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Earthworm activity in a simulated landfill cover soil shifts the community composition of active methanotrophs

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Abstract

Landfills represent a major source of methane into the atmosphere. In a previous study, we demonstrated that earthworm activity in landfill cover soil can increase soil methane oxidation capacity ~~(Héry et al., 2008)~~. In this study, a simulated landfill cover soil mesocosm (1 m x 0.15 m) was used to observe the influence of earthworms (*Eisenia veneta*) on the active methanotroph community composition, by analyzing the expression of the *pmoA* gene, which is responsible for methane oxidation. mRNA-based *pmoA* microarray analysis revealed that earthworm activity in landfill cover soil stimulated activity of type I methanotrophs (*Methylobacter*, *Methylomonas*, *Methylosarcina* spp.) compared to type II methanotrophs (particularly *Methylocystis* spp.). These results, along with previous studies of methanotrophs in landfill cover soil, can now be used to plan *in situ* field studies to integrate earthworm-induced methanotrophy with other landfill management practises in order to maximize soil methane oxidation and reduce methane emissions from landfills.

Keywords: landfills; earthworms; methanotrophs; *pmoA* microarray

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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 oxidize methane, forming biomass and CO_2. It is estimated that about 22 Tg of methane per year is oxidized in landfill cover soils (Reeburgh, 1996). Methanotrophy, the ability to utilize methane as a sole carbon and energy source, is recognized within two bacterial phyla, *Proteobacteria* and *Verrucomicrobia* (Trotsenko and Murrell, 2008; Op den Camp et al., 2009). Methanotrophic *Proteobacteria* are subdivided into type I and type II methanotrophs belonging to *Gammaproteobacteria* and *Alphaproteobacteria*, respectively. Methanotrophs use the enzyme methane monooxygenase (MMO) to catalyze the oxidation of methane to methanol. There are two types of MMO, a membrane-bound particulate MMO (pMMO) and a soluble MMO (sMMO) (reviewed in Trotsenko and Murrell, 2008; Semrau et al., 2010). *pmoA* (encoding the 27 kDa subunit of pMMO) and *mmoX* (encoding the α-subunit of the hydroxylase of sMMO) along with 16S rRNA genes have been successfully used as functional gene probes for detection of methanotrophs in the environment (reviewed in McDonald et al., 2008). Recently, an mRNA-based *pmoA* microarray method has been developed and applied to assess the distribution of active methanotrophs in the environment (Bodrossy et al., 2006).

Singer et al (2001) first demonstrated a link between earthworm bioturbation and methane depletion in a soil mesocosm study, where the authors reported a methane degradation coefficient of 2.5 x 10^{-4} s^{-1}. ~~In previous microcosm experiments, we~~Henry et al (2008)

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conclusively demonstrated ~~showed~~ that earthworms can mediate an increase in methane oxidation in landfill cover soil (Héry et al., 2008). Héry et al., (2008) used both DNA- and RNA-SIP ~~were used~~ to compare active bacterial communities oxidizing methane in earthworm-incubated and non-incubated landfill cover soils. Based on the results, it was hypothesized that a change in the composition of the active methanotroph population brought about by earthworm activity leads to increased methane oxidation activity.

Here we demonstrate the spatial and temporal shifts in the relative abundance of active methanotrophs brought about by earthworm activity. This was achieved by modifying the experimental system reported by Kightley et al. (1995) who established large scale laboratory soil cores to simulate landfill conditions. These were employed to simulate *in situ* conditions in a landfill such as methane emissions from lower soil profiles and to gather more data before establishing field scale *in situ* landfill trials on cover soils. The stimulatory effects of earthworms on soil methane oxidation were confirmed with previous flask-scale laboratory studies (Héry et al., 2008). Therefore in order to enable replicate soil sub-sampling for detecting active methanotroph populations and methane oxidation rate measurements at different soil depths, single column mesocosms (one each of earthworm-incubated and non-incubated soil column) were used in this experiment. The aims of this study were: (i) to assess the effect of earthworms on soil methane oxidation rates in a simulated landfill cover soil ~~with a larger-scale experimental system than our previous study~~ and (ii) to examine the effect of earthworm activity on the relative abundance of active methanotroph composition using an mRNA-based *pmoA* microarray analysis.

