Appendix F. Microbial methane oxidation in a blanket peat ditch-blocking experiment: evidence from ^{13}C tracer investigations

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1. Abstract

Peatlands contain the largest biosphere carbon stores in the northern hemisphere and are important regulators of greenhouse gas carbon dioxide (CO_2) and methane (CH_4) emissions. Peatland ditch (grip) blocking in the UK is often assumed to restore characteristic hydrological conditions, reduce aerobic decomposition of peat to CO_2, and enhance carbon storage capacity. The effects of re-wetting on peat-derived CH_4 efflux and the processes that contribute to it are, however, poorly resolved. In this study, we performed a series of laboratory and field experiments to improve understanding of peatland CH_4 production and consumption processes in ditch-drained and blocked-ditch systems. In the first investigation, we used peat samples from the experimental area in the Migneint blanket peatland to examine how ditch-blocking methods, distances from the ditch, and plant functional types affected CH_4 and CO_2 fluxes. We used $^{13}CH_4$ stable isotope tracer techniques to compare the CH_4 oxidation activity of low- and high-affinity methanotrophs in the peat. Our results showed no effects on overall CH_4 fluxes, but ^{13}C isotopic tracer studies revealed higher CH_4 oxidation potential in peat adjacent to reprofiled ditches compared to peat next to control (open) and dammed ditches. In addition, CH_4 oxidation potential increased with distance in the control and dammed ditch treatments, though this pattern was reversed in reprofiled plots. In a second set of tracer studies, we executed two $^{13}CH_4$ isotope pulse-labelling experiments in the field. These were designed to examine differences in CH_4 oxidation between the blocking treatments and in plots with and without *Sphagnum* moss. Results of these field experiments showed that CH_4 oxidation was active across all treatments and in *Sphagnum*-removal and control plots. **The implications of these findings are that neither peat microbial CH_4 production potential nor oxidation were strongly influenced by short term changes in ditch management conditions or by vegetation.**

2. Background, aim, and predictions

2.1 Background

Peatlands contain the largest soil carbon stocks in the northern hemisphere and are important sinks and sources of atmospheric carbon dioxide (CO_2) and net sources of atmospheric methane (CH_4) (**Baird *et al*. 2009**). From the 1950s onwards, many blanket peatlands in the UK were drained using ditches (grips) in an attempt to improve grazing. Over the last decade, concerns regarding the impacts of ditches on peatland hydrological and carbon-balance processes have led to calls for widespread blocking of ditches in an attempt to restore important peatland functions (**Armstrong *et al*. 2009**). Ditch blocking can be expected to raise the water table and thereby reduce aerobic decomposition of peat matter and the release of CO_2 (**Cooper *et al*., 2014**) so that a peatland becomes a smaller net source of atmospheric CO_2 or even a sink. However, a growing number of studies have shown significant increases in CH_4 release following restoration (e.g., **Waddington and Day, 2007**; **Wilson *et al*., 2009**; **Cooper *et al*., 2014**). As CH_4 has a 100-year global warming potential (GWP) about 28 times that of CO_2 (**Myhre *et al*., 2013**), it is important to investigate how different ditch-blocking methods affect CH_4 release to the atmosphere because the increase in CH_4 emissions following ditch blocking may reduce or even negate benefits from a reduction in CO_2 emissions or a net CO_2 uptake (*cf*. **Baird *et al*., 2009**).

Net peatland CH_4 emissions are a balance between microbial methanogenesis and methanotrophy. Methanogenesis (methane production) occurs under anaerobic conditions and is mediated by Euryarchaeota. Peatland organic matter is first fermented by microbes to form organic acids, alcohols, and methylated amines. These molecules are then chemically reduced and altered by microorganisms to form

CH_4, CO_2, and water (e.g., **Topp and Pattey, 1997**). Methanotrophy (methane oxidation) is the process whereby CH_4 is converted to formaldehyde and subsequently to CO_2. This requires oxygen as a final electron acceptor and thus occurs at oxic-anoxic interfaces by Alpha- and Gammaproteobacteria (**Conrad, 1996**; **Edwards *et al*., 1998**). In blanket peatlands, the main abiotic factor influencing the balance of these processes is water-table depth, a change in which will shift the location of the aerobic and anaerobic zones (**Freeman *et al*., 2002**; **Wilson *et al*., 2009**; **Cooper *et al*., 2014**). In peatlands, the upper zone in which the peat is periodically aerated (often called the acrotelm) is the habitat of diverse and active microbial communities involved in the regulation of CH_4 fluxes. For example, peats typically exposed to low levels of CH_4 can host an active community of methanotrophs with high affinities for CH_4 at atmospheric concentrations. When exposed to high levels of CH_4, as might occur with a rise in water table and more anaerobic breakdown of peat, microbes with low affinities for CH_4 can subsequently become more active consumers (**Bender and Conrad, 1992**).

Peatland vegetation type also influences CH_4 emissions (**Levy *et al*. 2012**; **Gray *et al*., 2013**). For example, roots are able to carry oxygen below the water table and thereby enhance CH_4 oxidation activity by methanotrophs. Conversely, the exudation of organic compounds from roots provides substrate for methanogens and may enhance CH_4 production (**Saarnio *et al*., 2004**). In addition, aerenchymatous tissues, present in some vascular plants and particularly some graminoids, can create a conduit for enhanced transport and emission of gaseous CH_4 from the rhizosphere to the atmosphere (**Watson *et al*., 1997**; **Frenzel and Rudolph, 1998**; **Greenup *et al*., 2000**). **Raghoebarsing *et al*. (2005)** showed that, in some waterlogged *Sphagnum* species, bacteria living within the outer cortex can convert CH_4 to CO_2, which can thereafter be sequestered by the moss (i.e., used in photosynthesis). Since then, several studies have shown a loose association of methane-oxidizing bacteria with many *Sphagnum* species which, following photosynthesis, provide a source of oxygen for these bacteria in an otherwise low-oxygen environment (e.g., **Larmola *et al*., 2010**; **Kip *et al*., 2011**; **Van Winden *et al*., 2012**; **Stępniewska *et al*., 2013**). Ericoid shrubs such as *Calluna vulgaris* (L.) Hull*.,* another abundant plant type in UK peatlands, are known to release recalcitrant litter and phenolic exudates in addition to having shallow roots hosting mycorrhizae capable of quickly taking up nutrients near the peat surface, all of which may act to reduce decomposition by microbes (**Jalal *et al*., 1982**; **Read *et al*., 2004**). Ericoids can also shade and thereby reduce rates of photosynthesis in neighbouring plants (**Ward *et al*., 2009, 2013**). The carbon flux may be further modified by changes in the vegetation composition following ditch blocking, with some studies suggesting a shift towards domination by graminoids (cotton grasses, in particular) (e.g., **Cooper *et al*., 2014**). Gaps in our understanding of how the interaction of water table and vegetation type in ditch-blocked peatlands affect CH_4 and CO_2 fluxes clearly need to be addressed.

2.2 Aim and predictions

We examined CH_4 production and oxidation processes in peat sampled from the area between the ditches in the experimental area (see section 2 of the main report and section 2 of Appendix D (**Green *et al*., 2016)**). The 12 experimental ditches were either left as unblocked controls or were blocked using dams (hereafter termed 'dammed') or using a combination of damming and infilling (hereafter termed 'reprofiled'), the latter done using peat scraped from the peatland surface adjacent to the ditches. For the first series of experiments, we collected peat from three distances away from each ditch (edge of ditch – 0 m[1], and 1 and 3 m). We also examined the potential effects of dominant and characteristic peatland plant functional types (i.e., bryophyte, graminoid, and ericoid shrub) on these processes at the same distances from the ditch. This combined approach was used to examine whether effects on water table induced by the ditch management treatment were as influential as the effects of vegetation type on the biotic and abiotic conditions of the acrotelm. A sub-sample of peat from each core sample was then used in three separate laboratory incubation experiments to examine differences in CH_4 production, high-affinity CH_4 oxidation, and low-affinity CH_4 oxidation activities. A stable isotope tracer approach was employed in both of the CH_4 oxidation experiments to determine the conversion of $^{13}CH_4$ to $^{13}CO_2$.

[1] In other parts of the final report, including Appendices C and D '0' is used to denote within-ditch (in the ditch channel). Here it is used to denote the edge of the ditch (upper part of the ditch bank).

For field experiments, we used $^{13}CH_4$ isotopes as tracers to investigate *in situ* CH_4 oxidation. In the first field experiment, using cylindrical collars (702 cm^2) previously installed for the gas flux monitoring (see **Green *et al*. (2016)**, Appendix D), we 'pulsed' a plot adjacent to each of the 12 ditches with $^{13}CH_4$ to quantify variance in methane oxidation amongst and between the ditch-blocking treatments. In a second field experiment, we used $^{13}CH_4$ to pulse intact plots and plots from which *Sphagnum* had been removed, to better understand the roles of *Sphagnum cuspidatum* Ehrh. ex Hoffm. and *Sphagnum fallax* (H. Klinggraff) H. Klinggraff and associated methanotrophs in CH_4 oxidation.

