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Ecology of methane oxidising bacteria in soil

Ph.D. Thesis

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"I want to bring out the secrets of nature and apply them for the happiness of man . I don't know of any better service to offer for the short time we are in the world."

Thomas Edison , American inventor

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Chapter 1

Summary

Methane oxidizing bacteria (methanotrophs) are ubiquitous, acting as bio-filters for greenhouse gas methane in the environments. Hence, methanotrophs play a central role in eco-processes affecting climate change (e.g. global warming). Even though methanotrophs were intensively studied in the last decades, little is known about the feasibility of comparing the experimental results across the different laboratories performing the same procedures. Similarly, little is known about how methanotroph function and community composition are influenced by the overall microbial diversities; their resistance and resilience against disturbances regarding their community structure, abundance and function.

In this Ph.D. study, the impacts of inter- and intra-laboratory variations of environmental DNA extraction were evaluated by methanotroph community analysis. The differences in yield and quality of extracted DNA were significantly different between laboratories even though the same protocol of DNA extraction and same soil samples were applied in this study. Subsequently the extraction differences also influenced methanotroph community composition and abundance analysis.

Methanotrophs were applied as a model system in a mesocosm study. Different total microbial and methanotroph community assemblies were applied as starting materials and the dynamics of the methanotroph and bacterial community compositions as well as methane oxidation as function were followed over a period of 15 weeks. The results showed that methanotrophs recolonized from the upper inoculum layers. Surprisingly, different microbial assembly histories did not affect methanotroph community diversity, while did significantly influence total microbial diversity. The recovery and succession of

methanotroph communities followed a similar pattern between different microbial assemblies. Furthermore, flood and drought as disturbances were applied to the same mesocosms to assess the response and resilience of methanotroph community composition and function to and against these perturbations. Methanotroph compositions showed remarkable resistance to both disturbances even though they were embedded in different microbial communities. Methanotroph abundance and function (methane oxidation potentials) indicated high level resilience against the varying of moisture content.

Keywords: methanotroph, DNA extraction, mesocosm, microbial assembly, resistance, resilience

Zusammenfassung

Das Treibhausgas Methan dient als Nährstoffquelle für Methanoxidierende Bakterien (Methanotrophe). Daher spielen Methanotrophe eine zentrale Rolle in ökologischen Prozessen, die auch Einfluss auf das Klima haben (Klimaerwärmung). Obwohl Methanotrophe in den letzten Jahrzenten Gegenstand intensiver Untersuchungen waren, ist wenig über die Aussagekraft der einzelnen Untersuchungen bekannt, da identische Prozeduren in unterschiedlichen Laboratorien zu unterschiedlichen Ergebnissen führen können. Ebenso ist wenig darüber bekannt, wie Funktion und Organismenzusammensetzung der Methanotrophen durch die allgemeine Vielfalt der mikrobiellen Zusammensetzung beeinflusst wird, ihre Resistenz und Widerstandskraft gegenüber Störungen in Gemeinschaftsstruktur, Häufigkeit und Funktion.

In dieser Ph.D.-Arbeit wurde der Einfluss von unterschiedlichen umwelttechnischen DNS-Extraktionen, sowohl innerhalb der Labore als auch zwischen den unterschiedlichen Laboren, auf die Methanotrophen-Gemeinschafts-Analyse untersucht. Obwohl das gleiche Protokoll der DNA-Extraktion auf identische Bodenproben angewandt wurde fanden, wurden signifikante Unterschiede in Ausbeute und Qualität der extrahierten DNS gefunden. Ebenfalls stellte sich heraus, dass unterschiedliche Extraktionsprotokolle sowohl die Zusammensetzung der Spezies als auch die Häufigkeitsanalyse beeinflussten.

Methanotrophe dienten als Modellsystem in einer Mesocosmos-Studie. Über einen Zeitraum von 15 Wochen wurden verschiedene Zusammensetzungen von gesamten und methanotrophen Gemeinschaften untersucht. Gegenstand der Untersuchungen waren bakterielle Zusammensetzung und funktionelle Methanoxidation. Die Resultate zeigten, dass Methanotrophe, ausgehend von den oberen Inokulationsschichten, tiefere

Bodenschichten rekolonisierten. Unerwarteter Weise hatten unterschiedliche mikrobielle Zusammensetzungen keinen Einfluss auf die Diversität innerhalb methanotrophen, während ein signifikanter Einfluss auf die mikrobielle Gesamtzusammensetzung beobachtet werden konnte. Ausbeute und Sukzession der einzelnen methanotrophen Gemeinschaften zeigten über verschiedene mikrobielle Zusammensetzungen ein vergleichbares Verhalten.

In einem weiteren Ansatz wurden die gleichen Mesocosmen Flut- und Dürrestörungen ausgesetzt, um die Resistenz der methanotrophen Gemeinschaften gegen und Adaption an diese Bedingungen zu untersuchen. Die methanotrophen Gemeinschaften zeigten, unabhängig von der mikrobiellen Gesamtzusammensetzung, eine bemerkenswerte Resistenz gegenüber beiden Bedingungen. Ebenso wiesen Häufigkeit und Funktion der Methanotrophen (Methanoxidationspotentiale) auf eine hohe Widerstandsfähigkeit gegen wechselnde Feuchtegehalte des Bodens hin.

Schlüsselwörter: Methanotrophe, DNS Extraktion, Mesocosmos, Mikrobieller Verband, Resistenz, Widerstandsfähigkeit

Chapter 1

General Introduction

1.1 Methane, its source and sink

Since an Italian physicist, Alessandro Volta (1745-1827), first identified methane (CH_4) from the bubbles coming from marsh at the late 18th century until nowadays, methane concentrations in the atmosphere increased from around 715 parts per billion (ppb) in 1750 to over 1787 ppb in 2008 (Dlugokencky et al., 2009). Though atmospheric CH_4 concentrations are still much lower than those of carbon dioxide (CO_2) (around 386 parts per million, ppm) (Reay et al., 2010), CH_4 is much more effective in trapping infrared radiation (heat) from the atmosphere resulting in the second largest contributor to global warming followed to CO_2. Methane concentrations in total are more than twice compared to the pre-industrial time, however, the annual abundance of methane was rather constant (varying by < 0.4 %) since 1999 (Steele et al., 1992; Dlugokencky et al., 1998; Dlugokencky et al., 2003). Until recently, concerns were raised up again due to the renewed growth of CH_4 started from 2007 (Rigby et al., 2008; Dlugokencky et al., 2009). Studies also showed methane could influence other atmosphere constituents such as OH radicals in order to increase its own lifetime in atmosphere (Lelieveld et al., 1993).

Methane emitted to the atmosphere comes from both natural and anthropogenic sources. Annual CH_4 productions around 320 million tons are from anthropogenic activities, such as ruminants, gas or oil industry, rice agriculture, landfill, waste, coal mining and biomass burning (IPCC, 2007). Natural emissions around 250 million tons of CH_4 per year are accounted for microorganism: methanogenesis, from wetlands, termites, ocean, wild animals, and hydrates.

Recently, it was reported by Keppler and colleagues (Keppler et al., 2006) that there was another methane source directly from terrestrial plants under aerobic conditions and they estimated it would increase the global methane budget around 62-236 million tons per year. However, this finding is still under debate.

The major sink of atmospheric CH_4 is chemical oxidation by OH radicals in the troposphere which contributes to about 90 % methane removal. A small part is either reacted with free chlorine or lost to the stratosphere. Biological methane oxidation in soils indicates an additional important sink (ICPP, 2007) performed by a group of bacteria referred as methane oxidation bacteria (MOB) or methanotrophs which act as natural filters before methane reaches atmosphere.

1.2 Methanotrophs

Methylotrophs are a diverse group of microorganisms which can use reduced carbon compounds without carbon-carbon bonds as their carbon and energy source. Methanotrophs as a subgroup of methylotrophs are often characterized as the microorganisms which could utilize methane as their sole carbon and energy source (Hanson and Hanson, 1996), recently facultative methanotrophs were reported (see 1.2.2). Methane oxidation occurs in both anaerobic and aerobic environments.

1.2.1 Anaerobic methanotrophs

It is estimated that there is 85-300 million T CH_4 per year coming from the ocean of which more than 90 % is consumed by anaerobic oxidation of methane (AOM) (Reeburgh, 2007). The process of AOM was largely unknown until Hinrichs (Hinrichs et al., 1999) and his colleagues

had the first evidence of anaerobic methane oxidation by archaea, indicated by archaeal lipids depleted in the carbon isotope ^{13}C. It has been demonstrated that AOM was coupled to sulfate reduction. There are three known archaeal groups responsible for AOM: ANME-1, ANME-2 and ANME-3 (Knittel et al., 2005). ANME seem to be ubiquitous, present in marine environments as well as in lakes, limnic sediments, landfills, aquifer and soils (Stein et al., 2001; López-Archilla et al., 2007; Jiang et al., 2008; Steinberg and Regan, 2008). The presence of gene homologues methyl-coenzyme M reductase (*mcr*), the key enzyme for methane synthesis in ANME suggests that AOM might be performed by reverse methanogenesis reactions (Krüger and Frenzel, 2003; Hallam et al., 2004). However, so far, no pure culture or defined consortium of ANME has been isolated and the biochemical pathways of AOM remain unknown (Lazar et al., 2011).

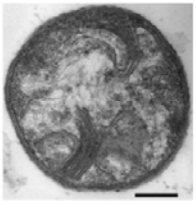
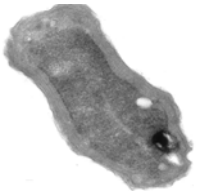
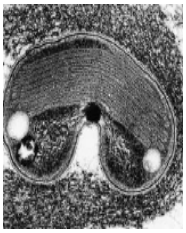
Theoretically, AOM can also be coupled to alternative electron acceptors iron (Fe^{3+}), manganese (Mn^{4+}) and nitrate (NO^{3-}) which are energetically more favorable than sulfate. However, only Raghoebarsing and his colleagues found experimental evidences (Raghoebarsing et al., 2006). They enriched a culture which could oxidize methane anaerobically using NO^{3-} as electron acceptor from anoxic sediments. Approximately 80 % of the enrichments consist of bacteria belonging to the NC 10 phylum whereas 10 % was composed of archaea related to ANME-2. Ettwig and her co-workers proved the bacteria could carry out AOM without archaea (Ettwig et al., 2008). AOM coupled with manganese and iron has been found as well in methane-seep sediments (Beal et al., 2009).

1.2.2 Aerobic methanotrophs

For a long time, methanotrophs were known as strictly aerobic, gram-negative bacteria, using O_2 as electron acceptors. Aerobic methane oxidation is carried by the aerobic methanotrophs belonging to the *Proteobacteria* or the *Verrucomicobia* (Table 1). Proteobacterial methanotrophs could be further divided into type I (*γ-Proteobacteria*, family *Methylococcaeae*) and type II (*α-Proteobacteria*, family *Methylocystaceae* or *Beijerinkiaceae*) according to their morphologies, physiologies and phylogenies (Whittenbury et al., 1970; Trotsenko et al., 2008). Type I consists *Methylomonas, Methylobacter, Methylomicrobium, Methylosarcina, Methylosphaera, Methylosoma, Methylothermus, Methylohalobium,Methylocaldum, Methylococcus, Crenothrix* and *Clonothrix*, type II consists *Methylosinus* and *Methylocystis*. The genera *Methylocapsa* and *Methylocella* belonging to the family *Beijerinkiaceae* share several characteristics with type II, but the major phospholipid fatty acids (PLFA) profiles differ from those of type II. Moreover, *Methylocapsa* owns a unique intracytoplasmic membrane arrangement referred in Table 1 as type III.

The phylum *Verrucomicrobia* were recently discovered (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008) at the extreme environments growing at low pH around 1 to 2 and high temperatures over 55^{o}C. Complete genome sequencing of one isolate suggested that the genes essential for methanotrophy came from the *Proteobacteria* by horizontal gene transfer (Hou et al., 2008). In addition, besides of *Proteobateria* and *Verrucomicrobia*, there are a number of environmental sequences retrieved from culture-independent studies, which could not be assigned to either type I or type II. In this study, type I and type II classifications are solely based on phylogenetic analysis.

Table 1 Phylogenetic, morphological and physiological characteristics of aerobic methanotrophs. Abbreviations: PLFA: Phospholipid fatty acids; RuMP pathway: Ribulose monophosphate pathway; p.d.: poorly developed. The pictures of intercytoplasmatic membranes were adopted from the following publications: A: (Wartiainen et al., 2006a); B: (Wartiainen et al., 2006b); C: (Dedysh et al., 2002); D:(Dunfield et al., 2007).

	ɤ-Proteobacteria (Type I)	α-Proteobacteria (Type II)		Verrucomicrobia
Family	Methylococcaceae	Methylocystaceae	Beijerinckiaceae	Verrucomicrobiaceae
Genera	*Methylomonas* *Methylobacter* *Methylomicrobiu* *Methylosarcina* *Methylosphaera* *Methylosoma* *Methylothermus* *Methylohalobiu* *Methylocaldum* *Methylococcus* *Crenothrix Clonothrix*	*Methylosinus* *Methylocystis*	*Methylocapsa* *Methylocella*	*Methylacidiphilum*
Carbon assimilation pathway	RuMP pathway	Serine pathway	Serine pathway	Alternative serine pathway
Major PLFAs	C16:1ω7c, C16:1ω8c, C16:0, C14:0	C18:1 ω8c, C18:1 ω7c, C18:2 ω7c,12c:0	C18:1 ω7c	C18:0, C16:0, C15:0, C14:0
Intracytoplasmic membranes	Type I (A)	Type II (B)	TypeIII (C)/ p.d.	Different (D)
Pictures of intracytoplasmic membranes	A  *Methylobacter tundripaludum* SV96	B  *Methylocystis rosea sp. nov.*	C  *Methylocapsa acidiphila*	D  *Verrucomicrobia* isolate V4

Until 2005, methanotrophs were regarded as obligate, utilizing only one-carbon compounds for growth. In 2005, it was reported that *Methylocella* could utilize multi-carbon compounds besides methane (Dedysh et al., 2005). Two filamentous methanotrophs *Crenothrix polyspora* and

Clonothrix fusca (Theisen and Murrell, 2005), as well as some *Methylocystis* strains were also proved as facultative MOB (Im and Semrau, 2011).

In aerobic methane oxidation, methane is oxidized via methanol, formaldehyde and formate to carbon dioxide as the final product in the dissimilatory pathway (Figure 1). Formaldehyde is incorporated to the biomass via either ribulose monophosphate (RuBP) pathway (*ɤ-proteobacteria*) or serine pathway (*α-proteobacteria*). *Methylocaldum* and *Methylococcus* possess both RuBP and serine pathway, normally are regarded as type Ib. In addition, some methanotrophs can fix CO_2 via the RuBP pathway as well.

The first crucial step from methane to methanol is catalyzed by the key enzyme methane monooxygenase (MMO). MMO as the principal defining enzyme of MOB, exits in two forms, particulate membrane MMO (pMMO) and soluble cytoplasmic form (sMMO). In methanotrophs which form is expressed is regulated by the availability of copper ions. The copper-containing pMMO is expressed when methanotrophs grow in high copper-to-biomass ratio environments (Murrell and Radajewski, 2000). All methanotrophs possess pMMO except for the facultative type II methanotroph *Methylocella*, which has only sMMO. Compared to pMMO, sMMO has wider range substrate spectrums. The pMMO contains three subunits encoded the genes *pmo C, A* and *B* of about 22, 49 and 27 kDa in size. According to structural studies, the subunit encoded by the *pmoA* gene is considered to contain the active site (Murrell and Radajewski, 2000). sMMO is encoded by a six-gene operon *mmoXYBZDC* (Stainthorpe et al., 1990).

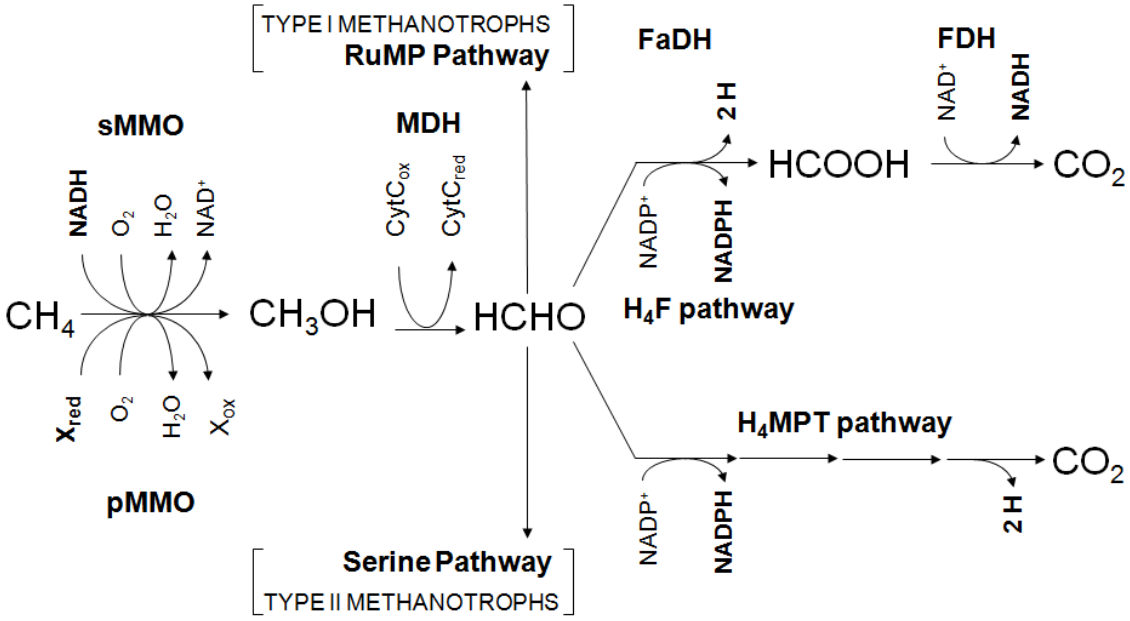


Figure 1: Assimilatory and dissimilatory methane oxidation pathways. Abbreviations: Cytc = Cytochrome c; X = $NADP^+$ or cytochrome linked; MDH: methanol dehydrogenase; FaDH: formaldehyde dehydrogenase; FDH: formate dehydrogenase; H_4F: tetrahydrofolate; H_4MPT: tetrahydromethanopterin.

1.3. Ecology of aerobic methanotrophs

Methanotrophs are ubiquitous, present even in the extreme environments. They could be detected by both cultivation and cultivation-independent methods. According to methane affinity, methanotrophs could be categorized to high affinity and low affinity. High affinity methanotroph could oxidize methane at atmospheric level from the atmosphere and they prefer living in well aerated environments. Low affinity methanotrophs oxidize methane before reaching the atmosphere and they live at or near the oxic-anoxic interfaces.

Rice wetlands were estimated contribute approximately 15 % of the global methane emissions (IPCC, 2007) and the influence is increasing due to the increasing demand of the global rice supply. Numerous studies show both type I and type II MOB live in the rice fields (Qiu et al., 2008). The diversity and abundance of methanotrophs are not affected by the rice varieties or geographical distribution pattern (Lüke et al., 2010), but environmental factors, such as oxygen availability, nitrogen fertilization (Bodelier and Laanbroek, 2004), pH, and growth period of rice (Eller and Frenzel, 2001).

Landfills are not only the source of methane but the sink as well. Amounts of methane oxidized in landfill soils were estimated in the range from 10 % to 100 % (Whalen et al., 1990; Christophersen and Kjeldsen, 2001). Like rice fields, both type I and type II have been found in landfill soils, and *Methylosinus* and *Methylocystis* are dominant. The factors which could shape MOB distributions and populations are oxygen, temperatuere, pH, moisture and earthworms (Gebert et al., 2003; Hery et al., 2007).

Wetlands are also an important methane source. Methanotrophs act as filters for methane, thereby reducing methane emissions from the wetlands. Peat bogs, occupying 3% of total land area, contain both aerobic and anaerobic methanotrophs. Kip et al. (Kip et al., 2009) studied aerobic methanotrophs living in and on the *Sphagnum* mosses in peatland ecosystem. Fluorescence *in situ* hybridization (FISH) experiments showed there were symbiotic methanotrophs in and on the mosses. This symbiont was shown to belong to the *Alphaproteobacteria* with highest homology to *Methlycella* and *Methylocapsa.* spp.

Freshwater sediments represent another important source of methane. It is generally estimated that type I MOB are dominant in these environments (Costello et al., 2002; Rahalkar and Schink, 2007). Studies on methanotrophs in marine environments are rare.

Upland and forest soils are regarded as major sinks for atmospheric methane. Methanotrophs detected from these environments are so called high-affinity MOB as mentioned before. Up to now, these putative methanotrophs have only been defined by the environmental clones grouped as the upland soil cluster (USC) e.g. USC-ɤ and USC-α (Knief et al., 2003; Knief and Dunfield, 2005). Recently, some methanotrophs possessing a second different copy of pMMO were shown to be able to oxidize methane at atmospheric level (Baani and Liesack, 2008). However, the role and mechanism of this second copy pMMO are still unclear.

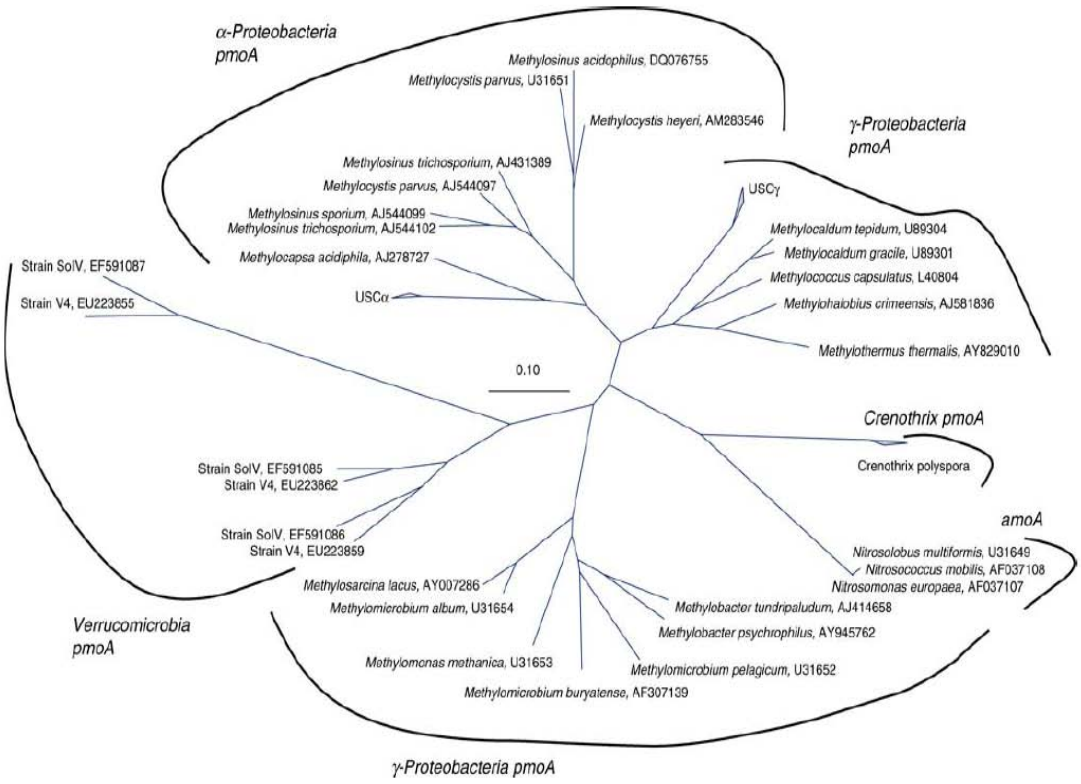


Fig.2. Neighbour joining phylogenetic tree of *pmoA* (Chen, 2010).

1.4. Phylogeny markers

The 16S rRNA gene is the most frequently used phylogenetic marker to determine microbial evolutionary relationships and for microbial diversity studies. Due to the large database and its conservative nature, the 16S rRNA gene is also widely used in the analysis of methanotrophs. However, methanotrophs are rather diverse; hence it is rather difficult to design specific 16S rRNA primers. Recently, Chen (Chen et al., 2008a) designed 16S rRNA gene primer sets targeting type I and type II methanotrophs, however specificity is not warranted. An alternative method is the analysis of functional genes which are unique to the physiology and metabolism of the target microorganisms. A unique enzyme for methanotrophs is methane monooxygenase, which is in charge of the first step of methane oxidation. The genes *pmoA* and *mmoX* encode a subunit of the pMMO and the sMMO, respectively. They are highly conserved and can therefore be also applied to study phylogenetic relationships or the diversity of methanotrophs (Horz et al., 2001). Consequently, phylogeny based on *pmoA* and *mmoX* is mostly congruent with 16S rRNA gene based phylogeny (Holmes et al., 1999; Kolb et al., 2003). The *pmoA* gene is present in all the methanotrophs except for *Methylocella spp.,* as a result, the *pmoA* sequence database is much larger than the *mmoX* database. Different *pmoA* primer combinations target different groups of MOB (Holmes et al., 1995; Costello and Lidstrom, 1999; Bourne et al., 2001), e.g. the A189f-A682r primer set was used to amplify *pmoA* and the related *amoA* gene (encoding ammonium monooxygenase); the A189f-A661r primer set targets most *pmoA* genes, but does not address the "high-affinity" methanotrophs (Bourne et al., 2001), which can be detected by other primer systems.

1.5. Most frequently used molecular techniques to study the ecology of methanotrophs

1.5.1 Denaturing gradient gel electrophoresis (DGGE)

Both, 16S rRNA genes and *pmoA* were applied as targets in DGGE for analyzing methanotroph communities. This method is based on the electrophoretic separation of DNA fragments, which have the same length but different sequences (Laprise et al., 1998). However, the use of degenerate primers, which are sometimes needed to cover methanotroph diversity, often generates multiple bands for a single organism. Chen *et al*. (Chen et al., 2008b) recently designed new 16S rDNA primer sets, which could cover almost all known methanotrophs (excluding *Verrucomicrobia*) and applied them to study MOB diversity in landfill soils. Up to now only a few studies have used these primer sets. Lin *et al*. (Lin et al., 2005) recently designed a new *pmoA*-based non-degenerate primer (mb661_nd) to avoid multiple band production and successfully used it for the detection MOB in an alkaline soda lake.

1.5.2 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is alternative method to fingerprint methanotroph communities and is often regarded to be rapid, sensitive, semi-quantitative and highly reproducible. Since Horz *et al*. (Horz et al., 2001) first applied *pmoA*-based T-RFLP analysis to study the diversity of methanotrophs on rice roots, there have been a number of studies based on this method (Gebert et al., 2009; Lüke et al., 2010).

1.5.3 Stable isotope probing (SIP)

Compared to the fingerprint methods, SIP is a method to identify the active microorganisms responsible for selected environmental functions *in situ*. In SIP, growth substrates labelled with

stable isotopes such as ^{13}C, ^{15}N are added to environmental samples, resulting in these elements being used as carbon or nitrogen source and incorporated into DNA (Radajewski et al., 2000), RNA (Manefield et al., 2002), phospholipids fatty acids (PLFA) (Boschker and Middelburg, 2002) or proteins (Jehmlich et al., 2008). The labelled nucleotides, PFLA or proteins can be separated by CsCl density gradient centrifugation and used in various downstream analyses.

DNA, RNA and PFLA SIP have been applied in methanotroph diversity studies. DNA/RNA or PFLA-SIP have been extensively used by Murrell and his co-workers to discover the methanotrophic bacteria in different habitats, e.g. the Movile Cave (Hutchens et al., 2004), Transbaikal soda lake sediments (Lin et al., 2004), landfill cover soil (Cébron et al., 2007), acidic peatlands (Chen et al., 2008b), alkaline soil (Han, 2009), pine forest soil (Bengtson et al., 2009). One of the key limitations of DNA-SIP are the long incubation times and "cross-feeding" problems. Recently this shortcomings have been improved by shorter incubation times and lower substrate concentrations (Chen et al., 2008b). RNA-SIP may be more sensitive than DNA-SIP because RNA synthesis is faster than DNA synthesis. Compared to nucleotides-SIP, PLFA-SIP needs much less stable isotope which could reduce "cross-feeding". Due to its sensitivity, PFLA-SIP is the preferred method to detect "high-affinity" MOB. However, the limitation of PLFA-SIP is that it is difficult to analyze one particular group of microbes in environments, which contain complex communities (Boschker and Middelburg, 2002). For MOB, it is only possible to detect organisms at the genus level. Bodelier *et al*. (Bodelier et al., 2009b) enlarged biomarker PLFAs database, which increased phylogeny resolutions of type II methanotrophs.

1.5.4. Sequence analysis

Cloning and Sanger sequencing technology are widely used in microbial ecology studies. Methanotroph community diversity and single methanotroph genome analysis were largely investigated by Sanger sequencing (Murrell and Radajewski, 2000). Next generation high throughput sequencing (HTS) such as 454 pyrosequencing and Illumina sequencing technologies have recently revolutionized microbial community analysis. HTS allows to generate massive sequence data in order to get sufficient depth to resolve biological patterns. Pyrosequencing is a DNA sequencing method based on a "sequencing by synthesis" principle. Unlike Sanger sequencing, it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotide (Ronaghi et al., 1998). This technology has been successfully applied for studying microbial 16S rRNA gene diversity in a number of environments (Roesch et al., 2007; Hamady et al., 2009; Kunin et al., 2010), as well as for methanotroph communities composition analysis. The 16S rRNA gene as pyrosequencing tag has been used for studying anaerobic methane oxidizers (ANME) communities in cold seep sediments (Roalkvam et al., 2011). The *pmoA* gene has been usually chosen as amplicon pyrosequencing tag for aerobic methanotroph communities studies (Kip et al., 2011; Lüke and Frenzel, 2011). Compared to the 16S rRNA gene, the choice of protein encoding genes has great advantage. It could be directly detected in amino acid alignment if reading frame shifted (Lüke and Frenzel, 2011).

1.5.5 Methanotroph diagnostic microarray analysis

Microarrays consist of an orderly arrangement of probes (oligonucleotides, DNA fragments, proteins, sugars) attached to a solid surface. Microarrays were initially used as tools for studying

genome-wide expression analysis. To date, microbial diagnostic microarrays (MDM) have been developed and widely applied to the microbial ecology study as well. MDMs, relying on sequence complementarities usually contain oligonucleotide probes, which are specific for a given strain, subspecies, species, genus or higher taxon (Bodrossy et al., 2003). The main advantages of MDM are high-throughput, parallelism, short time to result in high-reproducibility and high resolution. According to the nature of the marker gene, MDMs can be further classified into phylogenetic arrays (Brodie et al., 2006) and functional MDMs. The 16S rRNA gene is the marker of choice for phylogenetic arrays, however in many cases, it could not reach species-specific resolution due to the high conservation of the gene. Functional MDMs on the other hand show better resolutions but are limited by the coverage of published sequence databases. In methanotroph studies, *pmoA*-based functional MDMs have been successfully employed. Since Wu *et al*. (Wu et al., 2001) developed a functional gene array using probes targeting *nirS, nirK, amoA* and *pmoA*, a more specific microarray has been developed and repeatedly updated for methanotrophs (Stralis-Pavese et al., 2004).

This *pmoA*-based microarray (Bodrossy et al., 2003) employs short oligonucleoties (18 to 27 nucleotides) as probes. Fluorescently labelled nucleic acids of unknown samples (targets) are hybridized to the probes on the array, which can reliably discriminate a perfect match target from two (in some cases one) mismatches target. The microarray analysis can be performed based on both DNA and mRNA levels. This array only consisted of 59 probes designed and fully validated against the *pmoA* genes of all known MOB and *amoA* genes of ammonia-oxidizing bacteria at that stage when it was developed by Bodrossy *et al*. in 2003. Up to date, with all the new *pmoA* environmental clones and sequences, which are available in the database, the newest version of

pmoA-array already possesses 209 fully validated probes (data not published) for *pmoA* and *amoA* genes covering all pMMO-possessing aerobic methanotrophs including the newest phylum *Verrucomicrobiales.* This MDM allows rapid analysis of methanotroph communities at the genus and species levels, with one researcher being able to analyze at least 30 samples per week from DNA preparation to results. The same process would take a month with the clone library and sequencing method. The *pmoA*-array has been widely applied to study methanotroph diversity (Abell et al., 2009; Bodelier et al., 2009a; Gebert et al., 2009) .