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2. METHODS

2.1 Sampling site and soil collection

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, predominantly grass above the cover soil, was cleared before collecting soil samples. The soil samples were collected to a depth of 30 cm and indigenous earthworms were removed before use avoiding significant perturbation to the soil structure. Soil moisture content at the time of sampling was 27.1 ± 2.2%, which was 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.

2.2 Simulated landfill cover soil

Landfill conditions were simulated by adapting soil columns used by Kightley *et al.* (1995) with minor modifications (Figure 1). Columns (1 m 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 which a sampling needle (0.8 x 40 mm needle; fitted with an airtight valve) was pushed into the column, which allow gas samples to be withdrawn by 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 (approximately 7 kg) of landfill cover soil, placed 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 de-ionized water. About 75 *Eisenia veneta* earthworms (WormsDirect UK, Essex, UK), approximately 53 g, were added to one column ("+ worms") while no earthworms were added to another column ("control"). Earthworm guts were evacuated, as described in Héry et al., (2008) before addition into the columns. No exogenous food source was provided for the earthworms for the duration of the

experiment. Columns were maintained at 20°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. Methane concentration was monitored at regular intervals at different depths of the soil column using a Pye Unicam series 204 gas chromatograph (GC) fitted with a flame ionization detector by withdrawing 0.2 ml of gas and injecting it into the GC. Soil moisture content was monitored throughout the experiment at each soil depth and maintained at *in situ* levels by adding de-ionized water. During the experiment, the landfill gas mixture was added continuously and when methane concentration levels fell below detection limits, the top end-cap was opened to replenish oxygen and also to prevent CO_2 build up.

2.3 Assessment of soil methane oxidation rates

Soil methane oxidation rates in between "+worms" and "control" soil columns were compared at different time intervals; time I (2 weeks after earthworm addition but without CH_4 addition), at 20 cm soil depth and time II (7 weeks after earthworm addition ~~and~~that includes one week of CH_4 exposure) at 10, 20 and 30 cm soil depth. Assessment of methane oxidation rates was carried out in triplicate with 5g of soil sub-samples in 120 ml serum bottles with a headspace methane concentration of 1% (v/v). The rates of methane oxidation were determined by measuring the decrease in headspace CH_4 concentrations at regular intervals by GC analysis.

2.4 Nucleic acid extraction, cDNA synthesis and *pmoA* microarray analysis

Soil samples for molecular biological analysis were collected in triplicate from time I (20 cm depth) and time II sampling (10, 20 and 30 cm depth) and stored at -80°C. Detailed protocols

for nucleic acid extraction, cDNA synthesis, *pmoA* PCR amplification and microarray analysis are provided in the supplementary information (Supplementary Information 1).

3. RESULTS

3.1 Comparison of soil methane oxidation rates

At time I, soil sub-samples (20 cm depth) from the "+worms" soil column exhibited higher CH_4 oxidation rates than "control" soil column (Figure 2a). At time II, soil sub-samples from all three depths (10, 20 and 30 cm) in the "+worms" column exhibited greater CH_4 oxidation rates compared to soil sub-samples from the "control" column (Figure 2b).

3.2 Analysis of methanotroph community composition

PCR products for *pmoA* were obtained with DNA templates from all soil samples and *pmoA* transcripts were detected by RT-PCR from RNA templates from all soil samples. *pmoA*-based microarray hybridization profiles (obtained with both DNA and mRNA) were analyzed to compare methanotroph diversity (DNA) to that of the active methanotrophs (RNA) in both soil columns (Figures 3 and 4).

3.3 DNA-based analysis using the *pmoA* microarray

Similar hybridisation signal patterns were observed between different DNA samples from "+worms" and "control" soil columns (at different soil depths from time I and II). DNA-based hybridisation signals for type Ia methanotroph probes were dominated by *pmoA* affiliated to 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 have contributed to the greater signal intensity with probe Mmb_562 (data not shown). For probes targeting type II methanotrophs, the hybridisation signal was dominated by *pmoA* sequences affiliated to the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459) (Figure 4).

