Methane Turnover in Desert Soils

Doctoral thesis

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by

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To my father

"... I find that the plains of Patagonia frequently cross before my eyes; yet these plains are pronounced by all wretched and useless. They can be described only by negative characters; without habitations, without water, without trees, without mountains, they support merely a few dwarf plants. Why, then, and the case is not peculiar to myself, have these arid wastes taken so firm a hold on my memory?"

Charles Darwin
The Voyage of the Beagle, 1839

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Deserts cover about a third of the land surface on Earth. However, despite their size, their ecology – and particularly their microbial ecology – is far less understood than the ecology of more humid regions. Previous studies have indicated that desert soils might be involved in the production and consumption of methane, an important greenhouse gas. The turnover of atmospheric gases involves many microorganisms, and methane is no exception – it is both produced and consumed by microbes. Despite the extensive research methane has been subjected to, a rigorous study striving to elucidate methane turnover patterns in arid regions and aiming to detect the active organisms involved has not been conducted so far.

This work comprises three parts. The first part deals with biogeographical patterns of soil microbial communities along a steep rainfall gradient in Israel ranging from less than 100 to more than 900 mm yr^{-1}. We show that community profiles of both *Archaea* and *Bacteria* do not change continuously along the gradient, but rather cluster into three groups that we have defined as arid, semi-arid and Mediterranean. These three categories demonstrate a qualitative difference in the microbiology of arid soil compared to more humid regions.

In the second part we show that pristine arid soils in the Negev Desert, Israel, are sinks for atmospheric methane, but that disturbed sites and pristine hyper-arid sites are probably not. The methanotrophic activity was located in a narrow layer in the soil down to about 20 cm depth. Interestingly, the biological soil crust (BSC) which is typically the most active layer in desert soils showed no methane uptake activity and was apparently devoid of methanotrophs. Transcripts of the key methanotrophic gene – encoding for the particulate methane monooxygenase (PMMO) – were detected in the active soils and their sequences showed that they are affiliated with two clusters of uncultured methanotrophs: USCγ and JR3. Based on a correlation of the relative abundance of each methanotroph to the methane oxidation rate we concluded that JR3 is the dominant atmospheric methane oxidizer in this arid system.

The third part deals with methanogenesis in upland soils with a focus on drylands. Following previous work we show that many upland soils,

sampled globally, possess a methanogenic potential, when incubated anoxically, despite being aerated most of the time. Only two active methanogens were detected – *Methanosarcina* and *Methanocella* – which appear to be universal upland soil methanogens. Under these conditions, acetoclastic methanogenesis, mediated by *Methanosarcina*, was the dominant methanogenic pathway and cell numbers of *Methanosarcina* were well correlated with methane production rates.

Lastly, we show that the BSC was the source for methanogenic activity in arid soils while the deeper layers showed little or no methanogenic potential. When the BSC was incubated in a wet state in microcosms and in the presence of oxygen methanogens could still grow and methane was still produced albeit at relatively low amounts. Both methanogens expressed the gene encoding for the oxygen detoxifying enzyme catalase giving at least some explanation to their ability to remain viable in the presence of oxygen. Under these conditions, *Methanocella* was the dominant methanogen and most methane was produced from H_2/CO_2, indicating niche differentiation between the two methanogens.

The findings of this work suggest that under standard dry conditions pristine arid soils are a net sink for atmospheric methane but that following a rain event they might turn into net sources.

Wüsten bedecken circa ein Drittel der Erdoberfläche. Trotz dieser Ausmaße ist ihre Ökologie – insbesondere ihre mikrobielle Ökologie – weit weniger erforscht als die Ökologie feuchter Gebiete. Einige Studien deuten an, dass Wüstenböden an der Produktion und dem Verbrauch von Methan – einem wichtigen Treibhausgas – beteiligt sein konnen. Mikroorganismen sind verantwortlich für den Umsatz atmosphärischer Gase. Methan stellt hierbei keine Ausnahme dar. Es wird sowohl von Mikroben produziert, als auch umgesetzt. Trotz umfangreicher Forschung sind grundlegende Untersuchungen der Methanumsetzung in ariden Gebieten und den aktiv beteiligten Organismen bisher ausgeblieben.

Diese Arbeit besteht aus drei Teilen. Der erste Teil beschäftigt sich mit biogeographischen Verteilungsmustern mikrobieller Bodengemeinschaften entlang eines ansteigenden Niederschlagsradientens in Israel (100 bis über 900 mm Niederschlag yr^{-1}). Es konnte gezeigt werden, dass sich die Zusammensetzung von Bakterien- und Archeengemeinschaften nicht kontinuierlich über den Gradienten ändert, sondern sich eher in drei Gruppen zusammenfassen lässt, welche als arid, semi-arid und mediterran definiert wurden. Diese drei Kategorieren demonstrieren einen qualitativen Unterschied in der Mikrobiologie arider Böden im Vergleich zu feuchten Regionen.

Im zweiten Teil zeigen wir, dass ursprüngliche aride Böden der Negev Wüste in Israel eine Senke für atmosphärisches Methan darstellen, während anthropogen beeinflusste, ebenso wie ursprüngliche hyper-aride Böden dies nicht zu sein scheinen. Die methanotrophe Aktivität wurde in einer schmalen Schicht in ca. 20 cm Bodentiefe gefunden. Interessanterweise zeigte die Oberflächenkruste, welche die typischerweise die aktivste Schicht im Wüstenboden darstellt, keine Methanaufnahme und methanotrophe Bakterien konnten in dieser Schicht nicht nachgewiesen werden. Transkripte des *pmoA* Gens, welches eine Untereinheit des Schlüsselenzyms der Methanoxidation kodiert, wurden in den aktiven Bodenschichten detektiert und die phylogenetische Analyse zeigte eine Zugehörigkeit zu den zwei Umweltclustern USCg und JR3. Die Korrelation von relativen Sequenzhäufigkeiten mit Methanoxidationsraten deutet darauf hin, dass

bislang unkultivierte Bakterien des Clusters JR3 die dominant aktiven Methanoxidierer in diesem ariden System sind.

Der dritte Teil dieser Arbeit beschäftigt sich mit der Methanogenese in *upland soils* (gut durchlüftete Böden) mit dem Fokus auf trockene Böden. Anknüpfend an frühere Arbeiten zeigen wir, dass viele *upland soils*, global verteilt, ein methanogenes Potential besitzen, wenn sie anoxisch inkubiert werden, auch wenn sie in unter natürlichen Bedingungen überwiegend gut durchlüftet vorkommen. Nur zwei aktive methanogene Archeen wurden detektiert – *Methanosarcina* und *Methanocella* – die in *upland soils* allgemein verbreitet zu sein scheinen. Unter den getesteten Bedingungen stellte die acetoclastische Methanogenese – katalysiert von *Methanosarcina* - den dominanten methanogenen Stoffwechselweg dar und Zellzahlen korrelierten gut mit der Methanproduktionsrate.

Zuletzt zeigen wir, dass die Metanogenese in ariden Böden in der Oberflächenkruste stattfindet und tiefere Schichten kaum oder nur wenig metanogenes Potential aufweisen. Selbst bei Inkubation der Oberflächenkruste in Anwesenheit von Sauerstoff zeigten die Methanogenen noch Wachstum und Methan wurde produziert, wenn auch in vergleichsweise niedrigen Raten. Beide nachgewiesenen methanogenen Arten besitzen Katalase-Aktivität, was zumindest zum Teil ein Überleben in sauerstoffreicher Umgebung erklären könnte. Unter diesen Bedingungen dominierte *Methanocella* als methanogene Art und das Meiste Methan wurde über H_2/CO_2 produziert. Dies weist auf eine Nischendifferenzierung zwischen den beiden Methanogenen hin.

Die Ergebnisse dieser Arbeit lassen annehmen, dass ursprüngliche aride Böden unter trockenen Bedingungen Senken für atmosphärisches Methan darstellen, jedoch könnten Regenfälle diese Senken in Methanquellen verwandeln.

Chapter 1 |

Introduction

1.1 Atmospheric methane

Methane is the simplest possible hydrocarbon; it is very light and flammable, and readily volatile. The discovery of methane is attributed to Allesandro Volta. In 1778 he collected gas which emerged when the shallow sediment of Lake Maggiore, Italy, was disturbed and observed that it is very flammable. The experiment was later repeated in 1822 by John Dalton who termed the gas 'carbonated hydrogen' (Dalton, 2005). From a human perspective, methane is of interest for two main reasons: first, burning methane can be utilized for the production of energy, such as in electrical generators. Second, it possesses greenhouse properties and thus contributes to global warming, which is rightly considered one of the greatest threats humanity is currently facing (Houghton, 2005). In fact, methane is the third most important greenhouse gas, after water vapours and CO_2, making it a subject of interest for extensive research. Traditionally it was assumed that methane is approximately 25 times more potent than CO_2 as a greenhouse gas over a 100-years time horizon, but recent modelling which accounted for direct and indirect interactions with aerosols now estimate that it is 26-41 times more potent than CO_2 as a greenhouse gas (Shindell et al., 2009).

Methane has probably existed on planet Earth soon after its formation some 4.5 billion years ago due to early volcanic activities. However, it apparently did not accumulate in significant amounts and contributed little to the developing atmosphere (Kasting, 1993; Canfield et al., 2006). Today, geological methane seeps still play an important ecological role in some marine environments and contribute partly to the atmospheric methane pool, but overall geological methane sources contribute little to the global

budget (Reeburgh, 2007; Alperin and Hoehler, 2010). Starting from the emergence of methanogenesis – probably in the mid-Archean era – and up to our present times, most methane produced on earth (up to 80%), and as a consequence most methane in the atmosphere, is of biogenic origin (Bréas et al., 2001). From the mid-Archean and up to the early Proterozoic era, prior to the oxidation the atmosphere, methane accumulated substantially in the atmosphere, up to 10^5 – 10^6 ppbv, and played a much greater role as a greenhouse gas (Catling et al., 2001). The only known way where biogenic methane is formed in nature is through the anaerobic methanogenesis process, for which a single guild of microorganisms – the methanogenic Archaea – is responsible.

Present levels of atmospheric methane are 1,774 ± 1.8 ppbv making methane the most abundant hydrocarbon and the 7th most abundant gas in the atmosphere (excluding water; Forster et al., 2007). This present concentration, however, is not all the result of natural ecosystem processes. For the past 800,000 years, methane levels in the atmosphere varied between 350-800 ppbv (Spahni et al., 2005; Loulergue et al., 2008) but in the past 250 years or so they have increased dramatically up to the present concentration, primarily due to increasing anthropogenic activity (Forster et al., 2007).

Natural wetlands are, according to most estimates, the largest source of atmospheric methane, emitting over 200 Tg CH_4 yr^{-1}, yet the combined contribution of human induced activities is larger (**Figure 1.1**). These comprise the largest part of the present global budget and include primarily cattle raising, rice agriculture, and fossil fuel mining which, along with other activities, emit some 350 tons of methane into the atmosphere annually. An additional source of methane of global importance has been recently recognized – non-biogenic methane emission from plant leaves (Keppler et al., 2006). It is currently believed that this methane is formed by photochemical cleavage of methyl groups from the pectin found in leaf tissues by UV radiation (McLeod et al., 2008). Reliable estimates of the magnitude of this source on a global scale are presently still missing and it is therefore not yet incorporated into most global methane balances.

While it is believed that the major sources and sinks of methane are known, there are great uncertainties regarding the exact magnitude of each of them (**Figure 1.1**). These uncertainties are manifested in the often large differences between global models (Potter et al., 1996; Lelieveld et al., 1998; Bréas et al., 2001). Particularly, the magnitude of the different sources is debatable and the existence of a missing source has been postulated. One possible missing source is plant leaves as described above, but others have suggested earlier that it lies in wetlands and water reservoirs in the Amazon Basin (Melack et al., 2004; Frankenberg et al., 2005). In addition, although methane has been increasing at an average rate of 4.4 ppbv yr^{-1}, a large interannual variability in methane emissions has been observed and also long term changes (Blake and Rowland, 1986). The rise in atmospheric methane concentrations seems to have been slowing down since the late 80's (Wang et al., 2004; Dlugokencky et al., 1998) and has virtually ceased since 1999 (Dlugokencky et al., 2003) probably due to changes in anthropogenic activity (Bousquet et al., 2006; Rigby et al., 2008; Worthy et al., 2009). Whether the rise in atmospheric methane has really ceased is still in question and a recent study suggests a renewed increase (Rigby et al., 2008).

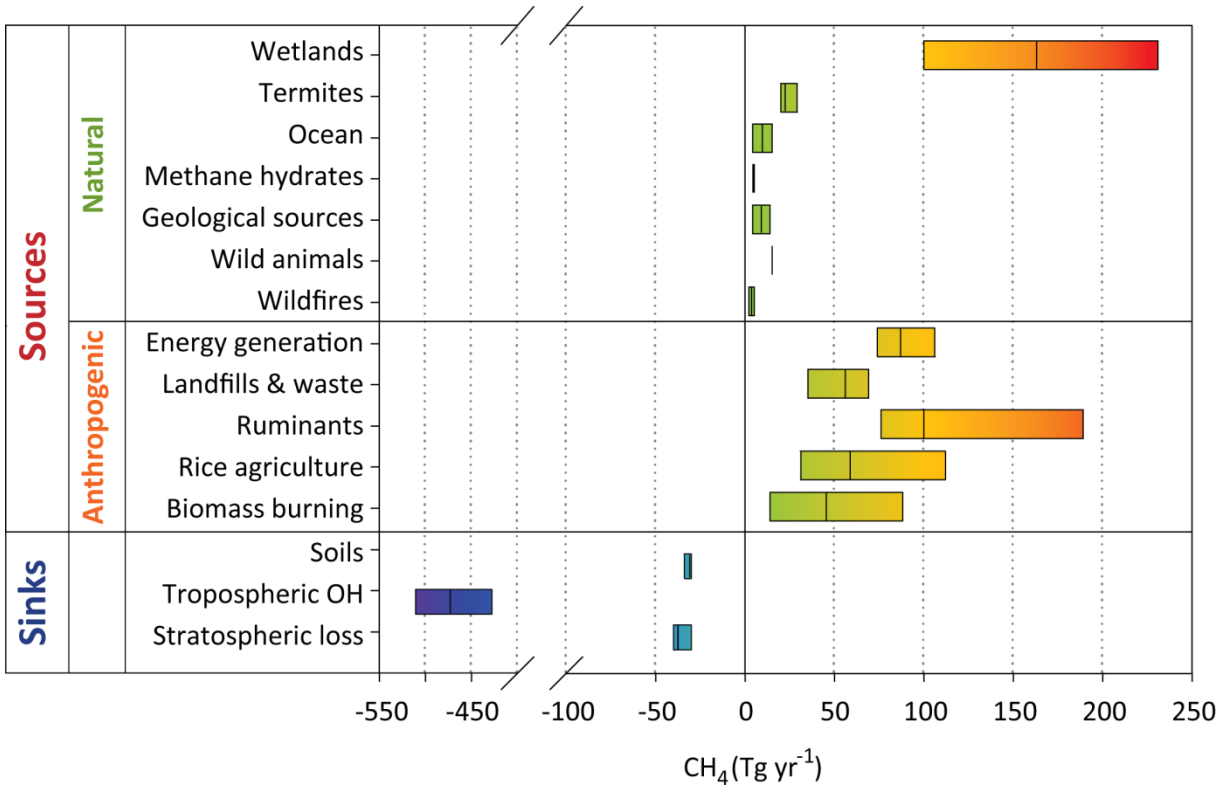

Figure 1.1| Sources and sinks of atmospheric methane. Bar widths represent estimate ranges, the line within each bar represents the mean. Total budget is estimated at 574 ± 91 Tg yr^{-1}; present atmospheric concentration of methane is approximately 1.8 ppmv. Data: Denman et al., 2007.

It is estimated that about one to two percent of all photosynthetically fixed carbon is ultimately degraded into biogenic methane. A minor part of it gets trapped and buried and exits the carbon cycle, but most if it diffuses to the oxic zone. In the intestines of ruminants and termites methane is released directly into the air, but in soil and freshwater sediments, where an oxic/anoxic interface exists, a large part of that methane is consumed by methane oxidizing bacteria (methanotrophs) and only some of it ends up in the atmosphere. The fate of most geological methane is similar. Geological methane which is released due to human activities such as fossil fuel mining is almost directly and fully released into the atmosphere, but the methane which bubbles naturally in oceanic methane seeps and mud volcanoes is mostly oxidised by anaerobic methane oxidisers (Knittel and Boetius, 2009). Eventually, it is estimated, only about 42% of the methane formed on earth from either biological or geological sources reaches the atmosphere while the rest is consumed by microorganisms (Reeburgh, 2006). In these

environments, the difference in activity between methanogens and methanotrophs and their independent population dynamics, is what determines their magnitude as methane sources (Conrad, 1996).

1.2 Methanogenic Archaea

Methanogens are a guild of microorganisms that produce methane as part of their energy metabolism. All known methanogens are part of the *Euryarchaeota* phylum; they are strict anaerobes and can only use methanogenesis for energy production (Whitman et al., 2006). Unlike other archaeal groups, and in fact most prokaryotes, methanogens are relatively easy to cultivate and a cultured representative exists for virtually every phylogenetic group of methanogens. Methanogens were the first (and for a long time, the only) cultivated representatives of the domain Archaea. Indeed, it was the polymorphic characteristics of the ribosomal RNA of methanogenic species of *Methanobacterium* and *Methanosarcina* which were used by Woese and Fox to postulate the tripartite structure of the Tree of Life in their seminal work from 1977.

Although all methanogens belong to the *Euryarchaeota* their diversity within this single phylum is quite extensive and deeply branching – a further evidence for the primordial emergence of this form of life. Currently there are 30 recognized genera of methanogens (including *Candidatus Methanoregula*) which in turn belong to 11 families and 6 orders spread throughout the euryarchaeal phylogenetic tree: *Methanomicrobiales*, *Methanocellales*, *Methanosarcinales*, *Methanobacteriales*, *Methanococcales* and *Methanopyrales*.

In somewhat of a contrast to their extensive phylogenetic diversity, the metabolic diversity of methanogens is rather limited. Methanogens take a pivotal role in anaerobic degradation of organic matter, yet they are unable to utilize most degradation products and depend on secondary fermenters and syntrophs to provide them with substrates. Methanogenic pathways are often classified into three types: hydrogenotrophic (CO_2 reduction), C1 compounds demethylation and acetate cleavage (**Table 1.1**).

Table 1.1| Most common methanogenic reactions. After Hedderich and Whitman (2006)

Reaction	*$\Delta G^{o'}$ (kJ/mol CH_4)*
Hydrogenotrophic	
$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$	-130
$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	-120
$CO_2 + 4$ (isorpoanol) $\rightarrow CH_4 + 4\ CH_3COOH + 2\ H_2O$	-37
C_1 compounds demethylation	
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-113
$4\ CH_3OH \rightarrow 3CH_4 + CO_2 + H_2O$	-103
$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$	-74
$2\ (CH_3)_2S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$	-49
Acetate cleavage	
$CH_3COOH \rightarrow CH_4 + CO_2$	-33

Different pathways are used by different methanogens and the distribution is not random but rather linked to phylogenetic origin. Thauer and colleagues (2008) proposed to classify methanogens, according to their ecophysiology, into those that posses cytochromes and those that do not. The cytochromes containing methanogens are from the order *Methanosarcinales*, they are characterized by high growth yields and high threshold for hydrogen (when a hydrogenotrophic pathway exists). With the exception of the family *Methanocellaceae* which are strict hydrogenotrophs, all cytochromes containing methanogens are able to produce methane from methylated compounds and/or from acetate. Methanogens which do not contain cytochromes are all strict hydrogenotrophs (with the exception of *Methanosphaera*), have lower threshold values for H_2 but also lower growth yields.

The first type of methanogenesis uses CO_2 as an electron acceptor and hydrogen is normally the electron donor (hydrogenotrophic methanogenesis). Since CO_2 is usually abundant in anoxic environments, hydrogen concentration usually limits this reaction. Methanogenesis based on H_2/CO_2 is the most commonly found pathway in nature and is common among all methanogens. In some environments such as in ruminants and termites it is the dominant pathway and probably the only one (Liu and Whitman, 2008). Methanogenesis from formate is performed in a similar way to H_2/CO_2 based methanogenesis since formate is first converted to hydrogen and CO_2 intracellularly, and it seems to be restricted to methanogens without cytochromes. In addition, few methanogens are also able to utilize secondary alcohols such as isopropanol and isobutanol as well as ethanol as hydrogen

donors for CO_2 reduction. Methanogenesis through demethylation of C1 compounds is found only in members of the family *Methanosarcinaceae* (cytochromes containing methanogens), and for most genera it is an obligatory pathway. A methanogen outside the *Methanosarcinaceae* family, classified in this metabolic group, is the genus *Methanosphaera* (of the *Methanobacteriaceae*) which can utilize methanol in addition to H_2/CO_2 (Thauer et al., 2008). The last type is methanogenesis through acetate cleavage into methane and CO_2 (acetoclastic methanogenesis). This type is to be found only in two genera of cytochromes containing methanogens – *Methanosarcina* and *Methanosaeta*, for the latter this pathway is obligatory (Liu and Whitman, 2008).

Despite the different pathways, the biochemical machinery involved in methane production shares key components amongst all methanogens (**Figure 1.2**). The components of the biochemical pathways of methanogenesis through CO_2 reduction and demethylation of C1 compounds are nearly identical (but the step order in the pathway is reversed). These two pathways are somewhat different from the acetoclastic one, but they share the coenzymes tetrahydromethanopterin (H_4MPT; although it is used to bind different functional groups) and F_{420}-hydrogenase. Above all, all methanogenic pathways converge into a single final step – the reduction of the methyl group bound to the coenzyme M (CH_3-S-CoM) by a hydrogen bound to coenzyme B (H-S-CoB; Thauer 1998). The reaction is catalyzed by the enzyme methyl coenzyme reductase M which is homologous in all methanogens and is relatively well conserved. Because it is common to all methanogens and is conserved, the *mcr* gene, or more precisely it's α subunit – *mcrA*, makes a good functional genetic marker and is often used to identify methanogens in the environment through molecular means (Luton et al., 2002).

Methanogens are key components in nearly all anoxic environments. When oxygen and other electron acceptors such as Fe^{3+}, NO^{3-}, SO_4^{2-} are absent methane production acts as the sole terminal sink for electrons and is the rate limiting step for all upstream reactions. In the absence of methanogens, hydrogen and acetate quickly accumulate and many anaerobic fermentation reactions become thermodynamically unfavorable. In

this case, the entire degradation cascade ceases or is reduced to minimum. It is therefore not surprising that methanogens are found in abundance in most anoxic environments around the world (Liu and Whitman, 2008).
In most environments, the dominant methanogenic pathways are acetoclastic and hydrogenotrophic while methane formation from methylated compounds and secondary alcohols is marginal, primarily due to substrate limitation (Conrad, 2005). Therefore, in environmental modeling of methanogenic pathways often only the acetoclastic and hydrogenotrophic ones are taken into account while the others are neglected, and so was done in this work as well. Important exceptions to this rule are marine sediments and hypersaline mats. In these environments, sulfate is usually abundant and sulfate reducers outcompete methanogens for hydrogen and acetate (Martens and Berner, 1974; Cappenberg and Prins, 1974). It was therefore puzzling for several years to find active methanogenesis in these sediments (Oremland et al., 1982). As it turned out, methanogenesis was occurring primarily from methylated compounds such as trimethylamine, compounds which cannot be utilized by sulfate reducers or not as effectively as methanogens (Hippe et al., 1979). Trimethylamine is a degradation product of the compatible solute glycine-betain. These compounds are found in abundance in the sediments of saline water bodies since it is used by fish, algae and cyanobacteria to maintain intercellular osmotic pressure (Oremland, 1988).

In non-saline environments where methanogenesis from compatible solutes is marginal, the complete degradation of organic carbon (usually polysaccharides) should theoretically lead to two thirds of the methane being formed from acetate and only a third from H_2/CO_2 (Conrad, 1999). In many environments, a deviation is observed from this classical ratio most probably due to either homoacetogenesis (less hydrogenotrophic methanogenesis) and/or incomplete degradation of organic carbon (less acetoclastic methanogenesis; Conrad et al., 2009).

Competition for substrates is the main reason why methanogens require a highly reduced environment to thrive. As it turned out in several studies, the redox potential of the environment in itself does not hamper methanogenesis significantly, up to about 400 mV (Fetzer and Conrad,

1993; Yu et al., 2007). This is not the case, however, for oxygen. As with most anaerobes, methanogens too cannot cope with the damage caused by reactive oxygen species to their membranes, proteins and nucleic acids (Storz et al., 1990). Additionally, the F_{420}-hydrogenase, a crucial electron transporter in methanogenesis, is particularly sensitive to oxygen (Schönheit et al., 1981). For these reasons it was considered for years that methanogens could only be found in anoxic and highly reduced environments such as those mentioned above. Indeed, all isolation strategies for methanogens include strong reducing agents and keeping the media from oxygen contamination is often tricky (Atlas, 2010).

But in 1995 Peters and Conrad reported that samples of upland soils (soils which are aerate throughout most of the year) taken from various parts around the world, representing different ecosystems, could exhibit methanogenic potential (as well as sulfate reduction and homoacetogenesis). Sample types ranged from temperate forest to savanna and desert soils; though overall the number of samples was very small and did not include true replicates. While their experiments were performed under anoxic/highly reduced conditions, viable methanogens could nevertheless be detected in these soils and could be readily activated with just the addition of water, even after being exposed to oxygen for long periods in the field and then stored in a dry state at room temperature for periods ranging from several months to nearly nine years. The core methanogenic population in these samples was small and methanogenesis was apparently limited to some extent by population size and not only competition (Peters and Conrad, 1996). Only few researchers followed up on these experiments, trying to reproduce the observations and in addition to detect the methanogens which are involved in these process (West and Schmidt, 2002; Teh et al., 2005; Nicol et al., 2003; Radl et al., 2007; Gattinger et al., 2007). In many of these cases, however, the authors focused on soils which are heavily impacted by grazing and thus attributed most of the methanogenic activity to the effect livestock had on the soil by enriching it with nutrients from urine and manure and by inoculating it with rumen microflora. In contrast, we hypothesized that the occurrence of methanogens in aerated soils and their ability to survive long periods of exposure to oxygen might indicate that they

are also active in nature under certain conditions and that at least some are native to aerated soils.

1.3 Methane oxidizing bacteria

Biological methane oxidation is the primary mechanism in nature by which methane is degraded and the carbon is recycled. It is now agreed that methane is oxidised in nature in both aerobic and anaerobic pathways and that both types of methane oxidation are of global significance. The first methane oxidizing bacteria was isolated already in 1906 by N. L. Söhngen but only in 1970, following the work of Whittenbury and his colleagues, could a large set of pure cultures of methanotrophs from various sources be generated and maintained (Dalton, 2005). Today there are over two hundred isolates of aerobic methane oxidisers from 17 different genera (Bowman et al., 1993; Lüke, 2010).

In contrast to aerobic methane oxidation, anaerobic oxidation of methane (AOM) has only recently been recognized and presently no cultured representative exists. The first indications for the occurrence of AOM coupled to sulphate reduction came in the mid 70's and early 80's (Reeburgh, 1976; Zehnder and Brock, 1980). It was initially thought that methanogens were responsible for the process which they performed simultaneously with methane production, but the rates measured in the lab for methanogens could not account for the fluxes measured in the field (Zehnder and Brock, 1979). It took more than 20 years for the first molecular evidence to appear tying AOM to an unknown group of *Euryarchaeota* which are closely related to *Methanosarcinales* and *Methanomicrobiales* (Hinrichs et al., 1999). Later it could be shown microscopically that anaerobic methane oxidisers (termed ANME) live in aggregates with sulphate reducing bacteria (Boetius et al., 2000). While it is currently recognized that ANME are phylogenetically different from methanogens it appears that Zehnder and Brock were not entirely wrong in their prediction since ANME contain the *mcr* gene and apparently use a reverse methanogenic cycle for methane oxidation (Shima and Thauer, 2005). Recently, a methane oxidizing bacteria that uses nitrite as an electron acceptor and produces oxygen was isolated from anoxic freshwater sediment

in the Netherland (Ettwig et al., 2010). Currently, however, there is no indication for the existence of anaerobic methane oxidation in soils and these microbial groups are therefore not discussed in this work.

Aerobic methanotrophs are a guild of phylogenetically different bacteria which oxidise methane for both energy and carbon assimilation (Mancinelli, 1995). They are all obligatory aerobes and most of them are also obligatory methane oxidisers (Bowman, 2006). Aerobic methanotrophs are a subset of a larger guild known as methylotrophs which metabolize a variety of C1 compounds. The basic taxonomy of the aerobic methanotrophs was established in 1970 with the seminal work of Whittenbury and his colleagues. The distinction remains in use till today though with modifications and reservations as more and more exceptional methanotrophs are discovered (Semrau et al., 2010). Accordingly, methanotrophs are classified as either type I or type II based to the structure of their phylogeny, internal membrane, membrane lipids composition and their resting stages. The 'classical' aerobic methanotrophs lie all within the proteobacteria phylum. Those classified as type I all belong to the family *Methylococcaceae* of the *γ–Proteobacteria* and include genera such as *Methylomonas Methylobacter* and *Methylomicrobium* while type II methanotrophs lie within the *α–Proteobacteria* and include the families *Methylocystaceae* (e.g. *Methylosinus*) and *Beijerinckiaceae* (e.g. *Methylocapsa*). Type I is in itself further divided into type Ia and type Ib with the aforementioned genera classified as type Ia. Type Ib (initially termed type x) comprises of *Methylococcus* and *Methylocaldum* which also belong to the γ–Proteobacteria but posses several key traits which differ from type Ia methanotrophs. Among these are some differences in the biochemical machinery for methane oxidation (see below), high G+C content and high optimal growth temperatures (Hanson and Hanson, 1996). A recent addition to these classical methanotrophs is the isolation of thermophilic and highly acidophilic aerobic methane oxidisers from hot springs in New Zeeland, Italy and Russia. These isolates are members of the Verrucomicrobia phylum rather than the Proteobacteria (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008). These new isolates were classified under the new genus

Methylacidiphilum and so far seem to be highly adapted and restricted to high temperature/low pH environments (Camp et al., 2009).

Types I and II methanotrophs use different biochemical pathways to oxidise methane but they all use the same initial steps. The first step in the aerobic oxidation of methane is its oxidation to methanol by the methane monooxygenase (MMO) which comes in two forms. All known methanotrophs apart from *Methylocella* possess the membrane bound type of the enzyme – the particulate methane monooxygenase (pMMO) - which is embedded in the intricate system of internal membranes of the cell (Dedysh et al., 2000). The second type – the soluble methane monooxygenase (sMMO) – is a cytoplasmatic type and exists only in some species (Murrell et al., 2000). In those species which possess both types of the enzyme it was found that the availability of copper ions (which are required for the synthesis of pMMO) regulates the differential expression of these enzymes (Stanley et al., 1983; Nielsen et al., 1996; Semrau et al., 2010). Because pMMO is found in nearly all methanotrophs and because it is very conserved one of its subunits - the *pmoA* subunit (27 kDa) – is the most commonly used genetic marker for the detection of methanotrophs (Murrell et al., 1998). The next steps in the pathway are also identical for both type I and II – conversion of methanol to formaldehyde then to formate and finally to CO_2. The more prominent biochemical distinctions between the different methanotrophic types (and the Verrucomicrobia) relates to the pathway in which carbon is assimilated by the cell. Type II and Verrucomicrobia methanotrophs use primarily different versions of the serine cycle to assimilate formaldehyde into cellular carbon, while Type I methanotrophs use primarily the RuMP pathway (though there's some level of expression of genes of the serine pathway in type Ib methanotrophs Hanson and Hanson, 1996; Camp et al., 2009). Some species of methanotrophs are also able to fix carbon using the Calvin-Benson-Bassham cycle (Trotsenko and Murrell, 2008).

An additional distinction made with regards to methanotrophs is related to their ecophysiology. Upland soils are a biological sink of atmospheric methane and consume approximately 30 Tg per year (**Figure 1.1**). To be able to consume methane at such trace amounts these methanotrophs must possess a methane monooxygenase with a low Km

property. Indeed, Bender and Conrad determined the apparent Km for various upland soils and reported values around 50 nM and a threshold value of down to 0.2 ppmv of methane (Bender and Conrad, 1992). These values are much lower than those known for methanotrophs from pure culture studies which are normally in the μM range (Knief and Dunfield, 2005). On the basis of these observations it was postulated that two types of methanotrophs exist: low affinity methanotrophs which are adapted to high methane concentration end encompass all cultivated strains and high affinity methanotrophs which are able to oxidise atmospheric methane and which are not present in culture collections (Conrad, 1999). This dichotomy has been somewhat undermined by the alternative notion that some known methanotrophs might hold both qualities by possessing two sets of MMOs with different Km values (Knief and Dunfield, 2005). As a support for this alternative theory it was found that many type II methanotrophs possess an alternative MMO operon, termed *pmoCAB2*, which has only a low similarity to the known *pmoCAB1* operon (Yimga et al., 2003; Ricke et al., 2004). Later, Baani and Liesack (2008) could show that in *Methylocystis* sp. strain SC2 the *pmoCAB1* was responsible for the low affinity methane oxidation activity while its counterpart *pmoCAB2* operon showed a high affinity methane oxidation property. Nevertheless, while *Methylocystis* and other similar type II methanotrophs are abundant in upland soils, most *pmoA* sequences detected in soils with active atmospheric methane uptake form clusters (e.g. upland soil cluster alpha, USCα) that are different from those of the known methanotrophs (Holmes et al., 1999; Henckel et al., 1999; Knief et al., 2003; Kolb et al., 2005). It therefore remains to be discovered whether these upland soil *pmoA* sequences are alternative operons of known methanotrophs or belong to novel species (Kolb, 2009).

1.4 Interactions between methanogens and methanotrophs in soil

Oxygen has low solubility in water and its diffusive flux in most wet environments is much lower than its consumption rate by heterotrophic microorganisms. Because of these traits, wet environments (salt and fresh water bodies, wetland soils etc.) tend to develop a typical structure by which the sediment is almost entirely anoxic and trace amounts of oxygen may or may not be present in the topmost few millimetres (Fenchel et al., 1998a). Once oxygen is detectable, either in the sediment or the water column, its concentration rises steadily from the bottom along the water column up to the air-water interface where it might be at saturation for that temperature or at hyper-saturation (if photosynthesis is taking place in the water column). In the anoxic sediment anaerobic degradation processes take place degrading the carbon which originates from photosynthetic activity in the upper layers of the water column, by plants and microorganisms, or from land. The specific oxygen diffusion rates and the availability of alternative electron acceptors determine the redox potential of the sediment and the specific nature of the anaerobic degradation processes which take place (Fenchel et al., 1998b). Two types of sequential patterns, a spatial and a temporal, are acknowledged with respect to redox reactions in anoxic sediments. On the spatial level, vertical layers are formed according to the dominant electron acceptor, with oxygen at the topmost layer. Once oxygen is depleted alternative electron acceptors become dominant in the deeper layers according to their redox potential and their availability. These are usually NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^{2-}, in this order (Zehnder and Stumm, 1988). The same process is seen temporally when oxic soils get flooded, for the same thermodynamic reasons, with oxygen being depleted first followed by the depletion of available electron acceptors. Methanogenesis (based on acetate or H_2/CO_2) being the least thermodynamically favourable is found at the bottom layer of the sediments or last in a sequential reduction process (Yao et al., 1999).

Since methanogens are unable to degrade organic polymers or utilize short chain fatty acids (SCFA) they rely on a cascade of anaerobic

degradation processes for methanogenesis (**Figure 1.2**; Zinder, 1993). These start from the secretion of hydrolytic enzymes by fermenting bacteria (anaerobic heterotrophs) hydrolyzing polymers (such as polysaccharides) to monomers. The monomers are then further fermented to H_2, acetate and SCFA. H_2 and acetate can be directly used by methanogens but fatty acids need to be further degraded. The degradation of SCFA is usually thermodynamically unfavourable under standard conditions, but it is nevertheless achieved by an association of syntrophic bacteria and methanogens. The latter utilize hydrogen directly transferred to them by syntrophs thereby reducing its concentration to a minimum and making the degradation of SCFA by syntrophs energetically possible (Stams and Plugge, 2009). In addition to these reactions, homoacetogenesis might be occurring in parallel, generating acetate from H_2/CO_2, though this is energetically unfavourable when hydrogenotrophic methanogenesis occurs (Siriwongrungson et al., 2007). Lastly, acetate itself might be consumed syntrophically by syntrophic acetate-oxidizing bacteria, usually at high temperatures (Liu and Conrad, 2010).

Methane which is formed in these anoxic sediments is transported to the atmosphere either through plant aerenchyma or by direct diffusion and bubbling through the sediment and the water column (Conrad, 2004). In the second transport mechanism, methane reaches the oxic-anoxic interface where it is consumed by methanotrophs (Damgaard et al., 1998; Gilbert and Frenzel, 1998).

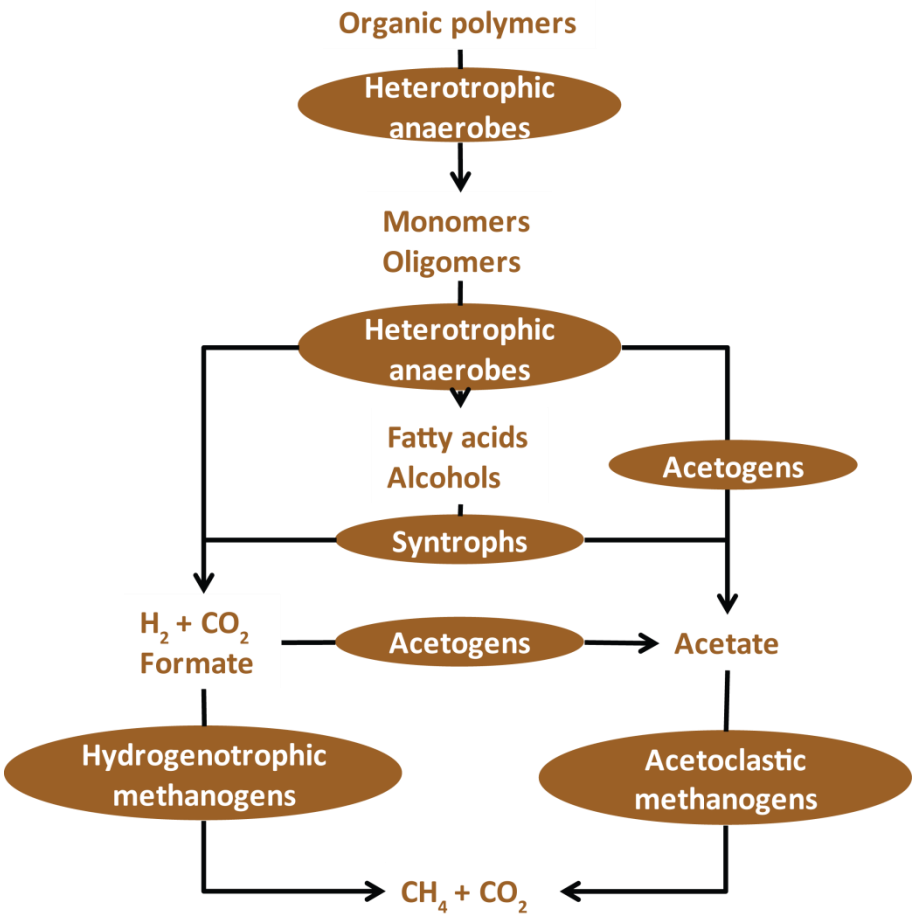

Figure 1.2| Anaerobic degradation cascade of organic matter. Text in brown represents substrates or products. Ellipses represent different microbial guilds. Adapted from Liu and Whitman (2008).

In upland soils, such a layered structure of redox gradient does not exist. Throughout most of the year upland soils are drained and oxygen penetrates deeply into the soil by direct diffusion from the atmosphere and also from plant roots. Degradation of organic matter is primarily performed by aerobic heterotrophs which convert sugars directly into H_2O/CO_2. As discussed, upland soils constitute a net global sink for atmospheric methane consuming about 5% of the annual budget. The rate of atmospheric methane oxidation in upland soils is apparently site specific and also varies with time and depth. Field measurements in various ecosystems have confirmed this to be a universal phenomenon (Yavitt et al., 1990; King and Adamsen, 1992; Henckel et al., 2000; Knief et al., 2005; Kolb et al., 2005; Horz et al., 2005; Tate et al., 2007; Striegl et al., 1992). Despite the many field measurements performed, not all regions have been as intensely studied and some ecosystems, primarily drylands are significantly

underrepresented (Smith et al., 2000). Soil type and land use as well as season all affect oxidation rates (through temperature and soil water content (Adamsen and King, 1993; Reay et al., 2001, 2005; Menyailo and Hungate, 2003). Upland soils do get temporarily anoxic, though, when water displaces air in the soil pores. While it has been shown in principle that the same sequential reduction processes can occur in upland soils, when becoming anoxic, and that even methane can be formed, this behaviour has not been confirmed under field conditions and the ecological significance of this phenomenon is unknown (Peters and Conrad, 1996)

1.5 Dryland ecosystems

What are deserts?

