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Remarkable Recovery and Colonization Behaviour of Methane Oxidizing Bacteria in Soil After Disturbance Is Controlled by Methane Source Only

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Abstract Little is understood about the relationship between microbial assemblage history, the composition and function of specific functional guilds and the ecosystem functions they provide. To learn more about this relationship we used methane oxidizing bacteria (MOB) as model organisms and performed soil microcosm experiments comprised of identical soil substrates, hosting distinct overall microbial diversities (i.e., full, reduced and zero total microbial and MOB diversities). After inoculation with undisturbed soil, the recovery of MOB activity, MOB diversity and total bacterial diversity were followed over 3 months by methane oxidation potential measurements and analyses targeting *pmoA* and 16S rRNA genes. Measurement of methane oxidation potential demonstrated different recovery rates across the different treatments. Despite different starting microbial diversities, the recovery and succession of the MOB communities followed a similar pattern across the different treatment microcosms. In this study we found that edaphic parameters were the dominant factor shaping microbial communities over time and that the starting microbial community played only a minor role in shaping MOB microbial community

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Introduction

Recent developments in molecular techniques for microbial ecology have enabled the rapid, high-resolution analysis of the phylogenetic and functional diversity of microbial communities. However, due to the complexity of microbial communities and their interactions with the environment, the relationship between microbial diversity and ecosystem function still remains largely uncharacterised [1, 2]. Our study investigated the relationship between the diversity of the total microbial community, the diversity of a selected functional guild, and its corresponding ecosystem function.

The biological mitigation of methane is mediated by microbes, both under anaerobic and aerobic conditions [3]. Aerobic methane oxidizing bacteria (MOB) are ubiquitous in nature and are often studied as model organisms due to their unique characteristics [4]. Aerobic MOB (also called methanotrophs) belong to the Alpha- and the Gamma-Proteobacteria [5] or the Verrucomicrobia [6, 7]. Proteobacterial MOB are traditionally categorized into "type I" and "type II", based on cell morphology and physiology [5]. Type I MOB belong to the Gamma Proteobacteria and can be further divided into type Ia and type Ib according to both their phylogeny as well as their physiological and biochemical characteristics. "Type II" MOB belong to Alpha Proteobacteria. Both types oxidize methane via methanol and formaldehyde to carbon dioxide or assimilate carbon into cell biomass. The first step of the methane oxidation pathway is catalyzed by the enzyme methane monooxygenase. There are two types of this enzyme, a particulate methane monooxygenase (pMMO) and a soluble methane monooxygenase (sMMO). All methanotrophs contain the pMMO, except for the members of the genera *Methylocella*, *Methyloferula* and *Methylocapsa* [5]. The sMMO is found in the above two genera and in some strains of *Methylosinus*,

Methylocapsa, *Methylococcus*, *Methylovolum* and *Methylomonas*. The *pmoA* gene encodes subunit A of pMMO and is often used as a functional marker gene due to the congruence of the *pmoA*- and the 16S rRNA-based phylogenies among MOB [8].

The main factors affecting MOB community composition in soils are methane and oxygen concentrations [5]. These, in turn are strongly influenced by water content and porosity (influencing aeration) and organic matter content and composition (influencing methane production rates in lower layers) [9]. Other factors known to influence MOB community composition include the concentrations of ammonia and nitrate (N supply) [9], bioavailable copper (required for the expression and activity of the pMMO) and iron (required for the expression and activity of the sMMO); temperature, salinity and pH [5]. Based on differences in their physiology, methanotroph clades occupy different environmental niches [5] and react differently to environmental changes. It is therefore important to understand their diversity in order to be able to predict and possibly control methane emissions from various environments.

Competition for space and nutrients as well as prey–predator interactions play important roles in shaping microbial community structures [10]. Two contradicting theories exist on the evolution of microbial communities [11]. One theory states that environmental conditions shape microbial communities, whereby the microbial assembly history has little to no effect on the outcome. According to this theory, different microbial communities converge towards the same final composition if exposed to the same environmental conditions. The other theory states that microbial communities are primarily shaped by their assembly history, with the effect of the environment only having secondary effects. Results have been found supporting both of these contradicting theories [12–14].

The link between community assembly and ecosystem function remains largely unknown. Fukami and Morin [15] observed that the relationship between productivity and biodiversity depends on the community assembly history. Cardinale [16] demonstrated that communities with higher diversity could take advantage of niche partitioning, further supporting the diversity of the community through a capacity to capture a greater proportion of the available nutrients. It has been also reported that bacterial community structures are unrelated to ecosystem functioning due to the presence of generalist species able cope with a wide range of environmental conditions [17].

Soil microbial communities are generally regarded as functionally redundant due to their vast diversity [2]. Structural and functional redundancies relay to a microbial community the ability to maintain functional and structural characteristics in the face of potential environmental changes or perturbations. Debates exist as to whether higher diversity results in more stable community function. A number of studies have suggested that the greater microbial community evenness or diversity may lead to increased functional stability [18, 19]. However, other studies suggest that the nature of the perturbation also influences the interaction between diversity and functional stability [20–22].

The objective of the present study was to determine how different initial microbial assemblages ultimately influence the composition and succession of MOB communities. To exclude the influence of soil characteristics we built microcosms containing identical soil but hosting microbial communities of different starting complexities. We observed MOB as a model functional group over a period of 3 months. The aim was to test the hypothesis that there is a significant interaction between the diversity of the total microbial community and the function of a selected microbial guild, in this case MOB.

Materials and Methods

Sampling Site

Soil samples were collected on 22 October 2009 in the area Ewijkse Waard, the Netherlands (51°88′N, 5°73′E), a river floodplain described in detail earlier [23, 24]. The soil texture was silt–clay–loam, pH was 7.4, and soil moisture content was 29.5 %. The soil was sieved (<2 mm), air-dried, sieved again (<2 mm), and thoroughly homogenised for further treatment.