3.4 mRNA-based analysis using the *pmoA* microarray

Analysis based on mRNA encoding *pmoA* (active methanotrophs) revealed a different hybridisation pattern compared to the DNA-based microarray analysis (Figure 4). Pronounced differences in the active methanotroph composition between "control" and "+worms" soil columns were observed at time II. The hybridisation signal pattern with RNA samples for the "control" soil column was similar to that observed with DNA, with strong signal intensities for the probes targeting 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), respectively. However, when profiles of the "+worms" RNA samples at time II were analysed, very weak (20 cm depth) or no (10 and 30 cm depth) hybridisation signals were observed with *pmoA* probes targeting the genus *Methylocystis* (Mcy233, Mcy413, Mcy522, Mcy264, Mcy270 and Mcy459) (Figure 3). These probes exhibited relatively strong signal intensities for "+worms" DNA samples (Figure 4).

Moreover, differences between "+worms" and "control" RNA samples were also observed in the hybridisation pattern with type Ia methanotroph *pmoA* probes in samples taken at time II (Figure 3 and 4). 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 detected in "control" RNA samples. Based on DNA-based analysis at time II, no hybridisation signals were detected for the probe Mb_A557 in either "control" or "+worms" samples (Figure 4). 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 (Figures 3 and 4). 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 by the appearance of a hybridization signal for the other type Ia generic probe Ia193 in the "+worms" RNA samples (Figure 3).

4. DISCUSSION

The bio-turbation activity of earthworm as they move through soil, bring about profound changes to soil microbial community and in particularly their functional diversity. Microcosm experiments at the scale of the laboratory flask are limited by the fact that they cannot recreate earthworm activity in soil. By scaling up to soil cores~~d~~ we were able to improve on previous study (Héry et al., 2008), by producing condition which are more representative of landfill soil covers. Such insight will help generate data on the effect of earthworm activity on soil methane oxidation and active methanotroph populations, which will help direct the design and implementation of field scale trials.

Results from this study confirmed that earthworm activity in soil not only increased the soil methane oxidation capacity but also had a significant impact on the composition of active methanotrophs. The first sampling (Time I) corresponded to the timescale used for soil earthworm incubation in flask-scale microcosm-based studies (Héry et al., 2008). This timescale was selected to confirm that the methane oxidation results were congruent, with a

reproducible greater methane oxidation capacity obtained for earthworm-incubated soil. We observed a lag phase in both "control" and "+worms" sub-samples used for methane oxidation assays. It has been suggested that methanotrophic activity requires a lag phase to recover after rewetting (Scheutz and Kjeldsen, 2004) and was also observed in other studies using this landfill cover soil (Héry et al., 2008; Kumaresan et al., 2009). In the present study, an increase in methane oxidation rate was also observed at time II in the "+worms" soil column. We observed a shift in function, i.e. methane oxidation rates, alongside significant changes in the relative abundance of *pmoA* transcripts from methanotroph populations at time II, with greater 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. Microarray hybridisation signal patterns with DNA and RNA revealed that the methanotrophs present were not necessarily be active, indicating preferences for suitable environmental conditions for their activity.

4.1 Potential interactions between earthworms and methanotrophs

Earthworms provide a constant low supply of nitrogen (N)-containing waste in their casts and burrow linings (Needham, 1957; Buse, 1990). Previous studies have revealed greater nitrate concentrations in earthworm-incubated soils and this was attributed to the stimulation of nitrifiers in the soil (Mulongoy and Bedoret, 1989; Parkin and Berry, 1999; Héry et al., 2008). This additional N availability in the presence of earthworms might relieve N-limitation for cell growth (Bodelier and Laanbroek, 2004) and could be responsible for the greater soil methane oxidation rates observed within the "+worms" column. Moreover, type I methanotrophs are known to be stimulated by the addition of N whilst type II methanotroph activity might dominate under nitrogen-limited conditions as many of them can fix N_2

(Murrell and Dalton, 1983). 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.

