Microbial regulation of biogeochemical cycles: evidence from a study on methane flux and land-use change

Loïc Nazaries [a,b], Yao Pan [c,d], Levente Bodrossy [e], Elizabeth M. Baggs [f], Peter Millard [g], J. Colin Murrell [h], Brajesh K. Singh [a,#]

Supplementary section

Materials and methods

Field site description

Bad à Cheo is situated near Thurso, in Northern Scotland, along the A9 (national grid reference ND169503; 58°25'47.35"N, 3°25'48.27"W) and is part of the Rumster forest. It had been the subject of detailed hydrochemical studies (1). The study site is composed of an open bog of deep blanket peat dominated by a mixture of peat moss (*Sphagnum* spp.), deergrass (*Trichophorum cespitosum*) and cotton-grass (*Eriophorum* spp.). Adjacent to the bog, experimental forestry plots were drained, ploughed and planted in 1968 and 1988 with a mixture of Sitka spruce (*Picea sitchensis*) and lodgepole pine (*Pinus contorta*) (2). Therefore, at the time of this study, the young and old pine forests were about 20- and 40-years old, respectively. Because of the age difference, the younger pine forest lay on much wetter ground compared to the older pine forest. Also, at the time of sampling, natural colonisation of the bog by conifers was observed.

The Glensaugh Research Station is part of the James Hutton Institute (formerly known as the Macaulay Land Use Research Institute) and is located in Laurencekirk, Aberdeenshire, Scotland, UK (national grid reference NO671782; 56°53'55.91"N, 2°33'0.28"W). The study site is an agroforestry plot that was used to study experimental planting and grazing management between 1988 and 2001 (Glensaugh Agroforestry Demonstration,

http://www.macaulay.ac.uk/aboutus/researchstations/agroforestry.html). The site is a pasture occupied by ewes and lambs with regular fertiliser applications during each grazing season. In 1988, a subplot was planted with Scots pine (*Pinus sylvestris*). Therefore, the pine forest was about 20-years old at the time of this study.

The Craggan forest is also described elsewhere (3, 4). It is located in Moray, near the Spey River, Scotland, UK (national grid reference NJ190322; 57°22'20.50"N, 3°20'45.42"W) and was originally used in 1978 in a study testing the durability of changes caused by *Betula* spp. on moorland (3). Open *Calluna*-dominated moorland is adjacent to a natural chronosequence of birch trees (*Betula* spp.) aged about 53, 62 and 88 years. During the colonisation phase, heather (*Calluna vulgaris*) was replaced by wavy hairgrass (*Deschampsia flexuosa*) and bilberry (*Vaccinium myrtillus*) before long-term establishment of birch woodland (5, 6). For our study, soil samples were taken from the 62-year-old stand (young birch forest) and the 88-year-old stands (old birch forest) only. It is worth noting that the moorland was cleared of trees in 1974 but has since been progressively naturally colonised by birch. Also, due to the old age of the 88-year-old stands, few trees were left standing and alive, with mainly colonial bentgrass (*Agrostis capillaris*) present as understorey vegetation. The Craggan site was situated on the slope of a hill.

The Tulchan Estate is described in more detail elsewhere (3, 5). Briefly, the study site is located on the Tulchan Estate, Speyside, Scotland, UK (national grid reference NJ154373; 57°24'42.78"N, 3°26'28.65"W). The site contains a natural heather moorland-birch woodland chronosequence. The open *Calluna*-dominated moorland is adjacent to two stands of birch trees (*Betula pubescens*) following natural invasion of the heathland in *ca.* 1953 (young birch forests, 55-year-old) and *ca.* 1943 (old birch forests, 65-year-old) (7). Like in Tulchan, similar changes in vegetation occurred (5).

Table S1. Sites and land uses for this study.

For each land use, there was n=4 replicates per seasonal sampling. N/A means non-applicable.

Site	Land use	Age of the forest	National grid reference (GPS coordinates)
Bad à Cheo	Bog Young Pine Old Pine	N/A 20 years 40 years	ND169503 (58°25'47.35"N, 3°25'48.27"W)
Glensaugh	Grassland Young Pine	N/A 20 years	NO671782 (56°53'55.91"N, 2°33'0.28"W)
Craggan	Moorland Young Birch Old Birch	N/A 62 years 88 years	NJ190322 (57°22'20.50"N, 3°20'45.42"W)
Tulchan	Moorland Young Birch Old Birch	N/A 55 years 65 years	NJ154373 (57°24'42.78"N, 3°26'28.65"W)

Soil sampling

The sampling procedure was the same for each site and similar to a method used in a previous study (8). In brief, stainless steel rings (10 cm diameter, 0-10 cm depth) were used to extract soil cores after removal of the L and FH layers. For each site, twelve replicates per habitat were sampled at random, and were randomly grouped in four sets of three cores for measurement of net CH_4 fluxes (see below). Therefore, n=4 for each habitat, for each site, for each season (total n=176). Within a few hours of sampling, the soil cores were taken to the laboratory and left overnight in an environment-controlled chamber (minimum 70% humidity). For each seasonal experiment, the temperature of the chamber was set using a value close to the air temperature of the site at the time of sampling: 5°C in winter, 10°C in spring, 15°C in summer and 20°C in autumn. The following day, measurements of net CH_4 fluxes were performed and the soil cores were then stored at 4ºC.

Also, during summer, smaller intact cores (5 cm diameter, 0-5 cm depth) were taken in triplicate from each habitat from each site for bulk density, porosity and water retention analysis (total n=33).

