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A laboratory study of the long-term impacts of a methane pulse event on a soil microbial community

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Abstract

Aim: Methane is a potent greenhouse gas and soils can act as both a source and sink. The presence of a methane flux can promote an increase in methanotrophs; however, broader changes to the soil community are not well documented. Shifts within the differing methanotrophic niches are also poorly understood. This work explores the resistance and resilience of a soil microbial community over 18 months after exposure to methane pulses.

Methods: Quantitative PCR (qPCR) of genes involved in methanotrophy (*pmoA, mmoX,* and *Methylocella*-specific *mmoX*), 16S rRNA gene sequencing and methane oxidation rate measurements were undertaken immediately after the pulse and after 5, 9, 12, and 18 months.

Conclusions: Compared to the control, the pulse altered the methanotrophic community, which remained disturbed throughout the experiment. Stimulation of methanotrophs resulted in increases in methane oxidation rates which declined through time. The relative abundance of *pmoA* increased in response to the methane pulse, while *mmoX* was greater in the control. The broader microbial community was also disturbed by the methane pulse.

Impact Statement

This work highlights how methane pulses can alter soil bacterial communities and describes the complexity of soil methanotroph responses when exposed to methane. The study revealed that changes to the microbial community and methane oxidation potential after a methane pulse are long-lasting, and that the changes to community structure persist longer than changes to methane oxidation potential. An important implication of this finding is that once exposed to elevated methane, the soil microbial community retains the ability to mitigate methane emissions for a prolonged period after methane production stops. This could have a beneficial impact at sites exposed to intermittent methane exposure. The use of multiple approaches, including changes to microbial community composition and their potential to oxidize methane, is useful when exploring methanotrophic niches and interactions which act as sink in soils for methane.

Keywords: methanotrophs; soil; molecular ecology; stress, resistance and resilience

Introduction

Soil microbial communities are a cornerstone in nutrient cycling and soil health. These communities are dynamic and respond rapidly to changes in their environment. A better understanding of how soil microbial communities respond to disturbance and how, or if, they recover, is needed. Disturbances are events that result in a community change due to population mortality and shifts in relative abundance (Shade et al. 2012). Disturbances can occur over different timescales: a sudden change that persists over the long term (a press), a sudden short-term event that dissipates (a pulse), or an event which gradually accumulates over time (a ramp) (Lake 2000).

Soil contamination is a disturbance which can provide both positive and negative selection pressures on microorganisms. The introduction of new substrates for metabolism can produce an advantage for the specialists (individual taxa or consortia) that can degrade them. Conversely, the presence of contaminants in toxic concentrations can elicit a strong negative selection pressure upon members of the community sensitive to that substance. The presence of a methane or a natural gas leakage is a possible disturbance, as this is anticipated to change the community composition. If the leakage persists and is large enough, it will cause an increase in the relative abundance of methanotrophs and alkanotrophs (Farhan Ul Haque et al. 2018, Farhan Ul Haque et al. 2019). In other words, an additional source of methane would provide a positive selection pressure on these groups of microorganisms.

Given the importance of methane as a potent greenhouse gas, there is a need to accurately estimate methane fluxes to and from the atmosphere (Saunois et al. 2020). The use of microbial indicators may be a useful tool to identify soil methane fluxes; previous work has used methanotrophs to identify leakage from potential hydrocarbon reservoirs (Miqueletto et al. 2011, Zhang et al. 2014, Liu et al. 2016). However, the ability of soil microbial communities to recover from a methane disturbance is poorly understood and it is plausible that the community might remain in a disturbed state for a prolonged period after the flux has ended, thus leading to false positives when trying to identify soils with elevated methane emissions. Conversely, this ability to retain a 'record' of recent leakage might be useful for detecting leaks that are intermittent, which therefore might be missed by gas detection methods. An understanding of methanotrophic community structure and functioning may also lead to a stronger understanding of the ca-

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pacity for soils to act as a sink for methane. Furthermore, identifying patterns of change in the broader soil microbial communities, driven by methane fluxes, could improve understanding of the potential for other environmental impacts of methane fluxes, which may develop through changes to nutrient cycling rates or other ecosystem services provided by microorganisms.

Classically, methanotrophs have been considered as being capable of growth only through the oxidation of methane (i.e. obligate) and have been split into two broad types based on membrane structure and carbon assimilation pathways. The Type I methanotrophs, are ϒ-proteobacteria which utilize the ribulose monophosphate pathway (RuMP) for carbon fixation (Hanson and Hanson 1996) and make up all the genera in the Methylococcaceae family. The Type II methanotrophs are α-proteobacteria and utilize the serine pathway for carbon fixation and are from the Methylocystaceae family (Hanson and Hanson 1996). Dedysh et al. (2000) first isolated the *Methylocella* genus, a group of facultative methanotrophs capable of growth on variety of multi-carbon compounds such as ethanol and acetate (Dedysh et al. 2005). The *Methyocella* genus is in the Beijerinckiaceae family and are α-proteobacteria using the serine pathway, although differences in cell structure had led to them being classified as separate from the classical Type II genera (Semrau et al. 2010).

The majority of methanotrophs have a membrane-bound particulate methane monooxygenase (pMMO) (McDonald et al. 2008). Some methanotrophs also possess a soluble methane monooxygenase (sMMO), with a different catalytic centre (di-iron not copper) and are not bound to a membrane (Kopp and Lippard 2002, Hakemian and Rosenzweig 2007). Both the pMMO and sMMO have conserved genes for their catalytic centres, *pmoA* and *mmoX*, respectively, for which primer sets for PCR have been published. *Methyocella* has not been reported to possess pMMO, and the complete genome of *M. silvestris* BL2 has none of the genes encoding pMMO (Chen et al. 2010). Furthermore, the sMMO reported within the Beijerinckiaceae family is distinct from that seen in the obligate methanotrophs (Dedysh et al. 1998) and can be amplified through PCR using primers that exclude *mmoX* genes from other genera (Rahman et al. 2011, Farhan Ul Haque et al. 2018).

This work was designed to explore changes in community composition in response to an artificially imposed methane flux and to assess the long-term stability of the new community's structure. A soil microbial community was repeatedly exposed to 2.5% methane repeatedly over eight weeks. The soil was subsequently monitored for a further 18 months to allow the soil community to recover with periodic analysis of the soil microbial community using qPCR and 16S rRNA gene sequencing as well as methane oxidation rate analysis. It was predicted that exposure to methane would increase the relative abundance of methanotrophs and methanotrophic activity (i.e. maximum potential rate of methane oxidation) as well as affecting the broader microbial community. With increasing time following methane exposure, the relative abundance of methanotrophs and methanotrophic activity were expected to decrease.

Materials and methods

Soil was collected, homogenized and then exposed to either a methane-enriched or a normal-laboratory (control) atmosphere in laboratory mesocosms. Post exposure, the mesocosms' soils were aliquoted into pots and the soil communities allowed to recover. Periodically, three pots of soil, from both mesocosms, were destructively sampled to measure methanotroph abundance and methane oxidation rates. The key steps of this study and sub-sampling strategy are illustrated in Fig. 1.

Soil collection and homogenization

Soil was collected from a farm site at the University of Nottingham's Sutton Bonington campus, England (52° 50′ 0.24′′ N 1° 14′ 58.56′′ W). The field had been most recently used to graze sheep. A W-survey was completed to confirm there was no detectable methane flux from the site using a portable gas fluxmeter (West Systems, It.) (S1). Soil was a dark brown, sand-rich (coarse) loam which began to transition to a more gravel-rich layer below about 25 cm depth. Soil was collected from 5 to 30 cm depth and left to dry at room temperature for eight weeks. The dried soil was sieved at 1 cm. Soil had a pH of 7.26 after homogenization.

