Methane oxidizing bacteria at the oxic-anoxic interface: taxon-specific activity and resilience

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“Wer noch nie einen Fehler gemacht hat,

hat sich noch nie an etwas Neuem versucht”

-Albert Einstein, Physiker

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Summary

The methanotrophic bacteria are the only known biological sink for the third most important greenhouse gas methane, performing an important ecosystem function influencing global climate change. In the soil surface layer of water logged soils aerobic methanotrophs thrive at the oxic-anoxic interface attenuating the amount of potentially emitted methane. The highly diverse methanotroph community is shaping the interface characterized by overlapping methane and oxygen gradients by their own activity. However, how the different methanotrophs physically share their microhabitat at the interface is unknown. Here we highly resolved the vertical distribution and activity of methanotrophs at the interface. To assess the structure of the present and active methanotroph community the particulate methane monooxygenase gene *pmoA* was used as a functional and phylogenetic marker. For quantification of *pmoA* genes and transcripts a new method, the competitive-(reverse transcriptase)-t-RFLP was established. Only a subset of the methanotroph community was shown to be active and the major activity was confined to a small zone around the interface. The predominantly active methanotrophs were affiliated to *Methylobacter* and no transcripts of type II methanotrophs (*Methylosinus, Methylocystis*) were found. Hence, different species within this guild exploited different niches in the same microenvironment.

Furthermore, the methanotrophs resistance to desciccation (up to 18 years) was tested. Longer-lasting droughts reduced methanotrophic diversity, and adversely affected methane oxidation upon rewetting. Type I methanotrophs showed relatively higher *pmoA* gene expression, while type II were more resistant to desiccation.

Finally, we showed that high methane source strength aids the ability of the methanotroph community to recover from the effect of a strong disturbance. However, recovery of the type II community was independent of *pmoA* gene expression and source strength indicating facultative growth.

Overall, the methanotroph community in the soil surface layer is highly diverse. This work contributed to understand the high and apparently redundant

diversity by unravelling niche differentiation at the fine spatial resolution and evaluating the effect of different source strength. Moreover, the range of alternative substrates used for growth seems to be another important factor in the environment.

Zusammenfassung

Die Methanotrophen Bakterien sind die einzige bekannte biologische Senke für das drittwichtigste Treibhausgas, Methan. Damit erfüllen sie eine wichtige ökologische Funktion, die direkten Einfluss auf den globalen Klimawandel hat. Durch ihr Wachstum an der oxisch-anoxischen Grenzschicht in der Oberfläche von wassergesättigten Böden reduzieren aerobe Methanotrophe die Menge an potentiell emittiertem Methan. Außerdem führt ihre Aktivität zur Entstehung der durch überlappende Methan- und Sauerstoff-Gradienten gekennzeichneten Grenzschicht.

Wie die verschiedenen Methanotrophen in dem Mikrohabitat an der oxisch-anoxischen Grenzschicht koexistieren, ist unbekannt. Zur Aufklärung haben wir hier sehr hochaufgelöst die vertikale Verteilung und Aktivität von Methanotrophen an der Grenzschicht untersucht. Die Zusammensetzung der gegenwärtigen und der aktiven methanotrophen Gemeinschaft wurde mittels eines der für die Membran-gebundene Methan-Monooxygenase kodierenden Gene (*pmoA*) aufgelöst, welches als funktioneller und phylogenetischer Marker diente. Zur Quantifizierung der *pmoA* Gen- und Transkript-Anzahl wurde eine neue Methode, die kompetitive-(Reverse Transkriptase)-t-RFLP, etabliert. Nur für einen Teil der methanotrophen Gemeischaft konnte Aktivität nachgewiesen werden. Diese ist jedoch auf eine kleine Zone um die Grenzfläche herum beschränkt. Die aktive Gemeinschaft wurde von *Methylobacter* verwandten Typ I Methanotrophen dominiert, während keine Typ II-spezifischen Transkripte (*Methylosinus*, *Methylocystis*) gefunden wurden. Obwohl also verschiedene Arten dieser funktionellen Gruppe im selben Mikrohabitat vorkommen besetzen sie doch offensichtlich verschiedene Nischen.

Darüber hinaus wurde die Beständigkeit der Methanotrophen gegen Austrocknung getestet. Hierbei führten länger anhaltende Trockenzeiten (bis 18 Jahre) zu einer verringerten Diversität der methanotrophen Gemeinschaft und minderten die Methanoxidationsraten nach erneuter Vernässung des Bodens. Die Typ I Methanotrophen zeigten eine relativ hohe *pmoA* Genexpression, während Typ II resistenter gegen Austrocknung waren.

Schließlich haben wir gezeigt, dass ein hoher Methan-Fluss die Fähigkeit der methanotrophen Gemeinschaft verbessert, sich von den Auswirkungen einer Störung zu erholen. Die Reaktion der Typ II Methanotrophen war allerdings unabhängig von *pmoA* Genexpression und dem Methan-Fluss, was auf fakultatives Wachstum hindeutet.

Insgesamt ist die methanotrophe Gemeinschaft in der Oberflächenschicht des Bodens sehr divers. Diese Arbeit hat durch eine räumlich hoch aufgelöste Analyse der Einnischung und Beurteilung der Wirkung von verschieden starken Methan-Flüssen dazu beigetragen die Bedeutung der hohen scheinbar redundanten Vielfalt zu verstehen. Die Verwendung alternativen Substrate für das Wachstum scheint darüber hinaus ein weiterer wichtiger Faktor zur bevölkerung neuer Nischen zu sein.

1 Introduction

1.1 Atmospheric methane cycle

The importance of methane for the greenhouse effect is based on its high global warming potential which is ~33 times higher compared to CO_2 (Shindell et al., 2009). Therefore methane, beside its low abundance is the third most important greenhouse gas after CO_2 and water vapour. The mixing ratio changed from a pre-industrial value of 715 ppb in 1750 to a concentration of 1,774 ppb in 2005, resulting in the second largest contribution to global warming after carbon dioxide (Intergovernmental Panel on Climate Change, 2007). Ice core studies have indicated that consistently lower concentrations were present in the atmosphere over the last 650,000 years, varying between 400 ppb and 770 ppb (Spahni et al., 2005). Although the total concentration more than doubled during the industrial era, the annual growth rates decreased substantially from about 1% to nearly zero since 1999 (Blake and Rowland, 1988; Dlugokencky et al., 1998; Dlugokencky et al., 2003). Several controversial theories have been proposed to explain the decreased growth rates and the contribution of human activities (Bekki et al., 1994; Bousquet et al., 2006; Hansen et al., 2000; Lelieveld et al., 1998; Lowe et al., 1997; Worthy et al., 2009). Nevertheless, a study from Rigby and colleagues (2008) indicates a renewed growth of methane in the atmosphere. Wetlands and rice fields are the most important biological methane sources with 23% and 21% share of the global methane budget (Chen and Prinn, 2005). Additional sources of atmospheric methane are ruminants, termites, oceans, freshwater sediments, landfills, tank bromeliads and fossil methane released during fossil fuel extraction (Chen and Prinn, 2005; Cicerone and Oremland, 1988; Martinson et al., 2010; Wuebbles and Hayhoe, 2002). Biogenic methane is produced by methanogenic archaea under anaerobic conditions from acetate or carbon dioxide and molecular hydrogen (Conrad and Frenzel, 2002; Thauer et al., 2008). Overall 75% of atmospheric methane are of microbial origin (Chen and Prinn, 2005), which is also

why studies on the biological sources and sinks of methane are of large ecological importance.

1.2 Methanotrophs

Methylotrophs are a diverse group of prokaryotic microorganisms capable of reducing single carbon compounds for growth (Lidstrom, 1992). The methanotrophs are a subgroup of the methylotrophs and are generally characterized by their ability to use methane as their sole carbon and energy source. They were thought to be restricted to methane and methanol as substrates; however, at least for some strains this was already shown not to be the case (see below). Methanotrophic bacteria are found in three phyla; Proteobacteria, Verrucomicrobia and NC10. The traditionally classified methanotrophs belong to the phylum Proteobacteria. More recently methanotrophs in the phylum Verrucomicrobia have been discovered but they seem to be restricted to extreme environments (Dunfield et al., 2007; Islam et al., 2008; Pol et al., 2007). The novel phylum, NC10 represents bacteria capable of aerobic methane oxidation coupled to denitrification under anoxic conditions (Ettwig et al., 2010). Moreover, ammonia oxidizers were also shown to be able to convert methane to methanol by an enzyme homologous to the methane monooxygenase of methanotrophs. It seems, however, that they cannot grow using this process (Hyman and Wood, 1983; Jones and Morita, 1983).

Aerobic methanotrophs

Based on phylogenetic, morphological and physiological characteristics the proteobacterial methanotrophs are divided into two groups, type I and type II (Bowman, 2000; Trotsenko and Murrell, 2008; Whittenbury et al., 1975). Type I methanotrophs belong to the γ-Proteobacteria with the family of *Methylococaceae* containing 15 different genera (Table 1). Type I methanotrophs are further divided into type Ia (e.g. *Methylobacter*, *Methylomicrobium*, *Methylomonas* and *Methylosarcina*) and type Ib including the genera *Methylococcus*, *Methylocaldum* and *Methylogaea* described as type X in previous publications (Bowman, 2000; Geymonat et al., 2011; Hanson and Hanson, 1996). Type II methanotrophs (α-Proteobacteria) are

represented by the families of *Methylocystaceae* and *Beijerinckiaceae* with the genera *Methylocystis*, *Methylosinus* and *Methylocella*, *Methylocapsa*, *Methyloferula*, respectively (Dedysh et al., 2000; Dedysh et al., 2002; Horz et al., 2001; Vorobev et al., 2011; Table 1). An important feature to distinguish type I and type II methanotrophs is their carbon assimilation pathway. The type Ia methanotrophs assimilate formaldehyde using the Ribulose monophosphate pathway (RuMP), while type II methanotrophs use the serine cycle (Trotsenko and Murrell, 2008). However, the type Ib species possess not only enzymes catalyzing the metabolic reactions of the RuMP, but also enzymes of the serine pathway (Trotsenko and Murrell, 2008). Another characteristic for the classification of the methanotrophs is the arrangement of the membranes containing the particulate methane monooxygenase. In type I methanotrophs, the membranes form stacks of round membrane vesicles, whereas the membranes of type II methanotrophs extend parallel to the cell wall (Hanson and Hanson, 1996; Takeda, 1988; Table1). *Methylocapsa*; however, possesses a different intracytoplasmic membrane arrangement referred to as membrane type III (Dedysh et al., 2002; Table 1).

Recently methanotrophs were discovered in the Phylum of the Verrucomicrobia (Dunfield et al., 2007; Islam et al., 2008; Pol et al., 2007). These are the first known obligate methanotrophs, which do not belong to the Proteobacteria and form a new genus (*Methylacidiphilum*). However, these methanotrophs seem to be restricted to extreme environments growing at low pH (2-2.5) and high temperatures above 50 °C. Since methanotrophs have been detected in many terrestrial and aquatic habitats, they can be regarded as ubiquitous (Hanson and Hanson, 1996). Besides the rather extreme Verrucomicrobia species also some Proteobacteria (*Methylococcus*, *Methylocaldum* and *Methylothermus*) were reported to exhibit a thermotolerant or slightly thermophilic lifestyle growing up to temperatures around 70°C (Bodrossy et al., 1999; Tsubota et al., 2005). On the other hand there are also some methanotrophs (*Methylobacter* and *Methylocella*) adapted to cold environments with a temperature range of 0-30°C (Berestovskaya et al., 2002; Trotsenko and Khmelenina, 2005; Wartiainen et al., 2006). Some *Methylomicrobium*

species that can grow up to a NaCl concentration of 1.1-1.5 M (Khmelenina et al., 1997) form together with *Methylohalobius crimeensis* the group of halotolerant methanotrophs (Kalyuzhnaya et al., 2008). With a growth optimum around 1-1.5 M NaCl and tolerating up to 2.5 M NaCl *Methylohalobius crimeensis* is even halophilic (Heyer et al., 2005). The acidophilic *Methylocella* and *Methylocapsa* species that were both isolated from acidic bogs can grow at a Ph range between 7.2 to 4.2 (Dedysh et al., 2000; Dedysh et al., 2002).

Until the isolation of *Methylocella palustris* by Dedysh and colleague (Dedysh et al., 2005) methanotrophs were thought to be obligate methylotrophs restricted to the utilization of methane, methanol and a narrow range of C1 compounds (e.g. Bowman, 2000). With the characterization of *Methylocella* Dedysh (2005) and colleague gave first proof for the existence of facultative methanotrophs able to utilize multi carbon compounds. *Crenothrix polyspora* a sheathed γ-Proteobacteria was identified to be another possible candidate for a facultative methanotroph (Stoecker et al., 2006). More recently pMMO-possessing methanotroph of the genus *Methylocapsa* as well as some *Methylocystis* species were demonstrated to be able to grow on acetate as sole substrate (Belova et al., 2011; Dunfield et al., 2010). The aforementioned examples of facultative lifestyle in methanotrophs indicate that broader substrate utilization might be more common in methanotrophs as previously thought.

Table 1: Phylogenetic, morphological and physiological characteristics of aerobic methanotrophs. Facultative and putative facultative methanotrophs are highlighted in bold. The pictures of intercytoplasmatic membrane arrangements were adopted from the following publications: A: Wartiainen et al., 2006; B: Dalton, 2005; C: Dedysh et al., 2002. PLFAs - Phospholipid fatty acids; RuMP pathway - Ribulose monophosphate pathway; p.d. - poorly developed. Adopted from Lüke, 2009

	γ-Proteobacteria (Type I)	α-Proteobacteria (Type II)		Verrucomicrobia
Family	*Methylococcaceae*	*Methylocystaceae*	*Beijerinckiaceae*	*Verrucomicrobiaceae*
Genera	*Methylomonas* *Methylobacter* *Methylomicrobium* *Methylosarcina* *Methylosphaera* *Methylosoma* *Methylomarinum* *Methylovulum* *Methylogaea* *Methylococcus* *Methylocaldum* *Methylothermus* *Methylohalobium* ***Crenothrix*** ***Clonothrix***	*Methylosinus* ***Methylocystis***	*Methylocapsa* ***Methylocella*** *Methyloferula*	*Methylacidiphilum*
Resting stages	*Azotobacter*-type cysts/none	Cysts/Exospores	Exospores/*Azotobacter* -type cysts	
Intracytoplasmic membranes	Type I (**A**)	Type II (**B**)	Type III (**C**)/p.d.	different
Major PLFAs	C16:1ω7c, C16:1ω8c, C16:0, C14:0	C18:1ω8c, C18:1ω7c, C18:2ω7c,12c	C18:1ω7c	C18:0, C16:0, aC15:0, C14:0,
Activity of				
sMMO	Yes/No	Yes/No	Yes/No	No
pMMO	Yes	Yes	Yes/No	Yes
Carbon Assimilation Pathway	RuMP pathway	Serine cycle	Serine cycle	Alternative serine cycle

Intracytoplasmic Membrane Arrangements

A — 0.2 µm

Methylobacter tundripaludum

B — 0.5 µm

Type II methanotroph

C — 0.5 µm

Methylocapsa acidiphila

The aerobic methanotrophs oxidize methane as shown in Figure 1 via the intermediates methanol, formaldehyde, and formate to carbon dioxide. The first step, the oxidation of methane to methanol, is catalysed by the key enzyme of this pathway, the methane monooxygenase (MMO). This enzyme occurs in two forms: the membrane bound or particulate (pMMO) and the cytosolic or soluble form (sMMO). The pMMO is found in almost all the methanotrophs, except *Methylocella* and *Methyloferula* (Dedysh et al., 2000; Vorobev et al., 2011). The sMMO is only found in some type II and type Ib methanotrophs, such as *Methylococcus capsulatus* and *Methylosinus* (Hanson and Hanson, 1996). In methanotrophs possessing both forms of the MMO gene expression of the pMMO and sMMO is regulated by media copper content; the iron containing sMMO is expressed when copper-to-biomass ratios in the cell are low (Murrell et al., 2000; Nielsen et al., 1996; Prior and Dalton, 1985; Stanley et al., 1983). The pMMO has a limited substrate spectrum ranging from methane and short-chained alkanes up to alkenes five carbons in length. In contrast the sMMO catalyses the oxidation of a wide spectrum of substrates including a variety of alkanes, alkenes and aromatics (Trotsenko and Murrell, 2008).

In different upland soils methane oxidation kinetics measured were showing a much higher apparent affinity for methane than observed for pure cultures of methanotrophs or wetland soils (Bender and Conrad, 1992; Bender and Conrad, 1993; Benstead and King, 1997; Gulledge et al., 1998). These upland soils act as a net sink of atmospheric methane; the oxidation of trace concentrations of methane is attributed to high affinity methanotrophs (Bender and Conrad, 1992). The organisms and mechanisms involved high affinity methane oxidation are still unknown (Dunfield and Reay, 2007). However, some uncultured methanotrophs were found to be abundant and active in these soils and were suggested as promising candidates (Holmes et al., 1999; Knief et al., 2003). Recently, Baani and Liesack (2008) showed *Methylocystis* sp. strain SC2 to contain a second pMMO (pMMO2) that is constitutively expressed and enabled it to consume methane at atmospheric concentrations.

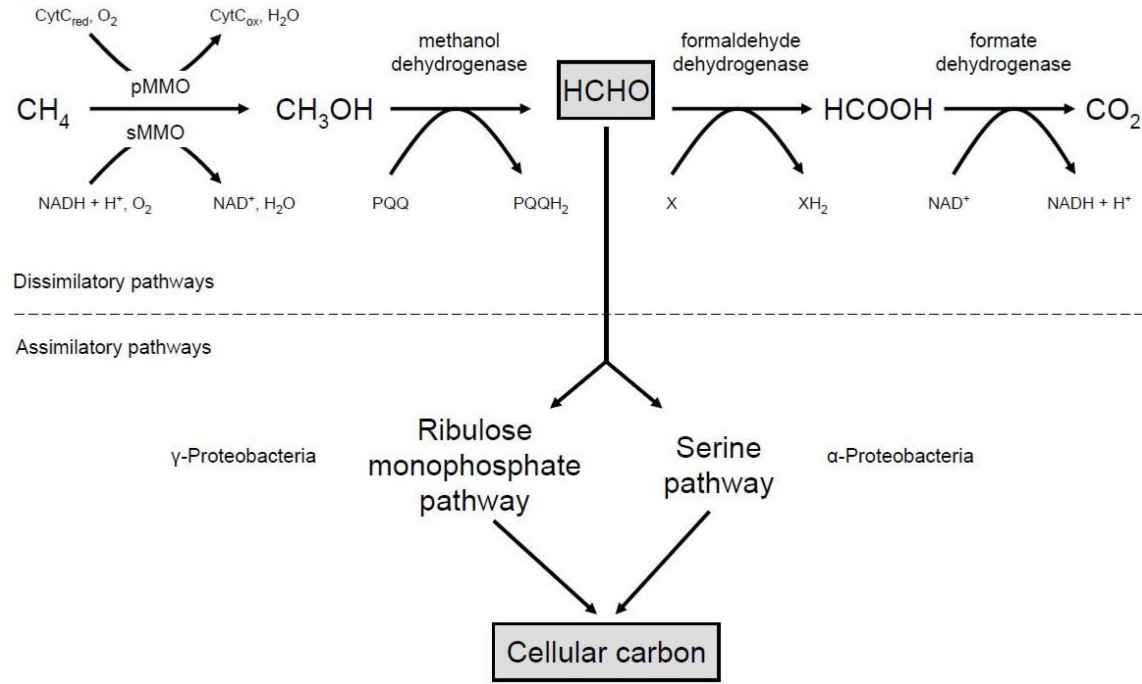

Figure 1: Assimilatory and dissimilatory methane oxidation pathways. Abbreviations: Cytc =Cytochrome c; PQQ = pyrroloquinoline quinine; X = NADP+ or cytochrome linked. Modified from Hanson and Hanson 1996; Lüke, 2009; Mancinelli, 1995. Adopted from Ho, 2010

Anaerobic methanotrophs

In contrast to the above introduced aerobic methane oxidation, yet another form, the anaerobic methane oxidation was postulated already almost 4 decades ago, when the first studies demonstrated sulfate-dependent methane oxidation in anoxic marine sediments (Barnes and Goldberg, 1976; Martens and Berner, 1974; Reeburgh, 1976). The mechanisms behind this remained enigmatic for a long time and are even today not fully deciphered. The current hypothesis was proposed by Hoehler et al. (1994) suggesting sulfate dependent methane oxidation to be performed by a consortium of sulfate reducing bacteria and methanogenic archaea. In this syntrophic association the sulfate reducing bacteria are believed to efficiently remove the H_2; maintaining sufficiently low levels of H_2 for the net reversal of methanogenesis to become thermodynamically feasible (Hoehler et al., 1994; Hoehler, 1996). Recently, Thauer (2011) demonstrated that all but one of the enzymes involved in the reduction of CO_2 with H_2 to methane in methanogenic archaea catalyze their back reactions at specific rates sufficiently high to be involved in anaerobic oxidation of methane with sulfate. So far three distinct groups of methanotrophic archaea (ANME-1, ANME-2 and

ANME-3) are described (Niemann et al., 2006; Orphan et al., 2002). All three are related to *Methanosarcinales* and *Methanomicrobiales*, two orders of methanogens with many cultivated representatives. However, even though there are many attempts to isolate them so far there are only enrichments (Jagersma et al., 2012; Zhang et al., 2011). Sulfate reducing bacteria, mostly *Desulfosarcina*/*Desulfococcus*, were found in close physical association with the methanogenic archaea (Knittel and Boetius, 2009). Recently, ANME-1 organisms were suggested to assimilate inorganic carbon while growing on methane and should be classified as methane-oxidizing chemoorganoautotrophs (Kellermann et al., 2012).

Theoretically methane oxidation can be coupled to many other alternative electron acceptors such as Fe^{3+}, Mn^{4+} and NO^{3-}. All of the above are also energetically more feasible than sulfate (Caldwell et al., 2008). However, it was only in recent years that evidence was found for those processes to occur in nature. Raghoebarsing and colleagues (2006) demonstrated that methane oxidation coupled to denitrification occurs in anoxic sediment by enrichment of a microbial consortium from canal sediment in the Netherlands. The bacterial part of the consortium *Candidatus* Methylomirabilis oxyfera was shown to be able to perform the anaerobic oxidation without the archaea belonging to the ANME-2 cluster (Ettwig et al., 2009). Recently, *Ca*. M. oxyfera was shown to produce its own supply of oxygen in an intra-aerobic metabolism by metabolizing nitrite via nitric oxide into oxygen and dinitrogen gas (Ettwig et al., 2010; Wu et al., 2011). Beal and colleagues (2009) demonstrated that microbial populations of marine-seep sediment oxidize CH4 in the presence of Fe(III) or Mn(IV). However, both occur at much slower rates than sulfate-dependent anaerobic methane oxidation, although they are substantially more energetically feasible. Besides the tremendous amount of research conducted the anaerobic methane oxidation still holds a lot of secrets. Besides the successful amplification of NC10 *pmoA* sequences using a specific primer set (Luesken et al., 2011), there is so far no indication for anaerobic methane oxidation in paddy soil.

1.3 The paddy soil surface as model system

About 60% of overall methane emitted originates from anthropogenic sources (Intergovernmental Panel on Climate Change, 2007); while in 1700 AD before industrial revolution only approximately 12% were of anthropogenic origin (Lassey et al., 2007). The main reason for the strong post-industrial increase in atmospheric methane concentration is the intensification of agriculture and animal husbandry (Wuebbles and Hayhoe, 2002). In particular we focus on rice as one of the most cultivated crop plants. Worldwide, approximately 160 million hectares (FAO, 2012) are cultivated with rice; of which a large proportion (75 %) is grown under permanently flooded conditions (Liesack et al., 2000). Methane emission of rice fields corresponds to approximately 20 % of the total annual methane emissions of 500-600 Tg yr^{-1} (Chen and Prinn, 2005; Lelieveld et al., 1998; Wang et al., 2004). The importance of methane emission in rice agriculture becomes even more apparent if the projected increase in rice production for this century from current 700 Tg (FAO, 2012) to 1000 Tg is taken into account (Neue, 1997). In rice fields methane is produced as terminal product in the anaerobic degradation of organic matter in anoxic parts of the soil. The emission from rice fields is the net balance of methane production and aerobic methane oxidation (Conrad and Rothfuss, 1991). Three basically different ways of methane transport are known: plant vascular transport, ebullition, and diffusion through soil (Conrad, 1996). At oxic-anoxic interfaces as in the soil surface layer or on rice roots methane is oxidized by the methanotrophic bacteria to carbon dioxide. In rice fields 10 % to 50 % of the total methane produced are internally re-oxidized (Conrad and Frenzel, 2002; Frenzel, 2000; Groot et al., 2003). Up to 80 % of the methane potentially emitted by diffusion is oxidized by the methanotrophs (Conrad and Rothfuss, 1991). As the production of rice will increase due to continued population growth, the importance of rice fields as methane source will follow. Furthermore, insights gained on the ecology of methanotrophs in rice fields can be transferred to methanotroph communities in other waterlogged soils. This tremendous ecological importance and its role as a model system make the study of methanotrophic communities in rice fields so important.

1.4 *pmoA* as marker gene

The 16S rRNA gene is by far the most commonly used phylogenetic marker gene to study microbial ecology. However, when functional guilds of bacteria are studied, genes coding for enzymes catalyzing specific key processes are often an adequate alternative to the 16S rRNA gene. The enzyme unique to methanotrophs is the methane monooxygenase. The *pmoA* gene encodes a subunit of the pMMO (see 1.2) and is the most frequently targeted gene for methanotroph diversity studies (Dumont and Murrell, 2005; McDonald et al., 2008). Many of its characteristics make the *pmoA* gene a suitable phylogenetic marker for methanotrophs in environmental samples. First, the *pmoA* gene is present in almost all methanotrophic bacteria (see 1.2). Second, the methanotroph phylogeny based on the *pmoA* gene is very similar to the 16S rRNA based phylogeny (Kolb, 2003). Finally, *pmoA* gene expression can be used as a proxy for methanotrophic activity (Bodrossy et al., 2006; Bürgmann et al., 2001; Chen et al., 2007; Chen et al., 2008).

The A189f and mb661r *pmoA* specific primers used in the following work are known to discriminate against sequences of the *amoA* gene (Costello and Lidstrom, 1999; Holmes et al., 1995); a *pmoA* homologous encoding the α-subunit of ammonia monooxygenase. Furthermore, A189f and mb661r detect the largest methanotroph diversity of all common *pmoA* specific primers (Bourne et al., 2001; McDonald et al., 2008).

1.5 Aims of this study

The paddy soil is one of the most extensively studied methanotrophic environments. The large scale spatial structure of the community and the effect of different disturbances as well as methane concentrations on the resilience of methanotrophs have already been studied. However, only little is known on the community structure and localisation of methanotrophs in the oxic-anoxic interface and the effect of the energy flow on the resilience of the community. Therefore, here we use new experimental tools (e.g. microcosm setup and cryosection sub-sampling) to focus on the very interface itself and employ new molecular tools (e.g. competitive t-RFLP and next generation sequencing) to address the following questions:

Chapter 2: One millimetre makes the difference: high-resolution analysis of methanotrophs and their specific activity at the oxic–anoxic interface in a flooded paddy soil

Here we assessed the diversity and activity of the methanotrophs in more detail to gain deeper insight in the community composition and activity of the methanotrophs at the very oxic-anoxic interface. So far over 30 species equivalent operational taxonomical units (OTUs) were found in Paddy soil. *How do these OTUs physically share their microhabitat?*

Chapter 3: Recovery of paddy soil methanotrophs from drought

Desiccation in rice paddies is a regular event during the agricultural cycle; affecting the methanotrophic community and activity. To persist during times of unfavourable environmental conditions methanotrophs form different resting stages. *But how resistant are those resting stages to extended drought periods up to 18 years? And how do they recover from drought upon re-wetting?*

Chapter 4: Effect of energy flow on the susceptibility of aerobic methanotrophic communities to disturbance

Compared to the methanotroph communities in the high methane environments the high affinity methanotrophs in upland soil seem to be much more vulnerable to disturbances. A major difference between those two environments is the methane source strength. *Do the source strength and therefore, the energy flow supplied to a community influence the ability of the community to recover from a simulated die-off? And do the different energy flows favour different methanotrophs?*

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2 One millimetre makes the difference: high-resolution analysis of methane-oxidizing bacteria and their specific activity at the oxic–anoxic interface in a flooded paddy soil

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2.1 Abstract

Aerobic methane-oxidizing bacteria (MOB) use a restricted substrate range, yet >30 species-equivalent operational taxonomical units (OTUs) are found in one paddy soil. How these OTUs physically share their microhabitat is unknown. Here we highly resolved the vertical distribution of MOB and their activity. Using microcosms and cryosectioning, we sub-sampled the top 3 mm of a water-saturated soil at near *in situ* conditions in 100-µm steps. We assessed the community structure and activity using the particulate methane monooxygenase gene *pmoA* as a functional and phylogenetic marker by terminal restriction fragment length polymorphism (t-RFLP), a *pmoA*-specific diagnostic microarray, and cloning and sequencing. *pmoA* genes and transcripts were quantified using competitive (RT)-PCR combined with t-RFLP. Only a subset of the methanotroph community was active. Oxygen microprofiles showed that 89 % of total respiration was confined to a 0.67-mm-thick zone immediately above the oxic–anoxic interface, most probably driven by methane oxidation. In this zone, a *Methylobacter*-affiliated OTU was highly active with up to 18 *pmoA* transcripts per cell and seemed to be adapted to oxygen and methane concentrations in the micromolar range. Analysis of transcripts with a *pmoA*-specific microarray found a *Methylosarcina*-affiliated OTU associated with the surface zone. High oxygen but only nanomolar methane concentrations at the surface suggested an adaptation of this

OTU to oligotrophic conditions. No transcripts of type II methanotrophs (*Methylosinus, Methylocystis*) were found, which indicated that this group was represented by resting stages only. Hence, different OTUs within a single guild shared the same microenvironment and exploited different niches.