1.5.6 Quantification of methanotrophs

The most probable number (MPN) technique has been widely used in the past for quantifying methanotrophs in the environment. The method is limited by the fact that only a fraction of microorganisms can be cultivated. Quantitative, real-time PCR is widely used to quantify microbes from enviromental samples. Quantitative PCR (qPCR) uses fluorescent dyes (e.g. SYBR green) or oligonucleotide probes (e.g. Taq man probes) (Heid et al., 1996; Giglio et al., 2003). Kolb *et al.* (Kolb et al., 2003) developed a quantitative real-time PCR assay for different types of methanotrophs using SYBR green and *pmoA* specific primers. This assay has been subsequently used to quantify the methanotrophs, however, it has been suggested that this assay might underestimate methanotroph populations (Kolb et al., 2003). Nevertheless, there are no upgraded primers for quantifying different groups of MOB. Real-time PCR targeting 16S rRNA genes has been applied for methanotroph quantification as well (Halet et al., 2006), however, the primers used in this study were not specific for methanotrophs and thus likely overestimated MOB populations.

1.6 Methanotroph and biodiversity - ecosystem function (BEF)

Ecosystems consist of regulated biogeochemical processes conducted by all creatures. As the catalysts for all the biogeochemical reactions, microorganisms play a crucial role in ecosystems. Microorganisms are regarded as the major source of the total biomass on earth, therefore, they should be considered in biodiversity conservation. However, due to microbes' vast numbers and that they are usually assumed to be redundant and inextinguishable, microorganisms are often neglected in BEF studies. The relationships between the microbial world and different ecosystems are largely unknown.

BEF studies are normally involved in debates of species richness and evenness, resistance and resilience. Species richness and evenness refer to the absolute amount and proportion of species, respectively. Resistance is defined as the degree to which community composition remains unchanged after disturbance while resilience is defined as the rate of to return from disturbance. There are studies demonstrating that the species are the first factor to stabilize the eco-functions (Cardinale et al., 2002). However, recent studies have shown that not only species richness but also evenness make an important contribution to the stability of a system (Wittebolle et al., 2009). Microbial communities have been exposed to different disturbances in a number of studies (e.g. limited or enriched nutrients, temperature, moisture content). These studies indicated that different assemblies of microbial communities have different sensitivities to disturbances (Allison and Martiny, 2008). Microbial communities have been shown to be resilient (Degens, 1998; Griffiths et al., 2001), however, there are also studies suggesting that the resilience of microbes might need time to be observed (Allison and Martiny, 2008).

Due to the complexity of the microbial world, debates of all these hypotheses will continue. A model system the complexity of which could be reduced but still could link eco-function to the model is needed. Methanotrophs as a well defined and studied group of organisms and with a functional link to methane oxidation represent an ideal model system to study and understand the causal relationship between microbes and BEF.

1.7 Aims of the study

Methanotrophs have been well studied during the last decades. Many studies still focus on exploring the diversity and ecology of methanotroph communities in different habitats. However, there are few studies, in particular using methanotrophs as a model system, trying to unveil the link between microbial community structures and BEF. Therefore, the major objectives of this study were: i) to evaluate the feasibility to compare different experimental results from different laboratories using methanotroph as model organisms; ii) to assess the effects of different microbial assembly histories on diversity, functional stability, recovery and re-colonization of methanotrophs; and iii) to address the influence of disturbances on the diversity and functional stability of methanotroph communities and the overall microbial community. The thesis is divided into 3 main chapters in addition to a general introduction and final conclusions.

Chapter 2 addresses the impact of inter- as well as intra laboratory variation on reproducibility of microbial community composition analyses. Standardization of DNA extraction has been shown as the first crucial step for towards molecular ecology studies quantitatively comparable across laboratories and studies.

Chapter 3 addresses microbial community assembly history and its effect on methanotroph community composition. Methanotrophs have been employed in mesocosms studies to investigate whether the total microbial community assembly would influence methanotroph communities and methane oxidation.

Chapter 4 the influence of flood and drought disturbances on methanotroph communities. Drought and flood stresses have been applied to mescosms in order to study the response of the methanotroph communities and methane oxidation to these disturbances, their resilience and recovery.

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Chapter 2

Assessing the impact of inter- as well as intra laboratory variation on reproducibility of microbial community composition analyses

Running Head: Reproducibility of microbial community composition analyses

Abstract

With the advent of molecular biological techniques, especially next generation sequencing and metagenomics, the number of microbial biogeography studies is rapidly increasing. However, these studies involve the synthesis of data generated by different laboratories using different protocols, chemicals etc. with all inherent biases. The aim of this study was to assess inter- as well as intra laboratory variation in the assessed microbial community composition when standardized protocols are applied to a single soil sample. Aliquots from a homogenized soil sample from a rice field in Italy were sent to 5 participating laboratories and subsequently, DNA was extracted by two investigators per laboratory using an identical protocol. Subsequently, all DNA samples were sent to one laboratory to perform DNA quantification, quantitative PCR (QPCR), microarray and DGGE analyses of methanotrophic communities. Yields, as well as purity of DNA was significantly different between laboratories but in some cases also between investigators within the same laboratory. The differences in yield and quality of the extracted DNA were reflected in QPCR, microarray as well as in DGGE results of methanotrophic communities originating from the same soil sample. Diversity indices (Shannon-Wiener, evenness, richness) differed significantly between laboratories. The observed differences have implications for every project where microbial communities are compared in different habitats and assessed even within the same laboratory. To be able to make these comparisons and derive correct conclusions from the results, intra-laboratory variation should be assessed. Standardization of DNA extraction protocols and possible use of internal standards in inter-laboratory comparisons may help in rendering a "quantifiable" bias.

Introduction

Microorganisms comprise a major part of the total biomass of organisms inhabiting Earth (Whitman et al., 1998) and biodiversity (Gans et al., 2005; Sogin et al., 2006; Roesch et al., 2007; Rusch et al., 2007). They play a critical role in biogeochemical processes and ecosystem functioning (Falkowski et al., 2008). However, our knowledge of the ecology and functioning of environmental microbial communities is still far from complete. This is because of our inability to grow the majority of environmental microbes under laboratory conditions. The introduction of many culture-independent DNA- and RNA-based techniques has led to a revolution in environmental microbiology, yielding a wealth of information on community composition in an ever growing range of habitats. Phylogenetic as well as functional micro arrays (Yergeau et al., 2009), and metagenomic techniques (Roesch et al., 2007; von Mering et al., 2007) enable in depth analyses and comparison of whole microbial communities in a high throughput manner.

The collective goal of all environmental microbial ecology studies is to come to an overall understanding of microbial community composition, dynamics and functioning and the mechanisms regulating these parameters, on a level comparable to our comprehension of plant and animal communities. Reaching this goal will necessitate the integrated analyses of all data generated in different labs and from different habitats. The first step in most if not all environmental microbial community studies is the extraction of total community DNA from environments in a way that it reflects the *in situ* community composition as close as possible. Numerous methods, protocols and commercial kits have been developed to improve and optimize the quantity and the quality of extracted community DNA from a wide range of natural environments (Picard et al., 1992; Berthelet et al., 1996; Purdy et al., 1996; Miller et al., 1999; Burgmann et al., 2001), especially not for complex habitats like soil. Beside the challenge of

lysing all cells in environmental matrices, the incomplete removal of compounds that interfere with downstream molecular processing render the development of a bias free protocol a shear impossible mission. Studies assessing the bias of DNA extraction with different methods and different kits on subsequent microbial community profiling analysis (Frostegard et al., 1999; Carrigg et al., 2007; Feinstein et al., 2009) reveal that the perfect protocol to fit all the types of environments is not feasible (Thakuria et al., 2008). However, in light of the global biodiversity debate, assessing local and global patterns of microbial diversity including their controlling factors (Fierer and Jackson, 2006; Martiny et al., 2006), necessitates the comparison of data collected in multiple habitats processed in different laboratories.

In contrast to other scientific disciplines, assessing the reliability of analyses performed in various laboratories using identical protocols applied on the same samples is not a common practice in environmental microbiology. Inter-laboratory comparisons (ring analysis) were applied in food control, veterinary, forensic and soil study commonly to evaluate, for example, Salmonella diagnostic accuracy (Malorny et al., 2003), virus isolation (Ferris et al., 2006), ELISA methods (Batten et al., 2008), mtDNA sequencing (Montesino et al., 2007) and soil microbial biomass C (Beck et al., 1997) , QPCR (Cheng et al., 2009). Ring analyses to assess the reproducibility of DNA extraction and subsequent community composition determination between different laboratories, have not been carried out in environmental microbial ecology.

Microbial communities which have been intensively investigated using molecular techniques are aerobic methane oxidizing bacteria (MOB) which can be found in a wide variety of environments (McDonald et al., 2008) (e.g. air, soils, sediments, lakes, marine). The unique contribution of this group of bacteria to the global methane cycle, rendered the diversity and ecology of MOB to be a hot topic for decades (Dumont and Murrell, 2005; Caldwell et al., 2008;

Trotsenko and Murrell, 2008; White et al., 2008;Op den Camp HJM, 2009). By using methane single source of carbon and energy, these microbes represent the only biological sink of the greenhouse methane under aerobic conditions (Conrad, 2007). The need for filling the knowledge gap regarding this group of bacteria led to the emergence of multifarious molecular approaches to analyze the MOB communities (Horz et al., 2001; Bodrossy et al., 2003; Bodelier et al., 2005; Lin et al., 2005; Yin et al., 2007; McDonald et al., 2008; Bodelier, 2009). Aerobic MOB belong to the Gamma- and Alpha proteobacteria and the Verrucomicrobia (Conrad, 2007; Op den Camp HJM, 2009) and have features that enable linking function and identity. Using methane as carbon and energy source facilitates the application of stable isotope probing of diagnostic lipids (Mohanty et al., 2006; Bodelier et al., 2009) and of RNA/DNA (e.g. Noll et al., 2008). Besides this, the key gene in methane oxidation (methanemonooxygenase subunit A, *pmoA*) reflects the phylogeny of these bacteria, facilitating a direct link between methane consumption and taxonomy. These features have made this group of microbes as a "model" group for studies in environmental microbial ecology. Combined with the broad distribution and high environmental relevance, this group is highly suited to perform a ring-analyses on reproducibility of DNA extraction and subsequent community profiling.

In the present study, five independent laboratories from Norway, Finland, Netherlands, Germany and Austria extracted DNA from the same rice field soil sample, using identical protocols and performed by two different investigators per laboratory. Subsequently, the extracted DNA was sent to one laboratory where DNA quantification, QPCR, microarray and DGGE analysis was performed by one and the same person. The impact of inter- as well as intra laboratory variation of DNA extraction is discussed and recommendations for comparative studies presented.

Materials and methods

Soil

Soil samples were collected in rice fields in Vercelli, Italy on March 30th 2009 before flooding. The characteristics of this rice field soil has been described earlier (Kruger et al., 2001). Soil was frozen and freeze-dried in the laboratory of the Max-Planck Institute for terrestrial microbiology (Marburg, Germany). After homogenizing by pestle and mortar and sieving (2mm), equal portions of 10 gram were send to four other laboratories involved in this study in the Netherlands, Finland, Austria and Norway.

DNA extraction

DNA extraction was executed using a modification of the method described by Yeates and Gillings (Yeates and Gillings, 1998), based on the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA). In every laboratory two investigators processed 4 replicate soil samples in parallel, on the same day using identical chemicals and machinery.

Soil (0.3 g) and 780 µl lysis buffer [200 mM $NaPO_4$ pH 7.0; 1% CTAB; 1.5 M NaCl; 2% Polyvinylpyrrolidone K30; 5 mg ml-1 lysozyme (added right before use)] was added into a multimix FastPrep tube and incubated at 37 °C for 30 min. MT buffer (122 µl) was added and tubes were shaken in the FastPrep instrument (MP, Biomedicals,LLC, Solon, OH, USA) for 30 s at 5.5 m s^{-1}. Subsequently, samples were centrifuged for 15 min at 10000 rpm and 700 µl supernatant were collected. The pellet was re-extracted with by adding lysis buffer (500 µl) and 50 µl MT buffer to the FastPrep tubes, shaken in the FastPrep instrument for 30 seconds at 5.5 m s^{-1} again followed by the transfer of the second 700 µl of supernatant into separate Eppendorf tubes. At this step, 2 × 700 µl supernatant was obtained from each sample. 5 µl of 10 mg ml^{-1} freshly made proteinase K was added to each tube. Tubes were incubated at 65 °C for 30 min.

Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by a chloroform-isoamyl alcohol (24:1) extraction. 125 µl of 7.5 M potassium acetate was added, samples were incubated on ice for 5 min and then centrifuged at 10000 rpm for 10 min. Supernatants (2 × 700 µl per soil sample) were transferred to new tubes, 700 µl Binding Matrix was added and tubes were mixed for 5 min on a rotator. Binding Matrix, with bound DNA, was pelleted by 1 min centrifugation at 10000 rpm. The supernatant was discarded and pellet was resuspended in 500 µl wash buffer. The resulting suspension was added into a Spinfilter, and centrifuged for 1 min at 10000 rpm. The eluate was discarded and the pellet was washed again in 500 µl wash buffer. After discarding the second eluate, the Spinfilter was centrifuged for another 10 s to dry the pellet. The filter was taken into a new tube and 50 µl of TE pH 8.0 was added. The filter was incubated at room temperature for 1 min and centrifuged for 1 min. The filter was re-eluted in the same way with 50 µl of TE pH 8.0. The eluate collected in the catch tube contained the purified DNA. The DNA was subsequently lyophilised at -40 ºC and shipped within 24 hours to the laboratory of the Netherlands Institute of Ecology (Nieuwersluis, the Netherlands) for further processing. Upon arrival, all lyophilized DNA samples were dissolved in equal amounts (100µl) of water. After which the analyses described below were executed.

DNA quantification

DNA concentration was assessed using 2 independent methods, NanoDrop and Picogreen. NanoDrop Spectrophotometer (ND-1000, Nanodrop Technology, Wilmington, DE, USA) gives DNA concentration as well as purity indices such as A260/280, A260/230 and A320.

The PicoGreen (Molecular Probes, Eugene, OR, USA) assay kit uses an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA in solution. A standard

curve was constructed by a dilution series of 0, 0.002, 0.001, 0.01, 0.1 and 2 ng/μl of standard lambda phage DNA (100 ng/μl). For each sample, duplicate 50 μl aliquots of the DNA extracts supplemented with 50 μl of 1× PicoGreen solution were transferred to a 96-well microtitre plate. The plate was shaken for 5 min; fluorescence of DNA extracts was measured at 520 nm after excitation at 480 nm by microplate reader (BioTek, USA). DNA concentration was calculated from the standard curve. During the whole procedure, all samples were protected from light.

Diagnostic pmoA microarray analyses

Microarray analysis was carried out using a modification of the method of (Stralis-Pavese et al., 2004) and described as follows:

Targets preparation

The PCR amplification was based on a two step semi-nested protocol. The first step PCR comprised 25 μl of 2×Premix F (EPICENTRE Biotechnologies, USA), 25 pmol of primer A-189f and A-682r (Holmes et al., 1995) each, 1 unit of Taq polymerase (Invitrogen, USA), 50ng of genomic DNA as template, in total volume of 50 μl. PCR was performed using a touchdown protocol with an initial incubation of 5 min at 94 ^{o}C, followed by 25 cycles of 1 min at 94 ^{o}C, 1 min at the annealing temperature and 1 min at 72 ^{o}C, followed by a final incubation of 10 min at 72 ^{o}C. The annealing temperature was lowered from 62 ^{o}C to 52 ^{o}C over the first 11 cycles after which it was maintained for further 24 cycles at 52 ^{o}C. 5 μl of 1/100 diluted PCR product from the first step was used as template in a subsequent nested amplification with primers A-189f and T7-661r. The second step PCR was performed by total 25 cycles with an initial incubation of 5 min at 94 ^{o}C, followed by 1 min at 94 ^{o}C, 1 min at the annealing temperature and 1 min at 72 ^{o}C, followed by a final incubation of 10 min at 72 ^{o}C. The annealing temperature was lowered from

62 ^{o}C to 52 ^{o}C over the first 11 cycles after which it was maintained for further 14 cycles at 52 ^{o}C.

In vitro transcription

In vitro transcription was carried under RNase-free conditions, the procedure was as follows: 7 μl purified PCR product (50 ng μl $^{-1}$)(by Qiagen kit), 4 μl 5×T7 RNA polymerase buffer, 2 ml DTT(100 mM), 0.5 ml RNAsin (40 U μl^{-1}) (Promega), 1 μl each of ATP, CTP, GTP (10 mM), 0.5 ml UTP (10 mM), 1 μl T7 RNA polymerase (40 U ml^{-1}) (Invitrogen) and 1 μl Cy3-UTP(5 mM) (GE) were added into a 1.5 ml tube and incubated at 37^{o}C for 4 h. RNA was purified immediately based on the RNeasy Mini Kit (Qiagen): 80 μl DEPC treated water were added to IVT mixture. Followed adding 350 μl RLT and 250 μl ethanol, mixed thoroughly. Sample were transferred to an Rneasy mini tube. Add 500 μl RPE, at 10000rpm centrifuged 15 sec. Add another 500 μl RPE, at 10000 rpm centrifuged 2 min. Purified RNA was eluted into 50 μl dH_2O. Purified RNA was fragmented by incubating with 9.5 mM $ZnCl_2$ and 24 mM Tris.Cl (pH7.4) at 60 ^{o}C for 30 min. Fragmentation was stopped by the addition of 12 mM EDTA pH 8.0 to the reaction and putting it on ice. RNAsin (1 μl 40 U μl^{-1}) was added to the fragmented target.

Hybridization

Hybridization was carried out in Belly Dancer which was preheated to 55 ^{o}C for at least one hour. For each hybridization, 62 μl DEPC treated water, 1 μl 10% SDS, 30 μl 20 ×SSC, 2 μl 50 × Denhardt's reagent and 5 μl target RNA were added into a 1.5 mL tube and incubated at 65 ^{o}C for 1 min. Preheated hybridization mixture were applied onto assembled slides with the HybriWell (Grace BioLabs). In the hybridization chamber, the slides were incubated at 55 ^{o}C at maximum bending and lowest r.p.m. overnight. In the washing procedure following the hybridization, the slides were washed by shaking at room temperature for 5 min in 2×SSC, 0.1 %

(w/v) SDS, twice for 5 min in 0.2×SSC and finally for 5 min in 0.1×SSC. Slides were dried individually using an air gun.

Scanning and data analysis

Hybridized slides were scanned at 10 μm resolution with a GenePix 4000 laser scanner (Axon, Foster City, CA, USA) at wavelengths of 532 nm. Florescent images analyzed with the GenePix software (Axon). Micosoft Excel was used for statistical analysis and presentation of results.

Quantitative PCR (QPCR)

Three methanotrophic sub-groups (see table 1) were quantified by *pmoA*-based quantitative PCR based on the assays described by (Kolb et al., 2003). The type Ia and II assays were carried out as described (Bodelier, 2009). For the type Ib assay, DNA standards were prepared by dilution of a known amount of PCR product amplified from a reference clone by using the 189-468 primer set (Kolb et al., 2003). 25 μl reaction containing 12.5 μl 2 ×SYBR green mix (AB gene), 2.5 μl of diluted DNA template and 0.8 mM each of primers. The samples were diluted accurately to 1 ng / μl. The thermal cycle started with an initial denaturation at 95 ^{o}C for 15 min, followed by 45 cycles of denaturation at 95 ^{o}C for 20 sec, annealing at 64 ^{o}C for 20 sec, and extension at 72 ^{o}C for 45 sec. Data were acquired at 84 ^{o}C, DNA melting curve analysis at temperature ranging from 70 ^{o}C to 99 ^{o}C. All of three assays were performed with a Rotor Gene 6000 thermal cycling system (Corbett Research, Eight Mile Plains, Qld, Australia), where samples were added to aliquots of the master mixture using a CAS-1200 (Corbett Robotics Eight Mile Plains, Qld, Australia) liquid handling system. Every sample was performed in duplicate.

Quantification analysis was performed by the RotorGene software. Cell numbers were calculated assuming a PCR product length of 412 bp, 279 bp and 423 bp for type Ia, type Ib and type II methanotrophs respectively.

16S rDNA PCR-DGGE

DGGE analysis was performed as described (Bodelier et al., 2005) except the following exception. PCR reaction mixture contained 25 μl of 2×Premix F (EPICENTRE Biotechnologies, USA), 25 pmol of each primer, and 1 unit of Taq polymerase (Invitrogen, USA), 50 ng of genomic DNA as template. 1 μl of first step PCR product was added to the second step. The gels were loaded with 2 μl of loading buffer and 23 μl of PCR product. Phoretix Software (Nonlinear dynamics, UK) was used for DGGE intensity analysis.

Statistical analyses

Effects of country and investigator on DNA extraction method, DNA concentration, purity and QPCR were analyzed using nested ANOVA design, where the investigator was nested within country. Post-hoc comparisons between countries were analyzed using the unequal N HSD test to account for the unequal number of replicates in the Norwegian dataset. All data were checked for normality and homogeneity of variances and if necessary log transformed. The analysis was carried out using the STATISTICA software package version 8.0 (Statsoft Inc., Tulsa, US).

Analyses of microarray data

The normalized signal intensities as derived from the scanned microarrays formed the matrix which was used in non-metric Multidimensional Scaling (MDS) analyses. The input of MDS

analyses were Bray-Curtis similarity matrices generated using Log (x+1) transformed signal intensity values to even out the contribution of very rare and very dominant probe signals. The MDS analyses results in a 2-dimensional plot were the distance between samples indicates the similarity of these samples relative to other samples in the plot. The accuracy of the 2-dimensional representation is indicated by the "Stress" value (Kruskall's stress formula). Stress values <0.1 indicate a good ordination with no prospect of misleading interpretation. Stress values <0.2 still give a good 2-dimensional representation where not too much reliance should be put on the detail. In this case other methods of representation should be used in parallel, like clustering analyses. The ANOSIM procedure was used to test whether microarray community profiles obtained in different laboratories differed. All MDS analyses were performed using the Primer-E software (Plymouth Marine Laboratory, Plymouth, UK). All theoretical aspects of the used cluster and MDS analyses are described by (Clarke and Warwick, 2001). Due to repetition of single sample DNA extraction in different countries the effect of country on positive microarray probes were tested with linear mixed effect model, which fits for observations taken on related individuals. Analysis was made with add-on package nlme (Pinheiro et al. 2009) in R 2.10.1. (R Development Core Team 2010).

Results

DNA concentration and quality

DNA concentration was analyzed using two different methods which are NanoDrop and picogreen assays (Figure 1). It is obvious that the DNA concentrations measured with the NanoDrop assay are on average double the amount measured with picogreen. Besides this, for

both assays there were significant effects of laboratory as well as investigator (Figure 1 and supplementary Table 1). Laboratories in the Netherlands and Finland extracted more DNA than the other labs while in 3 laboratories there were significant differences between investigators (Figure 3B, supplementary Table 1). Differences between labs were also observed in the purity of the DNA (i.e. A260/280). (Figure 2, and supplementary Table 1). However, yield and purity only correlated for the picogreen method and not for DNA concentrations assessed by the nanodrop method (supplementary Table 4). An investigator effect on DNA purity was only observed in the Austrian lab (Figure 2).

Quantitative PCR

To test the effect of different DNA concentration measurements on QPCR results, both methods were compared on the samples processed in Austria (Figure 3). QPCR assays targeted three groups of MOB, Type Ia, Type Ib and Type II. In all assays numbers of MOB were significantly higher when initial target input amounts were based on the NanoDrop than picogreen method (Figure 3 and supplementary Table 4). Except for Type II *pmoA*-copy numbers, there were also significant differences between the investigators (supplementary Table 4). In Figure 4 the QPCR data for all laboratories were presented for three different groups of MOB. Again there were significant differences between countries which were largest for type Ia where the numbers from the Norwegian and the Austrian lab were lower than the rest (Figure 4, supplementary Table 3). For all QPCR assays, there was no investigator effect (supplementary Table 3). Copy numbers of all assays correlated strongly with the DNA purity (i.e. 260nm/280nm ratio) and DNA concentration based on the picogreen assay (supplementary Table 4).

pmoA Microarray analysis

The diversity of the MOB was assessed using a *pmoA* diagnostic microarray. In analogue to the QPCR analysis, microarray analysis was carried out with Austrian samples using initial target concentration based on Nanodrop as well as Picrogreen DNA concentration assays. The non-metric multidimensional scaling plot in Figure 5 displays the dissimilarity between methanotrophic community composition in rice soil samples as extracted in 1 laboratory. What is graphically obvious is also confirmed by ANOSIM test; community profiles based on different DNA concentrations assays differ significantly from each other (Figure 5).

Micro array community profiles generated in the various laboratories based on Nanodrop DNA target concentrations, it is obvious that different microarray community profiles were generated in various laboratories (Figure 6). Global ANOSIM for 'laboratory' effect was highly significant ($p<0.001$), while there was no effect of investigator. Linear mixed effect model analyses indicated that only the profiles from the laboratories A and B did not differ much from each other (supplementary Table 5). Overall, 56 probes showed positive signals. DNA from laboratory D gave greater higher signal intensities for eight probes when compared to laboratories A and B. On the other hand, DNA from laboratories C and E gave 15 and 14 significantly reduced probe intensities, respectively. Over all laboratories and investigators, 59% of potentially positive probes were not detected or showed reduced intensity at least once. Most of these changes happened within species-specific type II probes (67% out of 21 probes were affected). However, group-specific probes for type II methanotrophs were consistently positive, while type Ia and type Ib specific probes differed between laboratories (see Supplementary Table 5).

DGGE analysis

The DGGE patterns are rather similar in terms of the number of bands (i.e. MOB species) detected (Supplementary Figure 1). However, the intensity of the bands differs. Since equal amounts of extracted DNA were added to the PCR, and equal volumes of PCR product were loaded on the gels, the intensity differences can be related to the amount of targets in the extracts. There is difference between countries as well as between investigators. Finland, also having the highest amount of extracted DNA has the highest band intensities, for type I as well as for type II. Countries with the biggest investigators difference (Austria and Netherlands) as observed with *pmoA*- based analyses also display highest variability using 16S-based PCR-DGGE.

Discussion

Source of variation

The data presented in this paper clearly demonstrate that even the application of identical protocols to a single soil sample leads to significantly different community composition. It is well known that different DNA extraction protocols differ in their efficiency of liberating and purifying DNA from various environmental matrices with consequences for microbial community composition e.g. (Ning et al., 2009). This study adds another component of variation being the laboratory or the researcher carrying out the analyses. Applying the same protocol on the same sample and the same successive treatments, different labs with different investigators ended up with different MOB community structure profiles. Different methods of assessing the community composition as well as different markers (16S as well as *pmoA*) confirmed the conclusion that the origin of the differences can only reside in the DNA extraction procedure.

The dependency of the outcome of microbial diversity analyses on laboratories and even on individual researcher has serious implications for any project or meta-analyses where results from different laboratories have to be compared.

Since all analyses following the DNA extraction were carried out in the same lab, using the same equipment and chemicals and executed by the same person, the source of variation between laboratories comes from the extraction procedure. One explanation may be that not all laboratories used the same bead-beating machine. This may have caused the rather low DNA extraction yield and purity in the Norwegian lab which used a different bead beater than the other laboratories involved. The bead beating has been found to increase the yield of soil DNA (Krsek and Wellington, 1999), however, so far there are no reports presenting data on the DNA extraction efficiency as a function of the type of bead beater.

However, labs using the same bead beating machine (e.g. B, C and D) also arrive at different results. In this case the source of variation may reside in the chemicals (buffers, enzymes, solvents etc) that were used in the extraction procedures. Various brands of chemicals may introduce some variations. However, the intra-laboratory comparisons, carried out by two investigators using exactly the same equipment as well as chemicals, also yielded significant differences in some labs. The latter can only be caused by variation in parts of the protocol that are subject to handling variation by the investigator, which can only be the pipetting routine. In the protocol, there are phase separation steps where the DNA is in the upper phase of the extract. The upper layer has to be removed without taking anything from the lower layers containing the contaminants. The efficiency of the removal of the upper phase will determine the DNA yield in 1:1 ratio. This step requires pipetting practice and may very well differ between investigators. Nevertheless, the effect between laboratories is still bigger than between investigators. Hence,

the observed variation is a mix of local differences in equipment or chemicals and also by the skills of the person carrying out the analyses.

In addition to the phase separation step, the quality of phenol used in this part of the protocol may affect the results. Phenol (pKa 9.95) is a sensitive and reactive organic compound having hydroxyl group in a benzol backbone. It has a crucial role in the phase separation step by removing proteins, polyphenols and polysaccharides from the aquatic phase where DNA resides. If the phenol is not in optimal condition, the quality of the extracted DNA will be lower. In our study all laboratories used the same mixing ratios of phenol, chloroform and isoamyl alcohol in the phase separation step, except for laboratory D which used water saturated phenol with pH 4.0 that contained 8-Hydroxyquinoline stabilizer. The other laboratories used Tris-HCl buffered phenol with pH 7.0-8.0 and without stabilizer. The difference in pH itself is not crucial but the storage at low pH in the presence of the stabilizer preserves the phenol better possibly leading to higher purity of DNA and higher values of microarray probes in case of laboratory D.

A critical factor investigated in this study was the method of DNA concentration measurement. The often used NanoDrop method is very fast and small aliquots are needed. However, it measures DNA as well as RNA and it is affected by the impurities in the extract. We observed that this method gave double amount of DNA than the Picogreen method which only detects double stranded DNA. The consequence of this possible overestimation by the NanoDrop method is that when starting to dilute the DNA in order to add a fixed amount to the PCR, then the extract is diluted more than it would be when using the concentration measured with picogreen. The former may lead to lowering of the influence of remaining PCR inhibiting compounds. This can of course contribute to inter-laboratory differences when different DNA concentration measurements were used. Which assay performs better and can be therefore

recommended is difficult to say. There are reports that above concentrations of 10 ng.µl^{-1} both assays perform similarly, while below this concentration picogreen performs better (Aranda Iv et al., 2009). The reproducibility is in the same range for both assays indicated by the coefficient of variation (Supplementary Figure 2) and the positive correlation (Supplementary Table 4).

Consequences for MOB community structure assessment

There are no inter- as well as intra-laboratory studies on methanotrophic community composition at present to compare our results to. However, it is obvious that quantitative comparisons between MOB communities analysed in different laboratories and even carried out by different investigators are problematic. DNA extraction yields and purity will be significantly different as are the subsequent QPCR results. Diversity measures considering quantitative data may be compromised. The Shannon diversity index based on the richness and evenness (microarray analyses) shows that there are significant differences between laboratories (Figure 7). These differences already start at the very beginning of every molecular investigation, at the DNA extraction procedure. The purity of DNA (A260/280 ratio) correlated positively with the Shannon and evenness indices well as MOB abundance as assessed by QPCR, indicating that yield as well as purity influence the downstream results. In addition, higher single probe intensities in case of eight probes of laboratory D could be connected to the higher purity of the DNA (supplementary Table 5). Comparing diversity and/or abundance between habitats can be almost impossible without having some information on the bias introduced at the extraction stage. A strategy overcoming these problems may be using internal controls during DNA extraction as previously described (Mumy and Findlay, 2004; Petersen and Dahllof, 2005; Ramette, 2009).However, the relative abundance as measured by QPCR stayed rather constant between labs. Hence, the ratio between numbers of type Ia, Ib and II was not significantly

affected by laboratory nor investigator and was also not correlated to DNA quantity and quality (Supplementary Table 4). Therefore statements like "in this habitat there is more type II than in another habitat" or "this habitat is more diverse than another" cannot be made when analyses have been carried out in different laboratories or different investigators without a proper assessment of lab or investigator bias. What is valid to do is testing the effects of environmental change (assessed in different labs) on normalized relative abundances of MOB, which were not affected by DNA purity and yield. In a recent study it was shown that denitrification gene abundance was correlated to activity and environmental parameters only when relative abundances were used in the analyses (Philippot et al., 2009). This may be caused by the fact that relative abundance as measured with independent assays is not influenced by biases in DNA extraction. Also changes in community structure as assessed in one laboratory are valid results because the same bias will be introduced in every time point, assuming that DNA yield, purity and the investigator are identical.

Conclusions

Quality and quantity of extracted DNA from a single soil sample using identical protocols can differ between laboratories and investigators resulting in significant bias in downstream molecular analyses. Comparing results from different experiments necessitates assessing this bias. Reducing the bias to a minimum we recommend performing all DNA extraction in one laboratory using identical chemicals and machinery. In large scale project with multiple research groups involved, all downstream processing steps should be executed using PCR ingredients purchased with the same company, preferably from the same production batch. However, in many projects these criteria are difficult to meet. An alternative strategy would be developing an internal control system which gives information on the inter- and intra laboratory biases. Internal

standards should not only help validating the quality of downstream processing, but preferably the DNA extraction step. In this way a correction factor might be developed allowing a reproducible interpretation of molecular data. For *post-hoc* meta-analyses not meeting these requirements, however, only robust algorithms should be used avoiding over-interpretation of differences.

