Molecular Ecology of Methanotrophs in a Landfill cover soil

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Abbreviations

ANME	Anaerobic methanotrophs
ANMS	Ammonium nitrate mineral salts
bp	Base pairs
cDNA	Complementary DNA
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetraacetic acid
FISH	Fluorescent *in situ* hybridisation
GC	Gas chromatography
MDA	Multiple displacement amplification
MPN	Most probable number
mRNA	Messenger RNA
MS	Mass spectrometry
NMS	Nitrate mineral salts
OTU	Operational taxonomic unit
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
pMMO	Particulate methane monooxygenase
RDP	Ribosomal database project
RFLP	Restriction fragment length polymorphism
RuMP	Ribulose monophosphate
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SIP	Stable isotope probing
sMMO	Soluble methane monooxygenase
sp.	Species
spp.	Species (pl.)
TCA	Tricarboxylic acid
TE	Tris EDTA
T-RFLP	Terminal restriction fragment length polymorphism
Tris	Tris (hydroxymethyl) methylamine
µ	Micro

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Declaration

I declare that the work presented in this thesis was conducted by me under direct supervision of Professor J. Colin Murrell, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the work presented here has been previously submitted for any other degree. Some of the thesis and data from Chapters 3 and 6 have been published:

Kumaresan D, Abell G, Bodrossy L, Stralis-Pavese N and Murrell JC (2009). Spatial and temporal methanotroph diversity in a landfill cover soil are differentially related to soil abiotic factors. *Environ Microbiol Reports*, 10.1111/j.1758-2229.2009.00059.x

Hery M, Singer AC, **Kumaresan D**, Bodrossy, L, Stralis-Pavese N, Prosser JI, Thompson I, Murrell JC (2008). Effect of earthworms on community structure of active methanotrophs population in a landfill cover soil. *ISME J* 2:92–104.

Abstract

Landfills are a major anthropogenic source of methane and understanding the factors influencing the activity and diversity of methane oxidizing bacteria (methanotrophs) in landfill cover soil is critical to devise better landfill cover soil management strategies. A detailed study was carried out to investigate the effect of earthworms on soil methane oxidation potential and community structure of active methanotrophs in a landfill cover soil. Earthworms were found increase soil methane oxidation potential by 15% ± 7%. However, no substantial shifts in the community structure of active methanotrophs were observed. A *Bacteroidetes*-related bacterium was identified only in active bacterial community of earthworm-incubated landfill cover soil. However, its role in methane cycling is uncertain. In a subsequent study, a larger experimental system was used to simulate *in situ* landfill conditions and also to mimic the *in situ* environmental heterogeneity. A mRNA-based microarray analysis revealed that earthworm activity in landfill cover soil stimulates activity and diversity of Type I methanotrophs compared to Type II methanotrophs.

Understanding spatio-temporal distribution pattern of microorganisms and the factors influencing their distribution pattern are integral for a better understanding of microbial functions in ecosystems. A *pmoA*-based microarray analysis of methanotroph community structure in a landfill cover soil revealed a temporal shift in methanotroph populations across different seasons. In the case of spatial distribution, only minor differences in methanotroph community structure were observed with no recognizable patterns. Correlation analysis between soil abiotic parameters (total C, N, NH_4^+, NO_3^- and water content) and distribution of methanotrophs revealed a lack of conclusive evidence for any distinct correlation pattern between measured abiotic parameters and methanotroph community structure, suggesting that complex interactions of several physic-chemical parameters shape methanotroph diversity and activity in landfill cover soils.

A study was designed to investigate the shift in functional diversity of methanotrophs when microniches created by soil aggregates are physically altered. mRNA-based analysis of the bacterial transcription activity revealed an effect of physical disruption on active methanotrophs. The result emphasized that a change in a particular microbial niche need not be accompanied by an immediate change to the bacterial functional diversity and it depends on the ability of the bacterial communities to respond to the perturbation and perform the ecosystem function.

DNA-SIP and mRNA based microarray techniques were compared for the assessment of active methanotroph community structure. Results from this study indicated that assessment of active methanotroph community structure by both the techniques were congruent. This suggested that the mRNA based microarray technique could be used to study active methanotroph community structure in situations where SIP experiments are not practical. However, both DNA-SIP and mRNA-microarray have their advantages and limitations and the selection of appropriate technique to assess active community structure depends on the nature of the study.

Chapter 1

Introduction

1. Introduction

1.1. Methane and its role as a greenhouse gas

Methane (CH_4) is next only to carbon dioxide (CO_2) in importance as a greenhouse gas. It has an atmospheric lifetime of 12 years (approximately) and its atmospheric concentration has tripled since pre-industrial times (IPCC., 2001). The average atmospheric concentration of methane is increasing annually at a rate of 1% due to anthropogenic and natural emissions (Blake and Rowland, 1988). Though in terms of absolute quantities (CH_4 emitted is lower than CO_2 emissions) it is far more effective in absorbing infrared radiation and has a global warming potential of about 23 times that of carbon dioxide. Lelieveld *et al*. (1993) predicted that increase in atmospheric methane concentration will decrease hydroxyl (OH) radical concentrations and thus increase the lifetime of methane in the atmosphere. However, a decrease in the rate of methane emissions would have a positive feedback on the decrease of methane in the atmosphere as a result of the increase in the atmospheric concentrations of OH radicals.

1.1.1 Sources and sinks of methane

Methane emissions into the atmosphere are contributed by both natural and anthropogenic sources. Frankenberg *et al*.(2005) estimated that about 50% of the total atmospheric methane is from anthropogenic sources, such as fossil fuel production, landfill sites, animal husbandry, rice cultivation and biomass burning. Natural sources of methane include wetlands, gas hydrates, permafrost, termites, oceans and freshwater bodies. Wetlands are the major natural source of methane contributing about 110 Tg CH_4 yr^{-1} (1Tg = 10^{12}g) (Wahlen, 1993). Emissions from these sources can considerably vary in space and time (Frankenberg *et al.*, 2005). It

was believed that anaerobic production by methanogeneic archaea was the only biological source of methane. Interestingly, Keppler and colleagues (2006) using stable carbon isotopes reported that methane is readily formed *in situ* in terrestrial plants under oxic conditions. The authors estimated a contribution of 62-236 Tg yr^{-1} of methane and if it is the case, the global methane budget should be revised. The mechanism of methane generation in plants is still unknown. The notion of plants as a source of methane has been recently challenged by Nisbet and co-workers (Nisbet *et al.*, 2009). The authors argued that plants do not contain a known biochemical pathway to produce methane and suggested that under high UV stress conditions there may be spontaneous breakdown of plant material, which could release methane. Moreover, plants could take up and release water containing dissolved methane through transpiration. Further studies are required to understand the contribution of terrestrial plants to the global methane budget.

Oxidation by OH radicals in the troposphere can convert methane into CO_2 and H_2O and can account for 85% of the global removal of methane (Wahlen, 1993). Biological oxidation of methane significantly contributes to the removal of methane from the environment. Soils are the major biological sink as a result of microbial methane oxidation and are estimated to remove 10-15 Tg of methane, annually (IPCC., 2001). Natural forests and upland soils are most active in the oxidation of atmospheric methane (Holmes *et al.*, 1999; Bull *et al.*, 2000; Knief *et al.*, 2003; Kolb *et al.*, 2005). Methane oxidation has also been reported in extreme environments such as deserts (Striegl *et al.*, 1992), tundra soils (Whalen and Reeburgh, 1990) and the surface water of the oceans (Conrad and Seiler, 1988).

1.1.2 Landfill sites – Role in methane global budget

Landfills can serve both as a source and sink of methane. Landfill sites generate landfill gas, due to the anaerobic decomposition of organic materials (wastes). Landfill gas consists of approximately 55-60% (v/v) methane and 40-45% (v/v) carbon dioxide (**Figure 1.1**). In addition to methane and carbon dioxide, landfill gas can also contain high number of different volatile organic components (VOCs), including C1-C5 hydrocarbons, sulphur compounds, halogenated and aromatic hydrocarbons (Rettenberger and Stegmann, 1996; Allen *et al.*, 1997). These trace components originate either from hazardous materials deposited in the landfill or from biological/chemical degradation of materials disposed in the landfill. Landfills are estimated to release between 9 and 70 Tg of CH_4 yr^{-1} into the atmosphere (Lelieveld *et al.*, 1998). Engineering solutions such as landfill gas extraction systems have been designed in new landfill sites to collect and destroy or recover energy. However, in old landfill sites, with landfill cover soils, microbial methane oxidation in the aerobic portions of a landfill cover soil limits methane emissions to the atmosphere (Oremland and Culbertson, 1992; Lelieveld *et al.*, 1998). In landfill cover soils, methane and oxygen counter gradients may appear due to emission of methane from the deep layers and diffusion of oxygen from atmosphere. Amounts of methane oxidized in landfill soils are estimated in the range of 10 to 50%, based on laboratory studies (Whalen *et al.*, 1990; De Visscher *et al.*, 1999) and up to 100% in field based studies (Christophersen *et al.*, 2001). Methane oxidation in landfill cover soil and the factors influencing methane oxidation have been the subject of several studies (Whalen *et al.*, 1990; Nozhevnikova *et al.*, 1993; Bogner *et al.*, 1995; Kightley *et al.*, 1995; Boeckx *et al.*, 1996; Borjesson *et al.*, 1998; De Visscher *et al.*, 1999; Borjesson *et al.*, 2004).

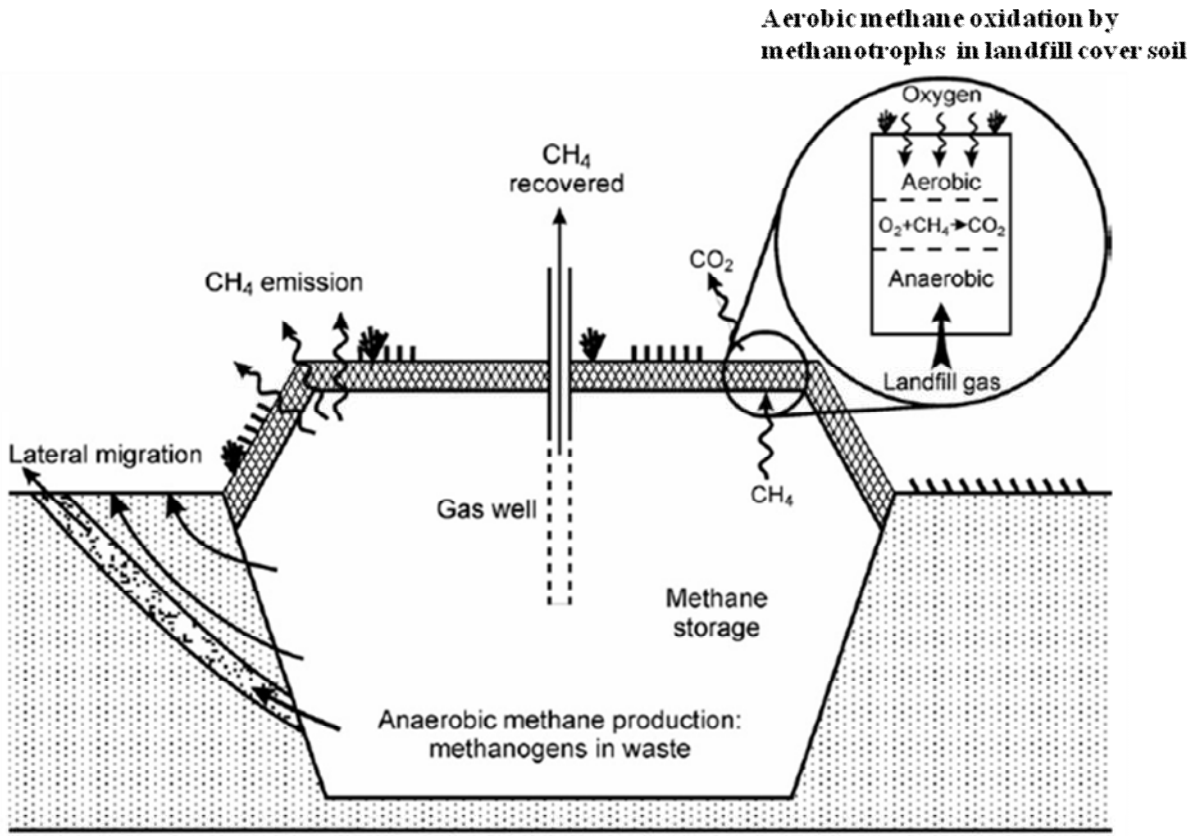

Figure 1.1 Methane cycling in landfill. Taken from Scheutz *et al.*(2009).

1.2 Microbial role in methane cycling

Methanogens, from the domain Archaea (Woese *et al.*, 1990), are the key organisms responsible for methane production in a wide range of ecosystems ranging from the human gut to deep high-temperature (>100 $^{\circ}$C) and high-pressure hydrothermal vents. They are obligate anaerobes with restricted nutrition and are mostly associated with members of anaerobic consortia (Raskin *et al.*, 1994). Methanogenesis occurs under strictly anaerobic conditions, such as in deep layers of landfill sites, and it mainly uses the substrates acetate, CO_2 and H_2. The type of substrate utilized depends on the genera of the methanogens present, with approximately 70 % of the methane produced being derived from acetate and approximately 30% from CO_2 and H_2 (Conrad and Klose, 1999).

Microbial methane oxidation can occur in both aerobic and anaerobic conditions. Anaerobic methane oxidation has been detected in marine sediments, particularly in methane seeps and vents and in anoxic waters. Microorganisms carrying out the anaerobic methane oxidation have not yet been cultivated and only Archaea are found to be involved in this process. Studies have reported coupling of anaerobic methane oxidation with sulphate reduction (Hinrichs *et al.*, 1999) and denitrification (Raghoebarsing *et al.*, 2006). Microorganisms involved in this process have been identified as ANME-1 Archaea, distantly related to *Methanosarcinales* and *Methanomicrobiales* (Hinrichs et al., 1999), ANME-2 (Boetius *et al.*, 2000) and ANME-3 (Knittel *et al.*, 2005).

Microorganisms performing aerobic methane oxidation include methanotrophs, belonging to α, γ *Proteobacteria, Verrucomicrobia,* and also the ammonia oxidizers (which can perform co-oxidation of methane) (Hanson and

Hanson, 1996). Whilst studies have demonstrated that ammonia oxidizers such as *Nitrosococcus* (Jones and Morita, 1983) and *Nitrospira* (Jiang and Bakken, 1999) can oxidize methane, their contribution to methane oxidation in the environment appears to be irrelevant (Jiang and Bakken, 1999). A significant proportion of the methane produced by methanogens is oxidized by methanotrophs in the environment. There are two types of methanotrophs based on their affinity for methane; high affinity methanotrophs that can oxidize methane at atmospheric methane concentrations (~1.7 ppmv) and low affinity methanotrophs (Bender and Conrad, 1992). Bender and Conrad (1992) reported the role of high affinity methanotrophs that were adapted to low atmosphere mixing ratios of methane. However, methanotrophs utilizing atmospheric methane have not been isolated yet and information on these methanotrophs is based on the use of biomarkers such as phospholipid fatty acid (PLFA) and *pmoA* (encoding a key subunit of particulate methane monooxygenase, pMMO). Using these biomarkers, studies have indicated that both α and γ *Proteobacteria* are involved in the atmospheric methane oxidation process in a wide range of environments (Holmes *et al.*, 1999; Bull *et al.*, 2000; Knief *et al.*, 2003; Kolb *et al.*, 2005). Low affinity methanotrophs have been well studied and a number of strains have been isolated in pure cultures.

1.3 Classification of methanotrophs

Methanotrophs are classified into either Type I and Type II methanotrophs on the basis of their carbon assimilation pathways, cell morphology, GC content of their DNA, intracytoplasmic membrane arrangements, nitrogen fixing capability and resting stages (reviewed in Trotsenko and Murrell, 2008) (**Figure 1.3**). Type I methanotrophs belonging to the γ-*Proteobacteria* include the genera *Methylobacter, Methylomonas, Methylomicrobium, Methylocaldum, Methylosphaera,*

Methylothermus, Methylosarcina, Methylohalobius, Methylosoma and *Methylococcus*. Type II methanotrophs include the genera *Methylocystis, Methylosinu, Methylocella and Methylocapsa*, belonging to the α *Proteobacteria* (**Figure 1.2**).

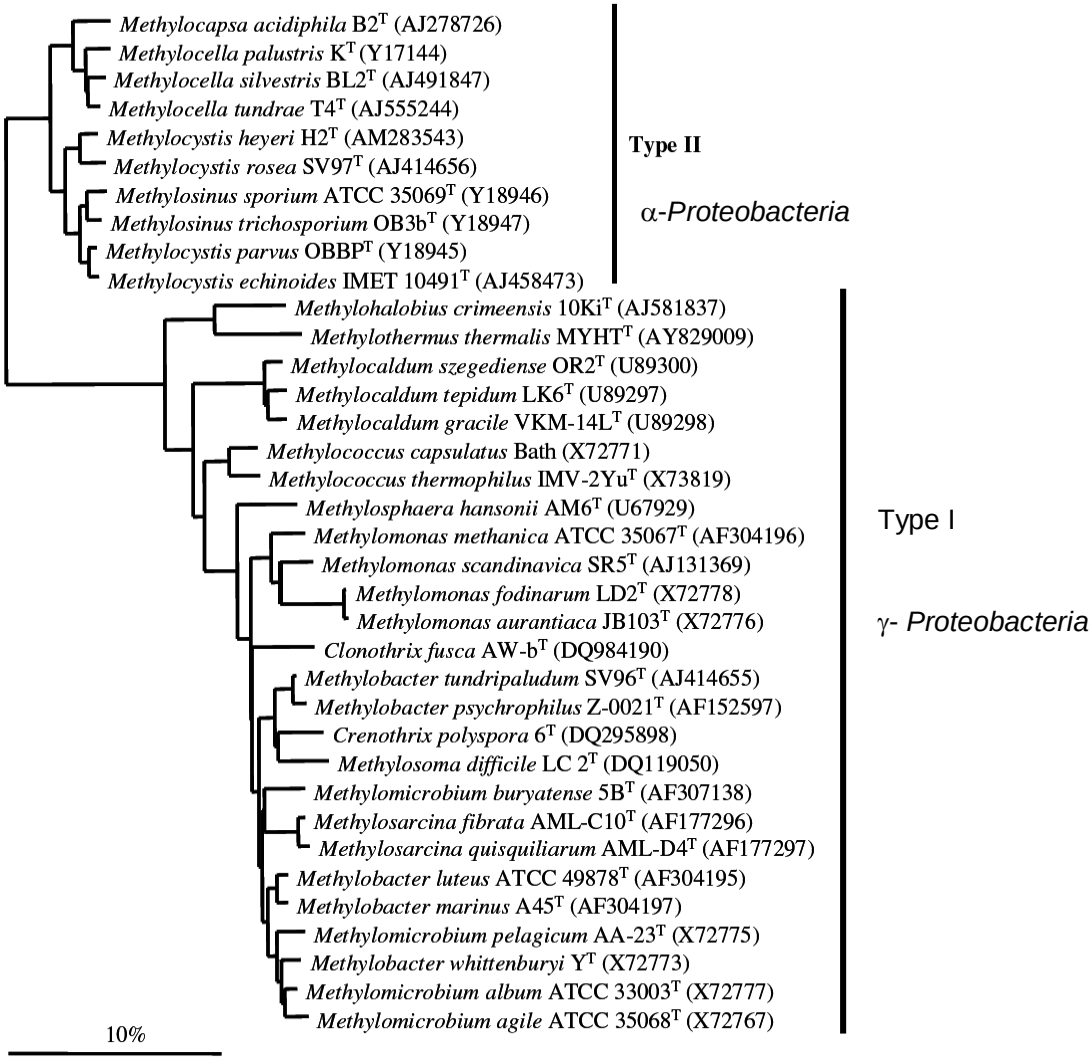

Figure 1.2 Phylogenetic tree based on the analysis of the 16S rRNA gene sequences of the type strains of Type I and II methanotrophs. The dendrogram was produced using the Neighbor joining method based on 1245 base pairs of aligned sequence. The phylogenetic tree was rooted to *Methylobacterium extorquens* (AF531770). The bar represents 0.1 substitution per nucleotide position. (McDonald *et al.*, 2008)

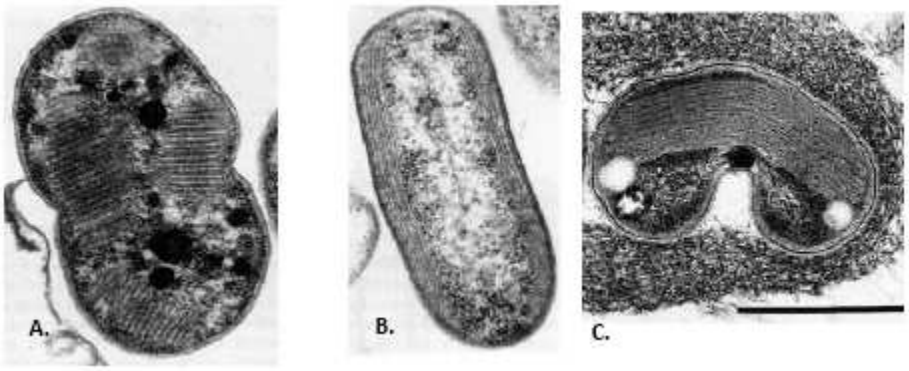

Figure 1.3 Electron micrographs of methanotrophs showing the different types of membrane arrangement. A: Section of *Methylomonas methanica* showing the membrane system found in Type I organisms. B: Section of *Methylocystis parvus* showing the membrane system found in Type II organisms (taken from Green, 1992). C: Section of *Methylocapsa acidophila* strain $B2^T$ (taken from Dedysh *et al.*, 2002).

1.3.1 Novel methanotrophs

Recent studies have reported two filamentous methane oxidizers, one with a novel *pmoA*, *Crenothrix polyspora* (Stoecker *et al.*, 2006) and another with a conventional *pmoA*, *Clonothrix polyspora* (Vigliotta *et al.*, 2007) (**Figure 1.4**). Both belongs to the γ-*Proteobacteria* and are closely related to Type I methanotrophs. Moreover, three new isolates belonging to the *Verrucomicrobia* phylum have been identified as methane oxidizers (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). These methanotrophs are both thermophilic and acidophilic and are reported to contain three divergent pmoCAB operons. *pmoA* sequences from methane oxidizing *Verrucomicrobia* are unusual with Dunfield *et al.*, (2007) suggesting that *Verrucomicrobia* methanotrophs may have diverged from *Proteobacteria* a long time ago and it is unlikely that *pmoA* genes were acquired by horizontal gene transfer. Characteristics of all known methanotrophs genera are listed in Table 1.1.

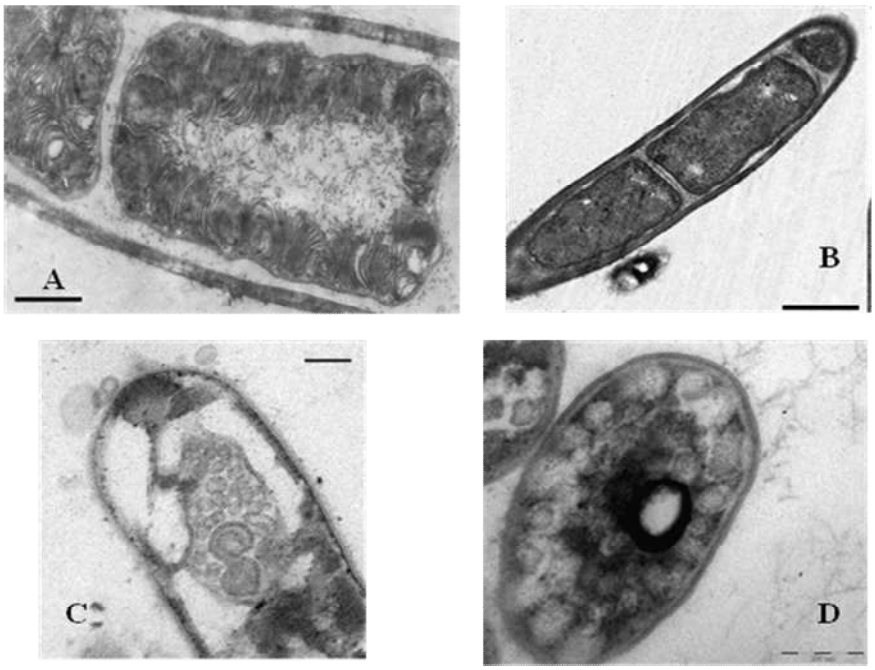

Figure 1.4 Electron micrographs of novel methanotrophs. **A.** Section of a sheathed *Crenothrix polyspora* filament. (bar 0.5 µm) (Stoecker *et al.*, 2006). **B.** Section of *Clonothrix fusca* (Bar 1 µm) (Vigliotta *et al.*, 2007). **C.** *Verrucomicrobia* isolate V4 (bar 100 nm) (Dunfield *et al.*, 2007) and **D.** *Verrucomicrobia* isolate SolV(bar 200 nm) (Pol *et al.*, 2007).

Table 1.1 Methanotroph genera and their characteristics.

Genus name	Phylogeny	MMO type	C_1 assimilation	ICM type	N_2 fixation	(mol % GC content)	Major PLFA	Reference
Methylobacter	γ *Proteobacteria*	pMMO	RuMP	Type I	No	49-54	16:1	1, 2, 3, 4
Methylosoma	γ *Proteobacteria*	pMMO	not known	Type I	Yes	49.9	16:1	22
Methylomicrobium	γ *Proteobacteria*	pMMO +/– sMMO	RuMP	Type I	No	49-60	16:1	7, 8, 9, 10, 11, 12, 13
Methylomonas	γ *Proteobacteria*	pMMO +/– sMMO	RuMP	Type I	some	51-59	16:1	1, 2, 14, 15, 16
Methylosarcina	γ *Proteobacteria*	pMMO	RuMP	Type I	No	54	16:1	17
Methylosphaera	γ *Proteobacteria*	pMMO	RuMP	ND	Yes	43-46	16:1	18
Methylococcus	γ *Proteobacteria*	pMMO + sMMO	RuMP/Serine	Type I	Yes	59-66	16:1	1, 5
Methylocaldum	γ *Proteobacteria*	pMMO	RuMP/Serine	Type I	No	57	16:1	6
Methylothermus	γ *Proteobacteria*	pMMO	RuMP	Type I	No	62.5	18:1/16:0	23
Methylohalobius	γ *Proteobacteria*	pMMO	RuMP	Type I	No	58.7	18:1	24
Methylocystis	α *Proteobacteria*	pMMO +/– sMMO	Serine	Type II	Yes	62-67	18:1	1, 2
Methylosinus	α *Proteobacteria*	pMMO + sMMO	Serine	Type II	Yes	63-67	18:1	1, 2
Methylocella	α *Proteobacteria*	sMMO	Serine	NA	yes	60-61	18:1	19, 20
Methylocapsa	α *Proteobacteria*	pMMO	Serine	Type III	Yes	63.1	18:1	21
Crenothrix	α *Proteobacteria*	pMMO	ND	Type I	ND	ND	ND	25
Clonotrix	α *Proteobacteria*	pMMO	ND	Type I	ND	ND	ND	26
"Methylokorus"	*Verrucomicrobia*	pMMO	Serine, RuMP?	Type IV?	No	ND	ND	27
"Acidimethylosilex"	*Verrucomicrobia*	pMMO	ND	Type IV?	No	ND	C18:0	28
"Methyloacida"	*Verrucomicrobia*	ND	ND	Type IV?	Yes	ND	ND	29

ND, not determined; NA, not applicable; ICMs are very limited in this genus. 1.(Whittenbury *et al.*, 1970b); 2. (Bowman *et al.*, 1993); 3.(Omelchenko *et al.*, 1996); 4.(Kalyuzhnaya *et al.*, 1998); 5.(Malashenko *et al.*, 1975); 6.(Bodrossy *et al.*, 1997); 7.(Bowman *et al.*, 1995); 8.(Sieburth *et al.*, 1987); 9.(Fuse *et al.*, 1998); 10.(Khmelenina *et al.*, 1997) 11.(Kalyuzhnaya *et al.*, 1999a); 12.(Sorokin *et al.*, 2000); 13.(Kaluzhnaya *et al.*, 2001); 14.(Whittenbury and Krieg, 1984); 15.(Omelchenko *et al.*, 1996); 16.(Kalyuzhnaya *et al.*, 1999b) 17.(Wise *et al.*, 2001); 18.(Bowman *et al.*, 1997); 19.(Dedysh *et al.*, 2000); 20. (Dunfield *et al.*, 2003); 21.(Dedysh *et al.*, 2002); 22.(Rahalkar *et al.*, 2007); 23.(Tsubota *et al.*, 2005); 24.(Heyer *et al.*, 2005); 25.(Stoecker *et al.*, 2006); 26. (Vigliotta *et al.*, 2007); 27.(Pol *et al.*, 2007); 28.(Dunfield *et al.*, 2007); 29.(Islam *et al.*, 2008). Adapted from Chen (2008)

1.4 Methane oxidation pathway

Methanotrophs use the enzyme methane monooxygenase (MMO) to catalyze the oxidation of methane to methanol (**Figure 1.5**). MMO are present either as particulate MMO (pMMO) or as soluble MMO (sMMO) (reviewed in Trotsenko and Murrell, 2008). Methanol is further oxidized to formaldehyde by the enzyme methanol dehydrogenase (MDH), which is present in all known Gram negative methylotrophs (Anthony, 1982). Formaldehyde can either be assimilated to biomass through different pathways or oxidized into CO_2 to produce energy and reducing power. Methanotrophs use two distinct pathways to assimilate formaldehyde into biomass, the ribulose monophosphate pathway (RuMP) in Type I methanotrophs or the serine pathway in Type II methanotrophs, to provide metabolites for biosynthesis (reviewed in Trotsenko and Murrell, 2008). Formaldehyde dehydrogenase oxidizes formaldehyde into formate, which is then subsequently oxidized into CO_2 and H_2O by formate dehydrogenase. Multiple enzyme systems are known to be involved in the oxidation of formaldehyde to formate in methylotrophs (Anthony, 1982), which includes NAD(P)-linked aldehyde dehydrogenases (may or may not require reduced glutathione or other factors) and dye-linked dehydrogenases measured by the reduction of dyes, such as 2.6-dichlorophenol (DiSpirito *et al.*, 1992). Methanotrophs such as *Methylococcus capsulatus* Bath can assimilate CO_2 using ribulose 1, 5-biphosphate carboxylase/oxygenase (Rubisco) via the Calvin Benson cycle (Stanley and Dalton, 1982).

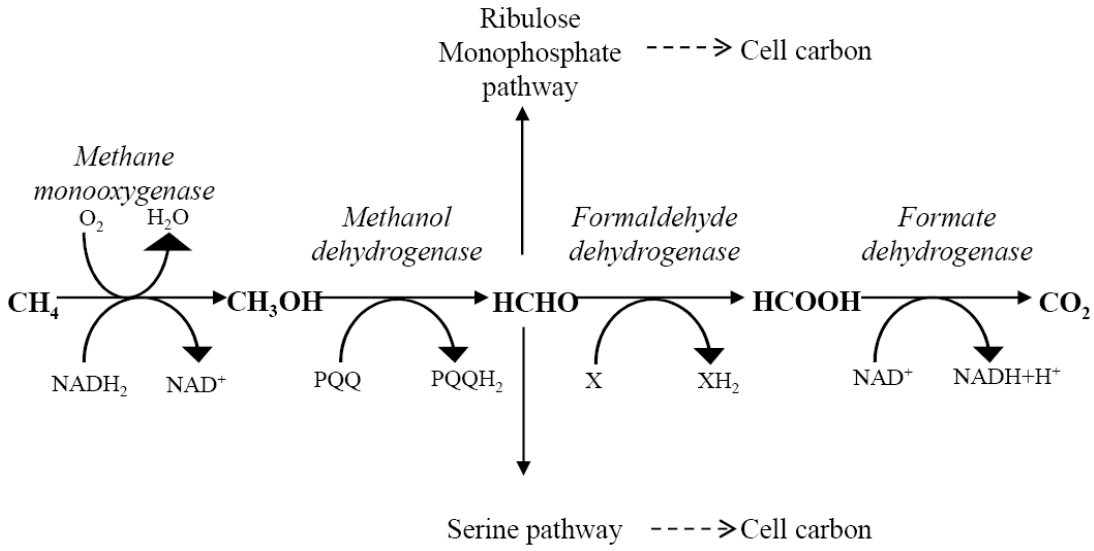

Figure 1.5 Methane oxidation and formaldehyde assimilation pathways in methanotrophs.

1.5 Methane monoxygenase

Methane monoxygenase is the key enzyme involved in the oxidation of methane by methanotrophs. There are two forms of MMO, one as a soluble MMO (sMMO) present in the cytoplasm and the other as a membrane bound particulate MMO (pMMO). pMMO is known to be present in all known methanotrophs except the facultative acidophilic methanotroph, *Methylocella*, isolated from a peat bog (Dedysh *et al.*, 2005; Theisen and Murrell, 2005). pMMO and sMMO appear to be unrelated without any similarities in both structural characteristics and sequences (Semrau *et al.*, 1995). The enzymes have different substrate specificity. sMMO has a broad substrate specificity and can co-oxidize a wide range of alkanes, alkenes and aromatic compounds (Sullivan *et al.*, 1998), whereas pMMO exhibits a narrow substrate specificity and can only co-oxidize a few short chain alkanes, alkenes (up to five carbons) and ammonia (Murrell *et al.*, 2000).

1.5.1 Particulate methane monooxygenase

pMMO is a membrane bound enzyme responsible for methane oxidation in all known methanotrophs except *Methylocella*. Recent studies suggest that methanotrophs use pMMO for *in situ* methane oxidation (Kolb *et al.*, 2005; Chen *et al.*, 2007). Although pMMO is the dominant form of MMO, it is not as well studied as sMMO, owing to difficulties in purifying the protein in its active form. Smith and Dalton (1989) partially purified this protein from *Methylococcus capsulatus* Bath after growing the organism under high copper conditions. By using dodecyl β-D maltoside as the solubilising agent, the authors successfully purified the pMMO complex (hydroxylase and reductase). However, further attempts to purify individual components were unsuccessful. Improvement in solubilisation procedures led to the identification of the pMMO complex, which consists of two components, the hydroxylase (pMMOH) comprising three subunits (α, β and γ with approximate masses of 47, 24 and 22 kDa subunits, respectively) and a putative reductase (pMMOR) consisting of 63 and 8 kDa proteins (Zahn and DiSpirito, 1996; Basu *et al.*, 2003). The genes that encode pMMO have been cloned and sequenced from a number of methanotrophs, including *Methylococcus capsulatus*, *Methylobacter albus* BG8 (Semrau *et al.*, 1995), *Methylosinus trichosporium* OB3b and *Methylocystis* sp. Strain M (Gilbert *et al.*, 2000). The genes are clustered on the genome in the order of *pmo*CAB with a σ^{70} promoter ahead of *pmoC* (**Figure 1.6**) (Gilbert *et al.*, 2000). Whilst some methanotrophs contain almost identical copies of *pmo*CAB, some Type II methanotrophs are known to have highly divergent copies of pMMO, such as *Methylocystis* sp. SC2 (Dunfield *et al.*, 2002). Recently discovered filamentous methanotrophs (Stoecker *et al.*, 2006; Vigliotta *et al.*, 2007) and methanotrophs from

Verrucomicrobia phylum (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008) are also known to have highly divergent *pmoA* sequences.

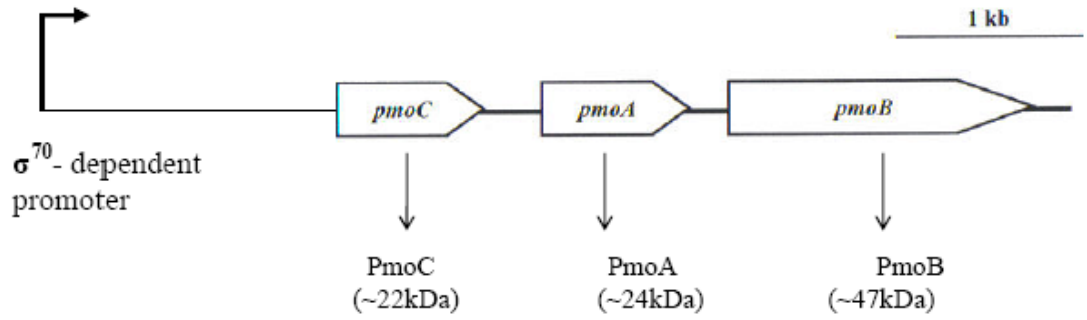

Figure 1.6 Gene cluster for pMMO from methanotrophs. Transcription of the operon occurs from a σ^{70}-dependent promoter (Gilbert *et al.*, 2000).

pMMO is a copper containing enzyme and the activity of the enzyme is directly related to the copper/total membrane protein ratio. All purified pMMO preparations revealed the presence of copper, ranging from 2 to 15 copper ions per αβγ complex (Zahn and DiSpirito, 1996; Basu *et al.*, 2003; Lieberman *et al.*, 2003; Yu *et al.*, 2003). Moreover, there are also discrepancies over the presence and amount of iron. While some studies have reported that no iron was present in the active pMMO, other studies have reported the presence of ~0.5 – 2 irons per αβγ complex. Zahn and DiSpirito (1996) suggested that the catalytic center might contain both copper and iron (2:2 ion ratio) with additional 6-8 copper ions bound to the copper binding compound, referred as methanobactin. *In vitro* studies have further confirmed the role of methanobactin in pMMO activity. Despite the progress on study of pMMO, further work is needed to understand copper and iron to αβγ ratios. The models for the metal centres proposed by various studies before and after the determination of pMMO crystal structure from *Methylococcus capsulatus* have been reviewed by Rosenzweig (2008).

1.5.2 Soluble methane monooxygenase

Soluble MMO is more stable than pMMO and has been purified from several methanotrophs and extensively studied. Previously, sMMO was believed to be present only in the Type II methanotroph genera *Methylosinus* and *Methylocystis* and the Type I methanotroph *Methylococcus capsulatus* Bath. However, it has subsequently been found in a marine *Methylomicrobium* and a *Methylomonas* strain (both Type I methanotrophs) and in the genus *Methylocella* (Dedysh *et al.*, 1998; Fuse *et al.*, 1998; Shigematsu *et al.*, 1999). sMMO in *Methylocella* strains is similar to those in Type II methanotrophs (Dedysh *et al.*, 2000) and has been characterised by Theisen *et al,* (2005). sMMO is a non-haem iron containing enzyme complex that consists of three main components, a hydroxylase ($\alpha_2\beta_2\gamma_2$) containing the active site, a NADH-dependent reductase (MmoC) that shuttle electrons to the hydroxylase and a coupling protein (protein B, MmoB). The Crystal structure of the hydroxylase has been resolved from *Methylococcus capsulatus* Bath (**Figure 1.7**) (Rosenzweig *et al.*, 1993) and *Methylosinus trichosporium* OB3b (Elango *et al.*, 1997).

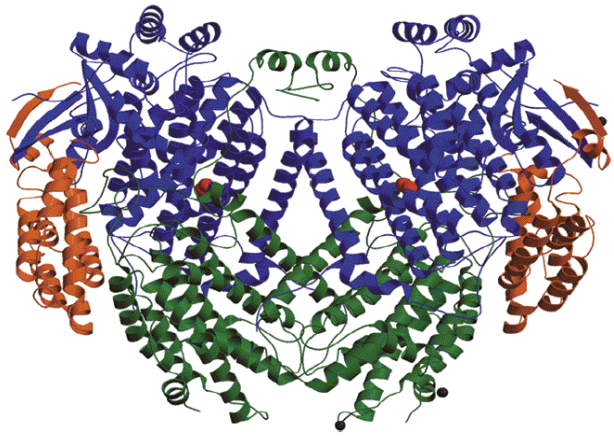

Figure 1.7 Crystal structure of the hydroxylase component of sMMO. The α subunit is in blue, β subunit is in green and γ subunit is in orange. Binuclear iron centres are in red on the α subunit. Taken from Rosenzweig *et al*. (1993).

The gene clusters (*mmoXYBZDC*) encoding sMMO enzymes have been cloned and sequenced from *Methylococcus capsulatus* Bath (Stainthorpe et al., 1990), *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991), *Methylocystis sp.* strain M (McDonald *et al.*, 1997), *Methylocystis sp.*strain W114 (Grosse *et al.*, 1999) and *Methylomonas sp.* strains KSPIII and KSWIII (Shigematsu *et al.*, 1999). The *mmoX, Y* and *Z* encode the α, β and γ subunits of the hydroxylase, while *mmoB* encodes protein B, *mmoC* encodes the reductase and *mmoD* encodes a polypeptide of uncertain function (**Figure 1.8**). In some methanotrophs, *mmoR* and *mmoG* have been identified at either upstream or downstream of *mmo*XYBZDC cluster. *mmoR* encodes a σ^{54}-dependent transcriptional regulator and *mmoG* encodes a homolog of the chaperone GroEL (Csáki *et al.*, 2003; Stafford *et al.*, 2003).

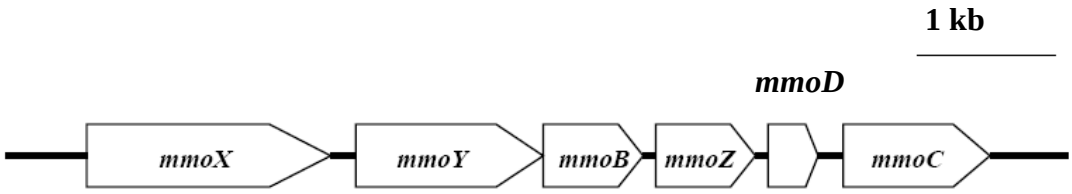

Figure 1.8 The gene cluster for soluble methane monooxygenase from methanotrophs (adapted from Murrell *et al.*, 2000)

1.5.3 The copper switch

In methanotrophs that have the genes for both pMMO and sMMO, their expression depends on the copper to biomass ratio during growth. At high copper to biomass ratio, pMMO is expressed, while at low levels of copper ions in the growth medium sMMO is expressed. Studies have reported that no pMMO transcripts were detected under low copper conditions and the transcription of sMMO is repressed by the addition of copper in the medium. However, Choi *et al*. (2003) reported that

though sMMO is not transcribed under high copper conditions, pMMO transcripts were detected under low copper to biomass medium and thereby questioning whether pMMO is constitutively transcribed. Ali (2006), by using a GFP-fused reporter, demonstrated that indeed pMMO was constitutively expressed from a σ^{70}-dependent promoter, whilst sMMO is transcribed only under low copper growth conditions.

The copper switch is not a simple on/off progress and it has been suggested that it is also regulated at the post-transcription level. Structural models of pMMO confirmed that copper is located in the active pocket of the enzyme (Lieberman and Rosenzweig, 2005). Although pMMO is constitutively expressed independent of the copper concentration, pMMO activity is significantly enhanced by copper ions. In the case of sMMO expression, it is regulated by via a σ^{54} promoter and only transcribed when copper ions are unavailable. Moreover, transcription of *mmoR* and *mmoG* is not regulated by copper at the transcriptional level (Ali, 2006). In the facultative methanotroph, *Methylocella silvestris*, sMMO transcription is repressed by substrates other than methane (e.g. acetate) instead of copper (Theisen, 2005).

In summary, at low copper to biomass ratio sMMO is expressed, whereas pMMO is also transcribed although the enzyme is not active due to the absence of copper at the active site. At high copper to biomass ratio, copper will be incorporated into the active site of pMMO and hence used for methane oxidation by methanotrophs. sMMO transcription in this condition is probably repressed by copper ions by an unknown mechanism.

1.6 Ecology of methanotrophs

Methane oxidizing bacteria are found in different environments and are particularly abundant at oxic/anoxic interfaces of methanogenic environments such as wetlands, landfills, rice paddies, and aquatic sediments (Hanson and Hanson, 1996). Type I and Type II methanotrophs are known to co-exist in the environment and their distribution might be affected by environmental factors such as oxygen-methane gradient, nitrogen and pH (Amaral and Knowles, 1995; Dedysh *et al.*, 2001). Whilst most methanotroph isolates are known to be mesophilic they are also known to thrive in extreme environments. Moderately thermophilic methanotrophs such as *Methylococcus capsulatus* and *Methylothermus thermalis* (growing at 45 – 55 ^{o}C) have been isolated from hot springs along with *Methylocaldum sp*. Recently reported *Verrucomicrobia* methanotrophs (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008) are both thermophilic and acidophilic, which can grow at pH 1.0 and at a temperature of 65^{o}C (Op den Camp *et al.*, 2009). Halophilic and alkaliphilic methanotrophs have been found by using both cultivation dependent and independent methods. Using stable isotope probing (SIP; discussed in section 1.8), Lin *et al.* (2004) detected *Methylobacter*, *Methylomonas*, *Methylosinus* and *Methylocapsa* in a soda lake sediments. Psychrophilic methanotrophs, such as *Methylobacter psychrophilus*, *Methylosphaera hansonii* and *Methylomonas scandinavica* have been isolated from different environments. These methanotrophs belong to *Gammaproteobacteria* and can grow between 5 – 15 ^{o}C (Pacheco-Oliver *et al.*, 2002).

1.6.1 Enrichment, isolation and cultivation

Methanotrophs have been traditionally studied in laboratory using cultivation based strategies. Methanotrophs from different habitats have been isolated, with isolates representing a wide range of physiological adaptations. Nitrate mineral salts (NMS) and ammonium nitrate mineral salts (ANMS) (Whittenbury *et al.*, 1970b) were used for enrichment and isolation of methanotrophic strains. Modifications to the conventional culturing methods, such as reduction of mineral salts concentration and pH of the medium have contributed to the isolation of acidophilic methanotrophs, *Methylocella* and *Methylocapsa*. Since some methanotrophs do not grow on agar plates, Dedysh *et al.* (2002; 2007) used serial dilution of enrichment cultures to isolate strains of *Methylocapsa* and *Methylocystis heyeri*. Growth conditions such as temperature, pH, salt concentration, N source and the use of copper free medium can select for different methanotrophs. Methane and oxygen concentrations are known to influence the competition between *Gammaproteobacteria* and *Alphaproteobacteria* (Graham *et al.*, 1993). Hence it is critical to carefully monitor the growth conditions while isolating methanotrophs from environmental samples.

It has been recognized that only a fraction of microorganisms have been cultivated and strains in laboratory culture collection only account for a small fraction of the microorganisms present in the environment. Moreover, the strains isolated may not even represent the dominant microorganism in the environment. It has been estimated that <5% of the soil microorganisms have been cultivated (Amann *et al.*, 1995; Felske *et al.*, 1999) and the media used to isolate microorganisms often introduce a selection bias (Gray and Head, 2001). Methanotrophs are difficult to cultivate and the difficulty of isolating methanotrophs

lead to the use of cultivation-independent methods for studying methanotrophs in the environment (reviewed in Murrell and Radajewski, 2000; McDonald *et al.*, 2008).

1.7 Phylogenetic markers

1.7.1 16S rRNA gene based probes

Owing to the large database of 16S rRNA gene sequences and the conserved nature of this gene, it is the most obvious marker for detecting methanotrophs in the environment. Probes and PCR primers designed to target 16S rRNA gene from different genera or species of methanotrophs have been used to detect methanotrophs in environmental samples (**Table 1.2**). Phylogeny of methanotrophs is rather diverse, being located in both α and γ *Proteobacteria* and hence it is difficult to design primers or probes targeting all methanotrophs. Recently Chen *et al*. (2007) designed 16S rRNA gene primer sets targeting Type I and Type II methanotrophs. However, the authors cautioned that there might be non-specific amplification with the primers and care should be taken in interpreting the results.

Table 1.2 List of 16S rRNA gene probes targeting methanotrophs

Type I methanotroph probes			
Name	**Sequence (5'-3')**	**Target**	**Reference**
10γ	GGTCCGAAGATCCCCCGCTT	RuMP pathway methylotrophs	(Tsien *et al.*, 1990)
1035-RuMP	GATTCTCTGGATGTCAAGGG	RuMP pathway methanotrophs	(Brusseau *et al.*, 1994)
Mb1007[a]	CACTCTACGATCTCTCACAG	*Methylobacter* (*Methylomicrobium*)[a]	(Holmes *et al.*, 1995a)
Mc1005	CCGCATCTCTGCAGGAT	*Methylococcus*	(Holmes *et al.*, 1995a)
Mm1007	CACTCCGCTATCTCTAACAG	*Methylomonas*	(Holmes *et al.*, 1995a)
MethT1dF	CCTTCGGGMGCYGACGAGT	Type I methanotrophs	(Wise *et al.*, 1999)
MethT1bR	GATTCYMTGSATGTCAAGG	Type I methanotrophs	(Wise *et al.*, 1999)
Type 1b	GTCAGCGCCCGAAGGCCT	Type I methanotrophs	(Auman *et al.*, 2000)
Gm633	AGTTACCCAGTATCAAATGC	*Methylobacter* and *Methylomicrobium*	(Gulledge *et al.*, 2001)
Gm705[c]	CTGGTGTTCCTTCAGATC	γ-methanotrophs except *Methylocaldum*	(Gulledge *et al.*, 2001)
Mlb482	GGTGCTTCTTCTAAAGGTAATGT	*Methylobacter*	(Gulledge *et al.*, 2001)
Mlb662[d]	CCTGAAATTCCACTCTCCTCTA	*Methylobacter*	(Gulledge *et al.*, 2001)
Mmb482	GGTGCTTCTTCTATAGGTAATGT	*Methylomicrobium*	(Gulledge *et al.*, 2001)
Mlm482	GGTGCTTCTTGTATAGGTAATGT	*Methylomonas*	(Gulledge *et al.*, 2001)
Mlm732a	GTTTTAGTCCAGGGAGCCG	*Methylomonas* group A	(Gulledge *et al.*, 2001)
Mlm732b	GTTTGAGTCCAGGGAGCCG	*Methylomonas* group C	(Gulledge *et al.*, 2001)
Mlc123	CACAACAAGGCAGATTCCTACG	*Methylococcus*	(Gulledge *et al.*, 2001)
Mlc1436	CCCTCCTTGCGGTTAGACTACCTA	*Methylococcus*	(Gulledge *et al.*, 2001)
Mcd77	GCCACCCACCGGTTACCCGGC	*Methylocaldum*	(Gulledge *et al.*, 2001)
Mγ84	CCACTCGTCAGCGCCCGA	Type I methanotrophs	(Eller *et al.*, 2001)
Mγ669[d]	GCTACACCTGAAATTCCACTC	*Methylobacter* and *Methylomonas*	(Eller *et al.*, 2001)
Mγ983	TGGATGTCAAGGGTAGGT	Type I methanotrophs	(Eller *et al.*, 2001)
Mγ993	ACAGATTCTCTGGATGTC	Type I methanotrophs	(Eller *et al.*, 2001)
Mγ1004[a]	TACGATCTCTCACAGATT	*Methylomicrobium*	(Eller *et al.*, 2001)
Mh996r	CACTCTACTATCTCTAACGG	*Methylosphaera*	(Kalyuzhnaya *et al.*, 2002)
Type IF	ATGCTTAACACATGCAAGTCGAACG	Type I methanotrophs	(Chen *et al.*, 2007)
Type IR	CCACTGGTGTTCCTTCMGAT	Type I methanotrophs	(Chen *et al.*, 2007)

Type II methanotroph probes			
Name	**Sequence (5'-3')**	**Target**	
9α	CCCTGAGTTATTCCGAAC	Serine pathway methylotrophs	(Tsien *et al.*, 1990)
1034-Ser	CCATACCGGACATGTCAAAAGC	Serine pathway methanotrophs	(Brusseau *et al.*, 1994)
Ms1020	CCCTTGCGGAAGGAAGTC	*Methylosinus*	(Holmes *et al.*, 1995a)
Type 2b	CATACCGGRCATGTCAAAAGC	Type II methanotrophs	(Costello and Lidstrom, 1999)
MethT2R	CATCTCTGRCSAYCATACCGG	Type II methanotrophs	(Wise *et al.*, 1999)
Am455[b]	CTTATCCAGGTACCGTCATTATCGTCCC	α-methanotrophs	(Gulledge *et al.*, 2001)
Am976	GTCAAAAGCTGGTAAGGTTC	α-methanotrophs	(Gulledge *et al.*, 2001)
Mα464	TTATCCAGGTACCGTCATTA	Type II methanotrophs	(Eller *et al.*, 2001)
Mcell-1026	GTTCTCGCCACCCGAAGT	*Methylocella palustris*	(Dedysh *et al.*, 2001)
AcidM-181	TCTTTCTCCTTGCGGACG	*Methylocella palustris* and *Methylocapsa acidiphila*	(Dedysh *et al.*, 2001)
Mcaps-1032	CACCTGTGTCCCTGGCTC	*Methylocapsa acidiphila*	(Dedysh *et al.*, 2003)
Msint-1268	TGGAGATTTGCTCCGGGT	*Methylosinus trichosporium*	(Dedysh *et al.*, 2003)
Msins-647	TCTCCCGGACTCTAGACC	*Methylosinus sporium*	(Dedysh *et al.*, 2003)
Mcyst-1432	CGGTTGGCGAAACGCCTT	All *Methylocystis* spp.	(Dedysh *et al.*, 2003)
Type IIF	GGGAMGATAATGACGGTACCWGGA	Type II methanotrophs	(Chen *et al.*, 2007)
Type IIR	GTCAARAGCTGGTAAGGTTC	Type II methanotrophs	(Chen *et al.*, 2007)

[a]Also called Mmb1007 and primer Mγ1004 has an identical 15 bp overlap with Mb1007; [b]Primer Mα450 is identical to part of Am455; [c]Primer Mγ705 is identical to Gm705; [d]Primer Mγ669 has an identical 15 bp overlap with Mlb662. MethT1dF/ MethT1bR excludes *Methylocaldum spp.* and MethT2R excludes *Methylocella spp.* (Adapted from Chen, 2008)

1.7.2 Functional gene based probes

Functional genes unique to the physiology and metabolism of organisms of interest have been used in molecular ecology studies to complement 16S rRNA gene based probes. By narrowing down the target to the studied functional group, functional gene probes enable higher sensitivity of detection in complex environments. Moreover, it allows the identification of putative uncultivated members of the functional group based on the presence of a homologous gene sequence. Functional genes for enzymes, such as methane monooxygenase (pMMO and sMMO; genes *pmoA* and *mmoX*, respectively) and methanol dehydrogenase (MDH; gene *mxaF*), involved in the methane oxidation pathway, have been used for detection of methanotrophs in environmental samples (McDonald *et al.*, 2008). *pmoA* genes have been cloned and sequenced from a large number of methanotrophs and have been extensively used to target methanotrophs including novel filamentous and *Verrucomicrobia* methanotrophs with the exception of *Methylocella* spp. (**Table 1.3**) (McDonald *et al.*, 2008). The sMMO gene cluster has also been sequenced from a number of methanotrophs and primers have been developed to target *mmoX* containing methanotrophs in the environmental samples (**Table 1.4**). The primer set 206f/886r (Hutchens *et al.*, 2004) was reported to have broader *mmoX* targets than other primer sets. However, the data set of *mmoX* sequences are still relatively small and it should be used along with 16S rRNA or *pmoA* based analysis to detect methanotrophs in the environment. The phylogenies of *pmoA* and *mmoX* have been shown to be congruent with 16S rRNA gene based phylogeny (Holmes *et al.*, 1999; Kolb *et al.*, 2003).

Primer sets have also been designed to target the large subunit of MDH (*mxaF*), which is also present in some methylotrophs (**Table 1.5**). Recent findings

revealed that some methylotroph strains (Kalyuzhnaya *et al.*, 2006a; Kalyuzhnaya *et al.*, 2006b; Kane *et al.*, 2007) might possess an alternative methanol oxidation system instead of a conventional methanol dehydrogenase. Therefore the results obtained with *mxaF* primers should be interpreted with caution. Other functional genes such as *nifH* (encoding dinitrogen reductase, a key component of the nitrogenase enzyme complex) (Auman *et al.*, 2001; Dedysh *et al.*, 2004) and *fhcD* (encoding the D subunit of the formyl transferase/hydrolase complex, part of the H_4MPT-linked C_1-transfer pathway) (Kalyuzhnaya *et al.*, 2004) have been used to detect methanotrophs in environmental samples. The pros and cons of different 16S rRNA gene and functional gene based primers designed to detect methanotrophs in the environment has been reviewed in detail by McDonald *et al.*(2008).

Table 1.3 PCR primers used for amplification of *pmoA* genes from environmental samples

Name	Sequence (5'-3')	Product (bp)	References
A189f[a]/	GGNGACTGGGACTTCTGG/	525	(Holmes *et al.*, 1995b)
A682r	GAASGCNGAGAAGAASGC		
mb661	CCGGMGCAACGTCYTTACC	510[b]	(Costello and Lidstrom, 1999)
pmof1/	GGGGGAACTTCTGGGGITGGAC/	330	(Cheng *et al.*, 1999)
pmor	GGGGGRCIACGTCITTACCGAA		
pmof2/pmor	TTCTAYCCDRRCAACTGGCC	178	(Cheng *et al.*, 1999)
pmoA206f/	GGNGACTGGGACTTCTGGATCGACTTCAAGGATCG/	530	(Tchawa Yimga *et al.*, 2003)
pmoA703b	GAASGCNGAGAAGAASGCGGCGACCGGAACGACGT		
A650r*	ACGTCCTTACCGAAGGT	478[b]	(Bourne *et al.*, 2001)
mb661r_nd	CCGGCGCAACGTCCTTACC	510[b]	(Lin *et al.*, 2005)
*pmoA*for/	TTCTGGGGNTGGACNTAYTTYCC/	281	(Steinkamp *et al.*, 2001)
*pmoA*rev	CCNGARTAYATHMGNATGGTNGA		
f326/r643	TGGGGYTGGACCTAYTTCC/	358	(Fjellbirkeland *et al.*, 2001)
	CCGGCRCRACGTCCTTACC		
Mb601 R[c]	ACRTAGTGGTAACCTTGYAA	432[b]	(Kolb *et al.*, 2003)
Mc468 R[c]	GCSGTGAACAGGTAGCTGCC	299[b]	(Kolb *et al.*, 2003)
II 223 F[c]/ II646 R[c]	CGTCGTATGTGGCCGAC	444	(Kolb *et al.*, 2003)
	CGTGCCGCGCTCGACCATGYG		
Mcap630	CTCGACGATGCGGAGATATT	461[b]	(Kolb *et al.*, 2003)
Forest675 R[c]	CCYACSACATCCTTACCGAA	506[b]	(Kolb *et al.*, 2003)
Mb661 R[c]	GGTAARGACGTTGCNCCGG	491[b]	(Kolb *et al.*, 2003)

[a] A189f is also known as A189gc. [b] When used in PCR with primer A189f. [c] Primers designed for real time PCR quantification of subsets of methanotrophs (II 223 F[c]/ II646 R targets *Methylosinus* group; Mc468r targets *Methylococcus* group; Mcap630 targets *Methylocapsa*; Mb601r targets *Methylobacter/Methylosarcina* group and Forest 675r targets forest clones). A682r detects amoA sequences when used in PCR with A189f. *No amplification of *pmoA* sequences from *Methylomicrobium album* (BG8) and *Methylosphaera hansonii* with a possible bias towards *Methylococcus capsulatus* when used in PCR with A189f. Adapted from McDonald *et al.*(2008).