Initial mesocosms

Two mesocosms were built: a control mesocosm flushed with laboratory atmosphere and one flushed with 2.5% methane in air mix (Calgaz, UK). Homogenized soil was rewetted to 20% w/w moisture and packed, gently, into the two large mesocosm systems ready for gassing, with ∼10 kg of soil in each mesocosm. Mesocosms were constructed from 1500 by 110 mm soil pipe, sealed using adapters and caps (Fig. 2), the integrated seals were made gas-tight by coating them with silicone grease. The caps had a Swagelok screw fitting and valve inserted, secured from either side with a rubber seal, with silicone sealant applied as a glue. Inside the cap a moistened sponge was fitted to prevent blockage of the inlet/outlets and to protect the soil from desiccation. Once constructed, both systems were pressurized and held at ∼250 kPa for one minute to test for leaks.

Flushing was achieved by venting gas into the system from one end and allowing it to exhaust from the other end of the pipe through an open valve. Both gas streams were run through a narrow rubber tube coiled in bucket of water at room temperature to maintain constant gas temperatures. Gas flow was measured using a bubble trap; the entire (empty) volume of the soil pipe (∼12 L) was displaced over 12 to 13 mins.

Both mesocosms were flushed every two to three days for four weeks. To simulate a pulse event that tapered off, for a further four weeks the mesocosms were flushed every seven days. To avoid possible introduction of elevated methane in the control treatment, the mesocosm receiving laboratory atmosphere was always flushed and sealed before the methane mesocosm. Once all flushes were complete, mesocosms were dismantled individually and the soil mixed before aliquoting into pots. Each pot contained 300 g of soil and was left open to the atmosphere. Both the gassing of mesocosms and storage of pots were completed in the laboratory with ambient temperature control set to 20°C.

Methanotroph relative abundances

Throughout the experimental work, soil DNA extractions were carried out using FastDNA™ Spin kits (MP-Biomedicals, USA) following the manufacturer's instructions. Three samples from the original field soil (B- -1), the newly homogenized soil (B-0), and the soils collected immediately after the

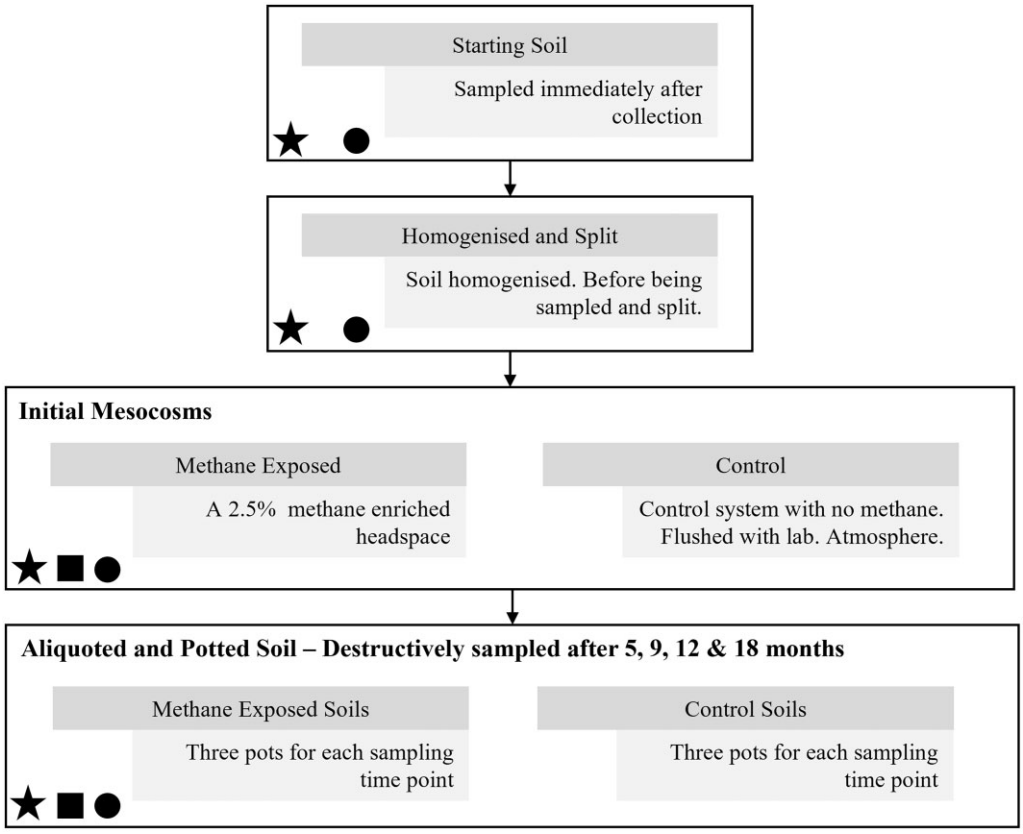

Figure 1. Key sections of the experimental work and the samples collected at that point. Coloured circles represent analyses carried out on the samples collected at that point. Stars: DNA extracts used for 16S rRNA gene sequencing. Squares: methane oxidation rate measurement using gas sampling for chromatography. Circles: DNA extracts collected for qPCR. The same potted soils sampled for DNA extracts were also used for the rate microcosm experiments for each time point.

dismantling of the mesocosms were collected. The potted soil undergoing destructive sampling was also used for DNA extractions, yielding one DNA sample per pot. DNA samples were stored at −80°C to enable all qPCR assays to be completed simultaneously.

Three soil samples were used to test for PCR inhibition using a method described previously (Bott et al. 2023). Based on the inhibition tests, the samples were diluted 1:25 with ultrapure water. Using all three extracts, a linear model suggested that a 1:90 dilution would achieve optimal amplification efficiency (linear trendline: $y = -0.0047 \times +1.4244$, $R^2 = 0.71$). Using the trendline, a 1:25 dilution was predicted to yield an average efficiency of ∼130%, which was considered the best compromise between efficiency and over-dilution.

To measure the relative abundance of methanotrophs, qPCR assays of *pmoA*, *mmoX*, and 16S rRNA were completed using a Bio-Rad CFX96 controlled by CFX Manager™ (V1.2). The SSoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad, USA) was used. For *mmoX*, two primer sets were used; one primer set amplified *mmoX* from the obligate methanotrophs [mmoX206f/mmoX886r—Hutchens et al. (2004)], and the second amplified *mmoX* from facultative *Methylocella* spp. [mmoXLF2/mmoXLR—Rahman et al. (2011) and Farhan Ul Haque et al. (2018)]. The *pmoA* gene was amplified using the A189f/mb661 primers (Costello and Lidstrom 1999) and the 16S rRNA gene using 341f/543r (Juck et al. 2000, Nossa et al. 2010). Complete PCR conditions are described in the Supplementary material (S2). The copy number of the functional gene was divided by the 16S rRNA gene copy number to estimate relative abundance, with the 16S rRNA gene copy number treated as an estimate of the total bacterial population.

Effect of time and the methane pulse on the relative abundances of individual methanotrophy genes was explored using a two-way multivariate analysis of variance (MANOVA) with base statistics functions in R (Ver 4.0.5). The three relative abundances of the methanotrophy gene targets were summed and a one-way ANOVA used to test for differences between mesocosm factors, excluding the baseline samples. Homogeneity of variances was tested using Levene's test. For all tests, significance was accepted at $P < 0.05$.