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Legends to the figures

Figure 1: DNA concentration as analysed with NanoDrop or picogreen, showing the comparisons between laboratories (panel A) and between investigators in the various laboratories (panel B). Different letters in panel A indicate significant differences between countries ($P<0.05$, unequal HSD test). In panel B * indicates significant difference between investigators within 1 laboratory (nested ANOVA, unequal HSD post-hoc comparison) and + indicates significant differences as assessed with students's T-test (+ $p<0.05$; +++ $p<0.001$). (FI = Finland; NL = Netherlands; AU = Austria; GE = Germany; NO = Norway).

Figure 2: Purity of extracted DNA measured as the 260/280 Absorbance ratio. Different letters indicate significant differences between laboratories from different countries (Unequal N HSD test, $p<0.05$). The arrow indicates significant difference between investigators within the respective laboratory. (FI = Finland; NL = Netherlands; AU = Austria; GE = Germany; NO = Norway).

Figure 3: Effect of DNA quantitation method on *pmoA* gene copy numbers in rice soil assessed using 3 different assays targeting sub-groups of methane-oxidizing bacteria, executed in 1 laboratory only. Aus A and B indicate the results of the 2 different investigators from the Austrian lab.

Figure 4: *pmoA* gene copy numbers in rice soil of three different sub-groups of methane-oxidizing bacteria as assessed in laboratories in 5 different countries. Different letters per sub-

group of MOB indicate significant differences between laboratories from different countries (Unequal N HSD test, $p<0.05$, n=8, except for NO lab n=6). (FI = Finland; NL = Netherlands; AU = Austria; GE = Germany; NO = Norway).

Figure 5: Non-metric multidimensional scaling plot using log-transformed Bray-Curtis dissimilarity matrices based on signal intensity values of *pmoA* micro array analyses, performed on the basis of Nanodrop or Picrogreen DNA quantitation method. Distances between symbols represent relative dissimilarity between MOB communities. Analyses of similarity (ANOSIM) resulted in a significant difference between MOB community structure when based on different DNA concentration measurements (n = 8).

Figure 6: Non-metric multidimensional scaling plot using log-transformed Bray-Curtis dissimilarity matrices based on signal intensity values of the *pmoA* micro array analyses, subjected on DNA extracted in 5 different laboratories. Distances between symbols represent relative dissimilarity between MOB communities. Analyses of similarity (ANOSIM) resulted in a significant difference between MOB community structure analysed in different countries. Only samples from Finland and the Netherlands did not differ from each other (n = 8, except for Norway n = 6).

Figure 7: Shannon index, species richness (i.e. positive probes) and evenness of MOB communities as assessed by *pmoA* micro array analyses, subjected on DNA extracted in 5

different laboratories. Different letters indicate statistically significant differences (Unequal HSD test, $p<0.05$, $n = 8$, except for Norway $n = 6$).

Figure 1

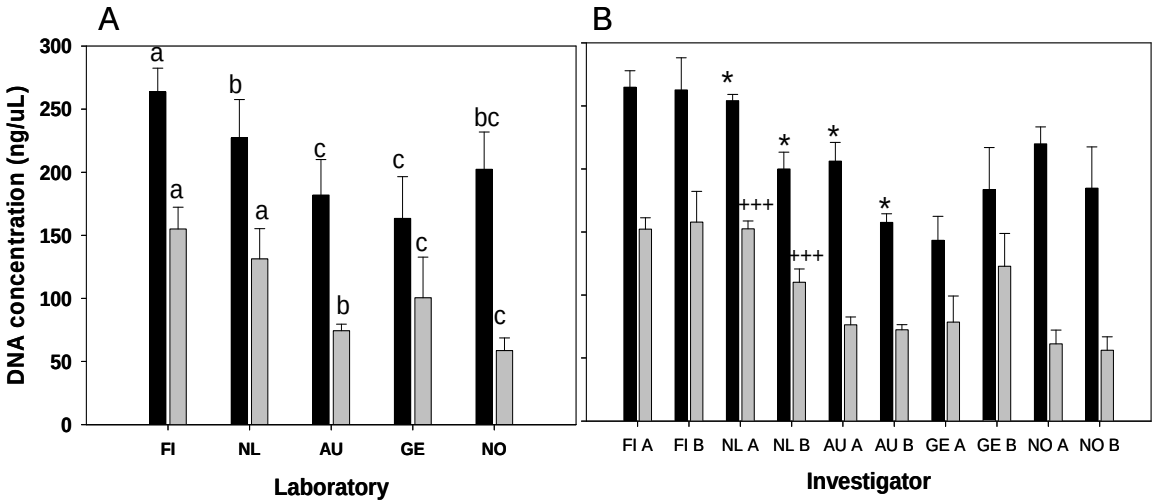


Figure 2

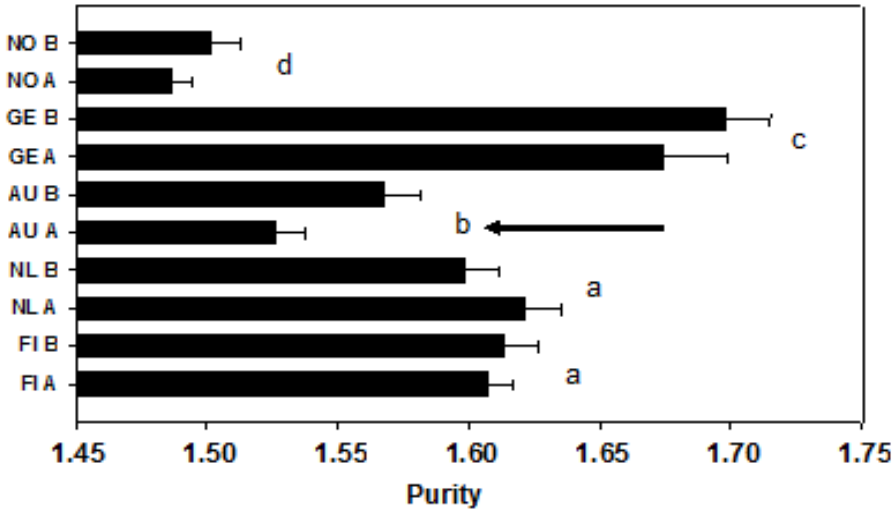


Figure 3

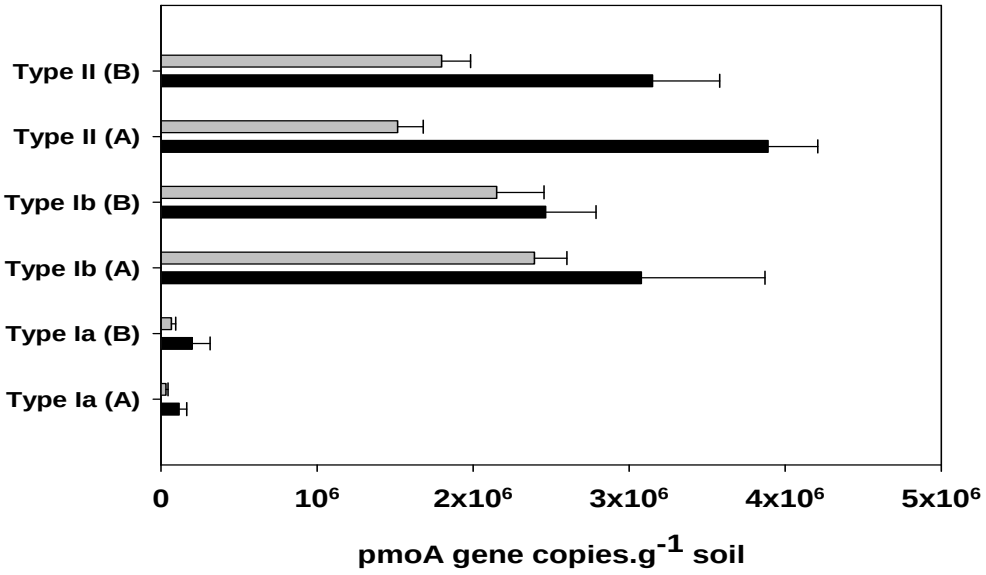


Figure 4

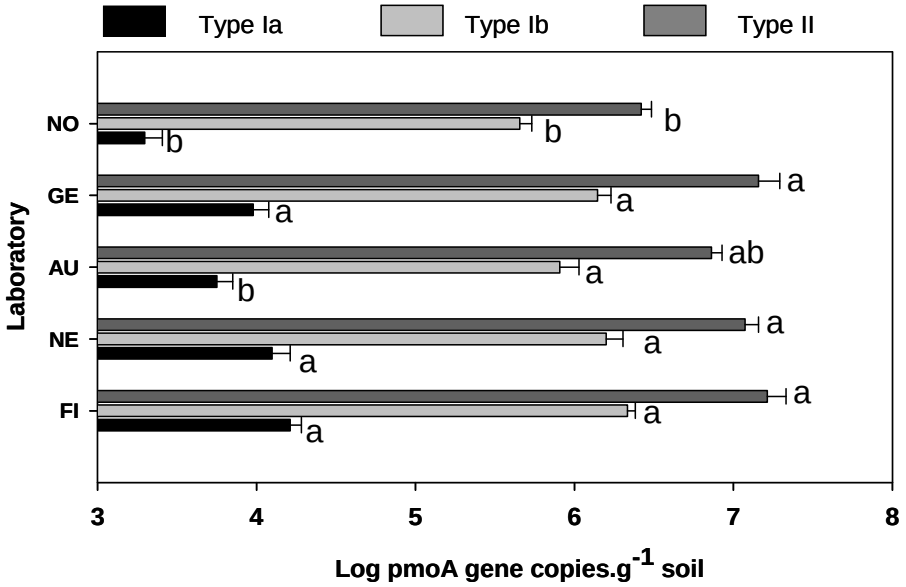


Figure 5

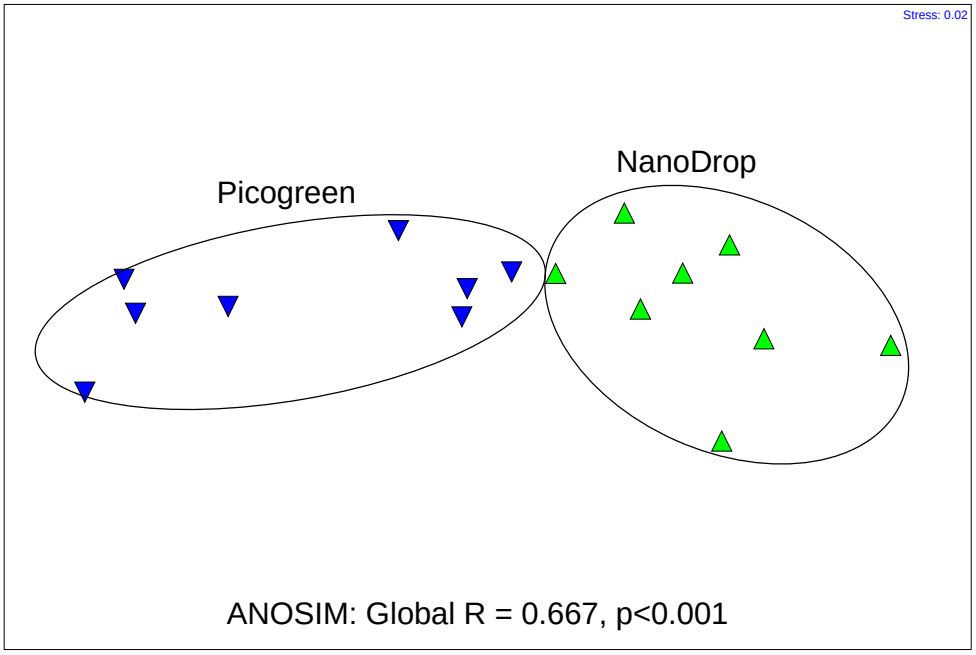


Figure 6

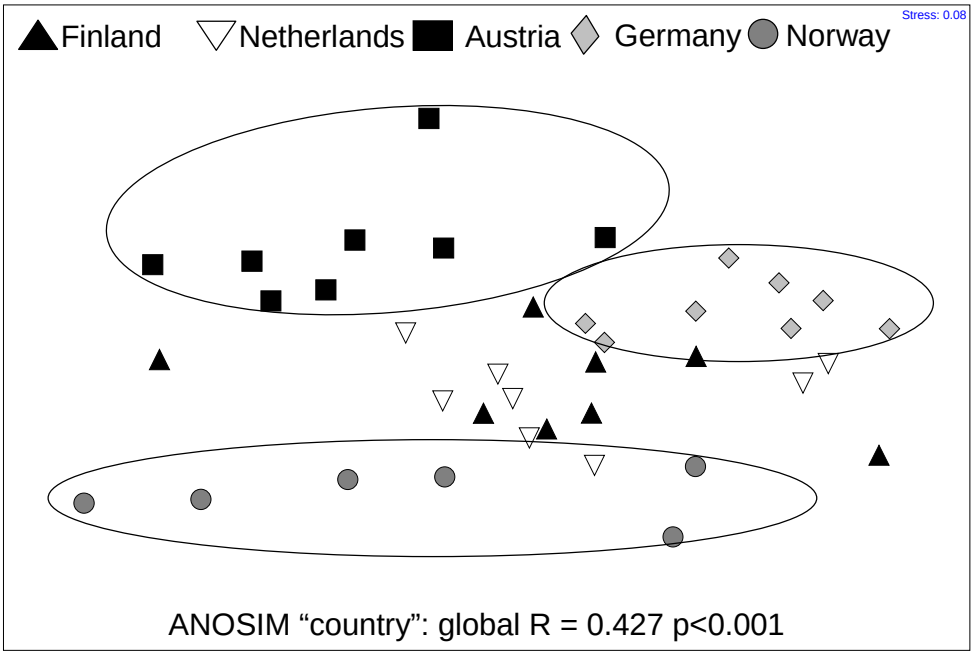
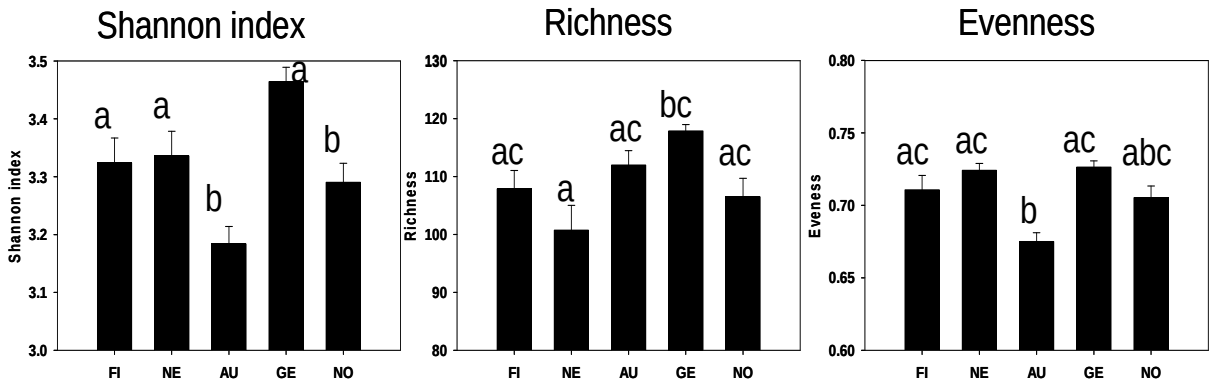


Figure 7


Shannon index
Shannon index
3.5
3.4
3.3
3.2
3.1
3.0
a
a
b
a
b
FI
NE
AU
GE
NO
Richness
Richness
130
120
110
100
90
80
ac
a
ac
bc
ac
FI
NE
AU
GE
NO
Evenness
Eveness
0.80
0.75
0.70
0.65
0.60
ac
ac
b
ac
abc
FI
NE
AU
GE
NO

Supplementary Table 1: Nested ANOVA analyses assessing the effects of Laboratory and investigator on DNA concentration and purity of DNA extracted from rice soils.

n=8, except for Norwegian laboratory n= 6.

	Df	F	P
DNA concentration (Nanodrop)			
Laboratory	(4,28)	31.911	0.00000
Investigator (nested in Laboratory)	(5,28)	8.1431	0.00008
DNA concentration (Picogreen)			
Laboratory (log picogreen)	(4,28)	52.822,	0.00000
Investigator (nested in Laboratory)	(5,28)	25.889	0.00000
Ratio (260/280)			
Laboratory	(4,28)	170.90	0.00000
Investigator (nested in Laboratory)	(5,28)	5.469	0.00124

Supplementary Table 2: Nested ANOVA analyses assessing the effects of DNA concentration analyses method and investigator on *pmoA* copy numbers in rice soil assessed for 3 different methanotrophic sub-groups, as carried out I 1 laboratory only.

Nanodrop VS picogreen Nested ANOVA comparison

Table 2	Df	F	P
qPCR log Type Ia			
Method	(1,12)	39.463	0.00004
Investigator (nested in method)	(2,12)	4.5655	0.03354
qPCR log Type Ib			
Method	(1,12)	257.79	0.00000
Investigator(nested in method)	(2,12)	8.8261	0.00439
qPCR log TypeII			
Method	(1,12)	4.8229	0.04847
Investigator (nested in method)	(2,12)	1.9383	0.18644

n=8

Supplementary Table 3: Nested ANOVA analyses assessing the effects of Laboratory and investigator performing DNA extraction on *pmoA* copy numbers in rice soil assessed for 3 different methanotrophic sub-groups.

Table 3	Df	F	P
qPCR log TypeIa			
Laboratory	(4,28)	11.104	0.00002
Investigator (laboratory)	(5,28)	0.63934	0.67156
qPCR Type Ib			
Laboratory	(4,28)	4.4563	0.00652
Investigator(laboratory)	(5,28)	0.87399	0.51105
qPCR log TypeII			
Laboratory	(4,28)	8.3562	0.00014
Investigator (Laboratory)	(5,28)	0.57984	0.71504

n=8, except for Norwegian laboratory n= 6.

Supplementary Table 4

Variables	Shannon	Eveness	Richness	Nanodrop	Picogreen	260/280	TIa	TIb	TII	II/Ia	Ib/Ia	II/Ib	Ia193	Ia575	Ib459	Ib559	II590
Shannon[1]																	
Eveness[1]	**0.84**																
Richness[1]	**0.36**	-0.05															
Nanodrop[2]	0.05	0.18	-0.27														
Picrogreen[2]	0.30	**0.44**	-0.08	**0.67**													
260/280[3]	**0.54**	**0.54**	0.22	-0.06	**0.60**												
TIa[4]	0.13	0.27	0.05	**0.39**	**0.72**	**0.56**											
TIb[4]	0.21	0.29	0.09	**0.42**	**0.67**	**0.53**	**0.91**										
TII[4]	0.30	**0.34**	0.22	0.27	**0.65**	**0.62**	**0.60**	**0.56**									
Ratio II/Ia[5]	0.13	-0.04	0.25	-0.18	-0.24	-0.07	**-0.56**	**-0.52**	0.25								
Ratio Ib/Ia[5]	0.04	-0.09	0.04	-0.02	**-0.35**	-0.24	**-0.45**	-.10	**-0.39**	0.16							
Ratio II/Ib[5]	0.07	-0.01	0.23	-0.15	-0.02	0.07	-0.24	**-0.41**	**0.48**	**0.81**	**-0.37**						
Ia193[6]	**0.80**	**0.76**	0.14	0.22	0.25	0.28	0.09	0.14	0.11	-0.01	0.09	-0.07					
Ia575[6]	**0.46**	**0.64**	**-0.34**	**0.46**	0.30	0.02	0.04	0.08	0.01	-0.09	0.06	-0.13	**0.77**				
Ib459[6]	**0.44**	**0.59**	-0.26	0.24	**0.53**	**0.45**	0.30	**0.32**	**0.51**	0.01	-0.31	0.17	**0.33**	**0.44**			
Ib590[6]	**0.73**	**0.82**	-0.07	**0.36**	**0.63**	**0.60**	**0.40**	**0.37**	**0.46**	-0.06	-0.29	0.06	**0.66**	**0.61**	**0.67**		
II590[6]	**0.67**	**0.64**	0.16	0.09	0.30	**0.49**	0.13	0.18	**0.33**	0.15	-0.00	0.18	**0.62**	**0.40**	**0.42**	**0.67**	
II630[6]	0.27	0.25	0.05	0.23	0.18	0.16	0.16	0.24	**0.37**	0.15	-0.03	0.20	0.28	0.28	**0.37**	**0.35**	**0.63**

Pearson Rank correlation coefficients (n= 38, bold is significant correlation $p<0.05$)

[1]: Shannon, richness and eveness are based on *pmoA*- based diagnostic micro array analyses.

[2]: DNA concentration as determined by Nanodrop or Picrogreen.

[3]: Ratio spectrophotometer values at wavelengths 260 and 280nm as measured using the Nanodrop device.

[4]: QPCR assays Ia, Ib and II

[5]: Ratio of *pmoA* copy numbers of type Ia, typeIb and TypeII MOB.

[6]: Signals of group probes for TypeIa, Ib and II as detected by *pmoA*-based diagnostic micro array.

Supplementary Table 5

Contribution of individual probes to the dissimilarity in methanotrophic community composition in rice soil after DNA extraction in 5 different laboratories. Comparison of extraction in five different laboratories were made with linear mixed effect model with nlme add-on package in R 2.10.1. Yellow color represents that probe has not changed between laboratories and green and red color represents positive or negative change respectively compared to Finland sample.

Abberviations: FI = Finland; NL = Netherlands; AU = Austria; GE = Germany; NO = Norway

	FI	NL	AU	GE	NO	* $P < 0.01$, ** $P < 0.001$
Mb292						*Methylobacter*
O_Mb282						*Methylobacter*
P_Mb_SL#3-300			**			*Methylobacter*
P_Mb_C11-403						*Methylobacter*
P_Mm531			*			*Methylomonas*
Mm275			**			*Methylomonas*
P_Mm451			**			*Methylomonas*
Mmb303						*Methylomicrobium album*
O_Mmb562						*Mmb. album* and *Methylosarcina*
LP20-644						*Methylomicrobium*-related clones
O_Ia575			**			Type I a (*M.bacter-M.monas-M.microbium-M.sarcina)*
P_JRC4-432						Japanese rice cluster #4
P_MclS402				*		*Methylocaldum szegediense*
Mcl408						*Methylocaldum*
P_501-375 *			**		**	*Methylococcus*- related marine and freshwater sediment clones
O_501-286					**	*Methylococcus*- related marine and freshwater sediment clones
P_USC3-305				*		Upland soil cluster #3
Mc396						*Methylococcus*
O_fw1-641						fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones
P_fw1-286 *			**		**	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones
P_LW21-374						LW21 group
P_JRC3-535						Japanese Rice Cluster #3
O_M90-574						*M.coccus-M.caldum* related marine and freshwater sediment clones
O_M90-253						*M.coccus-M.caldum* related marine and freshwater sediment clones
Ib453					*	Type I b (*M.thermus-M.coccus-M.caldum* and related)
Ib559 *			**		*	Type I b (*M.thermus-M.coccus-M.caldum* and related)
P_DS3-446			**			Deep sea cluster #3
P_Mcy233					*	*Methylocystis*
O_Mcy413					**	*Methylocystis*

O_Mcy522						*Methlocystis* A + peat clones
P_Mcy270					*	*Methylocystis*
P_Mcy459						*Methylocystis*
O_Mcy255 *			**		**	*M.cystis B*
P_McyM309				**		*M.cystis strain M and related*
P_McyB304 *			*		**	*M.cystis B (parvus/echinoides/*strain M)
P_MsT214						*Methylosinus trichosporium* OB3b and rel.
P_MsT343						
O_Msi269				**		*Methylosinus trichosporium*
P_MsS314				*		*Methylosinus sporium*
P_Msi263			*			*Methylosinus sporium* + 1 *Msi.trichosporium subclaster*
P_Msi423						*Methylosinus*
O_Msi294			*			*Methylosinus*
Msi232					*	*M.sinus*+ most *M.cystis* -considered as additional type II probe
Peat264					**	peat clones
O_II509						Type II
O_II630						Type II
P_NMcy1-247 *			*		**	Novel *pmoA* copy of *M.cystis #1* (?)
P_NMsiT-271						Novel *pmoA* copy of *M.sinus trichpsporium* (?)
O_RA14-594				**		RA14 related clones
P_RA14-591						RA14 related clones
B2all343						*Methylocapsa* and related clones
O_B2all341				*		*Methylocapsa* and related clones
P_TUSC409					**	Tropical Upland Soil
NsNv363 *		*	*			*Nitrosospira-Nitrosovibrio*
gp391				**		environmental clones of uncertain identity

56 positively detected probe

Green probes were not changed at all.

* marked probes were changed in at least two out five countries.

Probe changed in at least two out five countries:

All probes

49 out of 56 probe were not changed (P < 0.01)	87,5	% were not changed
7 out of 56 probes were changed (P < 0.01)	12,5	% were changed

Type Ia probes

11 out of 11 probe were not changed (P < 0.01)	100,0	% were not changed
0 out of 11 probes were changed (P < 0.01)	0,0	% were changed

Type Ib probes

12 out of 15 probe were not changed (P < 0.01)	80,0	% were not changed
3 out of 15 probes were changed (P < 0.01)	20,0	% were changed

Type II probes

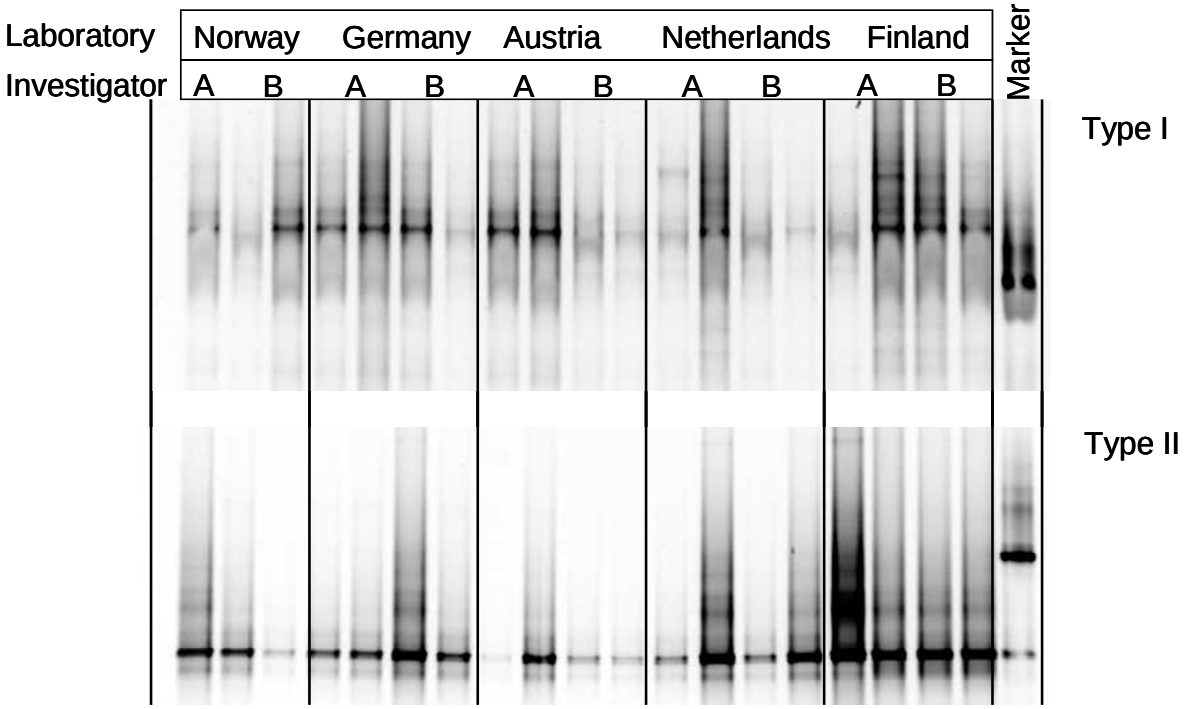
19 out of 21 probe were not changed (P < 0.01)	90,5	% were not changed

2 out of 21 probes were changed (P < 0.01)	9,5	% were changed

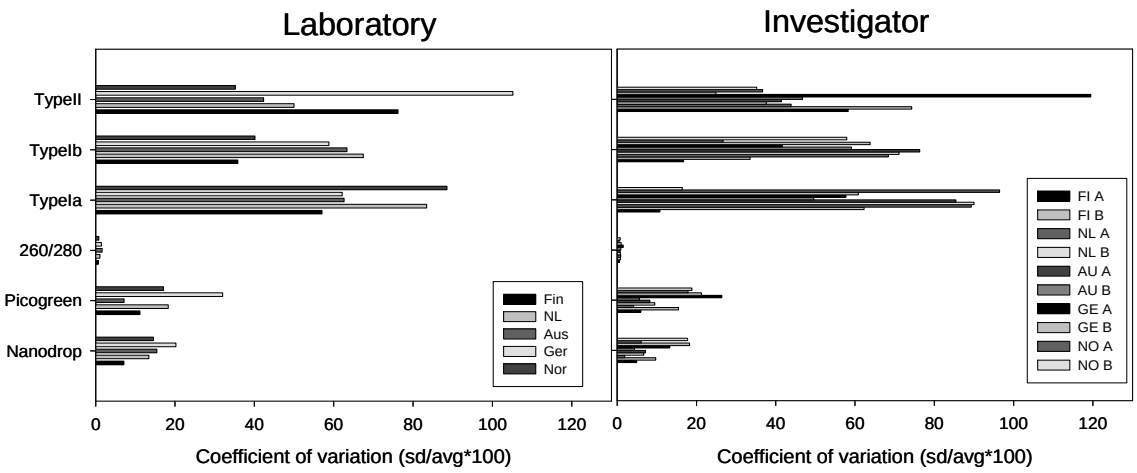
Probe changed in at least one out five countries:

All probes		
23 out of 56 probe were not changed (P < 0.01)	41,1	% were not changed
33 out of 56 probes were changed (P < 0.01)	58,9	% were changed
Type Ia probes		
6 out of 11 probe were not changed (P < 0.01)	54,5	% were not changed
5 out of 11 probes were changed (P < 0.01)	45,5	% were changed
Type Ib probes		
8 out of 15 probe were not changed (P < 0.01)	53,3	% were not changed
7 out of 15 probes were changed (P < 0.01)	46,7	% were changed
Type II probes		
7 out of 21 probe were not changed (P < 0.01)	33,3	% were not changed
14 out of 21 probes were changed (P < 0.01)	66,7	% were changed

Supplementary Figure 1



Supplementary Figure 2



Chapter 3

Convergent methanotroph communities develop in soils of differing life histories

Keywords: methanotroph / methane oxidation / *pmoA* / succession

Abstract

Little is understood about the relationship between microbial assembly history and ecosystem functions. To learn more about this relationship we used methane oxidizing bacteria as model organisms and performed a soil mesocosm experiment comprising four identical soil substrates, each hosting a distinct microbial and methanotroph diversity, i.e., full microbial diversity (control); reduced microbial and methanotroph diversity; and gamma sterilised soil. After inoculation with undisturbed soil, the recovery of methanotroph activity, communities and total microbial structures were followed over three months by methane oxidation potential measurements and analyses targeting *pmoA* and 16S rRNA genes. We found that methane oxidation potentials showed different recovery rates according to the various microbial community assemblies. Despite different starting microbial diversities, the recovery and succession of the methanotroph communities followed a similar pattern across the different treatment mesocosms. In this study we found that identical non-biological environmental conditions exerted an effect on the development and succession of methanotroph community structure strongly exceeding that of different microbial assembly histories.

Introduction

With the development and the application of the molecular techniques in microbial ecology, we can now identify bacterial community composition and obtain a better understanding on their functional diversity. However, members of complex microbial communities interact in various ways to contribute certain functions to their environment. Therefore, the relationship between microbial diversity and ecosystem function still remains largely uncharacterised (Torsvik and Øvreås, 2002).

As the only biogenic sink for methane in the aerobic ambient, methanotrophs are ubiquitous in nature. Due to their unique characteristics, methanotrophs have been often studied as model bacteria (Conrad and Donald, 2007). Aerobic methane oxidizing bacteria (methanotrophs) belong to the *Alpha-*, *Gammaprotebacteria* (Hanson and Hanson, 1996) or the *Verrucomicrobia* (Dunfield et al., 2007; Islam et al., 2008), recently discovered in extreme environments. Traditionally, gammaproteobacterial representatives or 'type I' methanotrophs, can be further divided into type Ia and type Ib according to their physiological, biochemical and phylogenetic characteristics. 'Type II' methanotrophs belong to *Alphaproteobacteria*. Both types oxidize methane via methanol and formaldehyde to carbon oxide or assimilate carbon to biomass. The first step in the pathway involves the enzyme methane monooxygenase (MMO), which is either a soluble methane monooxygenase (sMMO) or a membrane-bounded particulate methane monooxygenase (pMMO). Compared to the sMMO which can only be found in some methanotrophs, pMMO (encoded by *pmoA*) is present in all characterized methanotrophs except for *Methylocella* spp. The *pmoA* gene encodes a subunit of pMMO and is often used as a phylogenetic marker due to its congruence with the 16S rRNA-based phylogeny when studying methanotrophs (Holmes et al., 1995). Competition for space, competition for nutrients and prey-predator interactions play

important roles in shaping microbial community structures (Little et al., 2008). Two contradicting theories exist on the evolution of microbial communities (Chase, 2003). One theory states that environmental conditions shape microbial communities, whereby the microbial assembly history has little to no effect on the outcome. According to this theory, different microbial communities converge towards the same final composition if exposed to the same environmental conditions. The other theory states that microbial communities are primarily shaped by their assembly history, with the effect of the environment only having secondary effect. Results have been found supporting both of these contradicting theories (Chase, 2010; Caruso et al., 2011; Langenheder and Szekely, 2011).