The overarching aim of these experiments was to improve understanding of the biotic processes involved in CH_4 and CO_2 production and consumption/uptake under the different ditch treatments and vegetation types. For the laboratory experiments, due to the theoretically higher water tables and anaerobic conditions in areas between blocked ditches, it was predicted that **(1)** peat cores from areas between blocked ditches would have more active *methanogen* communities and thereby have higher rates of CH_4 production than samples from control (open, unblocked) ditches in the CH_4 production assays, and that the control ditches would have higher rates of CH_4 consumption in the CH_4 oxidation assays due to more active *methanotroph* communities. For the same reasons, we predicted **(2)** there would be higher CH_4 production and lower CH_4 consumption from cores sampled nearer to the blocked ditches in comparison to those sampled further away. We also predicted that **(3)** plants influence microbial communities in the peat leading to different levels of CH_4 production and oxidation under different plant functional types.

For the field experiments, it was predicted that **(4)** areas between blocked ditches would have lower $^{13}CH_4$ oxidation than areas between control or open ditches, again due to higher water-table levels. In the *Sphagnum*-removal experiment, we predicted that **(5)** the association with methane-oxidizing bacteria would lead to higher levels of $^{13}CH_4$ oxidation in plots containing *Sphagnum* than in those with the moss removed.

For a final investigation, we used microbial phospholipid fatty acid (PLFAs) biomarkers in peat samples to characterise microbial communities under each plant functional type at each ditch. A component of living microbial cell membranes, PLFA biomarkers can be characteristic of different taxa and thus provide an insight into the structure of the active peat fungal and bacterial communities that contribute to both CH_4 oxidation and production processes (**Buyer and Sasser, 2012**). We predicted that **(6)** the community composition, as shown in PLFAs, would differ between the different ditch blocking treatments and plant functional types.

3. Materials and methods

3.1 Laboratory incubation experiments

This series of controlled laboratory experiments using samples taken from the experimental site was undertaken to examine whether effects of ditch management and vegetation influenced CH_4 production and consumption processes within the peat.

3.1.1 Field sampling

Samples for the laboratory experiments were collected in early May 2012. At each ditch, peat cores were taken at three distances perpendicularly away from the edge of the ditch (i.e., 0, 1, and 3 m) from under three different dominant plant types (*Sphagnum* sp., *Eriophorum vaginatum* L., and C*alluna vulgaris*), giving a total of 108 cores. The latter two distances match those used in the second set of collars used for the gas flux monitoring (see Appendix D; **Green *et al*. (2016)**). To extract the cores, roots were first cut around the circumference of polyvinyl chloride (PVC) corers (15 cm depth, 5 cm internal diameter) to allow the latter to be inserted to 15cm depth without damaging the peat structure. The whole core was then removed using a

small shovel and pliers. After collection, the cores were sealed in labelled plastic bags and stored in field-moist conditions at 4°C until analysis.

3.1.2 Sample preparation and basic analyses

The peat samples were removed from the PVC tubes and cut to 10 cm depth (from surface). Large roots were removed and individual samples were then weighed (fresh weight) and manually mixed for 30 seconds in plastic bags. A 10 g subsample of each peat sample was taken and dried at 105°C to determine dry weight and bulk density. The dried sample was then milled and analysed for total carbon and nitrogen content using a Truspec C:N analyser (Leco Instruments (UK) Ltd). The remaining fresh peat samples were stored at 4°C prior to use in the CH_4 production and oxidation process experiments.

3.1.3 Methane production assay

This experiment was designed to determine the activity of microbial methanogens in the peat samples, using an approach similar to **Gupta *et al*. (2013)**. To begin, fresh homogenized peat samples were weighed out (10 g) in labelled chambers (400 mL Lock & Lock® containers with self-sealing rubber Suba-Seal® septa in the lids). Fifty mL of deionised water was then added to each container to just cover the peat, creating a peat slurry. The containers were then flushed with nitrogen gas for 20 seconds (to ensure anaerobic conditions), sealed, and a positive pressure created by injecting 40 mL of nitrogen gas through the septum. A headspace gas sample of 10 mL was extracted from each chamber and injected into evacuated 3 mL Exetainer® gas sampling vials (Labco Ltd, UK), representing Day 0. The chambers were then incubated at 14°C (selected to be within the summer seasonal range for the field site) in a controlled temperature room and placed on a 'Lab-Shaker' (Adolf Kühner AG, Switzerland) at 60 rpm, with headspace samples taken again after 1, 3, and 7 days.

3.1.4 High affinity methane oxidation assay

The purpose of this experiment was to examine the activity of methanotrophs in the peat with a high affinity for CH_4. Here, chambers were set up as in the methane production assay (section 3.1.3), but compressed air was used instead of nitrogen for flushing and injecting for positive pressure. Then, 99 atom% $^{13}CH_4$ (Isotech Inc, Ohio, USA) was injected to allow for a headspace concentration of 10 ppm (as used in **Crossman *et al*. (2004)**). The chambers were placed on a Lab-Shaker at 60 rpm in a 14°C controlled temperature room and a 10 mL gas sample was taken at 0, 4, 24, and 48 hours.

3.1.5 Low affinity methane oxidation assay

This assay was designed to examine the activity of methanotrophs with a low affinity for CH_4 by injecting a higher concentration of $^{13}CH_4$ than in the high affinity oxidation experiment. The chambers were set up exactly as in the high affinity experiment but with 99 atom% $^{13}CH_4$ injected for a headspace concentration of 10,000 ppm (as used in **Crossman *et al*. (2004)**). The chambers were placed on a Lab-Shaker at 60 rpm in a 14°C controlled temperature room and a 10 mL gas sample was taken at 0, 1, 3, and 7 days.

3.2 Field ^{13}C pulsing experiments

This series of field experiments used labelled ^{13}C-CH_4 to examine differences amongst the ditch management treatments and to improve understanding of the role of *Sphagnum cuspidatum* and *Sphagnum fallax* in methane oxidation.

3.2.1 $^{13}CH_4$ pulse-labelling of ditch-blocking experiment

We used a novel approach to investigate peatland CH_4 oxidation *in situ* using isotopically labelled $^{13}CH_4$. In this study, we examined and compared oxidation in the three ditch-blocking treatments. A closed static

chamber approach (see that used for similar habitat by **Ward *et al*. (2007)**) was used both for labelling and subsequent measurements of net gas fluxes. Chamber lids (30 cm diameter, 35 cm height, and 19 L volume) consisted of a plastic pipe (10 cm height) and a plastic plant cloche (Haxnicks, UK) fitted with a self-sealing rubber septum for label injection and gas sampling. To conduct tests, we used the pre-installed collars located 1 m away from each ditch (see Appendix D; **Green *et al*. (2016)**) as a base ring and a dark chamber lid, sealed with a rubberized band. To pulse-label the plots, 99 atom% $^{13}CH_4$ (Isotec Inc, Ohio, USA) was transferred from a lecture bottle (a small compressed gas cylinder) to a 0.5 L Tedlar sampling bag (SKC Inc, UK) and 100 mL was subsequently injected into the chamber headspace *via* the rubber septum. After five minutes enclosure time, 10 and 20 mL headspace samples were taken and injected into 3 mL and 12 mL Exetainer® vials (Labco Ltd, UK), respectively. Further samples were taken after 1, 2, 4, and 24 hours. Chamber soil surface temperature measurements were made using HOBO Pendant® data loggers (Onset Computer Corporation, Massachusetts, USA) and air temperature measurements were made using data from the on-site automatic weather station (see section 2 of the main part of the report).

3.2.2 $^{13}CH_4$ pulse-labelling *Sphagnum*-removal plots

Different plant types can play different roles in carbon cycling in peatlands. This experiment was conducted to examine CH_4 oxidation in plots with and without *Sphagnum cuspidatum* and *Sphagnum fallax* using a similar approach to that employed in section 3.2.1. In an area of peatland beyond the experimental area where previously eroded peat has naturally recolonised with *Sphagnum* and other peatland plant species, four pairs of plots containing only *Sphagnum* were selected. At each plot, a PVC pipe collar was inserted 20 cm into the peat with 10 cm remaining above ground level to act as a base ring for pulsing and sampling. After a 24-hour settling period, pre-experimental natural abundance $^{13}CO_2$ isotope samples were collected by placing dark chamber lids with rubberized seals on the base rings and taking 10 and 20 mL gas samples at 0, 15, 30, 45, and 60 minutes. The chamber lids were then removed and the entire *Sphagnum* aboveground biomass was removed from one plot of each pair. Gas sampling was repeated to compare natural $^{13}CO_2$ abundance in plots with and without *Sphagnum* species. Pulse-labelling and subsequent gas sampling were then performed as in section 3.2.1. Temperature measurements were made as in section 3.2.1.