2.2 Introduction

Methane is, next to water vapour and carbon dioxide, the most important greenhouse gas (Intergovernmental Panel on Climate Change 2007), with natural wetlands and irrigated rice fields together emitting about one-third of the total (Conrad 2009). Their contribution would be even higher without the activity of aerobic methane-oxidizing bacteria (MOB), which act as a biofilter, mitigating emissions to the atmosphere (Reeburgh et al 1993). MOB use methane as the sole source of carbon and energy, provided oxygen is available (Trotsenko and Murrell 2008). Owing to this dual dependency, they thrive at oxic–anoxic interfaces, where both substrates are supplied (Brune et al 2000). In flooded soils and sediments, these interfaces are located at the soil surface and in the rhizosphere of macrophytes when present (Bodelier et al 2006, Bosse and Frenzel 1997). Rhizospheric MOB in both paddy fields and natural wetlands have been often studied (Calhoun and King 1997, Eller and Frenzel 2001, Shrestha et al 2008, Sorrell et al 2002, Vishwakarma et al 2009), but work at the soil surface has been mainly focused on process measurements (Bosse et al 1993, Conrad and Rothfuss 1991, Frenzel et al 1992). The soil surface is characterized by sharp counter-gradients of oxidized and reduced species. Where these gradients overlap, ≥ 90% of potentially emitted methane is oxidized (Conrad and Rothfuss 1991, Frenzel et al 1990).

MOB can be divided into two major groups, type I and type II, being equivalent to the families *Methylococcaceae* (γ-Proteobacteria), and *Methylocystaceae* and *Bejerinckiaceae* (α-Proteobacteria), respectively. The key enzyme of all MOB is methane monooxygenase (MMO), and the overwhelming majority of cultivated MOB possess a membrane-bound MMO (particulate MMO, pMMO). Only the genera *Methylocella* and *Methyloferula* lack this enzyme and instead have a soluble MMO (sMMO; Dedysh et al 2000, Dedysh 2009, Vorobev et al 2011). The *pmoA* gene, which

encodes the β-subunit of pMMO, is an excellent functional marker for studying MOB in most environments (Dumont and Murrell 2005, McDonald and Murrell 1997, McDonald et al 2008). Its phylogeny reflects very well that of the 16S rRNA gene (Degelmann et al 2010, Kolb et al 2003). Type I MOB can be further divided into type Ia (e.g. *Methylomonas*, *Methylobacter*, *Methylosarcina* and *Methylomicrobium*), and type Ib (e.g. *Methylococcus* and *Methylocaldum*). Recently, MOB belonging to the phylum Verrucomicrobia have been isolated, but these seem to be restricted to extreme environments (Dunfield et al 2007, Op den Camp et al 2009, Pol et al 2007).

Sanger-and pyro-sequencing have provided a large database of high-quality *pmoA* sequences (Lüke et al 2010, Lüke and Frenzel 2011). Based on *pmoA* phylogeny, type II MOB form a coherent cluster well represented by cultivated strains. Many distinct groups lacking cultivated representatives have been allocated to type I MOB. Numerous sequences are located at an intermediate position between the *pmoA* gene of MOB and the *amoA* gene of ammonia oxidizers. The substrate of the enzymes encoded by these sequences remains uncertain, with the exception of a few proven methane (Dunfield et al 2007, Stoecker et al 2006) and alkane oxidizers (Coleman et al 2012, Sayavedra-Soto et al 2011).

More than 30 operational taxonomical units (OTUs) corresponding to the species level have been found in a single paddy soil (Lüke et al 2010). This raises the question whether and how their niches are separated, and whether all these individual OTUs really contribute to overall methane oxidation. However, not all of these 30 OTUs need to be active at the same time as methanotrophs form resting stages (Whittenbury et al 1970a, Whittenbury et al 1970b). Indeed, the development and activity of methanotroph communities has been suggested to vary depending on methane flow (Krause et al 2012), nitrogen supply (Bodelier et al 2000a, Bodelier et al 2000b, Graham et al 1993, Noll et al 2008, Rudd et al 1976), disturbance (Ho et al 2011) and grazing (Murase and Frenzel 2008). At the macroscale, MOB community structure within a paddy field varies randomly, probably as ploughing prevents the development of explicit spatial patterns (Krause et al 2009). Rice roots select for specific communities, thereby favouring the growth of certain OTUs (Lüke et al 2011).

Another aspect of spatial organization, however, has not yet been addressed — the community structure at the microscale. We focused on the soil surface and hypothesized that activity would be highest right at the oxic–anoxic interface, which would potentially separate MOB according to substrate availability, e.g. high methane/low oxygen in deeper layers and low methane/high oxygen in shallower layers.

The study of gradient organisms requires a physical model that mimics naturally occurring gradients. With MOB, these are primarily the counter-gradients of oxygen and methane (Gilbert and Frenzel 1998). We constructed microcosms that allow incubation of the top 3 mm of a water-saturated soil at near *in situ* conditions (Murase and Frenzel 2007). When methane was supplied from below and air was supplied from above, a functioning methanotrophic community developed within a few days, oxidizing virtually all the methane that otherwise would have passed through this soil layer. We used cryosectioning (Murase et al 2006) to subsample the soil from top to bottom in 100-µm steps. Focusing on *pmoA* as a functional and phylogenetic marker, we analysed genes and transcripts along this depth profile, using *pmoA* transcripts as a proxy for species-specific activity. The community structure was assessed by terminal restriction fragment length polymorphism (t-RFLP), by a *pmoA*-specific diagnostic microarray (Bodrossy *et al*., 2003), and by cloning and sequencing. Oxygen microprofiles were used to model the depth-dependent oxygen consumption rate (Berg *et al*., 1998). Rates were correlated to copy numbers of *pmoA* genes and transcripts. Transcripts were quantified using a combination of competitive RT-PCR and t-RFLP.

In addition to *pmoA*, we used *mmoX* encoding for a subunit of the sMMO to search for genes and transcripts of aerobic MOB that lack a *pmoA*. Three different primer sets were applied to DNA and RNA extracted from microcosms incubated for two, four and six weeks, respectively. While this design allows to cover potential successional changes, the chosen primer sets are expected to target a wide range of *mmoX* diversity.

2.3 Materials and methods

Soil microcosm incubation and sampling

The construction and setup of the microcosms have been described previously (Murase and Frenzel, 2007). Briefly, 14 g dry rice field soil from Vercelli (Italy) was saturated with 7 ml demineralized water and incubated on a polytetrafluoroethylene membrane, which divided the microcosm into an upper and a lower compartment. The upper compartment contained oxygen at atmospheric concentrations, while the lower chamber was connected to an external reservoir (volume 1 L) with nitrogen gas supplemented with methane (15%).

We set up four microscosms (1–4). During incubation, methane and oxygen concentrations were monitored by gas chromatography. Methane was added regularly to the reservoir keeping concentration stable (standard error 0.16 % CH_4, n = 18). The microcosms were incubated in the dark for 14 days at 25 °C. Present and active methanotroph populations show some succession, but most changes occur from 25 days onwards (Krause et al 2010). Prior to sampling, vertical oxygen profiles were determined using an oxygen microelectrode (OX50, Unisense, Aarhus, Denmark). The microcosms were then shock-frozen in liquid nitrogen and stored at −80 °C until further analysis. For sub-sampling, the frozen soil was attached to a pre-cooled stage with Tissue-Tek O.C.T. Compound (Sakura Finetek, Staufen, Germany). A cryotome (cryostat HM500M, MICROM, Walldorf, Germany) was used to prepare subsamples 100 µm thick (30 sub-samples per microcosm). The subsamples were stored in 500 µl RNAlater-ICE (Ambion, Austin, Tex., USA) at −20 °C for subsequent nucleic acid extraction.

Nucleic acid extraction

DNA and RNA were extracted following the protocol of Lueders *et al.* (Lueders et al 2004) with minor modifications (Krause *et al.*, 2010). RNA was prepared by digestion of 1 mg total nucleic acid with RQ1 RNase-free DNase (Promega, Madison, Wisc., USA) and subsequent purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. *pmoA* transcripts were enriched using the

mRNA-ONLY Prokaryotic mRNA Isolation Kit (Epicentre Biotechnologies, Madison, Wisc., USA) and again purified with the RNeasy Mini Kit (Qiagen).

cDNA synthesis and pmoA amplification

cDNA was synthesized and *pmoA* was amplified using the One-step Access RT-PCR System (Promega) with the forward primer A189f (5’-GGN GAC TGG GAC TTC TGG; Holmes et al., 1995) and the reverse primer mb661r (5’-CCG GMG CAA CGT CYT TAC C; Costello and Lidstrom, 1999). For t-RFLP, the forward primer was FAM-labelled. For microarray analysis, the reverse primer contained the T7 promoter site (Bodrossy et al 2003). One µl purified template RNA was mixed with 5 µl AMV/Tfl 5× reaction buffer (Promega), 0.01 mg bovine serum albumin (Roche), 2.5 nmol of each dNTP (Promega), 8 pmol of each primer, 25 nmol $MgSO_4$ (Promega), 5% (v/v) DMSO, 20 U RNasin Plus (Promega), 2.5 U Tfl DNA polymerase (Promega), 2.5 U AMV reverse transcriptase (Promega) and molecular-grade water (Sigma-Aldrich, Munich, Germany) in a total volume of 25 µl. Reactions without AMV reverse transcriptase were used to check for DNA contamination. The first strand of cDNA was synthesized at 45 °C in 45 min, followed by 2 min at 94 °C to inactivate the AMV reverse transcriptase. The template was amplified in 35 cycles (30 s at 94 °C, 1 min at 55 °C, 1 min at 68 °C, final elongation 7 min at 68 °C). PCR products were checked on a 1% agarose gel and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). Genomic copies of the *pmoA* gene were amplified following the same protocol, but without the initial cDNA synthesis step.

Cloning and sequencing

Cloning and sequencing was done as described before (Lüke et al., 2010).

t-RFLP analysis

The purified PCR product (100 ng) was digested with FastDigest MspI enzyme (Fermentas, St. Leon-Rot, Germany) at 37 °C for 6 min. Digested samples were purified with Post-Reaction Clean-Up Spin Columns (Sigma-Aldrich) according to the manufacturer's instructions. Two µl of each purified sample was mixed with 11 µl Hi-Di Formamide (Applied Biosystems, Foster City, Calif., USA) and 0.2 µl of an internal DNA fragment length standard (MapMarker 1000, 50−1,000 bp, x-rhodamine, Eurogentec, Ougree, Belgium) and denatured for 2 min at 94 °C. The terminal restriction fragments were separated and detected with capillary electrophoresis and an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems; 30 min at 15 kV and 9 µA). The t-RF patterns were analysed with GeneMapper Version 4.0 (Applied Biosystems).

Microarray analysis

In vitro transcription, fragmentation, hybridization, scanning and data analysis were performed as described elsewhere (Stralis-Pavese et al 2004, Stralis-Pavese et al 2011).

Competitive t-RFLP

A competitive PCR (cPCR) assay (Han and Semrau 2004) was adapted to quantify copy numbers of the *pmoA* gene and its transcripts. The assay was optimized for the most abundant and active OTU. A vector-born copy of an environmental *Methylobacter*-related *pmoA* gene (accession number) was used for standard preparation. Primers were A189f_T7 (5’-TAA TAC GAC TCA CTA TAG GGG GNG ACT GGG ACT TCT GG) and Inner-rev-661 (5’-CCG GMG CAA CGT CYT TAC CAC TCA GGA GTA CCA GTT CTT). Concentrations of DNA and RNA standards were determined using RiboGreen and PicoGreen, respectively (Molecular Probes Inc., Eugene, Ore., USA). For each sample, a minimum of three PCR or RT-PCR amplifications was performed as described above. Each reaction contained equal amounts of the environmental template, but varying standard concentrations. After amplification, PCR products were processed and analysed by t-RFLP. We regressed the logarithms of standard-to-sample ratios to the

logarithms of standard added; the amount of standard at the equivalence point equalled the unknown copy number (Freeman et al 1999). For further details and an example see Supplement 1.

Statistical analysis

t-RFLP data were analysed as described before (Krause et al 2010). Briefly, the terminal restriction fragments (t-RFs) were binned to OTUs based on an *in silico* analysis of about 500 sequences from field and greenhouse experiments of Vercelli soil (Lüke et al 2010). t-RF profiles were standardized (Dunbar et al 2001) and expressed as fractions. For microarray analysis, signals were standardized (i) against the mean of the overall array intensities (Lüke et al 2011) and (ii) against an experimentally determined reference value for positive detection (Bodrossy et al 2003). Statistical analysis and graphics were done in R (R Development Core Team 2011). Analysis of similarity (ANOSIM) and non-metric multidimensional scaling (NMDS, using the function *metaMDS*) were done with the *vegan* package, version 2.1-0 (Oksanen et al 2011).

Soil microcosms for mmoX analyses

To evaluate the potential role of sMMO, we used another eight microscosms that were installed and incubated as described above. Two microcosms were sacrificed after two weeks, and three microcosms each after four and six weeks, respectively. Soil was sampled in 0.5 g aliquots, shock-frozen in liquid nitrogen, and stored at -80°C till further analysis. DNA and RNA were simultaneously extracted and prepared as described above. RNA samples were reverse transcribed with random hexamer primers and SuperScript III reverse transcriptase (Invitrogen, Darmstadt, Germany). Amplification of *mmoX* gene and transcript sequences was done using (i) primer set mmoX206f/886r (Hutchens et al 2004), (ii) primer set mmoXf92/r1430 (Islam et al 2008, McDonald et al 1995), and (iii) primer set mmoXLF/LR (Rahman et al 2011). Primer set mmoX206f/886r covers a fairly wide range of *mmoX* diversity, while mmoXf92/r1430 includes verrucomicrobial sequences. Primer set mmoXLF/LR is specific for *Methylocella.* To check for cDNA quality, we amplified both rRNA and

pmoA with primer sets 8F/1392R (Amann et al 1995) and A189f/682r (Holmes et al 1995), respectively. We got products from all samples. Amplicons generated with mmoX206f/886r from DNA extracted from microcosms after two and six weeks of incubation were cloned and sequenced as described above. Sequencing was carried out by GATC (GATC Biotech AG, Konstanz, Germany). Phylogenetic trees were constructed from sequence data using the ARB software package (Ludwig et al 2004).

Sequences

pmoA sequence data have been submitted to EMBL under accession numbers HE805099–HE805112. *mmoX* sequence data have been submitted to GenBank under accession numbers JQ889714 - JQ889792.

2.4 Results

Methane oxidation and t-RFLP analysis

We followed methane concentrations over time in both the lower and upper compartments of the microcosms. The lower compartment with the methane source simulated the methanogenic soil layer. The upper compartment, or headspace, was replenished with air every 2 days. Initially, in all microcosms, up to 3% methane accumulated in the headspace, but after 5 days of incubation, accumulation ceased resulting in an average headspace concentration of 228 ppm_v CH_4 which indicated the presence of an active and efficient methanotrophic community.

After 2 weeks of incubation, the oxic–anoxic interface in all microcosms stabilized between 1.5 and 1.7 mm (Figure 1A). After 14 days, the soil of four microcosms was shock-frozen with liquid nitrogen, removed intact, mounted in a cryotome, and sub-sampled in 100-µm layers by sectioning. Thirty subsamples per microcosm were analysed (total n = 120).

For an initial overview, we analysed the genes and transcripts by *pmoA* t-RFLP. We assigned OTUs based on an *in silico* analysis of 500 *pmoA* clone sequences plus another 3,500 sequences retrieved by pyrosequencing (Lüke et al 2011, Lüke and Frenzel 2011). The assignments were supported by 15 sequences generated from cloned mRNA (another 80 clones were derived from rRNA, see below). Figure 1 shows

a synopsis of the average oxygen microprofile and the DNA and mRNA-based t-RFLP profiles from microcosm 1. The DNA-based pattern was diverse, with dominating fragments identified as type Ia (*Methylobacter*) and Ib, and as type II (*Methylocystis* and *Methylosinus*). The type-II-specific fragment was dominant below 2.2 mm depth, while fragments assigned to type Ib MOB had their highest relative abundance around the oxic–anoxic interface between 1.0 and 2.0 mm depth (Figure 1B). This increasing dominance of type-I-specific fragments was even more pronounced in the *pmoA* transcripts (Figure 1C). *Methylobacter*-related tRFs dominated around the oxic–anoxic interface, but also in the upper 0.5 mm. Based on t-RFLP, type II MOB did not transcribe the *pmoA* gene. This was consistent with cloning and sequencing of *pmoA* transcripts: type-II-specific sequences were lacking. A considerable fraction of t-RFs was not derived from *pmoA* transcripts but rather from rRNA, as observed previously with *pmoA* RT-PCR (Krause et al 2010). These false-positive t-RFs became most obvious below the oxic–anoxic interface, which suggested an extremely low number of target molecules in the anoxic soil.

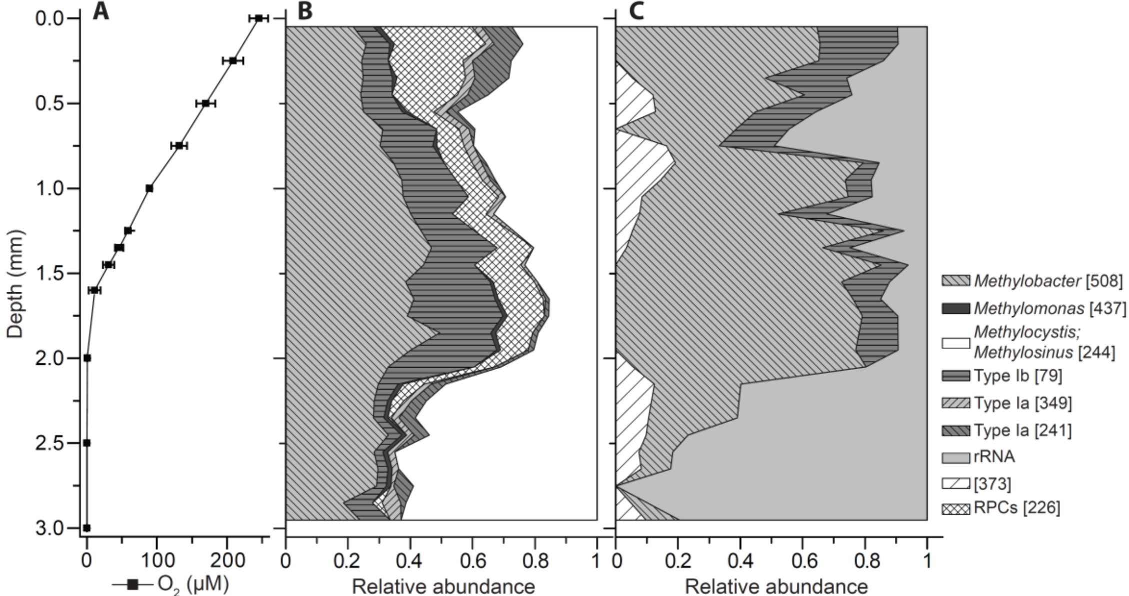

Figure 1: Vertical profiles of oxygen concentration and of terminal restriction fragments (t-RFs) derived from *pmoA* genes and their transcripts. Data are from microcosm 1. (A) Average oxygen profile (± se, n = 4) measured with a microelectrode. Relative abundance of *pmoA* t-RFs derived from (B) DNA and (C) RNA. The phylogenetic affiliation of t-RFs is given together with their size in brackets. rRNA: combined relative abundances of different tRFs derived from unspecific reverse and amplification of 16S and 23S rRNA sequences; RPCs: rice paddy clusters (Lüke et al., 2010).

The *pmoA* gene diversity along the depth profiles was consistent between all four microcosms. On average, the dominating methanotroph groups (type II and type Ia) showed an alternating pattern with a predominance of type Ia around the oxic–anoxic interface (Figure 2). Also the transcript analysis was consistent with the results described for microcosm 1: a lack of type II, a pronounced dominance of type Ia around the oxic–anoxic interface, and a high fraction of false-positive products at depths where no aerobic methane oxidation was expected.

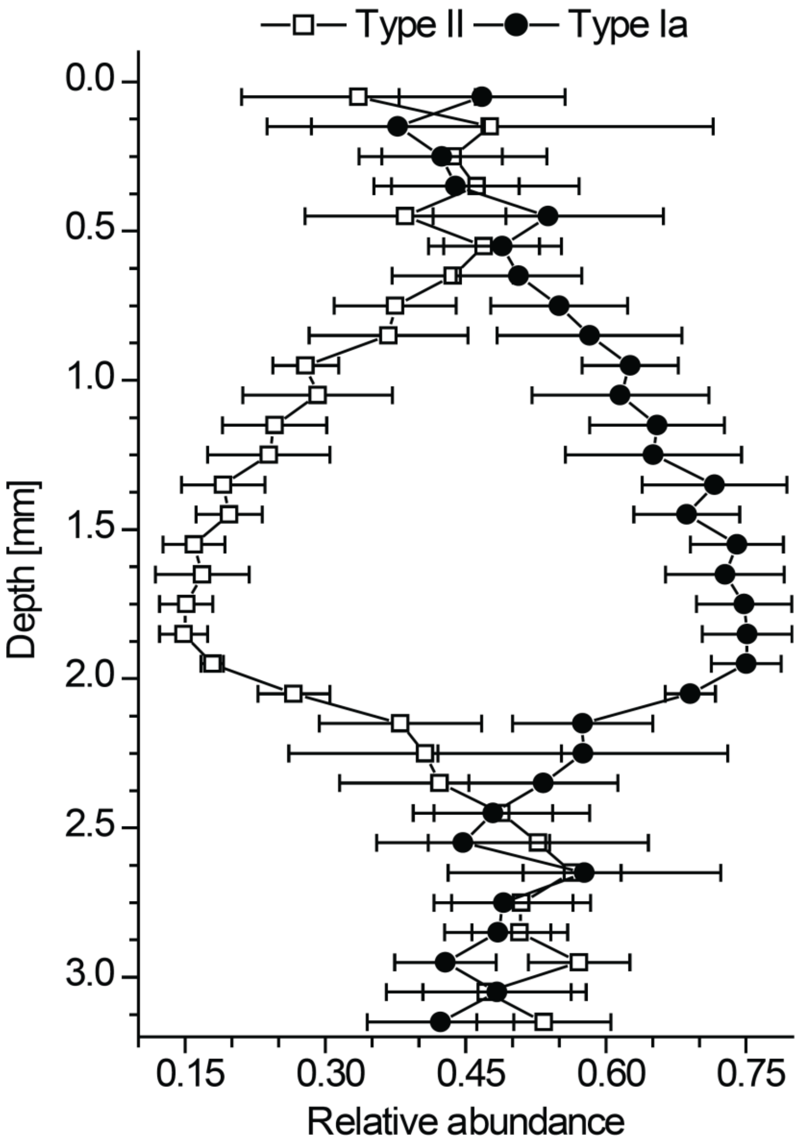

Figure 2: Vertical differentiation of the DNA-based population structure in all four microcosms. Mean relative abundances of type II and type Ia MOB (± se, n = 4). Type II corresponds to tRF 244; type Ia is the sum of all other fragments. Unlike RT-PCR, the DNA-based analysis did not suffer from unspecific amplification of rRNA genes.

Quantification of transcripts by competitive t-RFLP

Neither the *pmoA* microarray nor classic t-RFLP (Horz et al 2001) allow quantification *sensu stricto,* even if they are often regarded as semi-quantitative. To quantify copy numbers of the *pmoA* gene and its transcripts, we combined competitive PCR or RT-PCR with t-RFLP, respectively. We focused on a fragment affiliated to *Methylobacter* that showed high relative abundances at the oxic–anoxic interface (tRF 508, Figure 1B). This OTU reached copy numbers up to 3.73×10^8 *pmoA* genes per gram fresh weight of soil, and a maximum of 9.5 transcripts per genomic *pmoA* copy at the oxic–anoxic interface (Figure 3). Based on this distribution and further supported by microarray data (Figure 4, see below), we defined three depth zones: surface zone, oxic–anoxic interface, and anoxic zone (Table 1). The average numbers of pmoA transcripts in the highly active oxic–anoxic interface were one order of magnitude higher than in the surface and anoxic zones (Table 1). The highest transcript:gene copy ratios coincided with the area of highest oxygen consumption, as calculated from the oxygen microprofiles (Figure 3).

Microarray analysis of pmoA transcripts

We analysed the transcript patterns of the four microcosms using non-metric multidimensional scaling (NMDS; Figure 4). Each of the three depth zones formed a distinct cluster with significant differences to each other (ANOSIM, Bray-Curtis dissimilarity, $p < 0.001$). Probes used for ordination and a few others yielding obvious spatial patterns are listed in Supplement 1.

The original microarray data from all four microcosms (1–4) provided additional information (Supplement 2). Hybridization signals for type-II-specific probes were observed mostly in the oxic–anoxic interface, but the signals were faint. The only type II probe that gave a stronger signal (P_MM_MsT343) is unspecific, i.e. it binds also to many type Ib sequences. This signal coincided with that of the general probes for type Ib (Ib453 and Ib559).

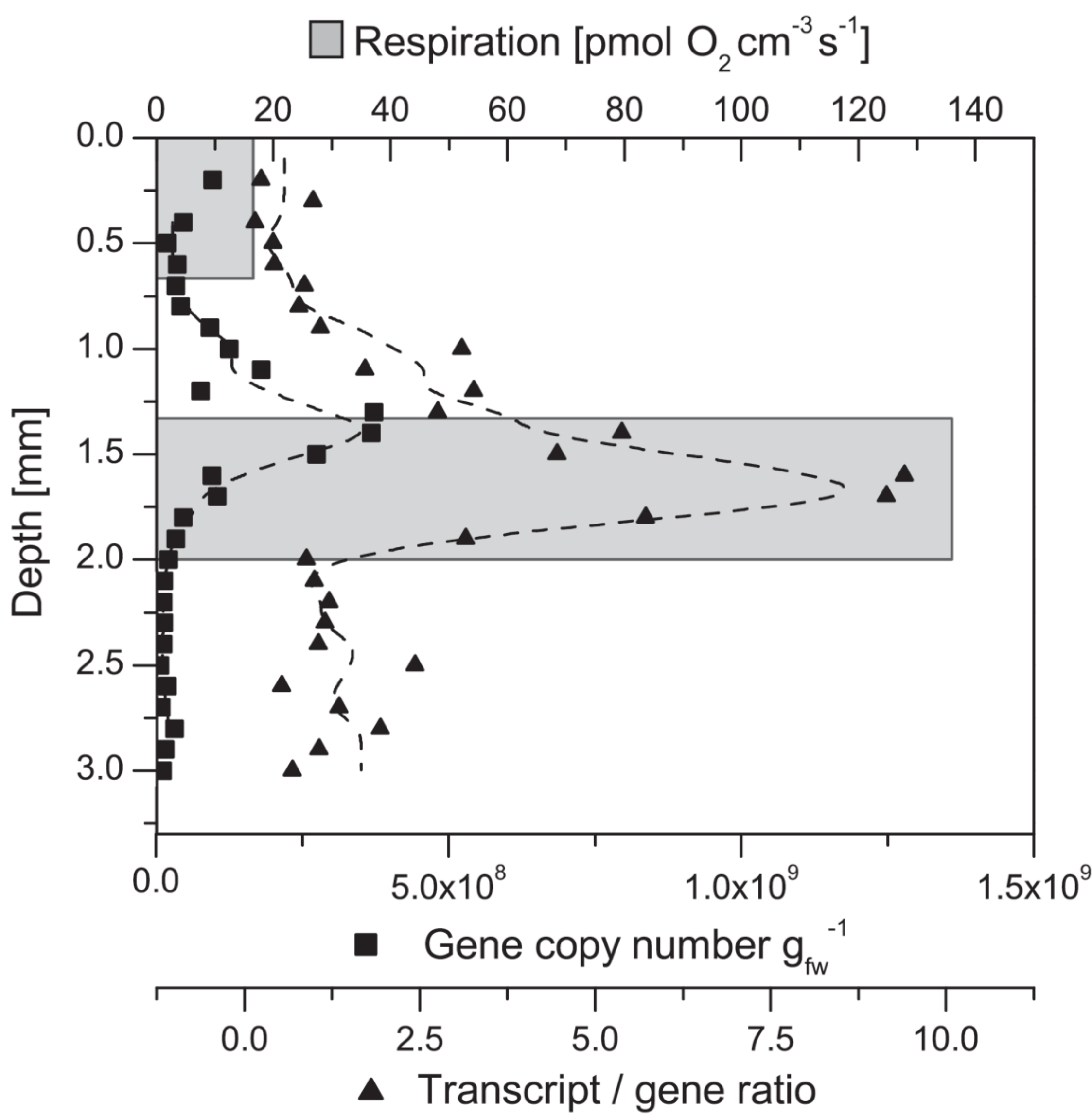

Figure 3: Competitive t-RFLP analysis of a *Methylobacter*-related fragment (t-RF 508). Copy numbers of the *pmoA* gene and the *pmoA* RNA/DNA ratio are shown with Sawitzky-Golay smoothers (n = 5). The oxygen consumption rate was calculated from the microprofile shown in Figure 1A using Berg's program PROFILE ver. 1.0 (Berg et al 1998), considering the top 2 mm.

The general probes for type Ia (O_Ia193, O_Ia575) gave strong signals not only at the oxic–anoxic interface, but also in the anoxic zone. Furthermore, probe Mb271 gave signals in all three depth zones, but most signals in microcosms 1 and 2 were in the oxic–anoxic interface. In three out of the four microcosms, the upper 0.4 mm was characterized by a strong signal of probe O_Mmb562, which is indicative for *Methylosarcina*. Also the largely redundant probes Mmb303 and Mmb304 (Supplement 2) gave consistently high signals, which suggested that the activity of *Methylosarcina* was indeed high in the surface zone (Supplement 3, microcosms 2–4).