The link between community assembly and ecosystem function remains largely unknown. Fukami and Morin (Fukami and Morin, 2003) observed that the relationship between productivity and biodiversity depends on the community assembly history. Cardinale (Cardinale, 2011) demonstrated that communities with higher diversity could take greater advantage of niche partitioning, resulting in a more diverse community able to capture a greater proportion of available nutrients. It has been also reported that bacterial community structures are unrelated to ecosystem functioning due to the presence of generalist species, which can cope with a wide range of environmental conditions (Langenheder et al., 2005).

Soil microbial communities are generally regarded as functionally redundant due to their vast diversity. Structural and functional redundancy relay a microbial community the ability to maintain its functional and structural characteristics in the face of potential environmental changes or perturbations. There are debates as to whether higher diversity results in more stable community functions. Studies have suggested that the greater microbial community evenness or diversity may lead to more functional stability (Degens et al., 2001; Wittebolle et al., 2009). However, some studies suggest that the source of perturbation also influences the

interaction between diversity and functional stability (Mohanty et al., 2006; Bressan et al., 2008).

The objective of the present study was to determine how different microbial assemblies determine the composition and succession of methanotroph communities. To exclude the influence of soil characteristics we built mesocosms containing the same soils but hosting microbial communities of different complexities. We observed methanotrophic bacteria as a functional model group over a period of three months. With this experiment our aim was to test the hypothesis that there is a significant interaction between the diversity of the total microbial community and the function of a selected microbial clade, the methanotrophs.

Materials and Methods

Sampling site

Soil samples were collected on the 22nd of October 2009 in the area Ewijkse Waarrd (51º88'N, 5º73'E), a river floodplain described in detail earlier (Kemnitz et al., 2004). The soil texture was silt-clay-loam, pH was 7.4, soil moisture content was 29.5 %. The soil was sieved (< 2 mm), air dried, sieved again (< 2 mm), and mixed thoroughly for further treatment.

Mesocosm experiment

Eighty kilograms of thoroughly homogenised dry soil was divided equally to four parts. One aliquot used as control was kept at 4 ^{o}C without any further treatment ('UN'). Another 20 kg aliquot was sterilized by gamma-irradiation (28 kGy; ^{60}Co) ('GAMMA'); the sterility of the soil was confirmed by lack of PCR amplification of the 16S rRNA gene from a DNA

extraction. Furthermore, no colony forming units were formed after plating water extract from 2 g soil on LB agar. The remaining 40 kg soil was treated by acetylene ('ACET'), and then divided into two 20 kg aliquots, one of which was stored at 4 ^{o}C until further use, the other one was also subjected to kanamycin treatment ('KAN'). For the acetylene treatment, 40 kg of dried and sieved soil was divided into 2 aliquots of 20 kg and placed each into a 20 l plastic bag with 5 l headspace. Each bag was flushed with 5 l acetylene (≥99.6 % pure, Linde Gas, Austria) and incubated for 2 days. After 2 days, soil aliquots were aerated for 8 hours, and then flushed with 5 l acetylene again. The same treatment was repeated twice. At the end of the treatment, the two 20 kg aliquots were pooled and thoroughly mixed. No methane oxidation was detected in the acetylene treated soil, even after allowing 7 days which for recovery and regrowth of surviving methanotrophs. For kanamycin treatment, 1 l of 10 mg/l kanamycin solution (10 mg kanamycin was dissolved in 1 ml DMSO, and then diluted into 1 l of H_2O) was added to the 20 kg dried soil aliquot by spraying and mixing, resulting in a final concentration of 0.5 mg kanamycin / g soil. Soil was incubated for 13 days at room temperature and the change in the microbial community composition was checked via bacterial 16S rRNA gene-based T-RFLP analysis as described below.

Mesocosms were built from two plastic boxes, each 60 cm x 45 cm x 25 cm in size. The top box contained the soil, while the lower part served as a reservoir for methane to diffuse into the soil through the perforation applied to the bottom of the top part. The bottom box (connected to the top box airtight) was flushed with a gas mixture of 20% (v/v) methane and 80% (v/v) N_2 at a gas flow rate of 70 ml/min. The methane concentration measured at the soil surface was around 1 % (v/v) throughout the experiment. Four mesocosms were constructed and filled with each of the 20 kg aliquots of soil (representing four different treatments). Mesocosms were kept in a temperature regulated room with natural lighting between 20 to 25

ºC. Approximately 200-250 ml water was sprayed onto each mesocosm every day to keep the moisture in the range of 35±3 % (w/w). The soil in the mesocosms was approximately 20 cm deep.

As soon as mesocosms were created and filled with treated soil, each mesocosm was inoculated by applying 1 kg untreated soil in a thin layer on top of the treated soil. At the start of the experiment, 10 g soil was sampled from each treatment (time point 0). For all subsequent sampling times, three cores (2.5 cm in diameter, 20 cm in length) were collected from each mesocosm. The soil cores were divided into 4 layers (5 cm each; layer A: 0-5 cm; layer B: 5-10 cm; layer C: 10-15 cm; layer D: 15-20 cm). Layers were pooled, homogenized, directly followed by DNA extraction and further analyses. Mesocosms were sampled 7 times, at days 0, 7, 26, 41, 67, 91 and 105 (corresponding to time points t0–t6).

Methane oxidation assay

The assay was performed as described earlier (Steenbergh et al., 2009) with modifications. Each assay was performed in triplicate. In short, for each incubation, 1 gram of soil was suspended in 10 ml MilliQ water (Millipore) in a 150 ml flask capped with a rubber stopper. Pure methane (1.4 ml) was added to the bottle. Slurries were incubated in the dark at room temperature on a shaker (100 rpm). The methane concentration in the headspace was measured by GC-FID analysis (Fisons HR 8060). Activity was calculated by considering results at 0 h and 24 h.

DNA extraction

DNA extraction was performed using a modification of a method based on the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA) (Yeates and Gillings, 1998). Soil (0.3 g) and 780 µl lysis buffer [200 mM NaPO4 pH 7.0; 1% CTAB; 1.5 M NaCl; 2% Polyvinyl-pyrrolidone K30; 5 mg ml-1 lysozyme (added right before use)] was added into a multimix FastPrep E tube and incubated at 37 ^{o}C for 30 min. MT buffer (122 µl) was added and tubes were shaken in the FastPrep instrument (MP, Biomedicals,LLC, Solon, OH, USA) for 30 s at 5.5 m s-1. Subsequently, samples were centrifuged for 20 min at 13000 rpm and 700 µl supernatant were collected. 5 µl of 10 mg ml-1 freshly made proteinase K was added to the tube. Tubes were incubated at 65 ^{o}C for 30 min. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by a chloroform-isoamyl alcohol (24:1) extraction. Supernatants were transferred to new tubes, 600 µl Binding Matrix was added and tubes were mixed for 5 min on a rotator. Binding Matrix, with bound DNA, was pelleted by 1 min centrifugation at 13000 rpm. Supernatants were discarded and pellets were resuspended in 500 µl wash buffer. The resulting suspensions were added to a spinfilter (supplied with the kit), and centrifuged for 1 min at 10000 rpm. The eluate was discarded and the pellet was washed again in 500 µl wash buffer. After discarding the second eluate, the spinfilter was centrifuged for another 10 s to dry the pellet. The filter was taken into a new tube and 50 µl of TE pH 8.0 was added. The filter was incubated at room temperature for 1 min and centrifuged for 1 min. The eluate collected in the catch tube contained the purified DNA which was then used for the analyses described below. DNA concentrations were estimated using a NanoDrop spectrophotometer (Thermo Scientific, DE, USA).

Diagnostic pmoA microarray analysis

PCR amplification of the *pmoA* gene was based on a two-step semi-nested protocol. The first step PCR comprised 25 μl of 2×Premix F (EPICENTRE Biotechnologies, USA), 25 pmol of primer A-189f and A-682r (Holmes et al., 1995) each, 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 50 ng of genomic DNA as template, in total volume of 50 μl. PCR was performed using a touchdown protocol with an initial incubation of 5 min at 94 ºC, followed by 25 cycles of 1 min at 94 ºC, 1 min at the annealing temperature and 1 min at 72 ºC, followed by a final incubation of 10 min at 72 ºC. The annealing temperature was lowered from 62 ºC to 52 ºC over the first 11 cycles after which it was maintained for further 24 cycles at 52 ºC. 5 μl of 1/100 diluted PCR product from the first step was used as template in a subsequent nested amplification with primers A-189f and T7-661r. The second step PCR was performed by total 25 cycles with an initial incubation of 5 min at 94 ºC, followed by 1 min at 94 ºC, 1 min at the annealing temperature and 1 min at 72 ºC, followed by a final incubation of 10 min at 72 ºC. The annealing temperature was lowered from 62 ºC to 52 ºC over the first 11 cycles after which it was maintained for further 14 cycles at 52 ºC.

In vitro transcription was carried under RNAse-free conditions, the procedure was as follows: 7 μl purified PCR product (50 ng μl^{-1}), 4 μl 5×T7 RNA polymerase buffer, 2 ml DTT(100 mM), 0.5 ml RNAsin (40 U μl^{-1}) (Promega, Madison, WI, USA), 1 μl each of ATP, CTP, GTP (10 mM), 0.5 ml UTP (10 mM), 1 μl T7 RNA polymerase (40 U ml^{-1}) (Invitrogen Carlsbad, CA, USA) and 1 μl Cy3-UTP(5 mM) (GE, Fairfield, USA) were added into a 1.5 ml tube and incubated at 37 ºC for 4 h. RNA was purified immediately based on the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA): 80 μl DEPC treated water were added to IVT mixture. Followed adding 350 μl RLT and 250 μl ethanol, mixed thoroughly. Samples were transferred to an RNeasy mini tube. Add 500 μl RPE, at 10000rpm centrifuged 15 sec. Add

another 500 µl RPE, at 10000 rpm centrifuged 2 min. Purified RNA was eluted into 50 µl dH_2O.

Purified RNA was fragmented by incubating with 9.5 mM $ZnCl_2$ and 24 mM Tris.Cl (pH7.4) at 60 ºC for 30 min. Fragmentation was stopped by the addition of 12 mM EDTA pH 8.0 to the reaction and putting it on ice. RNAsin (1 µl 40 U μl^{-1}) was added to the fragmented target.

Hybridization was carried out using a Belly Dancer (Stovall Life Science, Greensboro, NC, USA) equipped with a custom made aluminium block serving as dry, temperature controlled platform for the slides, preheated to 55 ºC for at least one hour. For each hybridization, 62 µl DEPC treated water, 1 µl 10% SDS, 30 µl 20 ×SSC, 2 µl 50 × Denhardt's reagent and 5 µl target RNA were added into a 1.5 ml tube and incubated at 65ºC for 1 min. Preheated hybridization mixture were applied onto assembled slides with the HybriWell (Grace BioLabs, Bend, OR, USA). Slides were also preheated on the aluminium block. Slides were hybridized overnight at 55 ºC in the BellyDancer, set at maximum bending and lowest rpm, providing slow mixing within the chamber by the air bubbles formed. Following the hybridization, slides were washed by shaking at room temperature for 5 min in 2×SSC, 0.1 % (w/v) SDS, twice for 5 min in 0.2×SSC and finally for 5 min in 0.1×SSC. Slides were dried individually using an air gun.

Hybridized slides were scanned at 10 µm resolution with a GenePix 4000 laser scanner (Axon, Foster City, CA, USA) at wavelengths of 532 nm. Florescent images analysed with the GenePix software (Axon). Microsoft Excel was used for statistical analysis and presentation of results.

Hybridization between a probe and a target was considered in analyses if the signal was at least 1.5% of the strongest signal obtained for that probe with the validation set of reference strains/clones (for details see (Bodrossy et al., 2003)).

pmoA-based quantitative PCR (qPCR) assay

qPCR assays were performed using a method based on (Kolb et al., 2003), carried out as shown in Table 1. Each assay was performed in duplicate. Plasmid DNA from cloned pmoA was used as to generate a standard curve of known copy number in the assays. qPCR was performed in 25 μl volumes using 96-well PCR plates (VWR GmbH, Vienna, Austria) with the optical cover (AB). PCR master mix contained 12.5 μl iQ SYBR Green Supermix (Bio-Rad), 1 μl 5ng/ul DNA template and 2.5 μl of each forward and reverse primers. DNase and RNase-free water were added to a final volume of 25 μl.

16S rRNA gene-based T-RFLP assay

Bacterial 16S rRNA genes were amplified with the FAM-labeled forward primer 8fM (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1406r (5'-ACGGGCG GTGTGT(AG)C-3') (Liu et al., 1997). Each 50 μl reaction mixture contained 25 μl 2×Premix F (EPICENTRE Biotechnologies, Madison, Wisconsin, USA), 20 pmoles of each primer, and 1 U Taq polymerase (Invitrogen, Carlsbad, California, USA), 50ng of genomic DNA as template. Cycling conditions were as follows: an initial denaturation of 5 min at 94 ^{o}C; followed by 35 cycles of 1 min of denaturation at 94 ^{o}C, 45 s at 55 ^{o}C, 1 min at 72 ^{o}C; and a final extension at 72 ^{o}C for 10 min.

Four independent PCRs were performed for each sample, and PCR products were combined and purified with a Qiaquick PCR cleanup kit (Qiagen Inc., Chatsworth, CA, USA). PCR products (150 ng) were digested by mixing with 10 U of *Rsa*I enzyme (Invitrogen, Carlsbad, California, USA) and 1 µl Tango buffer filled up to 20 µl with PCR grade water and incubated at 37 ^{o}C for 4 h. The enzyme was inactivated at 65∞C for 20 min. Purification was performed using Sephadex G-25 (GE, Fairfield, USA) columns. Five µl of each purified sample was mixed with 0.5 µl of DNA fragment length standard (MapMarker 1000, Eurogentec, Seraing, Belgium) and 14.5 µl Hi-Di formamide (Applied Biosystems, Foster City, CA, USA). Samples were denatured for 2 min at 94 ^{o}C and T-RFLP analysis was carried out using the GeneScan ABIPrism 310 (Applied Biosystems, Foster City, CA, USA). Peaks derived from primer dimers were excluded from analysis after cross-checking with a negative control. Analysis of T-RFLP profiles were based on peak heights.

Statistical analysis

T-RFLP profiles were binned using the macro Treeflap in Microsoft Excel (http://www.sci.monash.edu.au/wsc/ staff/walsh/treeflap.xls). T-RF standardization was performed as described in (Dunbar et al., 2001). T-RFLP patterns were regarded as total bacterial community profiles. Methanotroph community (derived from microarray analyses) and total microbial community structures were analysed using Permanova, PERMDISP and Canonical analysis of principal coordinates (CAP) analysis, using the Primer PERMANOVA+ software package (Primer-E, Plymouth, UK). The correlation between methanotroph communities and methane oxidation potentials were analysed in R v. 2.12.2 (R Development Core Team, 2011) using the vegan v. 1.17-8 package.

Results

Analysis of treatments

Soil used in the mesocosms was subject to four different treatments, UN, ACET, KAN and GAMMA representing untreated (control), acetylene treatment, acetylene and kanamycin treatment and gamma irradiation treatment, respectively. Prior to the specific treatments, soil was sieved, dried and sieved again (While this treatment itself introduced considerable perturbation to the microbial community, it was necessary to achieve a homogenous, reproducible experimental system). Following acetylene treatment, no methane oxidation was detected after 6 days of incubation, which would have allowed for the recovery of any reversibly inactivated methanotrophs under a constant methane stream. Thirteen days following kanamycin treatment, the number of TRFs detected was reduced to 18±3 compared to 26±2 detected in the untreated soil. The number of cultivable colonies on LB agar plates was reduced from 195±25 cfu/g soil to 75±25 cfu/g soil. Plating of soil treated by gamma-irradiation on LB agar plates and incubating at 30 ºC for 48 hrs revealed no colonies.

Methane oxidation potential

Methane oxidation (MO_x) potentials recovered at different rates in the four different treatment mesocosms (Fig.1). MO_x potentials varied between the different soil layers in each of the treatment mesocosms. The maximum MO_x potential was detected in the upper soil layers (0-10 cm, layers A and B) in all treatments over the first 41 days with the exception of the untreated (control) samples. It has to be noted that the top 5 cm layer (layer A) included an approximately 0.3 cm inoculum of untreated (control) soil in all four treatment mesocosms. In the second half of the experiment (from 67 days onwards), the bottom layers

(D), closest to the CH_4 source, showed the highest MO_x potentials in all treatments. MO_x potentials in treatments ACET and KAN reached almost the same level as the untreated samples by day 26, whilst MO_x potentials in the GAMMA treatment matched the untreated samples after 67 days.

Methanotroph abundance

Abundance of methanotrophs was inferred from pmoA copy numbers as assessed by qPCR analysis and indicated that type II MOB dominated most of the methanotroph communities in all treatment mesocosms (mean value of 8.7×10^3 – 8.0×10^9 g $^{-1}$ wet soil) (Fig. 2-4). The mean abundance of type Ia MOB was 8.7×10^3 – 1.2×10^9 pmoA copies g $^{-1}$ wet soil. Abundance of type Ib MOB were lower (mean abundance ranged from 3.0×10^3 – 2.6×10^8 copies g $^{-1}$ wet soil). Type Ia MOB increased by one to two orders of magnitude by day 7 in all layers across all four mesocosms. The abundance of both, type Ib and type II MOB, increased by day 7, but at a lower and more variable rate. The ratios between the abundances of the three major clades of methanotrophs (Type Ia, Type Ib and Type II) varied greatly. In particular, at day 41, the MOB ratio between II:Ib was the highest (193 - 14164) during the time course of the experiment. At this time point type II MOB reached the highest abundance and type Ib MOB reached the lowest abundance, whilst MO_x potentials were the lowest in the treatments UN, ACET and KAN.

Methanotroph community structure

Microarray analysis was carried out using a semi-nested PCR approach (Abell et al., 2009), effectively excluding ammonia oxidisers and deep branching novel/unknown *pmoA*/*amoA*

sequences from the analysis (Supplementary Figure S1). Microarray results demonstrated positive signals for probes targeting both type I (type Ia and Ib) and type II MOB (Fig. 5) in all treatments. Strongest signals were associated with probes targeting type II MOB, which indicated that methanotroph communities were dominated by *Methylocystis* related phylotypes (probes Mcy459, Mcy264, Mcy270, Mcy413, Mcy255, McyB304, Mcy522, and Mcy233) and a novel, deep branching, uncultivated clade of type II methanotrophs (probe ARC2-518) (Fig. 5). The signals of the probes targeting *Methylocystis* were somewhat lower and varied considerably at day 0 and 7 compared to all of the later sampling time points. Signals for probes targeting type II peat clones (probe peat264) and different clades of *Methylosinus* (probes MsiS475, Msi263, MsiS314, MsiT214, Msi294) indicated that these groups were not detectable in the original MOB community, but showed positive signals in all different treatments at different time points and layers (Fig. 5). The signals of probes targeting type Ia methanotrophs were generally weaker than type II (*Methylocystis*) probes, indicating that this group of MOB was less abundant. Probes Mb380, Mb271, Mb_SL#3-300, LF1a-456, Mm229, Mmb562 and MsQ290 targeted *pmoA* from *Methylobacter*, *Methylomonas* and *Methylomicrobium/Methylosarcina*, respectively and were positive in most samples throughout the experiment. LP10-424, which targets a deep branch of *Methylobacter*, was positive at day 0, but was not detected in any of the subsequent samples. Probes LF1a-456, Mmb303, Mmb304 targeting a subclade of *Methylobacter* and *Methylomicrobium*, respectively, were positive at different time points in all treatments but not in the initial starting community (Fig. 5).

The methanotroph community in the GAMMA treatment showed more temporal variation than the other treatments. This was indicated by the appearance and disappearance of strong signals for different type Ia methanotrophs (probes BB51-299, Mb267, Mb292; Mmb303,

Mmb304) and by the disappearance of signals for *Methylocystis* clade B from all the deeper layers (probes McyB304 and Mcy255).

PERMANOVA and canonical analysis of principal coordinates (CAP) analysis demonstrated a significant effect of time on methanotroph community structure (Fig. 6, correction is 82%). PERMANOVA analysis showed a layer effect on methanotroph community structure when regarding the layer nested within the treatment factor, but no treatments effect when regarding time and layer factors nested within treatment factor (Table 2). Probes fw1-641, fw1-639, NMsiT-271, LW21-374, ARC2-518, NMsi1-469, SWI1-377, LP20-644, Mcy522, Mcy264, Mcy413 distinguished different sampling times (Pearson correlation greater than 0.6). Mcy522, Mcy264, Mcy413, NMsiT-271 and ARC2-518 had weaker signals at day 0 and day 7 in all treatments. The signal of probe fw1-641 was weaker at day 7 in the UN, KAN and GAMMA treatment, at day 91 in the KAN and GAMMA, and there was no positive signal in the treatment D at day 0. The signals of probe fw1-639 were stronger in all treatments at day 26, 41, 67 and day 105. Probe LW21-374 signals were stronger at day 41, 67 and 105 in all four mesocosms. Probe signals of SWI1-377 were weaker in control soil at day 7 and day 67, were weaker in treatment ACET soil at day 7, 41 and day 67, in treatment C soil were weaker at day 7 and day 67, in treatment D soil were weaker at day 0, 7, 26 and day 41. LP20-644 signals were stronger in UN, KAN and GAMMA treatment at day 41 and day 105, whereas in treatment ACET the signals were only stronger at day 41.

Total microbial community structure

From several samples 16S rRNA gene PCR products were not obtained after repeated attempts including samples from GAMMA at day 0 and day 41, from ACET at day 41 and

from UN at day 7. While many T-RF peaks were present in all four treatments, several unique peaks were found in various treatment (Supplementary Table 2.). In particular, the GAMMA treatment showed a large number of unique peaks.

PERMANOVA analysis demonstrated significant treatment, time and layer effects on the whole microbial community structures when time and layer factors were nested within treatment (Table 3). CAP analysis (Fig. 7) also showed a clear time effect on total microbial community structure. PERMDISP analysis on the total microbial community structures showed that in the GAMMA treatment the mean Bray-Curtis distance of the individual samples to the centroid was the largest amongst treatments (49.4 in treatment GAMMA versus 30.3, 31.8 and 34.1 in treatments UN, ACET and KAN, respectively). No relationship was found between total microbial community and methanotroph community structure.

Relationship between microbial communities and MO_x potentials

No significant correlation was found between $MO_{\underline{x}}$ potentials and MOB or whole bacterial community composition in the control treatment. In treatment KAN, MO_x was correlated with MOB diversity but not whole bacterial community diversity. In the treatments ACET and GAMMA MO_x correlated with MOB diversity composition as well as with whole microbial community composition (Table 4). We found positive relationships between the abundances of type Ia and type Ib methanotrophs and MO_x potentials. No correlation was found between the abundance of type II methanotrophs and MO_x potentials (Fig. 8).

Discussion

In this study we investigated the interaction between the total soil bacterial community and a well defined functional group, methane oxidizing bacteria. In particular, we wanted to understand how reduced total microbial diversity influences the recovery of function (i.e. methane oxidation) and the succession of the methanotrophic bacteria. We selected a river floodplain site, which hosts a diverse methane oxidizing community, encompassing a range of type Ia, type Ib and type II methanotrophs (Bodelier et al., unpublished results). The four different treatments applied aimed at generating soils with highly similar chemical and physical properties but different microbial numbers and total microbial diversities.

Treatment with acetylene was made to irreversibly inactivate methanotrophs, while leaving the vast majority of the total microbial community intact. The lack of any methane oxidation in samples treated with acetylene following 6 days incubation with methane in a shaken slurry confirmed that methanotrophs were irreversibly inactivated. This was further confirmed by the lack of methane oxidation at day 0 of the mesocosm experiment (prior to inoculation with untreated soil). Previous results suggested that acetylene treatment may inactivate methanotrophs in a reversible way (Bodelier and Frenzel, 1999). Therefore we applied three cycles of acetylene treatment and thorough aeration, allowing for proper interaction of all methane monooxygenase enzymes with acetylene and oxygen. Further reduction of the microbial community was achieved by treatment with kanamycin, whereas gamma radiation resulted in complete eradication of detectable microbial life. Although the latter was confirmed by PCR analysis of 16S rRNA genes as well as by plating, it cannot be excluded, that DNA derived from dead cells may have contributed to a minor extent to results obtained from DNA-based analysis.

Methane oxidation potential in the control mesocosm was low (0.06 mol CH_4 g^{-1}WWsoil h^{-1}) at day 0 but increased to 0.4±0.04 μmol CH_4 g^{-1} WWsoil h^{-1} in all four layers by day 7, indicating a fast recovery of methane oxidizing bacteria following the perturbation caused by sieving and drying. In both soils treated with acetylene (treatment ACET and KAN) the rate of recovery was slower compared to the control treatment. From day 7 to day 26 methanotroph activity in the upper two layers recovered faster than the lower two layers, indicating re-colonisation by methanotroph communities from the upper inoculation layer. In the gamma- irradiated mesocosm MO_x potentials were detected only in the upper layer until day 41. This mesocosm showed slower MO_x recovery compared to all other soils with higher initial microbial diversities. However, following a slower initial recovery in all four layers, the MO_x potential in this treatment increased by day 67 beyond that detected in the other three treatments. We hypothesize that this reflects a reduced competitive exclusion (due to gamma irradiation) leading to an improved adaptation of methanotrophs to substantially altered environmental conditions. When comparing methane oxidation potentials of top and bottom layers, they were roughly equal or higher in the top layers up to day 41. From day 67 on, methane oxidation potentials migrated towards the bottom layers.

Microarray as well as qPCR results indicated that type II methane oxidizing bacteria were dominant across all time points and treatments. Type II MOB copy numbers were initially higher compared to type I (type Ia and type Ib) methanotrophs, however, they did not increase substantially over time, suggesting that methane may not be the main driving factor in their proliferation. Type I methanotroph numbers increased with time and their abundances were significantly related to the methane oxidation potential suggesting that at least a substantial part of the increased MO_x potential was due to proliferation of type Ia methanotrophs. This rapid increase in type I copy numbers following a low initial abundance

is in agreement with reports describing type I methanotrophs as r strategists (Steenbergh et al., 2009).

The genus *Methylocystis* is a highly abundant member of the methanotroph community in most soil, often showing little or no response in numbers due to changes in environmental conditions (Eller and Frenzel, 2001; Gebert et al., 2009). Here, we report distinct responses of the two main clades (clade A and B) of *Methylocystis* due to changes. Two probes, McyB304 and Mcy255, which target *Methylocystis* clade B were less dominant in the bottom layers in all four treatments. The rest of the *Methylocystis* probes (targeting either *Methylocystis* clade A or the entire genus) did not show such a pattern. The bottom layers were exposed to a higher concentration of methane compared to the upper layers due to their closer proximity to the methane source, suggesting that methane and/or oxygen availability played an important role in differentiating the ecological niches of the two clades of *Methylocystis*. The same clade was also remarkably less abundant and in many cases not detected in the gamma-irradiated soil. Abell *et al.* (Abell et al., 2009) also found that in an Alpine meadow soil the presence and relative abundance of *Methylocystis* clade B was more variable compared to *Methylocystis* clade A. In an experiment simulating disturbance-induced mortality reducing methanotroph and total microbial community, Ho and co-workers (Ho et al., 2011) demonstrated a rapid increase in type II abundance in the range of three orders of magnitude, and a temporal shift in the methanotroph community from type I to type II methanotrophs. We did not observe the same strong increase in type II abundance. In contrast to our experimental setup, Ho *et al.* applied homogenous inoculation mixing sterile and untreated (inoculums) soils and kept microcosms in Petri dishes within anaerobic jars. We hypothesize that the contrasting results are due to the difference in the experimental conditions and that the competitive advantage of type I vs. type II methanotrophs is defined by a complex

interaction of environmental conditions, some of which are hidden behind these differences in the experimental setup.

Type Ib methanotrophs are typically isolated from and detected in mud/sediment samples (Siljanen et al., 2011). While preliminary analyses indicated a high abundance and diversity of type Ib methanotrophs in the floodplain used in this study, they were detected only in low relative abundance in this experiment. The samples were tested after 3 days of sieving and air drying at which point the microarray analysis indicated the presence of *Methylocaldum* with strong signals for the probes targeting this group (MclS402, MclS394, MclS400, Mcl404 and Mcl408), however, after 13 days of drying and sieving (before establishing the mesocosms) this group was no longer detected (data not shown). It should be noted that the detection sensitivity of the microarray is limited at approximately 5% relative abundance. In practical terms, the absence of positive signal for type Ib methanotrophs does not indicate the total absence of this group, only that their relative abundance was below 5% within the total methanotroph community. We hypothesize that this decrease in type Ib methanotroph abundance and the lack of their full recovery indicated a high sensitivity of type Ib methanotrophs to severe drought and/or aeration (which was experienced by the entire community during drying and sieving of the soil).

We found a similar succession in methane oxidizing communities in all four mesocosms despite the strong differences in the initial total microbial communities. In regard to methanotroph communities no significant differences between treatments were found. This may at least in part be due to the selective pressure applied. The constant, relatively high methane flow has apparently had a stronger influence on the methanotroph community than the different treatments and their indirect effects via the differences introduced into the total microbial communities. In contrast, succession of total microbial communities under the

same conditions resulted in different community structures. The soil, which was sterilised by gamma-irradiation, developed a more diverse and more disperse total microbial community (shown by PERMDISP analysis). PERMDISP as a measure of beta diversity (Anderson et al., 2006) suggested that higher beta diversity observed in gamma treatment was also associated with higher methane oxidation potential. This is also consistent with the often observed positive correlation between productivity and beta diversity (Chase and Leibold, 2002; Chase, 2010; Langenheder et al., 2011). Furthermore, the presence of many unique T-RFs indicates the presence of bacteria or bacterial groups, which were able to manifest under these conditions but probably were out-competed in other treatments. We hypothesize that the observed higher methane oxidation potentials and methanotroph abundance are due to a reduced competition with other microbes for shared resources. In this (gamma irradiation) treatment, the initial eradication of all microbial life generated an open ecological space enabling the development of a different total microbial community suiting the new environmental conditions with methane being a major carbon source.

Acknowledgements

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Table 1. Primer combinations for qPCR assays used in this study (Kolb et al., 2003).

Target	**Primers**	**Sequences**	**Conc. (pM)**	**PCR thermal profile ***
Type Ia	A189f	5'-GGNGACTGGGACTTCTGG	500	94ºC, 25s; 54ºC, 20s; 72ºC, 45s; 80ºC, 15s
	Mb601r	5′-ACRTAGTGGTAACCTTGYAA	500	
Type Ib	A189f	5'-GGNGACTGGGACTTCTGG	750	94ºC, 25s; 66ºC, 20s; 72ºC, 45s; 84ºC, 15s
	Mc468r	5'-GCSGTGAACAGGTAGCTGCC	750	
Type II	II223f	5'-CGTCGTATGTGGCCGAC	625	94ºC, 25s; 66ºC, 20s; 72ºC, 45s; 84ºC, 15s
	II446r	5'-CGTGCCGCGCTCGACCATGYG	625	

*Thermal profile showing temperature and time for denaturation; annealing; elongation and data acquisition. Deviations from the protocol published by Kolb and co-workers are underlined.

Table 2. PERMANOVA test on the microarray data standardisation (square root). Universal and control probes were excluded from the analysis. Only probes with a signal above 1.5% of reference value at least in one sample were kept. Treatments were regarded as fixed factors. Time and layer factors were nested in treatment factors. Number of permutations was 999. The permutation method uses unrestricted permutation of raw data. Sum of squares type was type III (partial). Monte Carlo test was applied in this test. "Tr" refers to treatment. "Ti" refers to time and "La" refers to layer. Significant effects ($p<0.05$) are shaded.

Source	Pseudo-F	CV (√)	P
Tr	1.65	12.28	0.014
Ti (Tr)	6.27	30.85	0.001
La (Tr)	2.30	11.98	0.011

Table 3. PERMANOVA test on T-RFLP data after standardisation (square root). Treatments were regarded as fixed factors. Time and layer factors were nested in treatment factors. Number of permutations was 999. The permutation method uses unrestricted permutation of raw data. Sum of squares type was type III (partial). Monte Carlo test was applied in this test. Significant effects ($p<0.05$) are shaded.