3.3 Microbial phospholipid fatty acid extraction

Microbial phospholipid fatty acid (PLFA) biomarkers were extracted to examine differences in peat bacterial and fungal community structure across ditch-blocking treatments and beneath different plant functional types. In November 2013, peat cores (5 cm diameter × 15 cm deep) were collected from beneath each of the previously-considered plant functional types (i.e., *Sphagnum*, *Eriophorum*, and *Calluna*) at each ditch, giving a total of 36 cores. Peat samples from these cores were then frozen at -20°C until needed for PLFA extraction and analysis. Following a modified **Bligh and Dyer (1959)** method, lipids were first extracted from freeze-dried, finely ground peat samples using combinations of chloroform, methanol, and a citrate buffer (**Frostegård *et al.*, 1991**). Following **Zelles and Bai (1993)**, in the solid phase extraction, lipids were separated using an elution series (neutral lipids via chloroform, glycolipids via acetone, followed by collection of phospholipids from the remaining sample using a methanol eluent). Finally, methanolic KOH was used for mild alkaline methylation of the phospholipids to produce fatty acid methyl esters for analysis. Internal standards included C13 (methyl tridecanoate) and C19 (methyl nonadecanoate).

3.4 Gas concentration calculations, quality control, and statistical analyses

3.4.1 Gas concentration calculations

All gas samples were analysed for CH_4, CO_2, and N_2O by injecting 2.25 mL samples, using an HTA autosampler, into a PerkinElmer Autosystem XL gas chromatograph (GC) with a methaniser, flame ionisation and electron capture detectors, and argon carrier gas. Sample peak areas were calculated and part per million (ppm) concentrations determined using three calibrated gas standard mixtures (BOC

Industrial Gases, UK). If necessary, samples were corrected for instrumental drift. Gas concentration data from the GC were transformed from parts per million to µg CH_4-C or µg CO_2-C g^{-1} hr^{-1} (where g is the dry weight of the peat).

3.4.2 Isotopic analysis

For the CH_4 oxidation incubations (sections 3.1.4 and 3.1.5) and $^{13}CH_4$ field pulsing experiments (sections 3.2.1 and 3.2.2), a subset of vials was analysed for $^{13}CO_2$ at the NERC Stable Isotope Facility at Lancaster. For $^{13}CO_2$ analysis, $\delta^{13}C$‰ values of CO_2 were measured using an Isoprime Ltd Tracegas Preconcentrator coupled to an Isoprime Ltd isotope ratio mass spectrometer. Gas samples were purged from 12 mL Exetainers through a double-needle sampler into a helium carrier stream (20 mL min^{-1}), which is passed through a H_2O / CO_2 scrubber ($Mg(ClO_4)_2$, Ascarite) and a cold trap cooled by liquid nitrogen. The isotope ratio of the resultant CO_2 was compared to pulses of known reference CO_2 (BOC Industrial Gases, UK). The instrument was calibrated on each day of analysis. Blanks were run prior to each analytical batch, in addition to a quality control reference CO_2 standard and duplicate analysis after every 15th sample. Precision was better than or equal to ± 0.2‰.

3.4.3 ^{13}C calculations

Isotopic data were calculated as µg $^{13}CH_4$-C oxidised g^{-1} hr^{-1} in the laboratory incubation experiments and as µg $^{13}CH_4$-C oxidised m^{-2} hr^{-1} in the field-pulsing experiments. Isotope mass balance calculations were used to calculate the $^{13}CH_4$ oxidation rate (**McNamara *et al.*, 2006**):

$$\delta^{13}\mathrm{C}-\mathrm{CH}_{4\ \mathrm{head}} = \frac{(\mu\mathrm{g\ CH_4{-}C_{background}\ x\ }\delta^{13}\mathrm{C_{background}}) + (\mu\mathrm{g\ CH_4{-}C_{label}\ x\ }\delta^{13}\mathrm{C_{label}})}{(\mu\mathrm{g\ CH_4{-}C_{background}} + \mu\mathrm{g\ CH_4{-}C_{label}})} \quad (1),$$

$$\mu\mathrm{g}\ ^{13}\mathrm{C}-\mathrm{CO}_{2\ \mathrm{from\ label}} = \frac{(\delta^{13}\mathrm{C{-}CO_{2\ assay}} - \delta^{13}\mathrm{C{-}CO_{2\ background}}) \times 100 \times \mu\mathrm{g\ CO_2{-}C_{head}}}{(\delta^{13}\mathrm{C{-}CH_{4\ head}} - \delta^{13}\mathrm{C{-}CO_{2\ background}})} \quad (2).$$

Equation (1): δ^{13}C-$CH_{4\ head}$ is the headspace δ^{13}C value of CH_4, µg CH_4-$C_{background}$ is the background mass CH_4-C in the headspace based on atmospheric concentrations and headspace volume, $\delta^{13}C_{background}$ is the estimated δ^{13}C value for background CH_4 levels (-47‰; **McNamara *et al.* (2006)**), µg CH_4-C_{label} is the mass of CH_4-C remaining from the added label (assumed to be the mass of CH_4-C calculated from the measured sample concentration, as natural CH_4 concentrations were negligible in comparison), and $\delta^{13}C_{label}$ is that of 99 atom% $^{13}CH_4$ (8809024‰). Equation (2): µg ^{13}C-$CO_{2\ from\ label}$ is the mass of $^{13}CO_2$ produced from $^{13}CH_4$ oxidation, δ^{13}C-$CO_{2\ assay}$ is the δ^{13}C value obtained for the labelled sample by mass spectrometry, δ^{13}C-$CO_{2\ background}$ is that for background measurements (approximately -9 at 0 hours, changing to -22 at all time-points after 1 hour (Simon Oakley (CEH Lancaster), unpublished data), and µg CO_2-C_{head} is the mass of CO_2 in the headspace (calculated from the measured sample concentration).

3.4.4 Microbial PLFA analysis

Once suspended in hexane, 1 µL PLFA samples were analysed on an Agilent Technologies (UK) 6890 GC equipped with a CP-Sil 5CB fused-silica capillary column (50 m × 0.32 mm internal diameter × 0.25 µm). A daily system check used an external standard consisting of 0.1 µg each of C16:0 (methyl palmitate), C18:0 (methyl stearate), C21:0 (methyl heneicosanoate), and C23:0 (methyl tricosanoate) in 1 µL hexane. Sample PLFA peaks were identified using known relative retention times, calculated as a proportion of the internal standards, and converted to nmol PLFA g^{-1}. Bacterial PLFAs were identified by terminal and mid-chain branched fatty acids (15:0i, 15:0a, 16:0i, 17:0i, 17:0a) or cyclopropyl saturated and monosaturated fatty acids (16:1ω7, 7,cy-17:0, 18:1ω7, 7,8cy-19:0), while fungal PLFAs were identified as 18:2ω6,9. The concentration of total PLFAs was calculated using all identified peaks (**Whitaker *et al.*, 2014**).

3.4.5 Statistical analyses

Data were collated in Microsoft Excel and all statistical analyses were performed using the R software package (http://www.R-project.org/). For the laboratory microcosm incubation experiments, data were tested for normality and square-rooted to improve this (except in the isotopic data from the low affinity oxidation experiment, which were not improved after transformation). Analysis of variance (ANOVA) tests and Tukey's HSD (honest significant difference) post-hoc tests were then run to determine differences in flux based on blocking treatment, distance from the ditch, plant functional type, and factor interactions.

In the analysis for the field ^{13}C pulsed experiments, we chose to perform non-parametric tests which do not require a large sample size or make any distributional assumptions. For the $^{13}CH_4$ pulse of the 12 ditches, the Kruskal-Wallis Rank Sum test was used to determine differences in fluxes based on blocking treatment. For the *Sphagnum*-removal $^{13}CH_4$ pulse, the Wilcoxon Signed Rank test was used to determine differences in fluxes between treatments.

With the PLFA analysis, ANOVA tests were performed to determine differences in PLFA composition based on ditch treatment, plant functional type, and factor interactions.