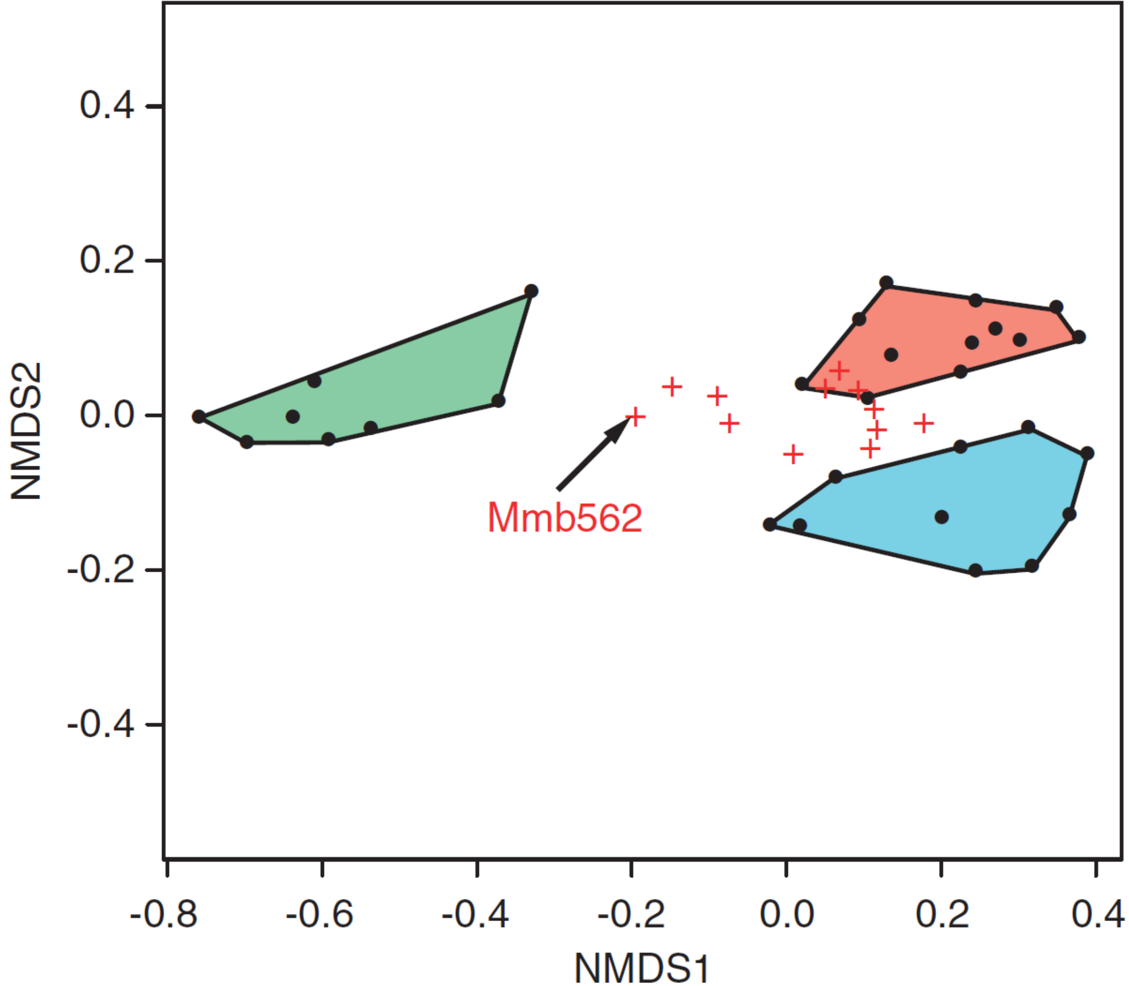

Figure 4: Non-metric multidimensional scaling (NMDS; Bray-Curtis dissimilarities; stress = 0.0689; R^2 = 0.997; linear fit, R^2 = 0.989) of transcript patterns derived from hybridization to a *pmoA* microarray; average values from four microcosms. Layers are shown as black symbols. The green, red and blue hulls visualize the three different zones (surface, 0–0.8 mm; oxic–anoxic interface, 0.8–2 mm, and anoxic, 2–3 mm), respectively. The differences between the zones are highly significant (ANOSIM, Bray-Curtis dissimilarity, $p < 0.001$). Red symbols mark the different probes. Probe O_Mmb562, which separated the surface zone from the other two zones, is specific for *Methylosarcina*. The original data from each microcosm are documented in Supplement 3, and probes considered for the ordination together with the rational for selecting them are given in Supplement 2.

To evaluate the potential role of sMMO, we sampled two to three replicate microcosms after two, four and six weeks, respectively. All *mmoX* sequences retrieved could be affiliated with type II MOB of the genera *Methylosinus* and *Methylocystis* (Supplement 4). No transcripts were found, while a *Methylocella*-specific assay even failed to produce products from DNA suggesting that this genus is missing (Supplement 4).

Table 1 Average number of *pmoA* gene copies and transcripts in the three different depth zones defined in Figure 4. p-values are for t-tests comparing averages per depth zone (n.a. = not applicable).

Depth zone [mm]	*pmoA* copies			*pmoA* transcripts		
		p-value			p-value	
	[10^7 g^{-1}]	Anoxic–oxic interface	Surface	[10^7 g^{-1}]	Anoxic–oxic interface	Surface
Surface [0–0.8]	4.6	< 0.05	n.a	1.3	< 0.01	n.a
Anoxic–oxic interface [0.8–2]	16.1	n.a	<0.05	44.9	n.a.	< 0.01
Anoxic [2–3]	1.5	< 0.01	<0.005	2.8	< 0.001	0.3

2.5 Discussion

Previous experiments with gradient microcosms have already shown that focusing on the active layer allows processes and interactions to be analysed in unparalleled detail (Krause et al 2010, Murase and Frenzel 2007). While only 3 mm thick, the soil layer in the microcosm was considerably thinner and allowed a stronger focus on the organisms of interest than in many other experiments (Dumont et al 2011, Siljanen et al 2011). Even working at a resolution of one centimetre dilutes the active layer with the microbial seed bank in the bulk soil and limits interpretability, regardless if major soil compartments are sampled separately (Eller et al 2005). Dividing the soil further into 100-µm-thick layers brought an unprecedented resolution that was sufficient not only to analyse the vertical position of different OTUs, but also physiological differences within one OTU. While applied here to a laboratory system, this technique can also be adapted to retrieve real-time snapshots from sediment surfaces using freeze cores (Macumber et al 2011).

Using the *pmoA* gene as a functional and phylogenetic marker for MOB, we analysed genes and transcripts at the sub-millimetre scale along a depth profile from the soil surface down into the anoxic zone. As predicted from oxygen microprofiles, methanotrophic activity was indeed located at the oxic–anoxic interface. Using *pmoA* transcripts as a proxy for species-specific activity, we correlated the transcript-to-gene ratio of specific methanotroph taxa to methane-driven respiration.

Transcripts of *mmoX*, a gene encoding for a subunit of sMMO found as a second monooxygenase in some MOB, could not be detected, even if the gene was found (Supplement 4). MOB of the genus *Methylocella* lack *pmoA*, but has *mmoX* (Dedysh 2009). This genus was previously thought to be acidophilic, but could recently be detected in circum-neutral environments, too (Rahman et al 2011). However, it was undetectable in the paddy soil under study (Supplement 4). Hence, *pmoA* is a reliable functional and phylogenetic marker for this microcosm experiment.

Concentration profiles and activity

At the oxic–anoxic interface, oxygen is not only used for the direct mineralization of organic matter, but also for the re-oxidation of end-products from anaerobic processes (Brune et al 2000). In freshwater environments, methane is most important and may be the dominant oxidation substrate at the interface. Our microcosms were designed to model this situation, but can in principle be re-configured to focus on other redox processes or to study the interaction between different competing processes.

The 16 oxygen microprofiles measured (four per microcosm) showed the same characteristics: a near-linear decrease in oxygen concentration down to a depth of about 1.3 mm, followed by a pronounced curvature before the concentration reached zero at about 1.8 mm depth (Figure 1A). The concomitant methane concentrations at the oxic–anoxic interface were most probably in the lower micromolar range (Gilbert and Frenzel 1998). The methane sensors built for previous work (Gilbert and Frenzel 1998, Rothfuss et al 1994) do not achieve the sub-millimetre resolution required for this experiment. While further miniaturization is feasible, the detection limit would decrease proportionally, making a sensitive analysis impossible. Membrane-inlet mass spectrometry (Beckmann et al 2004, Lloyd et al 1986, Lloyd et al 1996) suffers from the same constraints, and the development of a microscale biosensor for methane (Damgaard and Revsbech 1997) has been discontinued. Hence, interpretation has to rely on oxygen microprofiles alone.

We calculated oxygen consumption using Berg's program PROFILE ver. 1.0 (Berg et al 1998) and a sediment diffusion coefficient measured in this paddy soil (Noll et al 2005, Rothfuss and Conrad 1994). The algorithm calculates the zone-specific respiration rate using Fick's second law (Figure 3). Assuming constant porosity, the respiration rate at the surface was modest, followed by an area where no significant respiration took place. High values contributing 89% of the total oxygen respiration were calculated for the zone above the interface (Figure 3). Assuming a stoichiometry of CH_4:O_2 = 1:2, the total respiration of 36.6 nmol O_2 cm^{-2} h^{-1} corresponded to a methane oxidation rate of 18.3 nmol CH_4 cm^{-2} h^{-1}.

t-RFLP patterns and quantification

In community profiling studies of MOB, t-RFLP analysis targeting the *pmoA* gene has a long tradition (Hoffmann et al 2002, Horz et al 2001, Mohanty et al 2007). The results, however, depend on the choice of primer sets. Compared to the 682r reverse primer (Holmes et al 1995), the reverse primer mb661r (Bourne et al 2001, Costello and Lidstrom 1999) covers methanotroph diversity, but not the homologous *amoA* gene encoding for a subunit of ammonium monooxygenase. Furthermore, primer mb661r seems to be superior for resolving type I diversity (Bourne et al 2001, Lüke et al 2010).

When we compared DNA- and RNA-based community profiles, we found striking differences between the extant (DNA-based) and active (RNA-based) populations (Figure 1). The extant population changed consistently with depth in all four microcosms studied (Figure 2). Virtually no transcripts of type II MOB were found, but a *Methylobacter*-like OTU (tRF 508) was most prominent around the oxic–anoxic interface (Figure 1B & C). Taking transcription as a proxy for activity, this dominance suggested a corresponding localization of *Methylobacter*-specific methane oxidation. This conclusion, however, depends largely on the high fraction of unspecific tRFs derived from ribosomal RNA. While this large fraction of false-positive tRFs suggested an extremely low content of *pmoA* mRNA compared to rRNA, it is only a tentative measure. We therefore adapted a competitive PCR assay (Han and Semrau 2004) and used it to quantify the most prominent *Methylobacter*-like OTU (tRF 508; Figure 3).

Competitive PCR has rarely been applied in microbial ecology (Han and Semrau 2004, Saleh-Lakha et al 2005), but it has certain advantages, in particular if combined with t-RFLP. T-RFLP alone gives only relative values. Provided adequate standards can be constructed, t-RFLP can easily be modified to retrieve truly quantitative data. In addition, RT-cPCR helps in overcoming the variability inherent to the RT step (Freeman et al 1999). In the particular case of *pmoA*, RT-cPCR helped in circumventing the problem with unspecific RT-PCR products; the latter had restriction sites that were different from those of the OTU of interest. Some limitations of t-RFLP still apply, e.g. a limited phylogenetic resolution and the co-occurrence of particular t-RFs in more than one phylotype. Hence, a decent sequence database is a must. However, cPCR

combined with t-RFLP has the major advantage that not only quantification, but also other tasks such as community analysis (t-RFLP, microarray) and sequencing can be based on the same assays, whereas covering different MOB types by qPCR requires different primer sets (Kolb et al 2003, Kolb et al 2005).

In the anoxic zone below 2 mm, the *pmoA* copy number was low and may correspond to the initial numbers present in the dry soil when the experiment was started. The slightly higher copy number in the top zone suggested that growth had taken place there at least for some time (Table 1). The maximum transcript:gene ratio coincided with the highest respiration rate, but was localized slightly deeper in the soil than the maximum *pmoA* copy number (Figure 3). If we consider two *pmoCAB* operons per cell (Semrau et al 1995), the *Methylobacter*-affiliated OTU had up to 18 transcripts per cell (Figure 3). The half-life of *pmoA* mRNA is unknown. The half-life of other mRNAs may be as short as 30 s, but could also be much longer depending on the environment and the growth state (Arraiano et al 2010, Steglich et al 2010). However, we are confident that we preserved the *in situ* mRNA content by shock-freezing the soil with liquid nitrogen when still in the microcosm.

The soil surface in the microcosms was characterized by high oxygen (Figure 1) and low methane concentrations. Before we sampled the soil for molecular analysis, 318 ppm_v methane had accumulated in the headspace, which corresponds to 400 nM methane in the pore water near the soil surface. Hence, MOB living in the top layers of the microcosms were exposed to this or a slightly higher methane concentration immediately before shock-freezing. However, the *Methylobacter*-affiliated OTU had less than one transcript per cell (Table 1), which suggested that this situation was rather unfavourable. In the anoxic zone, however, the *Methylobacter*-affiliated OTU had nearly four transcripts per cell (Table 1). Our microcosm design aimed at producing a planar system varying only with depth. Microelectrode measurements have a high spatial resolution (< 0.1 mm, corresponding to ca. 0.53 nL), whereas the molecular data refer to the entire area of the microcosm (28.3 cm^2, 0.28 mL per layer). However, the 16 oxygen profiles were so reproducible that we can rule out spatial heterogeneities. Hence, factors other than locally deviating oxygen penetration depth

must be responsible for the high transcript:gene ratio below the oxic–anoxic interface. The nitrite-reducing methanotroph, "*Candidatus* Methylomirabilis oxyfera" is able to generate O_2 from NO via a dismutase (Ettwig et al 2010, Strous 2011). However, we have no indication that *Methylobacter* possesses this trait, and nitrite was not detectable in another microcosm experiment with the same soil (Krause et al 2010). Recently developed microelectrodes (Revsbech et al 2009, Revsbech et al 2011) have demonstrated nanomolar oxygen concentrations in areas that have been considered anoxic so far. However, the design of our microcosms includes a trap to remove any oxygen that might have diffused into the lower compartment (Murase and Frenzel 2007). Hence, oxygen may have been present in trace amounts in the methane-rich 'anoxic' zone below the oxic–anoxic interface, but diffusive transport of oxygen to MOB must have been negligible, if it occurred at all. Therefore, the mRNA:DNA ratio points to a differentiated physiological status of the cells depending on depth and a surprisingly high number of transcripts in the anoxic zone.

Microarray analyses

Although the microarray analysis leads to essentially the same conclusions as t-RFLP analysis, the higher phylogenetic resolution of the microarrays may allow differentiation down to the species level (Stralis-Pavese et al 2011). First developed for DNA-based analyses, the microarray has been successfully applied to study *pmoA* transcripts (Bodrossy et al 2006, Chen et al 2007, Krause et al 2010). Here we used it to refine the transcript analysis (Figure 4, Supplement 3). The microarray design follows a multiple-probes approach. This has significant advantages and may help in detecting groups not yet covered by a specific probe by another, more conserved and general probe. A certain degree of redundancy also helps to exclude false-positive hybridization signals, but may introduce a bias in ordination analyses if a particular phylotype hybridizes with more than one probe. Hence, we used only mutually exclusive probes with a well-defined phylogenetic coverage for ordination analysis. These probes and a few others showing obvious spatial patterns are listed in Supplemental Table 1. The ordination (NMDS, Figure 4) gave a clear separation between the surface, oxic–anoxic interface, and bottom zones.

The only signal that could be associated with type II MOB was most probably false positive since the respective probe hybridizes also with different rice paddy clusters (Lüke et al 2010) belonging to type I (Supplement 3); the general probes for type II gave no signal. Type II MOB have been described as more abundant than type I but as contributing to methane oxidation mainly under high methane concentrations, while type I may thrive if the methane source strength decreases (Henckel et al 2000). This, however, may be a misconception: even at a high source strength, i.e. high methane production rates, methanotrophic activity shapes counter-gradients, resulting in a steady state with concomitantly low oxygen and methane concentrations at the oxic–anoxic interface. Thus, not methane concentration but rather the energy flow through a population may be the decisive factor (Krause et al 2012). Type II MOB may occasionally become active in such a situation, but the controlling factor(s) are unknown (Krause et al 2010). However, extinguishing 97.5% of all microbiota stimulates the exponential growth of type II MOB, which eventually become by far the dominant group (Ho et al 2011). Hence, thanks to their robust resting stages (Whittenbury et al 1970a, Whittenbury et al 1970b), type II MOB may apply a sit-and-wait strategy, taking advantage of changing situations that may be a catastrophe for others.

Type I MOB may have benefited from the rather constant conditions in the microcosms. Probes indicative for *Methylobacter* gave positive signals in the oxic–anoxic interface and the anoxic zone (Supplemental Figure S1), but much less so in the surface zone. This corresponds to the quantification with competitive PCR/t-RFLP (Figure 3), which revealed a higher mRNA:DNA ratio in the anoxic zone than in the surface zone. The role of type II MOB and *Methylobacter* is surprisingly similar to that found in a seasonal study on an alpine meadow (Abell et al 2009), in which type II MOB remained largely unaffected by season and environment but nevertheless represented the dominant MOB. *Methylobacter*-related MOB, however, were found to be responsible for the majority of methane oxidation.

Probes indicative for *Methylosarcina* gave significant signals in the surface zone (Supplemental Figure S1), which suggested activity at high oxygen

concentrations (245–132 µM) and low methane concentrations (ca. 400 nM). This is much higher than the atmospheric methane concentration (1.8 ppm_v, corresponding to 2.3 nM), but cultures of and sequences affiliated to *Methylosarcina* have so far only been retrieved from high-methane environments such as lake sediments, rice paddies and landfills (Henneberger et al 2012, Kalyuzhnaya et al 2005, Lüke and Frenzel 2011, Wise et al 2001). Apparent K_m constants in environments with high source strength are usually > 1 µM CH_4 (Conrad 1996), but some cultivated MOB, in particular *Methylocystis* strains, may grow for an extended period at 120 nM CH_4 and less (Knief and Dunfield 2005). For *Methylosarcina*-like MOB, however, activity at low methane concentrations has not yet been reported.

Conclusions

As shown before, only a restricted subset of a diverse methanotroph community was active, and most activity was confined to a zone 0.67 mm thick. However, our experiment showed how different OTUs within a single guild can share the same microenvironment, thereby exploiting different niches. We hypothesized that activity would be highest immediately at the oxic–anoxic interface separating MOB according to substrate availability. This was indeed the case, with a *Methylobacter*-affiliated OTU and dominating overall methane oxidation located at the oxic–anoxic interface. This OTU seems to be well adapted to the oxic–anoxic interface, where oxygen and, presumably, methane concentrations are in the micromolar range. This OTU was not active at the surface with its high oxygen but only nanomolar methane concentrations. In contrast, transcripts of a *Methylosarcina*-affiliated OTU were associated with this surface layer, which suggested an adaptation to oligotrophic conditions.

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2.8 Supplemental material

Supplement 1: competitive t-RFLP

A modified competitive PCR (cPCR; Gilliland, 1990; Han & Semrau, 2004) was used to quantify copy numbers of *pmoA* and its transcripts. Since in competitive t-RFLP (ct-RFLP) signal intensity depends only on the number of amplicons, the method is not affected by the size difference between standard and target. The standard was designed for the specific quantification of a particular OTU ensuring similar amplification efficiencies of standard and target. Furthermore, potentially inhibiting substances in environmental samples are affecting target and standard equally.
The construction of the internal standard was performed as described elsewhere (Semrau & Han, 2005). In short, a vector-born copy of an environmental *Methylobacter*-related *pmoA* was used. Primers for standard construction were A189f_T7 (5'-TAA TAC GAC TCA CTA TAG GGG GNG ACT GGG ACT TCT GG) and Inner-rev-661 (5'-CCG GMG CAA CGT CYT TAC CAC TCA GGA GTA CCA GTT CTT). Concentration of standards was determined using RiboGreen (RNA standard) and PicoGreen (DNA standard) kits according to the manufacturer's instructions (Molecular Probes Inc., Eugene, OR/USA). For each sample, a minimum of three PCRs was performed. Reactions were spiked with 1 µl DNA or RNA standard, respectively, ideally corresponding to the 0.1-, 1-, and 10-fold target concentration. After amplification, PCR products were analyzed by T-RFLP (Figure 1). Quantification was done by regressing log(target × standard-1) against log(standard concentration). The target copy number is given by the equivalence point, *i.e.* log(target × standard-1) = log (1) (Figure 2). The analysis was repeated with adjusted standard concentrations, if the target concentration was not covered appropriately.

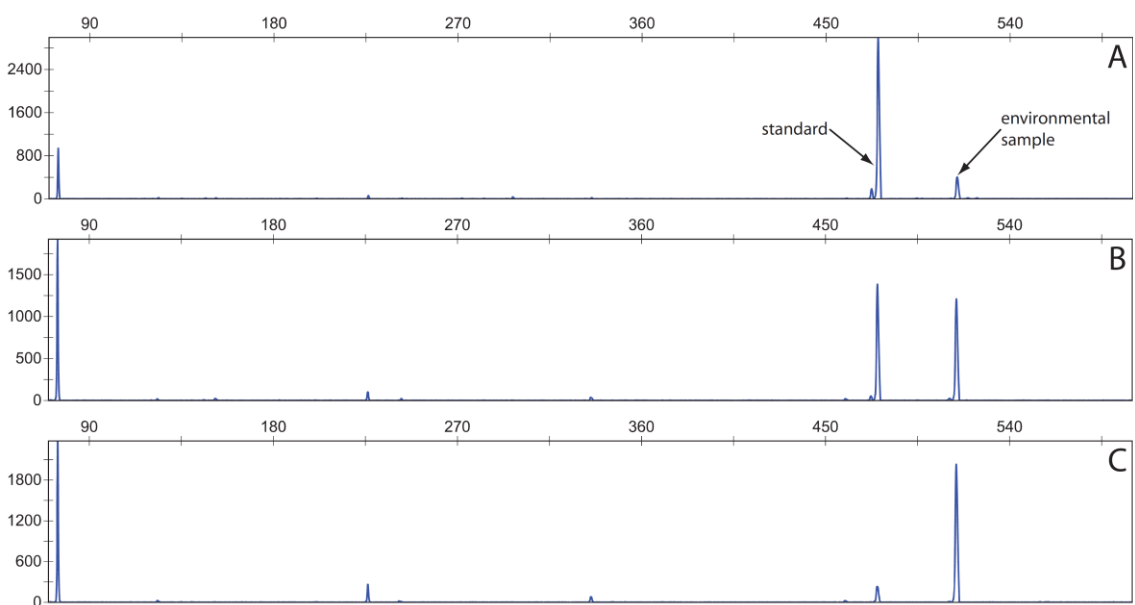

Figure 1: Example of ct-RFLP results. Product separation by t-RFLP allows a clear differentiation between t-RFs derived from the internal standard (475 bp) and the target (508 bp). Standard concentrations decrease from A to cover three orders of magnitude.

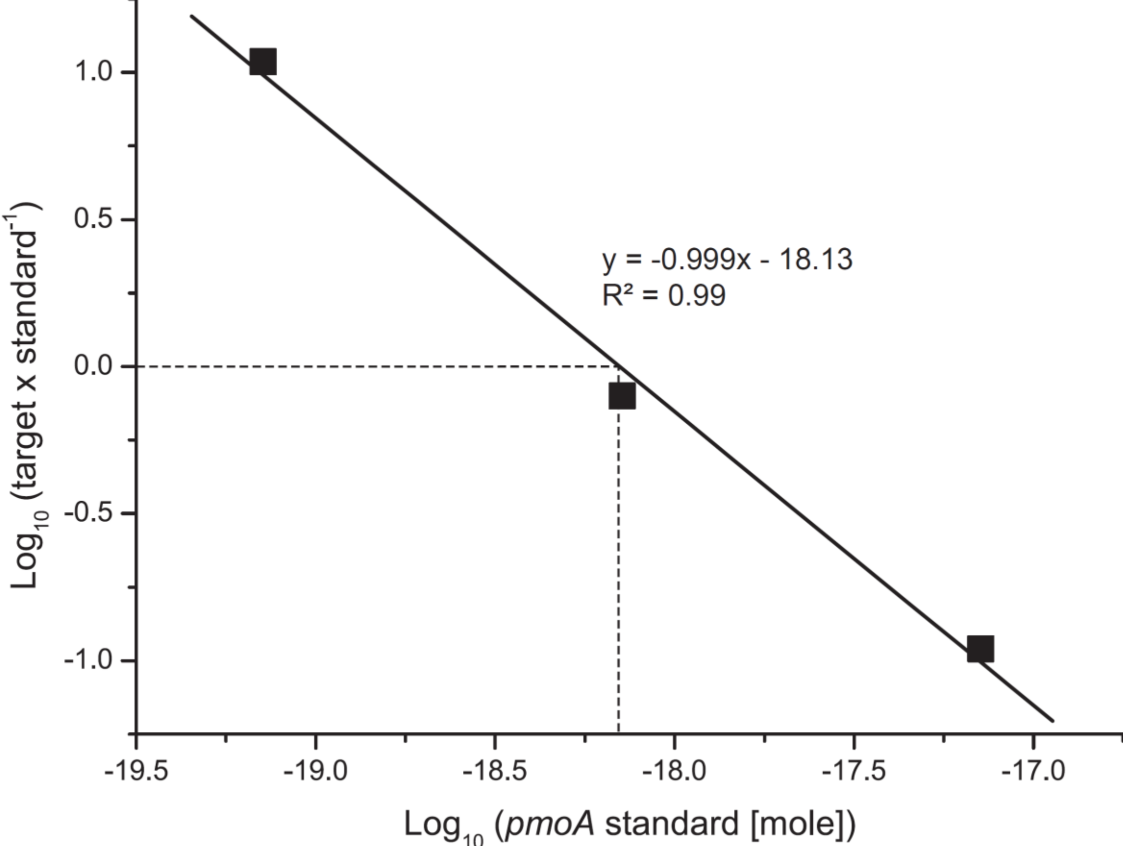

Figure 2: Quantification by ct-RFLP. A constant amount of target is mixed with varying amounts of standard. After PCR amplification and detection by t-RFLP, log(target × standard-1) is plotted against log(standard concentration). When the amount of product from environmental target and standard are equal ordination value is zero. Given similar amplification efficiency the initial amount of standard equals the initial amount of environmental sample in this point.

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Supplement 2

Affiliation of selected probes to phylogenetic groups of methane-oxidizing bacteria (MOB). MOB are subdivided according to *pmoA* phylogeny (Bodrossy et al 2003, Lüke and Frenzel 2011). Type I MOB are subdivided into types Ia, Ib, and Ic. Type II MOB are subdivided into types IIa (*Methylosinus* and *Methylocystis*) and IIb (*Methylocystis* and *Methylosinus pmoA-2*, USC-α and related sequences, cluster MO3 and *Methylocapsa*). Only mutually exclusive probes with a well-defined phylogenetic coverage were used for ordination analysis. A few other probes showing obvious spatial patterns are listed, but were excluded from analysis for reasons given below.

Species/Genus/Cluster	Probe name	Used for ordination	Remark
Type Ia			
Methylobacter	MbA557	Yes	
	Mb_SL.3-300	Yes	
	Mb271	No	Partial overlap with probe Mb_SL.3-300, large overlap with probe O_Mmb562
	MbA486	No	Nearly identical to probe MbA557; however, MbA557 shows a more consistent coverage than MbA486
	Mb380	No	Only in Microarray version 7.1b
	Mb460	Yes	
Methylomicrobium pelagicum	Mm_pel467	Yes	
Methylomonas	Mm275	Yes	
	Mmb303	No	Large overlap with probe O_Mmb562
	Mmb304	No	Only in Microarray version 7.1b
Methylomicrobium album/Methylosarcina lacus	O_Mmb562	Yes	
Type Ib			
Methylocaldum	Mcl408	Yes	
RPCs	O_501.286	Yes	
JRC-4	JRC4.432	Yes	
LW21	LW21.391	Yes	
Type Ic			
RPC2	SWI1.375	No	Only in Microarray version 7.1b
RPC2	SWI1.377	No	Only in Microarray version 7.1b
Type II			
Methylocystis	O_Mcy413	Yes	
Methylosinus	O_Msi294	Yes	
	MM_MsT343	No	Unspecific, binds also to many type Ib MOB sequences

Supplement 3

Heatmap of *pmoA* transcript analyses from four microcosms (MC1-4). Transcripts were amplified with primers A189f and mb661r and hybridized to *pmoA*-specific microarrays (Bodrossy et al 2003). Each line represents the average of three replicate arrays. The depth of a layer is indicated in millimeters to the right. The top row contains the probe names. Names differ slightly between microarray versions 6 and 7.1b used for MC1 & 2 and MC3 & 4, respectively. For consistency of probe names, we have omitted certain prefixes used in the older version. A few layers failed to give RT-PCR products, e.g. layers 0.05 to 0.25 of MC1, and have been omitted. White horizontal lines separate the three vertical zones identified in Figure 4. The color gradient is scaled proportional to the square root of the standardized signal intensity. Anything less than 0.05 and displayed in different shades of blue should be considered as background. Significant signals begin with cyan and may reach maximum values at dark read. Type I methanotrophs are subdivided into types Ia, Ib, and Ic (Bodrossy et al 2003, Lüke and Frenzel 2011). The microarray design follows a multiple probes approach. While this allows for identification of false positive hybridization signals, it may introduce a bias in ordination analyses, if a particular phylotype hybridizes against more than one probe. Hence, only the probes marked in red were used in the ordination (NMDS, Figure 3). Some of the other probes, however, showed obvious spatial patterns. These probes are marked in blue and are discussed in the text. Probe specificities are summarized in Supplemental Table 1.