Soil analyses

Field-moist 5.6-mm sieved soils were extracted with 1 M KCl for one hour and extracts were analysed colorimetrically for mineral N (NH_4^+-N and NO_3^--N). Moisture content was measured after drying the fresh soil samples in an oven at 105ºC overnight. Fresh soils were dried at 30ºC and then dry-sieved through a 2 mm-mesh sieve for pH and particle size analysis. pH was measured in water after mixing thoroughly the soil water slurry (1:2.5 suspension) for 30 minutes. Particle size distribution analysis was performed using laser diffraction on a Malvern Mastersizer 2000 particle size analyser fitted with a Malvern Hydro 2000G sample dispersion tank (Malvern, UK). A sub-sample of the dry-sieved soils was

83 milled (Retsch mill, 5 minutes at 60 strokes per second) for subsequent use for total C and N
84 analysis by combustion in a Thermo-Finnigan Elemental Analyser (FlashEA 1112 Series).
85 Small soil cores (5 cm diameter, 0-5 cm depth) were dried using hanging water columns for
86 measurement of soil bulk density, porosity and WFPS. The field-moist cores were saturated
87 with water and gravimetric soil water content was estimated after equilibrating the cores at
88 10, 50, 100 and 150 kPa suction pressures. WFPS was estimated as the ratio of the volumetric
89 soil moisture content to the total pore space, or porosity. Porosity was estimated to be
90 equivalent to the volumetric water content at water saturation. Volumetric water content was
91 calculated as the product of the gravimetric water content and the bulk density. Bulk density
92 corresponded to the oven-dry soil weight (105°C overnight) divided by the core volume.
93 Table S2 provides some of the physico-chemical properties of the soils under the different
94 habitats.
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Table S2. Chemical and physical soil properties.

The data are means ± s.e.m. (n=8 replicates – autumn and summer combined, except for bulk density, porosity and WFPS (n=4 – summer only)) of each habitat. For each soil characteristic, Greek letters (α, β, γ) indicate statistical differences between habitats within each site, according to multiple pairwise comparison ($P<0.05$).

Site	Habitat	pH	Total C ($g.kg^{-1}$)	Total N ($g.kg^{-1}$)	C:N ratio	NH_4^+-N ($mg.kg^{-1}$)	NO_3^--N ($mg.kg^{-1}$)	Moisture (%)	Particle size (% of total)			Bulk density ($g.cm^{-3}$)	Porosity (%)	WFPS at field capacity (at 50 kPa) (%)
									0.02-2.00 µm	2-20 µm	20-2000 µm			
Bad à Cheo	Bog	3.6±0.04 $^{\alpha}$	94±3	3.2±0.1 $^{\alpha}$	30±1	154±15 $^{\alpha}$	167±16	87±1 $^{\alpha}$	1.5±0.32 $^{\alpha}$	21±3 $^{\alpha}$	77±3 $^{\alpha}$	0.39±0.07	85±3	76±6
	Young Pine	3.5±0.04 $^{\beta}$	90±2	2.9±0.2 $^{\alpha\beta}$	31±1	89±7 $^{\beta}$	142±12	88±0 $^{\alpha}$	0.38±0.14 $^{\beta}$	10±2 $^{\beta}$	90±2 $^{\beta}$	0.25±0.04	91±2	71±8
	Old Pine	3.2±0.07 $^{\gamma}$	92±1	2.7±0.07 $^{\beta}$	34±1	81±7 $^{\beta}$	132±16	79±1 $^{\beta}$	0.40±0.17 $^{\beta}$	10±2 $^{\beta}$	90±2 $^{\beta}$	0.34±0.03	87±1	66±6
Glensaugh	Grassland	4.2±0.05 $^{\alpha}$	5.9±0.93 $^{\alpha}$	0.58±0.08 $^{\alpha}$	10±0 $^{\alpha}$	30±4 $^{\alpha}$	131±20	32±1	3.8±0.38	34±1 $^{\alpha}$	63±2 $^{\alpha}$	1.39±0.06	48±2	72±6
	Young Pine	3.9±0.04 $^{\beta}$	3.9±0.26 $^{\beta}$	0.36±0.03 $^{\beta}$	11±0 $^{\beta}$	17±1 $^{\beta}$	148±19	30±1	4.6±0.31	39±1 $^{\beta}$	56±2 $^{\beta}$	1.57±0.12	41±5	59±3
Craggan	Moorland	3.4±0.03	76±5 $^{\alpha}$	2.9±0.2 $^{\alpha}$	27±1 $^{\alpha}$	83±10 $^{\alpha}$	71±10 $^{\alpha}$	79±1 $^{\alpha}$	1.8±0.27	20±3	78±4	0.36±0.07	87±3	60±3 $^{\alpha}$
	Young Birch	3.4±0.04	45±7 $^{\beta}$	2.0±0.3 $^{\beta}$	22±1 $^{\beta}$	105±12 $^{\alpha\beta}$	35±4 $^{\beta}$	66±2 $^{\beta}$	3.5±0.42	36±3	60±3	0.38±0.01	86±1	69±2 $^{\alpha\beta}$
	Old Birch	3.4±0.01	63±5 $^{\alpha}$	3.0±0.3 $^{\alpha}$	22±1 $^{\beta}$	116±13 $^{\beta}$	37±6 $^{\beta}$	70±1 $^{\beta}$	2.9±0.69	33±8	64±8	0.49±0.05	82±2	72±1 $^{\beta}$
Tulchan	Moorland	3.5±0.03 $^{\alpha}$	79±4 $^{\alpha}$	3.2±0.2 $^{\alpha}$	25±1 $^{\alpha}$	84±13 $^{\alpha}$	131±19 $^{\alpha}$	85±1 $^{\alpha}$	0.55±0.17 $^{\alpha}$	11±2 $^{\alpha}$	88±2 $^{\alpha}$	0.26±0.02 $^{\alpha}$	90±1 $^{\alpha}$	64±3
	Young Birch	3.7±0.04 $^{\beta}$	6.6±0.82 $^{\beta}$	0.36±0.05 $^{\beta}$	19±1 $^{\beta}$	30±4 $^{\beta}$	23±3 $^{\beta}$	37±2 $^{\beta}$	3.9±0.05 $^{\beta}$	28±3 $^{\beta}$	68±3 $^{\beta}$	0.99±0.08 $^{\beta}$	63±3 $^{\beta}$	62±5
	Old Birch	3.6±0.03 $^{\alpha\beta}$	11±4 $^{\beta}$	0.51±0.15 $^{\beta}$	19±2 $^{\beta}$	38±5 $^{\beta}$	33±7 $^{\beta}$	42±3 $^{\beta}$	3.1±0.45 $^{\beta}$	24±2 $^{\beta}$	73±2 $^{\beta}$	1.48±0.22 $^{\beta}$	44±8 $^{\beta}$	62±9