Microcosm Experiment

Eighty kilograms of thoroughly homogenised dry soil was divided equally into four parts. One aliquot, used as control, was kept at 4 °C without any further treatment ("UN"). Another 20 kg aliquot was sterilized by gamma-irradiation (28 kGy; ^{60}Co) ("GAMMA"); the sterility of the soil was confirmed by lack of PCR amplification of the 16S rRNA gene from a DNA extraction and an absence of colony forming units following plating of a water extract from 2 g soil on LB agar. The remaining 40 kg of soil was treated by acetylene ("ACET"), and then divided into two 20-kg aliquots, one of which was stored at 4 °C until further use, the other one was also subjected to kanamycin treatment ("KAN"). For the acetylene treatment, 40 kg of dried and sieved soil was divided into two aliquots of 20 kg and each placed into a 20-l plastic bag with 5 l headspace. Each bag was flushed with 5 l acetylene (≥99.6 % pure, Linde Gas, Austria) and incubated for 2 days. After 2 days, soil aliquots were aerated for 8 h, and then flushed with 5 l acetylene again. The same treatment was repeated twice. At the end of the treatment, the two 20-kg aliquots were pooled and thoroughly mixed. No methane oxidation was detected in the acetylene treated soil, even after allowing 7 days for recovery and re-

growth of surviving MOB. For kanamycin treatment, 1 l of 10 mg/l kanamycin solution (10 mg kanamycin was dissolved in 1 ml DMSO, and then diluted into 1 l of H_2O) was added to the 20 kg dried soil aliquot by spraying and mixing, resulting in a final concentration of 0.5 mg kanamycin/g soil. Soil was incubated for 13 days at room temperature and the change in the microbial community composition was checked via bacterial 16S rRNA gene-based T-RFLP analysis as described below.

Microcosms were built from two plastic boxes, each 60×45×25 cm in size. The top box contained the soil, while the lower part served as a reservoir for methane to diffuse into the soil through the perforation applied to the bottom of the top part. The bottom box (connected to the top box via an airtight seal) was flushed with a gas mixture of 20 % (v/v) methane and 80 % (v/v) N_2 at a gas flow rate of 70 ml/min. The methane concentration measured at the soil surface was around 1 % (v/v) throughout the experiment. Four microcosms were constructed and filled with each of the 20 kg aliquots of soil (representing four different treatments). Microcosms were kept in a temperature regulated room (20–25 °C) with natural lighting. Approximately 200–250 ml water was sprayed onto each microcosm every day to keep the moisture in the range of 35±3 % (w/w). The soil in the microcosms was approximately 20 cm deep. Figure S1 illustrates the experimental setup.

At the start of the experiment, before creating the microcosms, 10 g soil was sampled from each treatment (time point 0). As soon as microcosms were created and filled with treated soil, each microcosm was inoculated by applying 1 kg untreated soil in a thin layer on top of the treated soil. For all subsequent sampling times, three cores (2.5 cm in diameter, 20 cm in length) were collected from each microcosm. The soil cores were divided into four layers (5 cm each; layer A, 0–5 cm; layer B, 5–10 cm; layer C, 10–15 cm; layer D, 15–20 cm). Individual layers of the three replicate cores were pooled, homogenized, directly followed by DNA extraction and further analyses. Microcosms were sampled seven times, at days 0, 7, 26, 41, 67, 91 and 105 (corresponding to time points t0, t1, t2, t3, t4, t5 and t6).

Methane Oxidation Assay

The assay was performed as described earlier [25] with modifications. Each assay was performed in triplicate. In short, for each incubation, 1 g soil was suspended in 10 ml MilliQ water (Millipore) in a 150-ml flask capped with a rubber stopper. Pure methane (1.4 ml, representing 10,000 ppmv headspace concentration) was added to the bottle. Slurries were incubated in the dark at room temperature on a shaker (100 rpm). The methane concentration in the headspace was measured by GC-FID analysis (Fisons HR 8060). Activity was calculated by considering results at 0 and 24 h.

DNA Extraction

DNA extraction was performed as described earlier using a modification of a method based on the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA) [39]. DNA concentrations were estimated using a NanoDrop spectrophotometer (Thermo Scientific, Delaware, USA).

Diagnostic *pmoA* Microarray Analysis

PCR amplification of the *pmoA* gene was based on a two-step semi-nested protocol. The first step PCR comprised 25 μl of 2×Premix F (Epicentre Biotechnologies, Madison, WI, USA), 25 pmol of primer A-189f and A-682r (Holmes et al. 1995) each, 1 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), 50 ng of genomic DNA as template, in a total volume of 50 μl. PCR was performed using a touchdown protocol: 5 min at 94°C; 25 cycles of 1 min at 94°C, 1 min at the annealing temperature and 1 min at 72°C; final 10 min at 72°C. The annealing temperature was lowered from 62°C to 52°C over the first 11 cycles after which it was maintained for further 24 cycles at 52°C. Then, 5 μl of 1/100 diluted PCR product from the first step was used as template in a subsequent nested amplification with primers A-189f and T7-661r. The second step PCR was performed using the protocol: by 5 min at 94°C; 25 cycles of1 min at 94°C, 1 min at the annealing temperature and 1 min at 72°C; final 10 min at 72°C. The annealing temperature was lowered from 62°C to 52°C over the first 11 cycles after which it was maintained for further 14 cycles at 52°C.

The resulting PCR product was used to generate fragmented, Cy3 labelled RNA target via in vitro transcription and the target was used for hybridization on *pmoA* microarray slides as described earlier [39]. Hybridized slides were scanned at 10 μm resolution with a GenePix 4000 laser scanner (Axon, Foster City, CA, USA) at wavelength of 532 nm. Florescent images analysed with the GenePix software (Axon). Microsoft Excel was used for statistical analysis and presentation of results.

Hybridization between a probe and a target was considered in analyses if the signal was at least 1.5 % of the strongest signal obtained for that probe with the validation set of reference strains/clones (for details, see Bodrossy et al. [26]).

pmoA-Based Quantitative PCR (qPCR) Assay

qPCR assays were performed using a method based on [27], carried out as shown in Table 1. Each assay was performed in duplicate. Plasmid DNA from cloned *pmoA* was used as to generate a standard curve of known copy number in the assays. qPCR was performed in 25 μl volumes using 96-well PCR plates (VWR GmbH, Vienna, Austria) with the optical cover (AB). PCR master mix contained 12.5 μl iQ

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

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

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

SYBR Green Supermix (Bio-Rad), 1 μl 5 ng/μl DNA template and 2.5 μl of 10 μM each forward and reverse primers. DNase and RNase-free water were added to a final volume of 25 μl.