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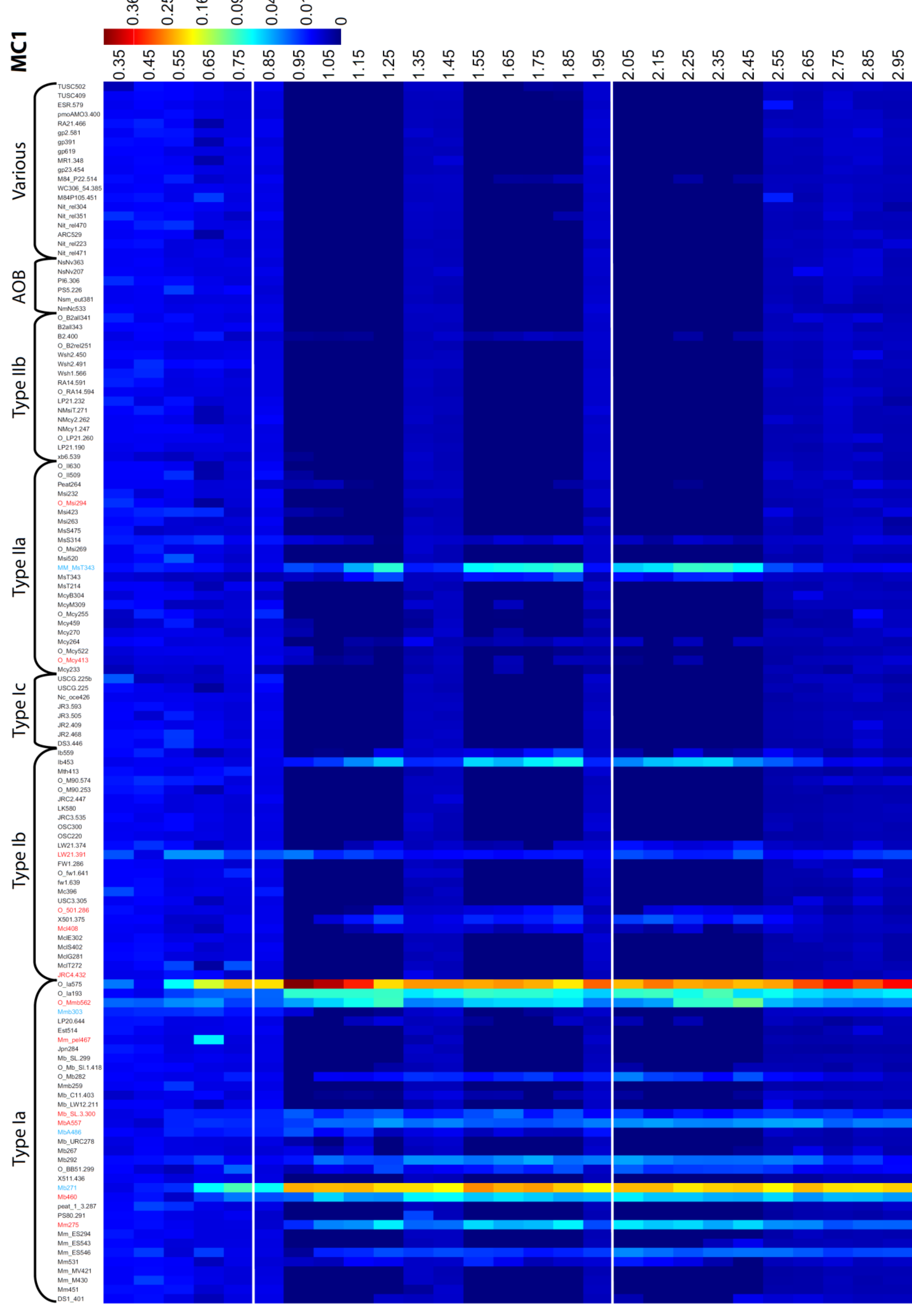

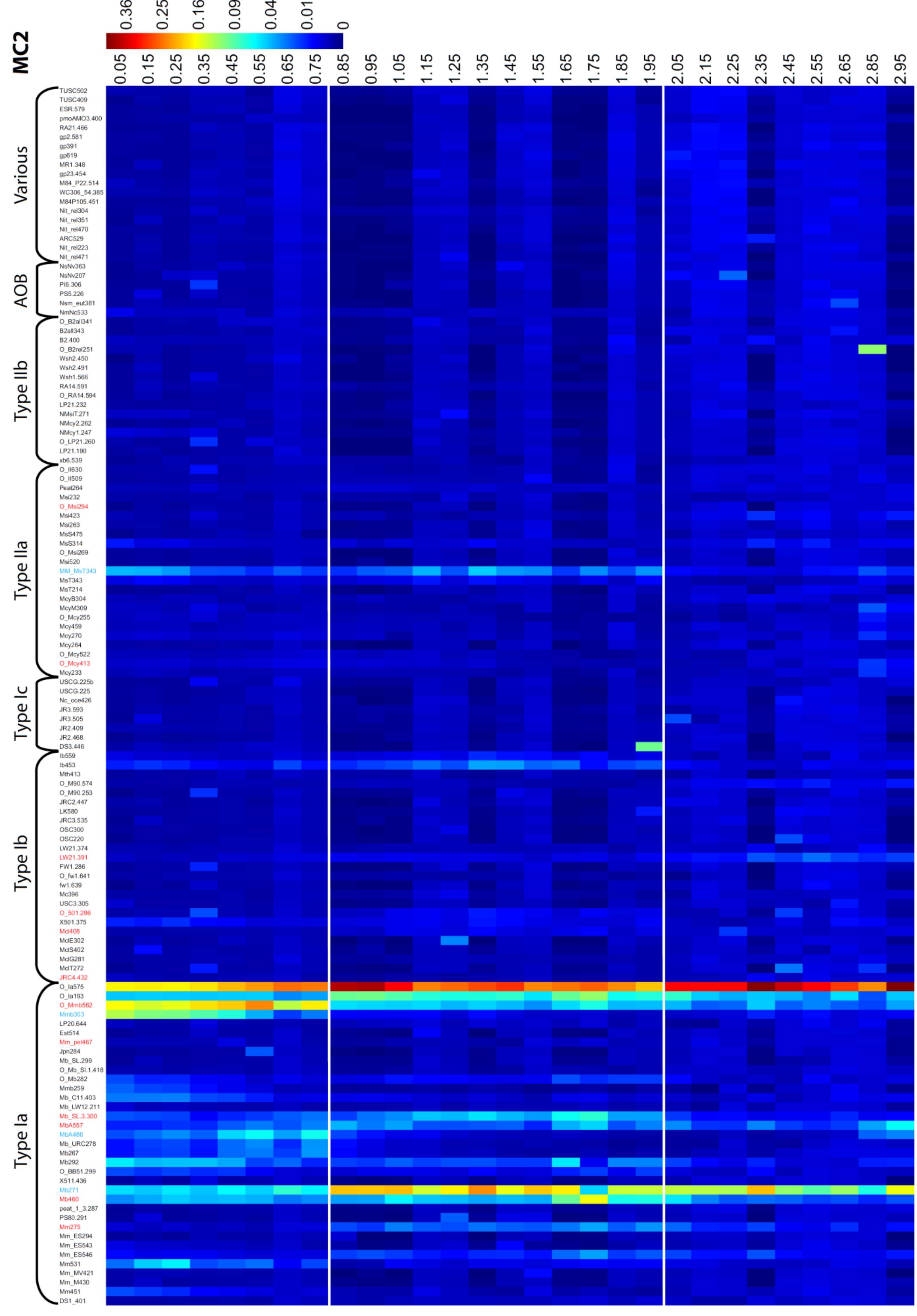
MC2
0.36
0.25
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0.09
0.04
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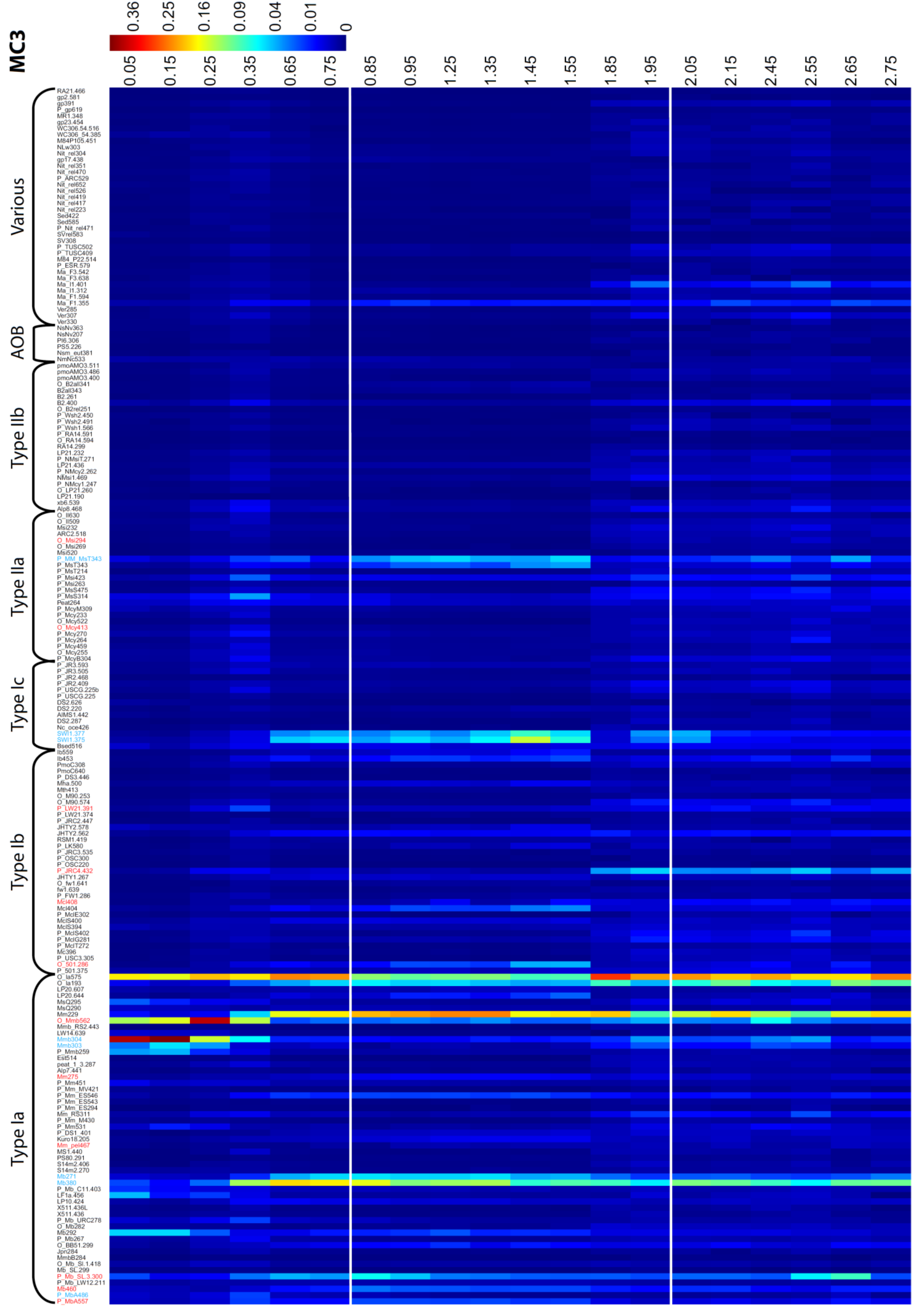
0
0.05 0.15 0.25 0.35 0.45 0.55 0.65 0.75 0.85 0.95 1.05 1.15 1.25 1.35 1.45 1.55 1.65 1.75 1.85 1.95 2.05 2.15 2.25 2.35 2.45 2.55 2.65 2.75 2.85 2.95
Various
AOB
Type IIb
Type IIa
Type Ic
Type Ib
Type Ia


MC3
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0.95
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Various
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Type IIb
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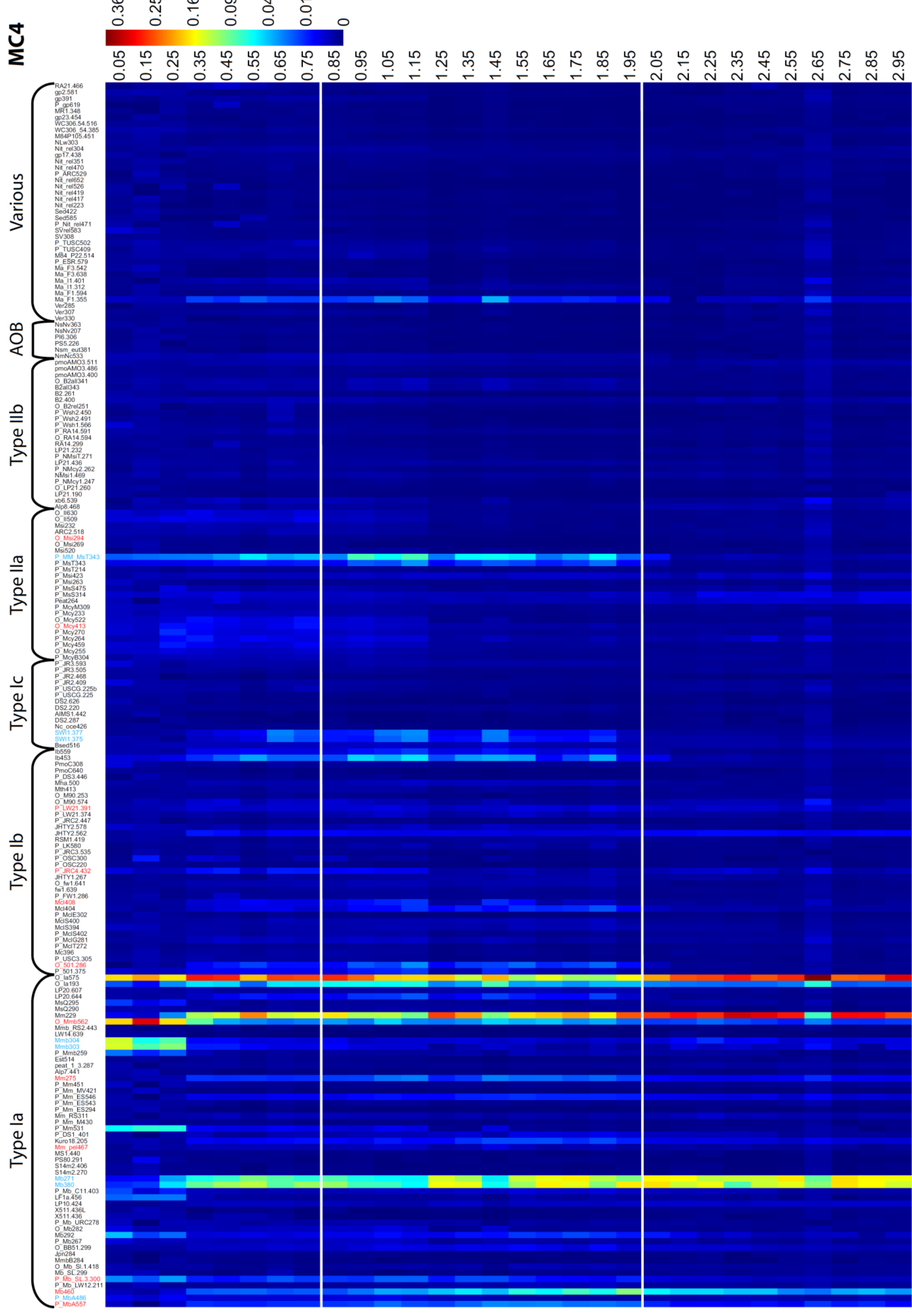
Type Ic
Type Ib
Type Ia


MC4
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Various
AOB
Type IIb
Type IIa
Type Ic
Type Ib
Type Ia

Supplement 4: mmoX

To evaluate the potential role of sMMO, we sampled two to three replicate microcosms each after two, four and six weeks, respectively. DNA and RNA were extracted, and RNA samples were reverse transcribed with random hexamer primers and SuperScript III reverse transcriptase (Invitrogen, Darmstadt, Germany). To check for cDNA quality, we amplified both rRNA and *pmoA* with primer sets 8F/1392R (Amann et al 1995) and A189f/682r (Holmes et al 1995), respectively. We got products from all samples. For amplification of *mmoX* genes and transcripts see Figure 1. A neighbor-joining tree showing the phylogenetic relationship of partial *mmoX* sequences is shown in Figure 2.

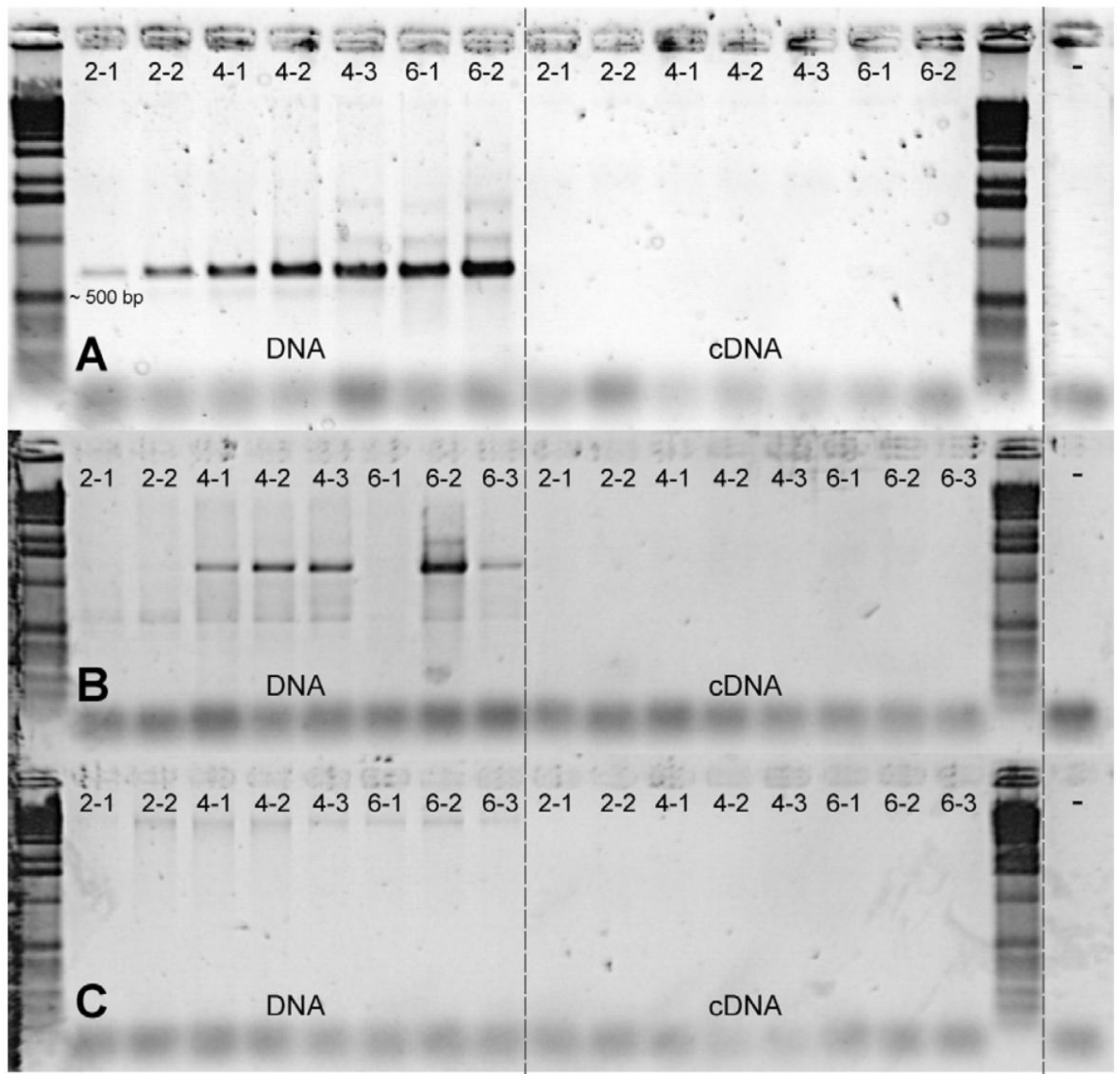

Figure 1: Amplification of *mmoX* gene and transcript sequences from the replicate microcosms after two, four, and six weeks of incubation using various primer sets. (A) primer set mmoX206f/886r (Hutchens et al 2004), (B) primer set mmoXf92/r1430 (Islam et al 2008, McDonald et al 1995), (C) primer set mmoXLF/LR (Rahman et al 2011). The first number on top of a lane gives the week, the second the replicate. Negative controls are labeled with '−'. Primer set mmoX206f/886r covers a fairly wide range of *mmoX* diversity, while mmoXf92/r1430 covers verrucomicrobial sequences, too. Primer set mmoXLF/LR is specific for *Methylocella.*

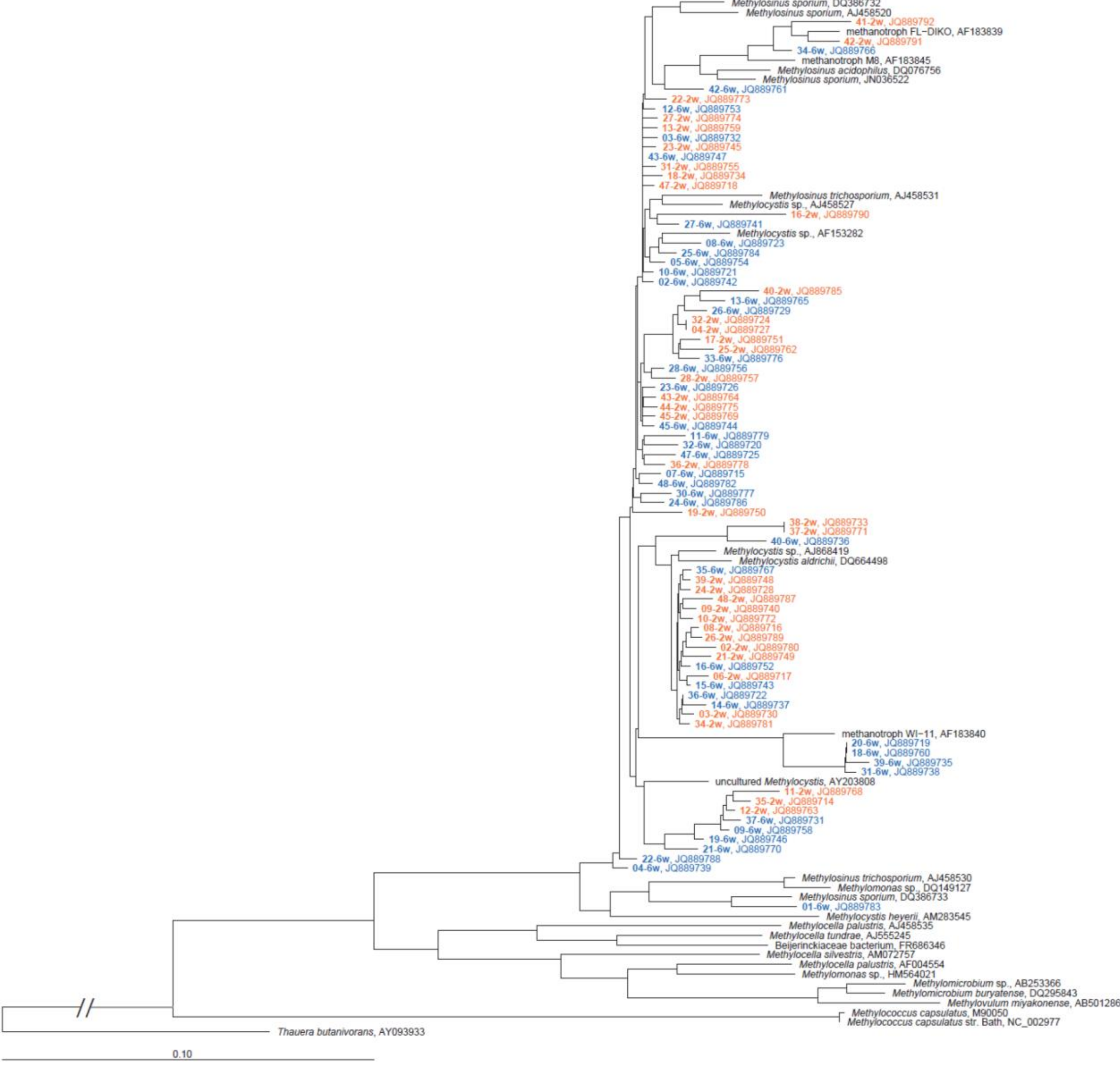

Figure 2: Neighbor-joining tree with Jukes–Cantor correction showing the phylogenetic relationship of partial *mmoX* sequences. The *mmoX* clones derived from DNA of microcosms after two and six weeks of incubation are in red and blue, respectively. The tree is rooted with the soluble butane monooxygenase gene of *Thauera butanivorans* (AY093933). The scale bar represents 10% nucleic acid sequence divergence. GenBank accession numbers are JQ889714 - JQ889792

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3 Recovery of paddy soil methanotrophs from drought

Sebastian Collet, Andreas Reim, Adrian Ho and Peter Frenzel

3.1 Abstract:

Air-dried paddy soils stored for 1 to 18 years were used to examine the resistance of methanotrophs to drought. Longer-lasting droughts reduced methanotrophic diversity, and adversely affected methane oxidation. Type I methanotrophs showed relatively higher *pmoA* gene expression, while type II were more resistant to desiccation.

3.2 Results and discussion

Wetland rice paddies are characterized by alternating drainage and flooding cycles. Therefore, aerobic proteobacterial methanotrophs from rice paddies are exposed to fluctuating substrate availability through changing water levels (Conrad and Frenzel, 2002; Ratering and Conrad, 1998). Moreover, after drainage, methanotrophs face a desiccation stress. However, they are able to form different resting stages: exospores, *Azotobacter*-type cysts, and lipoidal cysts, enabling them to persist for extended periods during unfavourable conditions (Whittenbury et al., 1970). Heat- and desiccation-resistant exospores are formed by *Methylosinus* species, which, together with *Methylocystis,* are traditionally named type II methanotrophs. *Methylocystis* may also form desiccation resistant lipoidal cysts. *Azotobacter*-type cysts are formed by some type I methanotrophs (*Methylobacter*), and are resistant to desiccation, too (Whittenbury et al., 1970), while other type I (*Methylocaldum*, *Methylosarcina*, *Methylomonas*, *Methylococcus*) form cysts that are similar to those of *Methylobacter*, but are not as desiccation resistant (Bodrossy et al., 1997; Whittenbury et al., 1970; Wise et al., 2001). To induce germination, an exposure to a sufficiently high methane

and oxygen concentration is necessary (Higgins et al., 1981; Rothfuss et al., 1997). Furthermore, heat stress is thought to trigger the transformation from dormant to active states, too (Ho and Frenzel, 2012; Whittenbury et al., 1970). Previously, studies have shown that disturbances lead to reduced diversity and evenness in methanotrophic communities (Bodelier et al., 2000; Wertz et al., 2007). However, disturbed communities were still able to oxidize methane at similar rates as undisturbed controls, and can even overcompensate losses caused by the disturbance (Ho et al., 2011).

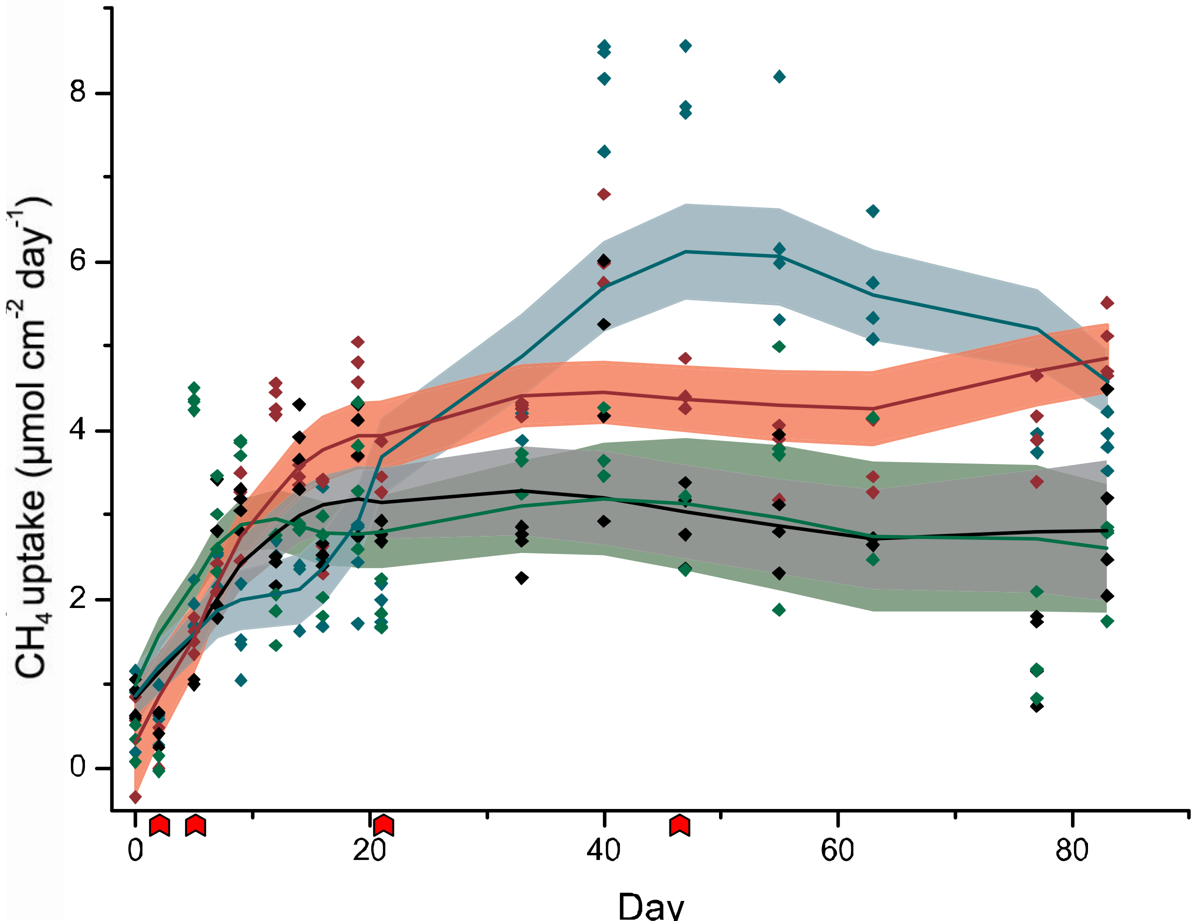

Figure 1: Effect of the length of desiccation on methane uptake rates. Individual measurements and smoothed (Fast Fourier transformation; *n*=3) average with the 95% confidence interval. Green, black, red and blue denote the soils from 1993, 1998, 2006 and 2010. The soil microcosms were prepared, incubated, and the flux measurement were performed as described previously (Ho et al., 2011). The red arrow heads indicate time points (2, 5, 21, and 47 days) where duplicate microcosms were sacrificed. Nucleic acid was extracted as described elsewhere (Krause, 2010), and subject to a *pmoA* specific diagnostic microarray analysis as described elsewhere with minor modification (Bodrossy et al., 2003; Ho et al., 2011).

