earlier in the case of the littoral sediment and in the same range as that of the profundal sediment (Sala and Guede, 2006).

qPCR designed to amplify type II MOB was not successful, suggesting that type II MOB were present in low abundance. The type I MOB qPCR assay also showed that type I MOB represented a major proportion of total MOB in terms of copy numbers. Various molecular approaches used so far, i.e., terminal restriction fragment length polymorphism using the *pmoA* gene (Pester et al., 2004; Röling, 2007), clone libraries based on *pmoA*, and the 16S rRNA gene, all have suggested low abundance of type II MOB in the littoral and profundal sediments (Röling, 2007) compared to that of type I MOB. Although all the type II MOB sequences deposited thus far from Lake Constance sediment were covered by the primers (Kolb et al., 2003), there could be yet-undescribed type II MOB present that were not covered by the existing primer sets. By using another molecular method, FISH, which is not based on PCR, we were able to document the presence of type II MOB which have also been cultivated from this site (Bussmann et al., 2004).

Few studies have focused on determining the abundances of methanotrophs in environments such as sediments and soils (Costello et al., 2002; Kolb et al., 2003; Kolb et al., 2005; Knief et al., 2006). In the profundal sediment of Lake Washington, 10^8 to 10^9 total MOB cells per g (dry weight) have been detected by phospholipid fatty acid analysis and by quantitative slot-blot hybridization (Costello et al., 2002). This is within the same range as our qPCR number for profundal sediment (1 x 10^8 cells per g of sediment [dry weight]). FISH and qPCR are the most recent techniques used for the quantification of methanotrophs (McDonald et al., 2008). Very few quantitative data for methanotrophs based on FISH are available, e.g., data for methanotrophs in rhizoplane soil (Eller et al., 2001) and Sphagnum peat bogs (Dedysh et al., 2001). In Siberian permafrost regions of the Lena Delta, similar numbers of methanotrophs were counted by FISH (Liebner and Wagner, 2007). Many freshwater habitats have been shown to be dominated by type I MOB, as observed in our study (Costello et al., 2002; Bodelier et al., 2005; Eller et al., 2005a).

Relating activity to cell numbers. We found low methane oxidation activity in the top 2 cm of the littoral sediment, followed by a zone of higher methane oxidation activity at approximately 2 to 4 cm. This is also the zone where the MOB abundance increased. Thus, we found a good correlation between increased cell numbers and increased methane oxidation activity (Fig. 2.4A). In profundal sediment, we observed a slight decrease in abundance of MOB with sediment depth, and a zone of high methane oxidation activity at the sediment subsurface (0.5 to 2 cm) (Fig. 2.4B).

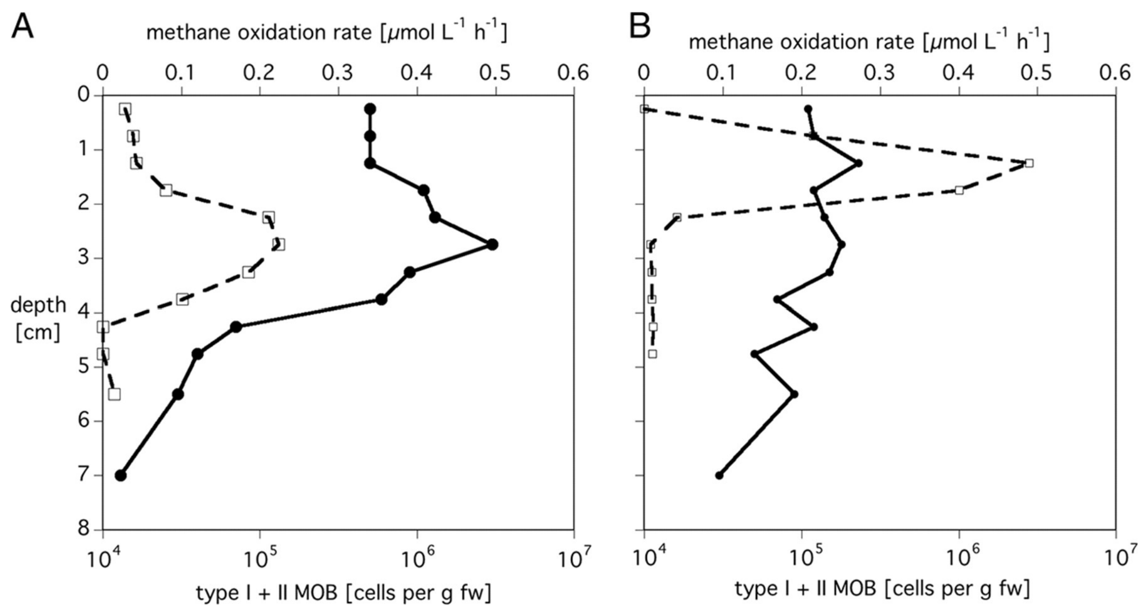

Fig. 2.4. Abundances of type I and II methanotrophs as determined by FISH (circles) together with the corresponding average methane oxidation rates (squares) for littoral (A) and profundal (B) sediments. fw, fresh weight.

The activity of a population is the product of its cell number (population size) and the activity per cell (Röling, 2007). We applied this concept to our data set. The average methane oxidation rate was estimated in 0.5-cm steps, similar to the sampling intervals in the determination of cell numbers by FISH. Analysis by cell numbers based on qPCR showed similar results. In the littoral sediment, the slope of the double logarithmic plot (log methane consumption rate versus log MOB cell number) was significantly different from zero, but it was not near 1 (0.44 ± 0.10, $P < 0.005$, $n = 10$). Thus, in the littoral sediment, the methane oxidation activity was controlled by changes in population size and by a changed activity per cell. In the profundal sediment, the slope of the double logarithmic plot did not differ significantly from zero (0.13 ± 0.07, $P = 0.08$, $n = 10$), indicating that in the profundal sediment, the methane oxidation rate was controlled mainly by a changed activity per cell and not by the number of MOB. The uncoupling of activity and cell number in the profundal sediment is probably due to lower growth efficiency. Growth efficiency of bacteria is positively influenced by grazing and nutrient addition (N and especially P) (Jansson et al., 2006; Ram and Sime-Ngando, 2008). In the littoral zone, with frequent sediment resuspension, nutrients can be released from deeper sediment layers (Güde et al., 2000), which could lead to a higher growth efficiency. Protozoa graze on MOB (Murase and Frenzel, 2007); however, no information on grazing or protozoan abundance in littoral sediments in comparison to that in profundal sediments of Lake Constance is available, and it remains

unclear if lower grazing activity in profundal sediment could explain the low growth efficiency.

Additionally, in the littoral sediment, maximum ratios of MOB over bacterial numbers (FISH/DAPI) coincided with the zone of high methane oxidation, whereas in the profundal sediment, the ratios decreased with depth, not influenced by the depth distribution of the methane oxidation rate. Ratios of MOB over bacterial numbers determined by qPCR did not show any conclusive pattern, which could be due the fact that these ratios were very low and the method was not sensitive enough to detect the small differences in this range accurately.

Depth zonation of methanotrophs. The high spatial resolution of MOB abundance together with the corresponding methane oxidation activity permitted definition of three zones in the sediment. In the surface zone, both oxygen and methane are present, and methane can be oxidized aerobically by MOB. In the second zone, most obvious in the littoral sediment, most of the methane was actually consumed where oxygen was not detectable. This was also where MOB were most abundant. In the deepest zone (deeper than 3 to 4 cm), we found MOB still in large numbers even though methane oxidation activity was not detectable.

In the littoral sediment, the noticeable disappearance of methane in the absence of oxygen in the second zone is hard to explain; sulfate-dependent oxidation of methane is energetically difficult under the conditions of low sulfate availability and low methane pressure prevailing there (Schink, 1997; Schink and Stams, 2001). One can argue that the described oxygen profiles were shifted artificially by transport and by the incubation conditions that prevented water flow and wave action at the sediment surface. Thus, *in-situ*, the oxygen would penetrate deeper into the sediment, as has been described repeatedly in studies on sandy marine sediments (Precht and Huettel, 2003). However, even with strong illumination of littoral cores, oxygen did not penetrate deeper than 6 mm into the sediment (Gerhardt et al., 2005), and in sediment cores incubated in a flume tank, oxygen penetrated to 4-mm depth at most (I. Bussmann, unpublished data). Bioturbation by *Tubificidae* or chironomid larvae can transport oxygen deeper into the sediment (Brune et al., 2000), but at least for chironomids, this occurs no deeper than 6 mm (Stief and de Beer, 2006). Comparisons of microelectrode measurements of oxygen in sediment cores with *in-situ* measurements revealed no significant difference of the oxygen penetration depth for profundal cores; in littoral cores, oxygen penetrated twice as deep in the *in-situ* measurement (Koschorreck et al., 2003). Thus, even after correcting for laboratory artifacts and bioturbation, oxygen could be available only in traces at 20- to 30-mm sediment depth, at which we found maximal methane oxidation and

maximal numbers of aerobic MOB (Fig. 2.4A). By analogy, aerobic methanotrophs in the Black Sea water column are thought to be responsible for methane oxidation at the chemocline, although no free oxygen could be detected at these water depths (Schubert et al., 2006). Thus, further studies are necessary to check for traces of oxygen in these respective depths or to check if alternatively nitrate could be involved as terminal electron acceptor (Raghoebarsing et al., 2006). In the profundal sediment, the profiles of oxygen and methane distribution showed maximal methane oxidation activity in a sediment layer at a depth of 0.8 to 1 cm, which is only a few mm below the measured oxygen penetration maximum. Again, we have to assume that *in-situ* oxygen penetrated deeper into the sediment than our measurements indicate.

In the deepest zone, MOB were present at high numbers, and they obviously did not oxidize methane at this depth. The presence of aerobic methanotrophs in anoxic zones has been documented several times by MPN counts (Roy et al., 1996; Eller et al., 2005a) and by cultivation-independent analyses (Carini et al., 2005). These bacteria may be in a dormant state, possibly thriving on endogenous storage material (Rahalkar et al., 2007). Nonetheless, they are able to respond quickly to environmental changes (Roslev and King, 1995) when oxygen or other electron acceptors become available again by bioturbation or sediment resuspension.

In sediments of Lake Constance, littoral and profundal type I MOB dominated over type II MOB. In the littoral sediment, the methane oxidation rate was controlled by cell number and activity per cell, while in the profundal sediment, the methane oxidation rate was controlled mainly by changing the activity per cell. For both sediments, we found a depth zonation of MOB, with maximal activities and highest MOB abundance at depth layers where oxygen was not detectable.

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Chapter 3

Activity and Diversity of Methanotrophic Bacteria at Methane Seeps in Eastern Lake Constance Sediments

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Abstract

The activity and community structure of aerobic methanotrophic communities was investigated at methane seeps (pockmarks) in the littoral and profundal zone of an oligotrophic freshwater lake (Lake Constance, Germany). Measurements of potential methane oxidation rates showed that sediments inside littoral pockmarks are hotspots of methane oxidation. Potential methane oxidation rates at littoral pockmark sites exceeded rates of the surrounding sediment by 2 orders of magnitude. T-RFLP analysis of the *pmoA*-gene revealed major differences in the methanotrophic community composition between littoral pockmarks and the surrounding sediments. Clone library analysis confirmed that one distinct *Methylobacter*-related group dominates the community at littoral pockmarks. In profundal sediments, the differences between pockmarks and surrounding sediments were found to be less pronounced.

Introduction

Methane is an effective greenhouse gas and accounts for 18% of the radiative forcing caused by long-lived greenhouse gases (IPCC, 2007). The relative contribution of different sources to the global atmospheric methane pool is being discussed (Conrad, 2009; Reay, 2010), and the importance of freshwater lakes is especially a matter of dispute (Bastviken et al., 2004; Etiope, 2004; DelSontro et al., 2010). Aerobic methane-oxidizing bacteria (MOB) play an important role in mitigating methane emission from freshwater environments, as they oxidize methane to carbon dioxide in the presence of oxygen (Rudd and Taylor, 1980). In Lake Constance, MOB oxidize aerobically more than 90% of the methane formed in deeper sediment layers by methanogenic archaea (Frenzel et al., 1990).

In undisturbed sediments stable gradients of methane and oxygen are formed (Rudd and Taylor, 1980). MOB shape these gradients by consuming methane and oxygen in a way that both substrates meet only in a narrow zone due to limited diffusive transport (Noll et al.,

2005; Rahalkar et al., 2009). With the establishment of such a gradient system, the bacterial community experiences constantly very low substrate concentrations, which select within the community for strains with higher substrate affinities (Noll et al., 2005).

Pockmarks are concave depressions of the sediment surface, which have been observed so far mainly in marine sediments (Hovland and Judd, 1988; Hovland et al., 2002; Judd and Hovland, 2007). Reports of pockmarks in freshwater habitats are still rare (Pickrill, 1993; Duck and Herbert, 2006). In the last years hundreds of pockmark-like structures were discovered in the South Eastern part of Lake Constance (Wessels et al., 2010). These pockmarks are round depressions of up to 8 m diameter and 0.5 to 1.5 m depth. At 40% of these sites, biogenic methane was observed to bubble out of the sediment (Bussmann et al., 2011). In shallow areas of the lake that exhibit enhanced productivity these gas bubbles can reach the water surface and emit methane to the atmosphere (Wessels et al., 2010).

Environmental conditions in sediments close to the gas-emitting sites are likely to differ drastically from the conditions in undisturbed sediment. Close to the gas emanation sites, both oxygen and methane are probably available in excess. Methane from gas bubbles can dissolve into the well-oxygenated water and diffuse into the sediments, leading to high local methane concentrations and potentially high diffusive methane fluxes. Additionally, water movements might be caused by the upwelling bubbles, which may increase convective mixing of both methane and oxygen. Therefore, methanotrophic bacteria situated at those sites likely encounter higher substrate concentrations there than in the surrounding sediment.

The community composition, diversity, abundance and distribution of MOB in the well-stratified and undisturbed profundal sediments as well as in the wind- and wave-exposed littoral sediments of Lake Constance have been investigated (Pester et al., 2004; Rahalkar and Schink, 2007; Rahalkar et al., 2009). In the present study we investigated the MOB community composition at pockmark sites and compared it to the communities in the surrounding sediment outside the pockmarks. In addition, the methane oxidation potential of the communities was measured to examine whether the escaping methane increases the methane oxidation activity of MOB communities at these sites.

Materials and methods

Sampling. Pockmarks that were previously located and mapped (Wessels et al., 2010) were relocated by use of the Global Positioning System (GPS), and a horizontally scanning sonar was employed for exact positioning of the sediment corers. At the first cruise on 26 April 2010, profundal pockmark samples (PP) were taken with a gravity multicorer with plastic

tubes of 60 mm inner diameter in the South-Eastern part of Lake Constance (47°31,37755N, 9°35,89464E) at 82 m water depth. Profundal reference samples (PR) were taken approx 50 m away (47°31.37151336N, 9°35.91289522E) also at 82 m water depth. The littoral pockmark (LP1) was sampled at 12 m water depth by scuba diving with plastic tubes of 80 mm inner diameter to sample the gas ebullition site reliably (47°29.97485222N, 9°35.70826669E) whereas the reference samples (LR1) were collected at 12 m water depth with the same corer used for profundal samples (47°29.95212802N, 9°36.13246003E).

At a second cruise on 15 June 2010, only littoral samples were taken by scuba diving. Two gas ebullition sites in pockmarks (LP2, LP3) were sampled with plastic tubes of 80 mm inner diameter (LP2, 47°29.97538772N, 9°35.7090691E and LP3, 47°29.97593976N, 9°35.7066863E). Reference sediment cores (LR2 and LR3) were taken by divers outside the respective pockmark with two 50 ml falcon tubes with a small hole in the bottom to release the overlying water during sampling. Samples taken with the multicorer were cut directly onboard and then cooled. Samples taken by divers were transported as undisturbed sediment core to the lab. All samples were cooled during transport to the laboratory.

Methane oxidation capacities. Samples for determination of methane oxidation capacities were stored overnight at 4°C and processed on the next day. Sediment material from the uppermost 4 cm of each core was suspended 1:2 (w/w) with sterile freshwater medium (Bussmann et al., 2004) and mixed well under nitrogen gas to prevent oxidative stress during methane starvation. Twenty milliliters slurry was prepared in 58-ml serum bottles under a headspace of 25% air (~5% O_2) and 75% nitrogen. Samples LR2 and LR3 were treated similarly, but 10 ml slurry was incubated in 23-ml serum bottles. Methane was added to the headspace to provide final concentrations of 0.05, 0.1, 0.2, 0.4, 0.7, 1.0, 2.0% (vol/vol) and, for incubation of sample LP1 also 4.0%. Vials were incubated at *in-situ* temperature (4°C for 1st cruise, 13°C for 2nd cruise) in a horizontal linear shaker. Initial methane oxidation rates were calculated from the determined methane concentration decrease over time. The rates for LP and profundal samples were measured over 5 to 8 h on the first day. On the second day, the gas phase was renewed and methane decrease was monitored again over 8 h. Maximum methane oxidation capacities (V_{max}s) and apparent half saturation constants ($S_{0.5}$s) were calculated with second-day datasets because the sampling intervals on the first day were chosen too long to get trustworthy results from some pockmark slurries (methane was consumed almost completely at low concentrations at the second data acquisition point). The methane decrease in LR samples was monitored up to 5 days. Methane was determined with a

Carlo Erba 6000 Vega Series 2 gas chromatograph (Carlo Erba Instruments) as described previously (Platen and Schink, 1987). To estimate $S_{0.5}$s and V_{max}s of the different sediment incubations, initial methane oxidation rates were plotted against the initial methane concentration, and curves were fitted using Origin software, version 6.0 (Microcal Software). Methane oxidation rates that were too low to be detected at high methane concentrations due to the high relative measurement errors were not analyzed any further. Oxygen measurements in a subset of samples during the sampling period never revealed complete oxygen depletion (<0.2% in the gas phase).