16S rRNA Gene-Based T-RFLP Assay

Bacterial 16S rRNA genes were amplified with the FAM-labeled forward primer 8fM (5′-AGAGTTTGATCMTGGC TCAG-3′) and the reverse primer 1406r (5′-ACGGGCGG TGTGT(AG)C-3′) [28]. Each 50 μl reaction mixture contained 25 μl 2× Premix F (Epicentre Biotechnologies), 20 pmol of each primer, and 1 U *Taq* polymerase (Invitrogen), 50 ng of genomic DNA as template. Cycling conditions were as follows: an initial denaturation of 5 min at 94°C; followed by 35 cycles of 1 min of denaturation at 94 °C, 45 s at 55 °C, 1 min at 72 °C; and a final extension at 72 °C for 10 min.

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

Statistical Analysis

T-RFLP profiles were binned using the macro Treeflap in Microsoft Excel (http://www.sci.monash.edu.au/wsc/staff/walsh/treeflap.xls). T-RF standardization was performed as described in [29]. T-RFLP patterns were regarded as total bacterial community profiles. MOB community (derived from microarray analyses) and total microbial community structures were analysed using Permanova, PERMDISP and Canonical analysis of principal coordinates (CAP) analysis, using the Primer PERMANOVA+software package (Primer-E, Plymouth, UK). The correlation between MOB communities and methane oxidation potential was analysed using a linear model in R v. 2.12.2 (R Development Core Team 2011) using the vegan v. 1.17-8 package. qPCR data was log transformed to meet the assumption of normality.

Univariate analysis of differences in MOB abundance (qPCR analysis of type Ia, type Ib and type II) as well as MO_x was performed using ANOVA. Relationships between gene abundance, diversity and MO_x variables were determined using spearman correlation. All univariate statistical analyses were performed in R v. 2.12.2 (R Development Core Team 2011) (at α=0.05)

Results

Analysis of Treatments

Soil used in the microcosms was subject to four different treatments as detailed below. UN denotes untreated, representing full diversity and functionality; ACET denotes acetylene-treated, representing full reduction of methane oxidation functionality, a collateral, potentially full reduction of ammonia oxidation functionality but limited effect on the overall microbial diversity; KAN denotes acetylene and kanamycin treated, representing full reduction of methane oxidation and potentially of ammonia oxidation functionality and partial reduction of overall microbial diversity; and GAMMA denotes gamma irradiated, representing full reduction of both methane oxidation functionality and overall microbial diversity (Fig. S1). Prior to the specific treatments, soil was sieved, dried and sieved again (while this treatment itself introduced considerable perturbation to the microbial community, it was necessary to achieve a homogenous, reproducible

experimental system). Following acetylene treatment, no methane oxidation was detected after 6 days of incubation to allow for the recovery of any reversibly inactivated MOB under a constant methane stream. Thirteen days following kanamycin treatment, the number of TRFs detected was reduced to 18±3 compared to 26±2 detected in the untreated soil. The number of cultivable colonies on LB agar plates was reduced from $1.95\pm0.25\times10^5$ to $7.5\pm0.25\times10^5$ cfu/g soil. Plating of soil treated by gamma-irradiation on LB agar plates and incubating at 30°C for 48 h revealed no colonies.

Methane Oxidation Potential

Potential 24-h methane oxidation (MO_x) recovered at different rates in the four different treatment microcosms are displayed in (Fig. 1). MO_x potentials varied between the different soil layers in each of the treatments. The maximum MO_x potential was detected in the upper soil layers (0–10 cm, layers A and B) in all treatments over the first 41 days with the exception of the untreated (control) samples. It must be noted that the top 5-cm layer (layer A) included an approximately 0.3 cm inoculum of untreated (control) soil in all four treatments. In the second half of the experiment (from 67 days onwards) the bottom layers (D), closest to the CH_4 source, showed the highest MO_x potentials in all treatments. MO_x potentials in treatments ACET and KAN reached almost the same level as the untreated samples by day 26, whilst MO_x potentials in the GAMMA treatment matched the untreated samples only by 67 days. There was a significant interactive effect of time and treatment on MO_x (ANOVA $F=3.871$; $p<0.0001$), whereby a generally slower increase of MO_x was observed in the GAMMA compared to the other three treatments (Fig. 1).

MOB Abundance

Abundance of MOB was inferred from *pmoA* gene copy numbers as assessed by qPCR analysis and indicated that type II MOB were generally the most abundant of the MOB communities in all treatments, closely followed by Type Ia (mean values of 8.7×10^3–8.0×10^9 and 8.7×10^3–1.2×10^9 *pmoA* copies g^{-1} wet soil, respectively) (Fig. 2). Abundance of type Ib MOB were lower (mean abundance ranged from 3.0×10^3 to 2.6×10^8 copies g^{-1} wet soil). Type Ia MOB increased by 1 to 2 orders of magnitude by day 7 in all layers across all four microcosms. The abundance of both, type Ib and type II MOB, increased by day 7, but to a lesser and more variable degree. The ratios between the abundances of the three major clades of MOB (Type Ia, Type Ib and Type II) varied substantially. In particular, at day 41, the MOB ratio between II:Ib was the highest (193–14,164) during the time course of the experiment. At this time point type II MOB reached the highest abundance and type Ib MOB reached the lowest abundance, whilst MO_x potentials were the lowest in three out of four treatments (UN, ACET and KAN). There was a significant effect of treatment for type Ia and type II abundance (ANOVA $F=3.19$ and 3.965; $p=0.0286$ and 0.00169, respectively) and of time for type Ia abundance ($F=4.301$; $p<0.001$). There was an interaction between time and treatment for type Ia abundance as well (ANOVA $F=2.192$; $p=0.0101$), whereby the increase in type Ia abundance was generally more pronounced for the GAMMA treatment compared to the others (Fig. 2a). Apart from the above, there was no significant effect found for any other combinations of factors and MOB abundances.