Source	Pseudo-F	CV (√)	P
Tr	1.20	4.00	0.267
Ti (Tr)	7.19	19.91	0.001
La (Tr)	1.53	4.45	0.011

Table 4. Pearson correlation between methane oxidation potential and T-RFLP profiles after standardisation and microarray data after removal of values for universal and control probes. Only signals larger than 1.5% of the reference values in different treatments were kept.

Treatment	Total microbial community ordination (T-RFLP)	Methanotroph community ordination (microarray)
Untreated	-	-
Acetylene	+++	+++
Acetylene+Kanamycin	-	+
Gamma irradiation	+++	+++

+++ indicates p<= 0.001, ++indicates p<= 0.01, +indicates p< = 0.05, - indicates no significant relation within the factor.

Figure legends

Fig. 1. Methane oxidation (MOx) potentials measured in different layers and at different time points. Fig. 1 A, B, C and D represent methane oxidation potentials in the treatments UN, ACET, KAN and GAMMA, respectively. "Days" represent time since the establishment and inoculation of mesocosms. Error bars represent standard error between replicates.

Fig. 2. *pmoA* gene copy numbers of type Ia methanotrophs at different time points and in different layers. Fig. 2A, B, C and D refer to treatments UN, ACET, KAN and GAMMA, respectively. "UN", "ACET", "KAN" and "GAMMA" represent treatment A, B ,C and D soils at day 0. "Days" represents the time of soil sampling in mesocosms.

Fig. 3. *pmoA* gene copy numbers of type Ib methanotrophs at different time points and in different layers. Fig. 2A, B, C and D refer to treatments UN, ACET, KAN and GAMMA, respectively. "UN", "ACET", "KAN" and "GAMMA" represent treatment A, B, C and D soils at day 0. "Days" represents the time of soil sampling in mesocosms.

Fig. 4. *pmoA* gene copy numbers of type II methanotrophs at different time points and in different layers. Fig. 2A, B, C and D refer to treatments UN, ACET, KAN and GAMMA, respectively. "UN", "ACET", "KAN" and "GAMMA" represent treatment A, B ,C and D soils at day 0. "Days" represents the time of soil sampling in mesocosms.

Fig. 5. Microarray analysis of the methanotroph community composition. t0-t7 denote sampling dates (t0: day 0; t1: day 7; t2: day 26; t3: day 41; t4: day 71; t5: day 91; t6: day 105). Results for t0 represent the community in the homogenised soil used to fill the mesocosms. Results for t0-t6 represent four layers of 5 cm depth each (top layer shown on top of corresponding section of the figure). Results of individual microarray experiments were first normalized to the positive universal methanotroph control probe mtrof173, then to the reference values determined for each individual probe (Bodrossy et al 2003). Results are represented as a heatmap; a value of 1.0 (see colour code bar) indicates maximum achievable signal for an individual probe, whereas a value of 0.1 indicates 10 % of that. Only probes with signals higher than 1.5% of their reference values are shown. Numbers above probe names correspond to numbers in Supplementary table S1.

Fig. 6. Canonical analysis of principal coordinates (CAP) analysis of *pmoA* microarray data performed using the Primer 6 package (Primer-E, UK) on standardized array data excluding universal and control probes, as well as probes never showing signals higher than 1.5 % of reference values. Microarray probes are regarded as variables. Probes shown on the graph indicate the correlation is larger than 0.6 using Pearson correlation. The graph highlights the difference in MOB community structure between 7 different time points. Leave one out Cross Validation (Anderson et al., 2008) gave 81% correct assignment to time groups.

Fig. 7. Canonical analysis of principal coordinates (CAP) analysis of total microbial community composition performed using the Primer 6 package (Primer-E, UK) on standardized T-RFLP data. T-RFLP peaks are regarded as variables, highlighted number

indicate the correlation is larger than 0.6 using person correlation test. Highlighting the difference in total microbial community structure between 7 different time points Leave one out Cross Validation (Anderson et al., 2008) gave 95 % correct assignment to time groups.

Fig. 8. Linear regression between methane oxidation potentials and methanotroph abundance. Figure A refers a positive correlation between methane oxidation potential and log Type Ia *pmoA* gene copies. (Slope=0.06, R^2= 0.28). Figure B refers a positive correlation between methane oxidation potential and log Type Ib *pmoA* gene copies (Slope=1.22, R^2= 0.04). Figure C does not refer a correlation between methane oxidation potential and log Type II *pmoA* gene copies (Slope=-1.57, R^2= 0.03).

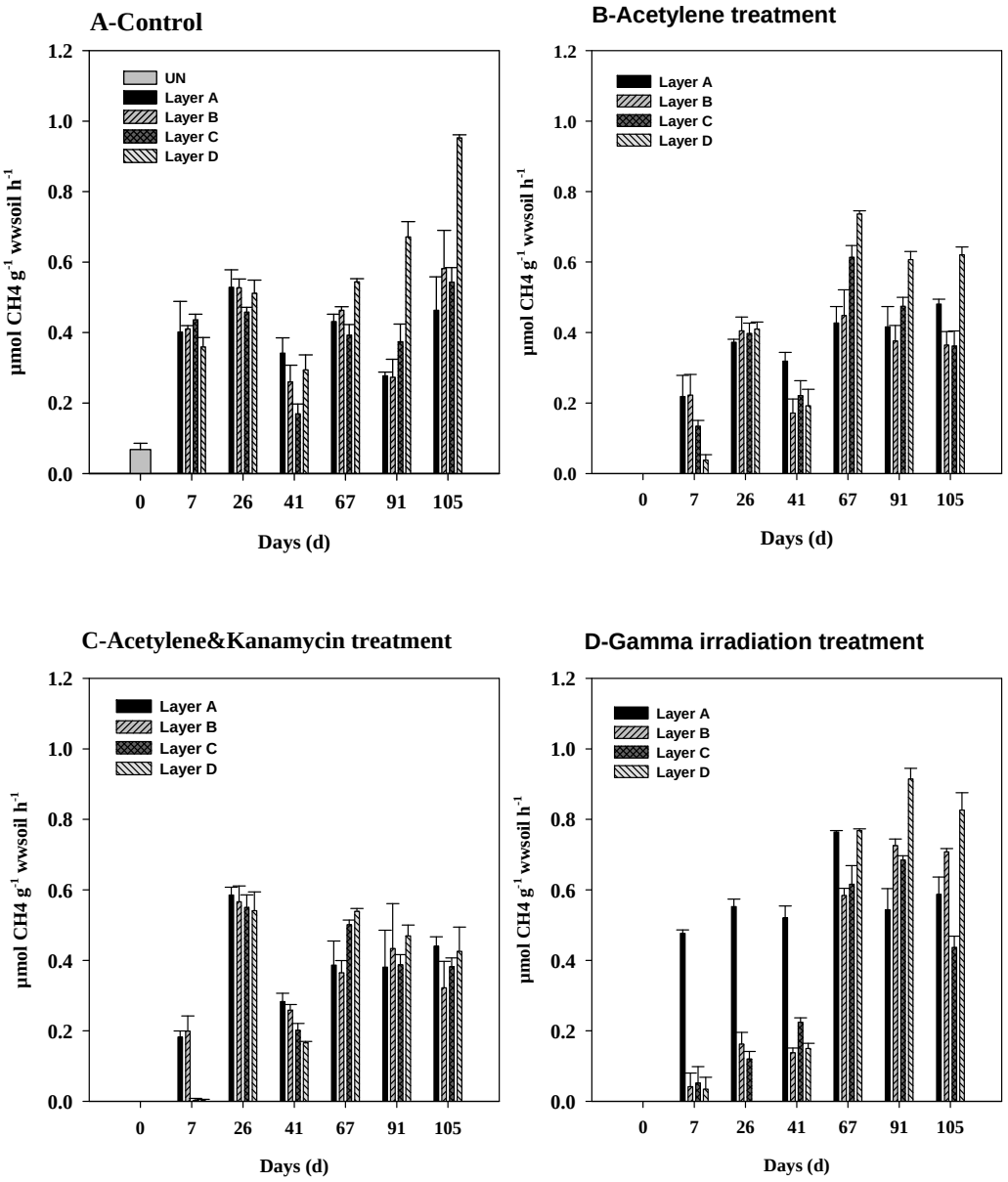

A-Control
B-Acetylene treatment
C-Acetylene&Kanamycin treatment
D-Gamma irradiation treatment
UN
Layer A
Layer B
Layer C
Layer D
µmol CH4 g-1 wwsoil h-1
Days (d)
0
7
26
41
67
91
105
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Fig. 1

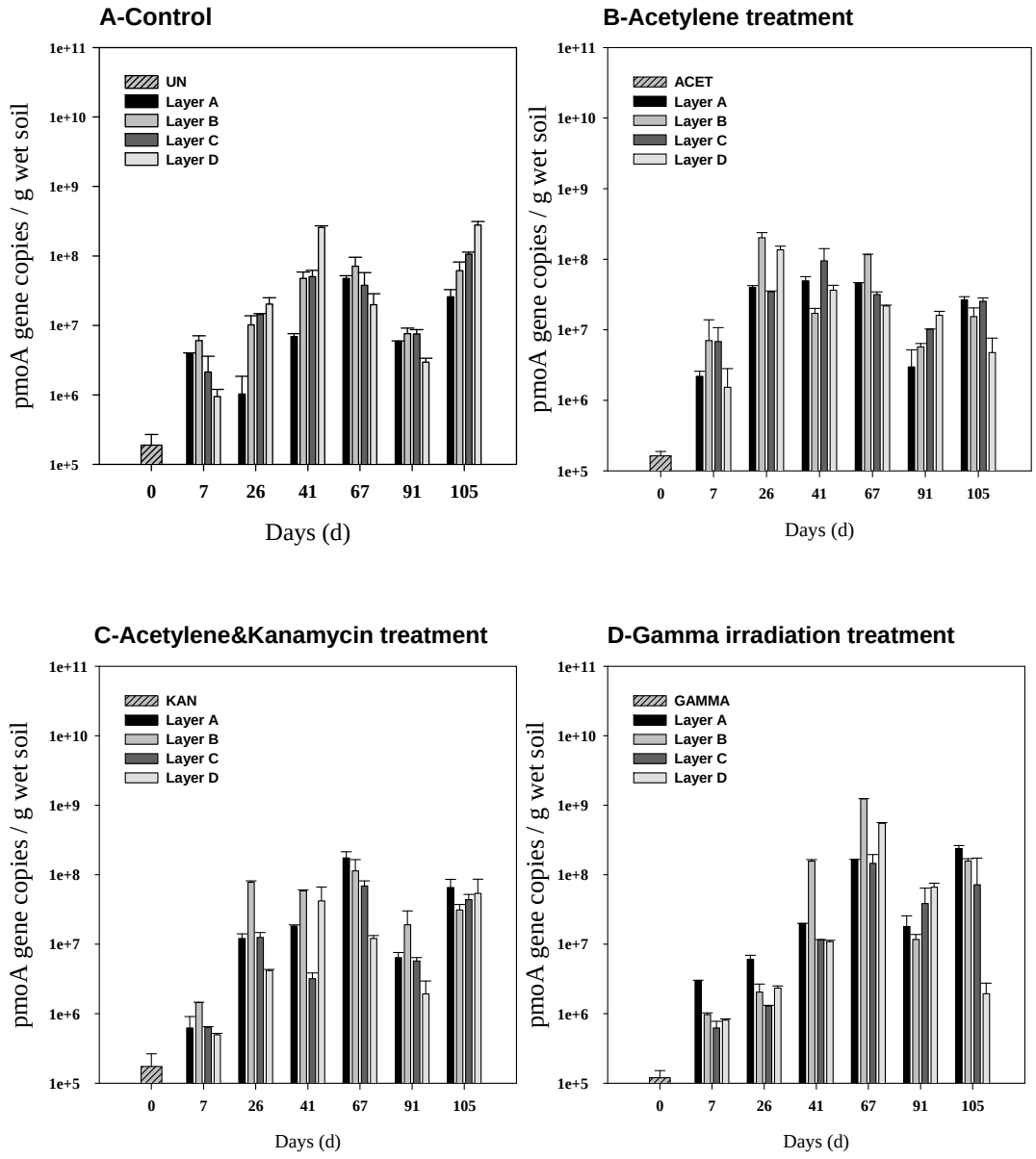

A-Control
B-Acetylene treatment
C-Acetylene&Kanamycin treatment
D-Gamma irradiation treatment
UN
ACET
KAN
GAMMA
Layer A
Layer B
Layer C
Layer D
pmoA gene copies / g wet soil
Days (d)
0
7
26
41
67
91
105
1e+5
1e+6
1e+7
1e+8
1e+9
1e+10
1e+11

Fig. 2

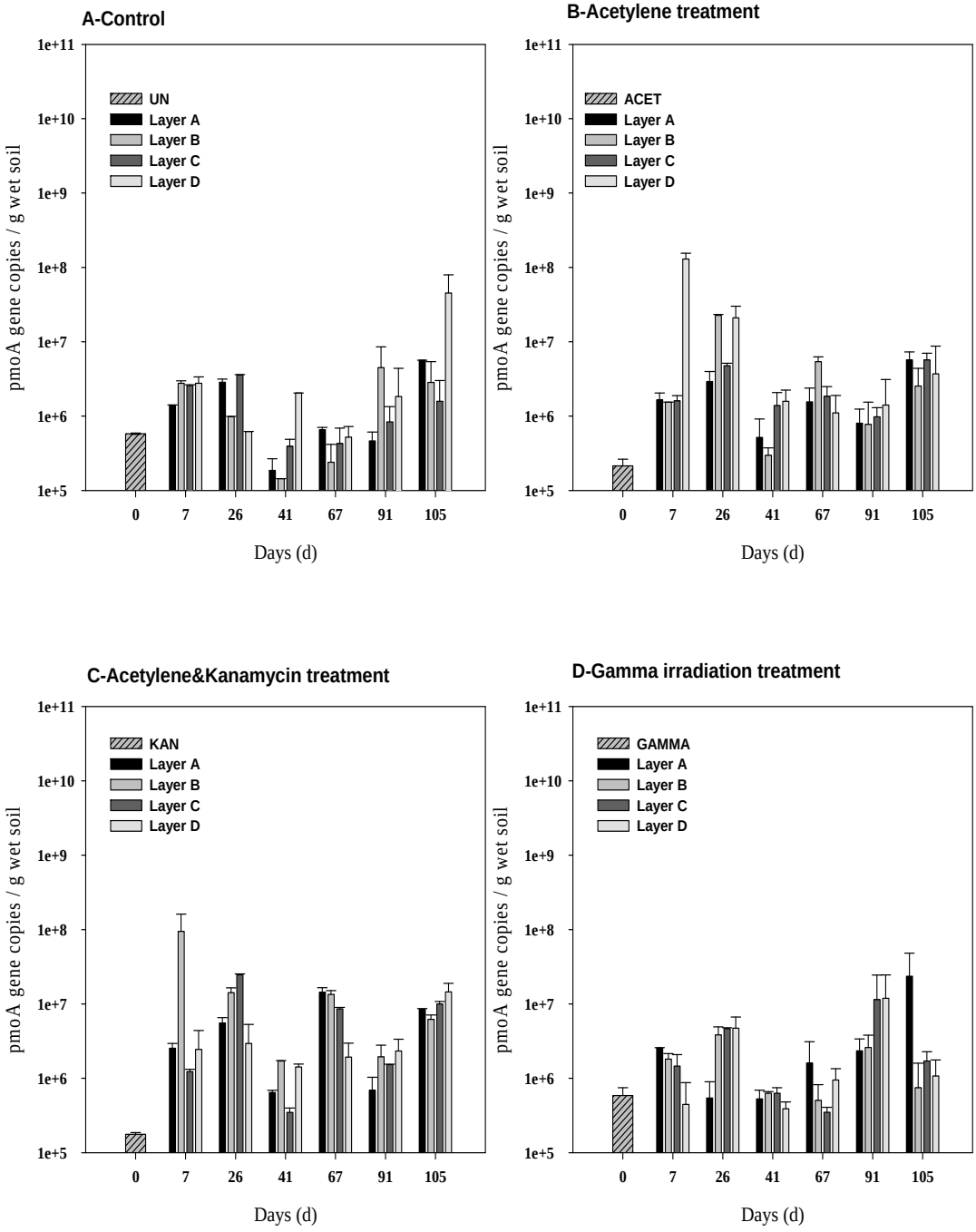

A-Control
B-Acetylene treatment
C-Acetylene&Kanamycin treatment
D-Gamma irradiation treatment
UN
ACET
KAN
GAMMA
Layer A
Layer B
Layer C
Layer D
pmoA gene copies / g wet soil
1e+11
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
0
7
26
41
67
91
105
Days (d)

Fig. 3

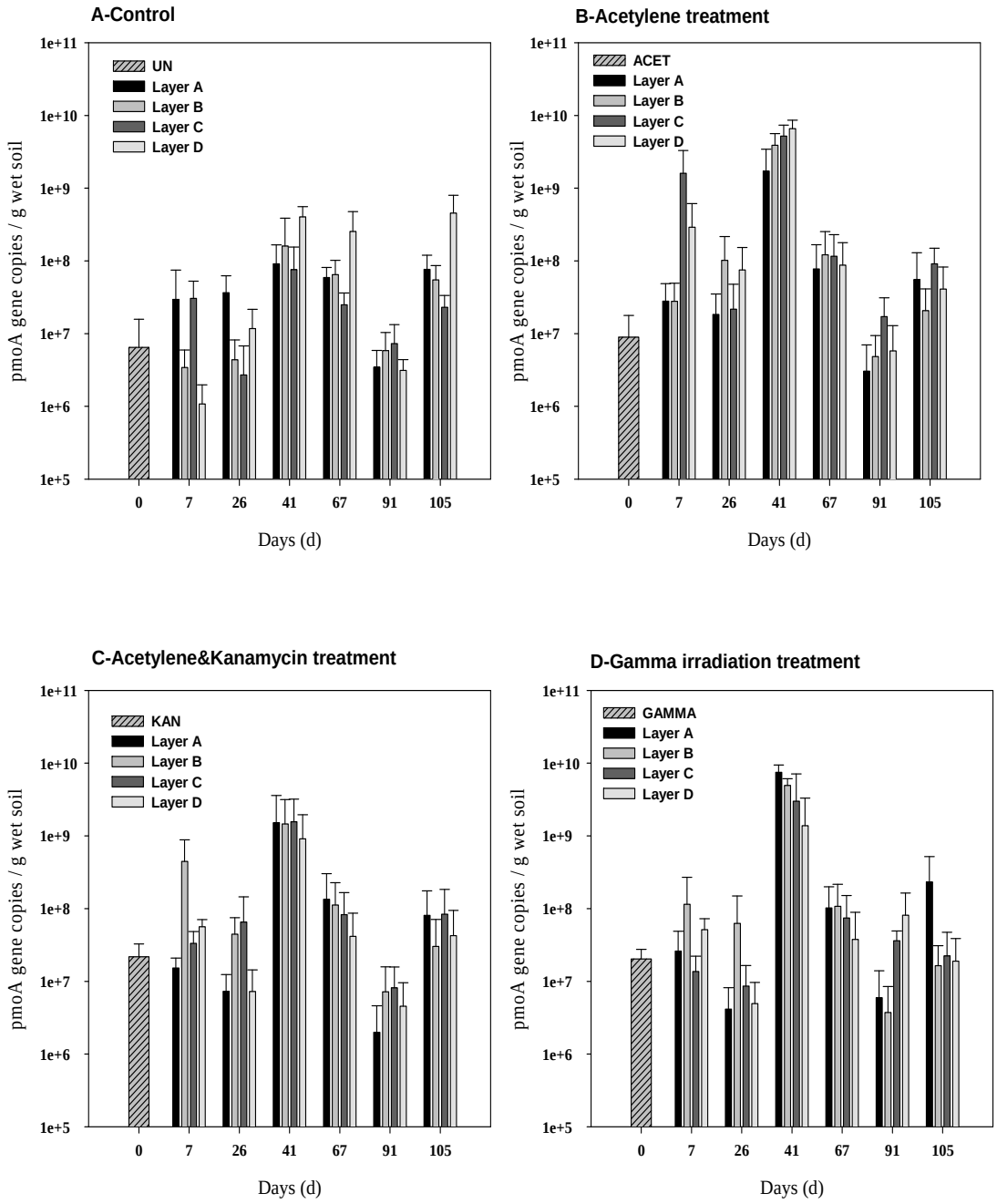
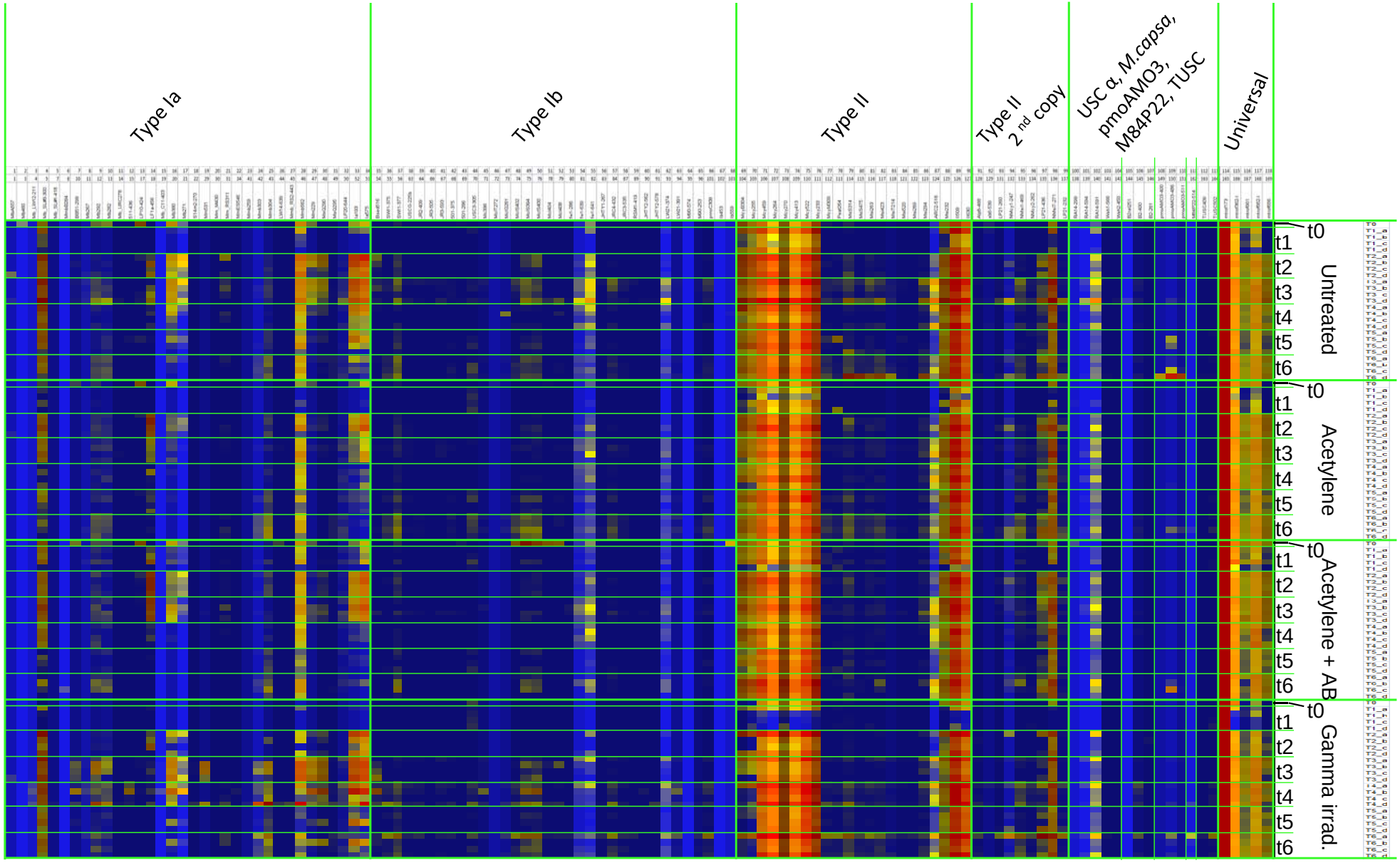

A-Control
UN
Layer A
Layer B
Layer C
Layer D
pmoA gene copies / g wet soil
1e+11
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
0
7
26
41
67
91
105
Days (d)
B-Acetylene treatment
ACET
Layer A
Layer B
Layer C
Layer D
C-Acetylene&Kanamycin treatment
KAN
D-Gamma irradiation treatment
GAMMA

Fig. 4


Type Ia
Type Ib
Type II
Type II
2 nd copy
USC α, M.capsa,
pmoAMO3,
M84P22, TUSC
Universal
t0
t1
t2
t3
t4
t5
t6
Untreated
Acetylene
Acetylene + AB
Gamma irrad.

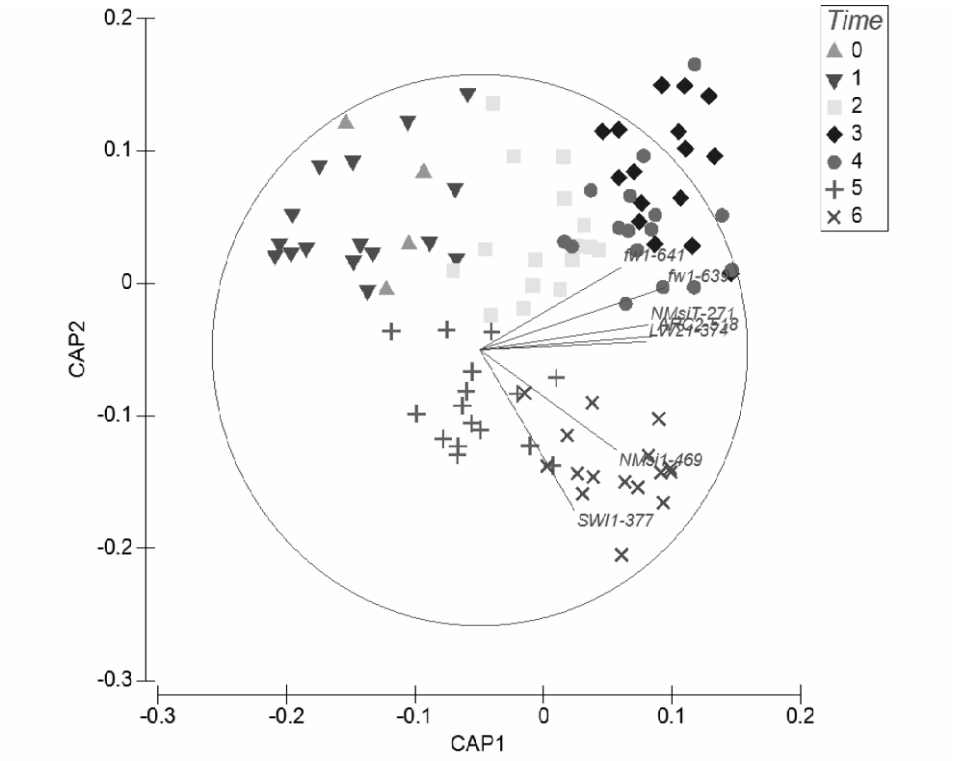

Time
0
1
2
3
4
5
6
fw1-641
fw1-635
NMsiT-271
LW21-374
NMsi1-469
SWI1-377
CAP1
CAP2
0.2
0.1
0
-0.1
-0.2
-0.3

Fig. 6

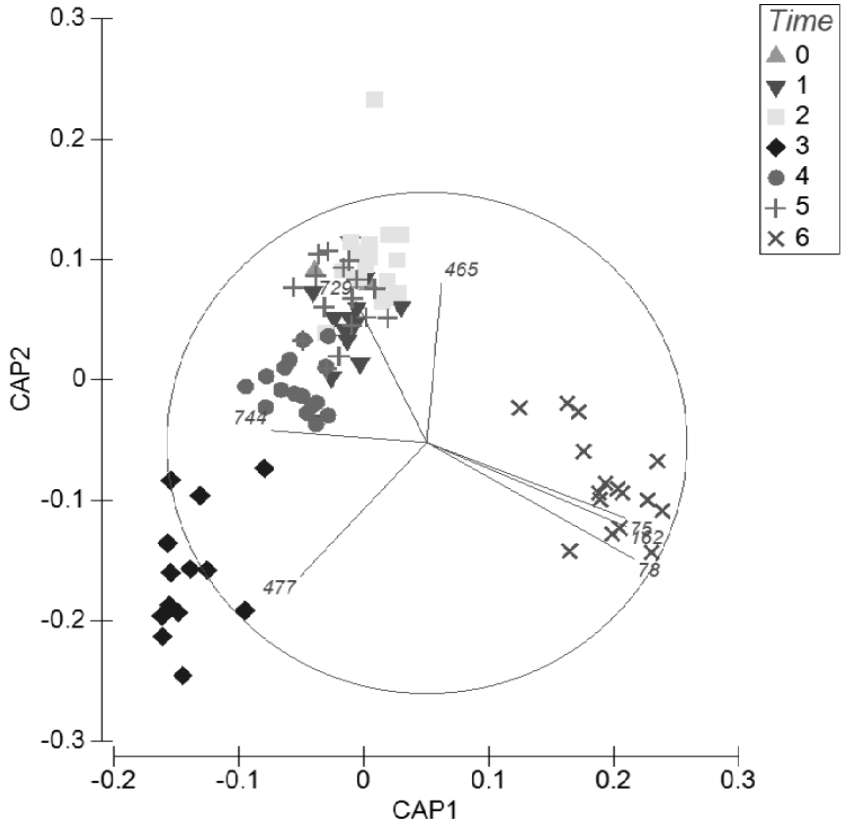

Time
0
1
2
3
4
5
6
465
729
744
477
75
102
78
CAP1
CAP2
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.2
-0.1
0
0.1
0.2
0.3