DNA extraction, T-RFLP and clone library. DNA was extracted in duplicate from undiluted sediment slurries of the uppermost 4 cm of each sediment core immediately after arrival at the lab (2 to 5 h after sampling). A NucleoSpin soil kit (Macherey-Nagel) was used according to the manufacturer's instructions and the products of duplicate extractions were pooled. Partial *pmoA* genes were amplified using the *pmoA* primer pair A189-mb661 (Costello and Lidstrom, 1999) to cover the maximum MOB diversity without amplification of *amoA* genes (Bourne et al., 2001). Terminal restriction fragment length polymorphism (T-RFLP) analysis was performed using two fluorescent primers (mb661 labeled with 6-carboxyfluorescein [mb661-FAM], A189 labeled with hexachloro-6-carboxyfluorescein [A189-HEX]) to increase the resolution of the T-RFLP analysis. PCR products were purified using a DNA Clean & Concentrator™-5 kit (Zymo Research).
For T-RFLP analysis, 200 ng DNA (or 17 µl PCR mixture for negative controls) was digested with 0.5 U of the restriction enzyme MspI (Fermentas) for 3 h at 37°C according to the manufacturer's instructions. After heat inactivation at 80°C for 20 min, all samples were stored at -20°C till analysis. T-RFs were size-separated in triplicate on an ABI PRISM® 3130xl genetic analyzer (Applied Biosystems) using an internal size standard (0.5 µl diluted in 10 µl water; GeneScan 500 ROX; Applied Biosystems) and 10 ng of digested DNA. Samples were denatured at 94°C for 5 min, immediately cooled on ice, and shortly centrifuged. T-RF sizes between 50 and 500 bp with peak heights of at least 30 fluorescence units were analyzed using GeneMapper software 4.0 (Applied Biosystems). T-RFs present in no-template controls or in only one replicate were excluded from analysis. Despite the localization of the MspI restriction site at the very end of the mb661-FAM primer, good T-RFLP profiles were also obtained in that direction. Thus MspI obviously cannot cut off the fluorophore at this site. T-RFLP analysis, including Additive Main Effects and Multiplicative Interaction Model (AMMI), and interaction principal component analysis (IPCA) was

performed using the T-REX program (version 1, revision 11; http://trex.biohpc.org/) (Culman et al., 2009). Peak height was chosen as the parameter, and data were aligned using a 1.7 bp threshold. No noise filtering was used because the filtering algorithm is not suitable for samples with a low number of T-RFs. The dataset was analyzed first using T-RFLP profiles from all sites as individual samples (environments). To check for the amount of variation that can be explained by the site characteristic pockmark in the littoral zone alone, a second analysis was performed using the three littoral pockmark sites and references as replicates for littoral pockmark and reference sites, respectively.

pmoA clone libraries were constructed using the TA cloning kit (Invitrogen), and the plasmid inserts of randomly picked clones were amplified using the M13 primer pair. Subsamples of the amplified products were digested with MspI (5 U; MBI Fermentas), separated by gel electrophoresis on a 2.5 to 4% agarose gel, and grouped according to their restriction patterns. Clones from each group were randomly selected for sequencing. Undigested PCR products were digested with another restriction enzyme TasI (Fermentas) and analyzed as mentioned above. Sequencing was carried out by GATC-Biotech Co., Konstanz, Germany. Quality of sequences was checked manually. Alignment and phylogenetic analysis was done using MEGA (version 4) software (Tamura et al., 2007) and the online-based software on www.phylogeny.fr. The tree shown was constructed using the Minimum Evolution method in MEGA (version 4) software selecting the pairwise deletion option (1,000 replications). Evolutionary distances were computed using the JTT matrix-based method. Clones were grouped according to their position in the phylogenetic tree.

qPCR. Quantitative real-time PCR (qPCR) was performed using 10 ng template DNA, SsoFast EvaGreen Supermix With Low ROX (Bio-Rad) and 50 nM of each Primer mb661 and A189 on an ABI 7500 fast machine. A primer concentration of 50 nM was chosen because higher concentrations caused an additional unspecific PCR product in some samples. A two-step PCR protocol was used with an initial denaturation at 98°C for 2 min, followed by 40 cycles of 98°C for 6 s and 30 s at 60°C for annealing, elongation and data acquisition. Melting curves were obtained at 60-95°C at 0.5% heating rate. A plasmid (PCR2.1; Invitrogen) containing a single copy of the *pmoA* gene (GenBank accession number HQ383803) was diluted 1:10 to 10^1 to 10^7 molecules per PCR mixture and used as standard in triplicates. Samples were analyzed in four parallel assays. PCR products were visualized via gel electrophoresis and ethidiumbromide staining to exclude formation of unspecific PCR products. Analysis was done with the LinRegPCR program (Ruijter et al., 2009) setting

individual amplification efficiencies for the different samples, but using one common threshold. Cell numbers were calculated using the obtained *pmoA* copy numbers and the amount of DNA extracted from 500 mg sediment (wet weight). Two copies of the *pmoA* gene were assumed to be present per cell (Kolb et al., 2003).

Nucleotide sequence accession numbers. Nucleotide sequences were deposited at the National Centre for Biotechnology Information under accession numbers HQ383711-HQ383812.

Results

Sampling. Undisturbed sediment cores were obtained with all sampling methods. Since the profundal sediments had to be sampled with a multicorer, it was not possible to sample a gas emanation site exactly inside the profundal pockmark. Littoral Sediment cores LP1 and LP3 showed disturbances in the sediment layers, most probably due to direct sampling of the gas emanation site. Also, some cavities were observed in deeper layers of those sediment cores during sample preparation. Core LP2 did not exhibit any indications of disturbance and is therefore likely to be taken from near proximity but not directly from the gas emanation site.

Methane oxidation capacities. To estimate the capacity for methane oxidation at pockmark sites, methane oxidation rates were measured at *in-situ* temperature over few days after sampling with slightly diluted sediment slurries. On average, there was an increase in methane oxidation rates on the second day to 179% of the first day activity in littoral samples, regardless of the initial methane concentration but with high variations between the corresponding vials of both days (standard deviation, ±104%). Profundal samples, however, showed a decrease in methane oxidation capacities between the first and the second day to a mean of 77% ± 52%, regardless of the initial methane concentration. Therefore, calculated V_{max}s have to be interpreted with caution. In addition, methane oxidation rates of LP samples (LP1, LP3) still increased with methane concentration even at high methane partial pressures to an extent that no definitive saturation of the methane oxidation rates was observed in those samples. This leads to error-prone estimates of V_{max} because the fitted curves have to be extrapolated (Fig. 3.1). Nonetheless, the calculated rates can serve as a measure for comparison between the different sites. The reference cores R2 and R3 showed only marginal methane oxidation capacities below 1 nmol ml^{-1} h^{-1} which was below the detection limit at higher methane concentrations. In littoral pockmark slurries LP1 and LP2, V_{max}s were 627 nmol ml^{-1} h^{-1} and 831 nmol ml^{-1} h^{-1}, respectively (Tab. 3.1).

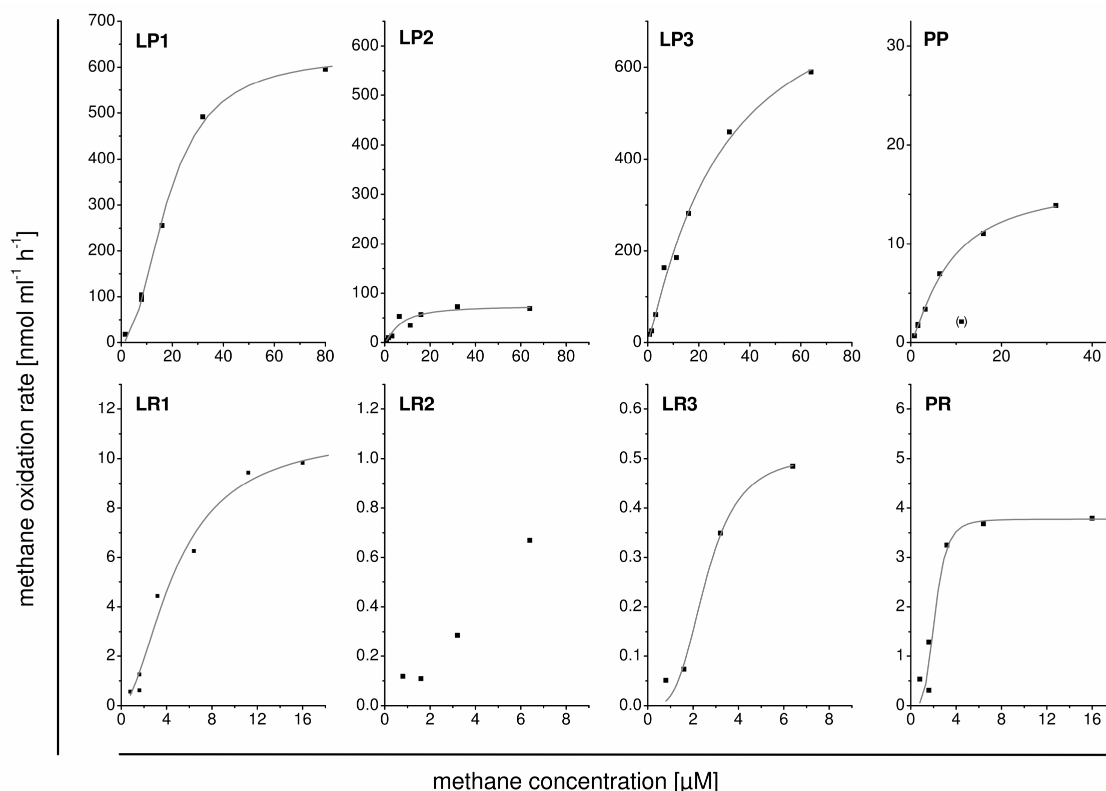

Fig. 3.1. Rates of methane oxidation by different sediment slurries plotted against dissolved methane concentration (LP: littoral pockmark; LR: littoral reference sediment outside the pockmark; PP: profundal pockmark; PR: profundal reference sediment outside the pockmark). Dots: measured values; lines: curve fits using the hill equation to estimate V_{max} and $S_{0.5}$ of the sediment slurries.

Compared to the reference sites outside the pockmarks, those values are higher by approximately two orders of magnitude (Tab. 3.1). In LP2 and the profundal cores (PP, PR), the differences were less pronounced and within 1 order of magnitude (a factor of 4.3 in profundal slurries). Calculated $S_{0.5}$s for methane were by a factor of 3 to 6 higher in pockmark sediment slurries from LP1 and LP3 than in the reference sediments (Tab. 3.1).

Tab. 3.1. Results of nonlinear curve fits of methane oxidation rates using the Hill equation[a] and cell numbers determined by qPCR

Sample	Chi^2	R^2	V_{max}[b]	$S_{0.5}$ [nM]	n	MOB
LP1	224	0.998	627 ± 21.7	18.3 ± 1.06	2.10 ± 0.17	$2.50 \pm 1.59 \cdot 10^9$
LP2	121	0.89	75.0 ± 14.6	6.76 ± 3.11	1.33 ± 0.61	$2.74 \pm 0.73 \cdot 10^7$
LP3	468	0.992	831 ± 138	28.4 ± 9.66	1.13 ± 0.17	$1.06 \pm 0.72 \cdot 10^9$
LR1	0.459	0.981	11.1 ± 1.46	4.81 ± 1.10	1.80 ± 0.42	$3.72 \pm 1.85 \cdot 10^7$
LR2	NA[d]	NA	<0.001	NA	NA	$1.53 \pm 0.53 \cdot 10^7$
LR3	0.002	0.986	0.51 ± 0.06	2.57 ± 0.36	3.40 ± 1.19	$3.16 \pm 0.77 \cdot 10^6$
PR	0.239	0.944	3.77 ± 0.37	2.15 ± 0.32	4.24 ± 1.81	$1.02 \pm 3.30 \cdot 10^8$
PP	0.055	0.998	16.4 ± 0.74	8.56 ± 0.85	1.28 ± 0.08	$7.27 \pm 2.12 \cdot 10^7$

[a] The hill equation is $y = V_{max} \cdot x^n \cdot (x^n+k^n)^{-1}$ where y is the methane oxidation rate, x is the methane concentration, k is the half saturation constant (i.e., $S_{0.5}$), and n is the Hill coefficient.
[b] in nmol ml_{sed}^{-1} h^{-1};
[c] per gram sediment wet weight;
[d] NA, not available

T-RFLP Analysis. Methanotrophic communities of all eight samples were compared using T-RFLP as a fast and easy fingerprint method. T-RFs with the forward primer A189-HEX (abbreviations consist of the prefix f and the T-RF size, in bp) were more diverse than T-RFs originating from the FAM-labeled reverse primer (prefix r), especially in the low-intensity T-RFs. While in samples from LR the T-RF f76 was dominant (59 ± 6%), T-RFs of the uncut PCR product (f513) showed the highest relative fluorescence intensity in profundal and LP samples (58 ± 2% and 75 ± 18%, respectively). T-RF f341 was found almost exclusively in LP samples, and f351 was most prominent in profundal samples (Tab. 3.2).

With the reverse primer, the uncut *pmoA* fragment r504 was also dominant in LP (67 ± 16%) and profundal (68 ± 2%) samples. The T-RFs r56 and r129 were present only in littoral samples, whereas the r64 fragment was present in all samples. In profundal samples, the T-RF r421 contributed to about 20% of the total fluorescence intensity and was found only in minor amounts at LR and never at LP sites (Tab. 3.3). Use of two labeled primers clearly enhanced the resolution of the T-RFLP analysis. Especially sequences yielding short T-RFs with one primer are especially likely to be split into different groups using the other primer. This can clearly be seen with T-RF f76, which is dominant in LR samples, but reverse T-RFs show multiple groups sharing a comparable contribution to total fluorescence intensity. Relative fluorescence intensities from the uncut PCR-Product varied up to 11% (PP and PR) between both fluorescent dyes of identical samples. This variation could be explained by T-RFs that were eliminated from the analysis because of properties mentioned in the Material and Methods section.

AMMI analysis revealed that 30.7% (0.3% noise) of the total variation among the T-RFLP profiles can be explained by the interaction of sampling location with T-RF patterns using all samples as individual environments in the analysis. The remaining variation can be contributed to differences between the replicate T-RFLP profiles. Sample heterogeneity was 1.45 in this analysis. When only littoral samples were analysed using the three different

Tab. 3.2. Relative fluorescence signals of T-RFs with fluorescence of forward primer A189-FAM from different sampling locations

Sample	76f	100f	146f	186f	190f	209f	218f	226f	233f	242f	257f	339f	341f	351f	373f	439f	513f
LP1	0.6		0.8	1.9	0.7	0.6	0.5		0.5				2.0			1.5	93.9
LP2	12.5			1.4		3.0				3.6			13.8			8.7	59.3
LP3	5.0			1.9	1.1	2.2	1.4			1.8	1.1		15.2			2.9	70.4
LR1	54.4					2.6		0.7		18.8			1.1	2.3		3.7	17.6
LR2	58.2	1.0				4.6	0.8	1.4		5.7		0.8	1.6		0.5	3.7	23.9
LR3	65.6	1.3				12.2		2.6		1.9		2.1			2.2	3.8	14.1
PP	21.8		1.9	1.5		2.8				2.1	1.1			10.8		2.1	59.3
PR	29.7					9.9				2.7	1.5			3.4		2.1	55.9

Tab. 3.3. Relative fluorescence signals of T-RFs with fluorescence of reverse primer mb661-HEX from different sampling locations (LP: littoral pockmark; LR: littoral reference sediment outside the pockmark; PP: profundal pockmark; PR: profundal reference sediment outside the pockmark).

Sample	56r	64r	69r	129r	155r	168r	257r	262r	275r	387r	421r	504r
LP1		5.7		2.3		8.5						86.0
LP2	8.6	16.8		14.6		8.6						54.8
LP3	3.6	7.1		7.0		9.0				13.5		61.2
LR1	18.3	32.2		16.6	6.2	1.9	4.5	3.2				22.3
LR2	9.1	18.4	2.7	39.5				4.5			2.0	27.2
LR3	5.3	26.5		32.5		4.3			11.0		5.5	24.2
PP		10.9			4.5						17.1	69.8
PR		9.7									27.3	66.9

samples from both environments (LP and LR) as replicates, 31.5% (0.3% noise) of the total variance was explained by the difference between pockmark and reference. Sample heterogeneity was reduced to 1.28 by exclusion of the profundal samples.

IPCA using all samples as individual sites clearly showed that T-RFLP profiles of samples from LP and LR sites clustered separately from each other and from T-RFLP profiles from profundal samples, which showed no clear differences between pockmark and reference sites (Fig. 3.2). Another interesting finding was that LP samples cluster more closely to profundal samples than to LR samples, at least in IPCA1 that explains most of the variance. This effect could be explained mainly by the high abundance of the uncut *pmoA* fragment in both samples and disappeared if presence of T-RFs was analysed only (data not shown).

***pmoA* clone libraries and phylogenetic analysis.** Four separate clone libraries were generated from the *pmoA* gene fragments amplified from DNA of the littoral and profundal pockmark and reference sediment. A total of 322 clones (LP, n = 101; LR, n = 72; PP, n = 67; PR, n = 82) were analyzed and grouped according to their RFLP patterns. Of the 101 sequences obtained (LP, n = 20; LR, n = 26; PP, n = 25; PR, n = 29) 1 was identified as a chimera, and was excluded from further analysis. Clone library analysis revealed a diverse methanotrophic community of Type 1 MOB. Most clones belonged to groups related to *Methylobacter* species (groups P-I to P-III, I and II, LP-I). Clones belonging to group V were also quite abundant and not closely related to any cultured MOB. Clones related to *Methylosarcina*/*Methylomicrobium* were found at all sites. No type 2 MOB or clones closely related to *Methylomonas* spp. were obtained from any sampling site. Most clones from the profundal pockmark, with only two exceptions, and all clones from PR clearly belonged to the type 1a MOB (Fig. 3.3). The majority of clones obtained from littoral sediments fell into two clone groups.

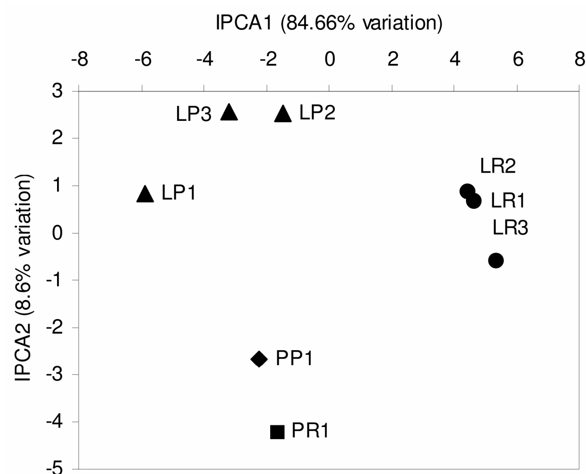

Fig. 3.2. Plot of the interaction principal component analysis of the individual sample datasets consisting of at least 5 replicate T-RFLP profiles from each site.

Most clone sequences from LP clustered in one group (LP-I) related to *Methylobacter* spp. but distinct from all clones from other sites, and there was no closely related sequence (<93% nucleotide sequence identity; <97% amino acid sequence identity) in the NCBI database. The closest matches in the database were clones from profundal sediment of Lake Washington near Seattle, Washington (GenBank accession number DQ067074) or a nondescribed clone from a Finnish boreal lake (GenBank accession number FN597117). On the other hand, many clones from the LR site could be assigned to type 1b MOB (group LR). This group was not detected at the other sites and belongs to a cluster that lacks any cultivated representative. The most related sequence was that of a clone from a Chinese rice field soil (GenBank accession number AM910094).

When the clones were grouped using a limit of 90% sequence similarity (corresponding to 97% sequence similarity of the 16s rRNA gene; Heyer et al., 2002), the clones were grouped into 7, 12, 12 and 10 different species for LP, LR, PP and PR, respectively. The total species richness was calculated using the Chao1 estimate (Hughes et al., 2001) and yielded estimated numbers of bacterial species of 12, 53, 29 and 19 for LP, LR, PP and PR, respectively, meaning that 19 to 37% of the estimated diversity was sampled. However, these values may overestimate the overall species richness because the clones were pre-selected according to their restriction patterns.

Linking T-RFLP data to clone library analysis. In general, the observed T-RF sizes were shifted compared to theoretical values by -3 bp at small T-RFs to +2 bp at bigger ones with FAM dye and with HEX-dye by -6 bp at small T-RFs to -8 bp at bigger ones, depending on

the size of the DNA fragment. The different apparent sizes of the uncut fragment labelled with both dyes (HEX, 513 bp; FAM, 504 bp; theoretical, 508 bp) originate from internal size corrections for the different dyes, performed by the analysis software, which created wrong results if both dyes are present. The shift of the T-RFs could be verified using selected sequenced clones for T-RFLP analysis. Restriction sites corresponding to all major T-RFs were detected in the sequences used to construct the phylogenetic tree. Restriction sites corresponding to T-RFs r69, f100, f146, f186, f190, f218, f233 and f257 were not present in these data, and no reverse T-RF at 300 bp was detected although some clones had the respective restriction site. Assignment of T-RFs to phylogenetic groups was possible only in few cases, because the same T-RFs were often shared by more than one phylogenetic group of MOB. However, *in silico* digestion of the clone sequences of the individual samples and the corresponding observed major T-RFLP patterns fitted well, indicating that the sequenced clones were indeed representatives of the T-RFLP patterns observed. T-RF 76f is shared by all Type 1b MOB, clone PP-II434 and group P-III. Additionally, all clones of the *Methylobacter* sp. LW12-related group could clearly be assigned to T-RF 421r (429 bp). However, also the reference strain *Methylocapsa acidophila* B2, a Type 2 MOB, could be assigned to this T-RF, but no phylogenetically related clone was detected. Only some clones from group LR and the related Type 1b MOB *Methylococcus capsulatus* and thermophilic methanotroph HB harbor the restriction site responsible for T-RF r129 (135 bp) allowing a clear identification of this group by T-RFLP analysis. Most clones from LP have no MspI restriction site which fits very well to the T-RFLP patterns (Tab. 3.2 and 3.3).

qPCR. Quantitative real-time PCR was used to estimate the abundance of methanotrophic bacteria at the different locations. This technique yielded no unspecific PCR products, thus allowing a quantification of the *pmoA* gene in all samples. In the littoral pockmark samples LP1 and LP3, MOB abundances were significantly higher than in the other samples ($2.5 \pm 1.6 \times 10^9$ and $1.1 \pm 0.7 \times 10^9$ cells per gram sediment wet weight, respectively). In profundal samples MOB numbers were one order of magnitude lower, with $1.0 \pm 0.3 \times 10^8$ and $7.3 \pm 2.1 \times 10^7$ cells per gram in PP and PR, respectively. Littoral reference samples and LP2 had the lowest abundances of methanotrophic bacteria in the range from 3.2×10^6 (LR3) to 3.7×10^7 (LR1) cells per gram sediment (wet weight) (Tab. 3.1). Melting curve analysis showed slightly variable melting temperatures of the PCR products between 82°C and 85°C for all samples, but all LR samples showed another maximum at 88 to 90°C (data not shown).