Here, we analysed the effect of long-term drought on the diversity and function of the methanotrophs. We used air-dried paddy soils that were stored for varying periods, up to almost two decades (Figure 1). The recovery of methanotrophic activity and community composition of the rewetted soils were monitored using gas flux measurements and a diagnostic microarray, respectively. Duration of the experiment was over 80 days. Due to the *post-hoc* nature of this experiment, no data on the initial community composition in the different soils is available. However, samples were taken from an area of 100 m radius in the lowlands of the rivers Po and Sesia (Vercelli, Italy). These paddy fields are planted to wetland rice for at least one century (Lüke et al., 2010). At this spatial scale methanotroph communities in Vercelli didn't show any geographical pattern (Krause et al., 2009). Therefore, we assume the structure of methanotroph community to have been largely the same at the different sampling times.

Methanotrophic activity was monitored as described before (Ho et al., 2011). Methane uptake in all soils was similar until around 3 weeks, where the younger (stored since 2006, and 2010) and older (stored since 1993 and 1998) soils diverged (Figure 1). From here on, methane uptake was significantly higher in the younger soils (two-sided KS-test, average days 19-83, $P \leq 0.05$). This may be a result of an increase of cell-specific activity (Steenbergh et al., 2010), but is more likely due to population growth (Ho et al., 2011). Consistent with the methane uptake rates, we saw an increase in the hybridization signals in the *pmoA* specific microarray analysis for the different subgroups of methanotrophs (mainly type Ib), indicating growth in population size over time (Figure 2). In particular, type II specific probes (Mcy413; McyM309; NMsiT-271; Table 1, Figure 2) showed relatively higher hybridisation signal earlier (5 days) during the incubation, indicating their ability to form drought resistant spores/cysts that can take 7 to 15 days to germinate (Higgins et al., 1981). Later during the incubation (21-47 days), type II specific probes showed an overall increase in the hybridization signal as would be expected from a growing community. This was further supported by the increase in the hybridization signal of type II *pmoA* transcripts at 47 days (probe Mcy413; McyM309; Figure 2).

On the contrary, type I specific probes showed lower hybridisation signals early (2 days) during the incubation, indicating that their resting stages were less resistant to drought. However, later on (5 days), type I methanotrophs represented by *Methylobacter* (probes Mb_SL#3-300; Mb380) and *Methylosarcina* (probe Mmb562) showed *pmoA* gene expression in all soils except the oldest, corresponding to a first increase of methane oxidation (Figure 2). This is consistent with a study by Henckel and colleagues (2001), where they showed that type I methanotrophs can grow to high population sizes in a matter of days under favourable conditions, and that type II population is relatively stable, but generally present. It is possible that the presence of *Methylobacter* after 5 days in the 1993 and 2006 soils were masked by a dominance of *Methylosinus* and *Methylocystis* species (type II); the microarray analysis has a relative detection limit of around 5% abundance (Bodrossy et al., 2003). Other probes specific for type I methanotrophs targeting members of the rice paddy clusters (probe fw1.641; LW21.374; M90.253), *Methylococcus* (probe Mc396), and *Methylocaldum* (probe Mcl404) showed no appreciable hybridisation signal in the *pmoA* transcript.

Based on the standardized microarray data, we tested the effect of drought duration at the community composition (*pmoA* gene) and potential activity (gene transcript). First, we estimated the diversity of the methanotrophic community after two days incubation as Shanon–Weaver's diversity (H'). The diversity in the old soils were lower (1993, H'=2.15; 1998, H'=1.59) than in the younger soils (2006, H'=2.59; 2010, H'=2.65), indicating a higher species richness and/or evenness of methanotrophs in the younger soils. Further, we applied canonical correspondence analysis (CCA) to the same data. Results showed a significant effect on community composition (DNA; ANOVA, 299 permutations, $P \leq 0.05$), and a trend when targeting potentially active community (RNA; ANOVA, 299 permutations, $P=0.1$). The soil age explained 14.2 % of the total inertia at community level, and 13.1 % on transcript level, respectively (Figure 3).

The methanotrophic population present in the seed bank (Eller and Frenzel, 2005), and environmental conditions favouring different types of methanotrophs seem to determine the composition of a community. The methanotrophic seed bank

in rice paddies are thought to be dominated by type II (Eller et al., 2005; Eller and Frenzel, 2001). Our findings were consistent, showing type I to be relatively low in abundance or even undetectable at the beginning of the incubation. However, *Methylobacter* were the first active type I methanotroph, expressing the *pmoA* gene after already 5 days; their DNA were detected soon after (Figure 2). Although type II methanotrophs were initially present at higher relative abundance, transcripts were not immediately detected. The different responses of methanotrophs to desiccation and subsequent recovery reflect specific traits (see review Ho, 2012), enabling different subgroups to prevail under different conditions.

In conclusion, the methanotrophic activity and community is evidently affected by drought. Usually, type I methanotrophs were less desiccation resistant and became depleted from the seed bank with on-going drought. However, some type I methanotrophs (*Methylobacter*) form desiccation resistant resting stages that recovered well from long-term drought after re-wetting. Recurring drainage and flooding regime in rice paddies may have accounted for the selection of a desiccation-resistant community. Therefore, we would expect methanotroph communities in permanently flooded environments, like limnic or marine sediments, to be more vulnerable to desiccation.

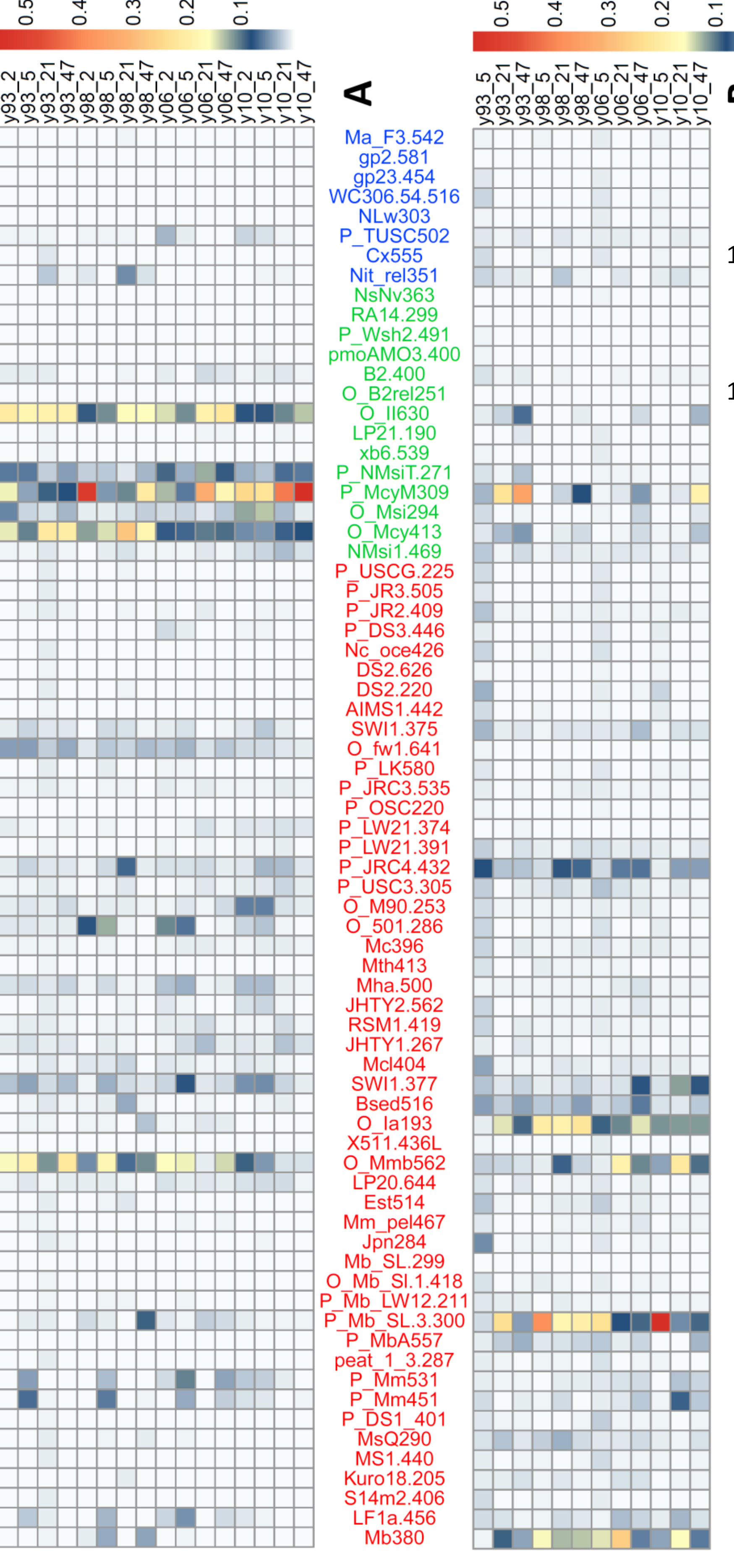

Figure 2: Heatmap showing the relative hybridization signals of the probes used for the canonical correspondence analysis shown in Figure 2. The two panels show the present (A) and the potentially active (B) community. Sample names are composed of soil age (1993, 1998, 2006 and 2010) and the incubation time (2, 5, 21 and 47 days). Red, green, and blue indicate the specificity of the probes for type I, type II, and other *pmoA* sequences, respectively

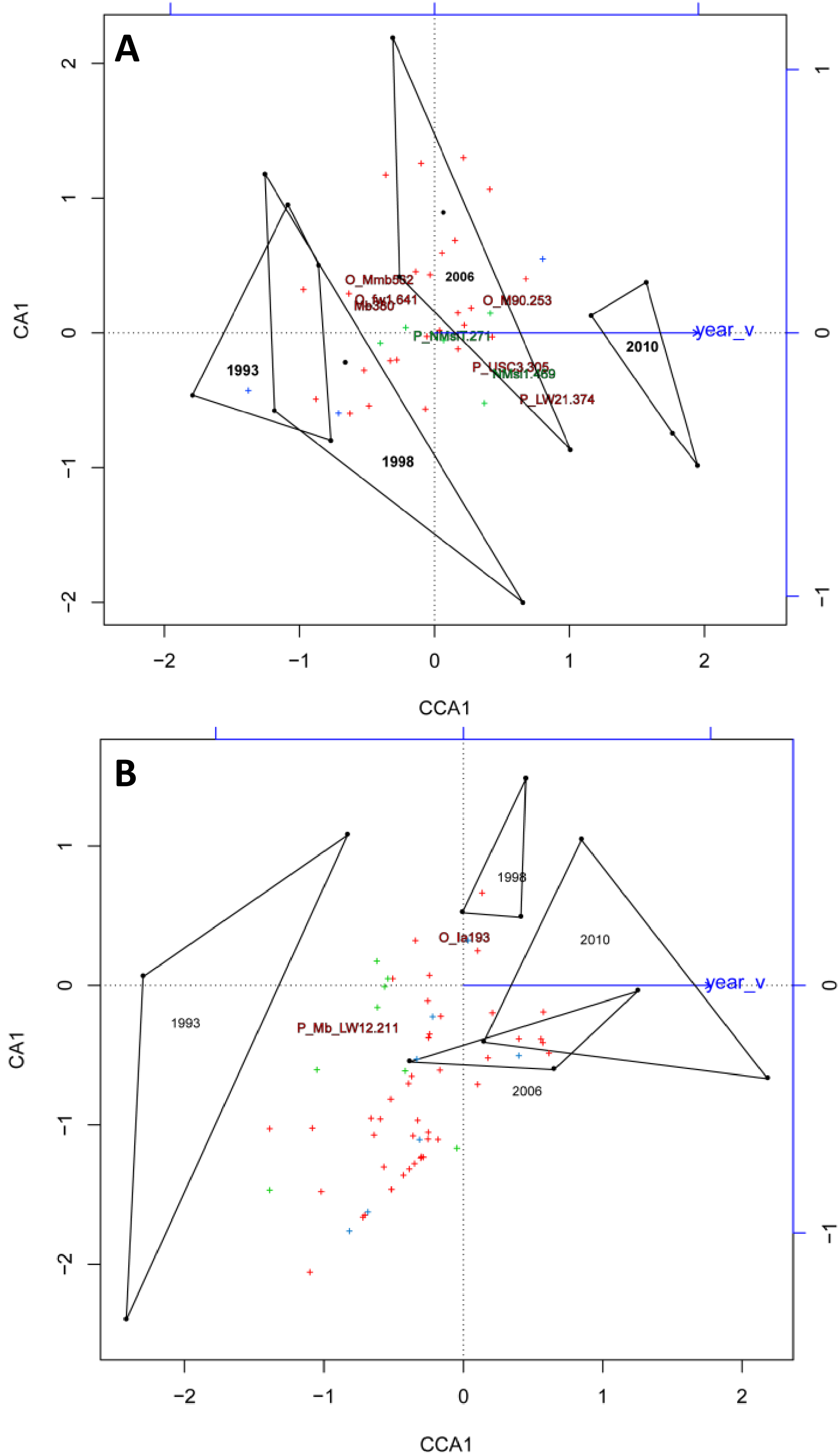
A
B
CA1
CCA1
year_v
1993
1998
2006
2010
O_Mmb562
O_M90.253
P_NMsIT.271
P_USC3.305
P_LW21.374
O_Ia193
P_Mb_LW12.211

Figure 3: Canonical correspondence analysis (CCA) of the methanotrophic community based on the microarray analysis of pmoA gene (A), and gene transcript (B). Probe selection was based on the subset defined by Krause et al (Krause et al., 2012) corrected by the removal of probes redundant for the samples analysed and addition of highly indicative probes (Dufrene and Legendre, 1997). The samples are shown as black symbols and joined by the hulls visualizing the different soil ages as indicated by the year of collection (1993, 1998, 2006, and 2010). The differences between the different aged soils are significant on the community level (P<0.01) while on transcript level only a tendency is shown (P<0.1, analysis of similarity, Bray-Curtis dissimilarity). The CCA analysis is conditioned against the incubation time with soil age as constraint. The coloured crosses mark the different probes. Probes which are indicative for one of the soils with a high probability (P<0.05) by indicator value analysis using the labdsv package in the R software environment are shown with their full name and discussed in the text. Red, green, and blue indicate the specificity of the probes for type I, type II, and other *pmoA* sequences, respectively. The relative hybridization signals for the probes used in the CCA are given in Figure 3.

Table 1: Overview of probes with a probability below 0.05 in the indicator species analysis as shown in Figure 2 and the groups covered by the probes.

Probe	Indicator value	Probability	Groups covered
fw1.641	0.4047	0.015	type Ib (RPCs and lake cluster)
Mmb562	0.4046	0.043	type I Methylosarcina
Mb380	0.7500	0.030	type Ia Methylobacter (Mbac)
NMsiT.271	0.4178	0.042	type II pmoA2
LW21.374	0.6971	0.007	type Ib (RPC1 & LW21)
NMsi1.469	0.6045	0.007	type II pmoA2
Mb_LW12.211	0.5681	0.015	type Ia (Mbac and LW12)
Ia193	0.4016	0.024	type Ia general probe
Mha.500	1.0000	0.022	type I Methylothermus

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4 Effect of energy flow on the susceptibility of aerobic methanotroph communities to disturbance

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4.1 Abstract

We used the aerobic methane oxidizing bacteria as a model group investigating the effect of disturbance on the community composition under different source strength. Previous studies suggested the resistance and resilience of methanotrophs to increase parallel to the energy flow. Disturbed and control paddy soil was incubated in microcosms, in methane-oxygen counter-gradients simulating the environmental conditions at the soil surface layer. Both high and low methane source strengths were provided. As a disturbance, we considered a simulated die-off event diluting native with gamma-ray sterilized soil. A *pmoA* microarray-based canonical correspondence analysis showed a significant effect of source strength as well as disturbance on community structure and taxon-specific activity. Quantitative analysis by ct-RFLP revealed type I methanotrophs to specifically benefit from an elevated energy flow. 16S cDNA amplicon pyrosequencing confirmed the dominance of type I in the active population. Growth of type II methanotrophs was unrelated to the methane source strength indicating facultative growth.

4.2 Introduction

Soils microbiota is highly diverse with one gram harbouring tens of thousands of different species (Fierer et al., 2007; Roesch et al., 2007). The different microbial communities in a soil fulfil complex ecosystem functions including degradation of organic matter, carbon and nutrient cycling and regulation of plant growth and primary production (Wall et al., 2012). Given their sheer abundance, the ability to react quickly to a changing environment and their high diversity, bacteria are assumed to have high functional redundancy levels and to be virtually inextinguishable (Ho et al., 2011b; Strickland et al., 2009; Wertz et al., 2006). However, some microbial clades were found to have strict habitat preferences (von Mering et al., 2007). Furthermore, community composition of bacterial groups has been shown to be susceptible to disturbances with community changes being reflected in changes of the ecosystem functioning (Allison and Martiny, 2008). To further investigate the effect of disturbances on the microbial diversity and physiological traits we used the guild of aerobic methanotrophs as a model. The main advantages of the aerobic methanotrophs is their well characterized physiology (Trotsenko and Murrell, 2008), their large, yet manageable, diversity (Lüke and Frenzel, 2011), and the sound number of molecular tools available to assess the community structure and diversity.

Aerobic methanotrophs are found in the phyla of Proteobacteria, Verrucomicrobia and recently in the candidate division 'NC10' (Ettwig et al., 2008). The methanotrophic Verrucomicrobia, however, are so far limited to extreme environments with high temperature and very low pH (Dunfield et al., 2007). Proteobacterial methanotrophs can be divided into type I and type II, corresponding to the families Methylococcaceae (type I, γ-Proteobacteria), Methylocystaceae and Bejerinckiaceae (type II, α-Proteobacteria; Semrau et al., 2010). The type I methanotrophs can be further divided into type Ia comprising amongst others the genera *Methylobacter*, *Methylomonas*, *Methylosarcina* and *Methylomicrobium*, and type Ib with *Methylococcus, Methylocaldum* and *Methylogaea*. The key enzymes in the methane oxidation pathway is the methane monooxygenase (MMO) existing as a membrane bound particulate (pMMO) or a soluble form (sMMO; Trotsenko and Murrell, 2008). All known methanotrophs possess pMMO except *Methylocella* and *Methyloferula* (Dedysh et al., 2000; Vorobev et al., 2011), while

sMMO is only found in some species. The *pmoA* gene encodes for the α-subunit of the pMMO. It has been shown to be highly conserved and to reflect the phylogeny of the 16S rRNA gene making it an excellent marker for studying methanotroph diversity (Degelmann et al., 2010; McDonald and Murrell, 1997).

As gradient organisms, which thrive at the very oxic-anoxic interface between the anoxic methanogenic bulk soil and the oxic soil surface, aerobic methanotrophs shape the environment generating overlapping methane and oxygen gradients (Gilbert and Frenzel, 1998; Reim et al., 2012). They perform an important ecosystem function by oxidizing a large portion (circa 50%) of the estimated global biogenic methane (Reeburgh, 2007). The local impact of methanotrophs can be even bigger, e.g. at the surface of flooded soils, where methane emission is reduced by up to 80% (Conrad and Rothfuss, 1991; Reim et al., 2012). Previous studies suggested that in methanotroph communities the vulnerability and resilience to disturbances correlates to the energy flow through a given system. Under high methane concentrations, methane oxidation is quite resistant to disturbance, but diversity and evenness decreased (Ho et al., 2011b). In contrast, methane oxidation in upland soils seems to be extremely vulnerable with recovery times up to decades after disturbance (Bodelier and Laanbroek, 2004; Nazaries, 2011; Prieme et al 1997). Under low methane conditions methanotrophs show much lower growth rates (Knief and Dunfield, 2005), which don't allow for sufficient compensation of population losses. Therefore, we expect the energy flow through a given habitat to be a key factor determining stability and hypothesize that the methanotroph community will be more resilient under high methane source strength.

We designed an experiment to test the effect of high and low source strengths on the recovery of a methanotroph community upon a simulated die-off. Italian rice field soil was incubated in gradient microcosms supplied with oxygen from above and with different methane mixing ratios from below, simulating the environmental conditions in the soil surface layer (Murase and Frenzel, 2007).

To simulate a severe die-off event of 97.5% of all bacteria we mixed fresh and sterilized soil in a ratio of 1:40. The significant effect on the composition of methanotrophic community and its recovery was already shown by Ho et al. (2010). However, with the high and low source strength we introduced an additional factor potentially influencing the efficiency of methanotrophs in recolonizing the free space.

The location of the oxic-anoxic interface in the soil surface layer was expected to change according to the different energy flow through the system (Murase and Frenzel, 2007). In the active zone forming around the oxic-anoxic interface we predicted a succession of methanotrophs with shifts from type I to type II as observed in other studies on methanotroph communities (Ho et al., 2011a; Krause et al., 2010).

During incubation the formation of the oxic-anoxic interface was followed with oxygen microelectrodes. Community structure and taxon-specific activity were analyzed using the *pmoA* gene and its transcript as a phylogenetic marker for methanotrophs, applying a diagnostic microarray (Bodrossy et al., 2003) and competitive PCR (Reim et al., 2012). The overall bacterial population was monitored using 16S cDNA amplicon pyrosequencing.

4.3 Material and Methods

Soil microcosm incubation and sampling

The soil was sampled from the experimental field of the CRA Agricultural Research Council, Rice Research Unit (Vercelli, Italy) in spring 2010. Soil parameters and agricultural practice have been reviewed earlier (Krüger et al., 2001). Gamma-ray sterilized soil (25 kGy; 60Co; McNamara et al., 2003) was prepared as described by Ho and colleagues (2011b). Fresh and sterilized soil was mixed in a ratio of 1:40, homogenized, and incubated in stainless steel microcosms. The construction and setup of the microcosms has been described previously (Murase and Frenzel, 2007). Briefly, fresh soil equivalent to 14 g of dry soil was mixed with sterilized water to reach a water content of 0.5 g water per g of dry soil for the control incubations. The 1:40 dilution was prepared in a similar fashion with 1 part fresh soil in 39 parts of sterilized soil. The homogenized soils were incubated on a polytetrafluoroethylene membrane dividing the microcosm into an upper and a lower compartment. Where the upper compartment contained oxygen at atmospheric concentrations and the lower compartment was connected to an external gas reservoir (volume 2 L). The gas mixture in the reservoir consisted of nitrogen gas and either 10 % or 0.35 % of methane for the high and low methane incubations, respectively. One disturbed and one undisturbed microcosm was connected guarantying identical incubation conditions. These microcosm pairs were connected to the same gas reservoir. In total, 24 microcosms (6 per treatment) were set up. Methane and oxygen concentrations were monitored regularly by gas chromatography. The microcosms were incubated for 3, 7 and 14 days at 25 °C in the dark. At each time point duplicates of the different incubations were sacrificed. Prior to sampling, vertical oxygen profiles were determined using an oxygen microelectrode (OX50, Unisense, Aarhus, Denmark). The microcosms were shock-frozen in liquid nitrogen and ground on dry ice for homogenization. Aliquots of 0.5 g were stored in 500 µl RNAlater-ICE (Ambion, Austin, Tex., USA) at −20 °C for subsequent nucleic acid extraction.

Nucleic acid extraction

Nucleic acids were extracted following the protocol of Lueders *et al.* (2004) with minor modifications (Krause et al., 2010). Total RNA was prepared by digestion of 1 µg total

nucleic acid with RQ1 RNase-free DNase (Promega, Madison, Wisc., USA) and subsequent purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

pmoA amplification

pmoA genes and transcripts were amplified using the A189f (5'-GGN GAC TGG GAC TTC TGG; Holmes et al., 1995) forward and the mb661r (5'-CCG GMG CAA CGT CYT TAC C; Costello and Lidstrom, 1999) reverse primer. The *pmoA* gene was amplified as described by Lüke and colleagues (2010) with some minor modifications in the amplification protocol. Template DNA was mixed with 2.5 U of Taq Polymerase (Invitrogen), 66 pmol of each primer, 0.02 mg bovine serum albumine (Roche), 5 % (v/v) DMSO, 25 µl Masteramp 2x PCR Premix F (Epicentre Biotechnologies) and filled up to 50 µl with molecular grade water (Sigma). The PCR was carried out with an initial denaturing step at 94°C for 2 min, followed by 30 cycles of 0.5 min at 94°C, 1 min at 55°C and 1 min at 68°C. Final elongation was performed for 7 min at 68°C. For *pmoA* transcript amplification the One-step Access RT-PCR System (Promega) was used as described elsewhere (Reim et al., 2012). Briefly, one µl purified template RNA was mixed with 5 µl AMV/Tfl 5× reaction buffer (Promega), 0.01 mg bovine serum albumin (Roche), 2.5 nmol of each dNTP (Promega), 8 pmol of each primer, 25 nmol $MgSO_4$ (Promega), 5% (v/v) DMSO, 20 U RNasin Plus (Promega), 2.5 U Tfl DNA polymerase (Promega), 2.5 U AMV reverse transcriptase (Promega) and molecular-grade water (Sigma-Aldrich, Munich, Germany) in a total volume of 25 µl. DNA contamination was excluded by performing control reactions without AMV reverse transcriptase. First strand of cDNA synthesis was done at 45 °C in 45 min, followed by 2 min at 94 °C to inactivate the AMV reverse transcriptase. The template was amplified in 35 cycles (30 s at 94 °C, 1 min at 55 °C, 1 min at 68 °C, final elongation 7 min at 68 °C). All PCR products were checked on a 1% agarose gel and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Competitive t-RFLP analysis

For the competitive t-RFLP analysis, the forward primer used in the amplification reactions was FAM-labelled. The whole procedure is described in detail by Reim and colleagues (2012). A minimum of three PCR or RT-PCR amplifications was performed per

sample as described above. Each reaction contained equal amounts of the environmental template and varying standard concentrations. After amplification, PCR products were processed and analysed by t-RFLP as described in an earlier study (Reim et al., 2012). The terminal restriction fragments were separated and detected with capillary electrophoresis on an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems; 30 min at 15 kV and 9 µA) and analysed with GeneMapper Version 4.0 (Applied Biosystems). The linear regression of the logarithms of standard-to-sample ratios to the logarithms of standard added was calculated, where the amount of standard at the equivalence point equals the unknown environmental copy number (Freeman et al., 1999).

Microarray analysis

For microarray analysis a T7 promoter site labelled reverse primer was used for amplification of the *pmoA* genes and transcripts, respectively (Bodrossy et al., 2003). *In vitro* transcription, fragmentation, hybridization, scanning and data analysis were performed as described elsewhere (Stralis-Pavese et al., 2004; Stralis-Pavese et al., 2011).

16s cDNA amplification and Pyrosequencing

16s rRNA of all samples was in-vitro transcribed into cDNA using random hexamer primers and M-MLV reverse transcriptase (Roche, Basel, Switzerland), according to manufacturer's instructions. In short, 8 µl of purified RNA was mixed with 2 pmol of the random hexamer primers (Roche, Basel, Switzerland), filled up to 10 µl, incubated for 5 minutes at 75°C and chilled on ice immediately. For cDNA synthesis 1 µl dNTPs (Promega, Madison, WI, USA), 4 µl M-MLV 5xreaction buffer (Promega, Madison, WI, USA), 0.4 µl Recombinant RNasin® Ribonuclease Inhibitor (Promega, Madison, WI, USA), 0.8 µl M-MLV reverse transcriptase (Promega, Madison, WI, USA), filled up to 20µl with Nuclease-Free Water and incubated at 37°C for 60 minutes. The 16s cDNA was amplified using the 343Fmod (TACGGGWGGCWGCA) and 784Rmod (GGGTMTCTAATCCBKTT) primer set (Köhler et al., 2012). Template cDNA (2 µl) was mixed with 5 µl 10X PCR buffer (Invitrogen, Darmstadt, Germany), 5 µl of dNTP mix (10mM each), 2.5 µl of each tagged primer (10 pmol / µl), 1.5 µl $MgCl_2$ (50 mM, Invitrogen, Darmstadt, Germany), 0.5 µl Taq DNA Polymerase (Invitrogen, Darmstadt, Germany) and molecular-grade water (Sigma-

Aldrich, Munich, Germany) in a total volume of 50 µl. After an initial denaturizing step at 94°C for 3 minutes the template was amplified in 25 cycles (1 min at 94 °C, 1 min at 57 °C, 1 min at 72 °C, final elongation 10 min at 72 °C). The amplicons were purified via gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Equimolar amounts of the tagged amplicons were mixed and sequenced in the Max Planck Genome Center Cologne (Collogne, Germany) using the 454 GS FLX Titanium chemistry (Roche, Mannheim, Germany).