Whether or not to set significance thresholds such as the often used $p = 0.05$ is the subject of considerable debate. Some argue strongly that a threshold is objective, while others point to the oddity of declaring something as non-significant if p marginally exceeds 0.05 and significant if it just equals or is just below 0.05; they argue that such thresholds are arbitrary (e.g., **Daly *et al*., 1995**) and prefer workers to refer to the p value as the significance probability and to consider the strength of evidence for and against a particular hypothesis. This view contrasts strongly with some web commentators (e.g., https://mchankins.wordpress.com/2013/04/21/still-not-significant-2/; last accessed on 02.02.2016). Generally, we adhere to use of $p = 0.05$ as a significance threshold. However, in keeping with the recommendations of **Daly *et al*. (1995)** we also consider results that might be regarded as being in a 'grey zone' of significance (broadly, $p = 0.05$-0.1).

4. Results

4.1 Controlled incubation experiments

4.1.1 Methane production assay

The results from this experiment and of the ANOVA are shown in Figures 4.1 and 4.2 and Table 4.1. Figure 4.1 shows that there were no significant differences between treatments in terms of CH_4 production. Reprofiled ditches had significantly higher CO_2 production than dammed ditches (1.76 and 1.34 µg CO_2-C g^{-1} hr^{-1}, respectively) and some reprofiled ditch samples from 3 m had high potential rates of CH_4 production, but high variability in these data resulted in a non-significant interaction (see Figure 4.2).

Table 4.1. *ANOVA results for CH_4 and CO_2 fluxes from methane production microcosm assays (bolding indicates a significant difference).*

	CH_4		CO_2	
Factor/Interaction	*F*	*p*	*F*	*p*
Treatment	2.120	0.126	**4.881**	**0.001**
Distance	1.826	0.167	0.435	0.648
Vegetation	0.014	0.986	1.496	0.230
Treatment* Distance	1.078	0.372	0.134	0.970
Treatment*Vegetation	0.736	0.570	0.737	0.569
Distance*Vegetation	0.143	0.966	1.508	0.207

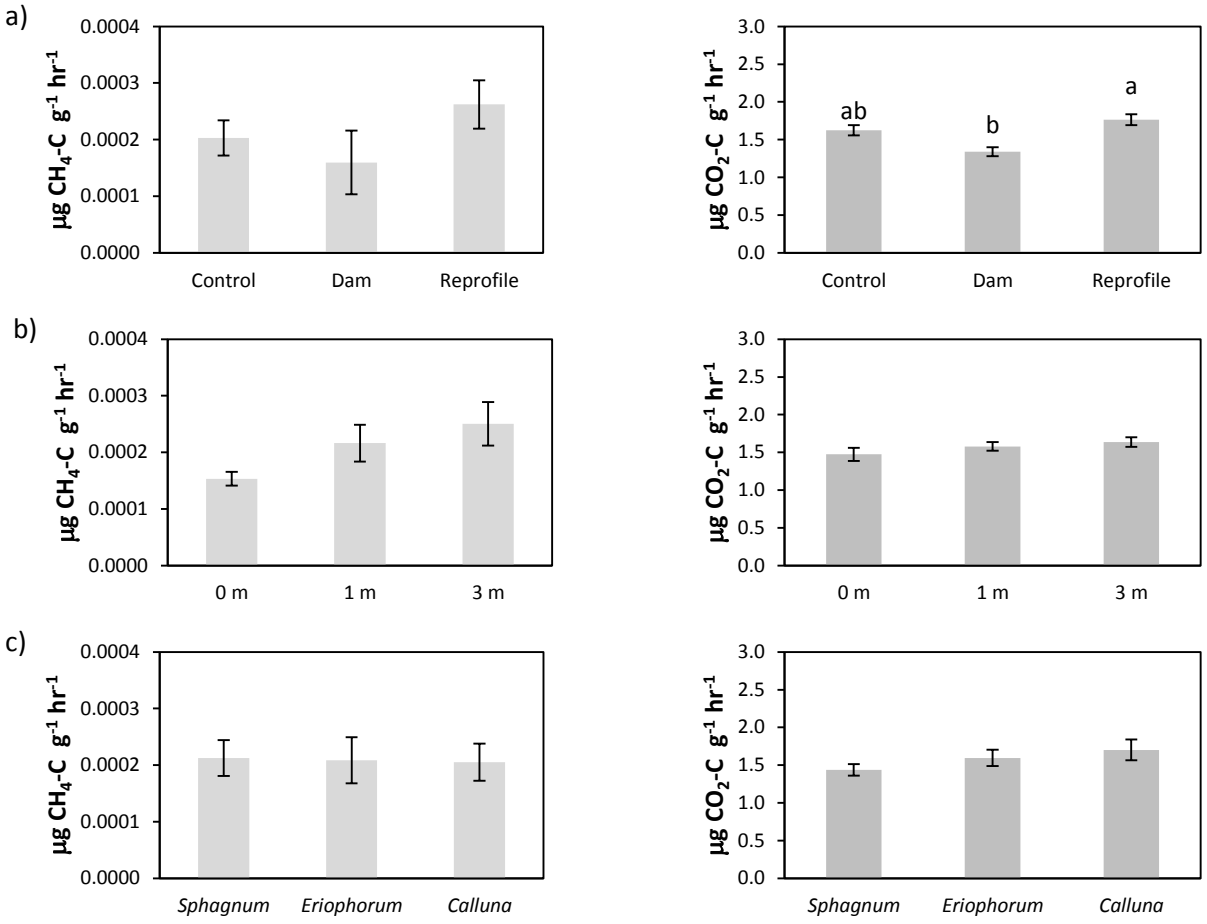

Figure 4.1: *Means (± SE) of CH_4 and CO_2 fluxes (µg C g^{-1} hr^{-1}) from methane production experiment based on a) treatment, b) distance from the ditch (m), and c) vegetation type. Statistically significant differences (*p < *0.05) are denoted using letters.*

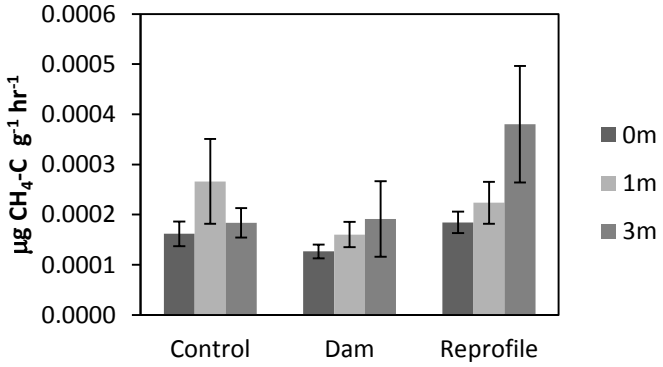

Figure 4.2: *Means (± SE) of CH_4 fluxes (µg C g^{-1} hr^{-1}) from the methane production experiment based on treatment type and distance from the ditch. There were no significant differences at* p < *0.05.*

4.1.2 High affinity methane oxidation assay

Table 4.2 shows the results of the ANOVA for the high affinity methane oxidation experiment. The overall flux results are presented in Figure 4.3. For $^{13}CH_4$ oxidation to $^{13}CO_2$, the samples from the control and reprofiled ditches oxidised more methane than dammed ditches (0.028, 0.026, and 0.021 µg $^{13}CH_4$-C g^{-1} hr^{-1}, respectively), as can be seen in Figure 4.4a. Furthermore, Figure 4.4b shows samples taken furthest from the ditch oxidised more methane (0.028 µg $^{13}CH_4$-C g^{-1} hr^{-1}) than those at 0 m (0.023 µg $^{13}CH_4$-C g^{-1} hr^{-1}) and 1 m (0.024 µg $^{13}CH_4$-C g^{-1} hr^{-1}) from the ditch.

Samples from the reprofiled ditches produced significantly more CO_2 than dammed ditches (2.25 and 1.71 µg CO_2-C g^{-1} hr^{-1}, respectively). On average, samples taken 3 m away from the ditches had higher rates of CO_2 production than those taken at 0 m (2.31 and 1.74 µg CO_2-C g^{-1} hr^{-1}, respectively). Reprofiled ditches and, to a lesser extent, control ditches had high rates of CO_2 production at 3 m, but this interaction was not significant (Figure 4.5). Reprofiled ditches also appeared to produce more CO_2 than dammed ditches in peat samples from each of the vegetation types, although again, this was not statistically significant (Figure 4.5).