MOB Community Structure

Microarray analysis was carried out using a semi-nested PCR approach [30], effectively excluding ammonia oxidisers and deep branching novel/unknown *pmoA*/*amoA* sequences from the analysis (Fig. S2 and Fig. 3). Microarray results demonstrated positive signals for probes targeting both type I (types Ia and Ib) and type II MOB (Fig. 3) in all treatments (see Fig. S2 for detailed information on probe specificities). Strongest signals were associated with probes targeting type II MOB, which indicated that MOB communities were

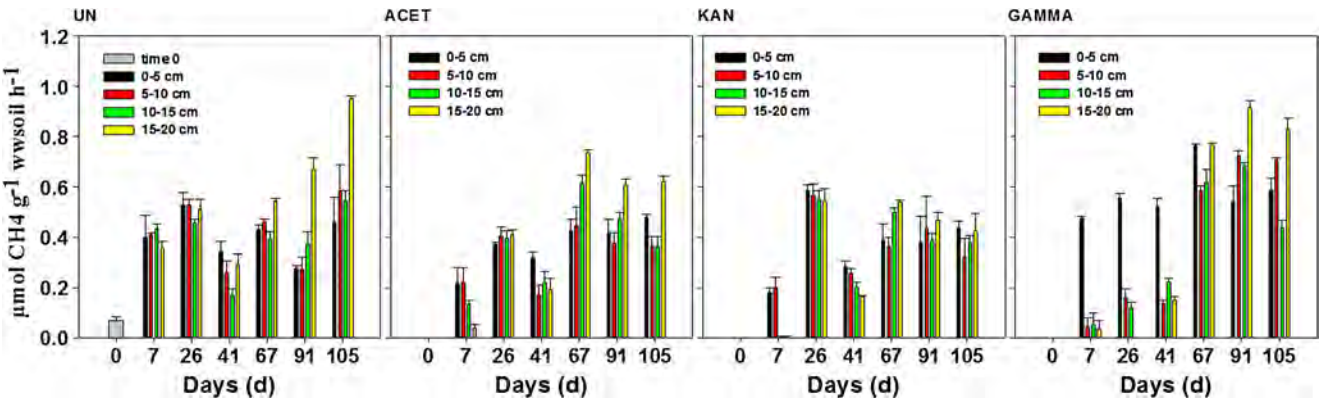

Fig. 1 Methane oxidation (MO_x) potentials under different treatments. Results represent the average of three replicate assays. *UN* denotes the methane oxidation potential of the untreated soil before the start of the experiment. Error bars represent standard error between replicates

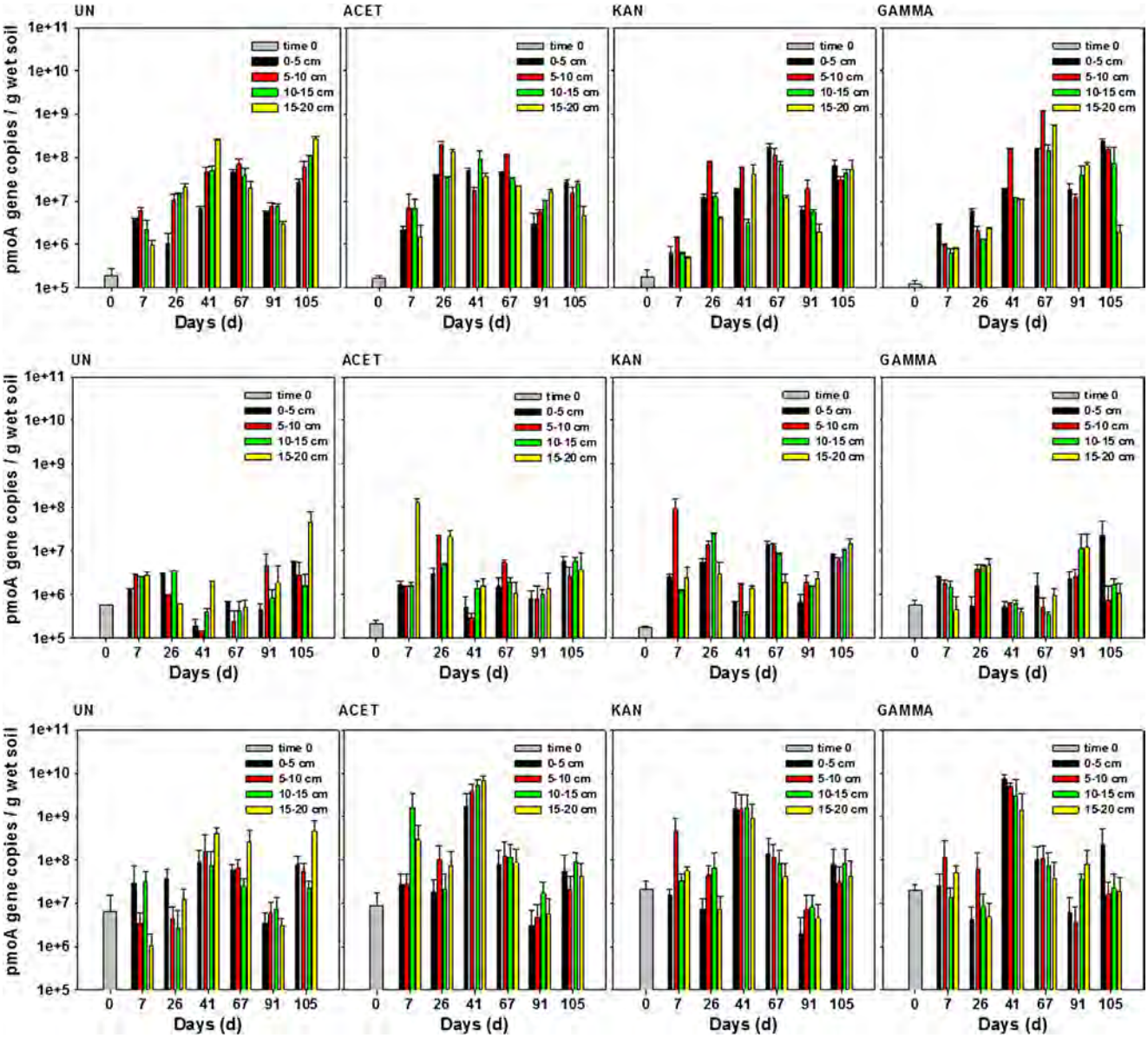

Fig. 2 *pmoA* gene copy numbers of type Ia (**a**), type Ib (**b**) and type II (**c**) MOB under different treatments. "UN", "ACET", "KAN" and "GAMMA" represent treatments A, B, C and D soils at day 0. "Layers" correspond to depth and "Days" represent time since the establishment and inoculation of microcosms