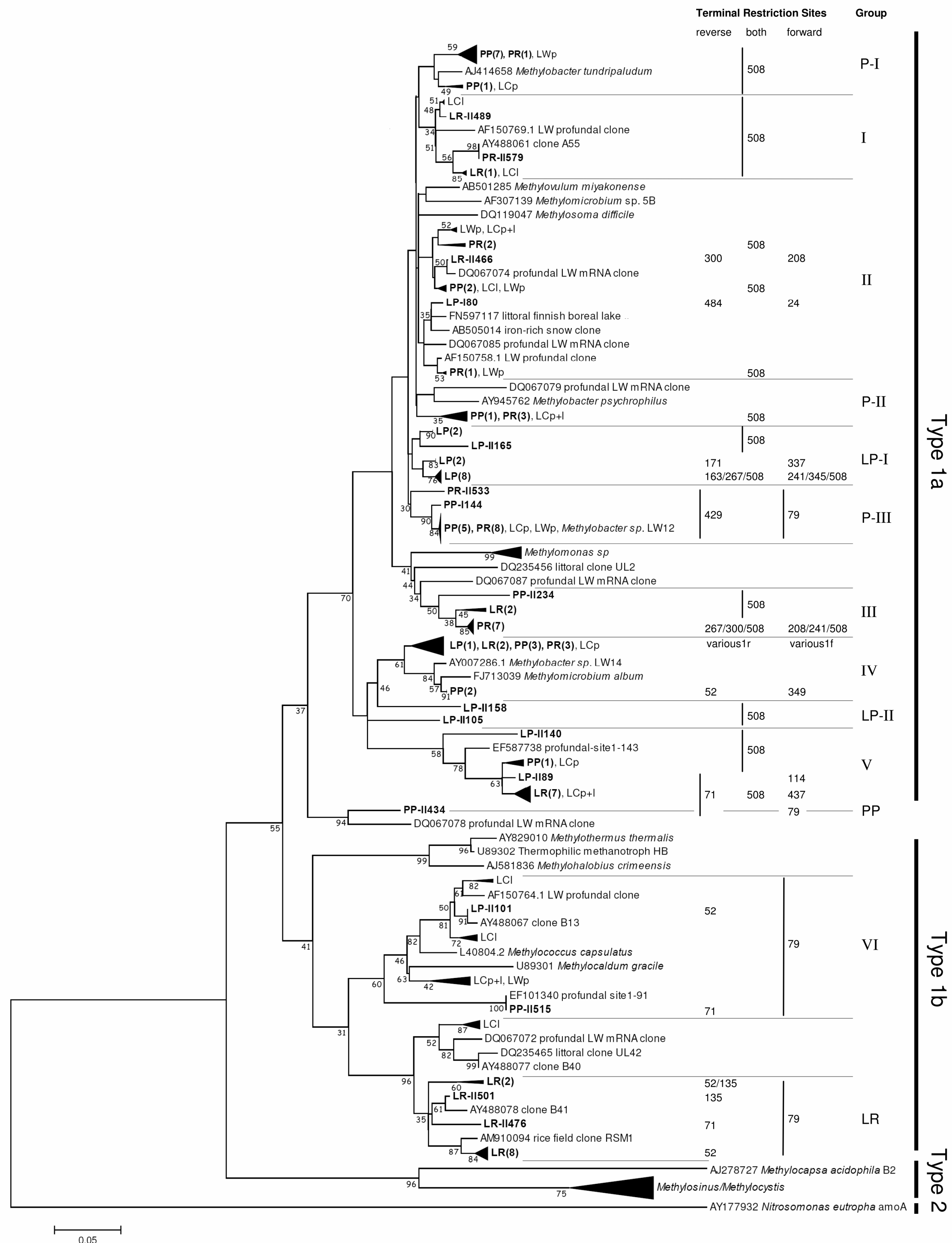

Fig. 3.3. Phylogenetic dendrogram of deduced PmoA sequences (169 amino acids). The tree was constructed using the minimum-evolution method in MEGA (version 4) software selecting the pairwise deletion option. Bootstrap values (1,000 replicates) are shown next to the branches. The evolutionary distances were calculated using the JTT matrix-based method. Clones obtained in this study are shown in boldface, numbers in parenthesis represent numbers of clones in a collapsed subtree. Theoretical T-RF sizes are given for reverse and forward primer, or in case of the uncut PCR product, for T-RFs shared by both primers. Groups are labeled as mentioned in the text. LW, Lake Washington; LC, Lake Constance; p, profundal; l, littoral; various1r, 71, 159, 267, 508 bp; various1f, 33, 158, 241, 437, 508 bp

Discussion

Methane oxidation capacities. Methane oxidation capacities or potential methane oxidation rates were determined to estimate the methanotrophic potential present in the respective sediment. Although they do not directly reflect methane oxidation rates *in-situ*, they can serve as a measure for abundance and activity of the methanotrophic communities present.

Methane oxidation capacities at pockmarks were found to be substantially higher than in the surrounding sediment, indicating that they can be considered as hotspots of methanotrophic activity in an otherwise oligotrophic environment. These differences are likely to be even higher at a higher spatial resolution, since the slurry for the experiments in this study was prepared from a total of 113 to 200 ml sediment, thus diluting the high methanotrophic activity that might be concentrated in a few ml at close proximity to the gas emanation site. Such spatial heterogeneity may also explain why methane oxidation capacities of core LP2 were lower by 1 order of magnitude compared to the other LP samples although all littoral samples were taken by trained scientific divers right at the respective gas emanation sites. Our data confirm earlier results derived from methane concentration profiles which indicated that *in-situ* methane oxidation rates at pockmarks were 20 times higher than in the surrounding sediment (Bussmann et al., 2011). Also the comparably small but still substantial differences between methane oxidation activities at the PP versus the PR site can be explained by sampling inaccuracies, even though the horizontal sonar was employed to direct the multicorer into the centre of the profundal pockmarks. These results are consistent with low geochemical differences observed for these sites (Bussmann et al., 2011).

The methane oxidation capacities measured inside LPs were comparable to those measured previously in sediment slurries taken in summertime from the Obere Güll, a shallow bay in Lake Constance (Bosse et al., 1993), where maximal methane oxidation capacities of about 1 µmol ml_{sed}^{-1} h^{-1} (where the subscript sed indicates sediment) and half-saturation constants of 3.5 to 11 µM were measured. These sediments are oversaturated with methane in summertime, since both methane production and methane oxidation are seasonally fluctuating processes even in the profundal of temperate lakes, with reported 5 to 20 –fold changes in Lake Constance (Thebrath et al., 1993; Schulz and Conrad, 1995).

Sediments of Lake Washington, a comparable, well investigated oligotrophic freshwater lake, showed maximal oxidation rates of 7.2 - 41.3 nmol ml_{sed}^{-1} h^{-1} at the sediment/water interface, and 0.27 - 4.5 nmol ml_{sed}^{-1} h^{-1} in deeper layers of profundal sediment (65 m water depth) (Lidstrom and Somers, 1984). Those values are similar to those obtained in our study for reference sediments outside the pockmarks (Tab. 3.1). However, $S_{0.5}$ values were slightly

higher in Lake Washington (9.5 ± 1.2 µM) compared to reference sediment in our study (2 to 5 µM). Another study (Auman et al., 2000) found significantly higher rates of 364 nmol ml_{sed}^{-1} h^{-1} in the uppermost 0.5 cm of profundal Lake Washington sediment and slightly lower rates below that zone, reaching methane oxidation capacities comparable to the rates found at littoral pockmark sites.

Qualitative analysis of methane-oxidizing communities. Molecular community analysis revealed a picture that corresponds well with the measurements of the methane oxidation capacities. T-RFLP analysis showed considerable differences between pockmark and reference sediments in the littoral zone and less pronounced differences between the profundal sediments. These patterns accounted for 30% variance in the AMMI analysis of the T-RFLP data, which is comparable to other environmental samples (Culman et al., 2008; Culman et al., 2009). Interestingly, the community composition in LP2, which showed clear differences in the methane oxidation capacities, was very similar to that of the other LP samples, confirming that samples were taken in close proximity to the gas emanation site. Unfortunately, we were not able to relate all T-RFs to defined phylogenetic groups with our dataset, like other studies (Shrestha et al., 2008; Krause et al., 2010; Lüke et al., 2010), because the same T-RFs were shared often by more than one phylogenetic group of MOB or pseudo T-RFs rendered a clear phylogenetic assignment barely possible (Egert and Friedrich, 2003). However differences in community structure of MOB between LP, LR, and profundal sites were clearly reproduced.

Clone library analysis verified the differences between MOB communities at the different sites and revealed the identity of MOB groups that form these differences. In the littoral zone, Type 1b MOB were abundant at the reference site whereas group LP-I appears to dominate the MOB community and to benefit from the conditions at the pockmark site. *In silico* digestion of the clones mirrors the major features of the obtained T-RFLP profiles, suggesting that the abundant MOB groups were covered in our analysis. A PCR bias towards Type 1 MOB has been reported for primer mb661 and might explain the lack of type II MOB affiliated sequences in our analyses, even though primer mb661 amplified also type II MOB from environmental samples (Bourne et al., 2001). Phylogenetic analysis of clone libraries obtained in this study were mostly congruent with previous studies performed for Lake Constance and Lake Washington (Costello and Lidstrom, 1999; Auman et al., 2000; Pester et al., 2004; Rahalkar and Schink, 2007). *Methylobacter*-like MOB appeared to be dominant, especially in the profundal part of the lake. In contrast to previous studies (Rahalkar and

Schink, 2007), Type 1b MOB constituted a significant part of the MOB community in littoral reference sediment, in particular, group LR, which is related to sequences often found in rice field soils (Qiu et al., 2008). However, a study using the A189-A682 primer pair (Holmes et al., 1995) found type II and type 1b MOB in littoral sediments obtained from Obere Güll (Pester et al., 2004). These variances in the community structure between different studies on Lake Constance can be explained by temporal variations or environmental conditions between different parts of the lake, e. g., input of allochthonous carbon and nutrients by the Rhine River (Sobek et al., 2009) or exposure to mechanical disturbance (Hofmann et al., 2008b). This might also be a reason for the observed similarities between LP and profundal sites, as the pockmark structure might provide some shelter against disturbing environmental factors. However, despite known correlations between MOB occurrence and environmental factors (Henckel et al., 2000b; Henckel et al., 2001; Macalady et al., 2002; Krause et al., 2010) or the results of direct competition experiments (Graham et al., 1993; Amaral and Knowles, 1995; Henckel et al., 2000b; Steenbergh et al., 2010) the physiological reasons for the observed distribution of different MOB strains in their environmental niches are still largely unknown.

Quantitative analysis of methane-oxidizing communities. The abundance of methanotrophic bacteria at the different sites correlated well with the observed methane oxidation capacities, suggesting that high methane oxidation capacities were caused by high MOB abundances. Absolute MOB cell numbers obtained in previous studies from Lake Constance and Lake Washington agree with those from our reference sediments (Costello et al., 2002; Rahalkar et al., 2009). When MOB cell numbers were calculated based on the methane oxidation capacities of the sediments using a maximum methane oxidation activity per cell of 0.5×10^{-6} to 3×10^{-6} nmol h^{-1} (Costello et al., 2002), cell numbers were, on average, more than 1 order of magnitude lower than cell numbers obtained by qPCR. This indicates that major parts of the MOB communities present in the sediments were inactive or resting. Similar results were obtained previously when MOB cell counts obtained by fluorescence *in-situ* hybridization and qPCR were compared (Rahalkar et al., 2009). Absolute copy numbers or cell numbers have to be treated with caution, because the use of external standards is error prone and the heterogeneity among environmental samples like sediments can cause variations in DNA extraction and PCR efficiencies. However, in this study MOB abundances differed by orders of magnitude between different samples, thus clearly identifying pockmarks as hot spots of methane oxidation and MOB abundance.

Melting curve analysis after qPCR showed slight differences between samples reflecting different *pmoA* sequences, and in LR samples an additional distinct peak occurred at increased temperatures that can be attributed to the higher GC content of the *pmoA* fragments obtained from Type 1b MOB. Therefore, melting curve analysis and especially high-resolution melting curve analysis (HRM) could serve as tools to access quickly the diversity of, e.g., *pmoA* genes also in environmental samples. In our study, it verified the differences in community composition obtained by clone library analysis.

Collectively, potential methane oxidation rates as well as various molecular methods identified pockmarks as hot spots of methanotrophic activity and abundance and as environmental niches in Lake Constance that are occupied by distinct strains of MOB, especially in the littoral zone. Furthermore, the results show once again that entire groups of MOB detected by molecular methods lack cultivated representatives and, thus, lack physiological information that might be crucial to understand the distribution and ecology of MOB in their natural environments.

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Chapter 4

Characterization and Phylogeny of a Novel Methanotroph, *Methyloglobulus morosus* gen. nov., spec. nov.

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In preparation

Abstract

A novel methanotroph, strain KoM1, was isolated from the profundal sediment of Lake Constance after an initial enrichment in opposing gradients of methane and oxygen. Strain KoM1 grows on methane or methanol as its sole source of carbon and energy. It is a Gram-negative methanotroph, often expressing red pigmentation. Cells are short rods and occur sometimes in pairs or short chains. Strain KoM1 grows preferably at reduced oxygen concentrations (<10%). It possesses the *nifH* gene indicating nitrogen fixation abilities, and it grows at neutral pH and at temperatures between 4 and 30°C. Phylogenetically, the closest relatives are *Methylovulum miyakonense* and *Methylosoma difficile* showing 90% 16S rRNA gene sequence identity. The major cellular fatty acids are summed feature 3 (presumably C16:1ω7c) and C16:1ω5c and the G+C content of the DNA is 47.7 mol%. Strain KoM1 was deposited at DSMZ and is described as the type strain of a novel species within a new genus, *Methyloglobulus morosus* gen. nov., sp. nov.

Introduction

Aerobic methane-oxidizing bacteria (MOB), also called methanotrophic bacteria, can use methane as their sole source of carbon and energy. They are widespread in nature and inhabit soils, fresh and marine waters, sediments, and all kinds of wetlands (Hanson and Hanson, 1996; Lüke, 2010). Upland soil methanotrophs are capable of using atmospheric methane concentrations and act as atmospheric methane sink (Holmes et al., 1999; Henckel et al., 2000a), whereas MOB inhabiting waterlogged sites often act as biofilters, which oxidize a significant part of the methane that is produced in the anoxic parts of these environments (Hanson and Hanson, 1996). The "classical" MOB belong either to the α-proteobacteria (type II MOB) with five described genera, or to the γ-proteobacteria (type I MOB) with 12 described genera (Murrell, 2010; Geymonat et al., in press; Iguchi et al., 2011; Vorob'ev et al., in press). Type I MOB can be subdivided into type Ia and type Ib (also called type X), the

latter sharing some traits with type II MOB (Hanson and Hanson, 1996). *Crenothrix polyspora* and *Clonothrix fusca* have also been reported to be methanotrophs and form another branch of γ–proteobacterial MOB (Stoecker et al., 2006; Vigliotta et al., 2007). More recently, some thermoacidophilic Verrucomicrobia have been isolated that also oxidize methane (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008), indicating that methanotrophy might be a more widespread trait among bacteria than previously thought.

In Lake Constance, an oligotrophic freshwater lake, methanotrophic bacterial communities have been investigated in detail by culture independent methods. The results indicated that type I methanotrophs are dominant in this habitat (Pester et al., 2004; Bussmann et al., 2006; Rahalkar and Schink, 2007; Rahalkar et al., 2009). This holds true also for other freshwater habitats (Lüke, 2010). However, these molecular investigations also revealed substantial differences in the community composition among different sites of Lake Constance such as littoral, profundal, or methane seep sediments (Rahalkar and Schink, 2007; Rahalkar et al., 2009; Deutzmann et al., 2011). The physiological properties determining these differences in community composition are largely unknown and need further research. To resolve the complex interactions between methanotrophs and their environment, which influence the community structure, it is essential to get access to cultured representatives of the MOB communities *in-situ*. In this study we present the isolation, characterization and taxonomic position of a novel methanotroph isolated from Lake Constance profundal sediment.

Materials and Methods

Isolation and maintenance of strain KoM1. *Methyloglobulus morosus* strain KoM1 was isolated from Lake Constance profundal sediment in summer 2006. One ml sample was taken from the upper 1.5 cm of an undisturbed sediment core and suspended in 9 ml N_2-gassed medium by vigorous mixing for 1 minute. Nitrate mineral salt medium (Whittenbury et al., 1970) was used, but with lower nitrate content (dNMS) (Rahalkar et al., 2007) containing per liter: 1 g $MgSO_4 \cdot 7 H_2O$; 0.2 g $CaCl_2$; 0.1g KNO_3. After autoclaving, 600 µM KH_2PO_4/Na_2HPO_4 Buffer (pH = 7.2), 10 mM HEPES buffer (pH = 7.2), 1 mg l^{-1} Sequestren® Fe 138 complex (Novartis Agro GmbH, Frankfurt), 1 ml l^{-1} of 7 vitamin solution (Widdel and Pfennig, 1981) and 1 ml l^{-1} trace element solution SL10 (Widdel, 1988) were added. Gradient enrichments (Bussmann et al., 2006) were set up from 1:10 serial dilutions of this slurry. Grown bands from the gradient tubes were cut out, resuspended in 1 ml medium and streaked on dNMS agarose plates (1.2% agarose). Red colonies were transferred into glass tubes which were closed with butyl rubber stoppers and sealed with aluminium crimp caps and contained 5

ml liquid dNMS medium and a gas phase consisting of 30% CH_4, 5% CO_2, 30% air, and 35% N_2. Repeated serial dilutions (1:2) were performed in glass tubes to purify the strain. In the first two dilution series, 0.01% tryptone was added to facilitate the growth of the strain, but was omitted in the last two dilution series to eliminate remaining contaminants. The pure culture was usually cultivated in glass tubes or bottles filled to 10% of the volume with dNMS medium and a gas phase consisting of 30% CH_4, 30% air, and 40 % N_2.

Substrate utilization. The utilization of different nitrogen sources was tested in 48 well plates (Nunc) in dNMS medium, but KNO_3 was replaced by KCl. The following nitrogen sources were tested in 0.1, 1, and 10 mM concentrations: NO_3^-, NO_2^-, NH_4^+, taurine, alanine, and urea. Yeast extract and tryptone were tested at 0.1%, 0.01% 0.001%. Optical density of multi well plates was measured using a GENios microtiterplate reader (Tecan).
Carbon substrates were tested in 48 well plates (Nunc) or in glass tubes with butyl rubber stoppers for volatile compounds. CH_4 was replaced by N_2 in the usual gas phase. The following carbon sources besides methane were tested: 10 mM methanol; 1 mM para-formaldehyde, 10 mM glucose, 0.1% and 0.01% yeast extract and 0.1% and 0.01% tryptone. The following substrates were tested in 1 mM or 10 mM concentration: formate, methionine, acetate, fructose, succinate, fumarate, glycerol, formamide, glycolic acid, glyoxylate, ethanol, taurine, alanine, and urea. Furthermore the following complex media were tested undiluted and 1:10 diluted: SOC, LB (Sambrook et al., 1989), nutrient broth (BD Difco™).

