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Evidence of Microbial Regulation of Biogeochemical Cycles from a Study on Methane Flux and Land Use Change

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Microbes play an essential role in ecosystem functions, including carrying out biogeochemical cycles, but are currently considered a black box in predictive models and all global biodiversity debates. This is due to (i) perceived temporal and spatial variations in microbial communities and (ii) lack of ecological theory explaining how microbes regulate ecosystem functions. Providing evidence of the microbial regulation of biogeochemical cycles is key for predicting ecosystem functions, including greenhouse gas fluxes, under current and future climate scenarios. Using functional measures, stable-isotope probing, and molecular methods, we show that microbial (community diversity and function) response to land use change is stable over time. We investigated the change in net methane flux and associated microbial communities due to afforestation of bog, grassland, and moorland. Afforestation resulted in the stable and consistent enhancement in sink of atmospheric methane at all sites. This change in function was linked to a niche-specific separation of microbial communities (methanotrophs). The results suggest that ecological theories developed for macroecology may explain the microbial regulation of the methane cycle. Our findings provide support for the explicit consideration of microbial data in ecosystem/climate models to improve predictions of biogeochemical cycles.

Soil microbial communities are among the most diverse and complex natural communities and are responsible for many ecologically and economically important ecosystem processes (1). For example, soil microbes carry out key steps in global biogeochemical cycles, and their activities influence primary productivity, plant and animal diversity, and Earth's climate, such as greenhouse gas emissions (2). Despite their essential role in ecosystem function, microbial communities are considered a black box in predictive ecosystem and climate models. This neglect is mainly because (i) microbial communities are regarded as being omnipresent and functionally redundant, (ii) there is a lack of theoretical approaches to disentangle microbial regulation of ecosystem functions from other biotic and abiotic drivers, and (iii) temporal and spatial variation in environmental microbes is considered too large to be meaningful in the predictive models. However, detailed studies on the biodiversity-ecosystem function relationship for plant communities have demonstrated that both the magnitude and stability of ecosystem functions are sensitive to loss of diversity. On the other hand, more-diverse plant communities appear to be more productive (in terms of biomass) and more stable in the face of disturbance (3–5). Two hypotheses to explain the underlying basis of the relationship between ecosystem processes and diversity have been put forward. The "complementarity hypothesis" states that biotic interactions and niche differentiation collectively result in a positive biodiversity-ecosystem function relationship in plant communities, whereas the "selection hypothesis" suggests that the magnitude of the process may be the result of the presence of one to a few particularly productive (key) species (3, 5, 6). For plants, the evidence from a number of pioneering studies overwhelmingly supports the complementarity hypothesis. For the microbial community-ecosystem function relationship, distinguishing between these two competing hypotheses is key in order to determine whether all functional microbial communities, or only selected species, need to be conserved or restored to maintain soil functions. Additionally, if either of these hypotheses clearly demonstrates the microbial regulation of the biogeochemical cycle, such knowledge may be further used to develop parameterized microbial data for incorporation into predictive models, as has been done for plant communities (7–11).

However, understanding the role of soil microbial communities in biodiversity-ecosystem function is made more complex due to the diversity of functions mediated by microbes, whether they are rare or abundant and whether they mediate specific or general processes. For example, organic matter decomposition is carried out by a large number of microbial species while xenobiotic degradation capability is restricted to more-specialized species. Should the biodiversity-ecosystem function relationship also hold for the microbial community, diversity loss would have a greater effect on some functions than on others. Moreover, the global importance of microbes in soil function, combined with the lack of knowledge on how the variability in composition and functioning of these communities is affected, necessitates a detailed examination of consistent microbial response to disturbance over time and space. This is a fundamental requirement in order to under-

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stand and predict how microbial community and ecosystem functions will respond to global changes (12, 13). Here we used land use change (tree growth) as a treatment to provide evidence that both microbial community structure and the rate of biogeochemical cycles are stable over time and space. Our results provide correlative evidence that ecological theories developed for macroorganisms, such as plants and animals (macroecology), may explain the microbial regulation of ecosystem functions. We used methane (CH_4) flux and associated microflora (methanogens and methanotrophs) in our study because this function is restricted to selected microbial taxa which are well characterized. This allowed us to test our hypothesis without manipulating species diversity or constructing artificial consortia, which has huge confounding effects due to the unculturability of the majority (>99%) of environmental microbes (14, 15).

MATERIALS AND METHODS

A full description of the field sites and methodologies is provided in the supplemental material.

Field site description. Four sites were investigated in Scotland (see Table S1 in the supplemental material). As a result, three types of land use change were examined: afforestation of bog with pine trees (Bad à Cheo), conversion of grassland to pine forest (Glensaugh), and natural colonization of moorland by birch stands (Craggan and Tulchan). For ease of analysis, afforestation of bog and grassland (at Bad à Cheo and Glensaugh) and tree invasion (at Craggan and Tulchan) were referred to as "tree growth" in this paper to describe the general change in land use. Craggan and Tulchan were sites with similar habitats and were ~7 km apart, whereas the Bad à Cheo and Glensaugh sites both had pine woodland and were ~178 km apart.

