Response and resilience of methanotrophs to disturbances

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Pledge

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Marburg, 01 October 2010

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Summary

Methanotrophic bacteria are the only known biological sink for the greenhouse gas methane. Therefore, methanotrophs play a key function in carbon cycling, an important biogeochemical process that affects global climate change. Yet, little is known of their vulnerability and resilience to disturbances. Driven by the gap of knowledge, this PhD thesis is a seminal study focusing on the recovery of methanotrophs from disturbances with respect to population dynamics, diversity and functioning. Two model disturbances were tested; disturbance-induced mortality and heat shock. While the former model disturbance represents a non-selective form of disturbance, the heat shock treatment may select for sub-populations of thermo-tolerant methanotrophs. Overall, methanotrophs are shown to be remarkably resilient to induced disturbances, compensating and even over-compensating for methane uptake during recovery. Type II methanotrophs, known to be present in high abundance as resting cells, appear to become more important during disturbances.

Furthermore, the establishment and subsequent development of the methanotrophic community and activity were studied along a rice paddy chronosequence. With the influx of anthropogenic influences once a rice paddy is formed, the methanotrophic community structure is anticipated to undergo a dramatic change which in turn, may affect the activity. It appears that the young and ancient rice paddies do not show clear divergence, suggesting that the methane oxidizing community was soon established after a rice paddy is formed. However, the selection of the best adapted sub-

population needs time. Accordingly, long term rice agriculture allows for higher methane uptake, and may select for a methanotroph sub-population that remains active. The predominant methanotrophs found in the Chinese rice paddies are type II, mainly *Methylocystis* species, and type Ib (RPC-1). However, type Ib seems to be the active dominant sub-population. This and previous studies suggest specific adaptation of type Ib to rice paddy environments. Interestingly, novel sequences phylogenetically grouped between *pmoA* and *amoA* were detected.

Overall, paddy soil methanotrophs are not only able to recover from disturbances, but are apparently showing specific adaptation to rice paddy environments, demonstrating their resilience in face of perturbation.

Zusammenfassung

Methanotrophe Bakterien stellen die einzig bekannte biologische Senke für das Treibhausgas Methan dar. Sie katalysieren somit eine Schlüsselfunktion im globalen Kohlenstoffkreislauf mit direktem Einfluss auf den weltweiten Klimawandel. Dennoch ist erst wenig über die Anfälligkeit bzw. Widerstandsfähigkeit dieser Bakteriengruppe gegenüber Umweltstörungen bekannt. In dieser Doktorarbeit wurden grundlegende Untersuchungen zur Regenerierung methanotropher Bakteriengemeinschaften nach Umweltstörungen durchgeführt. Als Modelstörgrößen wurden die durch äußere Einflüsse bedingte Sterblichkeit sowie die Hitzeschockbehandlung getestet. Die Regenerierung wurde anhand der methanotropher Gesamtdiversität, Populationsdynamiken und Methanoxidationsraten verfolgt. Hinsichtlich ihrer Funktion Methan zu oxidieren, zeigten sich die Bakteriengemeinschaften auffallend resistent gegenüber den indizierten Störungen: Die Methanoxidationsraten stiegen im Verlauf der Regenerierung sogar über die Ausgangsrate an. Die Diversität nahm jedoch drastisch ab und Typ II methanotrophe Bakterien dominierten bei Beendigung der Experimente. Die Ergebnisse zeigen, dass Typ II methanotrophe Bakterien, die eine mengenmäßig bedeutsame Rolle in Reisfeldern spielen, vor allem nach Störungen eine wichtige Funktion einnehmen.

Im zweiten Teil dieser Doktorarbeit wurden die Besiedlung und die nachfolgende Entwicklung methanotropher Gemeinschaften und ihrer Aktivität anhand einer Chronosequenz chinesischer Reisfelder untersucht. Der Beginn der Reiskultivierung mit den einhergehenden anthropogenen Einflüssen

könnte eine nachdrückliche Änderung der Bakteriengemeinschaft und ihrer Aktivität zur Folge haben. Die methanotrophen Gemeinschaften im jüngsten und ältesten Reisfeldboden unterschieden sich jedoch nicht bedeutsam voneinander, was darauf hindeutet, dass sich die spezifische Gemeinschaft in Reisfeldern vergleichsweise schnell etabliert. Die Selektion einer am besten abgepassten Subpopulation dauert womöglich dennoch etwas länger: Die Methanoxidationsraten in den einzelnen Reisfeldböden zeigten eine positive Korrelation zu dem entsprechenden Alter des Bodens.

Vorwiegend detektiert wurden in allen Böden Typ II methanotrophe Bakterien, hauptsächlich der Gattung *Methylocystis*, und Typ Ib methanotrophe Bakterien des RPC-1 (Rice Paddy Cluster-1). Die Typ Ib scheinen jedoch die aktive Subpopulation zu stellen. Diese und vorhergehende Arbeiten deuten auf eine spezifische Anpassung von Typ Ib methanotrophen Bakterien an das Reisfeldhabitat hin. Interessanterweise wurden weiterhin neuartige *pmoA* Sequenzen gefunden, die phylogenetisch zwischen den methanotrophen und den Ammonium oxidierenden Bakterien gruppieren.

Zusammenfassend lässt sich schlussfolgern, dass methanotrophe Bakterien bemerkenswert resistent gegenüber äußerer Störung sind. Im Reisfeldhabitat konnte weiterhin eine sehr spezifische Gemeinschaft detektiert werden, was ihre schnelle Anpassungsfähigkeit demonstriert.

1. Introduction

1.1 Methane cycle

Methane, next to water vapor and carbon dioxide, is the third most important greenhouse gas. Atmospheric methane concentrations have been increasing from pre-industrial values of around 715 ppbv to 1770 ppbv in 2005 (Intergovernmental Panel on Climate Change, 2007). Although total methane concentration increased by more than two fold, the annual methane growth rate decreased from 12 ppb $year^{-1}$ in the 1980s to 4 ppb $year^{-1}$ since 1999 (Bousquet *et al*., 2006). Following almost a decade of slight fluctuations in methane concentrations, renewed growth of atmospheric methane has been reported (Rigby et al., 2008).

Methane growth rate is determined by the balance of sources and sinks. Non-biogenic methane sources include combustion of fossil fuels, biomass burning and geological sources account for 25% of the total budget of around 500-600 Tg methane $year^{-1}$ (Conrad, 2009). The biggest contribution of methane (around 69%), however, originates from microbial metabolism, specifically methane production by methanogenic archaea. Among biogenic sources, wetlands, livestocks and rice paddies are the major methane contributors. Methane sources are balanced by methane sinks. The largest methane sink, accounting for >80% of the total, is the photochemical reaction of methane with hydroxyl radicals in the troposphere. Two other sinks are diffusion of methane into the stratosphere and microbial methane oxidation by

methanotrophs. The later is the only known biological methane sink on Earth. Microbial methane oxidation is responsible for methane uptake in upland soils, and has a filter function at oxic-anoxic interfaces to attenuate methane emission in wetland ecosystems.

1.2 Rice paddies as anthropogenic methane source

Rice paddies contribute around 10% to the global methane budget (Conrad, 2009). Rice is an important food source for more than 50% of the world's population. One of the largest global rice producers is China, contributing approximately 30% of total world rice (FAOSTAT, 2005). It is inevitable that rice production will need to increase to sustain growing human population in decades to come.

Methane turnover in rice paddies is regulated by the availability of organic carbon source and oxygen. Oxygen is the thermodynamically favorable electron acceptor. In the absence of oxygen, alternative electron acceptors are reduced depending on their redox potential in the following order NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^{2-} (Liesack *et al*., 2000). These electron acceptors are regenerated at oxic-anoxic interfaces (Figure 1). While organic carbon is available through decaying plant materials or from root exudates, oxygen is usually limited in flooded rice paddies where oxygen penetration depth is restricted to a few millimeters from the surface of the soil (Frenzel *et al*., 1992). However, oxygen is supplied deeper in the bulk soil via the aerenchyma of the rice plants which act as a gas conduit (Frenzel *et al*.,

1992). Conversely, methane generated in the deeper anoxic soil can be emitted into the atmosphere via the aerenchyma. Methane which has not escaped through the aerenchyma diffuses upwards in the soil and serves as substrate for methane oxidizing bacteria (methanotrophs) at the soil-floodwater interface where as much as 90% of potentially emitted methane may be oxidized (Kajan and Frenzel, 1999). Therefore, net methane emission from rice paddies results from a balance of methane production and methane oxidation.

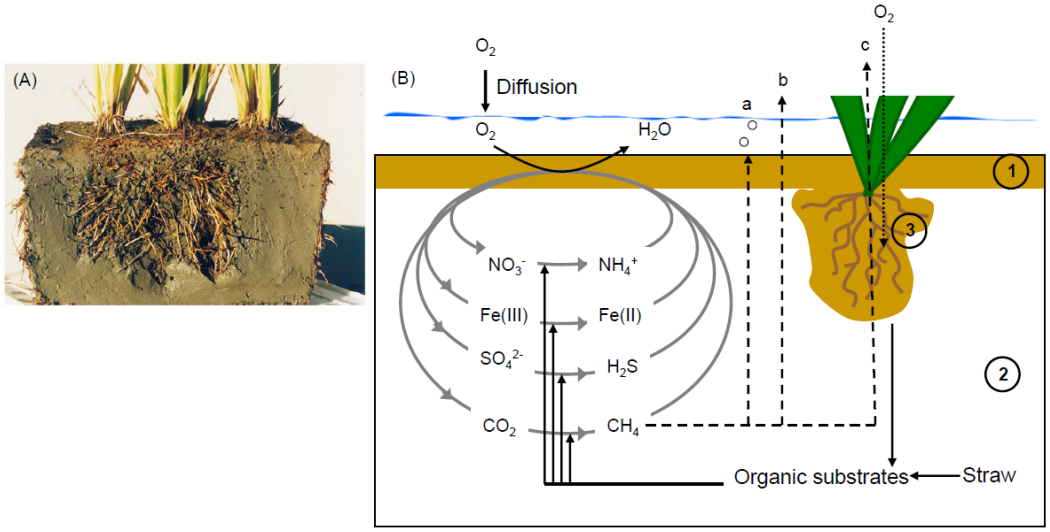

Figure 1: (A) Cross –section of a drained rice microcosm showing two main compartments: root zones and non-rooted bulk soil. (B) Schematic diagram showing the cross-section as main habitats for active microorganisms involved in redox cycling in flooded rice paddies. The potential methane emission pathways are also given: a = ebullition; b = diffusion; c = plant mediated transport via rice plant aerenchyma. 1= oxic surface soil; 2= anoxic non-rooted bulk soil; 3= root zones (figure not drawn to scale). Adapted from Conrad and Frenzel, 2002; Liesack, 2000.

Agricultural practices from implementation of water management strategies (Neue, 1997) to fertilization (Bodelier *et al.,* 2000; Mohanty *et al*., 2006; Qiu *et al*., 2008; Shrestha *et al*., 2008) and the choice of rice cultivars (Bilek *et al*., 1999; Lüke, 2009; Neue, 1997) are shown to affect methane oxidation potential and ultimately, methane emission from rice paddies. The time course of methane genesis and emission had been studied before (see reviews Conrad and Frenzel, 2002; Liesack 2000). However, little is known about the establishment and long term development of the methanotrophic community and activity in rice paddies. Therefore, the biogeochemistry and microbial aspects of rice paddy evolution warrant further attention.

1.3 Methanotrophs

Methylotrophs represent a diverse group of microorganisms capable of reducing single carbon compounds for growth. A sub-group of methylotrophs, the methanotrophs, are generally characterized by their unique ability to utilize methane as their sole carbon and energy source; however, there are some exceptions (see below). Methanotrophs belong to three phyla; *Proteobacteria*, *Verrucomicrobia* and NC10. Traditionally, methanotrophs fall within the phylum *Proteobacteria*. Only recently had methanotrophs in the phylum *Verrucomicrobia* been discovered and they seemed to be restricted to extreme environments (Dunfield *et al*., 2007; Pol *et al*., 2007; Islam *et al*., 2008). The novel phylum, NC10 represents bacteria capable of anaerobic methane oxidation coupled to denitrification (Ettwig *et al*., 2009).

Aerobic methane oxidizers

Cultured members of methanotrophs in phyla *Proteobacteria* and *Verrucomicrobia* are known to be aerobic methane oxidizers, using O_2 as electron acceptors. Methanotrophs in *Proteobacteria* have been traditionally grouped into type I (family *Methylococcaeae*) and type II (family *Methylocystaceae* or *Beijerinkiaceae*) based on morphological, physiological and phylogenetical characteristics (Whittenburg *et al*., 1975; Bowman, 2000; Trotsenko and Murrell, 2008; Bodelier *et al*., 2009). Type I consists of type Ia (e.g. *Methylobacter*, *Methylomicrobium, Methylomonas and Methylosarcina*) and type Ib (also named type X: *Methylococcus* and *Methylocaldum*), while type II consists of 2 genera: *Methylocystis* and *Methylosinus. Methylocapsa* and *Methylocella*, both acidophilic methanotrophs, belong to family *Beijerinkiaceae* and falling within the class α-proteobacteria which also constitutes type II, but are not monophyletic with *Methylocystis* and *Methylosinus* (Dedysh *et al*., 2002; 2005). Major phospholipid fatty acids (PLFA) of *Methylocapsa* and *Methylocella* differ from those of type II. In addition to *Proteobacteria*, three recently discovered methanotrophs were grouped within the phylum *Verrucomicrobia*. These methanotrophs, however, appear to be restricted to extreme environments showing growth at low pH (2-2.5) and high temperatures exceeding 50°C (Dunfield *et al*., 2007; Pol *et al*., 2007; Islam *et al*., 2008).

Methanotrophs are found to inhabit in a wide range of environments. Besides *Verrucomicrobia*, some methanotrophs in *Proteobacteria* (*Methylococcus*,

Methylocaldum and *Methylothermus*) have been reported to be thermotolerant or moderately thermophilic growing up to 70°C (Bodrossy *et al*., 1999; Tsubota *et al*., 2005). At the other end of the temperature spectrum, some methanotrophs (*Methylobacter* and *Methylocella*) are adapted to cold environments with a temperature range of 5-15°C (Berestovskaya *et al*., 2002; Wartiainen *et al*., 2006). Halophilic methanotrophs are represented by *Methylohalobius crimeensis* showing optimum growth at 1-1.5 M NaCl, tolerating NaCl concentrations up to 2.5 M (Heyer *et al*., 2005). Some halotolerant methanotrophs (*Methylomicrobium*) are capable of growth in 1.1-1.5 M NaCl (Kalyuzhnaya *et al*., 2008). While known *Verrucomicrobia* species typify extremely acidophilic methanotrophs, moderately acidophilic methanotrophs include genera *Methylocapsa* and *Methylocella* growing at a pH range of 4.2 – 7.2 (Dedysh *et al*., 2002; 2005). The discovery of methanotrophs from diverse environments testifies to the ubiquity of this functional guild.

The narrow substrate range of methanotrophs are not as restrictive as previously thought. The first evidence of facultative methanotrophs was provided with the characterization of *Methylocella* utilizing multicarbon compounds (Dedysh *et al*., 2005). In addition, *C. polyspora*, a methane oxidizer was found to be a likely candidate for facultative methanotrophy (Stoecker *et al*., 2006). Recently, some *Methylocystis* species have been shown to grow on acetate as their sole substrate (Belova *et al*., 2010). These previously unrecognized broader substrate utilization among methanotrophs indicates that facultative methanotrophy may not be unusual exceptions.

In aerobic methane oxidation, methane is initially oxidized to methanol and via the intermediates formaldehyde and formate, to carbon dioxide as the final product in the dissimilatory pathway (Figure 2). In the assimilatory pathway, formaldehyde is incorporated into cell biomass via the ribulose monophosphate pathway (type I) or serine pathway (type II). Methane monooxygenase (MMO) is the key enzyme catalyzing the initial conversion of methane to methanol and is present in either a particulate (pMMO) or soluble (sMMO) form. Copper is essential for regulating the expression of both enzymes (Kim *et al*., 2004). All known methanotrophs possess pMMO except *Methylcella palustris* (Dedysh *et al*., 2000); however, sMMO is restricted to some species. In contrast to pMMO which has a narrow carbon substrate spectrum (alkanes and alkenes up to C_5), sMMO is able to oxidize a wider range of compounds including aliphatic and aromatic hydrocarbons and their halogenated derivatives (Trotsenko and Murrell, 2008), and is of interest for removal of contaminants in bioremediation applications (Shigematsu *et al*., 1999).

The existence of 'high-affinity' methanotrophs in environments with low methane mixing ratio was postulated by Bender and Conrad (1992). So far, these putative methanotrophs have only been defined by environmental gene sequences grouped within a distinct cluster usually referred as the upland soil cluster (USC) (Knief *et al*., 2003; Knief and Dunfield, 2005). Recently, it was shown that a *Methylocystis* sp. harboring a second pMMO (pMMO2) was able to oxidize methane and grow at trace atmospheric levels for over three months (Baani and Liesack, 2008).

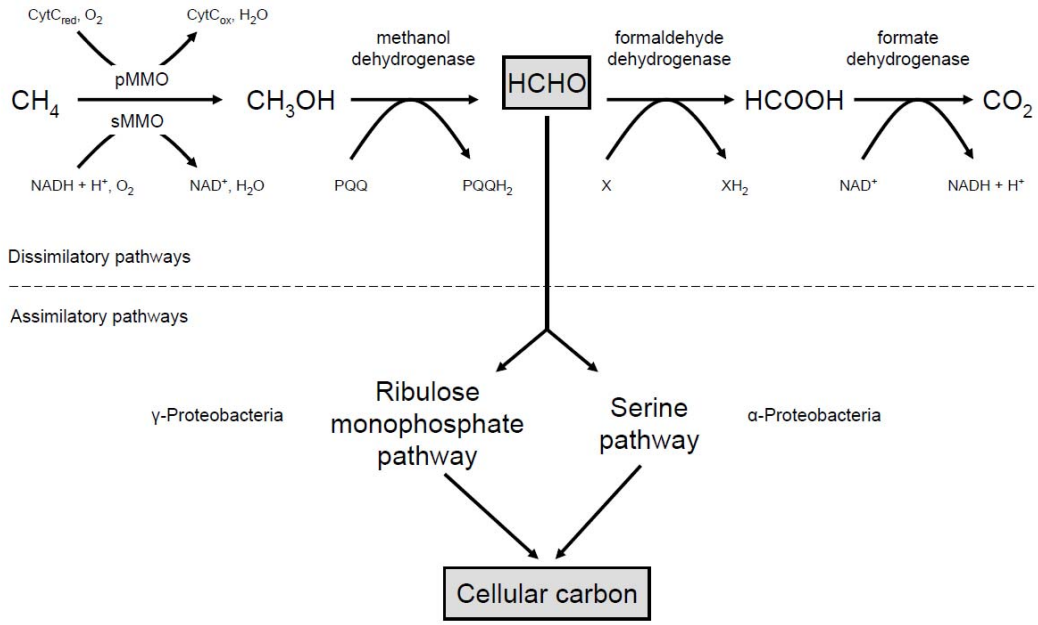

Figure 2: Assimilatory and dissimilatory methane oxidation pathways. Abbreviations: Cytc = Cytochrome c; PQQ = pyrroloquinoline quinine; X = $NADP^+$ or cytochrome linked. Modified from Hanson and Hanson 1996; Lüke, 2009; Mancinelli, 1995.

Anaerobic methane oxidizers

The existence of anaerobic methane oxidation have long been postulated based on methane concentration profiles, radiotracer experiments and stable isotope analysis indicating methane consumption in anoxic marine sediments; but, the microorganisms mediating this reaction had escaped isolation (Valentine and Reeburgh, 2000). The first microorganisms apparently mediating anaerobic methane oxidation were shown to be a consortium of archaea and sulphate reducing bacteria when visualized by fluorescent *in situ* hybridization (FISH) (Boetius *et al*., 2000). So far, three clusters of methanotrophic archaea (ANME-1, ANME-2 and ANME-3) have

been described and found to be related to methanogens of the order *Methanosarcinales* and *Methanomicrobiales* (Knittel *et al*., 2005; Orphan *et al*., 2002). The presence of gene homologues of the enzyme methyl-coenzyme M reductase (mcr), a key enzyme for methane synthesis in ANME suggests that anaerobic methane oxidation is supposedly performed by a reverse reaction of methanogenesis (Hallam *et al*., 2004; Shima and Thauer, 2005). Furthermore, nickel proteins resembling cofactor F_{430} of mcr were isolated from microbial mats exhibiting anaerobic methane oxidation (Krüger *et al*., 2003). However, the detailed biochemical mechanism of this reaction remains unclear.

It is also unclear whether methanotrophic archaea are capable of anaerobic methane oxidation by themselves or in syntrophy with sulphate reducers (Boetius *et al*., 2000). In theory, anaerobic methane oxidation can be coupled to alternative electron acceptors (Fe^{3+}, Mn^{4+} and NO_3^-) which are energetically more favorable that sulphate. Recently, empirical evidence supports the idea that anaerobic methane oxidation could take place within a sole microbial species. Methane oxidation coupled to denitrification was discovered in an enrichment culture from anoxic sediment where approximately 10% of the consortium consisted of archaea related to ANME-2, the rest being bacteria belonging to the candidate division NC10 (Raghoebarsing *et al*., 2006). As it turned out, methane oxidation was later found to be catalysed exclusively by the bacteria candidate division NC10 (Ettwig *et al*., 2008). Even more recently, coupling of anaerobic methane oxidation to manganese and iron have been found in methane-seep sediment

(Beal *et al*., 2009). Anaerobic methane oxidation remains an enigmatic process and interesting study. To date, there is no evidence for the existence of anaerobic methane oxidation in rice paddies.