Table 4.2. *ANOVA results for high affinity CH_4 oxidation experiment – CH_4 and CO_2 fluxes and conversion of $^{13}CH_4$ to $^{13}CO_2$.*

	CH_4		CO_2		$^{13}CH_4 \rightarrow {}^{13}CO_2$	
Factor/Interaction	*F*	*p*	*F*	*p*	*F*	*p*
Treatment	0.744	0.478	**4.508**	**0.014**	**8.197**	**0.001**
Distance	2.269	0.109	**4.701**	**0.012**	**6.066**	**0.003**
Vegetation	1.212	0.302	1.245	0.293	0.428	0.653
Treatment* Distance	1.043	0.390	1.940	0.111	1.335	0.263
Treatment*Vegetation	1.289	0.280	0.183	0.947	1.742	0.148
Distance*Vegetation	0.078	0.989	1.162	0.333	0.986	0.420

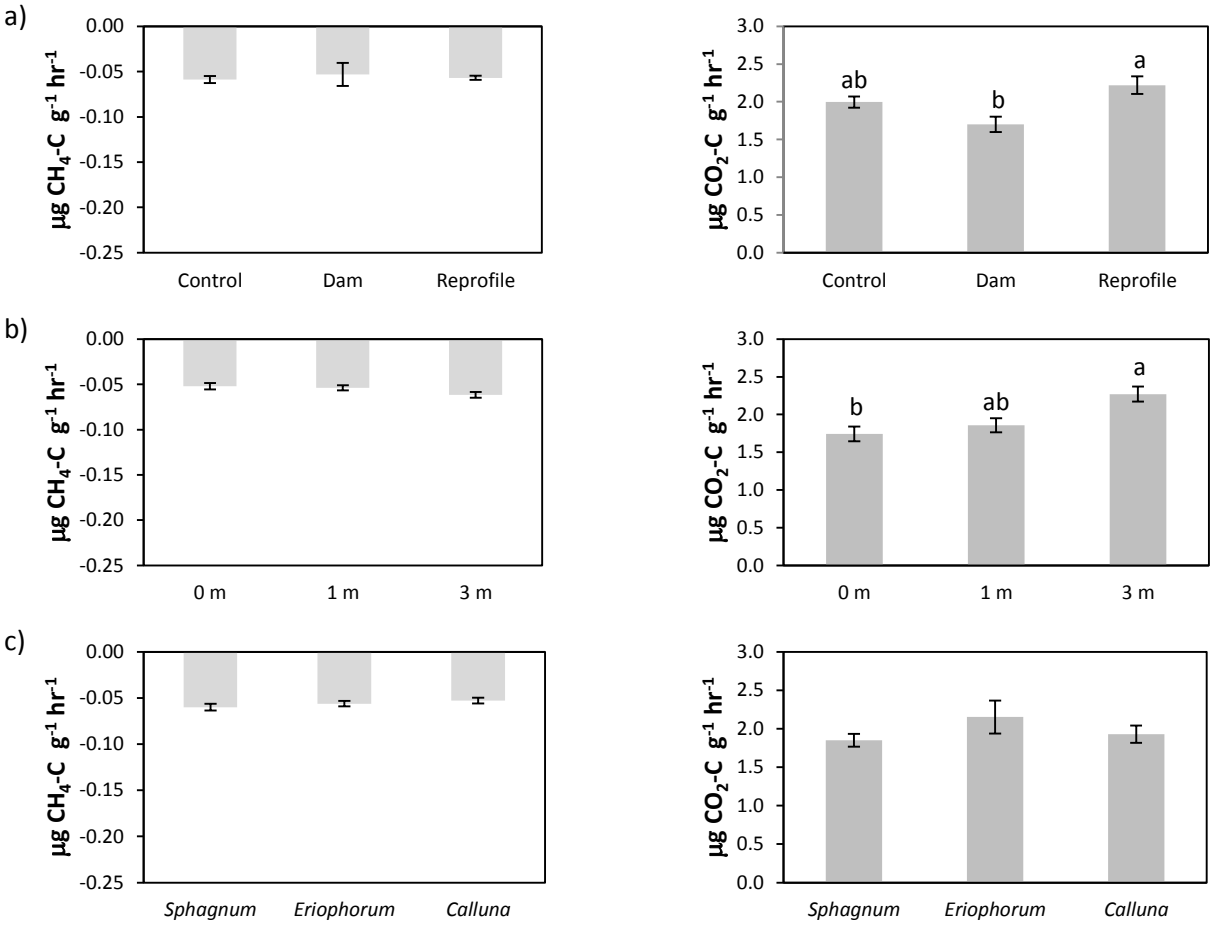

Figure 4.3 *Means (± SE) of overall CH_4 and CO_2 fluxes (µg C g^{-1} hr^{-1}) from the high affinity oxidation experiment based on a) treatment type, b) distance from the ditch (m), and c) vegetation type. Statistically significant differences (p < 0.05) are denoted using letters.*

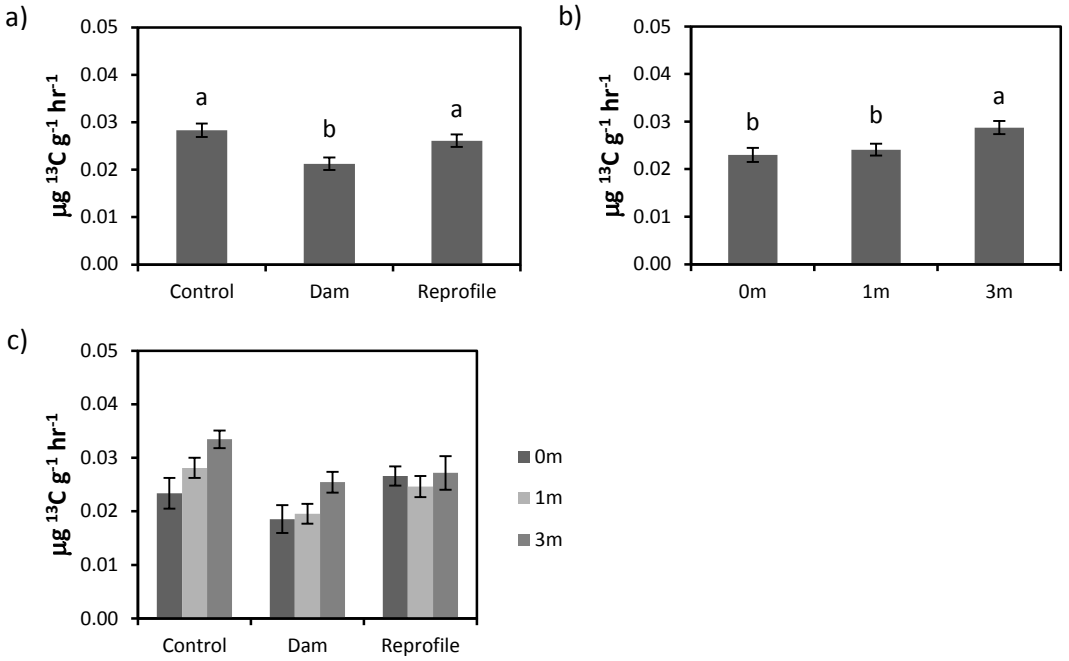

Figure 4.4. *Means (± SE) of $^{13}CH_4$ oxidation (µg C g^{-1} hr^{-1}) from the high affinity methane oxidation experiment based on a) treatment type, b) distance from the ditch, and c) both treatment type and distance from the ditch. Statistically significant differences (*p *< 0.05) are denoted using letters.*

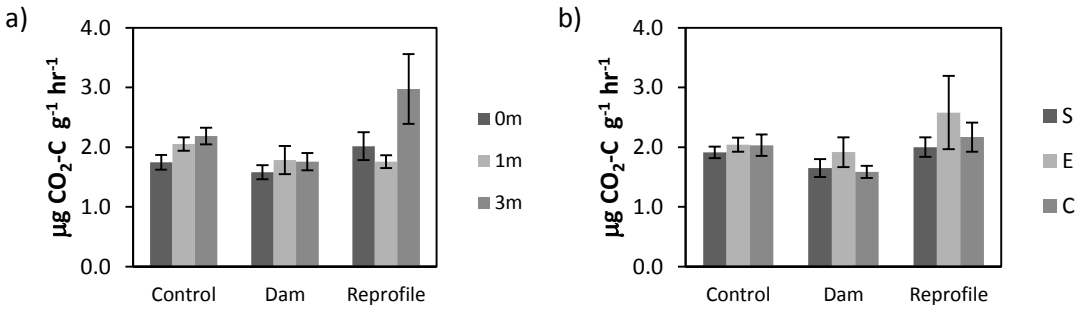

Figure 4.5. *Means (± SE) of overall CO_2 fluxes (µg C g^{-1} hr^{-1}) from the high affinity methane oxidation experiment based on treatment type and a) distance from the ditch and b) vegetation type (where S =* Sphagnum*, E =* Eriophorum*, and C =* Calluna*). There were no significant differences at* p *< 0.05.*

4.1.3 Low affinity methane oxidation assay

The results for overall fluxes and isotopic methane oxidation are presented in Table 4.3. In this experiment, samples taken 3 m from the ditch consumed more CH_4 than those at 0 m and 1 m from the ditch (-0.18, -0.15, and -0.16 µg CH_4-C g^{-1} hr^{-1}, respectively; see Figure 4.6). The response in $^{13}CH_4$ oxidation to both treatment type and chamber distance is shown in Figure 4.7 where the trend for increasing oxidation with increasing distance from the ditch in both control and dammed ditches is reversed in reprofiled ditches (this interaction between treatment and distance was significant – see Table 4.3).