Deserts (semiarid, arid and hyperarid regions) span over 44 mil. km^2 which make up 33% of the earth's land surface. Together with dry sub-humid areas these regions are defined as drylands and make up 44% of the land surface (Verstraete and Schwartz, 1991). This estimation excludes polar deserts which span over another 5.5 million km^2 and are not covered in this work. The most distinctive feature of deserts, and what in fact defines them, is water deficiency. This is most often measured by the aridity indices which are also used to classify drylands. A common index – AI_U – determined by UNESCO is defined as the ratio of precipitation *(P)* to potential evapotranspiration (*PET*; Middleton and Thomas, 1997). In addition to the classifications described above deserts are further divided into subtropical deserts, cold winter deserts, and cool coastal deserts. In English (and the romance languages) the noun 'desert' is related to the verb of the same name – to abandon – both stemming from the Latin *dēserere* – to forsake. But deserts are not entirely forsaken, in fact half of the world's countries are in part or entirely located in drylands environments and they are home to nearly 40% of the human population (Ffolliott et al., 2003).

Desertification

All deserts on Earth are currently expanding at their margins in a process termed desertification. Much of this is attributed to human activity, either indirectly through climate change or directly through unsustainable land use practices such as logging, overgrazing and cultivation of unsuitable crops (Cloudsley-Thompson, 1988; Dregne, 1991). Once soil degradation has started it often exhibits positive feedback cycles such as invasion of desert species and nutrient loss through fluvial and aeolian processes which perpetuate the desertification process and even exacerbate it (Schlesinger et al., 1990; Thomas et al., 2005). Because of that and because of the detrimental effects of desertification on human populations global efforts are made to study the process and combat it (Kassas, 1995; UNCCD, 2009)

Stress factors

The desert environment is perceived by us as an extreme environment. For humans and animals solar radiation is strong and temperatures are high, plants are scarce and so food is difficult to obtain, and water resources are rarely encountered. It's no wonder then that deserts always have a low density of human settlements, animals and plants (but not low diversity). However, is this also the case for soil bacteria? If we examine these stress factors from the bacteria's perspective we see that most of them are irrelevant or do not represent extreme conditions. While exposure to direct sun radiation is detrimental to microorganisms for much of the same reasons that it is for plants and animals – namely mutagenesis by UV radiation – it is primarily not a problem to any cell living just a few μm below ground as soil particles effectively screen out radiation. Those living on top do have to deal with it and indeed many desert dwelling bacteria were found to possess various 'sunscreen' pigments (Bowker et al., 2002; Belnap et al., 2007). This point can be demonstrated easily by simply spreading some top soil on an agar plate. While a sample taken from, say, a forest soil would produce primarily white and similarly looking colonies a desert soil sample would produce a variety of colourful colonies which can effectively deal with high solar radiation. High temperatures might be the first association for

most people when it comes to deserts but for most microorganisms they could hardly be more comfortable. Once again soil acts as a strong buffering matrix and temperatures tend to be very mild throughout the year even just a few millimetres below ground. Low plant coverage in deserts is the primary reason for low levels of nutrients in desert soils, primarily of carbon and nitrogen. Soil dwelling cyanobacteria, microalgae, lichens and mosses are responsible instead for much of the primary production in desert environments and in some cases they can even outperform plants in terms of net ecosystem carbon fixation (Lange, 2002). Cyanobacteria are also responsible for nitrogen fixation in deserts and in some cases are even the primary nitrogen fixers. The one true limiting factor for all life forms in deserts including microorganisms is water availability. Precipitation in deserts is very limited and tends to be highly unpredictable both in time and space. Low air moisture and high radiation dry the soil very quickly after a rain event had occurred giving plants and microorganism a narrow window of time to complete their life cycles. Water potential in desert soils tends to be very low and is the primary factor limiting microbial growth in the soil. Interestingly, low water potential is perhaps the only environmental stress factor for which bacteria are not the most tolerant organisms. While certain halophilic bacterial strains can tolerate water potential down to approximately -40 MPa, some yeasts and fungi can survive desiccation down to even -70 MPa (Skujins, 1984). Because the primary water resource – rain – is very limited, marginal water resources such as dew gain more importance. Dew alone was found to reactivate 80% of the activity of photosystem II in a dry cyanobacterial crust from Hopq desert in China while light and temperature accounted only for the remaining 20% (Rao et al., 2009). For supporting biomass growth in the cyanobacterial crust, however, liquid water seems to be necessary (Lange et al., 1993).

Distinctive features of desert biomes

Patchiness

Water scarcity limits plant proliferation. This generates in semiarid and arid ecosystems a typical pattern of landscape patchiness by which 'islands' of shrubs or low trees are scattered across the landscape, in a more or less ordered pattern, and between them is barren soil where annuals might grow in rainy years (Whitford, 2002). Patches are first formed primarily by fluvial processes causing spatial heterogeneity on the small geographic scale and are later maintained by both physical – fluvial and aeolian – as well as biological processes in a positive feedback cycle (Ludwig and Tongway, 1995). Garcia-Moya and McKell coined in 1970 the term 'islands of fertility' to describe the contrast between shrub and intershrub patches. At first the term referred only to the accumulation of nitrogen under the canopies of shrubs in deserts compared to the surrounding area (Charley and West, 1975; Charley and West, 1977) but later it was found that also organic carbon and in fact virtually all nutrients are significantly more concentrated under shrub canopies in deserts (Barth and Klemmedson, 1982; Virginia and Jarrell, 1983; Whitford et al., 1997).

But an ecological vacuum rarely exists on earth and the barren soil areas in the interspaces between shrub patches are not left abiotic, instead they allow the development of the most distinctive feature of desert soils – the biological soil crust.

Biological soil crusts

The most unique and interesting feature of desert soils, for microbiologists at least, is the biological soil crust (BSC). Soils in more temperate regions are usually comprised of different layers formed as a result of two inverse gradients – a decreasing level of organic matter from top to bottom and an increasing degree of bedrock erosion from bottom to top. The typical desert soils (Ardisols, Entisols and sometimes Vertisols) are usually comprised of a bulk of undifferentiated eroded bedrock with only a low degree of soil development (cambic horizon) with minerals differentiated along the profile

according to solubility (Sombroek, 1987). The topmost few millimetres of the soil are significantly different. This layer is densely colonized by microorganisms which interact with each other in complex ways and form a mat. These microorganisms secrete polymeric substances (mostly polysaccharides) which aggregate the soil and create a physically separate layer. The specific microbial members of different BSCs differ from one location to another but they all rely on a combination of at least some of the following groups: cyanobacteria, microalgae, fungi, lichens and mosses. Which of the groups is dominant is a function of climatic region and precipitation, but they all rely on photosynthesis as a primary source of energy and carbon.

BSCs are in theory not restricted to deserts; what really limits their development is the presence of plants. Most of the soil on earth is covered by plants unless water availability restricts them, such as in deserts (or if it is covered by ice or snow). Also, in some ecosystems such as coastal sand dunes, plants have difficulty establishing themselves and BSCs are formed (Belnap et al., 2002). BSCs are typically a few millimetres in thickness; they form a solid crust on top of the soil that tends to brake easily when the soil is dry. Photosynthetic activity occurs when the BSC is wet at the top millimetre and pH tends to be somewhat higher in this section. Oxygen levels vary between night and day when the crust is active. In the dark, oxygen levels drop sharply from atmospheric levels to zero in a linear fashion and it penetrates only to approximately 1-2 mm depth. The crust is anoxic below that layer and so is the bulk soil underneath. During daytime, intense photosynthetic activity takes place and oxygen penetrates much deeper. The oxygen profile is not linear but rather tends to increase in concentration at the top micrometres and the soil in this part is hyperoxic (Garcia-Pichel and Belnap, 1996). BSCs have a considerable mechanical strength thus providing protection to their microbial inhabitants. A developed BSC provides resistance to weathering and reduces soil migration by wind (Gillette et al., 1980; Neuman et al., 1996), but usually also reduces infiltration of water (Kidron, 2007). BSCs are however very sensitive to mechanical destruction by animals and humans (Beymer and Klopatek, 1992).

Some members of BSCs are nitrogen fixers. Particularly, the cyanobacteria genera *Nostoc* and *Scytonema* are common in mature crusts and active nitrogen fixers (Belnap, 2002; Abed et al., 2010). In some ecosystems, particularly in arid ones, nitrogen fixation by cyanobacteria can be the primary nitrogen source for the soil (Evans and Ehleringer, 1993). The preservation of nitrogen in the soil depends on the BSC remaining intact and considerable nitrogen losses from dryland soils were measured after disturbance (Evans and Ehleringer, 1993; Evans and Belnap, 1999). In parallel, BSCs are also inhabited by nitrifying and denitrifying bacteria and nitrogen loss through nitrification/denitrification has also been reported (Nejidat, 2005; Johnson et al., 2005; Angel et al., 2009).

Development of a BSC is a successional process. The initial colonisers are filamentous cyanobacteria, most commonly of the genera *Phormidium*, *Oscillatoria*, *Microcoleus* and *Schizothrix*. These colonise the subsurface and produce copious amounts of exopolysaccharides which coagulate soil particles and assist in generating a uniform crust layer (Mazor et al., 1996). They are later joined by microalgae and also by saprophytic fungi living on the secretions and debris of the former colonisers. At the end of this stage a physically stable crust has been formed and colonization of the surface can begin. Typical surface colonising cyanobacteria are dark pigmented, most commonly *Nostoc* and *Scytonema* types (Garcia-Pichel, 2002). If the crust reaches advanced development stages, and is not disturbed, then over the years lichens will be able to establish shifting the crust community from cyanobacteria dominated to lichens dominated, or in more humid regions – mosses dominated (Belnap and Eldridge, 2002).

Recovery of disturbed crusts may take years. Kidron and colleagues measured full recovery of chlorophyll a, proteins and carbohydrates after 8 to 9 years in arid sand dunes in the eastern part of the Negev Desert, Israel, receiving 95 mm rain per year. Recovery of mosses took far longer and required 17-22 years (Kidron et al., 2008). This process can be speeded up, however, by inoculating the soil with cyanobacterial cultures (Wang et al., 2009)

1.6 Aims of this study

Drylands span over 33% of the earth's land surface and are expanding due to desertification, yet they are much underrepresented in scientific research. The soil structure and composition in drylands is distinct from that in more humid regions. Nevertheless, as far as microbial ecology is concerned, only few studies characterized the general microbial population in desert soils, and no comparative study was done on the differences and similarities between arid and temperate soils.

Extensive literature on specific parts of the microbial community in drylands exists; it primarily focuses on cyanobacteria, algae and fungi living in the biological soil crust. Very little is known about other prokaryotes living in dryland soils. Moreover, much of this body of research is old and precedes the molecular paradigm in microbial ecology and thus is largely not comparable to present works.

Desert soils have been shown to be involved in the consumption of atmospheric methane and also to have methanogenic potential, but extent of these phenomena and the microorganisms which are involved in these processes are unknown.

In this work I tried shedding some light on the microbial inhabitants of deserts, particularly on their involvement in the turnover of the greenhouse gas methane. Specifically, the following questions were addressed:

Chapter 2| Biogeography of soil *Archaea* and *Bacteria* along a steep rainfall gradient

Biogeography is a well described ecological phenomenon in plants and animals and the forces which shape it are largely known, but as for *Bacteria* and *Archaea* the existence of biogeography is still a debatable question.
Do Bacteria and Archaea communities follow large scale geographic gradients of precipitation or are they only affected by local conditions on the microscopic level?

Chapter 3| In situ measurement of methane fluxes and analysis of transcribed particulate methane monooxygenase in desert soils

Methane uptake by upland soils has been repeatedly measured in virtually all aerated soils on earth, yet for dryland environments only a single study exists. *How ubiquitous is methane uptake in desert soils? Does the activity vary with land use? Which methanotrophs are responsible for methane uptake in dryland ecosystems?*

Chapter 4| Methanogenic *Archaea* are globally ubiquitous in aerated soils and become active under anoxic conditions

Methanogenesis was shown to occur in many types of aerated soils when incubated anoxically but the identity of the methanogens is unknown. *Which methanogens are present in aerated soils? Are they different at different sites or are there universal species?*

Chapter 5| Activation of Methanogenesis in Arid Biological Soil Crusts despite the Presence of Oxygen

The occurrence of methanogenic activity in desert crust soils under anoxic conditions proves only a potential activity. *Does methanogenesis occur in desert crusts even when they exposed to oxygen? Is the community composition affected by the presence of oxygen?*

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Chapter 2 |

Biogeography of Soil *Archaea* and *Bacteria* along a Steep Precipitation Gradient

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RA, OG and MIMS designed the study; RA, OG and EDU designed the sampling scheme and performed the sampling; RA performed all lab experiments, design of Matlab codes and data analyses; RA, MIMS and OG wrote the manuscript.

2.1 Abstract

For centuries, biodiversity has spellbound biologists focusing mainly on macroorganism's diversity and almost neglecting the geographic mediated dynamics of microbial communities. We surveyed the diversity of soil *Bacteria* and *Archaea* along a steep precipitation gradient ranging from the Negev Desert in the south of Israel (<100 mm annual rain) to the Mediterranean forests in the north (>900 mm annual rain). Soil samples were retrieved from triplicate plots at five long-term ecological research stations, collected from two types of patches: plant interspaces and underneath the predominant perennial at each site. The molecular fingerprint of each soil sample was taken using terminal restriction length polymorphism of the 16S rRNA gene to evaluate the bacterial and archaeal community composition and diversity within and across sites.

The difference in community compositions was not statistically significant within sites (P = 0.33 and 0.77 for *Bacteria* and *Archaea*, respectively), but it differed profoundly by ecosystem type. These differences could largely be explained by the precipitation gradient combined with the vegetation cover: the archaeal and bacterial operational taxonomic units were unique to each climatic region, that is, arid, semiarid and Mediterranean (P = 0.0001 for both domains), as well as patch type (P = 0.009 and 0.02 for *Bacteria* and *Archaea*, respectively). Our results suggest that unlike macroorganisms that are more diverse in the Mediterranean ecosystems compared with the desert sites, archaeal and bacterial diversities are not constrained by precipitation. However, the community composition is unique to the climate and vegetation cover that delineates each ecosystem.

2.2 Introduction

For centuries, ecologists have observed the profound differences between dry and temperate regions with respect to vegetation cover and animal abundance. Studies on how communities respond to precipitation gradients led to the search for diversity patterns across a wide variety of taxonomic groups, including annuals, trees, mammals, birds, reptiles and insects (Hawkins et al., 2003). Emerging patterns showed a general increase in the diversity of plants and animals with an increase in available water. The bulk of these studies established an ecological paradigm claiming that an increase in water availability is followed by elevated diversity and abundance of biological communities.

Although patterns of variation in species richness along precipitation gradients have been studied extensively, much less is known about the manner in which, and the extent to which, precipitation interacts with landscape diversity in determining patterns of species richness (van Rensburg et al., 2002; Gardezi and Gonzalez, 2008). On a local scale, studies of community and landscape ecology of both macro- and microorganisms have indicated a relationship between species richness, internal structure of the habitat and landscape heterogeneity as the main controllers of biodiversity. In water-limited environments, one of the most widely accepted theories is the 'fertility' or 'resource island' hypothesis, which states that shrubs create heterogeneity in soils by localizing soil fertility under their canopies (Schlesinger et al., 1996). Indeed, heterotrophic *Bacteria* (Herman et al., 1995) and protozoa (Robinson et al., 2002) have been found to be more abundant under shrubs than in their interspaces. The links between microbial biogeography, local diversity of microorganisms and the factors that shape them represent largely unexplored territory. Here, we integrated a study of local-scale microbial diversity in bare soil and under a plant canopy with that of distinct climatic regions.

In the last three decades, microbial ecologists have experienced a quantum leap in the study of microbial ecosystems independent of their ability to culture the resident species. Microbiologists have gone to remote corners of the earth to analyze the microbial inhabitants of every

environment. Of the microbial groups that are abundant in the soil, *Bacteria* have been the most extensively studied. Nevertheless, our understanding of the spatial distribution patterns of bacterial diversity is limited, mainly because most studies are limited to local scales (Navarro-Gonzalez et al., 2003; Zhou et al., 2004). Recent large-scale surveys have revealed that different ecosystems support unique microbial populations (Zhou et al., 2002; Fierer and Jackson, 2006; Green and Bohannan, 2006; Vishniac, 2006; Adler and Levine, 2007), giving rise to the notion that microbial populations can exhibit geographic distribution. These emerging microbial distribution patterns suggest that the ecological rules followed by macroorganisms do not necessarily apply to microorganisms (Fierer and Jackson, 2006; Green and Bohannan, 2006; Bryant et al., 2008).

The majority of microbial biogeography studies have focused on the bacterial domain (Fierer, 2008). However, members of the *Archaea* domain, once thought to be present only in extreme environments, have been found to be significant or even major components in mundane habitats such as ocean waters, freshwater sediments and soils (Kent and Triplett, 2002; Chaban et al., 2006). Archaeal diversity has been relatively well documented in rice paddy soils (Grosskopf et al., 1998) and peat bogs (Hoj et al., 2008), and has also been reported in temperate, tropical and agricultural soils (Kent and Triplett, 2002). Those studies were confined mainly to local scales, but a handful of researchers have examined biodiversity patterns of *Archaea* along spatial or temporal gradients (Ochsenreiter et al., 2003; Nemergut et al., 2005; Walsh et al., 2005; Oline et al., 2006; Hansel et al., 2008). In this study, we compare and contrast the diversity patterns of soil *Archaea* with those of *Bacteria*. We explored the diversity of these domains on local and regional scales, addressing their richness and community composition. A prudent hypothesis would be that each domain is characterized by distinct patterns of diversity, with *Archaea* having a unique distribution pattern, as they occupy specific soil niches, whereas *Bacteria* are more widely distributed and thus are subjected to biogeographical patterns. Alternatively, niche occupation and abundance of *Archaea* in the soil might not be a determinant, in which case the forces structuring biodiversity across the precipitation gradient would be the same for both domains, resulting in

similar phylogenetic biogeographical patterns. To test these hypotheses, we adopted an approach that examines local and regional relationships with respect to microbial biodiversity.

As mentioned above, traditional precipitation diversity studies relied heavily on the sampling and identification of large number of plant and animal species, and were focused on how patterns of richness, abundance and phenotype change with water availability. As culturing techniques are currently limited for most *Bacteria* and *Archaea*, microbial ecologists use molecular techniques that are dependent on the universal marker gene encoding for the 16S rRNA to document microbial presence at every level, from division to strain. However, comprehensive sequencing of the 16S rRNA gene in soil samples is both labour-intensive and expensive. Consequently, true replication and statistical characterization of microbial diversity in an environment, performed as demanded by plant ecologists, for instance, are rarely achieved. In this study, we addressed questions of local- and regional-scale distribution and diversity patterns by using a multiscale nested sampling approach. Five long-term ecological research (LTER) sites ranging from the Negev Desert in the south of Israel (with less than 100 mm annual rain) to the Mediterranean forests in the north (with over 900 mm annual rain) were examined and the diversity patterns of their soil *Bacteria* and *Archaea* were elucidated. Our nested sampling scheme consisted of the following: (i) triplicates of approximately 1000 m^2 plots in each site; (ii) two patches in each plot, one under the canopy of the predominant perennial (woody patch) and one in the perennial interspace (open patch) and (iii) a composite of eight soil samples taken from each patch type, at each plot. This scheme enabled us to compare patches within each plot, plots within each site and the different sites, answering, at least in part, the requirements for a comprehensive ecological survey.

2.3 Results

We surveyed the diversity of soil *Bacteria* and *Archaea* along a steep precipitation gradient ranging from an arid area with less than 100 mm annual rain to a meso-Mediterranean forest receiving over 900 mm precipitation (**Table 2.1** and **Supplementary Figure 2.1**). Thirty soil samples (each a composite of eight samples) were retrieved from five LTER stations, collected from open and woody patches at each site. Using TRFLP analysis of the 16S rRNA-encoding gene, the molecular fingerprint of each soil sample was taken to evaluate the diversity and composition of the microbial community within and across sites. Here, we present the patterns obtained when the bacterial and archaeal small subunit rRNA gene amplicons were digested with a single enzyme (TaqI for *Bacteria* and MseI for *Archaea*). Analyses of the 16S rRNA gene fragments digested with the additional enzymes used in this study (see Experimental procedure) are presented in **Supplementary Figure 2.2**.

Bacterial and archaeal community distribution

To observe the differences in the overall distribution patterns of both the bacterial and archaeal communities in each sample, the rank and relative abundance of the TRFs were calculated and plotted (**Figure 2.1**). A Kolmogorov–Smirnov test was performed on all possible sample pairs (separately for each domain) to test whether they are derived from the same distribution. The test showed that in 83.7% and 85.5% of the cases, for *Bacteria* and *Archaea*, respectively, any two random samples were drawn from the same distribution at a 0.05 confidence level. Moreover, for both domains, only two samples showed distinctly different distributions from the rest of the samples (**Supplementary Table 2.1**).

Table 2.1| Characteristics of the sites and values of major physico-chemical parameters of the sampled soils.
For each site values are presented as means for open/woody soil patches.

Climate	*Location (coordinates, elevation)*	*Average precipi-tation (mm yr¹)*	*WC* (%)*	*pH***	*Na^+*	*Ca^{2+}*	*Mg^{2+}*	*NO_3^--N*	*NH_4^+-N*	*$P_{tot.}$*	*K^+*	*Calcium carbonate (%)*	*OM† (%)*	*Predominant perennials*
Arid	Avdat (30°47' N/34°46' E, 600-700 m)	100	1.9/ 1.8	7.9/ 8.0	110.5 /40.2	55.7/ 35.4	10.5/ 8.4	4.0/ 9.4	3.4/ 6.3	0.03/ 0.04	9.4/ 19.1	33.0/ 33.7	0.6/ 0.8	*Hammada scoparia*†† and *Zygophyllum dumosum*
Semi-arid	Lehavim (31°20' N/34°45' E, 350-500 m)	300	2.6/ 3.8	7.2/ 7.0	18.8/ 19.3	129/ 263	19.5/ 44.5	2.8/ 3.5	49.0/ 62.5	0.07/ 0.09	7.7/ 14.1	17.0/ 16.3	2.0/ 3.4	*Sarcopotarium spinosum*[3] and *Thymelaea hirsute*
Dry-Mediterranean	Adulam (31°40' N/34°50' E, 200-300 m)	400	9.6/ 14.7	7.3/ 7.2	33.5/ 39.0	310/ 1051	30.2/ 162.6	5.9/ 3.7	21.0/ 27.5	0.09/ 0.57	6.9/ 63.7	14.0/ 6.7	6.1/ 19.9	*Quercus calliprino*[3] and *Pistacia palaestina*
Mediterranean	Ramat Hanadiv (32°30' N/34°55' E, 100-200 m)	600	6.0/ 20.4	7.0/ 7.1	13.7/ 45.8	143/ 325	29.5/ 70.0	6.7/ 6.5	40.8/ 51.0	0.09/ 0.02	9.9/ 34.5	1.7/ 1.7	4.9/ 10.5	*Phillyrea latifolia*[3] and *Quercus calliprinos*
Meso-Mediterranean	Mt. Meron (32°00' N/35° 20'E, 700-900 m)	900	12.1/ 23.6	7.0/ 6.9	7.4/ 15.3	136/ 309	52.1/ 120.0	8.2/ 2.7	54.6/ 98.6	0.09/ 0.13	12.8/ 37.2	1.7/ 2.3	7.3/ 10.9	*Quercus calliprino*[3] and *Quercus boissier*

* Gravimetric water content.

** All soil parameters except pH are given in mg kg^{-1} unless stated otherwise.

† Organic matter.

†† The woody patches samples were taken from under the canopy of the underlined plant species.

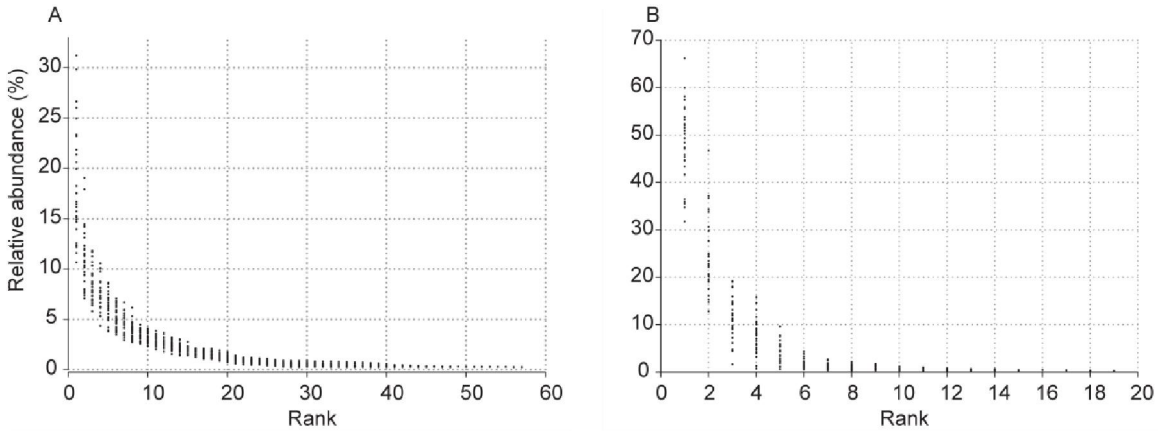


Figure 2.1| Rank-abundance plots of the TRFLP profiles of (A) *Bacteria* and (B) *Archaea*. The y axis shows the relative abundance of each TRF, whereas the x axis is the ordinal rank of the TRFs from most abundant (1) to least abundant (n).

Bacterial and archaeal diversity and community composition in woody and open patches

We tested the effect of patch type (using one categorical dummy variable) on the microbial community composition (**Supplementary Table 2.2**). The analysis showed that both *Bacteria* and *Archaea* are distributed according to the patch type (P = 0.0091 and 0.0202, respectively). This indicates that the perennial plant influences the soil's microbial communities and that this factor is responsible for a sizable portion of the variability in the community structure (7.3% and 9.8% of the variance in the data for *Bacteria* and *Archaea*, respectively). The analysis was block designed so that permutations were only allowed within each station (reflecting the six composite soil samples). Both bacterial and archaeal scores of the first canonical axis were plotted versus the patch variable, showing two distinct groups clustered according to patch type (**Figure 2.2**).

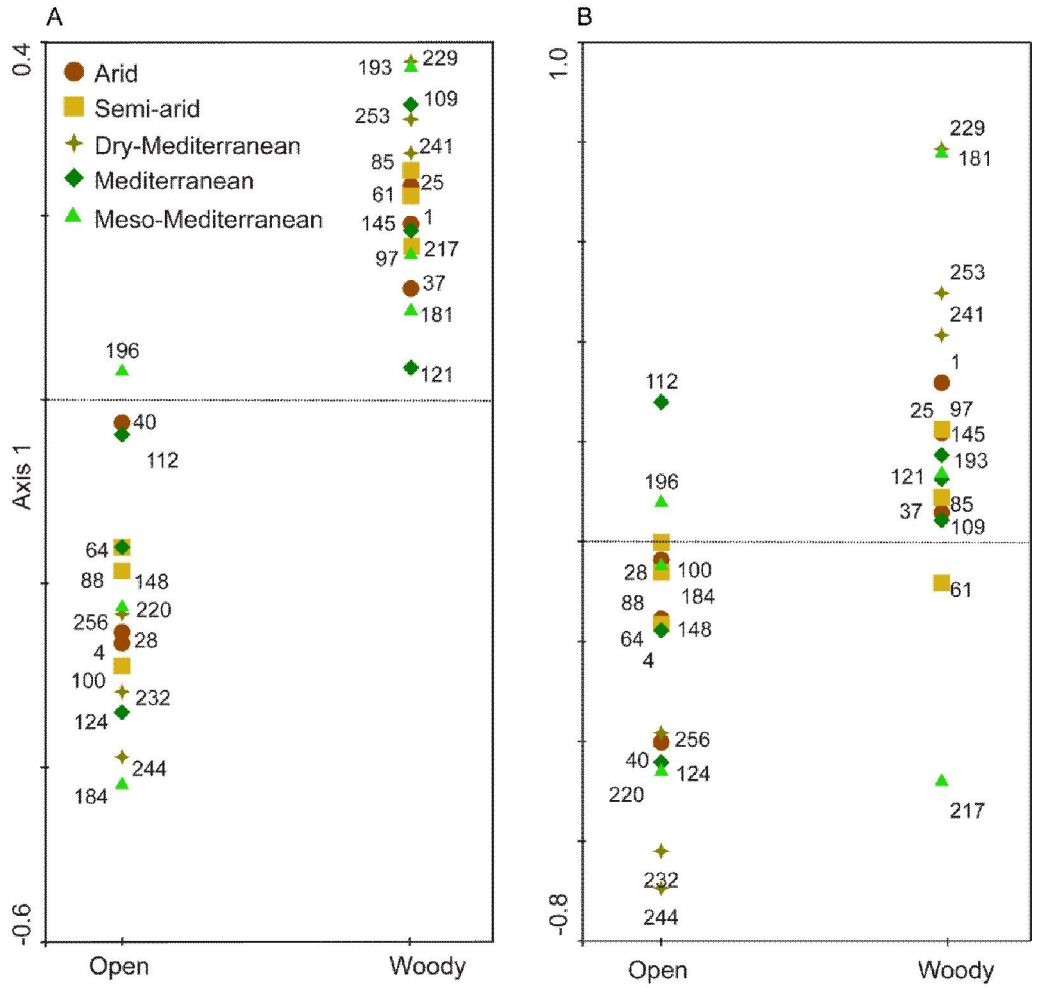


Figure 2.2| First non-canonical axis of the redundancy analysis (RDA) of (A) *Bacteria* and (B) *Archaea* TRFLP profiles versus patch type (open and woody) for each sample. The axes explain 7.3% and 9.8% of the variability in the data for *Bacteria* and *Archaea*, respectively, and the difference in the community between patches is significant at levels P = 0.0091 and 0.0202 for *Bacteria* and *Archaea*, respectively.

Bacterial and archaeal community composition within and between stations

We tested the effects of all samples taken from the triplicate plots within each station (15 categorical dummy variables, three in each of the five stations) on community composition. The analysis was block designed so that permutations were only allowed within each station (corresponding to six samples). Neither *Bacteria* nor *Archaea* showed any significant differences in their distribution between the replica plots within stations (P = 0.33 and 0.77, respectively). These results indicated that the soil samples taken at a single station from three different plots are indeed replicates.

Our next step was to test the effect of stations (five categorical dummy variables) on community composition. The analysis was performed with unrestricted permutations between the stations. The community compositions of both *Bacteria* and *Archaea* were significantly different between different stations (P = 0.0001, R^2 = 0.174 and P = 0.0001, R^2 = 0.386, respectively), suggesting that the community composition is biogeographically structured. Bacterial and archaeal diversity across the precipitation gradient **Figure 2.3** reflects the results of two-way cluster analysis of the samples (rows) and TRFs (columns). The consensus profiles of both *Bacteria* and *Archaea* were used in this cluster analysis, corresponding to the TRFs generated by two of the restriction enzymes used in this study (see Experimental procedure): TaqI for 16S rRNA fragments amplified with the bacterial primers (**Figure 2.3A**) and MseI for fragments amplified using the archaeal primers (**Figure 2.3B**). Each individual square in the central coloured matrix represents the relative abundance (indicated by colour) of a single TRF. The top right Scree plot represents the distance between each of the two levels of clustering versus cluster number.

Analyses of both *Bacteria* and *Archaea* showed a clear clustering of the arid soil samples (marked dark brown) and, to a lesser extent, of the semiarid samples (marked light brown). Interestingly, the three Mediterranean stations (marked bronze green, dark green and light green) did not cluster: the bacterial heat map showed two major clusters at both ends, whereas the archaeal TRFs amplified from the Mediterranean soil samples clustered together.

Heat maps were constructed on the basis of the bacterial and archaeal 16S rRNA-encoding gene-amplified fragments digested with HhaI, HpyCH4IV, TaqI and MboI (**Supplementary Figure 2.2**). The bacterial TRFs (**Supplementary Figures 2.2A and B**) followed the pattern described above, unlike the archaeal TRFs (**Supplementary Figures 2.2C and D**) that clustered into dissimilar patterns.

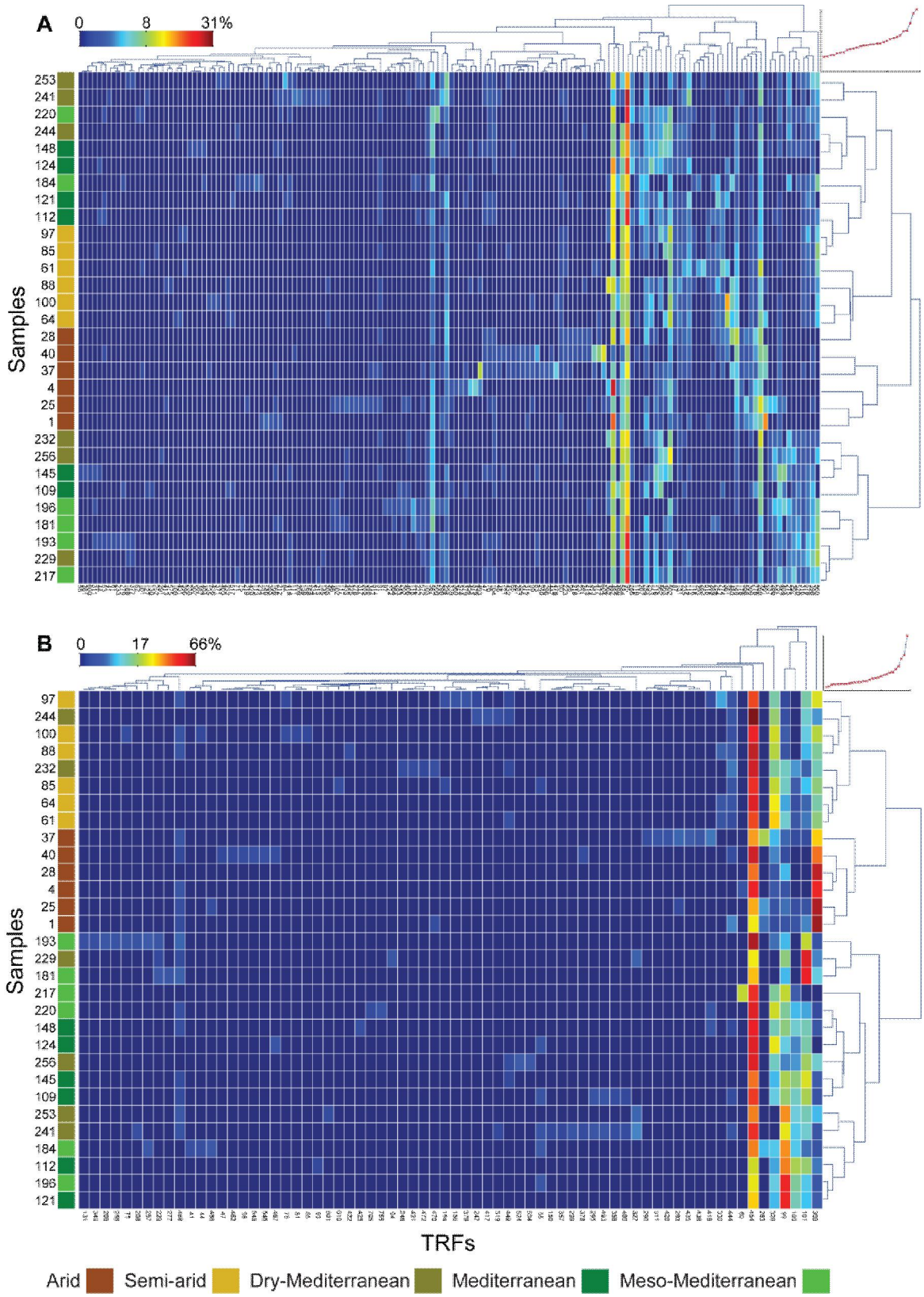


Figure 2.3| Two-way cluster analysis of consensus TRFLP profiles of (A) *Bacteria* and (B) *Archaea*. Each row in the heat map represents a sample, and each column represents a TRF. Columns are clustered according to samples, whereas the rows are clustered in accordance with the TRFs generated by restriction of each soil

sample's amplified 16S rRNA fragment using the restriction enzymes TaqI for *Bacteria* (A) and MseI for *Archaea* (B). The colour-coded column to the left of the heat map corresponds to the origin of each sample (see legend at the bottom for colour coding). Heat map colours represent the relative abundance of the TRFs. Clustering was performed on a Euclidean distance matrix of the standardized and transformed TRFLP profiles (see Experimental procedure). Scree plots (top right of each map) show the distance between each of the two hierarchical clusters versus cluster number.

Relationship between abiotic factors and bacterial and archaeal communities

Eleven physicochemical factors were measured in the soil samples used for the analyses of *Bacteria* and *Archaea* community composition (**Table 2.1**). The standardized values of these parameters were correlated to the bacterial and archaeal TRF scores using a partial RDA model.

In a forward selection method, only water content, organic carbon and calcium carbonate correlated significantly with the community profiles of both domains ($P = 0.0001$). In addition, the bacterial distribution correlated to Mg^{2+} and nitrate ($P = 0.0051$ and 0.0152, respectively). All the above parameters (except Mg^{2+} for the *Bacteria* domain) were also found to be significant in a model that excluded the effect of the sites (i.e., allowing permutation testing only within sites). The analysis results indicated that the effect of these parameters is evident not only at a regional, but also at a local scale (data not shown). However, although showing significant correlations, all the above-mentioned parameters were also strongly auto-correlated (data not sown), making it difficult to determine which is the driving force of microbial soil diversity in this setting. Of these parameters, water content was found to have the strongest fit to the bacterial and archaeal community structures. **Figure 2.4** shows how the communities of both domains are positioned along the water-content concentration axis. The *Bacteria* and *Archaea* communities amplified from arid environment soil samples clustered at the low end of the soil water content, whereas the Mediterranean communities clustered along the higher end. Much like in the cluster analysis (**Figure 2.3**), the community compositions of *Bacteria* and *Archaea* did not strictly follow the water-content gradient, but rather formed three separate clusters of arid, semiarid and Mediterranean communities (**Supplementary Figure 2.3**).

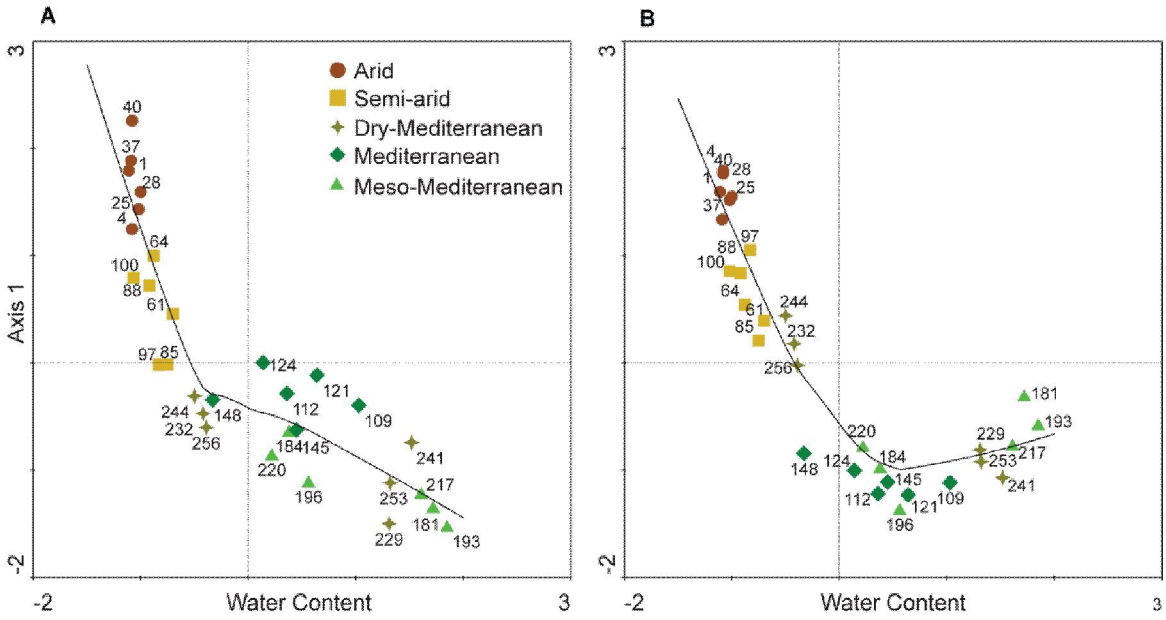


Figure 2.4| First non-canonical axis of the redundancy analysis (RDA) of (A) *Bacteria* and (B) *Archaea* TRFLP profiles versus the standardized (Z-score) values of the water content of each sample. Locally weighted scatter plot smoothing (LOESS) fit was performed with a local linear model and a span of α = 0.67. The axes explain 15% and 29%, respectively, of the variability in the data for *Bacteria* and *Archaea*.