Statistical analysis

Microarray hybridization signals of a subset of the probes representing the whole phylogeny of methanotrophs covered by the microarray (Krause et al., 2012) were standardized (i) against the mean of the overall array intensities (Lüke et al., 2011) and (ii) against an experimentally determined reference value for positive detection (Bodrossy et al 2003). Statistical analysis and graphics were done in R (R Development Core Team, 2012). Analysis of similarity (ANOSIM) and canonical correspondence analysis (CCA) were done using the *vegan* package, version 2.0-4 (Oksanen et al., 2012). The indicator species analysis was performed according to Dufrene and Legendre (1997) using the labdsv package, version 1.5-0 (Roberts, 2012).

The pyrosequencing data was analysed using the mothur software platform version 1.27.0 (Schloss et al., 2009). Sequencing errors were corrected using PyroNoise as implemented in mothur (Quince et al., 2009). Sequences were trimmed, grouped, aligned and classified against the greengenes reference database in mothur (DeSantis et al., 2006). Sequences from replicate microcosms were combined for analysis of alpha diversity and relative abundance. Diversity was assessed using Good's nonparametric coverage estimator and the inverse Simpson index. The relative abundance was calculated from all sequences at phylum and subphylum level for the Proteobacteria. Additionally, all sequences classified as *Myxococcacea*, Chloroflexi, Bdellovibrionales, *Beijerinckiaceae*, *Methylocystaceae* and Methylococcales were added to a 16S SILVA reference database using the ARB software package (Ludwig et al., 2004) and checked for correct classification.

4.4 Results

The methane concentration in the upper compartment was measured as proxy for the activity of the methanotrophs in the soil microcosm incubations. Passage of methane through the soil layer has ceased in all incubations by the sixth day. The initial amount of residual methane accumulating in the upper compartment was highest in the disturbed incubations under high methane (980 ppm_v h^{-1}) and decreased to 3.4 ppm_v h^{-1} on the sixth day. In the low methane incubations only around 1.5 ppm_v h^{-1} methane accumulated on day one and decreased below detection limit later (Figure 1). Therefore, methane-oxidation can assumed to be active in all incubation from the sixth day of incubation on. Formation of the oxic-anoxic interface was verified by oxygen-microelectrode measurements. After three days of incubation oxygen was detected down to a depth of 4 mm in all microcosms. Interface location shifted upwards in the high methane incubations with incubation time (2 mm at day 14); while in the low methane incubations the oxic-anoxic interface stabilized around 4 mm (Figure 2).

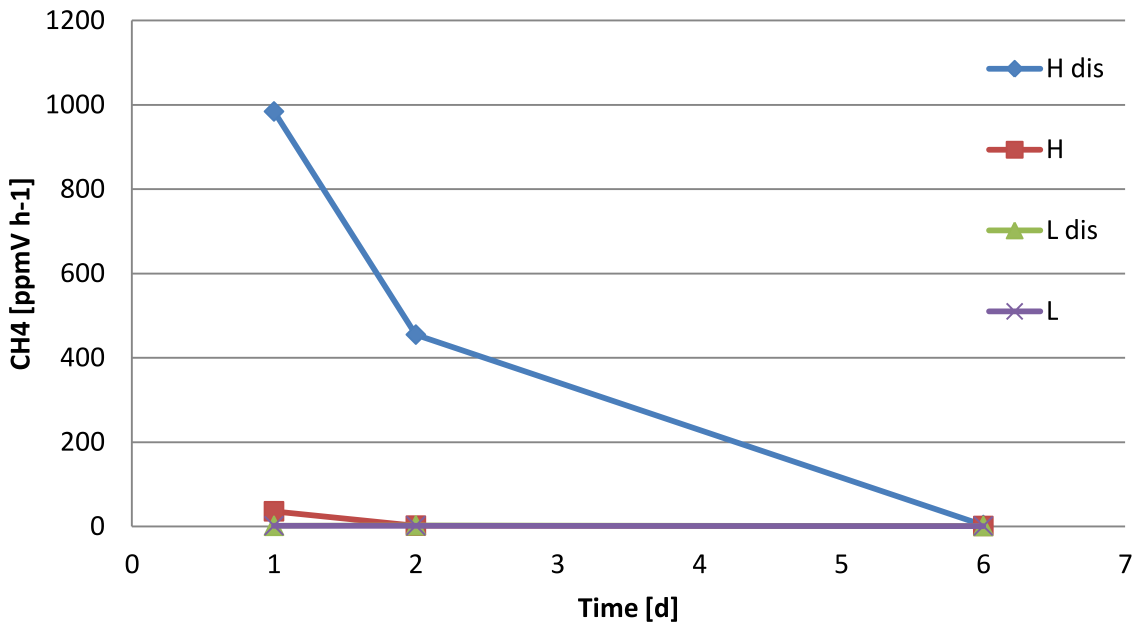

Figure 1: Methane accumulation in the upper compartment of the microcosms during the first days of the incubation.

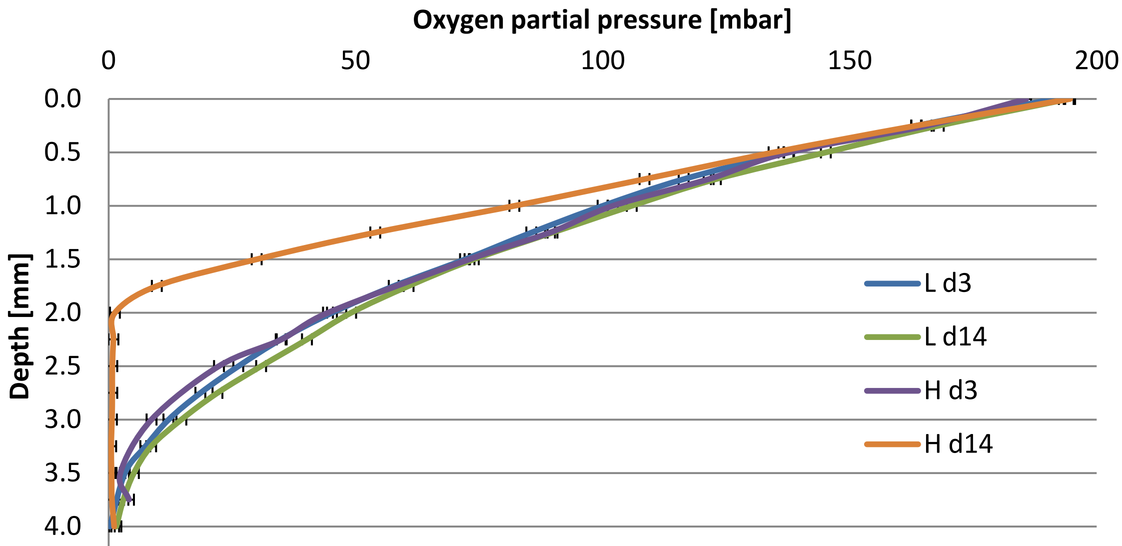

Figure 2: Oxygen profiles measured in the undisturbed incubations after different incubation time. The labels indicate the methane concentrations in the incubations (high / H and low / L) as well as the incubation time in days (3, 7 and 14). Profiles were measured using an oxygen-microsensor (Unisens, Aarhus, Denmark) in duplicates in duplicate microcosms (standard deviation, n=4).

To follow the recovery of the different groups of methanotrophs we sacrificed duplicate microcosms of each treatment on three time points throughout incubation. Apparent and active community composition was analysed by competitive t-RFLP and *pmoA*-specific microarray analysis.

To verify the influence of the different source strengths as well as the disturbance we used constrained correspondence analysis (CCA) to visualize the effect on the apparent and the active methanotroph community in the different incubations (Figure 3 and Figure 4). The CCA was calculated based on hybridization patterns retrieved from the *pmoA* specific microarray analysis. To focus on the effect of source strength and disturbance we conditioned the analysis for incubation time (Oksanen, 2012). On DNA level we found that the methanotroph community present is strongly influenced by the source strength (ANOVA, P=0.005) as well as the disturbance applied (ANOVA, P=0.02). The samples from the different incubations form distinct clusters. To see if there are methanotrophs indicative for either high or low methane fluxes we performed indicator species analysis (Dufrene and Legendre, 1997). The indicator species analysis exclusively reported probes specific for the low methane incubations. The type I probes (JHTY1.267, O_fw1.641, O_M90.253 and P_JRC3.535) were indicative for clusters containing high numbers of type Ib clones from paddy soil like FW1 and RPC1 (Table 1). Additionally two

type II specific probes were identified to be indicative with a high probability (P_NMsiT.271 and Msi232; Table 1). The P-NMsiT.271 probe is specific for type II *pmoA2* while Msi232 covers *Methylosinus pmoA* copies. Though most were not identified in the indicator species analysis the separation of the high and the low methane incubation samples in general can be attributed to type I methanotrophs specific probes (Figure 3 and Figure 4).

A similar trend is observed on the level of the active community. The split of the samples according the different treatments is even more pronounced although here no type II specific probes were found to be indicative for either of the incubations (Figure 4). However, one of the type I methanotroph specific probes (P_Mb_SL.3.300) splitting the high from the low methane incubations was highly significant in the analysis and is indicative for *Methylobacter* related sequences.

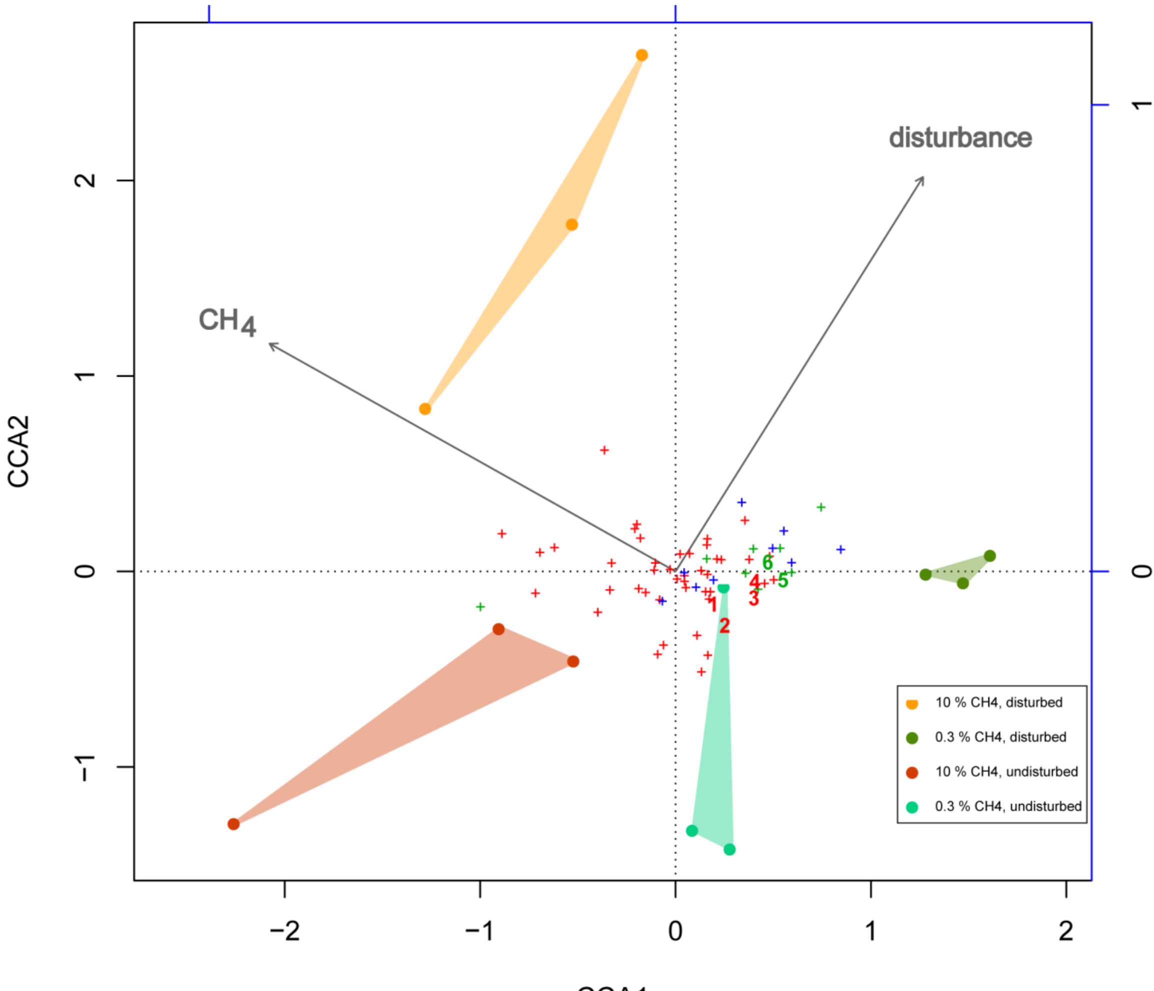

Figure 3: Constrained correspondence analysis based on the microarray *pmoA* gene hybridization patterns. For probe selection see Krause *et al.* 2012. Sample points are averages of replicate incubations for the three time points per treatment. The coloured crosses mark the different probes. Probes which are indicative for one of the source strengths with a high probability (P≤0.01) are represented by number (1, P_JRC3.535; 2,

O_M90.253; 3, O_fw1.641; 4, JHTY1.267; 5, P_NMsiT.271; 6, Msi232). Indicator values as well as the clusters targeted by these probes are shown in Table 1. The colour codes the specificity of the probes for type I, type II and other *pmoA* sequences (red, green and blue).Data was conditioned on incubation time to show the effect of the source strength (CH4) and the application of the disturbance. The two constrains together explain around 34 % of the total inertia and are both significant (CH_4, P=0.005; disturbance, P=0.02).

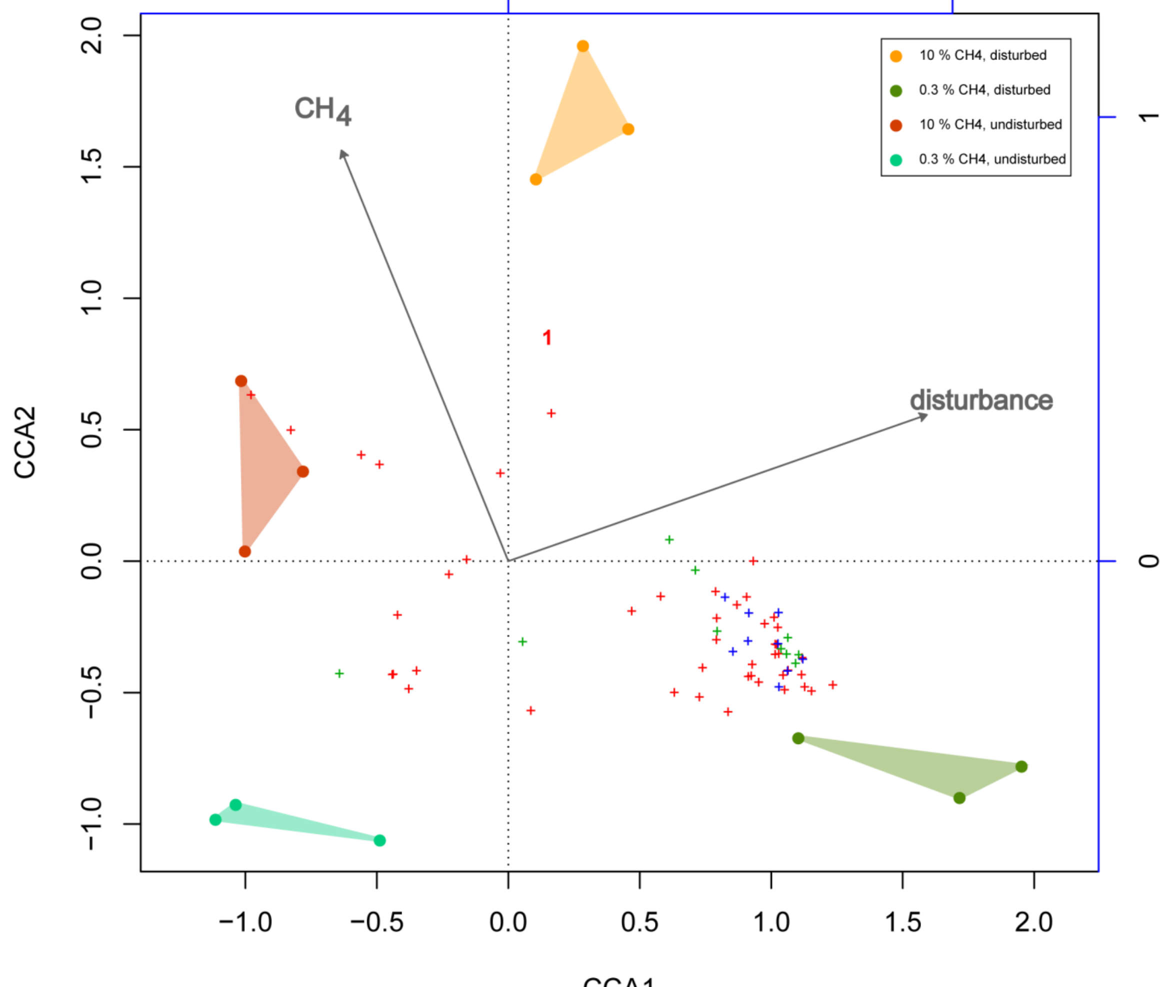

Figure 4: Constrained correspondence analysis based on the microarray *pmoA* transcript hybridization patterns. For probe selection see Krause *et al.* 2012. Sample points are averages of replicate incubations for the three time points per treatment. The coloured crosses mark the different probes. Only one probe was indicative for high source strength with a high probability (P≤0.01) and is represented by number (1, P_Mb_SL.3.300). Indicator values as well as the clusters targeted by these probes are shown in Table 1. The colour codes the specificity of the probes for type I, type II and other *pmoA* sequences (red, green and blue). Data was conditioned on incubation time to show the effect of the source strength (CH4) and the application of the disturbance. The two constrains together explain around 46 % of the total inertia and are both significant (CH_4, P=0.005; disturbance, P=0.005).

Table 1: Probes indicative for the high and low methane incubations on *pmoA* genes and transcripts. The indicator species values for probes with significant values (P< 0.01) and the according groups covered by the probes are given. Indicator species analysis was done based on the *pmoA* specific Microarray hybridisation patterns of a subset of probes, selected according to Krause and colleagues (2012), using the R software and the labdsv package.

pmoA	CH_4	Probe	Indicator value	Genus/species/cluster targeted	
DNA	**low**	P_NMsiT.271	0.7533	**Type II**	pmoA-2b
		JHTY1.267	0.7087	**Type Ib**	JHTY (JRC-4) Methylogaea like
		O_fw1.641	0.7162	**Type Ib**	FW1
		O_M90.253	0.6949	**Type Ib**	RPC-1a
		P_JRC3.535	0.6456	**Type Ib**	JRC-3a
		Msi232	0.7023	**Type II**	Methylosinus
RNA	**high**	P_Mb_SL.3.300	0.8697	**Type Ia**	*Methylobacter* sp. LW12 related

The quantification of the type I and type II methanotroph community with competitive t-RFLP (ct-RFLP) showed, that the type I methanotrophs reacted to an increase in the source strength of the substrate methane. Compared to the incubations under low methane we observed faster growth in the undisturbed high methane incubations (Figure 5). Under disturbed conditions the pattern is similar: high methane concentrations lead to higher *pmoA* copy numbers in equivalent periods of time. After 14 days of incubation the size of the type I community in high methane incubations was similar, but in the undisturbed incubations the type I peak at the third day (6.74×10^8 *pmoA* copies per gram soil). While in the low methane incubations the *pmoA* copy number increasing almost linearly over time (Figure 5). The type II community size in the undisturbed incubations increased as well. However, the growth observed was independent of the source strength. In both the low and the high undisturbed methane incubations, the size of type II community increased, from 5.52×10^8 to 9.66×10^8 and 8.78×10^8 *pmoA* copies per gram soil, between the first and seventh day, respectively. The effect of the disturbance itself was more prominent, since no net growth of the type II was observed in these incubations regardless of the source strength applied.

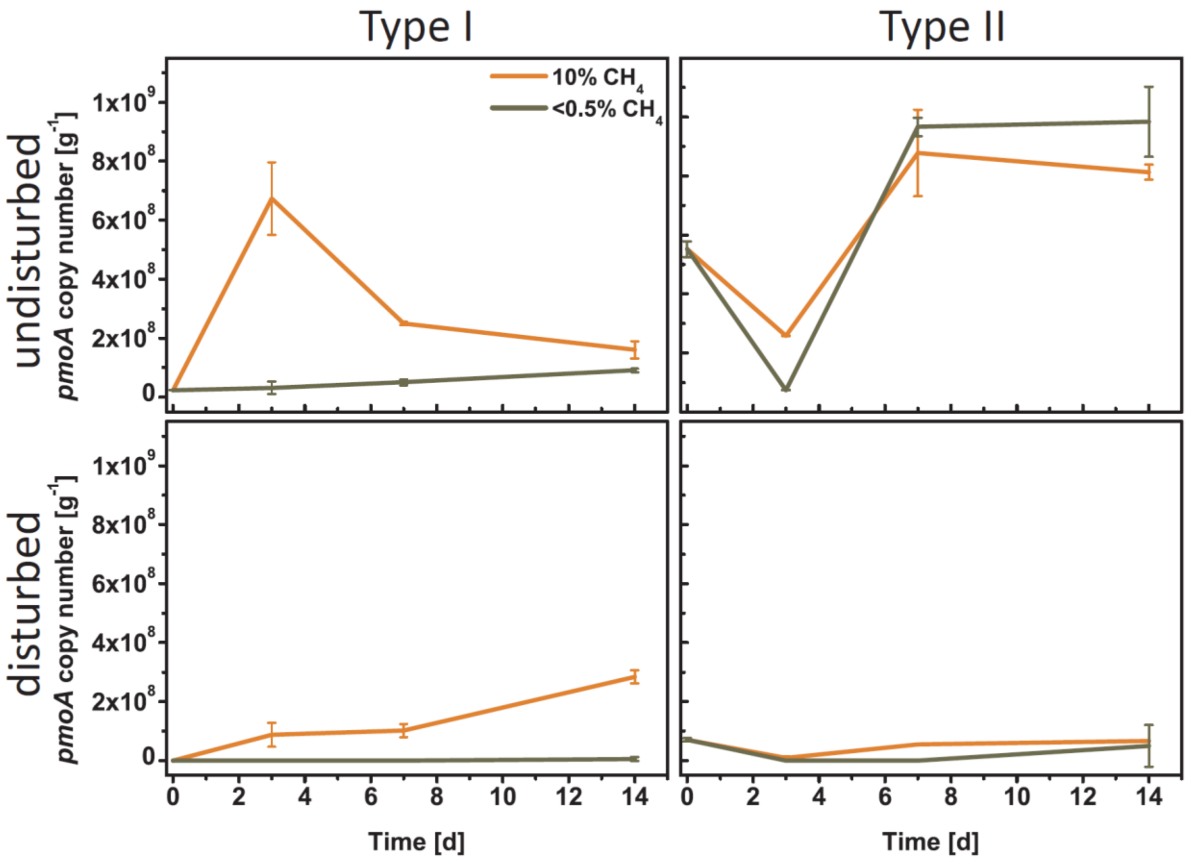

Figure 5: Effect of the source strength on the type I and type II community size. Copy numbers of the *pmoA* gene were quantified by competitive t-RFLP.

The *pmoA* transcript shows a similar pattern as the *pmoA* genes. The increases in *pmoA* gene copy numbers observed for the type I are generally supported by the detection of *pmoA* transcripts (Figure 6). The strong increase in *pmoA* copy number observed for the type I in the undisturbed high methane incubations on the third day coincide with a peak in the *pmoA* transcript number (2.12 x 10^8 *pmoA* copies per gram soil) in the same sample (Figure 5 and Figure 6). The amount of type II *pmoA* transcripts detected is always much lower and doesn't fit the *pmoA* gene pattern. For example on day 7 where we observed a strong increase in the type II *pmoA* copy number in the undisturbed high and low methane incubations only comparatively low numbers of *pmoA* transcript (7.96 x 10^6 and 5.72 x 10^6 *pmoA* copies per gram soil) were found to be present (Figure 5 and Figure 6).

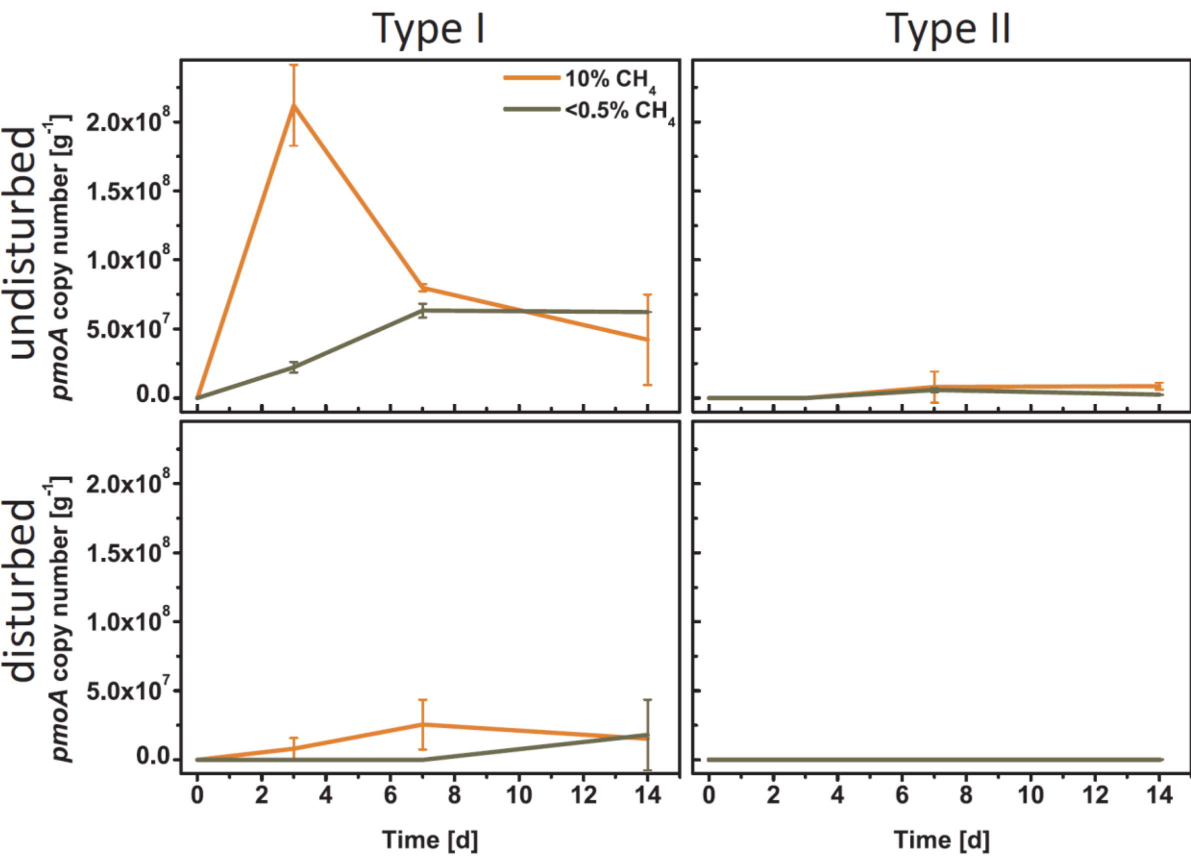

Figure 6: Effect of the source strength on the type I and type II active community. Copy numbers of the *pmoA* transcripts were quantified by competitive t-RFLP.

To verify the data retrieved by ct-RFLP on the methanotroph active community we performed 16S cDNA amplicon pyrosequencing. On the subphylum level all samples had a coverage greater than 99% indicating sufficient sampling depth. Diversity assessed by the inverse Simpson index was significantly lower in the disturbed incubations (ANOVA; P=0.04). The methane source strength (ANOVA; P=0.20) and the incubation time (ANOVA; P=0.32) on the other hand had no significant effect on the diversity. About 7% of the 42000 processed 16S cDNA sequences were classified as methanotrophs (Table 2). Type Ia *Methylobacter* sequences reached up to 18.5 % relative abundance (sample d3 H-u; Table 2). Type Ib (*Methylocaldum* and *Methylococcus*) specific sequences were not detected in either of the treatments and type II specific 16S sequences were almost entirely classified as *Methylocystis* (Table 2).