Gas fluxes measurements

Headspace gas samples were taken under dim light using closed PVC chambers (~9 L) fitted with a gas sampling tube and a 3-way tap. Out of the twelve replicates from each habitat, three soil cores per chamber were used, so for each habitat n=4. Before starting any measurements, the soil cores were unwrapped and left in the open chamber for 2-3 hours. Immediately after locking the lid of the chambers (T_0), air (12 mL) was sampled from the chamber's headspace using a plastic syringe fitted with Luer lock (Fisher Scientific, UK) and 3-way tap, and quickly injected into a pre-evacuated 12-mL glass Exetainer (Labco Ltd, UK) using a Luer syringe needle 24 mm, 25G (Fisher Scientific, UK). Headspace sampling was repeated after 30, 60 and 90 minutes (T_{30}, T_{60} and T_{90}, respectively).

Using a gas-tight precision injection glass syringe, an air sample (1 mL) was taken from the Exetainer and injected into the column of the gas chromatograph. A 20-ppm CH_4 standard (CryoService Limited, UK) was run every 20 samples to check for accuracy. Precision was 3.31% with a method-detection limit of 0.19 ppm. The atmospheric CH_4 concentrations (at T_0, T_{30}, T_{60} and T_{90}) of the unknown samples were calculated by comparing the peak area from the chromatogram to the peak area of the CH_4 standard. The results were then used to estimate the CH_4 flux inside the chamber headspace as follows (9, 10):

$$F = \rho \frac{V}{A} \times \frac{\Delta c}{\Delta t}$$

where F is the CH_4-C flux ($\mu g.m^{-2}.h^{-1}$); V the volume of the chamber (m^3); A the base area of the chamber (m^2); $\Delta c/\Delta t$ the average rate of change of CH_4 concentration (ppmv) with time (h); ρ the density of CH_4–C ($kg.m^{-3}$) at the corresponding experimental temperature. The gas density ρ was calculated as follows:

$$\rho = \frac{P \times M}{R \times T}$$

where P is the air pressure (1 atm); M the molecular weight of CH_4-C ($g.mol^{-1}$); R the universal gas constant (0.082057 $atm.L.mol^{-1}.K^{-1}$); T the experimental air temperature (K).

Analysis of methanotrophic community by molecular ecology approaches

PCR conditions

The amplification of the *pmo*A genes used the following optimised master mix (final concentrations given): 1x NH_4^+ reaction buffer, 6 mM $MgCl_2$, 50 µM of each deoxynucleotide, 0.02 $U.\mu L^{-1}$ BioTaq™ DNA polymerase (all reagents from Bioline, UK), 0.3 $\mu g.\mu L^{-1}$ bovine serum albumin (Roche diagnostic, UK), 0.3 µM of each primer and 3 $ng.\mu L^{-1}$ of DNA template.

An optimised touchdown PCR program was used: initial denaturation at 95ºC for 7 min, denaturation at 94ºC for 1 min, annealing at 65ºC for 1.5 min, extension at 72ºC for 1 min for 15 cycles with a decrement of 0.8ºC/cycle of the annealing temperature, and then denaturation at 94ºC for 1 min, annealing at 53ºC for 1 min, extension at 72ºC for 1 min for 20 cycles, and a final extension at 72ºC for 10 min. PCRs were performed on a DYAD™ DNA Engine® Peltier thermal cycler (MJ Research, USA). Purity and size of the PCR amplicons were checked by loading 5 µL of each reaction mix on a 1% (w/v) agarose gel stained with ethidium bromide, and observed under UV light. PCR products were purified using the UltraClean-htp™ 96-well PCR Clean-up™ kit (MoBio, USA) according to the manufacturer's instructions, except that DNA was eluted in 35 µL instead of the recommended 100 µL, in order to increase the final concentration. Concentrations of the purified PCR products were then measured on the Nano-Drop® ND-1000.

Terminal-restriction fragment length polymorphism (T-RFLP) analysis

In a 10-µL reaction mix, the final concentrations of the different components were the following: 10 $ng.\mu L^{-1}$ of DNA template, 1x of enzyme solution, 1x of enzyme buffer and 0.1 $\mu g.\mu L^{-1}$ of bovine serum albumin (all reagents from Promega, UK). Samples were then digested for 3 hours at 37ºC on a DYAD™ thermal cycler, and the enzymatic reaction was stopped by an incubation at 95ºC for 15 min. Aliquots of digested PCR products (1 µL) were

transferred onto a MicroAmp® optical 96-well plate (Applied Biosystems, UK) and mixed with 12 µL of Hi-Di™ formamide. 0.3 µl of LIZ-labelled GeneScan™-500 internal size standard (all reagents from Applied Biosystems, UK) was added and the reaction was denatured at 95°C for 5 min.