1.4 The *pmoA* gene marker

The 16S rRNA gene is commonly used as a phylogenetic marker for studying microbial ecology. An alternative to the 16S rRNA gene are the genes encoding for enzymes catalyzing specific key processes (functional genes). In methanotrophs, the *pmoA* gene encodes for the particulate methane monooxygenase, pMMO, and is frequently used as a functional gene marker. The *pmoA* is highly conserved and has been found to correspond to the 16S rRNA phylogeny (Kolb *et al*., 2003), making *pmoA* a suitable alternative to the 16S rRNA gene.

The *pmoA* is a subunit of the pMMO gene cluster comprising of pmoCAB. Results from pure culture studies show that pmoCAB are present in duplicate gene copies (Semrau *et al*., 1995). Recently, an additional *pmoA* gene, referred to as *pmoA2* was found to be present in several type II methanotrophs (Yimga *et al*., 2003). The *pmoA2* appears to be constitutively expressed and is responsible for oxidizing methane at atmospheric concentrations (Baani and Liesack, 2008). Besides the *pmoA*, the *mmoX* gene encoding for the soluble methane monooxygenase and *mxaF* encoding for the methanol dehydrogenase had been used for targeting methanotrophs (Horz *et al*., 2001).

For assessing *pmoA* gene diversity, the choice of primer sets (A189f-A682r, A189f-mb661 and A189f-A650) is an important criterion to consider. The A189f-A682r primer set, for instance, amplifies both *pmoA* and *amoA* sequences, reflecting the high degree of sequence identity between *pmoA* and *amoA* (Holmes *et al*., 1995). While the A189f-mb661r primer set demonstrated the largest recovery of *pmoA* gene diversity, it failed to amplify sequences related to 'high-affinity' methanotrophs which were retrieved using the A89f-A650 primer set (Bourne *et al*., 2001). Therefore, a combination of primer sets is required to access *pmoA* gene diversity.

1.5 Methanotrophs as model microorganisms to study biodiversity and ecosystem functioning (BEF)

Prokaryotic microbes can catalyze all biogeochemical cycles and have done so before the advent of eukaryotes. Hence, microorganisms play a critical role in biogeochemical cycles. Overall, microbial diversity may be very high (Spain *et al*., 2009; Torsvik and Øvreås, 2002) and have been assumed to be functionally redundant and omnipresent, and therefore, virtually inextinguishable. Owning to their vast diversity and persistence, microbes are often neglected in BEF studies. The majority of these studies use higher plants or invertebrates as model systems while only few studies have dealt with belowground processes mediated by soil microbial communities. However, recent work indicates that microbes may well be sensitive to environmental disturbances (Allison and Martiny, 2008; Wittebolle *et al*., 2009).

The BEF discussion considers two multi-facetted concepts comprising species richness and evenness, and resilience and resistance, respectively. Species richness and evenness refer to the number and proportion of species, respectively. Resilience is defined here as the rate of a microbial composition to return to its undisturbed composition after disturbance, while resistance is the 'degree to which microbial composition remains unchanged in the face of disturbance' (Allison and Martiny, 2008). It is thought that higher species diversity begets superior ecosystem functioning (Cardinale *et al.,* 2002; Marquard *et al.,* 2009; Wohl *et al.*, 2004). However, experiments with constructed communities have demonstrated that not only species richness but also their evenness may be essential for the functional stability of a microbial system (Wittebolle *et al*., 2009). In contrast, other studies have shown that biodiversity has little or no impact, or inconsistent and varied effects on ecosystem processes (Bradford *et al.*, 2002; Naeem and Li, 1997; Wardle *et al.*, 2000).

Recovery needs time: the resilience of microbes to disturbances may not be observed immediately (Allison and Martiny, 2008). While short term effects of disturbances on the sensitivity of a microbial community are frequently reported, only few studies explicitly focused on the recovery of the community composition over a longer time course. Community composition changes have been associated with changes in ecosystem process rates (Allison and Martiny, 2008). Therefore, the recovery of the community composition is essential when considering the resilience of ecosystem functioning. The community composition or functioning, however, may be radically altered

considering the conversion of an indigenous to a man-made ecosystem such as during the formation of rice paddies. In this scenario, microbes adapt to the new environment. With physiological flexibility and rapid evolution rates, it is plausible to expect a selective adaptation of the microbial population to the new abiotic condition.

The soil microbial community has been shown to be resilient, recovering in soil respiration and litter decomposition after induced disturbances (Degens, 1998; Griffiths *et al*., 2001). These, however, are general mineralization processes that could be performed by a broad spectrum of soil microorganisms. Therefore, functional guilds with a well-defined and restricted substrate usage are prime candidates for BEF studies. These prime candidates include nitrifiers (Le Roux *et al*., 2006; Wertz *et al*., 2007) and methanotrophs (King, 1997; Horz *et al*., 2005).

1.6 Aims of study

Methanotrophs have been intensively studied. Different aspects of methanotrophy in rice paddies covering population diversity (Lüke *et al*., 2010), and effects of temperature (Mohanty *et al*., 2007), and agriculture management practices (Lüke, 2010; Mohanty *et al*., 2006; Noll *et al*., 2008; Shrestha *et al*., 2010) have been documented. However, the vulnerability and resilience of paddy soil methanotrophs to disturbances is largely unknown. Therefore, this PhD thesis addresses the following questions:

Chapter 2: Recovery of methanotrophs from disturbance: population dynamics, evenness, and functioning.

Stochastic events resulting in die-offs of microbial populations occur in nature. Simulating die-offs, disturbance-induced mortality represents a non-selective form of disturbance. *Are methanotrophs capable of colonizing the 'space' left by perished microorganisms? How changes methanotrophic community structure, diversity and evenness upon disturbance? How does disturbance affect methane uptake?*

Chapter 3: Response and resilience of methanotrophs to heat shock.

A strong temperature increase (heat shock) is known to occur in rice paddies, particularly on the surface soil of recently planted rice saplings. Heat shock may affect sub-populations of methanotrophs by selecting for those that are

thermo-tolerant. *How changes methanotrophic community structure and diversity upon heat shock? How does heat shock affect methane uptake?*

*Chapter 4***:** Aging well: methanotrophic potential and methanotroph along a a chronosequence of 2000 years.

On going rice agriculture, while shaping the soil properties may also shape the methanotroph population, leaving an imprint on the community structure. *How does on-going rice agriculture affect the methanotrophic community structure and methane uptake? Are sub-populations of methanotrophs showing specific adaptation to rice paddy environments? How diverse are paddy soil methanotrophs?*

Chapter 5: Methanotrophic activity and composition in young and ancient rice paddies.

In contrast to soil microcosms, the pilot scale experimental set up simulating *in-situ* conditions may provide a more holistic and comprehensive view when comparing the methane oxidizing potential, and methanotrophic community in young and ancient rice paddies. *How divergent are the young and ancient rice paddies with regard to methanotrophic activity and composition? How changes methanotrophic activity and community upon fertilization?*

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2. Recovery of methanotrophs from disturbance: population dynamics, evenness, and functioning

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2.1 Abstract

Biodiversity is claimed being essential for ecosystem functioning, but threatened by anthropogenic disturbances. Prokaryotes have been assumed to be functionally redundant and virtually inextinguishable. However, recent work indicates that microbes may well be sensitive to environmental disturbance. Focusing on methane oxidizing bacteria as model organisms, we simulated disturbance-induced mortality by mixing native with sterilized paddy soil in two ratios, 1:4 and 1:40, representing moderate and severe die-offs. Disturbed microcosms were compared to an untreated control. Recovery of activity and populations was followed over four months by methane uptake measurements, *pmoA*-qPCR, *pmoA*-based T-RFLP (terminal restriction fragment length polymorphism), and a *pmoA*-based diagnostic microarray. Diversity and evenness of methanotrophs decreased in disturbed microcosms, but functioning was not compromised. We consistently observed distinctive temporal shifts between type I and type II methanotrophs, and a rapid population growth leading to even higher cell numbers comparing disturbed microcosms to the control. Overcompensating mortality suggested

that population size in the control was limited by competition with other bacteria. Overall, methanotrophs showed a remarkable ability to compensate for die-offs.

2.2 Introduction

Earth's biodiversity is declining forcing mankind to consider how ecosystems' stability and services will change in future (Loreau *et al.* 2001; Mccann 2000). The biodiversity-stability discussion combines two multi-facetted concepts comprising species number and evenness, and resistance and resilience, respectively (Ives and Carpenter 2007). Prokaryotic microbes can catalyze all biogeochemical cycles and have done so before the advent of eukaryotes. They have been assumed to be functionally redundant and virtually inextinguishable. However, recent work indicates that microbes may well be sensitive to environmental disturbance (Allison and Martiny 2008). Experiments with constructed communities have demonstrated that not only the number of species but also their evenness may be important for the functional stability of a microbial system (Wittebolle *et al.* 2009). Overall, soil microbial diversity is indeed very high (Spain *et al.* 2009; Torsvik *et al.* 2002). Therefore, functional guilds with a well-defined substrate usage are prime candidates not only to study potentially endangered microbial functions, but also to contribute to the general discussion about biodiversity and ecosystem functioning. These prime candidates include nitrifiers (Le Roux *et al.* 2006; Wertz *et al.* 2007) and methanotrophs (Horz *et al.* 2005).

Methanotrophs are able to use methane both for catabolism and anabolism. They have a well-known physiology (Trotsenko and Murrell 2008) and a large yet tractable phylogenetic diversity (Lüke *et al.* 2010; McDonald *et al.* 2008). Methane is an important greenhouse gas contributing substantially to radiative forcing (Intergovernmental Panel on Climate Change 2007). Methanotrophs serve as a biological filter significantly mitigating methane emissions from wetlands, rice fields and landfills, and are the only biological sink to atmospheric methane (Conrad 2009; Reeburgh *et al.* 1993). Traditionally, methanotrophs have been divided into two subgroups; type I and type II. Type I can be further divided into type Ia (e.g. *Methylobacter*, *Methylomicrobium, Methylomonas and Methylosarcina*) and type Ib (*Methylococcus* and *Methylocaldum*) (Bodrossy *et al.* 2003). The key enzyme of all methanotrophs is the methane monooxygenase (MMO) existing either as a particulate membrane bound (pMMO) or a soluble form (sMMO) (Trotsenko and Murrell 2008). While all known methanotrophs except *Methylocella palustris* (Dedysh *et al.* 2000) possess pMMO, sMMO is restricted to only some species. The *pmoA* gene encodes a subunit of pMMO and has been shown to be highly conserved often being used as a marker gene in cultivation independent studies (Lüke *et al.* 2010; McDonald *et al.* 2008).

Here we investigated the response of methanotrophs to a model disturbance causing a partial die-off of all microbial populations. We addressed the following questions: (i) how changes methanotrophic community structure, diversity and evenness upon disturbance, and (ii) how does disturbance affect methane uptake rates. We incubated microcosms with untreated and

sterilized paddy soil in different ratios over four months and followed methane uptake rates with time. Temporal succession of methanotrophs was monitored by *pmoA*-based T-RFLP, and different subgroups of methanotrophs were enumerated by qPCR. For higher phylogenetic resolution, a *pmoA*-based diagnostic microarray was used allowing to identify methanotrophs down to the genus level (Bodrossy *et al.* 2003).

2.3 Experimental procedure

Soil and soil microcosms

Soil was sampled in a rice field at the C.R.A. Agricultural Research Council, Rice Research Unit (Vercelli, Italy) in 2006. Soil parameters and agricultural practice have been described previously (Krüger *et al.* 2001). Soil was air-dried and part of it was sterilized by γ-irradiation (25 kGy; ^{60}Co) (McNamara *et al.* 2003; Murase *et al.* 2006). Sterility of γ -irradiated soil was checked by following CO_2 release from water-saturated soil incubated in serum bottles. CO_2 release was minute (3%) as compared to non-sterile controls. Untreated and sterilized soil was homogenized and mixed in ratios of 1:4 and 1:40, representing a moderate and severe disturbance, respectively. Microcosms containing untreated soil were used as controls. Dry soil (20 g) was filled in sterile Petri dishes and saturated with autoclaved distilled water (0.45 ml · g dry soil^{-1}). These microcosms were incubated up to four months in gas tight jars under an atmosphere of 10 vol.% CH_4 in air at 25°C in the dark. The atmosphere was replenished every third day. Microcosms representing the

same treatment were incubated together in the same jar. Microcosms were sacrificed at intervals and soil was homogenized before being stored in aliquots at -20°C for not more than ten days.

DNA Extraction from soil samples

Total DNA was extracted in triplicates from soil using the Q-Biogene soil extraction kit (MP, Heidelberg). The extraction was performed according to the manufacturer's instructions with the following modification: DNA was subjected to additional washing steps with 5.5 M guanidine thiocyanate to remove humic acids. DNA extracts were stored at -20°C for further analysis.

Porewater Analysis

Total NH_4^+ concentration in water-logged soil was measured fluorometrically (Murase *et al.* 2006). Anion concentrations (SO_4^{2-}, NO_2^-, NO_3^-) were determined by ion chromatography (Bak *et al.* 1991) after centrifuging an aliquot of wet soil at 14000 x g for 10 minutes followed by filtration of the supernatant (0.2 µm; Whatman, Dassel, Germany). Nitrate was only detected in minute concentrations (<0.06 mM) and disappeared rapidly; nitrite was below detection limit (<0.005 mM).

Oxygen profiles and methane fluxes

O_2 was measured with a microsensor (Unisense, Aarhus, Denmark; (Revsbech 1989)). To measure methane uptake rates, microcosms were removed from the jars and incubated individually in flux chambers (volume 172 ml; 3-5 % CH_4 in air). Rates were determined from linear regression by following the decrease of methane with time (5-6 hours). Methane was measured by gas chromatography with a flame ionization detector (SRI-9300A; SRI Instruments, Torrance, CA).

pmoA-based qPCR assays

All qPCR assays were performed as described previously (Kolb *et al.* 2003). We focused on TYPEII, MBAC and MCOC assays targeting the dominant groups of methanotrophs in Vercelli paddy soils. In a pilot experiment, MCAP and FOREST assays targeting *Methylocapsa* and sequences retrieved from forest soils (USC-α), respectively, failed to detect methanotrophs in Vercelli paddy soil; they were not applied further. qPCR assays were performed with an iCycler IQ Thermocycler (Bio-Rad, München, Germany). Each assay was performed in triplicate with primers, primers concentration and PCR profiles as described (Kolb *et al.* 2003). In addition, total 16S rRNA (EUBAC assay) was enumerated according to Stubner (2002). Details and minor modifications are summarized in Table 1. DNA from clones was used as standard for calibrating the assays. qPCR was carried out in 25 µl volumes using ABgene 96 wells PCR plates (VWR International GmbH, Darmstadt, Germany). The

plates were sealed with iCycler IQ optical tape (Bio-Rad). PCR master mix containing 12.5 µl iQ SYBR Green Supermix (Bio-Rad) and 0.4 µl of forward and reverse primers each was added to 5.0 µl DNA template. The DNA template was diluted to obtain optimal target copy numbers (Kolb *et al.* 2003). Bovine serum albumin (0.8 g; Roche) was added to the EUBAC assay to relief inhibition of amplification (Kreader 1996). DNase- and RNase-free water was added to a final volume of 25 µl.

Table 1: Primer combinations for qPCR assays used in this study.

Primer set	Forward/reverse primer (nM)	PCR thermal profile[1]	Data Acquisition	qPCR assay	Reference
A189f	800	94°C, 25s;	82°C, 10s	MBAC	Kolb *et al.*,
Mb601r	800	54.5°C, 20s;			2003
		72°C, 45s			
A189f	1000	94°C, 25s;	82°C, 10s	MCOC	Kolb *et al.*,
Mc468r	333	64.0°C, 20s;			2003
		72°C, 45s			
II223f	800	94°C, 25s;	83°C, 10s	TYPEII	Kolb *et al.*,
II646r	800	69.5°C, 20s;			2003
		72°C, 45s			
519f	330	94°C, 25s;	72°C, 10s	EUBAC	Stubner, 2002
907r	330	50°C, 20s;			
		72°C, 45s			

[1] Thermal profile showing temperature and time for denaturation, annealing and elongation.

pmoA-based T-RFLP analysis

The detailed T-RFLP protocol had been described before (Lüke *et al.* 2010). In short, the *pmoA* gene was amplified using the FAM-labeled forward primer A189f and the reverse primer A682r (Holmes *et al.* 1995). PCR amplicons were digested using the restriction endonuclease MspI and T-RFs were separated using the ABIPrism 310 (Applied Biosystems). The GeneScan 3.71 software (Applied Biosystems) was used to determine the length of fluorescently labeled T-RFs by comparison with the internal standard (MapMarker 1000; Bioventures). Peaks derived from primer dimers were excluded from analysis after cross-checking with a negative control. T-RFLP analysis for each time point was performed in duplicates.

Diagnostic Microarray

Diagnostic microarray analysis was performed according to the procedure developed by Bodrossy and colleagues (Bodrossy *et al.* 2003) with some modifications. The A189f/T7-A682r primer combination was used to prepare the target for the microarray probes. Five-fold diluted Cy3 was added into the *in vitro* transcription reaction and hybridization was performed over night in a hybridization oven (ThermoLifeSciences, Egelsbach, Germany) with the shaking platform set at maximum speed.

Statistics

Statistical analyses were done in *R* ver. 2.10.1 (R Development Core Team 2009) using packages *vegan* ver. 1.18-2 for community ecology analysis (Oksanen *et al.* 2010) and *ggplot2* ver. 0.8.5 for statistical graphics (Wickham 2009). T-RFLP profiles were standardized prior to analysis (Dunbar *et al.* 2001). Probe signals were scaled to maximum signal intensity as revealed from previous validations with clone DNA (Bodrossy *et al.* 2003; Stralis-Pavese *et al.* 2004) and limited to 1, if higher. To calculate Shannon-Weaver's diversity $H' = \Sigma\ p_i\ log\ p_i$, all probes were summed up and the fraction *p* per probe *i* was calculated.

2.4 Results

Methane uptake rate and environmental parameters

Moderate disturbance showed no adverse effect on methane uptake rates. However, methane uptake rates in severely disturbed microcosms first dropped below the control level, but increased afterwards for two months attaining a maximum twice as high as in the other microcosms (Figure 1). Compared to the control, methane uptake rates in the severely disturbed microcosms were significantly lower at the beginning (day 4), but became significantly higher already at day 14 and remained so till day 77 (two-sided KS-test, $p \leq 0.01$). The moderate disturbance resulted in a somehow intermediate response but still with significantly lower (days 14 – 77, $p \leq 0.01$)

methane uptake rates than in the severely disturbed microcosms. After four months incubation, methane uptake rates for control and moderately disturbed microcosms were virtually identical while microcosms exposed to severe disturbance exhibited a slightly lower rate.

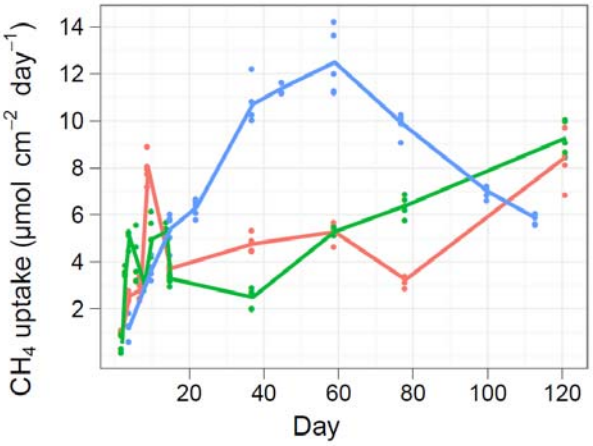

Figure 1: Effects of disturbance on methane uptake rate (mean and individual measurements from four microcosms). Red, green and blue denotes control, moderate and severe disturbances, respectively.

Oxygen penetration depth decreased rapidly during the first 10 (disturbance) to 18 days (control) being later restricted to the uppermost 0.4 mm (Figure S1). The initial rapid change in O_2 penetration depth in disturbed microcosms reflected the faster increase in methanotroph cell numbers as confirmed by qPCR (see below). Porewater sulphate was high at 1.9 - 2.3 mM at the beginning, but had virtually disappeared at day 15 while total soil ammonium showed a cyclic pattern. (Figure S2).

Population dynamics and community structure

The coverage of the qPCR assays was checked *in silico* using a database with 579 clone sequences retrieved from Vercelli (Lüke *et al.* 2010). The assays MBAC, MCOC and TYPEII covered 93, 91, and 83% out of 164, 136, and 279 clones, respectively. The *pmoA2* genotypes retrieved from Vercelli (Yimga *et al.* 2003) are not covered by any of the assays. Hence, the assays provide a fairly complete estimate of the target groups.

We observed a rapid initial growth of type II methanotrophs in severely disturbed microcosms, while growth of type I (MBAC and MCOC) was retarded (Figure 2). On the long term, however, all communities became dominated by type II. Eventually, methanotrophs reached even higher copy numbers in disturbed microcosms nearly equaling the copy numbers of the eubacterial 16S rRNA gene.