2.4 Discussion

A major goal in biogeography and ecology is to understand the causes of taxonomic diversity gradients. Such gradients occur on spatial scales ranging from a few centimetres (Carson et al., 2009) to thousands of kilometres (Fierer and Jackson, 2006). For microorganisms, research has primarily focused on local scales (Fierer, 2008); however, the drivers of diversity and their relative influence may differ with scale, and understanding diversity gradients may require analyses of their variation relative to various spatial scales. To the best of our knowledge, this study is the first to link local and regional scales of bacterial and archaeal community diversities. We tested both domains within each of the five LTER sites, using a scheme that enabled us to examine a triplicate composite of eight soil samples in each patch at each site; this procedure ensured that the samples reflect the entire plot. Statistical analysis of bacterial and archaeal fingerprints in this sampling scheme revealed that the differences in diversity within sites are not statistically significant, unlike the diversity between sites across the precipitation gradient. This encompasses our most surprising result, that is, the spatial patterns of OTU diversity for *Archaea* and *Bacteria* are very similar in structure, despite the profound biological differences between these two domains.

Fingerprinting methods, such as TRFLP, are robust and can be applied to a large number of samples; however, the TRFLP technique entails two major drawbacks. The first is inherent to all known fingerprinting techniques and concerns their detection limit; abundant species are well represented, whereas the rare species remain unseen. Consequently, the majority of species in a highly diversified environment, such as soil remains undetected and hence taxa–area relationships within microbial communities are difficult to decipher (Curtis et al., 2006; Woodcock et al., 2006). However, theoretical modelling has indicated that if significant shifts in microbial community are spatially correlated, as shown in this report, then the models will yield closer estimates reflecting the ‘true’ taxa–area relationship (Woodcock et al., 2006). The other drawback concerns the choice of enzymes for restricting the amplified 16S rRNA fragment, which strongly influences the observed TRFs

and thus the emerging diversity patterns (Schutte et al., 2008). Although the enzymes used in our study were chosen in accordance with *in silico* analysis of the RDP database (see Experimental procedure), the outcome varied between the two domains: archaeal and bacterial TRFs were each analyzed using three distinct restriction enzymes, however, in contrast to *Bacteria*, archaeal TRF clusters of the enzymes TaqI and MboI did not follow the same biogeographical structure across the precipitation gradient (compare **Figure 2.3b** with **Supplementary Figures 2.1c** and **d**). This observation could be attributed to the choice of the restriction enzymes.

Both domains followed similar biogeographical patterns (**Figure 2.2**), their diversity apparently unrelated to variables that typically govern plant and animal diversity. Diversity gradients of macroorganisms have been described on different scales in relation to latitude, climate, productivity and temperature, documenting the generality of the latitudinal diversity gradient, with stronger and steeper diversity gradients on regional as opposed to local scales (Hawkins et al., 2003). Those studies have shown a positive relationship between annual precipitation (an index of productivity in arid regions), species richness and phylogenetic composition. For instance, in grasslands, the number of species per square meter was shown to increase by one with each 100 mm increase in precipitation (Cornwell and Grubb, 2003; Adler and Levine, 2007); the diversity and community organization of North American ants (Keil et al., 2008) and rodents (Bowers et al., 1987) were shown to be tightly correlated to annual precipitation, and a survey across Western Europe and Northern Africa showed that water availability limits the richness of Odonata (dragonfly) species (Keil et al., 2008). In contrast to macroorganisms, our results showed similar diversity and richness of the soil bacterial and archaeal communities across sites, whereas the taxonomic composition differed by ecosystem type. The species abundance distribution of the 30 soil samples showed a similar pattern for bacterial and archaeal communities: domination of a few of the more abundant OTUs, whereas most of the OTUs are relatively rare, exemplifying the classic 'long tail' phenomenon (Fuhrman, 2009).

The microbial communities in the arid, semiarid and Mediterranean sites were significantly different (P = 0.0001), whereas the microbial communities

within the Mediterranean sites (although the annual precipitation differed markedly, at 400, 500 and 900 mm per year) shared key characteristics, with no significant differences among them (P = 0.079 and 0.244 for *Bacteria* and *Archaea*, respectively). The clustering of the microbial communities according to the ecosystem (arid, semiarid and Mediterranean) rather than strictly according to the precipitation gradient could be largely explained by a combination of precipitation, as reflected by the soil water content (**Figure 2.4**) and vegetation cover as reflected by the soil organic matter content (**Table 2.1**). It has been suggested that microbial biogeographical patterns are shaped by environmental factors (Fierer, 2008). For instance, pH has been found to be the best predictor of the continent-scale patterns exhibited by soil *Bacteria* (Fierer and Jackson, 2006). Diversity of Antarctic soil *Bacteria* changed along a temperature gradient, yet was comparable in locations with dense vegetation cover (Yergeau et al., 2007), and the diversity of soil microbial community assemblages in the Chihuahuan Desert followed the precipitation patterns (Clark et al., 2009). In this study, numerous factors were measured for each of the 30 soil samples (**Table 2.1**) including pH, salinity, calcium carbonate and nutrients (e.g. phosphorus, nitrogen, carbon, magnesium and potassium), yet the distribution pattern of both *Bacteria* and *Archaea* correlated mainly with soil water content (**Figure 2.4**), organic matter that is stored in the soil and calcium carbonate. We suggest that precipitation and vegetation cover are the major factors shaping the structure of the soil microbial community in the arid, semiarid and Mediterranean sites. Indeed, patch types were found to vary in both bacterial and archaeal communities, with different OTUs found in the open areas and under the plant canopies (**Figure 2.2**). We speculate that the structures of the bacterial and archaeal communities were comparable among the three Mediterranean sites because of a combination of selection pressure exerted by plants and the protection from environmental fluctuations provided by the vegetation. However, in the exposed arid and semiarid sites, where vegetation is scarce and the open patches are devoid of plants, the resource islands support distinct microbial communities (Herman et al., 1995).

Numerous studies have shown the strong correlation between precipitation and macroorganism richness and diversity, especially in water-limited regions (Hawkins et al., 2003). Until recently, however, microorganisms' spatial diversity has received little attention, as the requisite sampling and analysis efforts were unrealistic considering the number of *Bacteria* in a gram of soil (Schloss and Handelsman, 2006). The introduction of quick and reproducible fingerprinting techniques 12 years ago (Liu et al., 1997; Fisher and Triplett, 1999) has enabled microbiologists to compare large number of soil samples and move beyond local-scale observations. Here, we examined local and regional diversity patterns of both *Bacteria* and *Archaea* and found that the two domains cluster in a similar manner. The fingerprint-based analysis suggests that separate evolutionary and ecological processes have directed the biogeography of micro- and macroorganisms, resulting in distinct patterns. Further work is needed to elucidate the following: (i) whether these biogeographical patterns are stable over time; (ii) the phylogenetic patterns in the three separate ecosystems delineated here and (iii) the functional groups within each community. Such comprehensive examination would improve our understanding of the spatial and temporal patterns of microbial life in different habitats and provide a link to the full breadth of the ecosystems.

2.5 Experimental procedure

Site description

Sampling was performed in May and June of 2007 at five LTER stations in Israel (http://lter.bgu.ac.il/) located in areas with mean annual precipitation ranging from 100 to 900 mm per year (**Supplementary Figure 2.1**). At each station, sampling was performed in triplicate plots of 4025 m^2, all fenced and thus protected from grazing livestock and undisturbed by human activity.

At each plot, eight randomly selected subsamples were taken from the bare soil in the interspaces between the dominant perennial plants (open patch) and under the perennial canopy (woody patch). The predominant perennial was singled out at each station) and we sampled under its canopy alone. The eight subsamples of each patch type at each plot were composited to represent an average for that site, resulting in a total of six composite soil samples per station (**Supplementary Figure 2.1**).

Soil collection and physicochemical characterization

After crust and litter removal, the top 5 cm of the soil was collected into sterile Whirl-Pak sample bags (Nasco, Fort Atkinson, WI, USA) and placed in a cooler. The samples were transported to the laboratory and homogenized within 24 h of sampling. A 50 g subsample of each soil sample was stored at -80 °C for molecular analysis, whereas the rest was used for physicochemical analysis.

Soil chemical analysis was performed according to standard methods for soil analyses (SSSA, 1996): soil water content by gravimetric method; percentage organic matter by dichromate oxidation method; pH and electrical conductivity in saturated soil extract (SSE); sodium, calcium and magnesium in SSE by inductively coupled plasma spectroscopy; sodium adsorption ratio by calculation from Na^+ and Ca^{2+} + Mg^{2+} concentrations; total phosphate by the 'Olsen method' (sodium bicarbonate extract); K^+ in SSE by flame spectrophotometer; nitrogen as nitrate in aqueous extract;

nitrogen as ammonium in KCl solution extract (including adsorbed nitrogen); percentage of calcium carbonate by hydrochloric acid digestion.

As different units were used to measure the various physicochemical parameters, they all had to be brought into an equal range before any analysis. In addition, the distribution in each factor had to approach normality to better meet the assumptions of the statistical models. Testing different transformation techniques showed standardization (Z-score) to yield the best results in terms of eliminating scale differences and achieving normality under Kolmogorov–Smirnov test.

DNA extraction, PCR amplification and TRFLP analysis

Bacterial and archaeal community fingerprints were obtained using terminal restriction fragment length polymorphism (TRFLP; Liu et al., 1997). DNA was extracted from triplicate soil subsamples, each consisting of 0.25 g (wet weight), using the PowerSoil DNA Isolation Kit (MoBio, West Carlsbad, CA, USA).

Polymerase chain reaction (PCR) amplification was performed with the primer pairs 341F (Ishii and Fukui, 2001): 50 -CCTACGGGAGGCAGCAI-30 and 908R (Lane et al., 1985): 5'-CCGTCAATTCMTTTGAGTTI-3' targeting *Bacteria*, and 109F (Grosskopf et al., 1998): 5'-ACKGCTCAGTAACACGI-3' and 934R (Stahl and Amann, 1991): 5'-GTGCTCCCCCGCCAATTCCI-3' targeting *Archaea*. All primers were modified by the addition of inosine at the 3' end in an attempt to broaden their target scope (Ben-Dov et al., 2006). In both primer pairs, the forward primer was labelled with the fluorescent dye 6-FAM (6-carboxyfluorescein; Metabion, Martinsried, Germany) at the 5' end. PCRs were conducted in triplicates of 50 μl to minimize reaction bias. In addition, the following steps were taken to minimize some of the artefact effects of PCR, such as the appearance of chimeras and pseudo-terminal restriction fragments (TRFs; Egert and Friedrich, 2003): (i) the number of PCR cycles was reduced to 24 and 25 for *Bacteria* and *Archaea*, respectively, and elongation time was extended to 3min; (ii) before cleanup and digestion with restriction enzymes, amplified DNA samples were treated with mung bean exonuclease (TaKara, Shiga, Japan) according to the manufacturer's instructions. Each PCR contained 2.5 μl 10 × buffer (Bioneer, Daejeon,

South Korea), 0.25 mM of each dNTP (Larova, Teltow, Germany), 2.5 mM MgCl2, 0.5 µM of each primer (Metabion), 1 µg µl $^{-1}$ BSA (New England Biolabs (NEB), Ipswich, MA, USA), 2.5 units Taq DNA polymerase (HyLabs, Rehovot, Israel) and 1 µl DNA template. The PCRs were carried out as follows: after an initial 5min denaturation step at 95 °C, 24 or 25 cycles (for *Bacteria* and *Archaea*, respectively) were run at 94 °C for 45 s, 45 °C for 1 min and 72 °C for 3 min, followed by a final elongation step at 72 °C for 10 min. After amplification, the triplicate PCRs were pooled, treated with mung bean exonuclease and purified using a PCR purification kit (Bioneer). The purified PCR products were digested with the restriction enzymes TaqI, HhaI (TaKara) and HpyCH4IV (NEB) for samples amplified using the bacterial primers (341F/908R). The restriction enzymes TaqI, MseI and MboI (TaKara) were used for the amplicons generated with the archaeal primers (109F/934R). For each enzyme, digestions were performed in reactions of 20 µl containing 2 µl of digestion buffer (TaKara), 20 units of restriction enzyme and approximately 200 ng of the purified PCR product. Digestion was followed by precipitation using standard ethanol precipitation with Pellet Paint (Novagen, Darmstadt, Germany), and resuspension in double-distilled water. These samples were analyzed with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The peaks in each profile were related to specific fragment lengths based on a size marker (70–500 MapMarker, BioVentures, Murfreesboro, TN, USA). Data were retrieved using Peak Scanner software v1.0 (Applied Biosystems). Each sample was loaded at least twice and the profiles were treated as replicates.

Data manipulation and statistical analysis

Raw TRFLP data cannot be used directly for analysis, and therefore the following standardization and normalization procedures were applied prior to all statistical analyses. The size in base pairs of each peak (TRF) was used to indicate an operational taxonomic unit (OTU), whereas the area under the peak was used to determine its relative abundance in the profile. The TRFLP patterns of the replicates (method replicates) of each sample were standardized as described elsewhere (Dunbar et al., 2001). Profiles were then aligned and a consensus profile was computed for each sample from its

replicates by eliminating non reproducible peaks and averaging shared peaks. The procedure was then applied again to standardize the consensus profiles and they were aligned to generate a sample-by-species matrix, which was used in subsequent analyses. The above procedure was repeated for each restriction enzyme separately. For a better fit of the data set to the assumptions of the statistical models, two additional transformations were applied. (i) To deal with possible skewness of the data set, a Log (x + 1) transformation was applied; this greatly improved the overall performance of the samples in a Kolmogorov–Smirnov test for normality (data not shown). (ii) To deal with the problem of null values in the matrix, it was transformed to give Hellinger distances between the samples when Euclidean distances were computed (Legendre and Gallagher, 2001). The matrix was tested under a detrended correspondence analysis (DCA) model and the length of the first gradient was found to be less than 4 SD, and hence linear models were constructed (data not shown). A two-way cluster analysis was simultaneously performed on the TRFs and the samples, using Euclidean distances and Ward's linkage. A Scree plot showing the distance between the clusters as a function of clustering order was used to determine the relevant number of clusters. To test for the differences between species distributions, the standardized TRFLP data of each sample were ordered from the most abundant to the least abundant TRF (rank abundance) and a Kolmogorov–Smirnov test was performed on every pair of samples to test whether they were drawn from the same distribution.

All standardization and normalization procedures were performed using MATLAB 7 (http://www.mathworks.com) and the codes are available at http://www.staff.uni-marburg.de/~angel. Cluster and distribution analyses were computed using MATLAB. Hypothesis testing was performed using block-design redundancy analysis (RDA) and tested using Monte Carlo permutation tests (ter Braak and Smilauer, 1998). Correlations to physicochemical characteristics were performed using RDA with forward selection procedure (ter Braak and Smilauer, 1998). All RDA models were computed using Canoco 4.53 (http://www.canoco.com).

2.6 Supplementary material

Supplementary Table 2.1| Pairwise Kolmogorov-Smirnov tests for the bacterial (A) and archaeal (B) TRFLP profiles

A

	1	4	25	28	37	40	61	64	85	88	97	100	109	112	121	124	145	148	181	184	193	196	217	220	229	232	241	244	253
4	0.034																												
25	0.021	0.000																											
28	0.483	0.047	0.012																										
37	0.043	0.000	0.542	0.032																									
40	0.093	0.000	0.466	0.039	0.286																								
61	0.402	0.060	0.155	0.719	0.080	0.171																							
64	0.491	0.051	0.382	0.597	0.063	0.114	0.997																						
85	0.432	0.041	0.116	0.703	0.134	0.072	0.724	0.768																					
88	0.064	0.009	0.609	0.087	0.129	0.290	0.397	0.625	0.888																				
97	0.587	0.067	0.128	0.953	0.212	0.152	0.414	0.442	0.995	0.433																			
100	0.863	0.083	0.132	0.746	0.141	0.106	0.976	0.977	0.878	0.307	0.769																		
109	0.025	0.003	0.961	0.065	0.299	0.494	0.555	0.551	0.556	0.911	0.230	0.145																	
112	0.064	0.032	0.362	0.395	0.225	0.311	0.097	0.183	0.900	0.218	0.636	0.484	0.444																
121	0.111	0.008	0.308	0.247	0.354	0.609	0.397	0.603	0.900	0.981	0.614	0.642	0.749	0.514															
124	0.058	0.861	0.000	0.035	0.000	0.000	0.048	0.051	0.076	0.014	0.027	0.132	0.004	0.037	0.011														
145	0.011	0.003	0.922	0.056	0.545	0.379	0.432	0.497	0.353	0.949	0.218	0.148	1.000	0.243	0.740	0.002													
148	0.009	0.001	0.953	0.018	0.677	0.518	0.273	0.419	0.213	0.688	0.181	0.124	0.967	0.588	0.403	0.001	0.841												
181	0.055	0.045	0.167	0.076	0.060	0.167	0.653	0.588	0.863	0.997	0.393	0.265	0.712	0.474	0.931	0.045	0.639	0.423											
184	0.014	0.004	0.755	0.065	0.374	0.527	0.477	0.542	0.696	0.997	0.353	0.173	0.943	0.425	0.848	0.005	0.879	0.871	0.863										
193	0.000	0.000	0.180	0.000	0.163	0.012	0.015	0.016	0.034	0.261	0.004	0.002	0.318	0.003	0.114	0.000	0.485	0.079	0.197	0.154									
196	0.222	0.035	0.166	0.460	0.125	0.134	0.984	0.994	0.997	0.683	0.875	0.890	0.535	0.357	0.792	0.047	0.485	0.241	0.746	0.458	0.018								
217	0.133	0.009	0.359	0.298	0.109	0.229	0.893	0.961	0.940	0.953	0.871	0.711	0.881	0.540	0.937	0.025	0.830	0.554	0.941	0.944	0.073	0.851							
220	0.124	0.003	0.495	0.134	0.091	0.218	0.145	0.248	0.798	0.964	0.628	0.500	0.765	0.740	0.994	0.009	0.893	0.580	0.949	0.584	0.117	0.485	0.691						
229	0.090	0.071	0.215	0.208	0.013	0.039	0.418	0.750	0.811	0.922	0.535	0.567	0.602	0.330	0.637	0.071	0.353	0.173	0.997	0.471	0.118	0.756	0.739	0.717					
232	0.810	0.031	0.102	0.834	0.193	0.185	0.795	0.674	0.878	0.208	0.975	0.931	0.174	0.517	0.479	0.039	0.164	0.152	0.298	0.165	0.003	0.767	0.545	0.515	0.257				
241	0.008	0.000	0.691	0.037	0.654	0.513	0.084	0.210	0.231	0.496	0.152	0.104	0.475	0.336	0.775	0.001	0.603	0.971	0.244	0.591	0.095	0.196	0.243	0.380	0.058	0.129			
244	0.936	0.191	0.049	0.910	0.058	0.059	0.795	0.913	0.841	0.308	0.837	0.972	0.111	0.355	0.517	0.226	0.099	0.079	0.307	0.116	0.003	0.767	0.570	0.515	0.429	0.581	0.080		
253	0.041	0.006	0.624	0.100	0.361	0.503	0.605	0.497	0.832	0.986	0.319	0.342	0.998	0.377	0.897	0.014	0.893	0.885	0.897	0.879	0.296	0.865	0.946	0.893	0.384	0.233	0.735	0.261	
256	0.575	0.056	0.211	0.544	0.051	0.140	0.996	0.981	0.368	0.500	0.426	0.984	0.219	0.158	0.376	0.071	0.351	0.315	0.653	0.400	0.019	0.836	0.734	0.219	0.726	0.593	0.163	0.714	0.502

= $P<.01$ = $.01 \leq P<.05$ = $P<.1$

B

	1	4	25	28	37	40	61	64	85	88	97	100	109	112	121	124	145	148	181	184	193	196	217	220	229	232	241	244	253
4	0.425																												
25	0.958	0.180																											
28	0.062	0.338	0.062																										
37	0.966	0.483	0.750	0.043																									
40	0.073	0.017	0.073	0.030	0.009																								
61	0.690	0.586	0.315	0.385	0.508	0.014																							
64	0.316	0.871	0.316	0.340	0.767	0.006	1.000																						
85	0.603	0.561	0.603	0.339	0.457	0.073	0.967	0.975																					
88	0.279	0.748	0.093	0.225	0.723	0.004	0.929	0.987	0.638																				
97	0.684	0.222	0.935	0.084	0.862	0.209	0.380	0.132	0.583	0.106																			
100	0.435	0.171	0.435	0.179	0.504	0.281	0.296	0.101	0.587	0.081	0.998																		
109	0.852	0.112	0.978	0.254	0.536	0.066	0.192	0.150	0.421	0.045	0.890	0.947																	
112	0.080	0.176	0.044	0.198	0.013	0.010	0.254	0.468	0.363	0.254	0.034	0.048	0.080																
121	0.018	0.209	0.018	0.657	0.009	0.006	0.305	0.193	0.425	0.107	0.023	0.070	0.112	0.847															
124	0.232	0.550	0.140	0.534	0.103	0.010	0.749	0.951	0.894	0.749	0.169	0.129	0.080	0.810	0.847														
145	0.209	0.443	0.209	0.180	0.132	0.044	0.748	0.883	0.954	0.748	0.132	0.173	0.268	0.907	0.526	0.980													
148	0.603	0.901	0.603	0.339	0.457	0.010	0.997	0.975	0.958	0.967	0.399	0.435	0.421	0.140	0.425	0.894	0.620												
181	0.363	0.176	0.140	0.534	0.264	0.010	0.608	0.907	0.725	0.749	0.034	0.129	0.080	0.810	0.847	1.000	0.980	0.363											
184	0.471	0.465	0.168	0.141	0.704	0.001	0.645	0.920	0.824	0.978	0.079	0.147	0.083	0.129	0.070	0.229	0.431	0.400	0.297										
193	0.539	0.706	0.539	0.025	0.953	0.003	0.335	0.569	0.277	0.428	0.564	0.249	0.342	0.007	0.012	0.059	0.167	0.277	0.121	0.353									
196	0.080	0.550	0.080	0.534	0.045	0.010	0.608	0.543	0.533	0.352	0.079	0.129	0.080	0.810	0.993	1.000	0.951	0.894	0.810	0.040	0.023								
217	0.032	0.259	0.032	0.821	0.019	0.012	0.150	0.078	0.095	0.048	0.008	0.045	0.032	0.669	0.259	0.136	0.499	0.032	0.136	0.016	0.010	0.136							
220	0.603	0.779	0.603	0.156	0.750	0.073	0.967	0.678	0.958	0.638	0.684	0.435	0.852	0.363	0.180	0.725	0.620	0.958	0.363	0.478	0.539	0.232	0.095						
229	0.790	0.871	0.405	0.340	0.767	0.044	0.970	1.000	0.975	0.987	0.132	0.389	0.268	0.468	0.193	0.543	0.883	0.790	0.907	0.920	0.377	0.543	0.250	0.790					
232	0.916	0.645	0.858	0.179	0.732	0.281	0.473	0.520	0.889	0.473	0.646	0.736	0.909	0.094	0.070	0.523	0.196	0.435	0.576	0.374	0.330	0.262	0.005	0.858	0.616				
241	0.667	0.197	0.991	0.041	0.434	0.121	0.334	0.109	0.253	0.084	0.951	0.710	0.693	0.008	0.007	0.078	0.040	0.134	0.008	0.022	0.263	0.019	0.004	0.433	0.082	0.275			
244	0.958	0.180	0.958	0.156	0.750	0.010	0.315	0.316	0.958	0.093	0.684	0.435	0.852	0.140	0.063	0.363	0.405	0.603	0.140	0.168	0.539	0.140	0.032	0.958	0.405	0.435	0.433		
253	0.269	0.152	0.112	0.180	0.132	0.006	0.559	0.883	0.678	0.622	0.022	0.101	0.059	0.621	0.932	0.994	0.883	0.316	0.980	0.431	0.092	0.951	0.078	0.316	0.883	0.520	0.005	0.112	
256	0.279	0.366	0.093	0.385	0.158	0.004	0.519	0.861	0.628	0.929	0.016	0.081	0.045	0.471	0.305	0.959	0.748	0.279	0.994	0.603	0.072	0.749	0.048	0.279	0.748	0.473	0.003	0.093	0.987

= $P<.01$ = $.01 \leq P<.05$ = $P<.1$

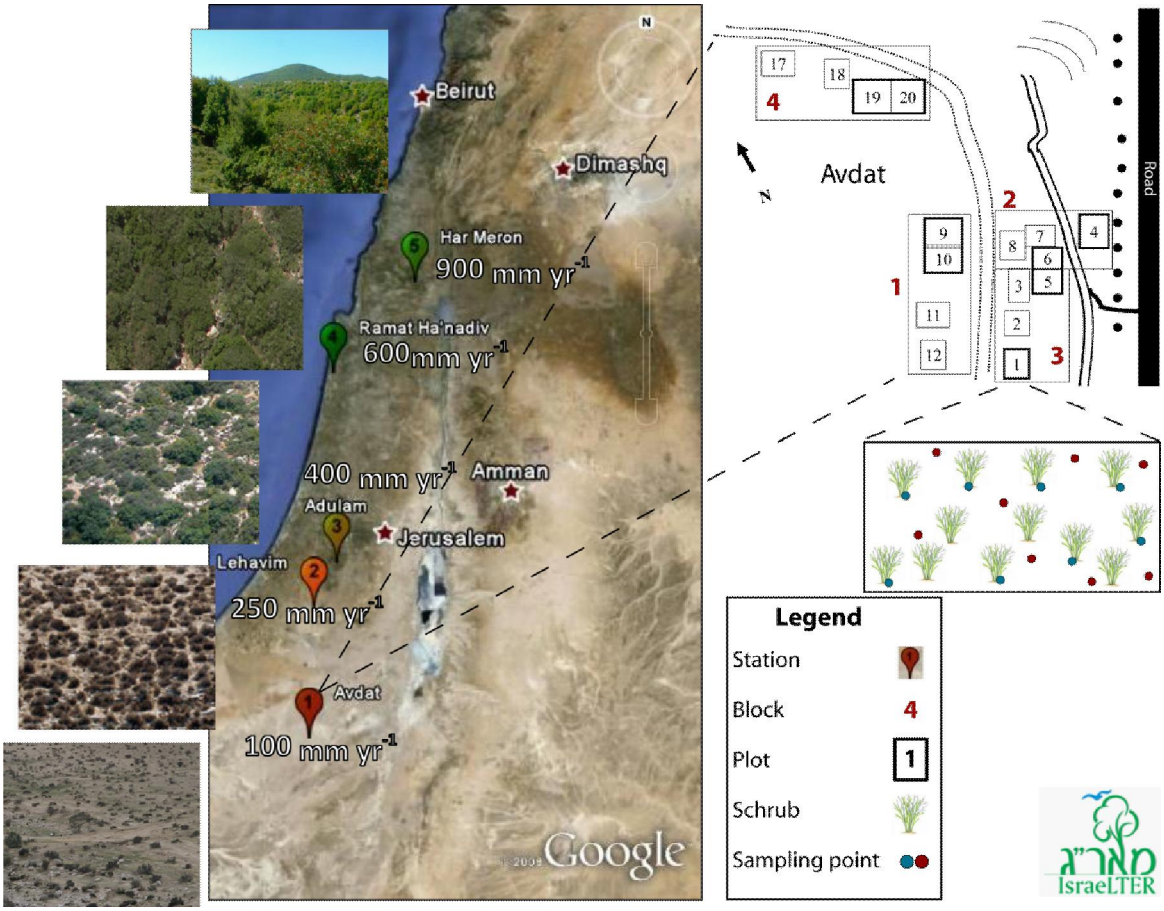
P = Chance that two sets of data are drawn from the same distribution

Supplementary Table 2.2| Statistical tests for the effect of different geographic scales on the community compositions (see text for methods description)

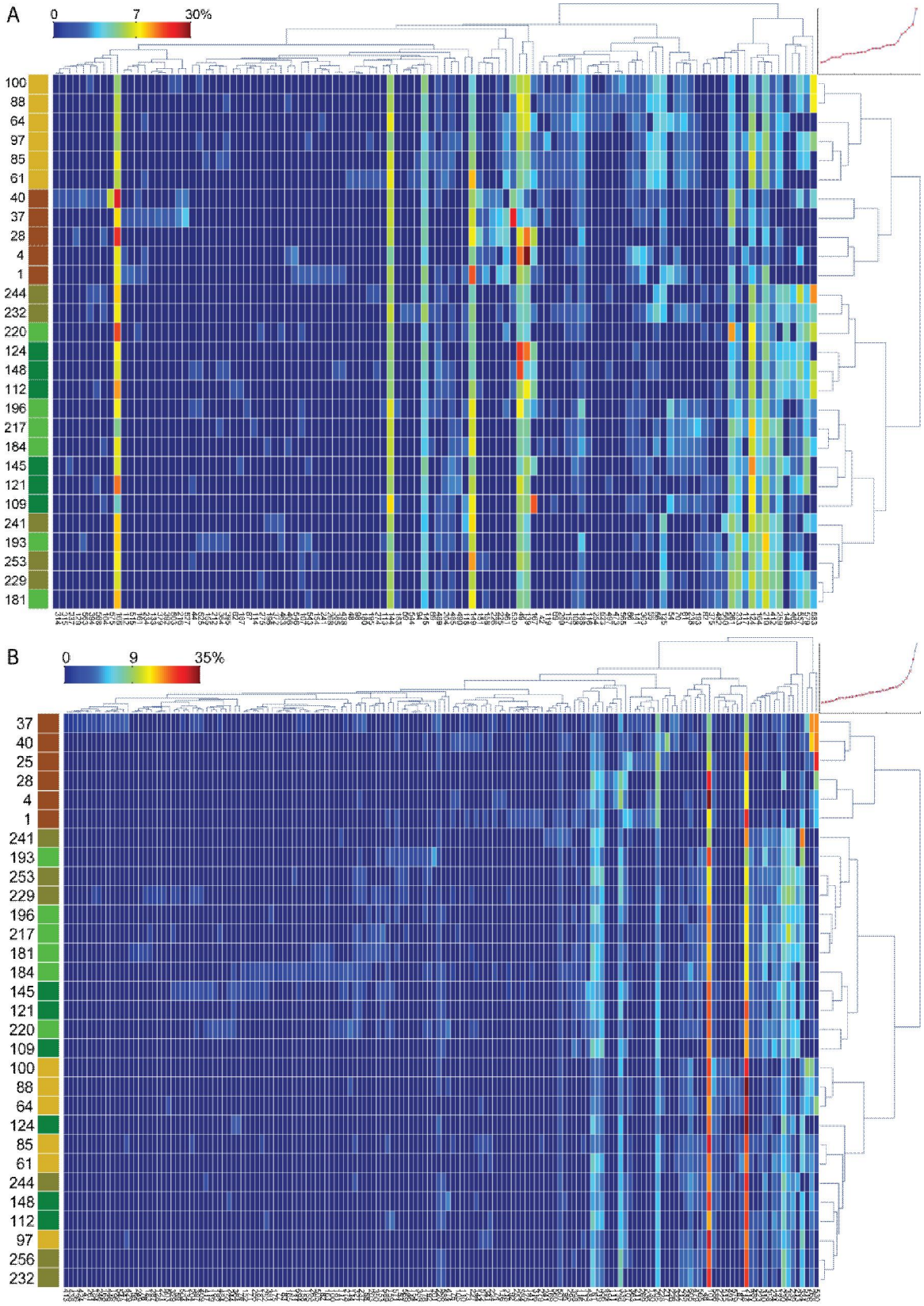
			Bacteria		*Archaea*	
Test	*Matrix*	*Permutations*	*Significance**	*Variance***	*Significance*[a]	*Variance*[b]
Sites	Correlation	Unrestricted	F = 5.279 *P* = 0.0001	17.4%	F = 15.736 *P* = 0.0001	38.6%
Plots	Covariance	Only within stations	F = 1.763 *P* = 0.3297	-	F = 2.455 *P* = 0.7683	-
Patch type	Covariance	Only within stations	F = 1.878 *P* = 0.0091	7.3%	F = 2.596 *P* = 0.0202	9.8%

* Significance of the first canonical axis

** Variance of the species data explained by the first canonical axis



Supplementary Figure 2.1| Schematic description of the sampling design. Five LTER sites are located across the precipitation gradient in Israel. In each site three plots were sampled all fenced and maintained such that they are undisturbed by human activity (represented by the black-filled square). In each plot eight soil samples were taken from two patch types: under the dominant perennial canopy (marked green) or at the interspaces between plants (marked grey).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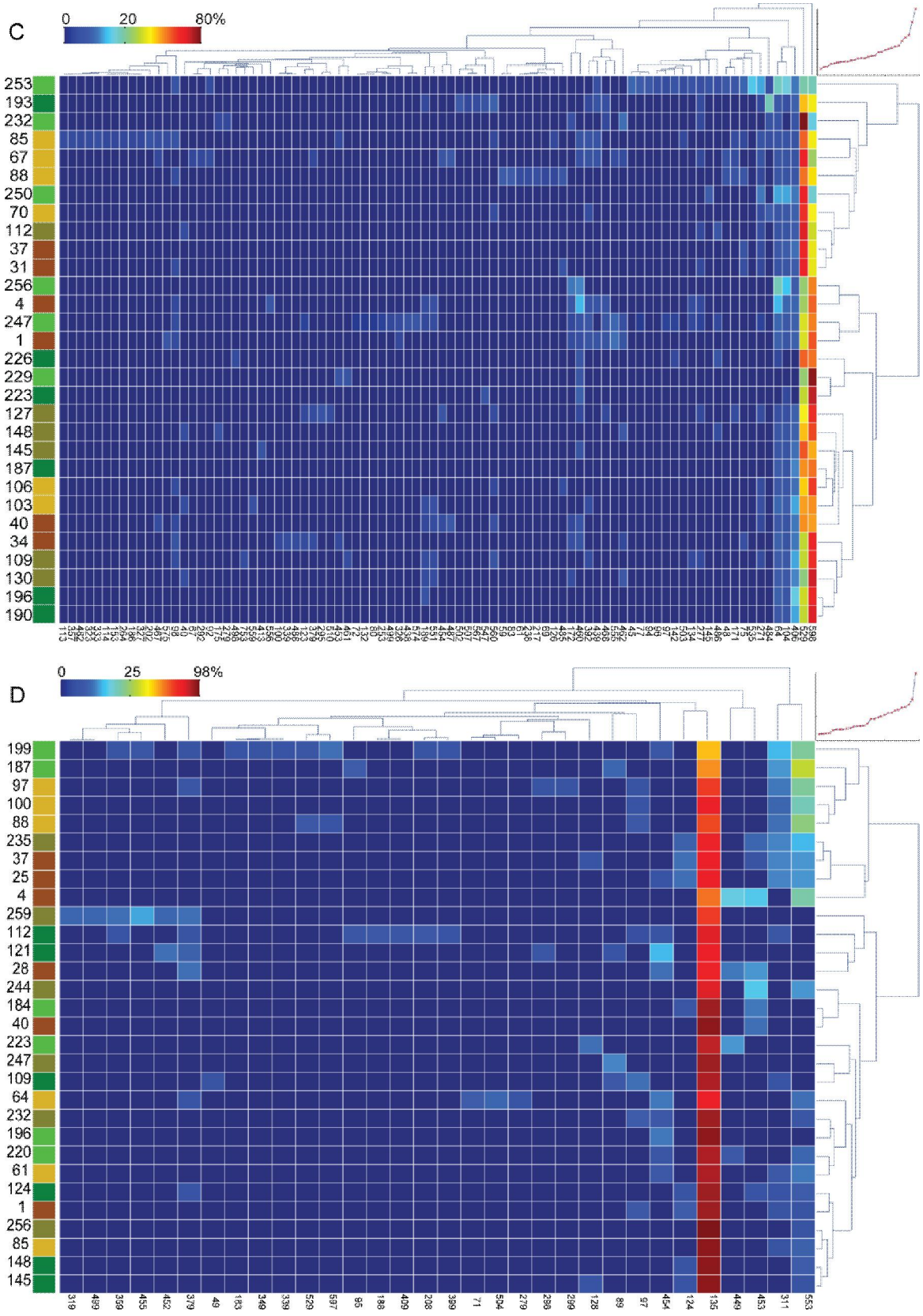
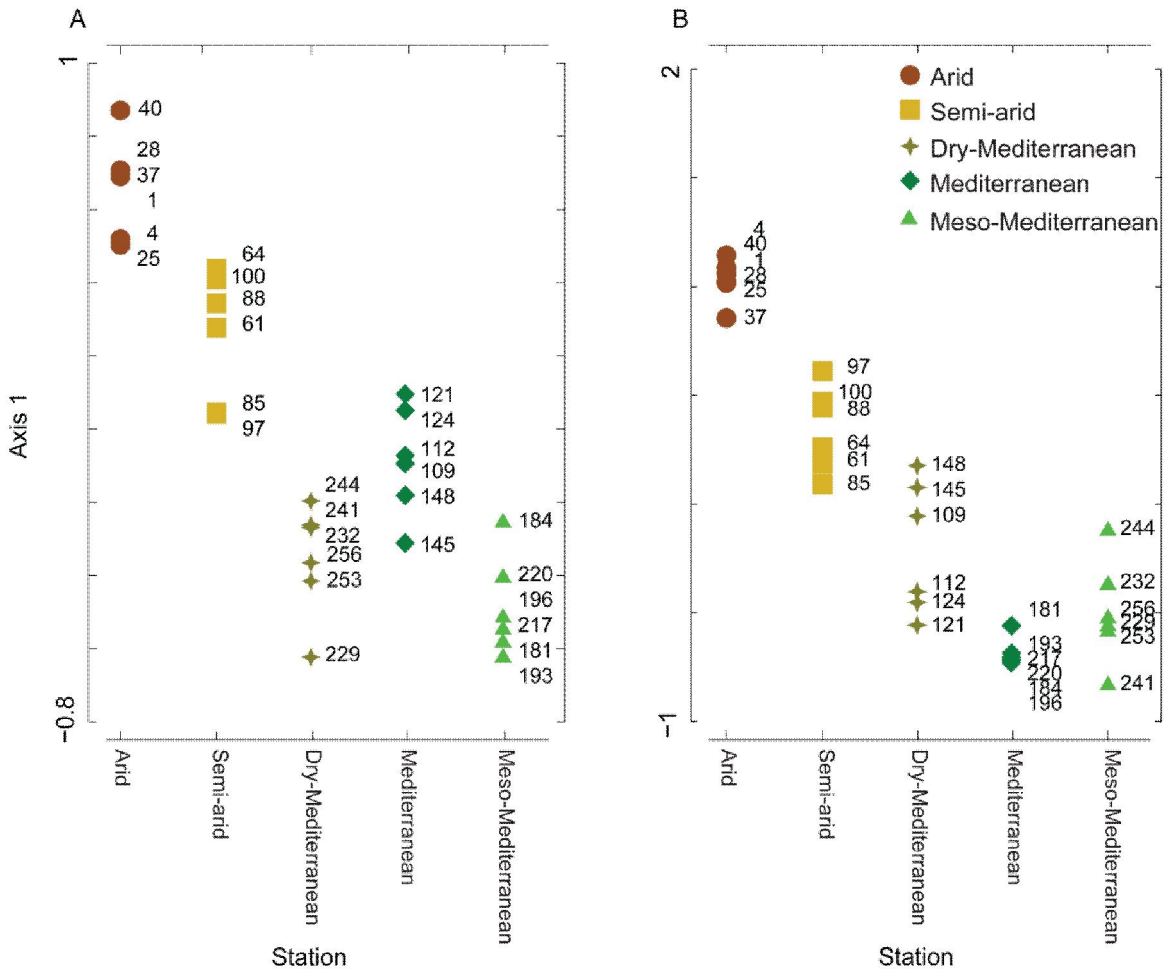


Supplementary Figure 2.2| Two-way cluster analysis of consensus TRFLP profiles of *Bacteria* and *Archaea*. Each row in the heat map represents a sample, and each column represents a TRF. Columns are clustered according to samples while the rows

are clustered in accordance to the TRFs generated by restriction of each soil sample's amplified 16S rRNA fragment using the restriction enzymes (A) *HhaI* and (B) *HpyCH4IV* for *Bacteria* and (C) *TaqI* and (D) *MboI* for *Archaea*. The colour-coded column to the left of the heat map corresponds to the origin of each sample (see legend at the bottom for colour coding). Heat map colours represent relative abundance of the TRFs. Clustering was done on a Euclidean distance matrix of the standardized and transformed TRFLP profiles (see *Experimental procedure*). Scree plots (top right of each map) show the distance between each two hierarchical clusters vs. cluster number.