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

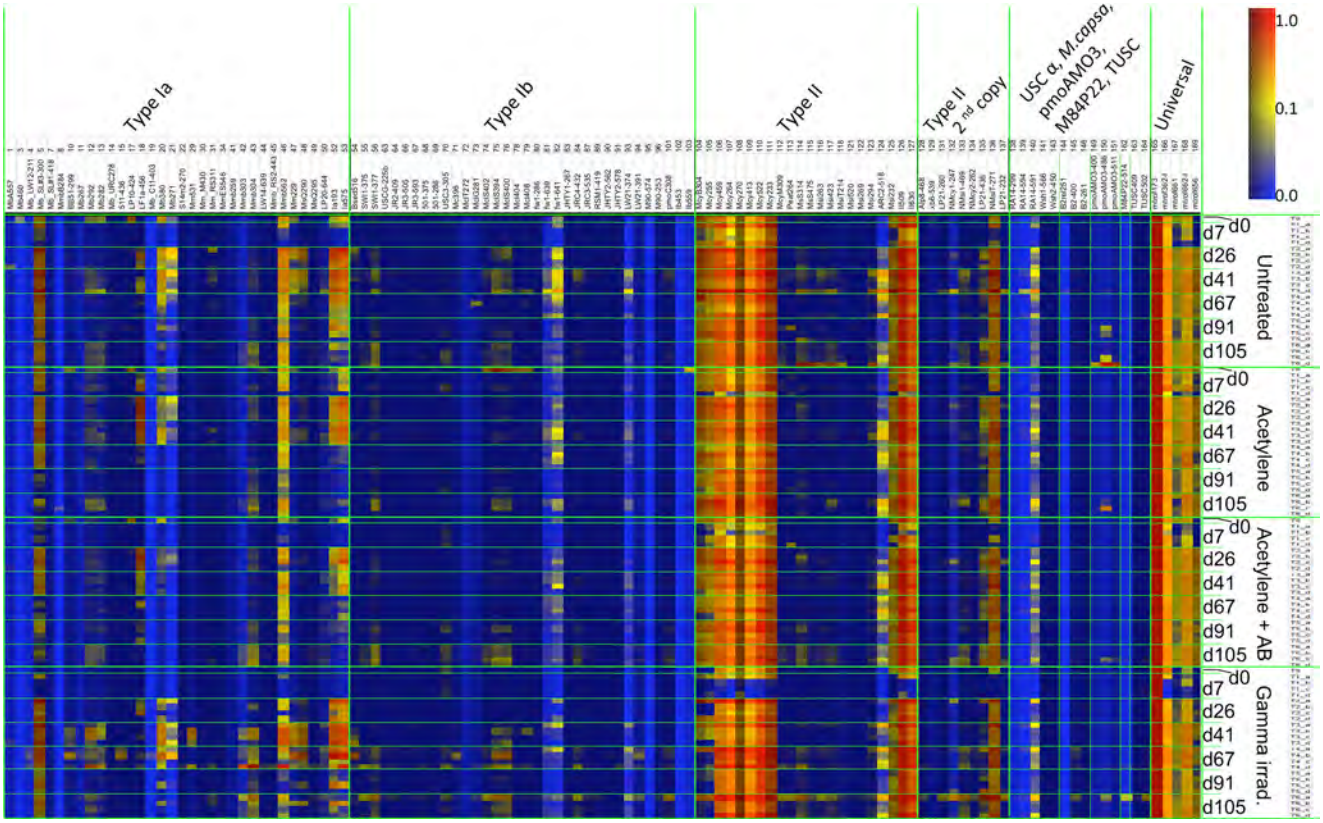

Fig. 3 Microarray analysis of the MOB community composition. t0–t7 denote sampling dates (*t0* day 0, *t1* day 7, *t2* day 26, *t3* day 41, *t4* day 71, *t5* day 91, *t6* day 105). Results for t0 represent the community in the homogenised soil used to fill the microcosms. For t1–t6, separate results are shown for four different layers of 5 cm depth each (*top layer* shown on top of corresponding section of the figure). Results of individual microarray experiments were first normalized to the positive universal methanotroph control probe mtrof173, then to the reference values determined for each individual probe (Bodrossy et al. 2003). Results are represented as a heatmap; a value of 1.0 (see colour code bar) indicates maximum achievable signal for an individual probe, whereas a value of 0.1 indicates 10 % of that. Only probes with signals higher than 1.5 % of their reference values are shown. Numbers above probe names correspond to numbers in Table S2

The MOB community in the GAMMA treatment showed more temporal variation than the other treatments. This was indicated by the variability in strong signals for different type Ia MOB (probes BB51-299, Mb267, Mb292; Mmb303, Mmb304) and by the fade in signals for *Methylocystis* clade B from all the deeper layers (probes McyB304 and Mcy255) during the experiment.