Reprofiled ditches produced more CO_2 than dammed and control ditches (1.65, 1.32, and 1.38 µg CO_2-C g^{-1} hr^{-1}, respectively, $p < 0.05$), but no other significant differences were evident.

Table 4.3: *ANOVA results for low affinity CH_4 oxidation experiment – CH_4 and CO_2 fluxes and conversion of $^{13}CH_4$ to $^{13}CO_2$.*

	CH_4		CO_2		$^{13}CH_4 \rightarrow {}^{13}CO_2$	
Factor/Interaction	*F*	*p*	*F*	*p*	*F*	*p*
Treatment	1.689	0.191	**7.568**	**0.001**	2.066	0.133
Distance	**5.346**	**0.006**	2.163	0.121	1.668	0.195
Vegetation	1.263	0.288	0.626	0.537	0.462	0.632
Treatment*Distance	1.084	0.369	0.949	0.440	**2.864**	**0.028**
Treatment*Vegetation	1.391	0.244	0.347	0.845	1.064	0.380
Distance*Vegetation	0.303	0.875	0.593	0.669	0.943	0.444

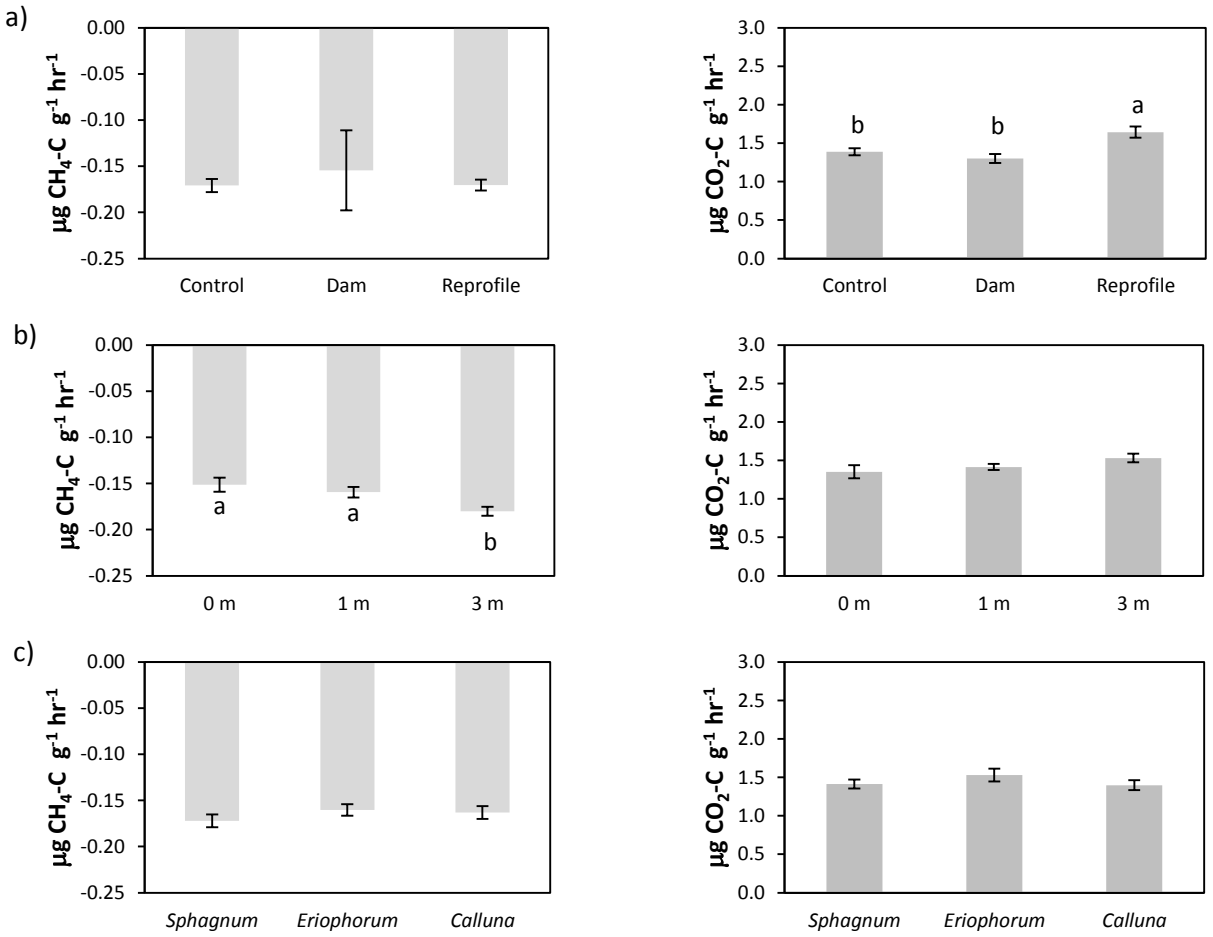

Figure 4.6. *Means (± SE) of overall CH_4 and CO_2 fluxes (µg C g^{-1} hr^{-1}) from the low affinity methane oxidation experiment based on a) treatment type, b) distance from the ditch (m), and c) vegetation type. Statistically significant differences (*p *< 0.05) are denoted using letters.*

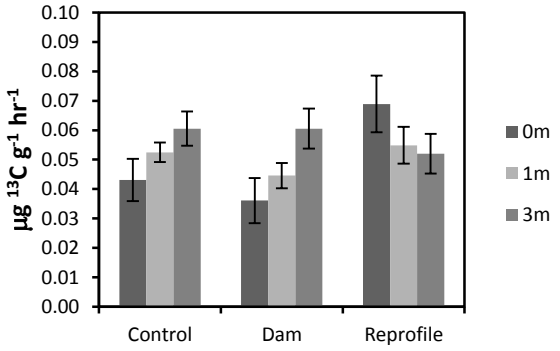

Figure 4.7. *Means (± SE) of $^{13}CH_4$ oxidised (µg ^{13}C g^{-1} hr^{-1}) throughout the low affinity methane oxidation experiment based on treatment type and distance from the ditch.*

4.2 Field pulsing experiments

4.2.1 $^{13}CH_4$ pulse-labelling of ditch-blocking experiment

The results for $^{13}CH_4$ oxidation rates in this experiment can be seen in Figure 4.8. There was high variation and no significant difference amongst fluxes for each ditch type. Highest variance in methane oxidation rates were observed adjacent to reprofiled treatment ditches. Some plots showed evidence of leakage (i.e., rapid declines in added $^{13}CH_4$ concentrations accompanied by small or negligible changes in CO_2 concentrations were taken to reflect chamber atmosphere leakage – beneath the collars); following their removal from the dataset, results again showed high variation and no significant differences amongst fluxes.

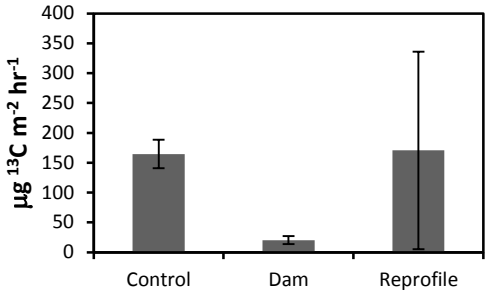

Figure 4.8. *Means (± SE) of $^{13}CH_4$ oxidised (µg ^{13}C m^{-2} hr^{-1}) for each ditch-blocking treatment after pulse labelling in the field. There were no significant differences at* $p < 0.05$.

4.2.2 $^{13}CH_4$ pulse-labelling *Sphagnum*-removal plots

The removal of the moss led to an overall decline in CO_2 fluxes, although this was only marginally significant (Figure 4.9). No significant change was observed for CH_4 fluxes (Figure 4.9). Figure 4.10 shows an apparent trend for reduced CH_4 consumption in bare peat plots following labelling, although this is not significant. It also shows no significant treatment effect in results for the oxidation of $^{13}CH_4$ following pulse-labelling. As shown in Table 4.4, CH_4 oxidation was higher in bare plots in three of the four plot pairs, but high variation in the results meant this effect was not significant.