Table 1.4 PCR primers used for amplification of *mmoX* genes from environmental samples

Name	Sequence (5'-3')	Product (bp)	Reference
mmoXf882/[a]	GGCTCCAAGTTCAAGGTCGAGC/	535	(McDonald *et al.*, 1995)
mmoXr1403	TGGCACTCGTAGCGCTCCGGCTCG		
mmoX1/	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT/GGCTCGACCT	369	(Miguez *et al.*, 1997)
mmoX2	TGAACTTGGAGCCATACTCG		
536f/	CGCTGTGGAAGGGCATGAAGCG/	341	(Fuse *et al.*, 1998)
877r	GCTCGACCTTGAACTTGGAGCC		
mmoXr901	TGGGTSAARACSTGGAACCGCTGGGT	396[b]	(Shigematsu *et al.*, 1999)
A166f/	ACCAAGGARCARTTCAAG/	1230	(Auman *et al.*, 2000)
B1401r	TGGCACTCRTARCGCTC		
534f/	CCGCTGTGGAAGGGCATGAA/	863	(Horz *et al.*, 2001)
1393r	CACTCGTAGCGCTCCGGCTC		
met1/	ACCAAGGAGCAGTTC/		(Baker *et al.*, 2001)
met4	TCCAGAAGGGGTTGTT		
mmoX206f/	ATCGCBAARGAATAYGCSCG/	719	(Hutchens *et al.*, 2004)
mmoX886r	ACCCANGGCTCGACYTTGAA		

[a] Primer *mmoX1* was located 2008-2037 and *mmoX2* was located 2347 – 2376. Primers A166f and B1401r are also known as mmoXA and mmoXD. [b] When used in PCR with primer *mmoX1*. Adapted from McDonald *et al.*(2008).

Table 1.5 PCR primers used for amplification of *mxaF* genes from environmental samples

Name	Sequence (5'-3')	Product (bp)	Reference
1003F/	GCGGCACCAACTGGGGCTGGT	550	(McDonald and Murrell, 1997)
1561R	GGGCAGCATGAAGGGCTCCC		
mxaF_for/	TGGAACGAGACCATGCGTC	455	(Moosvi *et al.*, 2005)
mxaF_rev	CATGCAGATGTGGTTGATGC		
mxaF-f769	TGGGAGGGCGAYGCCTGGAA[b]		(Dedysh *et al.*, 2005)
mxaF-1392	CTTSGGGCCCGGATACATG		(Dedysh *et al.*, 2005)
mxaF-1585	CTTCCASAGNAGKTCRCCNGTGTC		(Dedysh *et al.*, 2005)
mxaF-1690	CCCGGCCARCCGCCGAC		(Dedysh *et al.*, 2005)
1555R	CATGAABGGCTCCCARTCCAT	544[a]	(Neufeld *et al.*, 2007c)

[a] When used with primer 1003F. [b] mxaf-f769/1561r were used to amplify a *mxaF* fragment from *Albibacter methylovorans* (Dedysh *et al*., 2005). Adapted from McDonald *et al*.(2008).

1.8 Molecular ecology techniques to study methanotrophs

Denaturing gradient gel electrophoresis (DGGE)

DGGE was originally used for the detection of mutations within the human genome, and was later developed by Muyzer *et al.* (1993) and has been used extensively in microbial ecology studies (Schäfer and Muyzer, 2001). This method facilitates the electrophoretic separation of DNA fragments that are of the same length but have different sequences (Gray, 1999). DGGE has been extensively used to analyze the diversity of methanotroph 16S rRNA genes from the environment (Henckel *et al.*, 1999; Henckel *et al.*, 2000a; Eller and Frenzel, 2001; Chen *et al.*, 2007). Distribution of functional genes *pmoA* (Henckel *et al.*, 1999; Horz *et al.*, 2001; Knief *et al.*, 2003; Lin *et al.*, 2005), *mmoX* (Iwamoto *et al.*, 2000) and *mxaF* (Henckel *et al.*, 1999; Fjellbirkeland *et al.*, 2001) in the environment has also been studied using DGGE. However, the use of degenerate primers for functional genes may generate multiple bands for individual organisms during electrophoresis (McDonald *et al.*, 2008).

Terminal restriction fragment length polymorphism

T-RFLP is a rapid and sensitive fingerprinting technique that separates the restriction fragments of PCR amplicons according to size rather than sequence heterogeneity. This method avoids the bias that may occur during cloning and it can also produce semi-quantitative data. Horz and colleagues (2001) demonstrated the first application of *pmoA* T-RFLP to study methanotroph diversity on roots of submerged rice plants and the technique has been subsequently used in a number of studies (Horz *et al.*, 2002; Bussmann *et al.*, 2004; Horz *et al.*, 2005). However, it

should be noted that T-RFLP is yet to be used with 16S rRNA genes or other functional genes targeting methanotrophs.

Microbial diagnostic microarray

Microarrays were initially used as tools for studying genome-wide expression analysis. However, recently they have been developed for microbial diagnostic applications. Microbial diagnostic microarrays (MDM) contain nucleic acid probe sets targeting specific strain, subspecies, genus or high taxon (Bodrossy and Sessitsch, 2004). MDM offer a high-throughput tool for parallel analysis of complex gene mixtures in a single assay. Wu *et al* .(2001) developed a functional gene array that targeted functional genes involved in nitrogen cycling, such as *nirS, nirK, amoA* and *pmoA*. This study demonstrated the potential use of diagnostic microarrays to study microbial community structure in environments. Bodrossy and colleagues (2003) developed a microarray to specifically detect methanotrophs. This microarray consisted of 59 validated oligonucleotide probes (short oligonucleotides i.e., 18 to 27 nucleotides) targeting *pmoA* genes of all known methanotrophs and *amoA* of the ammonia oxidizing bacteria. This microarray has been upgraded (Stralis-Pavese *et al.*, 2004) and the latest version contain 138 probes (L. Bodrossy, unpublished data). Recently, an mRNA-based *pmoA* microarray was successfully developed (Bodrossy *et al.*, 2006) and used to study the community structure of active methanotrophs (Bodrossy *et al.*, 2006; Chen *et al.*, 2008a).

Quantification of methanotrophs

The Most probable number (MPN) technique has been widely used in the past for quantifying methanotrophs in the environment. MPN-based analyses are limited by the fact that only methanotrophs that can be cultivated using specific

medium can be quantified. Recently cultivation independent methods, such as fluorescent *in situ* hybridisation (FISH) and real-time PCR have been used to quantify methanotrophs in environmental samples.

FISH allows the detection of organisms in their natural environment and enables the phylogenetic identification of organisms in mixed communities without cultivation (Amann *et al.*, 1995). FISH relies on the specific annealing of a fluorescently labelled oligonucleotide probe to its complementary sequence in a fixed bacterial sample, which can be visualised using epifluorescence or confocal laser scanning microscopy (Delong, 1989). FISH targeting the 16S rRNA genes has been successfully used to analyze methanotrophs in the environment (Dedysh *et al.*, 2001; Dedysh *et al.*, 2003; Raghoebarsing *et al.*, 2005). However, one of the key limitations of FISH analysis is that it cannot be used with all environmental samples, particularly soil samples. Kolb *et al.* (2003) developed a quantitative real-time PCR assay for methanotrophs using using SYBR green and *pmoA* specific primers targeting five different groups of methanotrophs in real-time PCR. This assay has been subsequently used to quantify the methanotroph community in different environments (Kolb *et al.*, 2003; Kolb *et al.*, 2005; Knief *et al.*, 2006). It has been suggested that the *pmoA* primer set (a189F/mb661r) may underestimate methanotroph population and is not ideal for real-time PCR (Kolb *et al.*, 2003). Real-time PCR targeting 16S rRNA genes from methanotrophs has been also used to target methanotrophs (Halet *et al.*, 2006), however the primers used in the study was not specific for methanotrophs and they might overestimate methanotroph population.

Stable isotope probing – Linking function with diversity

Microorganisms are vital to the function of all ecosystems and they are probably more diverse than any other organisms. Cloning and fingerprinting techniques, through retrieval of sequences, provide us with an insight into the microbial community structure. However, it was not possible to identify and study the active organisms contributing to key processes in ecosystems. Recently there has been considerable focus to develop techniques that can link microbial diversity with a particular function.

Stable isotope probing is an elegant method that allows the identity of an organism tobe linked with its function under *in situ* conditions (Radajewski *et al.*, 2000; Dumont and Murrell, 2005). The method relies on the incorporation of stable isotopes (such as ^{13}C, ^{15}N) into DNA (Radajewski *et al.*, 2000), RNA (Manefield *et al.*, 2002), PLFA (Boschker *et al.*, 1998) or protein (Jehmlich *et al.*, 2008) followed by the separation and identification of those labelled molecules to identify the organism involved in the particular function (**Figure 1.9**). SIP was first performed to detect ^{13}C-labelled PLFAs in methanotrophs and acetate utilizing bacteria from lake sediments. The principle of SIP is based on the low natural abundance of ^{13}C, approximately only 1%. The addition of ^{13}C-labelled (>99%) substrate to an environmental sample will result in ^{13}C labelling of the actively metabolizing bacteria, as the bacteria uses the ^{13}C-lablled substrate as carbon substrate and incorporates into DNA during synthesis and replication. The ^{13}C-labelled DNA can then be separated from the ^{12}C-DNA of the bacteria by density gradient ultracentrifugation (Dumont and Murrell, 2005). DNA-SIP is a cultivation-independent method enabling the identification of uncultivated organisms (Whitby *et al.*, 2005) and also offers access to genomic information of novel organisms

involved in a particular ecosystem function (Dumont *et al.*, 2006; Neufeld *et al.*, 2008). One of the key limitations of DNA-SIP is the long incubation times required for DNA replication and incorporation of the ^{13}C-label into newly synthesized DNA, leading to "cross-feeding" problem. Recent studies have overcome this problem and performed SIP experiments with short incubation times and low concentration of substrates (Cadisch *et al.*, 2005; Chen *et al.*, 2008b; Neufeld *et al.*, 2008).

SIP has been also carried out with RNA. RNA synthesis occurs at a faster rate than DNA synthesis and hence RNA-SIP may have a greater sensitivity than DNA-SIP (Manefield *et al.*, 2002; Lueders *et al.*, 2004). Since the amount of isotope incorporation needed is considerably less than DNA-SIP experiments, near *in situ* concentrations can be used thereby minimizing potential bias caused by high substrate concentrations. RNA-SIP has been used extensively to study active bacterial populations in the environment (**Table 1.6**). Unlike DNA-SIP, in RNA-SIP experiments only 16S rRNA gene based analysis can be performed. Lueders *et al.*(2004) utilized both DNA- and RNA-SIP to analyze the incorporation of $^{13}CH_3OH$ in rice field soil microcosms. RNA-SIP allowed the identification of methylotrophs that were initially active in the microcosm, whilst DNA-SIP allowed the identification of primary methanol utilizing communities and their food-web interactions with fungi.

^{15}N-labelled substrates have also been used to identify communities metabolizing various N-compounds (Cadisch *et al.*, 2005; Buckley *et al.*, 2007a, b). The separation of DNA bands between ^{15}N-DNA and ^{14}N-DNA was only 4mm compared to the 10mm separation obtained with ^{13}C substrates. Therefore, a second round of centrifugation was used for effective separation of the bands (Buckley *et al.*, 2007a, b). Recently Schwartz (2007) used $H_2{}^{18}O$ to label actively growing cells

in soil samples and demonstrated efficient separation of labelled DNA from the background.

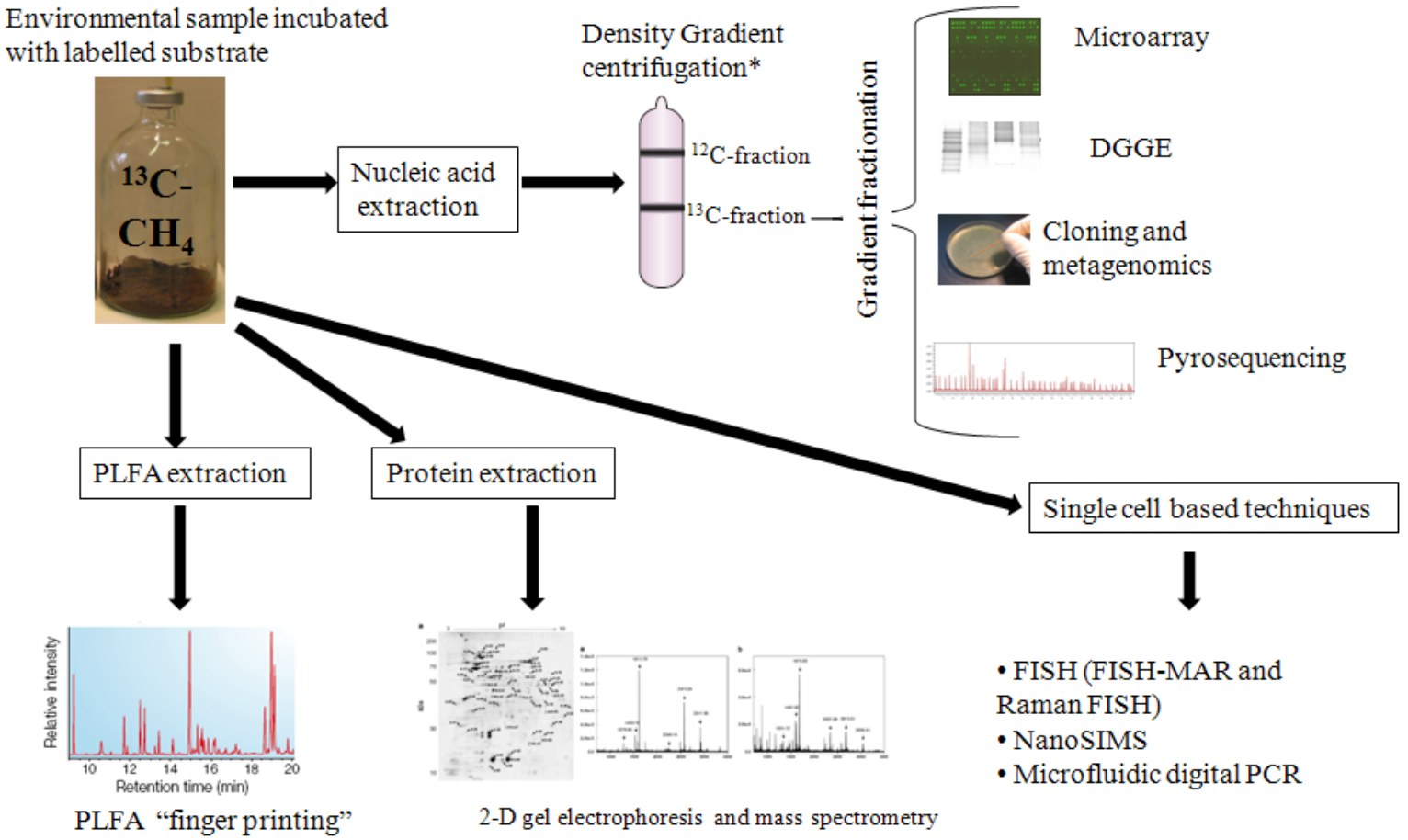

Figure 1.9 Schematic representation of a SIP experiment to study active microorganisms. *Density gradient ultracentrifugation for DNA is carried out with CsCl solution and for RNA with CsTFA solution.

Table 1.6 Recent studies using DNA/RNA SIP to identify active microorganisms

Substrate	Habitat	Phylogenetic groups identified	Marker genes	Reference
$^{13}CH_4$	Landfill soil originally from a peat bog	*Methylobacter*; *Methylomonas*; *Methylocystis*; *Methylocella*	16S rRNA; *pmoA*; *mmoX*	(Cebron *et al.*, 2007a)
$^{13}CH_4$	Landfill soil	*Methylobacter*; *Methylomicrobium*; *Methylocystis*	16S rRNA; *pmoA*	(Cebron *et al.*, 2007b)
$^{13}CH_4$	Rice field soil	*Methylococcaceae*; *Methylocystaceae*; *Lobosea*; *Heterolobosea*; *Colpodea*; *Cercozoa*	16S rRNA; 18S rRNA	(Murase and Frenzel, 2007)
$^{13}CH_4$	Forest soil	*Methylocystis*	16S rRNA, *pmoA*	(Dumont *et al.*, 2006)
$^{13}CH_4$	Cave water and microbial mat	Type I and Type II methanotrophs; *Hyphomicrobium*; *Bdellovibrio*; *Thiobacili*, *Methylophilu*s	16S rRNA; *pmoA*; *mmoX*; *mxaF*	(Hutchens *et al.*, 2004)
$^{13}CH_4$	Soda lake sediment	Type I methanotrophs; *Methylophilaceae*	16S rRNA; *pmoA*; *mmoX*;	(Lin *et al.*, 2004)
$^{13}CH_4$	Peat soil	*Methylosinus/Methylocystis*; RA-14 group; *Methylobacter/Methylomonas*; novel β-*Proteobacteria*	16S rRNA; *pmoA*; *mmoX*; *mxaF*	(Morris *et al.*, 2002)
$^{13}CH_3OH$	Coastal sea water	*Methylophaga*	16S rRNA; *mxaF*	(Neufeld *et al.*, 2008)
$^{13}CH_3OH$, ^{13}C-methylamine	Coastal sea water	*Methylophaga*; novel *Gammaproteobacteria*	16S rRNA; *mxaF*	(Neufeld *et al.*, 2007c)
$^{13}CH_3OH$	Activated sludge	*Methylophilaceae*; *Hyphomicrobiaceae*	16S rRNA; *nirS*; *nirK*	(Osaka *et al.*, 2006)
^{13}C-labelled methanol, methylamine, formaldehyde, formate	Lake sediment	*Methylophylaceae*; *Sphingomonadales Methylophylaceae*; *Methylophylaceae*; *Holophaga /Geothrix Xanthomonadaceae*; *Holophaga /Geothrix*; *Gemmatimonadetes*	16S rRNA; *pmoA*; *fae*	(Nercessian *et al.*, 2005)
$^{13}CH_3OH$	Rice field soil	*Methylobacterium*; *Methylophilaceae*	16S rRNA	(Lueders *et al.*, 2004)
$^{13}CH_3OH$	Active sludge	*Methylophilaceae*	16S rRNA	(Ginige *et al.*, 2004)
$^{13}CH_3OH$	Forest soil	*Methylocella*; *Methylocapsa*; *Methylocystis*; *Rhodoblastus*; *Acidobacterium*	16S rRNA; *mxaF*	(Radajewski *et al.*, 2002)
$^{13}CH_3OH$	Forest soil	*Methylocella/Methylocapsa*; *Acidobacterium*-related	16S rRNA; *mxaF*	(Radajewski *et al.*, 2000)
. $^{13}CH_3Cl$	Soil	*Hyphomicrobium*; *Aminobacter*	*cmuA*	(Borodina *et al.*, 2005)
$^{13}CH_3Br$	Soil	*Burkholderia*	16S rRNA; *cmuA*	(Miller *et al.*, 2004)
$^{13}CH_3Cl$	Soil	*Rhodobacter*; *Lysobacter*; *Nocardioides*	16S rRNA; *cmuA*	(Miller *et al.*, 2004)

$^{13}K_2CO_3$	Lake sediment	*Nitrosomonas*	16S rRNA	(Whitby *et al.*, 2001)
$^{13}HCO_3^-$	Water sediment	*Nitrosomonas*; *Nitrospira*	16S rRNA	(Freitag *et al.*, 2006)
$^{15}N_2$	Soil	*Rhizobiales*; *Methylosinus*; *Methylocystis*; novel bacteria	*nifH*	(Buckley *et al.*, 2007a)
$^{15}N_2$	Soil	*Rhizobiales*; *Actinobacteria*; *Alphaproteobacteria*	16S rRNA, *nifH*	(Buckley *et al.*, 2007b)
$^{13}CO_2$	Plant root	Extremely diverse Bacteria and Fungi	16S rRNA; 18S rRNA	(Vandenkoornhuyse *et al.*, 2007)
$^{13}CO_2$	Rice root	*Azospirillum*; *Burkholderiaceae*; *Clostridia*; *Comamonas*	16S rRNA	(Lu *et al.*, 2006)
$^{13}CO_2$	Rice root	*Methanosarcinaceae*; rice cluster-1 Archaea; *Methanobacteriales*	16S rRNA	(Lu *et al.*, 2005)
$^{13}CO_2$	Rice soil	Rice cluster-1 Archaea; *Methanosarcineae*; *Methanomicrobiaceae*; *Methanosaetaceae*	16S rRNA	(Lu and Conrad, 2005)
$^{13}CO_2$	Upland grassland soil	*Sphingomonas*; *Mycobacterium*; *Sistotrema*; *Rhodotorula*	16S rRNA	(Rangel-Castro *et al.*, 2005)
^{13}C-acetate	Lake sediment	*Methanosaeta concilii*; *Rhodocyclales; Nitrosomonadales; 'Magnetobacterium bavaricum'*; *Thermodesulfovibrio yellowstonii*	16S rRNA	(Schwarz *et al.*, 2007)
^{13}C-acetate	Rice field soil	*Geobacter*; *Anaeromyxobacter*	16S rRNA	(Hori *et al.*, 2007)
^{13}C-acetate	Arsenic contaminated aquifer sediments	*Sulfurospirillum*; *Desulfotomaculum*; *Geobacter*	16S rRNA; *arrA*	(Lear *et al.*, 2007)
^{13}C-acetate	Soil	*Syntrophus*; *Propionibacterium*; *Geobacter*; *Methanosaeta*; *Methanosarcina*	16S rRNA	(Chauhan and Ogram, 2006)
^{13}C-acetate	Activated sludge	*Comamonadaceae*; *Rhodocyclaceae*; *Rhodobacteraceae*	16S rRNA; *nirS*; *nirK*	(Osaka *et al.*, 2006)
^{13}C-acetate	Activated sludge	*Comamonadaceae*; *Rhodocyclaceae*	16S rRNA	(Ginige *et al.*, 2005)
^{13}C-acetate	Groundwater	*δ-proteobacteria*, mainly *Geobacter*	16S rRNA	(Chang *et al.*, 2005)
^{13}C-pyrene	PAH[a]-contaminated soil	Uncultivated γ-*Proteobacteria*	16S rRNA	(Jones *et al.*, 2008)
^{13}C-acetate + perchloroethene	Pristine river sediment	*Dehalococcoides*	16S rRNA	(Kittelmann and Friedrich, 2008)
$^{13}C_6$-benzene	Coal gasification soil	*Deltaproteobacteria*; *Clostridia*; *Actinobacteria*	16S rRNA	(Kunapuli *et al.*, 2007)
^{13}C-polychlorinated biphenyls	Pine tree soil	*Pseudonocardia*; *Kirbella*; *Nocardiodes*; *Sphingomonas*	16S rRNA; ARHD[b]	(Leigh *et al.*, 2007)
^{13}C-phenanthrene,^{13}C-pyrene	PAH-contaminated soil	*Acidovorax*	16S rRNA	(Singleton *et al.*, 2007)

^{13}C-labelled 2,4-dichlorophenoxyacetic acid	Agriculture soil	*β-Proteobacteria* related to *Ramlibacter* (*Comamonadaceae*)	16S rRNA	(Cupples and Sims, 2007)
^{13}C-pyrene	bioreactor-treated soil	*Sphingomonas*; uncultivated β- and γ-*Proteobacteria*	16S rRNA	(Singleton *et al.*, 2006)
$^{13}C_6$-benzene	Gasoline contaminated groundwater	*Azoarcus*	16S rRNA	(Kasai *et al.*, 2006)
$^{12}C_6$ salicylate; ^{13}C naphthalene phenanthrene	Bioreactor treating PAH-contaminated soil	*Acidovorax*.; *Pseudomonas*; *Ralstonia*	16S rRNA	(Singleton *et al.*, 2005)
^{13}C-labelled naphthalene and glucose	Soil	*Acidovoras*; *Pseudomonas*; *Intrasporangium*	16S rRNA	(Yu and Chu, 2005)
$^{13}C_7$-benzoate	Marine sediment or contaminated sediment	-	*nosZ*	(Gallagher, 2005)
^{13}C-phenol	Activated sludge	*Acidovorax*	16S rRNA	(Manefield *et al.*, 2005)
^{13}C-pentachlorophenol	Pristine grassland soil	*Pseudomonas*; *Burkholderia*; *Sphingomonas*	16S rRNA	(Mahmood *et al.*, 2005)
$^{13}C_6$ naphthalene	Coal tar waste contaminated aquifer	*Polaromonas naphthalenivorans*	16S rRNA	(Jeon *et al.*, 2003)
^{13}C-phenol $^{13}C_6$ naphthalene ^{13}C-caffeine	Soil	*Pseudomonas, Pantoea, Acinetobacter, Enterobacter, Stenotrophomonas*; *Alcaligenes* *Pseudomonas, Acinetobacter*; *Variovorax* *Acinetobacter, Enterobacter, Stenotrophomonas*; *Pantoea*	16S rRNA	(Padmanabhan *et al.*, 2003)
^{13}C-phenol	Bioreactor	*Thauera*	16S rRNA	(Manefield *et al.*, 2002)

Lipid biomarkers and PLFA – SIP

Lipids found in cellular membrane with other biomarkers such as sterols, hopanoic acids, ether lipids can be used to differentiate organisms like bacteria, fungi and algae (Boschker and Middelburg, 2002). Phospholipids fatty acids (PLFAs) have been extensively used as microbial biomarker and are relatively easy to extract from natural samples. They are known to remain intact only in viable cells and by using them as biomarkers it can reflect rapid changes in the microbial population in the environment. Being a key component in microbial cell membrane they are known to respond to the changes in external and intra-cellular environment. Moreover, the rapid turnover of PLFAs in environment also allows us to identify microbes and detect any changes in microbial populations. However, one of the shortcomings of analyzing total microbial PLFAs from environment was that it offered a complex PLFA "fingerprint" (30-50 compounds) and was difficult to analyze particular contribution by a group of microbial population to the fingerprint profile (Boschker and Middelburg, 2002). Use of stable isotopes such as ^{13}C-CH_4 or acetate as substrates and subsequent analysis of the isotope labelled PLFA revealed the phylogentic identity of microbes actively utilizing the substrate. Boschker et al., (1998) demonstrated the first use of isotope labelling of PLFAs to demonstrate that Type I methanotrophs dominate methane oxidation at a freshwater site using ^{13}C-CH_4 as substrate. Moreover the authors also revealed that *Desulfotomaculum acetoxidans* was the predominant organism involved in the sulfur cycle in the estuarine and brackish sediments by using ^{13}C-acetate. Since then a large number of labelled substrates (such as bicarbonates, CO_2, toluene, propionate) in different environments has been used to understand the role of microbes in different environments (see review by Evershed et al., 2006).

One of the major contributions of PLFA-SIP is to link function with microbial diversity particularly with methanotrophic bacteria in natural environments. The presence of distinct PLFAs in methanotrophic bacteria allows us to differentiate between Type I (16 carbon fatty acids: 16:0, 16:1) and Type II (monosaturated 18 carbon fatty acids: 18:1ω9c, 18:1ω8) methanotrophs and from all other organisms (Hanson and Hanson, 1996). PLFA-SIP has been successfully used to investigate methanotroph population in sediments (Boschker et al., 1998), soils (Knief *et al.*, 2003; Chen *et al.*, 2008a; Maxfield *et al.*, 2008; Shrestha *et al.*, 2008), microbial mat (Blumenberg et al., 2005) and peat bogs (Raghoebarsing et al., 2005). Owing to the sensitivity of the PLFA-SIP technique (the need for low incorporation of labelled substrate), it has been a preferred tool to study high affinity methanotrophs which oxidize methane at atmospheric concentration (2ppmv) (Bull et al., 2000; Maxfield et al., 2006, 2008). Moreover, PLFA-SIP needs significantly lower amounts of stable isotope incorporation in the microbial biomass than DNA-and RNA-SIP. This eliminates any possibility of the "cross-feeding" phenomenon which potentially is one of the main drawbacks of DNA-SIP.

PLFA-SIP has also been extensively used to study plant-microorganisms interactions either in laboratory incubations or in situ studies using ^{13}C-CO_2 pulsing labelling of growing plants (Treonis et al., 2004; Prosser et al., 2006; Williams et al., 2006; Lu et al., 2007). A key limitation of PLFA-SIP technique is that even in well-characterised microbial groups such as methanotrophs and sulphate reducers with extensive PLFAs database, it is only possible to detect organisms at the genus level. Recent studies by Dedysh *et al*. (2007) and Dunfield *et al*. (2007) have revealed some discrepancies in the PLFAs present in methanotrophs. Dedysh *et al*. (2007) demonstrated that the Type II methanotroph *Methylocystis heyeri* contains 16:1ω8c

PLFA which was previously considered to be a signature PLFA for Type I methanotrophs. However, studies have overcome this limitation by combining PLFA-SIP with other complimentary techniques. Chen *et al*., (2008a) successfully combined PLFA-SIP and mRNA-based analysis to investigate the active methanotroph community structure in peatlands and moorlands under the plant cover of Sphagnum*/Eriphorum* and *Calluna* , respectively. By combining PLFA-SIP with mRNA based analysis, the authors were able to harness the sensitivity of the PLFA-SIP while getting a better taxonomic resolution with mRNA-based analysis. A recent study by Bodelier *et al*. (2009) performed a reanalysis of PLFAs as biomarkers for methanotrophs. The results from this study significantly enhanced the resolution Type II methanotroph PLFAs, which could result in taxonomic assignment with higher confidence.

Protein-based SIP

Identification of proteins involved in the metabolic process of interest offers a direct link to the molecular function. Moreover, proteins provide phylogenetic and functional information on the microbial communities. Jehmlich *et al.* (2008) developed and demonstrated the application of protein-SIP by studying toluene metabolizing bacterial community by the analysis of labelled proteins. As the use of isotope labelling expands with new substrates and novel environments, SIP in combination with other complimentary techniques will continue to provide new insights into microbial ecology.

FISH-MAR

FISH-MAR combines the use of radioactive labelling with FISH, allowing to link taxonomy with a particular microbial function. FISH-MAR can be applied to

study the ecophysiology of microorganisms of interest for which specific FISH probes can be designed or available. This technique can also be used to identify and quantify microorganisms involved in a particular function in the environment. FISH-MAR allows detection of organisms at the single cell level by the identification of 16S rRNA or 23S rRNA based probes (Wagner *et al.*, 2006). The use of FISH-MAR was demonstrated by Lee *et al.* (1999) and Ouverney and Fuhrman (1999). One of the disadvantages of FISH-MAR is that it is of limited use in certain environments such as soils. DNA-and RNA-SIP can be used as complementary techniques with FISH-MAR to offer different insights and options for the analyses of microbial communities (Wagner *et al.*, 2006; Neufeld *et al.*, 2007a). SIP and downstream analyses allow retrieval of 16S rRNA sequences from active microorganisms, which can be used to design FISH probes. These probes can be used for hybridisation and visualization of particular microorganisms in the environmental sample.

Isotope array

Isotope array offers a high throughput microbial screening method to detect active microorganisms in the environment. The principle of this technique involves the identification of radioactively labelled microorganisms using rRNA-targeted oligonucleotide probes in a microarray format (Adamczyk *et al.*, 2003). The obvious advantage of this method over SIP is its ability for direct detection of labelled RNA from active organisms, without any unlabelled background. Moreover, it is a PCR-independent method and hence avoids the potential biases associated with PCR (Wagner *et al.*, 2006). The effectiveness of this technique depends on the availability of suitable microarrays and their performance, particularly their specificity and sensitivity. Also the application depends on the sufficient extraction of RNA from the sample (Neufeld *et al.*, 2007a).

Nano-secondary ion mass spectrometry (Nano-SIMS)

Secondary ion mass spectrometry (SIMS) technique links high-resolution microscopy with isotopic analysis thereby providing spatially resolved information on the molecular and isotopic compositions of materials. This technique is used to analyze the composition of solid surfaces and thin films by sputtering the surface of the specimen with focused primary ion beam and analyzing the ejected secondary ions. The size of the ion beam (~10-15 mm) in SIMS used to sputter biomass and generate secondary ions exceeded the average diameter of a bacterial cell (~1 mm), thereby limiting its use in microbial sciences. The emergence of Nano-SIMS made it possible to determine the chemical, radioisotopic or stable isotopic composition of biomass at the sub-micron level (Lechene *et al.*, 2006; Kuypers, 2007; Lechene *et al.*, 2007; Li *et al.*, 2008). Lechene *et al.*(2007), with the use of NanoSIMS and $^{15}N_2$, showed that bacterial cells living in the gills of shipworms can fix N and provide the host with N source. Unlike FISH-MAR, stable isotopes (^{13}C and ^{15}N) can be used for analyses with NanoSIMS and the technique is much more sensitive than MAR (Lechene *et al.*, 2006). Recently Li *et al.* (2008) combined NanoSIMS with FISH to link microbial metabolic function with identity at the single cell level. However, the key drawback of this technique is the exorbitant cost of the equipment.

RAMAN microscopy and RAMAN-FISH

Raman microscopy examines the scattering of laser light by the chemical bonds of molecules within microbial cells. Recently (Huang *et al.*, 2007) combined SIP, FISH and Raman microscopy to link individual bacterial cells with the metabolism of a specific carbon source. Stable isotope incorporated into cell compounds exhibited key red-shifted peaks and the authors observed a good correlation between the shift

ratio and ^{13}C-content within the cell (**Figure 1.10**). Raman-FISH can track the incorporation of ^{13}C-label into multiple cell biomarkers and can enable direct analysis of ^{13}C-incorporation at a single cell level. However, it should be noted that sensitivity of Raman-FISH is not comparable to FISH-MAR owing to the use of stable isotope rather than radioactive isotopes and are currently limited to samples that can be analyzed by FISH (Huang *et al.*, 2007). The application of Raman spectroscopy and NanoSIMS in microbial ecology studies has been recently reviewed in Wagner (2009).

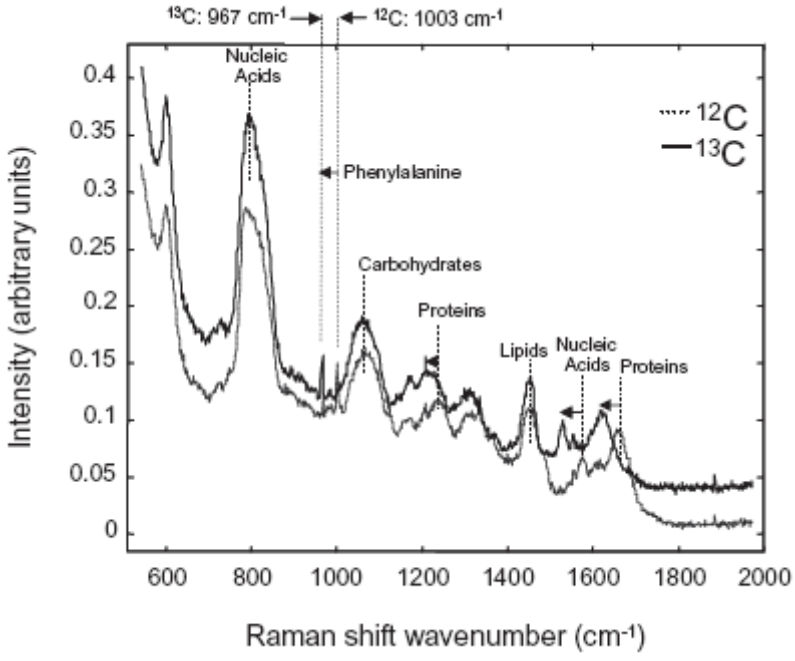

Figure 1.10 Raman spectra obtained from cells of *Pseudomonas fluorescens* SBW25 grown in media containing ^{12}C- and ^{13}C-glucose. Adapted from Huang *et al.*(2007).

Microfluidic digital PCR

Microfluidic digital PCR are performed on devices containing nano-scale components capable of handling low volumes (microliter to nanoliter) of liquids or gases. Owing to small reaction volumes, microfluidic systems are cost effective and

have the potential for automation and high throughput analysis. Microfluidic digital PCR is capable of performing multiple PCR of a segregate single bacterial cell in the microfluidic chamber and consequently allows one to link bacterial identity to certain genotypes (Ottesen *et al.*, 2006). Since MD-PCR facilitates direct access to multiple genes from a single organism from the environment, it has a great potential in the study of atmospheric methane oxidizers for which the known marker is the *pmoA* gene.

Metagenomics and genomics

"Metagenomics is the culture independent genomic analysis of microbial communities" (Handelsman, 2004) and has been applied to study uncultivated microbes in the environment. Recent developments in metagenomics have enabled entire operons to be cloned and sequenced from methanotrophs (Ricke *et al.*, 2005; Dumont *et al.*, 2006). Dumont *et al.* (2006) combined SIP with metagenomic analysis to retrieve an entire pMMO operon from a forest soil that has undergone SIP experiment using $^{13}CH_4$ as the substrate.

Recently the complete genome sequence of *Methylococcus capsulatus* Bath, was published and draft of the genome sequence of *Methylocella silvestris* BL2 has just become available. Joint Genome Institute is currently sequencing the genomes of two other methanotrophs, *Methylomicrobium album* BG8 and *Methylosinus trichosporium* OB3b (http://www.jgi.doe.gov/sequencing/why/99919.html). The availability of genomic and metagenomic sequences will provide information on the physiological mechanisms behind methane oxidation and how methane oxidation is regulated under different environmental conditions.

1.9 Methane Ecology (METHECO)

"The role of microbial diversity in the dynamics and stability of global methane consumption: microbial methane oxidation as a model system for microbial ecology (METHECO)" is one of the collaborative research projects (CRP's) funded by the European Science Foundation (ESF). Brief synopsis about the project is given below;

"Methane oxidising bacteria (MOB) and their functioning in a well defined set of environmental conditions and habitats will be used as a model system to understand basic questions in microbial ecology as well as to gain deeper insight into the function of this environmentally very important functional group. The overall aim of the CRP is to investigate how microbial and plant diversity influences the environmentally highly relevant process of methane oxidation and how changes in biodiversity affect this function and its resilience against environmental perturbations. Collecting a large dataset (diversity as well as function) in a highly standardised manner in a representative European set of habitats will yield a blue print for any future assessment or predictions of functional stability of microbial communities to environmental perturbation foreseen in the near future. Focusing on this ecosystem function and on this functional group of bacteria will make it possible to answer questions pertaining to both the role of biodiversity in stabilising and maintaining methane oxidation and to general issues of microbial diversity and ecology. The objectives of the CRP are to: (i) define meaningful taxonomic units which describe microbial diversity in a selected set of habitats (ii) assess the effects of natural and anthropogenic perturbations on diversity, functional stability, recovery and re-colonization of these habitats by MOB (iii) determine the role of plant and microbial (protozoa and non-MOB prokaryotes) diversity in the former objective (iv)

standardise methodology and interpretation in order to use the dataset obtained as a model for environmental microbial ecology."
(http://www.esf.org/activities/eurocores/programmes/eurodiversity/projects/metheco.html)

Standardised experimental methodologies were used across network laboratories in different ecosystems (**Figure 1.11**) to generate and interpret data relevant to methanotroph ecology. Based within the framework of the METHECO project, the following aims were investigated;

- To assess the effect of soil sample size on the assessment of methanotroph community structure using a *pmoA* based microarray (**Chapter 5**).
- To characterize the spatio-temporal distribution pattern of methanotroph populations and its relationship with abiotic parameters (**Chapter 6**).
- To identify whether there are any shifts in activity and functional diversity of methanotrophs when microniches created by soil aggregates are physically disturbed (**Chapter 7**).

Figure 1.11 Different ecosystem used to study methanotroph ecology across different network laboratories under the METHECO project.

1.10 Project Aims

The overall aims of this project were i) to understand the influence of earthworms on soil methane oxidation and methanotroph community structure and ii) to characterize the *in situ* spatial and temporal distribution pattern of methanotrophs and the relationship between abiotic parameters and methanotroph distribution.

Interactions between earthworm and methanotrophs

i. To assess the effect of earthworms on landfill cover soil methane oxidation and active bacterial community structure by using stable isotope probing.
ii. To use an experimental system reflecting *in situ* conditions in the landfill cover soil to study the effect of earthworms on spatial and temporal shifts in the active methanotroph populations by using a mRNA-based microarray.

***In situ* spatio-temporal distribution of methanotrophs**

i. To understand the effect of soil sample size in assessing methanotroph community structure using a *pmoA* microarray.
ii. To characterize *in situ* spatial and temporal distribution of methanotrophs in landfill cover soil and study the relationship between abiotic parameters (particularly total C, N, NH_4^+, NO_3^-, and water content) and the spatio-temporal distribution of methanotrophs.
iii. To identify whether there are any shifts in activity and functional diversity of methanotrophs when microniches created by soil aggregates are physically disturbed.
iv. To compare DNA-SIP and mRNA-based analysis to study the community structure of active methanotrophs by using a *pmoA* microarray.

Chapter 2

Materials and Methods

2.1 Landfill sampling site, soil collection and soil analysis

Landfill cover soil samples were collected from a local landfill site in Ufton, UK (latitude 52° 15' 0 N; longitude 1° 25' 60 W). The vegetation above the cover soil, predominantly grass, was cleared before collecting soil samples. Soil samples were collected up to a depth of 30cm and homogenised. Soil samples were air-dried (for 48 hours at room temperature), sieved (2-mm mesh) and subsequently stored at 4^{o}C until further use. Moisture content for both bulk and sieved soil was determined gravimetrically by drying soil samples at 80^{o}C until constant soil weight was observed. The pH of the soil was measured with soil 1g of soil was suspended in either 10ml of water and the pH of the suspension was measured using a pH meter (Hanna Instruments) (Chen *et al.*, 2008a). For experiments involving the study on effect of earthworms on soil methane oxidation, soil samples were used 2-3 weeks after collection, so that there was no residual effect from *in situ* earthworms.

Soil analyses were carried out at Macaulay Land Use Research Institute (MLURI), Aberdeen. Ammonia and nitrate were analysed following extraction in 1M KCl, with a detection limit of 0.10 mg NH_4^+ kg $soil^{-1}$ and 0.07 mg NO_3^- kg $soil^{-1}$. Total carbon and nitrogen were determined using Dumas combustion with a detection limit of 0.02% C/w and 0.03% N/w, based on 15 mg soil sample.

2.2 Soil methane oxidation potential assessment

Assessment of soil methane oxidation potential was carried out with 5g of soil sub-samples in 120 ml serum vial bottles. Sterile water was added to the soil to restore the original soil moisture content at the time of sampling. The bottles were sealed using butyl rubber septa and aluminium crimps. The soil samples were

allowed to equilibrate in the dark for 24 hours at 20^{o}C, before injecting methane (1% - 2% v/v) into the headspace. Methane oxidation potentials were determined by measuring the decrease in the headspace methane concentration at regular intervals using a Pye Unicam series 204 gas chromatograph. The soil methane oxidation experiments were performed in triplicate.

2.3 Nucleic acid extraction

DNA extraction from soils using kit

Soil DNA extraction was carried out using the Bio 101 FastDNA spin kit (QBiogene) according to the instruction manual.

DNA and RNA co-extraction from soils and RNA purification

DNA and RNA was directly co- extracted from soil samples following the method described by Burgmann *et al*. (2003) and Griffiths *et al*. (2000) modified by Chen *et al*. (2007). Briefly, 0.4 g of soil sample and 1.0 ml of extraction buffer (Appendix 1) in a lysing matrix E tube were processed in a FastPrep Ribolyser (QBiogene) for 45 s at 6 m/s. After bead beating, samples were put on ice for 5 min followed by centrifugation at 15 000 x g for 5 min. After centrifugation, 800 µl of the supernatant was extracted with 750 µl of a 1:1 mixture of phenol (pH 8.0) and chloroform–isoamyl alcohol (24:1) and again with 750 µl of chloroform–isoamyl alcohol. Total nucleic acid was precipitated for 1 h at 20^{o}C with 850 µl of RNase-free PEG solution (20% polyethylene glycol, 2.5M NaCl). After centrifugation (15 000 x g) for 30 min, the nucleic acid pellets were washed once with cold ethanol (70% v/v) which was followed by another centrifugation at 15 000 x g for 20 min. After centrifugation, the pellets were air dried at room temperature for 10 min and

then dissolved in 50 µl of diethyl pyrocarbonate (DEPC) - treated water. At this stage, the quality of nucleic acid was determined on a 1% (w/v) agarose gel.

DNA was separated from RNA using a Qiagen RNA/DNA mini kit (Qiagen) according to manufacturer's instruction and quantified using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE, USA). In order to remove any traces of DNA in RNA, total RNA was treated with 4U of DNase I (New England Biolabs Inc., Ipswich, MA, USA) at 37^{o}C for 2 h and then purified using a Qiagen RNeasy mini kit (Qiagen). RNA was quantified using a Nanodrop spectrophotometer (NanoDrop) and was confirmed to be DNA-free by amplification of 16S rRNA genes with universal primers 27f/907r (Lane, 1991; Muyzer *et al.*, 1993) (94^{o}C 1 min, 60 ^{o}C 1 min, 72 ^{o}C 1 min, 35 cycles).

2.4 Nucleic acid manipulation

Quantification of DNA and RNA

A Nanodrop spectrophotometer (NanoDrop) was used to quantify the concentration of DNA and RNA after extraction from soil samples.

Agarose gel electrophoresis

Agarose (Gibco BRL) gels were made and run in 1 × TBE using Flowgen Minigel Systems (Flowgen). For analyses of PCR products, 1 % (w/v) agarose gels in 1xTBE buffer were used. For analyzing RFLP patterns, DNA was visualised on a 2 % (w/v) agarose gel in order to resolve smaller DNA bands. Ethidium bromide (EtBr) was added to the gel at a final concentration of 0.5 µg/ml. Gel images were recorded with a gel documentation system (Gene genius®, Syngene, UK).

2.5 Restriction Fragment Length Polymorphism (RFLP)

Digestion of DNA and PCR products with restriction endonuclease was carried out according to the manufacturer's instructions (Fermentas, UK). Restriction digests were carried out with 10 – 100 ng of DNA/PCR product in a final volume of 10 µl, containing 5U of restriction enzymes and 1X digestion buffer. *Eco*RI enzyme was used to cut the plasmids and check that the inserted gene was of the correct size, and further digested with two different sets of enzymes to resolve different patterns, *Eco*RI/*Hinc*II/*Pvu* II for *pmoA* and *Eco*RI/*Hinc*II for *mmoX*. Digests were resolved on a 2% (w/v) agarose gel and grouped into OTUs (Operational Taxonomic Units) based on the restriction pattern. Representative clones from each OTU were sequenced uni-directionally with reverse primers to obtain their phylogenetic identity.

2.6 Polymerase Chain Reaction (PCR)

PCR amplification of target genes was performed in a total volume of 50 µl using a Tetrad thermocycler (Bio-Rad). Each PCR reaction contained 1× reaction buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 5 to 50 ng of DNA template and 2.5 units of Taq DNA polymerase (Fermentas, UK). A list of primers used in this study is given in table 2.1

Table 2.1 List of primers used in this study

Primers	Sequences (5'-3')	Target	References
27F	AGAGTTTGATCMTGGCTCAG	16S rRNA gene	(Lane, 1991)
907R	CCGTCAATTCMTTTGAGTTT	16S rRNA gene	(Lane, 1991)
341F_GC*	CCTACGGGAGGCAGCAG	16S rRNA gene	(Muyzer *et al.*, 1993)
518F_GC*	CCAGCAGCCGCGGTAAT	16S rRNA gene	(Muyzer *et al.*, 1993)
Type IF	ATGCTTAACACATGCAAGTCG AACG	16S rRNA of type I methanotrophs	(Chen *et al.*, 2007)
Type IR	CCACTGGTGTTCCTTCMGAT	16S rRNA of type I methanotrophs	(Chen *et al.*, 2007)
Type IIF	GGGAMGATAATGACGGTACC WGGA	16S rRNA of type II methanotrophs	(Chen *et al.*, 2007)
Type IIR#	GTCAARAGCTGGTAAGGTTC	16S rRNA of type II methanotrophs	(Chen *et al.*, 2007)
A189	GGNGACTGGGACTTCTGG	*amoA*/*pmoA*	(Holmes *et al.*, 1995b)
A682+	GAASGCNGAGAAGAASGC	*amoA*/*pmoA*	(Holmes *et al.*, 1995b)
mb661+	CCGGMGCAACGTCYTTACC	*pmoA*	(Costello and Lidstrom, 1999)
206F	ATCGCBAARGAATAYGCSCG	*mmoX*	(Hutchens *et al.*, 2004)
886R	ACCCANGGCTCGACYTTGAA	*mmoX*	(Hutchens *et al.*, 2004)

*a GC clamp was attached to the 5' end of the sequences:

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

This probe has one mismatch with Am 976 (Gulledge *et al*., 2001).
+ T7 promoter (TAATAC GACTCACTATAG) was added to this primer for when used for *pmoA* microarray analysis

2.7 Reverse transcription

SuperScript™ II Reverse Transcriptase kit (Invitrogen) was used to perform all the reverse transcription reactions. The reactions were carried out in a final volume of 12 µl containing 100 – 500 ng of purified RNA, 50 pmol gene-specific reverse primer and 1 µl of dNTP mix (10 mM each). This mixture was heated to 65°C for 5 min and then transferred to ice. To this reaction mixture, 4 µl of 5 x first-strand buffer, 2 µl 0.1M DL-1, 4-dithiothrethiol (DTT) and 1 µl (200 units) of SuperScript II reverse transcriptase were added to give the final volume of 20 µl. This mixture was then incubated at 42°C for 50 min, followed by 15 min incubation at 70°C to deactivate the RT enzyme. The resultant cDNA was used as a template (1 – 5 µl) for PCR amplification.

2.8 Cloning PCR products

The pCR2.1 TOPO TA cloning kit (Invitrogen) was used for cloning purified PCR products, according to the instruction manual.

2.9 DNA sequencing

DNA sequencing was carried out at University of Warwick molecular biology service using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The DNA sequences were analyzed using a 3730A (PE Applied Biosystems) automated sequencing system.

2.10 Phylogenetic analysis

Sequences were aligned to related sequences extracted from GenBank using MEGA version 3.1 (Kumar *et al.*, 2004). MEGA 3.1 was also used to estimate

evolutionary distances and to construct a phylogenetic tree. Several methods were used to construct trees: for *pmoA* and *mmoX* sequences, neighbour-joining with Kimura correction and maximum parsimony based on nucleotide sequence analysis, and neighbour joining with Poisson correction and maximum parsimony based on amino-acid derived sequences analysis; for 16S rRNA gene phylogeny, neighbour joining with Kimura correction and maximum parsimony. For all the genes, the different methods tested gave similar results. (Phylogenetic analyses was performed by Dr Marina Hery)

2.11 Denaturing Gradient Gel Electrophoresis (DGGE)

DCode^{TM} DGGE system (Bio-Rad) was used to analyze the difference in microbial community structure using amplified 16S rRNA PCR products with a GC clamp. DGGE gels were prepared at 6% - 10% (w/v) polyacrylamide with a denaturing gradient from 30% to 70% with 100% denaturant corresponding to 7.0M urea and 40% Ultrapure^{TM} formamide (Invitrogen). The gels were run for 14h at 85 V and at a constant temperature of 60^{o}C in 1X TAE buffer. 1:50,000 (v/v) SYBR green in 1 x TAE was used to stain the DGGE gels for 1 h before photographing using a FLA-5000 scanner (Fujifilm).

Bands of interest were excised by puncturing the gel with sterile P10 pipette tips. The tips were placed in 20 µl of sterile de-ionized water, usually in 1.5 ml microcentrifuge tubes, and incubated overnight at 4^{o}C. Re-amplification of the PCR product was done using 2-5 µl of the above solution, and the PCR products run again in a DGGE gel, along with the original samples, to check the band size. PCR products were then purified using a QIAquick gel extraction kit (Qiagen) before sequencing and identifying the phylogenetic affiliation of 16S rRNA genes.

2.12 Stable isotope probing (SIP)

DNA-SIP

DNA SIP was performed as described by Neufeld *et al*. (2007d) and Lueders *et al*. (2004). In brief, soil samples (5g) were incubated with 1% $^{13}CH_4$ (v/v) in a 120ml serum vial with the headspace methane concentration regularly monitored by GC measurements. After methane was consumed, the serum vials were opened to replenish oxygen and also to prevent CO_2 build up. After sufficient labelling, DNA was extracted from the soil using the methods described above. Total extracted DNA (~ two to four µg DNA) was added to caesium chloride solutions for density-gradient ultracentrifugation and subsequently gradient fractionation performed according to Neufeld *et al*. (2007d). The density of the caesium chloride gradient was measured using a digital refractometer (Reichert AR2000, Reichert Analytical Instruments). Fractionation by means of a pump (Watson Marlow Ltd, Cat. No. 101U/R) yielded 12 fractions of 400 µl from which the purified DNA was suspended in 50 µl of nuclease free water. The DNA from all of the fractions was then used as a template for amplification of 16S rRNA gene by PCR with 341F-GC/907R primers (Muyzer *et al.*, 1993) to confirm the presence of DNA in different fractions.

RNA-SIP

After sufficient labelling of $^{13}CH_4$, RNA was extracted according to the method described above. RNA SIP was then performed on total purified RNA as described by Whiteley *et al*. (2007). Purified RNA (500 ng) was added to caesium trifluoroacetate solution with a mean density of 1.825 g ml^{-1} for density gradient ultracentrifugation. After centrifugation, gradients were fractionated according to Neufeld *et al*. (2007d) resulting in 12 fractions of 400 µl for which the density was

checked using the digital refractrometer. The RNA from these fractions was precipitated using an equal volume of isopropanol and 3 µl of glycogen (20 mg ml^{-1}, Roche). 50 µl of nuclease-free water was used to resuspend the RNA in each fraction. Five µl of RNA sample were used for cDNA synthesis using the reverse primer 1492R as described in 2.4.6. Subsequent PCR amplification of 16S rRNA genes for DGGE analysis was done with bacterial 16S rRNA gene primers 341F-GC/907R (Muyzer *et al.*, 1993).

2.13 *pmoA* microarray

The microarray targeting the *pmoA* gene in methanotrophs was first developed by Bodrossy *et al*. (2003). This array encompasses probes that targets all known methanotrophs with a detection limit of 5% in a given DNA sample The *pmoA* microarray analyses in this study were performed as described by Stralis-Pavese *et al. (2004).* Oligonucleotide probes in the *pmoA* microarray are listed in Appendix 2.

PCR for *pmoA* microarray

pmoA genes were amplified from DNA using the primer set A189f/T7-mb661r (Bodrossy *et al.*, 2003). The T7 promoter attached to the 5' end of the reverse primer allows the T7 RNA polymerase to transcribe the DNA templates into RNA *in vitro*. PCR for each sample was carried out in triplicate. Reaction products were then combined and purified using a QIAquick spin column (Qiagen).

In vitro transcription of DNA

The reaction mix for *in vitro* transcription was prepared under RNAse free conditions. The final reaction volume of 20 µl contained 1 × T7 RNA polymerase buffer, 10 mM DTT, 20 U Rnasin (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.25 mM UTP, 1mM Cy3-UTP, 40 U T7 RNA polymerase (Gibco, BRL) and 350 ng of purified *pmoA* PCR product. The reactions were incubated at 37 °C for four hours. The resulting RNA from *in vitro* transcription was then purified using the Qiagen RNeasy kit. Purified RNA was eluted in 50 µl of DEPC-treated water and RNA concentration was determined using a Nano drop spectrophotometer.

Fragmentation of RNA

Purified RNA from *in vitro* transcription was incubated at 60°C for 30min with 10mM $ZnCl_2$ and 20 mM Tris-HCl (pH 7.4). The fragmentation reaction was stopped by the addition of EDTA (10mM, pH 8.0) and placing the reaction on ice. 40 U of RNAsin (Promega) was added to the fragmented target and the labelled RNA was stored at -20°C.

Hybridisation of RNA to microarrays

Hybridisation was carried out using a custom-made aluminium block that was used as an insert on a temperature controlled Belly Dancer (Stovall Life sciences, Greensboro, NC). The Belly Dancer was set at maximum inclination and the hybridisation block was preheated to 55°C for ~30minutes. Slides with pre-spotted *pmoA* probes were assembled with 200 µl HybriWell stick on hybridisation chambers (Grace Biolabs) and preheated on the hybridisation block. Meanwhile an incubator for microcentrifuge tubes was also heated to 65 °C and hybridisation

mixtures were preheated for 15 min. Hybridisation mixtures had a final volume of 200 µl, containing 0.01% SDS, 1 X Denhardt's reagent (Sigma), 6 × SSC, 124 µl DEPC-treated water and 10 µl target RNA. Preheated hybridisation mixture was added to each slide via the open port and incubated overnight at 55 °C at 30-40 rpm circulation and maximum bending in the Belly Dancer.