Supplementary Figure 2.3| First non-canonical axis of RDA analysis of (A) *Bacteria* and (B) *Archaea* TRFLP profiles vs. the sampled sites. The axes explain 17.4% and 38.6% of the variability in the data for *Bacteria* and *Archaea*, respectively.

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Chapter 3 |

In situ Measurement of Methane Fluxes and Analysis of Transcribed Particulate Methane Monooxygenase in Desert Soils

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Contributions:

RA and RC designed the study and the sampling scheme; RA performed the field work, the sampling, all lab experiments, design of Matlab codes and data analyses; RA and RC wrote the manuscript.

3.1 Abstract

Aerated soils are a biological sink for atmospheric methane. However, the activity of desert soils and the presence of methanotrophs in these soils have hardly been studied. We studied on-site atmospheric methane consumption rates as well as the diversity and expression of the *pmoA* gene, coding for a subunit of the particulate methane monooxygenase, in arid and hyperarid soils in the Negev Desert, Israel. Methane uptake was only detected in undisturbed soils in the arid region (~90 mm yr^{-1}) and vertical methane profiles in soil showed the active layer to be at 0–20 cm depth. No methane uptake was detected in the hyperarid soils (~20 mm yr^{-1}) as well as in disturbed soils in the arid region (i.e. agricultural field and a mini-catchment). Molecular analysis of the methanotrophic community using terminal restriction fragment length polymorphism (TRFLP) and cloning/sequencing of the *pmoA* gene detected methanotrophs in the active soils, whereas the inactive ones were dominated by sequences of the homologous gene *amoA*, coding for a subunit of the ammonia monooxygenase. Even in the active soils, methanotrophs (as well as in situ activity) could not be detected in the soil crust, which is the biologically most important layer in desert soils. All *pmoA* sequences belonged to yet uncultured strains. Transcript analysis showed dominance of sequences clustering within the JR3, formerly identified in Californian grassland soils. Our results show that although active methanotrophs are prevalent in arid soils they seem to be absent or inactive in hyperarid and disturbed arid soils. Furthermore, we postulate that methanotrophs of the yet uncultured JR3 cluster are the dominant atmospheric methane oxidizers in this ecosystem.

3.2 Introduction

Aerated soils constitute a global biological sink for atmospheric methane, a potent greenhouse gas whose atmospheric abundance has been steadily increasing since the beginning of the industrial revolution (Prather et al., 2001; Khalil, 2000). The magnitude of the soil methane sink has been estimated to be 30 Tg CH_4 yr^{-1} being 5% of the annual global methane budget (Prather et al., 2001; Dunfield, 2007). The site-specific methane uptake rates vary significantly and are affected by soil type and land use (Adamsen and King, 1993; Reay et al., 2001; 2005; Menyailo and Hungate, 2003). Thus, the estimate of the global sink strength for CH_4 uptake by soils has been criticized due to the large variation found in field measurements both spatially and temporarily. Smith and colleagues (2000) performed a set of measurements, compared it with field data from previous studies, and estimated the global biological methane sink to range from 7 to > 100 Tg CH_4 yr^{-1}. Although oxidation of atmospheric methane was measured in nearly all types of aerated soils including tundra (West and Schmidt, 1998), boreal, temperate and tropical forests (Yavitt et al., 1990; King and Adamsen, 1992; Henckel et al., 2000; Knief et al., 2005; Kolb et al., 2005), grasslands and cultivated soils (Horz et al., 2005; Tate et al., 2007), as well as deserts (Striegl et al., 1992), most data come from North America and northern Europe while data from other regions and ecosystems are limited or non-existent (Smith et al., 2000).

Methane uptake in soils is mediated by the methane-oxidizing bacteria, or simply methanotrophs, which oxidize methane for both energy (dissimilatory methane oxidation) and carbon assimilation (assimilatory methane oxidation; Mancinelli, 1995). Nearly all methanotrophs species are affiliated to the alpha and gamma subdivisions of the Proteobacteria (Bowman, 2006) and were traditionally thought to be exclusively limited to these phylogenetic groups. Recent work, however, demonstrated the existence of extreme thermo-acidophilic methanotrophs within the Verrucomicrobia phylum (e.g. Dunfield et al., 2007). Methanotrophs of the Proteobacteria are commonly divided into type I and type II based on their phylogeny, morphology and physiology (Bowman, 2006). A third group, type

X, is phylogenetically affiliated with type I but possesses several distinguishing biochemical features. The key enzyme in the oxidation of methane is the methane monooxygenase which is known to exist in two versions, a cytoplasmic one which is referred to as the soluble methane monooxygenase (sMMO) and a membrane-bound protein referred to as the particulate methane monooxygenase (pMMO; Murrell et al., 2000). The *pmoA* subunit (27 kDa) of the pMMO is the most commonly used genetic marker for the detection of methanotrophs as this form of methane monooxygenase is present in all methanotrophs except Methylocella, while the sMMO is limited to certain groups (Murrell et al., 1998).

Until recently it was believed that all cultured representatives of methanotrophs are able to oxidize methane only at far higher concentrations than our current atmospheric concentration. This notion has been revised with the discovery of a second *pmo* operon in some type II methanotrophs which encodes an isozyme of the pMMO that is able to oxidize methane at low and even atmospheric levels (Baani and Liesack, 2008). In addition, it had been noted that the dominant *pmoA* sequences detected in soils with active uptake of CH_4 from the atmosphere form clusters (e.g. upland soil cluster alpha, USCa) that are different from those of the known methanotrophs (Henckel et al., 1999; Holmes et al., 1999; Knief et al., 2003; Kolb et al., 2005). However, since the methanotrophs possessing these *pmoA* sequence clusters have not yet been cultured, the evidence for them being atmospheric methane oxidizers is only indirect.

Drylands (semiarid, arid and hyperarid regions) cover around 31–38% of the Earth's surface, with deserts (arid and hyperarid regions) alone covering 19–20% (Middleton and Thomas, 1997; Ffolliott et al., 2003). Half of the world's countries are in part or entirely located in dryland environments and they are home to nearly 40% of the human population (Ffolliott et al., 2003). Despite their significant size and importance drylands tend to be under-represented in research and are often less understood compared with other ecosystems. The importance of studying drylands stems not only from their current size but also from the fact that they expand through desertification (Navone and Abraham, 2006). Evidence exists of the involvement of desert

soils the turnover of atmospheric gases in general (e.g. Bitton, 2002) and of methane in particular (Striegl et al., 1992; Peters and Conrad, 1996).

The goal of this study was to characterize the diversity of methanotrophs, the expression of the *pmoA* gene as well as the spatial heterogeneity of methane uptake in arid and hyperarid environments. We hypothesized that water limitation conditions associated with arid and hyperarid environments might act as a strong selection force for specifically adapted methanotrophs and might also limit the extent of atmospheric methane oxidation in these soils. To this aim we performed field measurements of methane uptake and methane concentrations in soils of different habitats in arid and hyperarid parts of the Negev Desert in Israel. In addition, we characterized the methanotrophic community using the *pmoA* gene marker and its mRNA transcripts.

3.3 Results

Field measurements of methane uptake and methane vertical profiles

Of the five sites studied (**Table 3.1**) only one, the natural site at Avdat, showed a decrease in methane concentration over time inside the chambers, indicating uptake of atmospheric methane by the soil (**Figure 3.1**). This could be seen in the intershrub patches and also under shrub canopies (**Figure 3.1A**). Methane levels dropped from an initial atmospheric concentration of ~0.07 μmol per litre of air down to 0.033 ± 0.004 and 0.045 ± 0.01 μmol per litre of air in the open and shrub patches, respectively. Methane concentration in the chamber decreased logarithmically with time and thus matched first-order kinetics, as expected for methane uptake by soil (Bender and Conrad, 1993). The methane uptake rates at the Avdat natural site, as deduced from a linear approximation during the first 80 min, were 39.4 ± 16.79 and 62.4 ± 5.8 μmol CH_4 m^{-2} d^{-1} for the shrub and intershrub patches, respectively (**Table 3.2**). At all the other sites, closed chamber measurements showed no decrease in methane concentration over 80 min enclosure (**Figure 3.1B**).

Table 3.1| Characteristics of sites and soils*

Location	Site	Texture (%)			Water content (%)	pH	$N_{tot.}$, $C_{org.}$(%)		Predominant vegetation
		Sand	Silt	Clay			N	C	
Avdat, Negev Plateau, Israel	Natural field (30◦ 47'57N/ 34◦ 45'57E)	29	57.5	13.5	2.4	8.6	0.01	0.33	*Hamada scoparia*
	Liman (constructed mini-catchment) (30◦ 48'52N/ 34◦ 45'26E)	13.35	56.75	29.9	4.6	7.9	0.09	1.27	*Cupressus sempervirens* *Ziziphus spina-christi* *Balanites aegyptiaca*
Arava Valley South, Israel	Agricultural field (30◦ 52'33N/ 34◦ 48'16E)	33.9	47.8	18.3	3.0	8.5	-	0.28	
	Wadi (dry riverbed) (29◦ 58'44N/ 35◦ 05'16E)	88.2	7.51	4.29	-	8.4	-	0.06	
	Agricultural field (30◦ 04'11N/ 35◦ 08'45E)	91.5	5.81	2.69	ND	8.5	0.14	0.38	Spring onions

* All analyses presented here were preformed on the 0-10 cm soil fraction of each sample.
– Below limits of detection.
ND – not determined.

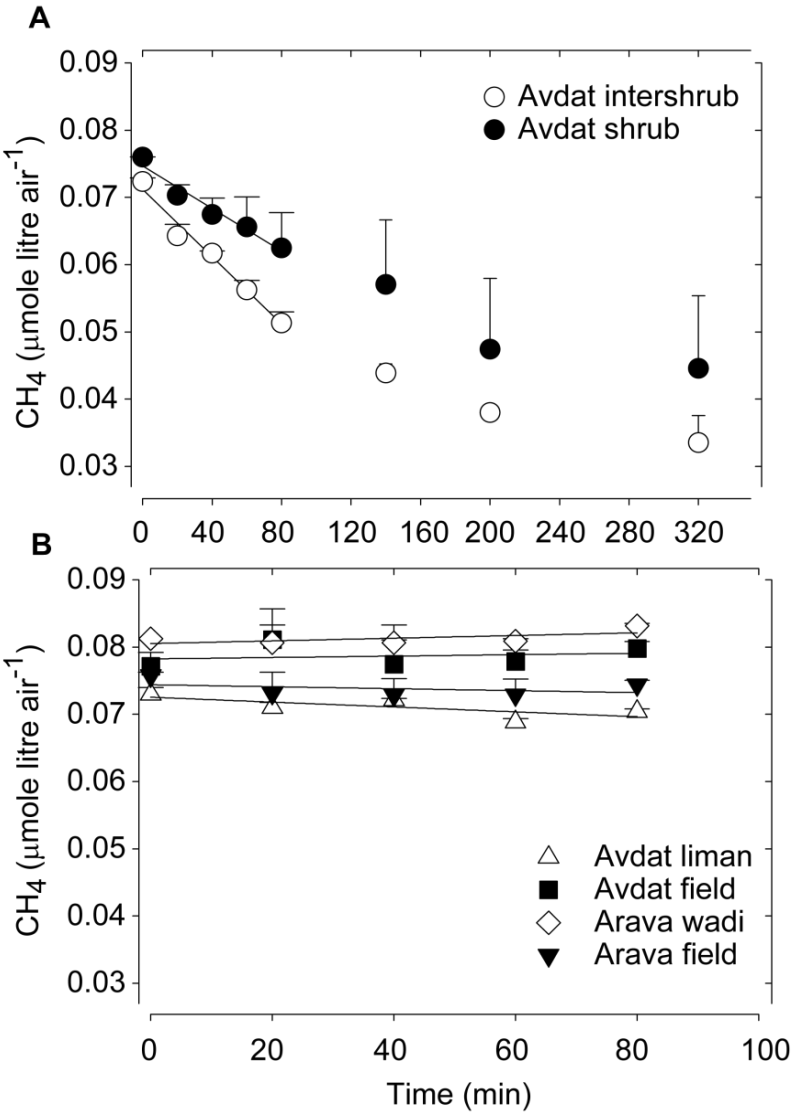

Figure 3.1| Methane uptake in the closed chamber experiments. Error bars represent the standard errors of measurements taken from two independent chambers. A. Measurements taken at Avdat. Regressions are given for the first 80 min (R^2 = 0.95 and 0.63 for intershrub and shrub, respectively). B. Measurements taken at Avdat and Arava excluding the natural site.

The vertical methane concentration profiles at the active sites (**Figure 3.2**) showed a sharp decrease in methane concentration down to 20 cm depth at the intershrub and shrub patches. Between 20 and 100 cm depth, the CH_4 concentration stabilized around 0.04 μmol per litre of air. The zone of decrease in CH_4 concentration points to the location of the active methanotrophic community. The vertical profile at the liman site showed a decrease in methane concentration within the soil layers down to 30 cm depth, below which it stabilized at around 0.025 μmol per litre of air. The

vertical profile measured in the agricultural field at Avdat also showed a decrease in methane concentration with soil depth, but unlike the previously mentioned profiles methane concentration decreased continuously down to about 0.04 μmol per litre of air at 100 cm depth. Lastly, the vertical profile measured in the wadi site at Arava showed no decrease in methane concentration with soil depth down to 110 cm.

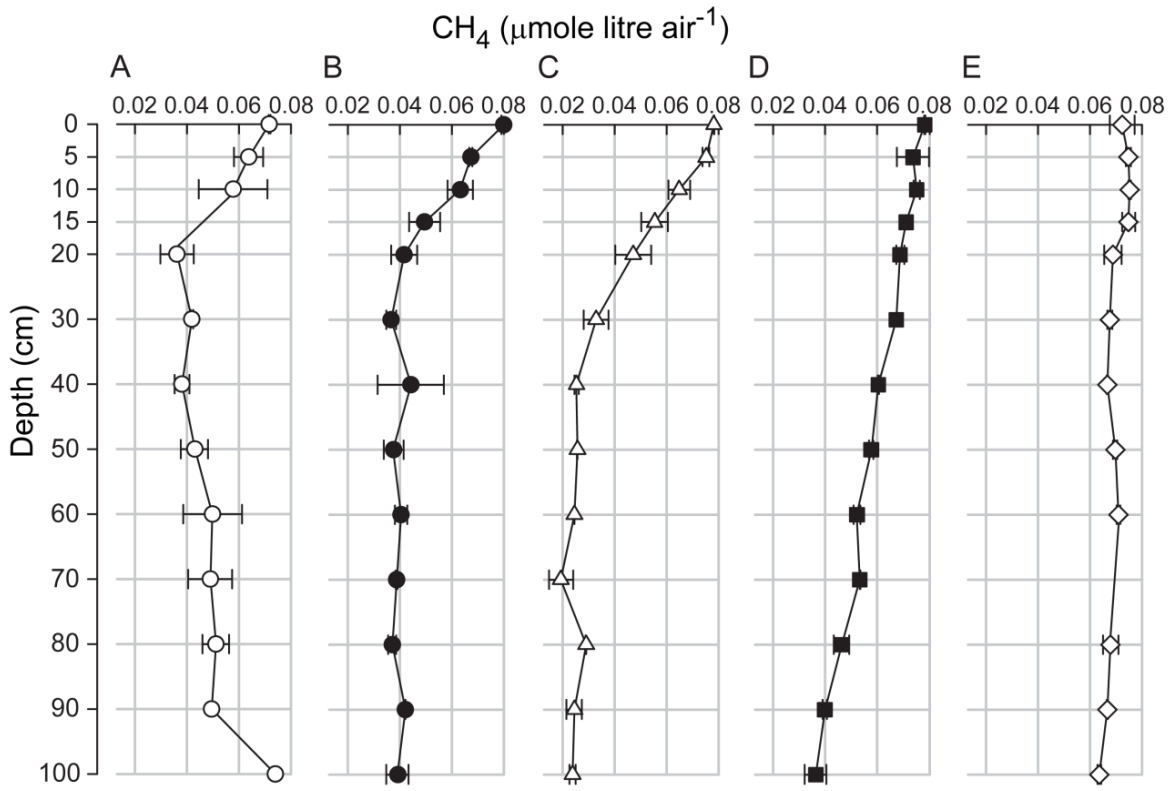

Figure 3.2| Vertical profiles of methane concentrations in the soil. (A) Avdat – intershrub, (B) Avdat – shrub, (C) Avdat – liman, (D) Avdat – agricultural field, (E) Arava – wadi. Vertical profile measurements could not be obtained for Arava – agricultural field. Error bars represent the standard errors of measurements taken from two independent cores.

Table 3.2| On site and *in situ* methane uptake rates of the soils

	Potential CH_4 uptake (μmole day^{-1} kg DW^{-1})			*On site CH_4 uptake (μmole day^{-1} m^{-2})*	*Predicted flux a (μmole day^{-1} m^{-2})*
Sample/replicate	*I*	*II*	*III*		
AV_IS_cr	-	-	-		
AV_IS_0-10	0.37	0.35	0.21	62.4 ± 5.8	42.0
AV_IS_10-20	0.26	0.20	0.20		
AV_S_cr	-	-	-		
AV_S_0-10	0.34	0.38	0.15	39.4 ± 16.8	34.2
AV_S_10-20	0.27	-	0.04		
AV_Lim_cr	-	-	-	-	
AV_Lim_0-10	-	-	-	-	32.5
AV_Lim_10-20	-	-	-	-	
AV_Ag_0-10	-	-	-	-	9.9
AV_Ag_10-20	-	-	-	-	
KT_W_cr	-	-	-	-	
KT_W_0-10	-	-	-	-	-
KT_W_10-20	-	-	-	-	
KT_Ag_0-10	-	-	-	-	ND
KT_Ag_10_20	-	-	-	-	

a. Fluxes were predicted from vertical CH_4 concentration profiles using Fick's first law of diffusion (see Experimental Procedure). DW – dry weight; – Below limits of detection; ND – not determined; AV – Avdat, KT – Arava, IS – intershrub, S – shrub, W – wadi, Ag – agricultural field, cr – crust, 0-10 and 10-20 – depth in cm.

A comparison between the values of the CH4 flux measured directly using the closed chamber and those estimated from the vertical profiles using Fick's first law shows a good agreement between the two methods for the natural site at Avdat (**Table 3.2**).

In contrast, the calculation for the liman site predicts a theoretical flux almost of the same magnitude as that at the natural site, but this flux was not detected by the closed chamber measurements. Also for the agricultural field at Avdat, the theoretical calculations indicate flux of CH_4 into the soil. However, this flux was below the detection limit of the closed chamber measurement of approximately 13 μmol d^{-1} m^{-2}.

Response of soil activity to addition of water

Shortly after application of water, the CH_4 uptake rate decreased dramatically compared with the base level measured at time zero (**Figure 3.3**). This was probably due to inhibition of diffusion as water filled the soil's pore spaces. Uptake rate returned to base level after 12 h and further increased after 24 h. After 36 and 48 h, uptake rates were still > 150% of the initial rate.

Potential methane oxidation of the soil samples

Methane consumption by soil samples mirrored the field observation with the closed chamber experiments. Thus, only soils from the natural site at Avdat showed methane consumption activity after 14 days of incubation, while all the other soil samples did not (**Table 3.2**). However, not all sections of the soil profile taken at the active sites showed potential methane oxidation activity. The samples taken at depths of 0–10 and 10–20 cm showed methane oxidation activity (AV_S_10–20 II being the only exception), but the samples taken from the surface crust layer showed no activity. This lack of activity probably indicates the absence of methanotrophs in the crust layer. The samples taken from the intershrub patches generally showed somewhat higher methane consumption rates than those taken from below the shrub (*Hamada scoparia*) canopies, and samples taken from the top

layer at 0–10 cm depth showed higher consumption rates than the deeper samples at 10–20 cm.

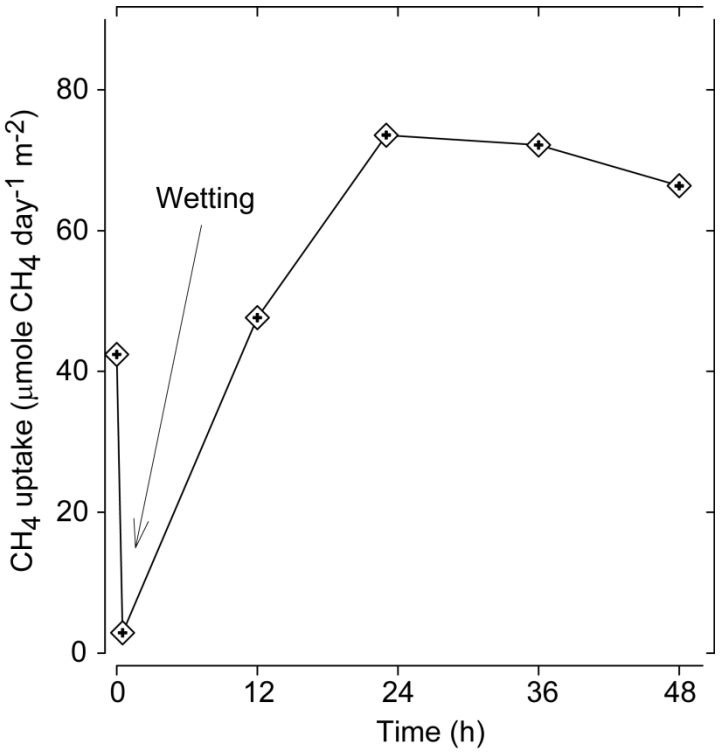

Figure 3.3| Artificial raining experiment on an intershrub patch at Avdat site. Each point represents the rate of methane uptake measured over 45 min.

Expression of the pmoA gene and diversity of the methanotrophic community

While amplification of *pmoA* transcripts was attempted in all soil samples, RT-PCR products (cDNA amplification) could only be detected in those samples showing methane oxidation activity, i.e. in the layers at 0–10 and 10–20 cm depth in the shrub and intershrub patches from the natural site at Avdat (**Figure 3.4**). In the other samples, *pmoA* transcripts could not be detected. In contrast, amplification of the *pmoA* gene using DNA instead of cDNA was successful for all samples at all depths tested (data not shown).

Terminal restriction fragment length polymorphism (TRFLP) analysis of the pmoA gene diversity was performed on DNA templates for all 16 samples and their replicates. In addition, TRFLP analysis was also performed on cDNA templates for those samples from which *pmoA* transcripts could be

amplified. The three replicate profiles of each sample were standardized and merged into one consensus profile to be used in the cluster analysis as described in Experimental procedure.

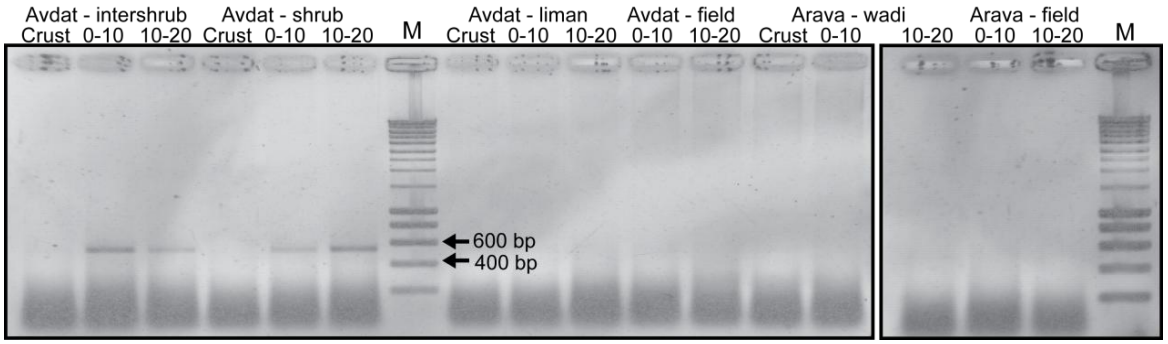

Figure 3.4| RT-PCR amplification products of transcripts of the pmoA gene using primers 189F and 682R. Sample names and depth from which they were taken are shown above the lanes. M – DNA marker.

Figure 3.5A shows the results of the two-way cluster analysis of the samples (horizontal clustering) and the terminal restriction fragments (TRFs; vertical clustering). The consensus profiles of both the DNA and the cDNA level TRFLP profiles were used in this cluster analysis. Each individual square in the central coloured matrix represents the relative abundance (indicated by the colour) of a single TRF. The top right scree plot (**Figure 3.5B**) represents the distance between each two levels of clustering versus the cluster number. **Figure 3.5A** shows a clear distinction between two major clusters – the bottom cluster which contains all inactive samples and the top cluster which contains all active samples. The inactive soils (no CH_4 oxidation potential) were dominated by the TRF of 40 bp with more than 80% of the total profile intensity in some of the samples. Other TRFs usually had a minor abundance, but in a few samples the TRFs of 173, 375 and 241 bp were also quite abundant. The second cluster – that of the active samples – was mainly dominated by peaks 375 and 173 bp and to a lesser extent also by peaks 241 and 115 bp, while the peak of 40 bp which dominated the former group could hardly be detected in these samples.

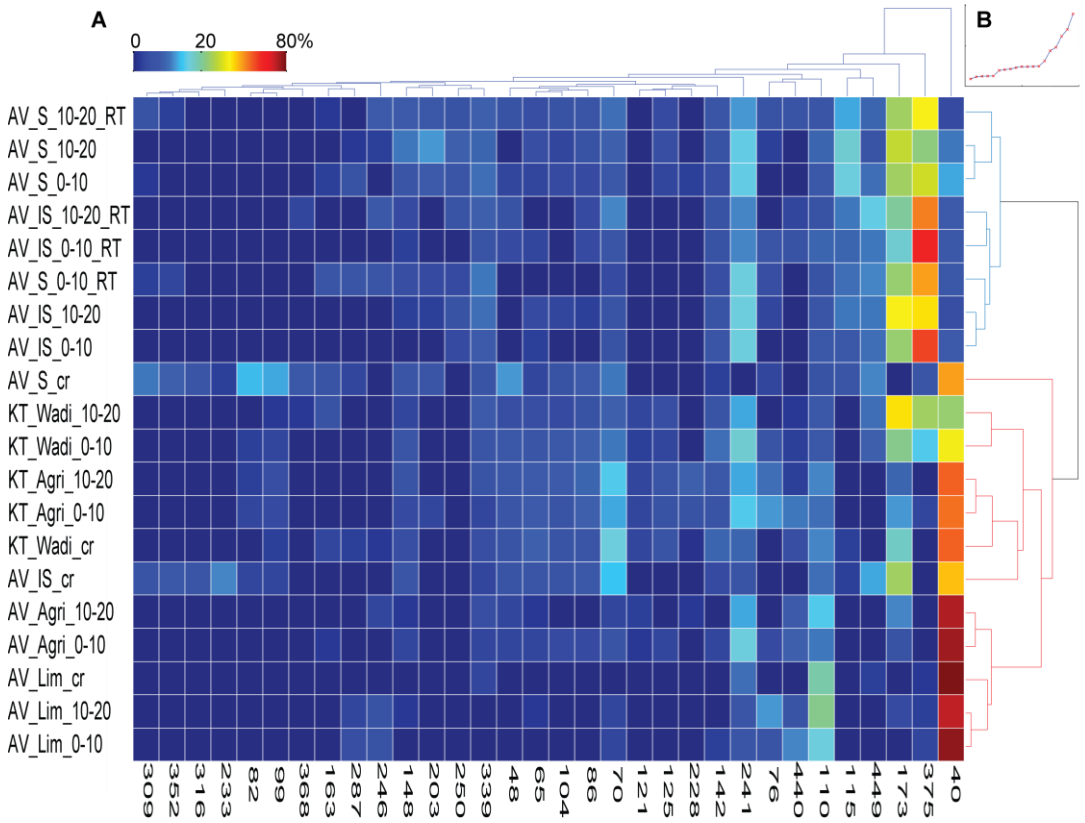

Figure 3.5| A. Two-way cluster analysis of the consensus profiles of samples (horizontal clustering) and TRFs (vertical clustering) with a heat map (middle). Each row represents a sample while each column – a TRF. Heat map colours represent relative abundance of the TRFs. The clustering was performed using weighted arithmetic average clustering (WPGMA) methods on Euclidean distance matrix of the standardized and transformed TRFLP profiles (see Experimental procedure). B. Scree plot showing the distance between each two hierarchical clusters versus cluster number. AV, Avdat; KT, Arava; S, shrub; IS, intershrub; Lim, liman; cr, crust; 0–10 and 10–20, depth in cm.

The peak of 375 bp tended to be more dominant in the intershrub samples compared with the shrub samples and also to be more dominant in the cDNA profiles compared with the DNA profiles of each sample.

For the taxonomic affiliation of the different TRFs, a phylogenetic maximum likelihood tree was constructed from the sequences (**Figure 3.6**). In parallel, an *in silico* analysis of the sequences was performed to determine their expected terminal restriction sites. This allowed the affiliation of individual TRFs to *pmoA* sequences. The TRFs of 40 and 110 bp, which dominated all inactive samples, were affiliated to *amoA* rather than *pmoA*.

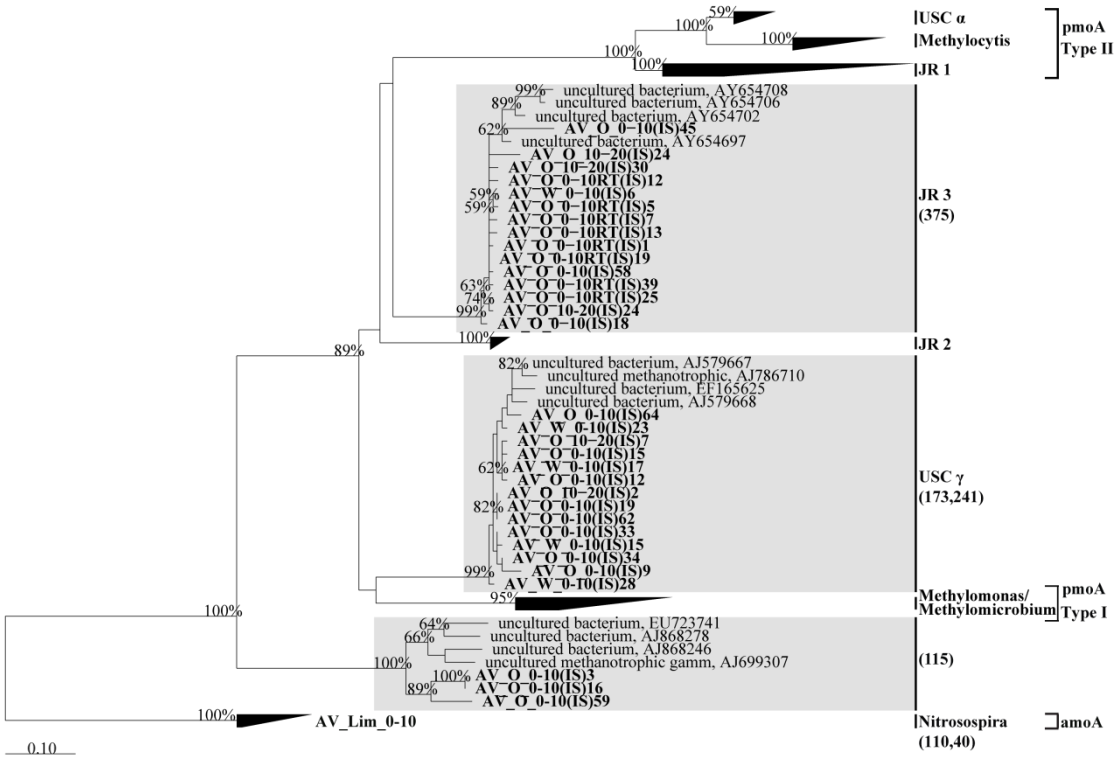

Figure 3.6| Maximum likelihood phylogenetic tree based on aligned amino acids of the *pmoA* gene. Amino acid composition was deduced from DNA sequence and the tree was calculated with RAxML 7.04 using rapid hill climbing algorithm and PROTMIXJTT evolutionary model. Names of the main pmoA clusters drawn in this tree are taken from Knief and colleagues (2003) and Horz and colleagues (2005) and the numbers below them are their respective TRF sizes. Numbers next to nodes indicate confidence values in bootstrap analysis. Names of sequences obtained in this study are marked in bold.

The corresponding sequences all clustered within the ammonia oxidizing genus *Nitrosospira*. The TRFs of 173 and 241 bp were both affiliated to USCγ, a cluster of yet uncultured methanotrophs frequently found in aerated soils (Knief et al., 2003). The sequences with either TRFs corresponding to USCγ did not form separate clusters in the phylogenetic tree. The peak of 375 bp, which dominated the active samples, was also affiliated with yet uncultured methanotrophs. Sequences of this cluster were formerly retrieved from Californian upland soils and termed JR3 (Horz et al., 2005).

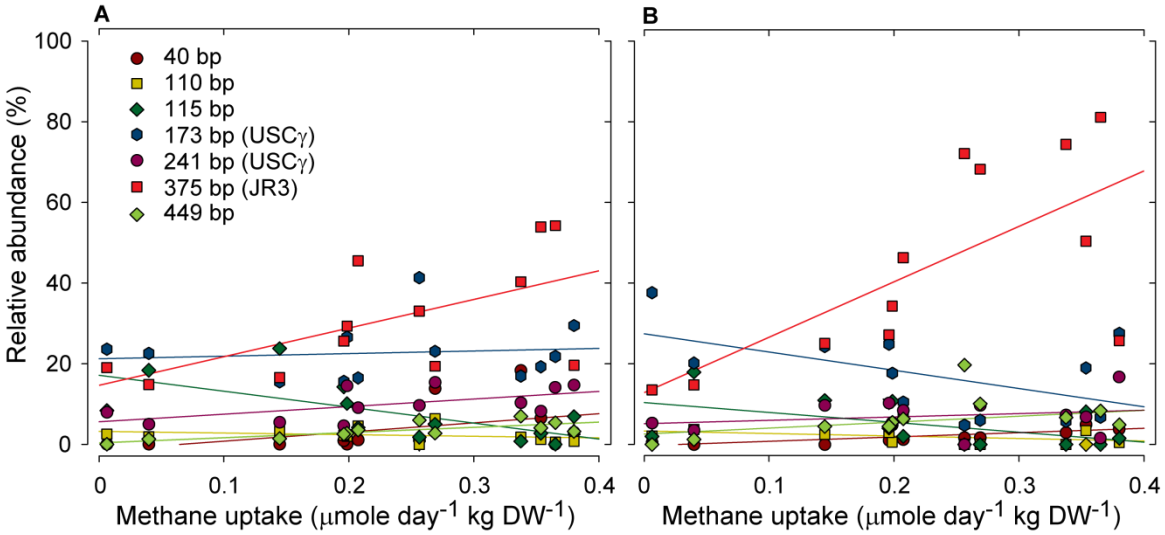

Figure 3.7| Species response curves showing the relative abundance of the seven most abundant TRFs in the TRFLP profiles of samples from Avdat shrub and intershrub at depths 0–10 and 10–20 cm as a function of their respective potential methane oxidation. A. TRFLP on DNA. B. TRFLP on cDNA. Some phylogenetic cluster names are given in brackets. Regressions for 375 bp (JR3): R^2 = 0.37, P = 0.03 for DNA, and R^2 = 0.48, P = 0.01 for cDNA.

In order to explore the correlation between structure and activity of the methanotrophic community, species response curves were plotted using the seven most abundant TRFs versus the potential methane oxidation activity at 0–10 and 10–20 cm depth of Avdat shrub and intershrub patches (**Figure 3.7**). On both the DNA and cDNA level, the strongest response was obtained for the 375 bp TRF, which corresponds to the JR3 methanotrophs. This was more pronounced on the cDNA level than the DNA level, as the slope of the curve was steeper and the fit better (R^2 = 0.48 and 0.37, respectively).

3.4 Discussion

We measured atmospheric methane uptake and characterized the active methanotrophic community in different soils from an arid and a hyperarid region in the Negev Desert, Israel. These soils included natural sites as well as disturbed sites, such as agricultural fields and a liman (constructed mini-catchment). Our field work showed that the natural site in the arid region (Avdat) of the Negev Desert was an active sink for atmospheric methane. The CH_4 uptake rates measured in shrub and intershrub patches were comparable to those measured by Striegl and colleagues (1992) in the Mojave Desert, USA, who reported average values ranging from 18.7 μmol CH_4 m^{-2} d^{-1} for dry soils to 116.6 μmol CH_4 m^{-2} d^{-1} for soils after precipitation. However, all the other sites in the Negev Desert, i.e. disturbed sites in the arid region and both disturbed and natural sites in the hyperarid region (Arava), did not exhibit CH_4 uptake from the atmosphere. Vertical profiles of CH_4 concentrations in the soil largely corroborated these results. For the Avdat natural site CH_4 concentrations decreased with soil depth indicating that the active layer was between 0 and 20 cm depth, which is in agreement with what has been reported for other soils such as temperate forests (Koschorreck and Conrad, 1993; Butterbach-Bahl and Papen, 2002), montane (Torn and Harte, 1996) and meadow soils (Bender and Conrad, 1994). In contrast, the hyperarid soils showed no decrease in CH_4 concentration down to 1 m depth being consistent with the lack of CH_4 uptake from the atmosphere. The Liman site in the arid region, on the other hand, exhibited a steep CH_4 gradient in the vertical soil profile, but no CH4 uptake. Right at the soil surface, the CH_4 gradient was close to zero. We assume that this was the reason why CH_4 was not taken up from the atmosphere. The lacking CH_4 gradient just below the soil surface may be an indication for a shallow zone of CH_4 production, which is reasonable due to the fact that the liman is periodically flooded. Indeed, we found a marked potential for CH_4 production in this soil layer (publication in preparation). In addition, it is also possible that the methanotrophic layer in this site lies below 20 cm depth. Indeed, the vertical profiles show a decrease in CH_4 concentration down to a depth of 40 cm, which is below the sampling layers.

If indeed methanotrophs reside at a deeper layer, this can explain our inability to detect any methane oxidation activity or *pmoA* genes in the soil samples taken. The agricultural field in the arid region showed a relatively flat gradient down to more than 1 m depth indicating that a zone with methanotrophic activity may exist in >1m depth, but this deep zonation did not result in detectable CH_4 uptake within the 80 min during which the flux chambers were closed. Cultivated and disturbed soils are known to show lower rates of methane oxidation compared with native soils; however, the reason is yet unclear (Jensen and Olsen, 1998; Knief et al., 2005). A possible explanation may be that the continuous ploughing of the soil disrupts the active layer of methanotrophs which then translocate to deeper soil causing a longer diffusion path and thus lower CH_4 flux.

We were also able to show a response to increased soil moisture of the natural soil in the arid region. After an initial drop in CH_4 uptake rate which was probably caused by diffusion limitation due to the saturation of the soil with water, CH_4 uptake increased with time. This observation is in agreement with a unimodal response of soil CH_4 uptake to water content (Schnell and King, 1996; Torn and Harte, 1996) resulting from the increase in metabolic activity with increasing soil water content on the one hand and the reduced methane diffusion coefficient on the other hand.

The results of the field observations were confirmed by potential CH_4 oxidation experiments. Here also, only the soil samples from the natural site at Avdat showed potential activity at 0–10 and 10–20 cm depth. Interestingly, this experiment showed lack of any CH_4 oxidation activity in the crust layer. This, together with the TRFLP profiles, which showed nearly no methanotrophs in the crust samples, provides strong evidence for the absence of methanotrophs in the crust layer. This is a surprising result, since the crust layer in drylands is thought to be the biologically most active and diverse layer in the soil (Skujins, 1991). However, many studies have shown that the top layers of soils usually contain a considerably lower activity compared with subsurface soil or lack it completely (Adamsen and King, 1993; Koschorreck and Conrad, 1993).

The primer set (189F-682R) which was used for amplification of *pmoA* gene of methanotrophs is known to target also some sequences of the *amoA*

gene present in ammonia oxidizers (Bourne et al., 2001). The attempts to obtain an amplification product from a DNA template using this primer set were successful for all samples regardless of whether they showed activity or not. However, the community composition in the samples being inactive in CH_4 oxidation was comprised almost solely of ammonia oxidizers (*amoA* gene). In the active soils, on the other hand, *amoA* accounted for less than 10% and *pmoA* for more than 90% of the total TRFLP profiles' intensity. The attempts to amplify transcripts using these primers were, in contrast, successful only for the samples with active CH_4 oxidation.