Fig. 7

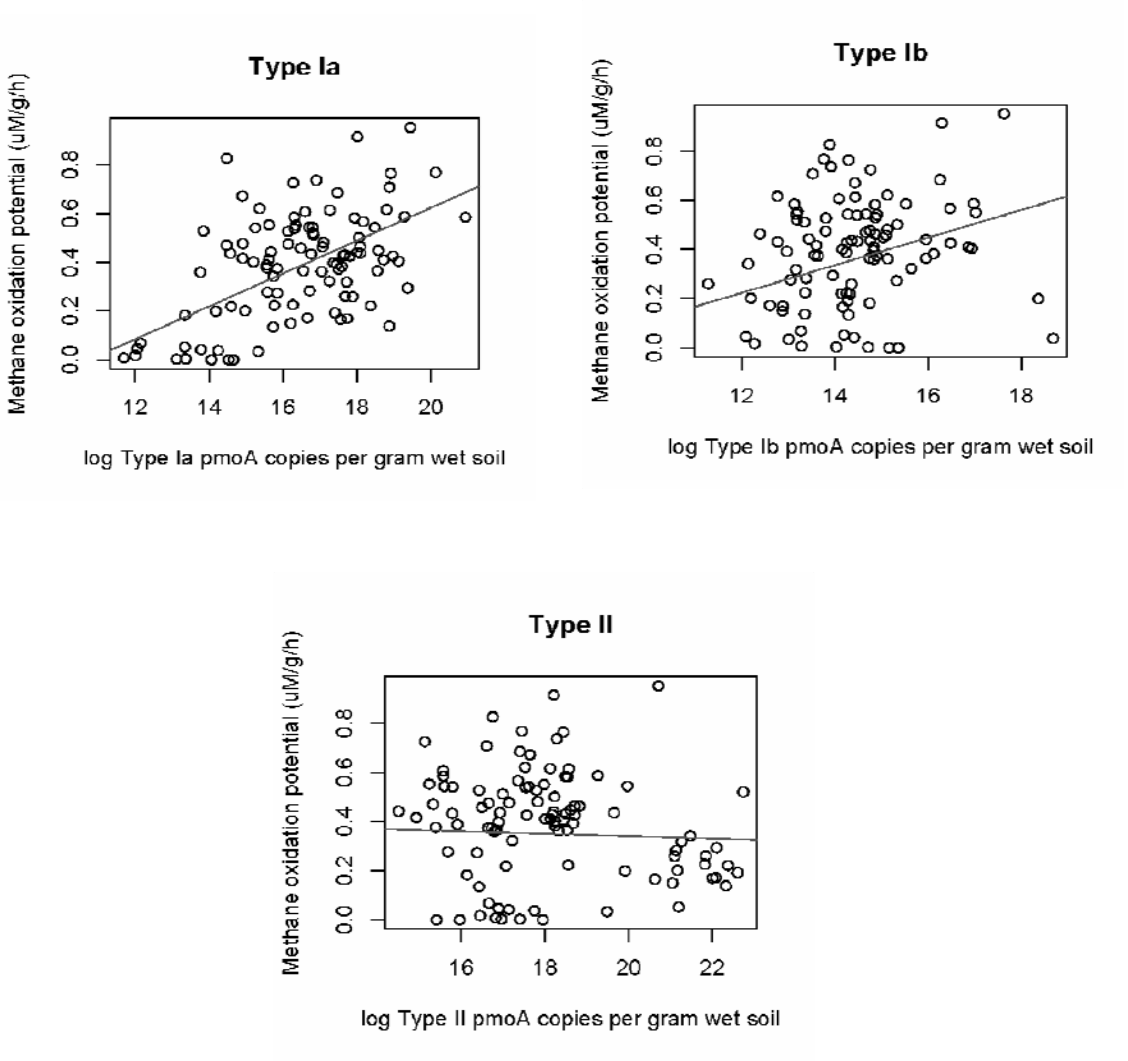
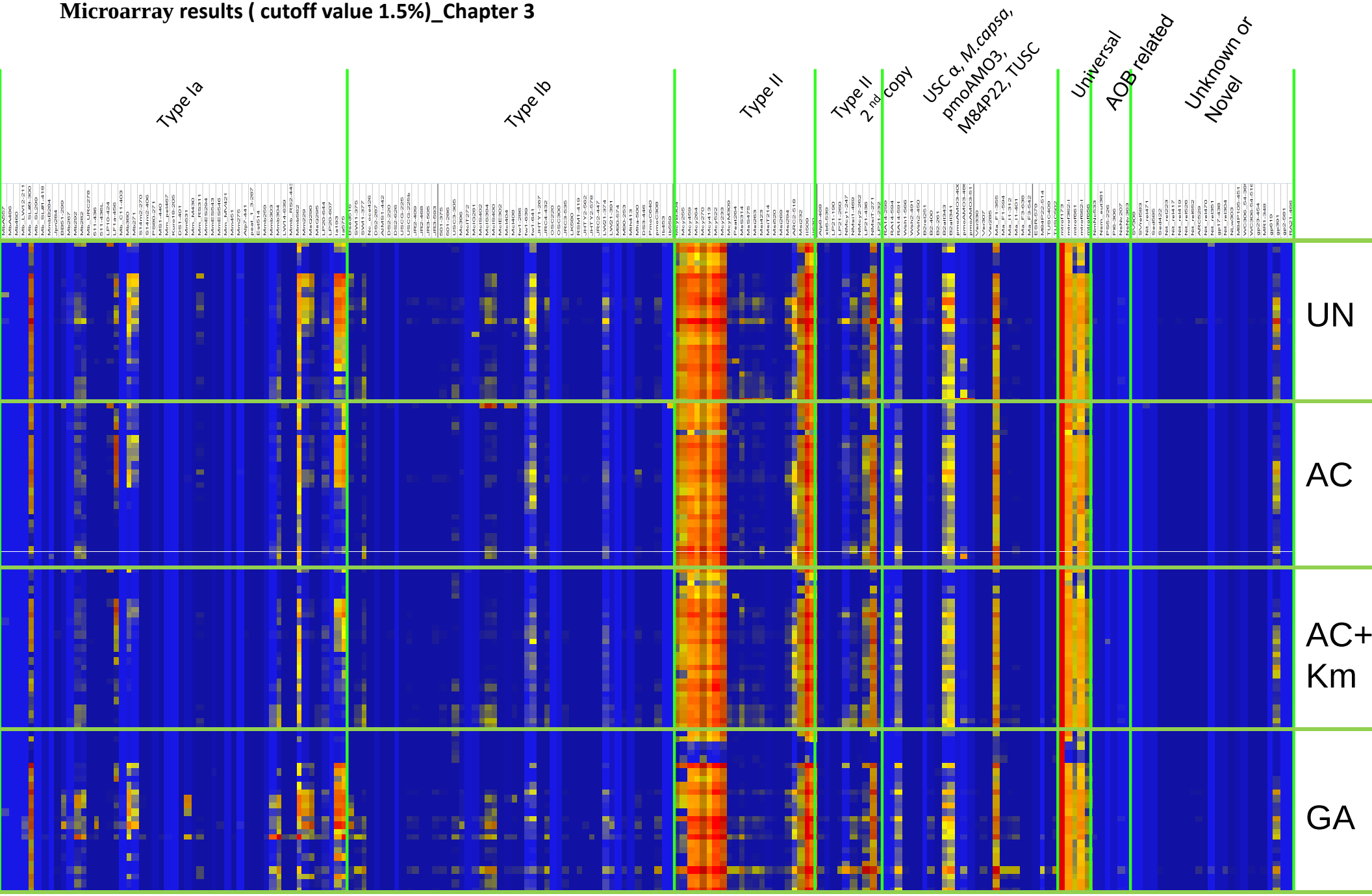

Type Ia
Methane oxidation potential (uM/g/h)
0.0 0.2 0.4 0.6 0.8
12 14 16 18 20
log Type Ia pmoA copies per gram wet soil
Type Ib
Methane oxidation potential (uM/g/h)
0.0 0.2 0.4 0.6 0.8
12 14 16 18
log Type Ib pmoA copies per gram wet soil
Type II
Methane oxidation potential (uM/g/h)
0.0 0.2 0.4 0.6 0.8
16 18 20 22
log Type II pmoA copies per gram wet soil

Fig. 8

Supplementary figure 1 - Microarray results with all probes



Supplementary Table 1

pmoA probe set

Order	Probe name	Intended specificity	Sequence 5' → 3'	L	GC%	T_m
1	MbA557	*Methylobacter*	CAATGGCATGATGTTCACTCTGGCT	25	48.0	61.5
2	MbA486	*Methylobacter*	AGCATGACATTGACAGCGGTTGTT	24	45.8	61.6
3	Mb460	*Methylobacter*	GACAGTTACAGCGGTAATCGGTGG	24	54.2	60.9
4	Mb_LW12-211	*Methylobacter*	CGTCTTTGGGTTACTGTTGTGCC	23	52.2	60.0
5	Mb_SL#3-300	*Methylobacter*	GGCGCTGTTGTTTGTGTATTGGGT	24	50.0	62.2
6	Mb_SL299	soda lake *Methylobacter* isolates and clones	GGGGTGCAACTCTGTGTATCTTAGG	25	52.0	60.5
7	Mb_SL#1-418	soda lake *Methylobacter* isolates and clones	GCGATCGTATTAGACGTTATCCTGATG	27	44.4	58.6
8	MmbB284	*Mmb. buryatense*	ATCGCATCGCTTGGGGTGCAA	21	57.1	62.5
9	Jpn284	clone Jpn 07061	ACCGTATCGCATGGGGTG	18	61.1	58.0
10	BB51-299	*Methylobacter*	CGGTTGTTTGTGTCTTAGGTCTG	23	47.8	57.2
11	Mb267	*Methylobacter*	GCATGCTTGTGGTTCCGTTAC	21	52.4	58.1
12	Mb292	*Methylobacter*	CCGTTACCGTCTGCCTTTCG	20	60.0	59.1
13	Mb282	*Methylobacter*	TTACCGTCTGCCTTTCGGC	19	57.9	58.6
14	Mb_URC278	*Methylobacter*	GTTCCGTTACAGACTGCCTTTCGG	24	54.2	61.3
15	511-436	*Methylobacter*	GTTTTGATGCTGTCTGGCAG	20	50.0	55.5
16	511-436L	*Methylobacter 511 group*	GUUUUGAUGCUGUCUGGCAGCA	22	50.0	60.0
17	LP10-424	*Methylobacter* LP 10 group	GTACTTGATTGTATCTTGATGCTGTCAG	28	39.3	55.7
18	LF1a-456	*Methylobacter* LF 1a group	CATGGTATTGACTGCTGTTATCGGTG	26	46.2	57.7
19	Mb_C11-403	*Methylobacter*	CAAACTTCATGCCTGGTGCTATCGT	25	48.0	61.4
20	Mb380	*M.bacter broad group A universal?*	CAGTAAATTTCTGCTTCCCTTCAAATCT	28	35.7	55.8
21	Mb271	*Methylobacter*	TTGTGGTGGCGTTACCGT	18	55.6	58.0
22	S14m2-270	*Marine type Ia cluster. S14m#2*	CTTATGGTACCGTTACAGATTGCCTTA	27	40.7	56.4
23	S14m2-406	*Marine type Ia cluster. S14m#2*	TTAATTCCTGGTGCAATTGCACTTGAC	27	40.7	58.3
24	PS80-271	clone PS-80	ACCAATAGGCGCAACACTTAGT	22	45.5	58.3
25	MS1-440	*Marine type Ia cluster. Marine sediment #1*	TGATGTTGTCTGGTAGCTTCACATTAAC	28	39.3	57.1
26	Mm_pel467	*Methylomicrobium pelagicum*	ACTGCGGTAATCGATGGTTTGGC	23	52.2	61.6
27	Kuro18-205	*Marine type Ia cluster. Kuro18*	AGACGTTTGTGGGTGACAGTTGC	23	52.2	60.0
28	DS1-401	Deep sea cluster #1	GCGCGGTAGTTTGTGTTATGGCT	23	52.2	61.7

29	Mm531	*Methylomonas*	CTCCATTGCACGTGCCTGTAGA	22	54.5	60.7
30	Mm_M430	*Methylomonas*	TGGACGTGATTTTGATGTTGGGCAA	25	44.0	61.6
31	Mm_RS311	*Mm.methanica. RS clade(10-286)*	CTGTTGTTGCTCTGATGCTGGG	22	54.5	58.6
32	MmES294	*Methylomonas*	CCAATCGGTGCAACAATTTCTGTAGT	26	42.3	59.8
33	MmES543	*Methylomonas*	GTGCCAGTTGAGTATAACGGCATGA	25	48.0	60.9
34	MmES546	*Methylomonas*	CCAGTTGAGTATAACGGCATGATGAT	26	42.3	58.7
35	Mm_MV421	*Methylomonas*	CTATCGTGCTGGATACAATCCTGATGT	27	44.4	60.0
36	Mm451	*Methylomonas*	CTGATGTTGGGTAACAGCATGACT	24	45.8	58.8
37	Mm275	*Methylomonas*	GTGGTGGAGATACCGTTTGCC	21	57.1	59.2
38	Alp7-441	*Alpine soil Methylomonas. Alp#7 (10-282)*	GATGTTAGGTAACAGCATGACACTGAC	27	44.4	57.4
39	peat_1_3-287	*Mehtylomonas*-related peat clones	AACTGCCTTTAGGCGCTACC	20	55.0	58.6
40	Est514	*Methylomicrobium*-related clones	AATTGGCCTATGGTTGCGCC	20	55.0	59.9
41	Mmb259	*Methylomicrobium album* + Landfill *M.microbia*	CTGTTCAAGCAGTTGTGTGGTATCG	25	48.0	59.8
42	Mmb303	*Methylomicrobium album*	CAATGCTGGCTGTTCTGGGC	20	60.0	60.3
43	Mmb304	*Methylomicrobium album* + Landfill *M.microbia* and related	ATGCTGGCTGTTCTGGGCTTG	21	57.1	60.6
44	LW14-639	*Methylomicrobium LW14 group*	AAAAGGUACUUGGAGAACCUUCGGU	25	44.0	60.0
45	Mmb_RS2-443	*Methylomicrobium. Mmb_RS2*	TGCTGGGCAACAGCATGCAGT	21	57.1	62.8
46	Mmb562	*Mmb. album* and *Methylosarcina*	ATGGTAATGACCCTGGCTGACTTG	24	50.0	60.6
47	Mm229	*Deep-branching M.monas (?) group (WHmb3 related group)*	CCAATCGTTGGAATCACTTTCCCAGC	26	50.0	60.2
48	MsQ290	*M.sarcina quisquilliarum related*	TGCCATTCGGCGCTGTAATTTCAGTA	26	46.2	60.8
49	MsQ295	*M.sarcina quisquilliarum*	CGGCGCGGTTCTTTCTGTACTG	22	59.1	60.6
50	LP20-644	*Methylomicrobium*-related clones	GTACACTGCGTACTTTCGGTAA	22	45.5	56.0
51	LP20-607	*LP20 group (Type Ia. deep branching-Mmb?)*	ACTGGTATGCCTGAATACATCCGTA	25	44.0	57.4
52	Ia193	Type I a (*M.bacter-M.monas-M.microbium)*	GACTGGAAAGATAGACGTCTATGGG	25	48.0	57.8
53	Ia575	Type I a (*M.bacter-M.monas-M.microbium-M.sarcina)*	TGGCTGACTTGCAAGGTTACCAC	23	52.2	61.3
54	Bsed516	*Marine sediment #2. Bsed*	AACTGGCCAATGGTTGCTCCA	21	52.4	59.9
55	SWI1-375	*Marine sediment #2. SW#1*	TGCTGGCGCTATGGGTTGG	19	63.2	60.9
56	SWI1-377	*Marine sediment #2. SW#1*	TGGCGCTATGGGTTGGGGTT	20	60.0	62.1
57	Nc_oce426	*Nitrosococcus oceani*	CTTGGATGCCATGCTTGCGA	20	55.0	59.8
58	DS2-287	*Deep sea #2. subgroup (N.coccus and Deep sea Type Ia 10-298)*	GAATCCCATTTGGCGCGACTTTGTG	25	52.0	61.0
59	AIMS1-442	*Deep sea #2. AIMS#1*	TTGTTGACAGGTAGCTATTTGGCAAC	26	42.3	57.7
60	DS2-220	*Deep sea #2. subgroup*	ACGGTGACTCCGATTGTGTGTAT	23	47.8	58.2

61	DS2-626	*Deep sea #2. subgroup*	ATTGCTGGTCTGCATCAGCCTG	22	54.5	60.2
62	USCG-225	Upland soil cluster Gamma	CTGACGCCGATCATGTGCAT	20	55.0	59.1
63	USCG-225b	Upland soil cluster Gamma	CTGACGCCGATCATGTGCATCA	22	54.5	61.2
64	JR2-409	JR cluster #2 (California upland grassland soil)	TTATTCCCGGCGCTATCATGATCG	24	50.0	60.5
65	JR2-468	JR cluster #2 (California upland grassland soil)	ACAGCCATAATTGGACCATTCTTCTG	26	42.3	59.2
66	JR3-505	JR cluster #3 (California upland grassland soil)	TGTATCCTACCAATTGGCCTCATCTG	26	46.2	60.1
67	JR3-593	JR cluster #3 (California upland grassland soil)	CTATCAGTATGTGCGGACAGGC	22	54.5	58.6
68	501-375	*Methylococcus*- related marine and freshwater sediment clones	CTTCCCGGTGAACTTCGTGTTCC	23	56.5	61.3
69	501-286	*Methylococcus*- related marine and freshwater sediment clones	GTCAGCCGTGGGGCGCCA	18	77.8	66.7
70	USC3-305	Upland soil cluster #3	CACGGTCTGCGTTCTGGC	18	66.7	59.5
71	Mc396	*Methylococcus*	CCCTGCCTCGCTGGTGCC	18	77.8	64.4
72	MclT272	*Methylocaldum tepidum*	GGCTTGGGAGCGGTTCCG	18	72.2	61.9
73	MclG281	*Methylocaldum gracile*	AAAGTTCCGCAACCCCTGGG	20	60.0	61.5
74	MclS402	*Methylocaldum szegediense*	GCGCTGTTGGTTCCGGGT	18	66.7	61.8
75	MclS394	*Methylocaldum szegediense* and related	TTCCCGGCGCTGTTGGTTCC	20	65.0	63.3
76	MclS400	*Methylocaldum szegediense* and related	CGGCGCTGTTGGTTCCGGGT	20	70.0	65.7
77	MclE302	*Methylocaldum* E10	CGCAACCATGGCCGTTCTG	19	63.2	60.3
78	Mcl404	*Mc.capsulatus-Mcl.tepidum-Mcl. Gracile-Mcl.Szeg* and related	TTTTGGTTCCGGGTGCGATTT	21	47.6	58.0
79	Mcl408	*Methylocaldum*	GGTTCCGGGTGCGATTTTG	19	57.9	57.8
80	fw1-286	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	ATCGTCAACCGTGGGGCG	18	66.7	61.1
81	fw1-639	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	GAAGGGCACGCTGCGTACG	19	68.4	62.0
82	fw1-641	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	AGGGCACGCTGCGTACGTT	19	63.2	63.3
83	JHTY1-267	*JH-TY#1*	TTGGTTGTGGGAAAACTTCCGT	22	45.5	57.4
84	JRC4-432	Japanese rice cluster #4	GACGTTGTCCTGGCTCTGAG	20	60.0	58.3
85	OSC220	Finnish organic soil clones and related	TCACCGTCGTACCTATCGTACTGG	24	54.2	60.8
86	OSC300	Finnish organic soil clones and related	GGCGCCACCGTATGTGTACTG	21	61.9	61.4
87	JRC3-535	Japanese Rice Cluster #3	CGTTCCACGTTCCGGTTGAG	20	60.0	59.3
88	LK580	fw-1 group + Lake Konstanz sediment cluster	CCGACATCATTGGCTACAACTATGT	25	44.0	58.7
89	RSM1-419	*RSM#1*	CCATTCTGCTCGACGTGGTTCT	22	54.5	59.4
90	JHTY2-562	*JH-TY#2*	ATGCTGTTGTCGATCGCCGACTTGC	25	56.0	63.6
91	JHTY2-578	*JH-TY#2*	CCGACTTGCAAGGCTACAACTATGTC	26	50.0	59.5
92	JRC2-447	Japanese Rice Cluster #2	CTGAGCACCAGCTACCTGTTCA	22	54.5	60.2
93	LW21-374	LW21 group	CTACTTCCCGATCACCATGTGCT	23	52.2	60.2
94	LW21-391	LW21 group	TGTGCTTCCCCTCGCAGATC	20	60.0	60.5

95	M90-574	*M.coccus-M.caldum* related marine and freshwater sediment clones	ATCGCCGACCTGCTGGGTTA	20	60.0	62.2
96	M90-253	*M.coccus-M.caldum* related marine and freshwater sediment clones	GCTGCTGTACAGGCGTTCCTG	21	61.9	61.7
97	Mth413	*Methylothermus*	CACATGGCGATCTTTTTAGACG TTG	25	44.0	58.3
98	Mha-500	*Methylohalobius - M.thermus and related ?*	TGATGTACCCGGGCAACTGGC	21	61.9	62.3
99	DS3-446	Deep sea cluster #3	AGCTGTCTGGCAGTTTCCTGTT CA	24	50.0	62.5
100	PmoC640	*PmoC*	AAGGGAACGCTTCGTACGTTTG G	23	52.2	59.8
101	pmoC308	*PmoC*	CCTGTGTGCTGGCGATTCTGCT	22	62.3	59.1
102	Ib453	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCAGCTACCTGTTCACCGC	20	65.0	61.7
103	Ib559	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCATGCTGATGTCGATTGCCG	22	59.1	62.5
104	McyB304	*M.cystis B (parvus/echinoides/*strain M)	CGTTTTCGCGGCTCTGGGC	19	68.4	62.7
105	Mcy255	*M.cystis B (parvus/echinoides/*strain M)	GGCGTCGCAGGCTTTCTGG	19	68.4	62.3
106	Mcy459	*Methylocystis*	GTGATCACGGCGATTGTTGGTT C	23	52.2	60.2
107	Mcy264	*Methylocystis*	CAGGCGTTCTGGTGGGTGAA	20	60.0	61.0
108	Mcy270	*Methylocystis*	TTCTGGTGGGTGAACTTCCGTC T	23	52.2	61.8
109	Mcy413	*Methylocystis*	TTCCGGCGATCTGGCTTGACG	21	61.9	63.2
110	Mcy522	*Methlocystis* A + peat clones	GGCGATTGCGGCGTTCCA	18	66.7	62.3
111	Mcy233	*Methylocystis*	ATTCTCGGCGTGACCTTCTGC	21	57.1	60.9
112	McyM309	*M.cystis strain M and related*	GGTTCTGGGCCTGATGATCGG	21	61.9	61.0
113	Peat264	peat clones	GGCGTTTTTCTGGGTCAACTTC C	23	52.2	60.3
114	MsS314	*Methylosinus sporium*	GGTTCTGGGTCTGCTCATCGG	21	61.9	60.8
115	MsS475	*Methylosinus sporium*	TGGTCGGCGCCCTGGGCT	18	77.8	68.3
116	Msi263	*Methylosinus sporium* + 1 *Msi.trichosporium subclaster*	GGCGTTCCTGTGGGAGAACTTC	22	59.1	61.2
117	Msi423	*Methylosinus*	CTGTGGCTGGACATCATCCTGC	22	59.1	61.4
118	MsiT214	*Methylosinus trichosporium* OB3b and rel.	TGGCCGACCGTGGTTCCG	18	72.2	63.5
119	MsiT343	*Methylosinus trichosporium* OB3b and rel.	TCAACCGCTACTGCAACTTCTG G	23	52.2	60.9
120	MM_MsiT343	*Methylosinus trichosporium* OB3b and rel. - MM control probe!	TCAACCGCTACTTCAACTTCTG G	23	47.8	58.5
121	Msi520	*Methylosinus trichosporium*	GCGATCGCGGCTCTGCA	17	70.6	61.6
122	Msi269	*Methylosinus trichosporium*	TCTTCTGGGAGAACTTCAAGCT GC	24	50.0	60.6
123	Msi294	*Methylosinus*	GTTCGGCGCGACCTTCGC	18	72.2	62.5
124	ARC2-518	*Deep branching type II clade ARC2 - Methylosinus trichosporium 15-084 group?*	GGCCGGCGATTGGTCAGTATCA	22	59.1	61.7
125	Msi232	*M.sinus*+ most *M.cystis* -considered as additional type II probe	ATCCTGGGCGTGACCTTCGC	20	65.0	63.3
126	II509	Type II	CGAACAACTGGCCGGCGAT	19	63.2	61.7

127	II630	Type II	CATGGTCGAGCGCGGCAC	18	72.2	62.4
128	Alp8-468	*Type II novel pmoA. Alpine cluster Alp#8*	CGCGCTCCTTGGCTCGTTGG	20	70.0	64.0
129	xb6-539	Novel *pmoA* copy of type II and related environmental clones	AGGCCGCCGAGGTCGAC	17	76.5	63.0
130	LP21-190	Novel *pmoA* copy of type II and related environmental clones	ATCGACTTCAAGGATCGCCG	20	55.0	58.2
131	LP21-260	Novel *pmoA* copy of type II and related environmental clones	CGCAGTCCTTCTTCTGGACG	20	60.0	58.6
132	NMcy1-247	Novel *pmoA* copy of *M.cystis #1* (?)	TCGACATCGTGCTGATGATCTCGG	24	54.2	62.1
133	NMsi1-469	Novel *pmoA* copy of *M.sinus*	GCGCTGGTCGGCTCCATGG	19	73.7	64.3
134	NMcy2-262	Novel *pmoA* copy of *M.cystis #2* (?)	CAGTCCTTCTTCTGGCAGAAGTTCC	25	52.0	60.9
135	LP21-436	*Mcy + Msi novel pmoA #1 groups*	GTGCTGATGATGTCGGGCAGCTGGC	25	64.0	66.1
136	NMsiT-271	Novel *pmoA* copy of *M.sinus trichpsporium* (?)	AGCGCTTCCGTCTGCCGAT	19	63.2	62.9
137	LP21-232	Novel *pmoA* copy of type II and related environmental clones	ATCGTCGCCATGTGCTTCGC	20	60.0	61.9
138	RA14-299	RA14 related clones	GCGCGACGTTCCTTTGTGTC	20	60.0	59.5
139	RA14-594	RA14 related clones	CCACAACGTTCGTACCTCGA	20	55.0	57.9
140	RA14-591	RA14 related clones	GGCTTCCACAACGTTCGTACCT	22	54.5	60.9
141	Wsh1-566	Watershed + flodded upland cluster 1	GCTCATGAGCTTGGCCGACATC	22	59.1	61.8
142	Wsh2-491	Watershed + flodded upland cluster 2	TCATTTGGCCAACCTCTCTCATTCC	25	48.0	60.9
143	Wsh2-450	Watershed + flodded upland cluster 2	CAAGAGCTGGATCATCACGATG	22	50.0	56.8
144	B2rel251	*Methylocapsa*-related clones	CCGCCGCGGCCCAGTATTA	19	68.4	63.4
145	B2-400	*Methylocapsa*	ACCTCTTTGGTCCCGGCTGC	20	65.0	63.4
146	B2-261	*Methylocapsa*	TCAGGCCTATTTCTGGGAAAGCT	23	47.8	58.3
147	B2all343	*Methylocapsa* and related clones	AACCGCTACACCAATTTCTGGGG	23	52.2	61.2
148	B2all341	*Methylocapsa* and related clones	TCAACCGCTACACCAATTTCTGGG	24	50.0	61.1
149	pmoAMO3-400	clone pmoA-MO3	ACCCAGATGATCCCGTCGGC	20	65.0	62.6
150	pmoAMO3-486	*MO3 group*	ggGATGGGGCCTTCTCATGTACC	23	60.9	61.5
151	pmoAMO3-511	*MO3 group*	AGCAACTGGCAGGTCCTCG	19	63.2	60.2
152	Ver330	*Verrucomicrobia. all pmoA1+pmoA2*	TGGTCAGTGGATGAATAGGTATTGGA	26	42.3	57.3
153	Ver307	*Verrucomicrobia. all pmoA2*	TTCAGCTGTGCCGGATTGTTTT	22	45.5	57.9
154	Ver285	*Verrucomicrobia. Ma.fum pmoA2+Ma.kam. pmoA2*	TAAAGCGCCTATAGGAGCAACCT	23	47.8	58.0
155	Ma_F1-355	*Ma.fum. pmoA1*	AACTTCTGGGGTTGGGGCACTT	22	54.5	61.5
156	Ma_F1-594	*Ma.fum. pmoA1*	TGAATACATCCGGACTTCTACCCC	24	50.0	57.9
157	Ma_I1-312	*Ma.inf. pmoA1*	AACCGTTGGGCTTTTCTTTGGC	22	50.0	59.1
158	Ma_I1-401	*Ma.inf. pmoA1*	AAACATTAATTCCCCAGGCTGTCGT	25	44.0	58.9
159	Ma_F3-638	*Ma.fum. pmoA3*	AAAGTGGGACTCTTCGGACCTT	22	50.0	58.1

160	Ma_F3-542	*Ma.fum. pmoA3*	AACCCTTAGAAGCCTTAGGCCA	22	50.0	58.1
161	ESR-579	ESR (Eastern Snake River) cluster	GACCTGATCGGATTCGAGAACATC	24	50.0	58.5
162	M84P22-514	environmental clones of uncertain identity	AACTGGGCCTGGCTGGG	17	70.6	61.0
163	TUSC409	Tropical Upland Soil Cluster #2	CGATCCCGGGCGCGATTC	18	72.2	61.8
164	TUSC502	Tropical Upland Soil Cluster #2	TCTTCTACTTCGGCAACTGGC	21	52.4	58.3
165	mtrof173	Universal	GGbGACTGGGACTTCTGG	18	66.7	57.4
166	mtrof362-I	Methanotrophs	TGGGGCTGGACCTACTTCC	19	63.2	59.5
167	mtrof661	Methanotrophs	GGTAARGACGTTGCKCCGG	19	63.2	60.4
168	mtrof662-I	Methanotrophs	GGTAAGGACGTTGCGCCGG	19	68.4	61.9
169	mtrof656	Methanotrophs	ACCTTCGGTAAGGACGT	17	52.9	53.2
170	NmNc533	*Nitrosomonas-Nitrosococcus*	CAACCCATTTGCCAATCGTTGTAG	24	45.8	58.6
171	Nsm_eut381	*Nitrosomonas eutropha*	CCACTCAATTTTGTAACCCCAGGTAT	26	42.3	59.0
172	PS5-226	*Nitrosomonas-Nitrosococcus* related clones	ACCCCGATTGTTGGGATGATGTA	23	47.8	59.9
173	Pl6-306	*Nitrosomonas-Nitrosococcus* related clones	GGCACTCTGTATCGTATGCCTGTTAG	26	50.0	60.5
174	NsNv207	*Nitrosospira-Nitrosovibrio*	TCAATGGTGGCCGGTGG	17	64.7	58.5
175	NsNv363	*Nitrosospira-Nitrosovibrio*	TACTGGTGGTCGCACTACCC	20	60.0	59.6
176	SV308	*Svalbard clade*	TGAGCATCTCTGGGCTTGTCGT	22	54.5	60.7
177	SVrel583	*Svalbard clade and related*	TACATGGGATTCACATTTGTGAGGAC	26	42.3	57.0
178	Nit_rel471	AOB related clones/probably methanotrophs	CGTTCGCGATGATGTTTGGTCC	22	54.5	60.1
179	Sed585	*Ssedi#1*	GGGCATTCGCGATGATGTTTTATCCGA	27	48.1	61.2
180	Sed422	*Ssedi#1 and related*	TGATCCTAGACTGCACCCTGTTG	23	52.2	58.5
181	Nit_rel223	AOB related clones/probably methanotrophs	GTCACACCGATCGTAGAGGT	20	55.0	56.9
182	Nit_rel417	*Arctic soil related #1. subgroup*	CGCGTTGATCTTTGATTGCACCCTGTT	27	48.1	61.8
183	Nit_rel419	*Arctic soil related #1. subgroup*	CGTTGATCCTTGATTGCACCCTGTT	25	48.0	59.8
184	Nit_rel526	*JRC#1+CCd#1 groups*	GCCATCAACCATTGGTTGCGGA	22	54.5	60.8
185	Nit_rel652	*Arctic soil MOB*	CGTACATTCGGTGGTCACACTG	22	54.5	57.9
186	ARC529	AOB related clones/probably methanotrophs	TAAGCAGCCGATGGTCGTGGAT	22	54.5	62.2
187	Nit_rel470	AOB related clones/probably methanotrophs	CGATATTCGGGGTATGGGCG	20	60.0	58.4
188	Nit_rel351	AOB related clones/probably methanotrophs	GTTTGCCTGGTACTGGTGGG	20	60.0	59.2
189	gp17-438	*environmental clones of uncertain identity - gp17*	ACTCTTATTGACCAGGAATTGGACCTTG	28	42.9	58.5
190	Nit_rel304	AOB related clones/probably methanotrophs - *Crenothrix* and related	CGCTCTGCATTCTGGCGCT	19	63.2	61.8
191	NLw303	*environmental clones of uncertain identity - NL wetland*	AACGATCACTATTCTGGCTCTTGCCTTT	28	42.9	60.1

192	M84P105-451	environmental clones of uncertain identity	AACAGCCTGACTGTCACCAG	20	55.0	58.1
193	WC306_54-385	environmental clones of uncertain identity	AACGAAGTACTGCCGGCAAC	20	55.0	59.2
194	WC306-54-516	environmental clones of uncertain identity	AACTGGCCGATTTTTGGCATGTT	23	43.5	58.4
195	gp23-454	environmental clones of uncertain identity	AACGCGCTGCTCACTGCG	18	66.7	62.3
196	MR1-348	environmental clones of uncertain identity	AATCTTCGGTTGGCACGGCT	20	55.0	61.1
197	gp619	environmental clones of uncertain identity	CGGAATATCTGCGCATCATCGAGC	24	54.2	61.5
198	gp391	environmental clones of uncertain identity	ATCTGGCCGGCGACCATG	18	66.7	61.1
199	gp2-581	environmental clones of uncertain identity	ACATGATCGGCTACGTGTATCCG	23	52.2	60.0
200	RA21-466	clone RA21 - environmental clone of uncertain identity	CGGCGTTCTTGGCGGCAT	18	66.7	62.4

Supplementary Table 2

T-RFLP peaks unique to the different treatments (across the entire time span of the experiment). Numbers indicate peak sizes in the T-RFLP profiles.

Treatment	**Unique Peaks**
Untreated	TRF 132, 225, 231, 328, 526 and 529
Acetylene + Kanamycin	TRF 79, 108, 205, 258, 427
Acetylene	TRF 241, 420
Gamma irradiation	TRF 94, 155, 170, 191, 265, 304, 438, 441, 452, 475, 540, 570, 591, 605, 607, 620, 625, 636, 662, 683, 736, 767, 794, and 802

Chapter 4

Resilience against and recovery from flood and drought stress by a floodplain methanotroph community

Keywords: methanotroph / methane oxidation / pmoA / Disturbance / resilience / recovery

Abstract

There is limited information and understanding on the role of microbial communities maintaining ecosystem function. Methane-oxidizing bacteria (MOB) were applied as model microorganisms in this study to investigate microbial resistance and resilience against perturbations. Our hypothesis was that, under sufficient methane supply, MOB communities showed high resistance and resilience against water stress. Drought and flooding were used as perturbations in a well-controlled mesocosm experiment. Following 35 days of perturbation treatment, we followed the recovery of function (methane oxidation potential) and community (structure and abundance) over 83 days. Methane oxidation potentials were suppressed by the perturbations but showed very rapid recovery. Positive linear correlations were found between MOB abundances and time. Neither drought nor flooding had significant influences on MOB community compositions or abundance even though MOB were embedded in different total bacterial communities.