PERMANOVA and canonical analysis of principal coordinates (CAP) analysis demonstrated a significant effect of time on MOB community structure (Fig. 4a). PERMANOVA analysis showed an effect of soil layer on MOB community structure when regarding the layer nested within the treatment factor, but no treatments effect when regarding time and layer factors nested within treatment factor (Table 2). Probes SWI1-377 (targeting marine sediment related type I clones); LP20-644 (targeting *M. microbium* related clones); fw1-641, fw1-639 and LW21-374 (targeting uncultivated type Ib clades); Mcy522, Mcy264, Mcy413, ARC2-518, NMsiT-271 and NMsi1-469 (targeting Methylocystis, a deep branching type II clade and novel *pmoA* copies of type II methanorophs) distinguished different sampling times (Pearson correlation greater than 0.6) (see Table S2 for detailed information on probe specificities). Mcy522, Mcy264, Mcy413, NMsiT-271 and ARC2-518 had weaker signals at day 0 and day 7 in all treatments. The signal of probe fw1-641 was weaker at day 7 in the UN, KAN and GAMMA treatment, at day 91 in the KAN and GAMMA, and there was no positive signal in treatment D at day 0. The signals of probe fw1-639 were stronger in all treatments at days 26, 41, 67 and 105. Probe LW21-374 signals were stronger at days 41, 67 and 105 in all four microcosms. Probe signals of SWI1-377 were weaker in control soil at day 7 and day 67, were weaker in treatment ACET soil at days 7, 41 and 67, in treatment C soil were weaker at day 7 and day 67, in treatment D soil were weaker at days 0, 7, 26 and 41. LP20-644 signals were stronger in UN, KAN and GAMMA treatment at day 41 and day 105, whereas in treatment ACET the signals were only stronger at day 41.

Total Microbial Community Structure

From several samples 16S rRNA gene PCR products were not obtained after repeated attempts including samples

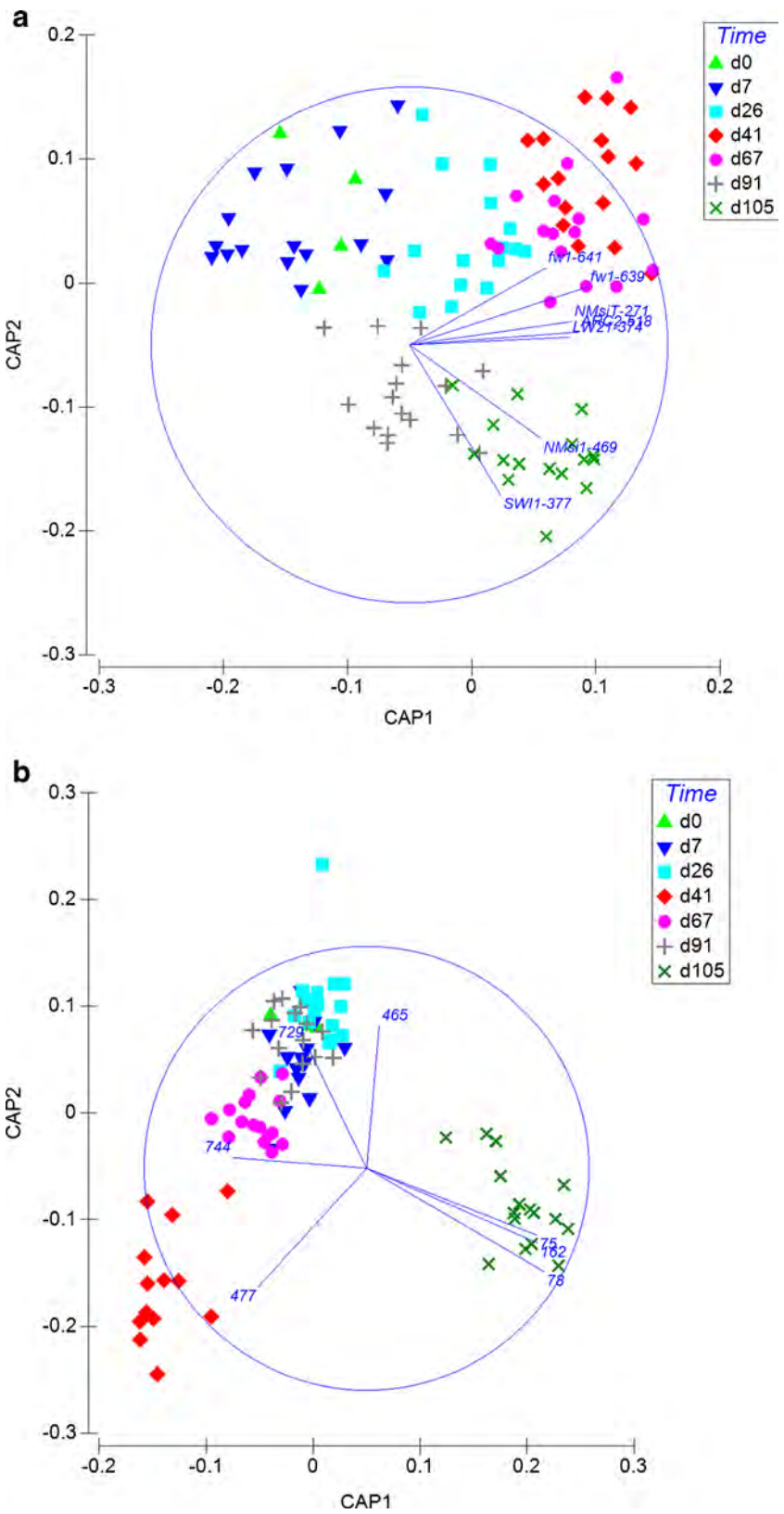

Fig. 4 Canonical analysis of principal coordinates (*CAP*) analyses of methanotroph and total microbial community compositions. **a** CAP analysis of *pmoA* microarray data performed using the Primer 6 package (Primer-E, UK) on standardized array data excluding universal and control probes, as well as probes never showing signals higher than 1.5 % of reference values. Microarray probes are regarded as variables. Probes shown on the graph indicate the correlation is larger than 0.6 using Pearson correlation test. The *graph* highlights the difference in MOB community structure between seven different time points. Leave one out Cross Validation [31] gave 81 % correct assignment to time groups. **b** CAP analysis of total microbial community composition performed using the Primer 6 package (Primer-E, UK) on standardized T-RFLP data. T-RFLP peaks are regarded as variables, *highlighted number*s indicate the correlation is larger than 0.6 using Pearson correlation test. Highlighting the difference in total microbial community structure between seven different time points. Leave one out Cross Validation [31] gave 95 % correct assignment to time groups

from GAMMA at day 0 and day 41, from ACET at day 41 and from UN at day 7. While many T-RF peaks were present in all four treatments, several unique peaks were found in various treatments (Table S1). In particular, the GAMMA treatment showed a large number of unique peaks.