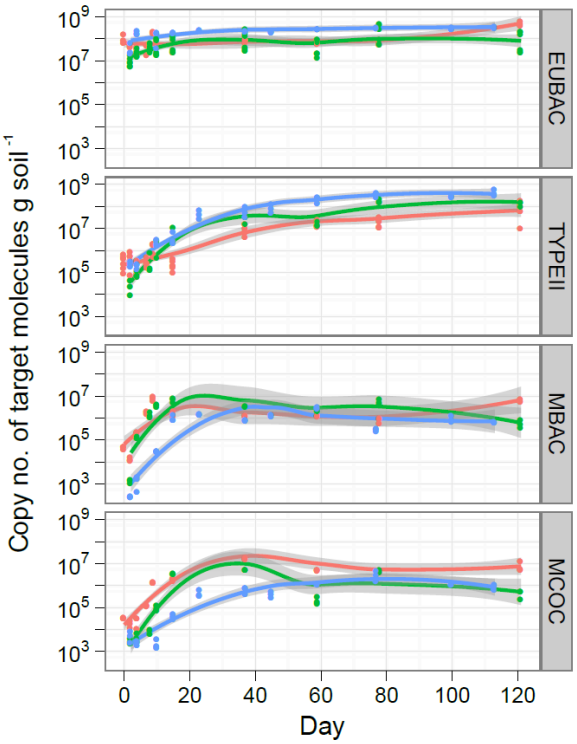

Figure 2: qPCR analysis of EUBAC, TYPEII, MBAC and MCOC assays. Assays were performed in triplicates at each time point. Red, green and blue denote control, moderate and severe disturbances, respectively. Local regression lines are surrounded by 95% confidence intervals (grey).

More details of community shifts became evident from *pmoA* T-RFLP (Figure 3). The assignment of T-RFs was based on a previous study on the same soil considering 500 clones (Lüke *et al.* 2010). Three dominant T-RFs were indicative for *pmoA* (type Ib: 79 bp; type II: 244bp; *pmoA2*: 278 bp). *pmoA2* encodes for a second isoenzyme in type II methanotrophs (Dunfield *et al.* 2002). The fraction of *pmoA2* increased notably in severely disturbed microcosm. Two fragments could be affiliated with *amoA* (46 and 114 bp). Additional minor T-RFs were summed up and did not show any significant temporal shifts accounting for <5% after ten days of incubation. The T-RFLP

analysis showed consistent shifts between type I and type II methanotrophs over all treatments suggesting a succession from type I to type II methanotrophs. After two months, the population had stabilized and the ratio of type I to type II methanotrophs was nearly the same as at the beginning of the experiment. However, type I methanotrophs were apparently less important in the disturbed microcosms.

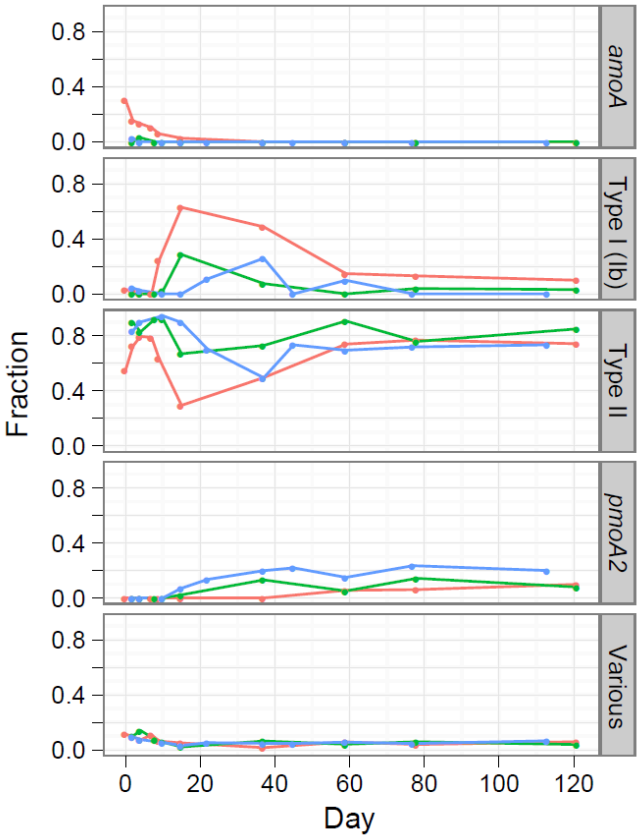

Figure 3: *pmoA*-based T-RFLP analysis of control, moderately and severely disturbed microcosms as denoted by red, green and blue, respectively (mean of two replicates from one microcosm). Dominant T-RFs were 79, 244 and 278 bp long and indicative for type Ib, type II and *pmoA2*, respectively. T-RFs 46 and 114 were pooled; they are indicative for *amoA*. Minor T-RFs were summed up and designated as 'Various'.

For a higher phylogenetic resolution, the *pmoA*-targeting microarray (Bodrossy *et al.* 2003) was used (Figure 4; Figure S3). The microarray revealed high signals for probes specific for *Methylocystis* (type II; probe Mcys413) with no obvious temporal changes. However, *Methylosinus* (type II; probe Msi294) was only detected in small amounts being nearly absent in the severely disturbed microcosm (Figure 4). *Methylosarcina*-like type Ia methanotrophs (probe Mmb562) and ammonia oxidizers (probe NsNv363) were only detected within the first days of incubation and seemed to play only minor roles in the disturbed microcosms. In contrast, signal intensity of type Ib methanotrophs belonging to the rice paddy cluster 1 (RPC-1; probe M90_253) (Lüke *et al.* 2010) and to *Methylocaldum*-related rice paddy clusters (probe 501_375) increased with time, but was undetectable or very low in severely disturbed microcosms. Like for the T-RFLP analysis, signals for probes targeting the *pmoA2* (LP21_190; NmsiT271) increased with time becoming even more important in the disturbed microcosms.

Based on the microarray data, the effect of disturbance was tested. We applied RDA (redundancy analysis) with treatment (control, moderate and severe disturbance) as constraints and found a significant effect (ANOVA, 999 permutations, $p \leq 0.001$) explaining 12% of total variance. Adding time as second constraint, treatment and time explained 12.3 and 20.7% of variance, respectively (ANOVA, 999 permutations, $p \leq 0.001$). Considering the interaction between treatment and time improved the ordination only marginally. Community patterns from controls and severely disturbed microcosm were well separated, while moderately disturbed microcosms

overlapped with both (Figure 5). As suggested by Figures 4 and S3, probes indicative for *pmoA2* contributed to the separation of severely disturbed microcosm (Figure 5).

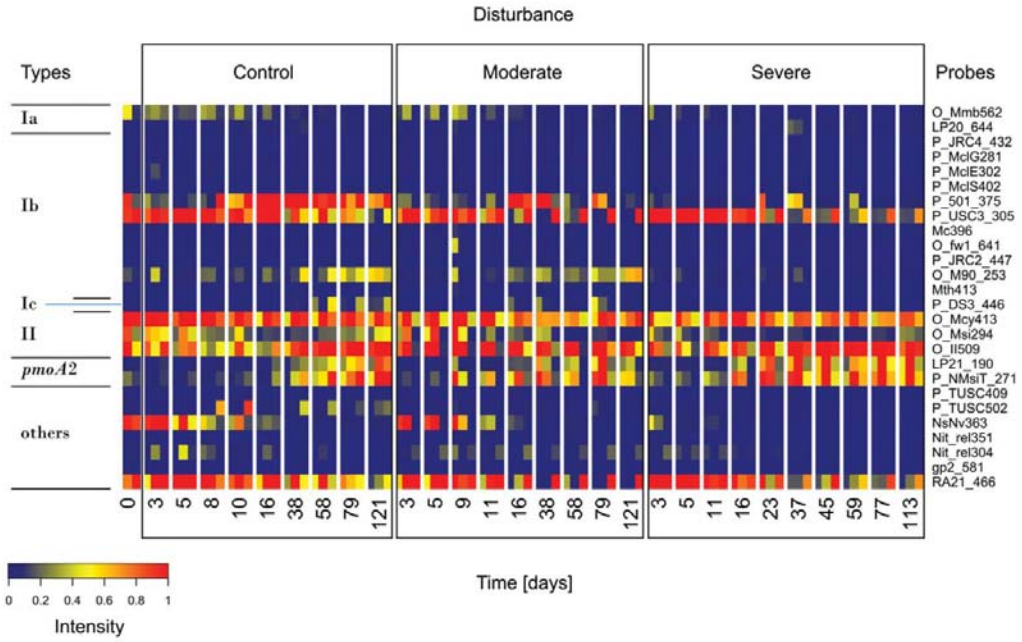

Figure 4: Microarray analysis of methanotroph communities in control, moderately and severely disturbed microcosms. Microarray results were normalized against reference values determined for each probe individually (Bodrossy *et al.* 2003), and relative fractions were calculated. Values of 1.0 and 0 indicate maximum signal intensity and no hybridization to the microarray probes, respectively. Only specific probes representing species or well defined environmental clusters within type Ia, type Ib, type II and *pmoA2* are shown. 'Others' represent probes targeting sequences that group phylogenetically between *pmoA* and *amoA*. Due to a lack of cultivated representatives in this intermediate group, it remains uncertain if the corresponding proteins catalyze methane or ammonium oxidation.

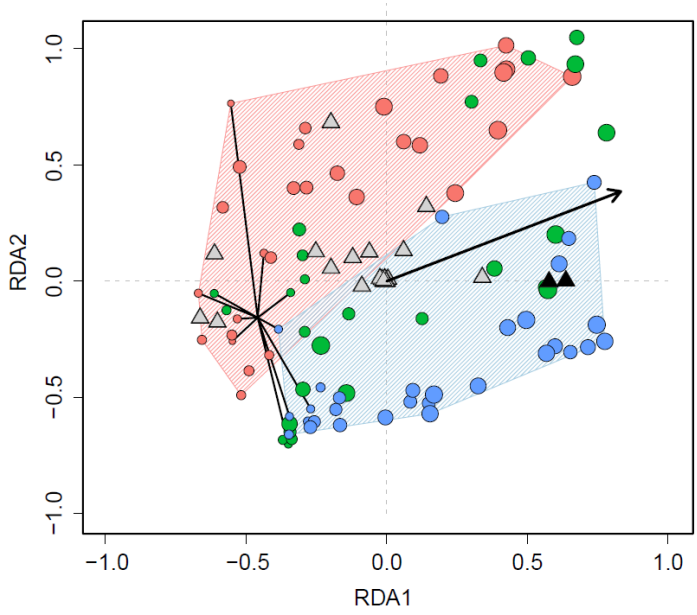

Figure 5: Microarray results as a biplot of a redundancy analysis (RDA) with treatment (red, green and blue denote control, moderate and severe disturbances, respectively) and time as constraints. The black arrow indicates the time gradient. Circles refer to microcosms. The area covered by control and severely disturbed microcosms is hatched with the corresponding colors. Circle size corresponds to increasing time. Samples taken at days 0 and 3 mark the beginning of the experiment and are connected by lines starting from the mean. Triangles represent probes. Probes indicative for *pmoA2* (LP21_190 and P_NMsiT_271) are marked in black

Diversity

Diversity *H'* has two components, number of taxa (here approximated by the number of positive probes) and evenness. One way to express evenness is by $V' = H' \cdot (ln\ s)^{-1}$ (s = number of species) (Pielou 1977). The time course of *V'*and *H'* was nearly identical (data not shown) and reflected the growth dynamics of different groups. Diversity in control and upon disturbance

showed drastic differences in particular after day 40 with low values in disturbed microcosms (Figure 6).

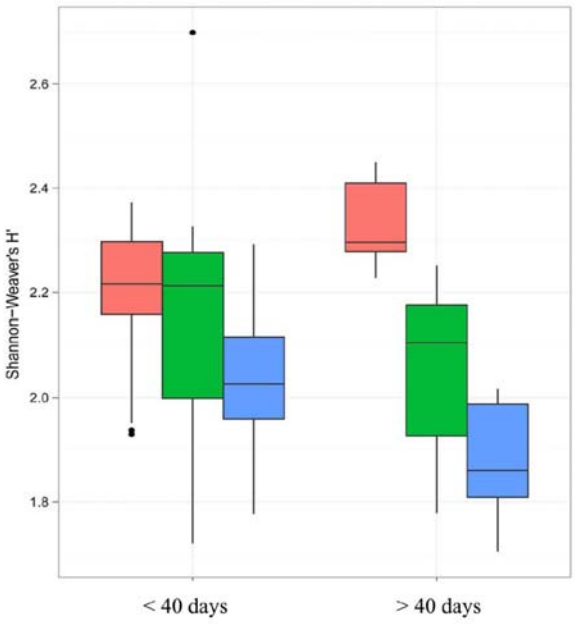

Figure 6: Box plot showing Shanon-Weaver's diversity H' based on *pmoA* microarray signals (refer to text). Red, green and blue denote control, moderate and severe disturbances, respectively.

2.5 Discussion

Here we show how a microbial guild recovers rapidly compensating for mortality. Growth of specific groups caused reduced diversity and evenness, but loss of functioning could even be over-compensated for some time. Three weeks after starting the experiment, methane uptake by the severely disturbed communities became even higher than in the control, however on the long term uptake rates became similar again (Figure 1).

The abiotic environment

Among potential abiotic controls, ammonium is a top candidate acting either as an inhibitor or a stimulator for methanotrophs and methane oxidation (Bodelier *et al.* 2000b; Bodelier and Laanbroek 2004; Bosse *et al.* 1993). The decrease of ammonium in the first 10-15 days was probably due to assimilation, while the subsequent increase could have been caused by mineralization enhanced by grazing (Figure S2). The role of protists as predators of soil bacteria and methanotrophs in particular has been shown before (Murase *et al.* 2006; Murase and Frenzel 2007; Murase and Frenzel 2008). qPCR, however, showed no net decrease of eubacteria or methanotrophs leaving the reason for these changes still open.

Population dynamics

The rapid increase of *pmoA* target numbers revealed by qPCR inferred successful re-colonization of the sterile soil and ruled out that extracellular DNA (Pietramellara *et al.* 2009) had any major effect on population analyses. Type II methanotrophs have often been regarded as largely inactive represented by resting stages, but target numbers increasing by two to three orders of magnitude (Figure 2) show clearly that upon disturbance they may take their chance. However, this does not imply a proportional contribution to methane oxidation. Cell-specific methane uptake rates calculated from Figures 1 & 2 decreased from 1-5 to 0.002-0.02 pmol $CH_4 \cdot h^{-1} \cdot cell^{-1}$ from

days 4 to 120, respectively, suggesting that a large fraction of cells became inactive with time re-filling the microbial seed bank.

To assess competition between methanotrophs and other bacteria, a qPCR assay targeting the eubacterial 16S rRNA gene was performed. It is suggested that methanotrophs contain two *pmoA* copies (Semrau *et al.* 1995). Average 16S rRNA copy numbers, however, have been shown to be high in bacteria thriving in recently flooded rice paddies, while they decrease during succession (Shrestha *et al.* 2007) reflecting changing life cycle strategies (Klappenbach *et al.* 2000). Therefore, assuming on average two copies per cell may overestimate cell numbers of eubacteria during early successions. Nevertheless, the ratio between the numerically dominating type II methanotrophs (*pmoA* copy number) and total eubacteria (16S rRNA gene copy number) shows how successfully methanotrophs over-compensate for mortality. They made up about 1-10% of total bacteria in controls, but reached apparently 100% upon disturbance (Figure 7). Hence, methanotrophs are not only limited by their main substrates methane and oxygen, but also by other nutrients and potentially even by space. Aerobic heterotrophs recovering from sub-lethal radiation doses grew to population levels significantly higher than in the untreated control (McNamara *et al.* 2007). Only few substrates were used upon disturbance, but the same substrate spectrum as in the control could be metabolized after recovery (McNamara *et al.* 2007). In summary, disturbed microbial communities show a remarkable ability to compensate for losses and to re-gain functionality.

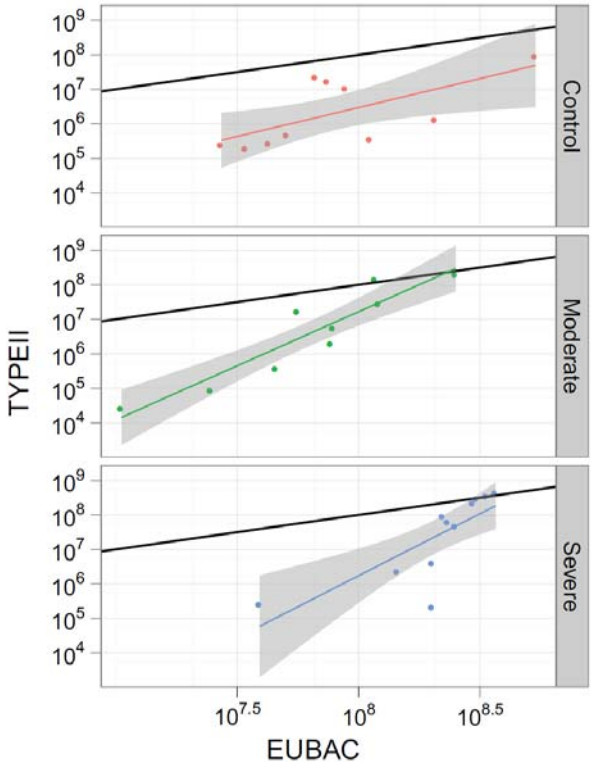

Figure 7: Ratio between the numerically dominating type II methanotrophs (*pmoA* copy number) and total eubacteria (16S rRNA gene copy). Assuming two copies each per cell, the black lines are drawn assuming that methanotrophs make up 1% of all bacteria. Red, green and blue denotes control, moderate and severe disturbances, respectively. Linear regressions are surrounded by 95% confidence intervals (grey). For details see text.

Community structure

We consistently observed temporal shifts from type I to type II methanotrophs by T-RFLP and qPCR analyses. T-RFLP is a rapid and reproducible method for monitoring community structure, but it lacks the high phylogenetic resolution provided by the *pmoA* microarray. A RDA of the microarray signals verified not only the temporal changes, but separated also the control and severely disturbed communities showing a significant treatment effect (Figure

5). The moderately disturbed microcosms took on an intermediate position. However, both T-RFLP and microarray lack exact quantitative qualities. Confirming trends already suggested by T-RFLP and microarray, the qPCR revealed the population dynamics of the main phylogenetic groups, but could not resolve details like the increase of *pmoA2* (Figures 3, S3). In summary, results were consistent in spite of the inherent limitations of the different methods, and lead to the same interpretation.

Both T-RFLP and microarray analyses detected a relative increase in *pmoA2*. The *pmoA2* encodes a subunit of a pMMO isoenzyme (pMMO2) (Dunfield *et al.* 2002). It was observed to be common among type II methanotrophs and was found to be functionally active using mRNA as proxy for activity (Dunfield *et al.* 2002; Yimga *et al.* 2003). The pMMO2 was constitutively expressed and oxidized methane at low mixing ratios, suggesting that *pmoA2* may play an important cellular function (Baani and Liesack 2008). The initial rapid increase of *pmoA2* upon severe disturbance (T-RFLP and microarray: Figures 3, 4) in parallel to an overall increase in *pmoA* target numbers (Figure 2) indicates that this was indeed caused by a quantitative increase of *pmoA2*. It is yet unknown, if all pMMO2 share the high affinity to methane that *per se* would not have provided a selective advantage in this experiment. However, other traits of the organisms carrying this gene may have been responsible for their success.

Diversity and evenness

Any disturbance from temperature shifts (Wertz *et al.* 2007) to fertilization (Bodelier *et al.* 2000a) may affect microbial diversity and performance. The most striking effect of a disturbance, however, is mortality. While different disturbances may affect different taxa or guilds in a specific way, our experiment used radiation killing non-selectively. We focused on the time after and simulated different mortalities by mixing sterile with untreated soil. What we call here a severe disturbance corresponds to killing 97.5% of all microbes. However, it is worth to compare this ratio to the absolute number of methanotrophs. With $5 \cdot 10^4$ to $5 \cdot 10^5$ methanotrophs per g untreated soil (Figure 2), we still have to expect $2.5 \cdot 10^4$ to $2.5 \cdot 10^5$ methanotrophs per microcosm. By design, this kind of disturbance could not have affected initial evenness, and it can be assumed having not affected species richness either, except the yet unrecovered "rare methanotrophic biosphere" (Lüke *et al.* 2010). Indeed, the number of positive probes in the microarray analysis was largely similar varying between 21 and 26. However, intensity changed, as did diversity and evenness that were calculated from relative probe intensities. Disturbance and low evenness had no detrimental effect on methane uptake rates that even could increase above the level in the control (Figures 1 & 6).

Concluding remarks

In conclusion, methanotrophs showed a remarkable ability compensating and even over-compensating for die-offs. However, recovery needs time: effects

of frequent disturbances hitting the community while evenness is low may accumulate shifting it to a different state. Calculated apparent cell-specific methane uptake rates decreased over time, suggesting that a fraction of the methanotrophic population became inactive and part of the microbial seed bank. The factors controlling this transition are still unknown, but are most probably not the main substrates oxygen and methane alone. We anticipate that future experiments will unravel how population dynamics of active microbes translate into the structure of a microbial seed bank. With methanotrophs, the seed bank may contain much more cells than are currently active (Eller *et al.* 2005) thus preserving and accumulating effects of repeated disturbance making historical contingencies an important component of the debate on biodiversity and stability.

Evenness of a microbial community has been found to be important for their functioning under environmental stress (Wittebolle *et al.* 2009). Here, we show that upon disturbance different growth rates may lead to an uneven community that nevertheless is functioning better than the control. Considering that only part of this community was active, linking diversity or evenness to functional resilience should consider not only population structure but also controls of activity.

Paddy soil methanotrophs are even able to overgrow other microorganisms after severe disturbance, provided methane is available. In contrast to our experiment, methanotrophs in upland soils are living at extremely low methane concentrations and have been found to be highly vulnerable

(Bodelier and Laanbroek 2004; Priemé *et al.* 1997). This may reflect particular physiological adaptations indicating that apparent functional redundancy can be constrained by other dimensions of an organism's niche. However, not only the qualitative aspect but also the energy flow through a microbial population and its effect on quantitative population dynamics deserves increased attention in biodiversity-ecosystem-functioning studies.