Introduction

Considering the rapid loss of biodiversity, biodiversity-ecosystem functioning studies (BEF) have been a key-issue in ecology and conservation studies (Gotelli and Colwell, 2001; Hooper et al., 2005; Barnosky et al., 2011). Despite the fact that microorganisms represent the largest source of biomass and biodiversity, they have been rarely considered in BEF and conservation studies. This may be due to a fundamental lack of knowledge of the functioning of microbial ecosystems, which are generally regarded as "black boxes" because of largely unknown and complex mechanisms involved (Bodelier, 2011). The unknowns within these "black boxes" comprise community, population and cell traits. Resistance (i.e. the degree to which a microbial community remains unchanged in the case of disturbance), resilience (i.e. the rate at which a microbial community returns to its original composition after being disturbed) and redundancy (i.e., if different taxa carry out the same function) are regarded as the three basic mechanisms, which maintain community functions and which have been poorly studied in microbial communities(Allison and Martiny, 2008).

In light of increasing anthropogenic disturbances and climate change and the associated possible functional impact on microbial communities (e.g. temperature increase, flooding, drying and nitrogen amendments, land use change) (Mohanty et al., 2006; Lesaulnier et al., 2008; Bell et al., 2009; Van Der Zaan et al.; Frazao et al., 2011) better functional understanding of microbial ecosystems is required. There have been numerous studies investigating the recovery of microbial diversity and eco-functions following the disturbances of stable ecosystems (Botton et al., 2006; Griffiths et al., 2008; Ho et al., 2011). Species richness and evenness of microbial communities have been reported to play an important role in their functional stability. However, there is still a large gap in our mechanistic understanding of the role of microbial diversity in

ecosystem functioning. The use of model organisms and processes such as methanotrophs (methane oxidizing bacteria, MOB)(Ho et al., 2011) or nitrifiers (ammonia oxidizing bacteria and archaea, AOB and AOA) (Wertz et al., 2007) involved in highly important environmental functions such as methane oxidation and nitrification may facilitate our understanding on how complex microbial communities are affected by disturbances.

Under aerobic conditions, methanotrophs are the only biological sink of the greenhouse gas methane. Most aerobic MOB utilize methane as sole carbon and energy sour (Hanson and Hanson, 1996; Conrad, 2007). Recently *Methylocella*, two filamentous methanotrophs *Crenothrix polyspora* and *Clonothrix fusca* as well as some *Methylocystis* strains were shown to have a facultative methanotroph lifestyle (Dedysh et al., 2005; Theisen and Murrell, 2005; Im et al., 2011). The first step in the methane oxidation pathway is catalyzed by either one of the two types of the enzyme methane monooxygenase (sMMO and pMMO). Traditionally, MOB were categorized as either *Alphaproteobacteria* (Type II) or *Gammaproteobacteria* (Type Ia and Type Ib). Recently, a new phylum of MOB belonging to the *Verrucomicrobia,* was discovered (Dunfield et al., 2007; Pol et al., 2007). Methanotrophs are a well defined and studied group of organisms with a functional link to methane oxidation and, as such, represent an ideal model system to study and understand the causal relationship between microbes and BEF. Based on the congruence between the phylogenies of the 16S rRNA gene and *pmoA*, encoding a subunit of pMMO, the latter gene is more often used as a phylogeny marker to study MOB (Conrad and Donald, 2007).

Flooding-drought cycle is a common stress for floodplain soils. Activity and diversity of methanotrophs are normally regulated by methane and oxygen concentrations, which vary during the flooding and drought cycle. Altered activity of methanotrophs under flooding and drought

stress might directly influence methane emissions, e.g., if flooding may alter the soil from a sink to a source of atmospheric methane (Bodelier et al., 2011). The aim of this study was to use MOB as a model group of microorganisms to study the recovery and resistance of MOB within different total microbial communities, from drought and flood disturbances.

Material and Methods

Construction of mesocosms

Mesocosms were built as described previously (as described in chapter 3). Briefly, four differently treated soils were used to create four mesocosms. These included untreated soil from a Dutch river floodplain (51º88'N, 5º73'E) as well as the same soil treated with i) acetylene, ii) acetylene together with kanamycin and iii) by gamma irradiation in order to obtain soils containing different numbers of bacteria/methanotrophs ($P<0.01$) and overall microbial diversities ($p<0.001$). All soils were inoculated with a thin layer (approximately 3 mm) of untreated soil after four treatments. Mesocosms were incubated for 105 days in 60 cm x 45 cm x 25 cm boxes separately in a climate controlled room (20 °C) with 20 % 70 ml/min methane flow from the bottom after inoculation with untreated soil. Soil moisture was maintained at approximately 30%. These treatments had the aim to study the development of microbial communities in soils with different life histories (see chapter 3). Briefly, the recovery and succession of the methanotroph communities followed a very similar, congruent pattern across the different treatments in spite of the difference in the starting overall microbial communities. These four mesocosms with very similar methanotroph communities and function, arising from different life histories, were considered as replicates in the present study investigating the resistance and resilience of methanotroph communities against water stress.

After 105 days incubation with soil moisture maintained at approximately 30%, each mesocosm was divided in three equal parts by introducing two concrete dividers. One part was watered regularly to keep the moisture around 30%; one part was not watered reaching a moisture of around 20%; and the last part was flooded and covered with plastic film leading to a moisture of around 47%. These perturbation treatments representing "drought" and "flooding", respectively, were carried out for 35 days with 20 % 70 ml/min methane flow. Then perturbation was stopped and the first sampling was performed ("day 0" referred as sampling T0). The plastic film covers were removed from the wet parts and the dry parts were watered again to reach a moisture of around 30%. Watering was continued in each part of the mesocosms with 100 ml H_2O once every two days keeping the moisture around 30%. Further samplings were performed at day 2, day 13, day 43 and day 85 after stopping perturbations, referred as sampling T1, sampling T2, sampling T3 and sampling T4, respectively. At each sampling time point, three cores (2.5 diameters x 20 cm long) were sampled from each part of all four mesocoms. Soil cores were divided into 2 layers (upper layer: 0-10 cm; lower layer: 10-20 cm). Each layer was homogenized and used for DNA extraction.

DNA extraction

DNA extraction was performed using a modification of the method described by Yeates and Gillings(Yeates et al., 1998), based on the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA). Soil (0.3 g) and 780 µllysis buffer [200 mM $NaPO_4$ pH 7.0; 1% CTAB; 1.5 M NaCl; 2% polyvinylpyrrolidone K30; 5 mg ml^{-1} lysozyme (added right before use)] were added into a multimixFastPrep E tube and incubated at 37 $^{\circ}$C for 30 min. MT buffer (122 µl) was added

and tubes were shaken in the FastPrep instrument (MP, Biomedicals, LLC, Solon, OH, USA) for 30 s at 5.5 m s^{-1}. Subsequently, samples were centrifuged for 20 min at 13000 rpm and 700 µl of supernatant were collected. Five µl of 10 mg ml^{-1} freshly made proteinase K was added to each tube. Tubes were incubated at 65 °C for 30 min. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by a chloroform-isoamyl alcohol (24:1) extraction. Supernatants were transferred to new tubes, 600 µl Binding Matrix was added and tubes were mixed for 5 min on a rotator. Binding Matrix, with bound DNA, was pelleted by 1 min centrifugation at 13000 rpm. Supernatant was discarded and pellets were resuspended in 500 µl wash buffer. Resulting suspensions were added into a spinfilter, and centrifuged for 1 min at 10000 rpm. The eluate was discarded and the pellet was washed again in 500 µl wash buffer. After discarding the second eluate, the spinfilter was centrifuged for another 10 s to dry the pellet. The filter was taken into a new tube and 50 µl of TE pH 8.0 was added. The filter was incubated at room temperature for 1 min and centrifuged for 1 min. The eluate collected in the catch tube contained the purified DNA which was then used for the analyses described below. DNA concentrations were estimated using a NanoDrop spectrophotometer (Thermo Scientific, DE, USA).

Diagnostic pmoA microarray analyses

PCR amplification of the *pmoA* gene was based on a two-step semi-nested protocol. The first step PCR comprised 25 µl of 2×Premix F (EPICENTRE Biotechnologies, USA), 25 pmol of primer A-189f and A-682r (Holmes et al., 1995) each, 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 50 ng of genomic DNA as template, in total volume of 50 µl. PCR was

performed using a touchdown protocol with an initial incubation of 5 min at 94 ºC, followed by 25 cycles of 1 min at 94 ºC, 1 min at the annealing temperature and 1 min at 72ºC, followed by a final incubation of 10 min at 72 ºC. The annealing temperature was lowered from 62 ºC to 52 ºC over the first 11 cycles after which it was maintained for further 24 cycles at 52 ºC. 5 µl of 1/100 diluted PCR product from the first step was used as template in a subsequent nested amplification with primers A-189f and T7-661r. The second step PCR was performed by total 25 cycles with an initial incubation of 5 min at 94ºC, followed by 1 min at 94ºC, 1 min at the annealing temperature and 1 min at 72 ºC, followed by a final incubation of 10 min at 72 ºC. The annealing temperature was lowered from 62 ºC to 52 ºC over the first 11 cycles after which it was maintained for further 14 cycles at 52 ºC.

In vitro transcription was carried under RNAse-free conditions, the procedure was as follows: 7 µl purified PCR product (50 ngμl^{-1}), 4 µl 5×T7 RNA polymerase buffer, 2 ml DTT(100 mM), 0.5 ml RNAsin (40 U μl^{-1}) (Promega, Madison, WI, USA), 1 µl each of ATP, CTP, GTP (10 mM), 0.5 ml UTP (10 mM), 1 µl T7 RNA polymerase (40 U ml^{-1}) (Invitrogen Carlsbad, CA, USA) and 1 µl Cy3-UTP(5 mM) (GE, Fairfield, USA) were added into a 1.5 ml tube and incubated at 37ºC for 4 h. RNA was purified immediately based on the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA): 80 µl DEPC treated water were added to IVT mixture. Followed adding 350 µl RLT and 250 µl ethanol, mixed thoroughly. Samples were transferred to an RNeasy mini tube. Add 500 µl RPE, at 10000rpm centrifuged 15 sec. Add another 500 µl RPE, at 10000 rpm centrifuged 2 min. Purified RNA was eluted into 50 µl dH_2O.

Purified RNA was fragmented by incubating with 9.5 mM $ZnCl_2$ and 24 mMTris.Cl (pH7.4) at 60ºC for 30 min. Fragmentation was stopped by the addition of 12 mM EDTA pH 8.0 to the reaction and putting it on ice. RNAsin (1 µl 40 U μl^{-1}) was added to the fragmented target.

Hybridization was carried out using a Belly Dancer (Stovall Life Science, Greensboro, NC, USA) equipped with a custom made aluminium block serving as dry, temperature controlled platform for the slides, preheated to 55ºC for at least one hour. For each hybridization, 62 µl DEPC treated water, 1 µl 10% SDS, 30 µl 20 ×SSC, 2 µl 50 × Denhardt's reagent and 5 µl target RNA were added into a 1.5 ml tube and incubated at 65ºC for 1 min. Preheated hybridization mixture were applied onto assembled slides with the HybriWell (Grace BioLabs, Bend, OR, USA). Slides were also preheated on the aluminium block. Slides were hybridized overnight at 55ºC in the BellyDancer, set at maximum bending and lowest rpm, providing slow mixing within the chamber by the air bubbles formed. Following the hybridization, slides were washed by shaking at room temperature for 5 min in 2×SSC, 0.1 % (w/v) SDS, twice for 5 min in 0.2×SSC and finally for 5 min in 0.1×SSC. Slides were dried individually using an air gun.

Hybridized slides were scanned at 10 µm resolution with a GenePix 4000 laser scanner (Axon, Foster City, CA, USA) at wavelengths of 532 nm. Florescent images analysed with the GenePix software (Axon). Microsoft Excel was used for statistical analysis and presentation of results.

Hybridization between a probe and a target was considered in analyses if the signal was at least 1.5% of the strongest signal obtained for that probe with the validation set of reference strains/clones (for details see (Bodrossy et al., 2003)

pmoA-based quantitative PCR (qPCR) assay

All qPCR assays were carried out as described by (Kolb et al., 2003) with the exception of the modifications indicated in Chapter 3 Table 1. Each assay was performed in duplicate. DNA from clones was used as standard for calibrating the assays. qPCR was performed in 25 µl volumes using 96-well PCR plates (VWR GmbH, Vienna, Austria) with the optical cover (Applied Biosystems, Foster City, CA, USA). PCR master mix containing 12.5 µliQ SYBR Green Supermix (Bio-Rad, CA, USA) , 1 µl 5ng/uL DNA template and 2.5 µl of each forward and reverse primers. DNase and RNase-free water was added to a final volume of 25 µl.

Methane oxidation assay

The assay was performed as described earlier (Steenbergh et al., 2009) with modifications. Each assay was performed in triplicate. In short, for each incubation 1 gram of soil was suspended in 10 mL Milli Q water (Millipore, MA, USA) in a 150 ml flask capped with a rubber stopper. Pure methane (1.4 ml) was added to the bottle. Slurries were incubated in the dark at room temperature on a shaker (100 rpm).The methane concentration in the headspace was measured by GC-FID analysis (Fisons HR 8060). Activity was calculated by considering results at 0 h and 24 h.

16SrRNA gene - based terminal restriction fragment length polymorphism (T-RFLP) analysis

Bacterial 16S rRNA genes were amplified with the FAM-labeled forward primer 8fM (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1406r (5'-

ACGGGCGGTGTGT(AG)C-3') (Liu et al., 1997). Each 50 µl reaction mixture contained 25 µl 2×Premix F (EPICENTRE Biotechnologies, Madison, Wisconsin, USA), 20 pmol of each primer, and 1 U Taq polymerase (Invitrogen, Carlsbad, CA,USA), and 50 ng of genomic DNA as template. Cycling conditions were as follows: an initial denaturation of 5 min at 94 °C; followed by 35 cycles of 1 min of denaturation at 94°C, 45 s at 55 °C, 1 min at 72 °C; and a final extension at 72 °C for 10min.

Four individual PCRs were performed for each sample, and then combined and purified with a Qiaquick PCR cleanup kit (Qiagen Inc., Chatsworth, CA, USA).PCR products (150 ng) were digested with 10 U of *Rsa*I enzyme (Invitrogen, Carlsbad, California, USA) in a reaction that contained 1µl Tango buffer (Invitrogen, Carlsbad, California, USA)made up to 20 µl with PCR grade water and incubated at 37 °C for 4 h, after which the enzyme was inactivated at 65 °C for 20 min. Purification was performed using SephadexG-25 (GE, Fairfield, USA) columns. 5 µl of each purified sample was mixed with 0.5 µl of DNA fragment length standard (MapMarker 500) and 14.5 µl Hi-Di formamide (Applied Biosystems, Foster City, CA, USA). Samples were denatured for 2 min at 94 °C and T-RFLP analysis was carried out using the GeneScan ABI Prism 310 (Applied Biosystems). Peaks derived from primer dimers were excluded from analysis after cross-checking with a negative control. T-RFLP profiles were based on peak heights.

Statistical analysis

T-RFLP profiles were compared by binning the peaks across profiles using the*treeflap* macro (http://www.sci.monash.edu.au/wsc/ staff/walsh/treeflap.xls). T-RF standardization was performed as described in Dunbar et al. (Dunbar et al., 2001). T-RF patterns were regarded as total bacterial community profiles. The methanotroph community (derived from microarray

analyses) and total microbial community composition were analyzed using ANOSIM and Permanova analysis (Primer6 + PERMANOVA, Primer-E ltd, Plymouth, UK). Methanotroph and total community diversity data were visualized using non-metric multidimensional scaling (MDS) ordination and canonical analysis of principal coordinates (CAP) analysis (Primer 6) respectively. R ver. 2.13.2 (R Development Core Team, 2011) was used for box plots diagrams showing methane oxidation potentials and *pmoA* copy numbers. Diversity indices (richness and evenness) were calculated with Primer 6 (Primer-E ltd, Plymouth, UK).

Results

Methane oxidation potential

The methane oxidation (MOx) potentials of both upper and lower layers were lower than control (sampling T0) levels after drought and flood disturbances among all samples (Fig. 1). In the lower layers MOx potentials from drought and flood disturbed soils increased by 100% ± 4% and 316% ± 3%, respectively, from sampling T0 to sampling T1. Two days following the treatments (samplingT1), MOx potentials of all lower layers in the disturbed samples had increased significantly ($p<0.001$). In the upper layers, MOx potentials increased by 32% ± 4% and 189 % ± 3% in drought- and flood-treated soils, respectively on sampling T1 compared to sampling T0. MOx in the flood-treated soil recovered faster than under drought conditions. At the last sampling day (sampling T4), MOx potentials of lower layers reached similar levels under drought and flood conditions.

Methanotroph abundance

During the time course of the experiment, the mean abundance of *pmoA* genes derived from type Ia varing from T0 to T4 ranged between $1.36\times10^{4}$and 9.22×10^{8} g^{-1} wetsoil, whereas the mean abundance of type Ib MOB*pmoA* was from $2.11\times10^{5}$to 2.35×10^{9} g^{-1} wet soil. The mean abundance of type II MOB *pmoA* varied between $4.70\times10^{5}$and 1.77×10^{10} g^{-1} wet soil (Fig. 2). There was no increase in type Ia methanotroph abundance (drought: mean value from 1.2×10^{7} dropped to 8.7×10^{6}; flood: mean value from 6.6×10^{6} increased to 7.0×10^{6}; control: mean value from 2.4×10^{7} increased to 9.5×10^{7}) at sampling T1 in any of the mesocosms. In contrast, an increase occurred in type Ib abundance in lower layers of drought (mean value increased from 7.4×10^{5} to 1.9×10^{6}) and flood (mean value increased from 7.1×10^{5} to 4.9×10^{6}) treated soil; and in upper layers of flood treated soil (mean value increased from 1.7×10^{6} to 1.6×10^{7}). Type II MOB abundance increased in all treatments (including the control soil) and layers at sampling T1 (drought: mean value increased from 3.5×10^{6} to 6.9×10^{7}; flood: mean value increased from 2.4×10^{6} to 6.9×10^{7}; control: mean value increased from 2.7×10^{7} to 1.4×10^{8}). In the lower layers, but not in the upper layers, of the drought and flood treatments we found a correlation between the abundance of all types of methanotrophs and time. A strong linear relationshipwas found between type Ia MOB abundance and time (drought: $R^2= 0.67$, $p< 0.001$, flood: $R^2= 0.54$, $p< 0.001$). To a minor extent this correlation was also seen in the control samples but the relationship was weaker than in the perturbed samples ($R^2=0.30$, $p< 0.05$). Similarly, the abundance of type Ib MOB in the lower layers correlated with time (drought: $R^2= 0.70$, $p<0.001$; flood: $R^2=0.58$, $p<0.001$; control: $R^2=0.38$, $p<0.01$). Furthermore, type II MOB *pmoA* copy numbers increased in lower layers ((copy number in log scale) drought: $R^2= 0.63$, $p<0.001$; flood: $R^2=0.60$, $p<0.001$; control: $R^2=0.40$, $p<0.01$). In the upper layers type II MOB copy numbers showed linear correlations with the time course in drought and flood treatments but not in control

samples (drought: R^2= 0.33, p< 0.01; flood: R^2=0.54, p<0.001). At the final sampling time (day 83), abundances of type Ia, Ib and II MOB from all lower layers of disturbed soil were higher than MOB copy numbers at sampling T0. These increases were significant, but no significant changes were found in the upper layers. ANOSIM analysis suggested perturbations had no effect on MOB abundances (R=0.031, p> 0.05).

Abundances of type Ia, type Ib and type II MOB showed significant correlations with methane oxidation potential (Pearson's r= 0.33, 0.21 and 0.23 respectively, all p< 0.05).

Methanotroph community composition analyses

Microarray evidenced the presence of type Ia, Ib and II methanotrophs in disturbed as well as control samples (Fig. 3). The signals of probes representing type II MOB were stronger than those targeting type Ia and type Ib probes. *Methylocystis* (probes Mcy459, Mcy264, Mcy270, Mcy413, Mcy255, McyB304, Mcy522, and Mcy233) appeared to be dominant within the MOB communities in all samples. Signals of *Methylocystis* probes displayed a decreasing trend with time compared to sampling T0. Similarly, signal intensities of probe ARC2-518 targeting a deep branching *Methylosinus*-related clade decreased from the first to the last sampling time . The signals of probe McyM309 associated with *Methylocystis* strain M, probes related with the different clades of *Methylosinus* (MsiS475, Msi263, MsiS314, MsiT214, Msi294, Msi423, Msi294, Msi232) and probes specific for peat clones (peat 264) increased with time. The signals of probes associated with type Ia MOB appeared sporadically. The signal of probe MbC11-403 targeting a clade of *Methylobacter* and the signal of probe LP20-607, targeting *Methylomicrobium* LP20 group appeared only under drought conditions of the fourth replicate mesocosm. Probes Mb_SL#3-300, Mb282 and Mb292 targeting *Methylobacter*, Mmb304 and

Mmb562 targeting *Methylomicrobium* appeared at all sampling points. Mb380 and Mb271, targeting *Methylobacter* appeared in three out of four mesocosms.. The probes targeting type Ib clade 501 (501-375 and 501-286) were only positive at day 0 in the upper layers of perturbed soils. The intensities of probes MclS394 and MclS400 specific for *Methylocaldum* were weak in all soils. The intensities of type Ib fw-1 clade probes fw1-639 and fw1-641 were stronger at day 0 than at later stages.

ANOSIM analysis (R=0.46, p=0.001) and PERMANOVA (Table 1) analysis suggested a significant time effect on the methanotroph community composition confirmed by CAP plot (Fig. 4). PERMANOVA (Table 1) also showed that there was a significant interaction between disturbances and layers. The Shannon diversity based on the richness and evenness (microarray analyses) and evenness indices showed that there were no significant differences between different disturbances on MOB diversities (all $p<0.05$). There was no significant correlation between MOB diversity evenness and methane oxidation potentials ($r = 0.034$, $r> 0.05$).

Composition of total microbial community structures

ANOSIM and PERMANOVA analysis (Table. 2) analysis revealed significant differences between five different sampling time points (R=0.322, p=0.001) confirmed by CAP analysis = (Fig. 5). ANOSIM (R= 0.093, p=0.001) and PERMANOVA (Table 2) analysis evidenced that layer was also a significant factor influencing community structure. There was no significant effect of disturbances on the structure of total microbial communities (R=0.001; p=0.49). There was no significant correlation between total microbial community evenness and methane oxidation potentials ($p=0.003$, $r > 0.05$).

Discussion

In this study we investigated the resistance and resilience of a specific functional clade, methane-oxidising bacteria, against flooding and drought. The study was performed in four mesocosms containing the same soil and very similar methanotroph communities, but containing microbial communities of different complexities (chapter 3).

After recovery of the soil microbial communities (chapter 3), soils were subjected to flooding and drought. Perturbation significantly reduced the methane oxidation potential of methanotrophs by app. 75% as compared to the control treatments. Furthermore our results indicated that the abundance of type Ia and type II methanotrophs decreased under drought or flooding conditions. However, microarray analysis evidenced that in contrast to methanotroph abundance, the composition of methane-oxidizing bacterial communities was not significantly affected by the disturbances. This observation is consistent with previous assumptions by Allison and Martiny (Allison and Martiny, 2008), who suggested that little change in microbial composition may be along with large changes in ecosystem process rates. This would suggest that changes (drought and flood) may directly influence the microbial numbers and functioning (methane oxidation potentials) without affecting microbial community (methanotrophs) composition. Similarly, Vanhala (Vanhala et al., 2011) showed that higher temperature accelerated soil organic matter decomposition but did not change microbial compositions. Very few studies addressed the effect of moisture content on methanotroph communities. However, it was often found that MOB compositions followed seasonal changes suggesting that MOB community compositions are different between summer (wetter) and winter (drier) (Abell et al.,

2009; Kumaresan et al., 2009). These temporal changes may indicate that longer exposures to lower and higher moisture conditions could influence the composition of MOB communities.

Methanotroph activity recovered very quickly. Already two days after perturbation methane oxidation potentials in the lower layers were close to that of the control treatment. The methane oxidation potential in all treatments and layers recovered to near control levels after 13 days. The soil samples from drought treatments showed slower recovery rates than the samples from flood treatments, which indicated that drought may cause more severe damage to MOB communities.

While methanotroph community compositions showed remarkable resistance against the perturbations applied, they changed with time, following a similar succession trajectory (Figs. 3 and 4). The observed changes were much stronger than those found due to inter-laboratory differences in a recent ring analysis study (Pan et al., 2010), minimizing the likelihood of a systematic artefact. If the observed changes are indeed real, the most likely explanation is that even after four months, methanotroph communities continue to change and adapt to their new environment (i.e., reduced soil structural heterogeneity, higher and more constant methane flow, relatively stable conditions of a climate controlled environment).

Microarray results showed that type II methane oxidizing bacteria were dominant throughout the experiment. The relative abundance of type Ia and type Ib methanotrophs increased with time. Generally, abundances of type Ia, type Ib and type II MOB correlated with methane oxidation potentials. The abundance of type I methanotrophs correlated with the methane oxidation potentials also before the perturbation started (see chapter 3) whereas the abundance of type II methanotrophs did not.

Moisture content is often regarded as one of the most important environmental effectors on microbial community compositions and functions (Tiemann and Billings, 2011; Brockett et al., 2012). High moisture content would impede oxygen permeating into the soil which could tamper with bacteria heavily relying on oxygen content such as methanotrophs. In our study, lower layers consistently showed higher methane oxidation potentials than upper layers. This may be due to higher methane input reaching the lower layers. *Methylosinus* appeared with time and became more abundant in the lower layers suggesting that *Methylosinus* favors high methane and lower oxygen concentrations (Scott et al., 1981). *Methylosinus* also appeared more often in drought treated samples than in flooded and control samples. The capacity to form endospores may be responsible for better survival rates under dry conditions (Whittenbury et al., 1970).

There are few studies on short term effects of soil water content on methanotroph communities. Fierer *et al.* (Fierer et al., 2003) found that a dry-wet disturbance influenced soil microbial diversity of an oak stand but not that of a grass site, because the latter was more exposed to moisture stress. Our findings support the latter observation as drought and flood did not cause significant changes in methanotroph and microbial diversity.

The behavior of methanotroph communities in this study was different from findings in other studies using other model organisms e.g. nitrifiers (Szukics et al., 2010) where rapid changes were observed in N cycling according to different water contents. This may be due to the fact that compared to methane oxidation; N cycling involves many more individual steps. Some of these, like NOx emission is promoted by dry conditions, whereas others like N_2O production is more likely to happen under high moisture conditions (Firestone and Davidson, 1989). Wittebolle et al. (Wittebolle et al., 2009) suggested that denitrifying bacterial communities characterized by a higher community evenness are more resistant to salinity stress. In contrast,

our study did not show such a significant correlation between the initial total microbial communities' evenness or initial MOB communities' evenness and MOB resistance to flood or drought (both $p>0.05$). Comparing to Wittebolle's system, we generated a more complex environment for methanotroph, which may lead to two opposite results.

In our study, neither methanotroph community nor overall microbial community composition were significantly affected by disturbance but both changed significantly with time. This shows that under the tested conditions communities, at least in terms of diversity, showed high resistance. Disturbance decreased the abundance of methanotrophs, which was accompanied by a decrease in function (methane oxidation potentials). In terms of abundance and function, methane oxidizing communities showed resilience as they adapted and at the end of the experiment did not behave differently to those of the control treatment.

Our study elucidated the influence of flood and drought stress on the floodplain methanotroph community embedded in different total microbial communities with a well controlled mesocosm system. Methanotroph abundance and methane oxidation potentials were suppressed by stress; however, both recovered rapidly, with approaching or reaching control levels within 2 or 13 days after perturbations were stopped. Our study showed that floodplain methanotroph community composition displayed a high level of resistance against varying moisture conditions while provided with sufficient methane supply. The level of resistance and resilience displayed was similar across our four replicate mesocosms despite the different initial microbial diversities present. This suggests the role of the total microbial community in the resistance and resilience of a functional guild may be limited / may display a high level of functional redundancy.

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Table 1.PERMANOVAtest on the microarray data after removal of values for universal and control probes, only keep the signals larger than 1.5 % of reference values. Disturbance is regarded a fixed factor. Time and layer factors are regarded as random factors.CV (√)denotes the square root of the component of variation (Anderson, 2008), a measure of the effect size in units of the community dissimilarities. Significant effects ($p<0.05$) are shaded. "Ti" refers to time, "Di" refers to disturbances and "La" refers to layer.

Source	Pseudo-F	CV (√)	P
Di	1.03	0.67	0.48
Ti	10.23	17.95	0.001
La	3.29	5.57	0.039
Ti * La	2.73	6.52	0.001
Ti * Di	0.79	-2.74	0.742
Di * La	1.34	2.25	0.274
Ti * Di *La	1.00	-0.46	0.456

Table 2.PERMANOVAtest on T-RFLP data after standardization. Disturbance is regarded a fix factor. Time and layer factors are regarded as random factors. CV (√) denotes the square root of the component of variation (Anderson, 2008), a measure of the effect size in units of the community dissimilarities. Significant effects ($p<0.05$) are shaded. "Ti" refers to time, "Di" refers to disturbances and "La" refers to layer.

Source	Pseudo-F	CV (√)	P
Di	1.4508	4.14	0.193
Ti	9.8853	24.18	0.001
La	4.9707	11.49	0.006

Fig. 1. Methane oxidation potential (MOx) at different time points and treatments. "D" represents disturbance by drought, "W" disturbance by flooding and "N" stands for the control treatment. "L" represents lower layers, "U" upper layers. Error bars represent standard error between replicates. Different lowercase alphabet letters represent different statistical differences.

Fig. 2. Figure A, B and C show *pmoA* gene copy numbers of type Ia, Ib and II methanotrophs at different time points and in different treatments and layers, respectively. D" represents disturbance by drought, "W" disturbance by flooding and "N" stands for the control treatment. "L" represents lower layers, "U" upper layers. Error bars represent standard error between replicates. Different lowercase alphabet letters represent different statistical differences.

Fig. 3. Methanotroph community analysis of each samples across the time. Results of individual microarray experiments were first normalized to the positive universal methanotroph control probe mtrof173, then to the reference values determined for each individual probe. Only signals larger than 1.5 % of reference values were chosen and displayed using GeneSpring software. A value of 1 (see colour code bar) indicates maximum achievable signal for an individual probe, while a value of 0.1 indicates 10 % signals (which, in turn, indicates that around 10% of the target hybridized to that probe

Fig. 4. Canonical analysis of principle coordinates (CAP) analysis of *pmoA* microarray data Highlighting the difference in MOB community diversity between 5 different time points, leave one out cross validation (Anderson et al., 2008) gave 87.5 % correct assignment to time points.

Fig. 5. Canonical analysis of principal coordinates (CAP) analysis of T-RFLP data performed using the Primer 6 package (Primer-E, UK) on standardized T-RFLP data. T-RFLP peaks are regarded as variables. Highlighting the difference in total microbial community structure

between 5 different time points leave one out Cross Validation(Anderson et al., 2008) gave 85.7 % correct assignment to time groups.