After overnight hybridisation the HybriWell chambers were removed, and the slides were immediately immersed and washed in 2 × SSC, 0.1 % (w/v) SDS at 20°C for 5min. The slides were then washed twice for 5 min, shaking at 20°C using 0.2 × SSC, 0.1 % (w/v) SDS and finally with 0.1 x SSC (5 min; shaking). Each slide was then dried individually using an airgun containing a cotton filter before storing in the dark at room temperature. Hybridised slides were scanned at 10 µm resolution with a GenePix 4000 laser scanner (Axon, Foster City, CA) at a wavelength of 532 nm. Results of individual microarrays were normalised and displayed using GeneSpring software (Agilent).

mRNA-based *pmoA* microarray analysis

mRNA based microarray analysis was performed as described by Bodrossy et al., (Bodrossy *et al.*, 2006). RNA from soil samples was extracted according the protocol described in section 2.3.2. Target generation for mRNA microarray was performed with 100 – 200ng of environmental RNA by generating cDNA using reverse primer mb661 as described in section 2.4.6. The resulting cDNA was used for PCR amplification with *pmoA* primers and subsequently used for microarray hybridisation.

2.14 Methane ecology (METHECO) project protocols

One of the core objectives of the METHECO project was to attempt and create common methodologies across different laboratories to assess methanotroph diversity in various habitats. A detailed description of the METHECO project and its objectives are given in Chapter 1.

Soil methane oxidation potential

Soil methane oxidation potential assessment was carried out for soil samples from different depths between 0-30cm at 5cm intervals with 1% (v/v) initial methane concentration as described by Knief *et al.*(2003). Briefly, 10 g of sieved soil samples were incubated with 1% CH_4 (v/v) in a 120 ml serum vial and the headspace methane concentration was measured at regular intervals using a GC.

Soil microbial community DNA extraction

In order to follow a common DNA extraction protocol across different laboratories, a modified DNA extraction protocol (by Bodrossy L and Siljanen H; unpublished) based on Yeates *et al.*(1998) was used to extract DNA from soil for experiments under the METHECO project. Briefly, 0.3 g freeze-dried and pre-homogenised soil (prepared in liquid nitrogen with a pestle and mortar) was added in a Multimix FastPrep tube (Lysing matrix E, FastDNA SPIN kit for soil). 780µl of lysis buffer (appendix 1) was added to the soil in the FastPrep tube and incubated at 37°C for 30 minutes. After incubation, 122 µl of MT buffer (FastDNA SPIN kit for soil) was added and the tubes were shaken in the FastPrep instrument for 30 sec at 5.5 m/s. The supernatant (700 µl) was collected from the tubes after centrifugation for 15 minutes at 10,000 rpm. 5 µl of 10 mg/ml fresh proteinase K added, followed

by incubation at 65°C for 30 min. After incubation, 300µl of phenol was added to each tube and vortexed for a few seconds followed by the addition of 300µl of Chloroform Isoamyl Alcohol (24:1). After brief vortexing to mix, the tubes were centrifuged for 5 minutes at 13,000 rpm. The supernatant was collected and extracted with 600 µl of Chloroform Isoamyl Alcohol (24:1). After extraction, 125 µl of 7.5 M potassium-acetate was added to the supernatent, incubated on ice for 5 min, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was then transferred to a new microcentrifuge tube followed by the addition of 700 µl of binding matrix (FastDNA SPIN kit for soil). The tubes were then placed on a rotator for 5 min to facilitate DNA binding, followed by centrifugation at 10,000 rpm for one min with the resulting supernatant discarded. The pellet was re-suspended in 500 µl of Wash Buffer and added into a spinfilter provided in the FastDNA SPIN kit and centrifuged for one minute at 10,000 rpm. After centrifugation, the eluate was discarded and the wash step was repeated. After the second wash step the tube was centrifuged for another 10 sec to completely dry the spinfilter. The spinfilter was then placed into a new collection tube, 50 µl of TE pH 8.0, added to the centre of the spin filter and incubated at 20^{o}C for a minute. The spin filter was then centrifuged for three minutes at 10,000 rpm to collect the DNA in the collection tube.

Comparison of *pmoA* primers and PCR strategy for microarray analysis

pmoA primer sets A189f – A682r and A189f-mb661r were compared for their coverage of methanotroph diversity as assessed by the *pmoA* microarray in this particular landfill cover soil. Also, a nested approach using a first round of PCR with primer pair A189-A682 and a second round of PCR with primer pair A189-mb661

was tested and compared for coverage of methanotroph diversity against direct PCR.

The details of the PCR reaction are given below:

Direct PCR: A189f -T7-A682r **or** A189f - T7-mb661

94°C 5 min.

94°C 1 min.
65°C 1 min.
72°C 1 min.
} Annealing temperature reduced by 1°C 11 cycles from 65 °C to 55 °C

94°C 1 min.
55°C 1 min.
72°C 1 min.
} 24 more cycles

72°C 10 min.

4°C pause

Total: 35 cycles

Nested PCR

35 cycles with A189f + T7-A682r, followed by 25 cycles with A189f + T7-mb661r.

94°C 5 min.

94°C 1 min.
65°C 1 min.
72°C 1 min.
} Annealing temperature reduced by 1°C 11 cycles from 65 °C to 55 °C

94°C 1 min.
55°C 1 min.
72°C 1 min.
} 14 more cycles

72°C 10 min.

4°C pause

Total: 25 Cycles

Strategy for *pmoA* microarray analysis

The scheme for *pmoA* microarray analysis of soil DNA samples for the METHECO project experiments are as described in **Figure 2.1**. Briefly, for each soil sample, DNA was extracted from three replicates, and PCR of *pmoA* was performed on each of the individual replicates. The individual PCR products were then pooled and used for microarray hybridisation experiments.

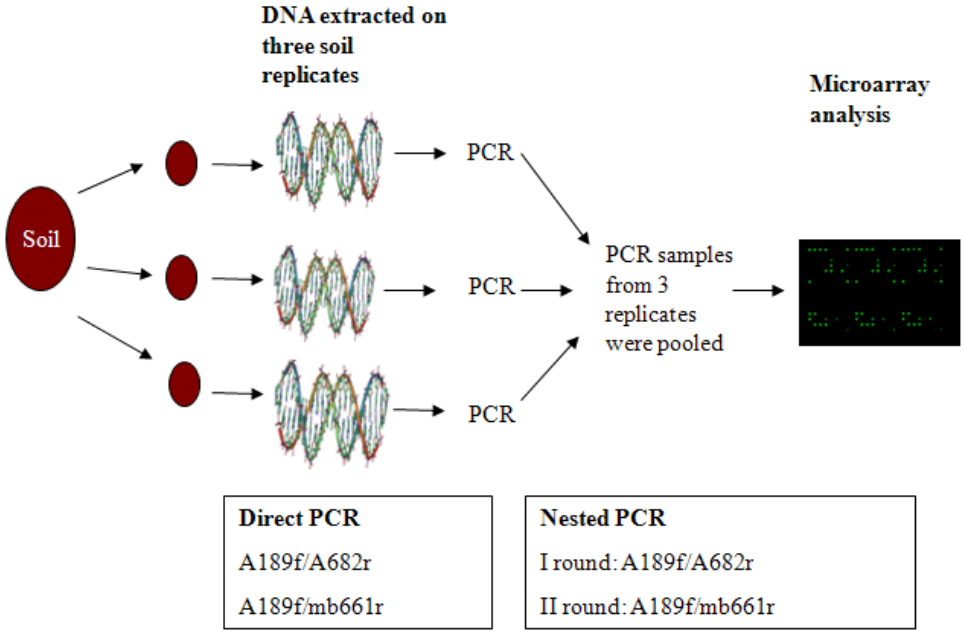

Figure 2.1 Schematic representation of DNA extraction and PCR strategy for microarray analysis

Statistical analysis

Microarray data used for statistical analysis comprised all probe signals with the exception of positive controls, universal probes and higher level probes. No negative values were included in the analysis. The multivariate analyses were conducted using the software Primer 6 (PRIMER-E Ltd, Plymouth UK). Standardized probe intensities were used for all analyses. Bray-Curtis similarity

metric was calculated using standardized data for samples (Kenkel and Orlóci, 1986; Minchin, 1987). All multivariate statistical tests were tested at $\alpha = 0.05$.

Univariate analysis of variance was tested at P<0.05%. For ANOVA, significant factors were then compared using Tukey *post-hoc* test. All tests were conducted at $\alpha = 0.05$. Correlation between environmental parameters and array probe signals were analysed using Pearsons product moment correlation in the SPSS software package (SPSS Inc., USA). Data that were not normally distributed were transformed (square root or Log). Positive and negative relationships are represented by positive and negative values. The values are arranged in a descending order and matched to their corresponding probes. The colours in the probe columns correspond to specific methanotroph groups. The probability for significance is P<0.1%. (Statistical analysis for chapter 5 was performed by Dr Paul Bodelier and for chapters 6 and 7 were performed by Dr Guy Abell)

Chapter 3

Effect of earthworms on the community structure of active methanotrophs in a landfill cover soil

3.1 Introduction

Landfills are a major anthropogenic source of methane (CH_4) and are estimated to contribute about 6 – 12% of global methane emissions to the atmosphere (Lelieveld et al., 1998). Engineering solutions such as landfill gas extraction systems have been used in new landfill sites to collect and recover methane before it is emitted into the atmosphere. However in old landfills, without gas extraction systems, methanotrophs present in the cover soils can oxidize methane, forming biomass and CO_2. It is estimated that about 22 Tg of CH_4 $year^{-1}$ is oxidized in the landfill cover soils (Reeburgh, 1996). Previous studies have reported high methane oxidation capacities in landfill cover soils (Whalen et al., 1990; Kightley et al., 1995; Borjesson et al., 1998; Streese and Stegmann, 2003) and have also focussed on the effect of environmental parameters that could affect the methane oxidation process (Jones and Nedwell, 1993; Chan and Parkin, 2000; Borjesson *et al.*, 2004; Scheutz and Kjeldsen, 2004).

Charles Darwin originally highlighted the role of earthworms in soil formation and the earthworm has been one of the most studied invertebrates for its impact on soil ecosystems. Earthworms as “soil engineers” exert a significant impact on soil properties (Needham, 1957; Binet and Trehen, 1992) and microbial function in soils through activities such as burrowing, casting and excretion of organic and nitrogenous compounds (Daniel and Anderson, 1992; Binet and Le Bayon, 1998; Haynes et al., 2003). Singer *et al.* (2001a) observed enhanced methane oxidation in earthworm-added soil while using methane as a tracer gas for a study on the impact of earthworms in soil PCB degradation. The study also demonstrated that earthworm-added soil had a homogenous PCB degradation profile compared to soil

without earthworms, possibly due to the bio-turbation effect of earthworms. Further studies, with two different earthworms, *Lumbricus rubellus* and *Eisenia veneta* in neutral pH soils demonstrated increased methane oxidation potential with earthworm-added soils (Singer A, unpublished). However, little was known about the influence of earthworms on functional diversity of methane oxidizing bacteria. Therefore, there was a need for better understanding of the underlying mechanism behind earthworm-enhanced soil methane oxidation and its effect on the bacterial community structure involved in methane oxidation. The aims of this study were

i. To identify the active methanotroph community in Ufton landfill cover soil using stable isotope probing.
ii. To assess the effect of earthworms on landfill cover soil methane oxidation and its effect on the community structure of active methanotrophs.

3.2 Soil sampling and microcosm set-up

Landfill cover soil were collected from a local landfill site in Ufton, UK (latitude 52° 15' 0 N; longitude 1° 25' 60 W). The vegetation, predominantly grass above the cover soil, was cleared before collecting soil samples. Soil samples were collected at a depth of 10 - 20 cm for this study. Soil was stored at 4^{o}C for one week before using it for experiments.

Figure 3.1 Soil sample collection at Ufton landfill

Soil earthworm-incubations were performed in plastic boxes (11 x 17 x 6 cm) with approximately 540g of air-dried, sieved (4 mm mesh size) landfill cover soil. The soil was maintained at 70% of its water–holding capacity (with 250 ml of de-ionized water) for 2-3 days before the addition of earthworms. Earthworms (*Eisenia veneta*) (Wormsdirect, UK) were incubated in Petri plates for 2 - 3 days to evacuate their gut. Three earthworms (1.9 +/- 0.2 g) were added per microcosm along with "no earthworm microcosms" as control. The microcosms were incubated at 19 ^{o}C for 17 days before sampling the soil for methane oxidation assessments. Methane

oxidation assessments were performed as described in Chapter 2, with 2% ^{13}C-CH_4 (v/v) in the headspace and incubated for 7 days at 19 ^{o}C. After methane incubations, soil samples were stored at -80 ^{o}C until molecular biology analysis was carried out. Methane oxidation potential assessments were carried out with seven replicates.

3.3 Results

3.3.1 Effect of earthworms on soil methane oxidation and physicochemical characteristics

Soil methane oxidation potentials were significantly higher in earthworm-incubated soil microcosm (+worms) than in microcosm without earthworms (control) (Fig 3.2). After 168h of methane incubation, +worms soil samples oxidized 67% ± 5% of added methane whereas control soil samples oxidized 52% ± 7% of added methane.

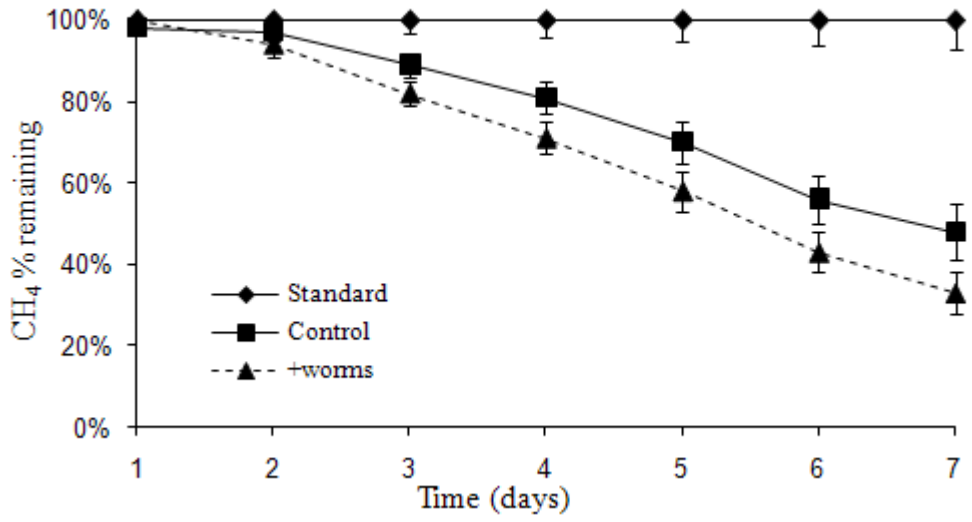

Figure 3.2 Comparison of methane oxidation in soil sub-samples from +worms and control soil microcosms. Standard indicates methane standards. Error bars represent standard error of seven replicates. (Methane oxidation potential assessments were carried out by Dr Andrew Singer)

Earthworm addition had an influence on soil composition, with higher silt and clay content being found in +worms soil compared to control soil. Although, similar total C and N contents were observed for control and +worms soil, differences were observed in NH_4^+ and NO_3^- content, with higher NH_4^+ in the control soil and higher NO_3^- in the +worms soil (Table 3.1).

Table 3.1 Comparison of soil physicochemical characteristics

	Particle size distribution (%)			**Chemical Analysis**			
	Clay	Silt	Sand	Total N (%/W)	Total C (%/W)	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})
Control	12	40	48	0.12	3.92	62.8	24.0
+Worms	16	49	35	0.16	3.89	23.4	71.1

3.3.2 Stable isotope probing analyses

Four soil replicates out of the seven soil replicates used for methane oxidation assessments were selected randomly for molecular biology analysis. The reproducibility of the four replicates for molecular biology analysis was confirmed by *pmoA* microarray analysis (Fig 3.3). Further SIP experiments and molecular biology analyses were performed on these four pooled samples. DNA-SIP was performed as described by Neufeld *et al*. (2007d) with DNA extracted from control and +worms samples. A control gradient containing ^{12}C- and ^{13}C- labelled *Methylococcus capsulatus* (Bath) was used in DNA-SIP procedures to confirm good separation of light and heavy DNA. After ultra-centrifugation, heavy and light DNA bands were visualized under UV and if the heavy DNA was not visible, its position

was determined in comparison to the control gradient containing *M. capsulatus* DNA. RNA-SIP experiment was performed as described by Héry *et al.* (2008) After centrifugation and fractionation, the densities of heavy and light RNA fractions were 1.80 and 1.77 g ml^{-1}, respectively.

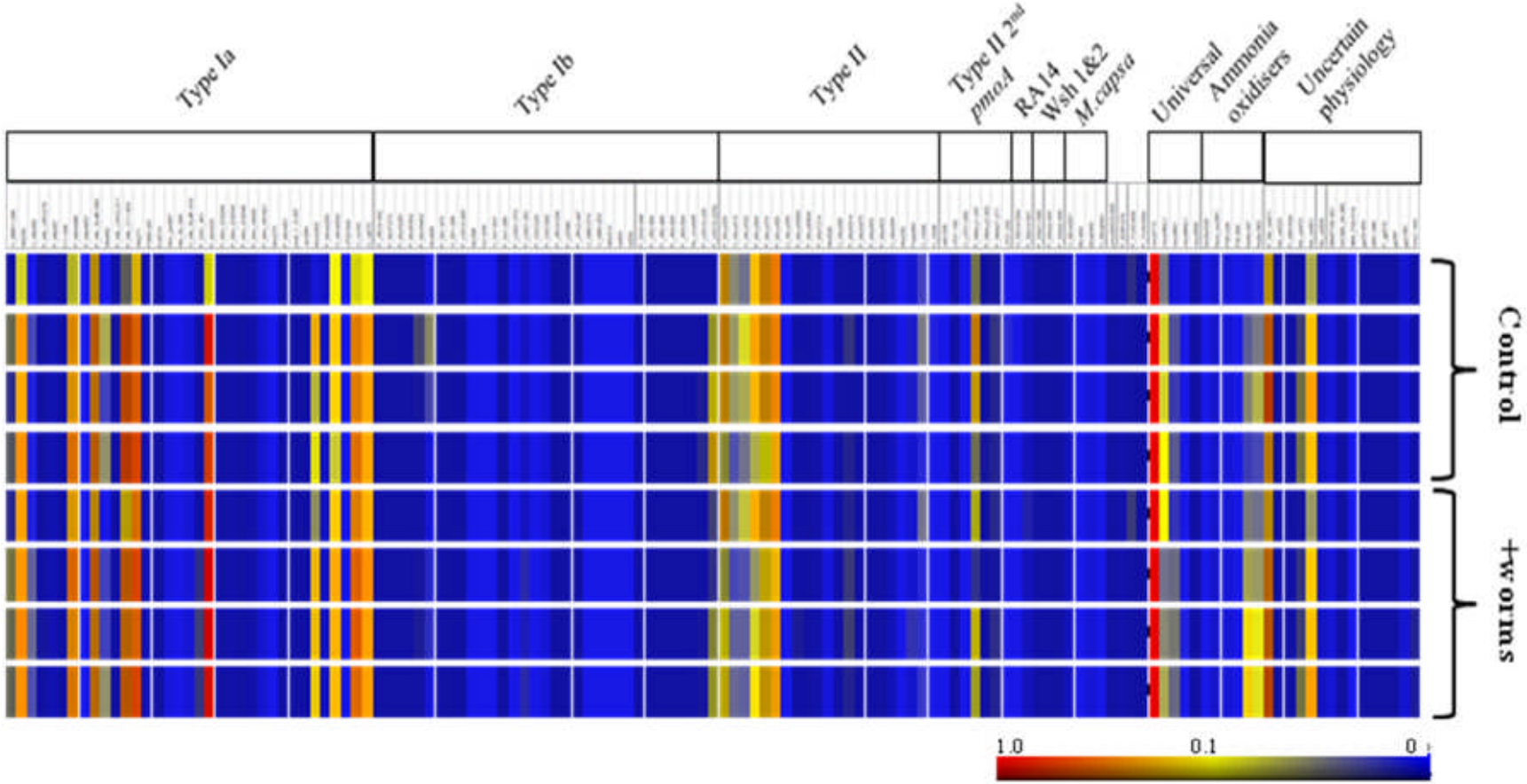

Figure 3.3 *pmoA* microarray analysis of methanotroph community structure based on DNA extracted from four replicates of control and +worms soil samples. The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to the probe.

3.3.3 Bacterial community structure analysis based on 16S rRNA genes

Bacterial community structure in control and +worms samples (both DNA and RNA) were compared using DGGE analysis with universal bacterial primers (341f-GC/907r) targeting the 16S rRNA gene. DGGE analysis of ^{12}C-DNA and RNA revealed complex but similar community profiles for both control and +worms samples (Fig 3.4). However, ^{13}C-DNA and RNA 16S rRNA gene DGGE profiles were dominated by few intense bands with two dominant bands present in both control and +worms samples and one dominant band specific to +worms samples. The dominant bands from both ^{13}C-DNA and RNA16S rRNA gene DGGE profiles were excised, sequenced and phylogenetic affiliations were determined. Sequences from two dominant bands common to both control and +worms samples were related to *Methylobacter*- and *Methylosarcina*- (Wise et al., 2001) related 16S rRNA gene sequences, both belonging to Type I methanotrophs (Fig 3.5). The sequence corresponding to the specific band in the +worms ^{13}C-DNA and RNA profiles were related to a *Bacteroidetes*-related 16S rRNA gene sequence. All three sequences 16S rRNA gene from the DGGE profiling with DNA shared 100% identity to the sequences from RNA DGGE profiles (Fig 3.5).

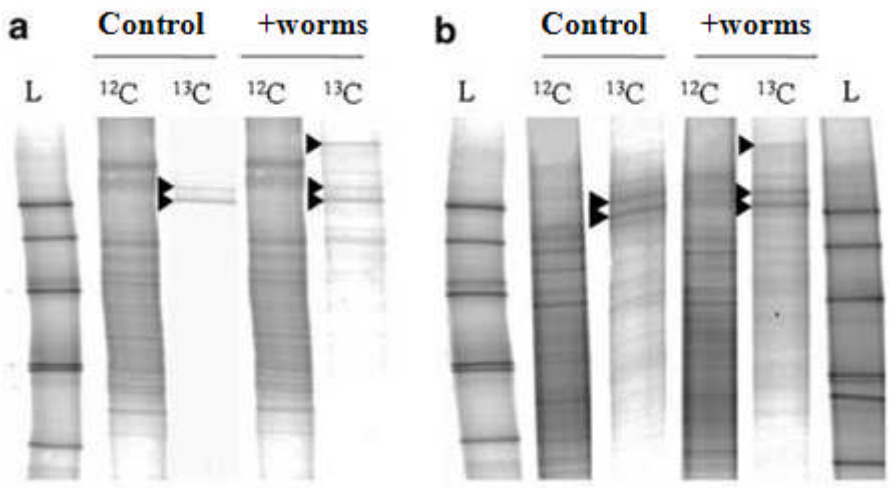

Figure 3.4 Comparison of bacterial community structure based on DGGE profiles targeting the bacterial 16S rRNA genes. (a) DGGE profiles from ^{12}C- and ^{13}C-DNA fractions and (b) DGGE profiles from ^{12}C- and ^{13}C- RNA fractions. L corresponds to a molecular mass ladder. Arrows indicate bands that have been excised and sequenced. (Bacterial DGGE analysis was performed by Dr Marina Hery)

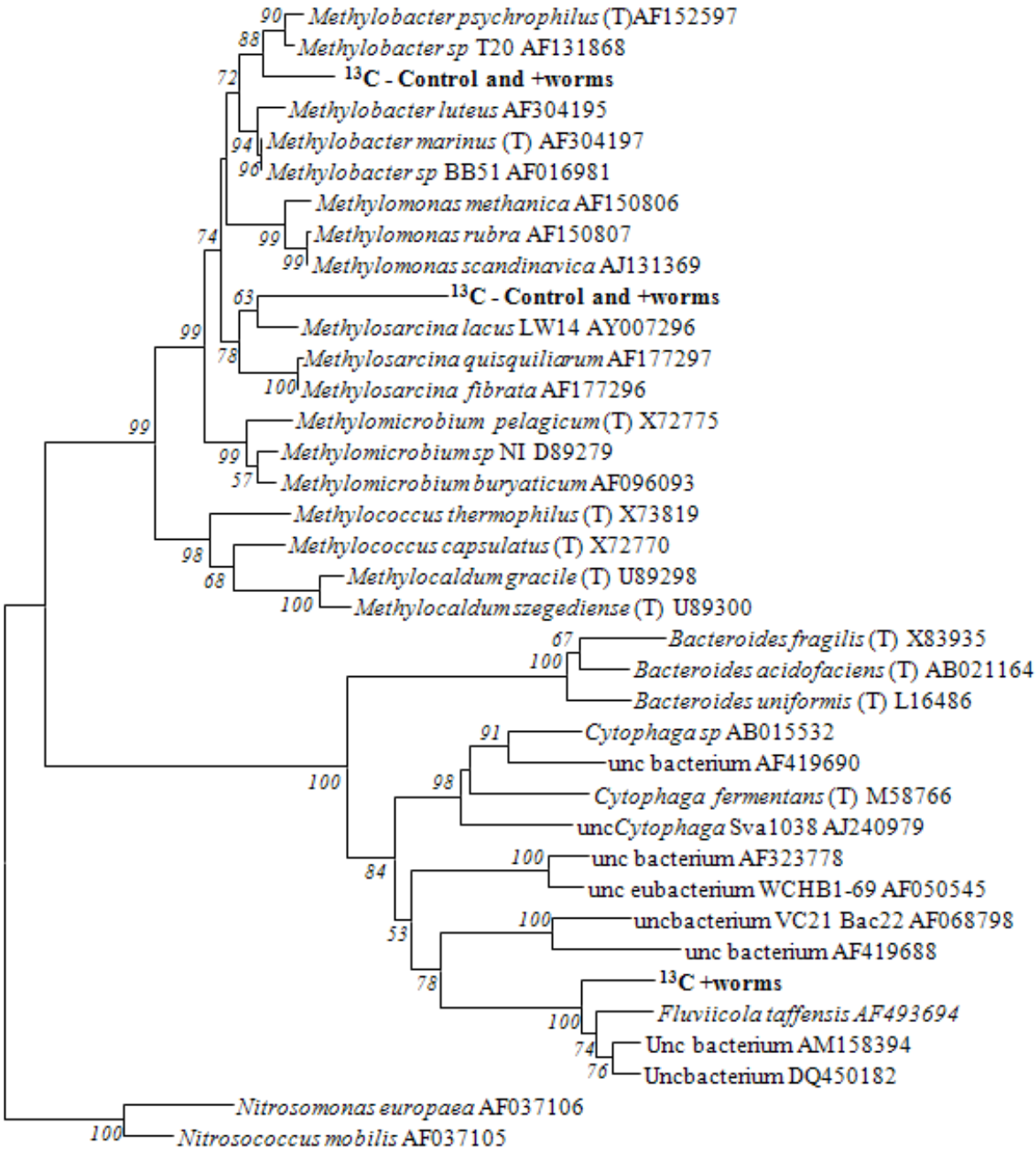

Figure 3.5 Neighbour-joining tree of partial bacterial 16S rRNA gene sequences (566 bp) from the DGGE bands obtained with both ^{13}C-DNA and ^{13}C-RNA samples. DGGE band derived sequences from this study are indicated by boldface type. Only bootstrap values >50% are indicated. Scale bar = 0.02 change per base position.

Type I and Type II methanotroph specific-16S rRNA gene primers (Chen et al., 2007) were used to compare methanotroph community structure between control and +worms with both DNA and RNA. Type I methanotroph specific DGGE analysis with ^{13}C-DNA and ^{13}C-RNA revealed two dominant bands in both control and +worms samples (Fig 3.6). The sequences from these two bands corresponded to *Methylobacter*- and *Methylosarcina*- related sequences (Fig 3.7). However, ^{12}C-DNA and ^{12}C RNA profiles revealed a complex fingerprint similar to the bacterial DGGE gels with minor differences in the band intensities between control and +worms ^{12}C- DNA. For Type II methanotroph specific DGGE analysis, similar profiles were observed for control and +worms samples with both ^{12}C- and ^{13}C-DNA and ^{13}C RNA (Fig 3.8). All the profiles were dominated by two intense bands, which were related to *Methylocystis*-related sequence. Sequences from both bands shared 99% identity (Fig 3.9).

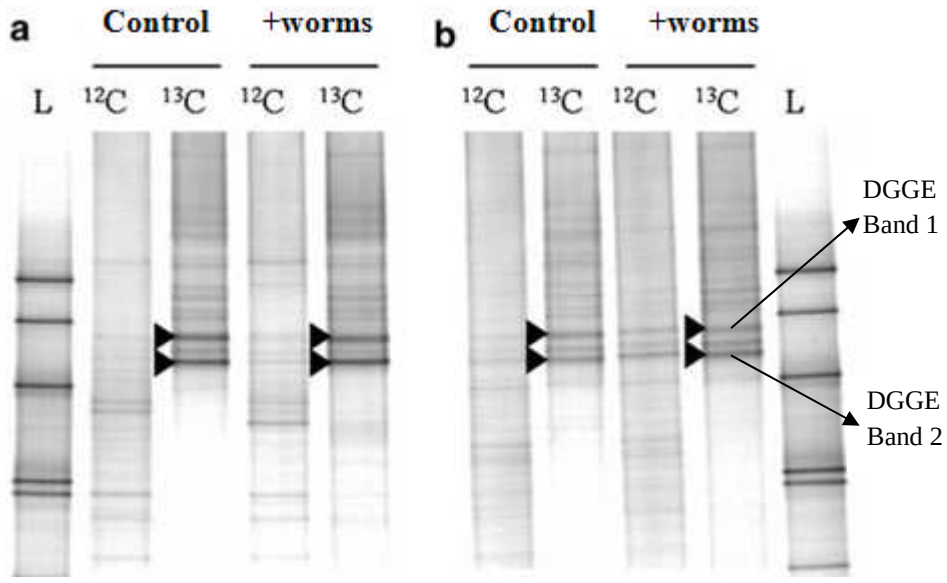

Figure 3.6 Comparison of Type I methanotroph community structure based on DGGE fingerprints targeting Type I methanotroph specific16S rRNA genes. **(a)** DGGE fingerprints from ^{12}C- and ^{13}C – DNA fractions and **(b)** DGGE fingerprints from ^{12}C- and ^{13}C- RNA fractions. L corresponds to a molecular mass ladder. Arrows indicate DGGE bands that have been sequenced.

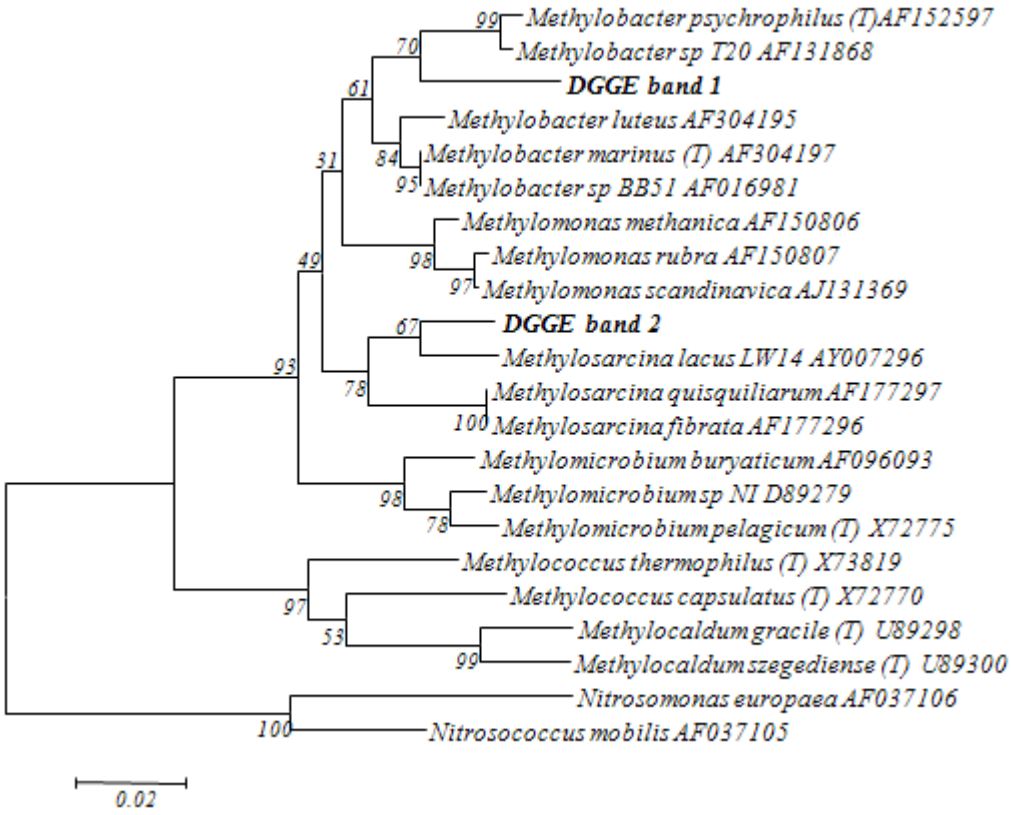

Figure 3.7 Neighbour-joining tree of Type I methanotroph 16S rRNA gene sequences from the DGGE bands. DGGE band derived sequences from this study are indicated by boldface type. Only bootstrap values >50% are indicated. Scale bar = 0.02 change per base position.

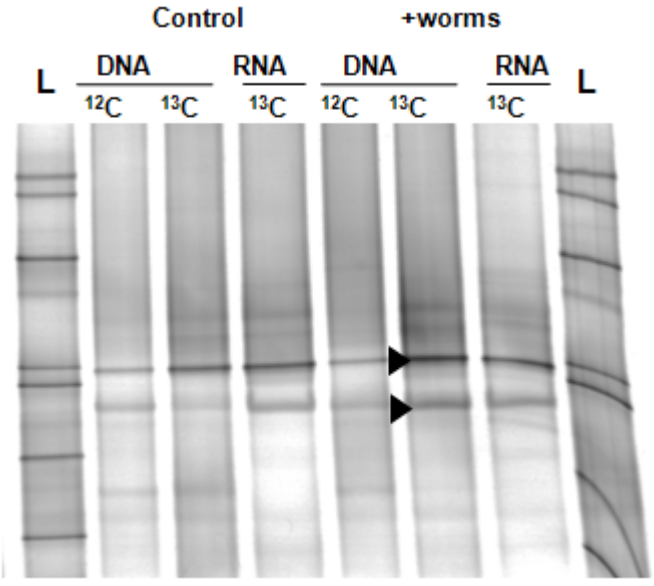

Figure 3.8 Comparison of Type II methanotroph community structure based on DGGE fingerprints targeting Type II methanotroph specific16S r RNA genes.

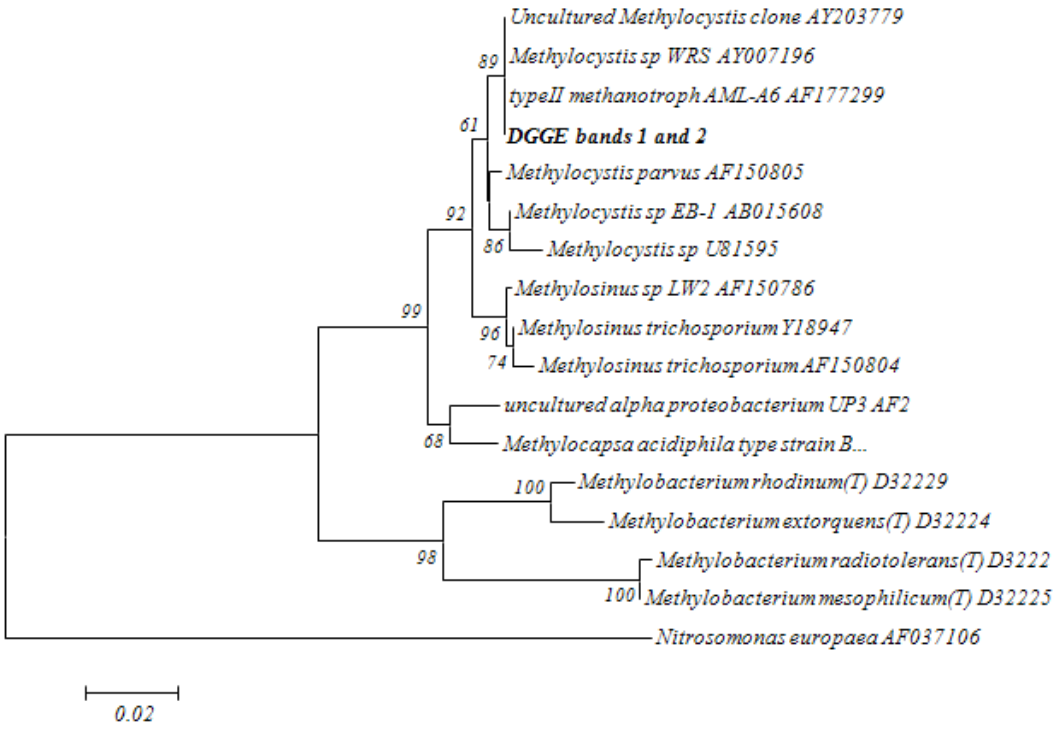

Figure 3.9 Neighbour-joining tree of Type II methanotroph 16S rRNA gene sequences from the DGGE bands. DGGE band derived sequences from this study are indicated by boldface type. Only bootstrap values >50% are indicated. Scale bar = 0.02 change per base position.

3.3.4 Functional gene-based analysis

Two key genes, *pmoA* and *mmoX*, can be used as functional gene markers for characterizing methanotroph diversity in the environment (reviewed by McDonald et al., 2008). Clone libraries were constructed using primers targeting both *pmoA* and *mmoX* genes from control and +worms ^{13}C-DNA samples. Bodrossy *et al*. (2003) developed a diagnostic microarray targeting *pmoA* of all known methanotrophs which has been used to characterize methanotroph community structure in various environments (Bodrossy *et al.*, 2003; Stralis-Pavese *et al.*, 2004; Cebron *et al.*, 2007a). In this study, the *pmoA*-based microarray was used to compare the methanotroph community structure between control and +worms heavy and light DNA and also to compliment the results from *pmoA* clone libraries.

pmoA-based methanotroph diversity

pmoA clones from control (45) and +worms (47) were grouped into 8 OTUs based on their restriction patterns and representative clones were sequenced (Fig 3.10). Phylogenetic analysis of the sequenced clones revealed that two dominant OTUs in both libraries corresponded to *Methylomicrobium/Methylosarcina* (OTU 1) and *Methylocystis* (OTU 2) – related *pmoA* sequences. The *pmoA* library from the control sample had 60% Type I and 40% Type II methanotroph *pmoA* sequences, whereas +worms sample consisted of 66% and 34% of Type I and II methanotroph *pmoA* sequences, respectively. Type I methanotrophs were represented by sequences from *Methylomicrobium/Methylosarcina* and *Methylobacter*- related sequences while Type II methanotrophs were only represented by sequences related to *Methylocystis* (Fig 3.11).

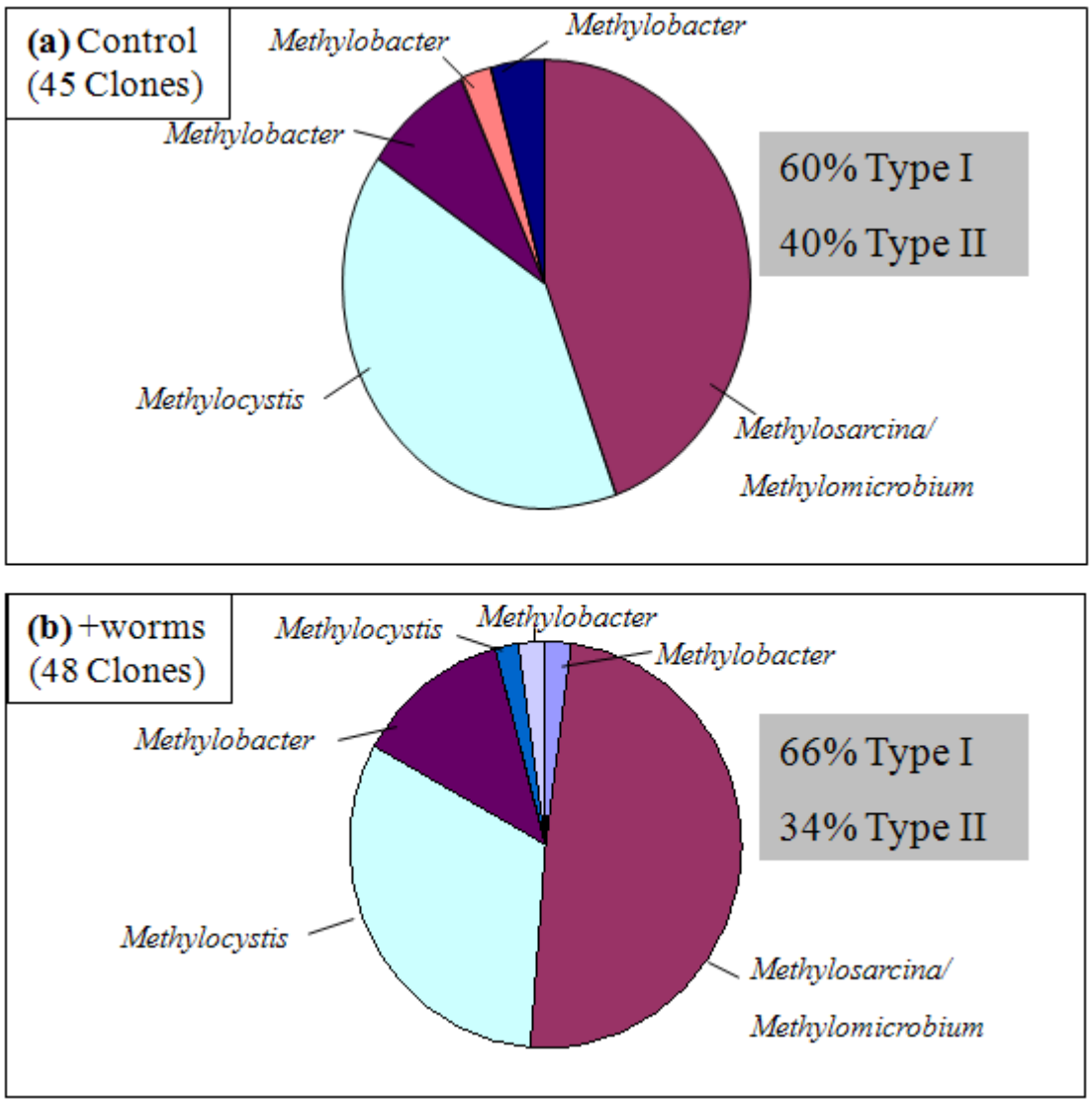

Figure 3.10 Graphical representation of the distribution of *pmoA* sequences based on restriction patterns (OTU's) in control (a) and +worms (b) ^{13}C-DNA samples.

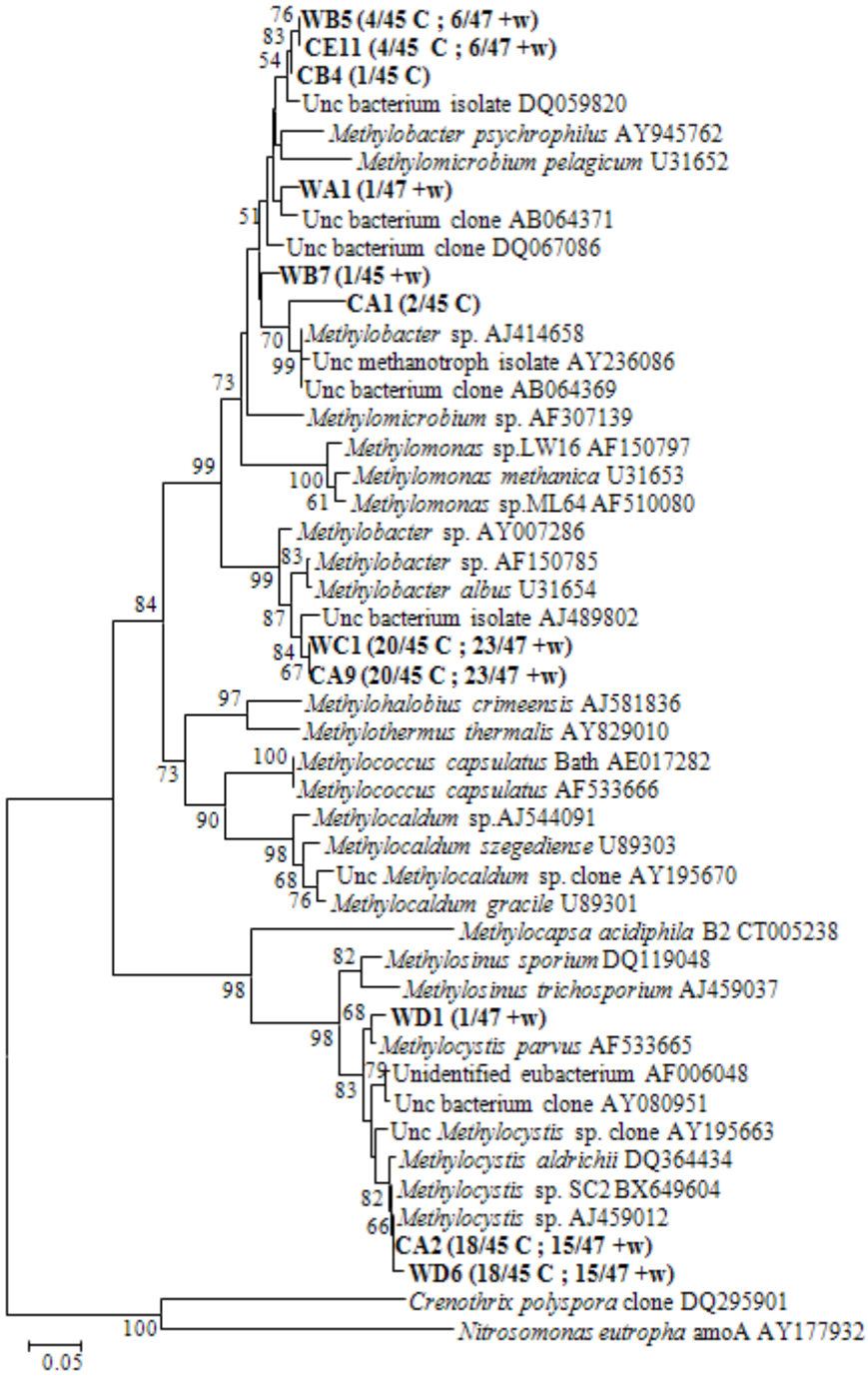

Figure 3.11 Neighbour-joining tree of partial PmoA sequences derived from the *pmoA* clone libraries (Poisson correction). The percentage of each operational taxonomic unit (OTU) among each library is indicated within parentheses, '+w' indicates worms library and 'C' indicates control library. Bootstrap values >50% are indicated. Scale bar = 0.05 change per base position.

pmoA microarray hybridization signal patterns revealed high diversity of *pmoA* sequences in both control and +worms samples (Fig 3.12). For *pmoA* probes targeting Type Ia methanotrophs, strong hybridization signals were obtained for the genera *Methylobacter, Methylomonas,* and *Methylomicrobium/Methylosarcina*. The probe Mmb_562 targets *pmoA* from both the genera *Methylomicrobium* and *Methylosarcina,* while probe Mmb_303 targets only *pmoA* from the genus *Methylomicrobium*. Although hybridisation signals were obtained for both probes (Mmb_562 and Mmb_303), the relative hybridisation signal intensity for the probe Mmb_303 was weaker than Mmb_562, suggesting that the genus *Methylosarcina* might contribute to the stronger signal intensity with probe Mmb_562. For Type Ib methanotrophs, weaker signal intensity was obtained for the probe targeting the genus *Methylocaldum* (McI408). However, for *Methylocaldum* and the Upland Soil Cluster Gamma (P_USCG-225), hybridization signals were obtained only with ^{12}C- and unfractionated DNA from both control and +worms samples. For Type II methanotrophs, strong hybridization signals were obtained for probes targeting the genera *Methylocystis* and *Methylosinus*. The high sensitivity of the *pmoA* microarray compared to the clone library analysis resulted in the detection of *pmoA* from genera *Methylocaldum*, *Methylomonas* and *Methylosinus*, which were not detected in *pmoA* clone libraries. Comparison of ^{13}C- DNA hybridization profile for both the samples revealed stronger hybridization signals for probes P_Mm531 (*Methylomonas*-specific probe), Mmb_303 (*Methylomicrobium*-specific probe) and P_MbSL#3-300 (*Methylobacter*-specific probe) with the +worms sample than the control sample.

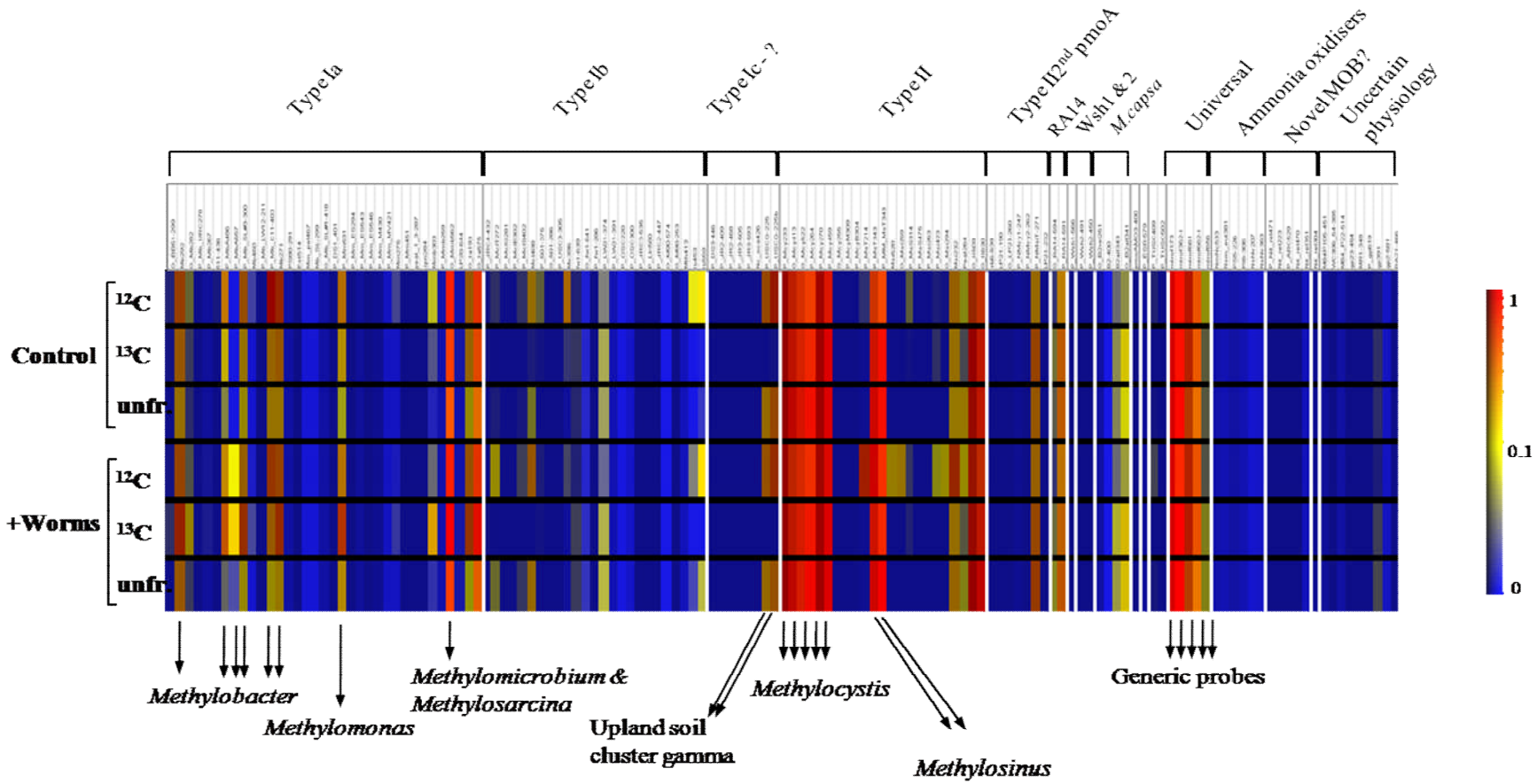

Figure 3.12 *pmoA* microarray analysis of methanotroph community structure based on DNA from control and +worms soil samples. The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to the probe. ^{12}C, ^{13}C and Unfr indicate ^{12}C-DNA, ^{13}C-DNA and unfractionated DNA, respectively.

***mmoX*-based methanotroph diversity**

RFLP analysis of *mmoX* clones (Control – 88 clones; +worms – 95 clones) revealed three distinct OTUs in both libraries (Fig 3.13). Sequencing of representative clones revealed that *mmoX* libraries were dominated by Type II methanotrophs-related *mmoX* sequences (OTU 1). The sequences from the dominant OTU (OTU 1), comprising 85 % of control and 81% of +worms clones, were related to the genus *Methylocystis*. The sequences from OTU 2 and OTU 3 were not affiliated to *mmoX* sequences from any known methanotrophs (Fig 3.14). Based on phylogenetic analysis, sequences from OTU 2 (control-7% and +worms-16%) and OTU 3 (Control-8% and +worms-3%) were related to Type I and Type II methanotrophs, respectively. These two OTUs may represent *mmoX* sequences from as yet uncultivated methanotrophs.

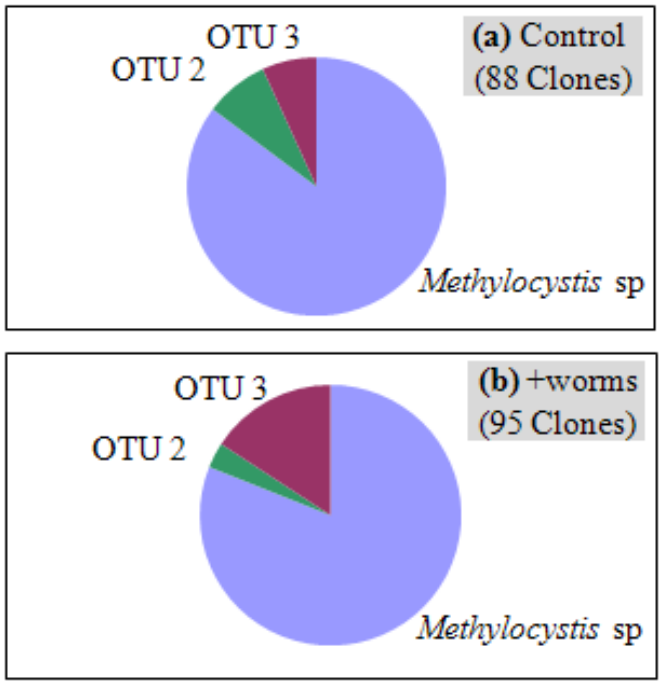

Figure 3.13 Graphical representation of the distribution of *mmoX* sequences based on restriction patterns in ^{13}C-DNA samples from (a) control and (b) +worms.

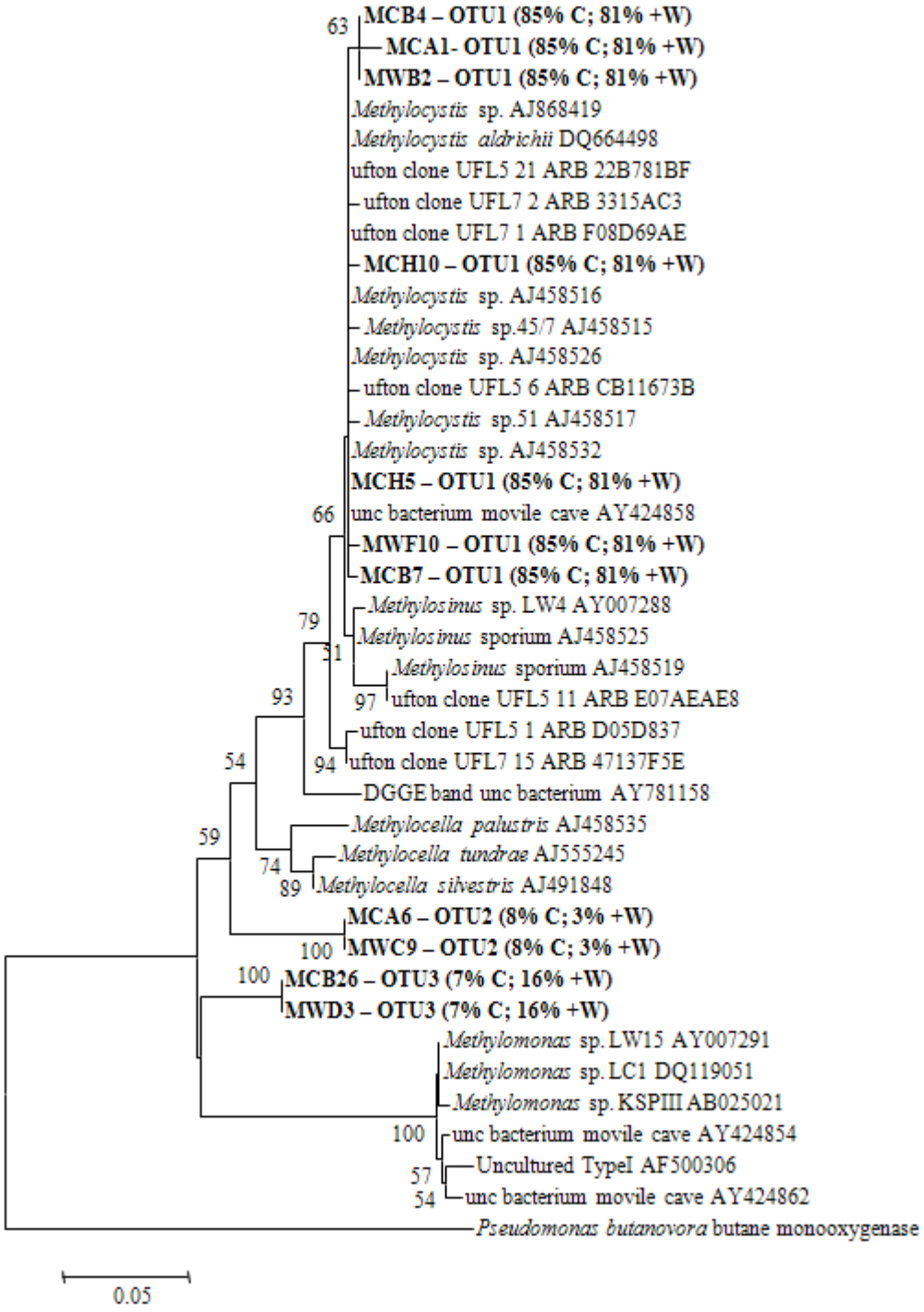

Figure 3.14 Neighbour-joining tree of partial MmoX derived sequences from the *mmoX* clone libraries (Poisson correction). The percentage of each operational taxonomic unit (OTU) among each library is indicated within parentheses, 'C'indicates control library and '+w' indicates +worms library. Bootstrap values >50% are indicated. Scale bar = 0.05 change per base position.