Differences in oxygen availability could also play an important role in altering the functional diversity of methanotrophs. 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. Earthworms burrowing activity is known to enhance gas diffusion through soil (Singer et al., 2001). The presence of earthworms in landfill cover soil would certainly increase the diffusion of oxygen through the soil profile. The increased diffusion and availability of oxygen for methanotrophs through earthworm burrows may have also contributed to the increase in methane oxidation rates in the "+worms" column (as observed at time II at all depths). Methanotrophs inhabiting niches created by earthworm burrows may encounter greater oxygen concentrations, stimulating type I methanotrophs and resulting in the increase in the relative abundance of *pmoA* transcripts from type Ia methanotrophs (*Methylomonas*, *Methylobacter* and *Methylosarcina*).

Previous studies have reported that type I methanotrophs respond more rapidly to changes in environmental conditions than type II methanotrophs (Graham et al., 1993; Henckel et al., 2000). In the "+worms" soil column, continuous disturbance by earthworm activity led to continuous disturbance in the prevailing soil environmental conditions. Type Ia methanotrophs (e.g. *Methylobacter, Methylomonas, Methylosarcina*), which can adapt better to a changing environment, might dominate methane oxidation activity under these conditions. The relatively stable "control" soil column, without any disturbance by earthworms, favoured growth of 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. Earthworm density is an important parameter that can affect microbial functions and composition via changes to soil properties. In this study we used 75 earthworms in 0.42m^{-2} (approximately 175 earthworms m^{-2}). Previous studies have reported earthworm densities of 19-103 m^{-2} in a forage plot (Hurisso et al., 2011)and 51-1005 m^{-2} (Piearce and Boone, 1998) on a landfill restoration site amended with papermill biosolids. Future *in situ* studies will have to focus on identifying a sustainable earthworm population density alongside optimal landfill management practises to maximize soil methane oxidation potential.

5. CONCLUSIONS

Understanding the factors influencing methanotroph activity in landfill cover soil is essential to optimize landfill management practises in order to maximize methane oxidation in cover soils and thereby reduce methane emissions from landfills. In accordance with our previous research (Héry et al., 2008), we confirmed on a much larger scale that the presence of earthworms in landfill cover soil stimulates soil methane oxidation rates. We demonstrated that earthworm activity in soil plays a major role in altering the relative abundance of active methanotroph composition, creating more favourable conditions for type Ia methanotrophs. The results indicate that earthworm activity alongside other environmental parameters (Borjesson et al., 2004; Scheutz and Kjeldsen, 2004) can affect methanotrophs activity in landfill cover soil. These data can now be used to plan future *in situ* field scale studies and attempts should be made to integrate earthworm-induced methanotrophy with other landfill management practises to reduce methane emissions from landfills.

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Figure Captions

Figure 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 approximately 53g biomass of earthworms. Landfill gas (60% CH_4 + 40% CO_2) was introduced into the columns from the inlet at the bottom of the soil columns. Soil sub-samples were taken from

soil sampling ports at regular intervals either for assessing methane oxidation rates or for nucleic acid isolation and methanotroph community analysis.

Figure 2 Graphical representation of CH_4 oxidation rates in soil sub-samples (5g) from "control" and "+worms" soil columns. Fig 2a represents CH_4 oxidation rate at time I at 20 cm depth in soil columns. Fig 2b represents CH_4 oxidation rates at time II for depths 10, 20 and 30 cm. C10, C20 and C30 represents soil depths 10, 20 and 30 cm, respectively in the "Control" soil column. W10, W20 and W30 represents soil depths 10, 20 and 30 cm, respectively in the "+worms" column. Error bars represent standard error of three replicates.

Figure 3 Microarray analysis of methanotroph community composition analysis based on RNA extracted from "control" and "+worms" soil samples from different soil depths at time I and II. 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. List of oligonucleotide probe set for pmoA microarray is given in supplementary information (SI 2).