Amplicon sequencing–16S rRNA gene

Soil DNA extracts were used for 16S rRNA gene amplicon sequencing. Extracts were pooled by time point and treatment (i.e. enhanced methane or control mesocosms) producing 12 samples for use in amplicon sequencing. Sequencing was completed on the Oxford Nanopore Technology (ONT) platform (Oxford, UK) with Flongle (V1-FLO-FLG001) flow cells. Libraries were prepared using ONT's 16S barcoding kit (SQK-RAB204). Recommended PCR reagents and protocols were used and are listed in Supplementary material (S3). Resulting PCR products were purified using the ChargeSwitch®

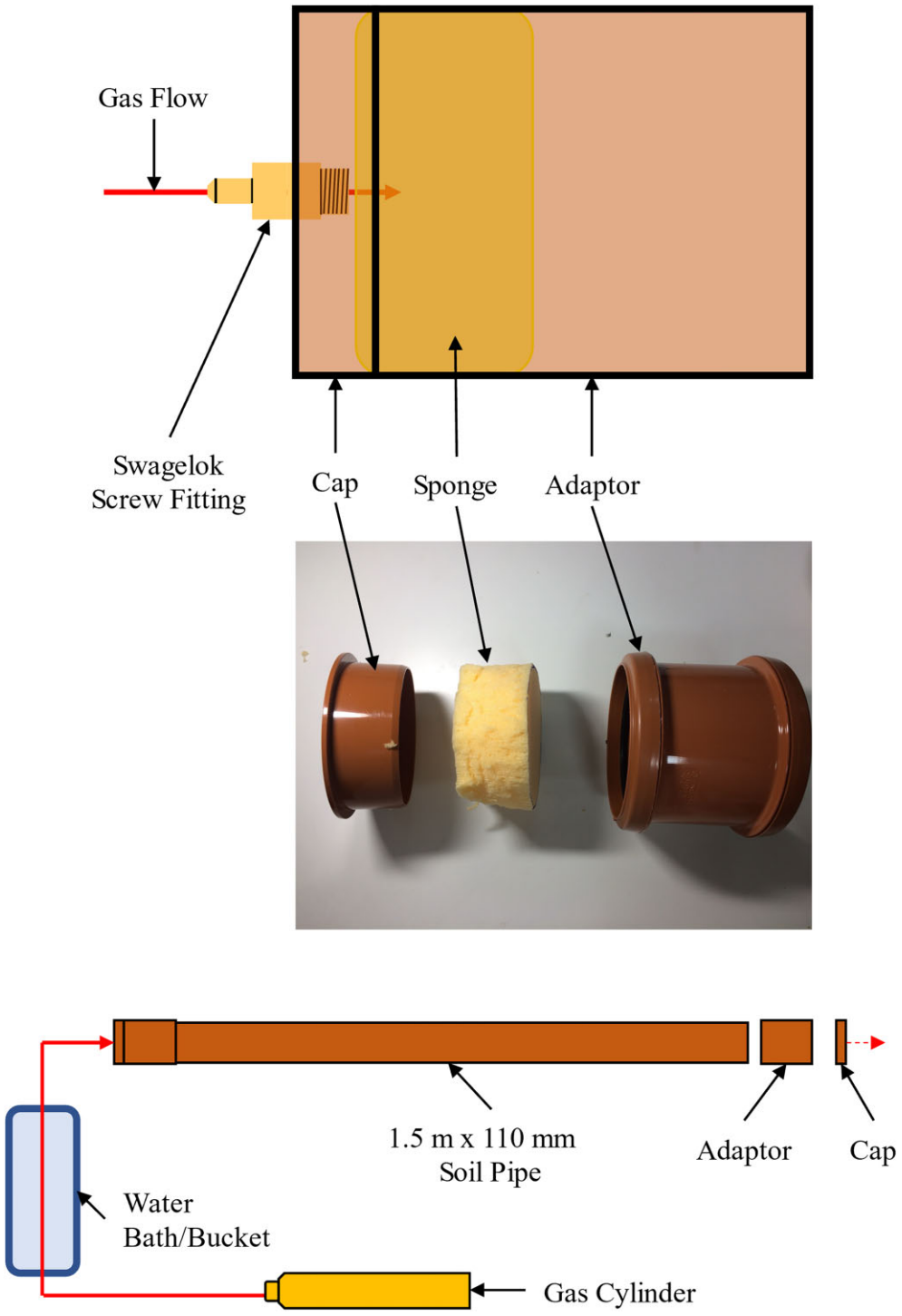

Figure 2. Components of the mesocosms used to create methane pulse. The sponge was moistened and packed into the cap. This was then pushed into the adaptor that fitted on the packed soil pipe. The internal seals within the adaptor were coated with silicone grease to improve the gas-tightness of the system.

PCR clean-up kit (Invitrogen, USA), before DNA concentration was quantified using a high specificity Qubit assay (Invitrogen, USA). Samples were pooled into one library with equal masses of DNA for each sample.

The ONT Flongle was run for 24 hours and managed by a MinIT® (ONT, UK) with live base-calling enabled using GUPPY (ONT, UK). Briefly, data were analysed through the following pipeline: FASTQ files from GUPPY were concatenated in R (Ver 4.0.5) to produce one file per sample. Following concatenation, the FASTQ files were processed in a conda environment (Python V3.9). Initial visualization of data quality was performed using FastQC [Ver. 0.11.9, Andrews (2015)]. Sequences were filtered for reads with lengths between 500 and 1750 bp using NanoFilt [Ver. 2.6.0, De

Coster et al. (2018)]. The first 50 bp were trimmed and an average minimum Q-score of 10 was applied. Filtered and trimmed sequences were assigned taxonomic labels using Kraken2 [V2.1.2, Wood et al. (2019)] with the MiniKraken reference database (V1-2019). The tab-separated output format from Kraken was converted to the BIOM format (hdf5) using Kraken-Biom (V1.0.1). The .BIOM format file was imported into the R environment for analysis with the phyloseq package [V1.34.0, McMurdie and Holmes (2013)].

The whole dataset was analysed at the family level using relative abundances. Exploratory analysis of the entire dataset was completed using a principal coordinates analysis (PCoA) without the baseline samples. To test for differences between mesocosm factor (methane or control) and time points, a PERMANOVA was completed using the vegan package [V2.6–4, Oksanen et al. (2020)], excluding the baseline samples, with Bray–Curtis distance matrices and run with 9999 permutations. Where a significant effect was observed, SIMPER using the vegan package [V2.6–4, Oksanen et al. (2020)] analysis was used to identify shifts in taxa with an arbitrary cut-off of 50% contribution.

To explore relative abundances of only methanotrophs within the 16S rRNA sequencing data, the dataset was trimmed to contain only three families with well-described methanotrophic taxa: Methylococcaceae, Methylocystaceae, and Beijerinckiaceae. A PCoA and PERMANOVA were completed on the trimmed data to explore relative changes in methanotrophs.

Methane oxidation rate measurement

Immediately after dismantling each mesocosm, the rate of methane oxidation was measured in microcosm systems. This was repeated after 5, 9, 12, and 18 months using the aliquoted potted soils. Triplicate microcosms, with a methane-enriched headspace (at 2.5%), were prepared for soil from both mesocosms. Three controls were set up, two with soil and one without soil. The controls with soil were not enriched with methane and instead tested for methane production within the microcosms. The soil in these controls was an equal mix from all three pots being sampled. The control without soil acted as a recovery standard.

Each microcosm consisted of 40 g of soil rewetted to 15% ± 1% w/w moisture content sealed in a 125 mL glass Wheaton® bottle with a crimped, self-healing, butyl rubber stopper. All glassware and butyl rubber stoppers were autoclaved prior to use. To test the seal, a vacuum was maintained for 30 seconds before flushing with 2.5% methane or laboratory atmosphere for 15 seconds. Flushing ceased and the microcosm's pressure allowed to equilibrate.

Immediately after flushing, a 1 mL sample of headspace was collected using a needle and syringe. Headspace samples were stored in evacuated, 3 mL, clear glass Exetainers® (Labco, UK). Headspace sampling continued for 6 days, samples were collected every 2 hours for the initial 6 hours, then every 3 hours until 12 hours had passed, then every 12 hours until 96 hours had elapsed and finally every 24 hours. Gas samples were analysed for methane concentration using gas chromatography (GC), (for complete method, see S4). The microcosms were stored out of direct sunlight at room temperature (~20°C).