3.4 Discussion

Landfill cover soils play a major role in limiting methane emissions to the atmosphere, predominantly through microbial oxidation of methane. Hence there is a need for a better understanding of the microorganisms involved in methane oxidation in landfill cover soil and of the factors influencing the diversity and activity of these microorganisms. This study identified the active methanotrophs in the Ufton landfill cover soil and also investigated the impact of earthworms on active bacterial community involved in methane oxidation.

Methanotroph diversity in the landfill cover soil

In this study, we used both DNA- and RNA-SIP to analyze the active bacterial community oxidizing methane in the landfill cover soil. A similar study using both DNA- and RNA-SIP was performed by Lueders *et al*. (2004) to investigate the temporal dynamics of methylotrophs in rice field soil. The authors suggested that RNA-SIP (after 6 days of incubation) revealed the initially active methylotrophs, whereas DNA-SIP (after 42 days incubation) revealed a specific enriched methylotroph. However, recent work by Neufeld *et al*. (2007d) has improved DNA-SIP sensitivity by optimizing the amount of substrate incorporation and the length of incubation times. In this study, 11 µmol of ^{13}C-CH_4 g^{-1} soil at 7 days of incubation were sufficient for efficient labelling of both DNA and RNA (as revealed by the quite similar bacterial 16S rRNA gene DGGE profile). However, it should be noted that a comparison between DNA and RNA-SIP at an earlier time point could have provided different results.

pmoA gene based analysis revealed a high methanotroph diversity, particularly with *pmoA* microarray analysis, with retrieval of *pmoA* sequences related

to both Type I and Type II methanotrophs. The presence of a high diversity of *pmoA* sequences from Type I and Type II methanotrophs in this landfill cover soil is similar to other landfill cover soils (Wise et al., 1999; Bodrossy et al., 2003; Uz et al., 2003; Crossman et al., 2004; Stralis-Pavese et al., 2004). However, the diversity of *mmoX* gene sequences was comparatively lower than that of *pmoA*, with more than 80% of the sequences related to the *mmoX* from genus *Methylocystis*. DGGE-based analysis of ^{13}C-DNA and RNA identified *Methylobacter*, *Methylosarcina* and *Methylocystis* as the active methanotrophs. These results are congruent with the *pmoA* hybridization signal pattern with ^{13}C-DNA. Previous SIP experiments in different environments such as peat soil (Morris et al., 2002) and Movile cave (Hutchens et al., 2004) identified a broad range of both Type I and Type II methanotrophs, whereas in some environments such as Russian soda lake sediments, it has been suggested that only Type I methanotrophs are active (Lin et al., 2004). *pmoA* microarray hybridization signals for *Methylocaldum* (McI408) and Upland soil cluster gamma probes (P_USCG-255 and P_USCG-255b) were obtained only with the ^{12}C-DNA and not with the ^{13}C-DNA samples, suggesting that although these methanotrophs are present they are not necessarily active.

Previous studies have concluded that 16S rRNA and *pmoA*-based analysis of methanotroph community structure gave similar results (Costello and Lidstrom, 1999; Horz et al., 2001). The results obtained from this study also confirm this observation. In particular, highly congruent results were obtained with the *pmoA* clone libraries and *pmoA* microarray hybridization patterns. However, microarray results revealed a more diverse methanotroph community structure than clone library analysis, indicating the sensitivity and suitability of this method for high throughput analysis of *pmoA* gene diversity in the environment. The poor representation of

methanotroph diversity in *pmoA* clone library analysis compared to *pmoA* microarray analysis might be due to the low number of clones analyzed in this study. Type I methanotroph-specific 16S rRNA gene DGGE profiles based on ^{12}C-DNA and ^{12}C-RNA revealed a complex and high diversity of Type I methanotrophs, whereas DGGE profiles based on ^{13}C-DNA and RNA revealed only a few distinct bands, suggesting that the dominant members of the community were not necessarily active. However, *pmoA* microarray results suggested that almost all of the methanotrophs detected (with ^{12}C-DNA) were active (with ^{13}C-DNA). This could be due to the lack of specificity of Type I methanotroph 16S rRNA gene primers when used to target Type I methanotrophs in a DNA or RNA template with complex bacterial community. In ^{12}C-DNA and RNA, the proportion of Type I methanotrophs among total bacteria might be too small to avoid amplification of non-methanotrophic bacteria (Héry et al., 2008).

Effect of earthworms on bacterial community structure

The distinct band present in the 16S rRNA gene DGGE profiles of ^{13}C-DNA and RNA of +worms sample corresponded to a *Bacteroidetes*-related bacterium and was the only difference observed between control and +worms samples using 16S rRNA gene based analysis. This is the first time that there is an indication of a possible role of *Bacteroidetes* in methane oxidation. Previous studies have reported *Bacteroidetes* in methane-rich environments (Scholten-Koerselman et al., 1986; Reed et al., 2002; Reed et al., 2006); however, there is a lack of evidence for possible methane oxidation capacity of *Bacteroidetes*. It is also possible that this result might be due to a cross-feeding phenomenon where the *Bacteroidetes*-related bacteria feeding a labelled by-product (such as methanol) produced by methanotrophs during methane oxidation. Alternatively, *Bacteroidetes* might have

fed on the dead ^{13}C-labelled methanotroph biomass due to food-web interactions. So far there is no conclusive evidence for the role of *Bacteroidetes* in methane oxidation and further investigations are necessary to understand the contribution (if any) of this bacterium to methane oxidation.

pmoA microarray analysis are semi-quantitative in nature, allowing us to compare the relative abundance of *pmoA* target sequences in environmental samples (Bodrossy et al., 2003). *pmoA* microarray hybridization signal patterns with ^{13}C-DNA indicated a higher relative abundance of Type Ia methanotrophs in +worms sample than in control sample. This can be suggested since the highest hybridization signals were observed for the generic Type Ia probe, O_Ia193 along with other *pmoA* probes targeting several genera of Type Ia methanotrophs such as *Methylomicrobium* (Mmb303), *Methylomonas* (P_Mm531) and *Methylobacter* (P_MbSL#3-300). Moreover, higher numbers of Type I methanotrophs-related sequences than Type II methanotroph-related sequences were found in the *pmoA* gene library with +worms sample. These results suggest that earthworms might stimulate the growth or activity of Type I methanotrophs. Previous studies have suggested that Type I methanotrophs might respond quickly to changing environmental conditions compared to Type II methanotrophs, possibly due to a higher growth rate (Graham *et al.*, 1993; Bodelier *et al.*, 2000; Henckel *et al.*, 2000a). This stimulation of activity could also be correlated with the increased nitrogen and/or nutrient availability directly linked earthworm activity (Needham, 1957; Buse, 1990). In this study, the relative amounts of ammonia and nitrate were influenced by the presence of earthworms. The possible interaction mechanisms between earthworms and methanotrophs are discussed in detail in Chapter 4. The higher nitrate content in the +worms soil compared to control soil might also indicate a possible stimulation of nitrifers. Earthworms

burrow walls (Parkin and Berry, 1999) and casts (Mulongoy and Bedoret, 1989) are known to harbour a higher number of nitrifiers compared to bulk soil. The enzyme ammonia monooxygenase in nitrifiers is evolutionarily related to methane monooxygenase and could possibly co-oxidize methane. However, the role of nitrifiers in methane cycling is unclear and further investigations will be necessary. In summary, use of SIP allowed us to identify the active methanotrophs in this landfill cover soil. It also showed that the earthworm-mediated increase in soil methane oxidation was only weakly correlated with a shift in the active methanotroph community structure. Further investigations are needed to understand the effect of earthworms on methanotroph activity and growth and also the possible role of nitrifiers in methane oxidation.

Chapter 4

Spatial and temporal shifts in active methanotroph diversity by earthworms in a simulated landfill cover soil

4.1 Introduction

Earthworms as “soil engineers” (Jones et al., 1994) exert a significant impact on soil properties (Binet and Trehen, 1992) and microbial functions in soil through their activities such as burrowing, casting and through their excretion of organic and nitrogenous compounds (Daniel and Anderson, 1992; Binet and Le Bayon, 1998; Singer *et al.*, 2001b; Haynes *et al.*, 2003). In our recent study (Héry et al., 2008), it has been shown that earthworms can mediate an increase in landfill cover soil methane (CH_4) oxidation. The results provided a promising incentive to harness the interaction between earthworms and methanotrophs in landfill cover soil to reduce methane emissions from landfills. Hery *et al*. (2008) employed DNA- and RNA-SIP to compare active bacterial communities oxidizing methane in earthworm-incubated and non-incubated landfill cover soils in soil microcosms. It was suggested that methane oxidation enhancement by earthworms in landfill cover soil was not correlated to any significant change in active bacterial community structure except for a *Bacteriodetes*-related bacterium in earthworm-incubated soils. Further it was hypothesized that a change in the relative abundance of active methanotroph population could contribute to the increase in activity.

Understanding the spatial and temporal shifts in functional diversity and relative abundance of active methanotrophs brought about by the complex and dynamic interaction of earthworms in soil needs an experimental system that could mimic the natural environment, in this case, a landfill environment. Also, the experimental system should provide the environmental heterogeneity over a sufficiently long time period that is observed under field conditions for a realistic representation of the interaction between different components in an ecosystem.

Microcosm studies have limitations in mimicking the environmental heterogeneity found in *in situ* conditions, which makes it difficult to extrapolate in terms of experimental design and execution of *in situ* field experiments. In this study the experimental system was adapted from soil cores previously used by Kightley *et al*. (1995) to simulate landfill conditions. This experimental system reflected *in situ* conditions in landfill i.e. methane seeping from the bottom of the landfill cover soil and also offered us the ability to resolve the impact of earthworms on the spatio-temporal shifts in active methanotrophs population alongside facilitating process measurements i.e. methane oxidation potentials.

Detection of functional genes via DNA based methodology does not imply bacterial transcriptional activity, since DNA may be stable in dormant and dead cells (Lindahl, 1993). Analysis of mRNA yields information on bacterial activity at the time of sampling and can also reflect the change in gene expression over varying environmental conditions (Bodrossy et al., 2006). An mRNA-based *pmoA* microarray has previously been used to detect the active methanotroph community structure in lysimeters simulating landfill soil (Bodrossy et al., 2006) and peat soils (Chen *et al.*, 2008a). It is also semi-quantitative in nature, allowing observation of changes in relative abundances within active methanotroph populations expressing *pmoA* (Bodrossy et al., 2006). The aims of this study were

i. To scale up the experimental system and confirm the effect of earthworms on methane oxidation potential in a simulated landfill cover soil.
ii. To examine the effect of earthworm on the spatial and temporal shifts in active methanotroph populations and the relative abundance of methanotrophs.

4.2 Simulated landfill cover soil

Soil samples were collected from the Ufton landfill cover soil (site description in Chapter 3) to a depth of 30 cm and before use indigenous earthworms were removed without significant perturbation of the soil structure. Soil moisture content at the time of sampling was 27.1 ± 2.2 %, determined gravimetrically by drying soil samples at 80^{o}C to constant weight. Soil was stored at 4^{o}C and used in for experiments 2-3 weeks after collection, to limit any residual effect from indigenous earthworms.

Landfill conditions were simulated by adapting soil columns used by Kightley *et al*. (1995) with minor modifications (**Figure 4.1**). Columns (1m height and 15 cm diameter) were constructed of polyvinyl chloride (PVC) with sampling ports at regular intervals. The ports were modified to facilitate gas and soil sampling at 10 cm intervals. For gas sampling ports, silicone bungs were fitted through to which a sampling needle (0.8 x 40 mm needle; fitted with an airtight valve) was pushed into the column allowing gas samples to be withdrawn using a syringe. The column was closed at both ends with gas tight PVC caps, fitted with rubber O-rings. The columns were tested for gas leaks before the start of the experiment, and then packed with 30 cm (~7 kg) of landfill cover soil on top of a perforated plate placed at the bottom of the column. Soil moisture content was restored to the original moisture content of the soil at the time of sampling by addition of the appropriate volume of de-ionized water. About 75 earthworms (*Eisenia veneta*), approximately 53 g, were added to one column (+ worms) while no earthworms were added to another column (control). No exogenous food source was provided for the earthworms for the duration of the experiment. Columns were maintained at ~20^{o}C. Landfill gas (60%

CH_4 + 40% CO_2), excluding any trace gas composition, was injected from the bottom of the closed columns to mimic landfill gas seeping from lower layers of landfill. The methane concentration in the landfill gas mixture at the time of injection was 1% (v/v) of the column volume (17.68 l). Methane concentration was monitored at regular intervals at different depths of the soil column using a Pye Unicam series 204 gas chromatograph (GC) by withdrawing 0.2 ml of gas and injecting it into the GC. Soil moisture content was monitored throughout the experiment (15 weeks) and the moisture content was maintained at *in situ* levels at the time of soil collection. During the experiment, the landfill gas mixture was added continuously and when methane levels fell below detection limits, the top end-cap was opened to replenish oxygen and also to prevent CO_2 build up. After 15 weeks, at the end of experiment, the soil columns were dismantled to measure the earthworm biomass. The biomass in the +worms column was 7.5g (about 90% reduction from the initial biomass). Soil physico-chemical analysis was done as described in Héry *et al.* (2008). Due to constraints pertaining to the experimental set-up, it was not possible to sample soil for physic-chemical analysis at all time intervals. It was revealed that after 15 weeks the +worms column had a significantly higher nitrate (NO_3^-) content than the control column at all soil depths (Table 4.1).

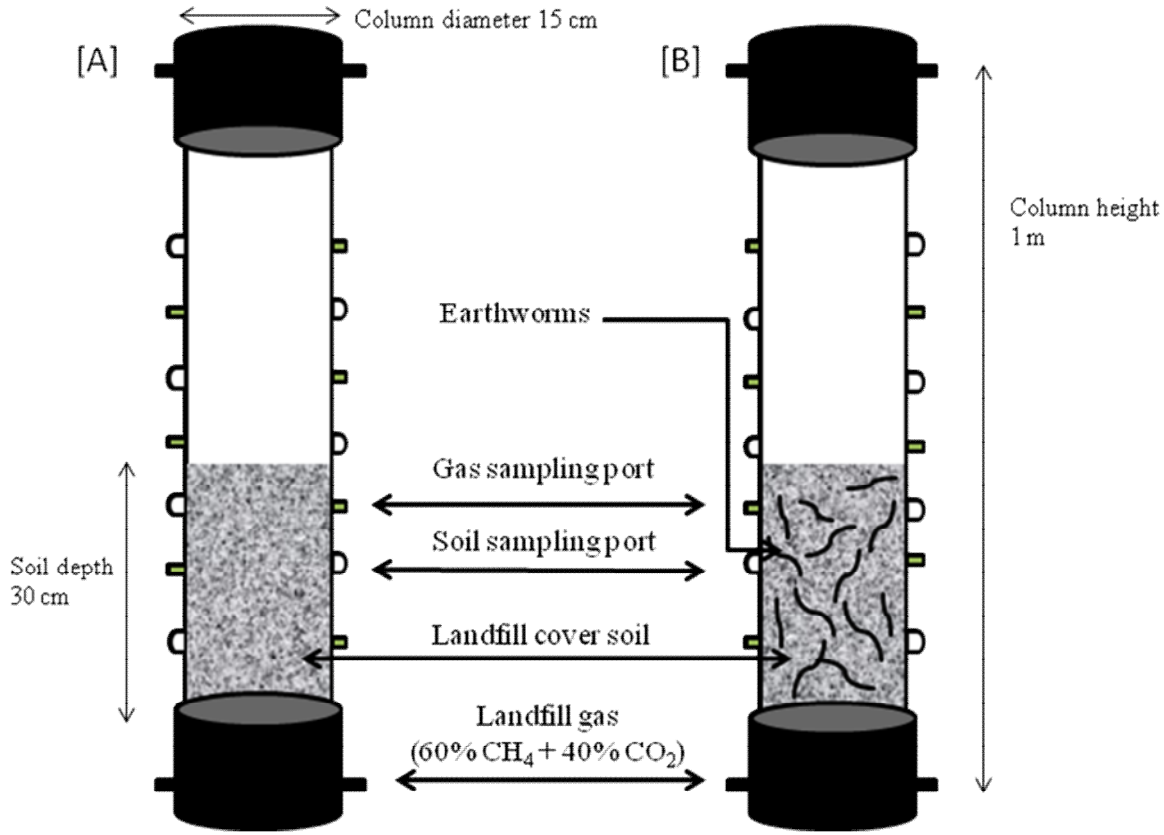

Figure 4.1 Schematic representation of soil columns used to simulate a landfill cover soil with or without earthworms. Column A had no earthworms while column B had ~53g biomass earthworms of biomass.

Table 4.1 Comparison of soil physiochemical properties between control and +worms soil

Sample	Soil depth cm	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	Total C (%)	Total N (%)
Control	10	20.49	197.4	2.42	0.16
	20	20.39	183.4	2.75	0.19
	30	12.66	168.4	2.82	0.19
+Worms	10	15.92	361.6	2.32	0.13
	20	18.97	513.7	2.59	0.13
	30	18.31	1058.0	2.50	0.19

4.3 Results

4.3.1 Comparison of soil methane oxidation potential

Soil methane oxidation potential were compared between +worms and control soil columns at different depths (10, 20 and 30 cm depth) and at different time intervals; time I (2 weeks after earthworm addition and without methane addition to the soil columns), time II (7 weeks after earthworm addition + one week of methane exposure) and time III (7 weeks after earthworm addition + 8 weeks of methane exposure).

Soil sub-samples (20 cm depth) from the +worms soil column exhibited higher methane oxidation potential than control soil column at time I (**Figure 4.2a**). At time II, soil sub-samples from all three depths (10, 20 and 30 cm) in the +worms column exhibited higher methane oxidation potential compared to soil sub-samples from the control column. However, there was little or no difference in methane

oxidation potentials between samples from different soil depths in both control and +worms soil columns (**Figure 4.2b**). At time III, no difference in methane oxidation potentials was observed between earthworm incubated and non-incubated soil columns. However, there were differences in methane oxidation potentials between different soil depths in both columns. Soil samples at 30 cm depth from both columns exhibited higher methane oxidation potentials compared to soil samples at 10 and 20 cm soil depth (**Figure 4.2c**).

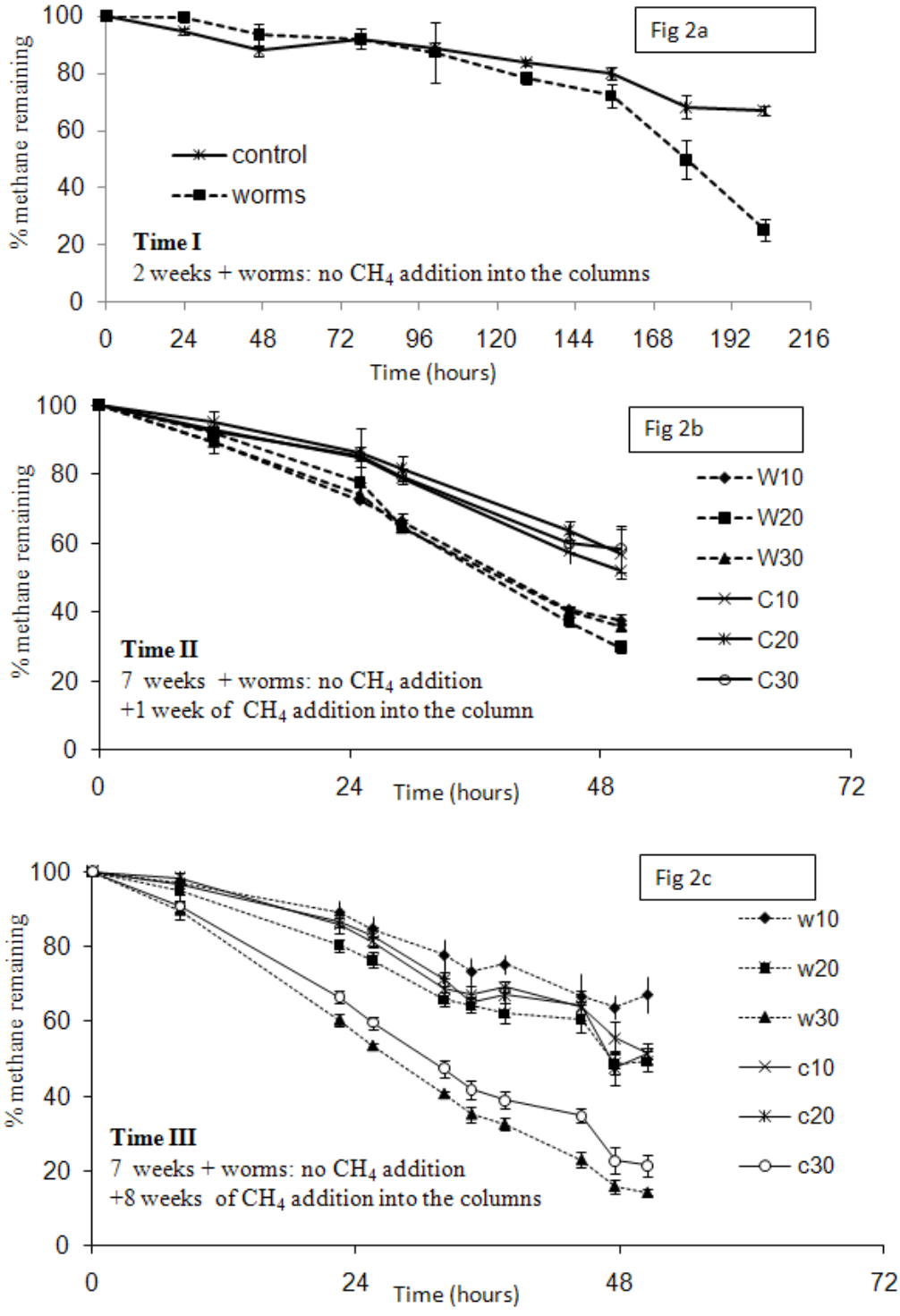

Figure 4.2 Graphical representation of methane oxidation potentials in soil sub-samples (5g) from control and +worms soil columns. Fig 4.2a represents methane oxidation rate at time I at 20 cm depth in soil columns. Fig 4.2b and 4.2c represent methane oxidation potentials at times II and III respectively, for depths 10, 20 and 30 cm. W10, W20 and W30 represent soil depths 10, 20 and 30 cm in +worms column. C10, C20 and C30 represent soil depths 10, 20 and 30 cm in –worms column (control). Error bars represent standard error of three replicates.

4.3.2 Analysis of methanotroph community structure

Nucleic acids (DNA and RNA) were extracted from the column soil samples at different time intervals and soil depth in duplicates. The reproducibility of the duplicate DNA extraction for molecular biology analysis was confirmed by bacterial 16S rRNA gene DGGE analysis (**Figure 4.3**). *pmoA*-based microarray hybridization profiles (both DNA and RNA) were analyzed to compare methanotroph diversity (DNA) with the diversity of active methanotrophs (RNA) in both of the soil columns.

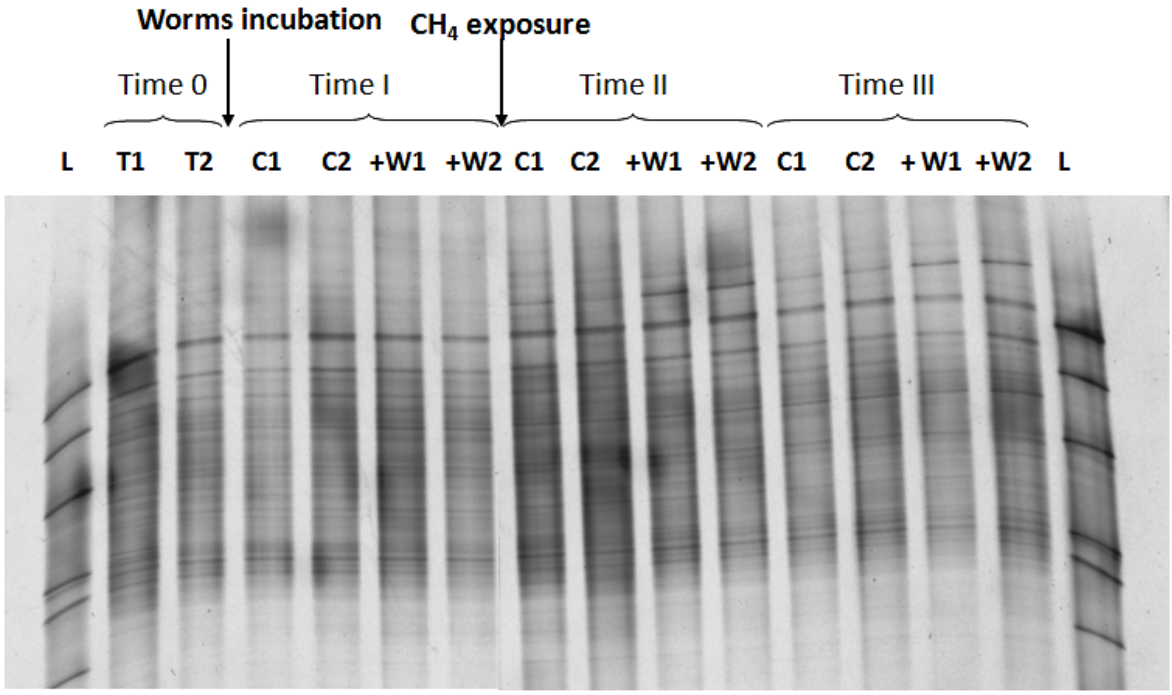

Figure 4.3 DGGE targeting the bacterial16S rRNA genes obtained for the DNA from control and +worms 20cm soil depth. C1 and C2 represent duplicate control samples. +W1 and +W2 represent duplicate +worms samples. T1 and T2 represent duplicate samples from landfill cover soil before the start of experiment. Lane L corresponds to a molecular mass ladder.

4.3.3 DNA-based analysis using the *pmoA* microarray

DNA-based hybridisation signal patterns revealed that there was a high diversity of *pmoA* sequences in all of the DNA samples from different depths and from different time points in both columns (**Figure 4.4**). Similar hybridisation pattern were observed between different DNA samples from +worms and control soil columns (at different depths and time intervals). DNA-based hybridisation signals for type Ia methanotroph probes were dominated by *pmoA* from the genera *Methylobacter* (Mb_292, Mb_C11-403, Mb_271), *Methylomonas* (Mm_531), and *Methylomicrobium/Methylosarcina* (Mmb_562 and Mmb_303). The probe Mmb_562 targets both the genera *Methylomicrobium* and *Methylosarcina,* while the probe Mmb_303 targets only the genus *Methylomicrobium*. Although hybridisation signals were obtained for both probes (Mmb_562 and Mmb_303), the relative hybridisation signal intensity for the probe Mmb_303 was weaker than Mmb_562, suggesting that the genus *Methylosarcina* might contribute to the higher signal intensity with probe Mmb_562. For probes targeting type II methanotrophs, the hybridisation signal was dominated by *pmoA* from the genus *Methylocystis* (probes Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459). Relatively low signal intensity was observed with type Ib methanotroph probes targeting *pmoA* from *Methylocaldum tepidum* (Mcl408), tropical upland soil cluster methanotrophs (USC3-305) and a uncultured methanotroph (501-375) for soil samples from both control and +worms columns. However, no apparent hybridisation pattern was observed between different samples with type Ib methanotroph probes.

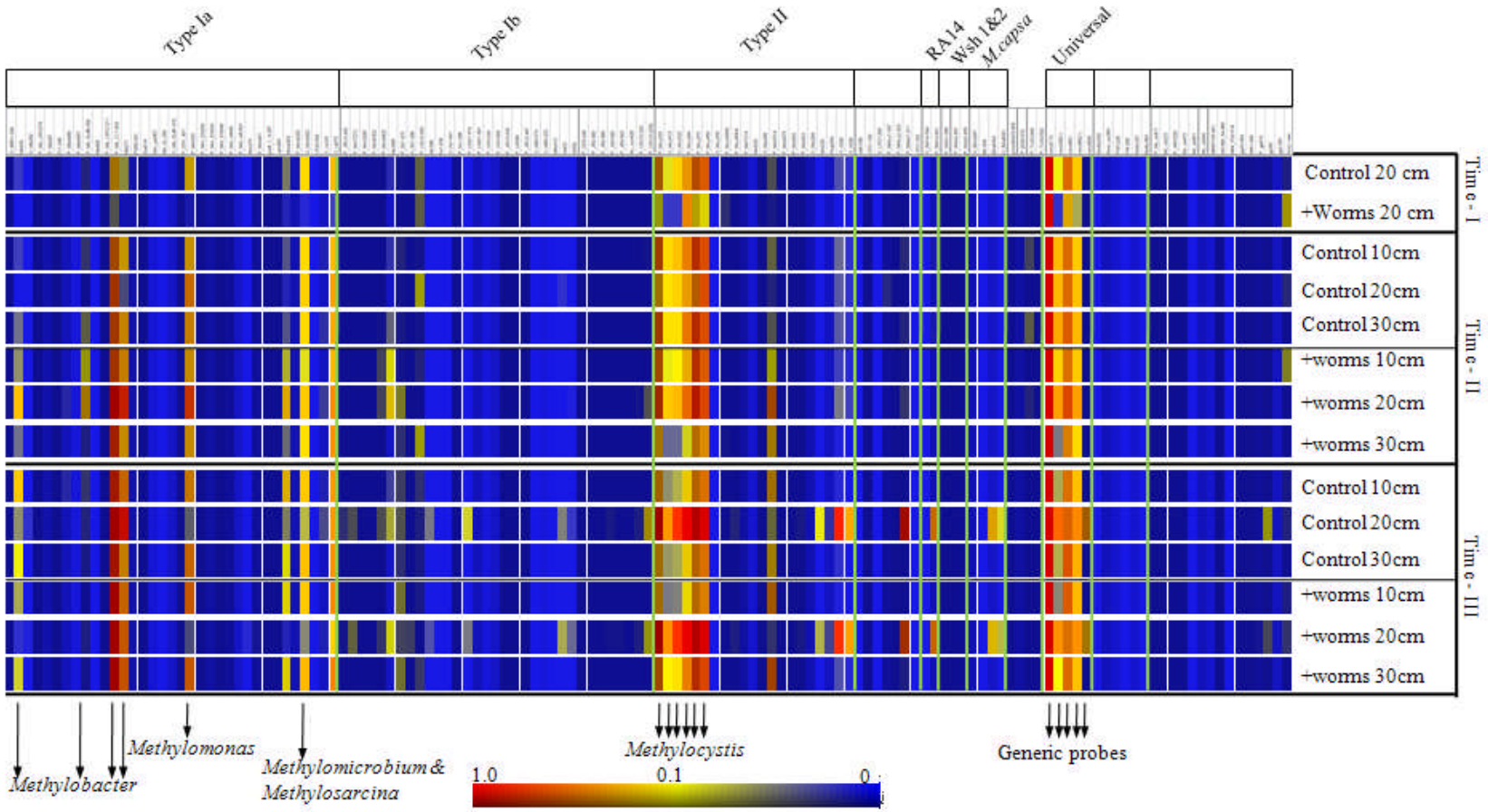

Figure 4.4 Microarray analysis of methanotroph community structure based on DNA extracted from control and +worms soil samples at different soil depths and time intervals. The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to each probe. 10, 20 and 30 cm represents the depth in the soil column from top to bottom.

4.3.4 mRNA - based analysis using the *pmoA* microarray

mRNA-based microarray analysis of the active methanotroph community structure revealed a different hybridisation pattern than for the DNA-based microarray analysis (**Figure 4.5**). Differences in the diversity of active methanotrophs between the control column and the +worms soil column was observed at time II. The hybridisation signal pattern with RNA samples for the control soil column was similar to that observed with DNA, with high signal intensities for the genera *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459), *Methylobacter* (Mb_C11_403 and Mb_271) and *Methylosarcina/ Methylomicrobium* (Mmb_562 and Mmb_303). However, when analyzing the hybridisation profile for the +worms RNA samples at time II, very weak (20 cm depth) or no (10 and 30cm depth) hybridisation signals were observed with *pmoA* probes targeting the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459) (**Figure 4.5**). These probes exhibited relatively high signal intensities for +worms DNA samples (**Figure 4.4** and **4.6**). However, at time III, when there was no effect of earthworms on soil methane oxidation potentials, strong hybridisation signals were once again observed for probes targeting the genus *Methylocystis* in the +worms column RNA samples. When comparing different soil depths at time III, a few differences in hybridisation patterns emerged, with relatively weak hybridisation signal intensity with RNA at 10 cm depth for the control column with probes targeting *pmoA* from the genus *Methylocystis*.

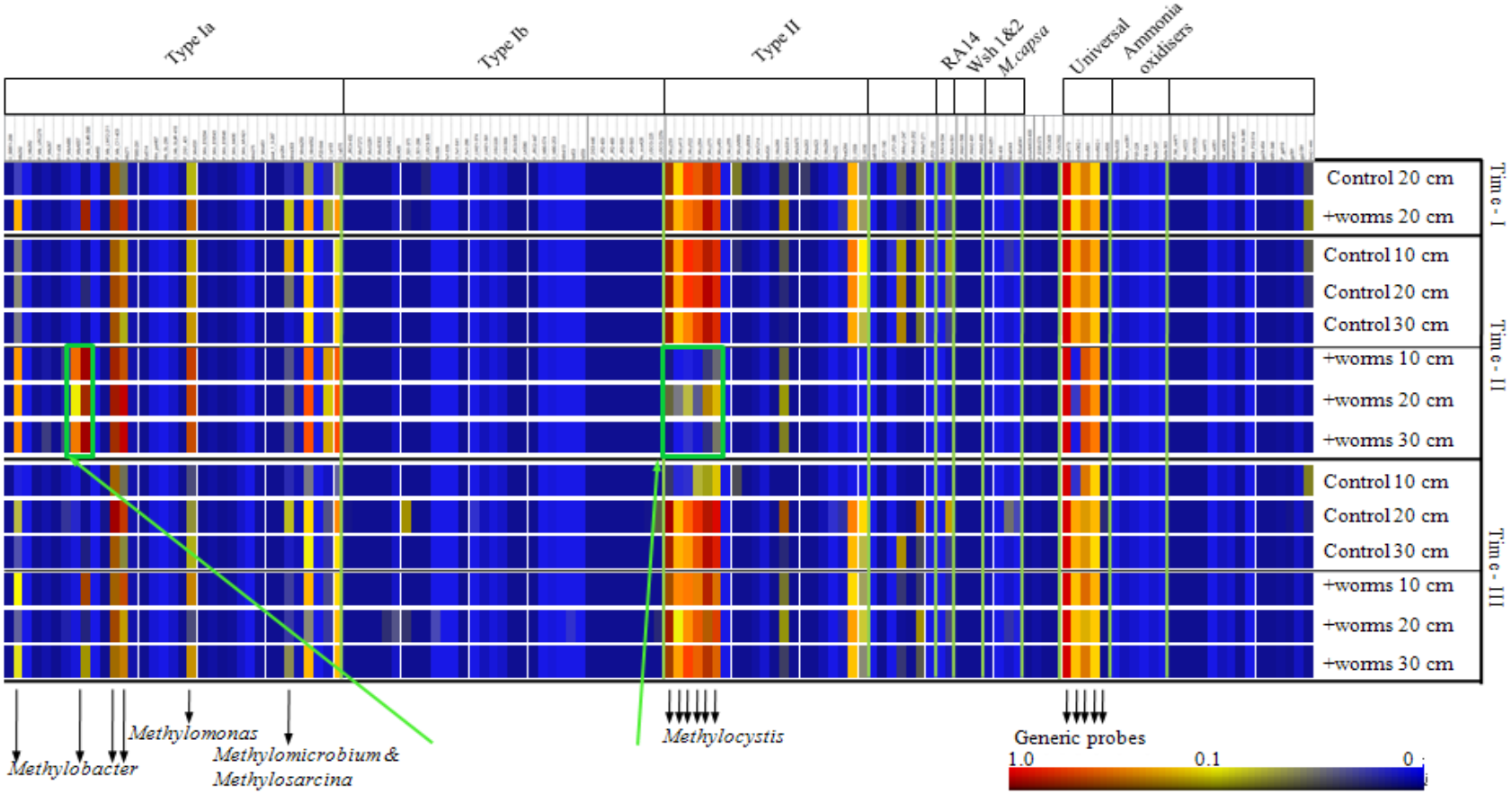

Figure 4.5 Microarray analysis of methanotroph community structure based on mRNA extracted from control and +worms soil samples at different soil depths and time intervals. The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to the probe. 10, 20 and 30 cm represents the depth in the soil column from top to bottom. The green coloured boxes indicate the significant differences in hybridisation profile between +worms and control RNA samples and also between DNA and RNA profile.

Moreover, differences were also observed in the hybridisation pattern with type Ia methanotroph probes between the +worms and control RNA samples at time II and also between RNA samples from different time intervals (time II vs. time III; **Figure 4.6**). At time II, strong hybridisation signals were observed in the +worms samples (for all soil depths) for probes Mb_A557 and Mb_SL#3-300 targeting *pmoA* from different sub-groups in the genus *Methylobacter*, while no hybridisation signals were observed in control RNA samples. However, at time III, no hybridisation signal was observed for the probe Mb_A557 with the +worms RNA samples (all depths), while for the probe Mb_SL#3-300, weak hybridisation signals were detected only with 10 and 30 cm in +worms RNA samples.

It is also interesting to note that with the DNA-based analysis at time II, no hybridisation signals were detected for the probe Mb_A557 in either control or +worms samples (**Figure 4.6**). For probes Mb_292 (*Methylobacter*), Mmb_562 (*Methylosarcina*) and Mm_531 (*Methylomonas*) stronger signal intensities were observed at time II for +worms RNA samples when compared to control RNA samples. At time III, these probes exhibited weaker signal intensities for both control and +worms RNA samples (all depths). Probes Mb_C11-403 and Mb_271 targeting *pmoA* of sub-groups in the genus *Methylobacter* exhibited similar hybridisation signal intensities with both DNA and RNA samples (both in the control and +worms columns) for all depths and all time intervals, indicating that there was little difference in the relative abundance of *pmoA* genes in these samples (Figures 4.5 and 4.6). The higher relative abundance of *pmoA* of type Ia methanotrophs in +worms RNA samples (all depths) compared to control RNA samples at time II is also supported by the stronger signal intensity of the generic type Ia probe Ia575 and of the appearance of a hybridization signal for the other type Ia generic probe Ia193 in

the +worms RNA samples. For type Ib methanotrophs, although *pmoA* hybridisation signals were detected with probes Mcl408, USC3-305 and 501-375 in the DNA-based analysis (**Figure 4.4**), no signals were detected with the RNA based analysis in any of the samples (**Figure 4.5**).

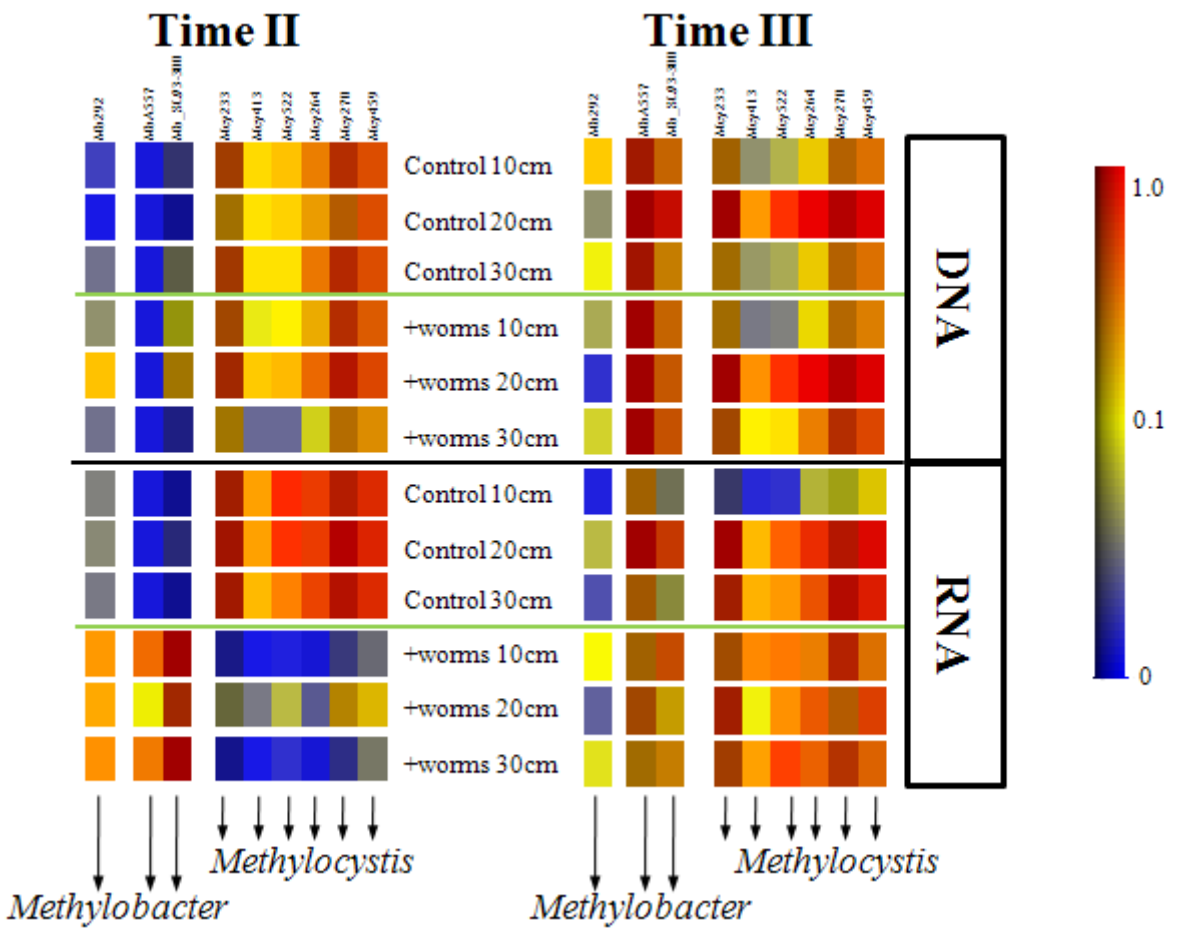

Figure 4.6 Microarray results representing significant differences in methanotroph community structure with DNA and RNA samples between control and +worms column (at time II and III at different soil depths). 10, 20 and 30 cm represents the depth in the soil column from top to bottom. The results include signals for selected *pmoA* probes that revealed differences between the treatments.

4.4 Discussion

In this study, a larger experimental system with a better spatial and temporal resolution was used to mimic *in situ* landfill conditions to study the effect of earthworm activity on relative abundance of active methanotrophs. Earthworms, owing to their movement across different depths in the soil profile, bring about profound changes to the habitat of the microbial populations and their function in soil. Microcosm experiments are limited by the fact that they cannot recreate earthworm activity in soil. By revising the scale of the experimental system from the previous study (Héry et al., 2008), this study offered a realistic opportunity to generate robust data on the effect of earthworm activity on soil methane oxidation and active methanotroph diversity in simulated landfill conditions. Recent advances in mRNA isolation protocols from soils (Burgmann et al., 2003; Chen et al., 2007) allowed the use of an mRNA – based microarray to observe the spatial and temporal shifts in active methanotroph populations. Results from previous studies revealed that *pmoA* microarray results are highly congruent and perhaps even more sensitive than *pmoA* clone library analysis (Bodrossy *et al.*, 2003; Bodrossy *et al.*, 2006; Chen *et al.*, 2008a; Héry *et al.*, 2008).

Influence of earthworm activity on methanotroph function and diversity

Results from this study confirmed that earthworm activity in soil increases soil methane oxidation capacity and also has a significant impact on the relative abundance of the active methanotroph population. Time I corresponds to the time scale used for soil earthworm incubation in the previous study (Héry et al., 2008). This time scale was selected to confirm that the methane oxidation results were congruent, with higher methane oxidation capacity for earthworm-incubated soil. We

observed a shift in function, i.e. methane oxidation potentials which coincided with earthworm activity in the soil column. Significant shifts in the relative abundance of active methanotroph populations were also observed at time II, with higher relative abundance of *pmoA* transcripts from Type Ia methanotrophs (*Methylobacter*, *Methylomonas Methylosarcina/Methylomicrobium*) compared to Type II methanotrophs, particularly *Methylocysis*-related genera, in the +worms column soil samples. However, at time III, when no more earthworm activity was detected, the relative abundance of *Methylocystis*-related genera was similar to the samples from the control soil column. The present study clearly shows that earthworms not only increase methane oxidation potentials but also play a significant role in altering the relative abundance of active methanotrophs. Different hybridisation signal patterns with DNA and RNA reveal that methanotrophs present need not necessarily be active, indicating preferences for suitable environmental conditions for their activity. The possible mechanisms of interaction between earthworm activity and methanotrophs which might have altered methane oxidation potentials and shifted the relative abundance of methanotrophs are discussed below.

Possible interaction mechanisms between earthworms and methanotrophs

Nitrogen input by earthworms in soil

Nitrogen (N) availability exerts a significant influence on methanotroph activity and diversity in environments (Graham et al., 1993; Bender and Conrad, 1995; Bodelier and Laanbroek, 2004). Earthworms provide a constant low supply of N-containing waste in their casts and burrow linings (Needham, 1957; Buse, 1990). In our study, earthworm incubated soil had higher nitrate concentration than soil without earthworms, which is consistent with earlier results (Mulongoy and Bedoret,

1989; Parkin and Berry, 1999; Héry et al., 2008). This additional N availability in the presence of earthworms could possibly relieve N-limitation for microbial (methanotroph) growth (Bodelier and Laanbroek, 2004) and be responsible for the higher soil methane oxidation potentials observed with the +worms column compared to the control column. Earthworms, by stimulating nitrifying population in soils, (Mulongoy and Bedoret, 1989; Parkin and Berry, 1999) might aid in the rapid conversion of NH_4^+ to NO_3^-. NO_3^- is more mobile than NH_4^+, particularly in landfill cover soils with high clay content, and the available nitrogen could be as NO_3^-, rather than NH_4^+, due to cation exchange at clay minerals (Bender and Conrad, 1995). Type I methanotrophs are known to be stimulated by the addition of N (Bodelier et al., 2000) while Type II methanotrophs might dominate under nitrogen-limited conditions as many of them can fix N_2 (Graham et al., 1993). The increase in relative abundance of *pmoA* from Type Ia methanotrophs over Type II methanotrophs in the +worms RNA samples at time II could reflect the N input and increased availability mediated by earthworms in the soil.

Earthworm impact on gas diffusion in soil

Earthworms, through burrowing affect gas diffusion through soil (Edwards and Bohlen, 1996; Singer *et al.*, 2001b). The presence of earthworms in landfill cover soil would certainly increase the diffusion of oxygen, which is a key factor influencing methane oxidation in landfill cover soil in the aerobic zone (Mancinelli, 1995; Stralis-Pavese et al., 2006). The increased diffusion and availability of oxygen for methanotrophs through earthworm burrows across soil depths may also contribute to the increase in methane oxidation potentials in the +worms column (at time II at all depths). Relative oxygen concentration could also play an important

role in altering the functional diversity of methanotrophs. Indeed, Amaral & Knowles (1995) suggested that Type II methanotrophs dominate methane oxidation at low oxygen concentrations while Type I methanotrophs dominate at relatively high oxygen concentrations. Methanotrophs in niches created by earthworm burrows may encounter higher oxygen concentrations, stimulating Type I methanotrophs and resulting in the higher relative abundance of *pmoA* transcripts from Type Ia methanotrophs (*Methylomonas*, *Methylobacter* and *Methylosarcina*).

Physical disturbance in soil by earthworm activity

Ecologists believe that physical disturbance is an important determinant of diversity. It has been suggested that if there is frequent disturbance, the community could be dominated by microorganisms which can quickly respond to niche changes due to the disturbance (Buckling et al., 2000). Previous studies have reported that Type I methanotrophs respond quickly to any changes in the environmental condition compared to Type II methanotrophs (Graham *et al.*, 1993; Henckel *et al.*, 2000b). In the +worms soil column, owing to continuous disturbance by earthworm activity, there will be a continuous change in soil environmental conditions and Type Ia methanotrophs (e.g. *Methylobacter, Methylomonas, Methylosarcina*) which adapt better to the changing environment will dominate methane oxidation activity. In the control soil column, without any disturbance by earthworms, the niche might be more favourable for Type II methanotrophs (*Methylocystis*-related genera) Although this might not be the primary factor driving changes in the active methanotroph population, this in conjunction with other factors, could aid changes in diversity and function.

Influence of earthworms on predator-prey relationship

Bacteria in soil ecosystems are susceptible to predation by higher organisms in soil with protozoa estimated to be the major predators of bacteria in soil (Mancinelli, 1995). Predation can influence soil bacterial community structure, activity, population size and turnover of nutrients. Previous studies suggest that selective grazing by protozoa on methanotrophs could affect methanotrophic community structure in wetland soils (Murase and Frenzel, 2007, 2008). Experiments have provided evidence that protozoa are a major food source for earthworms and earthworms affect the distribution of protozoan in soil with reduced abundance of amoebae and flagellates observed in cast material relative to soil (Miles, 1963; Bonkowski and Schaefer, 1997). It can be hypothesized that earthworms, by feeding on predators of methanotrophs in +worms soil column at time II, eliminate predation and thereby could have increased methane oxidation. However, we need more experimental evidence to look into the elimination of predators by earthworms that are particularly important to methanotroph populations. In summary, we confirmed that the presence of earthworms in landfill cover soil increases soil methane oxidation potentials and provide conclusive evidence that earthworm activity in soil plays a major role in altering the relative abundance of active methanotroph populations, creating more favourable conditions for Type Ia methanotrophs rather than Type II methanotrophs. Results from this study can now be used to plan future *in situ* field studies and attempt to integrate earthworm-induced methanotrophy with other landfill management practises to reduce methane emissions from landfills.

Chapter 5

Effect of soil sample size on the analysis of methanotroph community structure using a *pmoA*-based microarray

5.1 Introduction

Soil is a heterogeneous medium, with variations in physico-chemical and structural characteristics, harbouring an exceptionally high diversity of microbes (Torsvik and Ovreas, 2002). The advent of cultivation-independent techniques based on the analysis of nucleic acids has widened our knowledge of previously unknown microbial communities and their function in soil ecosystem. Most of these techniques are based on PCR and are reliant on the efficiency and specificity of the PCR primers designed to target the microbial community in the environment. Functional gene probes, particularly *pmoA*, have been successfully used to study methanotrophs in the environment (reviewed in McDonald *et al.*, 2008). Over the last two decades, a number of *pmoA* PCR primers have been designed and have been evaluated with different techniques for their efficiency to retrieve *pmoA* sequences from the environment (reviewed in McDonald *et al.*, 2008). In this study, we tested the efficiency of the primer sets, A189/A682 and A189/mb661 for retrieval of *pmoA* sequences in the landfill cover soil and subsequent methanotroph community structure analysis using a *pmoA* based microarray.

Soil sampling strategies for analysis of microbial community structure are critical to accurately represent microbial community structure in soil ecosystem (Litchfield *et al.*, 1975). It is a common practice to collect a large amount or multiple soil samples followed by pooling of collected samples to create a homogenous final sample, from which sub-samples are taken for the community structure analysis. The results obtained from this sub-sample are considered to be representative of the *in situ* community structure. It has been suggested that soil sample size and distance between the sampling locations are important for the assessment of microbial community structure in a heterogeneous soil ecosystem (Grundmann and Gourbiere,

1999). However, size of soil sample used for the assessment of microbial community structure using molecular ecology techniques is often ignored (Ranjard and Richaume, 2001). A survey of literature revealed that only a limited number of studies had focussed on this issue (Ellingsoe and Johnsen, 2002; Nicol *et al.*, 2003; Ranjard *et al.*, 2003; Kang and Mills, 2006).

Ellingsoe and Johnsen (2002) was the first to study the effect of soil sample size on the assessment of bacterial community structure evaluated on the basis of DGGE fingerprints, with four soil sample sizes (0.01, 0.1, 1.0 and 10.0 g). The authors reported that the size of samples did have an influence on the assessment of bacterial community structure. Based on the results, it was suggested that small sample sizes are suitable to perform an inventory of the microbial diversity whereas large sample sizes are suitable to give an overall assessment of the community structure. It was also reported that there was high variation in DGGE fingerprints between replicates from small sample size, whilst consistent fingerprints were found with 10 g replicate samples. In an another study, Ranjard *et al*. (2003) compared the influence of soil sample size (0.125 g to 4.0 g) on the assessment of bacterial community structure using automated ribosomal intergenic space analysis (ARISA) in three different soil types (sand, silt and clay soils). Bacterial ARISA fingerprints were found to be consistent across different sample sizes with no apparent influence of the soil sample size. Microbial diagnostic microarray has been successfully used for high throughput screening of methanotroph communities in environmental samples (Bodrossy *et al.*, 2003; Stralis-Pavese *et al.*, 2004; Bodrossy *et al.*, 2006; Cebron *et al.*, 2007a; Chen *et al.*, 2008a). Microarray based analysis is considered to be highly sensitive and can assess community structure at a higher taxonomic resolution than other techniques (Bodrossy and Sessitsch, 2004). The role of diagnostic microarray in microbial

ecology studies and its advantages over other techniques have been discussed in Chapter 1. However, there is a lack of specific information on the effect of soil sampling strategy on the assessment of community structure using a microbial diagnostic microarray such as the *pmoA*-based microarray. The aims of the work described in this chapter were:

i. To compare the efficiency of different *pmoA* primer sets and PCR strategies (direct vs semi-nested) in retrieving a broad diversity of methanotrophs, based on a microarray analysis.

ii. To investigate the effect of soil sample size in the assessment of methanotroph community structure using a *pmoA*-based microarray.

5.2 Comparison of *pmoA* primer sets and PCR strategy (direct vs semi-nested)

pmoA primer sets, A189f/mb661r and A189f/A682r, were compared for the retrieval of *pmoA* sequences in the landfill cover soil samples and subsequent analysis of methanotroph community structure using a *pmoA* microarray (**Figure 5.1**). Soil samples were collected at different soil depth intervals (0 – 10, 10 – 20 and 20 – 30 cm) and DNA extraction was performed in triplicate from each soil sample. PCR of *pmoA* genes was performed on each individual replicate DNA sample and the PCR products were pooled before microarray analysis. For comparison of PCR strategies, direct PCR with primer sets A189/mb661 and A189/A682 was compared to a semi-nested PCR, which consisted of a first round PCR with A189f/A682r and a second round PCR with A189f/mb661r. In the semi-nested PCR strategy, after the first round PCR, the PCR products were diluted with nuclease free water to 1:10 ratio and 1 µl of this diluted PCR product was used as a template for the second round PCR.

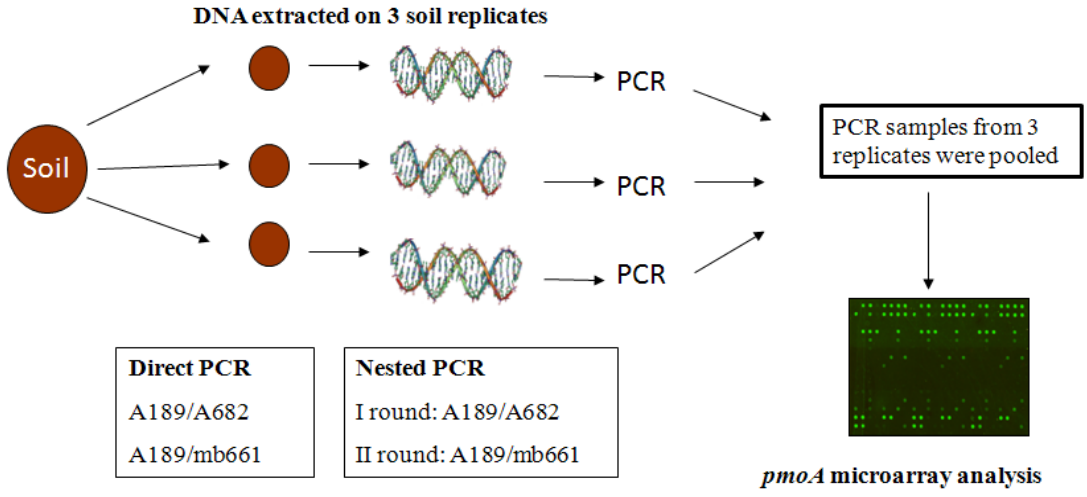

Figure 5.1 Schematic representation of the experimental methodology used to compare different *pmoA* primer sets and PCR strategy for the assessment of methanotroph community structure. For nested PCR strategy, 1 µl of 1/10 diluted PCR product from the first round were used as a template for the second round PCR.

5.3 Effect of soil sample size: DNA extraction, PCR and microarray analysis

The soil sampling strategy for this study has been schematically described in figure 5.2. *In situ* soil sample of different sizes (0.5, 5 and 50 g) from different soil depth intervals (0 – 10, 10 – 20, 20 – 30 cm) were compared in order to assess methanotroph community structure using a *pmoA*-based microarray. Three replicates samples were collected for each sample size. Soil samples, 5 and 50 g were manually homogenised by removing any stones without sieving the soil. DNA extraction was performed using the METHECO-DNA extraction protocol (Chapter 2). For 0.5 g *in situ* soil samples, it was used directly for DNA extraction whereas for 5 and 50 g *in situ* soil samples, three replicate 0.5 g soil sub-samples were used for DNA extraction. After DNA extraction, *pmoA* PCR was performed (PCR conditions described in chapter 2) on each individual replicates using the *pmoA* primer set, A189/T7-mb661. After PCR amplification, three individual replicates from each soil sample size were pooled and used for community structure analysis using a *pmoA*-based microarray.