Figure 4 Microarray results representing significant differences in methanotroph community composition with DNA and RNA samples between "control" and "+worms" column (at time II). 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.

Supplementary information

SI 1 Protocols for nucleic acid extraction, cDNA synthesis, *pmoA* PCR amplification and microarray analysis

SI 2 List of oligonucleotide probe set for *pmoA* microarray

Figure 1

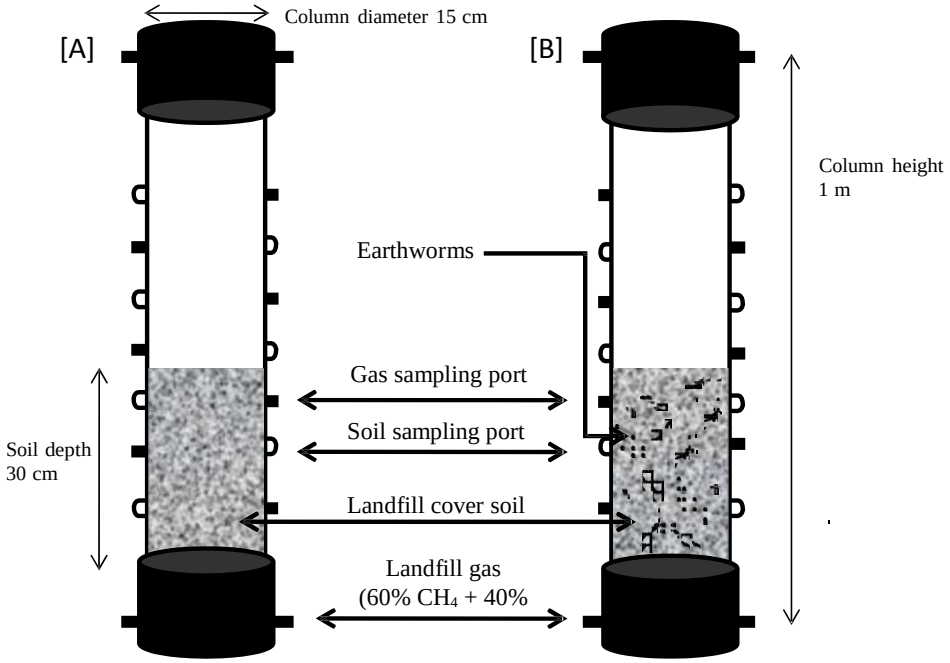


Figure 2

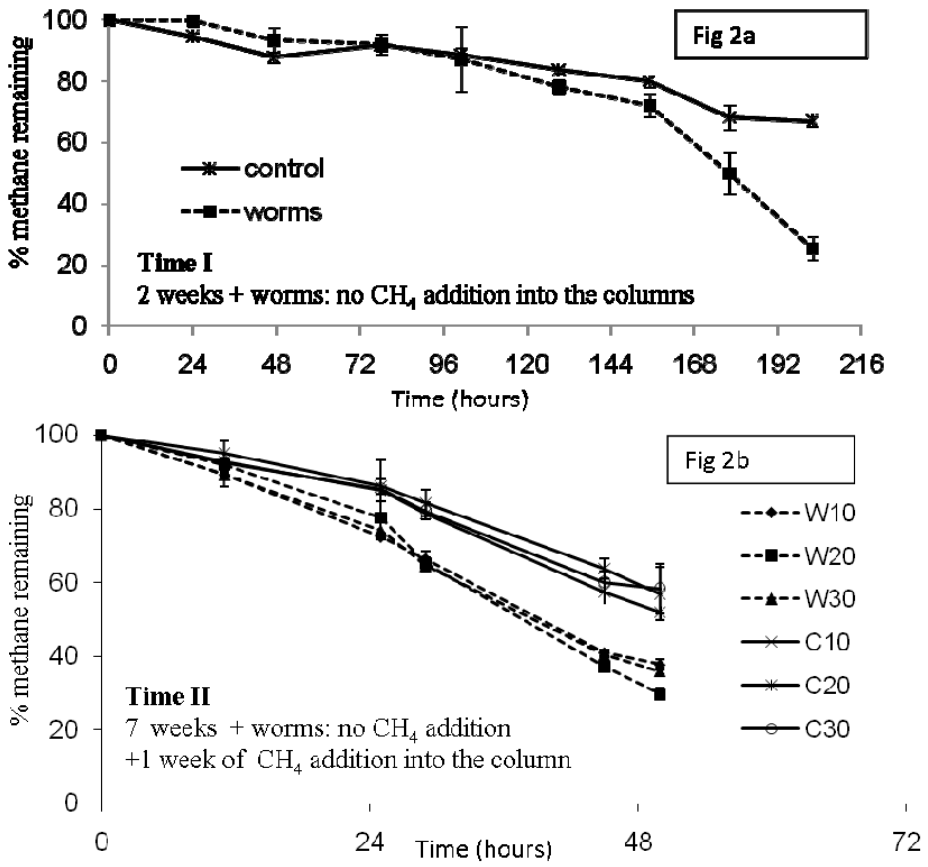


Fig 2a
% methane remaining
100
80
60
40
20
0
control
worms
Time I
2 weeks + worms: no CH_4 addition into the columns
0
24
48
72
96
120
144
168
192
216
Time (hours)
Fig 2b
% methane remaining
W10
W20
W30
C10
C20
C30
Time II
7 weeks + worms: no CH_4 addition
+1 week of CH_4 addition into the column
0
24
48
72
Time (hours)