Data from the GC analysis of headspace samples were used to estimate the maximum potential rates of methane oxidation. Data were normalized as a percentage of the starting value before subtracting this percentage from 100, i.e. the value at $T_0 = 0$. To estimate maximum potential oxidation rate, a logistic growth curve was fitted in R [Ver 4.0.5, R Core Team (2021)] using Equation 1 from Rockwood (2015).

$$N_t = \frac{K}{1 + \left(\frac{K - N_0}{N_0}\right) e^{-rt}}$$

Equation 1: logistic equation used to estimate maximum potential oxidation rate. N_0 gives the starting methane concentration. The maximum predicted rate of oxidation is given by r. The asymptote is denoted by K, which was fixed at a value of 100. The values of N_0 nd r were estimated by fitting the model to experimental data using R.

In R, a non-constrained logistic growth model was first fitted using the growthcurve package (Sprouffske and Wagner 2016). The fitted, K, r and N_0 values were used in a constrained model. The non-linear statistics function (from the base stats package) with the port algorithm was used enabling an upper bound to be fitted. Here, the asymptote equalled 100, as the dataset was a percentage. The final model was plotted to check accuracy and the r-values, with associated errors, were used for comparison between datasets.

Results

Soil was exposed in mesocosms to either methane or laboratory atmosphere. In the figures these are labelled 'C' for control and 'M' for methane. Subsequently the soil was left to recover in the lab and a total of five time points were destructively sampled. Time point is suffixed to the sample letter in the figures. Also analysed were two baseline samples, one of the soils immediately after being collected from the field (B—1) and a second sample of the soil after being dried and homogenized (B-0).

Relative abundance of methanotrophs

Relative abundances of *pmoA*, *mmoX*, and *Methylocella spp.*-specific *mmoX* were quantified using qPCR. Efficiency and R^2 values of the assays are given in the Supplementary material (S5). For the control mesocosm's soil, the final two time points (12 and 18 months) had *pmoA* copy numbers below the limit of detection (LoD) of the assay (<100 copies μL^{-1}) (Fig. 3). A two-way MANOVA, excluding the baseline samples, predicted no interaction between time and mesocosm for any of the genes (Table 1).

For *pmoA* and *mmoX*, both time and mesocosm treatment had a significant effect on relative abundance (Table 1). The relative abundance of *pmoA* was greater in the methane mesocosm ($F_{(1,20)} = 809.404$, $P < 0.001$ and Fig. 3). Both mesocosm soils saw decreases in *pmoA* relative abundance through time ($F_{(1,20)} = 5.739$, $P = 0.003$, Fig. 3). The detected significant effect of time on the obligate *mmoX* did not have a clear trend ($F_{(4,20)} = 3.49$, $P = 0.026$, Fig. 3). For both *mmoX* gene targets the control mesocosm soils had greater relative abundances compared to the methane-treated mesocosm (Table 1 and Fig. 3). The *Methylocella mmoX* was the most abundant of the methanotrophy gene targets, having a higher relative abundance than sum of the other *mmoX* and *pmoA* targets, but did not vary significantly with time (Table 1 and Fig. 3).

When the three qPCR targets were summed, as an indicator of total methanotrophs, the controls had a

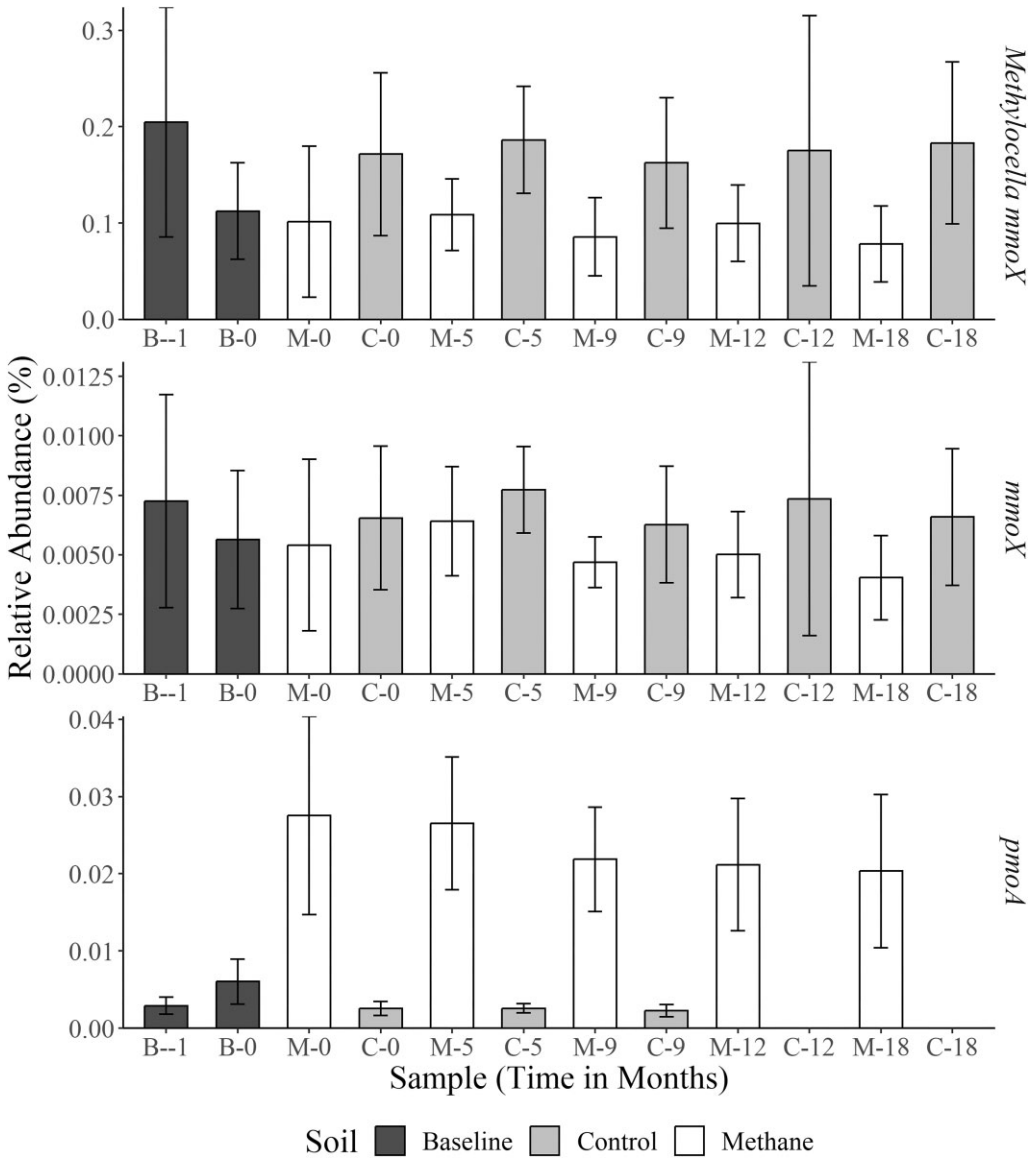

Figure 3. Relative abundances, expressed as percentages, for each of the methanotroph gene targets detected using qPCR. Error bars are the propagated error, from both the 16S rRNA gene and functional gene, derived from ± 2 SD.

Table 1. Results of the two-way MANOVA exploring the impact of time and differences between mesocosm.

Gene	Level	df	F	*P*
pmoA	Time	4,20	5.739	0.003∗
	Mesocosm	1,20	809.404	<0.001∗
	Mesocosm and time	4,20	2.396	0.085
mmoX	Time	4,20	3.496	0.026∗
	Mesocosm	1,20	32.303	<0.001∗
	Mesocosm and time	4,20	0.895	0.485
Methylocella spp. *mmoX*	Time	4,20	0.566	0.690
	Mesocosm	1,20	70.240	<0.001∗
	Mesocosm and time	4,20	0.398	0.808

MANOVA excluded the baseline soil. Reported F and *p* are rounded to 3 dp, ∗ indicates significance.

greater relative abundance of methanotrophs compared to the methane mesocosms ($F_{(1,8)} = 53.09$, $P < 0.001$, S6). The increased abundance of methanotrophs in the controls persisted throughout the duration of the experiment.