Effect of pH, temperature, oxygen tension and NaCl concentration on growth. NaCl tolerance was tested in 48 well plates (Nunc). 5 M NaCl solution was added to each well to the desired salt concentration. NaCl additions of 0, 20, 40, 60, 80, 100, 120, 140 mM were tested in 6 replicates each. The pH range supporting growth of strain KoM1 was determined in microtiter plates (8 replicates) and in glass tubes closed with butyl rubber stoppers (5 replicates). The dNMS Medium was buffered with a mixture of Tris, HEPES and MES (10 mM each) instead of HEPES alone to cover the whole pH range with the same buffer system. The pH range from pH = 4 to pH = 9 was tested in steps of 0.5. The temperature range supporting growth of strain KoM1 was examined in tubes incubated at 4, 10, 15, 20, 25, 30 and 37°C. Growth at different oxygen concentrations was assayed in tubes filled with dNMS medium. They were gassed with 30% CH_4, 5% CO_2, 65% N_2 and oxygen was added to final concentrations of 0%, 2.5%, 5%, 7.5%, 10%, 12.5%, and 15%.

Cellular fatty acid analysis and G+C content. Phospholipid fatty acid (PLFA) analyses were performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Strain KoM1 was grown in a 12 l bottle containing 8 l dNMS medium. The initial gas phase consisted of 20% air and 80% methane. Developing negative pressure was refilled semi-continuously with 33% CH_4 and 66% O_2. Cells were pelleted, freeze-dried, and sent for phospholipid fatty acid analysis. There, PLFAs were saponified, methylated, and the fatty acid methyl esters were subjected to GC. The GC elution profile of the fatty acid methyl esters was compared with the fatty acid patterns stored in the fatty acid database (TSBA40) of the Microbial Identification System (MIDI Inc.) using Sherlock software version 6.1 and qualitative and quantitative compositions of the pattern were given. G+C content was measured at the DSMZ as described previously (Mesbah et al., 1989). Presence of sterols has been tested previously (Martin-Creuzburg et al., 2011).

Molecular characterization of strain KoM1. DNA of strain KoM1 was extracted and the 16S rRNA gene was amplified using the primers 27f and 1492r (Weisburg et al., 1991). The *pmoA* gene encoding subunit A of particulate monooxygenase was amplified using the primers A189f and mb661 (Holmes et al., 1995; Costello and Lidstrom, 1999). The *nifH* gene was amplified with the primer pair PolF-PolR (Poly et al., 2001), and the *mxaF* gene coding for the α-subunit of methanol dehydrogenase was amplified using primers f1003 and r1561 as described previously (McDonald and Murrell, 1997). Presence of soluble methane monooxygenase was checked for by PCR targeting the *mmoX* gene with primers *mmoX*A–*mmoX*B (Auman et al., 2000). All amplicons were cloned using a TA cloning kit (Invitrogen) according to the manufacturer's instructions, and the plasmid insert of at least 2 clones was sequenced at GATC biotech GmbH (Konstanz). 16S rRNA gene sequences were aligned using the SINA web-aligner (http://www.arb-silva.de/aligner/) and deduced amino acid sequences of PmoA, MxaF and NifH were aligned using CLUSTAL W algorithm implemented in MEGA4 software (Tamura et al., 2007). Phylogenetic analysis was done with MEGA4 software and additionally with the ARB software package for 16S rRNA gene sequences. The shown phylogenetic tree of the 16S rRNA gene sequences was constructed using the Neighbor-Joining method, and evolutionary distances were computed using the maximum composite likelihood method in pairwise sequence comparisons as implemented in MEGA software version 4. The phylogenetic trees of PmoA, MxaF and NifH were constructed using the Minimum Evolution method choosing pairwise sequence comparisons. The evolutionary distances were computed using the JTT matrix-based method.

Presence of soluble methane monooxygenase was tested by a colorimetric assay (Graham et al., 1992). Nitrogen fixation ability was tested using the acetylene reduction assay (Dalton and Whittenbury, 1976) using cells grown in medium without bound nitrogen source. Cytochrome oxidase activity was tested with oxidase test strips (Fluka). Presence of catalase was tested by mixing a drop of 3% H_2O_2 solution with one drop of grown culture on a microscope slide and following gas bubble formation.

Results

Isolation and maintenance of strain KoM1. Strain KoM1 was enriched from a 1:10^{-4} dilution of profundal sediment of Lake Constance in gradient enrichments. After transfer to dNMS agarose plates red and white colonies were formed. It was not possible to purify the strain by repeated streaking of single red colonies, because whenever a single red colony was transferred and streaked on a new plate no new colonies formed. However, it was possible to transfer and grow non-pure colonies on new plates. Thus, the strain was purified by 1:2 serial dilutions in liquid medium. In liquid cultures, cells settle down and form a red to pink layer at the bottom. Strain KoM1 occasionally failed to grow in liquid cultures even if 5-10% of a grown culture was used as inoculum and exhibited different lag phases of up to two weeks, even in replicate treatments. Cells were short rods to cocci and were sometimes visible as pairs or short chains in growing cultures (Fig. 4.1, Tab 4.1). It was not possible to revive the strain after freezing in liquid nitrogen with glycerol or DMSO as cryoprotectant, but cultures could be stored at 4°C or room temperature for at least 6 months if excess methane was present in the culture.

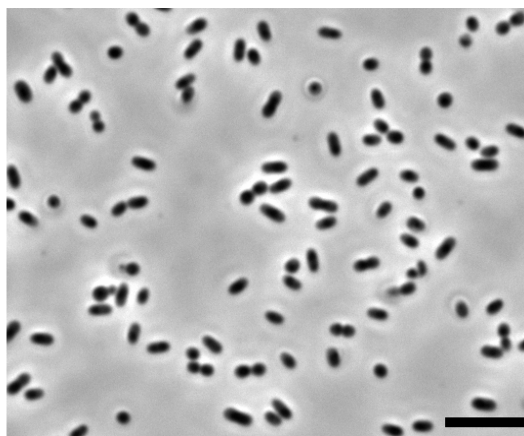

Fig. 4.1. Phase contrast micrograph of strain KoM1 grown on methane. Bar represents 5 µm.

Utilization of carbon and nitrogen sources. Only methane and methanol but no other tested carbon source was used as sole source of carbon and energy. 10 mM acetate inhibited growth of strain KoM1 even with methanol as co-substrate. Strain KoM1 used nitrate, nitrite, ammonia, urea, alanine, taurine, yeast extract or tryptone as nitrogen source, but nitrite, urea and to a lesser extent also taurine inhibited growth at 10 mM concentration.

Tab. 4.1. Comparison of strain KoM1 with other methanotrophic genera.

Characteristic	*Methylomonas*	*Methylobacter*	*Methylomicrobium*	*Methylosoma*	*Methylovulum*	strain KoM1
Cell morphology	Rods	Rods or cocci	Rods	Cocci, elliptical or rods	Cocci or short rods	short rods
Motility	+	Variable	+	-	-	-
Cyst formation	+	Variable	-	+	-	-
Pigmentation	Pink, white	Brown, yellow, pink	White	Pale pink	Pale brown	Red-pink
CH_4 oxidation	pMMO/sMMO	pMMO	pMMO/sMMO	pMMO	pMMO/sMMO	pMMO
nifH gene	Variable	Variable	-	+	+	+
G + C content	52-59	45-55	49-60	49.9	49.3	47.7

Effect of pH, temperature, oxygen and NaCl concentration on growth. Strain KoM1 grew at pH 5-8 in tubes and from pH 5 to pH 8.5 in microtiter plates with an optimum of pH 6-8 in both cases. The strain did not grow in the presence of citrate buffer. The temperature optimum for growth was 20°C, but the strain was able to grow from 4°C to 30°C. At 30°C the optical density (OD) at 600 nm decreased after reaching a value of 0.1 after one week, while at lower temperatures growth continued and reached higher final ODs. Growth was linear in these experiments and OD increased by $\Delta OD = 0.008\ d^{-1}$, $0.022\ d^{-1}$, and $0.012\ d^{-1}$ at 15°C, 20°C, and 25°C, respectively. Strain KoM1 grew best with 5-10% oxygen in the gas phase. Lower concentrations led to slower growth, and higher concentrations caused a higher proportion of non-growing replicates than at medium oxygen concentrations and increased the lag phase up to 1 week, indicating some metabolic imbalance or oxidative stress at the start of the incubations. NaCl had a negative effect on growth already at 20 mM concentration and inhibited growth completely above 100 mM concentration.

Absence of sMMO and presence of nitrogenase. PCR targeting the *mmoX* gene yielded no product and no indication of sMMO activity was detected after incubation in copper-free medium for 1 week in the colorimetric assay. The *nifH* gene was successfully amplified and nitrogenase activity was verified by acetylene reduction, but activity was low and required a few days of incubation. Strain KoM1 was cytochrome oxidase and catalase positive but activity of the latter was weak, which might reflect the preferred growth at low oxygen tensions.

Tab. 4.2. Cellular fatty acids of strain KoM1 in comparison to those of other type I methanotrophs. Values given are per cent of total. Data for the reference genera: *Methylomonas* (Bowman et al., 1991; Bowman et al., 1993), *Methylomicrobium* (Bowman et al., 1991), *Methylosarcina* (Wise et al., 2001; Kalyuzhnaya et al., 2005), *Methylobacter* (Bowman et al., 1991; Wartiainen et al., 2006), *Methylosoma* (Rahalkar et al., 2007) and *Methylovulum* (Iguchi et al., 2011) were published and summarized (Bodelier et al., 2009) previously.

Fatty acid	*Methylomonas*	*Methylomicrobium*	*Methylosarcina*	*Methylobacter*	*Methylosoma*	*Methylovulum*	strain KoM1
12:0			0 - 3.8		2.7		0.1
13:1 at 12-13							0.3
14:0	1.2 - 25.1	0.7 - 1.8	1.1 - 2.1	7.4 - 9.7	8.6	34.2	0.9
iC14:0	0 - 0.1						
C14:1ω7c	0 - 0.2						
15:0	0 - 1.2	0.2 - 7.3		0 - 4.2	0.8	3	0.7
iC15:0	0 - 2.5			0 - 0.4			
aC15:0	0 - 2.4						
16:0	5 - 18.1	11.3 - 12.5	9.9 - 19.7	4.6 - 8.4	8.5	46.9	6.8
16:0 2-OH							0.2
iC16:0	0 - 0.2						
iC16:0 3-OH							0.2
16:1 3-OH					1.3	8	1
16:1ω11c					2.4		
16:1ω8c	3 - 41.3	14.3 - 19.0	14.1 - 33.6	0 - 34.9			
16:1ω7c	**7.7 - 19.9**	**14.1 - 17.7**	**18.2 - 32.2**	**23.4 - 57.4**	**60**		**55.3***
16:1ω6c	4.5 - 5.9	7.8 - 14.4	6.7 - 9.1	4.4 - 8.2	15		5.8*
16:1ω5t	7.9 - 15.3	27.2 - 28.2	3 - 26.9	1.6 - 26.3			
16:1ω5c	**1.9 - 6.4**	**1.4 - 7.4**	**0 - 0.7**	**2.5 - 7.8**			**28.7**
17:1ω6c						6.4	

* Originally specified as summed feature 3 (C16:1ω7c and/or iC15:0 2-OH and/or 16:1 ω6c) which were not reliably identified by the MIDI system.

PLFA profiles. Strain KoM1 exhibited a unique PLFA pattern compared to other methanotrophs. Two peaks termed "summed feature 3 fatty acids", which contain C16:1ω7c, C15:0 iso 2-OH and C16:1ω6c, could not be identified reliably but were interpreted as C16:1ω7c and 16:1ω6c here, because they were the most probable PLFAs for MOB. Thus, regarding the PFLA patterns, strain KoM1 shows some similarity with *Methylosoma* and *Methylobacter* which also showed high amounts of C16:1ω7c and similar amounts of C15:0 and C16:0 fatty acids (Tab. 4.2). However, the high content of 16:1ω5c observed in strain KoM1 has not been described in other methanotrophs to date and clearly separates strain KoM1 from *Methylosoma* and *Methylobacter*. The PLFA pattern of *Methylovulum* differs significantly from that of strain KoM1. They share the fatty acids C14:0, C15:0, C16:0, and C16:1 3-OH, but in substantially different proportions. The G+C content of strain KoM1 was 47.7 mol% (Tab. 4.1) and, thus, comparable the G+C content of other mesophilic to cold adapted γ-proteobacterial MOB like *Methylobacter tundripaludum* (47%), *Methylobacter psychrophilus* (45.6%) *Methylovulum miyakonense* (49.3%) and *Methylosoma difficile* (49.7%). No sterols, but 2 unidentified hopanoids were detected in strain KoM1 (D. Martin-Creuzburg, pers comm.).

Phylogenetic characterization of strain KoM1. Analysis of the 16S rRNA gene clearly showed that strain KoM1 represents a phylogenetically distinct cluster of methanotrophs (Fig 4.2). The nearest neighbours are *Methylosoma difficile* and *Methylovulum miyakonense* which share 90.3% and 90.7% nucleotide identity of the 16S rRNS gene with strain KoM1, respectively. The phylogenetic analysis of the *pmoA* gene also reveals a distinct position of strain KoM1 within the type Ia MOB (Fig. 4.3). At the amino acid level, *Methylomicrobium japanense* NI and *Methylosarcina lacus* harbor PmoA sequences that share the highest identities with the one of strain KoM1, 87% and 86 %, respectively. The phylogenetically distinct position within the type I MOB became also obvious when the partial MxaF sequences were analyzed (Fig. 4.4). In contrast to the other investigated genes which cluster in the type Ia MOB, the NifH sequence of strain KoM1 clusters close to *Methylocaldum szegediense*, a type Ib MOB (Fig. 4.5). Thus, analysis of widely used phylogenetic markers clearly demonstrates the distinct position of strain KoM1 within the type I MOB. Due to these unique traits, we propose strain KoM1 as the type strain of a new genus and species.

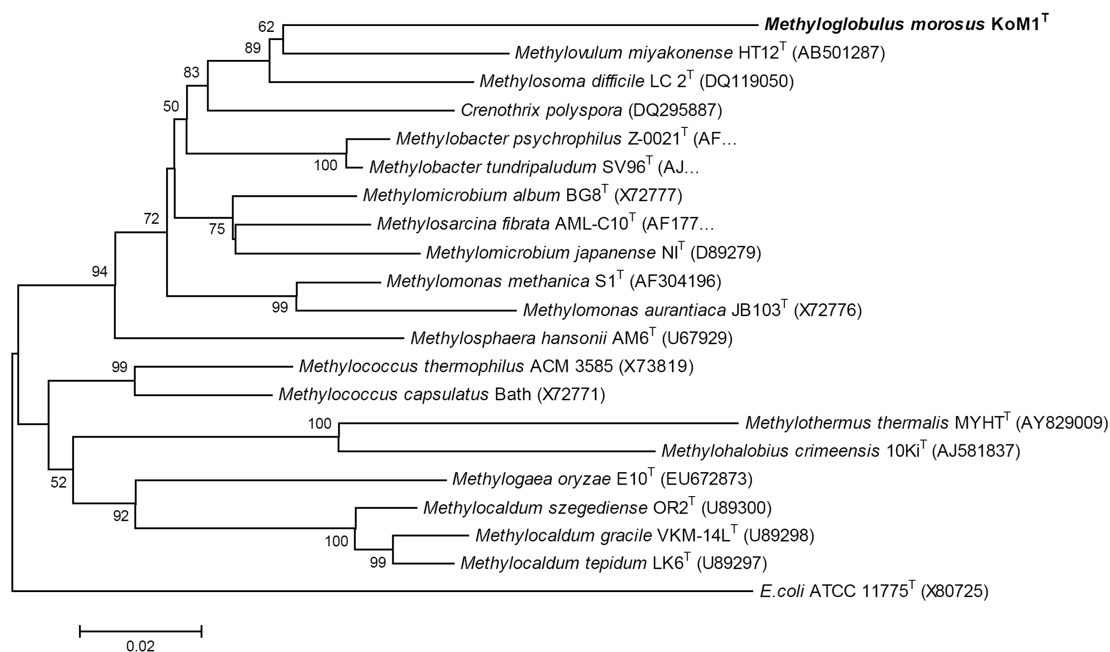

Fig. 4.2. Neighbor joining tree of the 16S rRNA gene sequences of strain KoM1 and other described methanotrophic isolates. *E. coli* was used as out-group. Genbank accession numbers are given in parenthesis. Bar represents 0.02 nucleotide exchanges per site. Bootstrap values (1000 replicates) less than 50% are not shown.

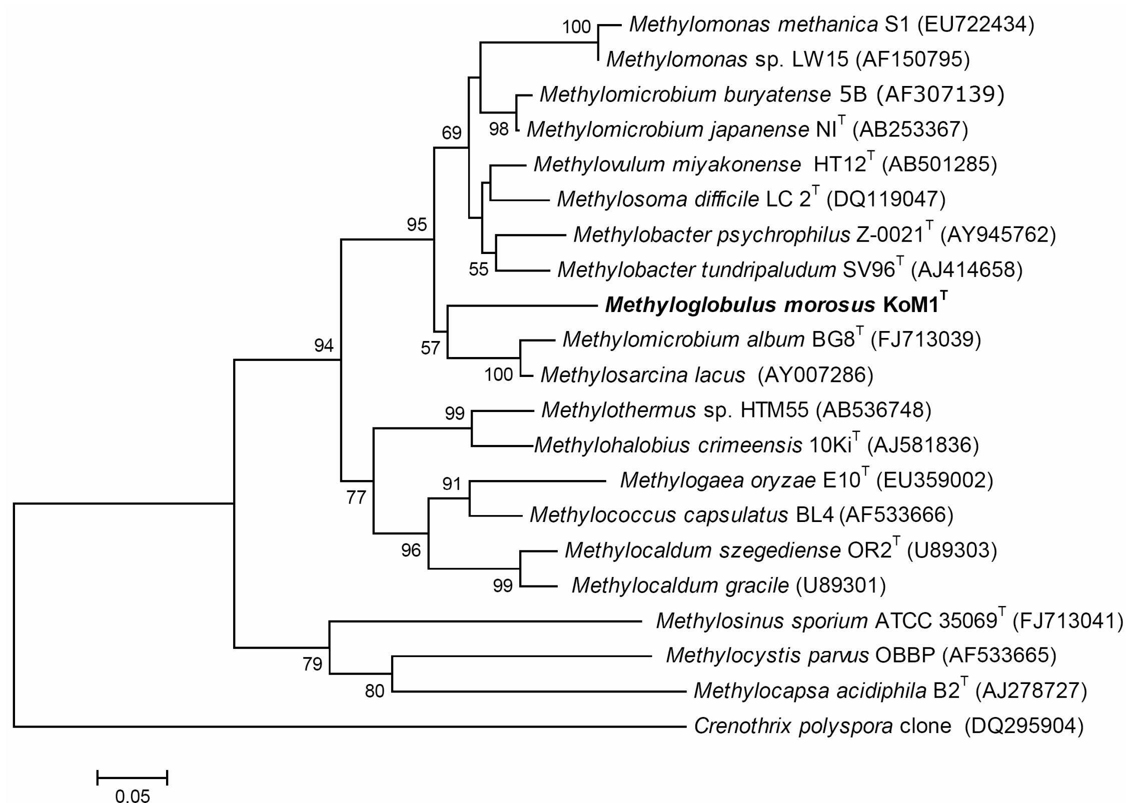

Fig. 4.3. Minimum evolution tree of the deduced PmoA amino acid sequences of strain KoM1 and other described methanotrophic isolates. Genbank accession numbers are given in parenthesis. Bar represents 0.05 amino acid exchanges per site. Bootstrap values (1000 replicates) less than 50% are not shown.