Terminal-restriction fragments (T-RFs) generated by the sequencer were analysed using the size-calling software GeneMapper™ 4.0 (Applied Biosystems, UK) and quantified by advanced mode using second order algorithm. T-RFs in a T-RFLP profile were selected by the software if their minimum peak height was above the noise observed with the negative control (usually above 25 relative fluorescence units).

Diagnostic *pmoA* microarray analysis

In vitro transcription

In vitro transcription was carried out under RNAase-free conditions. The procedure was as follows (20 µl final volume): 8 µL purified PCR product (50 $ng.\mu L^{-1}$), 4 µL 5x T7 RNA polymerase buffer, 2 µL DTT (100 mM), 0.5 µL RNAsin (40 $U.\mu L^{-1}$) (Promega), 1 µL of each ATP, CTP, GTP (10 mM), 0.5 µL UTP (10 mM), 1 µL T7 RNA polymerase (40 $U.\mu L^{-1}$) (Invitrogen) and 1 µL Cy3-UTP (5 mM) were added into a 1.5 mL Eppendorf tube and incubated at 37°C for 4 hours. RNA was purified immediately based on the RNeasy Mini Kit (Qiagen): 80 µL of DEPC-treated water were added to IVT mixture, followed by adding 350 µL of RLT and 250 µL of ethanol, and then mixed thoroughly. Samples were transferred to an Rneasy mini tube and 500 µL of RPE were added. Tubes were centrifuged at 10,000 rpm for 15 sec. Another 500 µL of RPE were added, and then centrifugation at 10,000 rpm for 2 min. Purified RNA was eluted into 50 µL of dH_2O. RNA yields and dye incorporation rates were measured by spectrophotometry. Purified RNA was fragmented by incubating with 9.5 mM $ZnCl_2$ and 24 mM TrisCl (pH7.4) at 60°C for 30 min. Fragmentation was stopped by the

addition of 12 mM EDTA (pH 8.0) to the reaction and putting it on ice. 1 µL of RNAsin (40 $U.\mu L^{-1}$) was added to the fragmented target.

Hybridisation

Hybridisation was carried out (in triplicate) in an aluminium block on a Belly Dancer (Stovall Life Sciences, USA), which was preheated to 55^{o}C for at least 1 hour. For each hybridisation, the following was added to a 1.5 mL Eppendorf tube (100 µl final volume) and incubated at 65^{o}C for 1 min: 62 µL of DEPC-treated water, 1 µL of 10% SDS, 30 µl of 20x SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0), 2 µl of 50x Denhardt's reagent (Sigma) and 5 µl of target RNA (corresponding to about 200 ng of RNA). Preheated hybridisation mixtures were applied onto the preheated slides containing the arrays. The assembled microarray slides were incubated overnight in the HybriWell hybridisation chambers (Grace BioLabs) at 55^{o}C at maximum bending and lowest rotation. Following hybridisation, the slides were washed by shaking at room temperature for 5 min in 2x SSC, 0.1% (w/v) SDS; twice for 5 min in 0.2x SSC and finally for 5 min in 0.1x SSC. Slides were dried using an airgun.

Scanning and data analysis

Hybridised slides were scanned at 10 µm resolution with a GenePix 4000 laser scanner (Axon, USA) at a wavelength of 532 nm. Fluorescent images were analyzed with the GenePix software (Axon, USA). Micosoft Excel was used for statistical analysis and presentation of results.

Results were normalised to a positive control. The hybridisation signal for each probe was expressed as a percentage of the signal (median of signal minus background) of the positive control probe mtrof173 on the same array (11). As each slide contained triplicate arrays, normalised signal intensities of the triplicate spots on a slide were used to determine average results and standard deviations. Hybridisation between a probe and a target was considered positive if the signal was at least 5% of the strongest signal obtained for that probe with the

validation set of reference strains/clones. For probes where no perfect match reference target was available or the strongest signal was less than 60 (% of the signal obtained for mtrof173), this reference value was arbitrarily set to 60. This was found to minimize false positive calls while not creating any false negative calls (12).

Identifying active methanotrophs by stable isotope probing of phospholipids fatty acids (PLFA-SIP)

Microcosm experiments and PLFA-SIP

Field-moist 5.6-mm sieved soils (10 g) were transferred into 125-mL Wheaton glass serum bottles (Sigma-Aldrich, UK), and left overnight in the dark at 20ºC. The following day, bottles were sealed and injected through the rubber septum with 2.5 mL of ^{13}C-CH_4 (>99 atom%, CK Gas, UK) from a ~5,000 ppm master mix in order to have a starting headspace concentration of ~100 ppm. Soils were incubated in the dark at 20ºC.

PLFA-SIP was performed on the autumn and summer soils only, and on all chamber replicate soils (n=4) from each habitat from each site (total n=88). The autumn samples were all incubated for 14 days whereas the summer samples were incubated until >90% of ^{13}C-CH_4 had been incorporated (between 4 and 32 days depending on the activity of the soils).

After incubation was complete, ^{13}C-enriched soils were kept frozen at -20ºC.

Compound-specific isotope analysis

The isotopic composition of individual PLFAs was determined using a GC Trace Ultra with combustion column attached via a GC Combustion III to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Finnigan, Germany). Samples (2 µL) were injected in splitless mode onto a J&W Scientific HP-5 column, 50 m length, id 0.2 mm with a film thickness of 0.33 µm (Agilent Technologies Inc, USA). All other running conditions were as described elsewhere (13). The carbon isotope ratios were calculated with respect to Vienna-

PDB ($\delta^{13}C_{V\text{-}PDB}$) through the use of a CO_2 reference gas injected with every sample and traceable to International Atomic Energy Agency reference material NBS 19 TS-Limestone. Repeated analysis, over a two-month period, of the $\delta^{13}C$ value of a C19:0 FAME internal standard gave a standard deviation of 1.11‰ (n=18).