Community composition

Soil DNA extracts were pooled by mesocosm factor and time, producing 12 samples for 16S rRNA gene amplicon sequencing. Following quality control steps and alignment, a total of 307 511 reads were generated (S7).

Stacked bar plots, at the family level, showed slight variation (Fig. 4a), furthermore separation of the mesocosm factors was revealed in the PCoA (Fig. 5a). The data from M-5 appeared to have a different community composition and clusters separately on the PCoA. Of the three families known to include methanotrophs none had a relative abundance greater than 1% (Fig. 4b). The overall separation between mesocosm treatments was supported by a PERMANOVA, with mesocosm observed to have a significance effect ($F_{(1,6)} = 2.190$, $P = 0.0322$); however, no interaction between time and mesocosm was detected, nor did time have a detectable effect (Table 2).

Follow-on SIMPER analysis using all taxa grouped at the family level indicated that the Methylococcaceae were in the 17 OTUs that contributed to the first 50% of the average dissimilarity between mesocosms ($P < 0.001$) (S8). The other 16 taxa predicted to contribute to the initial 50% were from a variety of different ecological niches. Contributions from two further taxa were reported with a high certainty, Hyphomicrobiaceae ($P = 0.022$) and Methylobacteriaceae ($P = 0.059$), both being greater in the methane mesocosm soils.

Differences in the relative abundances of three families containing well-described methanotrophs were apparent between the mesocosm factors and baseline soils (Fig. 4b).The methane mesocosm soils saw a large increase in the relative abundance of Methylococcaceae family compared to the control and baseline soils, with Methylocystaceae showing a small increase and Beijerinckiaceae showing no change or an increase at all time points except week 9 (Fig. 4b). These differences were also clear in the PCoA of the methanotroph-containing families (Fig. 5b), where the two mesocosms communities clearly separated. The PERMANOVA indicated that there significant dissimilarities were predicted between the two mesocosm factors ($F_{(1,6)} = 80.960$, $P = 0.001$, Table 3).

Methane oxidation rates

A total of 45 microcosms were set up over the course of 18 months to measure potential methane oxidation rates. No methane production was detected in the control microcosms without methane-enriched headspace. In the recovery controls, minor losses in methane concentration were detected in the final time points for the no soil controls; however, these losses were only seen after the point at which all methane had been removed from the test microcosms. The modelled maximum methane oxidation rates (r value) showed a slight decline over time within the soils from the methane mesocosms, while the rate within the control soils appeared to have an increasing trend leading to similar rates after 18 months (Fig. 6).

Clear shifts in the time taken to completely oxidize methane were evident. As the soils from the methane mesocosm recovered, the time taken for methane to be oxidized to the limit of detection (LoD) of 100 ppm increased (Fig. 7a). At 0 months (i.e. immediately after the initial mesocosms) methane concentration declined to the LoD after 36 hours, whereas after 18 months it took between 84 and 96 hours. For the control mesocosm soils time had little obvious effect with all replicates reaching the LoD between 96 and 120 hours after incubation started.

Discussion

The stability of a soil microbial community in response to a simulated methane pulse was explored. The broader community structure entered a disturbed state which appeared to persist throughout the 18 months, as indicated by both the qPCR and 16S rRNA sequencing. The abundance of *pmoA* genes increased in response to methane disturbance but the overall methanotrophic gene abundances were higher in the control soils. Once disturbed, the methanotrophic community structure did not return to the state observed in either the baseline or control soils.

Shifting methanotroph community

The higher relative abundance of the summed methanotrophy genes in samples from the control mesocosm was unexpected and driven by the greater abundance of the *Methylocella mmoX* gene in the controls. Although originally thought to be restricted to acidic soils, *Methylocella* are now known to be widely distributed in nature (Rahman et al. 2011). *Methylocella* are rarely reported to dominate methanotroph communities in soils, but they may have been overlooked by the reliance on primers that amplify the *pmoA* gene or *mmoX* primers that do not amplify this genus. Therefore, it is not clear how frequently *Methylocella* is the dominant genus in soils. Only the *pmoA* gene showed increased relative abundance after the methane pulse. Taken together, changes in the relative abundance of these genes suggest the methane pulse promoted growth of a small portion of the methanotrophic community and may have led to the decline in methanotrophs possessing *mmoX*. Prior work has also observed increases in *pmoA* abundance after incubation of soil in methane-enriched headspaces (Ho et al. 2011, Shiau et al. 2018), neither of these studies considered the response of *mmoX genes*. However, using the same primer sets as in this study, Farhan Ul Haque et al. (2018) reported a greater abundance of *pmoA* compared to *Methylocella mmoX* in acidic soils above two biogenic methane seeps.

Although the methanotrophic community compositions differed in the two experiments, the compositions remained stable over time. Both showed a gradual decline in the abundance of *pmoA* over the course of the experiments. The decline in the control soils should be interpreted cautiously given that *pmoA* was below the LoD of the assay for the final two time points in the controls. The increased relative abundance of the Methylococcaceae family in the methane microcosms and the SIMPER analysis identified this family as a possible driver of differences in bacterial community composition between mesocosm. The increase in Methylococcaceae is consistent with the increased relative abundance of *pmoA* seen in the qPCR data, as the obligate methanotrophs, those in Methylococcaceae and Methylocystaceae, all possess the *pmoA* gene.

Methanotrophic taxa within both the α- and Υ-proteobacteria have both pMMO and sMMO. It is therefore surprising that an increase in the relative copy number of *mmoX* was not seen in the methane mesocosm soil. It suggests that the possession of a *mmoX*, and therefore sMMO, does not always confer a competitive advantage. It is possible that if the soil had been copper limited or nitrogen rich that sMMO expression would have conferred a competitive edge, given the proposed copper catalytic centre in pMMO (Hakemian and Rosenzweig 2007, Semrau et al. 2010) and the potential for pMMO to be inhibited by ammonium and