2.6 Supplementary materials

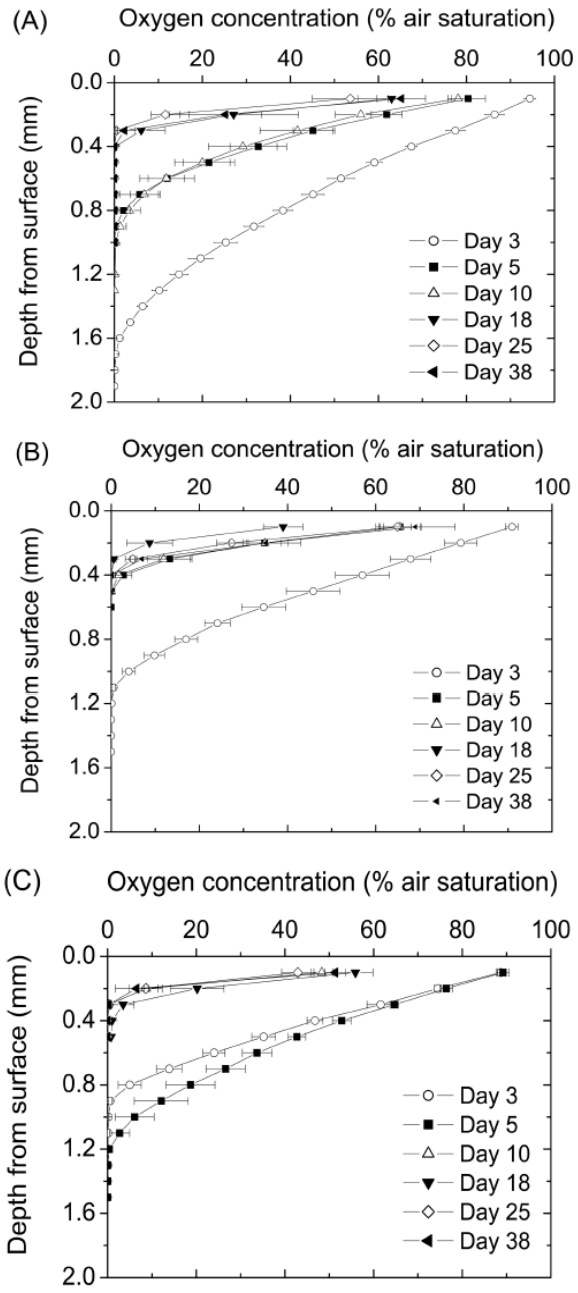

Figure S1: Effects of disturbance on O_2 penetration depth (mean ± SD, *n* = 3). A: control; B: moderate disturbance; C: severe disturbance.

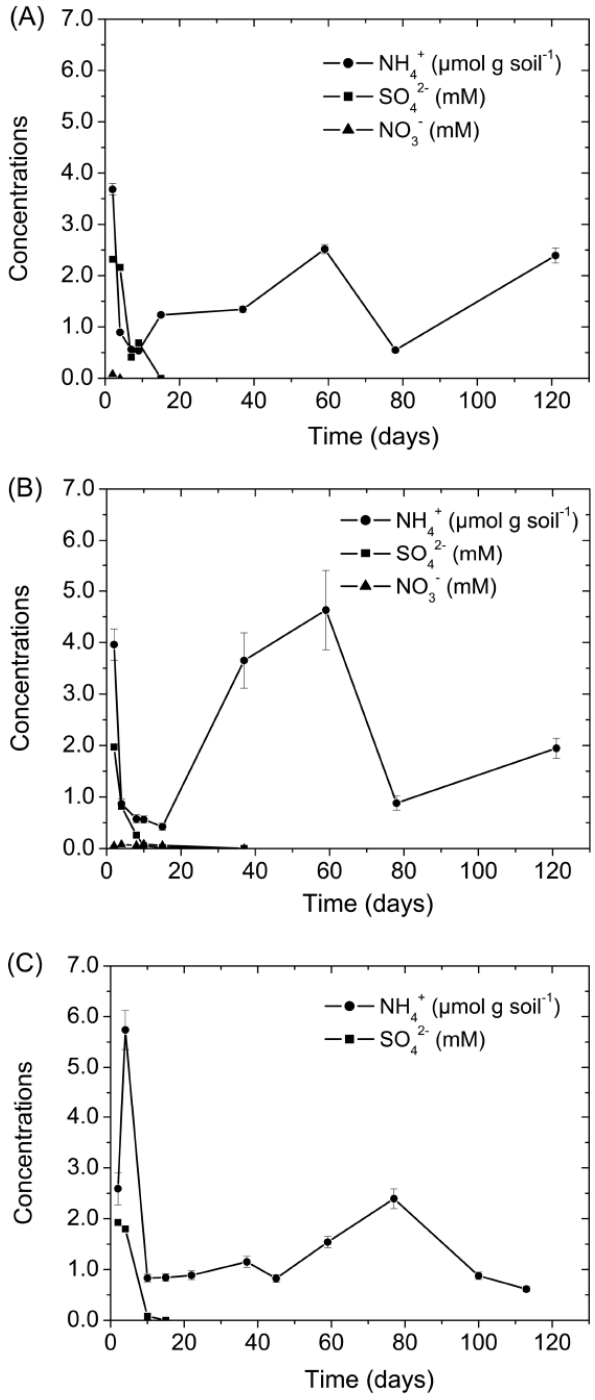

Figure S2: Temporal changes in nutrient concentration. Ammonium: mean ± SD, *n*=3. A: control; B: moderate disturbance; C: severe disturbance.

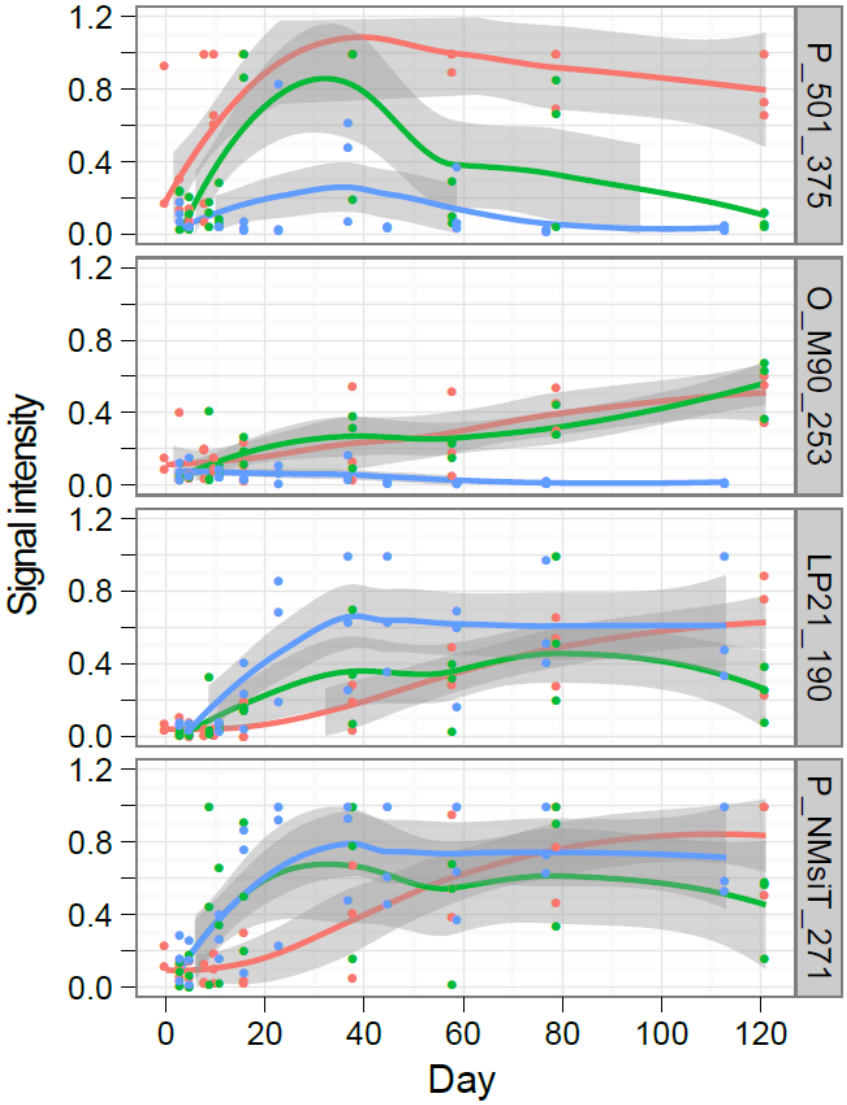

Figure S3: DNA-based microarray analysis. Probe signals were scaled to maximum signal intensity as revealed from validation with clone DNA (Bodrossy *et al.*, 2006; Stralis-Pavese *et al.*, 2004). Probes depicted here are specific for type Ib (P-501-375 and O_M90_253) and *pmoA2* (LP21_190 and P_NMsiT_271). For full coverage of all probes with positive signals refer to Figure 4. Hybridization was performed in triplicates. Red, green and blue denotes control, moderate and severe disturbances, respectively. Local regression lines are surrounded by 95% confidence intervals (grey).

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3. Response and resilience of paddy soil methanotrophs to heat shock.

Adrian Ho, and Peter Frenzel.
In preparation.

3.1 Abstract

Methane oxidation is a temperature-dependent process, with methane uptake rates positively correlated to temperature in paddy soils (Bender and Conrad, 1995). With no leaf canopy, the surface of recently planted rice paddies is exposed to higher temperatures than the subsurface horizons. Therefore, a strong temperature increase (heat shock) during the day can potentially affect the methanotrophic community, and its dynamics and functioning at the soil-floodwater interface. Here, simulating the surface of paddy fields, we examined the response and resilience of paddy soil methanotrophs to heat shock (37°C and 45°C). Recovery of activity and populations was followed over 2.5 months by methane uptake measurements, *pmoA*-based terminal restriction fragment length polymorphism (T-RFLP) and group-specific quantitative PCR (qPCR) analyses. While a heat shock at 37°C showed no deviation from the control (25°C), methane uptake 18 and 42 hours post-heat shock at 45°C was negatively affected. However, after 2.5 months, methane uptake was twice as high as in the control. Confirmed by T-RFLP and qPCR

analyses, type II methanotrophs increased over time, notably after heat shock at 45°C, while type I methanotrophs were negatively correlated to time and temperature. Overall, we observed community shifts, but methane uptake was not compromised indicating the resilience of paddy soil methanotrophs to heat shock.

3.2 Introduction

Rice paddies are anthropogenic methane sources, contributing around 10% to the total methane produced globally (Conrad, 2009). Methane emission from rice paddies will be higher if not for the role methanotrophs play to oxidize methane at oxic-anoxic interfaces such as in the rhizosphere (Eller *et al*., 2005), and at the soil-floodwater interface (Kajan and Frenzel, 1999). The soil-floodwater interface is subject to diurnal temperature fluctuations (Neue 1997), making a drastic temperature increase (heat shock) a potential disturbance that may affect both the methanotrophic activity and community composition. In contrast to previous work (Ho *et al*., 2010) where a non-selective model disturbance was applied to evaluate the resilience of methanotrophs, heat shock may select for thermo-tolerant methanotrophs during recovery. Moreover, the effects of long term soil incubation on the methanotrophic community had been studied before (King and Adamsen, 1992; Mohanty *et al*., 2007). Therefore, this work presents a unique model disturbance that may affect the methanotrophic community in a different way, resulting in divergent conclusions from previous work.

Taxonomically, methanotrophs have been traditionally divided into type I (phyla; *Gammaproteobacteria*) and type II (phyla; *Alphaproteobacteria*), with type I branching into type Ia and Ib. Type I and II methanotrophs are distinguished by their carbon assimilation pathways, phospholipid fatty acid contents or arrangement of intracytoplasmic membrane (Bodelier *et al*., 2009; Bowman, 2000; Trotsenko and Murrell, 2008). With the exception of some species (Belova *et al*., 2010; Op der Camp *et al*., 2009; Dedysh *et al*, 2000), the majority of methanotrophs fit into this conventional grouping. In contrast to generalists, methanotrophs have a narrow substrate spectrum, generally restricted to methane. The *pmoA* gene encoding for methane monooxygenase, a key enzyme for methane oxidation, is often used as a marker gene for detection of methanotrophs.

While microorganisms are better adapted to slowly increasing temperature e.g. seasonal temperature change, a rapid increase (heat shock) may induce a shift in community composition, and pose an adverse affect on process rates. With no or sparse leaf canopy during the early rice planting season, the soil-floodwater interface is exposed to higher temperatures than the subsurface (Schütz *et al*., 1990). While it is known that temperature selects for sub-populations of methanotrophs (Mohanty *et al*., 2007; Börjesson *et al*., 2004), the response of methanotrophic activity and composition to heat shock remains largely unknown. Here, simulating the soil-floodwater interface, we exposed paddy soil microcosms to heat shock (37°C and 45°C) for 18 and 42 hours (Figure 1), and followed the recovery of methanotrophic activity and composition.

3.3 Results and Discussion

Effects of temperature on methane uptake rates

Methane uptake was not compromised at 37°C (Figure 2). Indeed, this temperature was slightly above the optimum range (25°C – 35°C) for methane oxidation in various soils (Bender and Conrad, 1995). The subsurface soil (5 cm from surface) has been recorded to be 25°C, but will be higher on the surface (Schütz *et al*., 1990). Following exposure to 45°C, methane uptake was considerably lower than in the control. Nevertheless, 6 days post-heat shock, methane uptake recovered and reached twice as high as in the control after 2.5 months. Contrary to our observation, methane uptake was not detected in a time course experiment when incubated at 45°C (King and Adamsen, 1992; Mohanty *et al*., 2007). These studies, however, involved longer incubation periods and may have inactivated a huge portion of viable methanotrophs. Here, we observed low activity soon after exposure to elevated temperatures, but activity recovered within days, indicating the resilience of paddy soil methanotrophs to heat shock.

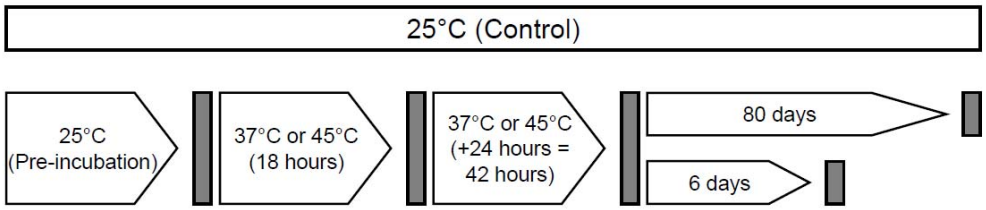

Figure 1: Schematic diagram of experimental set up. Grey bars denote triplicate methane uptake measurements, and subsequent sampling of the three microcosms for DNA extraction from soil.

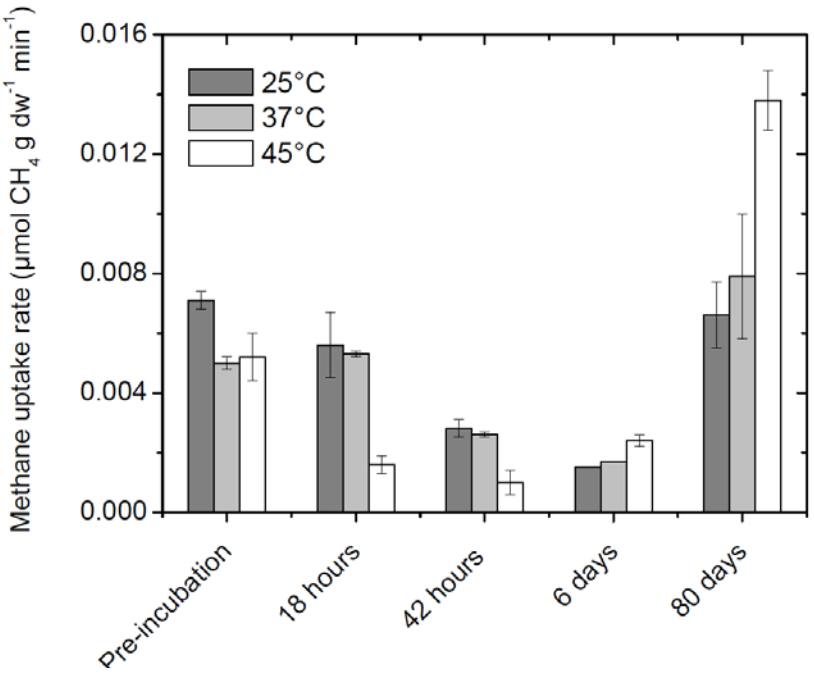

Figure 2: Response of methane uptake to heat shock (mean and standard deviation from 3 microcosms). The experimental procedure is given in the supplementary material.

Effects of temperature on methanotroph population dynamics

We consistently observed a distinctive population shift with type I dominating until 6 days post-heat shock and type II became prominent after 2.5 months, suggesting a succession of methanotroph population with time (Figures 3A and 4). The population shift was more notable after heat shock at 45°C where type II dominated the population after 6 days (Figure 3B). Based on the methane uptake rates and qPCR assays, the cell specific activity showed an increase from 42 hours post-heat shock at 6.5-7.5 x 10^{-3} to 1.6-4.0 x 10^{-2} pmol hr^{-1} $cell^{-1}$ after 2.5 months. The cell specific activity for the control was substantially lower than in the heat shock treatments. The increase in cell specific activity corresponded to the increase of type II methanotrophs, while type I population was relatively constant (MBAC) or decreased (MCOC) with time (Figure 4). Corroborating with previous work (Ho *et al*., 2010), it appears that type II methanotrophs seize their opportunity to proliferate in times of disturbance. As methanotrophs are able to form heat-resistant cysts or exospores (Bowman, 2000), a large portion of the population, in particular type II is assumed to be present as part of a microbial seed bank (Eller and Frenzel, 2001). This methanotroph pool may remain dormant until triggered. We speculate that a heat shock and the subsequent return to ambient temperature may have assisted the translation from dormant to active states, resulting in higher methane uptake.

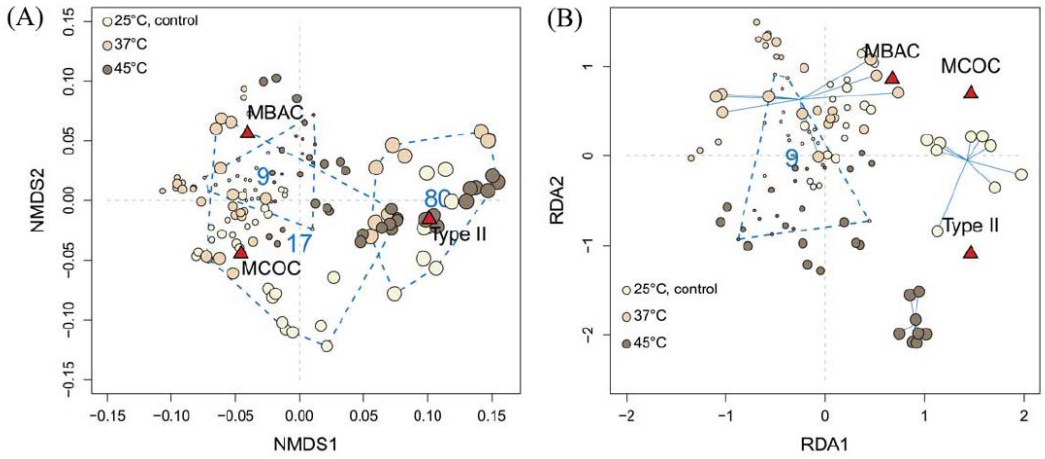

Figure 3: qPCR data as (A) Non-metric multidimensional scale analysis (NMDS) and (B) biplot of redundancy analysis (RDA) with treatment and time constraints. Yellow, light and dark brown denote control and heat shock at 37°C and 45°C, respectively. Circle size corresponds to increasing time point. Triangles indicate the qPCR assays; TYPEII, MBAC and MCOC. In the RDA, day 17 (6 days post-heat shock) is connected by lines originating from the mean.

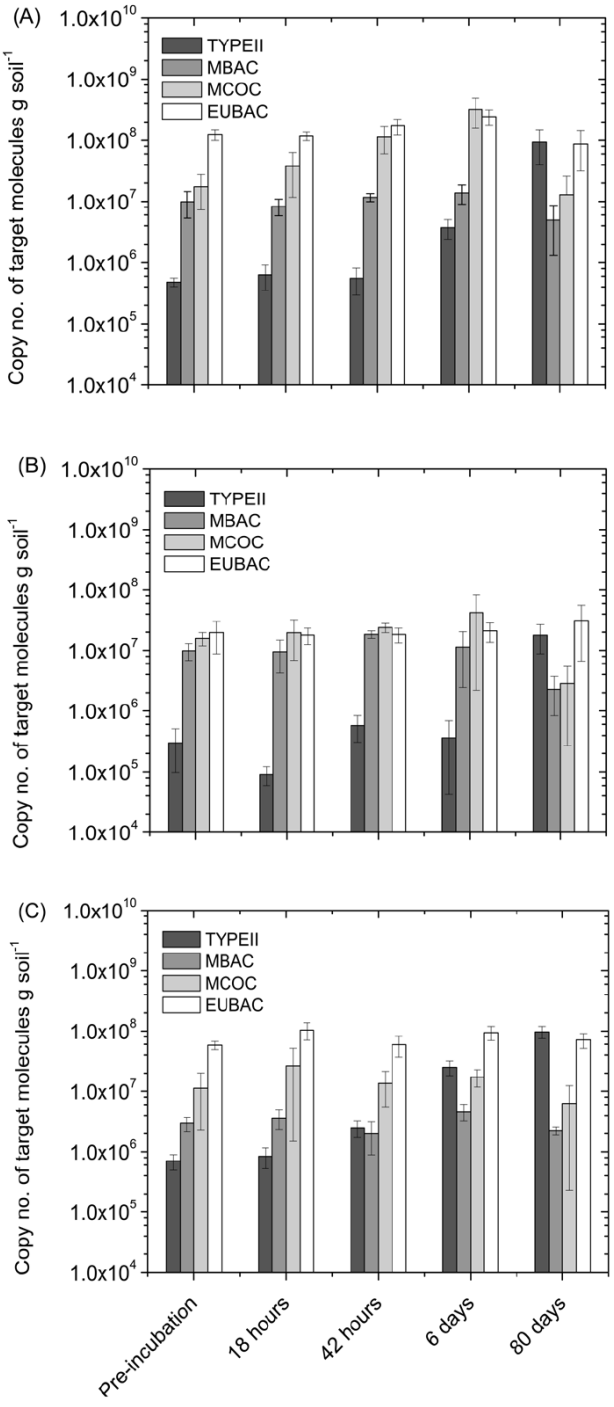

Figure 4: Analysis of qPCR assays showing the effect of heat shock; (A) control, (B) 37°C and (C) 45°C on sub-populations of methanotroph. Each time point is mean of 9 replicates from 3 microcosms.