We observed only a limited diversity of the *pmoA* gene in our soils. All *pmoA* sequences that were retrieved clustered in the phylogenetic tree within three different clusters of yet uncultivated methanotrophs, i.e. JR3, USCγ and a yet unclassified cluster. In the TRFLP profiles cluster JR3 was represented by a TRF of 375 bp of which the relative abundance reached > 50% in some of the profiles. This cluster was previously discovered in grassland soils in California, but only as a minor member of a diverse community of methanotrophs (Horz et al., 2005). These authors reported cluster JR1, JR2 and JR4 being the dominant group. Clusters JR1, JR2 and JR4 were not detected in our soils. Instead we observed cluster USCγ represented by the TRFs of 173 and 241 bp. USCγ reached over 30% in relative abundance in some profiles. This cluster was previously described in neutral and alkaline soils and was postulated to represent a high-affinity methane oxidizer (Knief et al., 2003). Finally, we observed a cluster represented by the TRF of 115 bp, reaching 20% in relative abundance in some profiles.

We assume that cluster JR3 is the most active methanotroph in the soils from the arid region of the Negev Desert. The relative abundance of cluster JR3 showed a significantly positive relation with the potential CH_4 oxidation activity. While this relation was shown for both DNA-based and cDNA-based TRFs, the stronger response was found for the latter. Since mRNA transcripts are assumed to be a proxy for metabolic activity, one may expect higher expression for the more active members of the community. While relative abundance in TRFLP profiles is not a quantitative measure of the template, it is reasonable to assume that the most active members in a

community will exceed the rest in relative abundance as well as in absolute abundance.

It is also worth mentioning that the TRFs of 375 and 173 bp were present in significant relative abundances at 0–10 and 10–20 cm depths at the site of Arava wadi. While these samples did not show any CH_4 uptake or potential CH_4 oxidation activity, nor were we able to detect *pmoA* transcripts in them, methanotrophs of the clusters JR3 and USCγ might be activated given the right conditions (e.g. after rain) and then be able to consume CH_4 and result in CH_4 uptake from the atmosphere.

Conclusion

The soil methane sink is of great importance for the global cycle of the greenhouse gas methane. However, the notion that aerated soils are usually a sink for atmospheric CH_4 is not necessarily true for soils in arid and hyperarid regions, where uptake of CH_4 from the atmosphere, potential CH_4 oxidation activity and transcription of the *pmoA* gene could be demonstrated only at some of the studied field sites. In addition, *pmoA* cluster JR3 may be the most important methane oxidizer for arid soils. Although the identity of these bacteria is yet unknown, the notion of their importance may be crucial for future understanding of factors governing their behaviour and that of the methane sink in arid soils.

3.5 Experimental procedure

Sites description

The arid and hyperarid regions which were studied are both located in the Negev Desert, Israel. Avdat – the arid region – is located on the Negev Desert Plateau in proximity to the town of Mitzpe Ramon. The area is elevated and hilly (approximately 800 m) with a mean annual precipitation (P) of 89.5 mm (between 1989 and 2007) and soil surface temperatures ranging from 9 °C (January) to 32 °C (July) [Israel Space Agency (English), http:// nasa.proj.ac.il/, and desert meteorology group, Ben-Gurion University of the Negev (Hebrew) http://bidr.bgu.ac.il/bidr/research/phys/meteorology/ index.html]. The potential evapotranspiration (PET) for this region is estimated at 1600–1800 mm yr^{-1} (Gvirtzman, 2002) giving an aridity index (AIU = P/PET) of 0.053 which classifies it as arid (Ffolliott et al., 2003). The landscape is characterized by patchy shrub vegetation dominated by the shrub *H. scoparia*. Three sites were measured and sampled in this region: (i) a natural site where both *H. scoparia* shrub and intershrub patches were measured and sampled, (ii) a constructed mini-catchment (Liman) planted with *Cupressus sempervirens*, *Zi*ziphus spina-christi and Balanites aegyptiaca trees, and (iii) an agricultural field.

Arava – the hyperarid region – is located in the southern part of the Arava Valley close to Kibbutz Ketura. The area is a sea-level valley with an average annual precipitation of 22.9 mm (between 1989 and 2007) and soil surface temperatures ranging from 27.1 °C (January) to 48.4 °C (July) [Israel Space Agency (English), and Arava R&D (Hebrew),
http:// www.arava.co.il/haklaut/]. The potential evapotranspiration for this region is estimated at 2400–2600 mm yr^{-1} (Gvirtzman, 2002) giving an aridity index of 0.009 which classifies it as hyperarid. The landscape is mostly barren with sparse *Accacia tortilis* trees. Two sites were measured and sampled in this region: (i) natural wadi soil close to a patch of *A. tortilis* trees and (ii) an agricultural field. The exact geographic coordinates for these sites are given in **Table 3.1**. Physicochemical characterization Gravimetric water content was determined by drying 50 g of soil at 110 °C until reaching

equilibrium of weight. pH was determined on a 1:1 dry soil/water mixture tilted for 2 h. Soil content of total carbon and total nitrogen were analysed using a CN analyser by the Analytical Chemical Laboratory of the Philipps-Universität, Marburg, after treatment with 10% HCl. Grain size distribution was determined using the laser diffraction method by the Chair of Physical Geography and Geoecology at the Aachen University. Physicochemical characteristics of the soil samples are given in **Table 3.1**.

Field measurements

Atmospheric methane flux was measured using the closed chamber method (Hutchinson and Livingston, 2001; Davidson et al., 2002): a steel frame of 36 by 36 cm was hammered 20 cm into the ground, and covered by a transparent 40 by 40 by 14 cm Plexiglas box. Degassed water was used in the interface between the cover and the frame to minimize gas exchange between the trapped air and the atmosphere. Two butyl rubber septa were installed on the top part of the box; one had a 12 cm syringe needle inserted in it to be used as a pressure vent while the other was used as a sampling port. The whole chamber was covered with an Emergency Isothermal Blanket to minimize temperature changes inside the chamber as well as to prevent any potential effects of direct sunlight. Vertical methane profiles in soil were determined using a gas sampling corer. The corer was made of a stainless steel tube (120 cm, 0.4″) to which a 1/16 inch stainless steel capillary was welded from the inside. The tube was capped on its bottom leaving a 1 cm edge of the capillary to minimize dead volume. A 5 cm punctured stainless steel tip was screwed to the bottom of the tube for protection. Gas samples of 20 ml were taken in duplicates from the chamber (directly) and the corer (after disposing one sample to rid the dead volume) using a plastic syringe. The sampled gas was immediately transferred to a 25 ml glass vial filled completely with saturated NaCl solution and capped with a butyl rubber stopper (the butyl stoppers had been boiled in water before usage). The solution was then partially displaced by the gas sample through a second needle. The vials were kept upside down in Styrofoam racks with the gas bubble in contact with the glass and the remaining NaCl solution only. Samples were analysed within 12 h from sampling using an

SRI gas chromatograph equipped with a flame ionization detector (SRI). The method's detection limit was estimated at 3×10^{-3} μmol L^{-1}. Predictions of CH_4 fluxes from the vertical concentration profiles were calculated using Fick's first law of diffusion:

$$jCH_4 = D_oCH_4\ D/D_o\ \Delta C_{CH4}, \quad (1)$$

where jCH_4 is the CH_4 consumption flux (mmol d^{-1} m^{-2}), DoCH_4 is the diffusion coefficient of CH_4 in air, D/Do is the normalized diffusivity in aggregated media, and ΔC_{CH4} is the change in the methane concentration in the relevant soil section (mmol cm^{-1}). DoCH_4 was taken from Potter and colleagues (1996). D/Do was estimated at 0.13 for dry medium/coarse soils, also following Potter and colleagues (1996).

Artificial raining experiment

One metal frame was hammered into the ground at an intershrub patch at Avdat natural site and methane flux was determined over 45 min as described above. Afterwards, 15 mm of deionized water were sprinkled on an area of 1 m^2 whose centre was the frame. The area was allowed to drain and a second measurement of methane flux was taken. The following measurements were taken at 12 h intervals. Sample collection, handling and transfer Samples of approximately 250 g where collected in triplicates, ~30 m apart, from each site at depths of 0–10 and 10–20 cm filled into Whirl-Pak® bags (Nasco). In addition, the topmost layer – the desert crust – was also collected except in the agricultural fields where it did not exist. For nucleic acid extraction, approximately 750 ml of soil from the same patches was collected into 1.5 ml tubes containing 1 ml of RNAlater® solution (Ambion). The tubes were stored at -80 °C upon arrival to the lab and were later transported on wet ice back to Germany.

Potential methane oxidation measurements

Ten grams of each replicate soil sample was incubated in a 120 ml glass serum bottle and was amended with 1 ml of sterile double-distilled water. The bottles were capped with butyl rubber stoppers and kept at 25 °C in the dark for 14 days. Gas samples were taken with a glass syringe and analysed for methane concentration using an SRI gas chromatograph equipped with a flame ionization detector.

Total nucleic acids extraction

Total nucleic acids were extracted from 0.5 g of the preincubated soil samples (preserved in RNAlater® solution) using a modified version of the method described by Bürgmann and colleagues (2003). For the extraction buffer, 2.5% sodium dodecyl sulfate (SDS) was used instead of hexadecyltrimethylammonium bromide (CTAB). For PCR amplification of DNA, the crude extract was used directly, whereas for cDNA synthesis RNA was purified the following way: following extraction of total nucleic acids, 87.5 ml of the crude extract was treated with DNase I (Qiagen) according to the manufacturer's instructions and was purified using RNeasy MinElute Cleanup Kit (Qiagen) and quantified using a NanoDrop 1000 spectrophotometer (Thermo). Complete DNA removal was verified by failure to obtain PCR amplification product with the purified RNA template using the conditions described below.

cDNA synthesis and PCR amplification

Complete cDNA was synthesized using ImProm-II™ Reverse Transcriptase (Promega) by the following procedure: 10 ng of purified RNA and 0.5 μg/μl of random hexamer primers were incubated at 70 °C for 5 min followed by 5 min chilling on ice. The following mixture was then directly added to the tubes: 4 μl of 5× ImProm-II™ Reaction Buffer, 3 mM MgCl2, 0.5 mM of each dNTP, 20 μg of bovine serum albumin (BSA; Roche) and 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega). The reaction tubes were incubated at 25 °C for 5 min followed by 52 °C for 1 h, and then denaturation at 70 °C for 15 min. The resulting cDNA product was used

directly as a template for consecutive PCR reactions. PCR amplification was performed identically for both DNA and cDNA templates. The primer pairs A189f and A682r (Holmes et al., 1995), targeting a segment of the pMMO gene, were used. This primer pair had been successfully used in previous studies to target upland soil methanotrophs not detected by other primers (McDonald et al., 2008). For TRFLP analysis the forward primer was labelled with the fluorescent dye 6-FAM (6-carboxyfluorescein). PCR reactions were conducted in triplicates of 50 μl which were later combined to minimize reaction bias. Each PCR reaction contained 5 μl of 10× AccuPrime™ PCR Buffer I or II (for the amplification of cDNA and DNA templates, respectively; Invitrogen), additional 1.5 mM MgCl2 (to a final concentration of 3 mM), 0.5 mM of each primer (Sigma), 50 μg of BSA (Roche), 1 μl of Taq DNA polymerase (Invitrogen) and 2 μl of template. The following programme was used: 94 °C for 4 min followed by 10 touch-down cycles of 94 °C for 1 min, 62 °C - 52 °C for 1 min and 68 °C for 1 min followed by 25 cycles of 94 °C for 1 min, 52 °C for 1 min and 68 °C for 1 min, and a single step of final elongation at 68 °C for 10 min. After amplification, the triplicate PCR reactions were pooled and loaded on 1.2% agarose gel stained with ethidium bromide. The right-sized fragments were excised and the DNA was eluted using GeneElute™ Gel Extraction Kit (Sigma) and quantified on a NanoDrop 1000 Spectrophotometer.

Cloning and sequencing and phylogenetic analysis

Samples Avdat-intershrub 0–10 cm (DNA and cDNA) and 10–20 (DNA), Avdat-shrub 0–10 cm (DNA), and 0–10 cm of the liman (DNA) were used for cloning. Libraries were constructed using cleaned PCR products and pGEM-T Easy cloning kit (Promega). Sequencing service was provided by ADIS (Cologne, Germany) using the primers T7f and M13r targeting flanking regions of the insert. A total of 134 clones were analysed: 25 from the AV_W_0–10_I_D library, 13 from the AV_Lim_0–10_I_D library, 51 from the AV_O_0–10_I_D library, 23 from the AV_O_0–10_I_RT library and 22 from the AV_O_10–20_III_D library. All sequences were deposited into the GenBank® (http://www.ncbi.nlm.nih.gov) and received the following Accession No.: FJ970508–FJ970626. Raw sequence data were analysed

using SeqMan software (DNAStar) where the primers and vector data were trimmed. Using ARB, sequences were converted to amino acid sequences and aligned against an ARB database of publicly available *pmoA* sequences obtained from EMBL Nucleotide Sequence Database. Reconstruction of a maximum likelihood phylogenetic tree was conducted with RAxML 7.04 using rapid hill climbing algorithm and PROTMIXJTT evolutionary model (http://icwww.epfl.ch/~stamatak/index-Dateien/Page443.htm; Stamatakis, 2006).

TRFLP analysis

Approximately 200 ng of cleaned PCR products were digested overnight at 37 °C with 20 U of the restriction enzyme MspI and 2 µl of 10× Buffer Tango™ (Fermentas). Following digestion, samples were desalted on SigmaSpin™ Post-Reaction Clean-up Columns (Sigma, Germany); aliquots of 3 ml were mixed with 10 ml of HiDi™ Formamide (Applied Biosystems) and 0.3 ml of the internal DNA standard MapMarker®1000 (BioVentures). The samples were denatured at 94 °C for 2 min and loaded into an ABI 3100 automated gene sequencer (Applied Biosystems) for separation of the TRFs. TRFLP data were retrieved by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems).

Analysis of TRFLP data and cluster analysis

Raw TRFLP cannot be used directly for statistical analysis. Therefore, the following standardization and normalization procedures were applied prior to all statistical analyses. The size in base pairs of each peak (TRF) was used to indicate an operational taxonomic unit (OTU) while the peak's area was used to determine its relative abundance in the profile. The TRFLP patterns of the triplicates of each sample were standardized as described elsewhere (Dunbar et al., 2001). Profiles were then aligned and a consensus profile was computed for each sample from its replicates by eliminating non-reproducible peaks and averaging shared peaks. The standardization procedure was then applied again to standardize the consensus profiles and they were aligned to generate a sample by species matrix which was used in

the cluster. TRFLP data set poses another problem by the presence of many null values in the matrix. These are treated as genuine zero value observations in the computation procedure and could lead to an overestimation of the similarity between samples. Following Legendre and Gallagher (2001) the matrix was therefore transformed to give Hellinger distances between the samples when Euclidean distances are computed. Cluster analysis was then performed on the transformed data set using a Euclidean distance matrix and the weighted arithmetic average clustering (WPGMA) clustering methods. All standardization and normalization procedures were performed using MATLAB (http://www.mathworks.com) and the codes are available at http://www.staff.uni-marburg.de/~angel.

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Chapter 4 |

Methanogenic *Archaea* are Globally Ubiquitous in Upland Soils and Become Active Under Anoxic Conditions

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Contributions:

RA and RC designed the study and the sampling scheme; RA, RC and others performed the sampling. RA and PC performed the soil incubations and isotopic measurements. RA performed all molecular and data analyses; RA and RC wrote the manuscript.

4.1 Abstract

Members of the domain *Archaea* were once considered to inhabit mostly extreme environments, a reminiscence of an ancient Earth in which they supposedly originated. However in recent years, evidence accumulated for the ubiquitous occurrence of *Archaea* (mostly *Crenarchaeota*) in many temperate environments, such as upland soils. The prototypical representatives of the *Euryarchaeota* – the methanogens – are particularly sensitive to oxygen and are thought to occur only in highly reduced, anoxic environments. In contrast to this textbook notion, we found methanogens of the genera *Methanosarcina* and *Methanocella* to be present in many types of upland (including dryland) soils sampled globally. These methanogens could be readily activated by incubating the soils as slurry under anoxic conditions, as seen by rapid methane production within a few days without any additional carbon source. Analysis of the archaeal 16S rRNA gene community profile and gene copy numbers indicated dominance of *Methanosarcina,* and their 16S rRNA gene copy numbers correlated with methane production rates. Analysis of the $\delta^{13}C$ of the methane further supported this as the dominant methanogenic pathway was in most cases acetoclastic, which *Methanocella* cannot perform. Sequences of the key methanogenic enzyme methyl-CoM reductase (*mcrA*) retrieved from the soil samples prior to incubation confirmed that *Methanosarcina* and *Methanocella* are the dominant methanogens, though some sequences of *Methanobrevibacter* and *Methanobacterium* were also detected. The global occurrence of only two methanogenic genera supports the hypothesis that these are autochthonous members of the upland soil biome and are well adapted to their environment.

4.2 Introduction

The methanogenic *Arachaea* (methanogens) are the only known organisms capable of producing methane. All known methanogens fall into the phylum *Euryarchaeota* where they form six distinct orders: *Methanomicrobiales*, *Methanocellales*, *Methanosarcinales*, *Methanobacteriales*, *Methanococcales* and *Methanopyralles*. The pathways for generating methane in methanogens vary and include: methanol and CO_2 reduction, acetate cleavage as well as methane production from a variety of methylated compounds. With the exception of marine sediments and hypersaline mats (Oremland, 1988), methanogenesis in natural systems is dominated by CO_2 reduction (hydrogenotrophic methanogenesis) and acetate cleavage (acetoclasitc methanogenesis) while other pathways can be neglected (Conrad, 2005).

Methanogens are strict anaerobes and methanogenesis was shown to be fully suppressed upon exposure to high levels of oxygen (Yuan et al., 2009). In addition, the typical energetic yields for hydrogenotrophic and acetoclasitc methanogenesis (ΔG = -131 and -36 kJ, respectively) are lower than those obtained by Iron, nitrate, and sulphate and reduction (ΔG = -228.3, -163.2 and -152.2 kJ, respectively) which compete with methanogens over hydrogen (Thauer et al., 1977, 1989). As a result, methanogenesis typically occurs only in highly reduced anoxic environments where oxygen is absent and alternative electron acceptors such as nitrate and sulphate are depleted (Whitman et al., 2006).

While the biochemical machinery in methanogens varies with the pathway used, few functional genes which encode for key enzymes in the production of methane are common to all known methanogens (Hedderich and Whitman, 2006). Of those, the methyl coenzyme reductase M which is responsible for the last step in all methanogenic pathways – the reduction of the methyl group bound to the Methyl-Coenzyme M – is typically used as functional gene marker for analysis of methanogenic communities (Luton et al., 2002)

In contrast to the notions presented above, Peters and Conrad (1995) demonstrated that several types of aerated soils could become methanogenic when incubated under anoxic conditions as slurry. Among these soils was a South African desert soil which was stored in dry state at room temperature

for nearly 10 years prior to incubation. In a further experiment they demonstrated that typical sequential reduction processes occurred in these soils with nitrate, iron, manganese and sulphate depleted prior to onset of methanogenesis (Peters and Conrad, 1996). The precursors for methanogenesis were, however, present in sufficient concentrations in the slurries before methanogenesis started indicating that the methanogenic population was limited by size. Few others have reported the occurrence of methanogenesis in aerated soils and/or the presence of methanogens. For example West and Schmidt (2002) were able to induce methanogenesis in alpine soils when incubated under H_2 and CO_2 enriched atmosphere (but not under pure N_2 atmosphere) and Teh and colleagues (2005) found methanogenesis occurring in tropical forest soils containing up to 19% O_2. In two other cases, researchers measured methanogenic activity in pasture soil and were able to retrieve sequences affiliated to methanogens (Radl et al., 2007; Nicol et al., 2003; Gattinger et al., 2007). However, the authors have attributed most of this activity to the effect livestock had on the soil by enriching with nutrients from urine and manure and by inoculating the soil with rumen microflora. Lastly, Poplawski and colleagues (2007) retrieved sequences of *Methanosarcina* and *Methanocella* in a Swedish barley field but have not attempted to measure potential methanogenic activity.

The discovery of potential methanogenesis in arid soils is of particular interest as it is least expected to be found where soil is dry and oxic for most of the year and where input of organic carbon is particularly low and grazing ruminants are scarce. Nevertheless, particularly for these reasons if active methanogens are to be found in arid soils it can demonstrate their autochthonous origin and ability to survive in anoxic microniches of aerated soils around the world.

Arid soils differ from other soils by their unique profile. Unlike most soils, soils in arid parts don't display a profile of layers representing different stages of bedrock erosion and a gradient of nutrient concentrations. Rather, the bulk soil is undifferentiated eroded bedrock poor in nutrients and is in turn covered by a densely populated mat of microorganisms termed the biological soil crust (BSC). The BSC is an association of microorganisms which is formed practically in any soil whose water budget restricts the

development of higher plants. The primary members and first colonizers of BSCs are cyanobacteria and microalgae who aggregate the soil using exopolysaccharide. Fungi and lichens are often also associated with mature BSCs and sometimes moss as well (Belnap et al., 2002).
An extensive literature exists on the above mentioned members of BSCs but studies (particularly molecular) on the prokaryotic inhabitants of this mat (other than cyanobacteria) are scarce (Garcia-Pichel, 2002).

Our goals in this study were to compare the methanogenic potential of different aerated soils collected globally and, more importantly, to uncover the identity of the active methanogens in these soils and the potential methanogenic pathways. We focused primarily on dryland soils and collected samples from dryland environments in Israel, Australia and Utah but also from a forest and a grassland meadow in Germany for comparison. We screened the soil samples for methanogenic potential and attempted to decipher the methanogenic pathways through stable isotopes analysis and the use of an inhibitor for acetoclasitc methanogenesis. We then analysed the identity of the archaeal population in these soils and quantified its size through specific qPCR essays.

4.3 Results

Methanogenic potential in upland soils

Of the 27 soil samples we tested 22 produced at least some methane during the incubation time (**Table 4.1**). We observed stark differences in the extent of the methanogenic potential of the soils which was expressed in up to four orders of magnitude difference in daily production rates. Of the 22 samples we classified 10 as having a strong methanogenic potential (highly methanogenic soils) meaning, they had methane production rates in the hundreds or thousands nmol gdw^{-1} d^{-1}. In most samples methane was detected within 7 – 14 days but a linear methane production began, on average, at day 21. Interestingly, methane production rates and lag time did not correlate with parameters such as precipitation, soils water content and organic matter which would seem to be most important for supporting methanogenesis. For examples, desert crust samples from the Negev Desert had more than double the rate of methane production than the meadow samples. With respect to the desert samples we observed that the biological soil crust (BSC) layer was the only truly active layer in the soil while those below had a very low or not methanogenic potential. The only exception to this is the Liman whose soil: 1. periodically behaves like pond sediments when the Liman is flooded; 2. builds up much quicker than the surrounding due to alluviation.

Isotopic analysis

In parallel to measuring potential methane production rates of the soils, we have also determined the isotopic signature of the carbon in the methane being produced. This was done in order to estimate the proportion of each of the two main methanogenic pathways – hydrogenotrophic and acetoclasitc – in our experiments. Additionally, those samples which we classified as highly methanogenic were also incubated with CH_3F in order to inhibit acetoclasitc methanogenesis. This allowed us to specifically determine the isotopic signature of the methane produced through hydrogenotrophic methanogenesis (δ_{mc}).

Table 4.1| Characteristics of the sites and values of major physico-chemical parameters of the soil samples

Location	*Site*	*Soil type (USDA)*[*]	*Sampling layer*	*pH*	C_{tot} N_{org} *(%)*	*Methane production rate (nmol gdw-1 d-1)*	Y_0[**] *(d)*	R^2	*Inhibited methane production rate (nmol gdw-1 d-1* †	Y_0 *(d)*	R^2
Avdat, Negev Plateau,	Natural field - Intershrub	Silt loam	BSC	8.3	0.46, 0.30	3081.6	20	0.99	629.02	24	0.96
	(30° 47' N 34° 45' E)		0-10 cm		0.33, 0.01	1.03	24	0.90			
			10-20 cm		0.38, 0.01	0.46	23	0.94			
	- " - Shrub	Silt loam	BSC	8.4	0.66, 0.05	3710.73	16	0.96	1793.76	21	0.96
			0-10 cm		0.34, 0.01	1.54	22	0.93			
			10-20 cm		0.26, BLD	1.23	23	0.82			
	Liman (constructed mini-catchment)	Silt loam	BSC	7.9	2.00, 0.15	5657.59	11	0.99	3471.14	15	1.00
	(30° 48' N 34° 45' E)		0-10 cm		1.27, 0.09	540.95	16	1.00			
			10-20 cm		0.96, 0.05	224.6	16	1.00			
	Agricultural field	Loam	0-10 cm	8.5	0.28, BLD	0.62	23	0.71			
	(30° 52' N 34° 48' E)		10-20 cm		0.25, 0.03	BLD					
Arava Valley South, Israel	Wadi (dry riverbed)	Sand	BSC	8.4	0.98, BLD	4.39	22	0.99			
	(29° 58' N 35° 05' E)		0-10 cm		0.06, BLD	BLD					
			10-20 cm		0.08, BLD	BLD					
	Agricultural field	Sand	0-10 cm	8.5	0.38, 0.14	BLD					
	(30° 04' N 35° 08' E)		10-20 cm		0.18, BLD	BLD					

Table 4.1| Continued

Location	*Site*	*Soil type (USDA)*[*]	*Sampling layer*	*pH*	C_{tot} N_{org} *(%)*	*Methane production rate (nmol gdw-1 d-1)*	Y_0[**] *(d)*	R^2	*Inhibited methane production rate (nmol gdw-1 d-1* †	Y_0 *(d)*	R^2
North Utah, USA	Natural field (41° 44' N 112° 20' E;	Silt Loam	0-10 cm	6.5	4.71, 0.37	222.98	52	0.95	23.3	28	1.00
	41° 42' N 112° 20' E;	Sandy Loam	0-10 cm	7.7	2.03, 0.11	138.35	13	0.93	49.53	26	0.99
	41° 40' N 113° 10' E;	Silt Loam	0-10 cm	8.2	2.61, 0.14	3.49	19	0.99			
	41° 51' N 113° 05' E;)	Silt Loam	0-10 cm	8.1	2.63, 0.16	8.98	62	0.94			
Hann River, Australia	Flood plain	Silt Loam	0-10 cm	4.8	0.72, 0.10	11.61	59	0.91			
		Loamy sand	0-10 cm	5	0.35, 0.43	374.5	38	0.93	62.88	37	0.96
		Loamy sand	0-10 cm	3.9	1.21, 0.10	0.63	38	0.97			
Giessen, Germany	Meadow	Silt Loam	0-7.5 cm	6	4.24, 0.40	1860.07	16	0.92	305.48	11	0.97
	(50° 32' N 8° 41' E)		7.5-15 cm		3.48, 0.35	1428.4	16	0.85	171.91	12	0.98
Marburg, Germany	Forest	Sandy Loam	0-10 cm	3.5	20.45, 0.91	1.2	55	0.99			
	(50° 48' N 8° 48' E)		0-10 cm			1.58	62	0.98			

* http://soils.usda.gov/technical/aids/investigations/texture/

** Initiation of linear methane production (extrapolated from linear regressions)

† Inhibition of acetoclastic methanogenesis using 2% CH_3F (see materials and methods).

BLD - below limits of detection; ND - not determined

The BSC samples from Israel showed a temporal trend by which methane began with a light isotopic signature of the carbon (-57‰ on average) and became heavier with time (up to -35‰ on average) while the isotopic signature of the carbon in the CO_2 became slightly lighter over time starting from -9 to -15‰ on average (**Table 4.2**). A similar phenomenon with respect to methane but not to CO_2 was observed for the samples from Utah while the other soil samples showed a relatively stable isotopic signature of both methane and CO_2 over time. Fractionation factors in all non-inhibited incubations were lower than those in the inhibited incubations (which were 1.050 – 1.076) but higher than those obtained for purely acetoclastic methanogenesis which typically range between 1.021-1.027 (Krzycki et al., 1987; Gelwicks et al., 1994) indicating a mixture of both pathways. To precisely determine the contribution of hydrogenotrophic and acetoclasitc methanogenesis knowledge of the exact isotopic signatures δma and δmc are required. We can, however, estimate the relative contribution of each pathway if we use the $\delta^{13}C\text{-}CH_4$ produced in the inhibited incubations as the specific signature for methane produced from H_2/CO_2 alone (δmc) and use the isotopic signature of the organic carbon in the soil to estimate the signature of methane produced from acetate. The measured $\delta^{13}C$ of the organic carbon in the soil samples (which is the substrate for acetate) differed somewhat between sites but ranged between -20.20 and -27.9. The fractionation factor for acetoclastic methanogenesis in pure cultures varies with respect to substrate concentration but ranges between $\varepsilon \approx 5.6$ and -25.6‰ (Goevert and Conrad, 2009). Since acetate concentrations were low in our incubations (relative to those used by Goevert and Conrad, 2009) we can expect a relatively high turnover and assume a low fractionation factor (-10‰). By using equation 3 and these assumptions we can thus estimate the fraction of hydrogenotrophic methanogenesis in the system (**Table 4.2**). In all cases methanogenesis began with a dominance of hydrogenotrophic methanogenesis but in samples from Israel and Utah this fraction dropped to about 10-20% only of the methane being produced.

Table 4.2| Stable carbon isotope analysis of the methane and CO_2

	$\delta^{13}C$-$C_{org.}$ (‰)		$\delta^{13}C$ (‰)				Apparent fractionation factor (α_{app})		δ_{ma} (‰) †	Fraction of H_2/CO_2 methanogenesis	
	Non-acidified	Acidified	CH_4 begin*	CH_4 end	CO_2 begin	CO_2 end	α begin	α end**		fH_2 begin	fH_2 end
Israel (Natural field)	-5.4	-21.1	-57.3 ± 4.9	-34.9 ± 2.5	-9.1 ± 2.1	-15.3 ± 1.2	1.053 ± 0.006	1.025 ± 0.004	-31.09	0.74	0.1
Israel + CH_3F			-66.3 ± 3.2	-68.7 ± 0.6	-7.5 ± 1.67	-12.2 ± 1.1	1.061 ± 0.003				
Utah (sample 1)	-26.4	-26.6	-74.4 ± 6.8	-44.4 ± 3.4	-24.8 ± 1.8	-24.9 ± 0.6	1.054 ± 0.010	1.027 ± 0.004	-35.66	1.00	0.25
Utah + CH_3F				-70.4 ± 3.6		-25.2 ± 0.4	1.049 ± 0.003				
Australia (sample 2)	-20.6	-20.4	-69.6 ± 4.4	-65.4 ± 2.7	-20.0 ± 0.1	-16.8 ± 0.7	1.036 ± 0.004		-30.20	0.72	0.67
Australia + CH_3F			-85.3 ± 6.0	-82.6 ± 0.9	-17.1 ± 0.6	-15.2 ± -6.2	1.076 ± 0.003				
Giessen (0-7.5 cm)	-28.3	-28.2	-80.3 ± 3.0	-79.7 ± 4.5	-23.0 ± 1.0	-21.3 ± 0.3	1.053 ± 0.005		-37.86	1.00	0.98
Giessen + CH_3F			-78.6 ± 2.0	-80.6 ± 2.0	-25.2 ± 1.0	-23.2 ± 0.5	1.065 ± 0.005				

* 'Begin' and 'end' refer to the first third and last two thirds of the incubation period, respectively.
** α end is only shown for the cases where it differed significantly from α begin.
† δma was calculated by assuming a -10‰ fractionation from organic carbon (see text).

Molecular characterization of the methanogenic community

We characterized the archaeal community in our soil samples through cloning and TRFLP profiling and compared the pre-incubated state with the post incubated of both inhibited and non-inhibited incubations. Surprisingly, the diversity of the methanogenic community in our samples was extremely poor and nearly identical although samples were obtained from different parts around the world. In all of our post-incubated samples we identified only *Methanosarcina* and *Methanocella* related sequences along with a variety of crenarchaeal sequences typically associated with aerated soils such as Soil Crenarchaeotic Group (SCG) 1.1b and occasionally sequences related to *Thermoplasmatales* (**Figure 4.1**). The latter two groups were detected as sole members of the archaeal communities in the samples prior to incubation while none of the methanogens could be detected in our 16S rRNA clone libraries or TRFLP profiles. Inhibition with CH_3F resulted in lower methane production rates but did not change the identity of the methanogenic community. Not only did we detect nothing but *Methanosarcina* and *Methanocella* as methanogenic members in our post-incubated samples, their within group sequence diversity was itself very low. For example, the average sequence similarity between *Methanosarcina* from Avdat BSC and Giessen meadow soil was only 1.81% ± 0.025 and that between the *Methanocella* members only 3.3% ± 0.1.

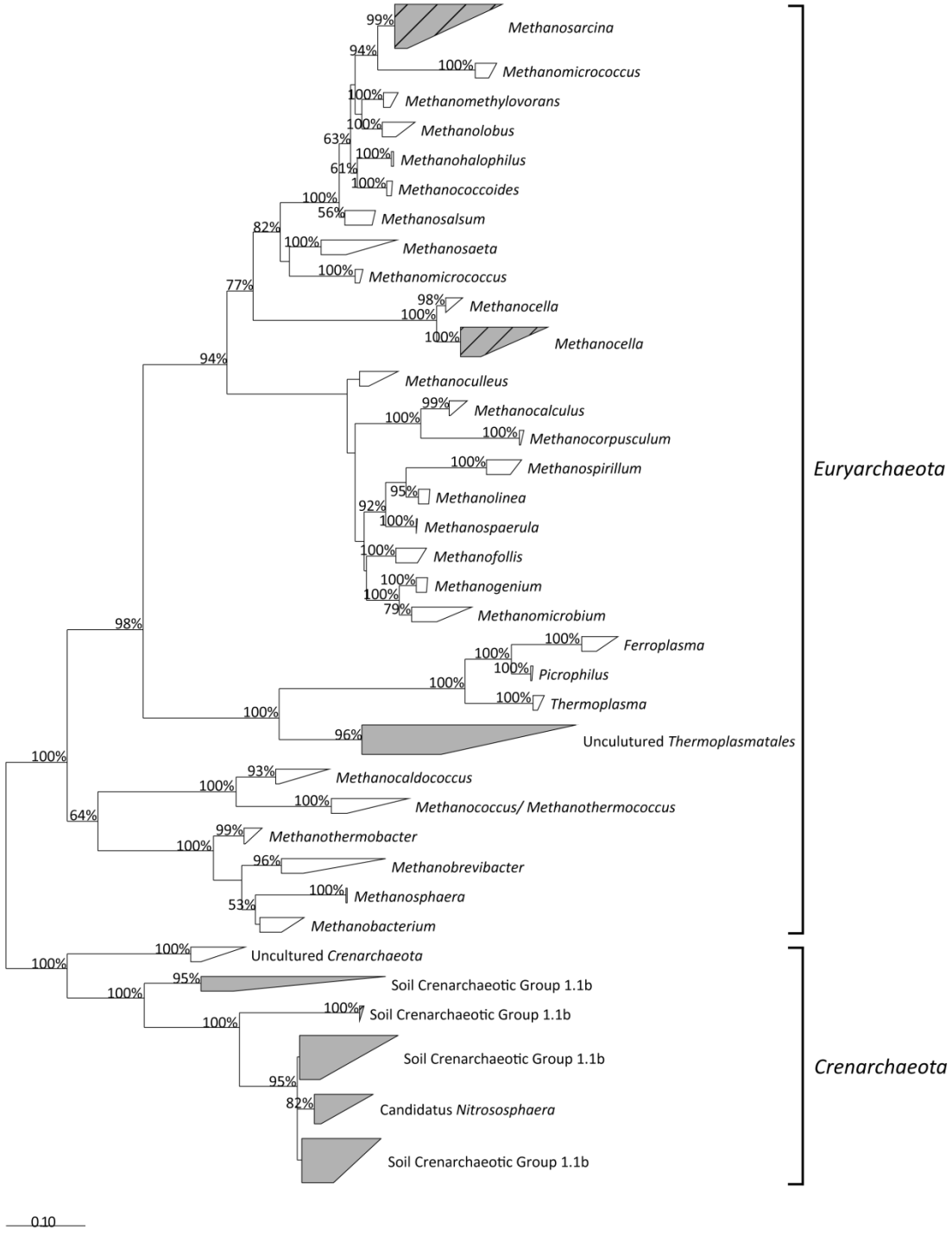

Figure 4.1| Maximum likelihood phylogenetic tree based on aligned partial archaeal 16S rRNA gene sequences. Sequences obtained in this study were aligned against the SILVA 102 database using the SINA aligner and the tree was calculated with RAxML 7.04 using rapid hill climbing algorithm and GTRMIX evolutionary model. Bootstrap values above 50% (out of a 100 trials) are displayed next to the nodes. Shaded clusters contain sequences that were only detected in the pre-incubated samples while sequences from the post- incubated samples cluster both into the shaded clusters and into the shaded with diagonal lines.

TRFLP analysis

We used TRFLP profiling to decipher relative changes in the archaeal community from *in situ* to post-incubated state and with respect to the effect of inhibition with CH_3F. 67 replicated TRFLP profiles are summarized in **Figure 4.2**. As expected, all pre-incubated samples (green circles) had similar 16S rRNA profiles on both DNA and RNA levels and clustered very tightly with respect to the primary principal component. Most post-incubated samples (red and blue circles) are to be found on the right to the ordinate indicating methanogenic activity and their approximate methane production rates can be deduced from the isolines. Those samples which did not demonstrate potential methanogenic activity remained close to the pre-incubated samples in these plots. The relative abundance of each of the archaeal types can be estimated from the perpendicular projection of each sample on the individual arrows while the length of each arrow indicates the variance (range of relative abundance values) of its respective archaeal type. *Methanosarcina* was more abundant than *Methanocella* in most methanogenic samples on the DNA level and seemed to have been overwhelmingly dominant on the RNA level. Moreover, the most methanogenic samples seem to have been those with the highest relative abundance of *Methanosarcina*. It seems that the inhibition with CH_3F, while reducing methanogenic activity, did not have an effect on the composition of the methanogenic community. Particularly, there does not seem to be an increase of *Methanocella* on the expense of *Methanosarcina* as would be expected from such inhibition.

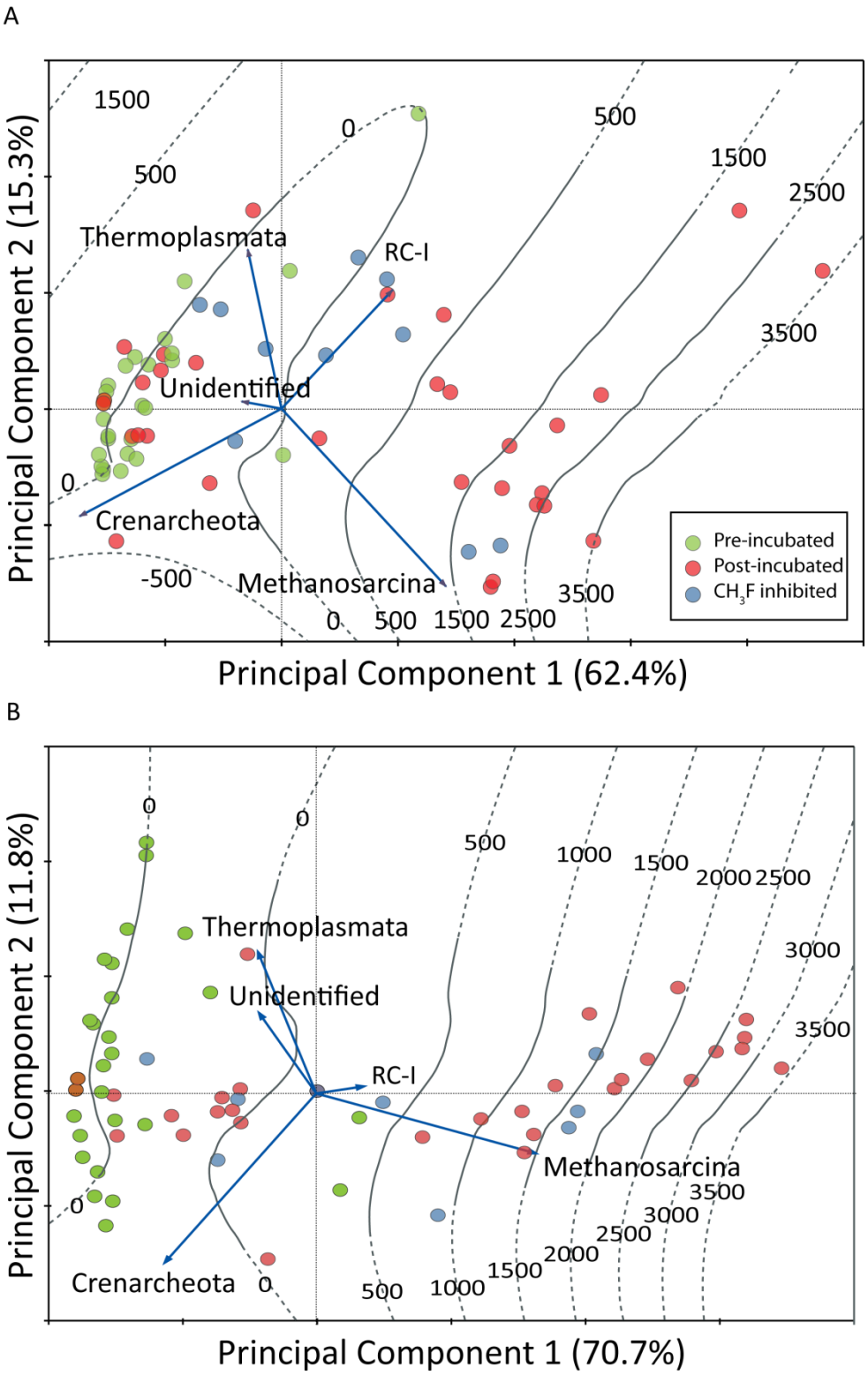

Figure 4.2| Principal component analysis plots of the archaeal community as deciphered from 16S rRNA gene. Circles indicate individual samples. The perpendicular projection of each sample on an arrow gives an estimation of the relative abundance of each archaeal type while its length indicates the species variance. CH_3F was used to inhibit acetoclastic methanogenesis. Fitted methane production rates are shown as isolines.