231 **Results**

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233 **Table S3. Linear regression of the net CH_4 fluxes from each site with some abiotic**
234 **properties of the soil.**

235 The data are P values from simple linear regression analyses. Values in bold represent
236 significance (α=0.05).

Abiotic property \ Site		Bad à Cheo	Glensaugh	Craggan	Tulchan
pH		0.443	**0.041**	0.709	0.054
Total C		0.625	0.293	0.014	**<0.001**
Total N		0.211	0.159	0.302	**<0.001**
C:N ratio		0.244	**0.012**	**0.010**	**0.012**
NH_4^+-N		**0.013**	**0.047**	0.663	**0.005**
NO_3^--N		0.537	0.959	0.095	**<0.001**
Moisture		0.202	0.211	**<0.001**	**<0.001**
Particle size	0.02-2.00 µm	**0.043**	0.655	0.343	**<0.001**
	2-20 µm	**0.018**	0.169	0.254	**0.001**
	20-2000 µm	**0.019**	0.216	0.258	**<0.001**
Bulk density		0.966	0.467	0.282	0.062
Porosity		0.966	0.218	0.282	0.062
WFPS		0.720	**0.006**	**0.004**	0.370

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It should be noted that the overall fluorescence detected in the T-RFLP profiles for the samples from the old pine forest at Bad à Cheo was very low due to the quality of the soil and the difficulty to extract DNA. Thus, T-RFLP results from the old pine forest at Bad à Cheo are not included (see Table S4 and Table S5).

Table S4. Relative (seasonal and annual) abundance (±s.e.m.) and phylogenetic affiliation of the most abundant T-RFs (digestion of *pmoA* with the restriction enzyme *Hha*I) found in soils (n=16 replicates for each habitat).

For each T-RF, statistical differences between seasons within each habitat are indicated by different Roman letters (a, b), while Greek letters (α, β) indicate statistical differences between habitats within each site, according to multiple pairwise comparison (α=0.05).

Site	Habitat	Season	T-RF ID (*enzyme*-bp)						T-RF relative total (%)
			Hha-32		*Hha*-129		*Hha*-81		
			Seasonal	Annual	Seasonal	Annual	Seasonal	Annual	
Bad à Cheo	Bog	Autumn	17±6	17±2	30±3	29±2	30±6	31±3	77
		Spring	17±3		25±2		33±5		
		Summer	19±2		28±7		26±6		
		Winter	17±3		33±6		34±7		
	Young Pine	Autumn	12±4	21±4	34±6	29±3	31±8	38±4	88
		Spring	15±7		29±7		48±12		
		Summer	23±8		36±3		38±5		
		Winter	34±7		19±5		35±5		
Glensaugh	Grassland	Autumn	58±21	61±7	15±8	9.0±2.3	12±12	3.8±2.7	74
		Spring	51±6		8.7±3.3		1.8±1.8		
		Summer	74±21		3.4±3.4		0		
		Winter	62±10		8.7±3.8		2.2±2.2		
	Young Pine	Autumn	75±8	71±6	9.4±1.3	11±1	2.7±2.7	0.8±0.78	83
		Spring	65±25		8.2±4.0		0		
		Summer	69±4		10±2		0		
		Winter	74±8		16±4		0		

Craggan	Moorland	Autumn	14±3	18±2 $^{\alpha}$	19±4	10±3 $^{\alpha}$	25±3	36±6 $^{\alpha\beta}$	64
		Spring	29±0		0		35±35		
		Summer	19±2		3.7±2.2		59±7		
		Winter	15±2		14±4		25±8		
	Young Birch	Autumn	19±7	45±8 $^{\beta}$	4.6±2.7	2.5±1.2 $^{\beta}$	67±2	46±7 $^{\alpha}$	94
		Spring	42±8		3.0±2.9		43±8		
		Summer	46±22		0		49±19		
		Winter	81±15		2.7±2.7		16±16		
	Old Birch	Autumn	64±19	58±9 $^{\beta}$	4.2±4.2	7.8±2.7 $^{\alpha\beta}$	13±8	17±6 $^{\beta}$	81
		Spring	37±18		8.4±8.4		25±16		
		Summer	65±5		13±6		12±10		
		Winter	68±22		6.4±3.7		17±11		
Tulchan	Moorland	Autumn	10±5	13±3 $^{\alpha}$	13±2	14±2 $^{\alpha}$	58±8	55±6 $^{\alpha}$	82
		Spring	21±12		9.8±3.4		53±18		
		Summer	6.5±1.8		14±6		73±6		
		Winter	14±2		18±3		38±6		
	Young Birch	Autumn	70±21	82±8 $^{\beta}$	2.8±1.6	2.7±1.0 $^{\beta}$	27±19	13±7 $^{\beta}$	98
		Spring	70±20		4.5±3.0		22±15		
		Summer	98±1		1.9±1.0		0		
		Winter	95±3		1.4±1.4		0.94±0.94		
	Old Birch	Autumn	58±13	61±9 $^{\beta}$	12±6	10±3 $^{\alpha}$	7.7±7.6 ^{a}	13±8 $^{\beta}$	84
		Spring †	0		0		90 ^{b}		
		Summer	68±19		12±6		3.1±3.0 ^{a}		
		Winter	73±11		10±5		5.3±4.3 ^{a}		
Associated organism			Distant relative of *Methylocapsa* sp./ USCα		Distant relative of *Methylocapsa* sp./ Cluster 5		Distant relative of *Methylocystaceae*		
Reference			Nazaries *et al.* (8) Singh *et al.* (14)						

Table S5. Effects of land-use change (or tree growth) and seasonal changes on the methanotrophic community (digestion of *pmoA* with the restriction enzyme *Hha*I).