Table 2 PERMANOVA test on the microarray data and T-RFLP data after standardisation (square root)

Source	Pseudo-F	CV (√)	*p*
(a) Microarray data (MOB community)			
Tr	1.20	4.00	0.267
Ti (Tr)	7.19	19.91	0.001
La (Tr)	1.53	4.45	0.011
(b) T-RFLP data (total microbial community)			
Tr	1.65	12.28	0.014
Ti (Tr)	6.27	30.85	0.001
La (Tr)	2.30	11.98	0.011

Only probes with a signal above 1.5 % of reference value at least in one sample were kept. Treatments were regarded as fixed factors. Time and layer factors were nested in treatment factors. Number of permutations was 999. The permutation method uses unrestricted permutation of raw data. Sum of squares type was type III (partial). Monte Carlo test was applied in this test. Significant effects ($p<0.05$) are shaded. For microarray data, universal and control probes were excluded from the analysis. Only probes with a signal above 1.5 % of reference value at least in one sample were kept

Tr refers to treatment; *Ti* refers to time; *La* refers to layer

PERMANOVA analysis demonstrated significant treatment, time and layer effects on the whole microbial community structures when time and layer factors were nested within treatment (Table 2). CAP analysis (Fig. 4b) also showed a clear time effect on total microbial community structure. PERMDISP analysis on the total microbial community structures showed that in the GAMMA treatment the mean Bray–Curtis distance of the individual samples to the centroid was the largest amongst the treatments (49.4 in treatment GAMMA versus 30.3, 31.8 and 34.1 in treatments UN, ACET and KAN, respectively). No relationship was found between total microbial community and MOB community structure.

Relationship Between Microbial Communities and MO_x Potentials

No significant correlation was found between MO_x potentials and MOB or whole bacterial community composition in the control treatment. In treatment KAN, MO_x was correlated with MOB diversity but not whole bacterial community diversity ($p<0.05$). In the treatments ACET and GAMMA MO_x correlated with MOB diversity composition as well as with whole microbial community composition ($p<0.001$). We found positive relationships between the abundances of type Ia, type Ib and type II MOB and MO_x potentials in the CONTROL treatment ($p<0.05$ for types Ia and Ib and $p<0.001$ for type II, respectively). We also found positive relationships between type Ia abundance and MO_x potentials in the KAN and GAMMA treatments (Table 3, Fig. S3).

Discussion

In this study we investigated the interaction between the total soil bacterial community and a defined functional group (MOB). In particular, we wanted to understand how reduced total microbial diversity influences the recovery of function (methane oxidation) and the succession of MOB. We selected a river floodplain site, which hosts a diverse methane oxidizing community, encompassing a range of type Ia, type Ib and type II MOB [24, 32]. The four different treatments applied resulted in soils with highly similar chemical and physical properties but varying microbial functionality, abundance and diversity. Previous results suggested that acetylene treatment may inactivate MOB in a reversible way [33]. Therefore we applied three cycles of acetylene treatment followed by thorough aeration, allowing for proper interaction of all methane monooxygenase enzymes with acetylene and oxygen.

Treatment with acetylene was applied to irreversibly inactivate MOB, while leaving the vast majority of the total microbial community intact. The lack of any methane oxidation in samples treated with acetylene following 6 days' incubation with methane in a shaken slurry confirmed that MOB were irreversibly inactivated. This was further confirmed by the lack of methane oxidation at day 0 of the microcosm experiment (prior to inoculation with untreated soil). Further reduction of the microbial community was achieved by treatment with kanamycin, an antibiotic with bactericidal potential at the applied concentrations [34]. Finally, gamma irradiation resulted in complete eradication of detectable microbial life.

Table 3 Generalised linear model of log(*pmoA* gene abundance) versus methane oxidation (MO_x) for each of the three major groups of MOB

	ACET		KAN		CONTROL		GAMMA	
	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*
Type Ia	0.09	0.105	0.32	0.001**	0.22	0.01*	0.44	<<0.001***
Type Ib	0.06	0.2	0.007	0.66	0.18	0.02	0.09	0.1
Type II	0.08	0.14	0.065	0.18	0.45	<<0.001***	0.12	0.063

R^2 values are reported for $n=28$. *$p<0.05$; **$p<0.01$; ***$p<0.001$

Although the latter was confirmed by PCR analysis of 16S rRNA genes as well as by plating, it cannot be excluded, that DNA derived from dead cells may have contributed to a minor extent to results obtained from DNA-based analysis.

Methane oxidation potential in the control microcosm was low (0.06 μmol CH_4 g^{-1} fw soil h^{-1}) at day 0 but increased to 0.4±0.04 μmol CH_4 g^{-1} wet soil h^{-1} in all four layers by day 7, indicating a fast recovery of MOB following the perturbation caused by sieving and drying. In both soils treated with acetylene (treatment ACET and KAN) the rate of recovery was slower compared to the control treatment. From day 7 to day 26, methanotroph activity in the upper two layers recovered faster than the lower two layers, indicating re-colonisation by MOB communities from the upper inoculation layer. In the gamma-irradiated microcosm MO_x potentials were detected only in the upper layer until day 41. This microcosm showed slower MO_x recovery compared to all other soils with higher initial microbial diversities. However, following a slower initial recovery in all four layers, the MO_x potential in this treatment increased by day 67 beyond that detected in the other three treatments. We hypothesize that this reflects reduced competition (due to gamma irradiation) leading to an overcompensation for loss of community members upon disturbance, a phenomenon common in plants as response to herbivory [35] as also observed in MOB communities in rice soils [36]. When comparing methane oxidation potentials of top and bottom layers, they were roughly equal or higher in the top layers up to day 41. From day 67 on, peak methane oxidation potentials migrated towards the bottom layers (and the source of methane).