More information on the population dynamics was provided by the T-RFLP analysis. The T-RFs were assigned based on previous work involving over 500 clones from the same soil (Lüke *et al*., 2010). Three dominant T-RFs detected were indicative for *pmoA* (type Ib: 74 bp; type I: 221; type II: 244 bp). In addition, T-RF 277 indicative for *pmoA2* was detected, but represents a small overall fraction (< 5%). *pmoA2* encodes for a subunit of a pMMO isoenzyme (pMMO2) (Baani and Liesack, 2008), and was found to be more significant in times of disturbance (Ho *et al*., 2010). Here, *pmoA2* showed a positive correlation to time, but was not significant in the treatment microcosms when compared to the control (Figures 5 and 6). Confirming the qPCR analysis, type II methanotrophs were positively correlated to time, increasing in relative abundance, and dominated the population post-heat shock (Figure 5 and 6). Previous studies suggest that methanotroph community composition is temperature-dependent with type I growing at lower temperatures, generally not exceeding 15°C (Börjesson *et al*., 2004) and type II favoring higher temperatures (Mohanty *et al*., 2007). Therefore, a cold shock at temperatures below 15°C may render a different response.

Overall, T-RFLP and qPCR analyses were generally congruent and pointed to the similar interpretation. Of interest are the high *pmoA* copy numbers found 6 and 80 days post-heat shock, apparently reaching 100% of total bacteria (EUBAC assay). While methanotrophs are assumed to possess two *pmoA* copies per cell (Semrau *et al*., 1995), the 16S rRNA gene copies vary. In re-setting the microbial succession following heat shock, fast growing bacteria with multiple 16S rRNA gene copies is anticipated to proliferate (Shrestha *et*

al., 2007). As such, a comparison of the *pmoA* and 16s rRNA gene copies is not possible. However, the high *pmoA*/16S rRNA gene ratio suggests the relative importance of methanotrophs during recovery.

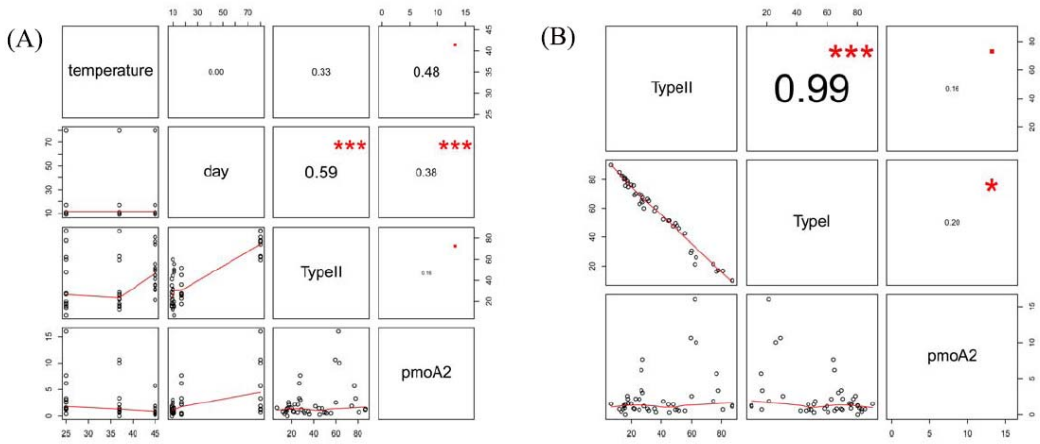

Figure 5: Spearman rank correlation coefficient for correlation between T-RFLP data (T-RFs indicative for type I and II methanotrophs, and *pmoA2*) with time and heat shock temperature (A), and among different T-RFs (B).

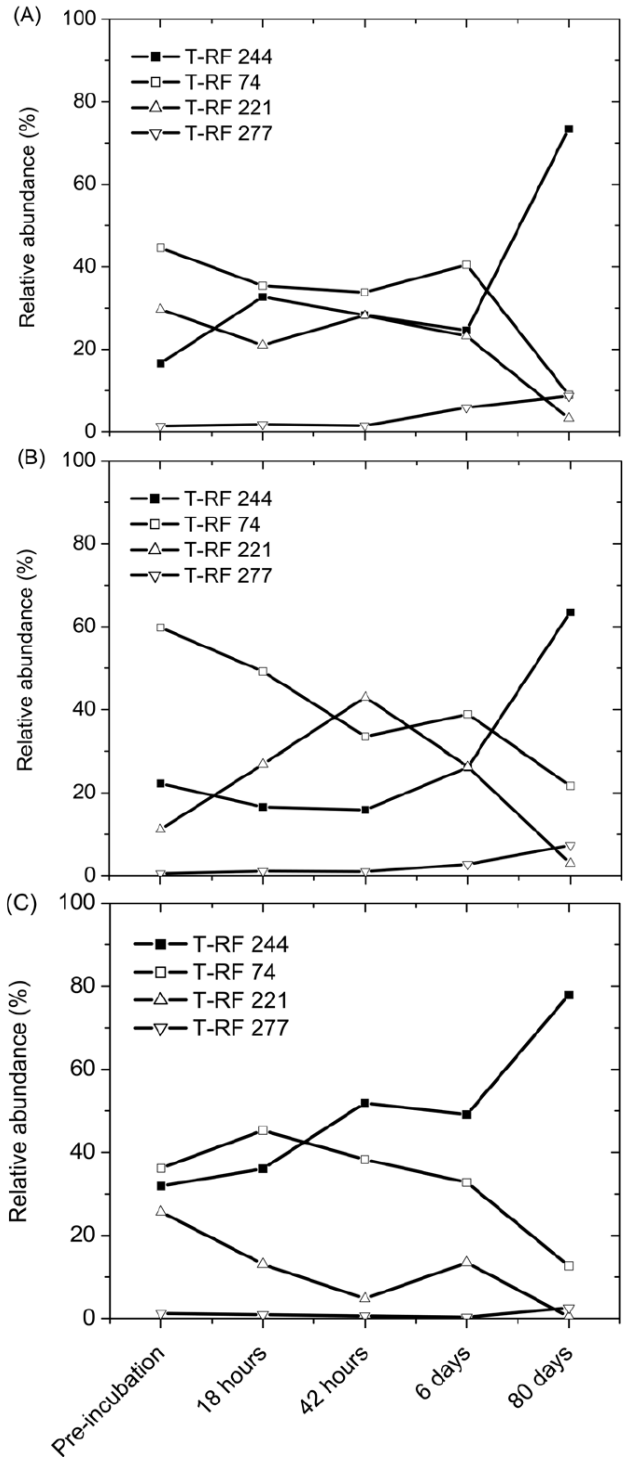

Figure 6: T-RFLP analysis showing the effect of heat shock; (A) control, (B) 37°C and (C) 45°C on methanotroph community dynamics. T-RFLP was carried out in triplicates for each time point as described in Lüke (2010).

Resilience of paddy soil methanotrophs

Methanotroph richness (number of T-RFs) did not appear to be adversely affected by the heat shock. However, a shift from the baseline community composition (pre-incubation), leading to a dominance of type II was observed. Although methane uptake was negatively affected immediately after heat shock at 45°C, it soon recovered within 6 days. In the long term, methane uptake was twice as high as in the control, inferring that not only methanotrophs are resilient to heat shock, but brief exposure to higher temperatures may have stimulated methanotrophic activity (discussed above).

This, and other study (Ho *et al*., 2010) show that paddy soil methanotrophs, supported by high energy flow, are remarkably resilient to disturbances. Nevertheless, the resilience may be strained if heat shock is continuously induced before full recovery; hence, having an accumulated effect on the methanotrophic activity. Similarly, prolonged periods of heat shock may pose an adverse effect on methane uptake. In long term incubations, methane uptake was not detected at 40°C and above (Mohanty *et al*., 2007). On the contrary, methanotrophs supported by low methane concentrations, the putative 'high affinity' methanotrophs are shown to be more vulnerable to disturbances (Bodelier and Laanbroek 2004; King 1997) and may have a different response altogether. Therefore, future work will focus on unraveling the resilience of methanotrophs living in low energy flow environments.

Conclusion

In conclusion, paddy soil methanotrophs are resilient to heat shock. While type I and II methanotrophs were detected, type II became dominant post-heat shock, particularly after 45°C heat shock. It appears that type II became more relevant, and may play an important role to guarantee functioning in face of disturbance.

3.4 Supplementary materials

Experimental procedure

Soil and soil microcosms

Soil was sampled in a rice field at the C.R.A. Agricultural Research Council, Rice Research Unit (Vercelli, Italy) in 2006, and was air-dried before storage in enclosed containers at ambient temperature. Soil parameters and agricultural practice at sampling site have been described before (Krüger *et al*., 2001). Soil (20 g) was filled in sterile Petri dishes and saturated with autoclaved distilled water (0.45 ml · g dry soil^{-1}). Water loss due to evapotranspiration was replaced. These microcosms were pre-incubated for 9 days in gas tight jars under an atmosphere of 10 vol.% CH_4 in air at 25°C in the dark before heat shock at 37°C or 45°C for 18 and 42 hours. After heat shock treatment, the microcosms were returned to 25°C. To ensure a constant supply of methane, the atmosphere in the gas tight jars were replenished every third day. Microcosms representing the same treatment were incubated together in the same jar. Microcosms were sacrificed at intervals and soil was homogenized before being stored in aliquots at -20°C till further analysis.

Methane uptake rate

Methane uptake rate was determined individually for each microcosm as described before (Ho *et al*., 2010). Briefly, pure methane (99.9%) was injected into the flux chambers and the decrease in methane concentration was followed over time (5-6 hours). Methane uptake rates were determined from linear regression. Methane was measured by gas chromatography with a flame ionization detector (SRI-9300A; SRI Instruments, Torrance, CA).

DNA Extraction from soil

Total DNA was extracted from each soil microcosm using the Q-Biogene soil extraction kit (MP, Heidelberg). The extraction was performed according to the manufacturer's instructions with a modification: DNA was subjected to additional washing steps with 5.5 M guanidine thiocyanate to remove humic acids. DNA extracts were stored at -20°C for further analysis.

pmoA-based qPCR assays

The detailed protocol for the qPCR assays; MBAC, MCOC, TYPEII and EUBAC had been described (Kolb *et al*., 2003; Stubner 2002) with some modifications (Ho *et al*., 2010). The coverage of the assays had been given (Ho *et al*., 2010). Each assay was performed in nine replicates originating from three soil microcosms. In short, the qPCR assays were performed with an iCycler IQ Thermocycler (Bio-Rad, München, Germany). The PCR reaction

consists 12.5 µl iQ SYBR Green Supermix (Bio-rad), appropriate concentration of forward and reverse primers, and 5.0 µl DNA template. DNase- and RNase-free water was added to a final volume of 25 µl. DNA from clones was used as standard for calibrating the assays.

pmoA-based T-RFLP analysis

The detailed T-RFLP protocol had been previously described (Lüke *et al*., 2010). Briefly, the *pmoA* gene was amplified in triplicates using the FAM-labeled forward primer A189f and the reverse primer A682r (Holmes *et al*., 1995). The amplicons were digested with the restriction enzyme MspI and the T-RFs were separated using the ABIPrism 310 (Applied Biosystems). The length of the fluorescently labeled T-RFs was determined using GeneScan 3.71 software (Applied Biosystems) by comparing with an internal standard (MapMarker 1000; Bioventures). T-RFLP from a negative control was used to identify, and omit peaks derived from primer dimers.

Statistics

Statistical analyses were performed in *R* ver. 2.10.1 (R Development Core Team 2009) using packages *vegan* ver. 1.18-2 for community ecology analysis (Oksanen *et al*., 2010) and *ggplot2* ver. 0.8.5 for statistical graphics (Wickham, 2009). Prior to statistical analyses, T-RFLP profiles were standardized (Dunbar et al., 2001).

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4. Aging well: methanotrophic potential and methanotroph along a chronosequence of 2000 years.

Adrian Ho, Claudia Lüke, Andrea Bannert, Cao Zhi-Hong and Peter Frenzel.

In preparation.

4.1 Abstract

Rice paddies are anthropogenic methane sources, contributing 10% to the global methane budget. The methane source strength would be higher if not for the role methanotrophs play to mitigate methane emission. Yet, little is known of the methanotrophic community dynamics and process rates during the formation of rice paddies, and with ongoing rice agriculture. Here, we analyzed the methanotrophic activity and community along a paddy soil chronosequence in China, ranging from recently reclaimed sites to paddies under permanent culture since 2000 years. Fresh soils were incubated under 10 vol.% CH_4 in air. Temporal changes in activity and population dynamics were followed by methane uptake measurement, *pmoA*-based terminal restriction length polymorphism (T-RFLP) and group-specific quantitative PCR (qPCR). The maximum methane uptake rate was found to be positively correlated to soil age. Our results inferred that long-term rice agriculture

imposes a pressure on different sub-populations of methanotrophs, and selects for the best adapted population. Cloning and sequencing the *pmoA* gene (a key gene in methane oxidation), the soils were found to harbor known and yet uncultivated putative methanotrophs.

4.2 Introduction

Rice is an important food source for more than 50% of the world's population. One of the largest global rice producers is China, contributing approximately 30% of total world rice (FAOSTAT, 2005). With rice production increasing to sustain the growing human population, it is inevitable that more rice paddies will be formed. Methane emission in rice paddies is a balance of methane production and aerobic methane oxidation. Aerobic methane oxidation is mediated by methanotrophs, and occurs at oxic-anoxic interfaces where methane and oxygen gradients overlap. A prime habitat for the methanotrophs is at the soil-floodwater interface where as much as 90% of potentially emitted methane may be oxidized in this area (Kajan and Frenzel, 1999). Hence, methanotrophs play an essential role mitigating methane emission from rice paddies.

Aerobic methanotrophs belong to the phyla *Verrucomicrobia* and *Proteobacteria*. Methanotrophic *Verrucomicrobia* have been discovered recently, and seem to be restricted to extreme environments (Dunfield *et al*., 2007; Pol *et al*., 2007; Islam *et al*., 2008). Cultured members of methanotrophs belonging to *Proteobacteria* have been traditionally grouped

into type I (family *Methylococcaeae*) and type II (family *Methylocystaceae* or *Beijerinkiaceae*). Type I consists of type Ia (e.g. *Methylobacter*, *Methylomicrobium, Methylomonas and Methylosarcina*) and type Ib (e.g. *Methylococcus* and *Methylocaldum*), while type II consists of 2 genera: *Methylocystis* and *Methylosinus. Methylocapsa* and *Methylocella*, both acidophilic methanotrophs belonging to family *Beijerinkiaceae* fall within the class α-proteobacteria which also constitutes type II, but are not monophyletic with *Methylocystis* and *Methylosinus* (Dedysh *et al*., 2005). Type I and II methanotrophs are distinguishable by their phylogeny, carbon assimilation pathway, arrangement of intracytoplasmic membrane (Bowman, 2000; Trotsenko and Murrell, 2008) and phospholipid fatty acid composition (Bodelier *et al*., 2009). Methane monooxygenase (MMO), the key enzyme for methane oxidation is present in a particulate (pMMO) or soluble (sMMO) form. While all known methanotrophs possess pMMO except *Methylcella palustris* (Dedysh *et al*., 2000), sMMO is restricted to only some species. The *pmoA* gene encodes for a subunit of pMMO, highly conserved, and used as a marker gene for detection of methanotrophs in culture-independent studies (Bourne *et al*, 2001).

Agricultural practices from implementation of water management strategies (Neue, 1997) to fertilization (Bodelier *et al.,* 2000; Qiu *et al*., 2008; Shrestha *et al*., 2008; 2010) and the choice of rice varieties (Neue, 1997; Lüke, 2009) may affect methane oxidation potential or the methanotrophic composition in rice paddies. However, the initial establishment, and the subsequent influence of agricultural practices on the methanotrophic community in the long term are

largely unknown. Focusing on a rice paddy chronosequence spanning 2000 years, we examined methanotrophic activity and community composition with ongoing rice cultivation. We anticipate long term rice agriculture to leave an imprint on the methanotrophic community composition, selecting for methanotroph sub-populations. Fresh soils were incubated under 10 vol.% CH_4 in air, and the methanotrophic activity and community dynamics were followed by measurements of methane uptake, *pmoA*-based T-RFLP and qPCR. Clone libraries were generated in order to verify and access methanotroph diversity.

4.3 Experimental procedure

Soil characterization, sampling procedure and soil microcosms

Paddy soil from a chronosequence (50, 100, 500, 1000 and 2000 years) were sampled from drained rice fields in Cixi, Zhejiang province, China (Cheng *et al*., 2009). In addition, sediment from a saltwater marsh (SW) was sampled. While all these fields were reclaimed from the sea, SW represents the start of this chronosequence. Selected characteristics of the paddy soils and SW are listed in Table 1. Methanotrophs in a paddy field show no spatial pattern (Krause *et al*., 2009). Nonetheless, sampling of paddy soils was performed in a spatial pattern, pooling five mixed samples from an area of 10m x 12m. Each mixed sample was comprised of seven sub-samples taken with an auger (5.5 cm diameter) from the upper 10 – 15 cm. Sediment from the SW

was sampled randomly and pooled (*n* = 5). The composite sample was homogenized and stored at 4°C until experimental set up.

Table 1: Sampling locations and selected soil parameters.

Soils	Geographic coordinates	Water content (%)§	pH	C/N	C_{org} (mg g^{-1})	Soil* Type	Soil* Texture	Vegetation coverage
SW	N 30°19.775' E 21°09.487'	29.74	7.4	34.64	-	Tidalic, Salic fluvisol	-	Reeds
50a	N 30°11.066' E 21°21.351'	28.84	6.9	10.75	15.27	Stagnic gleyic cambisol	-	Fallow
100a	N 30°09.827' E 21°20.955'	29.14	6.8	8.80	19.32	Gleyic cambisol	SiL	Fallow
500a	N 30°11.052' E 21°05.075'	25.85	6.3	8.68	15.18	Gleyic cambisol	SiL	Rape
1000a	N 30°09.760' E 21°06.953'	26.25	6.4	9.03	7.62	Endogleyic stagnosol	-	Fallow
2000a	N 30°05.455' E 21°26.738'	41.23	6.4	8.47	32.30	Endogleyic stagnosol	L	Young rice sapling

§Gravimetric water content was determined by drying fresh soil at 105°C for 3 hours.
*Classification according to Food and Agricultural Organization of the United Nations, FAO. Classification courtesy of Dr. Peter Schad, Munich.

Fresh soil (25g) was filled in sterile Petri dishes. The gravimetric water content (42 %) was standardized by adding autoclaved distilled water. These microcosms were incubated in gas tight jars under an atmosphere of 10 vol.% CH_4 in air at 25°C in the dark. The atmosphere was replenished every third day. Three microcosms were sacrificed at each time interval and soil was homogenized before being stored in aliquots at -20°C till further analysis.

Methane uptake rate measurement and soil nutrient content

Methane uptake rate was determined for individual microcosm from linear regression by following the decrease of methane (5-6 hours) in flux chamber (volume 172 ml; 3-5 % CH_4 in air). Methane was measured using gas chromatography with a flame ionization detector (SRI-9300A; SRI Instruments, Torrance, CA). Total soil NH_4^+ was measured flurometrically according to Murase (2006), and anion (SO_4^{2-}, NO_3^-) concentrations were determined according to Bak (1991). Phosphate and nitrite were below the detection limit (<0.005 mM).

DNA extraction

Total DNA was extracted from each microcosm using the Q-Biogene soil extraction kit (MP, Heidelberg, Germany) according to manufacturer's instructions. DNA was subjected to additional washing steps with 5.5M Guanidine Thiocyanate to remove humic acid before being stored at -20°C till further analysis.

pmoA-based qPCR assays

The qPCR assays were performed as previously described (Ho *et al*., 2010) based on work by Kolb (2003). Three assays; MBAC, MCOC and TYPEII targeting type Ia, type Ib and type II methanotrophs, respectively were performed in triplicates for each DNA extract. Futhermore, total eubacterial

16S rRNA gene (EUBAC assay) was enumerated according to Stubner (2002) with minor modifications (Ho *et al*., 2010). The qPCR assays were applied to the paddy soil chronosequence, and the 50a and 2000a soils in the time course experiment.

pmoA-based T-RFLP analysis

T-RFLP analysis was performed in triplicates for each time point as described before (Lüke *et al*, 2010). Briefly, the *pmoA* gene was amplified using a FAM-labeled forward primer, (A189f) and the reverse primer A682r (Holmes *et al*., 2005). PCR amplicons were digested with restriction endonuclease MspI. T-RFs were separated using an ABIPrism 310 (Applied Biosystems), and the length of the FAM-labeled T-RFs was determined by comparison with an internal standard (MapMarker 1000; Bioventures). Peaks from primer dimers - if present at all - were excluded after cross-checking with a negative control. The T-RFLP analysis was applied to the paddy soil chronosequence, and the 50a and 2000a soils in the time course experiment. Amplification of the *pmoA* gene was unsuccessful in the SW sample.