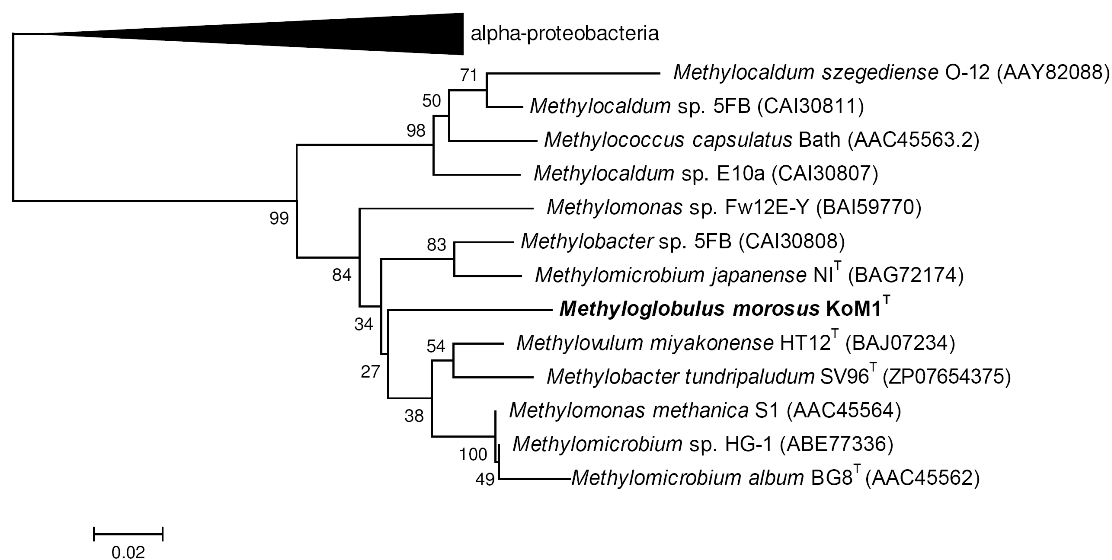

Fig. 4.4. Minimum evolution tree of the deduced MxaF amino acid sequences of strain KoM1 and other and described methanotrophic isolates (1000 replicates). Genbank accession numbers are given in parenthesis. Bar represents 0.02 amino acid exchanges per site.

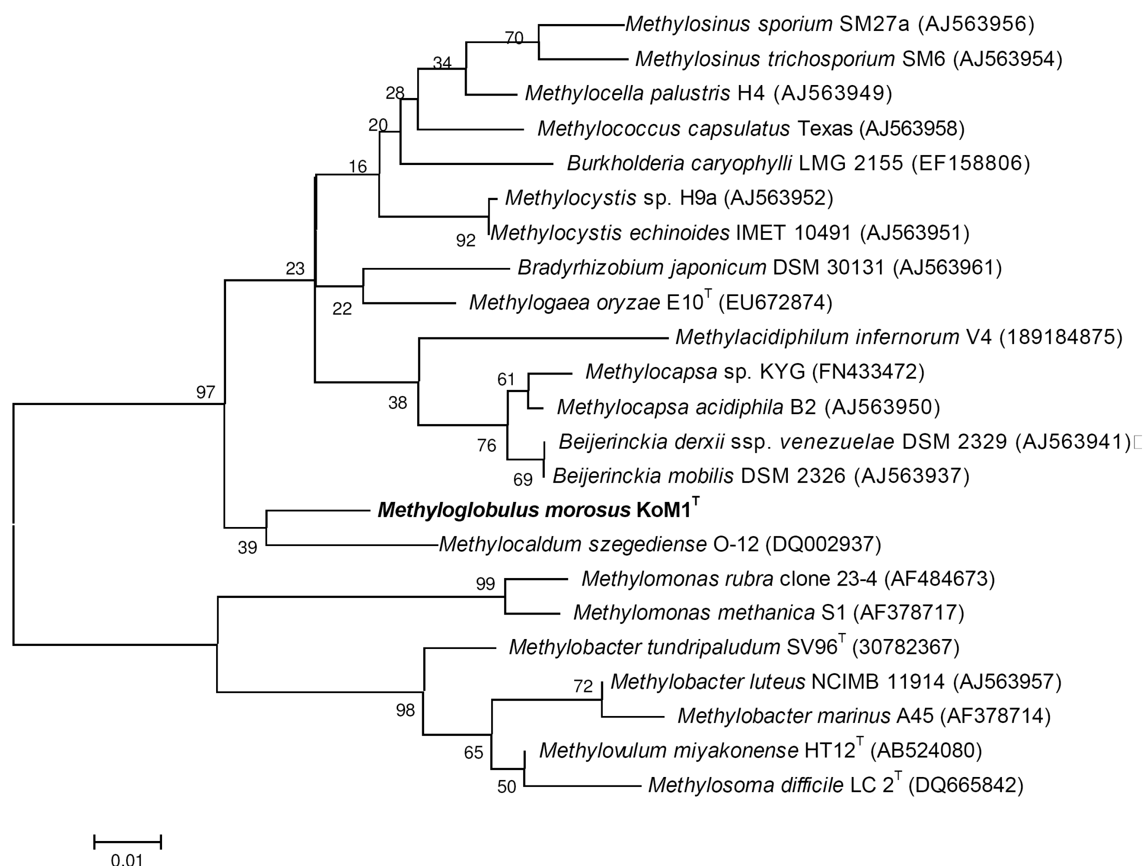

Fig. 4.5. Minimum evolution tree of the deduced NifH amino acid sequences of strain KoM1 and other described and related isolates (1000 replicates). Genbank accession numbers are given in parenthesis. Bar represents 0.01 amino acid exchanges per site.

Description of *Methyloglobulus* gen. nov.

Methyloglobulus (Me.thy.lo.glo'bu.lus. N.L. neut. n. methyl, the methyl group; L. masc. dim. n. globulus little ball, globule; N.L. masc. n. *Methyloglobulus* little round methyl-using bacterium).

Obligately methylotrophic, aerobic bacteria. Methanotrophic and Gram-negative. Phylogenetic traits place the genus into the type I methanotrophs of the family Methylococcaceae; the closest phylogenetic relatives on 16S rRNA gene basis are the genera *Methylosoma and Methylovulum.* The type species and only described species is *Methyloglobulus morosus.*

Description of *Methyloglobulus morosus* sp. nov.

Methyloglobulus morosus (mo.ro'sus. N.L. masc. adj. wayward, capricious, morose).

Description as for the genus. Short rod-shaped cells that are 0.6 µm in width and 0.8 – 1.5 µm in length. Reproduce by binary cell division; non–motile. Cells occur singly or in pairs, sometimes in short chains. Resting stages not detected. Do not possess soluble methane monooxygenase. Do not tolerate NaCl concentrations above 0.1 M. Optimum temperature for growth 20°C (range 4 to 30°C; no growth at 37°C). Grow from pH 5 to pH 8.5. Grow with methane or methanol as sole source of carbon and energy. Grow best at reduced (5-10%) oxygen tensions. Fixes atmospheric nitrogen; *nifH* gene present. Major cellular fatty acids are summed feature 3 (presumably C16:1ω7c) and C16:1ω5c. The G+C content of the DNA is 47.7 mol%. Habitats are cold oligotrophic freshwater sediments.

The type strain is strain KoM1 which was isolated from profundal sediment of the oligotrophic Lake Constance, Germany.

Acknowledgements

We thank Alfred Sulger and the crew of the RV Robert Lauterborn for their help and the opportunity to sample profundal sediment cores, and the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg (DFG) for funding in the frame of Sonderforschungsbereich 454 ("Littoral of Lake Constance").

Chapter 5

Anaerobic Oxidation of Methane in Sediments of an Oligotrophic Freshwater Lake (Lake Constance)

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Abstract

Anaerobic oxidation of methane (AOM) with sulfate as terminal electron acceptor has been reported for various environments including freshwater habitats, and recently also nitrate and nitrite were shown to act as electron acceptors for methane oxidation in eutrophic freshwater habitats. Radiotracer experiments with sediment material of Lake Constance, an oligotrophic freshwater lake, were performed to follow $^{14}CO_2$ formation from $^{14}CH_4$ in sediment incubations in the presence of different electron acceptors, namely nitrate, nitrite, sulfate, or oxygen. Whereas $^{14}CO_2$ formation without and with sulfate addition was negligible, addition of nitrate increased $^{14}CO_2$ formation significantly suggesting that AOM could be coupled to denitrification. Nonetheless, denitrification-dependent AOM rates remained at least one order of magnitude lower than rates of aerobic methane oxidation. Using molecular techniques, putative denitrifying methanotrophs belonging to the NC10 phylum were detected based on *pmoA* and 16S rRNA gene sequences. These findings show that sulfate-dependent AOM was insignificant in Lake constant sediments. However, AOM can be coupled to denitrification also in this oligotrophic freshwater habitat providing first indications that this might be a widespread process that plays an important role in mitigating methane emissions.

Introduction

Freshwater lakes account for 2-10% of the total emissions of the potent greenhouse gas methane (Bastviken et al., 2004) and are therefore an important part of the global methane cycle (Wuebbles and Hayhoe, 2002; IPCC, 2007). The major part of methane is formed biologically by methanogenic archaea in anoxic environments where alternative electron acceptors are lacking (Conrad, 2009). Some methane is lost from the sediments due to ebullition or mixing events (Bussmann, 2005; DelSontro et al., 2010), but most of it is readily oxidized by aerobic methanotrophic bacteria when reaching the oxic biosphere (Hanson and Hanson, 1996). Aerobic methanotrophs activate methane using molecular oxygen in a

monooxygenase reaction to cleave the strong C-H bond (King, 1992). Anaerobic oxidation of methane (AOM) with sulfate as electron acceptor is carried out by methanogen-like archaea, so-called anaerobic methanotrophic (ANME) archaea, in syntrophic cooperation with sulfate-reducing bacteria (Zehnder and Brock, 1980; Hoehler et al., 1994; Hinrichs et al., 1999; Boetius et al., 2000). Although no defined co-culture is available to date (Nauhaus et al., 2002; Nauhaus et al., 2007), metagenomic analysis (Hallam et al., 2004; Meyerdierks et al., 2010) and the discovery of an abundant, methyl-coenzyme M reductase like protein in microbial mats catalyzing AOM (Krüger et al., 2003) provided indications that sulfate dependent AOM operates in all probability as a reversal of methanogenesis. The energy gain in sulfate-dependent AOM according to equation 5.1 is close to the theoretical minimum for ATP synthesis $\Delta G°' = -20$ kJ mol^{-1} (Schink, 1997) which could hardly feed two organisms in a syntrophic cooperation.

$$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + HS^- + 2\,H_2O \qquad \Delta G°' = -21 \text{ kJ/mol} \qquad \text{Eqn. 5.1}$$

Therefore, this process is observed preferentially in marine environments at >800 m water depth and high methane pressures. AOM coupled to iron and manganese reduction (Beal et al., 2009) or humic compound reduction (Smemo and Yavitt, 2007) has been reported recently, but a direct coupling of these electron acceptors to AOM was not shown and the organisms responsible for these processes are unknown. However, the energy yield of AOM coupled to those proposed electron acceptors would be substantially higher than with sulfate, allowing the reactions to take place at lower substrate concentrations (Thauer and Shima, 2008). AOM can also be coupled to denitrification according to equation 5.2 (Raghoebarsing et al., 2006).

$$3\,CH_4 + 8\,NO_2^- + 8\,H^+ \rightarrow 3\,CO_2 + 4\,N_2 + 10\,H_2O \qquad \Delta G°' = -928 \text{ kJ/mol } CH_4 \qquad \text{Eqn. 5.2}$$

This process does not depend on a syntrophic cooperation with archaea (Ettwig et al., 2008) but is carried out by bacteria affiliated to the NC10 phylum, a phylum without any cultured representatives so far. Few enrichment cultures of this type have been obtained to date (Raghoebarsing et al., 2006; Ettwig et al., 2009; Hu et al., 2009), and a metagenome was assembled from two enrichments. It turned out that the denitrifying NC10 bacteria produce oxygen from nitrite via NO. (Ettwig et al., 2010). Thus, this type of methane oxidation takes place in anoxic environments, but the chemically challenging activation of methane does not proceed aerobically, and methane is activated through a methane monooxygenase reaction as in aerobic methanotrophs. A *pmo* gene cluster encoding for particulate methane monooxygenase has been identified in the metagenome and is actively transcribed and translated (Ettwig et al., 2010).

Sulfate-dependent AOM was reported mainly for marine environments (Boetius et al., 2000; Valentine and Reeburgh, 2000; Knittel and Boetius, 2009), and there is little evidence for AOM in freshwater habitats where it may often be masked by aerobic methane oxidation due to the close spatial proximity of the reactant transition zones (Strous, 2010). Sulfate-dependent AOM was reported for Lake Plußsee, a eutrophic lake (Eller et al., 2005b), rice paddies (Murase and Kimura, 1994a), peatlands (Smemo and Yavitt, 2007) and landfills (Grossman et al., 2002). AOM coupled to denitrification was reported for nutrient-rich habitats such as contaminated groundwater (Smith et al., 1991) and sewage sludge (Islas-Lima et al., 2004). Enrichments were obtained from eutrophic canals and ditches (Ettwig et al., 2009) and a mixed inoculum (Hu et al., 2009), but no data on the environmental distribution of this process are available, and direct measurements of denitrification-dependent AOM in freshwater samples are lacking. Some indications on the distribution of this process might be derived from 16S rRNA gene sequences affiliated to NC10 bacteria which have been obtained from various freshwater habitats (Ettwig et al., 2009). However, hardly any information is available on the distribution of methanotrophy in this uncultured phylum, and the presence of 16S rRNA gene sequences is not a solid indication for the presence of this process.

In Lake Constance, concentration profiles of methane and oxygen indicated that methane might be oxidized in Lake Constance sediments also anaerobically (Rahalkar et al., 2009), but micro-aerobic methane oxidation and temporal disturbances of the gradients could not be excluded. In the present study, we checked for AOM in the sediments of this lake, tested sulfate, nitrate, and nitrite as possible electron acceptors for AOM and searched for the responsible microorganisms by molecular methods.

Materials and Methods

Sediment sampling. Littoral sediment samples were collected with a sediment corer (Tessenow et al., 1975) with plastic tubes of 80 mm inner diameter from the lower infralittoral zone (“Litoralgarten”, 47°41’ N, 9°12’ E) of Lake Constance at a water depth of 2 to 3 m (Tab. 5.1). Profundal sediments were collected with a ship-born multicorer with the same plastic tubes from a depth of 80-120 m in upper Lake Constance between Wallhausen and Egg (Tab. 5.1). The profundal core used for the construction of the clone library was sampled in front of the Isle of Mainau 47°42’ N, 9°12’ E). All sediment cores were at least 20 cm long. The lower end of the core was closed with a plug without trapping of gas bubbles and the upper part was capped with a screw cap lid avoiding trapping of a gas bubble in the overlying

water to prevent resuspension of the sediment during transport. The closed sediment cores were transported to the laboratory avoiding percussions. Thus, the investigated sediment layer of 1-4 cm sediment depth was undisturbed until the core was cut in the anoxic tent where oxygen contamination of the investigated sediment layer could be excluded. All sediment cores for radiotracer experiments were collected between February 2009 and February 2010, immediately stored at 4°C, and experiments were started within 24 h after sampling.

Preparation of $^{14}CH_4$. A culture of *Methanospirillum hungatei* was grown in freshwater medium as described previously (Widdel, 1986) with some modifications (Müller et al., 2008), but 20 mM HEPES buffer (pH 7.2) was used instead of bicarbonate buffer. H_2/CO_2 (80/20) was added to an overpressure of 0.5 bar. After growth was visible nitrogen was bubbled through the culture to remove remnant CO_2. A 1:5 (v/v) mix of $^{14}CO_2$ and H_2 was added and nitrogen was supplied further to an overpressure of 0.5 bar. After 1 week, premixed H_2/CO_2 (80/20) was added to an overpressure of 0.5 bar. One week later the gas phase was removed by simultaneously adding medium. The gas phase was transferred into a 20 ml serum bottle filled with 1 M NaOH in dithionite-reduced freshwater medium containing 3 M NaCl (to decrease gas solubility in the liquid phase) while simultaneously some of the liquid phase was removed to release overpressure. The gas phase was then taken out with a syringe that contained hopcalite to remove traces of ^{14}CO (Harder, 1997) and injected again into a 20 ml serum bottle as described before, to trap $^{14}CO_2$. After an additional transfer the tracer gas was stored until further use. All transfers were carried out with pregassed (N_2 or He) one-way plastic syringes with a fitted luer-lock teflon valve. Resazurin was added as a redox indicator in all liquid phases.

Radiotracer experiments. Sediment cores were introduced into an anoxic tent and the uppermost 1 cm was removed to omit the oxic sediment layers from the experiment. Sediment from 1-4 cm depth of 1-3 sediment cores of the same location and sampling date was mixed and diluted with a few ml (at maximum one tenth of sediment volume) of freshwater medium (Widdel, 1986) to obtain a soft, viscous sediment slurry that could be transferred by a cut-off 3 ml or 5 ml plastic syringe. The slurry was split into different treatments and the desired electron acceptor (2 mM $NaNO_3$, 1.5 mM $NaNO_2$ or 2 mM $NaSO_4$) was added. Stock solutions were prepared freshly with double-distilled water, filter-sterilized, degassed by repeated vacuum/N_2 treatment, and sodium dithionite was added to secure anoxia. Three milliliter of the treated sediment was transferred with a cut-off plastic syringe into 9-ml serum

bottles, closed with black butyl rubber stoppers, and capped with aluminum crimp caps. The gas phase was then flushed with pure N_2 to remove the hydrogen present in the anoxic tent, and afterwards the tracer was added in a glove box gassed with N_2. The tracers were diluted with pure non-labeled methane to allow the addition of methane to an equivalent of 10 µmol per l slurry. Specific activities of the injected tracers were 1-2 x 10^6 dpm. All samples were incubated in a N_2-flushed plastic container at 4°C to mimic the *in-situ* temperatures. After incubation, samples were alkalinized with NaOH (0.5 M final conc.) and stored overnight at room temperature. Single vials for every measurement were used to avoid false-positive results due to oxygen contamination during sampling. One milliliter of the gas phase was removed with a one-way plastic syringe with a fitted luer-lock teflon valve for $^{14}CH_4$ radioactivity and CH_4 concentration measurements. Samples were bubbled with N_2 for 5 min to remove remaining $^{14}CH_4$, and the vial was connected via tubes and needles to three 5-ml scintillation vials filled with 2 ml of Carbosorb E (Perkin Elmer) in series as described previously (Zehnder and Brock, 1979). Tightness of the system was checked each time with soapwater and by injecting nitrogen gas into the vial before CO_2 trapping started. The slurry was acidified with 37% HCl until no gas formation was visible anymore, and was bubbled afterwards with nitrogen to flush remaining $^{14}CO_2$ into the trapping solution. An equal volume of Permafluor E+ (Perkin Elmer) was added, mixed, and the vial was stored overnight in the dark to reduce luminescence. Samples were analyzed in a LS 6100IC scintillation counter (Beckman). Initial maximum methane oxidation rates were estimated from the increase of CO_2 between start values and the highest observed values of $^{14}CO_2$ in the first days of the experiments. The effect of nitrate, ambient air, and no additional electron acceptor was tested on methane oxidation in all experiments; nitrite amendment was tested in experiment prof2 and both littoral sediments, and the effect of sulfate addition was investigated in experiments prof1 & 2 and both littoral sediments.

Samples of the gas phase used to determine the radioactivity of $^{14}CH_4$ were transferred into a 9 ml serum bottle filled with 7 ml toluene, incubated overnight at room temperature, transferred into a scintillation vial containing 10 ml LumaSafe Plus scintillation cocktail (Perkin Elmer), and analyzed as described before. Solubility of methane in toluene was calculated after published values (Zehnder et al., 1979). The other sample was transferred into a 9 ml serum bottle containing 3 ml saturated salt solution and stored upside down at -20°C until the methane concentration was determined. Methane was determined using a Carlo Erba 6000 Vega Series 2 gas chromatograph (Carlo Erba Instruments) as described previously

(Platen and Schink, 1987). Nitrate and nitrite were estimated with Merckoquant test strips (Merck) to estimate the time when nitrate and nitrite had disappeared completely.