Figure 1

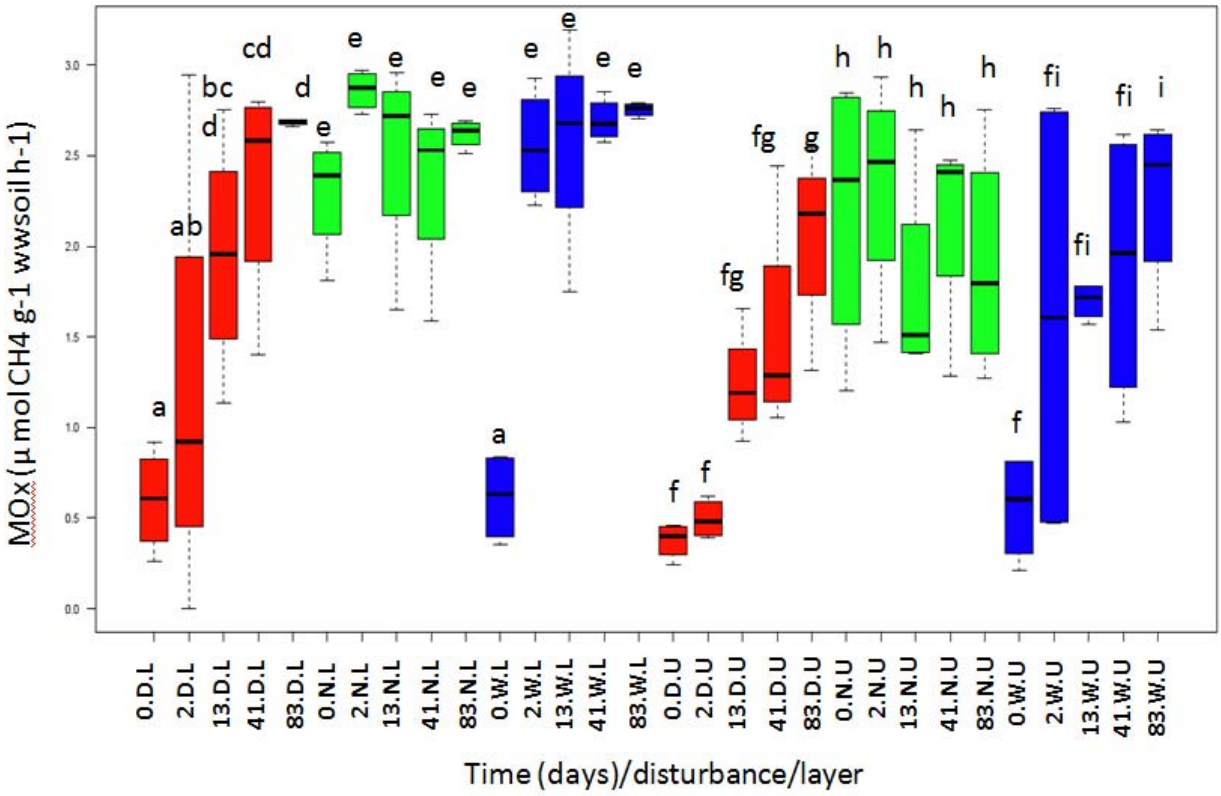
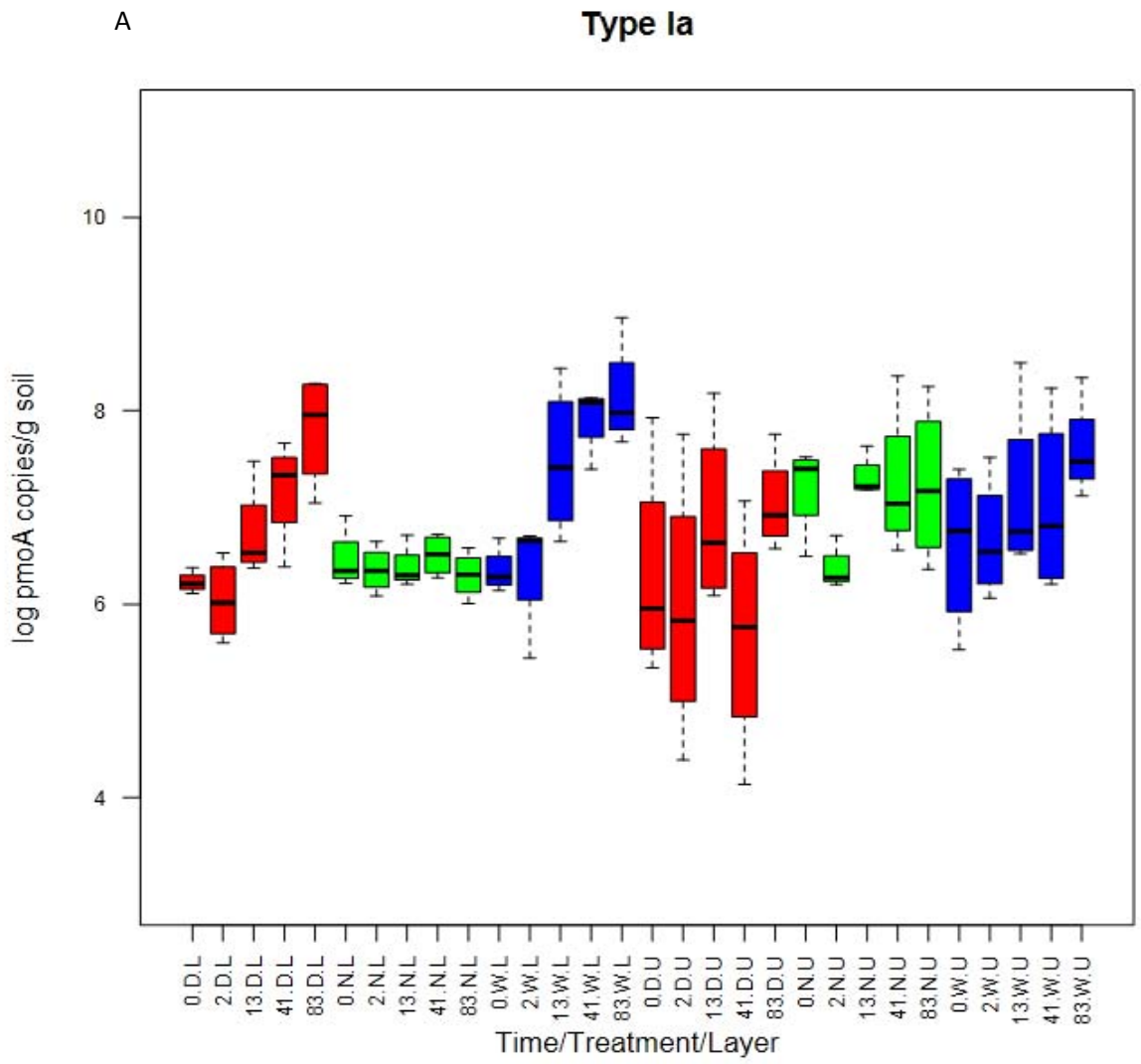
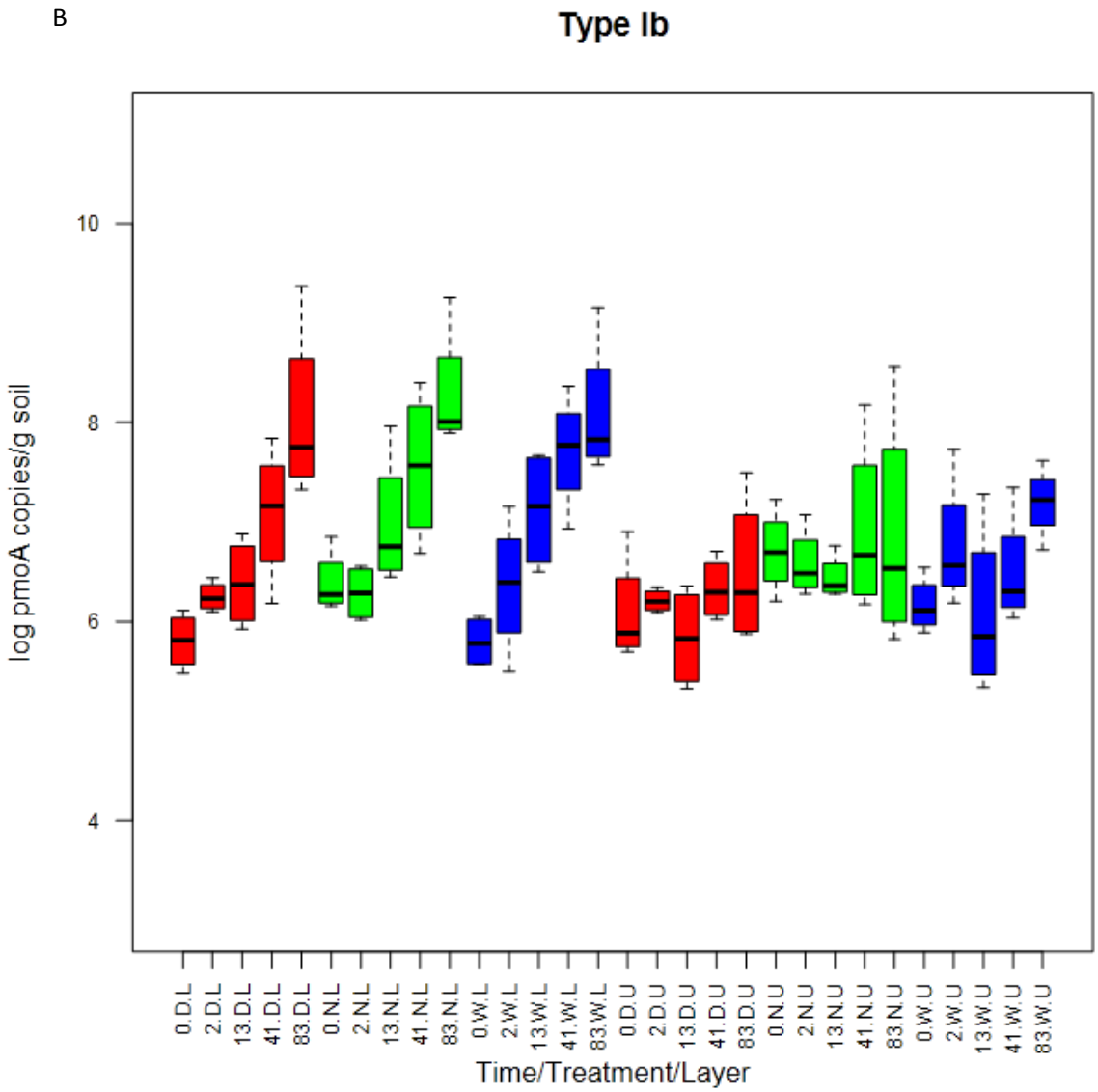

MOx (µ mol CH4 g-1 wwsoil h-1)
Time (days)/disturbance/layer
0.D.L
2.D.L
13.D.L
41.D.L
83.D.L
0.N.L
2.N.L
13.N.L
41.N.L
83.N.L
0.W.L
2.W.L
13.W.L
41.W.L
83.W.L
0.D.U
2.D.U
13.D.U
41.D.U
83.D.U
0.N.U
2.N.U
13.N.U
41.N.U
83.N.U
0.W.U
2.W.U
13.W.U
41.W.U
83.W.U

Figure 2


A
Type Ia
log pmoA copies/g soil
10
8
6
4
0.D.L
2.D.L
13.D.L
41.D.L
83.D.L
0.N.L
2.N.L
13.N.L
41.N.L
83.N.L
0.W.L
2.W.L
13.W.L
41.W.L
83.W.L
0.D.U
2.D.U
13.D.U
41.D.U
83.D.U
0.N.U
2.N.U
13.N.U
41.N.U
83.N.U
0.W.U
2.W.U
13.W.U
41.W.U
83.W.U
Time/Treatment/Layer

B


Type Ib
log pmoA copies/g soil
10
8
6
4
0.D.L
2.D.L
13.D.L
41.D.L
83.D.L
0.N.L
2.N.L
13.N.L
41.N.L
83.N.L
0.W.L
2.W.L
13.W.L
41.W.L
83.W.L
0.D.U
2.D.U
13.D.U
41.D.U
83.D.U
0.N.U
2.N.U
13.N.U
41.N.U
83.N.U
0.W.U
2.W.U
13.W.U
41.W.U
83.W.U
Time/Treatment/Layer

C

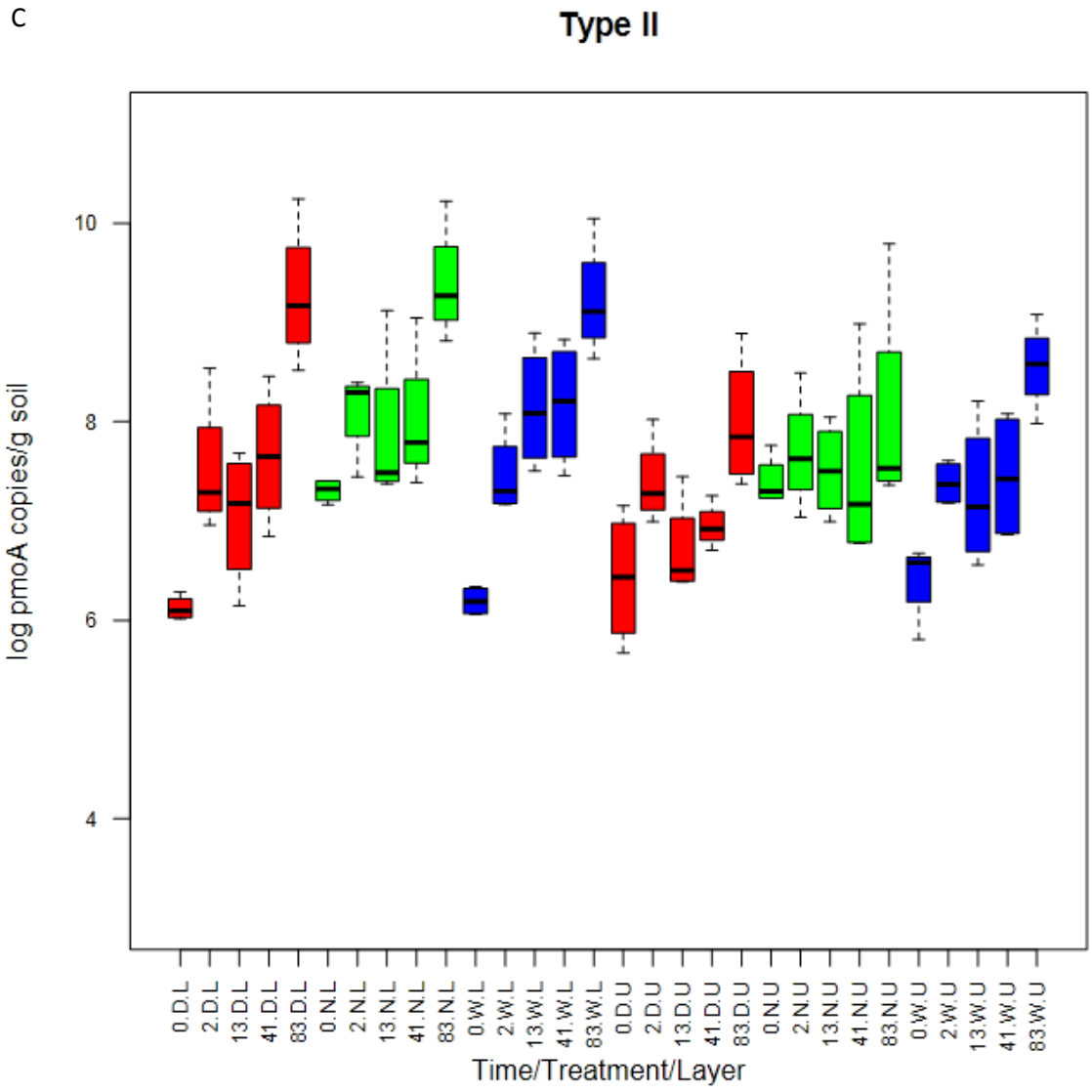

Type II
log pmoA copies/g soil
10
8
6
4
0.D.L
2.D.L
13.D.L
41.D.L
83.D.L
0.N.L
2.N.L
13.N.L
41.N.L
83.N.L
0.W.L
2.W.L
13.W.L
41.W.L
83.W.L
0.D.U
2.D.U
13.D.U
41.D.U
83.D.U
0.N.U
2.N.U
13.N.U
41.N.U
83.N.U
0.W.U
2.W.U
13.W.U
41.W.U
83.W.U
Time/Treatment/Layer

Figure 3

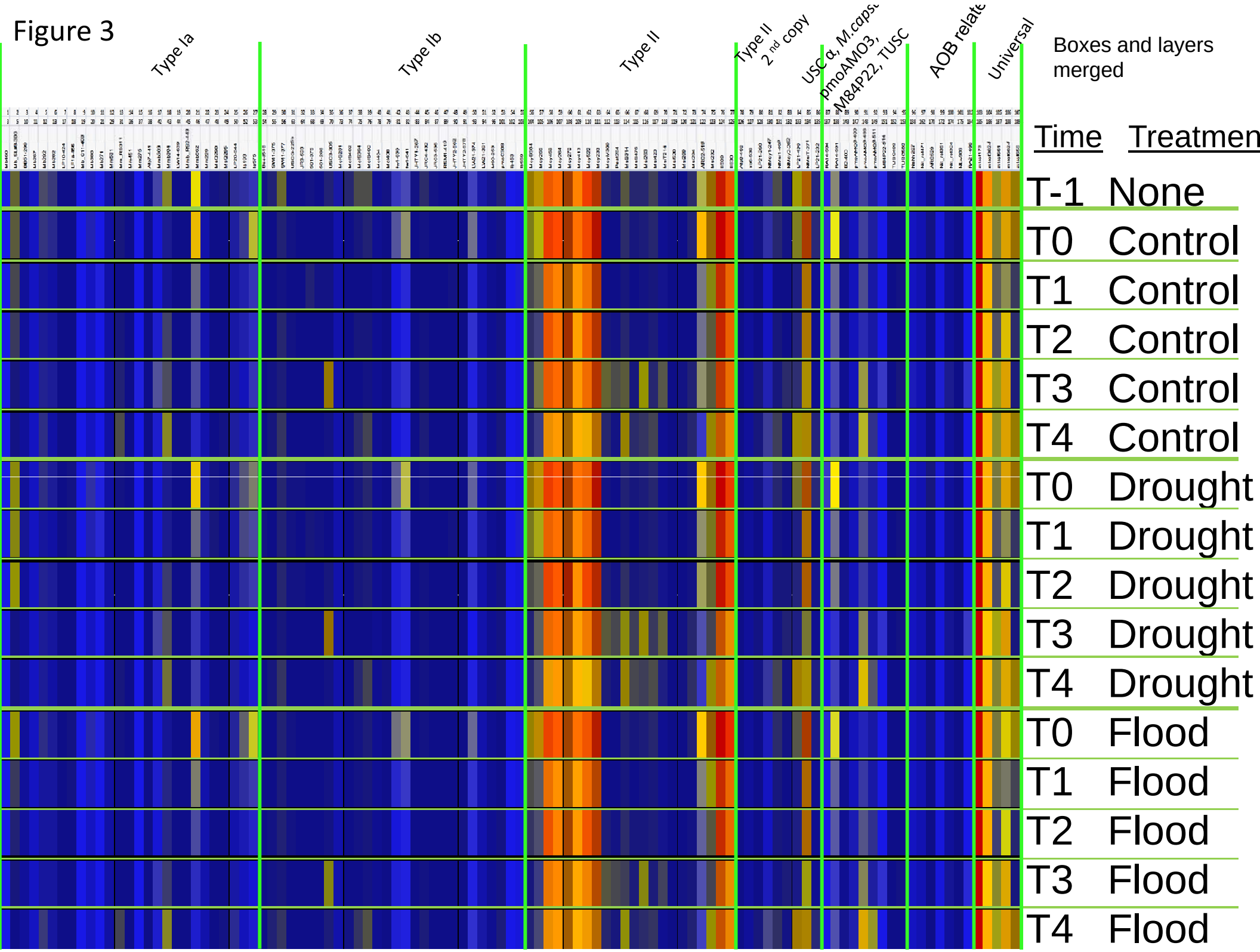

Type Ia
Type Ib
Type II
Type II 2nd copy
USC α, M.capsulatus pmoAMO3, M84P22, TUSC
AOB related
Universal
Boxes and layers merged
Time Treatmen
T-1 None
T0 Control
T1 Control
T2 Control
T3 Control
T4 Control
T0 Drought
T1 Drought
T2 Drought
T3 Drought
T4 Drought
T0 Flood
T1 Flood
T2 Flood
T3 Flood
T4 Flood

Figure 4

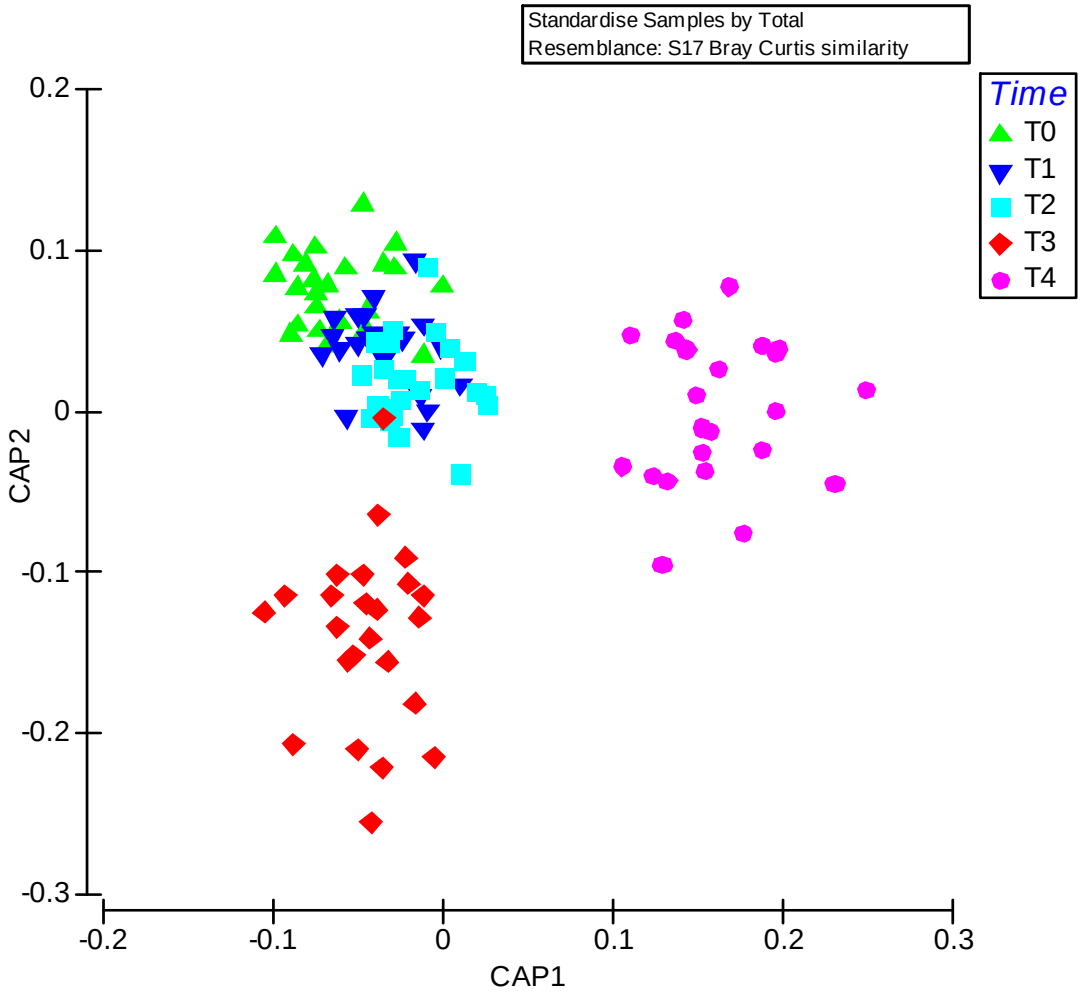
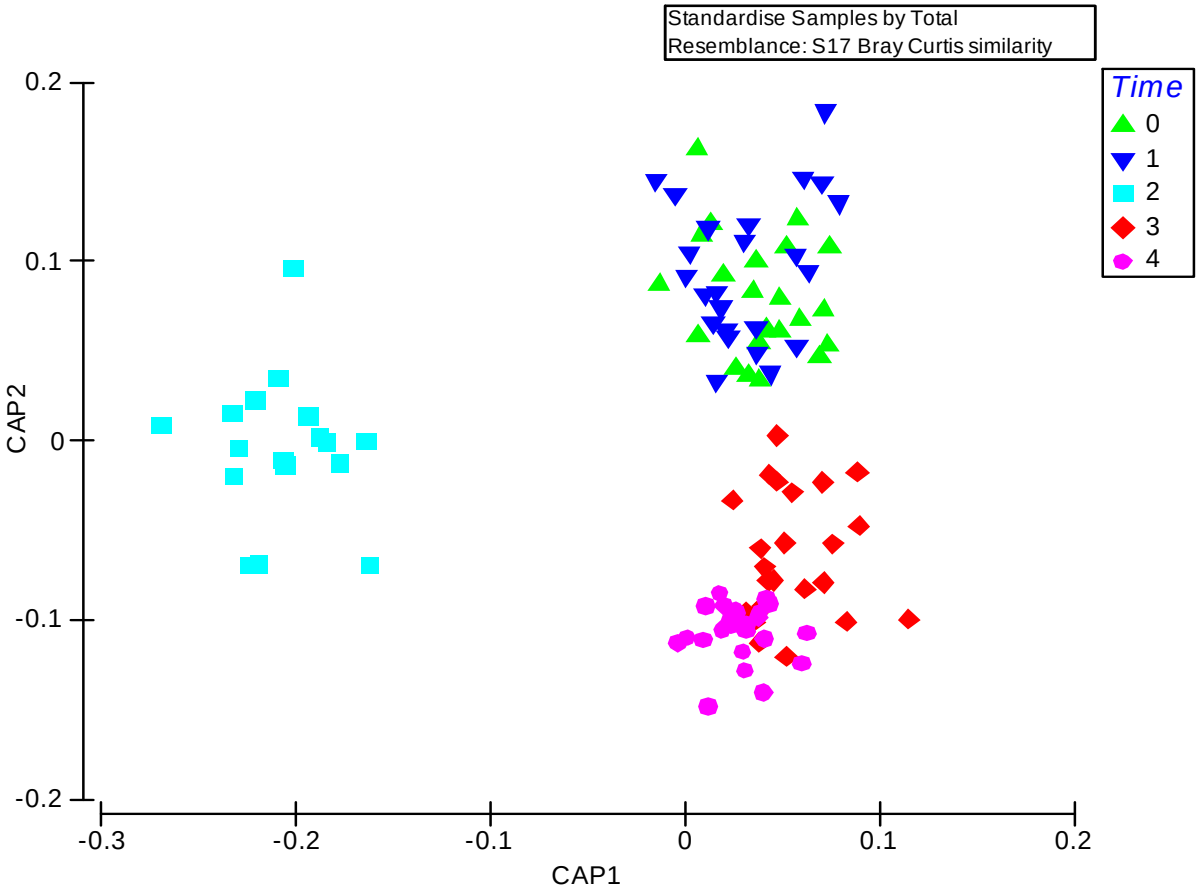

Standardise Samples by Total
Resemblance: S17 Bray Curtis similarity
Time
T0
T1
T2
T3
T4
0.2
0.1
0
-0.1
-0.2
-0.3
CAP2
-0.2
-0.1
0
0.1
0.2
0.3
CAP1

Figure 5


Standardise Samples by Total
Resemblance: S17 Bray Curtis similarity
Time
0
1
2
3
4
0.2
0.1
0
-0.1
-0.2
CAP2
-0.3
-0.2
-0.1
0
0.1
0.2
CAP1

Chapter 5

Final discussion and prospective

The main goal of this thesis was to gain insights into i) feasibility of data comparison in metagenomic studies across laboratories **(Chapter 2)**, ii) methanotroph community recovery and recolonization from different microbial communities assembly history regarding composition, abundance and function **(Chapter 3)**, iii) methanotroph community resistance and resilience against disturbances **(Chapter 4)**. In **Chapter 2** the variations from different investigators and different labs using the same DNA extraction protocol, followed by the same analysis carried out by the same investigator on aliquot samples were evaluated. In **Chapter 3**, mesocosm experiments were established using soils with four different levels of total microbial community complexities. Both the diversity and function of methanotrophs recovered. Methanotroph community compositions changed with time, following a similar pattern even though they were embedded in different total microbial communities. Total microbial community compositions changed with time as well; however, they did not come to be congruent. In **Chapter 4**, drought and flooding disturbances were applied to the mesocosms experiments, described in chapter 3. Neither disturbances affected methanotrophs or total microbial community compositions. Methane oxidation potentials were strongly reduced by drought and flooding, but recovered surprisingly quickly. After disturbances, MOB compositions changed with time following similar succession trajectories instead of going back to the original community composition.

5.1 Assessing intra- and inter-laboratories of microbial community composition analysis

It was shown that the quality and quantity of extracted DNA from a single soil sample using identical protocols can differ between laboratories and investigators resulting in significant bias MOB community structure. Comparing communities between various samples necessitates to assess this bias. Reducing the bias to a minimum, it is recommended to perform all DNA extraction in one lab, preferably using an automated procedure, including identical chemicals and cell-disruption machines. In large scale projects with multiple research groups involved, all downstream processing steps should be executed using PCR ingredients purchased with the same company, preferably from the same production batch. However, it is likely that in many cases and projects these criteria are difficult to meet. An alternative would be to develop an internal control system which gives information on the biases between labs. Hence, every involved laboratory adds a reference amount of target DNA in the extraction procedure. In this way an indication will be obtained of the biases between labs and a correction factor may be taking into the interpretation of the results.

5.2 Recovery of methanotrophs from different microbial assembly histories

This study followed the recovery of methanotroph community and function in soils of different life histories and overall microbial diversities, following inoculation with the original untreated soil. Methane oxidation potentials displayed different recovery rates according to the different microbial community assemblies. Gamma irradiation treatment which resulted in the lowest initial diversity of microbial communities showed the lowest methane oxidation recovery rate but exceeded over other treatments with time which suggested that methanotrophs diversity may adapt in a better way due to reduced competitive exclusion. The soil which was sterilized by

gamma irradiation treatment also demonstrated a more diverse and more disperse total microbial community. To the author's knowledge there is only one study done on MOB recovery from gamma irradiation treatment (Ho et al., 2011) though we set up a different experimental system. However, findings in this study confirmed Ho's results showing higher cell numbers of MOB in gamma irradiation treated soil compared to the control. The presence of many unique T-RFs in the gamma irradiation soil indicates the presence of bacteria or bacterial groups, which were able to manifest under these conditions but probably were out-competed in other treatments. In all treatments, type II methanotrophs were dominant throughout the experiment, but there was no positive correlation between type II abundance and MOx potentials. Type I MOB abundance increased with time and their copy numbers were positively related to MOx potentials, which was consistent with previous findings that type I MOB might be the active "workers" whereas type II MOB are often resting cells forming a microbial "seed bank" (Eller et al., 2005). A similar succession trajectory developed in methane oxidizing bacteria communities in all four mesocosms despite the strong differences in the initial total microbial communities. We assumed that selective pressure (relatively high methane flow) had a stronger influence on the methanotroph community than the different treatments. Total microbial community compositions ended up being significantlydifferent, which indicated that different bacterial species at the different stages depended on trade-offs between the species to colonize the available space versus their ability to compete with other species.

5.3 Recovery of methanotrophs from flood and drought disturbances

Drought and flooding disturbances were applied to the mesocosms following the experiments described in chapter 3. Disturbance decreased the abundance of methanotrophs, which was accompanied by a decrease in function (methane oxidation potential). However it did not influence methanotroph community compositions, which suggested that floodplain MOB diversity compositions, in this study, showed strong resistance to moisture variation. MOx potentials recovered rapidly following disturbances. However, soil from drought treatments showed slower recovery rates than soil samples from flood treatments, which indicated that drought may cause more severe damage to MOB communities. Both type I and type II MOB abundance increased during incubation and had positive correlations with MOx potentials. Interestedly type II MOB did not show correlation between abundance and MOx potential before disturbances **(Chapter 3)**. This may suggest that type II MOB need time to adapt to new environments in order to contribute to the oxidation of methane; this is in line with previous findings by Ho et al. (Ho et al., 2011) that there was a temporal shift of type II MOB abundance. There was no simple correlation between initial evenness of methanotroph or total microbial community and the resistance of methane oxidation potentials, in contrast with the theory that community evenness favors functionality under stress (Wittebolle et al., 2009). It should be pointed out that this experiment **(Chapter 4)** was following the experiment in chapter 3 where similar methanotroph communities developed in soils of different life histories and total microbial diversities, which indicated that the total microbial diversity surrounding methanotrophs did not significantly influence MOB resistance. We hypothesized that under high methane flow, total microbial community may have minor influence on MOB resistance.

5.4 Future prospective

This thesis work was part of the European research project METHECO "The role of microbial diversity in the dynamics and stability of global methane consumption: microbial methane oxidation as a model system for microbial ecology." In this work we focused on testing the feasibility to compare the experimental results between different laboratories **(Chapter 2)** and used methanotrphs as model organisms to study their behaviors linking to their function under different stresses **(Chapter 3 and 4)**. We raised several suggestions for inter-and intra-laboratory results comparison for the similar work. We found under steady methane flow, the community composition and function of methanotrophs ware remarkably robust against drastic changes in the overall microbial diversity as well as drought and flood stress. However, in our study only floodplain soils were examined. It is not known whether methanotrophs from other habitats would behave similarly or differently. There have been numerous studies on methanotroph communities in various habitats. However, there was little research focusing on how MOB maintain their functions. Researches into how methanotrophs from different habitats would cope with different treatments will lead to a better understanding of microbial world and their roles in maintaining the ecosystems.

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1. Activity and structure of methanotrophic communities in landfill cover soils. Gebert J, Singh BK, Pan Y, Bodrossy L. Environmental Microbiology Reports, 1(5), 414–423, 2009.
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