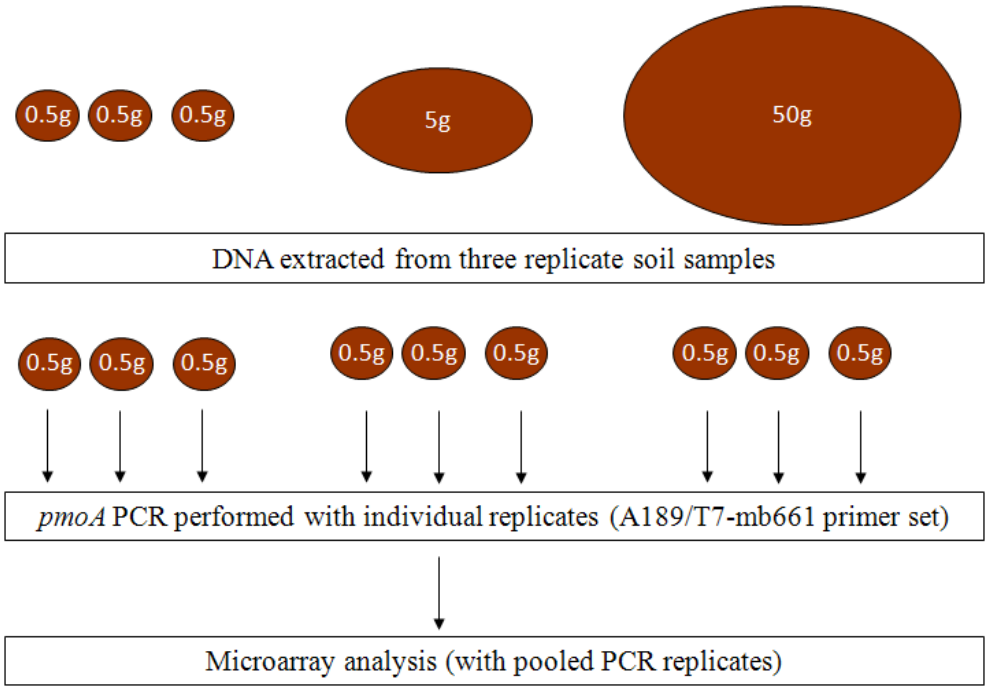

Figure 5.2 Schematic representation of the experimental methodology used to compare different soil sample size for the assessment of methanotroph community structure in a *pmoA*-based microarray.

5.4 Statistical analysis

Microarray data used for statistical analysis comprised all probe signals with the exception of positive controls, universal probes and higher level probes. No negative values were included in the analysis. The multivariate statistical analyses were employed to test for the effect of soil sample size (3 levels) at different soil depths on methanotroph community changes. The multivariate analyses were conducted using the software Primer 6 (PRIMER-E Ltd, Plymouth UK). Standardized probe intensities were used for all analyses. Bray-Curtis similarity metric was calculated using standardized data for samples (Kenkel and Orlóci, 1986; Minchin, 1987) and effect soil sample size on methanotroph community composition assessed using ANOSIM routines. All multivariate statistical tests were tested at $\alpha = 0.05$. (Statistical analysis for this study was performed by Dr Paul Bodelier).

5.5 Results

5.5.1 Comparison of *pmoA* primer sets and PCR strategy

Both primer sets A189/T7-A682 and A189/T7-mb661 yielded PCR products from all the soil DNA samples from the landfill cover soil. Hybridisation signal patterns for Type II methanotroph *pmoA* probes revealed similar hybridisation signals for both the primer sets (**Figure 5.3**). The Type II methanotroph population was dominated by the hybridisation signal for *pmoA* probes targeting the genus *Methylocystis* (probes Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459). However, hybridisation signals for Type Ia methanotroph *pmoA* probes revealed differences between the two primer sets. Hybridisation signals for *pmoA* probes targeting sub-groups of *Methylobacter* (Bb51-299, Mb292, MbA486, MbA557, Mb_C11-403 and Mb271) and *Methylomonas* (Mm531) were detected only with the primer set A189/mb661 and not with A189/A682. The primer set A189/A682 can amplify *amoA* sequences along with *pmoA* and hybridisation signals were detected for probes NsNv207, NsNv363 (both targeting *amoA* sequences from *Nitrosospira/Nitrosovibrio*), Nit_rel471 and Nit_rel351 (targeting AOB related clones), whereas no signals were detected with A189f/mb661r samples. Moreover, hybridisation signal for the probe targeting the novel *pmoA* copy from *Methylocystis* #1 (NMcy1-247) was detected only with the primer set A189f/A682r.

Comparison of hybridisation signal patterns for direct PCR (both A189/A682 and A189/mb661) and semi-nested PCR strategies revealed similar hybridisation signal for probes targeting *pmoA* sequences from Type II methanotrophs, particularly the genus *Methylocystis* (**Figure 5.3**). However, for semi-nested PCR samples, no hybridisation signals were detected for probes targeting *pmoA* sequences from Type

Ia methanotrophs whereas hybridisation signals were detected with direct PCR (only with A189/mb661 primer set). In summary, direct PCR with the primer set A189f/mb661r retrieved a broader diversity of *pmoA* sequences from methanotroph populations compared to direct PCR with the primer set A189/A682 or with a semi-nested PCR approach.

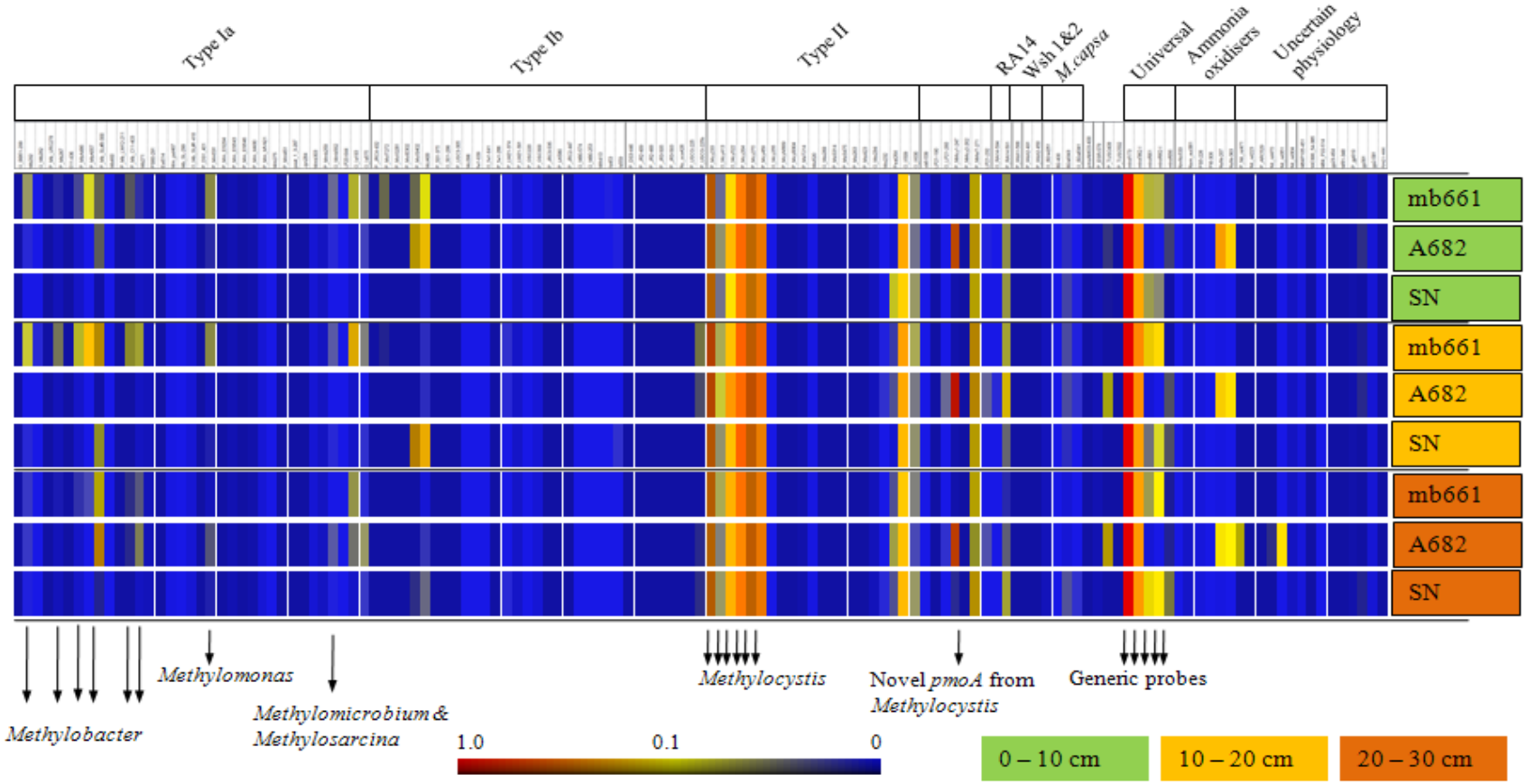

Figure 5.3 Comparison of methanotroph community structure between different primer sets and PCR strategies. mb661 and A682 represent the reverse primer used with A189 forward primer for the amplification of *pmoA* sequences in a direct PCR. SN indicates a semi-nested PCR strategy as described in Figure 5.1. 0 – 10 cm, 10 – 20 cm and 20 – 30 cm represents different soil depths. The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to the probe.

5.5.2 Effect of soil sample size on assessment of methanotroph community structure

The primer set, A189/mb661 was used to amplify *pmoA* sequences for determining the effect of soil sample size on the assessment of methanotroph community structure using a *pmoA*-based microarray (**Figure 5.4**). Strong hybridisation signals were detected for *pmoA* probes targeting the *pmoA* from Type II methanotroph genus *Methylocystis*, with no apparent differences between samples from different sample sizes. However, differences in signal intensities were observed between different sample size for the *pmoA* probe Msi423, targeting the genus *Methylosinus*. Samples from 0.5 g sample size revealed stronger signal intensity for the probe Msi423 compared to samples from 5 and 50 g sample sizes. Similarly, a broader diversity of *pmoA* sequences from Type Ia methanotrophs were detected with 0.5 g samples compared to 5 and 50 g samples. Hybridisation signals for probes Mb_SL-299, Mb_SL#1-418 (both targeting *pmoA* sequences related to soda lake *Methylobacter* isolates and clones) and Mmb303 (targeting *Methylomicrobium album*) were detected only with 0.5 g samples. Differences in probe signal intensities were observed between different sample sizes, with stronger signal intensities observed for probes Mcl408 (*Methylocaldum*), Mb292, Mb_C11-403 (sub-groups of *Methylobacter*), Msi423 (*Methylosinus*) with 0.5 g samples whilst weak or no signals were observed with 5 and 50 g samples.

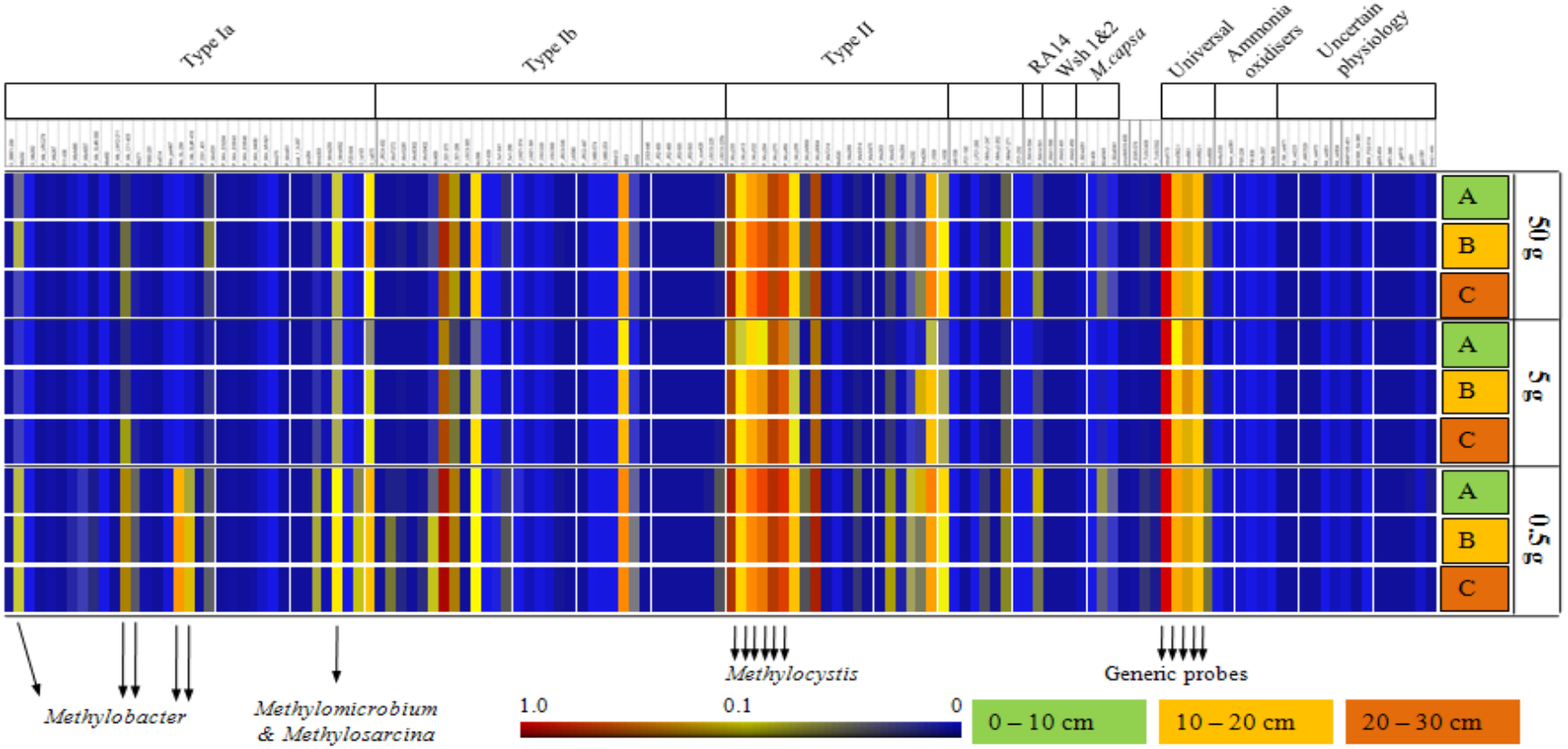

Figure 5.4 Comparison of methanotroph community structure between different soil sample sizes. 0 – 10 cm, 10 – 20 cm and 20 – 30 cm indicates different soil depths. 50 g, 5 g and 0.5 g indicates different amount of soil sample collected from the landfill cover soil. The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to the probe.

ANOSIM analysis revealed a significant effect of sample size on assessing methanotroph community structure using a *pmoA* based microarray (R = 0.646; p<0.004), supported by Multi-Dimensional Scaling (MDS) analysis. An MDS plot represents the similarity in methanotroph community structure between samples, with samples having similar community structure being clustered together (**Figure 5.5**.).

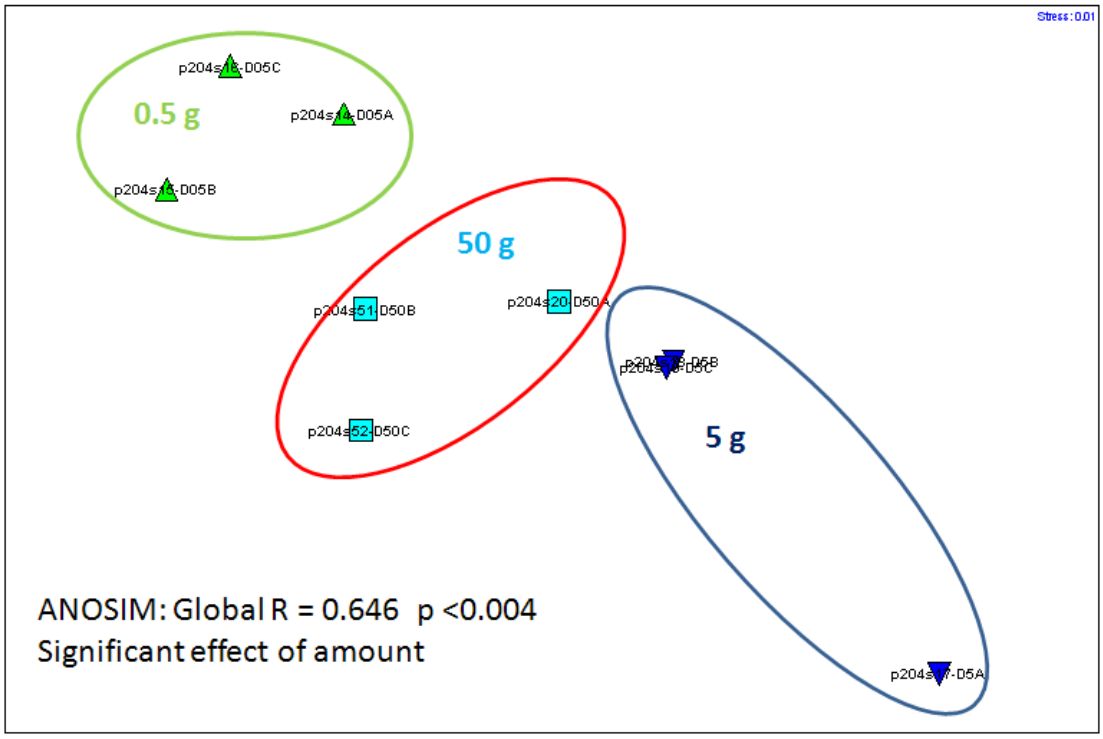

Figure 5.5 Non-metric multidimensional scaling plot based on the microarray data for different soil sample size used for assessing methanotroph community structure. A, B and C refers to soil depths 0 – 10 cm, 10 – 20 cm and 20 – 30 cm, respectively. 0.5 g, 5 g and 50 g represent different size of sample collected from the landfill cover soil.

5.6 Discussion

Microbial ecology has undergone profound changes after the advent of cultivation-independent techniques and its use to understand the structure and function of microbial communities in the environment. Microbial ecologists now have a suite of techniques, each with their own advantages and limitations, to study the microbial communities and depending on the question they seek to answer. At the centre of these techniques is the critical role of efficient nucleic acid extraction from environmental samples and issues associated with PCR, such as primer design, specificity and PCR conditions to amplify sequences from the extracted nucleic acid.

In a previous study, three different *pmoA* specific) primer sets (A189/A682, A189/mb661 and A189/A650 were compared by constructing clone libraries from three soil samples (Bourne *et al.,* 2001). Since the A189/A682 primer set was designed to amplify both *amoA* and *pmoA* sequences (Holmes *et al.,* 1995b), the authors reported retrieval of large number of *amoA* sequences (related to *Nitrosomonas europaea*) compared to *pmoA* sequences. In this study, microarray hybridisation for samples amplified with A189/A682 primer set revealed strong hybridisation signals for probes targeting *amoA* sequences related to *Nitrospira/Nitrovibrio*. Whilst strong hybridisation signals were detected for *pmoA* probes targeting Type II methanotrophs (particularly the genus *Methylocystis*), no hybridisation signals were detected for *pmoA* probes targeting Type Ia methanotrophs. During PCR amplification there is a chance that more abundant sequences in the sample are preferentially amplified compared to less abundant sequences (Ward *et al.,* 1992). In this landfill cover soil samples there is a possibility that *amoA* sequences are more abundant than *pmoA* sequences from Type Ia methanotrophs and hence the primer set A189/A682 might have preferentially

amplified *amoA* sequences. Reay *et al*.(2001) reported that this primer set might not be able to amplify *pmoA* sequences from all Type I methanotrophs. Moreover, Pacheco-Oliver *et al*. (2002) reported that the primer A682 excludes *pmoA* genes from *Methylocapsa* and other uncultivated methanotrophs. A broader diversity of methanotrophs was detected using the primer set A189/mb661 compared to A189/A682, particularly with Type Ia methanotrophs which was congruent to the result obtained by Bourne *et al*. (2001). Hybridisation signals were detected for *pmoA* probes targeting genera *Methylobacter* and *Methylomonas* that were not detected with A189/A682 primer set. Since the primer set A189/mb661 excludes amplification of *amoA* sequences, it was possible to detect *pmoA* sequences from Type Ia methanotroph that might be in low relative abundance compared to Type II methanotrophs. Based on the results obtained in this study, it is evident that primer set A189/mb661 retrieves a broader diversity of methanotrophs compared to A189/A682 from this landfill cover soil samples.

Whilst comparing direct versus semi-nested PCR strategy, the semi-nested PCR strategy retrieved a narrow diversity of methanotrophs, particularly Type Ia methanotrophs, when compared to direct PCR with A189/mb661. Horz *et al* (2005) employed a semi-nested approach to study methanotroph diversity in an upland grassland soil. It was reported that direct PCR with primer set A189/A682 revealed a high representation of *amoA* sequences, whilst the primer sets A189/mb661 and A189/A650 revealed a large number of non-specific amplifications. Hence the authors employed a semi-nested PCR approach to obtain high yields of *pmoA* amplicons. Based on our previous study (Chapter 3 and Héry *et al.*, 2008), use of the primer set A189/mb661 yielded no non-specific amplifications and the primer set was able to amplify *pmoA* from both Type I and Type II methanotrophs. Therefore,

based on the above results, a direct PCR with the primer set A189/mb661 was used for subsequent *pmoA* microarray analysis. However, the sensitivity of the *pmoA* primer sets to retrieve *pmoA* sequences from the environment might vary with the ecosystem and a reconnaissance study is essential to identify the suitable primer set for studying methanotroph diversity.

Sampling strategies for nucleic acid extraction from environmental samples are critical to understand the microbial communities in the environment and the sampling regime should be carefully designed to represent the ecosystem. One of the key factors that can influence the assessment of soil microbial community structure is the size of soil sample. Based on *pmoA* microarray and ANOSIM analysis, it was revealed there was a significant effect of soil sample size on retrieval of *pmoA* sequences and subsequent analysis of community structure using a diagnostic microarray. The major difference between the different soil sample sizes was the detection of *pmoA* sequences from Type Ia methanotrophs (genera *Methylobacter* and *Methylomonas*), with 0.5 g samples resulting in a broader diversity of Type Ia methanotrophs compared to 5 and 50 g samples. Owing to the averaging effect, it has been suggested that larger soil samples can be used to describe the overall community structure, since available community profiling techniques only detect the most abundant organisms in the sample. However, smaller soil samples can be used to perform a diversity inventory or discovering new bacterial strains (Grundmann and Gourbiere, 1999; Ellingsoe and Johnsen, 2002)

Soil is a heterogeneous medium consisting of numerous microenvironments, which can select for different microbial communities based on its physico-chemical characteristics (Ranjard and Richaume, 2001). It has been suggested that bacterial communities are associated with different sizes of soil aggregates (Hansel *et al.*,

2008). Therefore, it should be recognized that small sampling sizes might introduce a bias of sampling a particular soil aggregate size or microenvironment that might lead into misinterpretation of the overall community structure. Previous studies have reported that smaller soil sample sizes produced higher variability within replicates whereas larger sample size replicates produced consistent fingerprints. Moreover, it is a common practice to sieve the larger soil samples, which might actually introduce a bias when assessing the microbial community structure as the homogenization might not reflect the actual *in situ* heterogeneity.

Soil sample size for assessment of microbial community structure depends on the questions the study seeks to answer and the type of soil. Smaller sample size might be needed to uncover the extent of microbial diversity or for detection of novel or low abundance organisms. Moreover, handling and storage of numerous large soil samples can be expensive whereas small samples might benefit in terms of collection and storage. However, for studies which are directed at to observing community dynamics over space and time could use homogenized larger samples, which might represent the overall diversity. Moreover, the influence varies with the type of soil (sand, silt or clay soils) and it is recommended to perform an initial assessment on the effect of sample size on the particular environment, before a detailed study can be conducted.

Chapter 6

In situ spatial and temporal diversity of methanotroph community structure in a landfill cover soil

6.1 Introduction

Soil is a heterogeneous environment composed of many niches that harbour tremendous bacterial diversity (Curtis *et al.,* 2002). Bacterial diversity and abiotic factors such as soil particle size, porosity, water content, nutrient availability and pH can vary spatially from sub-millimeter scale to large geographic distances and also in time (Martiny *et al.*, 2006). While understanding spatio-temporal distribution patterns of bacterial communities and factors that influence these patterns and bacterial functions still remains a challenge (Torsvik and Ovreas, 2002), it is essential for a better understanding of biogeochemical cycling and ecosystem functioning (Green and Bohannan, 2006). It has been suggested that spatial isolation can influence bacterial community structure (Ranjard and Richaume, 2001; Sessitsch *et al.*, 2001). A number of studies have focussed on spatial heterogeneity of microorganisms in different soils (Grundmann and Debouzie, 2000; Nunan *et al.*, 2002; Mummey and Stahl, 2003; Fierer and Jackson, 2006). Franklin and Mills (2003) using amplified fragment length polymorphism (AFLP) and geostatistical variogram analysis reported autocorrelation of bacterial community structure at scales ranging from 30 cm to 6 m, depending on the extent of sampling. Analyzing bacterial community structure at a larger scale (across North and South America), Fierer and Jackson (2006) suggested that community structure was independent of the geographical distance but was influenced by soil pH. Recently, it has been suggested that an approach based on functional-traits within the context of environmental gradients might yield more insights into factors structuring microbial diversity rather than assessing total bacterial communities (McGill *et al.*, 2006; Green *et al.*, 2008). In this context, Philippot *et al*. (2009) used functional genes involved in denitrification as biomarkers to study field-scale spatial distribution

pattern of denitrifiers alongside denitrification activity and soil physicochemical properties.

Although, previous studies have characterized methanotroph communities in various landfill cover soils (Wise *et al.*, 1999; Uz *et al.*, 2003; Crossman *et al.*, 2004; Stralis-Pavese *et al.*, 2004; Chen *et al.*, 2007; Gebert *et al.*, 2008) there is a lack of knowledge on the spatio-temporal distribution of methanotrophs in landfill cover soils and the role of environmental heterogeneity on their activity and diversity. Variations in abiotic parameters such as CH_4 and O_2 availability, temperature, pH and nitrogen sources can cause shifts in methanotroph populations (Hanson and Hanson, 1996; Bodelier and Laanbroek, 2004). In this study we used a *pmoA*-based microarray (Bodrossy *et al.*, 2003) targeting *pmoA* from all known methanotrophs except from *Verrucomicrobia*, which as yet have only been found in high temperature, low pH environments (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008), to analyze the spatio-temporal distribution of methanotrophs in a landfill cover soil and also to identify any relationship between methanotroph community structure, methane oxidation potential and abiotic factors, particularly C/N ratio, NH_4^+ and NO_3^-. Variation in spatial methanotroph community structure was studied across five sites (5m nested square set-up) and three depths sampled at April 2007 (Apr 07) and temporal changes in comparison to three other seasons, September 06 (Sep 06), June 07 (Jun 07) and September 07 (Sep 07).

6.2 Soil sample collection and abiotic parameters measurement

Variation in spatial methanotroph community structure were analyzed across five sites (5m nested square set-up) and three depths sampled at April 2007 (Apr 07) and temporal changes in comparison to three other seasons, September 06 (Sep 06),

June 07 (Jun 07) and September 07 (Sep 07). Soil samples were analyzed for total N, total C, NH_4^+, NO_3^- (**Figure 6.1**) and moisture content (**Figure 6.6**) as described in Chapter 2.

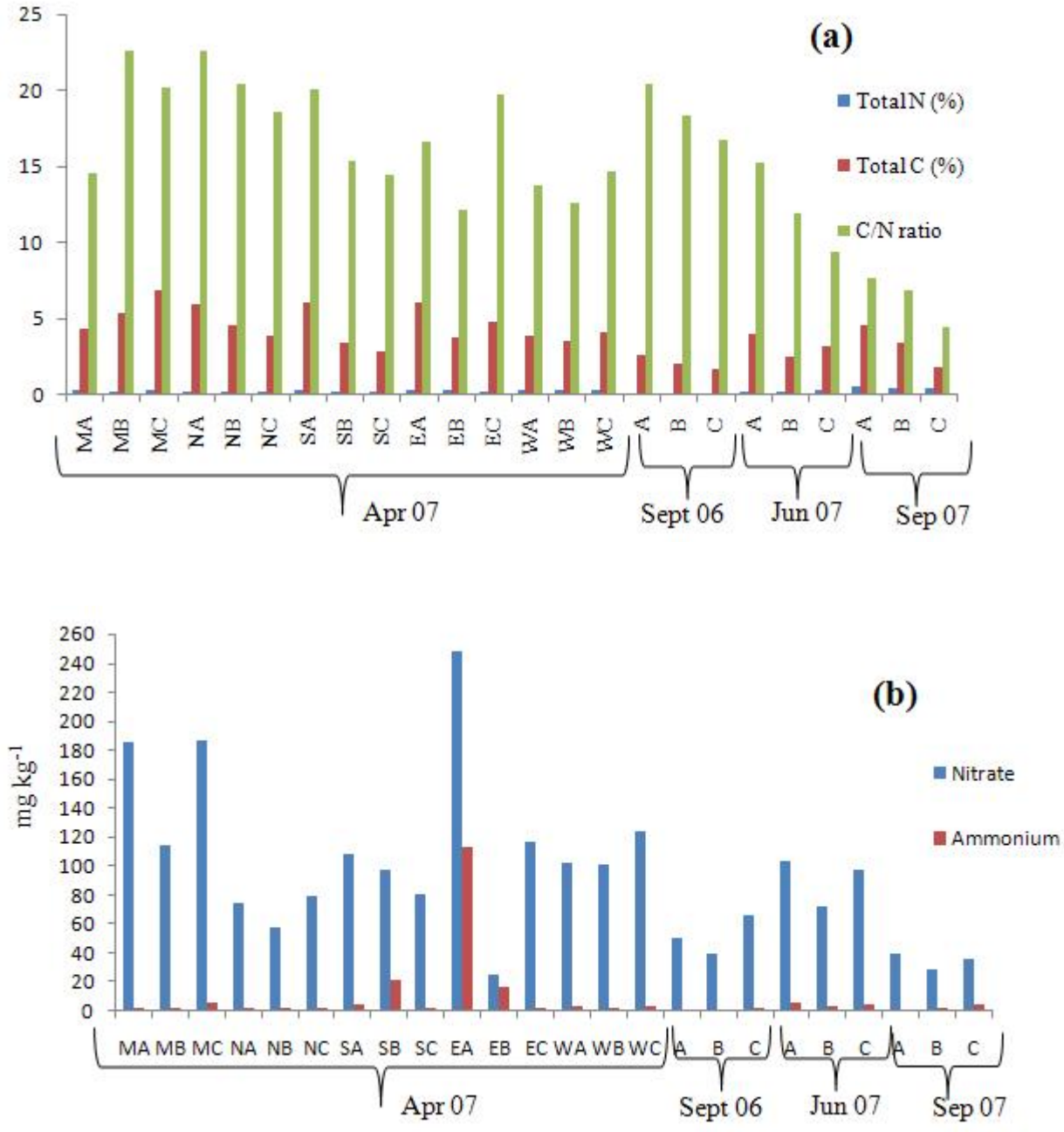

Figure 6.1 Graphical representation of soil abiotic parameters. (a) total C, total N and C/N ratio and (b) ammonium and nitrate from spatial and temporal soil samples. M, N, S, E and W represents sampling location with A, B and C representing 0 – 10 cm, 10 – 20 cm and 20 – 30 cm soil depths, respectively.

Rainfall and temperature data for each calendar month from September 2006 to September 2007 were collected from the British Atmospheric Data Centre (NERC) – MIDAS Land Surface Observation Stations Data (Table 6.1). These data were obtained from the weather station located at Wellesbourne, UK (~10 miles from Ufton landfill site). Data for December 2006 were not available.

Table 6.1 Rainfall and temperature data for each calendar month from September 2006 to September 2007. * Data for December 2006 were not available.

Month	**Total Rainfall month^{-1} (mm)**	**Average temperature day^{-1}**	
		Maximum Average temperature (^{0}C)	**Minimum average temperature (^{0}C)**
September 2006	95.8	22.13	13.04
October 2006	91.4	16.95	9.87
November 2006	68.2	12.09	4.20
December 2006 *	NA	NA	NA
January 2007	65.8	10.17	4.31
February 2007	62.2	9.41	2.58
March 2007	53.2	11.82	2.74
April 2007	6.8	17.79	5.42
May 2007	88.2	17.03	7.82
June 2007	106.8	20.36	11.13
July 2007	169.9	20.21	11.67
August 2007	26.2	21.57	10.93
September 2007	21.8	19.21	9.53

6.2.1 DNA extraction, *pmoA* PCR and microarray analysis

DNA was extracted from each soil sample in triplicate using a method described in Chapter 2 (METHECO DNA extraction protocol). PCR amplification of *pmoA* genes from DNA were performed as described in Chapter 2 on all the replicate DNA samples using the primer set A189f/T7-mb661r (Bourne *et al.*, 2001) with 50 ng of DNA as template. PCR products from three replicates of DNA samples were pooled for microarray analysis. Microarray analysis was performed as described in Chapter 2.

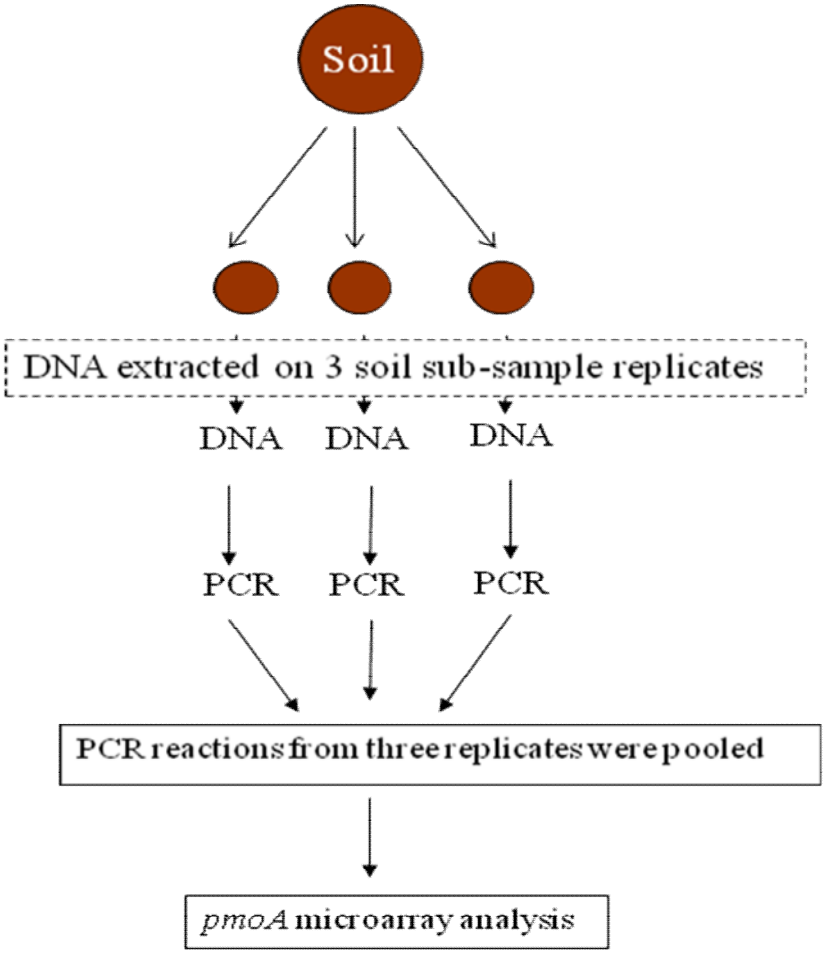

Figure 6.2 Schematic representation of the strategy for DNA extraction, PCR and *pmoA* microarray analysis for DNA samples from different sites and seasons.

6.3 Statistical analysis

The multivariate statistical analyses were employed to test for the effect of depth (5 levels), site (5 levels) and month (4 levels) on methanotroph community changes. The multivariate analyses were conducted using the software Primer 6 (PRIMER-E Ltd, Plymouth UK). Standardized probe intensities were used for all analyses. Bray-Curtis similarity metric was calculated using standardized data for samples representing different sites and months (Kenkel and Orlóci, 1986; Minchin, 1987) and effect of depth, site and season on methanotroph community composition assessed using PERMANOVA and ANOSIM routines. All multivariate statistical tests were tested at $\alpha = 0.05$. Univariate analysis of variance was tested at $P<0.05\%$. For ANOVA, significant factors were then compared using Tukey *post-hoc* test. All tests were conducted at $\alpha = 0.05$. Correlation between environmental parameters and array probe signals were analysed using Pearsons product moment correlation in the SPSS software package (SPSS Inc., USA). Data that were not normally distributed were transformed (square root or Log). Positive and negative relationships are represented by positive and negative values. The values are arranged in a descending order and matched to their corresponding probes. The colours in the probe columns correspond to specific methanotroph groups. The probability for significance is $P<0.1\%$. (Statistical analysis was performed by Dr Guy Abell)

6.4 Results and Discussion

6.4.1 Methanotroph community structure – temporal distribution

Seasonal differences in methanotroph community structure were observed, with *pmoA* microarray analysis revealing a high diversity of *pmoA* sequences belonging to both Type I and II methanotrophs across different seasons (**Figure 6.3**

and **6.5**). Strong hybridisation signals detected for *pmoA* probes targeting the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459) across all sampled seasons suggested that the genus *Methylocystis* (a Type II methanotroph) might be the dominant methanotroph. Seasonal variations in the relative abundance of Type Ia or Ib methanotroph *pmoA* sequences were also observed. Based on the hybridisation signals for probes targeting Type Ia methanotrophs, Jun 07 samples exhibited a lower diversity of Type Ia methanotroph *pmoA* sequences, with only weak signals being detected for probes Mmb562 (*Methylosarcina/Methylomicrobium*) and Mb_C11_403 (a sub-group of *Methylobacter*) compared to other seasons. For probes targeting *pmoA* from the genus *Methylocaldum* (Type Ib methanotroph; probes MclT272, MclS402 and Mcl408) strong hybridisation signal intensities were detected only with the samples from Apr 07 and Sep 07. Hybridisation signals for *pmoA* probes targeting the genus *Methylococcus* (501-375, 501-266 and Mc396) were relatively strong in seasons Apr 07 and Jun 07 compared to the other two seasons. However, signals for probes USCG-225 and USCG-225b targeting *pmoA* sequences from Upland soil cluster Gamma (USCG)(Knief *et al.*, 2003) were restricted only to samples from Apr 07. Sequences related to the novel *pmoA* of *Methylosinus trichosporium* (NMsiT-271) were detected with samples from all seasons with variations in signal intensities (it should be noted that, the PCR primer set, A189f-mb661r applied, excludes most of the novel *pmoA* copies of Type II methanotrophs).

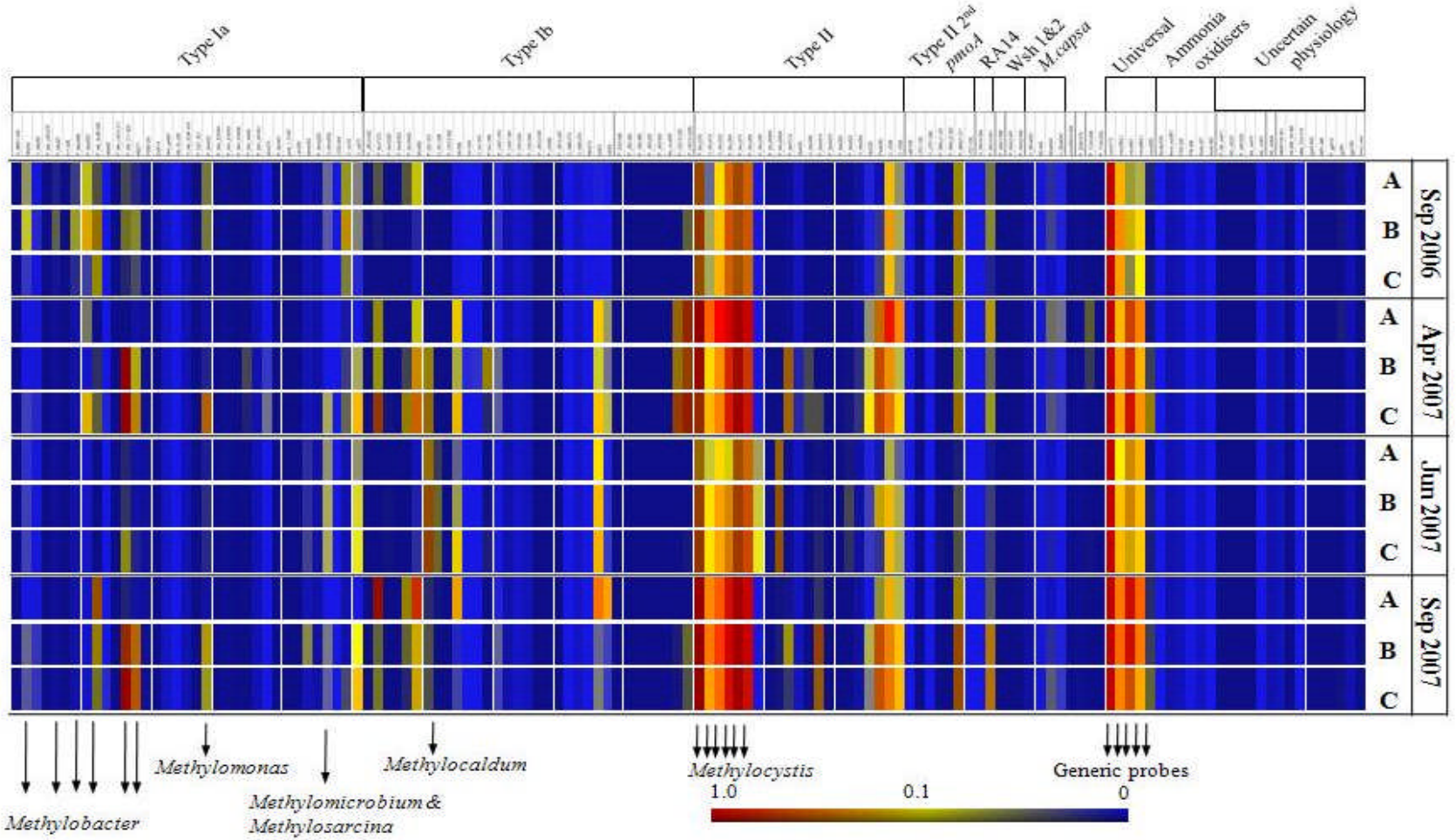

Figure 6.3 Microarray analysis representing methanotroph community structure from different seasons. A, B, and C refer to 0 – 10 cm, 10 – 20 cm and 20 – 30 cm soil depth, respectively. Month/year represents the time of sampling. Apr 07 samples represent SA, SB and SC samples from the spatial sampling set which was used for soil methane oxidation assay.

PERMANOVA analysis demonstrated a significant seasonal effect of methanotroph community structure but no significant effect of soil depth. ANOSIM analysis demonstrated a significant difference between temporal samples and the spatial sampling set (Apr 07), supported by Multi-Dimensional Scaling (MDS) analysis (**Figure 6.4**).

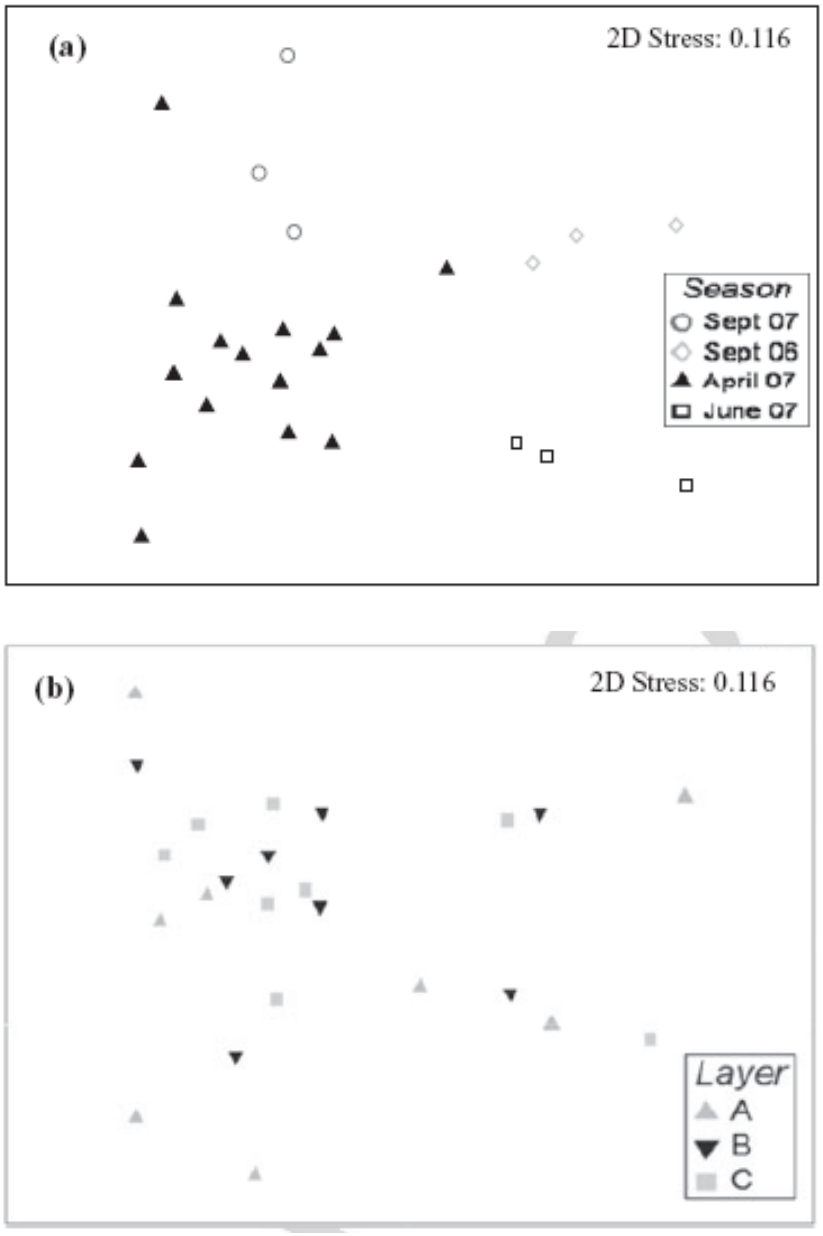

Figure 6.4 Non-metric multidimensional scaling plot based on the microarray data. (a) spatial and temporal methanotroph community structure and (b) methanotroph community structure at different soil depths. A, B and C refers to soil depths 0 – 10 cm, 10 – 20 cm and 20 – 30 cm, respectively. Spatial refers to samples from Apr 07 used for spatial diversity analysis.

MDS plots represent the similarity in methanotroph community structure between different samples, with samples having similar community structure clustered together. Sep 06, Jun 07 and Sep 07 were all significantly different to the spatial sampling set from Apr 07 (R= 0.83, 0.86, 0.503, respectively and all P<0.01), whilst there was no difference between layers (R=0.023, P=0.27). Shrestha *et al*. (2008), using *pmoA* gene and PLFA-SIP analyses, demonstrated that the activity and diversity of methanotrophs fluctuated over time, with different niches for Type I and II methanotrophs in a rice field ecosystem. Similar to this study, the authors also observed differences in soil methane oxidation capacity between different seasons (**Figure 6.6**). However, the differences in methane oxidation capacity in this study could not be correlated to any changes in methanotroph community structure across different seasons. A low diversity of Type Ia methanotrophs were observed at Jun 07, whereas the soil methane oxidation capacity was higher compared to other seasons (Sep 06 and 07), when there was a broader diversity of Type Ia methanotrophs. It might be possible that at this season i.e Jun 07 either *Methylocystis*, the dominant methanotroph, might have increased in relative abundance over Type Ia methanotrophs owing to favourable environmental conditions, or Type Ia methanotroph populations might be low in relative abundance and below the detection limit of the microarray. Strong signal intensities for *pmoA* probes targeting the genus *Methylocystis* across all seasons might indicate that either *Methylocystis* are present and are active across different seasons, enduring seasonal changes in the environment or the population are in a state of dormancy. *Methylocystis* spp. are known to form lipid cysts to survive unfavourable conditions (Whittenbury *et al.*, 1970a). Moreover, DNA may be stable in dormant and dead cells (Lindahl, 1993) and along with extra-cellular DNA, which remains adsorbed to

soil particles (Paget *et al.*, 1992), this might contribute to the DNA-based assessment of community structure. Use of mRNA-based analysis yields information on active bacterial transcription at the time of sampling. Owing to the low stability of mRNA, it is a significant challenge to recover intact mRNA (Hurt *et al.*, 2001). Recently, Chen et al., (2007) successfully extracted high quality mRNA extraction from soil, which enabled analysis of the expression of functional genes (*pmoA* and *mmoX*) encoding subunits of MMO. *In situ* analysis of methanotroph community structure in the future should employ functional gene-based analysis (DNA) alongside mRNA-based approaches to enable detection of methanotrophs that are present and active, respectively.

6.4.2 Methanotroph community structure – spatial distribution

Spatial patterning of bacterial communities, both vertical and horizontal, has been suggested to exist in soil over different scales (Green and Bohannan, 2006). Soil samples for analysis of spatial methanotroph diversity were collected during the season Apr 07 (**Figure 6.5**) As with results for different seasons with the temporal analysis of methanotroph populations, all samples from the spatial analysis revealed strong signal intensities for *pmoA* probes targeting the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459). In the case of Type Ia methanotroph probes, Mb_C11-403, Mb271 (sub-groups in genus *Methylobacter*), Mm531 (*Methylomonas*) and Mmb562 (*Methylomicrobium/Methylosarcina*), hybridisation signals were detected in all sites with varying signal intensities. The probe Mmb_562 targets both the genera *Methylomicrobium* and *Methylosarcina*, while the probe Mmb_303 targets only the genus *Methylomicrobium*. No hybridisation signals were obtained for the probe Mmb_303 suggesting that *pmoA*

sequences from the genus *Methylosarcina* contributed to the signal detected from the probe Mmb_562. Hybridisation signals for probes Mb292, MbA486 and MbA557 (all probes targeting sub-groups in the genus *Methylobacter*) were detected only in samples from site E and W, whereas hybridisation signals for probes targeting soda lake *Methylobacter* clones (Mb_SL_299 and Mb_SL#1-418) were detected only in sites M and W, indicating differential distribution of *Methylobacter* sub-groups. Hybridisation signals for probes targeting the genera *Methylocaldum* (MclT272, Mcl1408, Mcls402) and *Methylococcus* (501-375, Mc396) were detected in all sites with marginal differences in signal intensities. However, hybridisation signals for probes fw1-639, fw1-641 and fw1-286 targeting *pmoA* sequences from *Methylococcus-Methylocaldum*-related marine and freshwater sediment clones, were only detected at site M. *pmoA* sequences related to peat soil clones (peat264) and the novel *pmoA* copy of *M. trichosporium* (NMsiT-271) were retrieved from all sites, as indicated by the hybridisation signal for respective *pmoA* probes. Positive signals for the genus *Methylosinus* were in most cases inconclusive because of the lack of supporting signals from other probes of overlapping specificity and/or because of near-cutoff signals. The only exceptions are samples SA and SB with positive signals for *M. trichosporium* (probes MsT214 and Msi269).

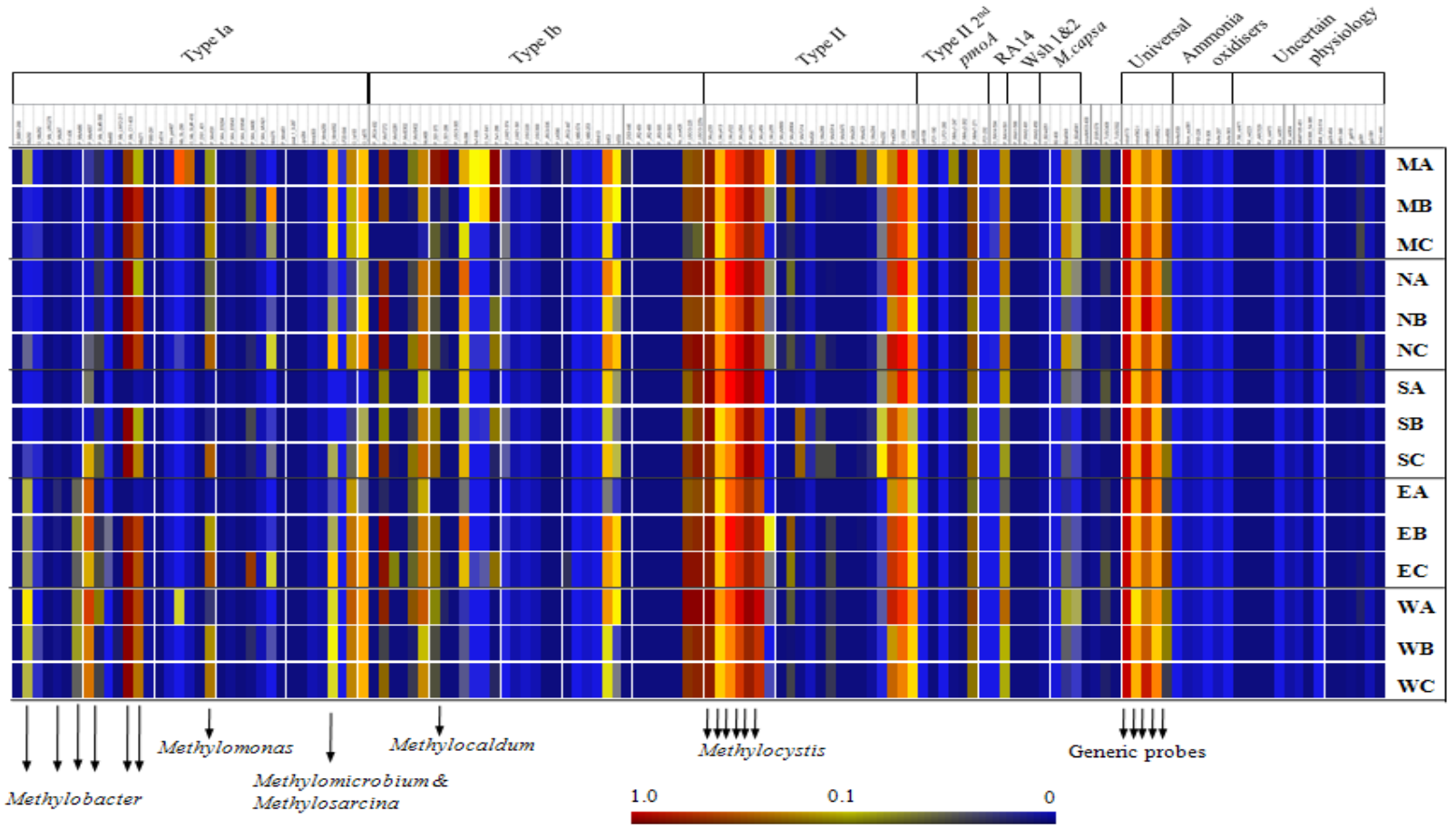

Figure 6.5 Microarray analysis representing methanotroph community structure from different spatial locations and soil depths. A, B, and C refer to 0 – 10 cm, 10 – 20 cm and 20 – 30 cm soil depths, respectively. Soil samples were collected in a five metre nested square set-up with M, N, S, E and W representing each sampling location.

Multidimensional scaling analysis of methanotroph community structure in the landfill cover soil represented limited spatial pattern at a 5 m scale (**Figure 6.4a**) and at different soil depth (**Figure 6.4b**). Other studies have used similar scales to assess spatial distribution of bacterial communities (Franklin and Mills, 2003; Ritz *et al.*, 2004; Philippot *et al.*, 2009). Results have reported both spatially independent (Felske and Akkermans, 1998; Fierer and Jackson, 2006) and dependent (Franklin and Mills, 2003; Ritz *et al.*, 2004) microbial community structure. In this study, only minor differences in methanotroph diversity were observed between spatial samples, with no recognizable pattern in the methanotroph community structure (both vertical and horizontal) was observed. Fierer and Jackson (2006) reported that bacterial distribution is controlled by soil pH rather than geographic distance. It might also be possible that the spatial scale used for sampling in this study might not be relevant in discriminating the distribution patterns there might be difference in spatial distribution at a larger scale or even at the microscale. Grundmann and Debouzie (2000), using a micro-sampling approach, suggested the existence of a spatial dependence for NO_2^- and NH_4^+ at a millimetre scale, which is closer to the bacterial micro-habitat. Variations in physical, chemical and biological properties at soil microsites could exert a profound influence on methanotroph diversity and activity and hence future studies should include sampling at the microscale level. Landfill cover soil can be a heterogenous environment. For example, methane emission rates can vary seasonally and spatially (Jones and Nedwell, 1993), with also spatial differences in soil abiotic factors. However, the results from this study indicated a spatially stable methanotroph community structure (at 5 m scale), while there is a shift in the community structure across different seasons in a landfill cover soil. It could be suggested that seasonal variations might have an overruling influence in

shaping the methanotroph community structure compared to other factors. In this study, we did not measure methane fluxes in the landfill and cannot correlate the impact of differential spatial and/or seasonal methane emission hot spots with methanotroph community structure in this landfill cover soil. Measurement of methane fluxes in future studies will be essential to identify the hot spots of methane emission to plan future sampling regimes.

6.4.3 Relationship of methanotroph diversity with abiotic factors and methane oxidation potential

Spatial patterning of microbial diversity can be influenced by environmental heterogeneity, with different community structure along an environmental gradient (Green and Bohannan, 2006). Soil abiotic factors (total C, total N, NH_4^+ and NO_3^-) were analysed in all the spatial and temporal soil samples (**Figure 6.1**). The landfill cover soil had a pH of 7.62 and a clay content of 12%. Comparison between different seasons using a one-way analysis of variance (ANOVA) with tukey *post-hoc* test to determine the differences between months revealed significant differences ($P<0.05\%$) between some abiotic factors. Krave and colleagues (2002) found no significant differences in bacterial community structure over time though there was a seasonal effect on pH, soil moisture and nutrient contents. However, in the case of spatial samples, no significant differences ($P<0.05\%$) were observed in abiotic factors between sites and different soil depths. Apr 07 recorded the lowest total rainfall $month^{-1}$ with 6.8mm followed by Sep 07 with 21.8mm, whereas Sep 06 and Jun 07 records were 95.8 mm and 106.8 mm, respectively.