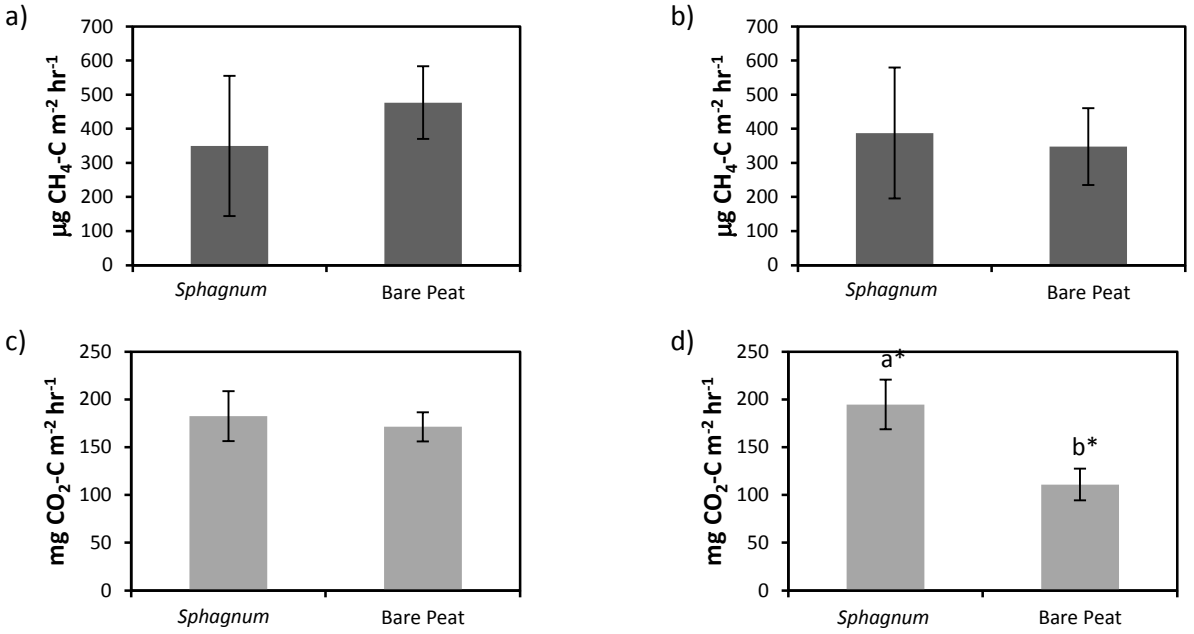

Figure 4.9. *Means (± SE) of CH_4 flux (µg C m^{-2} hr^{-1}) and CO_2 flux (mg C m^{-2} hr^{-1}) in plots a) and c) under natural conditions and b) and d) after* Sphagnum *removal (i.e., 'Bare Peat' in a) and c) denote the bare peat treatment before* Sphagnum *removal). There were no significant differences at* p *< 0.05; *however, d) was marginally significant at* p *= 0.057.*

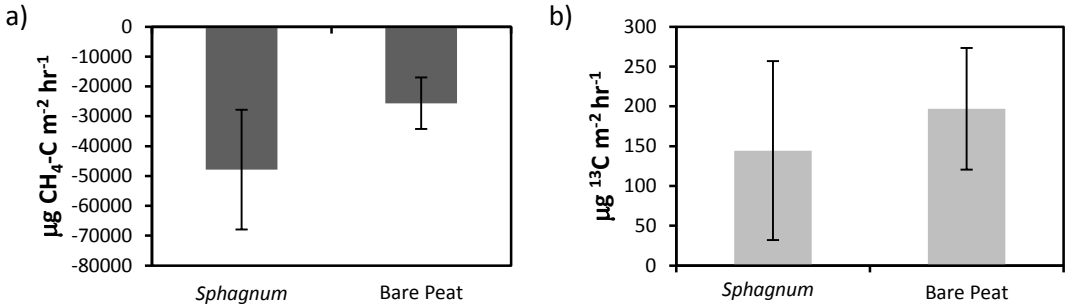

Figure 4.10. *Means (± SE) of a) overall CH_4 flux (ug CH_4 m^{-2} hr^{-1}) and b) $^{13}CH_4$ oxidised (ug ^{13}C m^{-2} hr^{-1}) for plots with* Sphagnum *or bare peat after pulsing in the field. There were no significant differences at* p *< 0.05.*

Table 4.4. *$^{13}CH_4$ oxidation rates (µg ^{13}C m^{-2} hr^{-1}) in paired plots following pulse-labelling in the field.*

	Sphagnum	Bare Peat
Pair 1	478.27	414.12
Pair 2	77.33	103.45
Pair 3	20.63	193.04
Pair 4	1.28	76.89

4.3 Phospholipid fatty acid analysis

The purpose of this analysis was to examine broad differences in PLFAs between the ditch treatments and plant functional types, namely in terms of total PLFAs and the ratio of fungal to bacterial PLFAs. The results are presented in Figures 4.11 and 4.12; however, no significant differences were found.

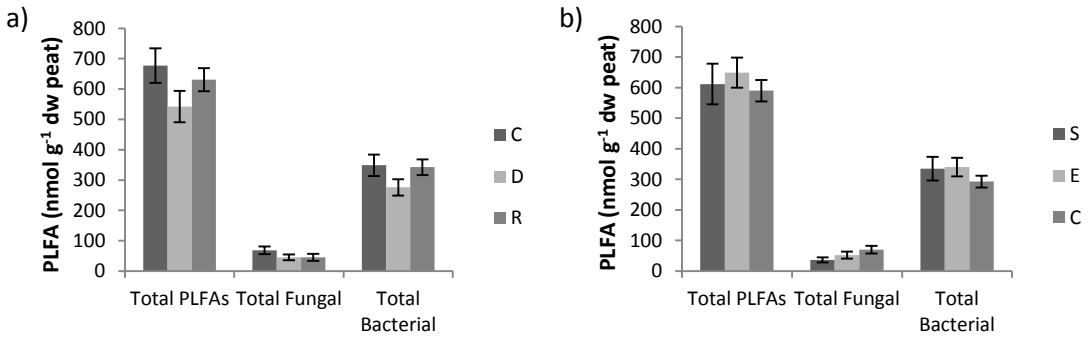

Figure 4.11. *Means (± SE) of total, total fungal, and total bacterial PLFAs based on a) treatment type (where C = control, D = dammed, R = reprofiled ditches) and b) plant type (where S =* Sphagnum*, E =* Eriophorum*, C =* Calluna*). There were no significant differences at* $p < 0.05$.

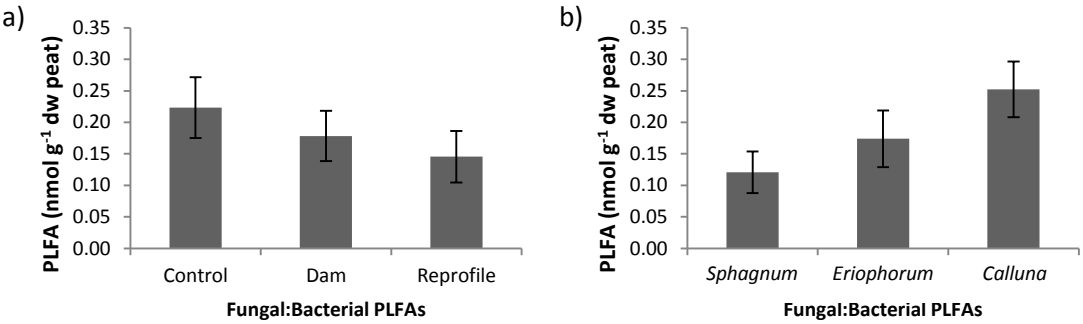

Figure 4.12. *Means (± SE) of fungal:bacterial PLFAs based on a) treatment type and b) plant type. There were no significant differences at* $p < 0.05$.

5. Discussion

5.1 Incubation experiments

Contrary to prediction (1), samples taken from peat between blocked ditches did not show higher CH_4 production potential than those from control ditches, nor were there differences in overall CH_4 fluxes in the oxidation assays. The only significant result in the methane production experiment was that peat from reprofiled ditches had 1.31 times higher CO_2 production than dammed ditches. This pattern was repeated in the high affinity methane oxidation assay, which also revealed higher methane oxidation in samples from reprofiled and control ditches in comparison to dammed ditches (1.24 and 1.33 times, respectively). Furthermore, in the low affinity methane oxidation assay, reprofiled ditches showed higher CO_2 production than both the dammed and control ditches (1.25 and 1.20 times, respectively). Thus, prediction (1) cannot be accepted, but together, the results demonstrate overall that the reprofiled ditches have a higher potential for methane oxidation than the dammed ditches and, perhaps to a lesser extent, the control ditches. It is unclear why this may be, and it is notable that reprofiled ditches did not show lower methane production, because aerobic conditions are considered to inhibit methanogenic archaea more than anoxia does methane oxidizers (**Freeman *et al.*, 2002**).