Cloning, sequencing and sequence analysis

The *pmoA* gene clone libraries for the 50a and 2000a soils were constructed using A189f-A682r and A189f-mb661r primer sets. The *pmoA* gene was amplified using the same primer concentrations and PCR thermal profile as for the T-RFLP. Ligation of *pmoA* gene amplicons was performed using

pGEM-T Easy Vector System (Promega, Germany) according to manufacturer's instructions, transformed into JM109 competent cells (Promega, Germany), and plated on LB plates prepared from imMedia^{TM} Amp Blue (Invitrogen, Carlsbad, CA, USA). Clones from the 50a soil (total 114 clones: 22 and 92 clones from A189f-mb682r and A189f-mb661r primer sets, respectively) and 2000a soils (total 147 clones: 53 and 94 clones from A189f-mb682r and A189f-mb661r primer sets, respectively) were randomly selected for comparative sequence analysis. Sequencing of inserts was performed at ADIS (MPI for Plant Breeding, Cologne, Germany) using T7 forward and M13rev-29 reverse primers targeting the vector sequence.

Comparative sequence analysis

The phylogenetic tree was constructed based on 146 deduced amino acids considering 114 and 147 clones from 50a and 2000a soils, respectively. The analysis was performed employing the neighbor joining method using the ARB software package (Ludwig *et al*., 2004).

4.4 Results

Methane uptake rate and porewater analysis

Methane uptake rate reached a maximum after 15-23 days from the start of the experiment. The maximum methane uptake rate was positively correlated to the age of the soils (Figure 1). In the SW where methane uptake rates were

relatively low and constant throughout the incubation period, the average methane uptake rate is given. Porewater analysis of bulk soil showed a decrease of sulphate with soil age (Figure S1). Nitrate was in the range of 0.4 -3.5 mM and was higher in the 100a and 1000a soils, but was below the detection limit (<0.005 mM) in SW. Total ammonium was notably higher in the 2000a soil.

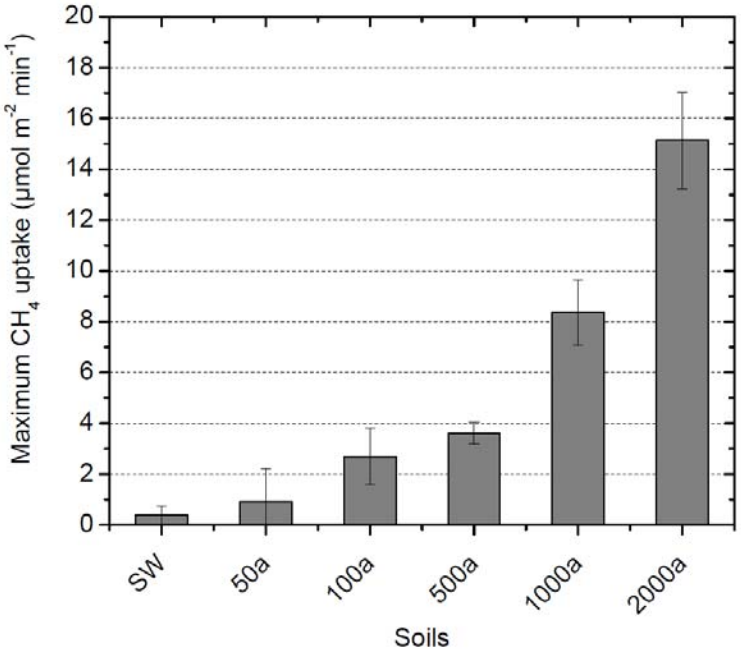

Figure 1: Maximum methane uptake rates (mean ± SD, *n* = 3).

Characterization of methanotroph abundance and community structure

The qPCR assays MBAC, MCOC and TYPEII covered 93, 91, and 83% out of 164, 136, and 279 clones, respectively. The coverage of these assays was checked *in silico* based on clone sequences retrieved from paddy soils (Ho *et al*., 2010). The lower detection limit for the assays was at 10^4 copy number of

target molecules g soil $^{-1}$. Type I and II methanotrophs were present at almost equal copy numbers except in the 2000a soil where type II dominated (Figure 2). In the time course experiment, a parallel increase in the methanotrophs covered by the MCOC assay with methane uptake rates was observed (Figure 3). This was most obvious in the 2000a soil.

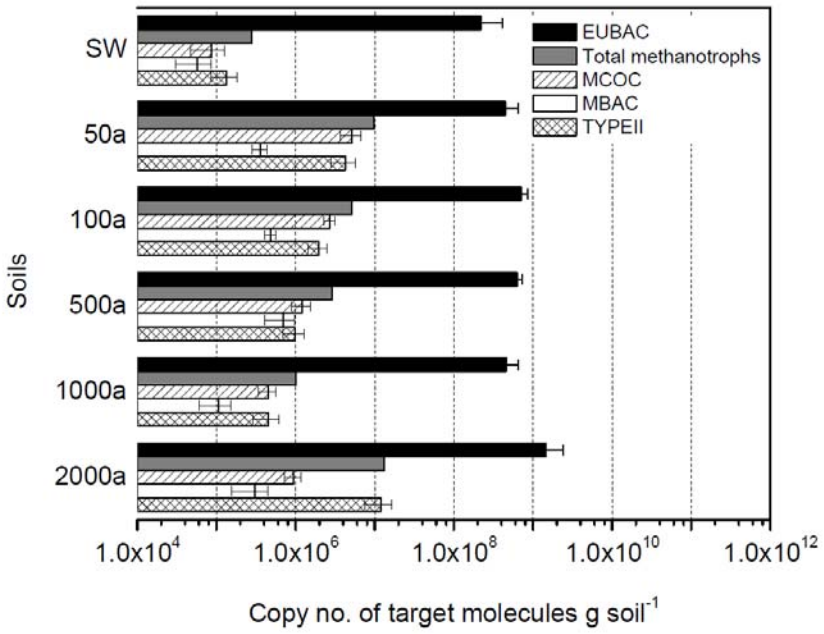

Figure 2: Analysis of qPCR assays in the paddy soil chronosequence (mean ± SD, *n* = 9).

Unfortunately, the T-RFLP analysis does not resolve type II taxa. However, it gives a fairly well differentiated insight in type I diversity (Figure 4). The T-RFs were assigned based on a comprehensive study considering 500 *pmoA* clones from paddy soils (Luke *et al*., 2010), and was further supported by an *in silico* analysis from clone sequences of the 50a and 2000a soils. Five T-RFs were indicative for type I *pmoA* (type Ia; 351 and 440 bp; type Ib: 74 bp and type I: 221 and 240 bp). In addition, T-RF 277 is indicative for *pmoA2*, a

gene coding for an isoenzyme that is present in some type II methanotrophs (Yimga *et al*., 2003). This fragment represented only a small fraction in the paddy soil chronosequence. T-RF 74 is indicative for type Ib and was dominant in the paddy soil chronosequence. This fragment increased with time in the microcosms with 2000a soil (Figure 4).

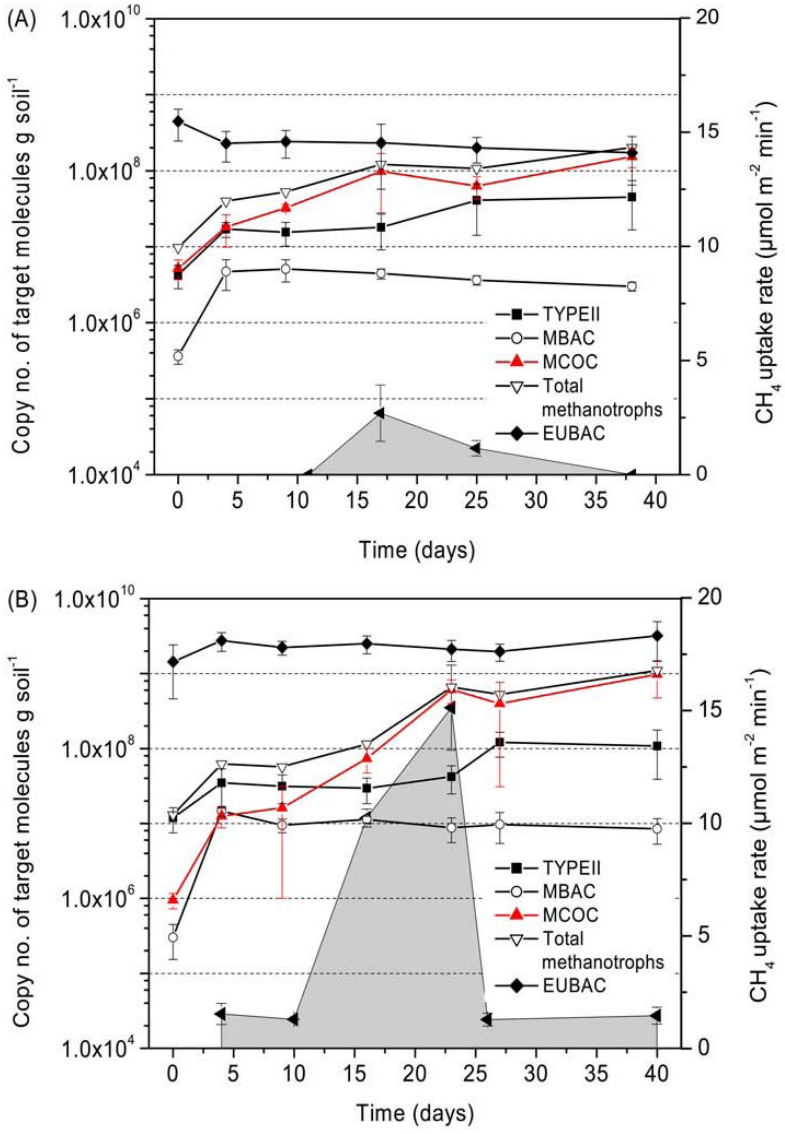

Figure 3: qPCR analysis showing the parallel increase in the MCOC assay (red) with methane uptake rates in the (A) 50a and (B) 2000a soils.

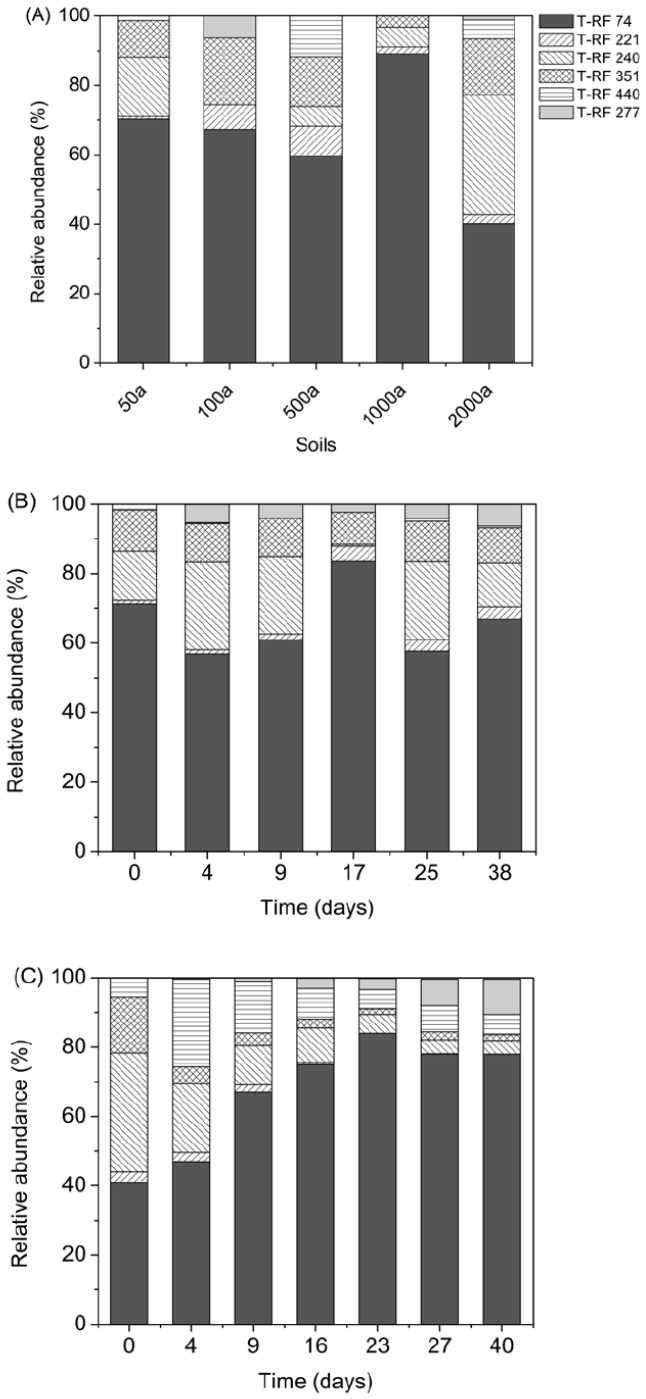

Figure 4: T-RFLP analysis showing type I methanotroph diversity (mean ± SD, *n* = 3) in the (A) paddy soil chronosequence, and temporal changes in the methanotrophic composition in the (B) 50a and (C) 2000a soils.

Cloning and sequence analysis

For a wider coverage of methanotroph diversity, two primer sets were used to construct the clone libraries (Bourne *et al*., 2001). Phylogenetic analysis of the *pmoA* sequences retrieved from the soils revealed the presence of known, and putative type I and II methanotrophs (Figure 5). In the 50a soil, sequences that were affiliated to type I include genera *Methylomonas* and *Methylosarcina*, and the yet uncultured rice paddy cluster (RPC-1; Lüke *et al*., 2010). Sequences related to type II belonged to the genus *Methylocystis*. Type I was relatively more diverse in the 2000a soil and was represented by sequences affiliated to genera *Methylococcus*, *Methylomonas* and *Methylosarcina*, while type II was represented by *Methylocystis* related sequences. Sequences affiliated to *amoA* were also retrieved due to the high homology of the *amoA* and *pmoA* genes (Holmes *et al*., 1995), constituting 16% of the total clones. Besides the *pmoA* gene sequences, the A189f-mb682r primer set enables the recovery of *amoA* and sequences in an intermediate position between the *pmoA* and *amoA*. *Crenothrix polyspora*-like sequences (>85% sequence identity), positioned between the *pmoA* and *amoA* genes were found, more frequently in the 2000a soils.

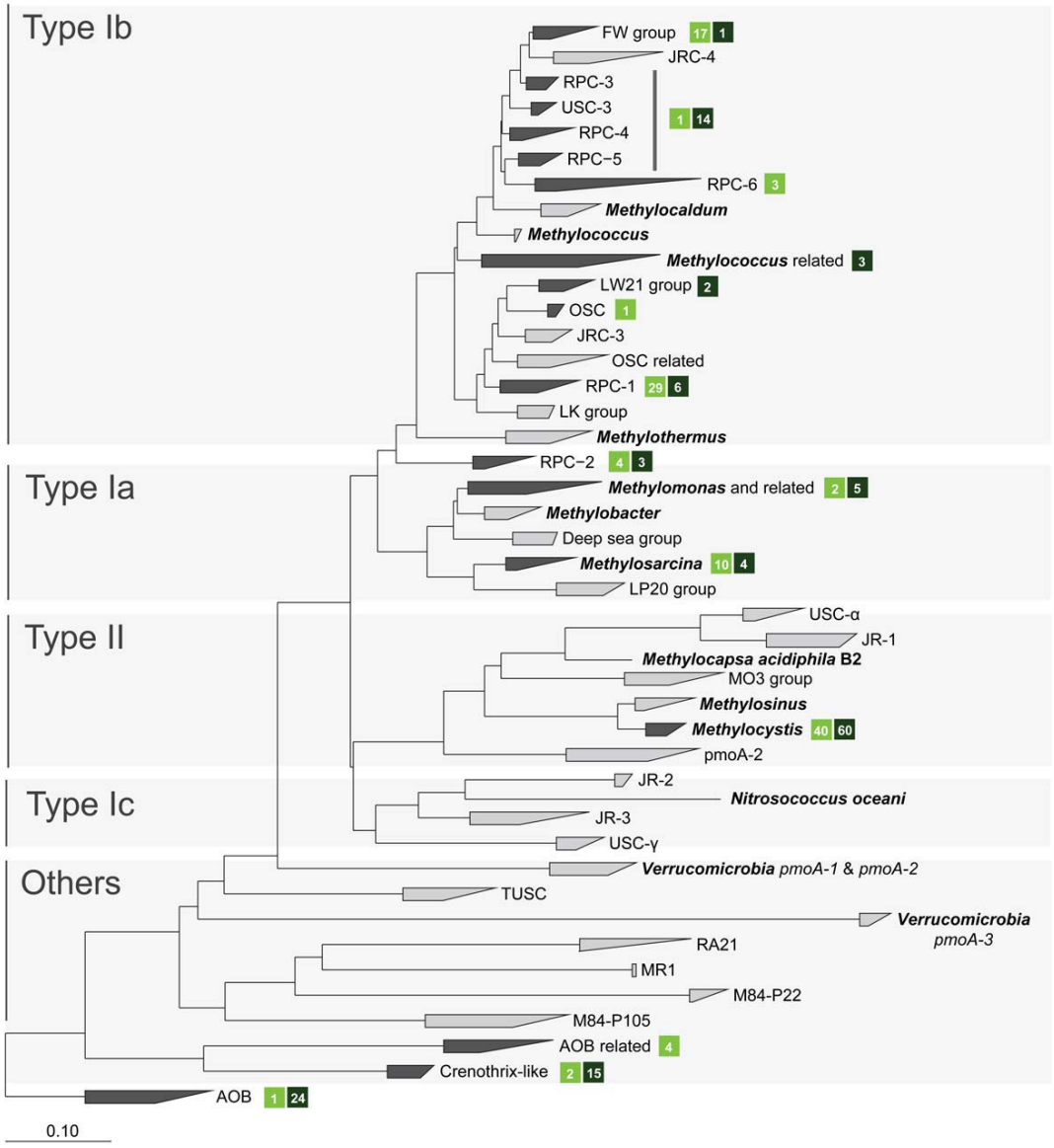

Figure 5: Phylogeny of the *pmoA* gene showing the distribution of methanotrophs and ammonium oxidizing bacteria (AOB) in the 50a (light green) and 2000a (dark green) soils. The figure in the box denotes the number of clones considered for the analysis. The Crenothrix - like sequences showed 92 – 94% sequence similarity to *Crenothrix polyspora* based on deduced amino acids, and were clustered together.

4.5 Discussion

With identical climate, fairly uniform agronomic practices, and marine deposits as starting material (Cheng *et al*., 2009), observed differences in methane oxidation potential and methanotrophic community composition were primarily an affect of soil age. It appears that long term rice agriculture allows for higher methane oxidation potential and may impose a pressure to select for sub-populations of methanotrophs. Accordingly, age appears to influence the methanotrophic activity and composition in rice paddies.

Methane oxidizing potential in rice paddy chronosequence

Maximum methane uptake rate increased monotonically with soil age. In the SW sample, virtually no methane uptake was detected. This corroborated with the relatively low number of methanotrophs present, slightly above the detection limit in the marine sediment (Figure 2). Therefore, considering only paddy soils, we showed the higher potential for methane oxidation with ongoing rice agriculture. However, the factors contributing to higher methane uptake remains unknown. Similarly, the reason for the final decline in methane uptake at fairly constant high cell numbers remains an open question (Figures 1, 3).

Paddy soil methanotrophs

Agronomic practices, while shaping the soil of recently converted rice paddies, may also shape the methanotrophic community. The parallel increase of the MCOC assay with methane uptake rates, while other assays remained relatively constant inferred that increasing methane uptake was a result of type Ib methanotrophs proliferation. The initial quantity of type Ib present was similar in the 50a and 2000a soils, but more rapid growth was observed in the 2000a soil (Figure 3), and was reflected in the T-RFLP analysis (Figure 4). In contrast, the community composition in the 50a soil was relatively constant, with no distinctive temporal changes (Figure 4). It appears that long term rice agriculture allows for faster growth, and may have also selected for a yet unresolved ecotype of type Ib.

Considering this and other studies on environments supported by high methane concentrations (Lüke *et al*., 2010; Chen *et al*., 2007; 2008), type Ib appears to be abundant, particularly in paddy soils. Besides *Methylococcus* and *Methylocaldum*, type Ib consists of various environmental clusters (e.g. RPC-1; Lüke *et al*., 2010) retrieved almost entirely from paddy soils. Moreover, using mRNA as proxy for activity, *pmoA* transcripts indicative for type Ib were found (Krause *et al*., 2010), suggesting that type Ib was actively oxidizing methane. In summary, we showed the close correlation of increasing type Ib to activity measurement, more pronounced in the ancient soil, indicating that with progressing age, prevailing conditions in rice paddies may select for sub-populations of active methanotrophs.