The data are *P* values corresponding to the first four IPC scores of the AMMI analyses, and were obtained by nested ANOVA and MANOVA. Within each column, statistical differences between seasons within each habitat are indicated by different Roman letters (a, b), while Greek letters (α, β) indicate statistical differences between habitats, according to multiple pairwise comparison (α=0.05).

		IPC 1	IPC 2	IPC 3	IPC 4	MANOVA
Bad à Cheo	% variation	42.0	26.5	14.2	9.3	
	Habitat	**0.008**	0.374	0.088	**0.007**	**<0.001**
	Habitat/Season	0.755	0.295	0.239	0.386	0.390
Bog	Autumn Spring Summer Winter	**a α**	**a α**	**a α**	**a α**	
Young Pine	Autumn Spring Summer Winter	**a β**	**a α**	**a α**	**a β**	
		IPC 1	**IPC 2**	**IPC 3**	**IPC 4**	**MANOVA**
Glensaugh	% variation	55.8	23.2	8.0	5.1	
	Habitat	0.840	**0.044**	**0.003**	**0.027**	**<0.001**
	Habitat/Season	0.476	**0.011**	0.208	0.507	**0.014**
Grassland	Autumn Spring Summer Winter	**a α**	**a** **a** **b** **a** **α**	**a α**	**a α**	
Young Pine	Autumn Spring Summer Winter	**a α**	**a β**	**a β**	**a β**	
		IPC 1	**IPC 2**	**IPC 3**	**IPC 4**	**MANOVA**
Craggan	% variation	47.9	24.5	10.8	7.2	
	Habitat	**<0.001**	**0.001**	0.063	0.089	**<0.001**
	Habitat/Season	0.116	0.334	0.852	**0.007**	**0.019**
Moorland	Autumn Spring Summer Winter	**a α**	**a α**	**a α**	**a** **b** **ab** **α** **a**	

Young Birch	Autumn Spring Summer Winter	**a α**	**a β**	**a α**	**a α**	
Old Birch	Autumn Spring Summer Winter	**a β**	**a β**	**a α**	**a α**	
		IPC 1	**IPC 2**	**IPC 3**	**IPC 4**	**MANOVA**
Tulchan	% variation	75.7	11.4	6.8	2.2	
	Habitat	**<0.001**	0.060	**0.037**	**0.026**	**<0.001**
	Habitat/Season	0.053	0.341	0.411	0.389	0.233
Moorland	Autumn Spring Summer Winter	**a α**	**a α**	**a αβ**	**a αβ**	
Young Birch	Autumn Spring Summer Winter	**a β**	**a α**	**a α**	**a α**	
Old Birch	Autumn Spring Summer Winter	**a β**	**a α**	**a β**	**a β**	

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Table S6. Effects of tree growth on the methanotrophic community (*pmoA* microarray).

The data presented are some of the *pmoA* probes that showed higher levels of hybridisation, and their statistical difference (Greek letters [α, β, γ]) between habitat within each site, according to multiple pairwise comparison ($P<0.05$). ND means that the probe showed no detectable hybridisation.

Site	Habitat	Probe										
		McyM309	**Mcy522**	**Mcy459**	**Mcy413**	**Msi233**	**Peat264**	**RA14-594**	**RA14-591**	**RA14-299**	**Wsh1-566**	**Wsh2-491**
Bad à Cheo	Bog	**α**	α	α	**α**	α	α	α	**α**	α	**α**	**α**
	Young Pine	**β**	α	α	**β**	α	α	α	**α**	α	**β**	**α**
	Old Pine	**β**	α	α	**β**	α	α	α	**β**	α	**γ**	**β**
Glensaugh	Grassland	ND	ND	α	α	ND	α	α	α	α	α	α
	Young Pine	ND	ND	α	α	ND	α	α	α	α	α	α
Craggan	Moorland	ND	α	α	α	α	α	**α**	**α**	**α**	α	ND
	Young Birch	ND	α	α	α	α	α	**α**	**α**	**αβ**	α	ND
	Old Birch	ND	α	α	α	α	α	**β**	**β**	**β**	α	ND
Tulchan	Moorland	ND	**α**	**α**	**α**	**α**	**α**	**α**	**α**	**α**	**α**	ND
	Young Birch	ND	**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**	ND
	Old Birch	ND	**β**	**β**	**β**	**β**	**αβ**	**αβ**	**αβ**	**αβ**	**β**	ND

Table S7. Effect of tree growth on the methanotrophic community (PCA from the *pmoA* microarray).

The data are *P* values corresponding to the first five PC scores of the probe hybridisation intensities, and were obtained by MANOVA. Within each column and for each site, results followed by different Greek letters (α, β, γ) are statistically different for each habitat, according to multiple pairwise comparison (α=0.05).