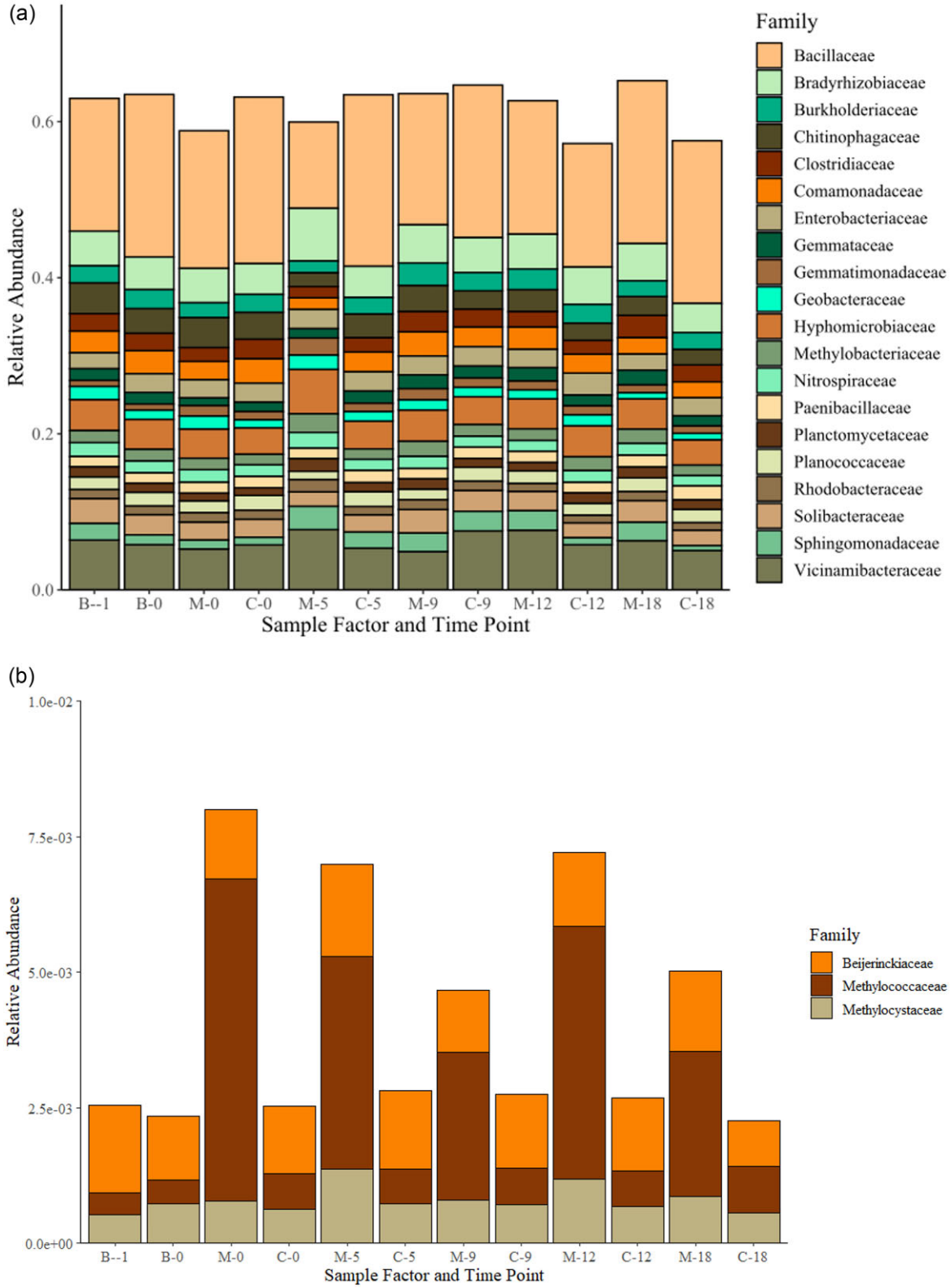

Figure 4. Relative abundances at different taxa level from the baseline and mesocosm soils. Plot A uses taxa with a relative abundance above 1×10^{-2}. Sample lists the factor and the time point, B- -1 = Baseline pre-homogenization, B-0 = Baseline post-homogenization. M = methane mesocosm, C = control mesocosm. Number after M and C is the number of months since mesocosm dismantlement. (a) Plot of the most abundant families. (b) Plot of relative abundance of the three families know to contain methanotrophs.

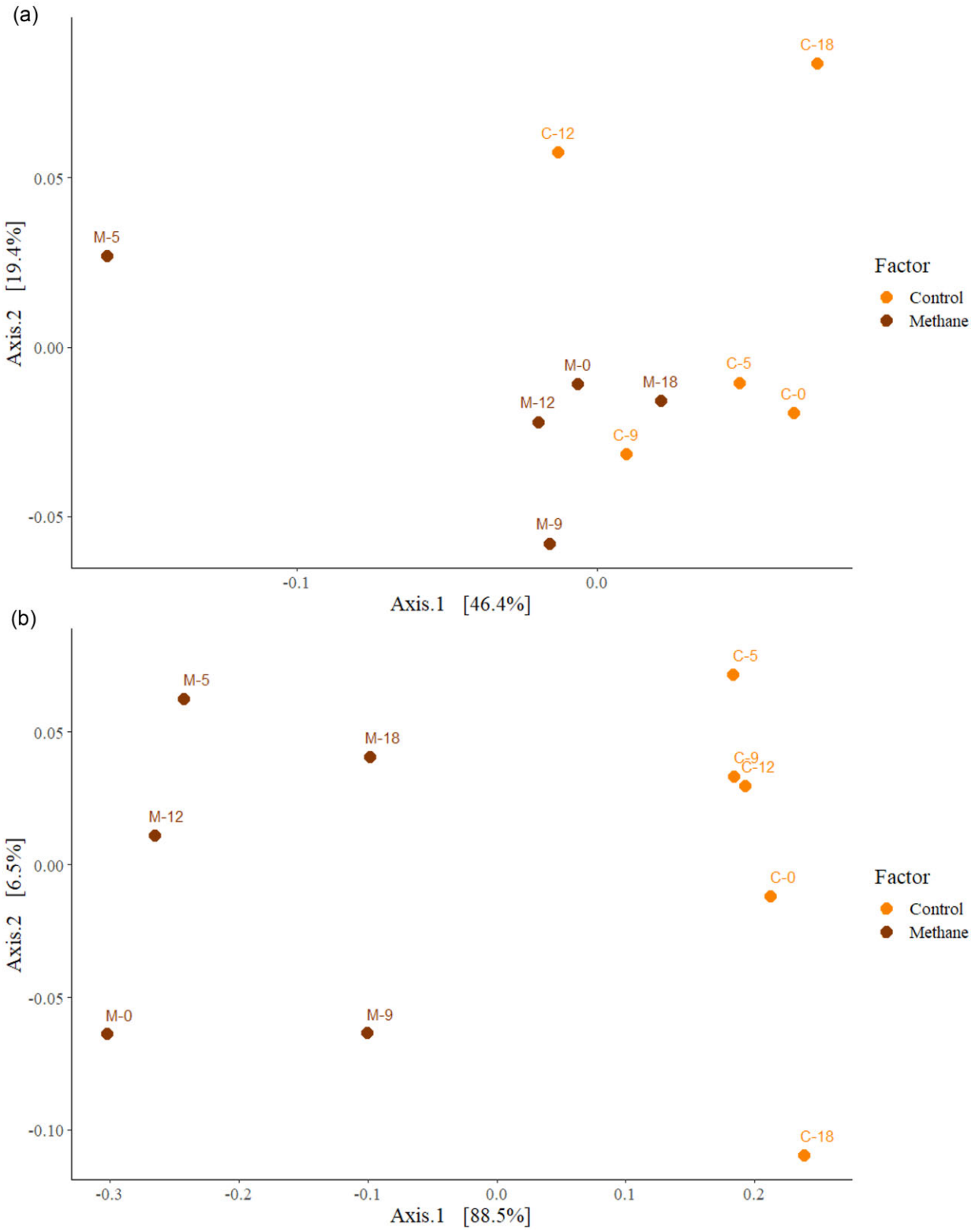

Figure 5. PCoA using the 16S rRNA gene sequencing data. Ordinations use Bray–Curtis dissimilarity distances on relative abundances. (a) All taxa, grouped at the family level. No clear separation of the samples, by either mesocosm factor or time point is seen. If M-5 is treated as an outlier and removed no impact is seen in the ordination. (b) Ordination using only the methanotroph-containing families.

nitrate (Nyerges and Stein 2009). The data here, supports the possibility that methanotrophs capable of utilizing both pMMO and sMMO exist in a separate ecological niche to methanotrophs with only pMMO. Further work is needed to better understand the competitive advantage possessed by methanotrophs with sMMO. A greater understanding of how the differing physiology of the Type I and Type II produces alternative life strategies is also needed.

Table 2. PERMANOVA results for effect of time and mesocosm on the 16S rRNA gene community composition.

Level	$F_{(1,6)}$	*P*
Time	1.158	0.343
Mesocosm	2.190	0.032∗
Mesocosm × time	1.240	0.300

The PERMANOVA was completed without the baseline samples, using Bray–Curtis distances and with 9999 permutations. Reported F and *P* are rounded to 3 dp, ∗ indicates significance.

Table 3. PERMANOVA results for effect of time and mesocosm on the methanotrophic families detected using 16S rRNA gene amplicon sequencing.

Level	$F_{(1,6)}$	*P*
Time	0.275	0.613
Mesocosm	80.960	0.001∗
Mesocosm × time	5.402	0.048∗

The PERMANOVA was completed without the baseline samples, using Bray–Curtis distances and with 9999 permutations. Reported F and *P* are rounded to 3 dp, ∗ indicates significance.

In terms of types, the three dominant methanotroph families identified by 16S rRNA sequencing belong to Type Ia (Methylococcaceae), Type IIa (Methylocystaceae) and Type IIb (Beijerinckiaceae). The qPCR results cannot be directly translated into types. With the exception of the *Methylocella mmoX* assay, which targets only organisms within the Beijerinckiaceae (Type IIb), the *pmoA* and obligate *mmoX* gene primers detect organisms belonging to both Type I and Type II organisms. There is conflicting evidence over the habitat preferences of Type I methanotrophs (the Methylococcaceae) compared to the Type II.