Molecular detection of NC10 bacteria. DNA was extracted from 2 × 500 mg sediment (fresh weight) with the NucleoSpin Soil Kit (Macherey-Nagel) using a BioSavant fast prep instrument (Bio 101) according to the manufacturers' instructions. The DNA concentration was measured photometrically at 260 nm using a BioPhotometer (Eppendorf). PCR was carried out using the NC10 specific primers 202F and 1043R as published previously (Ettwig et al., 2009), but after analysis of published NC10 sequences introducing two wobbles (NC10-1043Rdeg: 5'-TCTCCRCGYTCCCTTGCG-3' and NC10-202Fdeg 5'-RACCAAAGGRGGCGAGCG-3'). After adjustment of the PCR program to 94°C for 1 min followed by 32 cycles of 1 min at 94°C, 45 s at 67°C and 90 s at 72°C with a final elongation of 7 min at 72°C, PCR products of proper size were obtained directly from sediment DNA extracts and only sequences affiliated to the NC10 phylum were obtained. Primers for amplification of the *pmoA* gene were designed manually using a multiple alignment of Lake Constance *pmoA* clones and one *pmoA* sequence available from NC10 bacteria from the assembled genome of candidatus "*Methylomirabilis oxyfera",* Acc. No. FP565575.1, (Ettwig et al., 2010) with MEGA4 software (Tamura et al., 2007). Two primers were designed: NA437Rdeg: 5'-RAATGTTCGRAGCGTVCCBC-3' and NA555R: 5'-TCCCCATCCACACCCACCAG-3', which amplified only novel NC10 related *pmoA* genes but no known *pmoA* genes of aerobic methanotrophs from our samples. PCR targeting the *pmoA* gene was performed using primer A189f (Holmes et al., 1995) together with one of the newly designed primers and the PCR program described previously (Bussmann et al., 2006). 2-20 ng of extracted DNA was used for all PCR reactions. Pooled PCR products of at least 3 PCRs were purified with the DNA clean and concentrator Kit (Zymo research).

For construction of clone libraries the purified DNA was cloned using the TA cloning kit (Genaxxon) according to the manufacturers' instructions. Clones were picked and after a PCR using the M13 primer pair the product was sent for sequencing (GATC Biotech, Konstanz, Germany). A total of 42 16S rRNA sequences was obtained for profundal sediment and 23 sequences for littoral sediments. Rarefaction analysis of the clone libraries was carried out using aRarefactWin software (v. 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens; www.uga.edu/~strata/software/) and Chao1 estimators (Chao, 1987) were determined with EstimateS (Version 8.2, R. K. Colwell, http://purl.oclc.org/estimates) for each clone library. Nucleotide sequences were deposited at the National Centre for Biotechnology

Information under accession numbers HQ906501 - HQ906564 (16S sequences) and HQ906565 - HQ906579 (*pmoA* sequences).

Phylogenetic analysis. 16S rRNA gene sequences were aligned using the SINA web aligner (http://www.arb-silva.de/aligner/) and inferred PmoA sequences were aligned with the ClustalW algorithm implemented in Mega4. Phylogenetic trees were constructed with Mega4 software (Tamura et al., 2007). Different tree construction methods were compared and yielded similar results. The shown phylogenetic tree based on 16S rRNA gene sequences was constructed using the minimum evolution method choosing the pairwise deletion option. Evolutionary distances were computed using the Tajima-Nei method. There was a total of 875 positions in the final dataset. The shown tree based on PmoA sequences was constructed using the minimum evolution method choosing the pairwise deletion option. Evolutionary distances were computed using the JTT matrix-based method, with a total of 191 positions in the final dataset. The pairwise deletion option was chosen to allow the inclusion of shorter sequences because not many reference sequences were available. When short sequences were excluded and phylogenetic analysis was performed using the complete deletion option the same sequences clustered together but some deeper branching nodes changed, also indicated by low bootstrap values in the final tree (Fig. 5.3).

Results

Sampling. All sediment cores showed a defined stratification. Profundal sediment cores had a soft homogeneous yellow-brownish top layer and dark sulfidic fine-grained material at 3-5 cm depth. The littoral cores differed in their composition. One core (litt1) had a thin soft and beige surface layer of approximately 1 cm, then 0.5 cm of blackish sediment containing parts of mussel shells and consisting of very fine grey material, probably lake marl, below. The other littoral sediment core (litt2) and the core used for molecular work had a 2 cm thick layer of soft beige material and turned black in the deeper layers. *Chara* sp. grew on the sediment, and parts of mussel shells were visible throughout all investigated sediment layers.

Tab. 5.1. Sampling locations, sampling dates, and estimated maximum initial methane oxidation rates of Lake Constance sediment incubations.

habitat (depth)	abbreviation	sampling date	maximum initial methane oxidation		
			no addition	nitrate	ambient air
profundal (ca. 80 m)	prof1	17.02.2009	n.a.[1]	3.6	27
profundal (ca. 80 m)	prof2	06.10.2009	n.a.[1]	2.7	38
profundal (ca. 120 m)	prof3	09.02.2010	n.a.[1]	1.8	44
littoral (2-3 m)	litt1	16.11.2009	n.a.[1]	n.a.[1]	18
littoral (2-3 m)	litt2	14.01.2010	0.08	0.6	63

[1] Rates were not calculated if the $^{14}CO_2$ formation was within the background scatter of t=0 values and sterile controls. With sulfate as electron acceptor the $^{14}CO_2$ formation was within the background scatter in all experiments.
[2] with the given electron acceptors in nmol d^{-1} (ml sediment)$^{-1}$

Anaerobic oxidation of methane in sediment incubations. The influence of different electron acceptors on anaerobic oxidation of methane (AOM) was investigated using radiotracer experiments. Three independent experiments were performed with profundal sediments and two with littoral sediments (Tab. 5.1). All experiments investigating AOM in profundal sediments yielded similar results. Without addition of an external electron acceptor, $^{14}CO_2$ values remained within the background level in all profundal sediment incubations. Addition of sulfate did not enhance formation of $^{14}CO_2$ in any profundal sediment (Fig. 5.1). Nitrate caused a clear stimulation of $^{14}CO_2$ formation compared to untreated controls in all profundal sediment incubations (Fig. 5.1). Estimated AOM rates in nitrate-amended treatments ranged from 1.8 to 3.6 nmol d^{-1} (ml sediment)$^{-1}$ (Tab. 5.1). Nitrite addition led to slightly elevated $^{14}CO_2$ values in the profundal sediment tested, but due to high scatter (Fig. 5.1) no AOM rates were calculated. Control assays under air showed $^{14}CO_2$ formation rates that were about one order of magnitude higher than the nitrate-amended treatments. Results obtained with the two littoral sediment incubations differed in some cases. In the first experiment with littoral sediment (litt1), no enhanced formation of $^{14}CO_2$ was detectable when sulfate, nitrite or nitrate was added (Fig. 5.2). The second littoral sediment investigated (litt2) showed enhanced $^{14}CO_2$ formation without any addition, but no AOM was detectable with addition of sulfate or nitrite as electron acceptor (Fig. 5.2). On the other hand, nitrate addition enhanced $^{14}CO_2$ formation in this littoral sediment, although to a lower extent than in profundal sediments (Tab. 5.1). The oxic treatments showed $^{14}CO_2$ formation rates that were two orders of magnitude higher than the nitrate treatment in experiment litt2 (Tab. 5.1).

Nitrate-dependent AOM stopped in all experiments after a few days which coincided with the time when nitrate was depleted in non-labeled control vials (usually after 5 days, data not shown). Refeeding nitrate on day 15 in experiment prof2 did not cause a resumption of $^{14}CO_2$

formation. However, even in profundal sediments nitrate-dependent AOM accounted for less than 5% of the nitrate consumption in the treatments. High $^{14}CO_2$ formation was observed only in about 50% of the replicates even in positive experiments, whereas in the remaining vials only low or sometimes no $^{14}CO_2$ production was detectable.

Headspace methane concentrations were measured in experiments prof1, prof3, and litt2, and showed no changes over time in all anoxic profundal treatments. During incubation of the littoral sediment, however, methane increased from 0.15 µmol to 0.53 µmol and 0.37 µmol without addition and with addition of sulfate, respectively. No changes were observed in the nitrate and nitrite treatments. Methane concentrations decreased in the oxic treatments.

Diversity of NC10 bacteria. The 16S rRNA gene of bacteria allocated to the candidate division NC10 (NC10 bacteria) was successfully amplified directly from sediment DNA extracts. Clone libraries targeting the 16S rRNA gene with the specific primer pair were constructed from littoral and profundal sediments. The clone libraries contained only sequences belonging to the NC10 phylum, thus verifying the specificity of the primers. Clustering of the 16S clones (profundal: 42, littoral: 23 clones) to operational taxonomic units (OTU) using a 3% threshold resulted in 3 OTU for profundal sediment and 5 OTU for littoral sediment, with Chao1 richness estimators of 4.81 ± 1.34 and 5.25 ± 0.64, respectively.

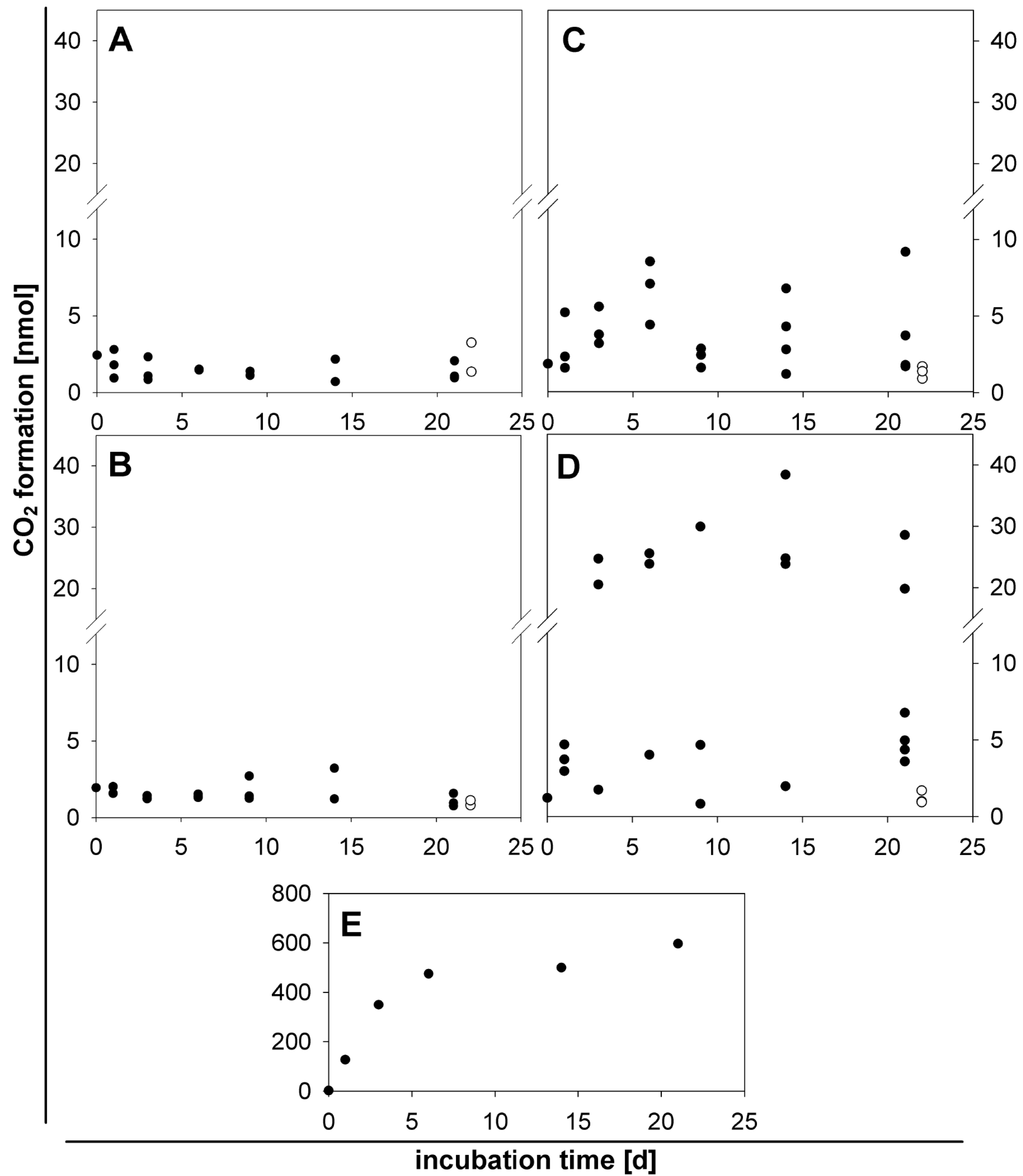

Fig. 5.1. CO_2 formation, calculated from $^{14}CO_2$ formation from $^{14}CH_4$, in incubations of Lake Constance profundal sediment. One of three experiments (prof2) is shown. The following electron acceptors were added: A) no added electron acceptor, B) 2 mM sulfate, C) 1.5 mM nitrite, D) 2 mM nitrate and E) ambient air. Filled symbols: sample values; open symbols: sterile controls.

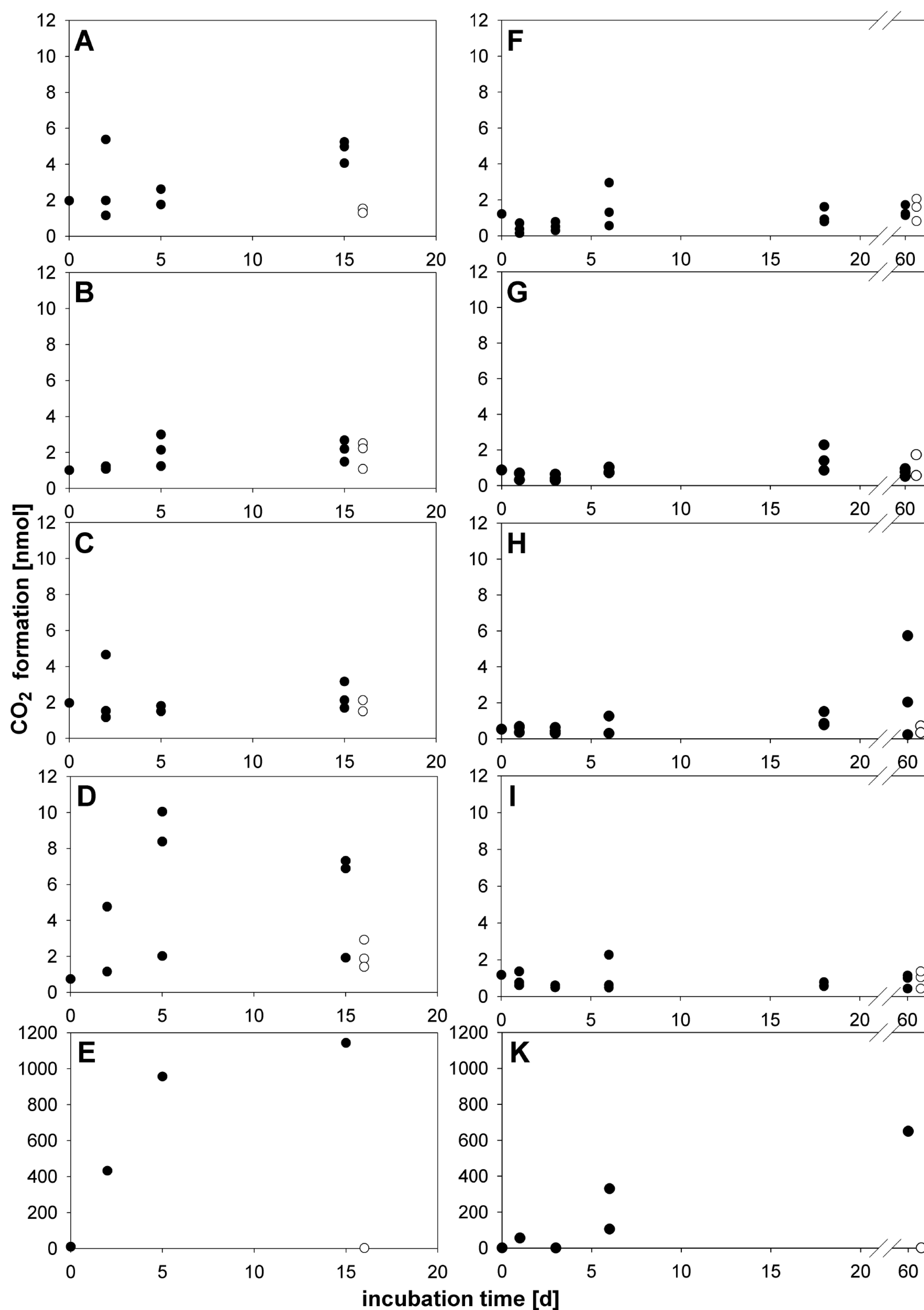

Fig. 5.2. CO_2 formation, calculated from $^{14}CO_2$ formation from $^{14}CH_4$, in incubations of Lake Constance littoral sediment. In experiment littoral 1 (A-E) and experiment littoral 2 (F-K) the following electron acceptors were added: A), F)no added electron acceptor, B), G) 2 mM sulfate, C), H) 1.5 mM nitrite, D), I) 2 mM nitrate and E), K) ambient air. Filled symbols: sample values; open symbols: sterile controls.

The obtained sequences could be assigned to two main groups of NC10 bacteria, namely, to groups A and B according to Ettwig (2009). Clones belonging to group A were obtained only from profundal sediments, whereas clones belonging to group B were obtained from both littoral and profundal sediments. All clones belonging to group A showed a maximum sequence diversity below 1% (average 0.4%), thus, they likely represent one species that accounts for more than 90% of the profundal clones. Group B clones were more diverse with 5% maximum sequence diversity (average 2.2%).

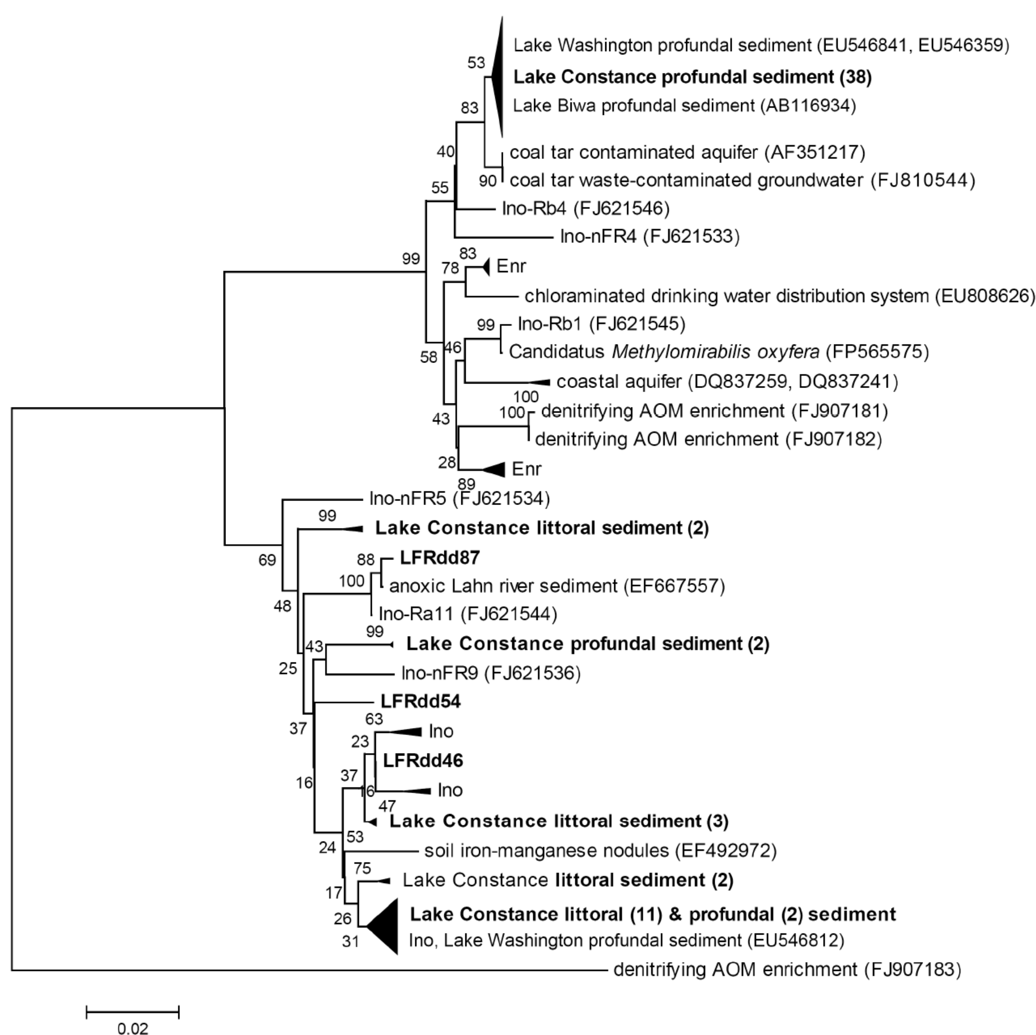

Fig. 5.3. Phylogenetic tree of the obtained NC10 bacterial 16S rRNA gene sequences from Lake Constance sediments. The tree was constructed using the minimum evolution method choosing the pair wise deletion option. Evolutionary distances were computed using the Tajima-Nei method. Bootstrap values were calculated from 1000 replicate trees. The scale bar represents the number of substitutions per site. Clones obtained in this study are shown in bold, number of clones or accession numbers are given in brackets. Ino: Sequences from the inoculum of a denitrifying AOM enrichment and Enr: sequences from the enrichment after 6 months (Ettwig et al., 2009)

The clones belonging to subgroup A of the NC10 phylum exhibited highest sequence similarity to Lake Washington and Lake Biwa sediment clones. These groups clustered separately but closely adjacent to the sequences from different denitrifying AOM enrichments and "candidatus *Methylomirabilis oxyfera"* (Fig. 5.3). Clones in group B were related to different environmental clones including the inoculum of a bioreactor and of other freshwater habitats.