Site/Habitat		PC 1	PC 2	PC 3	PC 4	PC 5	MANOVA
Bad à Cheo	**% variation**	74.23	15.93	6.07	2.22	0.66	
	P	**<0.001**	0.215	0.469	0.640	0.695	**0.003**
	Bog	**α**	α	α	α	α	
	Young Pine	**β**	α	α	α	α	
	Old Pine	**γ**	α	α	α	α	
Glensaugh	**% variation**	63.88	34.65	1.170	0.230	0.050	
	P	0.934	**0.003**	0.821	0.811	0.598	0.299
	Grassland	α	**α**	α	α	α	
	Young Pine	α	**β**	α	α	α	
Craggan	**% variation**	90.4	6.09	2.62	0.66	0.15	
	P	0.601	0.559	**<0.001**	0.125	0.758	**0.002**
	Moorland	α	α	**α**	α	α	
	Young Birch	α	α	**α**	α	α	
	Old Birch	α	α	**β**	α	α	
Tulchan	**% variation**	89.71	5.27	3.65	1.04	0.21	
	P	**0.011**	0.501	0.482	0.064	0.762	**<0.001**
	Moorland	**α**	α	α	α	α	
	Young Birch	**β**	α	α	α	α	
	Old Birch	**β**	α	α	α	α	

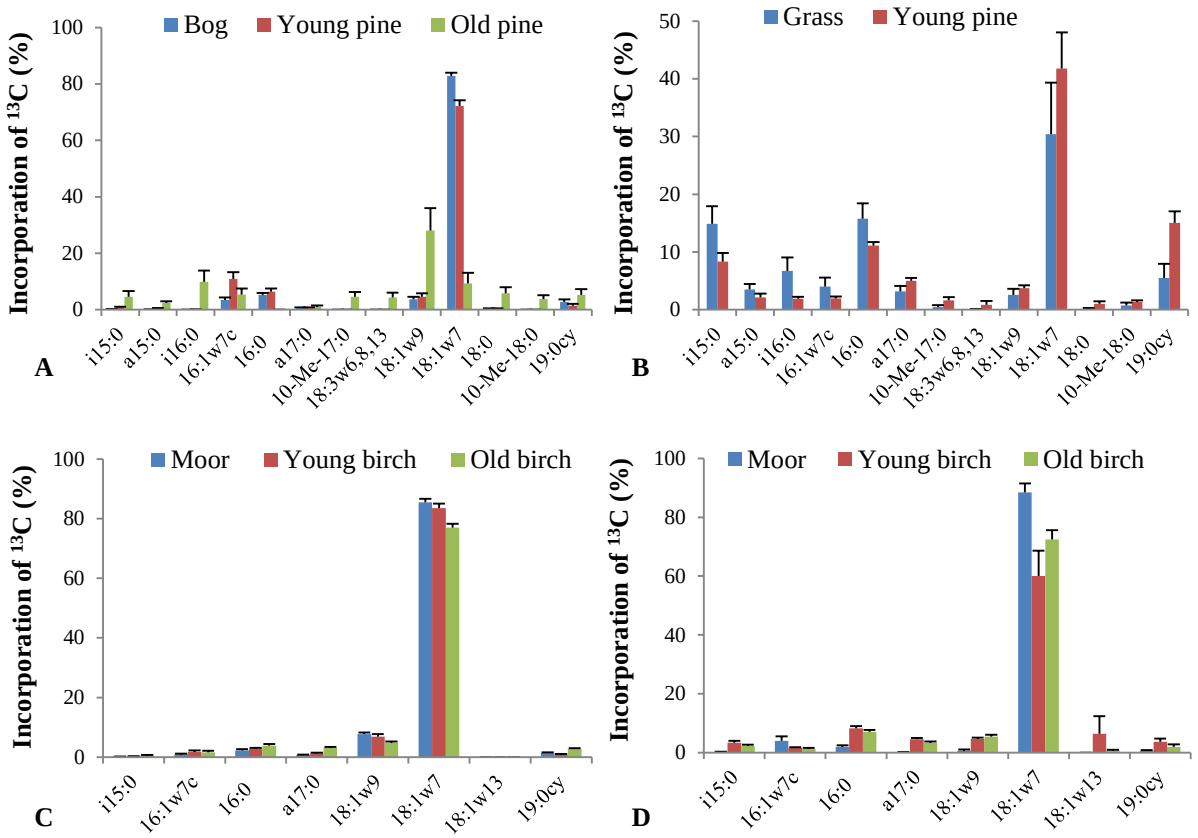

Fig. S1. Percentage of incorporation of ^{13}C within the PLFAs after incubation with ~100 ppm of ^{13}C-CH_4 at (A) Bad à Cheo, (B) Glensaugh, (C) Craggan and (D) Tulchan.

The data are seasonal average ± s.e.m. (n=8 replicates – autumn and summer combined) of the enriched PLFA content.