Type I methanotrophs have been described as having a competitive advantage under high oxygen and low methane conditions (Amaral and Knowles 1995). In hydromorphic soils, Type II appeared to be capable of growth at lower methane concentrations than Type I (Knief et al. 2006). Considering these studies, an increase in Type I methanotrophs would not be expected in response to a methane pulse. The observation in this study that Methylococcaceae became a larger proportion of the soil microbial community in response to the methane flux, while Methylocystaceae maintain a stable relative population size suggests that Type I is more responsive to a pulsed methane event than Type II. This is in line with studies that have suggested that the use of soil microcosms might lead to Type I methanotrophs becoming the most abundant methanotrophic community potentially due to Type I being more responsive to high methane concentrations and oxic conditions (Shiau et al. 2018). Furthermore, Henckel et al. (2000) observed that in incubations under a variety of CH_4:O_2 mixing ratios, the Type I population had a greater initial contribution to methane oxidation and increased in size faster than Type II. However, their population remained smaller than Type II and as incubation time increased Type II activity also increased. Ho et al. (2013), in their review using competitive-stress-ruderal (CSR) life strategy approach, summarized Type I organisms as a 'competitor to competitor-ruderal group.' This group rapidly adapts to increased methane and is the more active component of the methanotroph community, while Type II populations are generally more stable and capable of adapting to stress. These proposed strategies fit well with the data presented here, where a relative increase in Type I was seen in response to a pulse event, while Type II maintained a similar relative population size.

Broader microbial community changes

The 16S rRNA gene sequencing suggested that the broader microbial community composition also changed in the presence of a methane disturbance. However, these shifts appeared to be mostly limited to taxa with small relative abundances (<1% abundance). While methane oxidation and methanotroph proliferation are independent of other aerobic microorganisms and are generally considered an obligate life strategy (Conrad 2007, Semrau et al. 2010), there is evidence of interactions between methanotrophs and other heterotrophs. Previous work has suggested that methanotrophs can influence the wider bacterial community and vice versa (reviewed by Ho et al. 2016a). It is interesting to note that the two families, Hyphomicrobiaceae and Methylobacteriaceae, that were identified as influencing the dissimilarity between the mesocosms have both been reported as having methylotrophic genera, i.e. organisms that can oxidize C_1 compounds such as methanol. Increasing methanotroph populations have been reported alongside increased methylotroph populations, including *Hyphomicrobium* (a genera within Hyphomicrobiaceae), with the suggestion of metabolite sharing between the two (Kuloyo et al. 2020). Krause et al. (2017) also demonstrated, in a model community, that methane-derived methanol was removed by methylotrophic partners.

Methanotrophs are at the bottom of the microbial food chain, capable of fixing a gaseous carbon resource into readily accessible organic compounds (van der Ha et al. 2013). Here it is plausible that methanol and other methyl compounds, produced during the initial oxidation step of methane by methanotrophs, became available and led to an increased abundance of methylotrophs.

Potential methane oxidation rates

The methane oxidation rates were higher, and the lag times before methane oxidation rates reached maxima were shorter in the methane-treated microcosms. The shorter lag phase immediately after dismantling the initial mesocosms could be due to increased community size or activity. However, no reduction in relative population was seen in the sequencing dataset. Sabrekov et al. (2020) reported that *pmoA* gene numbers were not clearly related to methane oxidation rates across multiple soil profiles, with ephaptic factors probably complicating the relationship. It is plausible that the PCR amplified dormant community members and active methanotroph numbers had gradually declined. Methanotrophs are thought to enter dormant or resting states in response to stresses such as heat and desiccation (Whittenbury et al. 1970, Ho et al. 2016b, c), and it is possible that a proportion of the methanotrophic community could be in a dormant state in these experiments. Here, such resting states could be a survival strategy utilized by obligate methanotrophs to survive periods where methane is absent or low. However, the possibility of detecting dead bacterial cell DNA cannot be ruled out.

Community stability

A community's response to a disturbance can be framed using the terms resistance and resilience, which cumulatively

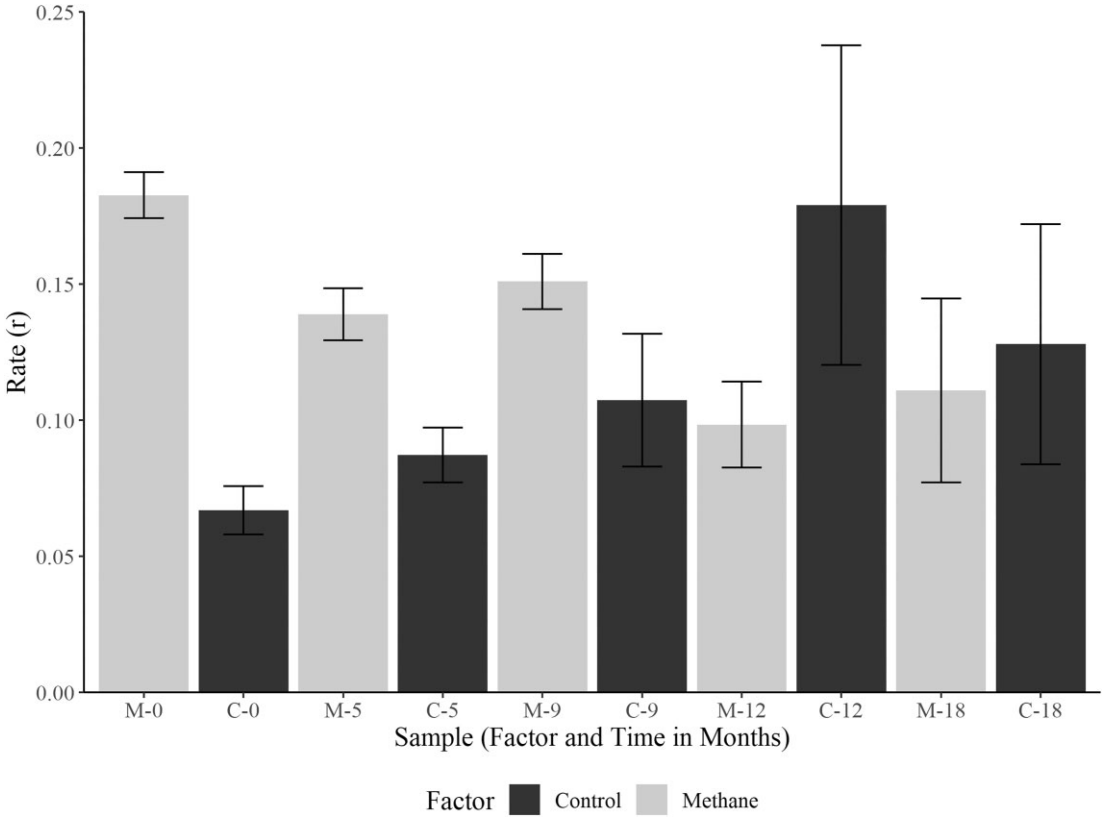

Figure 6. Estimated potential methane oxidation rates (r) for soils as they aged after mesocosm incubation. Rate is estimated using a logistic growth model, it should be noted that *r* is theoretically negative but due to transformation to enable estimation, it is presented as positive here. Error presented is ± 1SE as produced by the logistic model.

describe a community's temporal stability. Using the definitions from Shade et al. (2012) and Allison and Martiny (2008), resistance is the ability for a community to remain unchanged in the face of a disturbance, while resilience is the ability for a community to recover to its previous state after a disturbance.