Presence of the functional marker gene *pmoA* in Lake Constance sediments. *PmoA* genes were successfully amplified only from profundal, but not from littoral sediments using two newly designed reverse primers. With both primers, only PCR products of the expected size were obtained. Ten sequences were obtained using reverse primer NA437Rdeg, and five using the reverse primer NA555R. All sequences exhibited a maximum sequence diversity of 2.5% at the amino acid level and of 1.1% at the nucleotide level. Phylogenetic analysis showed that the sequences cluster closely with the two PmoA sequences known from NC10 bacteria, namely "candidatus *Methylomirabilis oxyfera"* strain Twente and strain Ooji (Fig 5.4). The sequences obtained from Lake Constance sediments share 3.5% - 5.5% amino acid identity with the sequences of *M. oxyfera*.

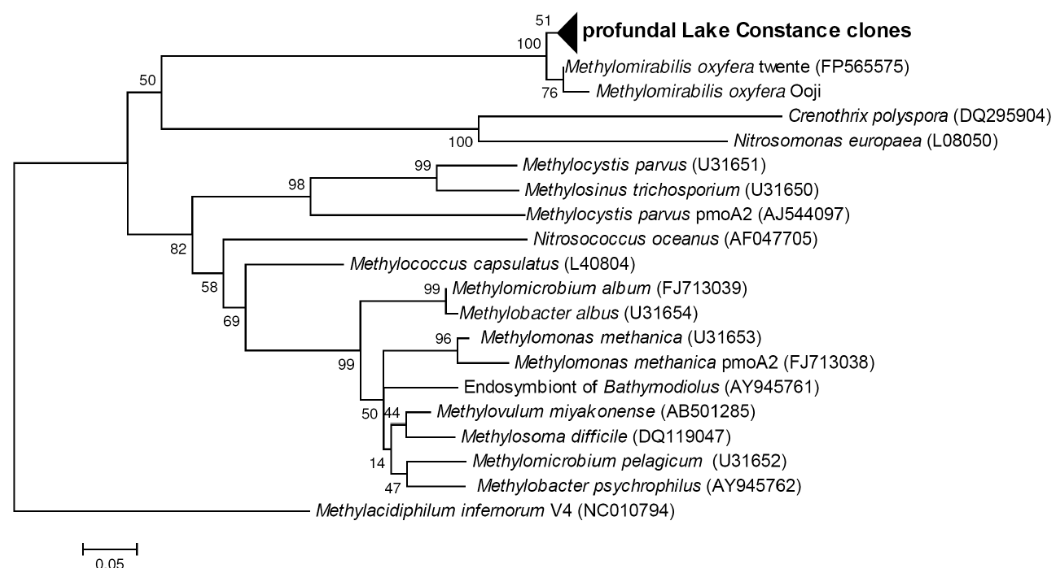

Fig. 5.4. Phylogenetic tree of the obtained NC10 bacterial PmoA sequences from Lake Constance sediments constructed using the minimum evolution method choosing the pairwise deletion option. Evolutionary distances were computed using the JTT matrix-based method. The scale bar represents the number of substitutions per site. Bootstrap values were calculated from 1000 replicate trees.

Discussion

Anaerobic oxidation of methane in sediment incubations. AOM was detected in profundal sediments only with nitrate and to a lesser extent with nitrite as electron acceptor. The lower rates with nitrite could be explained by the fact that nitrite at 1.5 mM concentration might be toxic to NC10 bacteria (Hu et al., 2010). On the other hand, denitrifying anaerobic methanotrophs have been shown to prefer nitrite over nitrate in enrichment cultures (Raghoebarsing et al., 2006; Hu et al., 2010). Although these bacteria have the enzymes to use nitrate directly (Ettwig et al., 2010), methane oxidation does not yield sufficient reducing equivalents to fuel a completely nitrate-dependent metabolism with molecular oxygen as intermediate for methane activation. The oxidation of methane to CO_2 yields 8 redox equivalents, but due to a monooxygenase reaction 4 redox equivalents are consumed to reduce O_2 to H_2O and the –OH group of methanol. Thus, the remaining 4 redox equivalents are sufficient to reduce 2 nitrite (consumes 2 redox equivalents) but not to reduce 2 nitrate (consumes 6 redox equivalents) to N_2 and O_2. The other 2 redox equivalents could be used for oxygen respiration (Wu et al., in press) which would lead to the stoichiometry of nitrite-dependent AOM previously observed (Eqn 5.1; Ettwig et al., 2010). In sediments, numerous denitrifying bacteria can provide nitrite from nitrate and denitrification was obvious in our incubations because nitrate disappeared completely, but at maximum 5% was consumed by denitrifying AOM. It was already hypothesized that NC10 bacteria may cooperate with unidentified bacteria which reduce nitrate to nitrite, or with ammonium-oxidizing bacteria (Hu et al., 2010; Zhu et al., 2010).

The high scatter of denitrification-coupled AOM in profundal sediments remains enigmatic, as the sediment appeared very homogeneous and had been mixed well after addition of the electron acceptor. Nonetheless, small differences in sediment composition or unknown factors might influence the competition for nitrate, or subsequently nitrite, between bacteria, thus affecting methane oxidation coupled to denitrification. Littoral sediments were more heterogeneous, and the small volume of 3 ml used in the replicate assays in this study may not be sufficient to produce identical data, considering the size of, e.g., plant roots and small invertebrates, which were likely not distributed evenly among the treatments and might cause significant differences among replicate samples. Furthermore, not much is known about the susceptibility of NC10 bacteria to environmental changes as the only physiological data come from enrichment cultures that ran continuously for several months before significant AOM rates were detected (Raghoebarsing et al., 2006; Ettwig et al., 2009; Hu et al., 2009). If NC10 bacteria depend on redox gradients at oxic/anoxic interfaces in their natural environments as

hypothesized by Zhu (2010) their activity might be restricted to a few millimeters, and conditions in our batch experiments may sustain their activity only for a short period of time. This might also be a reason why nitrate-dependent AOM was found only in one of the two littoral sediment samples. The plants and the thicker surface layer indicate that mechanical disturbances, e. g. by wave action, might be of minor importance at this site and therefore the geochemical gradients are more stable, whereas the other sediment was prone to mixing and did not provide a suitable habitat. Beyond this, plant roots are known to establish oxic-anoxic interfaces in sediments (Brune et al., 2000).

Aerobic methane oxidation rates obtained in our radiotracer experiments are comparable to rates measured before by conventional gas phase analysis (Deutzmann et al., 2011). However, substrate limitation caused by slow diffusive transport and the unnaturally high concentrations of the added electron acceptors prohibit exact calculations of methane oxidation rates *in-situ*. Thus, the potential for nitrate-dependent AOM was demonstrated, but a quantitative assessment of the importance of AOM *in-situ* demands further research.

The low rate of AOM in the absence of an external electron acceptor in the second littoral sediment sample might be a side effect of active methanogenesis in this sediment. Zehnder et al. observed up to 8% label exchange during methanogenesis in Lake Mendota sediment (Zehnder and Brock, 1980). In our case, the measured formation of 380 nmol methane would be by far sufficient to explain the observed formation of up to 6 nmol $^{14}CO_2$. This kind of “AOM” is always linked to methane production and was presumably not taking place in any other treatment. Furthermore, nitrate is known to inhibit methanogenesis (Klüber and Conrad, 1998), thus, AOM in nitrate treatments is probably independent of methanogenesis.

AOM coupled to sulfate reduction was not detectable in any of our experiments. Furthermore, previous studies did not detect ANME archaea in sediments of Lake Constance by clone library analysis (Rahalkar, 2007) or by fluorescence *in-situ* hybridization (M. Rahalkar, pers. comm.). AOM coupled to sulfate reduction has been reported for various environments (Knittel and Boetius, 2009) including freshwater habitats (Murase and Kimura, 1994b; Eller et al., 2005b). So far, these reports were based on indirect evidence, and in most cases an involvement of a further electron acceptor besides sulfate cannot be excluded with certainty. Considering that in shallow freshwater systems the methane partial pressure can hardly rise far beyond one atmosphere and considering the low sulfate concentrations in limnic systems, the energy gain of sulfate-dependent AOM is most likely insufficient to fuel a syntrophic binary methane-oxidizing association in these environments.

An AOM coupled to iron or manganese reduction as recently described (Beal et al., 2009) was not investigated in our study, but did obviously not take place at detectable rates although ferric iron is present in the investigated sediment layers of Lake Constance (Kappler et al., 2004). Also the proposed coupling of AOM to the reduction of humic compounds (Smemo and Yavitt, 2007) was not observed although humic compounds are present in Lake Constance sediments (Kappler et al., 2001).

Presence of denitrifying anaerobic methanotrophs (NC10 Bacteria). The presence of NC10 bacteria was verified using molecular methods, and these bacteria might be responsible for AOM coupled to denitrification in our samples. NC10 bacteria appear to be widespread in Lake Constance, as specific 16S rRNA-gene amplicons were obtained from DNA extracts taken at various locations in Lake Constance (data not shown). However, the community composition of NC10 bacteria appears to differ substantially between sites. The dominating clone sequences in profundal sediments form a very uniform cluster of group A NC10 bacteria as grouped by Ettwig et al (2009), and appears to be absent or low in abundance in littoral sediment. Furthermore, 16S rRNA gene sequences that are almost identical to the dominant profundal cluster described in our study have been detected before in profundal sediments of Lake Washington and the mesotrophic Lake Biwa, where this 16S sequence (AB116934) is also present as rRNA at sediment depths down to 8 cm (Koizumi et al., 2003). Interestingly, targeting the *pmoA*-gene, PCR products were obtained only from profundal samples in which group A members were detected. Furthermore, the low diversity of *pmoA* gene sequences coincides well with the low diversity of group A NC10 bacteria on 16S sequence basis, and both gene sequence clusters are similarly related to “candidatus *Methylomirabilis oxyfera”* (3.4-6.6% on 16S and 3.5% -5.5% on *pmoA* basis). Therefore we hypothesize that only representatives of group A of the NC10 bacteria are responsible for nitrate-dependent AOM in Lake Constance. This would also explain the lower rates of nitrate-coupled AOM in littoral sediments where group A of the NC10 bacteria was below detection limit. Additionally, only NC10 group A bacteria were enriched in various enrichments (Raghoebarsing et al., 2006; Ettwig et al., 2009; Hu et al., 2009). However, it cannot be ruled out that our *pmoA* primers have a too narrow target range to amplify the entire diversity of *pmoA* genes affiliated to the NC10 phylum because there are almost no references available. Despite the indications that NC10 bacteria of group A are responsible for denitrifying AOM in Lake Constance, the involvement of other yet unknown organisms cannot be excluded.

Consistent data on denitrifying AOM rates and the presence of the respective bacteria were obtained for profundal sediments of Lake Constance which provide constant environmental conditions, but not for the more disturbed littoral ones. The heterogeneity of littoral sediments was reflected in inconsistent data on denitrifying AOM rates, and no NC10 bacteria associated with denitrifying AOM have been detected. Thus, further research on the distribution of this process and the respective bacteria in heterogeneous environments and more extensive sampling might be required to allow general insights into their ecology.

Our study proves the presence of NC10 bacteria based on 16S rRNA gene and *pmoA* sequence analysis in an oligotrophic environment with nitrate concentrations below 75 µM (Rudolph et al., 1991; Petri, 2006) and shows that the recently discovered process of anaerobic methane oxidation coupled to denitrification can take place also in oligotrophic freshwater habitats like Lake Constance. Thus, we provide first evidence that this process might be widespread in freshwater habitats. However, further studies on other freshwater habitats have to follow to enable sound conclusions on the global importance of this methane sink which acts as a link between the carbon and the nitrogen cycle.

Acknowledgements

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Chapter 6

General Discussion

Despite their low share in eliminating atmospheric methane, methanotrophs play an important role in the global methane cycle. They act as biofilters in various habitats and efficiently mitigate methane emissions from anoxic environments to the atmosphere (Hanson and Hanson, 1996). However, our knowledge about these extraordinary bacteria and especially about their ecology is limited. In this study we combined several different strategies (cultivation and isolation, tracer experiments, rate measurements, high-resolution gradient measurements, molecular detection and quantification of MOB by marker genes) to gain insights into methane-oxidizing communities in Lake Constance and to add some important pieces to our picture of methane cycling in freshwater habitats.

Abundance, Distribution and Activity of MOB in Littoral and Profundal Sediments

To understand the ecology of bacteria it is necessary not only to investigate their physiology but also to know the environmental conditions they experience *in-situ*. Thus, we investigated the depth distribution of methanotrophic bacteria along the concentration gradients of their most important substrates: Methane and oxygen. Those gradients are very steep in freshwater sediments thus necessitating measurements with high spatial resolution. By applying oxygen microelectrodes and a diffusion-based methane microsensor (Bussmann and Schink, 2006) combined with the molecular detection of MOB by FISH and qPCR we detected a maximum of MOB abundance in the same sediment layer where the methane gradient indicated methane oxidation in littoral sediment. Surprisingly, this zone of methane oxidation was located below the zone where oxygen was available. In profundal sediments, this gap between methane and oxygen gradients was only a few millimeters and might be explained by small discrepancies between the gradients *in-situ* and after transfer to the laboratory, even though *in-situ* conditions were mimicked as far as possible. In littoral sediments the gap between methane oxidation zone and oxygen availability was too large to be explained by small gradient shifts due to differing incubation conditions or missing bioturbation. Two different circumstances could explain this phenomenon. Either methane had been oxidized anaerobically, which was investigated in chapter 5, or wave-induced sediment resuspension and pore water exchange had caused the observed methane gradients. As anaerobic oxidation of methane was found repeatedly only in profundal sediments and the detected AOM rate in littoral sediment was about 2 orders of magnitude lower than aerobic methane oxidation (chapter 5), AOM appears

not to be sufficient to explain the observed gradients. In fact, when a sediment core was incubated under laboratory conditions without disturbances for 2 weeks, the methane profile moved upwards until it met the oxygen profile a few mm below the sediment surface after 4-7 days (data not shown). It was also shown that oxygen penetrated approximately 2 times deeper into littoral sediment when measured *in-situ* in an acidic mining lake (Koschorreck et al., 2003). Lake Constance has a much larger surface area permitting stronger wind waves, and additionally ship waves are caused e.g. by the ferry boat from Konstanz to Meersburg. Ship waves have a huge impact on the littoral zone causing sediment resuspension and pore water interchange (Hofmann et al., 2008a) and have been shown to release methane from sediments at our sampling site (Hofmann et al., 2010). Thus, wave action is likely to be responsible for the distribution gradients observed. After sampling, oxygen can be used up rapidly by various processes, whereas methane cannot be oxidized at high rates under anoxic conditions and is therefore influenced mainly by diffusion processes. To investigate the disturbances of littoral sediment caused by waves in more detail, *in-situ* measurements of the oxygen distribution during wave action are in preparation. Additional measurements of methane gradients before and after ship wave induced resuspension events will help to quantify methane release from the sediments and will allow new insights into fluctuations of environmental conditions that bacteria have to face in littoral sediments.

Another finding of this study was that methanotrophs are abundant throughout the sediment column in littoral and profundal sediments of Lake Constance and, thus, experience more likely limiting oxygen than limiting methane concentrations. Oxygen is consumed rapidly by heterotrophic bacteria and is present at high concentrations only directly at the sediment surface. However, MOB have a high affinity for oxygen and outcompete heterotrophic bacteria at low oxygen concentrations (van Bodegom et al., 2001). Thus, MOB are presumably active in sediment layers where oxygen concentrations are usually low. Consistently, MOB prefer low oxygen concentrations also in stratified lakes, where highest methanotrophic activities have been detected at the oxic/anoxic boundary layer (Carini et al., 2005; Kankaala et al., 2006). Additionally, MOB are often present in anoxic environments (Rothfuss et al., 1997; Carini et al., 2005), but readily start to oxidize methane when environmental conditions are suitable (Rothfuss et al., 1997). They survive carbon limitation better under anoxic than under oxic conditions (Roslev and King, 1994), and the two recently isolated MOB from Lake Constance have difficulties to grow at ambient oxygen concentrations even if methane is present (Chapter 4; Rahalkar et al., 2007). Altogether, this indicates adaptations to low-oxygen environments that might be one important selective factor

influencing community composition of MOB at different sites. MOB present at sites like landfill cover soils and rice fields have to tolerate high concentrations of oxygen, as higher oxygen concentrations than in sediments have to be expected at these sites. In line with that, a recent sequence meta analysis showed a clear differentiation of MOB communities according to their environments (Lüke, 2010). Type Ia MOB dominate limnic systems like Lake Constance, whereas type II, type Ib and few distinct groups of type Ia MOB occur in landfill cover soils and rice fields (Lüke, 2010). Additionally, spore or cyst formation and resistance to desiccation might constitute important adaptations of MOB to these "high oxygen" habitats, which were not observed with our recent isolates from Lake Constance.

Abundance, Activity, and Community Composition of MOB at Methane Seeps

In addition to profundal and littoral sediments with their characteristic features, sediment of active methane-emitting pockmarks has been investigated (Deutzmann et al., 2011). Hundreds of these methane seeps have been detected in Eastern Lake Constance. At these sites, methane is bubbling out of the sediment in the center of up to 1.5 m deep and 8 meter wide depressions in the sediment. Thus, pockmarks likely cause environmental conditions that allow methane oxidation to take place without the diffusion-limitation present in the surrounding sediments (Rahalkar et al., 2009; Bussmann et al., 2011). In agreement with that, the methanotrophic communities at shallow (littoral) pockmarks exhibited distinct differences to those in the surrounding sediment. The lack of significant differences between MOB communities in profundal pockmarks and surrounding sediment was most likely caused by insufficient sampling accuracies at this depth as the influence of the gas emanation site reaches out only some decimeters into the surrounding sediment (Bussmann et al., 2011) and will not be discussed any further. At littoral pockmarks, one group of *Methylobacter* like MOB (group LP-I) dominated the clone library and therefore seemed to benefit from the high methane environment provided by the methane seep. However, the physiological adaptations of these strains enabling this predominance deserve further research, and the oxygen distribution in the pockmark sediments needs to be investigated to draw conclusions on their ecology and the special adaptations of MOB to these presumably "high flux" environments.