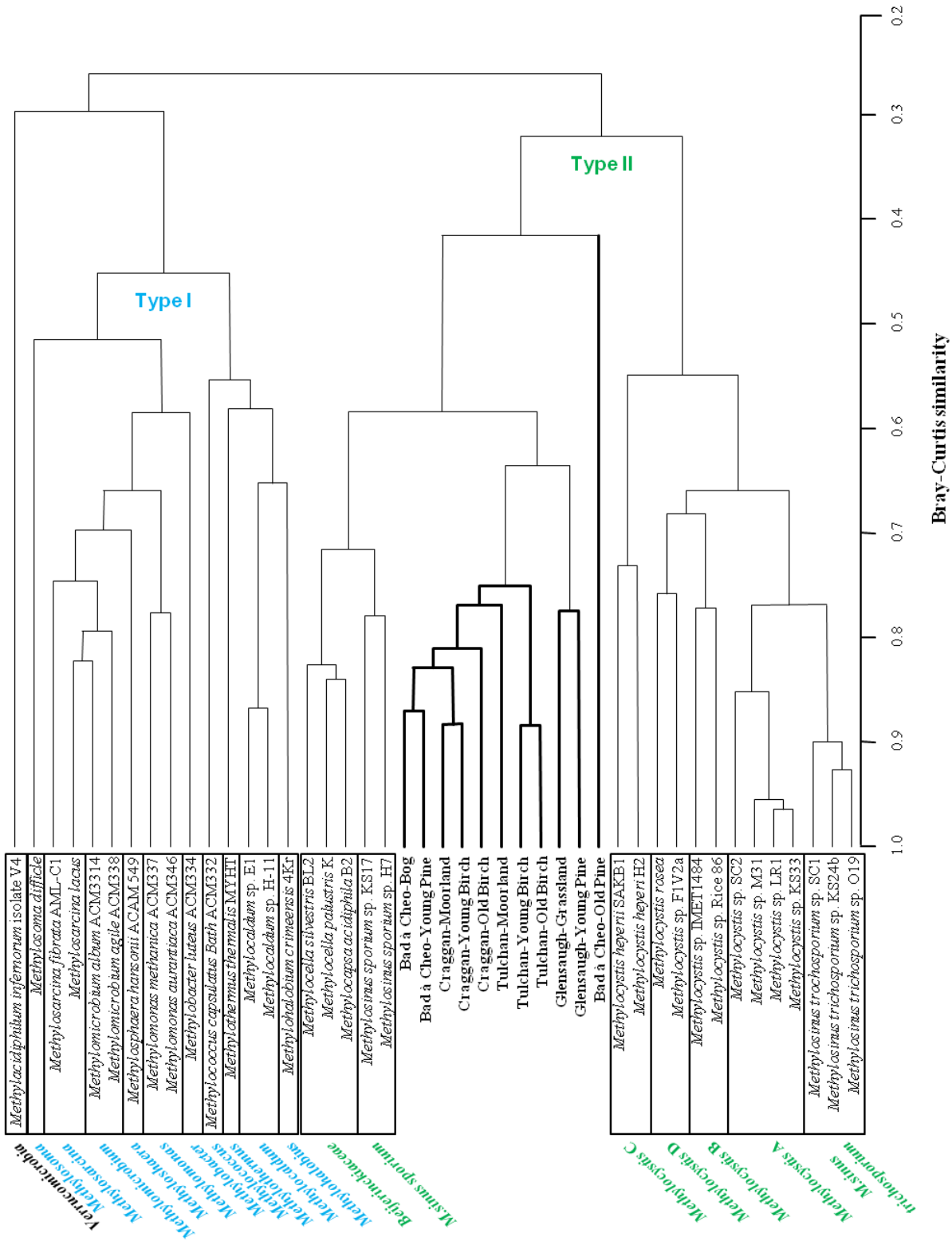

Fig. S2. Cluster analysis of the PLFA-SIP profiles (based on % of 13C-incorporation) of methanotrophs in the enriched (~100 ppm ^{13}C-CH_4) soils (n=8 replicates - autumn and summer combined).

The above dendrogram was built using data from this study, combined with data from the literature (15). Thus, the active methanotroph population present in the soils investigated could be affiliated with published methanotrophs. This is because PLFA-SIP allows for the identification of ^{13}C-labelled fatty acids produced by active methanotrophs feeding on ^{13}C-CH_4. The PLFA pattern of an environmental sample from a PLFA-SIP incubation with ^{13}C-CH_4 can then be compared to the PLFA content of a pure culture of methanotrophs. A Bray-Curtis similarity matrix was used, from the square-root transformation of the PLFA data (see Fig. S1), to perform a group average linking cluster analysis with GenStat® software.

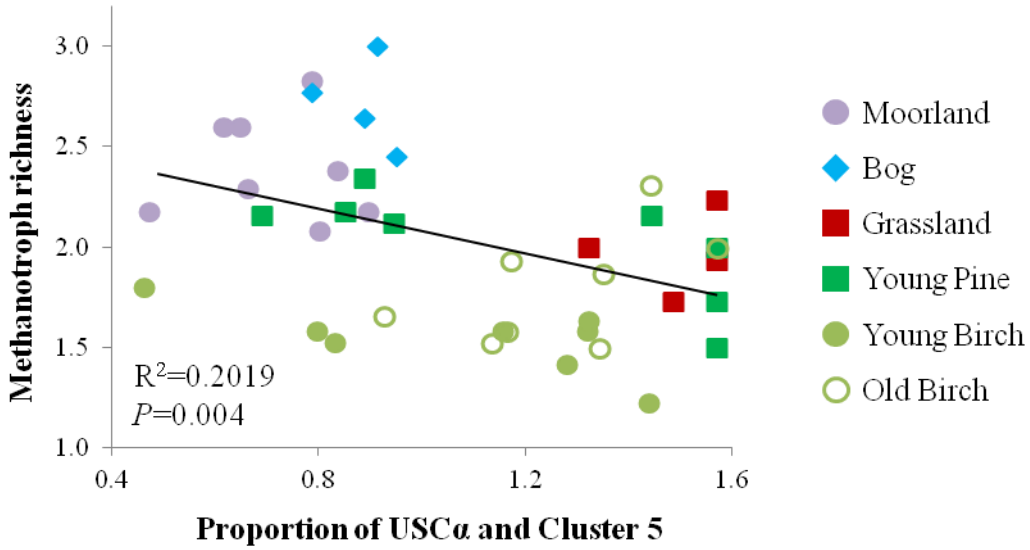

Fig. S3. Relationship between methanotroph richness and proportion of the USCα members associated with land-use change (n=40).

The methanotroph richness was calculated as the square-root transformation of the number of T-RFs present in each sample (among the 15 most abundant T-RFs of the T-RFLP profiles, which constituted >94% coverage). The proportion of USCα microorganisms was calculated as the angular transformation (arcsine of the square root) of the ratio of the relative abundance of the T-RFs specific to *Methylocapsa* sp. (USCα/Cluster 5 – T-RFs *Hha*-32 and *Hha*-129) to the sum of the T-RFs specific to USCα and the *Methylocystaceae* family (T-RF *Hha*-81). Refer to Table 1 in main text for T-RF reference values.

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