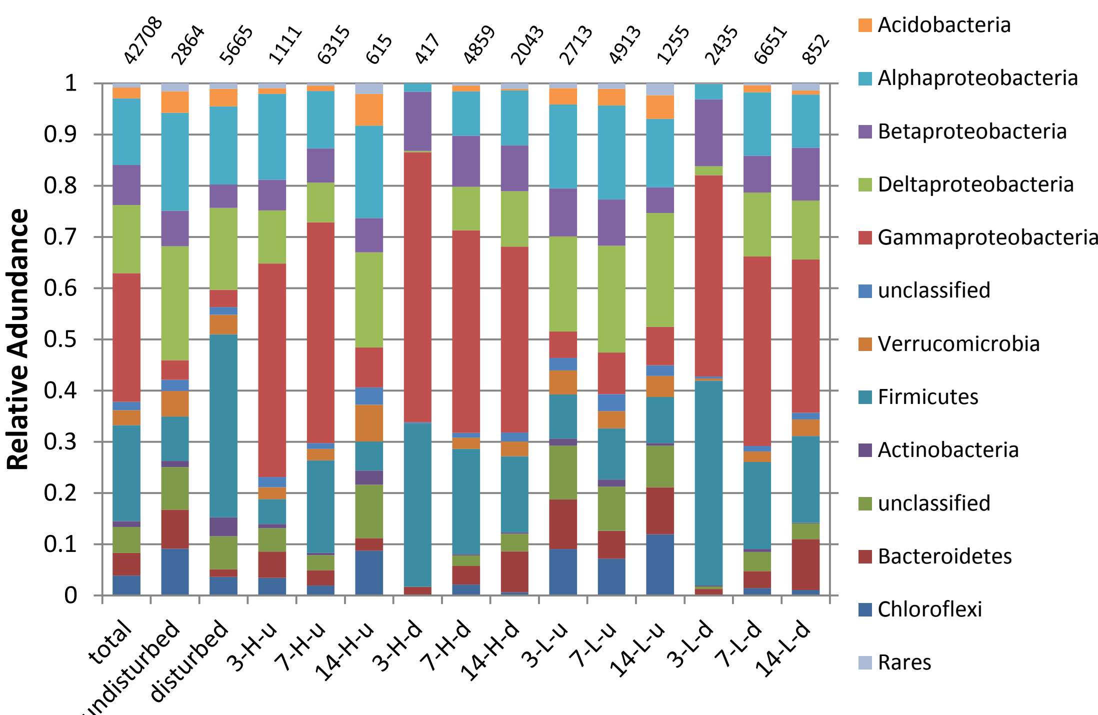

Figure 7: Taxonomic profiling of the 16S ribosomal RNA pyrosequencing data. The column ‘total’ contains all corrected and classified sequences. Phyla with less than 1% relative abundance in total were combined in the ‘Rares’ group. The sample labels are composed from incubation time (3, 7 and 14 days), methane concentration (**H**igh and **L**ow) and the application of the disturbance (**u**ndisturbed and **d**isturbed). On the top x-axis the number of sequences in the corresponding sample is given. All Samples (except 14_H_d) contain 16S cDNA sequence data from duplicate microcosms.

Table 2: Partial relative abundance data: only the taxonomic groups containing methanotrophs or possible predatory bacteria are shown. The classification was verified by phylogenetic analysis of the corresponding 16S cDNA sequences in the ARB software environment. Labels show incubation time (**d**ays **0**, **3**, **7** and **14**), methane concentration (**H**igh and **L**ow) and the application of the disturbance (**u**ndisturbed and **d**isturbed)

		d0	d0	d3	d7	d14	d3	d7	d14	d3	d7	d14	d3	d7	d14
taxon	**total**	**undisturbed**	**disturbed**	**H-u**	**H-u**	**H-u**	**H-d**	**H-d**	**H-d**	**L-u**	**L-u**	**L-u**	**L-d**	**L-d**	**L-d**
Bacteria[1]	1.0 (42708)	1. 0 (2864)	1. 0 (5665)	1. 0 (1111)	1. 0 (6315)	1. 0 (615)	1. 0 (417)	1. 0 (4859)	1. 0 (2043)	1. 0 (2713)	1. 0 (4913)	1. 0 (1255)	1. 0 (2435)	1. 0 (6651)	1. 0 (852)
Alphaproteobacteria	0.130	0.191	0.153	0.167	0.112	0.180	0.017	0.087	0.107	0.164	0.184	0.134	0.030	0.123	0.103
Rhizobiales	0.089	0.133	0.119	0.140	0.063	0.141	0.014	0.046	0.059	0.116	0.140	0.091	0.018	0.086	0.053
Beijerinckiaceae	0.001	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000
Methylocystaceae	0.025	0.028	0.030	0.087	0.021	0.072	0.002	0.006	0.003	0.030	0.045	0.022	0.000	0.023	0.000
Methylocystis	0.017	0.016	0.023	0.046	0.015	0.054	0.000	0.005	0.000	0.019	0.035	0.016	0.000	0.015	0.000
Methylosinus	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
unclassified	0.008	0.012	0.008	0.041	0.006	0.018	0.002	0.001	0.003	0.011	0.010	0.006	0.000	0.008	0.000
Gammaproteobacteria	0.251	0.038	0.034	0.417	0.431	0.078	0.528	0.395	0.363	0.052	0.081	0.075	0.393	0.370	0.299
Methylococcales	0.043	0.012	0.011	0.391	0.048	0.059	0.017	0.002	0.052	0.022	0.043	0.049	0.000	0.079	0.002
Methylococcaceae	0.043	0.012	0.011	0.391	0.048	0.059	0.017	0.002	0.052	0.022	0.043	0.049	0.000	0.079	0.002
Methylobacter	0.021	0.006	0.004	0.185	0.029	0.021	0.012	0.001	0.033	0.006	0.009	0.014	0.000	0.047	0.001
Methylocaldum	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Methylococcus	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
Methylomonas	0.003	0.001	0.000	0.029	0.005	0.005	0.002	0.000	0.003	0.002	0.001	0.002	0.000	0.007	0.000
Methylosarcina	0.006	0.002	0.002	0.064	0.004	0.010	0.000	0.000	0.001	0.004	0.013	0.005	0.000	0.008	0.000
unclassified	0.013	0.002	0.005	0.113	0.011	0.020	0.002	0.000	0.015	0.010	0.019	0.028	0.000	0.017	0.001
Deltaproteobacteria	0.133	0.222	0.160	0.104	0.077	0.185	0.002	0.085	0.108	0.185	0.209	0.222	0.018	0.125	0.115
Bdellovibrionales	0.003	0.002	0.002	0.001	0.004	0.007	0.000	0.001	0.001	0.001	0.004	0.004	0.000	0.005	0.000
Bacteriovoracaceae	0.001	0.001	0.001	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.001	0.000
Bdellovibrionaceae	0.002	0.001	0.001	0.001	0.004	0.005	0.000	0.001	0.001	0.001	0.002	0.002	0.000	0.004	0.000
Myxococcales	0.042	0.076	0.065	0.041	0.021	0.068	0.000	0.020	0.016	0.058	0.076	0.107	0.004	0.027	0.009
Chloroflexi	0.039	0.091	0.036	0.034	0.020	0.088	0.000	0.021	0.006	0.091	0.072	0.120	0.000	0.014	0.011

[1] given in parentheses is the numbers of sequences retrieved for each sample.

4.5 Discussion

The effects of energy flow on the apparent and active methanotrophic community in native and diluted paddy soil were studied. We show that a high and low source strength with the correlating energy flows favour different methanotrophic communities, indicating specification of the methanotrophs to different ecophysiological niches.

In all microcosms, methane accumulation in the upper compartment decreases (day 6; Figure 1), indicating methanotrophic activity at the oxic-anoxic interface (Reim et al., 2012). This was expected since the experimental design mimics *in-situ* conditions in the soil surface layer (Murase and Frenzel, 2007). However, the oxic-anoxic interface is located closer to the surface in high methane source strength incubations (Figure 2). Since the oxygen concentration was relatively constant, this was a result of the increased methane flux into the system. Hence, under both high and low methane source strengths, a dynamic steady state was reached indicating highly different methane turnover rates and that methane is a limiting factor for methanotrophic activity in the soil surface layer (Brune et al., 2000; Krause et al., 2012); the potential for methane oxidation is probably higher. Previous studies using a similar experimental setup showed complete methane oxidation under even higher source strength (Krause et al., 2012; Murase and Frenzel, 2007). Moreover, we showed in an earlier work that the increased metabolic rates necessary for the complete oxidation of the methane are achieved not only by an increase in the methanotrophic community size at the interface; but an enhanced species-specific activity indicated by a strong increase in the *pmoA* transcript to gene ratio (Reim et al., 2012). Hence, we expect the methanotrophs in the high methane incubations to recover faster from the application of the disturbance not only by growth but also by an increase in methanotrophic activity. Since a well-adapted subset of the present methanotrophic community will make use of the high source strength available, and lead to a change in detectable diversity and/or evenness of the apparent and active community. Indeed, we observe at both levels, a good separation of the communities in the treatment and reference microcosms using canonical correspondence analysis (CCA; Figure 3, Figure 4). Considering the underlying probe patterns, it is obvious that type I specific probes drive the separation of the high from the low methane treatments, while the type II specific probes are characteristic for the low methane incubations (Figure 3,

Figure 4). At the transcript level, the only highly indicative probe is *Methylobacter*-specific (type Ia), suggesting that the type I in particular, type Ia are the subpopulation reacting to the increased energy flows at the interface. Further, taking into account the results of the ct-RFLP, we can specify that the type I indeed not only recover faster under high energy flows, but also grow to high densities in the undisturbed treatments and show a higher activity in the *pmoA* transcription compared to type II methanotrophs (Figure 5, Figure 6). Moreover, the pyrosequencing data corroborate the results of the competitive t-RFLP (ct-RFLP) analysis. The relative abundance of the type I methanotrophic members of the γ-Proteobacteria is similar to the abundance quantified by ct-RFLP. The peak observed in the relative abundance of the type Ia (Table 2; d3 H-u; *Methylobacter*, *Methylomonas*, *Methylosarcina*) in the undisturbed high methane incubation correlates to the peak in the type I methanotrophs as quantified by ct-RFLP (Figure 5, Figure 6). This is consistent with the findings of an earlier study where *Methylobacter* were identified to be indicative for high methane source strength (Krause et al., 2012).

However, the role of the type II methanotrophs in the soil surface layer is still unclear. A growing number of studies detected relatively higher genomic type II *pmoA* copies than their corresponding transcripts (Bodrossy et al., 2006; Dumont et al., 2011; Krause et al., 2012; Reim et al., 2012). We found the lack in the type II *pmoA* transcripts to correlate to a low relative abundance in the 16S cDNA, indicating that type II methanotrophs might generally have lower expression rates than type I. However, the type II might as well have entered the stationary phase already by day 7, since the community size does not increase further between day 7 and day 14 (Figure 5), which would explain the low number of *pmoA* transcripts and 16S cDNA sequences.

The independence of the type II methanotroph *pmoA* copy numbers from methane source strength suggests that they use alternative substrates for growth. In earlier work, the methane flux over the soil layer in such microcosms was determined with 20.17 ± 1.57 and 1.09 ± 0.06 nmol CH_4 cm^{-2} h^{-1} (mean ± SE) at nominal source strengths of 20% and 2% (v/v), respectively (Krause et al., 2012). The methane requirement for cell duplication in *Methylocystis* sp. RP1, a type II methanotroph isolated from paddy soil (Gilbert, 1997), was determined to be 0.077 ± 0.004 pmol CH_4 per cell (mean ± SE; Reim, 2007). Based on these results we calculated the expected increase in type II *pmoA* copy number that could be supported by the high and low source strength

to be around $1.5x10^7$ and $4.3x10^5$ copy per gram soil, respectively (day 3 to day 7). However, these are only rough estimates and should therefore be treated with care. Nevertheless, the large discrepancy between expected and observed type II copy number support the suggested presence of facultative type II methanotrophs, using substrates other than methane for growth (Belova et al., 2012; Dedysh et al., 2005; Semrau et al., 2011).

Furthermore, type II methanotrophs possess a second isozyme particulate methane monooxygenase (pMMO2) that has been shown to oxidize methane down to trace levels (Baani and Liesack, 2008). Indeed probe P_NMsiT.271, which is specific for *pmoA*-2b, was indicative for low methane source strength incubations (Table 1, Figure 3); indicating that the presence of pMMO2 is advantageous for type II methanotrophs when methane concentration is low. Therefore, type II methanotrophs can potentially outcompete type I in certain environments (e.g. above the oxic-anoxic interface where methane concentration is extremely low). In our earlier work (Reim et al., 2012), we accessed the vertical microstructure of the methanotrophic community and found that type II were present in relatively high abundances above and below the interface. Since we only sampled the endpoint of the experiment (day 14) and detected no *pmoA* gene transcripts, we assumed them only to be present as resting cells. However, considering their growth observed in the present study we cannot exclude that they showed similar growth in the earlier experiment.

Both traits reviewed above, pMMO-2 possession and facultative growth could help explain type II methanotrophs independence of methane source strength; enabling them to grow apart from the oxic-anoxic interface as observed before (Reim, 2012).

Finally, the decrease in type I methanotroph population size observed in the type I genomic copy and transcript number after the third day in the undisturbed high methane incubations could be caused by grazing of predatory bacterial species or protists. Murase and Frenzel (2007) found *Myxobacteria*-related sequences to be labeled in a $^{13}CH_4$ SIP study, suggesting that predatory δ-Proteobacteria species may have preyed on the labeled methanotrophs. However, the obligate predators Bdellovibrio spp., Bacteriovorax spp. and Peridibacter spp. were only found in small numbers in all samples (Figure 7, Table 2). The relative abundance was highest in the undisturbed high methane incubations where they reached a relative abundance of up to 0.7 %. The relative

abundance of the Chloroflexi with 10 % in all of the samples is quite high (Figure 7, Table 2). However, none of the Sequences retrieved were classified as *Herpetosiphon* species which are the only known predatory Chloroflexi (Jurkevitch and Davidov, 2007; Quinn and Skerman, 1980). The only group containing predatory bacteria that was abundant enough in the undisturbed high methane incubations to have a significant effect on the methanotrophic community is the Myxococcales. Most of these myxobacteria are proteolytic and exhibit bacteriolytic activities, feeding among other substrates, on dead, and live bacterial cells alike (Jurkevitch and Davidov, 2007). However, the Myxococcales had an even higher relative abundance in the undisturbed low methane incubations where we observed a slow but constant growth of type I methanotrophs till the end of the incubation (Figure 5 and Table 2), indicating that grazing by myxobacteria is not the reason for the decrease in the type I population size in the high methane incubations. Nevertheless, we can still not rule out, that the type I methanotrophs were depleted by grazing. Since Murase and Frenzel (2008) showed that soil protozoan grazers specifically prefer type I over type II methanotrophs, indicating that they may exclusively feed on methanotrophs at the oxic-anoxic interface (Murase and Frenzel, 2007; Murase and Frenzel, 2008). However, testing the effect of protozoan grazers on the methanotrophic community would require further analysis (e.g. 18S pyrosequencing analysis).

In over 42000 sequences retrieved by 16S cDNA pyrosequencing, none were classified as *Methylocellales* or *Methyloferula*, and only 0.07 % of the sequences clustered inside the Beijerinckiaceae. Therefore, it is not unreasonable to assume that they are of no major importance in the paddy soil studied here, even though they were recently shown not to be as restricted to acidic environments as previously thought (Rahman et al., 2011). Hence, we conclude *pmoA* to be a valid functional marker to assess the methanotrophic community in the present study.

4.6 Conclusions

Here we demonstrated the methanotrophic community under high source strength to be more resilient; recovering faster from the simulated die-off. Even if this is only true for type I methanotrophs since as discussed, we cannot resolve the activity of the type II as a function of the methane concentration. In summary, we can conclude that the energy flow as a function of the source strength has a major impact on the

methanotrophic community composition. The methanotrophic community was shown to be functionally redundant in the paddy soil examined. Type I methanotrophs immediately reacted to bursts of methane relative to its source strength. Even upon disturbance, methane oxidation was restored in less than a week. While the type II methanotrophic community did not recover from the disturbance, but became the dominant group in the undisturbed incubations after one week independent of the source strength. Therefore, it seems that the stability of the ecosystem relies on the diversity of the functionally redundant members of the community reacting differently under varying source strength to the disturbance (Micheli et al., 1999).

4.7 References

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5 Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies

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5.1 Summary

Methane-oxidizing bacteria (MOB) possess the ability to use methane for energy generation and growth, thereby, providing a key ecosystem service that is highly relevant to the regulation of the global climate. MOB subgroups have different responses to key environmental controls, reflecting on their functional traits. Their unique features (C1-metabolism, unique lipids, and congruence between the 16S rRNA and *pmoA* gene phylogeny) have facilitated numerous environmental studies, which in combination with the availability of cultured representatives, yield the most comprehensive ecological picture of any known microbial functional guild. Here, we focus on the broad MOB subgroups (type I and type II MOB), and aim to conceptualize MOB functional traits and observational characteristics derived primarily from these environmental studies to be interpreted as microbial life strategies. We focus on the functional traits, and the conditions under which these traits will render different MOB subgroups a selective advantage. We hypothesize that type I and type II MOB generally have distinct life strategies, enabling them to predominate under different conditions and maintain functionality. The ecological characteristics implicated in their adopted life strategies are discussed, and incorporated into the Competitor-Stress tolerator-Ruderal (C-S-R) functional classification framework as put forward for plant communities. In this context, type I MOB can broadly be classified as competitor-ruderal (C-R) while type II MOB fit more within the stress tolerator categories. Finally, we provide an outlook on MOB

applications by exemplifying two approaches where their inferred life strategies could be exploited thereby, putting MOB into the context of microbial resource management.

5.2 Introduction: the role of MOB in the global methane cycle

Methane is the third most important greenhouse gas, after water and carbon dioxide, contributing substantially to radiative forcing (Intergovernmental Panel on Climate Change, 2007). The atmospheric methane concentration has been increasing for most of the past century, followed by a stabilization in the past decade. Recently, atmospheric methane concentration continued to rise again (Rigby *et al*., 2008). The stabilization has been related to lower fossil fuel emissions (Bousquet *et al*., 2006), while the recent anomalies are linked to changes in microbial processes (Kai *et al*., 2011). Hence, the acquisition of knowledge regarding the underlying methane sources and sinks, including methane-oxidizing bacteria (MOB) demands immediate attention. Methane accumulation rates are determined by the balance of sources and sinks. The most important methane source, approximately 70% of the total budget of 500-600 Tg methane year^{-1}, is the microbial production by methanogenic archaea in wetlands, areas associated with animal husbandry, and rice paddies (Intergovernmental Panel on Climate Change, 2007). The largest methane sink (> 80% of the total) is the photochemical reaction of methane with hydroxyl radicals in the troposphere, while diffusion of methane to the stratosphere and microbial methane oxidation account for the rest. Aerobic and nitrite-driven anaerobic methane oxidation are mediated by the MOB. A consortium seemingly comprised of methanogenic archaea and sulphate-reducing bacteria are thought to oxidize methane anaerobically in the marine ecosystem (Boetius *et al*., 2000; Orphan *et al*., 2002; Conrad, 2009; Orcutt *et al*., 2011). Recently, evidence was provided for the coupling of anaerobic methane oxidation to iron and manganese reduction in marine sediments (Beal *et al*., 2009), but the organism facilitating this process has not yet been isolated. In terrestrial environments, nitrite-driven anaerobic oxidation of methane may be an important methane sink, but is yet to be determined. To date, aerobic MOB are of high relevance to the global carbon cycle in terrestrial ecosystems, consuming atmospheric methane in non-flooded upland soils (Knief and Dunfield, 2005; Kolb, 2009), and attenuating methane emission from natural and anthropogenic wetlands (Brune *et al*., 2000).

Therefore, aerobic MOB provide a key ecosystem service, mitigating up to 50% of biologically produced methane (Conrad, 2009).

The ability to use methane as a carbon and energy source is virtually restricted to MOB. Hence, if MOB activity is disturbed, functionality cannot be compensated by the action of other microbial groups, making biological methane oxidation a potentially vulnerable microbial community trait. Considering that MOB are comprised of subgroups with distinct ecology and functional traits, a shift in the MOB community composition or diversity may affect methane oxidation rates (Steenbergh *et al*., 2010; P.L.E. Bodelier, unpublished). Among atmospheric methane oxidizers ('high-affinity' MOB), MOB diversity is directly correlated to methane consumption, and lowers the variability of this process (Levine *et al*., 2011). MOB composition and activity, therefore, is fundamental to the observed fluctuations in methane consumption, and subsequent emission. However, the utilization of atmospheric methane is not universally distributed, but is associated with specific MOB groups (e.g. upland soil clusters; Knief *et al*., 2003) without any cultured representatives; hence, limiting our knowledge on their functional traits. We focus, instead, on the 'low-affinity' MOB, known to be active at >40 ppm_v methane concentrations (Singh *et al*., 2010) which are detected in many high methane-emitting environments (e.g. rice paddies, landfills, lake sediments, and peatlands). The functional traits of MOB may mirror their life strategies. Here, we aim to determine these traits to conceptualize MOB life strategies for a better prediction of their response to environmental cues, and disturbances. Next, we determined whether this understanding could be applied to the context of microbial resource management.

5.3 Key players in methane oxidation in terrestrial ecosystems

Traditionally, aerobic MOB group into type I and type II MOB belonging to γ-Proteobacteria and α-Proteobacteria, respectively. Type I MOB, however, can be further divided into type Ia MOB (e.g. *Methylomonas*, *Methylobacter*, *Methylosarcina*, and *Methylomicrobium*) and type Ib MOB (e.g. *Methylococcus* and *Methylocaldum*) based on the *pmoA* gene phylogeny (Bodrossy *et al*., 2003; Lüke and Frenzel, 2011). Type I and type II MOB are distinguished in their phylogeny, physiology, biochemistry, and morphology (Trotsenko and Murrell, 2008; Semrau *et al*., 2010). Similarly, MOB can be

identified based on their distinctive polar lipid-derived fatty acids (PLFA) patterns (Bodelier *et al*., 2009). Outside the canonical MOB, novel MOB belonging to *Verrucomicrobia* and NC10 were recently discovered. Verrucomicrobial MOB are acidophilic, growing even at pH below 1 (Op den Camp *et al*., 2009), and may be prevalent in less hostile environments, but at present, their habitat range appears to be restricted to the environments from where they were isolated. The novel phylum NC10 is represented by a candidate bacterium *Methylomirabilis oxyfera*, capable of anaerobic methane oxidation coupled to denitrification (Ettwig *et al*., 2009; 2010; Strous, 2011). *M. oxyfera* apparently generates its own oxygen, subsequently used to oxidize methane. Of these three phyla, only proteobacterial MOB have been unequivocally proven to be functionally important in natural and anthropogenic terrestrial environments: lake sediments (Dumont *et al*., 2011), rice paddies (Bodelier *et al*., 2000; Noll *et al*., 2008; Qiu et al., 2008), landfills (Chen *et al*., 2007), peatlands (Chen *et al*., 2008; Kip *et al*., 2010), high artic wetlands (Graef *et al.*, 2011), and floodplains (Bodelier *et al*., 2012). Henceforth, the general term MOB will be used to refer to aerobic proteobacterial MOB. However, with the discoveries of MOB belonging to novel phyla, efforts should be considered for their detection in future environmental studies.

A moderately acidophilic MOB (optimum pH 5.0 - 5.5), *Methylocella* was demonstrated to grow on methane as well as other multicarbon compounds e.g. acetate, succinate, and pyruvate (Dedysh *et al*., 2005). *Methylocella* is not restricted to acidic environments as previously thought; its *mmoX* gene was detected in widespread environments with neutral or near neutral pH (e.g. rainforest soil, estuary sediment, Artic soil, and rice paddy soil; Rahman *et al*., 2011; Reim *et al*., 2012). Although *mmoX* gene was retrieved from a rice paddy soil, corresponding transcripts could not be detected, suggesting that the sMMO plays only a marginal role – if any – oxidizing methane in this environment (Reim *et al*., 2012). Recently, *Methylocystis* spp. known to be an obligate MOB, have been shown to consume acetate and ethanol for growth (Belova *et al*., 2011; Im *et al*., 2011). These bacteria, and *Methylocapsa*, also a proven facultative MOB (Dunfield *et al*., 2010), fall into α-Proteobacteria that use the serine cycle for carbon assimilation, while γ-Proteobacterial MOB assimilate carbon *via* the ribulose monophosphate pathway (Semrau *et al*., 2010). *Methylocella* and *Methylocapsa* belong to *Beijerinckiaceae*, but possess cytological and biochemical similarities with

Methylocystis. Interestingly, facultative MOB appear to be confined to the α-Proteobacteria, suggesting a more versatile substrate utilization than in the gammaproteobacterial MOB, and render them a survival strategy when methane availability is limited or fluctuates.

Discoveries of novel microorganisms oxidizing methane have pushed the boundary of MOB phylogeny. Therefore, the provisional grouping of MOB into type Ia MOB, type Ib MOB, and type II MOB, although still acceptable for proteobacterial MOB at present, may change in future. The key enzyme for methane oxidation is the methane monooxygenase (MMO), existing either as a particulate membrane bound (pMMO) or soluble (sMMO) form. Virtually all MOB possess the pMMO, with the exception of *Methylocella* and *Methyloferula* (Dedysh *et al*., 2000; Vorobev *et al*., 2011), while the sMMO is confined to some MOB. Copper regulates the expression of MMO in MOB that possess genes for both forms of the enzyme, stimulating the pMMO expression at high copper to biomass ratio, while repressing the sMMO (Stanley *et al*., 1983; Murrell *et al*., 2000; Knapp *et al*., 2007). The *pmoA* gene, present in duplicate copies in some MOB (Semrau *et al*., 1995), encodes for the β-subunit of the pMMO enzyme, is highly conserved, and has been generally found to correspond to the 16S rRNA gene phylogeny (Kolb *et al*., 2003), making *pmoA* an alternative to the 16S rRNA gene, and a suitable marker for culture-independent studies (McDonald *et al*., 2008).

5.4 Environmental control of MOB

Methane

Abiotic environmental factors affecting methane oxidation and the MOB have been reviewed (Conrad, 2007; Semrau *et al*., 2010). Among these, methane concentration and nitrogen availability are the most well studied factors, and are strong driving forces shaping MOB community composition and activity, asserting different responses in type I and type II MOB. A comprehensive list detailing MOB ecological characteristics possibly differentiating the functional traits belonging to type I and type II MOB are summarized (Table 1). Recently, a novel isoenzyme, pMMO2, was found in a MOB, and seems to be restricted within the type II *Methylocystis-Methylosinus* group (Yimga *et al*., 2003; Baani and Liesack, 2008). pMMO2 allows MOB to grow at low methane concentrations

(<100 ppm_v), but growth was not detected at atmospheric methane levels, whereas the conventional pMMO is typically expressed under higher methane concentrations (>600 ppm_v). Hence, some type II MOB may possess an advantage under methane depleted conditions, having the ability to withstand methane fluctuations. On the other hand, the 'low-affinity' MOB are found in many methane-emitting environments, and are represented by both type I and type II MOB.

Nitrogen

It was generally accepted that nitrogen fertilization had an inhibitory effect on methane oxidation, probably through competitive inhibition of the MMO by ammonia (Gulledge and Schimel, 1998; Bodelier and Laanbroek, 2004). However, Bodelier and colleagues (2000) found a stimulation of MOB activity and growth upon ammonium fertilization in a rice microcosm. Upon relief of nitrogen limiting conditions, MOB responded rapidly (within minutes) to nitrogen addition (Bodelier *et al*., 2000), suggesting a more direct mechanism affecting the MOB metabolism (Bodelier and Laanbroek, 2004). However, the effects of ammonium were not clear in a soil and rice microcosm study, respectively (Shrestha *et al*., 2010; Krause *et al*., 2012). Although repeatedly examined, the response of MOB activity to ammonium amendment is inconsistent, showing inhibition, stimulation, or no effect, suggesting that the variability observed was attributable to the inherent characteristics of the MOB composition, or the ammonium load tested. On the other hand, nitrite had been shown to differentially affect MOB, making it a potential inhibitory compound, particularly for type II MOB (Nyerges *et al*., 2010). Generally, nitrite exerts a toxic effect that leads to inhibition of methane uptake (Schnell and King, 1994), and is known to inhibit formate dehydrogenase (Jollie and Lipscomb, 1991). However, these effects are studied with pure cultures whereas under field conditions, the ability to denitrify (Campbell *et al*., 2011) may aid MOB to detoxify nitrite. The *nifH* gene encoding for the enzyme nitrogenase reductase was detected in both type I and type II MOB, but nitrogen fixation seems to be a characteristic of mainly type II MOB (Murrell and Dalton, 1983; Auman *et al*., 2001). At the community level, ammonium amendment was shown to selectively stimulate type I MOB in a rice paddy and forest soil, respectively (Bodelier *et al*., 2000; Mohanty *et al*., 2006; Noll *et al*., 2008). Although activity may vary, it is

becoming clear that MOB subgroups respond differently to nitrogen availability, indicating their level of tolerance to or dependency on nitrogen amendments.

5.5 Life strategies: type I and type II MOB

Accumulating evidence concerning the ecological characteristics of type I and type II MOB, and community level molecular analyses of MOB populations under different conditions suggest that the different MOB subgroups possess distinct traits, reflecting on their life strategies (Table 1). The detection of marker genes for MMO (e.g. *pmoA*, *mmoX*) is central for many molecular analyses and indicates the potential active community, taking into account the current and previous members contributing to the MOB seed bank, while retrieval of the corresponding gene transcript (mRNA) is typically considered to be a proxy for activity, and suggest the active population (Jones and Lennon, 2010). Experiments using stable isotope labelling, however, provide a direct link between function and microbial identity (Dumont and Murrell, 2005). Based on stable isotope (^{13}C-methane) labelling experiments, an apparent emerging pattern shows that type I MOB, although numerically less dominant than type II MOB, are predominantly active in many important habitats with high methane emissions (Chen *et al*., 2007; Noll *et al*., 2008; Qiu *et al*., 2008; Kip *et al*., 2010; Dumont *et al*., 2011; Graef *et al*., 2011). Moreover, type I MOB (*Methylobacter*) have been shown to be indicative of environments with a high methane source strength (Krause *et al*., 2012), and was predominant in an Artic tundra soil where virtually only type I MOB was detected (Liebner *et al*., 2009). Further evidence was demonstrated by Ho and colleagues (2011a), showing that the higher potential for methane oxidation corresponded well, particularly to the growth and activity of type Ib MOB in a rice paddy soil. Using soil from a river floodplain, incubations under methane showed a biphasic depletion curve of ‘initial’ and ‘induced’ uptake rates (Steenbergh *et al*., 2010). The ‘initial’ phase is generally considered to represent *in-situ* oxidation rates, whereas the ‘induced’ phase was shown to be contributed by an increase in MOB cell numbers and cell specific activity. Regardless, in both phases, the *pmoA* gene expression level and growth rates were significantly higher for type I MOB. Despite of the diverse environments, these studies provide strong evidence that generally, type I MOB are very responsive to high substrate availability, but when conditions are limiting or adverse, numbers are reduced quickly.