For samples taken at different distances from the ditch, it was predicted that in blocked ditches those closer to the ditch would have higher CH_4 production and lower CH_4 oxidation due to the raised water table created by blocking. In the methane production assay, samples taken 3 m from the ditch appeared to produce more CH_4 than those closer to the ditch (Figure 4.1b), but Figure 4.2 revealed that this was caused by high variation in fluxes from reprofiled ditches and it can be concluded that there was no significant effect of distance from the ditch. However, in the high affinity oxidation experiment, we can see that samples taken 3 m from the ditch had 1.22 times higher CH_4 oxidation (as demonstrated from the isotope tracers) and 1.30 times higher overall CO_2 production than those taken at 0 m (the ditch edge). In the low affinity oxidation experiment, samples from 3 m away also had 1.20 and 1.14 times greater overall CH_4

consumption than those from 0 m and 1 m, respectively, as demonstrated by the most negative overall CH_4 flux in Figure 4.6b. There is also a significant interaction effect seen in the low affinity oxidation assay (Table 4.3), where this pattern of higher methane oxidation in samples taken 3 m from the ditch is seen in both control and dammed plots, but reversed in reprofiled plots. Overall, our results demonstrate that microbial communities further from the ditch may exhibit more methanotrophic activity than those close to the ditch, which is in accordance with our prediction (2). However, the results of the low affinity CH_4 oxidation assay demonstrate that this may not be the case for reprofiled ditches. Our prediction was based on the assumption that the water table would be closer to the surface next to the ditch when blocked. However, the analysis of the water table data from wells placed at 1 and 3 m from the ditches (see Appendices C and D; **Holden *et al*. (2016)**; **Green *et al*. (2016)**, showed that there weren't any significant differences between the two distances for any of the blocking treatments. Therefore, while prediction (2) may be partially accepted, an explanation for our observations remains elusive. Further analysis of the 1 m and 3 m water table data in the months preceding the peat sampling may help shed light on any physical reasons for the differences in methanotrophy observed.

The influence of vegetation on rhizospheric conditions of the peat can be expected to have an effect on microbial communities, including those involved in methanogenesis and methane oxidation. In our laboratory incubation assays, there was no evidence of this influence in our CH_4 flux or isotope tracer results. One reason for this may be due to the sampling method in the field, where peat cores were taken from beneath small patches of each vegetation type. In many of the studies examining the effects of plant functional types, the plot compositions (whether individual species or a mixture) are created and left to adapt to treatments over time. For example, **Ward *et al*. (2009)** waited approximately 21 months after establishing a plant-removal experiment before taking gas measurements. Other studies of plant function examine vegetation in isolation; for example, **Raghoebarsing *et al*. (2005)** examined methanotrophs in *Sphagnum* from a peatland by first washing the moss thoroughly to remove any extra organic matter and debris. In this study, the cores were taken from intact areas of peat and there was no manipulated exclusion of the other plant species from the samples. It is thus possible that at any given sampling location (i.e., each ditch and distance from ditch) the roots of other species have influenced the samples, making them all similar to each other in that regard. Furthermore, most studies examining the effects of plant functional types include the plants in their study (e.g., **Greenup *et al*., 2000**; **Ward *et al*., 2009**; **Green and Baird, 2012**; **Cooper *et al*., 2014**), whereas we examined only the peat. Therefore, it could be expected that the differences in fluxes would not be as evident as when the system and plants are left intact. Whatever the reasons, in the case of this study, prediction (3) cannot be accepted.

5.2 Field pulsing experiments

5.2.1 $^{13}CH_4$ pulse-labelling of ditch-blocking experiment

The aim of this experiment was to examine *in-situ* CH_4 oxidation from the three blocking treatments (i.e. control (open), dammed, and reprofiled) using a ^{13}C pulse-labelling method that, to our knowledge, has not been previously performed in blanket peatlands. We found no significant differences in CH_4 oxidation rates between the blocking treatments. There are a number of possible explanations for this observation. First, it is well known that vegetation composition plays a critical role in peatland carbon dynamics. Following ditch blocking, *Eriophorum* is a common colonizing species, although this may change over longer time scales (**Cooper *et al*., 2014**). The collars all contained varying compositions of *Sphagnum, Calluna,* and *Eriophorum*. Differences in plant functional type composition likely played a role in the observed oxidation potentials. As well, the sample size was small (n = 12) as we performed labelling on one chamber from each between-ditch area. Finally, despite having positive linear CO_2 fluxes, the fluxes from four chambers were far lower than the other eight, leading us to conclude that they had leaked. Thus, when excluding one such chamber from each blocking type, comparison using Kruskal-Wallis Rank Sum test revealed no significant differences, possibly due to an insufficient sample size (n = 9) and variability in the data. Still, upon looking at the individual results for the well-sealed chambers, there may have been a tendency for control or reprofiled ditches to have higher CH_4 oxidation activity than dammed ditches but such a suggestion in the data can only be tested using additional investigations with greater replication and a larger sample size.

5.2.2 $^{13}CH_4$ pulse-labelling *Sphagnum*-removal plots

The purpose of this experiment was to further understand the role of *Sphagnum* in methane oxidation by using our $^{13}CH_4$ pulse-labelling method in the field. Our results showed a net emission of CH_4 from all intact plots. There was marginally significant (see section 3.4.5) decline in CO_2 flux following moss removal, as would be expected with a loss of respiring plant biomass. Pulse-labelling revealed no differences in $^{13}CH_4$ oxidation between treatments, which may largely be explained by high variation within treatments. High variation may have been caused by differences in water table depth, which has been shown to be the main factor controlling methanotrophic activity in *Sphagnum* (**Larmola *et al*., 2010**). The following interpretations have been made considering the limitations of the experimental setup. Although we found no overall significant effect of *Sphagnum* removal, there was substantially lower overall methane consumption (i.e., a less negative flux) in the removal plots, suggesting some level of methanotrophic activity occurred in the *Sphagnum* layer. We predicted (5) that moss removal would also remove *Sphagnum*-associated methanotrophs from plots and thus lead to lower CH_4 oxidation rates. However, there was no significant difference in calculated oxidation rates between plots and, when looking at values obtained for the paired plots, there was, if anything, the reverse effect (see Table 4.4). Isotope mass balancing took into account background levels of CO_2 fluxes when $^{13}CH_4$ oxidation rates were calculated (CH_4 fluxes were not included to simplify calculations – including them changed the final results by <0.01%). Assuming respiration was higher in the intact plots due to the presence of the moss, this may have diluted the system to produce artificially low rates of CH_4 oxidation in these plots. The degree to which methanotrophs in the *Sphagnum* are contributing to the CH_4 balance in this system does not appear to be any greater than the peat alone and thus prediction (5) is only partly accepted.

5.3 Phospholipid fatty acid analysis

PLFA biomarkers can be useful tools in comparing microbial communities in soils; however, our samples showed no significant differences in overall community structure and prediction (6) cannot be accepted. The reason for this might be explained in the same way as prediction (3), in that our peat samples were taken from beneath the plant functional type of interest, but these were in natural conditions with no root exclusion of other species prior to sampling. Therefore, it is possible that the samples were all somewhat similar to each other. Perhaps if plots had been created with plant removals, significant differences would have been seen between the plant types. A further step in PLFA analysis that could be useful in future studies would be to examine PLFAs following stable ($^{13}CH_4$) isotope probing (PLFA-SIP). The incorporation of labelled ^{13}C into methanotrophic PLFAs in cell membranes has been used in various studies and is a more useful tool in identifying which taxa and *types* of methanotrophs are active in the system (**Crossman *et al*., 2004**; **Chen *et al*., 2008**). Given that the incubation assays presented in this study showed a suggestion of higher oxidation in reprofiled ditches, PLFA-SIP might reveal differences in methanotrophic communities between the ditch-blocking treatments. However, given the results of our experiments, it is likely that variation in PLFAs across the landscape is greater than that between the treatments.

6. Conclusions

A series of laboratory and field assays were undertaken in an effort to improve understanding of the processes involved in CH_4 production and oxidation under different ditch-blocking treatments in a blanket peatland. Our overall CO_2 and CH_4 flux results were of the order of magnitude expected from this type of environment. Results suggest that there was some evidence that peat microbial communities associated with reprofiled ditches behave differently from those in peat adjacent to open (control) and dammed ditches, acting overall to oxidize more methane. Control ditches also showed some weak evidence of higher CH_4 oxidation rates than dammed ditches. However, these differences were small and insignificant in comparison to the spatial variability and differences in topography and nutrient status over the greater blanket peatland landscape. Another point to consider is a matter of timescale. The blocking took place just over 14 months before our first set of sampling. Although this is sufficient time for the system to begin

adapting to blocking, changes to the peatland (e.g., vegetation shifts) will likely continue for decades (**Cooper *et al.*, 2014**), making it difficult to predict changes in the balance of methanogenic and methanotrophic microbial communities in the future. **The implications of these findings are that neither peat microbial methane production potential nor oxidation were strongly influenced by short term changes in ditch management conditions/vegetation.**

7. Acknowledgements

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