Molecular characterisation using the *mcrA* marker gene

Our inability to detect methanogens in the 16S rRNA gene TRFLP profiles and clone libraries is most likely the result of their low relative abundance compared with the populations of *Crenarchaeota* and *Thremoplasmatales*. Any community member who comprises 1% or less of the community would be undetected in TRFLP profiles (due to method's threshold and standardization procedures) and also in the clone libraries (considering our sequencing efforts). We therefore attempted to perform analysis of the methanogenic community using the *mcrA* functional gene. While we were not able to amplify the gene in all our samples we did obtain PCR products from at least one sample in each site. The phylogenetic affiliation of these sequences in presented in **Figure 4.3**. The majority of the *mcrA* sequences belonged to either *Methanosarcina* or *Methanocella*, confirming our observation from the analysis of the 16S rRNA gene. We did, however, detect several sequences which belong to *Methanobrevibacter* and *Methanobacterium* which were not detected in the post incubated samples.

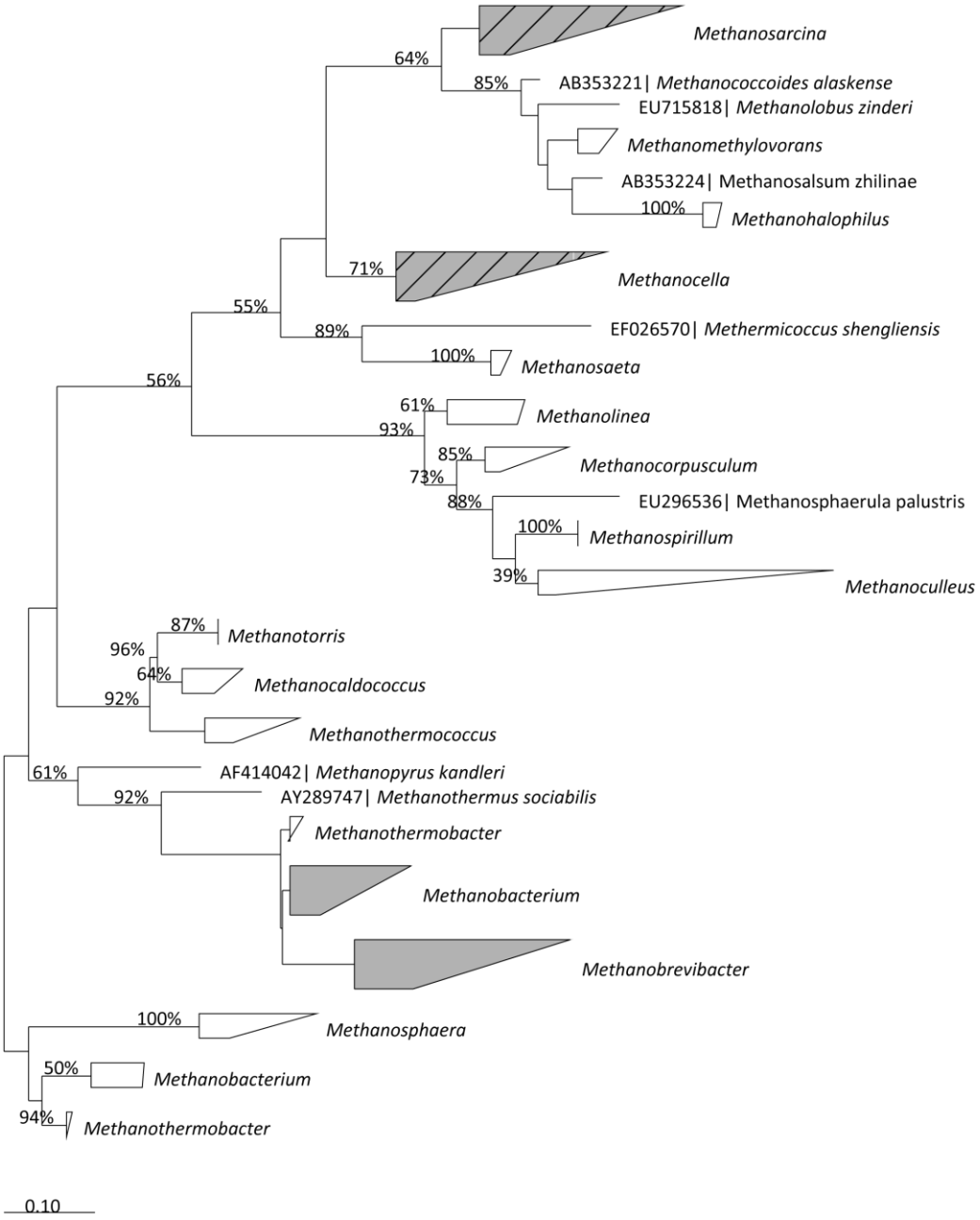

Figure 4.3| Maximum likelihood phylogenetic tree based on aligned partial amino acid sequences of the methyl coenzyme reductase M gene (*mcrA*). Amino acid composition was deduced from DNA sequences and the tree was calculated with RAxML 7.04 using rapid hill climbing algorithm and PROTMIX - JTT evolutionary model. Bootstrap values above 50% (out of a 100 trials) are displayed next to the nodes. Shaded clusters contain sequences that were only detected in the pre-incubated samples while sequences from the post- incubated samples cluster both into the shaded clusters and into the shaded with diagonal lines

Quantification of the methanogenic community

Copy numbers of archaeal 16S rRNA gene rose between 0.5 – 2 orders of magnitude during the incubation period in the highly methanogenic samples and were moderately correlated to methane production rates (**Figure 4.4A**). Both methanogen types were undetected in the in situ and non reactive samples (below 10^3 copies) but reached up to 10^9 copies gdw^{-1} after the incubation. Copy numbers of *Methanosarcina* and *Methanocella* 16S rRNA gene were within one order of magnitude from each other with those of *Methanosarcina* always exceeding *Methanocella* except in the inhibited samples (data not shown). Since both methanogen types contain 3 copies of the 16S rRNA gene in their genomes the actual number of methanogenic cells is a third of the gene copy numbers displayed in the figure. Both methanogen types were positively correlated to methane production rates but that of *Methanosarcina* was much better (**Figure 4.4C and D**). In contrast, gene copy numbers of *Crenarchaeota* did not change in most samples between the pre and post incubated states and showed no correlation to methane production rates (**Figure 4.4B**). Treatment with CH_3F had only a minor effect on the *Methanosarcina* population, by decreasing its copy numbers, and no apparent affect on the numbers of *Methanocella* (data not shown).

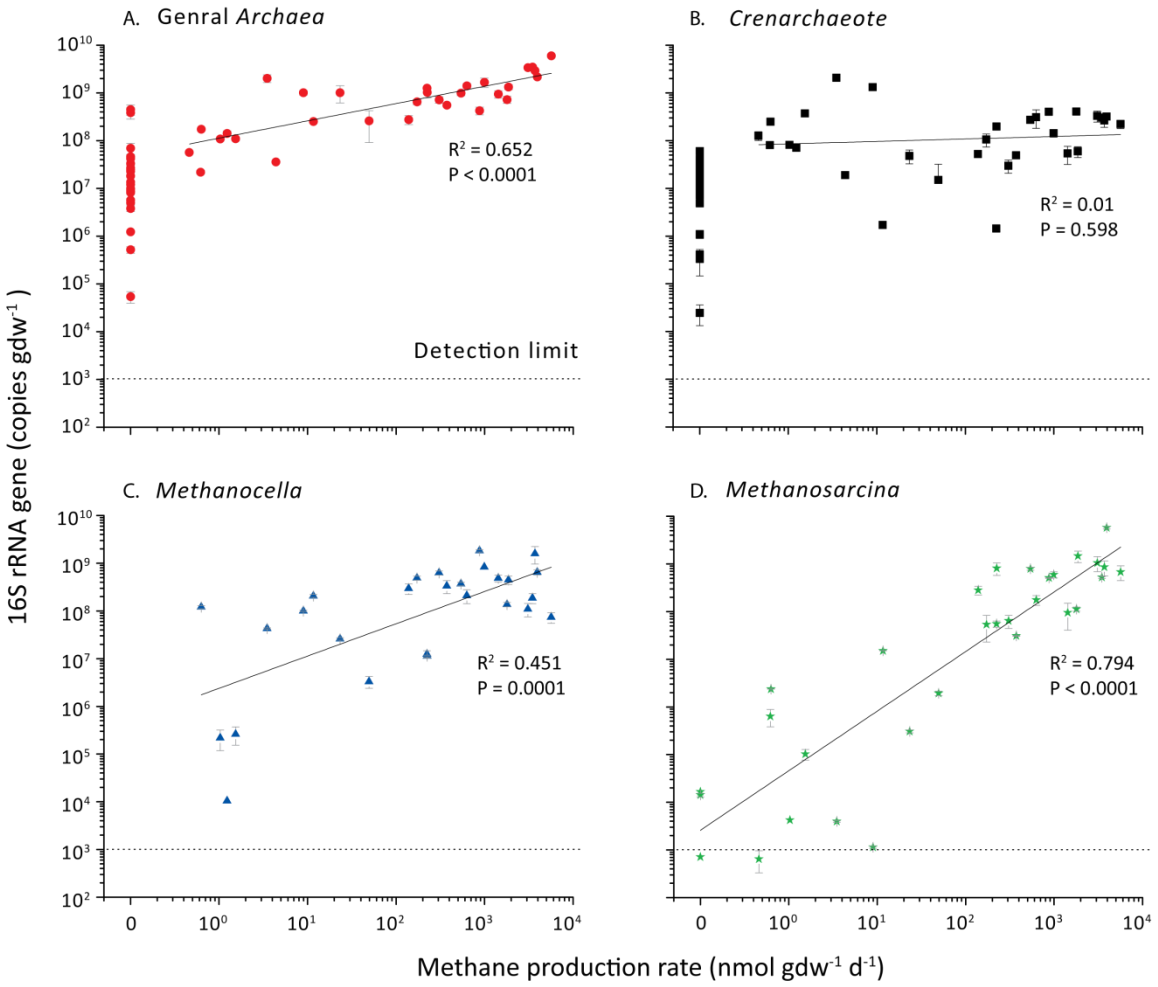

Figure 4.4| 16S rRNA gene copy numbers plotted against methane production rates. Zero on the X-axis indicates pre-incubated or non-reactive samples.

4.4 Discussion

When the domain *Archaea* was first described by Woese and Fox (1977) it was thought to be an ancient lineage of prokaryotes (hence the name). The slow rate of changes of the rRNA genes in *Archaea*, which gives this domain a basal location in the tree of life and short branch lengths (Pace, 2009), along with its predominance in extreme environments, particularly anoxic and hyperthermic (Woese et al., 1990; Takai and Horikoshi, 1999), led to the notion that *Archaea* had evolved early in the evolutionary history in a world which was hotter and whose atmosphere and hydrosphere were much more reduced. This notion has been challenged in recent years by two major lines of discovery. From biochemical and genetic studies of isolated archaea it became clear that the phylogenetic split of *Archaea* occurred later in the evolution, after the domain *Bacteria* had already split (Pace, 1997; Werner, 2008). Complementary to these studies, environmental surveys using molecular screening tools have been revealing more and more archaeal sequences in virtually all mesospheric environments including aerated soils, ocean water and freshwater bodies (Bintrim et al., 1997; DeLong, 1992; Schleper et al., 1997). It thus stems from these studies that *Archaea* are as cosmopolitan as *Bacteria*, adapted to their ecological niches and perform a variety of metabolic activities (DeLong, 1998; Schleper et al., 2005; Auguet et al., 2009).

In this study we have shown that methanogens are also ubiquitous in mesophillic, aerated soils around the world. While not all samples displayed methanogenic activity, at least one of each site did. Many of our samples had very low or no methanogenic potential and long lag times but some samples reacted very quickly and produced ample amounts of methane within a few days. Particularly intriguing is the fact that in the arid samples from Israel it was the topmost part of the soil – the biological soil crust (BSC) – which held nearly all of the methanogenic potential of the soil. While it is true that BSCs contain most of the microbial biomass and activity in desert soils (Garcia-Pichel and Belnap, 2002) it is also the layer most exposed to oxygen in the soil. In fact, since BSCs are mostly formed by photosynthetic microorganisms it becomes hyperoxic at parts when moist during day time (Garcia-Pichel and Belnap, 1996). The existence of soils with such high

methanogenic potential and fast response gives at least circumstantial evidence for the fact that they are also active, at least at certain times, under natural conditions and might comprise a yet unaccounted for global methane source.

Similar to many natural environments, we found a mixture of both acetoclastic and hydrogenotrophic methanogenesis in our incubations (Conrad, 1999), but the specific proportion of each pathway varied between samples and even within certain samples as incubation proceeded. All incubations began with methane being produced mostly or entirely out of hydrogen and CO_2 but in the case of the samples from Israel and Utah methanogenesis shifted to being predominantly acetoclasitc as the incubation proceeded. The ratio of acetoclasitc methanogenesis in these samples thus stabilized between 60-70%, which is very close to the ratio found in most natural environments (Conrad, 1999). Methanogenesis in the samples from Australia and Giessen, on the other hand, remained predominantly hydrogenotrophic throughout the incubation.

Although methanogenic potential thus seems to be a global trait of aerated soils (albeit of different characteristics), we have recovered a remarkably low diversity of methanogens in our samples. Of the 30 known methanogenic genera we found only two closely related genera in our post-incubated samples belonging to *Methanosarcina* and *Methanocella*, both types are of high ecological importance. *Methanosarcina* are, along with *Methanosaeta*, the only methanogens capable of performing acetoclasitc methanogenesis and are therefore the predominant methanogens in most natural environments (Liu and Whitman, 2008). *Methanocella* are a newly discovered genus, also globally distributed but colonises rice roots in particular (Conrad et al., 2006). They were also shown to be of high importance as one of the primary methane producers in rice fields, probably feeding on fermentation products of root exudates (Lu and Conrad, 2005). Although phylogenetically closely related to *Methanosarcina* and similar in their cellular structure, *Methanocella* species can only perform hydrogenotrophic methanogenesis (Sakai et al., 2010; Thauer et al., 2008).

In our samples, it appears that *Methanosarcina* were the dominant methanogens. Certainly this is the case in the samples from Israel and Utah

where acetoclasitc methanogenesis accounted for roughly two thirds of the newly formed methane, but also generally speaking *Methanosarcina* were had higher relative abundance in the TRFLP profiles and their absolute abundance correlated better with methane production rates. Similarly, *Methanosarcina* and *Methanocella* were also the methanogenic types which were found by Nicol and colleagues (2003) in a pasture soil in Scotland and by Poplawski and colleagues (2007) in a barley field in Sweden thus reinforcing our notion that these types are universal upland soil methanogens. In cases where heavy grazing was involved, the authors have also found *Methanosarcina* as dominant methanogens but along with it other types which could be directly associated to rumen microflora (Radl et al., 2007; Gattinger et al., 2007).

Our phylogenetic analysis of the pre-incubated soils using *mcrA* revealed also the presence of a few other methanogens which are closely affiliated with *Methanobrevibacter* and *Methanobacterium*. None of these Methanogens could be enriched in our incubations and it remains unclear whether these were viable cells. Nevertheless, their presence does imply that the diversity of the methanogenic community in these aerated soils is potentially wider.

The formation of biogenic methane when aerated soils are incubated anoxically testifies not only for the presence of active methanogens but also for the presence many other anaerobic microorganisms. Since methane is only an end product in a long cascade of anaerobic degradation processes, other viable anaerobic microorganisms starting from primary fermenters to secondary fermenters to acetogens and syntrophs need to be present in the soil and for it to be formed (Garcia et al., 2000; Stams and Plugge, 2009). It thus stems that upland soil host not just methanogens but entire consortia of anaerobes capable, at least potentially, to carry a full anaerobic degradation pathway.

Conclusion

Methanogens of the type *Methanosarcina* and *Methanocella* appear to be autochthonous and universal members of the biome in aerated soils. Despite being exposed to constant oxygen flux, a core population of these methanogens is able to survive and become rapidly active when conditions fit. Several key issues remain unanswered for the moment: 1. If and to what extent are upland soil methanogens active under natural conditions? (i.e. under an oxic atmosphere); 2. Is there a niche differentiation between *Methanosarcina* and *Methanocella* in aerated soils? 3. What are the factors that determine whether a soil would display a high methanogenic potential: lack of methanogens or very low initial population size, presence of inhibitors, absence or inhibition of one or more members of the microbial consortium required for anaerobic degradation such as syntrophs or acetogens?

4.5 Experimental procedure

Soil samples and soil characterization

Soil samples were collected from several sites around the world as listed in **Table 4.1**. The sampling done in the Negev Desert, Israel comprises our most comprehensive sampling. The sites were previously described by Angel and Conrad (2009). Samples were taken from the biological soil crust where it existed, from 0-10 cm below ground layer and from 10-20 cm below ground layer. Apart from the Negev Desert samples, samples from Utah and Australia were also from dryland environments while the samples obtained in Germany were from temperate forest and grassland.

Incubation conditions and gas measurements

For determining the methanogenic potential of the soils, samples were incubated under anoxic conditions the following way: 5 g of sample was placed in a 26 ml glass tube with 5 ml of sterile distilled deionised water (DDW) and the tube was capped with a pre-boiled butyl rubber stopper. The headspace was repeatedly evacuated and flushed with N_2 for 10 min and left with a 0.5 bar overpressure. Each sample was set up in triplicate and incubated at 25 ^{o}C in the dark for 48 days. Those samples which demonstrated methanogenic activity were later incubated again using fresh soil but supplemented with methyl-fluoride (CH_3F) in order to inhibit acetoclasitc methanogenesis (Janssen and Frenzel, 1997). Gas samples were taken from the tube headspaces every 6 days using a gas-tight pressure-lok® syringe (Vici) and analyzed immediately. Methane and CO_2 levels in the headspace were analyzed using a gas chromatograph (GC) equipped with a methanizer (Ni-catalyst at 350 ^{o}C, Chrompack) and a flame ionization detector (SRI).

Slurry sampling

After incubation was completed the tubes were opened and approximately 1 g of soil was sampled for nucleic acids extraction, immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

Analysis of stable carbon isotopes

The carbon isotope ratio ^{13}C:^{12}C was determined using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) against the V-PDB standard as described previously (Conrad et al., 2009). Isotopic calculations and estimation of the approximate fraction of hydrogenotrophic methanogenesis of the total methanogenesis were done after Conrad (2005). Briefly, the fraction of the newly formed methane between two time points is given by:

$$\delta_2 = f_n\delta_n + (1 - f_n)\delta_1 \quad [1]$$

where $\delta 1$, $\delta 2$ and δn are the isotopic signatures of the methane at times 1 and 2 and of the newly formed, respectively, while fn is the fraction of the newly formed methane at time 2.

The apparent fractionation factor for the conversion of CO_2 to CH_4 is given by:

$$\alpha_{app} = (\delta_{CO2} + 1000) / (\delta_{CH4} + 1000) \quad [2]$$

where δ_{CO2} and δ_{CH4} are the isotopic signatures of the carbon in CO_2 and CH_4, respectively. The relative fraction of H_2/CO_2-derived CH_4 in the total generated CH_4 was determined from

$$f_{H2} = (\delta_{CH4} - \delta_{ma}) / (\delta_{mc} - \delta_{ma}) \quad [3]$$

where δ_{ma} and δ_{mc} are the specific isotopic signatures of the carbon in methane produced solely from acetate and H_2/CO_2, respectively. $\delta^{13}C$ in the organic matter was analysed at the Institute for Soil Science and Forest Nutrition (IBW) at the University of Göttingen, Germany (courtesy of Heinz Flessa), using an elemental analyzer coupled to mass spectrometer. Measurements were done before and after acidification, the difference being due to carbonate (Nüsslein et al., 2003).

Nucleic acids extraction, RNA purification and cDNA synthesis

Total nucleic acids were extracted the following way: 0.5 g of frozen soil sample was placed in a Lysing Matrix E tube (MP). 375 µl SPB (10 mM sodium phosphate buffer, pH 8.0) and 125 µl TNS buffer (500 mM Tris HCL, 100 mM NaCl, 10% sodium dodecyl sulphate) were added to the tube; both solutions taken from Henckel and colleagues (1999) (Henckel et al., 1999). Then, 500 µl of Tris EDTA saturated phenol solution (pH 8.0; Fluka) were added to the tube and it was processed immediately in a FastPrep®-24 bead beating instrument for 30 s at 6.5 m s^{-1}. The tube was immediately transferred to ice and chilled for a minute and then centrifuged for 5 min at 14,000 rpm. Supernatant (including phenolic phase) was transferred to a 2 ml tube which was kept on ice and the beads tube containing the soil sample was retained. This process was repeated twice more with fresh aliquots of buffers and phenol solution. At the third time, the supernatant (excluding the phenolic phase) was distributed between the two 2 ml tubes used in to store the supernatant in the previous steps and the beads tube was discarded. Approx. 800 µl phenol/chloroform/isoamylalcohol (25:24:1; Fluka) were added to each of the tubes containing the extractant to a 2 ml total volume. The phases were homogenised by mixing and the tubes were centrifuged for 3 min at 14,000 rpm. The supernatants were transferred from each tube to fresh 2 ml tubes, and 1 volume of chloroform/ isoamylalcohol (24:1; Sigma-Aldric) was added to each. Phases were homogenised again by mixing and the tubes were centrifuged for 3 min at 14,000 rpm. The supernatants were transferred from each tube to Non-Stick RNase-free Microfuge Tubes (Ambion). To each tube 1 µl of glycogen (5 mg ml-1; Ambion) and 1.5 ml PEG precipitation solution (20% polyethylen glycol, 2.5 M NaCl) were added and the tubes were centrifuged for 90 min at 14,000 rpm and 4 °C. After centrifugation, the entire supernatant was discarded and the pellets were washed with ice cold 75% ethanol and centrifuged again for 20 min at 14,000 rpm and 4 °C. The entire supernatant was removed again, the pellets were dried under a stream of filtered N_2, then resuspended in 100 µl of low TE buffer (10mM Tris, 1mM EDTA) and the content of the two tubes was combined. In cases when the extractant had

colour it was further purified using MicroSpin S-200 HR (GE Healthcare). 10 µl of the extractant was diluted in 90 µl of low TE buffer and was used as DNA template. For synthesis of complete cDNA, 50 µl of nucleic acids extractant were digested with TURBO™ DNase (Ambion) and later purified using RNeasy® MinElute® Cleanup Kit (Qiagen) and quantified using a NanoDrop 1000 spectrophotometer (Thermo). Complete DNA removal was verified by failure to obtain PCR amplification product with the purified RNA template using the conditions described below. Complete cDNA was synthesized using ImProm-II™ Reverse Transcriptase (Promega) by the following procedure: 100 ng purified RNA and 0.5 µg/µl of random hexamer primers were incubated at 70°C for 5 min followed by 5 min chilling on ice. The following mixture was then directly added to the tubes: 4 µl of 5× ImProm-II™ Reaction Buffer, 3 mM $MgCl_2$, 1 mM of each dNTP, 20 µg of bovine serum albumin (BSA) (Roche), and 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega). The reaction tubes were incubated at 25°C for 5 min followed by 52°C for 1 h, and then denaturation at 70°C for 15 min. The resulting cDNA product was used directly as a template for consecutive PCR reactions. PCR amplifications were performed identically for both DNA and cDNA templates.

Primers and probes used in this study

Table S4.1 summarizes the primers and probes used in this study to monitor and quantify the archaeal population. For sequence affiliation, oligonucleotide design and evaluation, and reconstruction of phylogenetic trees we used the ARB software package (Ludwig et al. 2004). Analysis and oligonucleotide design of 16S rRNA-gene sequences were done using the Silva database (Pruesse et al. 2007; http://www.arb-silva.de/), while for analyses of *mcrA* sequences a specific ARB databases was built. The *mcrA* database was built using 5200 translated nucleic acid sequences which were obtained from EMBL (http://www.ebi.ac.uk/embl/) and aligned using Promals (Pei and Grishin 2007). The *mcrA* ARB database is available at: http://www.staff.uni-marburg.de/~angel/. The qPCR primers and probe targeting the 16S rRNA gene of *Methanocella* was designed using Primrose (Ashelford et al., 2002).

PCR amplification

For T-RFLP analysis, the forward primer was labelled with the fluorescent dye 6-FAM (*6 – carboxyfluorescein).* PCR reactions were conducted in triplicates of 50 µl which were later pooled to minimize reaction bias. Each PCR reaction contained 10 µl GoTaq®Flexi 5× Green Buffer (Promega), 1.5 mM $MgCl_2$, 40 mg BSA (Roche), 1.5 U of GoTaq® DNA polymerase (Promega) and 2 µl of template. For amplification of 16S rRNA gene, 0.5 µM of each primer were used while for amplification of *mcrA* 0.8 µM were used. The following programme was used for amplification of the 16S rRNA gene: 94 °C for 4 min followed by 28 cycles of 94 °C for 1 min, 52 °C for 30 s and 72 °C for 45 s and a single step of final elongation at 72 °C for 10 min. For amplification of *mcrA* the following programme was used: 94°C for 4 min followed by 5 touchdown cycles of 94 °C for 30 s, 60 °C - 1°C for 45 s and 72 °C for 30 s followed by 30 cycles of 94 °C for 30 s, 55°C for 30 s and 72 °C for 30 s, and a single step of final elongation at 72 °C for 10 min. After amplification, the PCR product was purified using GeneElute™ PCR purification Kit (Sigma) and quantified on a NanoDrop 1000 Spectrophotometer.

Cloning and sequencing and phylogenetic analysis

Active samples from the following locations were used for cloning of both 16S rRNA and *mcrA* genes: Avdat natural field, Liman – BSC; Giessen 0-7.5 cm; Hann River 0-10 cm. Libraries were constructed using purified PCR products and pGEM-T Easy cloning kit (Promega). Sequencing service was provided by ADIS (Germany) and GATC (Germany) using the primers T7f and M13r targeting flanking regions of the insert. A total of 250 clones containing 16S rRNA gene insert and 78 clones containing *mcrA* gene inserts were analyzed. All sequences were deposited into the GenBank® (www.ncbi.nlm.nih.gov) and received the following Accession Numbers: HQ268968 - HQ269217. Raw sequence data were analyzed using SeqMan software (DNAStar) where the primers and vector data were trimmed. 16S rRNA gene sequences were aligned using the SINA Webaligner (http://www.arb-silva.de/aligner/) against the Silva 102 database. *mcrA*

sequences were converted to amino acid sequences and aligned against the ARB database described above. Reconstruction of a maximum likelihood phylogenetic trees was conducted with RAxML 7.04 using rapid hill climbing algorithm and with GTRMIX and PROTMIX - JTT evolutionary models for 16S rRNA and *mcrA* genes, respectively (http://icwww.epfl.ch/~stamatak/index-Dateien/Page443.htm;Stamatakis 2006).

qPCR essays

qPCR was used in this study to quantify the numbers of 16S rRNA gene copies of the general archaeal, crenarchaeal, *Methanosarcina*, and *Methanocella* populations. All qPCR reactions were performed on an iCycler thermocycler equipped with a MyiQ™ detection system (Bio-Rad) and the data was analyzed using iQ5 Optical System software (Bio-Rad). For all essays we used standards containing known numbers of DNA copies of the target gene. Standards were serially diluted and used for construction of calibration curves in each reaction. A standard for the general archaeal and the *Methanosarcina* essays was prepared from a pure culture of *Mehtanosarcina thermophila* according to Lueders and colleagues (2004). Standards for *Crenaraeota* and *Methanocella* essays were prepared from clones containing 16S rRNA genes affiliated with either Crenaraeote or *Methanocella* as a plasmid insert the following way. The samples from Avdat Liman before and after incubation were used for cloning. DNA was amplified using the general primers ARC109F_mod and 1406R and cloned as described above. Clones containing the desired insert were identified through sequencing and the insert was amplified using the T7f and M13r primers. The PCR product was purified and quantified using Quant-iT™ PicoGreen®. The weight of a single amplicon was calculated from its sequence and that of the flanking regions (http://www.basic.northwestern.edu/biotools/oligocalc.html#helpMW) and the number of copies per microlitre was obtained by simple division of the total quantity by the weight of an amplicon. Total *Archaea* and *Crenarchaeota* essays were based on SYBR® Green. Each reaction was 25 µl in volume and contained the following mixture: 12.5 µl SYBR® Green JumpStart™ Taq ReadyMix™, 3 or 2 mM $MgCl_2$ (Total *Archaea* and

Crenarchaeota essays resp.), 0.8 ng μl^{-1} BSA (Ambion), 0.66 or 0.44 μM of each primer (Total *Archaea* and *Crenarchaeota* essays resp.), and 5 μl of template. For the two essays, the programme used was: 94 °C for 4 min followed by 40 cycles of 94 °C for 30 s, 66 or 63 °C for 30 s (Total *Archaea* and *Crenarchaeota* essays resp.), 72 °C for 30 s and 85 °C for 10 s for signal reading. The essays targeting the 16S rRNA gene of *Methanosarcina* and *Methanocella* were based on dual labelled probes. Each reaction was 25 μl in volume and contained the following mixture: 12.5 μl JumpStart™ Taq ReadyMix™, 4 mM $MgCl_2$, 0.8 ng μl^{-1} BSA (Ambion), 0.5 μM of each primer, 0.2 μM of the dual labelled probe and 5 μl of template. For the two essays, the programme used was: 94 °C for 4 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 60 s for annealing, extension and signal reading.

TRFLP analysis

Approximately 200 ng of purified PCR products were digested overnight at 37°C with 20 U of the restriction enzyme *TaqI* and 2 μl of 10× Buffer TaqI™ (Fermentas). Following digestion, samples were desalted using SigmaSpin™ Post-Reaction Clean-up Columns (Sigma, Germany), aliquots of 3 μl were mixed with 10 μl of HiDi™ Formamide (Applied Biosystems) and 0.3 μl of the internal DNA standard MapMarker®1000 (BioVentures). The samples were denatured at 94°C for 2 min and loaded into an ABI 3100 automated gene sequencer (Applied Biosystems) for separation of the TRFs. TRFLP data was retrieved by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems).

Data transformation and statistical analysis

Affiliation of TRFs to their respective archaeal taxa was done using *in silico* prediction of the restriction sites in the sequence data. For PCA analysis, TRFLP data was transformed and standardised as described previously (Angel et al., 2010). PCA plots were computed using Canoco 4.53 and plotted using CanoDraw 4.12 (http://www.canoco.com).

4.6 Supplementary material

Supplementary Table 4.1| Primers and probes used in this study

*Oligo. name**	*Target*	*Oligo. sequence (5'-3')*	*Position***	*GC (%)*	*Tm †*	*Amplicon size*	*Essay*	*Reference*
ARCH109 - F	*Archaea* 16S rRNA gene	AHDGCTCAGTAACACRT	109-125	42.2	56	826	TRFLP/ Sequencing	Miyashita et al. 2009
ARCH934 - R		GTGCTCCCCCGCCAATTCCT	915-934	65	68			Grosskopf et al. 1998
U1406 - R		ACGGGCGGTGTGTRC	1392-1406	70	63	1298	qPCR std.	Amann et al. 1995
ARCH364 - F	*Methanosarcinales* 16S rRNA gene	CGGGGYGCASCAGGGGCGAA	364-383	75-80	75	553	QPCR	Burggrafet al. 1997
MSL812 - F		GTAAACGATRYTCGCTAGGT	812-831	40-50	62			
MSL860 - P		AGGGAAGCCGTGAAGCGARCC	860-880	62-67	70	354	QPCR	Yu et al. 2005
MSL1159 - R		GGTCCCCACAGWGTACC	1143-1159	65	63			
MCL282 – F	*Methanocella*l 16S rRNA gene	ATCMGTACGGGTTGTGGG	282-299	56-61	65			
MCL609 – P		ATCCAGCGGCTTAACCGTTGGKCK	609-632	54-63	72	510	QPCR	This study
MCL832 – R		CACCTAGCGRGCATCGTTTAC	813-832	52-57	64			
771F	*Crenarchaeota* 16S rRNA gene	ACGGTGAGGGATGAAAGCT	753-771	53	63	203	QPCR	Ochsenreiter et al. 2003
957R		CGGCGTTGACTCCAATTG	957-974	56	62			
mlas-mod – F	Universal *mcrA* gene	GGYGGTGTMGGDTTCACMCARTA	976-998a	43-65	68	469	Sequencing / QPCR	Modified from: Steinberg and Regan 2009
mcrA-rev - R		CGTTCATBGCGTAGTTVGGRTAGT	1421-1444a	42-54	66			Steinberg and Regan 2009

* The following primer name suffixes are used: - F – forward primer, - R – reverse primer, - P – dual labelled probe.

** Position is based on the following: primers targeting the 16S rRNA gene - *E.coli*; primers targeting the *mcrA* gene - *Methanothermobacter thermautotrophicus mcrA* gene accession number: AAA73445 (following Steinberg and Regan, 2009);

† Calculated using Nearest Neighbour method with OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/).

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Chapter 5 |

Activation of Methanogenesis in Arid Biological Soil Crusts Despite the Presence of Oxygen

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RA and RC designed the study; RA performed all lab work and data analyses; DM assisted in statistical analysis; RA, DM and RC wrote the manuscript.

5.1 Abstract

Methanogenesis is traditionally thought to occur only in highly reduced, anoxic environments. Wetland and rice field soils are well known sources for atmospheric methane, while upland soils are considered sinks. Although methanogens have been detected in low numbers in some upland, and even in desert soils, it remains unclear whether they are active under natural oxic conditions, such as in biological soil crusts (BSCs) of arid regions. To answer this question we carried out a factorial experiment using microcosms under simulated natural conditions. The BSC on top of an arid soil was incubated under moist conditions in all possible combinations of flooding and drainage, light and dark, air and nitrogen headspace. In the light, oxygen was produced by photosynthesis. Methane production was detected in all the microcosms, though rates were much lower when oxygen was present. In addition, the $\delta^{13}C$ of the methane differed between the oxic/oxygenic and anoxic microcosms. While under anoxic conditions methane was mainly produced from acetate, it was almost entirely from H_2/CO_2 under oxic/oxygenic conditions. Only two genera of methanogens were identified in the BSC – *Methanosarcina* and *Methanocella*; their abundance and activity in transcribing the *mcrA* gene (coding for methyl-CoM reductase) was higher under anoxic than oxic/oxygenic conditions, respectively. Both methanogens also actively transcribed the oxygen detoxifying gene catalase. Since methanotrophs were not detectable in the BSC, all the methane produced was released into the atmosphere. Our findings point to a formerly unknown participation of desert soils in the global methane cycle.

5.2 Introduction

Methane is the third most important greenhouse gas on earth after water vapor and CO_2 (Forster et al., 2007). Traditionally, methane is thought to have 25 times the global warming potential (GWP) of CO_2, but recent models, which take into account direct and indirect interactions with aerosols, estimate its GWP to be as high as 26 to 41 times that of CO_2 over a 100-year horizon (Shindell et al., 2009). Of the 500 – 600 Tg CH_4 emitted annually into the atmosphere about 74% is biogenic, i.e. the product of methanogenesis (Denman et al., 2007). Despite the biogeochemical importance of methanogenesis as a terminal electron sink in anoxic environments, only one group of microorganisms, the methanogenic Archaea (methanogens), are able to produce methane. The methanogens themselves are phylogeneticaly divided into 5 families within the phylum Euryarchaea and are comprised of 31 known genera. Biogenic methane can be produced from a wide range of methylated compounds, but in most natural systems methane arises from two pathways only: reduction of CO_2 (hydrogenotrophic methanogenesis) and cleavage of acetate (acetoclastic methanogenesis) (Conrad, 2005). One exception is saline and hypersaline environments such as marine sediments and salt lakes where methanogenesis from methylated compounds such as trimethylamine can play a significant role (Oremland, 1988).

The traditional textbook notion is that methanogens are found only in highly reduced, anoxic environments such as wetlands, rice fields, lentic and marine sediments as well as in rumens and in the guts of termites. This notion is based on two features of the physiology of methanogens: 1) they are strict anaerobes and the presence of oxygen leads to the formation of reactive oxygen species (ROS) which cause damages to cell membranes, DNA and proteins (Storz et al., 1990). Particularly in methanogens, oxygen causes an irreversible dissociation of the F420-hydrogenase enzyme complex, a crucial electron transporter in methanogenesis (Schönheit et al., 1981). Indeed, methane production in an active rice paddy soil was shown to cease completely upon oxygen stress (Yuan et al., 2009). 2) Methanogens are poor competitors for hydrogen and acetate with nitrate, iron and sulfate reducers.

Thus, even in the absence of oxygen, hydrogenotrophic or acetoclastic methanogenesis only commences once most nitrate, ferric iron and sulfate in the system are depleted (Zehnder and Stumm, 1988). Nevertheless, it has been previously shown that many soils which are typically aerated, including a desert soil, can turn methanogenic when incubated under anoxic conditions as slurry (Peters and Conrad, 1995).

Deserts (semiarid, arid and hyperarid regions) span over 44 mil. km^2 and make up 33% of the earth's land surface (Verstraete and Schwartz, 1991). Desert soils are typically covered by a unique crust, of a few millimeters, densely colonized by microorganisms. These include primarily polysaccharide secreting/photosynthetic microorganisms such as cyanobacteria and microalgae but also fungi, lichens and mosses as well as an array of prokaryotic species about which little is known (Garcia-Pichel, 2002). These biological soil crusts (BSCs) are mostly inactive when dry but regain near full photosynthetic activity within hours to a few days upon wetting (Jeffries et al., 1993). As a result of their high microbial activity and of their compact structure oxygen becomes limiting very quickly in active BSCs and anoxic microniches are formed within it, where anaerobic degradation processes can potentially take place (Garcia-Pichel and Belnap, 1996). We hypothesized that despite being strict anaerobes, some methanogens are able to endure long periods of exposure to oxygen in the BSC when it is dry and take advantage of anoxic micro-niches and fresh organic matter which are formed after a rain event.

We used microcosms and simulated different natural conditions following a rain event to investigate a possible methanogenic activity BSCs, in particular when exposed to atmospheric oxygen levels. We set up a three-factorial experiment with the following treatments: microcosms were incubated under flooded or wet-drained conditions (denoted F and W resp. in the text and figures), in the light or dark (denoted L and D resp.), and under an oxic (air) or anoxic (N_2) gas headspace (denoted O and N resp.), in all possible combinations (**Figure S5.1**).

We followed gas evolution in the headspace of the microcosms as well as the signature of the stable carbon isotopes ($\delta^{13}C$) in the CO_2 and methane to deduce the pathway in which the latter was formed. In addition, we used

molecular techniques to identify the active methanogens, quantify their population size and transcription level using the 16S rRNA gene and the marker gene for methanogenesis – methyl coenzyme M reductase (*mcrA*). Finally, we quantified the expression of catalase (*katE*), which is part of the microbial oxygen detoxification mechanism.