BEST analysis (a combination of Bio-Env and BVSTEP procedures) were performed using the PRIMER-6 package, to understand correlations between the

similarity matrix of probe intensities and a secondary matrix of physical parameters. BEST analysis demonstrated no simple relationships between abiotic parameters and methanotroph community profiles ($R<0.21$), however, there were significant correlations between individual probe intensities and abiotic parameters (Table 6.2). Based on product moment correlation analysis, *Methylocystis* probes had a positive correlation with total N, total C and a negative correlation with NH_4^+ (Table S3). For *pmoA* probes targeting the genus *Methylobacter*, no significant correlation pattern was observed with any of the abiotic factors. Type Ib methanotroph genera *Methylocaldum* and *Methylococcus* probe signal intensities had a positive correlation with total N. *pmoA* probe signals for USCG had a positive correlation with total C, C/N ratio, NH_4^+ and NO_3^-, whereas it revealed a negative correlation with total N and water content. However, it should be noted that hybridisation signals for USCG probes were detected only in season Apr 07.

Table 6.2 Analysis of relationship between abiotic parameters and individual *pmoA* probe signal intensities.

Type Ia methanotrophs | **Type Ib methanotrophs** | **Upland Soil Cluster Gamma** | **Type II methanotrophs** | **Tropical Upland soil cluster**

Probes	Total N	Probes	Total C	Probes	C/N ratio	Probes	NH_4^+	Probes	NO_3^-	Probes	Water content
Mcy233	0.68	Mcy522	0.68	TUSC409	0.55	TUSC409	0.55	MbA557	0.26	Mb292	0.56
Mcy459	0.59	TUSC409	0.57	Mm275	0.40	fw1-639	0.40	Mb267	0.18	McyB304	0.40
Mcy413	0.58	Mcy413	0.46	USCG-225b	0.37	501-286	0.38	TUSC409	0.16	Mmb303	0.39
Mcy270	0.52	Mc396	0.46	USCG-225	0.37	fw1-286	0.38	MsT214	0.16	Mmb562	0.38
MsS314	0.47	Mcy264	0.45	fw1-639	0.36	fw1-641	0.36	MbA486	0.14	501-286	0.35
Mcy522	0.38	USCG-225	0.38	fw1-641	0.33	Msi423	0.34	USCG-225b	0.07	Mb267	0.31
Mmb303	0.33	Mcy459	0.37	fw1-286	0.29	USCG-225b	0.25	Mc396	0.04	MbA486	0.26
Mcl408	0.31	USCG-225b	0.36	Mb267	0.26	USCG-225	0.25	MclS402	0.03	Mb_SL#3-300	0.26
Mc396	0.31	Mcy270	0.36	Mcy522	0.25	Mmb562	0.24	USCG-225	0.03	Mcy255	0.24
Mcy264	0.30	Mcy255	0.29	MbA486	0.19	Mcy255	0.21	Mb292	0.01	Mb282	0.17
NMsiT-271	0.23	fw1-641	0.27	Mcy264	0.13	501-375	0.18	Mcy522	-0.02	MsS314	0.15
MclS402	0.19	fw1-639	0.26	MbA557	0.10	McyB304	0.15	Msi423	-0.03	Mb271	0.02
MclT272	0.16	MclT272	0.26	MclT272	0.10	MbA557	0.14	Mcl408	-0.05	Msi423	0.02
Mcy255	0.16	Mcy233	0.25	Mmb562	0.09	Mm275	0.12	Mb460	-0.05	501-375	0.01
Mb_SL#3-300	0.13	fw1-286	0.21	Mc396	0.08	Mcy522	0.11	Mcy233	-0.05	Mm531	-0.05
501-375	0.12	NMsiT-271	0.20	LW21-374	0.07	MbA486	0.10	LW21-374	-0.07	NMsiT-271	-0.13
McyB304	0.12	Mcl408	0.20	Mcy255	0.06	Mb292	0.06	Mcy264	-0.08	Mb460	-0.16
501-286	0.10	Mm275	0.19	Mm531	0.06	Mc396	0.03	fw1-286	-0.09	MbA557	-0.21
Mb460	0.07	501-286	0.11	Mb271	0.05	Mb_C11-403	-0.01	fw1-641	-0.09	Mb_C11-403	-0.29
LW21-374	0.03	McyB304	0.10	Mb_C11-403	0.04	Mcy264	-0.01	501-375	-0.11	MclS402	-0.30
Mb271	0.00	LW21-374	0.08	NMsiT-271	-0.01	Mb267	-0.02	fw1-639	-0.12	Mcy233	-0.31
Mb282	0.00	Mmb562	0.07	501-286	-0.03	Mm531	-0.05	501-286	-0.12	fw1-641	-0.33
MsT214	0.00	Mb271	0.05	Msi423	-0.06	MclS402	-0.05	Mcy270	-0.13	fw1-286	-0.35
Msi423	-0.04	Mb_C11-403	0.02	McyB304	-0.06	Mcy413	-0.07	Mm275	-0.13	LW21-374	-0.36
Mb_C11-403	-0.04	MbA557	0.01	Mcl408	-0.10	LW21-374	-0.08	Mcy255	-0.13	fw1-639	-0.38
Mmb562	-0.06	MclS402	0.00	Mcy413	-0.13	NMsiT-271	-0.10	Mcy413	-0.14	Mm275	-0.45
USCG-225	-0.08	Msi423	-0.03	Mb292	-0.14	MsT214	-0.11	Mcy459	-0.15	Mcl408	-0.47
Mb292	-0.08	501-375	-0.04	Mcy270	-0.16	Mb271	-0.12	Mb282	-0.16	P_MclT272	-0.48
TUSC409	-0.08	MbA486	-0.08	MclS402	-0.17	Mcy459	-0.13	Mb_C11-403	-0.18	MsT214	-0.56
USCG-225b	-0.10	Mm531	-0.10	Mb282	-0.19	Mcl408	-0.13	MclT272	-0.18	Mcy413	-0.57

fw1-641	-0.10	Mb460	-0.17	Mcy459	-0.21	Mcy270	-0.15	McyB304	-0.19	Mc396	-0.57
fw1-286	-0.11	Mb282	-0.22	MsT214	-0.23	Mb282	-0.16	MsS314	-0.20	Mcy459	-0.57
fw1-639	-0.13	Mb292	-0.23	501-375	-0.23	MclT272	-0.19	Mb271	-0.20	Mcy270	-0.61
MbA557	-0.15	MsT214	-0.23	Mb460	-0.25	Mcy233	-0.21	Mmb562	-0.24	Mcy522	-0.66
Mm531	-0.16	Mb267	-0.30	Mb_SL#3-300	-0.38	Mb460	-0.24	Mb_SL#3-300	-0.25	Mcy264	-0.71
Mm275	-0.23	Mmb303	-0.30	Mcy233	-0.39	Mmb303	-0.29	NMsiT-271	-0.28	TUSC409	-0.73
MbA486	-0.30	MsS314	-0.36	Mmb303	-0.57	MsS314	-0.47	Mmb303	-0.28	USCG-225	-0.89
Mb267	-0.55	Mb_SL#3-300	-0.39	MsS314	-0.75	Mb_SL#3-300	-0.50	Mm531	-0.30	USCG-225b	-0.89

Comparison of soil methane oxidation potential across different seasons revealed that soil samples from depth 0 – 10 cm from Apr 2007 exhibited the highest methane oxidation potential compared to other soil samples (**Figure 6.6**). Assessment of methane oxidation potential was carried out as described in Chapter 2 in triplicate with 10g of soil sub-samples in 120 ml serum vial bottles with a headspace methane concentration of 1% (v/v). In Apr and Jun 2007, the soil samples from 0 -10 cm soil depth exhibited the highest methane oxidation potential followed by soil samples from 10 – 20 cm and 20 – 30 cm soil depth. However, during Sep 2006 and 2007, the methane oxidation potential of soil samples from 0 – 10 cm soil depth exhibited lower oxidation potential compared to other two depths, while little or no differences were observed between 10 – 20 cm and 20 – 30 cm soil depth.

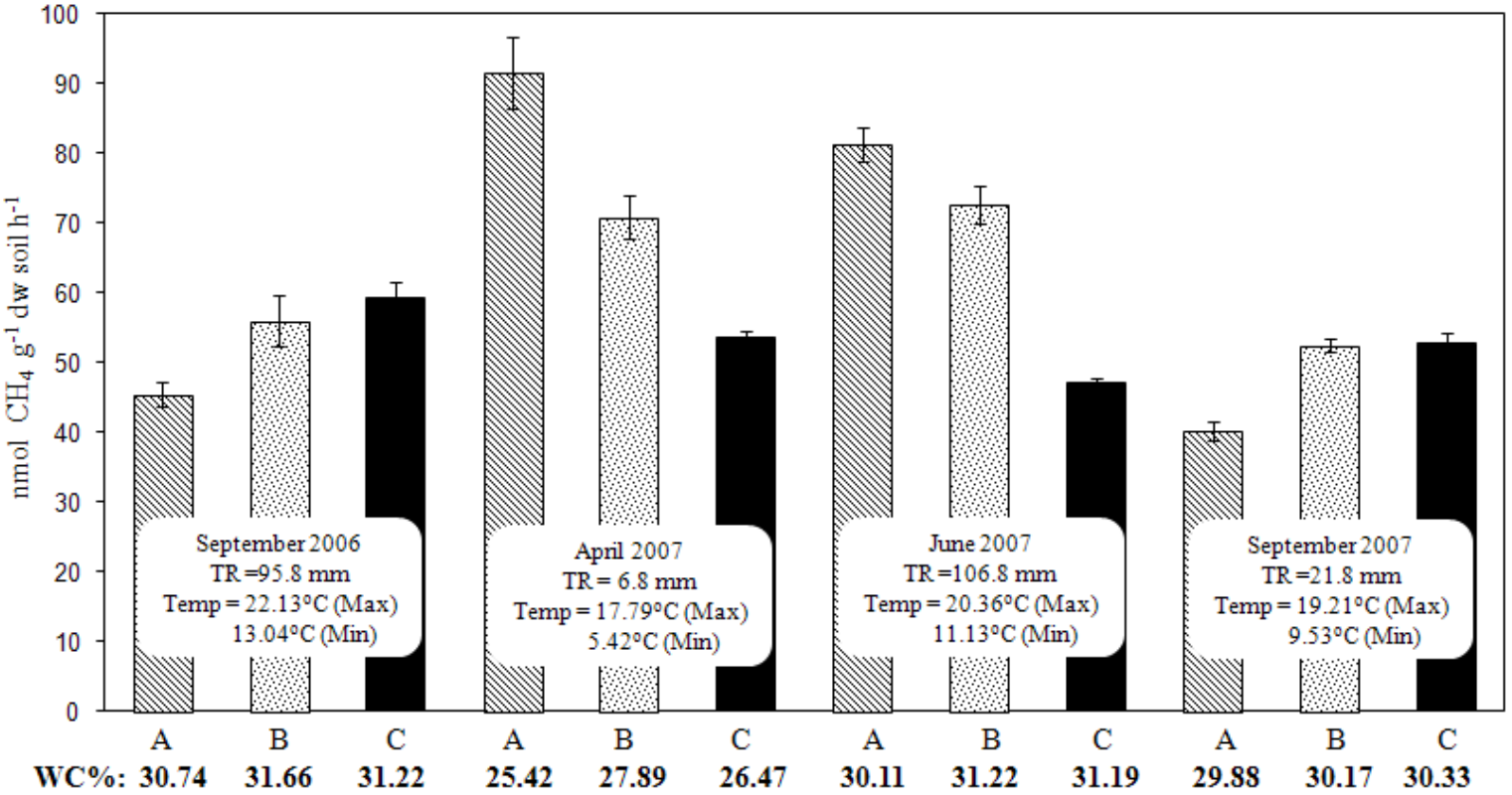

Figure 6.6 Graphical representation of the temporal differences in soil methane oxidation potential. The bars represent the methane oxidation potential. A, B, C represents 0 -10 cm, 10 – 20 cm, 20 – 30 cm soil depths. The month/year represent the time of soil sampling at landfill. "TR" refers to total rainfall month^{-1}, whereas "Temp" refers to average temperature day^{-1} (Max = maximum temperature and Min = minimum temperature). Monthly rainfall and temperature data from September 2006 to September 2007 are listed in Table S1. WC% denotes soil water content (in percentage). Error bars represent standard error between three replicates.

Analysis of correlation between water content and methane oxidation potential revealed no significant correlation ($P>0.05$). Methane oxidation potential observed in this study were about 10 fold lower than previously reported values, ranging from 0.998 to 25 μmol CH_4 g^{-1} dw soil h^{-1} (Nozhevnikova *et al.*, 1993; Kightley *et al.*, 1995; De Visscher *et al.*, 1999; Borjesson *et al.*, 2004). In the case of temporal samples, Jun 07 received the highest total monthly rainfall within the sampling periods (with the preceding month also receiving high total rainfall), with waterlogged conditions observed in the landfill. We might expect a lower diffusion of oxygen through soil depths at Jun 07 compared to other seasons. Amaral & Knowles (1995) suggested that Type II methanotrophs dominate methane oxidation at low oxygen concentrations, while Type I methanotrophs dominate at relatively high oxygen concentrations. Methanotroph community structure at Jun 07 revealed a lower relative abundance of Type Ia methanotrophs than Type II methanotrophs when compared to other seasons. Interestingly, correlation analysis with methane oxidation potential and *pmoA* probe signal intensities for temporal samples (Table 6.3) revealed that all probes targeting Type Ia methanotroph *pmoA* sequences had a negative correlation with methane oxidation potential. However, for all *pmoA* probes targeting the genus *Methylocystis* (except for the probe Mcy233) along with USCG *pmoA* probes revealed a positive correlation. This result is in contradiction to results obtained in previous studies (Henckel *et al.*, 2000a; Bodrossy *et al.*, 2006; Noll *et al.*, 2008), and use of techniques such as a mRNA-based *pmoA* microarray would give us more information on *in situ* activities of different methanotroph communities. No apparent correlation between soil methane oxidation potential with any measured abiotic factors was found, suggesting that a number of interacting

mechanisms between methanotrophs and abiotic factors might contribute to methane oxidation activity.

Table 6.3 Analysis of correlation between methane oxidation potential and individual probe signal intensities. *pmoA* probe cell colours indicate the different groups of methanotrophs.

Type Ia methanotrophs	Type Ib methanotrophs	Upland Soil Cluster Gamma	Type II methanotrophs

Probes	CH_4 oxidation potential
JRC4-432	0.52
USCG-225b	0.41
fw1-641	0.30
USCG-225	0.30
Mcy522	0.26
fw1-639	0.24
Mcy255	0.20
Mcy264	0.16
LP20-644	0.16
Mcy413	0.14
McyB304	0.13
501-375	0.12
Mc396	0.10
Mcy270	0.10
Mcy459	0.02
MsT214	-0.01
MbA557	-0.02
Mm275	-0.04
MbA486	-0.15
Mcy233	-0.15
Mb_C11-403	-0.18
Mmb562	-0.20
Mmb303	-0.20
Mb460	-0.23
LW21-374	-0.24
NMsiT-271	-0.32
Mb271	-0.34
MsS314	-0.38
Mm531	-0.41
Mb292	-0.41
MclT272	-0.42
Mcl408	-0.42
MclS402	-0.60
Mb_SL#3-300	-0.69

Previous studies have reported correlations between microbial community structure and environmental parameters such as salinity (Crump *et al.*, 2004), depth (Ovreas *et al.*, 1997) and oxygen (Franklin *et al.*, 1999). Type I and Type II methanotrophs are known to occupy different niches and heterogeneity in abiotic factors, such as nitrogen and oxygen availability, can influence methanotroph diversity and activity (Graham *et al.*, 1993; Bender and Conrad, 1995; Bodelier *et al.*, 2000; Henckel *et al.*, 2000a). In this study, we could not define any single measured factor responsible for driving methanotroph population. Methanotroph activity and diversity in the environment could be influenced by a complex set of interactions with different abiotic parameters and possibly individual methanotroph species respond to one or more different parameters in combination and/or in contrasting ways to the other species. Analyzing the impact of abiotic factors on community structure also depends on the taxonomic resolution used in the study. For example, in this study while considering the genus level, hybridisation signal patterns for *pmoA* probes targeting the genus *Methylocaldum* did not reveal any consistent pattern with that of the abiotic factors (Table 6.2). However, at a finer resolution, probes targeting specific species level within the genus *Methylocaldum* such as MclT272 (*M. tepidum*) and MclS402 (*M. szegediense*) revealed some correlation with abiotic parameters. Taxonomic resolution largely depends on the technique used to assess the microbial community structure and due consideration should be given while interpreting the results.

Microbial methane oxidation in the landfill cover soil is a significant sink for methane produced in the landfills. An integrated approach, correlating spatio-temporal distribution of methanotrophs with variations in environmental factors, is vital to design a successful landfill cover soil management strategy. In this study, it

was revealed that there was a temporal dynamics in methanotroph community structure, along with seasonal changes in abiotic factors. However, limited spatial patterning (vertical and horizontal) of methanotrophs and abiotic parameters were observed. We compared *pmoA* probes hybridisation signal intensity with the measured abiotic factors to determine the driving factors for methanotroph diversity and activity. Although, we found some relationship with the probe signals and abiotic factors, the evidence was inconclusive. These results emphasize the fact that methanotrophs cannot be treated as one discrete group of microorganisms when attempting to relate community structure with soil abiotic factors and indeed these factors affect the diversity differently, often in conflicting ways. *In situ* mRNA based analysis could provide with a better understanding on the role of abiotic factors in altering the diversity of active methanotrophs rather than focussing on methanotrophs present based on DNA analysis. Future studies must also include measurements of a wider range of abiotic factors such as *in situ* O_2 and CH_4 availability and *in situ* experiments, such as the effect of earthworm population density to increase methane oxidation potentials (Héry *et al.*, 2008), to understand the role of biotic and abiotic factors affecting methanotrophs activity and diversity.

Chapter 7

Understanding functional diversity of methanotrophs in soil microniches

7.1 Introduction

Soil, as a result of its structural organization in aggregates of different sizes and stability creates a composite of discontinuous microniches with variations in physico-chemical and structural characteristics (Torsvik and Ovreas, 2002). Distribution of pores, particles and organic matter determines the heterogeneous structural framework of the soil which provides microorganisms with their habitat. Bacterial activity in soil is often characterised by high spatial variability, which is determined by the existence of distinct microniches within the soil that influences the life-cycle and activity of bacteria (Young and Ritz, 1998; Ranjard and Richaume, 2001). Soil structure can be a key regulatory element in defining microbial habitats by influencing nutrients and substrate availability, competition, moisture content and grazing by predators (Focht, 1992; Wardle *et al.*, 1998). For example, in the context of soil structure, microbial decomposition processes in soil can be either aerobic or anaerobic as determined by the rate of gaseous exchange through pore space in a soil crumb (Young *et al.*, 1998). It has been suggested that microbial community composition varies with soil aggregate size and also as a function of location within the aggregates (Hansel *et al.*, 2008). Spatial arrangement of the soil structure can also determine the ecological interactions between microorganisms and their environment. This can determine the assemblage of microbes in soil by influencing the size, distribution and connectivity of soil pores, factors that are critical for the movement and dispersal of microorganisms, which can define the formation and maintenance of microbial communities.

Numerous endogenous and exogenous factors are known to affect soil aggregation and subsequently soil structure. Endogenous factors affecting soil aggregation include soil texture, clay mineralogy, nature of exchangeable cations,

quantity and quality of humus whereas exogenous factors include weather, biological processes, land use and management (Lal, 1991). Griffiths and Young (1994) suggested that intense drying of the top soil followed by sudden rewetting could lead to soil aggregate disintegration, which could subsequently cause changes in the soil structure. Soil fauna are reported to influence soil structure to varying degrees, earthworms and termites in particular can affect soil porosity, bulk density and infiltration, through their burrowing (Elkins *et al.*, 1986). Foraging, respiration and defecation by soil fauna can also affect the transport and transformation of soil organic carbon within pore spaces and thus influence the stability of microaggregates (Foster, 1988). Even microbes are known to have an impact on soil structure. Roberson *et al*. (1995) showed that high C:N ratio increased extracellular polysaccharides from microbes, which in turn increased soil structural stability. Although soil structure is an important factor controlling microbial diversity and activity in soil, there is a paucity of information on how changes to the soil structure influence the activity and diversity of bacterial communities, associated with a particular function such as soil methane oxidation.

The advent of stable isotope probing (SIP) (Boschker *et al.*, 1998; Radajewski *et al.*, 2000; Manefield *et al.*, 2002; Jehmlich *et al.*, 2008) and its subsequent applications has provided significant insights into "active" microbial populations and their role in biogeochemical cycling (reviewed in Dumont and Murrell, 2005) . mRNA-based analysis of bacterial transcription activities have been used successfully to yield information on active bacterial populations. An mRNA-based *pmoA* microarray has been previously used to describe active methanotroph community structure in lysimeters simulating landfill soil (Bodrossy *et al.*, 2006) and peat soil (Chen *et al.*, 2008a). By using mRNA instead of DNA, *pmoA* microarray

analysis can detect changes in relative abundance of *pmoA* transcripts from different methanotroph populations. Although, both DNA-SIP and mRNA based analysis are effective techniques to detect active bacterial populations, so far there has been no direct comparison between DNA-SIP and mRNA microarray techniques to detect the community structure of active methanotrophs in a defined environment.

Physical disruption to the soil structure results in disintegration of soil aggregates leading to the loss of soil microniches and thus microbial habitats. We hypothesise that if microniches created by soil aggregates are physically disrupted and the soil structure significantly changed, the activity and functional diversity of bacterial communities will also be altered. The aims of this study were:

(i) To identify whether there are any shifts in activity and functional diversity of methanotrophs when microniches created by soil aggregates are physically perturbed.

(ii) To compare DNA-SIP and mRNA based analysis to analyze the community structure of active methanotrophs using a *pmoA* microarray.

7.2 Soil sample collection and soil treatments

Landfill soil for this study was collected at April 2008. The moisture content of the soil was measured by oven drying the soil at 80^{o}C until constant weight (26.37% ± 1.28). The soil was stored at 4^{0}C until use. In order to process the soil for different levels of soil perturbation, large stones were removed and big clumps of soil were broken down manually with minimal disturbance to the soil structure. The soil was air-dried at room temperature (20^{o}C) for 2 days to facilitate sieving. After three days, soil clumps of 1.5-1.75 cm in diameter were used as the control soil treatment. The air-dried soil was sieved using a 2 mm mesh sieve (sieved soil treatment) and for ground soil treatment, the sieved soils were ground using a pestle and mortar (**Figure 7.1**). These soil treatments; control, sieved and ground were subsequently used for soil methane oxidation potential assessments. Before methane incubations, sub-samples of these soil treatments (time 0) were stored at -80^{o}C for subsequent nucleic acid extraction and molecular biological analysis.

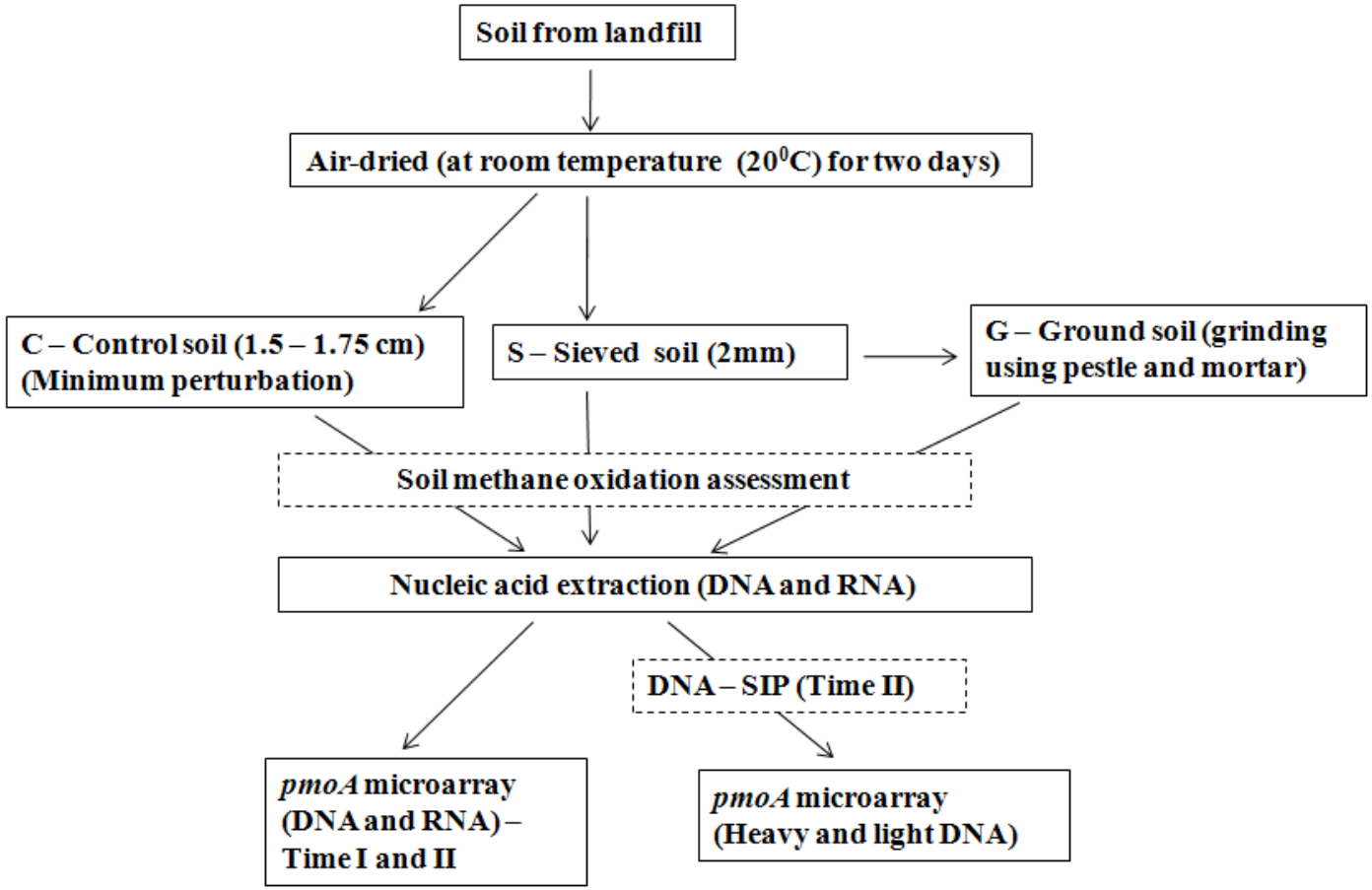

Figure 7.1 Schematic representation of the experimental methodology used in this study. Time I and II represents 120 hours and 293 hours after soil methane incubation.

7.2.1 Assessment of soil methane oxidation potential

Assessment of methane oxidation potential was carried out with 5g of soil sub-samples as described in Chapter 2. The moisture content of the soil was restored to the original soil moisture content at the time of sampling by adding de-ionized water. The experiment was performed in quadruplicate for each soil treatment; two of them were incubated with ^{13}C-CH_4 and other two with ^{12}C-CH_4 (as control for the SIP incubations). After 120 hours (time I), soil sub-samples of 1 g and after 293 hours (time II) all the soil samples from three soil treatments were stored at -80^{o}C for molecular biological analysis. Differences in methane consumption between soil treatments were tested using a 1-way ANOVA with Tukey's HSD Post hoc test using the SPSS 11.0 software package (SPSS Inc. USA). All tests were conducted at $\alpha = 0.01$. (Statistical analysis performed by Dr Guy Abell)

7.2.2 DNA-Stable isotope probing

Nucleic acids extracted from time II were used to compare mRNA-based microarray and DNA-SIP in assessing methanotroph community structure. One microgram of DNA extracted at time II (pooled DNA from ^{13}C-CH_4 replicates) was used for density gradient ultra-centrifugation followed by gradient fractionation, precipitation of DNA and buoyant density measurements of the fractions as described in Chapter 2.

7.3 Results

7.3.1 Methane oxidation potential of different soil treatments

Comparison of soil methane oxidation potential revealed that physical perturbation of the soil structure had a significant effect on the soil methane oxidation potential (**Figure 7.2**), with sieved and ground soils revealing higher methane oxidation potential than control soil ($F = 533.40$; $p<0.001$) (**Table 1**). All the soil treatments showed a lag phase before actively oxidizing methane and statistical analysis revealed significant differences in methane oxidized between three soil treatments, during the lag phase ($F=126.70$, $p<0.001$). Ground soil samples exhibited a longer lag phase compared to other two soil treatments. At time I (120 hours), sieved and ground soil treatments exhibited higher methane oxidation potential compared to the control soil treatment. ANOVA analysis revealed that at time I, methane oxidation by control soil was significantly different from both sieved and ground soils, whereas there was no significant difference between sieved and ground soils ($F=22.12$, $p<0.001$). After time I, there was an increase in the methane oxidation potential for sieved and ground soil samples, whereas the methane oxidation potential for the control soil samples remained unchanged. At time II, ground soil samples had higher total methane consumption (76.53 ± 0.003 µmol g^{-1} soil) compared to sieved (64.49 ± 1.53 µmol g^{-1} soil) and control (19.89 ± 1.46 µmol g^{-1} soil) soil samples. Methane oxidation between time intervals I and II and also for total methane consumption, all three soil treatments showed significant difference ($F=649.30$, $p<0.001$ and $F=533.40$, $p<0.001$). Although, sieved and ground soil exhibited similar methane oxidation between 120 h and 240 h, the methane oxidation potential for sieved soil was lower when compared to the ground soil after 240 hours of methane incubation.

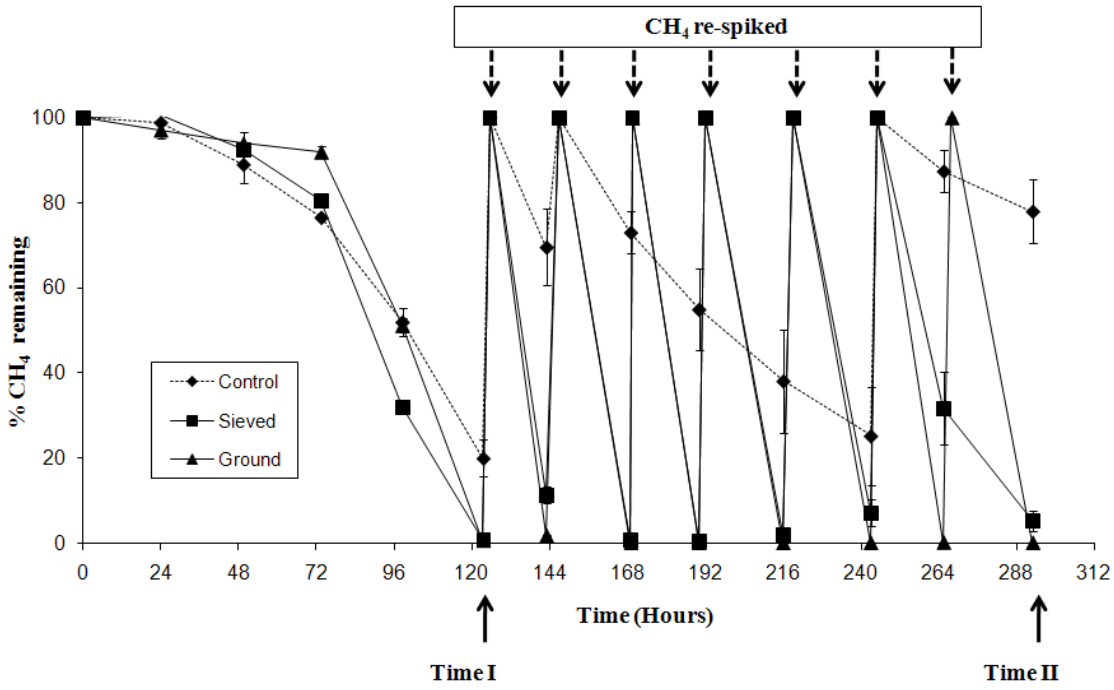

Figure 7.2 Comparison of soil methane oxidation between three soil treatments; control, sieved and ground soil. Time I and Time II represents the time of soil sampling for nucleic acid extraction and molecular biological analysis. Dashed arrows indicate the time points of methane re-spiking (Control – 120, 144 and 240 hours; Sieved and ground samples – at every 24 hours intervals after 120 hours).

Table 7.1 Statistical analysis representing the significance of difference in methane oxidation between different soil treatments at different time intervals. Different letters indicate significantly different mean methane oxidation values.

Soil treatment	**CH_4 oxidized at Time I**	**CH_4 oxidized between time I and time II**	**Total CH_4 oxidized**	**Lag time (at 73.5 hours)***
Control	a	a	a	a
Sieved	b	b	b	b
Ground	b	c	c	c
	F = 22.12 (p<0.001)	F = 649.30 (p<0.001)	F = 533.40 (p<0.001)	F = 126.70 (p<0.001)

* The analysis takes into account of CH_4 oxidation at 73.5 hours.

7.3.2 Methanotroph community structure based on DNA analysis

Soil treatments, i.e. sieving and grinding did not appear to have an impact on soil methanotroph community structure as revealed by similar hybridisation signals

for *pmoA* probes from all three soil samples before methane incubation at time 0 (**Figure 7.3**). Hybridisation signals at time 0 (for all three samples) revealed strong signal intensities for probes targeting *pmoA* sequences from the genus *Methylocystis* (Type II methanotroph; probes Mcy233, Mcy413,Mcy522, Mcy264, Mcy270 and Mcy459) and a novel Type II *pmoA* (NMsiT-271), while weak signals were observed for probes targeting the genera *Methylobacter* (Type Ia methanotroph; Mb-SL#3-300, Mb_C11-403), *Methylocaldum* (Type Ib methanotroph; MclT272, MclE302, Mcl408) and *Methylosinus* (Type II methanotroph; MsS314). After methane incubation, microarray hybridisation signal patterns at time I in all three soil samples revealed a higher diversity of *pmoA* sequences from type Ia methanotrophs, particularly within the genera *Methylobacter* (Mb292, Mb_C11-403, Mb271), *Methylomonas* (Mm531) and *Methylomicrobium/Methylosarcina* (Mmb303 and Mmb562). Comparison of results between different soil treatments at time I revealed minor differences in hybridisation signal patterns, with detection of weak signals for probes Mb282, Mb267, MbA486 and a strong signal for the probe MbA557 (all the probes targeting *Methylobacter* sub-groups) only with sieved soil sample. However at time II, signals for these probes were detected only in the ground soil sample with similar signal intensities and they were not detected either in sieved or control samples. For Type Ib methanotrophs, no signals were detected with probes targeting the genus *Methylocaldum* (MclT272, MclE302 and Mcl408) at time I and II, whereas weak signals were detected at time 0 from all three samples. Similar hybridisation profiles were observed with probes targeting Type II methanotrophs (for the genera *Methylocystis* and *Methylosinus*) at both time 0 and time I, with stronger signal intensity for the probe targeting the latter at time I. However, the major difference in hybridisation signal patterns was between time I and time II, particularly for probes

targeting the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459). These probes exhibited weaker signal intensities for sieved and ground soil samples, whereas stronger signals were detected from control samples.

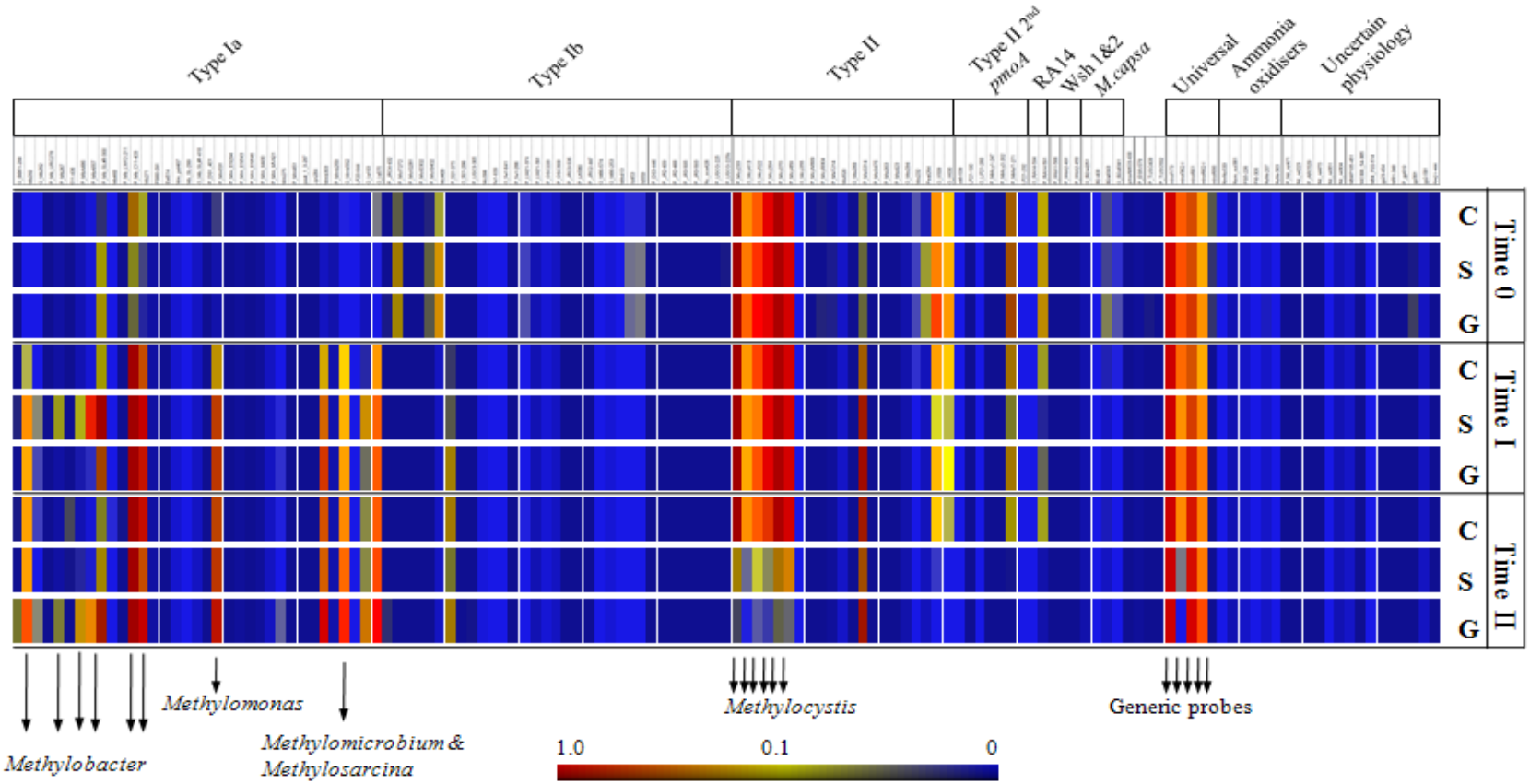

Figure 7.3 Microarray analysis representing hybridisation signal patterns for DNA from different soil treatments. C, S and G represents control, sieved and ground soil treatment. Time 0 indicate sampling time before methane incubation, whereas time I and II represents 120 hours and 293 hours after methane incubation, respectively. The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to the probe.

7.3.3 Active methanotroph community structure based on mRNA analysis

RNA hybridisation profiles were generally similar to DNA hybridisation profiles for all three samples at both time I and II, with minor differences between time RNA and DNA samples from time II (**Figure 7.4**). When compared to DNA samples at time II, no hybridisation signals were detected for probes Mb282, Mb267 and MbA486 (targeting sub-groups of *Methylobacter*) for ground RNA samples. Also weaker hybridisation signals were observed for probes Mb292 and Mmb303 (with all three RNA samples) in comparison to DNA samples. mRNA-based microarray results confirmed the lower relative abundance of *Methylocystis*-related *pmoA* transcripts at time II in ground and sieved samples compared to control sample.

7.3.4 Comparison of mRNA-based microarray and DNA-SIP analyses

In the DNA-SIP experiment, ^{13}C-DNA (heavy) was separated from the ^{12}C-DNA (light) using gradient ultracentrifugation and fractionation. The densities of the heavy (fraction 7 and 8) and light (fraction 11 and 12) DNA fraction were 1.725 g ml^{-1} and 1.704 g ml^{-1}, respectively. Comparison of hybridisation profile for heavy DNA and mRNA revealed congruent results with only minor variations in signal intensities for certain *pmoA* probes (**Figure 7.4**). Stronger signal intensity was detected with the probe Mmb303 for heavy DNA samples when compared to very weak signal intensity for RNA samples. Also for heavy DNA samples, hybridisation signals were detected for probes USC3-305 (Upland soil cluster Gamma), McyM309 (sub-group of genus *Methylocystis*), Msi263 (*Methylosinus*) and peat264 (environmental clones from peat), which were not detected with RNA samples.

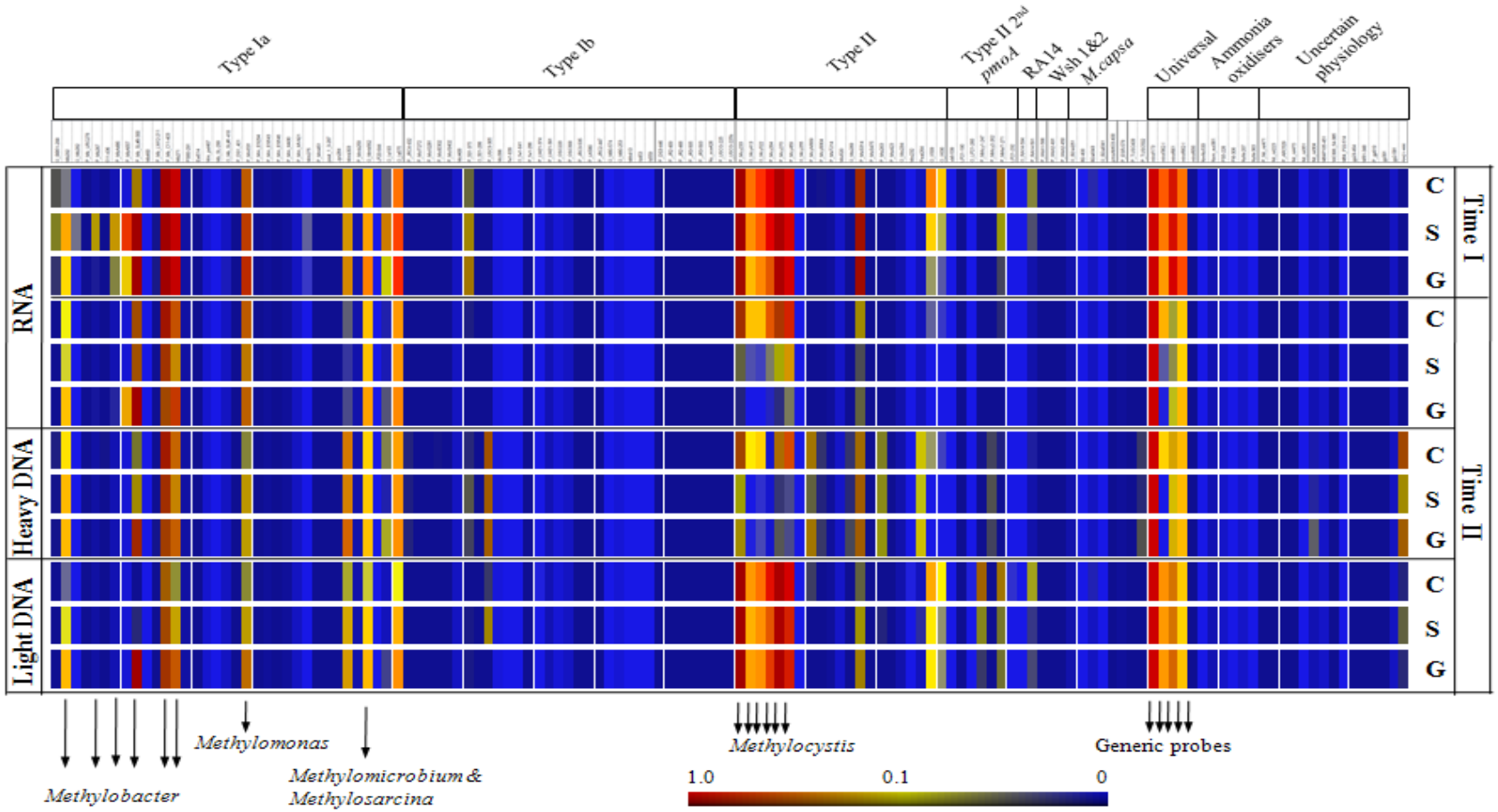

Figure 7.4 Microarray analysis representing active methanotroph community structure based on hybridisation signal patterns for RNA at time I & II and heavy and light DNA from SIP at time II. C, S and G represents control, sieved and ground soil treatment, respectively. Time I and II represents 120 hours and 293 hours after methane incubation, respectively.

7.5 Discussion

Recent studies confirm the preferential location of bacterial communities in microniches within a soil crumb and also their preference for different sizes of soil aggregates (Ranjard *et al.*, 2000a; Ranjard *et al.*, 2000b). Conrad (1996) suggested that even an individual soil crumb is likely to exhibit redox gradients; however these redox gradients are not well understood owing to methodological difficulties in measuring redox at the microscale. Soil crumbs can be highly heterogeneous and can be dominated either by aerobic or anaerobic metabolism which is determined by the rate of diffusion of gases (particularly oxygen) and microbial activity (Grant and Rochette, 1994; Renault and Stengel, 1994). In this study, we coupled activity measurements with bacterial community structure assessment to understand how physical perturbation to the soil structure affects soil microbial processes, particularly focussing on soil methane oxidation and methanotroph diversity.

Influence of soil perturbations on methanotroph activity

Soil treatments, sieving and grinding, resulted in the disturbance of the physical soil structure leading to the destruction of microniches created by the arrangement of soil aggregates. It can be assumed that a near homogenous condition was created with sieved and ground soil samples, whereas the control soil with minimal disturbance had the microniches intact at the start of methane incubations. The mode of physical disturbance used in this experiment resulted in a different range of particle sizes, with ground soil having a finer soil particle size compared to sieved and control samples. Decrease in particle size is associated with increase in surface area per gram of soil and hence the interfacial area available for bacterial colonization (Marshall, 1975). Sieved and ground soil samples have a higher surface area

compared to the control soil samples and hence offer a better opportunity for increased interactions between microorganisms and the liquid/gaseous phase. Previously, it has been showed that a decrease in the size of soil aggregates increased the rate of nitrification in soil (Hattori, 1973). Also the breakdown of aggregates could have led to the release of previously unavailable nutrients from the microniches created by soil aggregates.

Outer and inner fractions of soil crumbs offer widely different microbial habitats owing to different physico-chemical characteristics (Hattori *et al.*, 1976; Harris, 1994). Chenu *et al* (2001) suggested that the location of bacterial communities within the soil structure affects their response to substrate addition. Clay soils, such as the landfill cover soil used in this study, were reported to have pores that were <0.2 µm in diameter, whereas sandy soil had pores with diameters between 6 µm and 30 µm. Furthermore, the pores in clay soils are poorly interconnected as opposed to sandy soils in which the pores are well connected (Marshall, 1975). Lower methane oxidation potential in the control soil could be due to lower diffusion rates within the soil crumbs. It is also possible that methanotrophs present on the surface of the crumbs were responsible for the majority of the methane oxidation process, due to better access to their substrate. Oxygen plays a critical role in determining the methane oxidation process in methanotrophs, with level of oxygen availability influencing both activity and diversity of methanotrophs (Amaral and Knowles, 1995). In the case of the control soil, it is likely that there is a low diffusion of O_2 into the inner portions of soil crumbs resulting in anaerobic conditions in these microniches, conditions that are not favourable for aerobic methane oxidation. Anaerobic microsites within soil crumbs can exist as a consequence of O_2 consumption rates exceeding diffusive flux rates (Greenwood and

Goodman, 1967; Grant and Rochette, 1994; Renault and Stengel, 1994). However, differences in soil characteristics can be observed between clayey and sandy soils and therefore the effect of perturbation on soil bacterial communities to physical perturbation are bound to differ. The difference in methane oxidation activity between different soil treatments in this study could be due to several factors including increased availability of interfacial area, nutrient availability and rate of nutrient (especially gases) diffusion in sieved and ground soils.

Influence on active methanotroph community structure

Comparison of the community structure of active methanotrophs in different soil samples at time I by using an mRNA-based microarray revealed a similar community structure, indicating that disturbance of the soil structure did not have an effect on active methanotroph community structure at time I. Interestingly, at time II, both sieved and ground soil samples exhibited lower relative abundance of *pmoA* transcripts from Type II methanotrophs, particularly from the genus *Methylocystis*, when compared to Type I methanotrophs. Since the microarray analysis indicates the relative abundance of *pmoA* sequences, this could indicate either an increase in relative abundance of *pmoA* transcripts from Type I methanotrophs over Type II methanotrophs or a decrease in the transcription of *pmoA* from Type II methanotrophs. Microarray hybridisation pattern from heavy and light DNA from SIP analysis revealed that hybridisation signals for *pmoA* probes targeting sequences from Type II methanotrophs (particularly the genus *Methylocystis*) were detected only in the light DNA fraction and not in the heavy DNA fraction. This indicated that *Methylocystis* is present but they are not actively oxidizing methane at the time of sampling (time II). Based on both mRNA microarray and SIP results, it is clear that despite being present at time II in sieved and ground soils, methanotrophs from

the genus *Methylocystis* were not actively expressing *pmoA*. However, for control soil samples hybridisation signals for *pmoA* probes targeting sequences from the genus *Methylocystis* were detected both in RNA and heavy DNA indicating that they are indeed an active member.

Based on the microarray analysis results from time I and II, it could be hypothesized that the niche destructed (or created) by the perturbation of soil structure in sieved and ground soil samples favoured the activity of Type I methanotrophs as determined by bacterial transcription activity. However, the results also indicated that even though there was an onset of a favourable niche in sieved and ground soil samples for Type I methanotrophs, they required time to outcompete Type II methanotrophs as indicated by the detection of Type II methanotroph *pmoA* transcripts at time I in all soil samples and lack of them at time II, particularly with sieved and ground soils. This can indicate that a change in particular niche need not be accompanied by an immediate change to the active bacterial communities and it depends on the ability of the bacterial communities to respond to the perturbation and dominate the function. Chenu *et al*. (2001) emphasized that time is a critical factor when studying soil microhabitats as heterogeneous habitats can be created and probably disappear due to the nature and availability of substrates.

Type I and Type II methanotrophs are known to favour different ecological niches (Hanson and Hanson, 1996). Amaral & Knowles (1995) suggested that Type II methanotrophs dominate methane oxidation at low O_2 concentrations while Type I methanotrophs dominate at relatively high O_2 concentrations. Methanotrophs in microniches within soil crumbs in control soil would be expected to encounter lower O_2 concentrations compared to sieved and ground soils, owing to poor diffusion rates. This would most likely favour the activity of Type II methanotrophs, as

indicated by the higher relative abundance of *pmoA* transcripts from the genus *Methylocystis* within control samples, whereas homogenous O_2 diffusion in sieved and ground soils would have increased Type Ia methanotroph activity (*Methylomonas*, *Methylobacter* and *Methylosarcina*). Buckling *et al* (2000) suggested that physical disturbances could select for microorganisms that can respond quickly to the changes in their niche resulting from disturbance. In the case of methanotrophs, Type I methanotrophs are known to respond quickly to any changes in the environmental condition compared to Type II methanotrophs (Graham *et al.*, 1993; Henckel *et al.*, 2000a). The perturbation in the sieved and ground soil could have favoured the activity of Type I methanotrophs compared to Type II methanotrophs. Physical perturbation to the soil structure resulted in a change in active methanotroph community structure, however further studies are needed to elucidate the mechanisms that drives the change in community structure. Interestingly minimal difference was seen within the active community structure of Type I methanotrophs between control, sieved and ground soil. This indicates that the specific niches for the different Type I methanotrophs (*Methylobacter*, *Methylomonas*, *Methylomicrobium* and *Methylosarcina*) might not (solely) be defined by soil structure, or at least, not at the scale disrupted by the treatments applied.

DNA-SIP versus mRNA-based microarray analyses

Stable isotope probing (Boschker *et al.*, 1998; Radajewski *et al.*, 2000; Manefield *et al.*, 2002), has been a powerful tool for studies attempting to link function with microbial diversity in various environments (Morris *et al.*, 2002; Hutchens *et al.*, 2004; Cebron *et al.*, 2007a; Chen *et al.*, 2008a) and with diverse substrates (reviewed in Dumont and Murrell, 2005; Neufeld *et al.*, 2007a). However, whilst using a larger experimental set-up (mesocosms) with a longer time-scale, an exorbitant quantity of ^{13}C-labelled substrate is required to label active bacteria and one could also end up with a large number of samples for centrifugation and gradient fractionation, which could be laborious. Moreover, longer incubation time in DNA-SIP experiments could potentially lead to "cross-feeding" problems (Neufeld *et al.*, 2007b). Results from this study indicated that mRNA-based and DNA-SIP based assessment of active methanotroph community structure were generally congruent. This suggests that the mRNA based microarray technique could be used to study active methanotroph community structure in situations where SIP experiments are not practical. mRNA is very unstable in nature and has a very short life time (Rauhut and Klug, 1999) and is thus offering a suitable marker to identify active microorganisms immediately at the time of sampling. Results from previous studies have also revealed that *pmoA* microarray results are highly congruent or even more sensitive than *pmoA* clone library analysis (Bodrossy *et al.*, 2003; Bodrossy *et al.*, 2006; Chen *et al.*, 2008a; Héry *et al.*, 2008). Moreover, microarray based community structure assessments offers a high-throughput tool to screen of a large number of samples at a higher phylogenetic resolution, depending on the sensitivity of the probes used in the array (McDonald *et al.*, 2008). mRNA-based microarray analysis may be particularly useful to study the active diversity of high-affinity

methanotrophs, which oxidize methane at atmospheric concentrations (Bender and Conrad, 1992; Bull *et al.,* 2000). Long incubation time at low concentration of methane to label high-affinity methane oxidizers may lead to cross-feeding problems and hence the use of DNA-SIP is limited to study these organisms in the environment (Neufeld *et al.*, 2007b).

Unlike mRNA based detection, which allows us to detect bacterial transcription at the time of sampling, DNA-SIP can reveal the history of active bacterial populations by the incorporation of ^{13}C-labelled methane into their biomass. SIP can also be combined with metagenomics to retrieve large DNA fragments from uncultivated bacteria as demonstrated by Dumont *et al*. (2006). Moreover, probe design in microarrays if often limited to the detection of known organisms, whereas SIP can potentially detect any novel organisms, new genes and functions (Dumont and Murrell, 2005). Both DNA-SIP and mRNA-microarray have their advantages and limitations and the selection of the appropriate technique(s) to assess active community structure depends on the question the researcher seeks to answer.

Soil is an intricate environment with a variety of microniches, often determined by physical structure. In this study, we have shown that physical disturbance of the soil structure affected both function and active diversity of methanotrophs in a landfill cover soil. Physical disturbance of the soil structure favoured the activity of Type I methanotrophs over Type II methanotrophs. We hypothesise that a range of factors such as an increase in interfacial area, rate of diffusion of gases and increased nutrient availability could have influenced the function and diversity. These results should be carefully considered since soil microniches and their properties can vary depending on the soil composition (sand,

silt and clay) and aggregate organization and therefore the effects on bacterial functional diversity can vary between a sandy or clayey soil and also with the targeted functional group of bacteria. Moreover, this study also emphasizes the importance of the length of incubation time used to assess active bacterial communities in soil microcosms. In this study, we have shown that mRNA-based microarray and DNA-SIP based assessments provide congruent results when used to assess active methanotroph community structure.

Chapter 8

Final discussion

8.1 Final discussion

Landfills are a major anthropogenic source of methane. It has been widely accepted that limiting methane emissions from landfills is integral to the strategies seeking to reduce the effect of global climate change. Landfill gas extraction systems have been made mandatory in new landfill sites. However, research has to be focused on development of low-cost technologies to limit methane emissions from the existing old landfills (Scheutz *et al.*, 2009). To this extent, much of that research has been focused on optimizing landfill cover soil and increasing microbial methane oxidation in these cover soils to limit methane emissions. In this study, we have performed studies to address two important questions,

i. What is the effect of earthworms on soil methane oxidation and diversity of active methanotrophs in a landfill cover soil?
ii. Do methanotroph populations in landfill cover soil show a distinct spatio-temporal distribution pattern and is there an influence of abiotic parameters on these distribution patterns?

Interaction between earthworms and methanotrophs

Microcosm experiments combined with stable isotope probing technique allowed us to study the effect of earthworms on active bacterial community structure **(Chapter 3)**. Earthworms were found increase soil methane oxidation potential alongside marginally enhancing the activity of Type Ia (*Methylobacter, Methylosarcina and Methylomonas*) methanotrophs over Type II methanotrophs. Moreover, a distinct band was detected in the 16S rRNA gene DGGE profiles of ^{13}C-DNA and RNA of earthworms incubated soil sample that corresponded to a *Bacteroidetes*-related bacterium and was the only difference observed between

earthworm incubated and non-incubated soil samples. This is the first time that there is an indication of a possible role of *Bacteroidetes* in methane oxidation, which needs further investigation. However, it could be a "cross-feeding" phenomenon where the *Bacteroidetes*-related bacteria feeding a labelled by-product produced by methanotrophs during methane oxidation. Alternatively, *Bacteroidetes* might have fed on the dead ^{13}C-labelled methanotroph biomass due to food-web interactions.