Not only the qualitative composition of MOB differed at pockmark sites, compared to the surrounding sediment, but the abundance of MOB and the methanotrophic activity was more than 1 order of magnitude higher than in the surrounding sediment. Especially when taking into account that more than 120 active pockmarks have been identified in Eastern Lake Constance (Wessels et al., 2010), methane-derived carbon could constitute an important part

of the total carbon flux to higher trophic levels. Methane-derived carbon might play an important role in the benthic, but potentially also in the pelagic food web, of this otherwise oligotrophic environment. It has been reported for freshwater habitats that methane-derived carbon can contribute to up to 70% of the carbon biomass of benthic chironomid larvae (Jones et al., 2008). Up to 50% of the carbon biomass of pelagic copepods have been reported to originate from methane in stratified lakes with an anoxic hypolimnion (Jones and Grey, 2011), where methane oxidation taxes place in the water column at the oxic-anoxic interface. Furthermore, methane derived carbon could be tracked to higher trophic levels like fish and spiders due to its very light $\delta^{13}C$ isotopic signature (Jones and Grey, 2011). Future research on pockmarks should include detailed investigations on the importance of methanotrophs in mitigating methane emissions at pockmark sites and their influence on the entire lake methane emissions. Tracking of methane-derived carbon in the food web is needed to get an idea of the impact of these methane seeps on carbon cycling in Lake Constance.

Isolation of MOB

Another approach to understand the physiology of MOB from Lake Constance was isolating novel MOB. Many environmental surveys that use molecular techniques to detect certain genes or mRNAs in all kinds of habitats provide vast amounts of sequence data stored in public databases, but not much is known about the physiology of these bacteria. Therefore, one can speculate about their adaptations to their environments or their role *in-situ*. Even environmental genomics and other sophisticated "meta-omic" approaches have their limitations in predicting the function of an ecosystem because many gene and protein functions are unknown. Isolated representatives of given taxa provide the unique possibility to investigate the physiology of these bacteria und therefore enable more sound conclusions on their environmental function and adaptation. However, even at the phylum level, 13 out of nearly 40 defined divisions harbor no cultured representatives (Hugenholtz et al., 1998). Only a few per cent of the abundant bacteria in different habitats are culturable, and surely only a small fraction of the total bacterial diversity and of the different metabolic capacities have been discovered (Amann et al., 1995; Lemke and Leff, 2006; Stres, 2007). Thus, cultivation of novel bacteria is needed to gain insights in their yet unknown metabolic functions.

In the context of this work, a novel methanotrophic bacterium was isolated from profundal sediments of Lake Constance. Isolation of novel bacteria can be a challenging task, as already the name of a previously isolated methanotroph from Lake Constance indicates: *Methylosoma difficile* strain LC 2 (Rahalkar et al., 2007). The same was observed in this study. The novel

isolate, *Methyloglobulus morosus* strain KoM1 (chapter 4), failed to grow on plates in pure culture and was also sometimes difficult to maintain in liquid culture. If replicate vials were inoculated, growth was sometimes delayed for up to 10 days at oxygen concentrations above 12.5%. Also *M. difficile* seemed to face difficulties with high oxygen concentrations and did not grow in flasks if they were shaken within the first days of incubation (Rahalkar, pers. comm.). Additionally, both strains were initially enriched in opposing gradients of methane and oxygen (Bussmann et al., 2006; chapter 4) to mimic the low substrate concentrations they experience *in-situ* (Rahalkar et al., 2009). *M. difficile* and strain KoM1 also share similar prerequisites for growth regarding temperature and pH, and both possess the *nifH* gene indicating the capacity for nitrogen fixation. Altogether, this suggests that these bacteria share a similar ecology and habitat. However, according to sequence data available at the NCBI database, *M. difficile* and strain KoM1 (or to be precise, sequences similar to those of these two isolates) are not equally distributed in the environment. There are only 16S rRNA gene and *pmoA* gene sequences available in the NCBI database that are closely related to those of strain KoM1, but not a single one similar to those of *M. difficile*. Several uncultivated methanotrophs which harbor PmoA sequences similar to the one of strain KoM1 have been detected in environmental studies. Almost all clones that share more than 95% sequence identity with the PmoA sequence of strain KoM1 (which corresponds roughly to more than 97% 16S rRNA gene sequence identity considering the 1.9 times higher amino acid exchange rate of PmoA compared to the nucleotide exchange rate of the 16S rRNA gene; Lüke, 2010) originate from the following environments: Lake Constance sediments (Bussmann et al., 2004; Rahalkar and Schink, 2007), Zoige Wetland soil from Tibet, a boreal lake in Finnland (Siljanen et al., 2011), Eastern Snake River Plain Aquifer in Northern America (Erwin et al., 2005) or permafrost soil (Liebner et al., 2009). All these habitats share low average temperatures and likely never experience a high maximum temperature, which corresponds well with the temperature requirements of strain KoM1. Interestingly, only *pmoA* but no related 16S rRNA gene sequences showing high similarity to the ones of strain KoM1 were detected. The only exception was one 16S rRNA gene sequence from a high-mountain lake epilithic biofilm, which was 97% identical to the 16S rRNA gene sequence of strain KoM1. All other 16S rRNA gene sequences in the database share less than 93% identity with the one of strain KoM1. This could be attributed to a lack of 16S rRNA gene sequences of MOB due to the low abundance of MOB compared to other bacteria in natural environments, which renders the detection by general bacterial primers implausible. Additionally, commonly used MOB-specific 16S rRNA gene primers (McDonald et al., 2008) show mismatches to the 16S

rRNA gene sequence of strain KoM1 and *M. difficile*, which might prevent the detection of these MOB at the 16S rRNA gene level even in investigations which target MOB with specific primers. Thus, isolation of novel strains is not only important to understand their physiology, but also helps to obtain novel genomic information which can lead to improved tools to detect unknown MOB *in-situ*. Further investigations of strain KoM1 as a representative of a presumably cold-adapted genus of methanotrophs might lead to a better understanding of those methanotrophs, their ecology, and the mitigation of methane emissions at environments like Lake Constance sediments.

Anaerobic Methane Oxidation

Despite the intensive research on methane cycling in Lake Constance in the last decades, anaerobic oxidation of methane remained cryptic. High-resolution methane and oxygen profiles indicated a zone of anaerobic methane oxidation, and nitrate was proposed as feasible electron acceptor (Rahalkar et al., 2009). Sulfate has also been reported to act as electron acceptor for anaerobic methane oxidation in freshwater systems based on indirect evidence like gradient measurements and isotopic signatures (Eller et al., 2005b; Schubert et al., 2011). However, no incubation experiments showing net AOM and no clear evidence for the involvement of known ANME archaea in these habitats have been reported. Schubert et al. stated that the isotopic change in methane could also be caused by acetoclastic methanogenesis, but exclude this possibility because sulfate reducers outcompete methanogens when sufficient sulfate is present (Martens and Berner, 1974). However, methane production was reported for batch incubations of Lago di Cadagno sediment (Wagener et al., 1990) and coexistence of methanogens and sulfate reducers was reported at high sulfate concentrations (Dar et al., 2008) and even in marine systems (Gunnarsson and Rönnow, 1982). Despite the fact that sulfate concentrations in Lake Plußsee and especially in Lago di Cadagno are comparably high for freshwater lakes (1-2 mM), the energy gain of sulfate dependent AOM is very low ($\Delta G°' = -21$ kJ/mol). Given the minimum required energy change of $\Delta G°' = -20$ kJ mol^{-1} to form ATP (Schink and Stams, 2001) we doubt that *in-situ* conditions allow to fuel ANME-SRB consortia at the maximum methane partial pressures possible at shallow water depths. Thus, AOM in freshwater habitats is presumably coupled to electron acceptors of higher redox potentials, like iron(III), manganese(IV), or nitrate and nitrite. Methane oxidation coupled to iron or manganese reduction has not been reported for freshwater habitats, but methane oxidation coupled to denitrification was described for enrichments originating from nutrient-rich freshwater sediments (Raghoebarsing et al., 2006;

Ettwig et al., 2009; Hu et al., 2009). However, denitrifying methane oxidation has not been reported without prior enrichment or under oligotrophic conditions. Within the scope of this thesis, denitrifying AOM was detected for the first time in sediments of an oligotrophic freshwater lake. Nitrate was proven to stimulate methane oxidation in sediment slurries, and NC10 bacteria of subgroup A, which carry out this process, were shown to be present in Lake Constance (chapter 5). However, the electron balance of methane oxidation coupled to denitrification does not allow to oxidize methane with nitrate as the only electron acceptor if molecular oxygen is an intermediate (Fig. 6.1). Thus, if denitrifying methane oxidation is the only energy metabolism in the respective bacteria, this process has to be supplied not solely with nitrate but with nitrite, and the remaining reducing equivalents could be used by a terminal oxidase to reduce the remaining oxygen. This would result in the in the stoichiometry of nitrite dependent AOM observed previously (Eqn. 1.2). However, pure culture experiments are needed to resolve the metabolic flexibility of the NC10 bacteria and to gain further insights into this process.

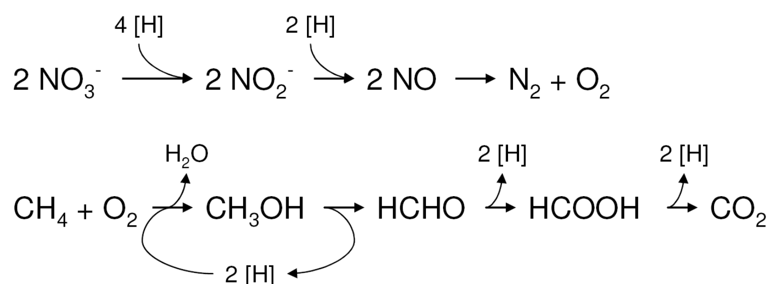

Fig. 6.1. Reaction scheme of methane oxidation coupled to denitrification with oxygen as intermediate.

Provided that the NC10 bacteria are obligate methanotrophs and do not use substrates besides methane as electron donor, and provided that molecular oxygen is the intermediate for methane activation, these bacteria have to be supplied, at least partly, with nitrite in their natural environments. This also happened in our slurry experiments, where nitrite formation from nitrate could be measured. Nitrite can be formed *in-situ* by incomplete nitrification from ammonia under oxic conditions or by incomplete denitrification from nitrate under anoxic conditions (Kuenen and Robertson, 1988). In freshwater sediments, these two processes occur in close proximity to each other, due to the steep gradients of oxygen and nitrate enabling nitrite accumulation to concentrations of several µM (Stief et al., 2002). Additionally, because the final product of the reductive branch of the energy conservation pathway is needed to

activate the oxidative part, NC10 bacteria might need a constant supply of methane and nitrite or a good regulation system to prevent running into a depletion of redox equivalents. Thus, NC10 bacteria likely depend on both, the presence of stable gradient systems and nitrite formation by other bacteria. This could explain (1) why they were successfully cultured only in continuous cultures with constant supply of methane and nitrite, (2) our finding that the methanotrophic activity in batch incubations could not be reactivated by refeeding with nitrate and (3) the occurrence of NC10 group A bacteria in well stratified profundal sediment, but not in the wave-exposed littoral zone. However, more studies on the environmental parameters associated with the occurrence of denitrifying methane oxidation and, thus, the occurrence of NC10 bacteria, are needed. As the molecular tools to investigate the ecology and distribution of NC10 bacteria are available (Ettwig et al., 2009; chapter 5), these studies will certainly follow soon and might provide insights into their importance in mitigating greenhouse gas emissions.

Summary

In this study, methane oxidation in Lake Constance and the respective microorganisms were investigated using a broad range of methods. High-resolution methane and oxygen profiles combined with molecular techniques (FISH, qPCR) were used to identify the distribution of methane oxidation activity and methanotrophic bacteria in the sediment column. Methane-oxidizing bacteria (MOB) were found to be evenly distributed in the sediment column of profundal sediments, and methane oxidation took place only in the narrow zone where methane and oxygen met at low concentrations. In littoral sediments, however, a maximum of MOB abundance and methane oxidation activity was detected at 1 to 4 cm sediment depth where oxygen was absent. The latter finding indicated either substantial mixing of the upper sediment layers of littoral sediments *in-situ* or a zone of anaerobic methane oxidation.

Because of these indications for AOM in littoral sediments, tracer experiments were performed to test this hypothesis. These experiments revealed the potential for nitrate-dependent AOM in sediments of oligotrophic Lake Constance, but could not detect sulfate-dependent AOM. Furthermore, NC10 bacteria related to candidatus "*Methylomirabilis oxyfera*", which catalyses AOM coupled to denitrification, were detected using 16S rRNA gene clone libraries. For the first time, *pmoA* gene sequences related to those of *M. oxyfera* were retrieved from environmental samples and support the presence of NC10 bacteria able to oxidize methane with nitrate or nitrite as electron acceptor in these sediments. AOM coupled to denitrification appears to depend on stable gradient systems and was found mainly in profundal but not in mixed littoral sediments.

To gain further insights into the methane-oxidizing communities from Lake Constance sediments opposing gradients of methane and oxygen were used to cultivate MOB, thus mimicking the low substrate concentrations they experience *in-situ*. This approach finally allowed the isolation of a novel oxygen-sensitive methanotroph (strain KoM1) from profundal sediment. Strain KoM1 represents a new species and genus and is distantly related to *Methylosoma*, which was previously isolated from Lake Constance.

In addition to the distinct environments of littoral and profundal sediments, active methane seeps, called pockmarks, were investigated in Eastern Lake Constance. High methane oxidation potentials as well as high MOB abundance designated littoral pockmark sediments as "hot spots" of methane oxidation in an otherwise oligotrophic environment. This was also reflected by the distinctly different MOB community at these sites compared to those in the surrounding sediments.

Altogether, this study demonstrates that methane oxidation occurs mainly at low oxygen concentrations *in-situ* and could even play a role under anoxic conditions. In line with previous studies, it was shown that MOB communities differ significantly even in different parts of the same lake, thus encouraging further research on the adaptations of MOB strains to different ecological niches.

Zusammenfassung

Im Rahmen dieser Arbeit wurden die Methanoxidation im Bodensee und die dafür verantwortlichen Mikroorganismen mit einer breite Palette von Methoden untersucht. Hochauflösende Methan- und Sauerstoffprofile wurden mit molekularen Methoden (FISH, qPCR) kombiniert, um die Verteilung der Methanoxidationsaktivität sowie der methanotrophen Bakterien in Abhängigkeit von der Sedimenttiefe zu untersuchen. MOB waren im Profundalsediment gleichmäßig über die Tiefe verteilt, und Methanoxidation fand nur in einer dünnen Schicht statt, in der sich sowohl Methan als auch Sauerstoff in geringen Konzentrationen trafen. Im Litoralsediment wurde jedoch ein Maximum der Methanotrophenanzahl und der Methanoxidationsaktivität in 1 bis 4 cm Sedimenttiefe festgestellt wo kein Sauerstoff mehr vorhanden war. Letzteres weist entweder auf beträchtliche Durchmischung der oberen litoralen Sedimentschichten *in-situ* oder auf anaerobe Methanoxidation hin.

Auf Grund dieser Anzeichen wurden Tracerexperimente durchgeführt, um die Hypothese der anaeroben Methanoxidation zu überprüfen. Diese Experimenten zeigten die Möglichkeit einer nitratabhängigen Methanoxidation im Sediment des oligotrophen Bodensees auf; es konnte jedoch keine sulfatabhängige Methanoxidation nachgewiesen werden. Außerdem wurden mit 16S-rRNA-Gen Klonbibliotheken Bakterien nachgewiesen, die mit Canditatus *„Methylomirabilis oxyfera“* verwandt sind. Dieses Bakterium katalysiert die an Denitrifikation gekoppelte Methanoxidation. Desweiteren wurden zum ersten mal *pmoA*-Gensequenzen aus Umweltproben erhalten, die ähnlich zu der von *M. oxyfera* sind. Dies ist ein zusätzlicher Hinweis darauf, dass NC10 Bakterien in diesen Sedimenten vorhanden sind, die Methan mit Nitrat oder Nitrit als Elektronenakzeptor oxidieren können. AOM, die an Denitrifikation gekoppelt ist, scheint auf stabile Gradientensysteme angewiesen zu sein und wurde hauptsächlich in Profundal-, jedoch nicht in durchmischten Litoralsedimenten nachgewiesen.

Um weitere Einblicke in die Methan oxidierende Bakteriengemeinschaft des Bodensees zu erhalten, wurden zur Kultivierung von neuartigen MOB gegenläufige Methan- und Sauerstoffgradienten genutzt. Dadurch konnten die geringen Substratkonzentrationen nachgestellt werden, die MOB *in-situ* erfahren. Diese Herangehensweise machte letztendlich die Isolierung eines neuartigen und sauerstoffempfindlichen Methanotrophen (Stamm KoM1) aus Profundalsediment möglich. Stamm KoM1 ist entfernt verwandt mit *Methylosoma,* einem früheren Isolat aus dem Bodensee, und repräsentiert eine neue Gattung und Art.

Zusätzlich zu den unterschiedlichen Habitaten des litoralen und profundalen Sediments wurden aktive Methanaustrittsstellen, sogenannte Pockmarks, im östlichen Teil des Bodensees untersucht. Hohe potentielle Methanoxidationsaktivitäten sowie eine hohe MOB Abundanz zeichneten die Sedimente der litoralen Pockmarks als „Hot Spots“ der Methanoxidation in einer sonst oligotrophen Umgebung aus. Dies spiegelte sich auch in den merklich unterschiedlichen MOB Gemeinschaften an diesen Stellen, verglichen mit dem umliegenden Sediment, wieder.

Zusammenfassend zeigt diese Arbeit, dass Methanoxidation *in-situ* hauptsächlich bei geringen Sauerstoffkonzentrationen stattfindet und sogar unter anaeroben Bedingungen eine Rolle spielen kann. In Übereinstimmung mit früheren Arbeiten wurde gezeigt, dass sich MOB-Gemeinschaften sogar in verschiedenen Teilen desselben Sees maßgeblich voneinander unterscheiden, was zur weiteren Erforschung der Anpassungen von MOB-Stämmen an unterschiedliche ökologische Nischen auffordert.

Record of Achievement

Unless stated otherwise all experiments in this work were performed and analyzed by myself or under my supervision. All experiments were developed by my supervisor Prof. Bernhard Schink and myself and planned by myself. Drafts for my first author manuscripts were written by myself and corrected by Prof. Dr. Bernhard Schink.

To the publication described in chapter 2, I contributed the experiments and parts of the manuscript dealing with fluorescence *in-situ* hybridization. The parts including gradient measurements and flux calculations were done by Ingeborg Bussmann, and all PCR based parts were performed and written by Monali Rahalkar.

The clone library and T-RFLP data on MOB communities of methane seep sediments (chapter 3) were generated by Susanne Wörner during her work for her bachelor thesis and were supervised, complemented and re-analyzed by myself.

Abgrenzung der Eigenleistung

Falls nicht anders angegeben wurden alle Experimente zu dieser Arbeit von mir selbst oder unter meiner Betreuung durchgeführt und ausgewertet. Das Konzept für die Arbeiten wurde von meinem Betreuer Prof. Bernhard Schink oder mir selbst entworfen und die Experimente wurden von mir geplant. Die Entwürfe der Manuskripte, bei denen ich Erstautor bin, wurden von mir geschrieben und von Prof. Dr. Bernhard Schink gegengelesen und korrigiert.

Zu der in Kapitel 2 vorgestellten Arbeit habe ich die Daten zur Fluoreszenz *in-situ* Hybridisierung beigesteuert und die entsprechenden Teile des Manuskripts geschrieben. Die Gradientenmessungen und Fluxrechnungen wurden von I. Bussmann, die PCR basierten Teile von Monali Rahalkar durchgeführt und im Manuskript beschrieben.

Die Klonbibliothek und die T-RFLP Daten über die Gemeinschaft der MOB an Methanaustrittsstellen (Kapitel 3) wurden von Susanne Wörner im Rahmen ihrer Bachelorarbeit beigetragen und von mir betreut, ergänzt und abschließend ausgewertet.

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