5.3 Results and Discussion

Methanogenesis under oxic atmosphere

Methane was detectable in the headspace of all microcosms seven days after the start of the experiment and its accumulation was linear after the 14th day, regardless of treatment (**Figure 5.1**). A strong, two orders of magnitude, difference in the methanogenic activity was seen between the oxic and the anoxic microcosms incubated in the dark. These anoxic treatments - FDN and WDN - accumulated methane at a rate of 3800 $\pm$ 400 and 1500 $\pm$ 400 nmol gdw^{-1} d^{-1}, respectively, while the corresponding oxic treatments – FDO and WDO – accumulated methane at a rate of 41.6 $\pm$ 12.4 and 9.2 $\pm$ 4.3 nmol gdw^{-1} d^{-1}, respectively. The microcosms incubated in the light showed an average methane production of 21.7 $\pm$ 3.7 nmol gdw^{-1} d^{-1}. This average rate was similar to that measured for the oxic microcosms in the dark, and indeed methane production rates between these treatments were not significantly different indicating no apparent effect of initial oxygen levels ($P = 0.66$ in a t-test).

Active production of oxygen due to photosynthesis was observed in the microcosms incubated in the light (**Figure S5.2A**). The oxygen fluxes modeled from vertical oxygen concentration profiles ranged between 10 - 20 nmol cm^{-2} s^{-1} (**Figure S5.3**). Oxygen in these microcosms penetrated down to about 1500 μm, i.e. half the depth of the crust, below which the soil was completely anoxic, while in the two oxic microcosms incubated in the dark oxygen penetrated somewhat deeper down to about 2-2.5 mm. Overall, oxygen penetration depth was in agreement with field measurements of a BSC following a rain event (Garcia-Pichel and Belnap, 1996). Most of the CO_2 released from the BSCs accumulated in the microcosm headspaces within the first week, but was much lower in the light treatment where it was most likely used for photosynthesis (**Figure S5.2B**). Hydrogen levels were, however, higher in the light than in the dark treatments (**Figure S5.2C**). In fact, hydrogen in the oxic dark treatment was below the detection limit throughout the experiment.

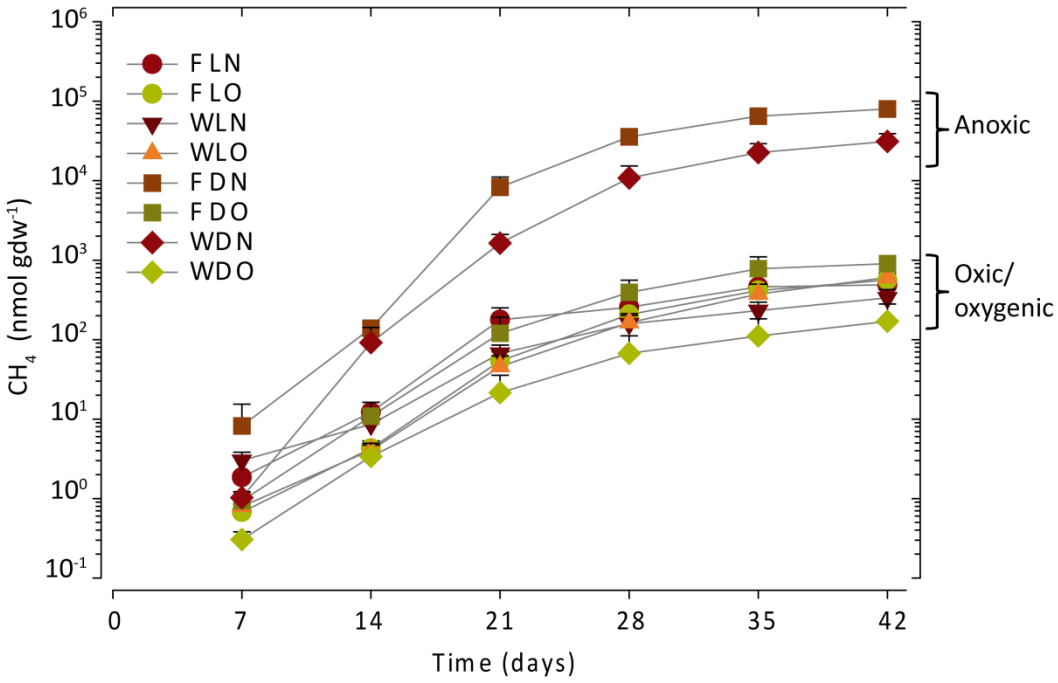

Figure 5.1| Accumulation of CH_4 in the headspace of the microcosms: mean ± 1 SE; n = 3. Treatment codes are as follows: flooded – F, wet-drained – W, light – L, dark – D, N_2 headspace – N, air (21% O_2) headspace – O.

Both light and oxygen treatments strongly reduced methane production while flooding increased it (**Table 5.1**). The effects of light and oxygen interacted strongly reflecting the fact that the effect of oxygen treatment on methane production was dependent on light (as the latter promoted photosynthesis in the BSCs). Methane production rates were strongly negatively correlated with the depth of the anoxic boundary (less than 1% O_2) but only weakly to water content (**Table S5.1**). This strong correlation demonstrates the well known negative effect of oxygen on the methanogenic process in the soil, which was the primary factor affecting methanogenesis in our experiment.

Table 5.1| ANOVA analyses (least squares) testing the effect of the various incubation conditions on methane production rates and ratios of gene and transcript copies

Source of variation	*Log methane production rate*			*16S MSL/ARC**			*msar/mcrA gen. DNA***			*msar/mcrA gen. cDNA†*		
	Mean square	*F*	*Sig. †† P > |F|*	*Mean square*	*F*	*Sig. P > |F|*	*Mean square*	*F*	*Sig. P > |F|*	*Mean square*	*F*	*Sig. P > |F|*
Light	6.45	59.61	<0.01	0.64	12.74	<0.01	0.80	8.70	0.01	0.36	8.25	0.01
Oxygen	5.82	53.82	<0.01	0.44	8.62	0.01	0.84	9.11	0.01	0.45	10.16	0.01
Flooding	1.32	12.25	0.03	0.02	0.39	0.54	0.00	0.00	0.99	0.13	2.98	0.10
Light * Oxygen	10.14	93.75	<0.01	0.15	3.02	0.10	0.00	0.00	0.96	0.84	19.20	<0.01
Light * Flooding	0.43	3.99	0.06	0.08	1.59	0.22	0.70	7.57	0.01	0.00	0.04	0.85
Oxygen * Flooding	0.02	0.20	0.66	0.01	0.15	0.70	0.15	1.63	0.22	0.53	12.04	<0.01
Light * Oxygen * Water	0.29	2.76	0.12	0.11	2.24	0.15	0.08	0.85	0.37	0.05	1.10	0.31
Error§	0.11			0.05			0.09			0.04		

* Ratio of 16S rRNA gene copies of *Methanosarcina* to general Archaea
** Ratio of *mcrA* gene copies of *Methanosarcina* to general *mcrA*
† Ratio of *mcrA* transcript copies of *Methanosarcina* to general *mcrA*
†† Values in red are significant at $P < 0.05$
§ Degrees of freedom for error in all tests: 16

Phylogenetic analysis of the *mcrA* gene revealed only two active methanogenic types

In contrast to other methanogenic environments, which typically host many methanogenic species simultaneously (Lueders et al., 2001; Banning et al., 2005; Denman et al., 2007; Orphan et al., 2008), the diversity in our microcosms was remarkably poor. Analysis of the *mcrA* gene sequences revealed only two very tight clusters of sequences closely related to either *Methanosarcina*, which produces methane from a variety of substrates including acetate and H_2/CO_2 (Liu and Whitman, 2008), or *Methanocella*, which is capable of hydrogenotrophic methanogenesis only (Sakai et al. 2008, 2010; **Figure 5.2**).

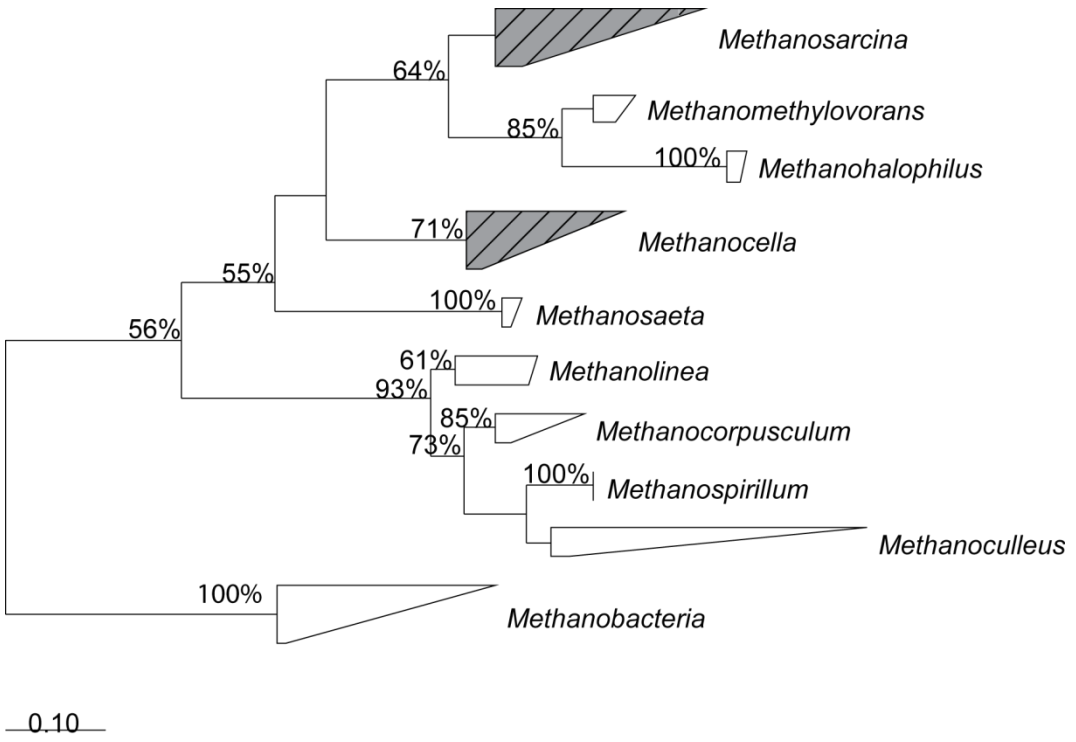

Figure 5.2| Maximum likelihood phylogenetic tree based on aligned partial amino acid sequences of the methyl coenzyme M reductase gene (*mcrA*). Amino acid composition was deduced from DNA sequences and the tree was calculated with RAxML 7.04. Bootstrap values above 50% (out of a 100 trials) are displayed next to the nodes. Shaded clusters with diagonal lines contain sequences that were detected in the microcosm soil samples.

Because of this low complexity of the methanogenic community we could individually quantify the 16S rRNA gene copies and *mcrA* gene and transcripts copies for the different methanogenic types as well as generally quantify the 16S rRNA gene of the archaeal community, and the total *mcrA* gene and transcript copies. We observed differences between individual treatment combinations, but by far the strongest effects were a smaller methanogenic community and lower transcription levels in the oxic/oxygenic microcosms (**Figure 5.3**).

16S rRNA and *mcrA* gene copies were in the range of 10^8 – 10^9 copies gdw^{-1} in the anoxic but only 10^3 – 10^7 in the oxic/oxygenic microcosms. In all treatments we observed an increase in the quantity of *mcrA* gene copies from 3.15 x 10^4 copies gdw^{-1} in the soil before incubation to at least 4.16 x 10^5 copies gdw^{-1} (a tenfold increase in the WLO treatment) and up to 1.15 x 10^9 copies gdw^{-1} in the FDN treatment (an increase of almost five orders of magnitude). Apart from a general effect of oxygen on the community size and gene expression, we noted a differential effect of oxygen on *Methanocella* and *Methanosarcina*. The ratio of *Methanosarcina* to total 16S rRNA gene copies was significantly lower in the light and oxygen-dark than in the anoxic treatments (**Table 5.1, Figure 5.3**). In contrast to the effect on methane production, flooding did not have a significant effect on the ratio of *Methanosarcina* to total 16S rRNA gene copies. The same trend was seen for the ratio of *Methanosarcina* versus the general *mcrA* gene and transcript copies.

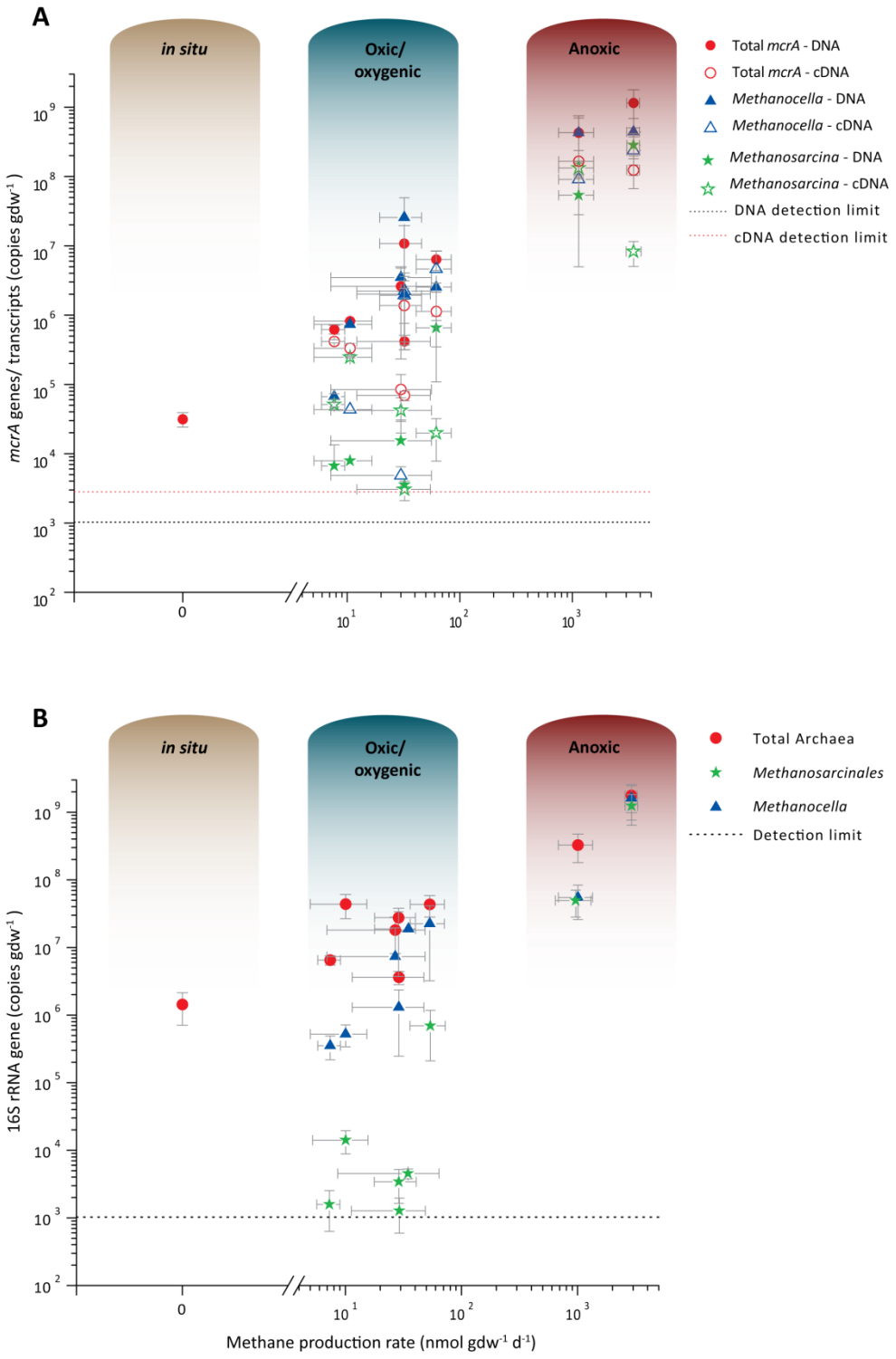

Figure 5.3| Gene and transcript copy numbers quantified using qPCR plotted against methane production rates: mean + 1 SE; n = 3. *In situ* refers to the dry BSC prior to any treatment. A. *mcrA* gene and transcript copy numbers. B. 16S rRNA gene copy numbers.

Stable isotope analysis reveals different methanogenic pathways under oxic and anoxic atmospheres. We also analyzed the stable isotope

signature of the carbon in methane and CO_2 (^{13}C: ^{12}C) to decipher the proportional contribution of different methanogenic pathways (Conrad, 2005). Our analysis of isotopic signatures (**Figure 5.4**) revealed two distinct clusters: all strictly anoxic microcosms had $\delta^{13}C$-nCH_4 (isotopic signature of the newly formed methane) values ranging from -63‰ on average in the first week of incubation and stabilizing at -35‰ on average throughout the rest of the incubation period, and $\delta^{13}C$-CO_2 values of -16 to -7‰. The oxic/oxygenic microcosms showed lighter isotopic signatures with average $\delta^{13}C$ values of -75‰ and -20‰ for methane and CO_2, respectively, and were stable over time. The difference between the two clusters in the isotopic signatures of the CO_2 (**Figure 5.4**) can be related to the difference in the signature of the organic carbon and the carbonate reservoir in the soil, which constituted up to 34% of the soil mass (Angel et al., 2010). The $\delta^{13}C$ of carbonate (-4.09‰) was heavier than that of organic carbon (-20.5‰). In the oxic/oxygenic microcosms CO_2 was probably produced from organic matter only. However, in the anoxic microcosms, the CO_2 was apparently also generated from the carbonate. The contribution of carbonate may be attributed to the higher acidity associated with anaerobic (compared to aerobic) degradation processes.

The $\delta^{13}C$ values of CO_2 and CH_4 allow the calculation of mean apparent fractionation factors ($\alpha_{app;}$ eq.2). The α_{app} for the strictly anoxic microcosms was 1.025 $\pm$ 0.002, which was much smaller than the α_{app} = 1.065 $\pm$ 0.003 obtained for the oxic/oxygenic microcosms, indicating different methanogenic pathways under the two treatments. The large fractionation factor obtained for the oxic/oxygenic microcosms is well within the range of 1.040 - 1.080, typically seen for hydrogenotrophic methanogenesis in pure cultures and in soils at moderate temperatures (20-30 ^{o}C; Conrad, 2005). Indeed, also for this soil we obtained a fractionation factor of 1.066 when acetoclastic methanogenesis was inhibited using CH_3F. We therefore conclude that in oxic/oxygenic microcosms CH_4 was almost entirely produced from H_2/CO_2. The nearly complete lack of acetoclastic methanogenesis in the oxic/oxygenic microcosms could be the result of competition with heterotrophs that oxidize acetate aerobically. Indeed, acetate concentrations were generally much lower in the pore water of

oxic/oxygenic than anoxic microcosms (and so were other fermentation products; **Table S5.2**).

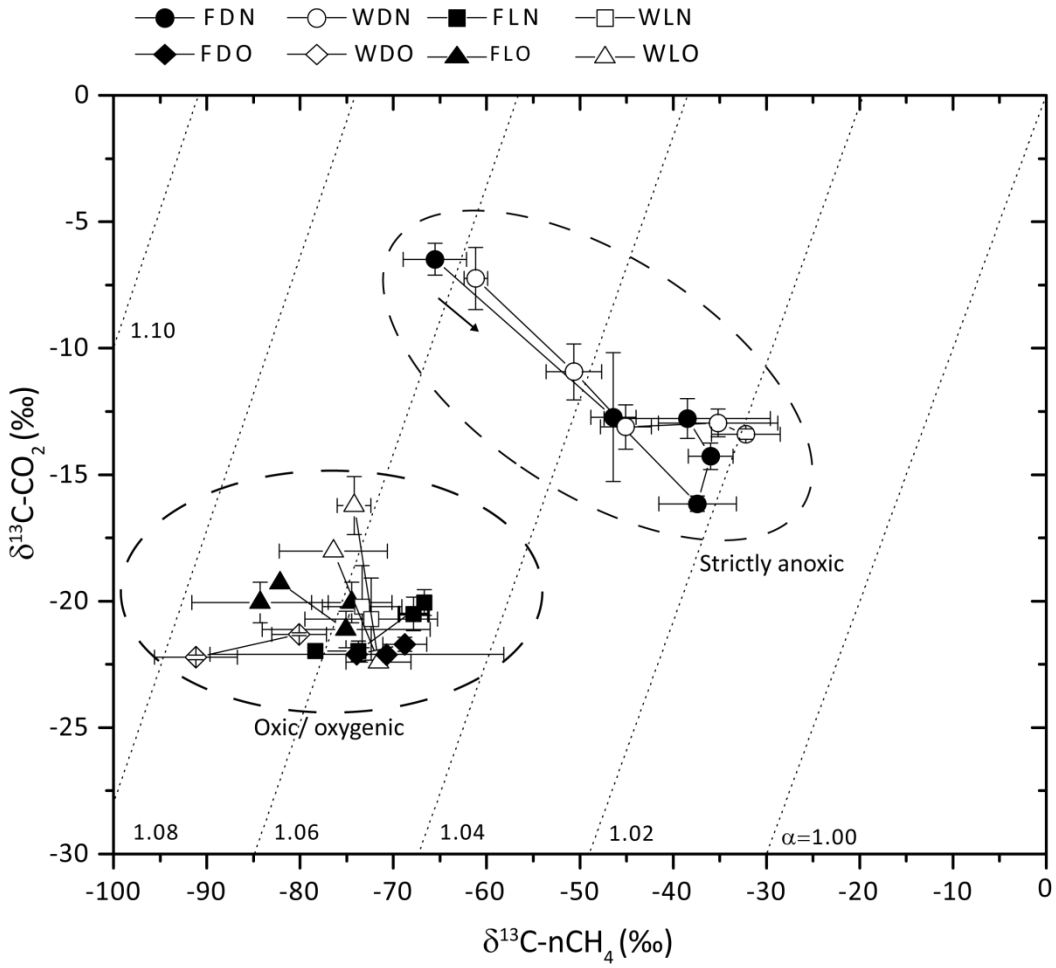

Figure 5.4| Values of $\delta^{13}C$ of CO_2 and the newly formed methane (see Materials and Methods): mean ± 1 SE; n = 3. Isolines represent different apparent fractionation factors (α_{app}; eq.2). "Strictly anoxic" refers only to the anoxic microcosms in the dark while "oxic/oxygenic" refers to all the rest. The arrow in the "strictly anoxic" circle points to the direction of temporal development. Treatment codes are as follows: flooded – F, wet-drained – W, light – L, dark – D, N2 headspace – N, air (21% O_2) headspace – O.

The fractionation factor of 1.025, obtained in the strictly anoxic microcosms, is similar to that of purely acetoclastic methanogenesis (Krzycki et al., 1987; Gelwicks et al., 1994). Therefore, we conclude that acetate contributed substantially to CH_4 production in these microcosms. To determine more precisely the specific contribution of acetoclastic and hydrogenotrophic methanogenesis to the total methane production in anoxic

microcosms, we made the following reasonable assumptions. We assumed that the isotopic signature of the methane in the oxic/oxygenic microcosms was characteristic for hydrogenotrophically produced methane. We further estimated the δ^{13}-C of acetoclastically produced methane from the δ^{13}-C the soil organic carbon (-20.5‰), assuming that the fractionation of organic C to acetate was only small, and that of acetate to methane was either zero or was -25.6‰ (Goevert and Conrad, 2009). By using equation 3 we could then confine the fraction of acetoclastic methanogenesis in the anoxic microcosms to 0.72-1.00 following the first week of incubation.

The BSC lacks methane oxidizing bacteria.

Our comparison of methane production rates and isotopic signatures is based on the assumption that all methane which had been produced was released into the headspace and none of it was oxidized by methane-oxidizing bacteria in the soil, which could potentially reduce the measured concentrations and alter the isotopic signature (Whiticar, 1999). We previously showed (Angel and Conrad, 2009) that active methanotrophs appear to reside only below the BSC, down to a depth of approx. 20 cm. No methane uptake activity and no transcription of the key enzyme in aerobic methane oxidation – the particulate methane monooxygenase (*pmmo*) – could be detected in the BSC itself. We have confirmed this observation also in this study as no *pmmo* transcripts could be detected in the microcosm samples by PCR.

BSC methanogens transcribe oxygen detoxification genes

For methanogens to be active in a system such as the BSC, which is exposed to atmospheric levels of oxygen throughout most of the year, when dry, and to a constant flux of oxygen, albeit at sub-atmospheric levels, when wet and active, they need to be able to efficiently detoxify reactive oxygen species (ROS). Indeed, it has been previously noted that both *Methanosaricna* and *Methanocella* contain a range of genes encoding enzymes that detoxify reactive oxygen species. These include enzymes such as catalase (*kat*), superoxic dismutase (*sod*), superoxic reductase (*sor*) and others (Erkel et al.,

2006). The metgenome sequence of RC-I strain MRE50 (now *Methanocella arvoryzae*) contained 7 different putative genes whose function is associated with detoxification of ROS (Erkel et al., 2006). Since *Methanosarcina* only contains 6 such genes, *Methanocella* is potentially the most oxygen-tolerant methanogen. We tested for the presence of catalase E (*KatE*) gene transcripts using katMsl and katRCI primer pairs for *Methanosarcina* and *Methanocella*, respectively (**Table S5.3**), and performed phylogenetic analysis. All sequences clustered tightly to their respective methanogen cultivar from which the primers were designed (**Figure S5.4**). Indeed, the *KatE* sequences retrieved from our microcosms showed a remarkable similarity to those of the cultivated methanogens with only a 1.8% and 4.7% difference in the amino acid sequence for *Methanocella* and *Methanosarcina,* respectively. By comparison, there is a 7.4% and 6.3% difference, respectively, in the *mcrA* sequence at the amino acid level. We compared also the relative expression (transcripts to genes) in differently treated microcosms with respect to the set level of oxygen using qPCR (**Table 5.2**). Our results show that while *katE* is being expressed we see no significant over expression in response to oxygen. This is in agreement with the results by Zhang and colleagues (2006) who reported no up regulation of catalase in *Methanosarcina barkeri* in response to air exposure (Zhang et al., 2006), but in contrast to Brioukhanov and colleagues (2006) who reported the opposite in response to oxidative stress.

Table 5.2| Differences in relative expression of *katE* (ΔΔCt*) in *Methanocella* and *Methanosarcina* between paired treatments. Mean ± 1 SE.

Treatment comparison	*Methanocella*	*Methanosarcina*
FLN - FLO	-2.2† ± 1.3	-5.6 ± 4.3
WLN - WLO	1.4 ± 2.3	-0.4 ± 0.9
FDN - FDO	1.7 ± 0.6	-1.5 ± 1.4
WDN - WDO	1.1 ± 1.6	-3.4 ± 3.0

* Each Ct unit represents a two-fold difference in expression.
† Positive values represent upregulation in the second matched treatment compared to the first. Treatment codes are as follows: flooded – F, wet-drained – W, light – L, dark – D, N_2 headspace – N, air (21% O_2) headspace – O.

Conclusions

The results presented show that while methanogens are strict anaerobes, at least some of them are more resilient than so far assumed. Former studies have demonstrated the ability of certain methanogenic cultures to endure desiccation and exposure to high levels of oxygen, probably in resting forms (Liu et al., 2008; Fetzer et al., 1993). Here we showed that *Methanosarcina* and *Methanocella* species, in particular, are able to tolerate long periods of desiccation in an arid soil and become metabolically active and start growing within just a few days after wetting.

It was previously shown that *Methanocella* are usually the most abundant and active methanogens in rice fields (Ramakrishnan et al., 2001; Lu and Conrad, 2005). It appears that they are also the dominant methanogens in BSCs. *Methanocella* and *Methanosarcina* spp. have apparently different ecological roles in nature. Although both are cytochrome-containing methanogens, they differ in their substrate range, threshold level to hydrogen concentrations and growth yield (Thauer et al., 2008). Our experiments showed differential activity and growth of either methanogen under different conditions and it is possible that niche differentiation or dominance of either methanogen under different natural conditions permits their coexistence in soil.

The production of biogenic methane in a BSC proves not just the activity of methanogens but indicates that of a whole cascade of anaerobes which constitute a formerly unrecognized part of the BSC biome. These include primary and secondary fermenters, syntrophs and maybe acetogens whose identity in these systems is yet to be elucidated but which are required for the different stages of the anaerobic degradation process (Zinder, 1993; Stams and Plugge, 2009). This array of microbes remains inactive during long periods when the soil is dry and saturated with oxygen but is apparently able to react quickly and take advantage of short periods when water is available and anoxic microniches can be formed.

Additionally, some hydrogen might be directly transferred from cyanobacteria to the methanogens and used as substrate for methanogenesis as occurs in some hypersaline mats (Hoehler et al., 2001). Plant litter constitutes most likely part of the organic substrate but primary producing microorganisms such as cyanobacteria probably also play a role in releasing fresh organic exudates into the soil even when water availability is very low (Wilske et al., 2008; Rao et al., 2009; Lange et al., 1994). While cyanobacteria have been shown to be activated by as little as 0.2 mm of rain or even fog or dew (Lange et al., 1992), it is currently not known what amount of water is required to activate the anaerobic part of the BSC. Our findings shed light on a new and unexpected function of arid soils and might point to a previously unknown contribution of biological soil crusts, and perhaps other aerated soils, to the global methane cycle.

5.4 Experimental procedure

Soil sampling and characterization

In April 2009 the top 3-4 millimeters of the soil comprising the biological soil crust in an arid site located in the northern Negev Desert in Israel were sampled. The soil is a calcareous silty loam and was previously characterized (Angel and Conrad, 2009).

Microcosm design and incubation conditions

Microcosms were designed after Murase and Frenzel (2007) with few technical modifications. In principle, the microcosm were gas-tight plastic vessels, which consisted of a lower compartment (approximately 60 ml) and an upper compartment (approximately 100 ml) separated by a 0.2 □m hydrophilic polyamide membrane (Whatman). Non-sieved, homogenized fractures of BSC (20 g, approximately 3 mm ø on average) were placed on top of the membrane and amended with sterile deionized water, thus generating a wet soil layer of approximately 3 mm, mimicking its actual thickness in the field. The bottom compartment of the microcosm contained either sterile deionized water ("flooded" treatment) or 0.1-0.3 mm quartz sand, baked (180 °C, 24 h), saturated with sterile deionized water and then drained ("wet-drained"). The upper compartment served as gas headspace, which was flushed with either N_2 ("anoxic") or 80% N_2, 21% O_2 ("oxic"). Oxygen was supplemented daily to maintain atmospheric levels ("oxic") or was flushed several times during the incubation with N_2 to maintain levels at below 5% O_2 ("anoxic" under light). Three replicate microcosms of each of the four possible combinations of treatments were incubated at 25° C in full darkness ("dark") or under constant light (3000 Lux) for 42 days ("light"; **Figure S5.1**).

Gas measurements

Gas samples were taken from the headspace of the microcosms at regular time intervals using a gas-tight pressure-lok® syringe (Vici) and analyzed immediately using a gas chromatograph (GC) and a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS). Methane production rates (nmol gdw^{-1} d^{-1}) were calculated for the entire incubation period using linear regression.

Stable isotopes analysis

The carbon isotope signature $\delta^{13}C$ was determined using GC-C-IRMS against the V-PDB standard as described previously (Ralf Conrad et al. 2009). δ13C in the organic matter was analysed at the Institute for Soil Science and Forest Nutrition (IBW) at the University of Göttingen, Germany (courtesy of Heinz Flessa), using an elemental analyzer coupled to mass spectrometer. Measurements were done before and after acidification, the difference being due to carbonate (Nüsslein et al., 2003).

Isotopic calculations and estimation of the approximate fraction of hydrogenotrophic methanogenesis of the total methanogenesis were done after Conrad (2005). Briefly, the fraction of the newly formed methane between two time points is given by:

$$\delta_2 = f_n\delta_n + (1 - f_n)\delta_1 \quad [1]$$

where δ1, δ2 and δn are the isotopic signatures of the methane at times 1 and 2 and of the newly formed, respectively, while *f*n is the fraction of the newly formed methane at time 2.

The apparent fractionation factor for the conversion of CO_2 to CH_4 is given by:

$$\alpha_{app} = (\delta_{CO2} + 1000) / (\delta_{CH4} + 1000) \quad [2]$$

where δ_{CO2} and δ_{CH4} are the isotopic signatures of the carbon in CO_2 and CH_4, respectively. The relative fraction of H_2/CO_2-derived CH_4 in the total generated CH_4 was determined from

$$f_{H2} = (\delta_{CH4} - \delta_{ma}) / (\delta_{mc} - \delta_{ma}) \quad [3]$$

where δ_{ma} and δ_{mc} are the specific isotopic signatures of the carbon in methane produced solely from acetate and H_2/CO_2, respectively. To determine δ_{mc}, 5 g of BSC was incubated with sterile deionized water (1:1) in a glass tube and supplemented with 3% CH_3F which gave complete inhibition of acetoclasitc methanogenesis (Janssen and Frenzel, 1997). The carbon isotope signature was determined during 42 days of incubation as described above.

Pore water analysis and oxygen profiles

After incubation was completed, microcosms were opened and approximately 1 g of soil was sampled for nucleic acid extraction, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Additionally, approximately 2 ml of pore water were collected and analyzed using high pressure liquid chromatography. Water content was determined gravimetrically and oxygen profiles in the soil were determined using an OX-50 glass microsensor (Unisense). The oxygen fluxes were modelled using the Profile V1.0 tool (Berg et al., 1998). The anoxic boundary was determined as the depth bellow which oxygen concentration was below 1% was.

Molecular characterization and quantification of archaeal 16S rRNA, mcrA and catalase genes

Total nucleic acids were extracted by disrupting 0.5 g of soil in a FastPrep®-24 bead beater in the presence of phosphate buffer, 10% SDS solution and phenol. Following phenol/chloroform purification a subsample was treated with DNase, and the RNA was purified. Random hexamer primers (0.5 μg) were used for complete cDNA synthesis which was used for amplification of the 16S rRNA and catalase (*KatE*) genes, while for amplification of the mcrA gene 2 pmol of the mcrA-rev primer (**Table S5.3**) were used for *mcrA* cDNA synthesis. All molecular characterizations were done using the primers listed in **Table S5.3**. Phylogenetic characterization of the methanogenic community was performed by amplifying and cloning the methyl coenzyme reductase M gene (*mcrA*) and catalase (*KatE*). Gene and transcript quantifications were done via qPCR (iCycler; Bio-Rad) using either SYBR®

Green or dual labelled probe technology. For more details see supplementary information.

Phylogenetic analysis

Phylogenetic analysis was based on aligned partial amino acid sequences of *mcrA* performed using PROMALS (Pei and Grishin, 2007). Amino acid composition was deduced from DNA sequences and the tree was calculated with RAxML 7.04 using rapid hill climbing algorithm and PROTMIX - JTT evolutionary model (Stamatakis, 2006).

Statistical analysis

The effects of the three treatments on methane production rates, 16S rRNA and *mcrA* gene copy ratios were analyzed by three-factorial analysis of variance using MATLAB (http://www.mathworks.com). Methane production rates and gene copy numbers obtained by QPCR were log transformed prior to analysis. As anoxic boundary, the depth where oxygen level dropped below 1% was used.

5.5 Supplementary material

Molecular characterization of archaeal 16S rRNA, mcrA and KatE genes.

Table S5.3 summarizes the primers and probes used in this study to monitor and quantify the Archaeal population. For reconstruction of phylogenetic trees we used the ARB software package (Ludwig et al., 2004). Analysis and oligonucleotide design of 16S rRNA gene sequences were done using the Silva database (Pruesse et al., 2007), while for analyses of *mcrA* and *KatE* gene sequences specific ARB databases were built. The *mcrA* database was built from 5200 translated nucleic acid sequences and aligned using Promals (Pei and Grishin, 2007). Catalase database was based on 26 translated nucleic acid sequences and aligned in ARB using the Clustal algorithm (Higgins and Sharp, 1988). All sequences were obtained from EMBL (http://www.ebi.ac.uk/embl/) and the databases are available at: http://www.staff.uni-marburg.de/~angel/. Reconstruction of the catalase gene maximum likelihood phylogenetic tree was conducted with RAxML 7.04 using rapid hill climbing algorithm and PROTMIX-JTT evolutionary model (Stamatakis, 2006). The new qPCR probe and PCR primers used in this study were designed using PRIMROSE (Ashelford et al., 2002). Primers for *katE* of *Methanocella* and *Methanosarcina* were designed from the catalase gene sequences of *Methanocella arvoryzae* (formerly RC-I_{MRE50}; CAJ36024) and *Methanosarcina mazei* (AAM32253), respectively. qPCR probe for *mcrA* of *Methanocella* was designed from the environmental sequences obtained in this study as well as from the *mcrA* gene sequence of *Methanocella arvoryzae* (formerly RC-I_{MRE50} ;YP_686530.1).

For sequencing of *mcrA* the primer pair mlas-mod – mcrA-rev was used and for *katE* the primer pairs katRCI and katMsr were used. As templates for both clone libraries we used DNA obtained from FDN and FLO treatments. Libraries were constructed using purified PCR products and pGEM-T Easy cloning kit (Promega). PCR amplifications were done using the following mixture: Each PCR reaction contained 5 µl 10X AccuPrime™ PCR Buffer II (Invitrogen), additional 1.5 mM $MgCl_2$ (to a final concentration of 3 mM), 0.5 µM of each primer (Sigma), 50 µg BSA (Roche), 1 µl of Taq DNA

polymerase (Invitrogen) and 2 µl of template. The following program was used for *katE* amplification: 94°C for 4 min followed by 10 touchdown cycles of 94°C for 1 min, 62°C - 1°C for 1 min and 68°C for 1 min followed by 25 cycles of 94°C for 1 min, 52°C for 1 min and 68°C for 1 min, and a single step of final elongation at 68°C for 10 min. For amplification of the *mcrA* gene the following program was used: 94°C for 4 min followed by 5 touchdown cycles of 94°C for 30 s, 60°C - 1°C for 45 s and 72°C for 30 s followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a single step of final elongation at 72°C for 10 min. Sanger sequencing services were provided by GATC (Germany). The primer M13r targeting flanking regions of the insert was used to sequence a total of 96 clones (48 from each library) of *mcrA* and 48 clones of *katE* (12 from each library and primer pair). Verification for lack of transcription of *pmmo* was done using RT-PCR as described previously (Angel and Conrad, 2009).

Gene and transcript quantification using qPCR

All qPCR reactions were performed on an iCycler thermocycler equipped with a MyiQ™ detection system and the data was analyzed using iQ5 Optical System software (Bio-Rad). Quantification of universal archaeal 16S rRNA gene, *mcrA* and *katE* copy numbers were based on SYBR® Green. Each reaction was 25 µl in volume and contained the following mixture: 12.5 µl SYBR® Green JumpStart™ Taq ReadyMix™, 3 mM $MgCl_2$, 0.8 µg $µl^{-1}$ BSA (Ambion), 0.66 (16S rRNA gene) or 0.5 (other) µM of each primer, and 5 or 2 µl of template (DNA and cDNA resp.). The program used was: 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 66 (16SrRNA gene) or 57°C (other) for 45 s, 72°C for 30 s and 84°C for 10 s for signal reading.
The essays targeting the 16S rRNA and *mcrA* genes of *Methanosarcina* and *Methanocella* were based on dual labelled probes. Each reaction was 25 µl in volume and contained the following mixture: 12.5 µl JumpStart™ Taq ReadyMix™, 4 mM $MgCl_2$, 0.8 µg $µl^{-1}$ BSA (Ambion), 0.5 µM of each primer, 0.2 µM of the dual labelled probe and 5 or 2 µl of template (DNA and cDNA resp.). For the 16S rRNA gene essays, the programme used was: 94°C for 5

min followed by 40 cycles of 94°C for 30 s, 57°C (*mcrA* gene) or 60°C (16S rRNA gene) for 45 s for annealing and 62°C for 30 s for elongation and signal reading. DNA standard for the universal archaeal and *Methanosarcina* 16S rRNA gene essays was prepared from a pure culture of *Mehtanosarcina thermophila* according to Lueders and colleagues (2004), while for *Methanocella* 16S rRNA gene essay it was prepared from a clone containing 16S rRNA gene affiliated with *Methanocella* as a plasmid insert. A standard for the universal *mcrA* essay and the *Methanosarcina mcrA* essays was prepared from a pure culture of *Mehtanosarcina thermophila*. For *Methanocella mcrA* essay, a clone containing an environmental sequence was used as template for a standard. For quantification of cDNA, mRNA transcripts of *mcrA* were synthesized from the clones used for making the DNA standard using Riboprobe® (Promega). Remaining DNA was digested using TURBO™ DNase (Ambion) and the RNA was purified using RNeasy® MinElute® Cleanup Kit (Qiagen). Pure RNA was quantified using Quant-iT™ RiboGreen® (Invitrogen) and cDNA was synthesized using mcrA-rev primer. The cDNAs were used as standards for quantification of *mcrA* transcripts.