Microcosm studies have limitations in mimicking the environmental heterogeneity found in *in situ* conditions, which makes it difficult to extrapolate in terms of experimental design and execution of *in situ* field experiments. Understanding the spatial and temporal shifts in functional diversity and relative abundance of active methanotrophs brought about by the complex and dynamic interaction of earthworms in soil needs an experimental system that could mimic the natural environment, in this case, a landfill environment. Therefore, we used an experimental system that could simulate *in situ* landfill conditions to study the effect of earthworms on methanotroph community structure (**Chapter 4**). Owing to the scale of the experimental system, it was not possible to use ^{13}C-labelled substrate to detect changes in the active methanotroph population. Hence an mRNA-based microarray was used to screen for any shifts in active methanotroph populations based on their transcription activity. mRNA-based microarray analysis revealed that earthworm activity in landfill cover soil stimulates activity and diversity of Type I methanotrophs (*Methylobacter*, *Methylomonas, Methylosarcina* spp.) compared to Type II methanotrophs (particularly *Methylocystis* spp.). This could be due to various factors such as the increase in nitrogen availability, increase in diffusion of gas through soil bioturbation effect and/or due to the physical disturbance in soil by earthworm activity, which selects for organisms that quickly responds to the

changes. Results from this study indicate that earthworm-induced methanotrophy can be successfully used to limit methane emissions from landfill by increasing the methane oxidation in cover soils. The results from this study can also be used to plan future *in situ* field studies and to integrate earthworm-induced methanotrophy with other landfill management practises to reduce methane emissions from landfills.

In situ spatio-temporal distribution of methanotrophs

Soil is a heterogeneous environment composed of many niches that harbour tremendous bacterial diversity (Curtis *et al.,* 2002). Bacterial diversity and abiotic factors such as soil particle size, porosity, water content, nutrient availability and pH can vary spatially from sub-millimeter scale to large geographic distances and also in time (Martiny *et al.,* 2006). While understanding spatio-temporal distribution patterns of bacterial communities and factors that influence these patterns and bacterial functions still remains a challenge (Torsvik and Ovreas, 2002), it is essential for a better understanding of microbial functions in ecosystems. Similar to other soil ecosystems, methanotrophs in landfill cover soil are also influenced by a range of environmental factors (Kightley *et al.*, 1995; Borjesson *et al.,* 1998; De Visscher *et al.,* 1999; Borjesson *et al.*, 2004; Scheutz and Kjeldsen, 2004). It is essential to understand the dynamics of methanotroph community structure and the factors influencing the community structure to devise better landfill management practises.

Preliminary studies included optimization of methodological issues such as the comparison of different *pmoA* primer sets, PCR strategies and different soil sample size for the assessment of methanotroph community structure using a *pmoA*-based microarray (**Chapter 5**). Direct PCR with A189/mb661 was found to retrieve a broader diversity of methanotrophs in this landfill cover soil compared to other

primer sets or a semi-nested PCR approach. A significant effect of soil sample size on the assessment of methanotroph community structure was revealed based on the *pmoA* microarray analysis and was supported by ANOSIM analysis. A small sample size of 0.5 g retrieved a broader methanotroph diversity compared to 5 and 50 g samples. However, this effect should not be generalised and it is possible that it might differ with different soil types and ecosystems. We recommend a reconnaissance study to find the suitable sample size based on the question the researchers seek to answer.

Previous studies have characterized methanotroph communities in various landfill cover soils (Wise *et al.*, 1999; Uz *et al.*, 2003; Crossman *et al.*, 2004; Stralis-Pavese *et al.*, 2004; Chen *et al.*, 2007; Gebert *et al.*, 2008). However, there is a lack of knowledge on the spatio-temporal distribution of methanotrophs in landfill cover soils and the role of environmental heterogeneity on their activity and diversity. We used a *pmoA*-based microarray to characterize the spatio-temporal distribution of methanotrophs in a landfill cover soil and also to identify any relationship between methanotroph community structure, methane oxidation potential and abiotic factors, particularly C/N ratio, NH_4^+ and NO_3^- (**Chapter 6**). In this study, it was revealed that there was a temporal dynamics in methanotroph community structure, along with seasonal changes in abiotic factors. However, limited spatial patterning (vertical and horizontal) of methanotrophs and abiotic parameters were observed. We compared *pmoA* probes hybridisation signal intensity with the measured abiotic factors to determine the driving factors for methanotroph diversity and activity. Although, we found some relationship with the *pmoA* probe signals and abiotic factors, the evidence was inconclusive. These results emphasize the fact that methanotrophs cannot be treated as one discrete group of microorganisms when

attempting to relate community structure with soil abiotic factors and indeed these factors affect the diversity differently, often in conflicting ways. *In situ* mRNA-based analysis could provide with a better understanding on the role of abiotic factors in altering the diversity of active methanotrophs, rather than focussing on methanotrophs present based on the analysis of DNA.

Soils, as a result of their structural organization in aggregates of different sizes and stability, create a composite of microniches differing in terms of physico-chemical and structural characteristics. Physical disruption to the soil structure results in disintegration of soil aggregates leading to the loss of soil microniches and thus microbial habitats. We hypothesised that if microniches created by soil aggregates are physically disrupted and the soil structure significantly changed, the activity and functional diversity of bacterial communities will also be altered. Based on the results obtained, we have shown that physical disturbance of the soil structure affected both function and active diversity of methanotrophs in a landfill cover soil. Physical disturbance of the soil structure favoured the activity of Type I methanotrophs over Type II methanotrophs. We hypothesise that a range of factors such as an increase in interfacial area, rate of diffusion and increased nutrient availability could have influenced the function and diversity. Moreover, this study also emphasizes the importance of the length of incubation time to assess active bacterial communities in soil microcosm.

Application of stable isotope probing has provided significant insights into active microbial populations and their biogeochemical cycling. However, whilst using a larger experimental set-up (mesocosms) with a longer time-scale, such as the soil cores used in chapter 4, exorbitant quantity of ^{13}C-labelled substrate is required

to label active bacterial population and could also end up with a large number of samples for ultra-centrifugation and gradient fractionation, which could be laborious. Moreover longer incubation time in DNA-SIP experiments could potentially lead to “cross feeding” problems. By using mRNA instead of DNA, *pmoA* microarray analysis can detect changes in relative abundances of *pmoA* transcripts from different methanotroph populations. We compared DNA-SIP and mRNA based microarray techniques for the assessment of active methanotroph community structure (**Chapter 7**). Results from this study indicated that assessment of active methanotroph community structure by both the techniques were congruent. This suggested that the mRNA based microarray technique could be used to study active methanotroph community structure in situations where SIP experiments are not practical. However, both DNA-SIP and mRNA-microarray have their advantages and limitations and the selection of appropriate technique to assess active community structure depends on the nature of the study (discussed in Chapter 7).

Analysis of microarray hybridisation signals for landfill cover soil samples over all of the above studies revealed an interesting finding. Hybridisation signals for *pmoA* probes targeting the genus *Methylocaldum* were detected in almost all of the *in situ* samples collected from the landfill cover soil. However, no hybridisation signals were detected in samples that were subjected to methane incubations in the laboratory and also have not been found to be an active methanotroph under laboratory methane incubation conditions. Therefore, future studies could focus on understanding the contribution of *Methylocaldum* in methane cycling in the landfill cover soil. It might be possible that laboratory incubation conditions are not suitable for their activity. In that case, *in situ* experiments are needed to study the ecology of *Methylocaldum* and factors that influence the activity of these organisms. Future

studies must also include measurements of a wider range of abiotic factors such as *in situ* O_2 and CH_4 availability to map out the hot spots of methane emissions in the landfill cover soil to devise better sampling strategies.

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Appendices

Appendix 1: Buffers, reagents and solutions

1. TE buffer (used for elution of DNA)

Tris-base	10 mM
EDTA (Na_2)	1 mM

pH adjusted to 8.0 using HCl

2. DNA extraction buffer

CTAB (hexadecyltrimethylammonium bromide)	0.2%
DTT	1 mM
Sodium phosphate buffer (pH 8.0)	0.2M
NaCl	0.1M
EDTA	50 mM

3. Lysis buffer for DNA extraction (METHECO)

$NaHPO_4$ (pH 7)	200mM
(39 ml 200 mM NaH_2PO_4 + 61 ml 200 mM Na_2HPO_4)	
NaCl	17.54g
CTAB	2g
PVP K30	4g
dH_2O	80ml

The pH was adjusted to 7.0 and the lysis buffer was autoclaved. Lysozyme was added to an aliquot of lysis buffer to final concentration of 5 mg/ml concentration just before use.

4. Electrophoresis solutions/buffers

Agarose gel-loading buffer (6 x)

Bromphenol blue	0.0125 g
Ficoll (type 400)	0.75 g
Distilled water	5 ml

10 × Tris borate EDTA (TBE) Buffer

Tris-base	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 ml

Made up to 1 litre with distilled water

5. Solutions for Denaturing Gradient Gel Electrophoresis

Gradient Dye Solution

Bromophenol blue (0.5 % w/v final concentration)	0.05 g
Xylene cyanol (0.5 % w/v final concentration)	0.05 g
1 X TAE buffer	10 ml

10 × gel loading solution

Glycerol (100 % v/v)	5 ml
Bromophenol blue (0.25 % w/v final concentration)	0.025 g
Xylene cyanol (0.25 % w/v final concentration)	0.025 g
Milli-Q water	5 ml

50 × Tris acetate EDTA buffer (TAE)

Tris-base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Made up to 1 litre with distilled water

DGGE acrylamide/bis-acrylamide solutions (6 %)	0 %	80 %
Acrylamide / bis-acrylamide (37.5: 1, 40% w/v (Sigma))	15 ml	15 ml
50 × TAE buffer	2 ml	2 ml
Urea (U)	-	33.6 g
Formamide (de-ionized) (F)	-	32 ml
Milli-Q water to	100 ml	100 ml

6. Solutions for microarrays

6 × SSC solution

NaCl	52.59 g
Sodium citrate	26.46 g

pH adjusted to 7.0 with a 10M NaOH and made up to 1 litre with distilled water

Appendix 2: Oligonucleotide probe set for *pmoA*-microarray

Name	Intended specificity	Sequence 5' → 3'	Length	GC%	T_m
BB51-302	*Methylobacter*	CGGTTGTTTGTGTCTTAGGTCTG	23	47.8	57.2
Mb292	*Methylobacter*	CCGTTACCGTCTGCCTTTCG	20	60.0	59.1
Mb282	*Methylobacter*	TTACCGTCTGCCTTTCGGC	19	57.9	58.6
Mb_URC278	*Methylobacter*	GTTCCGTTACAGACTGCCTTTCGG	24	54.2	61.3
Mb267	*Methylobacter*	GCATGCTTGTGGTTCCGTTAC	21	52.4	58.1
511-436	*Methylobacter*	GTTTTGATGCTGTCTGGCAG	20	50.0	55.5
MbA486	*Methylobacter*	AGCATGACATTGACAGCGGTTGTT	24	45.8	61.6
MbA557	*Methylobacter*	CAATGGCATGATGTTCACTCTGGCT	25	48.0	61.5
Mb_SL#3-300	*Methylobacter*	GGCGCTGTTGTTTGTGTATTGGGT	24	50.0	62.2
Mb460	*Methylobacter*	GACAGTTACAGCGGTAATCGGTGG	24	54.2	60.9
Mb_LW12-211	*Methylobacter*	CGTCTTTGGGTTACTGTTGTGCC	23	52.2	60.0
Mb_C11-403	*Methylobacter*	CAAACTTCATGCCTGGTGCTATCGT	25	48.0	61.4
Mb271	*Methylobacter*	TTGTGGTGGCGTTACCGT	18	55.6	58.0
PS80-291	clone PS-80	ACCAATAGGCGCAACACTTAGT	22	45.5	58.3
Est514	*Methylomicrobium*-related clones	AATTGGCCTATGGTTGCGCC	20	55.0	59.9
Mm_pel467	*Methylomicrobium pelagicum*	ACTGCGGTAATCGATGGTTTGGC	23	52.2	61.6
Mb_SL-299	soda lake *Methylobacter* isolates and clones	GGGGTGCAACTCTGTGTATCTTAGG	25	52.0	60.5
Mb_SL#1-418	soda lake *Methylobacter* isolates and clones	GCGATCGTATTAGACGTTATCCTGATG	27	44.4	58.6
DS1_401	Deep sea cluster #1	GCGCGGTAGTTTGTGTTATGGCT	23	52.2	61.7
Mm531	*Methylomonas*	CTCCATTGCACGTGCCTGTAGA	22	54.5	60.7
Mm_ES294	*Methylomonas*	CCAATCGGTGCAACAATTTCTGTAGT	26	42.3	59.8
Mm_ES543	*Methylomonas*	GTGCCAGTTGAGTATAACGGCATGA	25	48.0	60.9
Mm_ES546	*Methylomonas*	CCAGTTGAGTATAACGGCATGATGAT	26	42.3	58.7
Mm_M430	*Methylomonas*	TGGACGTGATTTTGATGTTGGGCAA	25	44.0	61.6
Mm_MV421	*Methylomonas*	CTATCGTGCTGGATACAATCCTGATGT	27	44.4	60.0
Mm275	*Methylomonas*	GTGGTGGAGATACCGTTTGCC	21	57.1	59.2
Mm451	*Methylomonas*	CTGATGTTGGGTAACAGCATGACT	24	45.8	58.8
peat_1_3-287	*Methylomonas*-related peat clones	AACTGCCTTTAGGCGCTACC	20	55.0	58.6
Jpn284	clone Jpn 07061	ACCGTATCGCATGGGGTG	18	61.1	58.0
Mmb303	*Methylomicrobium album*	CAATGCTGGCTGTTCTGGGC	20	60.0	60.3
Mmb259	*Methylomicrobium album* + Landfill *Methylomicrobium*	CTGTTCAAGCAGTTGTGTGGTATCG	25	48.0	59.8
Mmb562	*Mmb. album* and *Methylosarcina*	ATGGTAATGACCCTGGCTGACTTG	24	50.0	60.6
LP20-644	*Methylomicrobium*-related clones	GTACACTGCGTACTTTCGGTAA	22	45.5	56.0
Ia193	Type I a (*Methylobacter- Methylomonas- Methylomicrobium)*	GACTGGAAAGATAGACGTCTATGGG	25	48.0	57.8
Ia575	Type I a (*Methylobacter- Methylomonas- Methylomicrobium- Methylosarcina)*	TGGCTGACTTGCAAGGTTACCAC	23	52.2	61.3
JRC4-432	Japanese rice cluster #4	GACGTTGTCCTGGCTCTGAG	20	60.0	58.3
MclT272	*Methylocaldum tepidum*	GGCTTGGGAGCGGTTCCG	18	72.2	61.9
MclG281	*Methylocaldum gracile*	AAAGTTCCGCAACCCCTGGG	20	60.0	61.5
MclE302	*Methylocaldum* E10	CGCAACCATGGCCGTTCTG	19	63.2	60.3

MclS402	*Methylocaldum szegediense*	GCGCTGTTGGTTCCGGGT	18	66.7	61.8
Mcl408	*Methylocaldum*	GGTTCCGGGTGCGATTTTG	19	57.9	57.8
501-375	*Methylococcus*- related marine and freshwater sediment clones	CTTCCCGGTGAACTTCGTGTTCC	23	56.5	61.3
501-286	*Methylococcus*- related marine and freshwater sediment clones	GTCAGCCGTGGGGCGCCA	18	77.8	66.7
USC3-305	Upland soil cluster #3	CACGGTCTGCGTTCTGGC	18	66.7	59.5
Mc396	*Methylococcus*	CCCTGCCTCGCTGGTGCC	18	77.8	64.4
fw1-639	fw-1 group: *Methylococcus-Methylocaldum* related marine and freshwater sediment clones	GAAGGGCACGCTGCGTACG	19	68.4	62.0
fw1-641	fw-1 group: *Methylococcus-Methylocaldum* related marine and freshwater sediment clones	AGGGCACGCTGCGTACGTT	19	63.2	63.3
fw1-286	fw-1 group: *Methylococcus-Methylocaldum* related marine and freshwater sediment clones	ATCGTCAACCGTGGGGCG	18	66.7	61.1
LW21-374	LW21 group	CTACTTCCCGATCACCATGTGCT	23	52.2	60.2
LW21-391	LW21 group	TGTGCTTCCCCTCGCAGATC	20	60.0	60.5
OSC220	Finnish organic soil clones and related	TCACCGTCGTACCTATCGTACTGG	24	54.2	60.8
OSC300	Finnish organic soil clones and related	GGCGCCACCGTATGTGTACTG	21	61.9	61.4
JRC3-535	Japanese Rice Cluster #3	CGTTCCACGTTCCGGTTGAG	20	60.0	59.3
LK580	fw-1 group + Lake Konstanz sediment cluster	CCGACATCATTGGCTACAACTATGT	25	44.0	58.7
JRC2-447	Japanese Rice Cluster #2	CTGAGCACCAGCTACCTGTTCA	22	54.5	60.2
M90-574	*Methylococcus-Methylocaldum* related marine and freshwater sediment clones	ATCGCCGACCTGCTGGGTTA	20	60.0	62.2
M90-253	*Methylococcus-Methylocaldum* related marine and freshwater sediment clones	GCTGCTGTACAGGCGTTCCTG	21	61.9	61.7
Mth413	*Methylothermus*	CACATGGCGATCTTTTTAGACGTTG	25	44.0	58.3
Ib453	Type I b (*Methylothermus-Methylococcus-Methylocaldum* and related)	GGCAGCTACCTGTTCACCGC	20	65.0	61.7
Ib559	Type I b (*Methylothermus-Methylococcus-Methylocaldum* and related)	GGCATGCTGATGTCGATTGCCG	22	59.1	62.5
DS3-446	Deep sea cluster #3	AGCTGTCTGGCAGTTTCCTGTTCA	24	50.0	62.5
JR2-409	JR cluster #2 (California upland grassland soil)	TTATTCCCGGCGCTATCATGATCG	24	50.0	60.5
JR2-468	JR cluster #2 (California upland grassland soil)	ACAGCCATAATTGGACCATTCTTCTG	26	42.3	59.2
JR3-505	JR cluster #3 (California upland grassland soil)	TGTATCCTACCAATTGGCCTCATCTG	26	46.2	60.1
JR3-593	JR cluster #3 (California upland grassland soil)	CTATCAGTATGTGCGGACAGGC	22	54.5	58.6
Nc_oce426	*Nitrosococcus oceani*	CTTGGATGCCATGCTTGCGA	20	55.0	59.8
USCG-225	Upland soil cluster Gamma	CTGACGCCGATCATGTGCAT	20	55.0	59.1
USCG-225b	Upland soil cluster Gamma	CTGACGCCGATCATGTGCATCA	22	54.5	61.2
Mcy233	*Methylocystis*	ATTCTCGGCGTGACCTTCTGC	21	57.1	60.9
Mcy413	*Methylocystis*	TTCCGGCGATCTGGCTTGACG	21	61.9	63.2
Mcy522	*Methylocystis* A + peat clones	GGCGATTGCGGCGTTCCA	18	66.7	62.3
Mcy264	*Methylocystis*	CAGGCGTTCTGGTGGGTGAA	20	60.0	61.0
Mcy270	*Methylocystis*	TTCTGGTGGGTGAACTTCCGTCT	23	52.2	61.8

Mcy459	*Methylocystis*	GTGATCACGGCGATTGTTGGTTC	23	52.2	60.2
Mcy255	*Methylocystis B (parvus/echinoides*/strain M)	GGCGTCGCAGGCTTTCTGG	19	68.4	62.3
McyM309	*Methylocystis strain M and related*	GGTTCTGGGCCTGATGATCGG	21	61.9	61.0
McyB304	*Methylocystis B (parvus/echinoides*/strain M)	CGTTTTCGCGGCTCTGGGC	19	68.4	62.7
MsT214	*Methylosinus trichosporium* OB3b and rel.	TGGCCGACCGTGGTTCCG	18	72.2	63.5
Msi520	*Methylosinus trichosporium*	GCGATCGCGGCTCTGCA	17	70.6	61.6
Msi269	*Methylosinus trichosporium*	TCTTCTGGGAGAACTTCAAGCTGC	24	50.0	60.6
MsS314	*Methylosinus sporium*	GGTTCTGGGTCTGCTCATCGG	21	61.9	60.8
MsS475	*Methylosinus sporium*	TGGTCGGCGCCCTGGGCT	18	77.8	68.3
Msi263	*Methylosinus sporium* + 1 *Methylosinus trichosporium subcluster*	GGCGTTCCTGTGGGAGAACTTC	22	59.1	61.2
Msi423	*Methylosinus*	CTGTGGCTGGACATCATCCTGC	22	59.1	61.4
Msi294	*Methylosinus*	GTTCGGCGCGACCTTCGC	18	72.2	62.5
Msi232	*Methylosinus* + most *Methylocystis* -considered as additional type II probe	ATCCTGGGCGTGACCTTCGC	20	65.0	63.3
Peat264	peat clones	GGCGTTTTTCTGGGTCAACTTCC	23	52.2	60.3
II509	Type II	CGAACAACTGGCCGGCGAT	19	63.2	61.7
II630	Type II	CATGGTCGAGCGCGGCAC	18	72.2	62.4
xb6-539	Novel *pmoA* copy of type II and related environmental clones	AGGCCGCCGAGGTCGAC	17	76.5	63.0
LP21-190	Novel *pmoA* copy of type II and related environmental clones	ATCGACTTCAAGGATCGCCG	20	55.0	58.2
LP21-260	Novel *pmoA* copy of type II and related environmental clones	CGCAGTCCTTCTTCTGGACG	20	60.0	58.6
NMcy1-247	Novel *pmoA* copy of *Methylocystis*	TCGACATCGTGCTGATGATCTCGG	24	54.2	62.1
NMcy2-262	Novel *pmoA* copy of *Methylocystis*	CAGTCCTTCTTCTGGCAGAAGTTCC	25	52.0	60.9
NMsiT-271	Novel *pmoA* copy of *Methylosinus trichosporium*	AGCGCTTCCGTCTGCCGAT	19	63.2	62.9
LP21-232	Novel *pmoA* copy of type II and related environmental clones	ATCGTCGCCATGTGCTTCGC	20	60.0	61.9
RA14-594	RA14 related clones	CCACAACGTTCGTACCTCGA	20	55.0	57.9
RA14-591	RA14 related clones	GGCTTCCACAACGTTCGTACCT	22	54.5	60.9
Wsh1-566	Watershed + flooded upland cluster 1	GCTCATGAGCTTGGCCGACATC	22	59.1	61.8
Wsh2-491	Watershed + flooded upland cluster 2	TCATTTGGCCAACCTCTCTCATTCC	25	48.0	60.9
Wsh2-450	Watershed + flooded upland cluster 2	CAAGAGCTGGATCATCACGATG	22	50.0	56.8
B2rel251	*Methylocapsa*-related clones	CCGCCGCGGCCCAGTATTA	19	68.4	63.4
B2-400	*Methylocapsa*	ACCTCTTTGGTCCCGGCTGC	20	65.0	63.4
B2all343	*Methylocapsa* and related clones	AACCGCTACACCAATTTCTGGGG	23	52.2	61.2
B2all341	*Methylocapsa* and related clones	TCAACCGCTACACCAATTTCTGGG	24	50.0	61.1
pmoAMO3-400	clone *pmoA*-MO3	ACCCAGATGATCCCGTCGGC	20	65.0	62.6
ESR-579	ESR (Eastern Snake River) cluster	GACCTGATCGGATTCGAGAACATC	24	50.0	58.5
TUSC409	Tropical Upland Soil Cluster #2	CGATCCCGGGCGCGATTC	18	72.2	61.8
TUSC502	Tropical Upland Soil Cluster #2	TCTTCTACTTCGGCAACTGGC	21	52.4	58.3
mtrof173	Universal	GGbGACTGGGACTTCTGG	18	66.7	57.4
mtrof362-I	Methanotrophs	TGGGGCTGGACCTACTTCC	19	63.2	59.5

mtrof661	Methanotrophs	GGTAARGACGTTGCKCCGG	19	63.2	60.4
mtrof662-I	Methanotrophs	GGTAAGGACGTTGCGCCGG	19	68.4	61.9
mtrof656	Methanotrophs	ACCTTCGGTAAGGACGT	17	52.9	53.2
NmNc533	*Nitrosomonas-Nitrosococcus*	CAACCCATTTGCCAATCGTTGTAG	24	45.8	58.6
Nsm_eut381	*Nitrosomonas eutropha*	CCACTCAATTTTGTAACCCCAGGTAT	26	42.3	59.0
PS5-226	*Nitrosomonas-Nitrosococcus* related clones	ACCCCGATTGTTGGGATGATGTA	23	47.8	59.9
Pl6-306	*Nitrosomonas-Nitrosococcus* related clones	GGCACTCTGTATCGTATGCCTGTTAG	26	50.0	60.5
NsNv207	*Nitrosospira-Nitrosovibrio*	TCAATGGTGGCCGGTGG	17	64.7	58.5
NsNv363	*Nitrosospira-Nitrosovibrio*	TACTGGTGGTCGCACTACCC	20	60.0	59.6
Nit_rel471	AOB related clones/probably methanotrophs	CGTTCGCGATGATGTTTGGTCC	22	54.5	60.1
Nit_rel223	AOB related clones/probably methanotrophs	GTCACACCGATCGTAGAGGT	20	55.0	56.9
ARC529	AOB related clones/probably methanotrophs	TAAGCAGCCGATGGTCGTGGAT	22	54.5	62.2
Nit_rel470	AOB related clones/probably methanotrophs	CGATATTCGGGGTATGGGCG	20	60.0	58.4
Nit_rel351	AOB related clones/probably methanotrophs	GTTTGCCTGGTACTGGTGGG	20	60.0	59.2
Nit_rel304	AOB related clones/probably methanotrophs	CGCTCTGCATTCTGGCGCT	19	63.2	61.8
M84P105-451	environmental clones of uncertain identity	AACAGCCTGACTGTCACCAG	20	55.0	58.1
WC306_54-385	environmental clones of uncertain identity	AACGAAGTACTGCCGGCAAC	20	55.0	59.2
M84P22-514	environmental clones of uncertain identity	AACTGGGCCTGGCTGGG	17	70.6	61.0
gp23-454	environmental clones of uncertain identity	AACGCGCTGCTCACTGCG	18	66.7	62.3
MR1-348	environmental clones of uncertain identity	AATCTTCGGTTGGCACGGCT	20	55.0	61.1
gp619	environmental clones of uncertain identity	CGGAATATCTGCGCATCATCGAGC	24	54.2	61.5
gp391	environmental clones of uncertain identity	ATCTGGCCGGCGACCATG	18	66.7	61.1
gp2-581	environmental clones of uncertain identity	ACATGATCGGCTACGTGTATCCG	23	52.2	60.0
RA21-466	clone RA21 - environmental clone of uncertain identity	CGGCGTTCTTGGCGGCAT	18	66.7	62.4

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ORIGINAL ARTICLE

Effect of earthworms on the community structure of active methanotrophic bacteria in a landfill cover soil

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In the United Kingdom, landfills are the primary anthropogenic source of methane emissions. Methanotrophic bacteria present in landfill biocovers can significantly reduce methane emissions via their capacity to oxidize up to 100% of the methane produced. Several biotic and abiotic parameters regulate methane oxidation in soil, such as oxygen, moisture, methane concentration and temperature. Earthworm-mediated bioturbation has been linked to an increase in methanotrophy in a landfill biocover soil (AC Singer *et al.*, unpublished), but the mechanism of this trophic interaction remains unclear. The aims of this study were to determine the composition of the active methanotroph community and to investigate the interactions between earthworms and bacteria in this landfill biocover soil where the methane oxidation activity was significantly increased by the earthworms. Soil microcosms were incubated with ^{13}C-CH_4 and with or without earthworms. DNA and RNA were extracted to characterize the soil bacterial communities, with a particular emphasis on methanotroph populations, using phylogenetic (16S ribosomal RNA) and functional methane monooxygenase (*pmoA* and *mmoX*) gene probes, coupled with denaturing gradient-gel electrophoresis, clone libraries and *pmoA* microarray analyses. Stable isotope probing (SIP) using ^{13}C-CH_4 substrate allowed us to link microbial function with identity of bacteria via selective recovery of 'heavy' ^{13}C-labelled DNA or RNA and to assess the effect of earthworms on the active methanotroph populations. Both types I and II methanotrophs actively oxidized methane in the landfill soil studied. Results suggested that the earthworm-mediated increase in methane oxidation rate in the landfill soil was more likely to be due to the stimulation of bacterial growth or activity than to substantial shifts in the methanotroph community structure. A *Bacteroidetes*-related bacterium was identified only in the active bacterial community of earthworm-incubated soil but its capacity to actually oxidize methane has to be proven.
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Introduction

In landfill sites, anaerobic conditions induce high rates of microbially mediated methane production from the decomposition of organic wastes. In the United Kingdom, landfills represent the primary anthropogenic source of methane, the second largest contributor to global warming after CO_2. In landfills, which are not equipped with gas collection systems, biocover soils are used to limit methane emissions. Landfill biocover soils have the highest aerobic methane oxidation capacity reported so far in any environment (Whalen *et al.*, 1990; Bogner *et al.*, 1995; Kightley *et al.*, 1995; Borjesson *et al.*, 1998; Streese and Stegmann, 2003). Microorganisms indigenous to landfill caps have the capacity to degrade 10–100% of the methane emitted, thereby representing a major biological sink for this greenhouse gas (Hanson and Hanson, 1996; Spokas *et al.*, 2006). Biological methane oxidation to CO_2 can strongly reduce ($\sim$21-fold) climate forcing. Environmental parameters such as oxygenation and methane concentration, moisture content, pH, nitrogen sources and temperature can strongly influence this biological process in soil (Jones and Nedwell, 1993; Boeckx *et al.*, 1996; Chan and Parkin, 2000; Borjesson *et al.*, 2004; Scheutz and Kjeldsen, 2004). Another factor that might influence methane oxidation in soil is the presence of earthworms. Since

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they profoundly affect the physical and chemical properties of the soil, earthworms are commonly considered as efficient 'soil engineers' (Jones *et al.*, 1994). Earthworm burrowing contributes to soil aggregation, oxygenation and mixing. Furthermore, earthworms break down soil organic matter and plant deposition, thereby increasing nutrient turnover. Earthworms excrete several forms of organic and inorganic nitrogen that are used by endogenous and exogenous microorganisms (Needham, 1957; Binet and Trehen, 1992). Soil microbial community size and activity can be affected by earthworm activity (Daniel and Anderson, 1992; Binet and Le Bayon, 1998; Clapperton *et al.*, 2001; Singer *et al.*, 2001; Luepromchai *et al.*, 2002; Tiunov and Dobrovolskaya, 2002; Haynes *et al.*, 2003). However, little is known about the influence of earthworms on bacterial community structure (Schaefer *et al.*, 2005; Mummey *et al.*, 2006).

AC Singer *et al.* (unpublished) have recently shown an earthworm-mediated increase in methane oxidation rate in both pasture and landfill biocover soils. Improving the efficiency of microbially-mediated methane oxidation in landfill soil is crucial for limiting the emission of this greenhouse gas. The aims of the present work were (i) to determine the active methanotroph community in the landfill biocover soil and (ii) to gain insights into the mechanisms by which the earthworms enhance the methane oxidation in this biocover soil, by investigating their effect on the soil bacterial community.

In the last 10 years, novel cultivation techniques have enabled the isolation of previously uncultured methane oxidizers; the characterization of these new genera has led to an improved understanding of methanotroph taxonomy (Bowman *et al.*, 1993, 1997; Bodrossy *et al.*, 1997; Dedysh *et al.*, 1998, 2000, 2002; Wise *et al.*, 1999, 2001). Methanotrophs are currently classified as type I methanotrophs (comprising nine genera among the *γ-proteobacteria*) and type II methanotrophs (comprising four genera among the *α-proteobacteria*), according to their intracytoplasmic membrane structure, carbon assimilation pathways, fatty acid composition and phylogeny (Hanson and Hanson, 1996). Types I and II methanotrophs cohabit landfill soils (Wise *et al.*, 1999; Bodrossy *et al.*, 2003; Uz *et al.*, 2003; Crossman *et al.*, 2004; Stralis-Pavese *et al.*, 2004). All methanotrophs possess methane monooxygenase (MMO) that catalyses the first step of methane oxidation. This enzyme exists in two forms, a soluble, cytoplasmic form (sMMO) and a particulate, membrane-bound form (pMMO) (reviewed in Murrell *et al.*, 2000).

The development and application of suitable molecular tools have expanded our view of bacterial diversity in a wide range of natural environments. The 16S ribosomal RNA (16S rRNA) gene has been used for molecular characterization of natural populations of methanotrophs (Murrell *et al.*, 1998; Costello and Lidstrom, 1999; Noll *et al.*, 2005). Because the specific detection of methanotrophs based on their 16S rRNA gene sequence is not always accurate, functional genes of methanotrophs have been extensively targeted in environmental samples including *pmoA* (encoding the *β*-subunit of the particulate monooxygenase, pMMO) and *mmoX* (encoding the *α*-subunit of the soluble monooxygenase, sMMO). Since pMMO is present in all known methanotrophs with the exception of *Methylocella* (Dedysh *et al.*, 2000; Theisen *et al.*, 2005) and the phylogeny of *pmoA* is congruent with 16S rRNA phylogeny (Kolb *et al.*, 2003), *pmoA* is the most frequent target in molecular ecology studies of methanotrophs (see Dumont and Murrell, 2005 for a review). Recently, a *pmoA* microarray has been developed by Bodrossy and colleagues, which has proved to be particularly suitable for characterizing the diversity within methanotroph communities (Bodrossy *et al.*, 2003, 2006; Stralis-Pavese *et al.*, 2004).

Stable isotope probing (SIP) is a powerful molecular technique that directly links a defined metabolic process to members of bacterial communities. A ^{13}C-labelled substrate is added to samples from a natural environment and bacteria that actively assimilate this substrate incorporate the ^{13}C into their cellular material, including nucleic acids. The 'heavy'-labelled nucleic acids can then be separated from the 'light' nucleic acids by ultracentrifugation in a caesium chloride (DNA-SIP) or a caesium trifluoroacetate (RNA-SIP, Manefield *et al.*, 2002) gradient. The DNA-SIP technique, has provided valuable insights into the diversity and activity of methylotrophic bacteria (Radajewski *et al.*, 2000) and to methanotrophs in a peat soil (Morris *et al.*, 2002), acidic forest soil (Radajewski *et al.*, 2002), the cave environment (Hutchens *et al.*, 2004), soda lake sediments (Lin *et al.*, 2004) and a landfill soil (Cébron *et al.*, 2007). RNA is considered a much more sensitive marker than DNA because copy numbers are greater and activity of cells is linked directly to synthesis and turnover of RNA (Molin and Givskov, 1999). Furthermore, the isotope incorporation into RNA does not require cell division (for a review see Whiteley *et al.*, 2006).

In this study, SIP has been applied in combination with complementary molecular techniques to investigate the bacterial community structure in a landfill biocover soil and the possible effect of earthworms on these communities, based on 16S rRNA, *pmoA* and *mmoX* gene analyses. This work is the first report focusing on the effects of earthworms on active methanotroph communities.

Materials and methods

Landfill soil microcosms

Biocover soil was collected in Ufton Landfill (Warwickshire, UK), in December 2005. The sampling area was covered by grass that was removed

before soil was collected at a depth of 10–20 cm. The main physicochemical parameters of the soil (accurate to ±5%) are listed in Table 1. The soil was stored for 1 week at 4°C until use, at which time it was air-dried, sieved (4 mm mesh size) before packing into the wormery (see below).

Earthworms were incubated in plastic tubs (11 × 17 × 6 cm). Approximately 540 g of air-dried, sieved landfill soil, established and maintained at 70% of its water-holding capacity with 250 ml of deionized water was incubated for 2–3 days before the addition of earthworms. Earthworms were incubated in Petri plates for 24 h to evacuate their gut contents before adding to the wormery. Three *Eisenia veneta* (1.9 ± 0.2 g) were added per wormery, with a 'no earthworm' control prepared in parallel. Wormeries were incubated at 19°C in the dark for 17 days before destructive sampling for methane oxidation assays. Aliquots of soil (5 g) from the wormeries were distributed into 118 ml vial bottles. Seven replicate vials were prepared for both the earthworm and control treatments. Vials were spiked with methane to achieve 2% ^{13}C-CH_4 (v/v) in the headspace and were incubated at 19°C for 7 days. Methane concentration was measured by gas chromatography over this period. After 7 days incubation with ^{13}C-CH_4, soil samples were then stored at −20°C for SIP. Of the seven replicates used for the methane oxidation measurements, four were randomly selected for nucleic acid extraction and molecular analyses. The good reproducibility of these four replicates was confirmed by denaturing gradient-gel electrophoresis (DGGE) using 16S rRNA gene universal bacterial primers and by independent hybridizations on *pmoA* microarrays (data not shown). For the SIP experiments and clone library construction, the DNA and RNA of these four replicates were pooled, thereby reflecting the replication in the original samples, which were used in methane oxidation experiments.

Soil analysis

Earthworm-incubated soil and control soils were analysed at the end of the experiment, after 17 days incubation with or without earthworms, followed by 7 days incubation with 2% ^{13}C-CH_4.

Total carbon and nitrogen content were determined using Dumas combustion with a detection limit of 0.03% N/w and 0.02% C/w, based on a 15 mg sample. Nitrate and ammonia were analysed following extraction in 1 M KCl, with a limit of detection of 0.07 mg NO_3^- kg soil^{-1} and 0.10 mg NH_4^+ kg soil^{-1}. Particle size analysis was carried out using laser diffraction. The results are summarized in Table 1.

DNA and RNA extraction

DNA and RNA were coextracted directly from four soil replicates following the protocol described by Bürgmann *et al*. (2003) with minor modifications. Briefly, 0.4 g soil, 1 ml extraction buffer (0.2% hexadecyltrimethylammonium bromide (CTAB), 1 mM 1,4-dithio-DL-threitol [DTT], 0.2 M sodium phosphate buffer (pH 8.0), 0.1 M NaCl, 50 mM ethylenediamine tetraacetic acid (EDTA)) and lysing matrix E (Qbiogene Inc., Carlsbad, CA, USA) were processed in a bead beater (Bio 101/Savant, Farmingdale, NY, USA) for 45 s at 6 m s^{-1}. After bead beating, samples were put on ice for 5 min, centrifuged (16 000 *g*, 5 min) at 4°C and 800 μl of the supernatant was extracted with 750 μl of a 1:1 mixture of phenol (pH 8.0) and chloroform–isoamyl alcohol (CIA; 24:1) and then with 750 μl of CIA. Total nucleic acid was precipitated for 1 h at room temperature with 850 μl of RNase-free PEG solution (20% polyethylene glycol, 2.5 M NaCl). After centrifugation (16 000 *g*, 30 min), the nucleic acid pellets were washed once with cold ethanol (70% v/v). After further centrifugation (16 000 *g*, 20 min), the pellets were dried at 20°C for 10–20 min and then dissolved in 50 μl of RNase-free water. At this stage, the quality of nucleic acid was determined on a 1% (w/v) agarose gel. DNA was separated from RNA using a Qiagen RNA/DNA mini kit (Qiagen Inc., Valencia, CA, USA) and quantified using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE, USA). Total RNA was treated with 4 U of DNase I (New England Biolabs Inc., Ipswich, MA, USA) at 37°C for 2 h and then purified using a Qiagen RNeasy mini kit (Qiagen). RNA was quantified using a Nanodrop spectrophotometer and was confirmed to be DNA-free by amplification of 16S rRNA genes with universal primers 27f/907r (94°C 1 min, 60°C 1 min, 72°C 1 min, 35 cycles). No PCR products were obtained except with the appropriate controls.

Table 1 Main physicochemical properties of the soil

	Particle size distribution (%)			*Chemical analysis*			
	Clay	*Silt*	*Sand*	*Total N (%/w)*	*Total C (%/w)*	*NH_4 (mg kg^{-1})*	*NO_3 (mg kg^{-1})*
Control soil (−worms) (56.14 mg N/g soil)	12	40	48	0.12	3.92	62.8	24. 0
Earthworm-incubated soil (+worms) (32.6 mg N/g soil)	16	49	35	0.16	3.89	23.4	71.1

^{12}C and ^{13}C DNA recovery

DNA extracts from replicates were pooled. The gradients were prepared as described by Neufeld *et al*. (2007). One gram of caesium chloride (CsCl) was added to 5 μl of DNA (5 μg) diluted in 1 ml of H_2O. Then, 100 μl of ethidium bromide (10 mg ml^{-1}) was added to the DNA + CsCl solution in an ultracentrifuge tube (13 × 51 mm, Beckman, Fullerton, CA, USA). A control gradient was also prepared containing 2.5 μg each of ^{12}C- and ^{13}C-labelled DNA from *Methylococcus capsulatus* (Bath). Heavy and light DNA were then separated by centrifugation at 177 000 *g* (44 100 r.p.m. using a Beckman rotor VTi 65.2) for 40 h at 20°C.

After centrifugation, heavy and light DNA bands were visualized under UV (365 nm). Heavy DNA was not always visible under UV but its position in the tube was deduced by comparison with the control gradient. Light and heavy DNA were withdrawn gently from the gradient using a 1 ml syringe and hypodermic needle. Ethidium bromide was extracted from the DNA with an equal volume of butanol saturated with Tris-EDTA (TE) (10 mM Tris, 1 mM EDTA, pH 8) buffer (repeated twice). Then DNA was precipitated for 2 h at room temperature with two volumes of PEG solution (30% polyethylene glycol, 1.6 M NaCl) and 3 μl of glycogen to visualize the pellet. After centrifugation for 30 min at 16 000 *g* at 4°C, pellets were washed with 70% (v/v) ice-cold ethanol. After centrifugation for 15 min at 16 000 *g*, at 4°C, pellets were air-dried for 10–20 min and then dissolved in 40 μl H_2O.

^{12}C and ^{13}C RNA recovery

Replicates of RNA extracts were pooled and rRNA was resolved in a caesium trifluoroacetate (CsTFA) gradient with an average density of 1.795 g ml^{-1}. Centrifugation medium was prepared by mixing 4.655 ml of a 1.953 g ml^{-1} CsTFA stock solution (Amersham Biosciences, Piscataway, NJ, USA), 0.165 ml formamide, 0.680 ml of gradient buffer (GB; 100 mM Tris pH 7.8; 100 mM KCl; 1 mM EDTA) and RNA (500 ng) in a total volume of 5.5 ml (RNA volume was subtracted from GB volume). Heavy and light rRNAs were then separated by centrifugation at 130 000 *g* (37 800 r.p.m. using a Beckman rotor VTi 65.2) for 63 h at 20°C. Centrifuged gradients were fractionated from bottom to top into 12 equal fractions (~450 μl). A controlled flow rate was achieved by displacing the gradient medium with water at the top of the tube using a peristaltic pump at a flow rate of ~450 μl min^{-1}. The density of each fraction was checked by weighing 100 μl of each fraction by pipetting (measurement done in triplicate). RNA was precipitated with an equal volume of isopropanol and 3 μl of glycogen (to visualize the pellet). After centrifugation for 30 min at 16 000 *g* at 4°C, the pellets were washed with 500 μl of 70% (v/v) ice-cold ethanol. After centrifugation for 15 min at 16 000 *g* at 4°C, the pellets were air-dried for 10–20 min. RNA samples were then dissolved in 20 μl of RNase free-water.

16S rRNA gene amplification and denaturing gradient-gel electrophoresis

rRNA samples from the gradient were reverse transcribed using primer 1492r (Lane, 1991) and Superscript II reverse transcriptase (Invitrogen Inc., CA, USA) according to the manufacturer's instructions. The cDNA produced from the light and heavy rRNA recovered from the CsTFA gradient, and DNA samples recovered from CsCl gradient, were used as templates for PCR using three different primer sets. The universal bacterial primer set 341f-GC/907r (Muyzer *et al*., 1993) was used to amplify 16S rRNA genes from the bacterial community. Amplification was carried out with 30 cycles of 94°C for 1 min 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. A seminested PCR strategy (Y Chen *et al*., unpublished) was used to specifically amplify 16S rRNA genes of either type I or II methanotrophs. First-round amplification was done using type Ir (5′-CCACTGGTGTTCCTTCMGAT-3′) and type If (5′-ATGCTTAA CACATGCAAGTCGAACG-3′) or type IIr (5′-GTCAARAGCT GGTAAGGTTC-3′) and type IIf (5′-GGGAMGATAATGACGGT ACCWGGA-3′) primer sets, specifically targeting types I and II methanotrophs, respectively, using 30 cycles consisting of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 10 min. A second round of PCR was performed using 1 μl of the first PCR product as template, using 341f-GC (Muyzer *et al*., 1993) and the type Ir primer set for the type I methanotrophs and 518f-GC (Muyzer *et al*., 1993) and the type IIr primer set, for the type II methanotrophs. This second round of PCR was set up according to the procedure above but only for 25 cycles.

The PCR products containing a GC clamp were separated on 6% (w/v) polyacrylamide gels with a 30–70% urea/formamide-denaturing gradient. Gels were run at 85 V for 14 h at 60°C in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). Gels were then stained for 1 h with 1:10 000 (v/v) SYBR GREEN (Invitrogen, San Diego, CA, USA), rinsed with 1 × TAE and scanned with a storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Bands of interest were excised from the gel using cut pipette tips and DNA was dissolved at 4°C in 10 μl sterile H_2O overnight. Four microlitres of the dissolved DNA was used as a template for PCR amplification using primer set 341f and 907r. PCR products were then purified using shrimp alkaline phosphatase (SAP) and Exonuclease I (*Exo*I; Amersham Biosciences) as follows: for one reaction, 1.43 μl of SAP dilution buffer, 1 μl of SAP enzyme (1U μl^{-1}) and 0.075 μl of *Exo*I enzyme (20U μl^{-1}) were added to 20 μl of PCR product. Samples were incubated for 40 min at 37°C, followed by 15 min at 80°C and then they were stored at 4°C.

pmoA *and* mmoX *clone libraries*
Both *pmoA* and *mmoX* genes were amplified from the unfractionated pooled DNAs using primer sets mb661r (Costello and Lidstrom, 1999) and A189f (Holmes *et al.*, 1995) and 206F and 886R (Hutchens *et al.*, 2004), respectively, using 30 cycles at 95°C for 1 min, 55°C (*pmoA*) or 60°C (*mmoX*) for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10 min. For both genes, the size and purity of the PCR products were checked on 1% (w/v) agarose gels. PCR products were purified using the QIAGEN gel extraction kit and ligated into the PCR II vector (Invitrogen) according to the manufacturer's instructions. The positive recombinant clones were screened by direct amplification of the cloned inserts from transformant cells with vector-specific primers M13r and M13f. The clones with correct-sized inserts were digested for 1 h at 37°C using the restriction enzymes *Eco*RI–*Pvu*II–*Hin*cII for *pmoA* gene and *Eco*RI–*Hin*cII for *mmoX* gene. Digests were resolved on 2.5% (w/v) agarose gel and grouped into operational taxonomic units (OTUs), based on the restriction pattern obtained and representative clones for each OTU were sequenced.

pmoA *microarray experiments*
pmoA genes were amplified from unfractionated, light and heavy DNA using primer set A189f/T7-mb661r (Bourne *et al.*, 2001). The T7 promoter attached to the 5′- end of the reverse primer allowed the T7 RNA polymerase to transcribe the DNA templates into RNA *in vitro*. PCR amplification, *in vitro* transcription and hybridization protocol were as described by Bodrossy *et al.* (2003) and modified by Stralis-Pavese *et al.* (2004).

DNA sequencing and analysis
Sequencing of *pmoA* and *mmoX* clones was performed with the M13r (5′-CAGGAAACAGCTAT GAC-3′) primer targeting the multicloning site of the vector. Double-strand sequencing was performed directly (without any cloning step) on the reamplified and purified 16S rRNA–DGGE bands, using both the primers used for PCR amplification (907r/341f for universal bacterial 16S rRNA genes and types Ir/341f and IIr/518f for types I and II methanotroph 16S rRNA genes, respectively). DNA sequencing was performed using a Dye Terminator kit (PE Applied Biosystems, Warrington, UK). DNA sequences were analysed using a 373A automated sequencing system (PE Applied Biosystems). For each DGGE band excised from the gels, a unique sequence was obtained.

Sequences were submitted to a BLAST search (Altschul *et al.*, 1990) and checked for the presence of chimaeras. Chimaeras were not detected for the 16S rRNA gene–DGGE band sequences or for the *mmoX* sequences, but around 10% of the *pmoA* sequences were suspected to be chimaeras and were removed from the analysis. Sequences were aligned to related sequences extracted from GenBank using MEGA version 3.1 (Kumar *et al.*, 2004). MEGA 3.1 was also used to estimate evolutionary distances and to construct a phylogenetic tree. Several methods were used to construct trees: for *pmoA* and *mmoX* sequences, neighbour-joining with Kimura correction and maximum parsimony based on nucleotide sequence analysis, and neighbour-joining with Poisson correction and maximum parsimony based on amino-acid derived sequences analysis; for 16S rRNA gene phylogeny, neighbour-joining with Kimura correction and maximum parsimony. For all the genes, the different methods tested gave similar results.

Nucleotide sequence accession numbers
The GenBank accession numbers for the nucleotide sequences determined in this study are EF472919 to EF472921 for the 16S rRNA gene sequences, EF472933 to EF472943 for the *pmoA* sequences and EF472922 to EF472932 for the *mmoX* sequences.

Results

Effect of earthworms on methane oxidation in the landfill biocover soil and on soil characteristics
After incubation for 168 h with ^{13}C-methane, the earthworm-incubated soil removed significantly more methane (67%) than the control soil (52%, $n=15$, $P=0.007$; AC Singer *et al.*, unpublished).

The nitrate and ammonia contents of soil incubated with earthworms were respectively higher and lower than that in soil without earthworms. Total nitrogen and carbon contents were comparable. Earthworm-incubated soil consisted mainly of silt particles, while soil without earthworms consisted mainly of sand particles. The percentage of clay was generally higher (16%) in the earthworm-incubated soil (Table 1).

Bacterial community fingerprints
For RNA-SIP, the densities of the 'heavy' and 'light' fractions were 1.80 and 1.77 g ml^{-1}, respectively. DGGE analysis was performed on light and heavy DNA and RNA fractions recovered from the SIP experiments, using different primer sets targeting 16S RNA genes. Universal bacterial 16S rRNA gene PCR primers gave complex and similar DGGE profiles with ^{12}C-DNA and ^{12}C-RNA samples from the microcosms containing worms (+worms) and the corresponding control (−worms). Profiles corresponding to ^{13}C-DNA or RNA were less complex, comprising of only a few intense bands corresponding to the bacteria that have incorporated the ^{13}C in their nucleic acids. In the ^{13}C-RNA profiles, the ^{12}C-RNA background was still visible under the main ^{13}C bands. Two major DGGE bands were common to both −worms and +worms in the ^{13}C-DNA and ^{13}C-RNA DGGE profiles, whereas one

band was specific to the +worms in ^{13}C-DNA and ^{13}C-RNA profiles (Figure 1). For both DNA- and RNA-DGGE, these three bands were excised from the gels, sequenced, and their phylogenetic affiliations were determined. The sequences corresponding to the bands excised from the DNA-DGGE gel were similar to those corresponding to the bands excised from the RNA-DGGE gel (100% identity). The two DGGE bands common to both ^{13}C profiles of −worms and +worms samples corresponded to *Methylobacter*- and *Methylosarcina*- (Wise *et al.*, 2001) related 16S rRNA gene sequences, clustering among the type I methanotrophs. The band specific to the DGGE profiles obtained with ^{13}C-DNA and ^{13}C-RNA from the +worms microcosm sample corresponded to a *Bacteroidetes*-related 16S rRNA sequence (Figure 2).

Primers specifically targeting 16S rRNA genes from type I or II methanotrophs were used to investigate the effect of earthworms on methanotroph community structure. As observed with the universal bacterial 16S rRNA gene primers, the ^{13}C-DNA and ^{13}C-RNA profiles obtained with type I methanotroph-specific 16S rRNA gene primers were mainly composed of two intense bands (Figure 3) in contrast to the ^{12}C- profiles that appeared more complex. Sequencing of these 16S rRNA genes from the DGGE gels showed that the two bands dominating the ^{13}C profiles corresponded to the methanotroph sequences identified in the ^{13}C profiles using universal bacterial 16S rRNA gene primers: *Methylobacter*- and *Methylosarcina*-related 16S rRNA gene sequences. Whereas the DGGE profiles obtained with ^{13}C-DNA and ^{13}C-RNA from the −worms and +worms microcosms were similar, some slight differences were observed in the ^{12}C-DNA 16S rRNA gene DGGE profiles (Figure 3).

DGGE profiles obtained for the ^{12}C- and ^{13}C-DNA and RNA with type II methanotroph-specific 16S rRNA gene PCR primers were similar, for all the samples tested (data not shown), and contained two dominant bands. Sequencing of these bands excised from both heavy- and light-fraction DGGE profiles revealed that they corresponded to highly similar sequences (99% identity). Phylogenetic analysis assigned these two 16S rRNA gene sequences to *Methylocystis*-related bacteria (data not shown).

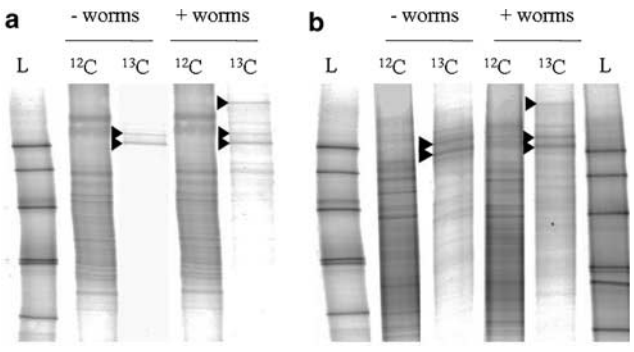

Figure 1 Denaturing gradient-gel electrophoresis (DGGE) targeting the bacterial 16S ribosomal RNA genes obtained for the ^{12}C- and ^{13}C-DNA fractions (**a**) and the ^{12}C- and ^{13}C-RNA fractions (**b**) from landfill cover soil samples incubated without (−worms) and with worms (+worms). Lane L corresponds to a molecular mass ladder. Black arrows indicate DGGE bands that have been sequenced.

pmoA *and* mmoX *diversity*

To complement the data obtained with 16S rRNA gene analyses, the distributions of *pmoA* and *mmoX*, two key genes for methanotrophy, were investigated in both −worms and +worms samples using complementary molecular biology techniques. Clone libraries were constructed for both genes using DNA extracted from −worms and +worms microcosm soil samples.

pmoA *diversity*

Around 10% of the *pmoA* sequences were suspected to be chimaeras and were removed from the clone

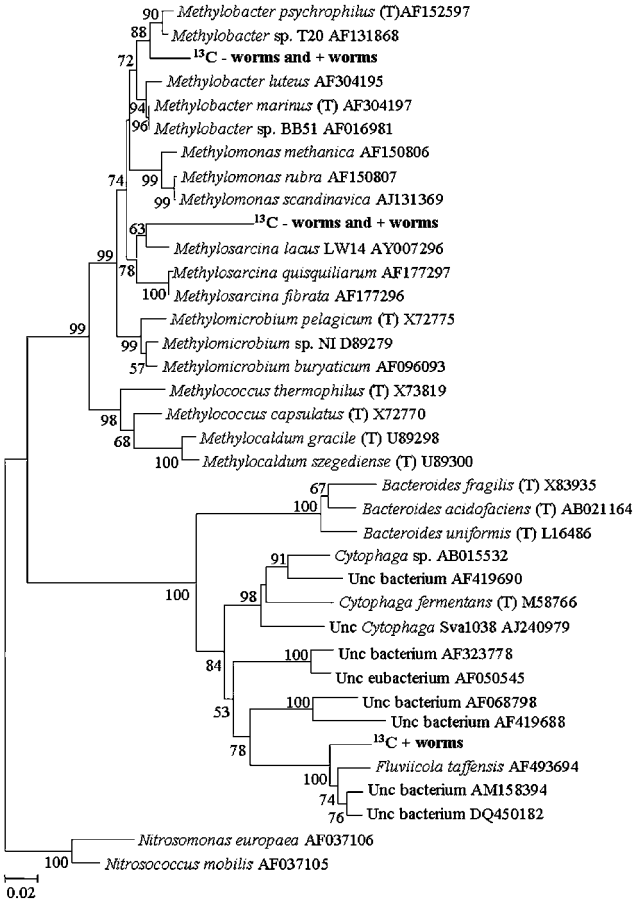

Figure 2 Phylogenetic neighbour-joining tree of partial 16S ribosomal RNA (16S rRNA) sequences, showing the relationship of sequences from denaturing gradient-gel electrophoresis (DGGE) bands obtained with both ^{13}C-DNA and ^{13}C-RNA samples to sequences of pure cultures and 16S rRNA gene sequences obtained in other cultivation-independent studies. DGGE band-derived sequences from this study are indicated by boldface type. Only bootstrap values >50% are indicated. Scale bar = 0.02 change per base position.

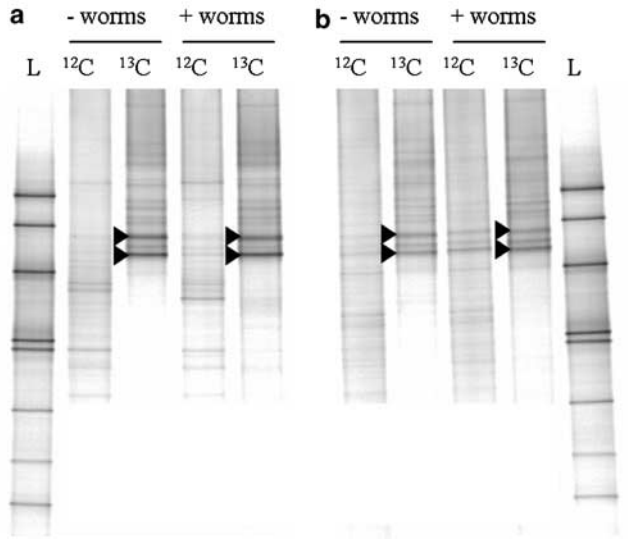

Figure 3 Denaturing gradient-gel electrophoresis (DGGE) specifically targeting the type I methanotroph 16S rRNA genes from the ^{12}C- and ^{13}C-DNA fractions (**a**) and from the ^{12}C- and ^{13}C-RNA fractions (**b**). Lane L corresponds to the molecular mass ladder. Black arrows indicate bands that have been sequenced.

library analysis. Forty-five *pmoA* clones obtained from −worms microcosm and 47 *pmoA* clones obtained from +worms microcosm clones were grouped into eight OTUs according to their restriction patterns. At least one representative of each OTU (two for OTUs containing more than five clones, three for OTUs containing more than ten clones) was sequenced. Phylogenetic analysis of *pmoA* sequences indicated that two OTUs were dominant in *pmoA* libraries constructed using DNA from both −worms and +worms libraries, corresponding to *Methylomicrobium*/*Methylosarcina* (OTU1) and *Methylocystis* (OTU2)-related *pmoA* sequences. In both libraries, type Ia *pmoA* sequences represented 60 and 66% of the *pmoA* clones in the −worms and +worms libraries, respectively, and were *Methylomicrobium*/*Methylosarcina*- and *Methylobacter*-related sequences. Type II methanotrophs accounted for 40 and 34% of the −worms and +worms clones, respectively, and were only represented by *Methylocystis*-related *pmoA* sequences (Figure 4). No *pmoA* sequences from the type Ib methanotroph genera *Methylocaldum*, *Methylothermus*, *Methylococcus* or the type Ia genus *Methylomonas* or the type II genus *Methylosinus* were found.