Microarray as well as qPCR results indicated that type II MOB were dominant across all time points and treatments. Type II MOB copy numbers were initially higher than type I (type Ia and type Ib) MOB, however, they did not increase substantially over time, suggesting that methane may not be the main driving factor in their proliferation. Type Ia MOB numbers increased with time suggesting that at least a substantial part of the increased MO_x potential was due to proliferation of type Ia MOB. This rapid increase in type Ia copy numbers following a low initial abundance is in agreement with reports describing type I MOB as r strategists [24, 25]. Another finding supporting the above is that Type Ia MOB abundances (and only type Ia) showed significant correlation to methane oxidation potential in KAN and GAMMA treatments. In these two treatments MOB had to re-colonise the soil while the competition for general resources with the overall microbial community (space, oxygen, other nutrients, etc.) was reduced (moderately by the KAN treatment and drastically by the GAMMA treatment). We did not find a similar correlation in the ACET treatment, and we hypothesize that the reason for this is that the competition for space, oxygen and other nutrients by non-methanotrophs was not removed and therefore the re-colonisation by MOB was driven by more complex dynamics. Finally, the abundance of all three major clades showed significant correlation to MO_x in the CONTROL treatment that represented a climax community with each group having already established niche specialization.

The genus *Methylocystis* is a highly abundant member of the MOB community in most soils, often showing little or no response in numbers due to changes in environmental conditions [37, 38]. Here, we report distinct responses of the two main clades of *Methylocystis* (clade A, containing *Mcy.* sp. KS3, *Mcy. echinoides* 491, *Mcy.* strain M; and clade B, containing *Mcy.* sp. SV97, *Mcy.* sp. SE12, *Mcy. minimus* 41, *Mcy. methanolicus* 39, *Mcy. parvus* OBBP) due to changes. Two probes, McyB304 and Mcy255, which target *Methylocystis* clade B were less dominant in the bottom layers in all four treatments. The probes targeting *Methylocystis* clade A did not display such a pattern. The bottom layers were exposed to a higher concentration of methane compared to the upper layers due to their closer proximity to the methane source, suggesting that methane and/or oxygen availability played an important role in differentiating the ecological niches of the two clades of *Methylocystis*. The same clade was also remarkably less abundant and in many cases not detected in the gamma-irradiated soil. Abell et al. [30] also found that in an Alpine meadow soil the presence and relative abundance of *Methylocystis* clade B was more variable compared to *Methylocystis* clade A. In an experiment simulating disturbance-induced mortality reducing MOB and total microbial community, Ho and co-workers [36] demonstrated a rapid increase in type II abundance in the range of three orders of magnitude, and a temporal shift in the MOB community from type I to type II MOB. We did not observe the same strong increase in type II abundance. In contrast to our experimental setup, Ho et al. applied homogenous inoculation mixing sterile and untreated (inoculums) soils and kept microcosms in Petri dishes within anaerobic jars. We hypothesize that the contrasting results are due to the differences in the experimental setups. In particular, in our experiment, MOB had to colonise across space, into areas which have potentially been colonised by other bacteria. In that sense, our experimental setup tested the colonising potential of MOB as well, which the paper by Ho et al. did not.

Type Ib MOB are typically isolated from and detected in mud/sediment samples [39]. While preliminary analyses indicated a high abundance and diversity of type Ib MOB in the floodplain used in this study, they were detected only in low relative abundance in this experiment. The samples were tested after 3 days following sieving and air drying at which point the microarray analysis indicated the presence of *Methylocaldum* with strong signals for the probes targeting this group (MclS402, MclS394, MclS400, Mcl404 and Mcl408), however, after 13 days following drying and sieving (before establishing the microcosms) this group was no longer

detected (data not shown). It should be noted that the detection sensitivity of the microarray is limited at approximately 5 % relative abundance. In practical terms, the lack of a positive signal for type Ib MOB does not indicate the total absence of this group, only that their relative abundance was below 5 % of the total MOB community. We hypothesize that this decrease in type Ib MOB abundance and the lack of their full recovery indicates a high sensitivity of type Ib MOB to severe drought and/or aeration (which was experienced by the entire community during drying and sieving of the soil).

We found a similar succession in methane oxidizing communities (Fig. 4a) in all four microcosms despite the strong differences in the initial total microbial communities. In regard to MOB communities no significant differences between treatments were found. This may at least in part be due to the selective pressure applied. The constant, relatively high methane flow has apparently had a stronger influence on the MOB community than the different treatments and their indirect effects via the differences introduced into the total microbial communities. In contrast, succession of total microbial communities under the same conditions resulted in different community structures. The soil, which was sterilised by gamma-irradiation, developed a more diverse and more disperse total microbial community (shown by PERMDISP analysis). PERMDISP as a measure of beta diversity [40] suggested that higher beta diversity observed in gamma treatment was also associated with higher methane oxidation potential. This is also consistent with the often observed positive correlation between productivity and beta diversity [12, 41, 42]. Furthermore, the presence of many unique T-RFs indicates the presence of bacteria or bacterial groups, which were able to manifest under these conditions but probably were outcompeted in other treatments. We hypothesize that the observed higher methane oxidation potentials and MOB abundance are due to a combination of two facts: (1) a reduced competition with other microbes for shared resources, and (2) steady supply of growth substrate. In this (gamma irradiation) treatment, the initial eradication of all microbial life generated an open ecological space enabling the development of a different total microbial community suiting the new environmental conditions with methane being a major carbon source.

While the MOB community composition followed a similar succession trajectory in all treatments, the correlation between the abundance of major MOB groups and MO_x did not. While the exact reason for this cannot be concluded from our results, it is likely that this result reflects the complex lifestyle of type II MOB. Interaction between MOB and other microbial guilds are likely to be involved in MO_x, as such the effect of treatment on the rest of the microbial community (as indicated by T-RFLP analysis) likely plays a role in this result. This interaction may be a dependence on syntrophic relationship between MOB and other microbial groups to enable full methane oxidation potential or a non-methanotrophic lifestyle under limited competition from heterotrophs [43, 44].

The project tested the initial hypothesis that there is a significant interaction between the diversity of the total microbial community and the function of a selected microbial guild, the MOB. The results obtained in this study indicated that, under steady supply of the growth substrate, the overall microbial diversity had no significant effect on the recovery or function of MOB, nor on the MOB community composition. This result needs to be interpreted with some care as in our experiment methane was the only carbon source supplied and it was supplied at relatively rates. Steady supply of other carbon sources, fuelling other functional guilds of the microbial community may result in a detectable influence of the overall microbial community composition.

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