Supplementary Table 5.1| Pearson correlation coefficients (r) between various measured parameters*

	mcrA general DNA	*mcrA general cDNA*	*mcrA mcell DNA*	*mcrA mcell cDNA*	*mcrA msar DNA*	*mcrA msar cDNA*	*16S Arc DNA*	*16S MCL DNA*	*16S MSL DNA*	*CH_4 production rate*	*Anoxic boundary (depth)*	*Water content*	*16S MCL / 16S tot*	*16S MSL / 16S tot*
mcrA general DNA	0.96	**												
mcrA mcell DNA	0.93	0.84												
mcrA mcell cDNA	0.71	0.62	0.86											
mcrA msar DNA	0.66	0.68	0.48	0.39										
mcrA msar cDNA	0.50	0.58	0.34	0.20	0.92									
16S Arc DNA	0.94	0.90	0.83	0.66	0.71	0.59								
16S MCL DNA	0.92	0.79	0.89	0.75	0.56	0.30	0.86							
16S MSL DNA	0.93	0.92	0.81	0.69	0.85	0.69	0.95	0.86						
CH_4 production rate	0.95	0.92	0.89	0.69	0.77	0.62	0.86	0.87	0.93					
Anoxic boundary	-0.89	-0.89	-0.85	-0.59	-0.69	-0.64	-0.79	-0.74	-0.83	-0.96				
Water content	0.45	0.26	0.31	0.05	0.27	0.06	0.45	0.65	0.38	0.39	-0.26			
16S MCL/16S tot	0.52	0.31	0.62	0.59	0.13	-0.20	0.33	0.77	0.38	0.51	-0.36	0.62		
16S MSL/16S tot	0.94	0.92	0.82	0.69	0.86	0.69	0.93	0.87	1.00	0.95	-0.86	0.39	0.41	
16S MSL/16S MCL	0.82	0.88	0.67	0.59	0.89	0.81	0.89	0.66	0.95	0.82	-0.75	0.15	0.10	0.93

* Parameters are coded as follows: mcrA - *mcrA* gene copies; 16S - 16S rRNA gene copies; general - total number; mcell or MCL - *Methanocella*; msar or MSL – *Methanosarcina*

** Values in red represent strong correlation ($\geq$ 0.75)

Supplementary Table 5.2| Major fermentation products (μM) in the pore water of the microcosms. Mean ± 1 SE.

*Treatment**	*Malate*	*Succinate*	*Lactate*	*Formate*	*Acetate*	*Propionate*	*Butyrate*
F L N	113.7 ± 16.2	2.7 ± 2.7	8.7 ± 8.7	9.2 ± 9.2	76.9 ± 42.8		83.5 ± 34.5
F L O	60.3 ± 9.2		6.5 ± 6.5		8.9 ± 8.9		67.5 ± 30.5
W L N	157.6						167.0
W L O	131.2 ± 10.0	7.8 ± 6.7	16.4 ± 16.4			5.2 ± 5.2	110.3 ± 101.5
F D N	49.0 ± 5.1	43.6 ± 5.3	4.7 ± 4.7	84.7 ± 52.2	303.8 ± 286.1	685.1 ± 685.1	293.4 ± 293.4
F D O	46.1 ± 5.5	42.8 ± 2.8		18.9 ± 12.9	69.8 ± 19.3		9.2 ± 9.2
W D N	58.7 ± 6.2	28.9 ± 4.0	3.7 ± 3.7		12205.8 ± 1181.3	5168.9 ± 631.8	1975.0 ± 954.5
W D O	59.2 ± 34.2	31.1 ± 7.7	14.3 ± 7.4		49.5 ± 20.56	7.9 ± 4.6	17.9 ± 15.7

* Treatment codes are as follows: flooded – F, drained – D, light – L, dark – d, N_2 atm. – N, 21% O_2 atm. – O.

Supplementary Table 5.3| Primers and Probes used in this study

*Oligo. name**	*Target*	*Oligo. sequence (5'-3')*	*Position***	*GC (%)*	*Tm†*	*Amplicon size*	*Essay*	*Reference*
ARC364 - F	Archaea 16S rRNA gene	CGGGGYGCASCAGGGGCGAA	364-383	75-80	75	553	QPCR	Burggraf et al. 1997
ARCH 934 - R		GTGCTCCCCCGCCAATTCCT	915-934	65	71			Grosskopf et al. 1998
MSL812 - F	Methanosarcinales 16S rRNA gene	GTAAACGATRYTCGCTAGGT	812-831	40-50	62	354	QPCR	Yu et al. 2005
MSL860 - P		AGGGAAGCCGTGAAGCGARCC	860-880	62-67	70			
MSL1159 - R		GGTCCCCACAGWGTACC	1143-1159	65	63			
MCL282 - F	*Methanocella*/ RC-I 16S rRNA gene	ATCMGTACGGGTTGTGGG	282-299	56-61	65	510	QPCR	**Chapter 4**
MCL609 - P		ATCCAGCGGCTTAACCGTTGGKCK	609-632	54-63	72			
MCL832 - R		CACCTAGCGRGCATCGTTTAC	813-832	52-57	64			
mlas-mod - F	Universal *mcrA* gene	GGYGGTGTMGGDTTCACMCARTA	976-998	43-65	68	469	PCR/ QPCR	Modified from: Steinberg and Regan 2009
mcrA-rev - R		CGTTCATBGCGTAGTTVGGRTAGT	1421-1444	42-54	66			Steinberg and Regan 2009
msar - P	*Methanosarcina mcrA* gene	TCTCTCWGGCTGGTAYCTCTCCAT GTAC	1269-1296	50-54	68		QPCR	Steinberg and Regan 2009
mcel/rc-I - P	*Methanocella mcrA* gene	CTVGGMTTCTWCGGBTACGACYT	1269-1296	43-61	66		QPCR	This study

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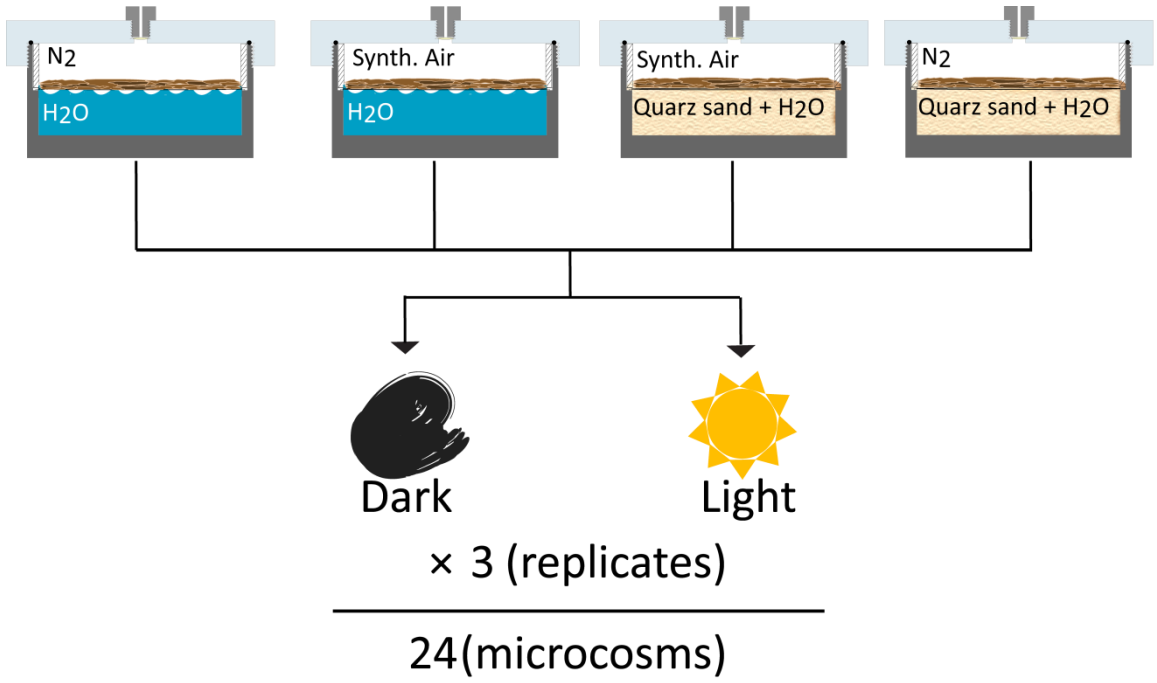
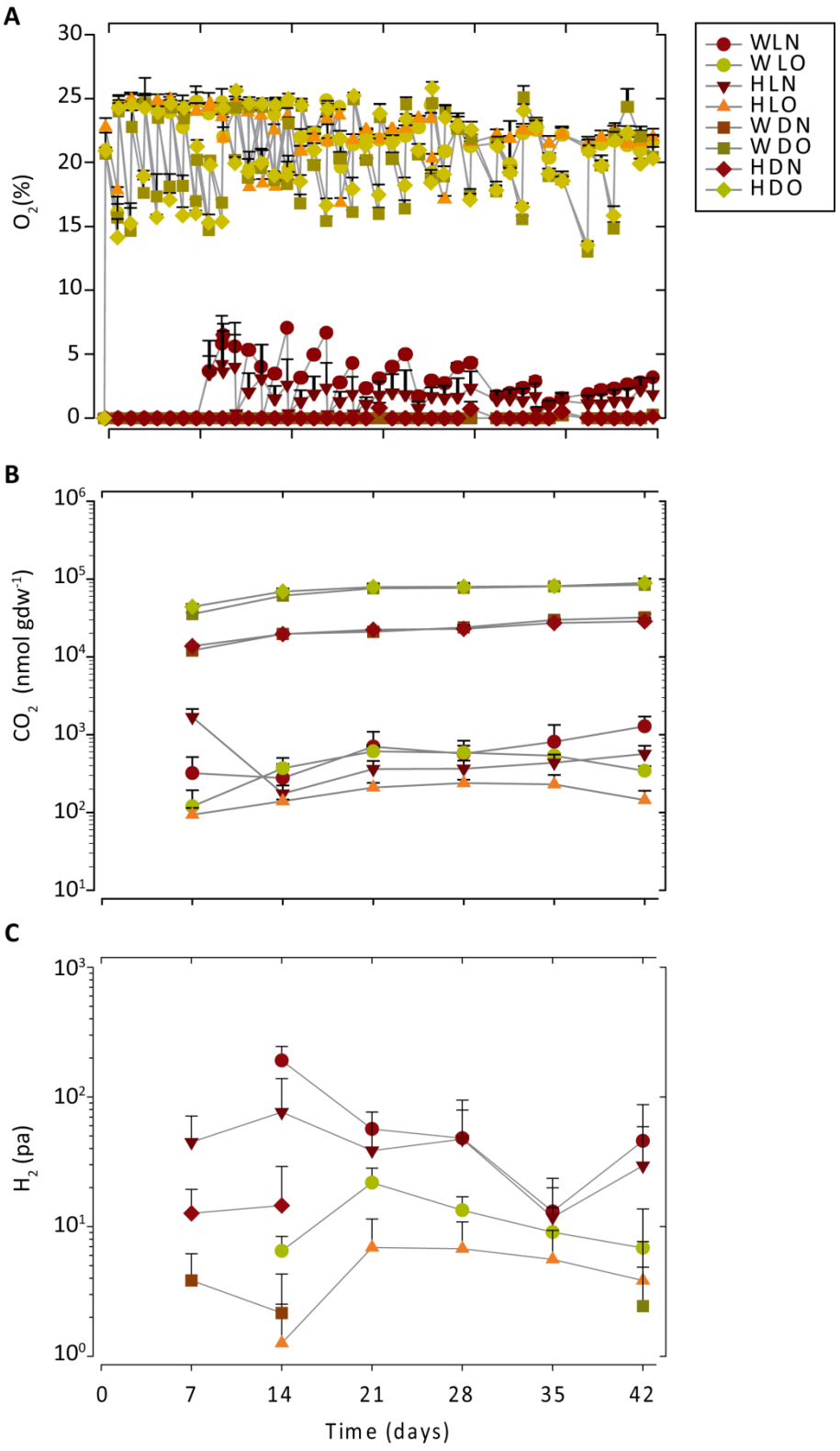
Supplementary Table 5.3| Continued

*Oligo. name**	*Target*	*Oligo. sequence (5'-3')*	*Position***	*GC (%)*	*Tm†*	*Amplicon size*	*Essay*	*Reference*
katRCI - F	*Methanocella arvoryzae KatE* gene	AAGCCACACACATAGACGTC	1321-1343	50	63	586	PCR/ QPCR	This study
katRCI - R		CATGATCATGTGGGCGTTCT	40-626	50	64			
katMsr - F	*Methanosarcina mazei KatE* gene	CAATTAGGTGAGCCGCTTCC	20-39	55	64	605	PCR/ QPCR	This study
katMsr - R		CCGCGGTCACCCATAATAAT	605-624	50	63			

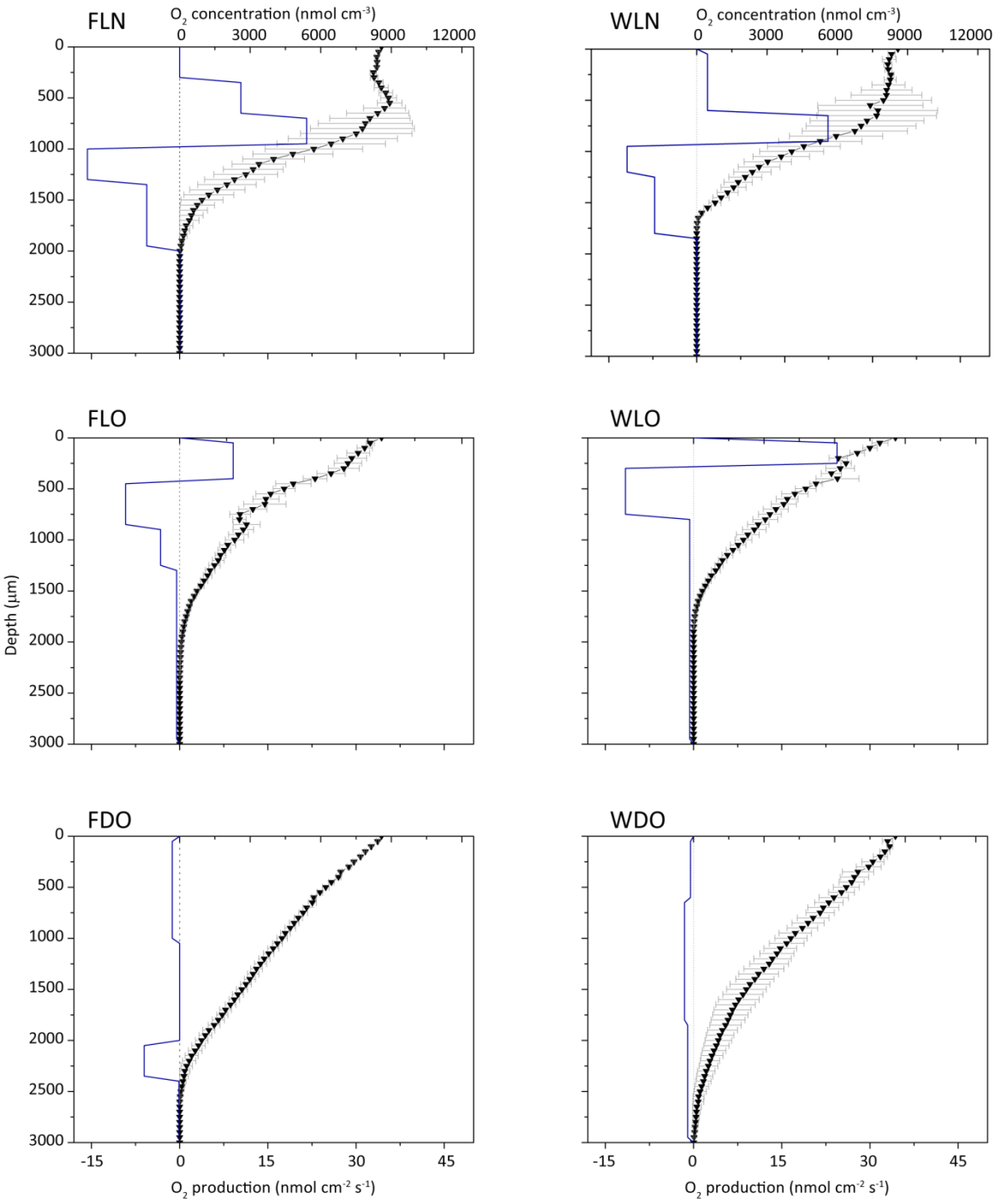
* The following primer name suffixes are used: - F – forward primer, - R – reverse primer, - P – dual labelled probe.
** Position is based on the following: primers targeting the 16S rRNA gene - *E.coli*; primers targeting the mcrA gene - *Methanothermobacter thermautotrophicus* mcrA gene accession number: AAA73445 (following Steinberg and Regan, 2009); KatRCI primers - *Methanocella* putative catalase gene (*KatE*), accession number: CAJ36024; KatMsl primers - *Methanosarcina mazei KatE* gene, accession number: AAM32253.
† Calculated using Nearest Neighbor method with OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) under the conditions described for each PCR/QPCR reaction in the Materials and Methods section



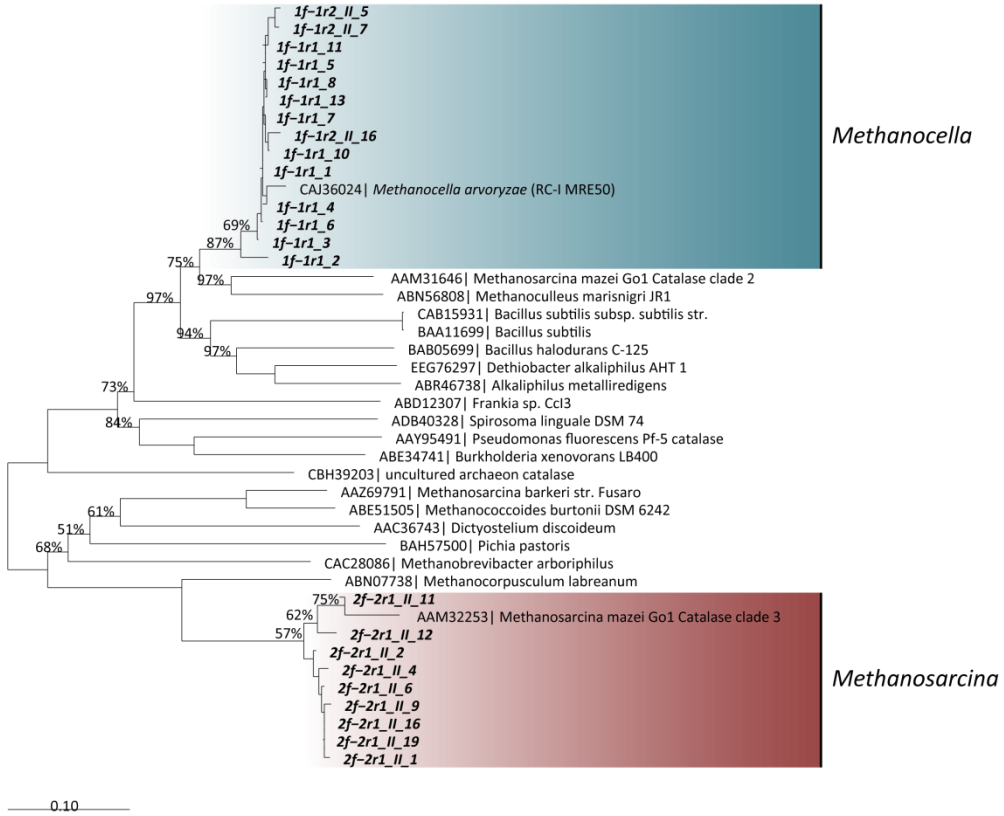
Supplementary Figure 5.1| A scheme depicting the different incubation conditions used in this experiment. The bottom compartment contained either water or drained wet sand. Biological soil crust samples were placed on top of a membrane allowing a flow of nutrients and water but not of cells. The headspace was flushed with either N_2 or with synthetic air (21% O_2/ 79% N_2). Microcosms were incubated either in the dark or under full light, in all possible combinations, in triplicates for 42 days.



Supplementary Figure 5.2| Evolution of: a. O_2, b. CO_2, c. H_2 in the microcosm headspaces during the incubation period: mean ± 1 SE; n = 3. Treatment codes are as follows: flooded – F, wet-drained – W, light – L, dark – D, N_2 atm. – N, 21% O_2 atm. – O.



Supplementary Figure 5.3| Oxygen profiles in the microcosms. Only oxic and oxygen producing treatments are shown. Black triangles represent concentration measurements: mean ± 1 SE; n = 3. Blue lines represent O_2 production zones modeled using Profile V1.0 (Berg et al., 1998). Treatment codes are as follows: flooded – F, wet-drained – W, light – L, dark – D, N_2 atm. – N, 21% O_2 atm. – O.



Supplementary Figure 5.4| Maximum likelihood phylogenetic tree based on aligned partial amino acid sequences of the catalase E gene (*katE*). Sequences were obtained using katRCI and katMsr primer pairs targeting the *katE* of *Methanocella* and *Methanosarcina,* respectively. Amino acid composition was deduced from DNA sequences and aligned against an ARB database of catalase sequences. The tree was calculated with RAxML 7.04 using rapid hill climbing algorithm and PROTMIX - JTT evolutionary model. Bootstrap values above 50% (out of a 100 trials) are displayed next to the nodes.

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Chapter 6 |

General Discussion and outlook

Deserts comprise a third of the Earth land surface but are nevertheless far less understood as ecosystems compared to other, more humid, regions. From a microbial ecology perspective there are indications that desert soil communities are unique, and their habitats are hence a source of untapped biodiversity (Garcia-Pichel, 2002). While there have been former indications for the involvement of desert soils in the turnover of the greenhouse gas methane to the best of my knowledge no one had previously studied the subject in depth.

This work explored the microbial diversity and the functional activity of microorganisms in desert soils. In particular, I was interested in detecting the consumption of atmospheric methane and the potential for production of that gas, which is one of the more potent greenhouse gases. To answer these questions I used a combination of field study techniques (measuring fluxes and sampling statistically representative set of samples) and lab experiments (which combined incubations and *in situ* analyses). I made use of an array of molecular techniques along with analyses of the isotopic signature of the carbon to detect and quantify the microbes involved in methane turnover and to decipher which of them is active and what pathways they use.

First, we were able to show that general community profiles of both *Bacteria* and *Archaea* in arid and semiarid soils are different from those in more humid regions forming three distinct clusters rather than a continuum along a precipitation gradient (**Chapter 2**). Our finding thus supported the hypothesis that desert soils, because of their distinctive features, harbour a qualitatively different microbial community. In the following part (**Chapter 3**) we demonstrated the ability of desert soils to consume atmospheric methane thus confirming past observations of Striegel and colleagues (1992). We

showed, however, that the hyper-arid site that was studied, in contrast to the arid site, did not consume atmospheric methane and did not harbour active methanotrophs. In addition, we have detected transcription of the particulate methane monooxygenase gene. The sequences of these transcribed monooxygenases were associated with putative high affinity methanotrophs.

In the third part of the work (**Chapters 4 and 5**) we studied the methanogenic potential and the archaeal diversity in upland soils. We showed that methanogenic potential is a global trait of upland soils and that two types of methanogens – *Methanosarcina* and *Methanocella* – are, apparently, universal upland soil methanogens. Biological soil crust (BSC) samples from the Negev Desert in Israel were shown to produce methane even in the presence of oxygen. The methanogenic community in these samples continuously expressed the gene for catalase, which apparently enables them to detoxify oxygen and survive under these conditions.

6.1 Methane cycle in desert soils – a proposed model

If both methane production and consumption occur in upland soils, in what way are they different from wetland soils? **Figure 6.1** illustrates the similarities and differences between two model soil systems: an arid soil such as the one studied at Negev Desert, Israel, and a simplified rice plant/rice field soil system which is the most established model for studying biogeochemical processes in wetland soils. When the soils are drained and atmospheric gases can diffuse no methane is produced in both soil types (**Figure 6.1 A**). This is the common state in desert soils while in rice fields such conditions occur only when the soil is drained and left to dry between crops. Under these conditions, the arid soil is a sink for atmospheric methane and consumption rate is at least partly dependent on water content. The rice field is also inhabited by methanotrophs but apparently, with the exception of upland rice fields (Singh et al., 1998), it is unable to consume methane at trace atmospheric levels and it therefore rarely acts as a net sink for atmospheric methane. The active layer for methane consumption in the arid soil is approximately within the upper 20-cm soil layer; below this depth methane concentration stabilizes at approximately 1

ppmv. Interestingly, the BSC is apparently devoid of methanotrophs and consequently does not consume atmospheric methane. This stands in contrast to the general notion that the BSC is the most biologically active layer of the soil. Indeed, other layers of desert soils have hardly been examined by microbiologists. It is virtually impossible to conclusively determine that something is absent and also to reason why it is absent, if only for epistemological reasons. Nevertheless, the lack, or at least the scarcity, of methanotrophs in the upper layer of upland soils has been previously reported (Henckel et al., 2000; Kolb et al., 2005). Higher ammonia levels in the upper soil layers which are known to inhibit methanotrophs have been proposed as an explanation for that, but considering the relatively low nitrogen levels in desert soils this could hardly serve as an argument in our case. Unlike desert soils, the upper layer of rice paddies is where methanotrophs preferentially reside since it is where the oxic/anoxic interface is located (Conrad and Frenzel, 2002).

The particulate methane monooxygenase (*pmoA*) sequences that were retrieved in the arid soil were always affiliated with the high affinity methanotrophs of the types USCγ and JR3, but 'classical', low affinity methanotrophs could not be detected. This is noteworthy as this is so far the only study on soil methanotrophs where no low affinity methanotrophs could be detected (Lüke, 2010). In rice fields, the diversity of methanotrophs is extensive and includes a large variety of type I and II methanotrophs, most of which are represented in culture collections (Lüke et al., 2010). Methanotrophs in rice fields are known to heavily colonise the root surfaces thus utilizing the oxygen which diffuses out of the plant – a logical strategy for aerobes living in anoxic soil. We detected no significant difference between methane oxidation rates of the soil under shrubs and in the inter-shrub patch, although the presence of methanotrophs on the root surfaces was not directly examined.

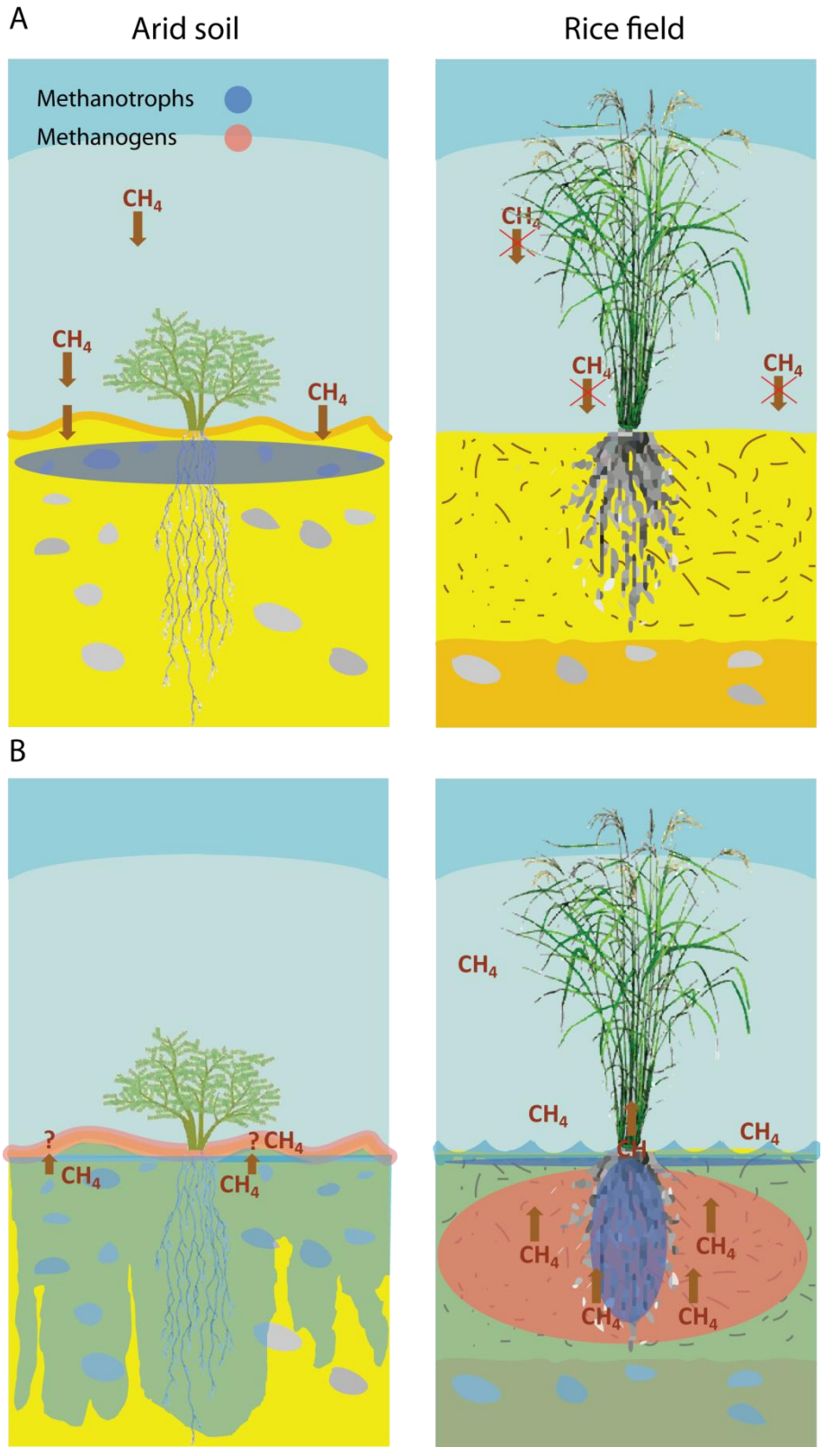

Figure 6.1| Dynamics of methane oxidation and production in a model arid soil and a rice field. A. drained soils. B. after wetting.

High affinity methanotrophs are also apparently very sensitive to land use changes and lower methane oxidation rates in agricultural fields compared with pristine sites have been repeatedly reported (e.g. Jensen and Olsen, 1998; Knief et al., 2005). Once again, high ammonia concentrations in the soil or the use of agrochemicals have been proposed as an explanation (Dunfield, 2007). Our results however, particularly the soil gas profile, suggest that the reason for that might be the mechanical disturbance caused by ploughing. Ploughing physically mixes soil layers and dislocates cells away from their preferred site of activity. Because of their low growth rates (an inevitable outcome of their limited supply of substrate) activity is only slowly recovered. In rice fields, soils are constantly mixed by ploughing, yet no effect on the activity of methanotrophs has been reported.

Several distinct clusters of 'high-affinity' methanotrophs have been detected in upland soils; among them are USCα, USCγ, RA21, MR1, JR2 and JR3, to name just a few. The occurrence of different clusters in different soils raises the question of what, if any, are the ecological forces driving their distribution. It has already been postulated in the past that, for instance, USCα (and *Methylocystis* species) are adapted to soils of low pH while USCγ and Cluster1 are adapted to soils of high pH (Knief et al., 2003; Kolb, 2009). Similarly, according to Horz and colleagues (2005), clusters JR1 and JR2 seem to be dominant among upland soil methanotrophs in a semi-arid site in California while other clusters (such as JR3) seem to be minor members. In our study, JR3 was the dominant type while the other clusters (JR1, JR2) were not detected. While so far not tested explicitly, a distribution of upland soil methanotroph clusters according to biogeographical parameters such as precipitation and soil type thus seems plausible.

When the arid soil becomes wet, usually after heavy rain, methane uptake ceases due to diffusion limitations (**Figure 6.1 B**). Our lab incubations have shown that the lower layer of the BSC becomes anoxic while the upper layer remains oxic due to atmospheric oxygen diffusion or photosynthesis by BSC primary producers. Anaerobic degradation processes commence at the bottom layer of the BSC and methane is detected within a week or so. In contrast to our observations regarding methanotrophs, methanogens are probably only present in the BSC since the deeper layers

(0-20 cm belowground) had little or no methanogenic potential. In rice field soil, the entire bulk of the soil and the root surfaces become methanogenic shortly after flooding when oxygen and alternative electron acceptors such as nitrate, ferric iron and sulphate are depleted. Rice fields normally possess a diverse community of methanogens including species of *Methanosarcina*, *Methanosaeta*, *Methanocella*, *Methanobrevibacter*, *Methanobacterium* and others (Grosskopf et al., 1998; Watanabe et al., 2006). A niche differentiation is recognized in the rice system: the bulk soil is dominated by *Methanosarcina* and *Methanosaeta* species and most methane which originates from this part of the soil is derived from acetate. The root surfaces, on the other hand, are primarily dominated by methanogens of the genus *Methanocella* and as a result most methane produced in this niche is H_2/CO_2 based (Lu and Conrad, 2005). The community structure in the BSC is far more limited. In fact, throughout the various upland soils (and layers) that were examined only *Methanocella* and *Methanosarcina* related sequences were found as active members of the methanogenic community. When the soils were incubated as anoxic slurries to detect methanogenic potential, acetoclastic methanogenesis was the dominant pathway for generating methane and consequently *Methanosarcina* outnumbered *Methanocella*. Under oxic conditions, however, it was found that methanogenesis, which was much less active, was almost entirely hydrogenotrophic, acetate was probably consumed by aerobic heterotrophs, and *Methanocella* often outnumbered *Methanosarcina*. Based on this these observations we hypothesize that a niche differentiation might exist between the two dominant methanogens. According to it, oxygen level in the soil determines the level of activity of each methanogen and the specific contribution of each pathway to the total methane being produced. When the BSC is mostly anoxic, acetate accumulates and *Methanosarcina* become dominant by converting it to methane. This can occur for instance when the soil water content and the overall metabolic activity in the BSC are high, but possibly also during night time when all photosynthetic microorganisms switch to net oxygen consumption. When soil oxygen level is higher, *Methanocella* become dominant and with it hydrogenotrophic methanogenesis becomes the primary pathway for methane production. This

can occur either because of competition with heterotrophs over acetate as proposed, but an alternative possibility is that *Methanocella* are better equipped for coping with oxygen as has been proposed before (Erkel et al., 2006; Conrad et al., 2006)

Methane which is produced in rice paddies quickly diffuses upwards. As discussed in **Chapter 1**, much of it is consumed by methanotrophs which colonise the rice roots and the top layer of the soil at the oxic/anoxic interface. This acts as an attenuating mechanism which decreases potential methane emission from rice fields (Conrad and Rothfuss, 1991). Methane in the arid soil is already produced at the topmost layer of the soil and the diffusion route to the atmosphere is short. Since this top layer – the BSC – apparently lacks methanotrophs, no attenuation can occur in this case. While field studies are still required to confirm methanogenesis in BSCs, it appears that methane which is produced in the BSC is released into the atmosphere in its entirety.

Quantifications of methanotrophs and methanogens in rice paddies using qPCR suggest that their cell densities range from 10^4 to 10^7 and 10^6 to 10^7 for methanotrophs and methanogens, respectively (Conrad and Frenzel, 2002). These numbers seem to be fairly stable throughout the growth season and even afterwards when the soil is dry (Ueki et al., 1997; Watanabe et al., 2007). In the arid soil, numbers of methanogens were estimated at 10^4 cells gdw^{-1} before incubations and rising up to 10^9 cells gdw^{-1} after incubations. While the cell density of methanotrophs was not directly quantified in this work, former studies estimate their numbers to be around 10^4-10^6 cells gdw^{-1} in upland soils (Kolb et al., 2005; Knief et al., 2006). Assuming oxidation rates of atmospheric methane are primarily a factor of cell density, we can estimate that the numbers of methanotrophs in the arid site which was studied are similar and probably somewhat lower to those mentioned above.

6.2 Summary and outlook

The analysis of the archaeal and bacterial communities in desert soils showed that they consist of different microbes compared to soils in more humid regions. I have shown that at least pristine arid soils have the capacity to consume atmospheric methane and that methanotrophs

belonging to a unique cluster are probably the agents performing this activity. The fact that a unique cluster of methanotrophs was found in the studied site as well as the patterns which stem from other works suggest that high affinity methanotrophs might correspond to biogeographic patters. Studying these patterns systematically and correlating them to methane oxidation rates and dynamics could enhance our understanding of the soil methane sink on a global scale.

The methanogens in arid soils are apparently not unique. *Methanosarcina* and *Methanocella* seem to be universal species, but this should not end the study on upland soil methanogenesis. The occurrence of methanogenesis in the BSC under oxic conditions can testify for the existence of an entire anaerobic biome. This formerly unrecognized side of the ecology of BSC opens paths for much further research. Further work is therefore needed to elucidate which are the players involved in the entire anaerobic degradation cascade which takes place in an active BSC. One possible approach to answer this question is metatranscriptomics. Recent advances in high throughput sequencing and microarray technology enable the simultaneous analysis of all mRNAs transcribed in an environmental sample (Urich et al., 2008; Yin et al., 2010). Using these approaches it might be possible to reconstruct much of the biochemical networks which govern the degradation process in a BSC which precede methanogenesis.

In summary, Methanotrophs and Methanogens are present and active in desert soils. With an estimated total microbial population size of 10^7-10^8 cells gdw^{-1} for the arid site at the Negev Desert (Bachar et al., 2010), methanotrophs and methanogens comprise only 0.01% of the total microbial population and they are therefore part of the 'rare biosphere' as defined by Pedros-Alio (2007). Still, when active, these microbes play important roles in their ecosystems and are able to influence atmospheric methane concentrations on a global level.

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List of abbreviations

16S-rRNA	small subunit of the ribosomal RNA
6-FAM	6-Carboxyfluoresceine
bp	base pairs
BSA	Bovine serum albumin
BSC	Biological soil crust
EDTA	Ethylenediaminetetraacetic acid
f_{H2}	Fraction of methane produced from H_2/CO_2
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
IC	Ion chromatography
IRMS	Isotope ratio mass spectrometer
katE	Catalase E
LTER	Long term ecological research
mcrA	Methane Co-M reductase α subunit
OTU	Operational taxonomic unit
PCA	Principal component analysis
pmoA	Particulate methane monooxygenase A subunit
qPCR	Quantitative PCR
RDA	Redundancy analysis
SCG	Soil crenarchaeotic group
SPB	Sodium phosphate buffer
TNS	Tris, HCl, SDS buffer
TRF	Terminal restriction fragment
TRFLP	Terminal restriction fragment length polymorphism
$\delta^{13}C$	Stable carbon isotope ratio relative to the international standard
δac	$\delta^{13}C$ of acetate
δmc	$\delta^{13}C$ of methane which originated from CO_2
ε und α	Isotope fractionation factors

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Curriculum Vitae

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EDUCATION

2007 - present:	**Doctoral studies** at the International Max Planck Research School for Environmental, Cellular and Molecular Microbiology; Max-Planck-Institute for Terrestrial Microbiology, Germany. **Supervisor: Prof. Dr. Ralf Conrad.**
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2007:	Fulbright Doctoral Fellowship (I declined).
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PROFESSIONAL EXPERIENCE

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List of publications and contribution to conferences

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Bachar, A, Al-Ashhab, A, Soares, MIM, Sklarz, MY, Angel, R, Ungar, ED, Gillor, O (2010) *Soil Microbial Abundance and Diversity Along a Low Precipitation Gradient*. Microbial Ecololgy **60**: 453-461

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International Society for Microbial Ecology 13th (2010)
Angel R , Sklartz M, Soares M, Conrad R, Gillor, O. *Biogeography of Active and Dormant Soil Microbial Communities Along a Steep Precipitation Gradient*

BAGECO 10 - Bacterial Genetics and Ecology (2009) Angel R and Conrad R. *in-situ Measurements of Methane Fluxes and Analysis of Transcribed Particulate Methane Monooxygenase in Desert Soils.*

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Angel R, Nagouker N, and Druschel G. *Community Analysis of Bacteria Using T-RFLP Technique across the Redox Gradient in the Sediment at St. Albans Bay, Lake Champlain VT, USA.*

Pledge

I certify that the present thesis entitled:

“Methane Turnover in Desert Soils”

was carried out without any unlawful means; no literature resources, reagents and technical devices were used other than those stated. This work has never been submitted before in this or similar format to any other university and has not been used before any examination.

Marburg, October 2010

Roey Angel

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