Figure 3

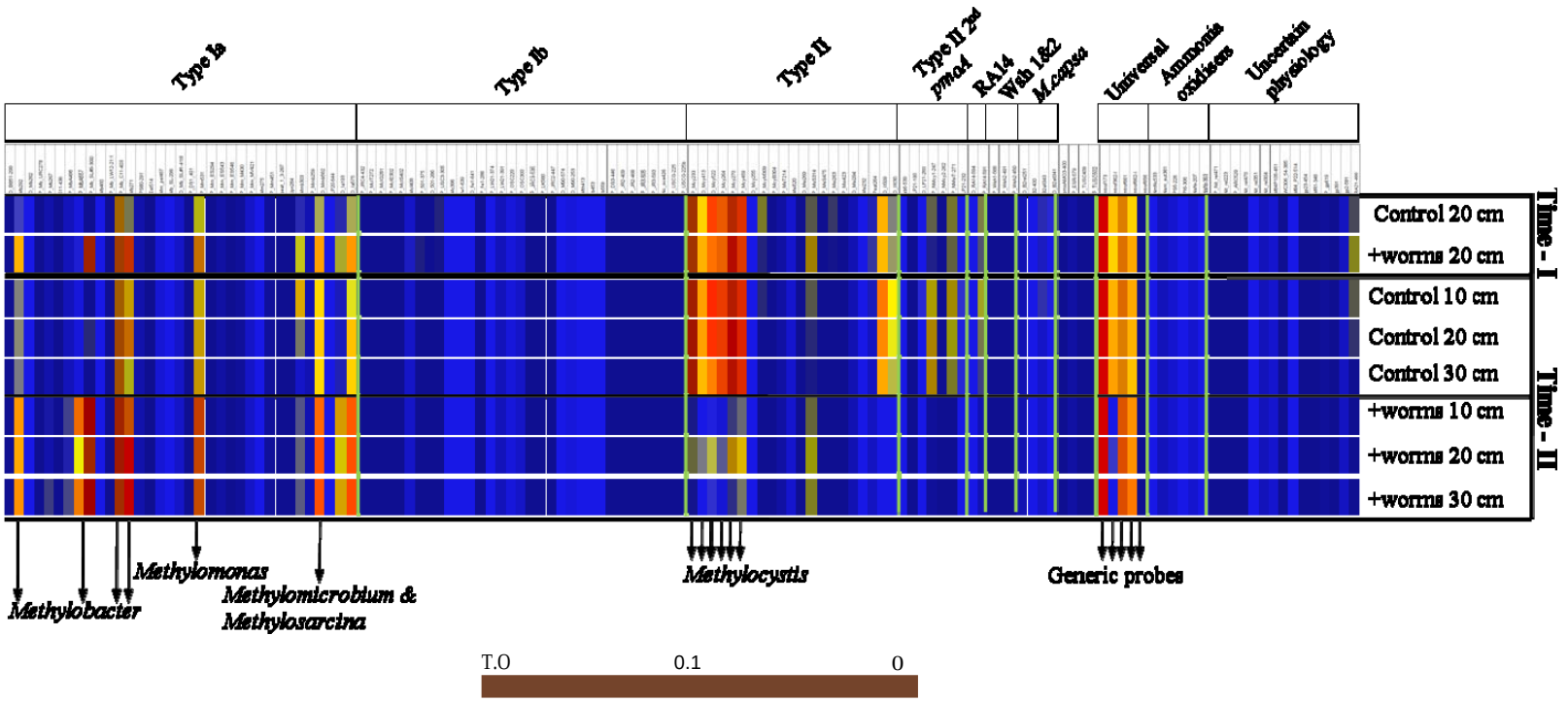
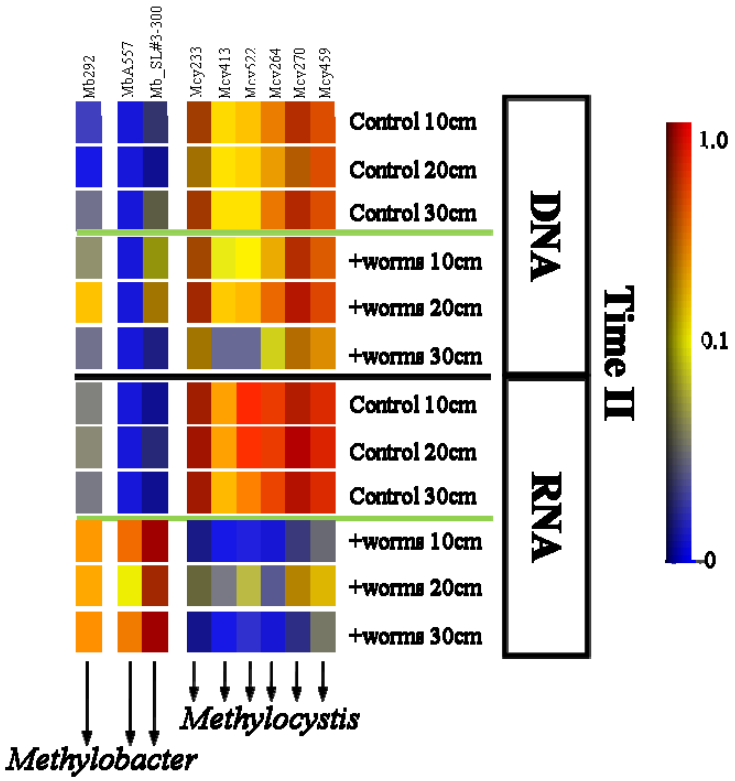

Type Ia
Type Ib
Type II
Type II 2nd pmoA
RA14
Wsh 1&2
M.capsu
Universal
Ammonia oxidisers
Uncertain physiology
Control 20 cm
+worms 20 cm
Control 10 cm
Control 20 cm
Control 30 cm
+worms 10 cm
+worms 20 cm
+worms 30 cm
Time - I
Time - II
Methylomonas
Methylomicrobium & Methylosarcina
Methylobacter
Methylocystis
Generic probes
T.0
0.1
0

Figure 4


Mb292
MbA557
Mb_SL#3-300
Mcy233
Mcy413
Mcy522
Mcy264
Mcy270
Mcy459
Control 10cm
Control 20cm
Control 30cm
+worms 10cm
+worms 20cm
+worms 30cm
Control 10cm
Control 20cm
Control 30cm
+worms 10cm
+worms 20cm
+worms 30cm
DNA
RNA
Time II
1.0
0.1
0
Methylocystis
Methylobacter