Table 1: Ecological characteristics of type I and type II proteobacterial MOB from various environments and pure culture studies.

MOB group	MOB genus	Source	Observational ecological characteristics	References
Gammaproteobacteria	*Methylohalobius* *Methylomicrobium*	Pure cultures from hypersaline lakes	Known to be halophilic or halotolerant.	Heyer and colleagues (2005) Kalyuzhnaya and colleagues (2008)
	Methylocaldum *Methylococcus* *Methylothermus*	Pure cultures	Known to be thermotolerant or moderately thermophilic.	Trotsenko and colleagues (2009)
	Methylobacter *Methylomonas* *Methylosarcina* *Methylococcus*	Rice paddy soil Landfills Peatlands Lake sediments Arctic wetlands Floodplains	Presence of conventional pMMO only, typically detected as the active population in methane-emitting environments.	Bodelier and colleagues (2000) Chen and colleagues (2007) Deines and colleagues (2007) Chen and colleagues (2008) Dumont and colleagues (2011) Graef and colleagues (2011) Bodelier and colleagues (2012)
	Methylobacter	Rice paddy soil	Indicative for MOB in environment with high methane source strength.	Ho and colleagues (2011a) Krause and colleagues (2012)
	Methylobacter *Methylomicrobium/Methylocaldum*	Rice paddy soil	Stimulation of activity and/or growth upon ammonium fertilization.	Bodelier and colleagues (2000) Mohanty and colleagues (2006) Noll and colleagues (2008)
		Rice paddy soil	Indicative for MOB in rice roots.	Bodelier and colleagues (2000) Luke and colleagues (2010) Shrestha and colleagues (2010) Luke and colleagues (2011)
		Rice paddy soil	Responsive to changes in oxygen concentration.	Henckel and colleagues (2000)
		Land fill soil Rice paddy soil	Rapid response to disturbances.	Hery and colleagues (2008) Ho and colleagues (2011b) Kumaresan and colleagues (2011)
	Methylobacter	Land fill soils Artic tundra soils	Favours lower temperatures.	Borjesson and colleagues (2004) Liebner and colleagues (2009) Graef and colleagues (2011)
Alphaproteobacteria	*Methylocystis* sp.	Pure cultures	Detection of pMMO2 capable of methane oxidation and growth at low methane concentrations (> 10 ppm_v).	Baani and Liesack (2008)
	Methylocystis-Methylosinus group	Pure cultures	Detection of *pmoA2* gene and gene transcripts.	Yimga and colleagues (2003)
	Methylocystis *Methylocella* *Methylocapsa*	Pure cultures	Proven to be facultative MOB.	Dedysh and colleagues (2005) Belova and colleagues (2011) Dunfield and colleagues (2010) Im and colleagues (2011)
	Methylocystis sp.	Pure cultures	Adverse effect on growth upon nitrite amendments.	Nyerges and colleagues (2010)
	Methylocystis *Methylocella* *Methylocapsa*	Pure cultures Sphagnum peatlands Acidic peatlands	Dominant in acidic environments. Known to be acidophilic and acidotolerant.	Dedysh and colleagues (2000) Dunfield and colleagues (2010) Kip and colleagues (2011)
	Methylocystis sp.	Rice paddy soil Pure cultures	Thought to form desiccation- and heat-resistant resting cells that revert to active state after heat induction.	Whittenbury and colleagues (1970) Ho and Frenzel (2012)
		Rice paddy soil	Thought to form resting cells, contributing to the microbial seed bank in the soil.	Eller and colleagues (2005) Krause and colleagues (2012)
	Methylocystis-Methylosinus group	Rice paddy soil	Become more important during recovery from disturbances	Ho and colleagues (2011b) Ho and Frenzel (2012)

Where available, MOB genera are given.

On the contrary, it is thought that the type II MOB population is relatively stable, and assumed to be present in a dormant state forming part of the microbial seed bank in the soil (Eller *et al*., 2005; Krause *et al*., 2012). Indeed, type II MOB generally form more desiccation- and heat-resistant resting cells than type I MOB (Whittenbury *et al.*, 1970). Here, we define dormancy as a state of reversible reduced metabolic activity and can be discriminated by not being able to detect the population at the gene transcription (mRNA) level. Accordingly, while the *pmoA* gene belonging to type II MOB was detected, the corresponding transcript was not retrieved or retrieved in relatively low levels, suggesting their presence, but inactive role in the soil (Bodrossy *et al.*, 2006; Krause *et al*., 2010). Although largely dormant, type II MOB became more important during recovery from disturbances or under fluctuating conditions. Results show that upon a disturbance-induced die-off, type II MOB population increased, and dominated the total MOB population after 40 days, while type I MOB showed a rapid response soon after the disturbance (Ho *et al*., 2011b). The initial relatively higher nutrient availability may have sustained type I MOB dominance (Mohanty *et al*., 2006; Krause *et al.*, 2010), but type II MOB, being less demanding, became more competitive later when nutrients were limiting (Graham *et al*., 1993). In another form of disturbance, the type II population numerically increased after a brief exposure to heat stress at 45°C, and subsequently led to a higher methane uptake rate than in the control (continuous incubation at 25°C; Ho and Frenzel, 2012). Hence, it was suggested that a brief exposure to elevated temperatures may have triggered the translation of type II MOB from dormant to metabolically active states (Whittenbury *et al*., 1970; Ho and Frenzel, 2012). Nevertheless, methane uptake was significantly lower in prolonged incubations at temperatures exceeding 40°C, likely due to the decreased activity of mesophilic MOB (Mohanty *et al*., 2007). Despite of the different disturbances simulated, type II MOB were persistent and recovered well, and appear to have a different adaptation strategy from type I MOB.

The traits of type I and type II MOB observed so far have often been interpreted as a reflection of the r- and k-selection theory (Steenbergh *et al*., 2009, Siljanen *et al*., 2011; Bodelier *et al*., 2012) , designating organisms to be evolutionary r-selected that invest in high reproductive success, and short life spans being most effective in unstable environments. K-selected organisms invest in maintaining numbers at carrying capacity of

the habitat, having low off-spring and growth rates typically displayed in stable habitats (MacArthur and Wilson, 1967). However, considering the knowledge gathered so far (see table 1), this 2-dimensional framework is designed for animal life-strategies, and do not represent MOB life strategies in an accurate way. The long-term survival of microbes under adverse conditions, their limited mobility in combination with their potential emergence from microbial seed banks makes their life strategies more similar to plants than animals.

The Competitor-Stress tolerator-Ruderal, C-S-R functional classification framework developed for plants (Grime, 1977) may be a more applicable framework, accounting for life strategies of type I and type II MOB, but also for microbial ecology in general. The scheme assumes that the combination of varying intensities of stress (i.e. factors restricting biomass production) and disturbance (i.e. factors leading to destruction of biomass) have led to three primary life strategies (competitors, stress-tolerators, and ruderals; Figure 1). With sufficient environmental knowledge (Table 1), we have adopted, and placed MOB into this scheme. Type II MOB were found to be persistent in inactive states, and became relevant during the recovery from disturbances. Besides, type II MOB show versatility in substrate utilization, and occur under non-favorable conditions (e.g. low pH). Considering these ecological observations, type II MOB are classified as stress tolerator (S) and stress tolerator-ruderal (S-R). Furthermore, since environmental data on the competitiveness of type II MOB is still lacking, we positioned them under competitor-stress tolerator (C-S) in the scheme. Type Ia MOB, responding rapidly to substrate availability and being the predominantly active community in many environments can thus be classified as competitors (C) and competitors-ruderals (C-R). The scheme allows more opportunities to accommodate the number of strategies displayed by MOB, and microbes in general. The three dimensions offer the definition of mixed strategies which is more suitable considering the metabolic flexibility of bacteria, and can provide a basis to predict and assess MOB distribution, prevalence, and response to disturbances/amendments.

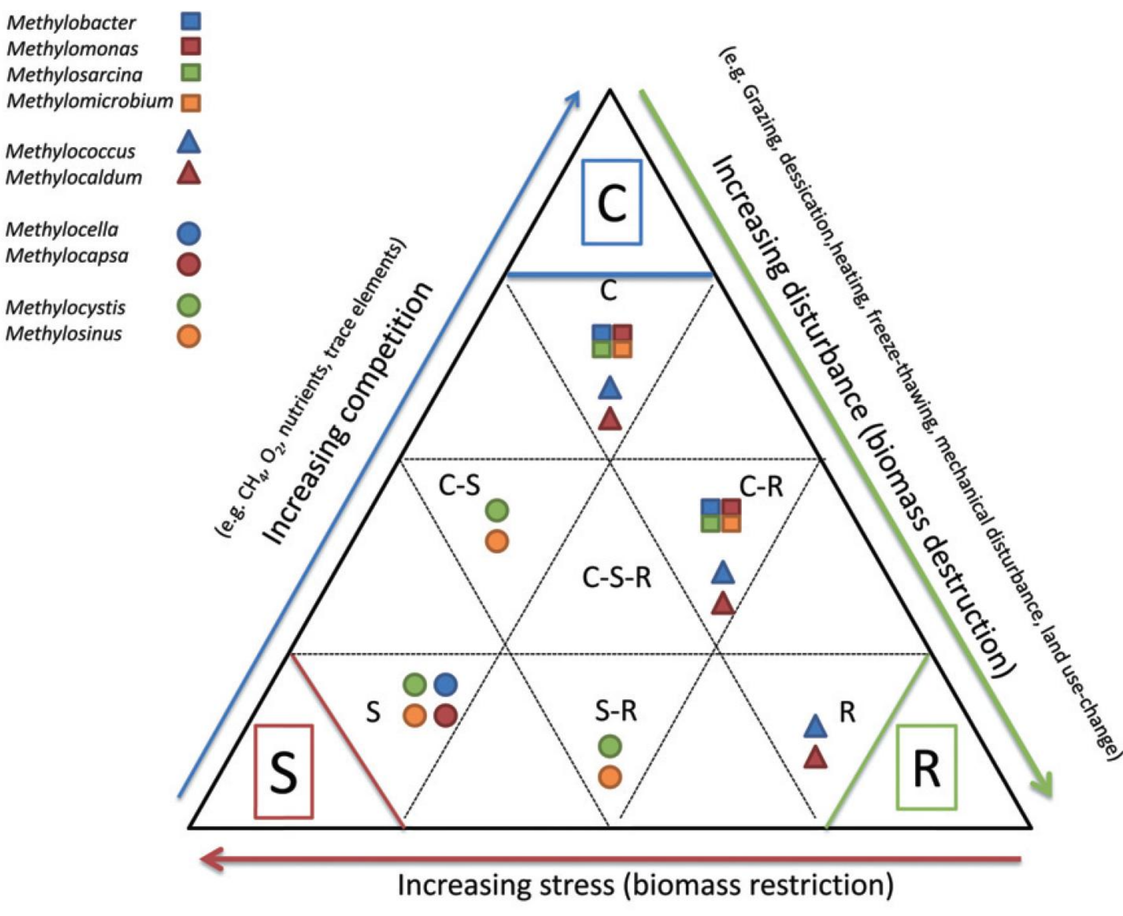

Figure 1: MOB within the three dimensions of C-S-R functional classification framework, conceptualizing MOB functional traits and observational characteristics to be interpreted as life strategies. The C-S-R framework allows the assignment of MOB life strategies, yet tractable in accommodating the versatility in bacterial metabolism.

5.6 Outlook: putting MOB into the context of microbial resource management

MOB have been studied extensively where knowledge on its ecology, and biotic and abiotic environmental variables controlling its activity, in combination to the availability of MOB cultured representatives from different subgroups provide a strong knowledge base to conceptualize the observational ecological characteristics and traits of MOB as life strategies. Next, we provide an outlook, capitalizing the scheme (Figure 1) to bridge the current knowledge to biotechnological applications. The challenge, however, is to structure and optimize the performance of the MOB community in respect to a desirable set of outputs. This strategy is called Microbial Resource Management (Verstraete *et al*., 2007). Two approaches will be exemplified to demonstrate the potential applications of mixed MOB cultures to cometabolically remove pollutants, and for the production of polyhydroxybutyrate (PHB).

MOB as pollutant degraders

The initial interest in using MOB to degrade pollutants stems from the broad substrate specificity of the sMMO. sMMO-expressing cell are able to oxidize a wide range of compounds including aliphatic and aromatic hydrocarbons, and their halogenated derivatives (Burrows *et al*., 1984; Trotsenko and Murrell, 2008). Similarly, pMMO-expressing cells, albeit possessing a narrower substrate range (pMMO oxidizes alkanes and alkenes up to C_5; Trotsenko and Murrell, 2008), have been found to degrade mixed pollutants in the laboratory as well as *in-situ* (Forrester *et al*., 2005; Lee *et al*., 2006; Paszczybski *et al*., 2011), and even out-perform sMMO-expressing cells under specific conditions in the laboratory (Lee *et al*., 2006). Hence, both pMMO- and/or sMMO-expressing cells are applicable for bioremediation. However, whether pMMO- or sMMO-expressing cells are more applicable depends on the inherent properties of the polluted site (e.g. level of copper bioavailability), and the pollutant to be degraded, among other factors (Lee *et al*., 2006; Semrau *et a*l., 2010). In the event that sMMO-expressing cells are favoured, enriching for, and subsequent stimulation of type II MOB, may be of interest, and can potentially be achieved by exposing a mixed community to heat as a pre-treatment. Type II MOB in particular, are generally more heat resistant (Whittenbury

et al., 1970). Conversely, it is not unreasonable to assume that type I MOB population could be repressed by manipulating the enrichment conditions (e.g. stimulate nutrient scarcity). Hence, by applying selection conditions favouring MOB adopting stress tolerator (S) strategies, the desired MOB population in a mixed community can be enriched to optimize processes to degrade pollutants.

Polyhydroxybutyrate (PHB) as an added value product accumulated in MOB

PHB is a polyhydroxyalkanoate (PHA) commonly accumulated in microorganisms in response to unbalanced growth conditions (e.g. nutrient limitation, presence of excess carbon; Salehizadeh and Loosdrecht, 2004). Hence, PHB can be produced from renewable resources, and is biodegradable, making it an attractive alternative to petrochemical plastics. So far, industrial scale microbial PHB production is largely restricted to using pure cultures of *Alcaligenes sp.* (e.g. *A. eutrophus*, and *A. latus*). Recently, however, using mixed microbial cultures to produce PHB is of interest as a step to reduce production cost, and avoid the necessity to operate under sterile conditions. In a novel approach using MOB mixed cultures as potential PHB factories, Pfluger and colleagues (2011) optimized the conditions in a fluidized bed reactor to select for type II MOB, known to accumulate PHB (Pieja *et al*., 2011). Results showed that type I MOB were favoured by ammonium amendments, while the key conditions favouring type II MOB were using N_2 as a nitrogen source, and having a low dissolved oxygen concentration. Besides being a source for biodegradable plastics, PHB in MOB can serve as a feedstock in aquaculture (De Schryver *et al*., 2011). The conditions required to cultivate aquatic organisms (e.g. fish, shellfish) involve frequent cycles of addition and depletion of nutrients, simulating a feast and famine regime needed for the accumulation of PHB (De Schryver and Verstraete, 2009). Hence, allowing cultivation of aquatic organisms and the production of their feedstock in parallel. Similarly, knowledge regarding the ecological characteristics and traits of MOB can be applied here to enrich for type II MOB (see above) to increase PHB production.

5.7 Concluding remarks

Our literature review shows that although MOB co-exist in the same environment, they possess distinct functional traits, reflecting on their life strategies, and may render a selective advantage under different conditions. In a concerted effort, MOB appears to weather disturbances well, and maintain functionality. However, the extent to which MOB are able to withstand the strain of disturbances before functionality is at risk, and the role of their traits in this matter is still unclear. MOB's resilience can be attributable to their ability to form resistant resting cells, among other traits, that allows their persistence under harsh conditions over long periods. In a seminal study based on a few MOB representatives (Whittenbury *et al*., 1970), it seems that only type II MOB are able to form resistant resting cells. In light of novel MOB discovered (Conrad, 2009), a more extensive survey to determine whether this trait is restricted to type II MOB can be considered. Moreover, potential factors inducing, and triggering MOB dormancy are of interest.

Similarly, isolation efforts could be increased to obtain the 'high-affinity' MOB associated with atmospheric methane uptake. A characterization of their biochemistry may unveil yet unknown functional traits and metabolic potential. Finally, to fully realize the genetic potential, and hence, understanding of the MOB ecology, a comparison of the available genomes of type I and type II MOB representatives is of high relevance. Only a few MOB genomes are currently available (e.g. *Methylococcus capsulatus* Bath: Ward *et al*., 2004; *Methylacidiphilum infernum*: Hou *et al*., 2008; *Methylocella silvertris*: Chen *et al*., 2010; *Methylosinus trichosporium* Strain OB3b: Stein *et al*., 2010; *Methylocystis* sp. Strain Rockwell; Stein *et al*., 2011; *Methylobacter tundripaludum* SV96: Svenning *et al*., 2011), but many are in the pipeline to be sequenced and annotated. Once these are available, we may be able to single out genes or groups of genes that are common, and associated to a particular trait. Finding the genes most crucial for survival and maintenance of methane consumption under various environmental conditions will pave the way for incorporating gene-traits into methane consumption models and in this way, optimizing methane consumption by choosing the right set of traits and associated strains. The knowledge amassed could then be applied to mixed MOB communities, for instance, by selecting a subgroup, as a strategy to optimize biotechnological applications.

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6 General discussion and outlook

In this thesis (i) the high resolution vertical profile of the methanotroph community in paddy soil surface layer was resolved and (ii) the effect of different unspecific disturbances on the paddy soil methanotroph community were addressed. **Chapter 2** focuses on the community composition and specific activity in the soil surface layer in a high resolution vertical profile. The community structure and activity was assessed using the particulate methane monooxygenase gene *pmoA* as a functional and phylogenetic marker by terminal restriction fragment length polymorphism (t-RFLP) and a *pmoA*-specific diagnostic microarray. Quantification of *pmoA* genes and transcripts was done using a newly developed competitive-(reverse transcriptase)-t-RFLP. In **Chapter 3** and **4** the resistance and resilience of the methanotrophic community to different disturbances was addressed. **Chapter 3** reviews the ability of methanotrophs to persist through and recover from long periods of drought upon rewetting; a challenge that –even though to a lesser extend- methanotrophs face on a regular basis due to the water management in the wetland rice agricultural cycle. In **Chapter 4** the effect of varying source strength on the ability to compensate for and recover from simulated disturbance induced mortality was studied. While the simulated die-off event is a non-selective disturbance affecting neither the diversity nor the evenness of the bacteria diversity the water stress over time selects for the sub-population best adapted to desiccation.

The aerobic methanotrophic bacteria show a high diversity in paddy soils (Lüke et al., 2010). Their classification is traditionally based on morphological and physiological characteristics as shown in **Chapter 1.2**. However, the recent discoveries of novel aerobic methanotrophs from various environments challenged this system. The novel methanotrophs so far are only restricted to rather extreme environments with mostly low pH like for example acidic bogs or hot mud volcanoes (Dedysh et al., 2002; Pol et al., 2007). The absence of the *Verrucomicrobia* species from the Italian paddy soil was already shown before with *Verrucomicrobia*-specific primer based PCR (Lüke, 2010). In **Chapter 2** we show also that the moderately acidophilic *Methylocella* or *Methyloferula* are of no importance in the paddy soil. Therefore, despite the high and redundant diversity the classical grouping into types Ia, Ib and II is still valid to characterise the communities studied in the Italian paddy soil used in this work.

During the rice growing seasons irrigated rice fields are known to be significant methane sources while in winter or drained periods in between the rice growing seasons the aerated fields can even act as sink for methane (Singh et al., 1998; Singh et al., 1999). However, sequences belonging to upland soil clusters that are assumed to be responsible for the consumption of atmospheric methane, for example, USC-α, USC-γ (Knief et al., 2003) and the clusters JR-1, JR-2 and JR-3 (Horz et al., 2005), were not detected so far (Lüke et al., 2011). Therefore, maybe other organisms have to be responsible for the oxidation of methane in these soils. Recently, Baani and Liesack (2008) showed that the second pMMO isoenzymes (pMMO2) that several type II methanotrophs contain enabled *Methylocystis* strain SC2 to oxidize methane close to atmospheric methane concentrations (Yimga et al., 2003). The apparent *K*m of pMMO2 in the *Methylocystis* strain SC2 corresponds well with the *K*m(app) values for methane oxidation measured in upland soils that consume atmospheric methane (Baani and Liesack, 2008). This could be an advantage for the type II methanotrophs containing *pmoA2* enabling methane oxidation over a wider substrate range in environments with constantly changing methane concentrations. In **Chapter 4** we observed strong growth of type II independent of the methane source strength supporting this hypothesis. Additionally, *pmoA2* was found indicative for low methane source strength incubations in **Chapter 4** also indicating that carrying the *pmoA2* copy is advantageous at low methane concentrations. However, the amount of methane supplied under low source strength is not sufficient to support

growth as strong as observed for the type II in **Chapter 4**. Therefore, other traits of the type II (e.g. facultative growth) could be the reason of the high type II abundance in paddy soils (**Chapter 2**, **3** and **4**).

Even the defined subgroup of type I methanotrophs shows niche differentiation on the micro scale spatial resolution. *Methylosarcina* was detected close to the surface of our microcosms in **Chapter 2** under oligotrophic conditions which might indicate that they as well possess an alternative methane monooxygenase (MMO). Although no evidence indicating the presence of pmoA2 in *Methylosarcina* is available. However, the gammaproteobacterial methanotrophs were shown to encode another sequence-divergent particulate monooxygenase (pXMO) with unknown substrate specificity and affinity (Tavormina et al., 2011). New results imply that the functional protein is a MMO (Hainbuch *et al*. in preparation).

Several studies identify the type Ib as dominant active subpopulation in paddy soils (Ho et al., 2011a; Lüke et al., 2010; Lüke et al., 2011; Lüke and Frenzel, 2011). Next to *Methylococcus* and *Methylocaldum*, type Ib consists of various environmental clusters retrieved almost entirely from paddy soils (Lüke et al., 2010). However, none of those studies were focusing on the methanotrophs in the soil surface layer. In studies that accessed the methanotroph community in soil surface so far type Ia *Methylobacter* related are always the most active community, which is in good agreement with our results (**Chapter 2, 3 and 4**; Chen et al., 2007; Krause et al., 2010; Krause et al., 2012). In the two studies evaluating the effect of the source strength on the methanotrophic community type I transcripts were identified to be indicative for high methane source strength (**Chapter 4,** Krause 2012). Indicating that the type Ia *Methylobacter* related methanotrophs are adapted to environments where both substrates methane and oxygen are available in high enough quantities to support the fast growth (**Chapter 2, 3 and 4**; Amaral and Knowles, 1995; Chen et al., 2008).

Due to the agricultural practice, with regular plowing and puddling, and the dominance of one plant species, the paddy soil is a homogenous environment exhibiting no large horizontal variance in methanotrophic community composition (Krause et al., 2009). Even after application of disturbance simulated die-off or long desiccation periods the methanotrophic communities recovers activity in the matter of days indicating the presence of a functionally redundant community stabilizing ecosystem function(**Chapter**

3 and 4; Ho et al., 2011b). However, stability relies on a species inventory as one key component of biodiversity (Krause 2012). In the paddy soil methanotrophs this inventory is believed to be represented by different resting stages stored in a microbial seed bank (Eller et al., 2005; Whittenbury et al., 1970); a reservoir of dormant individuals that can potentially be resuscitated in the future under different environmental conditions (Lennon and Jones, 2011). The concept of the microbial seed bank was first proposed by Eller and Frenzel (2005). In the dried paddy soil even after almost two decades of desiccation a sufficient amount of dormant cells survived to form a functional -though less diverse- community fulfilling the ecosystem function (**Chapter 3**). Nevertheless, functional redundancy does not rely on an inventory of viable species but also on the different response of the functionally redundant species to environmental conditions. Resulting compensatory dynamics of different methanotroph groups will stabilize the community function under changing environmental conditions, in disturbed communities or in different stages of a succession (Micheli et al., 1999).

So far the type II methanotroph population is usually assumed to be present in a dormant state forming part of the microbial seed bank in the soil and to generally form more desiccation resistant resting cells than type I (Eller et al., 2005; Krause et al., 2012; Whittenbury et al., 1970). Independent of the type of disturbance we found the type Ia to react fast under high source strength (**Chapter 3 and 4**). The type II followed later in succession independent of the source strength (**Chapter 3 and 4**; Ho et al., 2011b; Krause et al., 2010). Results indicating that methanotrophs can survive centuries under anoxic conditions in sediments are already around for more than two decades (Rothfuss et al., 1997). However, our results indicate that not only the type II but also type I form resting stages surviving desiccation under oxic conditions for up to almost two decades.

Moreover, a higher initial nutrient availability may have favored the dominance of the type I methanotrophs in the early incubations while the less demanding type II became dominant later when nutrients were limiting (Graham et al., 1993; Krause et al., 2010; Mohanty et al., 2006). However, the type II appear to have an adaptation strategy significantly different from type I methanotrophs.

So far the different traits observed for the type I and type II methanotrophs have often been interpreted as a reflection of the r- and k-selection theory (Bodelier et al., 2012; Krause et al., 2010; Siljanen et al., 2011; Steenbergh et al., 2010). The type I

methanotrophs exhibiting immediate growth under beneficial environmental conditions represent r-selected organisms, whereas type II methanotrophs slower reaction to beneficial environmental conditions and higher initial cell numbers indicate a k-selected strategy (Andrews and Harris, 1986). In **Chapter 5** the Competitor-Stress tolerator-Ruderal, C-S-R functional classification framework developed for plants (Grime, 1977) is discussed as a more applicable framework, accounting for life strategies of type I and type II MOB, but also for microbial ecology in general.

6.1 Outlook and concluding remarks

In this work we showed that the methanotrophs in the soil surface layer do not only form a diverse community fulfilling an important ecosystem function but do so even after long periods of adverse environmental conditions or under the effect of disturbance (**Chapter 3 and Chapter 4**). Even though the methanotrophs recovered well from both disturbances high energy flows boosting the resilience of the methanotroph community. The model disturbances employed here were both unspecific, affecting the whole bacterial community and required growth to compensate for the effect. To evaluate the short term effect of the source strength on the resilience of the methanotroph community we suggest application of a more specific disturbance directly influencing the metabolically active methanotrophic community. A potential model disturbance would be the use of acetylene, which was shown to effectively and irreversibly inhibit the methane monooxygenase (MMO; Prior and Dalton, 1985). In a study on paddy soil, a concentration as low as 1 µM (~10ppmv) was completely inhibiting methane oxidation in soil slurries (Bodelier and Frenzel, 1999). Therefore, the methanotrophs would have to synthesize MMO in order to regain the ability of methane oxidation. With de novo protein synthesis being an energetically expensive process, we expect higher source strength of their main substrate methane results in faster recovery of the bacterial groups affected.

The growing number of methanotrophic microorganisms, novel pathways and isoenzymes discovered in the last decade indicate that we only scratched the surface in the biochemical diversity of the methanotrophs. In Chapter 4 we presented results indicating facultative growth of the type II methanotrophs. To verify if the type II are really growing on alternative substrates we need to identify the microorganisms

metabolically active and responsible for the observed methane oxidation with a transcript independent technique. Stable isotope probing (SIP) using $^{13}CH_4$ as substrate could be used to identify the methanotrophs actively oxidizing methane incorporating the heavy carbon into their DNA or phospholipid fatty acids (Radajewski et al., 2000).

With respect to the newly identified methanotrophs there is not much known on their ecological significance and specific niche. However, NC10 related sequences were already detected in paddy soil (data not shown, see also Chapter1.2). Therefore, the high resolution vertical profiling method established in Chapter 2 could be used to show the localization and activity of the anaerobic bacteria in a methane-oxygen counter-gradient system.

The advent of new molecular techniques like next generation sequencing brought on an avalanche of new data. To evaluate and integrate these new results in the working models of the biodiversity and ecosystem function of the methanotrophs is undoubtedly challenging. Nevertheless, next generation sequencing techniques allow for in-depth analysis of the microbial communities. Especially, meta-transcriptomics of methanotroph communities in combination with SIP could provide further insight into the adaptations and niche differentiations of distinct species or clusters (Dumont et al 2011; Wang et al., 2009). Additionally pyrosequencing of the 18S rRNA would be an appropriate tool to determine if eukaryotic predators (e.g. protists and amoebae) are responsible for the decrease in type I methanotroph population size observed in **Chapter 4**.

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WISSENSCHAFTLICHE PUBLIKATIONEN

Reim,A., Lüke,C., Krause,S., Pratscher,J., and Frenzel,P. (2012) One millimetre makes the difference: high-resolution analysis of methane-oxidizing bacteria and their specific activity at the oxic-anoxic interface in a flooded paddy soil. *ISME. J.* **6:** 2128-2139.

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Reim,A., and Frenzel,P Effect of energy flow on the susceptibility of aerobic methanotroph communities to disturbance. **In preparation.**

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BEITRÄGE ZU WISSENSCHAFTLICHEN TAGUNGEN

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Ich versichere, dass ich meine Dissertation

'Methane oxidizing bacteria at the oxic-anoxic interface: taxon-specific activity and resilience'

selbstständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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