To complement data obtained from *pmoA* clone libraries, a *pmoA* microarray was used to investigate the diversity of this functional gene at different taxonomic levels. Hybridization signal patterns reflected the high diversity of *pmoA* sequences belonging to both types I and II groups retrieved in all of the DNA samples (Figure 5). For the type I methanotrophs, high hybridization signal intensities were observed for probes targeting the *pmoA* from the genera *Methylobacter*, *Methylomicrobium*

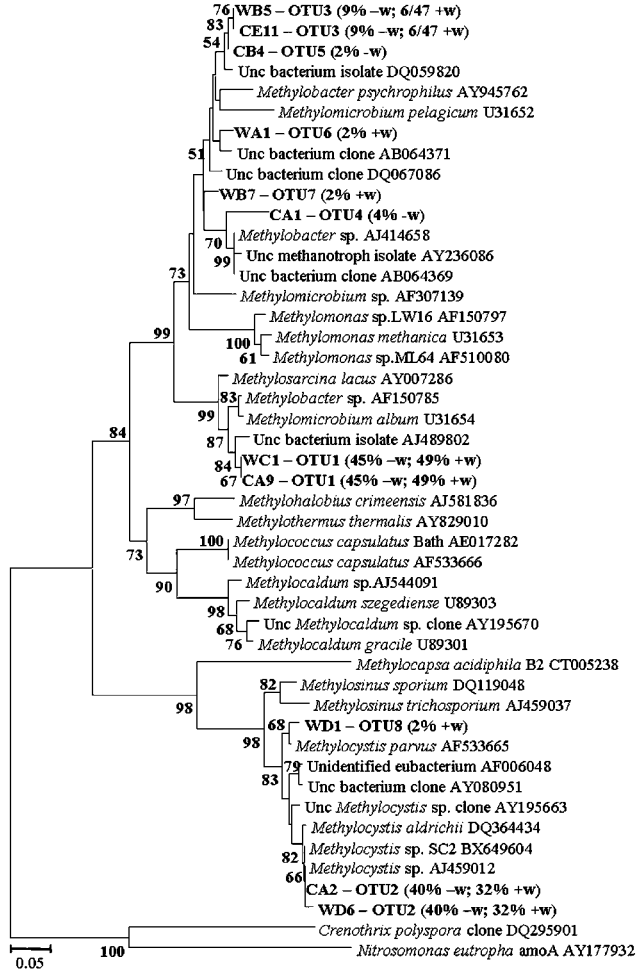

Figure 4 Phylogenetic neighbour-joining tree of partial PmoA sequences derived from the *pmoA* clone libraries (Poisson correction). The percentage of each operational taxonomic unit (OTU) among each library is indicated within parentheses, '−w' indicates −worms library and '+w' indicates +worms library. Only bootstrap values >50% are indicated. Scale bar = 0.05 change per base position.

and *Methylosarcina* (type Ia) and lower signal intensity was obtained for probe targeting genus *Methylocaldum* (type Ib). Since high signal intensity was obtained with the probe targeting both the genera *Methylomicrobium* and *Methylosarcina* (Mmb_562) and low signal intensity was obtained with the probe targeting only the genus *Methylomicrobium* (Mmb_303), it is likely that *Methylosarcina* and related bacteria are responsible for the high signal intensity obtained with probe Mmb_562. For the type II methanotrophs, very high hybridization signals were obtained for probes targeting the *pmoA* from the genera *Methylocystis* and *Methylosinus*, suggesting their relative high abundance in all of the samples. Interestingly, three genera that were not detected at all in the *pmoA* clone libraries have been detected with the *pmoA* microarray: *Methylomonas* (type Ia, probe P_Mm531), *Methylocaldum* (type Ib, probe Mcl408) and *Methylosinus* (type II). Two

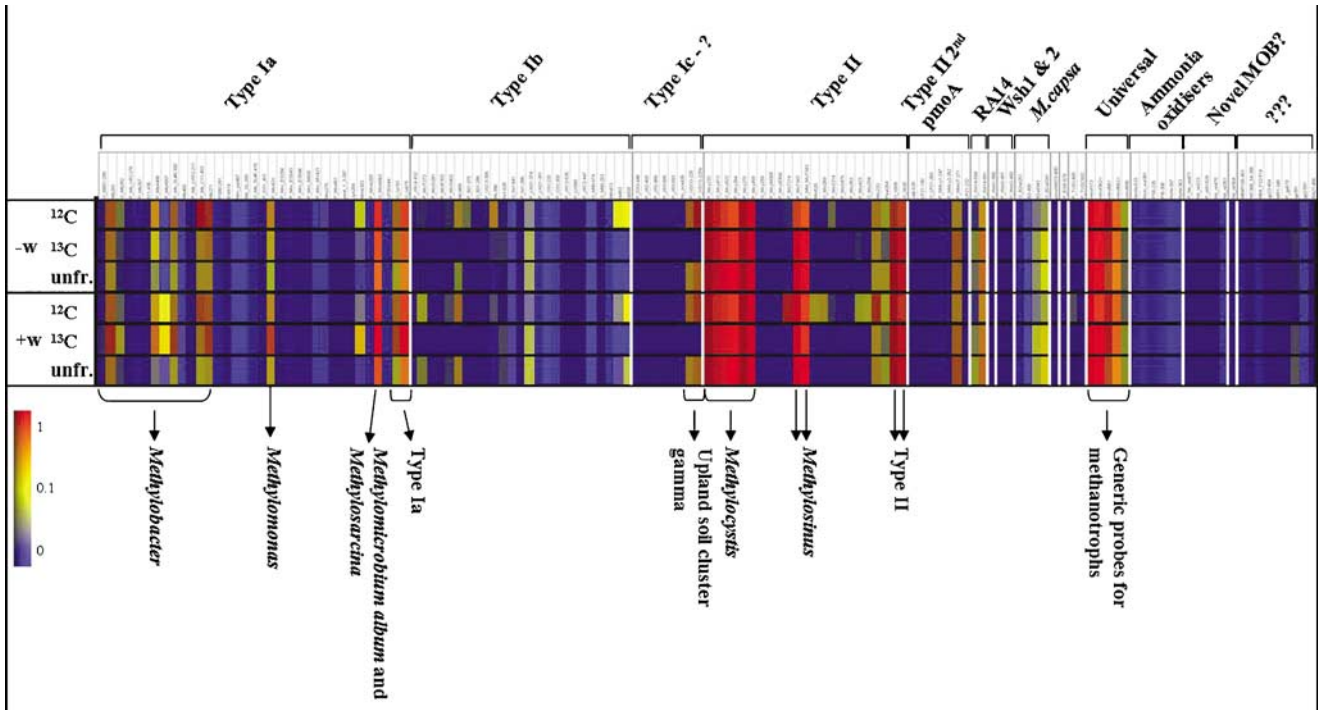

Figure 5 Microarray results showing hybridization patterns obtained for ^{12}C and ^{13}C DNA of both −worms and +worms samples with the microarray *pmoA* probe set. Relative signal intensities are indicated by the different colours as shown on the colour bar (a value of 1 corresponding to the maximum achievable signal for an individual probe).

probes, targeting *pmoA* from the genus *Methylocaldum* (McI408) and the Upland soil cluster Gamma (P_USCG-225), hybridized only with templates generated from the unfractionated DNA and the ^{12}C-DNA of both samples (not for the ^{13}C-DNA). Considering the ^{13}C-DNA samples, some probes targeting type Ia methanotrophs showed a stronger hybridization signal for the +worms sample than for the −worms sample: Mmb303 (*Methylomicrobium*-specific probe), P_Mm531 (*Methylomonas*-specific probe) and P_MbSL#3–300 (*Methylobacter*-specific probe). This increase in the relative abundance of type Ia methanotroph signals in the ^{13}C-DNA of the +worms sample is supported by the fact that a highest hybridization signal was also observed for the generalist probe for type Ia methanotrophs (O_Ia193).

mmoX *diversity*

Eighty-eight and 95 *mmoX* clones containing the correct size insert derived from DNA extracted from the −worms and +worms microcosms, respectively, were analysed by restriction fragment-length polymorphism (RFLP). These clones grouped into three distinct OTUs. The dominant OTU (OTU1), which represented 85 and 81% of the −worms and the +worms clones, respectively, corresponded to members of type II methanotrophs related to *Methylocystis* species (Figure 6). These *mmoX* sequences were also closely related to *mmoX* sequences previously recovered from the same Ufton landfill biocover soil (JC Murrell *et al.*, unpublished). OTU2 and OTU3 could not be affiliated to *mmoX* from any known methanotrophs, since the highest percentage of identity to other *mmoX* nucleotide sequences was 83%. However, based on phylogenetic analysis, OTU2, which represented 7 and 16% of the −worms and +worms clones, respectively, is probably related to type I methanotrophs; whereas OTU3, which represented 8 and 3% of the −worms and +worms clones, respectively, is probably related to type II methanotrophs. These two OTUs may correspond to *mmoX* sequences of uncultivated methanotrophs (Figure 6). Type II-related *mmoX* sequences were dominant in both libraries, whereas type I-related *mmoX* accounted for 7 and 16% of the −worms and +worms microcosm-derived clones, respectively.

Discussion

For a better control of methane emissions, landfill management practice requires a detailed knowledge of the microorganisms involved in methane oxidation and a better understanding of the environmental parameters that regulate this biological process. The mechanism by which earthworms enhance methane oxidation efficiency has been investigated, with particular emphasis on the active component of the bacterial methanotroph community.

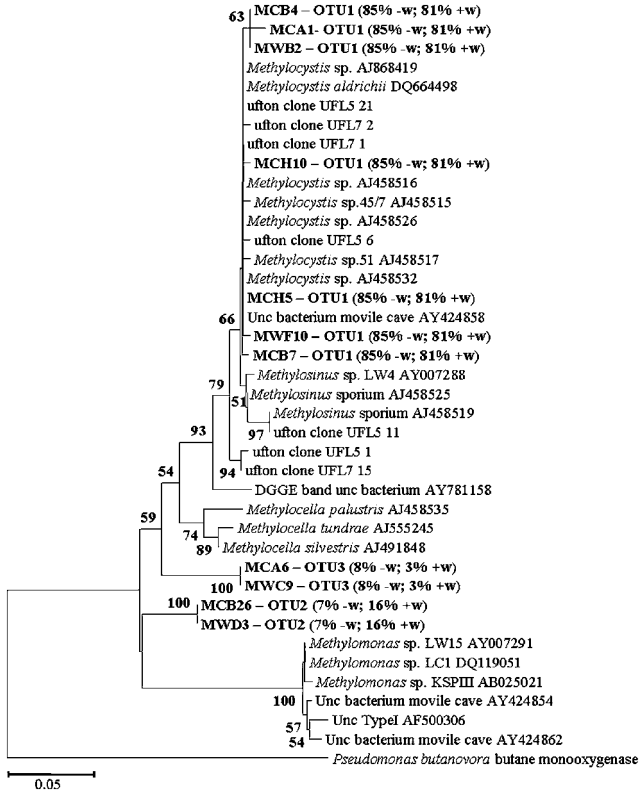

Figure 6 Phylogenetic neighbour-joining tree of partial MmoX-derived sequences from the *mmoX* clone libraries (Poisson correction). The percentage of each operational taxonomic unit (OTU) among each library is indicated within parentheses, '−w' indicates −worms library and '+w' indicates +worms library. Only bootstrap values >50% are indicated. Scale bar = 0.05 change per base position.

Methanotroph diversity in the landfill soil

On the basis of *pmoA* clone libraries and particularly on *pmoA* microarray results, a high diversity of *pmoA* sequences related to both types I and II methanotrophs has been retrieved as reported for other landfill biocover soils (Wise *et al.*, 1999; Bodrossy *et al.*, 2003; Uz *et al.*, 2003; Crossman *et al.*, 2004; Stralis-Pavese *et al.*, 2004). In the Ufton landfill biocover soil, types I and II methanotrophs seemed to be highly diverse with several genera being detected, in particular, after *pmoA* microarray analysis. Conversely, the diversity of *mmoX*-carrying methanotrophs was low, with more than 80% of the sequences relating to *Methylocystis* sp. sequences.

Active methanotrophs identified in ^{13}C-DNA and ^{13}C-RNA by DGGE were related to 16S rRNA from the type I genera *Methylobacter* and *Methylosarcina* and to 16S rRNA from the type II genus *Methylocystis*. These results are in agreement with *pmoA* hybridization patterns obtained with the ^{13}C-DNA. On the basis of previous SIP experiments, bacteria that actively oxidize methane were identified as belonging to a broad range of type I and type II methanotrophs in a peat soil (Morris *et al.*, 2002) and in Movile cave (Hutchens *et al.*, 2004), whereas it has been hypothesized that only type I methanotrophs were responsible for the majority of CH_4 oxidation in Russian soda lake sediments (Lin *et al.*, 2004).

The different molecular methods used in this study identified the same dominant and active methanotrophs. Other studies (Costello and Lidstrom, 1999; Horz *et al.*, 2001) have similarly concluded that 16S rRNA and *pmoA*-based methods gave similar methanotroph community structure profiles, both approaches being complementary. The congruent results obtained with the clone libraries and the microarray are confirmed by the fact that the dominant *pmoA* clones had perfect sequence matches with the most intensely hybridized *pmoA* probes (data not shown). However, microarrays gave a more complete view of the *pmoA* diversity in the soil samples studied, with a higher diversity of *pmoA* sequences retrieved, confirming the suitability of this high throughput method for assessing a wider diversity of *pmoA* genes present in the target environment (while *pmoA* gene libraries may only reveal the most abundant *pmoA* phylotypes). This difference observed in terms of the diversity recovered may also result from cloning bias and the low number of clones analysed, resulting in a poor representation of the methanotroph diversity in the clone libraries. Analysis of type I DGGE, ^{12}C-DNA and RNA profiles of both samples suggested a high diversity of type I, whereas only few distinct bands were observed in the ^{13}C DNA and RNA profiles, suggesting that the dominant member of the bacterial community was not necessarily active. On the contrary, *pmoA* microarray results showed only few differences between hybridization patterns for ^{12}C and ^{13}C samples, suggesting that almost all of the methanotrophs detected were active. This could be due to the lack of specificity of type I 16S rRNA gene primers when applied to a complex bacterial community. In ^{12}C-DNA or RNA, the proportion of type I methanotrophs among total bacteria might be too small to avoid the amplification of nonmethanotrophic bacteria. Hybridization of both ^{12}C- and ^{13}C-DNA to the *pmoA* microarray allowed a more precise discrimination between the active and the non-active methanotrophs. The hybridization signals obtained for the *Methylocaldum* (Mcl408) and Upland Soil Cluster gamma probes (P-USCG-225 and P-USCG-225b; Figure 5) only with the unfractionated and the ^{12}C-DNA, but not with the ^{13}C-DNA samples, suggested that these bacteria were present in the soil, but were not actively oxidizing methane.

In this study, RNA-SIP and DNA-SIP have been compared. Lueders *et al.* (2004) combined RNA- and DNA-SIP to monitor activation and temporal dynamics of methylotrophs in soil. The authors suggested that after 6 days of incubation with labelled substrate, RNA-SIP recovered the initially active bacteria, whereas a specific enriched methylotroph was identified after 42 days of incubation

using DNA-SIP. A recent publication also demonstrated the faster incorporation of label into RNAs (Manefield *et al.*, 2007). However, DNA-SIP sensitivity has been improved, and the amounts of substrate as well as the incubation times have been significantly optimized (Neufeld *et al.*, 2007). In our study, 7 days of incubation with 11 μmol of ^{13}C-CH_4 g^{-1} soil were sufficient for efficient labelling of both DNA and RNA and results obtained with DNA- and RNA-SIP based on 16S rRNA DGGE analyses were quite similar. This suggests that, after 7 days incubation with ^{13}C-CH_4, all the active methane-consuming bacteria synthesized DNA. However, it is not excluded that results would have been different if RNA- and DNA-SIP had been compared for successive earlier time points. Slight differences in ^{12}C profiles between RNA- and DNA-based DGGE profiles were observed, suggesting that the dominant member of the bacterial community was not necessarily active.

Earthworm effects on bacterial community structure

The *Bacteroidetes*-related bacterium identified in the ^{13}C-DNA and RNA of earthworm-incubated soil was the only obvious modification in the total bacterial community structure observed by DGGE. It is the first time that *Bacteroidetes* have been identified as potentially playing a role in methane oxidation. Even if *Bacteroidetes* have been identified in some methane-rich environments (Scholten-Koerselman *et al.*, 1986; Reed *et al.*, 2002, 2006), there is lack of evidence concerning the possible capacity of the *Bacteroidetes* we identified to oxidize methane. This result might be due to a cross-feeding phenomenon, that is the consumption by the *Bacteroidetes*-related bacteria of a labelled by-product produced by the methanotrophs during methane oxidation. An alternative hypothesis is that due to food web interactions, dead ^{13}C-labelled methanotrophs have been consumed by *Bacteroidetes*. Further investigations are necessary to determine if this bacterium can oxidize methane.

Microarray data are semiquantitative, thereby enabling the direct comparison of the relative abundance of target sequences (here *pmoA* sequences) in a number of environmental samples (discussed in Bodrossy *et al.*, 2003; Neufeld *et al.*, 2006). On the basis of *pmoA* microarray results obtained with ^{13}C-DNA, the relative abundance of type Ia methanotrophs appeared higher in the earthworm-incubated soil. This is suggested since the highest hybridization signals were observed for the generalist probe targeting the type Ia (O_Ia193) *pmoA* and other *pmoA* probes targeting several genera of type Ia methanotrophs (that is the *Methylomicrobium* probe Mmb303, *Methylomonas* probe P_Mm531 and *Methylobacter* probe P_MbSL#3–300). Furthermore, considering results from analysis of both *pmoA* and *mmoX* libraries, the percentage of type I methanotroph-related sequences was higher in the earthworm-incubated soil. These differences should not be considered as significant since clone libraries are not quantitative but this trend supported the microarray results, which indicate the greater abundance of *pmoA* sequence types in DNA or RNA samples, suggesting that earthworms might have stimulated growth or activity of type I methanotrophs. These finding are in agreement with the assumption that type I methanotrophs probably react faster to changing conditions than type II methanotrophs, owing to a higher growth rate (Graham *et al.*, 1993; Bodelier *et al.*, 2000; Henckel *et al.*, 2000). This growth or stimulation of activity could be correlated with a nitrogen supply and/or an improved nutrient availability directly linked to earthworm activity (Needham, 1957; Buse, 1990). In this study, the relative amounts of ammonia and nitrate were strongly influenced by the earthworm activity (Table 1). The highest nitrate content and the lowest ammonia content in the earthworm-incubated soil suggest a possible stimulation of nitrifier activity. A higher number of nitrifiers have been reported in earthworm burrow walls (Parkin and Berry, 1999), as well as in casts (Mulongoy and Bedoret, 1989) in comparison with the underlying soil, which was correlated to a higher content of mineral nitrogen in the soil. Nitrifiers possess the ammonia monooxygenase, an enzyme that is evolutionary related to the MMO, and can to some extent co-oxidize methane. However, the contribution of nitrifiers to the global methane cycle is unclear and sometimes controversial (Holmes *et al.*, 1995). Further investigations on the nitrifier communities will be necessary to determine the extent to which this group of bacteria could be involved in the earthworm-mediated increase in methane oxidation rates observed in the landfill soil studied.

Conclusions

We proposed the hypothesis that earthworms could stimulate the growth or the activity of methanotrophs. We showed that the earthworm-mediated increase of methane oxidation in the landfill biocover soil only weakly correlated with a shift in the structure of the active methanotroph population. Future work needs to focus on the relationship between this earthworm effect on enhanced methane oxidation in landfill cover soil and this effect on bacterial activity and growth. The possible contribution of an enriched population of nitrifying bacteria to methane oxidation also requires further investigation.

Acknowledgements

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Spatial and temporal diversity of methanotrophs in a landfill cover soil are differentially related to soil abiotic factors

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Summary

Methanotrophs present in landfill cover soil can limit methane emissions from landfill sites by oxidizing methane produced in landfill. Understanding the spatial and temporal distribution of populations of methanotrophs and the factors influencing their activity and diversity in landfill cover soil is critical to devise better landfill cover soil management strategies. *pmoA*-based microarray analyses of methanotroph community structure revealed a temporal shift in methanotroph populations across different seasons. Type II methanotrophs (particularly *Methylocystis* sp.) were found to be present across all seasons. Minor shifts in type I methanotroph populations were observed. In the case of spatial distribution, only minor differences in methanotroph community structure were observed with no recognizable patterns (both vertical and horizontal) at a 5 m scale. Correlation analysis between soil abiotic parameters (total C, N, NH_4^+, NO_3^- and water content) and distribution of methanotrophs revealed a lack of conclusive evidence for any distinct correlation pattern between measured abiotic parameters and methanotroph community structure, suggesting that complex interactions of several physico-chemical parameters shape methanotroph diversity and activity in landfill cover soils.

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Introduction

Landfills are a major anthropogenic source of methane (CH_4) and are estimated to contribute 6–12% of global CH_4 emissions to the atmosphere (Lelieveld *et al.*, 1998). In old landfills, without landfill gas extraction systems, landfill cover soils can limit CH_4 emissions to the atmosphere through microbial CH_4 oxidation. Methanotrophs present in these cover soils can oxidize CH_4, forming biomass and carbon dioxide (Hanson and Hanson, 1996). It is estimated that approximately 22 Tg of CH_4 $year^{-1}$ is oxidized in landfill cover soils (Reeburgh, 1996). Methanotrophs are a group of *Proteobacteria* known for their ability to utilize CH_4 as sole carbon and energy source. Methane monooxygenase (MMO) catalyses the oxidation of methane to methanol in methanotrophs. There are two distinct types of MMO, a membrane-bound particulate MMO (pMMO) and a soluble MMO (sMMO) (reviewed in Trotsenko and Murrell, 2008). Particulate MMO is present in all methanotrophs except for the genus *Methylocella* (Dedysh *et al.*, 2005). *pmoA* (encoding the 27 kDa subunit of pMMO) and *mmoX* (encoding the α-subunit of the hydroxylase of sMMO) have been successfully used as functional gene probes for detection of methanotrophs in the environment. Since the phylogeny of *pmoA* is congruent with 16S rRNA phylogeny, *pmoA* has been extensively used as a functional gene marker in molecular ecology studies to target methanotrophs (reviewed in McDonald *et al.*, 2008).

Soil is a heterogeneous environment composed of many niches that harbour tremendous bacterial diversity (Curtis *et al.*, 2002). Bacterial diversity and abiotic factors such as soil particle size, porosity, water content, nutrient availability and pH can vary spatially from sub-millimetre scale to large geographic distances and also in time (Martiny *et al.*, 2006). While understanding spatiotemporal distribution patterns of bacterial communities and factors that influence these patterns and bacterial functions still remains a challenge (Torsvik and Ovreas, 2002), it is essential for a better understanding of biogeochemical cycling and ecosystem functioning (Green and Bohannan, 2006). It has been suggested that spatial isolation can influence bacterial community structure (Ranjard and Richaume, 2001; Sessitsch *et al.*, 2001). A number of studies have focussed on spatial heterogeneity of

microorganisms in different soils (Grundmann and Debouzie, 2000; Nunan *et al.*, 2002; Mummey and Stahl, 2003; Fierer and Jackson, 2006). Franklin and Mills (2003) using amplified fragment length polymorphism (AFLP) and geostatistical variogram analysis reported autocorrelation of bacterial community structure at scales ranging from 30 cm to 6 m, depending on the extent of sampling. Analysing bacterial community structure at a larger scale (across North and South America), Fierer and Jackson (2006) suggested that community structure was independent of the geographical distance but was influenced by soil pH. Recently, it has been suggested that an approach based on functional traits within the context of environmental gradients might yield more insights into factors structuring microbial diversity rather than assessing total bacterial communities (McGill *et al.*, 2006; Green *et al.*, 2008). In this context, Philippot and colleagues (2009) used functional genes involved in denitrification as biomarkers to study field-scale spatial distribution pattern of denitrifiers alongside denitrification activity and soil physico-chemical properties.

Although previous studies have characterized methanotroph communities in various landfill cover soils (Wise *et al.*, 1999; Uz *et al.*, 2003; Crossman *et al.*, 2004; Stralis-Pavese *et al.*, 2004; Chen *et al.*, 2007; Gebert *et al.*, 2008) there is a lack of knowledge on the spatiotemporal distribution of methanotrophs in landfill cover soils and the role of environmental heterogeneity on their activity and diversity. Variations in abiotic parameters such as CH_4 and O_2 availability, temperature, pH and nitrogen sources can cause shifts in methanotroph populations (Hanson and Hanson, 1996; Bodelier and Laanbroek, 2004). In this study we used a *pmoA*-based microarray (Bodrossy *et al.*, 2003) targeting *pmoA* from all known methanotrophs except from *Verrucomicrobia*, which as yet have only been found in high temperature, low pH environments (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008), to analyse the spatiotemporal distribution of methanotrophs in a landfill cover soil and also to identify any relationship between methanotroph community structure, methane oxidation potential and abiotic factors, particularly C/N ratio, NH_4^+ and NO_3^-. Variation in spatial methanotroph community structure was studied across five sites (5 m nested square set-up) and three depths sampled at April 2007 (Apr 07) and temporal changes in comparison with three other seasons, September 2006 (Sep 06), June 2007 (Jun 07) and September 2007 (Sep 07).

Results and discussion

Methanotroph community structure – temporal distribution

Seasonal differences in methanotroph community structure were observed, with *pmoA* microarray analysis revealing a high diversity of *pmoA* sequences belonging to both type I and II methanotrophs across different seasons (Figs 1 and 2). Strong hybridization signals detected for *pmoA* probes targeting the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459) across all sampled seasons suggested that the genus *Methylocystis* (a type II methanotroph) might be the dominant methanotroph. Seasonal variations in the relative abundance of type Ia or Ib methanotroph *pmoA* sequences were also observed. Based on the hybridization signals for probes targeting type Ia methanotrophs, Jun 07 samples exhibited a lower diversity of type Ia methanotroph *pmoA* sequences, with only weak signals being detected for probes Mmb562 (*Methylosarcina/Methylomicrobium*) and Mb_C11_403 (a subgroup of *Methylobacter*) compared with other seasons. For probes targeting *pmoA* from the genus *Methylocaldum* (type Ib methanotroph; probes MclT272, MclS402 and Mcl408) strong hybridization signal intensities were detected only with the samples from Apr 07 and Sep 07. Hybridization signals for *pmoA* probes targeting the genus *Methylococcus* (501–375, 501–266 and Mc396) were relatively strong in seasons Apr 07 and Jun 07 compared with the other two seasons. However, signals for probes USCG-225 and USCG-225b targeting *pmoA* sequences from Upland soil cluster Gamma (USCG) (Knief *et al.*, 2003) were restricted only to samples from Apr 07. Sequences related to the novel *pmoA* of *Methylosinus trichosporium* (NMsiT-271) were detected with samples from all seasons with variations in signal intensities (it should be noted that the PCR primer set, A189-mb661 applied, excludes most of the novel *pmoA* copies of type II methanotrophs). Chen and colleagues (2007) reported a similar methanotroph community structure in a landfill soil from Roscommon (Ireland) which was dominated by *Methylobacter*, *Methylosarcina* and *Methylocystis*.

PERMANOVA analysis demonstrated a significant seasonal effect on methanotroph community structure but no significant effect of soil depth. ANOSIM analysis demonstrated a significant difference between temporal samples and the spatial sampling set (Apr 07), supported by multidimensional scaling (MDS) analysis (Fig. 4). Multidimensional scaling plots represent the similarity in methanotroph community structure between samples, with samples having similar community structure clustered together. Sep 06, Jun 07 and Sep 07 were all significantly different from the spatial sampling set from Apr 07 ($R = 0.83$, 0.86, 0.503, respectively, and all $P < 0.01$), while there was no difference between layers ($R = 0.023$, $P = 0.27$). Shrestha and colleagues (2008), using *pmoA* gene and PLFA-SIP analyses, demonstrated that the activity and diversity of methanotrophs fluctuated over time, with different niches for type I and II methanotrophs in a rice field ecosystem. Similar to this study, the authors also

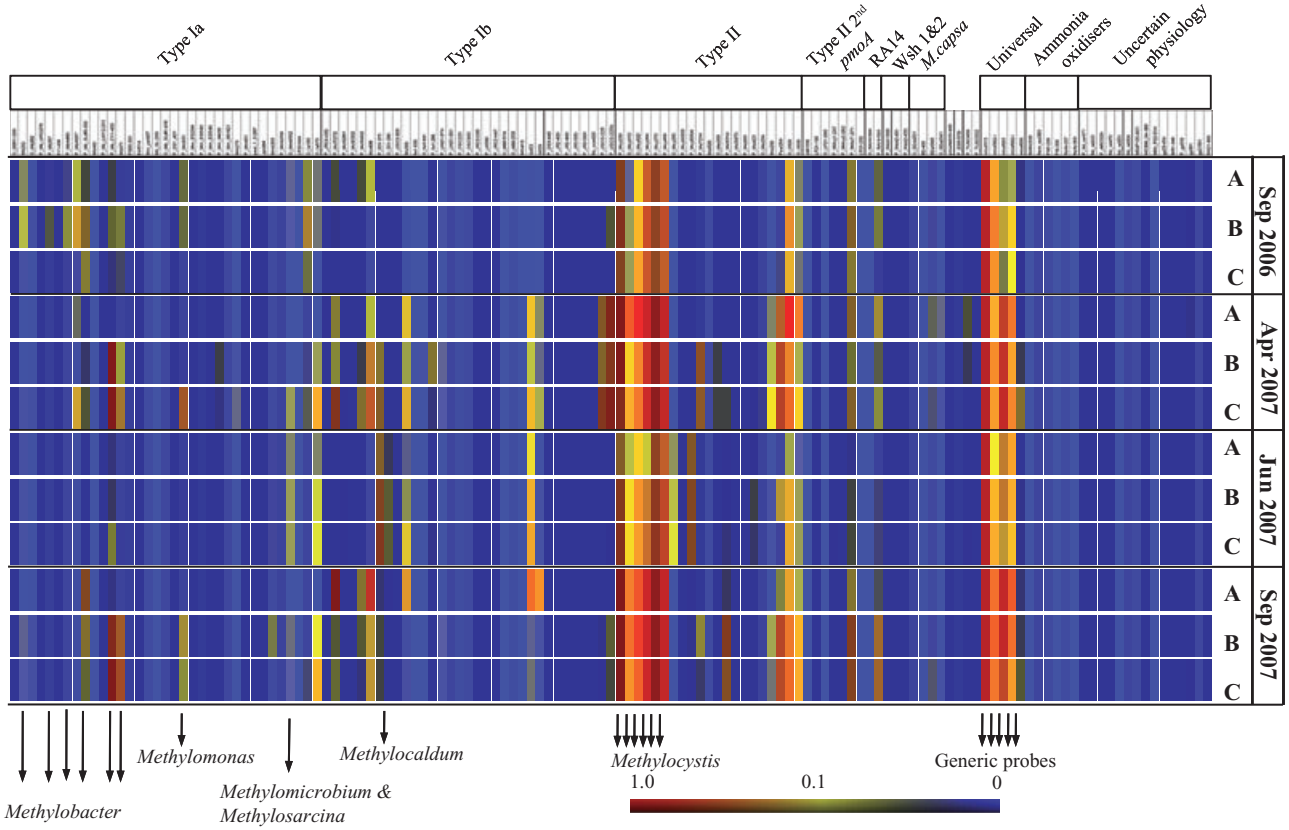

Fig. 1. Microarray analysis representing methanotroph community structure based on hybridization signals for *pmoA* sequences from different seasons. A, B and C refer to 0–10, 10–20 and 20–30 cm soil depth respectively. Month/year represents the time of sampling. Apr 07 samples represent SA, SB and SC samples from the spatial sampling set which was used for soil methane oxidation assays. DNA was extracted from each soil sample in triplicate (Stralis-Pavese *et al.*, 2004). PCR amplification of *pmoA* genes from DNA was performed as described by Héry and colleagues (2008). PCR products from three replicates of DNA samples were pooled for microarray analysis. *In vitro* transcription, RNA purification, microarray hybridization for DNA and scanning of slides were performed according to Bodrossy and colleagues (2003). The colour bar indicates the relative signal intensity with the value 1 indicating maximum signal and 0.1 indicating about 10% hybridization of the total PCR product to the probe.

observed differences in soil methane oxidation capacity between different seasons (Fig. 3). However, the differences in methane oxidation capacity in this study could not be correlated to any changes in methanotroph community structure across different seasons. A low diversity of type Ia methanotrophs was observed at Jun 07, whereas the soil methane oxidation capacity was higher compared with other seasons (Sept 06 and 07), when there was a broader diversity of type Ia methanotrophs. It might be possible that at this season, i.e. Jun 07, either *Methylocystis*, the dominant methanotroph, might have increased in relative abundance over type Ia methanotrophs owing to favourable environmental conditions, or type Ia methanotroph populations might be low in relative abundance and below the detection limit of the microarray. Strong signal intensities for *pmoA* probes targeting the genus *Methylocystis* across all seasons might indicate that either *Methylocystis* are present and are active across different seasons, enduring seasonal changes in the environment, or the population are in a state of dormancy. *Methylocystis* spp. are known to form lipid cysts to survive unfavourable conditions (Whittenbury *et al.*, 1970). Moreover, DNA may be stable in dormant and dead cells (Lindahl, 1993) and along with extracellular DNA, which remains adsorbed to soil particles (Paget *et al.*, 1992), this might contribute to the DNA-based assessment of community structure. Use of mRNA-based analysis yields information on active bacterial transcription at the time of sampling. Owing to the low stability of mRNA, it is a significant challenge to recover intact mRNA (Hurt *et al.*, 2001). Recently, Chen *et al.*, (2007) successfully extracted high-quality mRNA extraction from soil, which enabled analysis of the expression of functional genes (*pmoA* and *mmoX*) encoding subunits of MMO. *In situ* analysis of methanotroph community structure in the future should employ functional gene-based analysis (DNA) alongside mRNA-based approaches to enable detection of methanotrophs that are present and active, respectively.

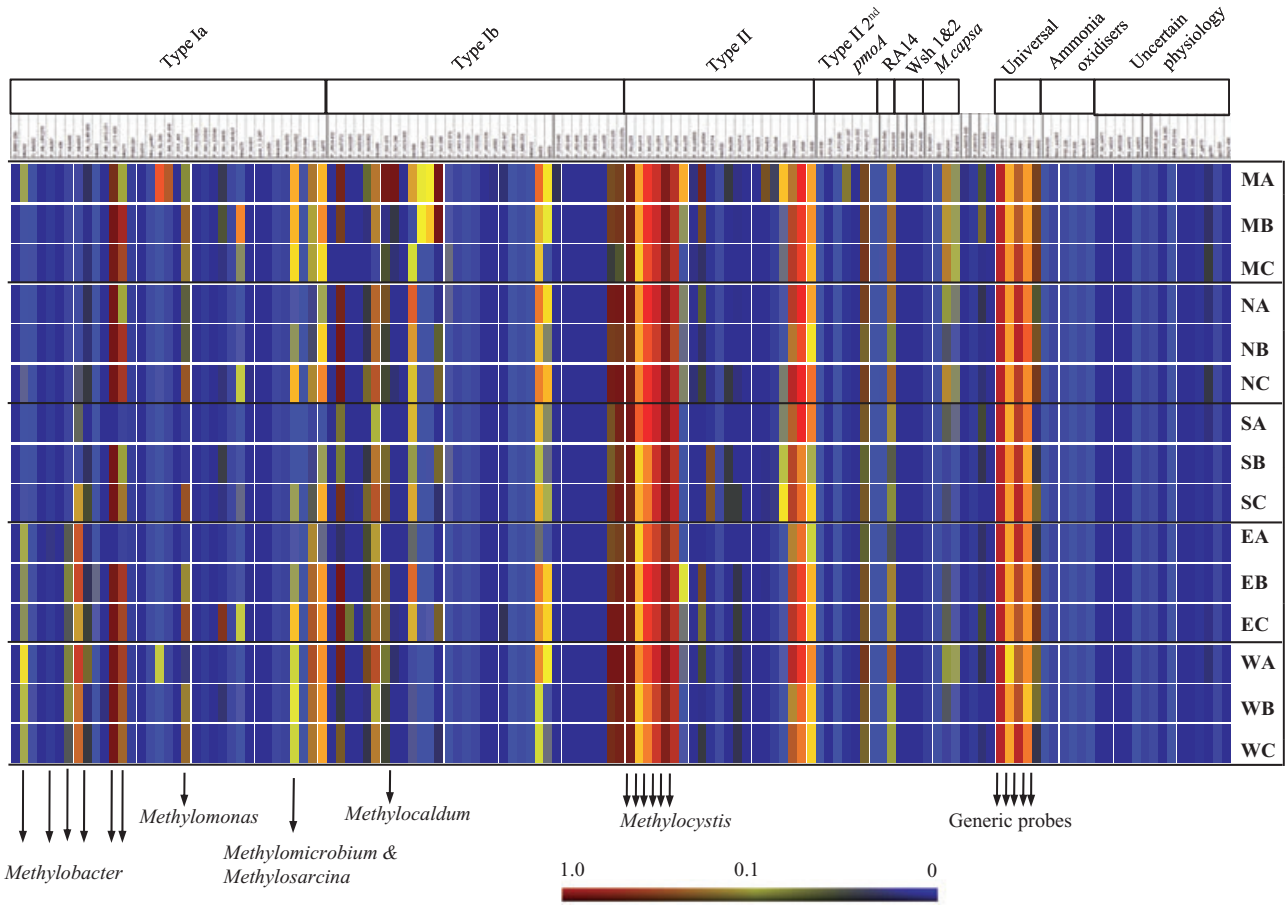

Fig. 2. Microarray analysis representing methanotroph community structure based on hybridization signal patterns for DNA from different spatial locations and soil depths. A, B and C refer to 0–10, 10–20 and 20–30 cm soil depths respectively. Soil samples were collected in a 5 m nested square set-up with M, N, S, E and W representing each sampling location.

Methanotroph community structure – spatial distribution

Spatial patterning of bacterial communities, both vertical and horizontal, has been suggested to exist in soil over different scales (Green and Bohannan, 2006). Soil samples for analysis of spatial methanotroph diversity were collected during the season Apr 07 (Fig. 2). As with results for different seasons with the temporal analysis of methanotroph populations, all samples from the spatial analysis revealed strong signal intensities for *pmoA* probes targeting the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459). In the case of type Ia methanotroph probes, Mb_C11-403, Mb271 (subgroups in genus *Methylobacter*), Mm531 (*Methylomonas*) and Mmb562 (*Methylomicrobium/Methylosarcina*), hybridization signals were detected in all sites with varying signal intensities. The probe Mmb_562 targets both the genera *Methylomicrobium* and *Methylosarcina*, while the probe Mmb_303 targets only the genus *Methylomicrobium*. No hybridization signals were obtained for the probe Mmb_303 suggesting that *pmoA* sequences from the genus *Methylosarcina* contributed to the signal detected from the probe Mmb_562. Hybridization signals for probes Mb292, MbA486 and MbA557 (all probes targeting subgroups in the genus *Methylobacter*) were detected only in samples from site E and W, whereas hybridization signals for probes targeting soda lake *Methylobacter* clones (Mb_SL_299 and Mb_SL#1-418) were detected only in sites M and W, indicating differential distribution of *Methylobacter* subgroups. Hybridization signals for probes targeting the genera *Methylocaldum* (MclT272, Mcl1408, Mcls402) and *Methylococcus* (501–75, Mc396) were detected in all sites with marginal differences in signal intensities. However, hybridization signals for probes fw1-639, fw1-641 and fw1-286 targeting *pmoA* sequences from *Methylococcus-Methylocaldum*-related marine and freshwater sediment clones were only detected at site M. *pmoA* sequences related to peat soil clones (peat264) and the novel *pmoA* copy of *M. trichosporium* (NMsiT-271) were retrieved from all sites, as indicated by the

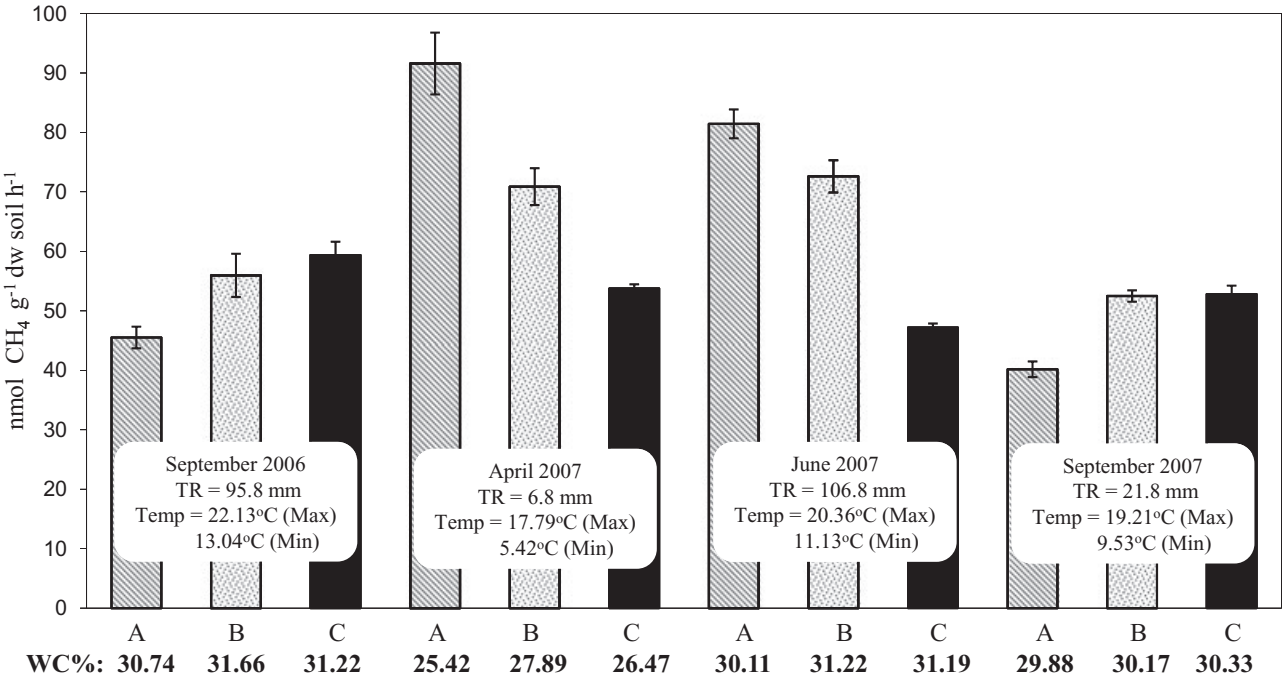

Fig. 3. Graphical representation of the temporal difference in soil methane oxidation potentials. The bars represent the CH_4 oxidation potential. A, B, C represents 0–10, 10–20, 20–30 cm soil depths. The month/year represents the time of soil sampling at landfill. 'TR' refers to total rainfall month^{-1}, whereas 'Temp' refers to average temperature day^{-1} (Max, maximum temperature; Min, minimum temperature). Monthly rainfall and temperature data from September 2006 to September 2007 are listed in Table S1. WC% denotes soil water content (in percentage). Assessment of methane oxidation potential was carried out as described in Héry and colleagues (2008) in triplicate with 10 g of soil subsamples in 120 ml serum vial bottles with a headspace CH_4 concentration of 1% (v/v). CH_4 concentrations were determined by gas chromatography at every 24 h interval with seven sampling points. Error bars represent standard error between three replicates.

hybridization signal for respective *pmoA* probes. Positive signals for the genus *Methylosinus* were in most cases inconclusive because of the lack of supporting signals from other probes of overlapping specificity and/or because of near-cut-off signals. The only exceptions are samples SA and SB with positive signals for *M. trichosporium* (probes MsT214 and Msi269).

Multidimensional scaling analysis of methanotroph community structure in the landfill cover soil represented limited spatial pattern at a 5 m scale (Fig. 4A) and at different soil depth (Fig. 4B). Other studies have used similar scales to assess spatial distribution of bacterial communities (Franklin and Mills, 2003; Ritz *et al*., 2004; Philippot *et al*., 2009). Results have reported both spatially independent (Felske and Akkermans, 1998; Fierer and Jackson, 2006) and dependent (Franklin and Mills, 2003; Ritz *et al*., 2004) microbial community structure. In this study, only minor differences in methanotroph diversity were observed between spatial samples, with no recognizable pattern in the methanotroph community structure (both vertical and horizontal) being observed. Fierer and Jackson (2006) reported that bacterial distribution is controlled by soil pH rather than geographic distance. It might also be possible that the spatial scale used for sampling in this study might not be relevant in discriminating the distribution patterns; there might be difference in spatial distribution at a larger scale or even at the microscale. Grundmann and Debouzie (2000), using a micro-sampling approach, suggested the existence of a spatial dependence for NO_2^- and NH_4^+ at a millimetre scale, which is closer to the bacterial microhabitat. Variations in physical, chemical and biological properties at soil microsites could exert a profound influence on methanotroph diversity and activity and hence future studies should include sampling at the microscale level. Landfill cover soil can be a heterogenous environment. For example, methane emission rates can vary seasonally and spatially (Jones and Nedwell, 1993), with also spatial differences in soil abiotic factors. However, the results from this study indicated a spatially stable methanotroph community structure (at 5 m scale), while there is a shift in the community structure across different seasons in a landfill cover soil. It could be suggested that seasonal variations might have an overruling influence in shaping the methanotroph community structure compared with other factors. In this study, we did not measure methane fluxes in the landfill and cannot correlate the impact of differential spatial and/or seasonal methane emission hot spots with methanotroph community structure in

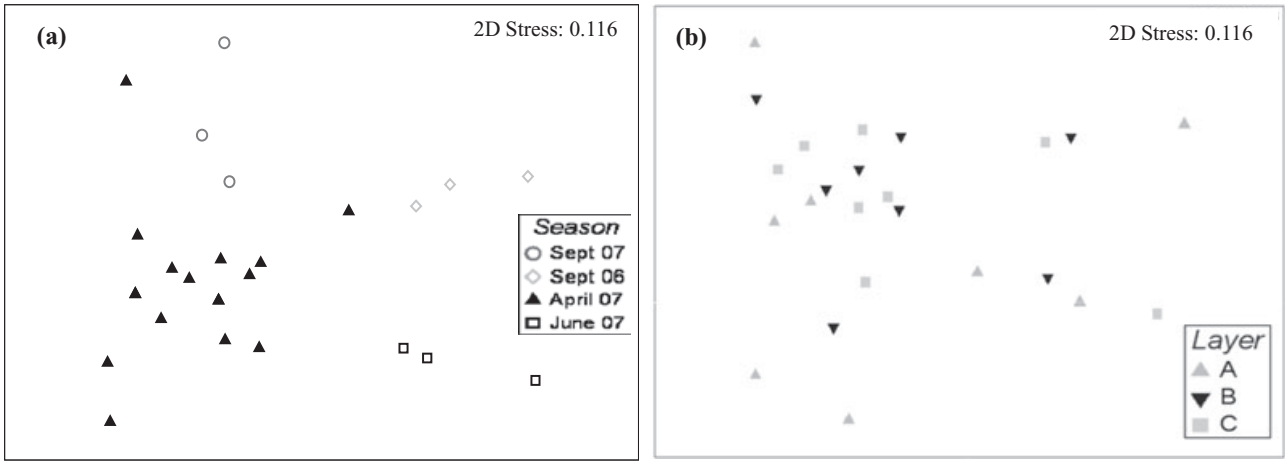

Fig. 4. Non-metric multidimensional scaling plot based on the microarray data for (A) spatial and temporal methanotroph community structure and (B) methanotroph community structure at different soil depths. A, B and C refers to soil depths 0–10, 10–20 and 20–30 cm, respectively. Spatial refers to samples from Apr 07 used for spatial diversity analysis. Microarray data used for statistical analysis comprised all probe signals with the exception of positive controls, universal probes and higher-level probes. No negative values were included in the analysis. The multivariate statistical analyses were employed to test for the effect of depth (five levels), site (five levels) and month (four levels) on methanotroph community changes. The multivariate analyses were conducted using the software Primer 6 (PRIMER-E, Plymouth, UK). Standardized probe intensities were used for all analyses. Bray-Curtis similarity metric was calculated using standardized data for samples representing different sites and months (Kenkel and Orlóci, 1986; Minchin, 1987) and effect of depth, site and season on methanotroph community composition assessed using PERMANOVA and ANOSIM routines. All multivariate statistical tests were tested at $\alpha = 0.05$.

this landfill cover soil. Measurement of methane fluxes in future studies will be essential to identify the hot spots of methane emission to plan future sampling regimes.

Relationship of methanotroph diversity with abiotic factors and CH_4 oxidation potential

Spatial patterning of microbial diversity can be influenced by environmental heterogeneity, with different community structure along an environmental gradient (Green and Bohannan, 2006). Soil abiotic factors (total C, total N, NH_4^+ and NO_3^-) were analysed in all the spatial and temporal soil samples (Fig. S1A and B). The landfill cover soil had a pH of 7.62 and a clay content of 12%. Comparison between different seasons using a one-way analysis of variance (ANOVA) with Tukey *post hoc* test to determine the differences between months revealed significant differences ($P < 0.05\%$) between some abiotic factors. Krave and colleagues (2002) found no significant differences in bacterial community structure over time though there was a seasonal effect on pH, soil moisture and nutrient contents. However, in the case of spatial samples, no significant differences ($P < 0.05\%$) were observed in abiotic factors between sites and different soil depths. Monthly rainfall and daily temperature (maximum and minimum temperature) data for the weather station at Wellesbourne (10 miles from Ufton landfill site) were collected from the British Atmospheric Data Centre (NERC) – MIDAS Land Surface Observation Stations Data (Table S1). Apr 07 recorded the lowest total rainfall month^{-1} with 6.8 mm followed by Sep 07 with 21.8 mm, whereas Sep 06 and Jun 07 records were 95.8 and 106.8 mm respectively.

BEST analysis (a combination of Bio-Env and BVSTEP procedures) were performed using the PRIMER-6 package, to understand correlations between the similarity matrix of probe intensities and a secondary matrix of physical parameters. BEST analysis demonstrated no simple relationships between abiotic parameters and methanotroph community profiles ($R < 0.21$); however, there were significant correlations between individual probe intensities and abiotic parameters (Table S2). Based on product moment correlation analysis, *Methylocystis* probes had a positive correlation with total N, total C and a negative correlation with NH_4^+ (Table S3). For *pmoA* probes targeting the genus *Methylobacter*, no significant correlation pattern was observed with any of the abiotic factors. Type Ib methanotroph genera *Methylocaldum* and *Methylococcus* probe signal intensities had a positive correlation with total N. *pmoA* probe signals for USCG had a positive correlation with total C, C/N ratio, NH_4^+ and NO_3^-, whereas it revealed a negative correlation with total N and water content. However it should be noted that hybridization signals for USCG probes were detected only in season Apr 07.

Comparison of soil methane oxidation potential across different seasons revealed that soil samples from depth 0–10 cm from Apr 2007 exhibited the highest methane oxidation potential compared with other soil samples. In

Apr and Jun 2007, the soil samples from 0–10 cm soil depth exhibited the highest methane oxidation potential followed by soil samples from 10–20 cm and 20–30 cm soil depth. However, during Sep 2006 and 2007, the methane oxidation potential of soil samples from 0–10 cm soil depth exhibited lower oxidation potential compared with other two depths, while little or no differences were observed between 10–20 cm and 20–30 cm soil depth. Analysis of correlation between water content and methane oxidation potential revealed no significant correlation ($P > 0.05$). Methane oxidation potentials observed in this study were about 10-fold lower than previously reported values, ranging from 0.998 to 25 μmol CH_4 g^{-1} dw soil h^{-1} (Nozhevnikova *et al.*, 1993; Kightley *et al.*, 1995; De Visscher *et al.*, 1999; Borjesson *et al.*, 2004). In the case of temporal samples, Jun 07 received the highest total monthly rainfall within the sampling periods (with the preceding month also receiving high total rainfall), with waterlogged conditions observed in the landfill (Table S1). We might expect a lower diffusion of oxygen through soil depths at Jun 07 compared with other seasons. Amaral and Knowles (1995) suggested that type II methanotrophs dominate methane oxidation at low oxygen concentrations, while type I methanotrophs dominate at relatively high oxygen concentrations. Methanotroph community structure at Jun 07 revealed a lower relative abundance of type Ia methanotrophs than type II methanotrophs when compared with other seasons. Interestingly, correlation analysis with methane oxidation potential and *pmoA* probe signal intensities for temporal samples (Table S3) revealed that all probes targeting type Ia methanotroph *pmoA* sequences had a negative correlation with methane oxidation potential. However, for all *pmoA* probes targeting the genus *Methylocystis* (except for the probe Mcy233) along with USCG *pmoA* probes revealed a positive correlation. This result is in contradiction to results obtained in previous studies (Henckel *et al.*, 2000; Bodrossy *et al.*, 2006; Noll *et al.*, 2008), and use of techniques such as a mRNA-based *pmoA* microarray would give us more information on *in situ* activities of different methanotroph communities. No apparent correlation between soil methane oxidation potential with any measured abiotic factors was found, suggesting that a number of interacting mechanisms between methanotrophs and abiotic factors might contribute to methane oxidation activity.

Previous studies have reported correlations between microbial community structure and environmental parameters such as salinity (Crump *et al.*, 2004), depth (Ovreas *et al.*, 1997) and oxygen (Franklin *et al.*, 1999). Type I and type II methanotrophs are known to occupy different niches and heterogeneity in abiotic factors, such as nitrogen and oxygen availability, can influence methanotroph diversity and activity (Graham *et al.*, 1993; Bender and Conrad, 1995; Bodelier *et al.*, 2000; Henckel *et al.*, 2000). In this study, we could not define any single measured factor responsible for driving methanotroph population. Methanotroph activity and diversity in the environment could be influenced by a complex set of interactions with different abiotic parameters and possibly individual methanotroph species respond to one or more different parameters in combination and/or in contrasting ways to the other species. Analysing the impact of abiotic factors on community structure also depends on the taxonomic resolution used in the study. For example, in this study while considering the genus level, hybridization signal patterns for *pmoA* probes targeting the genus *Methylocaldum* did not reveal any consistent pattern with that of the abiotic factors (Table S2). However, at a finer resolution, probes targeting specific species level within the genus *Methylocaldum* such as MclT272 (*M. tepidum*) and MclS402 (*M. szegediense*) revealed some correlation with abiotic parameters. Taxonomic resolution largely depends on the technique used to assess the microbial community structure and due consideration should be given while interpreting the results.

Conclusion

Microbial methane oxidation in the landfill cover soil is a significant sink for methane produced in the landfills. An integrated approach, correlating spatiotemporal distribution of methanotrophs with variations in environmental factors, is vital to design a successful landfill cover soil management strategy. In this study, it was revealed that there was a temporal dynamics in methanotroph community structure, along with seasonal changes in abiotic factors. However, limited spatial patterning (vertical and horizontal) of methanotrophs and abiotic parameters were observed. We compared *pmoA* probes hybridization signal intensity with the measured abiotic factors to determine the driving factors for methanotroph diversity and activity. Although we found some relationship with the probe signals and abiotic factors, the evidence was inconclusive. These results emphasize the fact that methanotrophs cannot be treated as one discrete group of microorganisms when attempting to relate community structure with soil abiotic factors and indeed these factors affect the diversity differently, often in conflicting ways. *In situ* mRNA-based analysis could provide with a better understanding on the role of abiotic factors in altering the diversity of active methanotrophs rather than focusing on methanotrophs present based on DNA analysis. Future studies must also include measurements of a wider range of abiotic factors such as *in situ* O_2 and CH_4 availability and *in situ* experiments, such as the effect of earthworm population density to increase methane oxidation rates

(Héry *et al*., 2008), to understand the role of biotic and abiotic factors affecting methanotrophs activity and diversity.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Graphical representation of abiotic parameters (A) total C, total N and C/N ratio and (B) ammonium and nitrate from spatial and temporal soil samples. Soil parameters were analysed as described by Héry and colleagues (2008).

Univariate analysis of variance was tested at $P < 0.05\%$. For ANOVA, significant factors were then compared using Tukey *post hoc* test. All tests were conducted at $\alpha = 0.05$

Table S1. Rainfall and temperature data for each calendar month from September 2006 to September 2007. These data were collected from the British Atmospheric Data Centre (NERC) – MIDAS Land Surface Observation Stations Data. These data were obtained from the weather station located at Wellesbourne, UK (~10 miles from Ufton landfill site). Data for December 2006 were not available.

Table S2. Analysis of relationship between abiotic parameters and individual probe signal intensities. *pmoA* probe cell colours indicate the different groups of methanotrophs. Correlation between environmental parameters and array probe signals were analysed using Pearsons product moment correlation in the SPSS software package (SPSS, USA). Data that were not normally distributed were transformed (square root or Log). Positive and negative relationships are represented by positive and negative values. The values are arranged in a descending order and matched to their corresponding probes. The colours in the probe columns correspond to specific methanotroph groups. The probability for significance is $P < 0.1\%$.

Table S3. Analysis of correlation between methane oxidation potential and *pmoA* probe signal intensities for methanotrophs temporal samples. *pmoA* probes cell colours indicate different group of methanotrophs.

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