Diversity of the pmoA gene

Sequence analysis of clone libraries revealed a diverse methanotrophic community and corroborated with the qPCR analysis, showing a higher abundance of type II in the 2000a soil. Consistent with other studies, the young and ancient soils harbored *pmoA* affiliated sequences that were dominant in other rice paddies (Lüke *et al*., 2010; Shresta *et al*., 2008; Horz *et al*., 2001; Hoffmann *et al*, 2002), indicating that the methanotrophic community was established soon after a rice paddy is formed. Furthermore, total methanotrophs enumerated, 10^6 – 10^7 cells g soil^{-1} assuming each cell contained two *pmoA* copies (Semrau *et al*., 1995) were consistent with other rice paddies from widespread geographical locations (Macalady *et al*., 2002; Eller *et al*., 2005; Vishwakarma *et al*., 2007). *Methylocystis* was the predominant type II methanotroph, and have been suggested to be present as resting cells (Eller *et al*., 2005). Some *Methylocystis* species are known to harbor a *pmoA2* gene encoding for the second pMMO (pMMO2) isoenzyme (Yimga *et al*., 2003). pMMO2 has been shown to oxidize methane at a lower mixing ratio comparable to atmospheric methane concentrations (Baani and Liesack, 2008). Therefore, the *pmoA2* gene suggests that some *Methylocystis* may find their niche in between rice growing seasons when the soil is drained.

In addition to type II, an uncultivated environmental cluster, RPC was detected in relatively high abundance, confirming the prevalence of these sequences in rice paddies (Lüke *et al*., 2010). RPC-1 is related to cultivated *Methylocaldum* and *Methylococcus* species (type Ib), and seems to be the

active dominant methanotroph sub-population. As anticipated, uncultivated environmental clusters previously detected from upland soils (USC-α and USC-γ) were virtually absent, and do not appear to play a significant role in rice paddies. Interestingly, a *C. polyspora*-like genotype was detected. These sequences are phylogenetically grouped between the *pmoA* and *amoA* gene, and had been reported to be present in other agricultural soils (Dörr *et al*., 2010; Jia *et al*., 2007).

Conclusion

As rice paddies are formed, the indigenous ecosystem is dramatically modified, resulting in a shift of the soil microbial community that may alter process rates. Our results inferred higher potential for methane oxidation with ongoing rice agriculture. Moreover, the abiotic environment in rice paddies may have left an imprint on the methanotrophic community, selecting for specifically adapted species. It appears that the methanotrophic community was soon established upon the formation of rice paddies, but the selection of the best adapted sub-populations requires time. To this end, type Ib appears to be favored in the older rice paddies. The majority of type Ib are represented by uncultured environmental clusters nearly entirely retrieved from rice paddies. The environmental cues that evoke the adaptive responses of methanotroph sub-populations are largely unknown. Hence, we anticipate that future work will identify contributing factors that are partial towards the proliferation of a methanotroph sub-population in rice paddies.

4.6 Supplementary materials

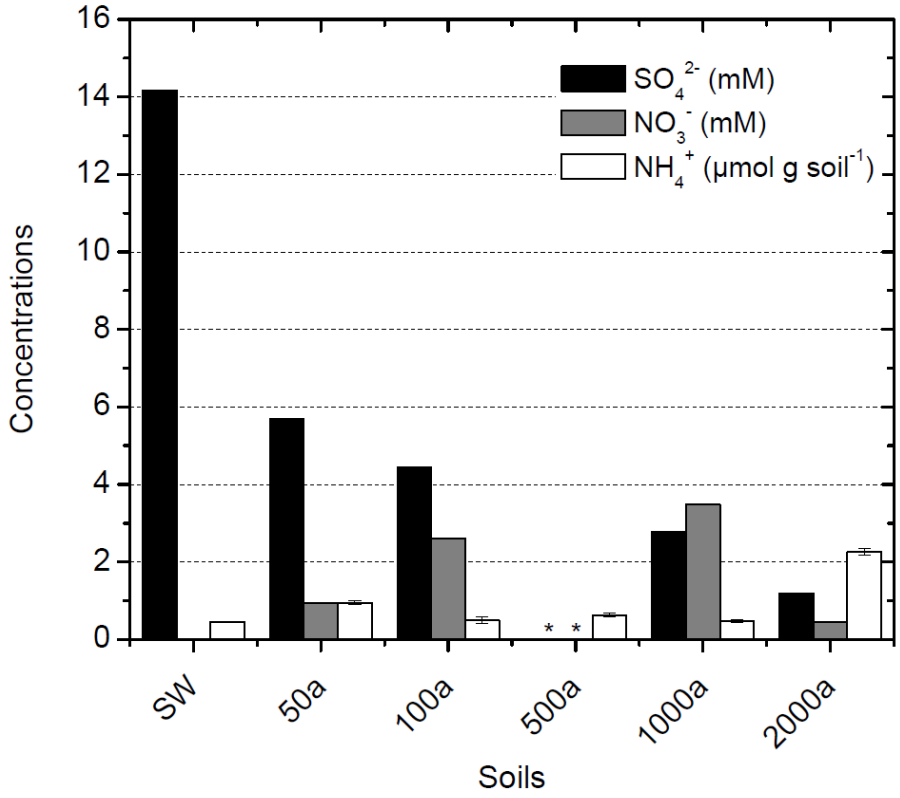

Figure S1: Anion (SO_4^{-2}, NO_3^-) and NH_4^+ (mean ± SD, *n* = 3) concentrations in the porewater of paddy soil chronosequence (* no porewater analysis possible).

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5. Methanotrophic activity and composition in young and ancient rice paddies.

Adrian Ho and Peter Frenzel.
In preparation.

5.1 Abstract

Rice paddies are biogenic methane sources, contributing around 10% to the methane produced globally (Conrad, 2009). Methane emission in rice paddies is attenuated by methanotrophs (Bosse and Frenzel, 1998). Yet, the effects of long term rice agriculture on methanotrophic activity and community composition is poorly understood. Here, a combination of flux measurements and *pmoA*-based terminal restriction fragment length polymorphism (T-RFLP) were used to compare a young (50a) with an ancient (2000a) paddy soil with regard to methane emission and oxidation capacity, the effects of nitrogen fertilization, and temporal changes in the methanotrophic community. Methane emission was higher from the young soil. Methane oxidation was detected in both soils during the early stage of rice growth, but became negligible later on. Moreover, fertilization had no effect on methane oxidation, and had little influence on methanotrophic community structure. The T-RFLP analysis indicated that type II methanotrophs were predominant in the bulk soil, while type I was represented in higher relative abundance in the roots,

suggesting niche differentiation. Overall, no clear divergence was observed in the young and ancient soils.

5.2 Introduction

Methane is, next to carbon dioxide, the most important greenhouse gas contributing significantly to global warming. Among the biogenic methane sources, rice paddies account for 10% of the total global methane emitted into the atmosphere (Conrad, 2009). It is inevitable that methane emission from rice paddies will rise with increasing rice production to sustain the growing human population in decades to come. Methane emission from rice paddies would be higher if not for the role methanotrophs play to oxidize methane at oxic-anoxic interfaces. Two hot spots for methanotrophic activity have been identified: the soil-floodwater interface and the roots of rice plants. Methanotrophs detected in the mainly anoxic bulk soil have been shown to be present as resting cells (Eller and Frenzel, 2001). Upon reclaiming a soil such as rice paddy, methanotrophs are exposed to agricultural practices (e.g. fertilization, ploughing, alternate redox cycles). Consequently, the composition could alter, selecting for the best adapted species. Therefore, comparing the young with the ancient soil may reveal a divergence in the methanotrophic community and activity.

Canonical methanotrophs fall within the phylum γ- or α-*Proteobacteria*, representing type I (family *Methylococcacceae*) and type II (family *Methylcystaceae* or *Beijerinkiaceae*) methanotrophs, respectively. Type I can

be further divided into type Ia or Ib (formerly type x). This grouping is based on morphological, physiological and phylogenetical characteristics (Bowman, 2000; Bodelier *et al*., 2009; Trotsenko and Murrell, 2008). Due to physiological differences, sub-groups of methanotrophs show preference to different environmental conditions (Lüke, 2009). Part of this difference has been suggested to be caused by methane and oxygen concentrations (Graham *et al*., 1993; Henckel *et al*., 2000). Therefore, methanotrophs have been observed to be active or predominate in different niches in rice paddies. The key enzyme for methane oxidation is the methane monooxygenase (MMO) which is present in a particulate (pMMO) or soluble (sMMO) form. While all cultured methanotrophs except *Methylocella palustris* possess the pMMO (Dedysh *et al*., 2000), sMMO is confined to some methanotrophs. The *pmoA* gene, encoding for a subunit of pMMO, is frequently used as a marker gene for detection of methanotrophs in molecular methods.

We simulated field conditions in the greenhouse, and investigated the methanotrophic activity and community changes over a rice growing season in a young (50a) and ancient (2000a) soil. The soils were compared with regard to methane emission and oxidation capacity, temporal changes in methanotrophic composition, and the effects of nitrogen fertilization. A combination of process measurements and *pmoA*-based T-RFLP were employed to determine *in-situ* methane emission and oxidation together with the methanotrophic community changes, respectively.

5.3 Experimental procedure

Sampling sites

Paddy soils cultivated for 50a and 2000a were sampled from drained rice paddies in Cixi, Zhejiang province, China, representing end points of a chronosequence. Pedological characterization and dating has been described before (Cheng *et al*., 2009). Samples were taken from the plough layer. After sampling, soil was shipped at field moisture to Germany.

Rice microcosms and aboveground plant development

Following germination, three rice saplings (local Chinese cultivar; *qian you yi hao*) were transplanted into each pot containing 3 kg soil. Time shown in figures hereafter refers to days after transplanting (dap). Rice plants were kept in the greenhouse at a temperature range of 25-30°C with a 12 hours photoperiod (average 90000 lux), and watered frequently to ensure a constant level of floodwater, approximately 4-6 cm above the soil surface. Fertilization was performed twice, during the initial preparation of soil and at 55 dap. Fertilizer solution was composed of urea (84 g), $Na_2HPO_4.2H_2O$ (32 g) and KCl (4.2 g) per litre deionized water, and 10 mls of fertilizer solution was added into each pot. Fertilizer amount corresponds to local agricultural practice per hectare paddy field. Bulk soil, rice roots and surface soil were sampled during tillering (41 dap), before fertilization (55 dap), after fertilization

(57 dap), at flowering (84 dap), and before harvest (117 dap). Samples were homogenized, frozen immediately, and stored at -20°C till further analysis.

The height of rice plants and number of tillers were measured regularly throughout the different rice growing stages.

In-situ methane flux measurements.

Methane emission was determined in four replicates in Perspex flux chambers (volume: 6360 or 3220 ml) over an hour using linear regression (Figure 1). After measurement of methane emission, potential methane production was determined in the same microcosms by inhibiting methane oxidation using 1% difluoromethane (CH_2F_2 99%, ICI Chemicals, UK), a specific inhibitor for methane oxidation (Miller e*t al*., 1998). Difluoromethane was added into the headspace of the flux chambers 30 mins prior to the first measurement. A built-in fan was used to prevent gradient formation in the chamber. Incubation of rice plant microcosms under higher concentrations (3% and 5%) of difluoromethane or for a longer period (overnight) did not have any affect on methane oxidation (data not shown). The rate of methane oxidation was determined by calculating the difference of methane production and emission. Methane was measured using gas chromatography with a flame ionization detector (SRI-9300A; SRI Instruments, Torrance, CA). Analysis of stable isotope $^{13}C/^{12}C$ in headspace gas was carried out using a gas chromatograph combustion-isotope ratio mass spectrometry (GCC-IRMS) as described before (Shrestha *et al*., 2008).

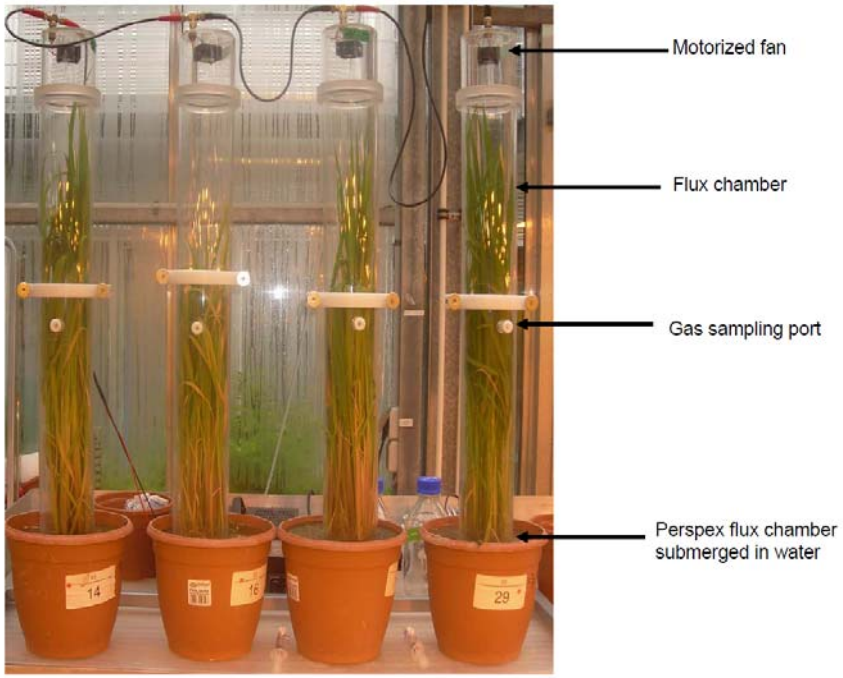

Figure 1: Experimental set up of rice microcosms during gas flux measurements.

Methane uptake in roots and soil surface.

Rice roots were rinsed to dislodge attached soil particles and placed in gas tight bottles. Methane (99.5%, Messer, Germany) was injected into the headspace, and the decrease of methane was followed over time. When methane was depleted, the headspace was flushed with air, and methane was re-filled. After incubation for approximately 90 – 100 hours, the roots were dried at 60°C overnight to determine the dry weight.

Surface soil, defined as soil located approximately 0-3 mm from the surface was collected from each pot, homogenized, and distributed to two sterile Petri dishes (20 g each). Prior to methane uptake measurements, the surface soil

was incubated for six days in gas tight jars under 10% methane in air in the dark at 25°C. Methane uptake measurements were performed individually for each microcosm in flux chambers as described before (Ho *et al*., 2010).

DNA extraction

DNA extraction from soil was performed using the Q-Biogene soil extraction kit (MP, Heidelberg, Germany) according to manufacturer's instructions with the following modification: DNA was subjected to 2 - 3 additional washing steps with 5.5M guanidine thiocyanate to remove humic acids. DNA extracts were stored at -20°C till further analysis.

pmoA-based T-RFLP analysis

T-RFLP analysis was performed in duplicates from each DNA extract as previously described (Lüke *et al*., 2010). Briefly, the *pmoA* gene was amplified with a FAM-labeled forward primer A189f and the reverse primer A682r. PCR amplicons were subjected to restriction endonuclease (MspI) digestion and T-RFs were separated using ABIPrism 310 (Biosystems). The length of the fluorescently labeled T-RFs was determined by comparison to an internal standard (MapMarker 1000; Bioventures) using GeneScan 3.71 software (Applied Biosystems).

5.4 Results and Discussion

Methane emission and oxidation in bulk soil, rice roots and surface soil

Methane emission rates increased throughout the rice growing period, with the young soil reaching four times higher values than the ancient soil after 84 dap (Figure 2). Increased in methane emission was reflected in the vegetative growth of rice plants (Figure 3). Rice tillers act as a conduit, transporting methane produced in the anoxic zone of the bulk soil into the atmosphere, and have been shown to be positively correlated to methane emission (Neue, 1997). Conversely, atmospheric oxygen could be transported to the root zones of the rice plants, making the roots a hot spot for methane oxidation (Frenzel *et al*., 1992). Nonetheless, the reason for higher methane emission seen in the young soil remains unclear but, it appears that the rice plant in the young soil invested more in vegetative growth (plant height).

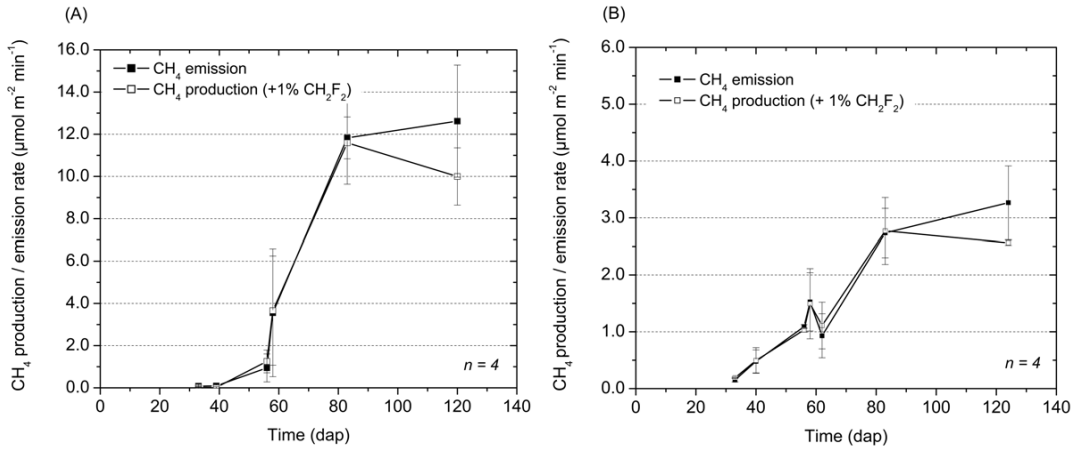

Figure 2: *In-situ* methane emission and production rates in (A) 50a and (B) 2000a microcosms (mean ± sd, *n* = 4). Note different scales in y-axis.

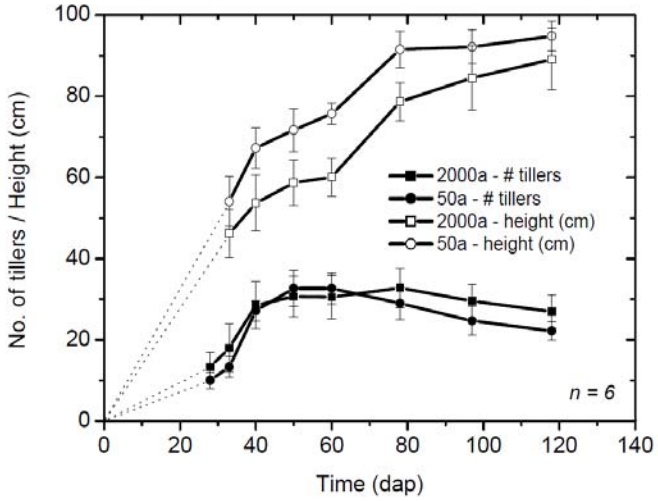

Figure 3: Aboveground vegetative development of rice plants (mean ± sd, *n* = 6).

Methane emission and production rates were nearly identical, indicating undetectable methane oxidation in both soils (Figure 2). The rice root is an important site for methane oxidation (Gilbert and Frenzel, 1995). The cumulative methane uptake in the roots was highest during the tillering stage at 41 dap (Figure 4), suggesting that root associated methane oxidation – if it happens – is restricted to the early rice growing period. Repeating the methane flux measurements in the second year after one rice growing season alternate with rape, low methane oxidation was detected at 29 dap. Based on the methane emission and production rates, methane oxidation in the young and ancient soil microcosms were found to be 0.30 ± 0.12 and 0.10 ± 0.02 μmol m^{-2} min^{-1} (mean ± sd, *n* = 2), respectively. It appears that the window for methane oxidation is narrow, occurring early in the rice growing season during tillering. Similarly, in Italian rice paddies, a relatively higher methane oxidation was detected during the early rice growing stage, but became less important later on (Eller and Frenzel, 2001). In the surface soil, methane uptake

changed with time and reached a maximum at 84 dap (Figure 5). Confirming previous studies (Frenzel *et al*., 1992; Gilbert *et al*., 1998; Shrestha *et al*., 2008), the roots and surface soil showed potential for methane oxidation, and are hot spots for the methanotrophs.

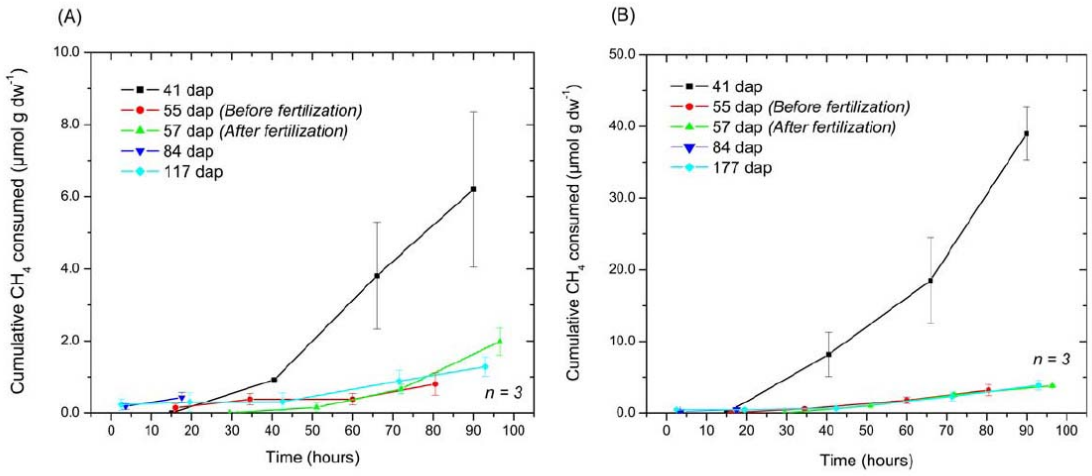

Figure 4: Cumulative methane consumed in the roots of rice planted in (A) 50a and (B) 2000a microcosms (mean ± sd, *n* = 3). Note different scales in y-axis.