The soil microbial community did enter a disturbed state when exposed to a methane flux. This new disturbed state persisted with the community demonstrating little resilience. In this work, the stability of storage conditions may have contributed to the stability of the microbial community, as has been observed in previous studies. Unlike field systems, with climatic changes and plant and invertebrate activity, the soil was held in an almost static state. The lack of this type of biological activity probably limited community changes in response to competition, predation, or other stresses. It is possible that had the soil been returned to the field, the exposure to new nutrients and the constant changes in conditions might have driven the recovery of the community.

The methanotrophic community also entered a disturbed state, with changes in relative composition and changes in potential methane oxidation rate. The change was seen primarily in the methanotrophs possessing pMMO. Once the disturbance was over, after dismantling the mesocosms, the methanotrophic community composition showed a low resilience and did not appear to return to the pre-disturbed state; however, there was some evidence for a greater resilience in terms of methanotrophic activity as changes in the methane oxidation rate did occur. This indicates that microbial soil functions, such as methane oxidation do not require the microbial community composition to return to a pre-disturbed state for specific activities to return to their original state. Work exploring temporal changes in methanotroph community structure has often focused on wetland systems. Studies around rice paddies, with seasonal changes in water level and plant cover, have reported changes in the ratio and activity of Type I:II (e.g. Macalady et al. 2002, Ma et al. 2013). Work on more stable environmental systems, like groundwater has observed that a methanotroph community can remain in a perturbed state for at least 250 days after methane injection (Cahill et al. 2017).

This study indicates that persistence of perturbed state may also occur in soils. This finding has important implications for detection of methane leaks, e.g. associated with drilling for hydrocarbons or natural gas storage. It has been proposed that microbial markers such as methanotrophs could be good candidates for detecting methane leakage. This work has shown that they could be particularly important where leakage is intermittent. Other detection methods such as using detectors to measure methane in soil gas rely upon leakage occurring at the time of testing, but the findings of this study indicate that the monitoring of methanotrophic populations has potential use as an indicator of past leakage due to the sustained response of these organisms to methane pulses.

Conclusion

Exposure to 2.5% methane over 8 weeks had a detectable effect on a soil bacterial community and the methanotrophic community also entered a disturbed state. While detectable,

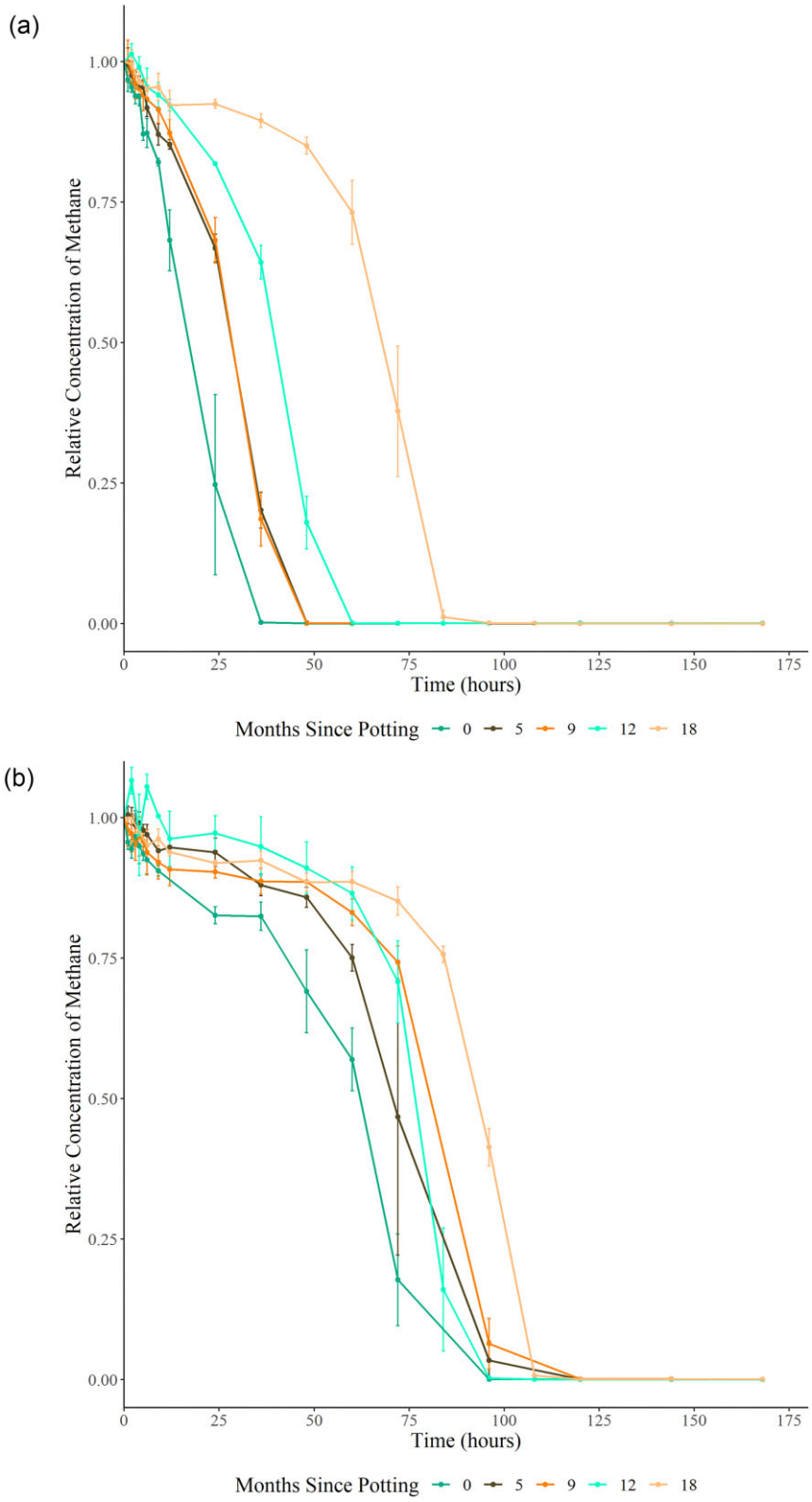

Figure 7. Relative methane concentrations against time from microcosm headspaces. Methane oxidation rates were measured six times as the soil from the initial mesocosms aged. Error bars are ± 1 SE. (a) Methane mesocosm soils. (b) Control mesocosm soils.

the disturbance in soil community appeared to be driven by changes in taxa with low relative abundances. The prediction that exposure to methane would increase the relative abundance of methanotrophs was only true for those carrying the *pmoA* gene and Type I methanotrophs. However, the relative abundance of the *mmoX* genes, which dominated the methanotrophy genes, was higher in the control soils compared to the methane-flushed soils, raising questions about methanotrophic habitat preferences and the interactions between differing groups of methanotrophs. Once perturbed, the soil methanotroph community remained in an altered state for at least 18 months compared to the control soils. Methanotrophic activity was higher following the methane pulse; however, the potential methane oxidizing activity of the community declined, with the lag time increasing and potential methane oxidation rate decreasing, suggesting that recovery to pre-disturbance had started but may take longer than 18 months. The methanotroph population showed a remarkable degree of resilience but it would need to be confirmed whether this, in part, could be explained by the PCR assays amplifying DNA from dormant cells.

Overall, this work has shown that the bacterial community composition, beyond those organisms directly responsible for methane oxidation, is affected by a methane pulse. Further work should include not only methanotrophic community dynamics but also interactions with the broader soil community to understanding the complexity of ecological networks within which methane oxidation occurs. This will improve understanding of potential impacts on other ecosystem services. Furthermore, it highlights the need for research that focuses on the conditions in which the different types of methanotroph are more able to oxidize methane; this will enable a greater understanding of their impact on soil greenhouse gas fluxes.

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Author contributions

Tom Bott (Conceptualization, Formal analysis, Investigation, Writing – original draft), George Shaw (Conceptualization, Funding acquisition, Supervision, Writing – review & editing), and Simon Gregory (Conceptualization, Funding acquisition, Supervision, Writing – review & editing)

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

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Data availability

Data is available on request from the corresponding authors. Sequencing data has been deposited at the NCBI under: PRJNA1132224.

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