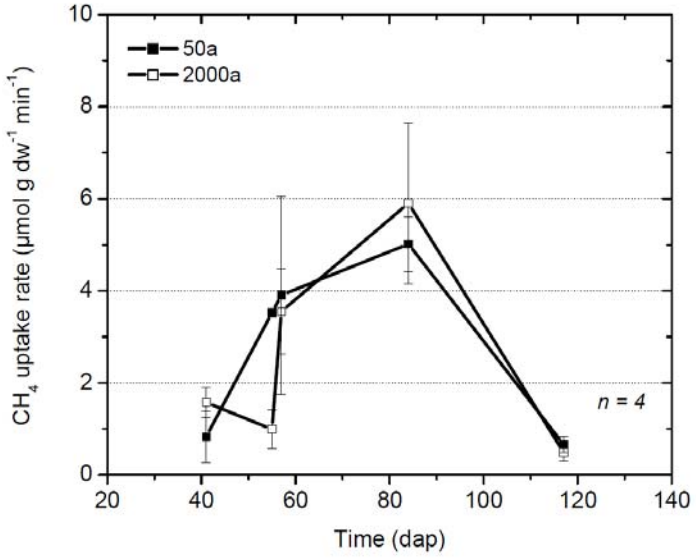

Figure 5: Methane uptake in the surface soil (mean ± sd, *n* = 4).

Effects of fertilization on methanotroph activity and community composition

Methanotrophic activity and composition is influenced by the input of fertilizers (Bodelier *et al*., 2000; Noll *et al*., 2008). However, methane oxidation was undetectable after fertilization in the flux measurements. While change in $\delta^{13}C$ value from around -90 to -40 would be consistent with a simulation of methane oxidation (Figure 6), it might also indicate a switch in methanogenesis pathways (Gelwicks *et al*., 1994). In contrast to low methane environment (Steudler *et al*., 1989), the addition of nitrogen fertilizers to rice paddies have been shown to increase methane oxidation rates (Bodelier *et al*., 2000; Krüger and Frenzel, 2003). In vitro experiments suggest a selective stimulation for type I methanotrophs (Noll *et al*., 2008). However, the positive effect of fertilization on methane oxidation is transient, and seems to occur in the root zones (Bodelier *et al*., 2000). In short, methanotrophs are not only controlled by their main substrates, methane and oxygen, but also by nitrogen amendments.

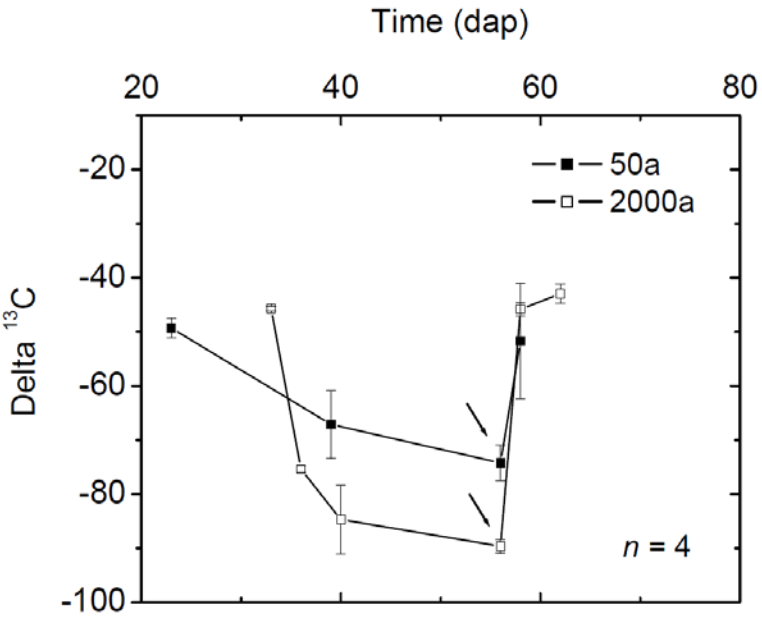

Figure 6: Analysis of $\delta^{13}C$ sampled from the headspace of flux chamber. Arrows denote time of fertilization.

T-RFLP analysis was employed to access and study the effect of fertilization on the methanotrophic community in the bulk soil and roots. Five major T-RFs indicative for methanotrophs were detected: T-RF 244: type II; T-RF 74: type Ib; T-RF 240 and T-RF 350: type I; T-RF 534: type Ia. Assignment of the T-RFs is based on a previous study considering 500 clones from rice paddies (Lüke *et al*, 2010). Due to the primer set used, the *amoA* gene (key gene for ammonium oxidation) was also amplified (Bourne *et al*., 2001), but was excluded from the analysis. The methanotrophic community in the bulk soil showed no temporal changes and was dominated by type II methanotrophs (Figure 7). However, slight difference was observed between both soils, with the ancient soil having a higher type I diversity (number of T-RFs). The methanotrophic community was more dynamic on the roots, showing a

predominance of type I early in the rice growing season, and type II increased in relative abundance over time (Figure 7). The changing composition of methanotrophs on the roots indicates active growth, and reflects on the activity detected early in the season. Later on, root associated methane oxidation was not measurable, and the community became dominated by type II as in the bulk soil. Overall, temporal changes of the methanotrophic community in the bulk soil and roots was dissimilar, with type I consistently present in higher relative abundance in the roots early in the season and type II dominating in the bulk soil, suggesting niche differentiation.

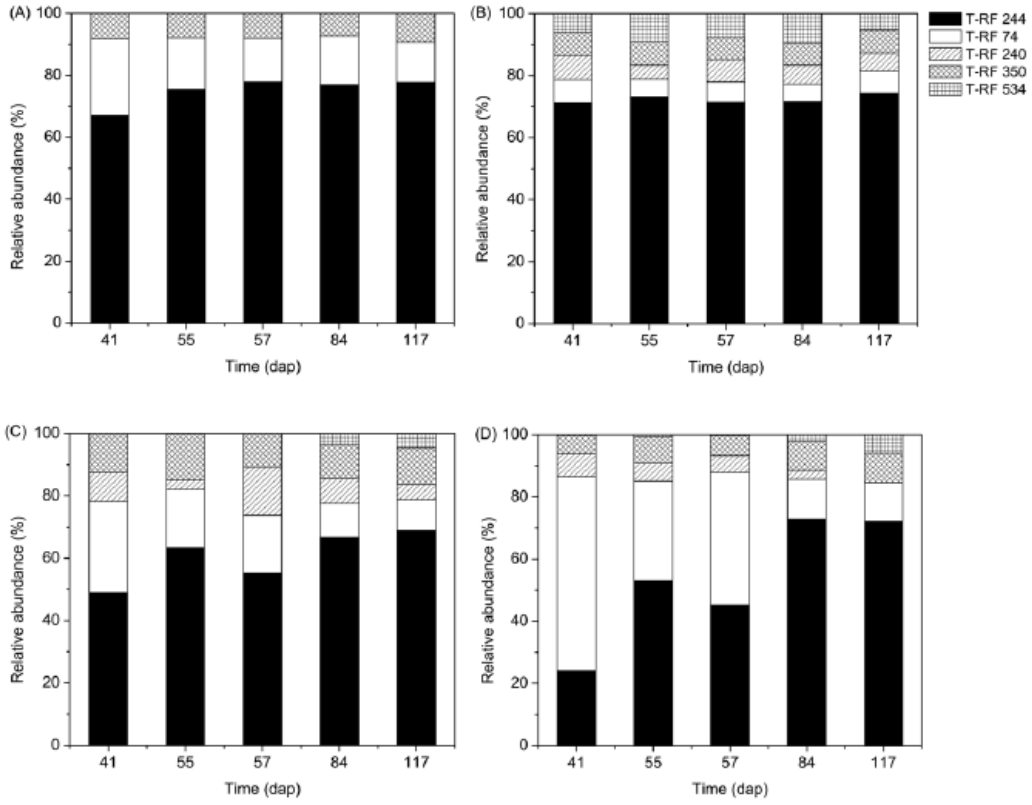

Figure 7: T-RFLP analysis of the methanotrophic community in the bulk soil of (A) 50a and (B) 2000a microcosms, and in rice roots of (C) 50a and (D) 2000a microcosms. T-RFLP was performed in 4 replicates from 2 rice microcosms.

Conclusion

The young soil achieved higher methane emission rate, but no clear differences was observed with regard to methane oxidation. Methane oxidation was only detected during the early rice growing stage, becoming negligible later on. It appears that fertilization had no major affect on the methanotrophic activity and community composition. While both type I and II methanotrophs were detected, T-RFLP analysis suggests that the roots favored type I, and type II was predominantly present in the bulk soil. In conclusion, no clear divergence was observed in the young and ancient soils.

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6. General discussion and outlook

This thesis addresses the (i) short and mid term recovery of paddy soil methanotrophs from disturbances and (ii) the long term development of the methanotrophic community and activity in rice paddies. In **Chapters 2** and **3**, the resilience of paddy soil methanotrophs was tested against two model disturbances: disturbance-induced mortality by mixing native with sterilized paddy soil in two ratios representing moderate and severe die-offs, and heat shock. While the former model disturbance represents a non-selective form of disturbance, the heat shock treatment may select for sub-populations of thermo-tolerant methanotrophs. In **Chapter 4**, the establishment and subsequent development of the methanotrophic community and activity along a Chinese rice paddy chronosequence was studied. After long term rice agriculture, the community is anticipated to adapt to the prevailing conditions in rice paddies. Hence, the chronosequence is an ideal site for studying the development of the methanotrophic community and activity with ongoing rice agriculture. **Chapter 5** is a pilot study considering the young (50a) and ancient (2000a) soils. Here, field condition was simulated in the greenhouse. The soils were compared with regard to methane emission and oxidation capacity, methanotrophic community dynamics, and effects of fertilization. Culture-independent approaches; *pmoA*-based T-RFLP, qPCR assays and a *pmoA*-based diagnostic microarray were employed in parallel with methane flux measurements to address the research questions.

6.1 Recovery of methanotrophs from disturbances: function

Species richness and evenness are considered among the most important measurements of diversity. By design, the model disturbances are unlikely to result in a reduction of species richness. However, species evenness may change upon disturbance and impose an effect on microbial mediated processes in the soil (Wittebolle *et al*., 2009). Here, **Chapters 2** and **3** showed that upon disturbance, different growth rates among methanotrophs led to an uneven community that nevertheless is functioning better than the control. On both disturbances, methane uptake recovered, and even reached a higher rate than in the control, indicating the remarkable resilience of paddy soil methanotrophs to disturbances.

On the contrary, previous studies demonstrated that methanotrophs oxidizing atmospheric methane are vulnerable to disturbances (Bodelier and Laanbroek, 2004; Prieme *et al*., 1997). This discrepancy could be partly explained by the extremely low energy flow in upland soils where methanotroph population size are small, around 10^5 cells g $soil^{-1}$ compared to 10^6 – 10^7 cells g $soil^{-1}$ in high-methane environments like rice paddies (Kolb *et al*., 2005; **Chapter 4**). Therefore, not only the qualitative aspect but also the energy flow through a microbial population and its effect on quantitative population dynamics has to be considered as buffer against disturbances.

So far, paddy soil methanotrophs have been shown to recover from short and mid term disturbances (**Chapters 2** and **3**). However, during the

establishment of rice paddies where a saltwater marsh is dammed and reclaimed for rice agriculture, the indigenous methanotroph population and methanotrophic activity is anticipated to be dramatically altered. Indeed, results showed a monotonic increase in maximum methane uptake with soil age, indicating the higher potential for methane oxidation in the ancient soils (**Chapter 4**). It appears that with ongoing rice agriculture, sub-populations of methanotrophs that are best adapted to the specific abiotic conditions may have remained active (**Chapter 4**), while others are present as resting cells, but may emerge from dormancy (Epstein, 2009). Therefore, the age of rice paddies in on-going rice agricultural sites may influence the methanotrophic activity and composition.

In the greenhouse study, *in-situ* methane oxidation was detected early during the rice growing period but became negligible later on (**Chapter 5**). Although the young soil achieved higher methane emission rates, no clear differences were observed for methane oxidation. Moreover, fertilization had no major impact on methane oxidation. Overall, results do not show clear divergence in the young and ancient soils with regard to methane oxidizing potential and methanotrophic composition (see below), indicating that the methane oxidizing community may well be established soon after a rice paddy is formed (**Chapters 4** and **5**).

Driven by the need to have a large number of replicates, the soil-floodwater interface was simulated in simplified soil microcosms (**Chapter 4**). In the greenhouse, however, a more complex environment was simulated, and other

factors (e.g. fertilization, rice plants) have to be considered affecting methane oxidation. Despite of the added complexity, methane uptake was detected on the surface soil (**Chapter 5**), demonstrating that the soil microcosm (**Chapter 4**) was an appropriate model to simulate the soil-floodwater interface.

6.2 Recovery of methanotrophs from disturbances: community composition

Although the diversity in the disturbed microcosms decreased, methane uptake was not adversely compromised, indicating that paddy soil methanotrophs were sufficiently diverse to allow for functional redundancy or a sub-population benefited from the disturbance, and increased in abundance. Interestingly, type II (in particular *Methylocystis*) increased in abundance following induced disturbances, suggesting the relative importance of this sub-population in the disturbed microcosms (**Chapters 2** and **3**). Type II methanotrophs are often regarded as largely inactive, represented by resting cells forming a microbial seed bank (Eller *et al*., 2005). The mechanism triggering the transition from dormant to active state remain to be elucidated, but the increase of type II show clearly that upon disturbance, they may seize their opportunity to proliferate. However, recovery needs time: effects of frequent disturbances hitting the community while evenness is low may accumulate shifting it to a different state.

Recently, some *Methylocystis* species have been shown to harbour the *pmoA2* gene encoding for a second pMMO (pMMO2). *pmoA2* have been

shown to be constitutively expressed and enables methane oxidation at lower mixing ratios (Baani and Liesack, 2008). Interestingly, the relative abundance of the *pmoA2* gene increased in parallel with overall increase in type II, indicating that this was likely a quantitative *pmoA2* increase. The increase of *pmoA2* was more pronounced during disturbance-induced mortality (**Chapter 2**), but was not obvious post-heat shock when compared to the control (**Chapter 3**). It is unknown, if all pMMO2 share the high affinity to methane that *per se* would not have provided a selective advantage in these experiments (**Chapters 2** and **3**). Nonetheless, other traits of the organisms carrying this gene may have been responsible for their success. During drainage in between rice growing seasons, methane concentrations will be low. Hence, carrying *pmoA2* may be advantageous, partly accounting for its abundance in paddy soils (**Chapters 2**, **3** and **4**).

Considering **Chapter 4** where a radical transformation in microbial community is anticipated, the methanotrophic composition is likely to be permanently altered. Similarly, other functional guilds (e.g. microorganisms responsible for nitrogen cycling) may have changed. While shaping the soil, long term rice agriculture may have also shaped the methanotrophic community, allowing faster growth of a methanotroph sub-population (**Chapter 4**). Consequently, this sub-population is favored in rice paddies. Generally, rice paddies are considered to be environments with low oxygen tension, and are subjected to temperature fluctuations, nitrogen source amendments, and intermittent periods of drainage and re-flooding (Liesack *et al*., 2000; Mohanty *et al*., 2006; Neue, 1997). Although the methanotrophic community may have

established soon after a rice paddy is formed (**Chapters 4** and **5**), the selection of the best adapted population needs time. The increase of type Ib methanotrophs in good correspondence with increasing methane uptake rates while other quantifiable sub-populations were relatively constant suggests that type Ib was the active dominant sub-population (see below). Therefore, long term rice agriculture may leave an imprint on the methanotrophic community.

6.3 Diversity of *pmoA* gene in paddy soils

Recent discoveries of novel aerobic methanotrophs from diverse environments and with broader substrate specificity than previously thought have challenged the conventional perception of methanotrophy. These novel methanotrophs are mainly restricted to extreme environments or acidic peat-lands, and have not been detected so far in rice paddies. Therefore, the grouping of methanotrophs into type Ia, Ib or II is still appropriate in rice paddies at present; however, this may change in future.

Sequence analysis of clone libraries revealed a diverse methanotrophic community from the Chinese paddy soils (**Chapter 4**). Consistent with other studies, the Chinese soils harbored *pmoA* related sequences that were also dominant in the Italian rice paddy (Lüke *et al*., 2010; Shresta *et al*., 2008; Horz *et al*., 2001; **Chapter 2**). Among type II methanotrophs, *Methylocystis* related sequences were consistently dominating, while *Methylosinus* affiliated sequences were virtually undetected or detected in relatively low abundance (Lüke *et al*., 2010; **Chapters 2** and **4**). Type II, in particular *Methylocystis*

species, have been shown here to be resilient, becoming more relevant during disturbances. The resilience and other traits (see above) may attribute to the success of *Methyocystis* in rice paddy environments.

In comparison to type II, type I methanotrophs showed a higher diversity (**Chapters 2** and **4**) with type Ib seemed to be the dominant active sub-population (**Chapter 4**). An uncultivated rice paddy cluster (RPC-1; Lüke *et al*., 2010) falling within type Ib was frequently detected in the clone library. In view of this and previous studies on environments with high methane concentrations (Chen *et al*., 2007; 2008; Lüke *et al*., 2010), RPC-1 was detected almost exclusively from paddy soils, suggesting specific adaptation to this environment (Lüke *et al*., 2010; **Chapter 4**).

Interestingly, a *C. polyspora*-like genotype was detected in the Chinese rice paddies, but more frequently in the ancient soil. The sequences detected in the Chinese rice paddies are phylogenetically grouped between the *pmoA* and *amoA* (key gene for ammonium oxidation) genes. As such, they share conserved amino acid residues with both the *pmoA* and *amoA* genes, and could not be affiliated to any of the two corresponding functions (Holmes *et al*., 1999). So far, similar sequences (> 95% sequence identity) have been reported to be present in other agricultural soils (Dörr *et al*., 2010; Jia *et al*., 2007), but no conclusions could be made due to the lack of information and pure culture. It appears that *C.polyspora*-like sequences may represent a novel cluster that had resisted cultivation so far. Therefore, this finding opens a promising area for future research.

6.4 Comparison of culture-independent approaches

The advent of culture-independent techniques has enabled a deeper and thorough analysis of environmental microbial communities. These methods are generally reliable and can be used in parallel to substantiate results. In addition to methane flux measurements, two or three of the PCR-based culture independent methods; *pmoA*-based T-RFLP, group-specific qPCR assays and a *pmoA*-based microarray were used in parallel to verify results (**Chapters 2, 3** and **4**). This provided a comprehensive data set.

T-RFLP proved to be a rapid and reproducible method for monitoring community structure, but it lacks phylogenetic resolution. While more laborious, the *pmoA* microarray provides a higher phylogenetic resolution, enabling the detection of methanotrophs down to the genus or species level (Bodrossy *et al*., 2003). However, both methods do not provide exact quantitative qualities. To overcome this, qPCR assays were employed to enumerate *pmoA* copy numbers. Therefore, relative and absolute quantitative methods were performed in parallel to confirm results.

Generally, analyses of the different culture-independent approaches were congruent. Observed population shifts were confirmed by both T-RFLP and qPCR analyses (**Chapters 3** and **4**), and were reflected in the microarray analysis (**Chapter 2**). However, when closely scrutinized, the ratio of type I / II methanotrophs was not always similar in the T-RFLP and qPCR analyses. This inconsistency may be attributed to biases in PCR selection caused by

degenerate primers (Luders and Friedrich, 2003). The reverse primer (A682r) employed here for the T-RFLP analysis contained four redundancies within its sequences, and could partly explain the discrepancies between both analyses. In spite of the inherent limitations of the different methods, results were generally consistent, and provided similar interpretation, indicating that different relative and absolute quantitative methods are comparable.

6.5 Summary

To summarize, paddy soil methanotrophs are remarkably resilient to induced disturbances, compensating and even over-compensating for methane uptake during recovery. The methanotrophic community appears to be established soon after a rice paddy is formed, with type Ib (RPC-1) and II (*Methylocystis*) found to be the predominant methanotrophs in this environment. The parallel increase of type Ib with methane uptake rates demonstrates the active role they play in methane oxidation. While type Ib appears to represent the dominant active sub-population, type II are known to be present as resting cells. However, type II became more relevant and may play a role to guarantee functioning during disturbances. Accordingly, age of on-going rice agricultural sites appears to influence the methane oxidizing capacity and selects for methanotroph sub-populations that are specifically adapted to rice paddy environments.

6.6 Outlook

Paddy soil methanotrophs have so far been shown to be resilient to one-off disturbances (**Chapters 2** and **3**). However, disturbances may hit the community repeatedly when diversity is still low, compromising the recovery of function and composition. The response and resilience of methanotrophs to repeated or multiple disturbances is virtually unknown. Furthermore, disturbances may affect the putative 'high-affinity' methanotrophs differently, resulting in divergent conclusions from the current work.

In the long term, results indicate that sub-populations of methanotrophs show specific adaptation to the rice paddy environments (**Chapter 4**). However, the traits enabling them to thrive successfully in rice paddies are conjectural, and remain to be substantiated. Similarly, the environmental cues that evoke the adaptive responses of methanotrophs are largely unknown. Moreover, the finding suggesting that the methanotrophic community was established soon after the formation of rice paddies warrants further attention. To this end, salt water marsh sediments could be used as starting material for a pilot study simulating *in-situ* conditions to follow the initial establishment of the methane oxidizing community when rice paddies are formed. Besides, the majority of the *pmoA* gene diversity is represented by uncultured environmental sequences. The RPC-1 and *C.polyspora*-like genotypes from this study are examples without cultured representatives. The lack of cultured representatives demonstrates the need to increase isolation efforts.

6.7 References

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