Soil sampling and soil analyses. Briefly, for each habitat from each site and at each season, 12 intact soil cores were collected in stainless steel rings (10-cm diameter, 0- to 10-cm depth). They were ~2 m apart from each other over 3 transects about 10 m apart and were grouped in triplicate for measurement of CH_4 fluxes ($n = 4$ replicates per season). Following gas measurements (see below), the soils were processed for physicochemical analysis as well as for biochemistry and molecular biology work. Chemical properties (pH, total C and N, mineral N [NH_4^+-N and NO_3^--N], and moisture) were measured periodically (autumn and summer). Physical properties (particle size, bulk density, and water-filled pore space [WFPS]) were measured once (summer). Soil property data from the different sites and habitats are presented in Table S2 in the supplemental material.

Function measurements and upscaling. Atmospheric CH_4 concentrations inside the headspace of the closed polyvinyl chloride (PVC) chambers were measured on a gas chromatograph (Trace GC; Thermo-Finnigan, Italy) using an analytical capillary column, PLOT Al_2O_3/KCl FS (length = 50 m; inner diameter [ID] = 0.53 mm; outer diameter [OD] = 0.70 mm; 10-μm-thick film) (Varian, United Kingdom), and a flame ionization detector (FID) for the detection of CH_4. Four headspace measurements were made at 30-min intervals over a 90-min period (t_0, t_{30}, t_{60}, and t_{90}). A linear model (16, 17) was then applied in order to estimate the CH_4 flux inside the chamber headspace.

Analysis of the functional microbial community using molecular ecology approaches. (i) DNA isolation. Soil (~250 mg) from each sample was used for DNA extraction using a PowerSoil-htp 96-well soil DNA isolation kit (Mobio). DNA concentrations were measured using a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies). We characterized the methanotrophic communities using the molecular marker from the functional gene *pmoA*. It encodes the putative active site of the particulate methane monooxygenase (pMMO) enzyme (18), which is specific to all known methanotrophs except *Methylocella* and *Methyloferula* spp. (19–21). We used primers targeting 16S rRNA genes for type II methanotrophs (22) to identify the relative abundance of methanotrophs which do not possess *pmoA* genes. Terminal restriction fragment length polymorphism (T-RFLP) analyses did not produce any T-RF assigned to *Methylocella* spp. The *pmoA* primers used in this study are described elsewhere (23). Methanogens were characterized by targeting methyl coenzyme M reductase (*mcrA*) genes using the primers and PCR conditions described previously (24).

(ii) T-RFLP analysis. The PCR products for the *pmoA* and *mcrA* genes for the T-RFLP analysis were fluorescently labeled with a forward primer (VIC-pmo189F and FAM-mcrA-F, respectively). A known amount of purified PCR amplicons (100 ng) was used with the restriction enzyme HhaI (25). T-RFLP analysis ($n = 4$ for each treatment and season) was carried out on an automated sequencer, an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems, United Kingdom). Only peaks between 30 and 550 bp were considered to avoid terminal restriction fragments (T-RFs) caused by primer-dimers and obtain fragments within the linear range of the internal size standard (26).

(iii) Diagnostic *pmoA* microarray analysis. In order to validate further our T-RFLP data, we performed diagnostic *pmoA* microarray analysis on all samples collected in summer ($n = 4$ for each treatment). Microarray analysis was carried out using a modified method of Stralis-Pavese et al. (27, 28). The different steps involved are briefly described here. The PCR products for the *pmoA* gene for the microarray assay were generated in the same way as detailed above except that no fluorescently labeled forward primer was used and the reverse primer (pmo650R) contained the T7 promoter site (5′-TAATACGACTCACTATAG-3′) at its 5′ end, which enabled T7 RNA polymerase-mediated *in vitro* transcription. Details of the *in vitro* transcription and hybridization procedures are in the supplemental material. Results were normalized to a positive-control probe, mtrof173. The hybridization signal for each probe was expressed as a percentage of the signal of the positive-control probe mtrof173 on the same array (29). The current version of the *pmoA* array contains 199 oligonucleotide probes targeting *pmoA* of methanotrophs, *amoA* (encoding a subunit of ammonia monooxygenase) of ammonia oxidizers, and other functionally related bacteria (28).

Identifying an active microbial community by PLFA-SIP. Microcosm experiments were designed to enrich fresh soils (~10 g) with $^{13}CH_4$ (~100 ppm) in 155-ml glass bottles, sealed with rubber septum, and crimped with an aluminum seal as described previously (26). Stable-isotope probing of phospholipid fatty acid (PLFA-SIP) was performed on the autumn and summer soils only ($n = 4$ for each treatment and each season). A subsample of all the ^{13}C-enriched soils was freeze-dried, milled, and used for extracting PLFAs (26, 30). Standard nomenclature was used for PLFAs (31). Quantification of PLFA contents was based on the normalized peak area of each PLFA, all of which were compared to the peak area of the C19:0 PLFA internal standard, accounting for the weight of soil used for the PLFA extraction (32).

Statistical analysis. (i) T-RFLP data processing. Raw data from GeneMapper were exported to be used with *T-REX*, an online software for the processing and analysis of T-RFLP data (http://trex.biohpc.org) (33). We used a standard approach for processing T-RFLP data (25, 33, 34). Briefly, quality control procedures included noise filtering and T-RF alignment (clustering threshold = 2 bp), and T-RFs were omitted if they occurred either in less than 2% of the total number of samples or with a relative abundance of less than 3% within a specific sample. This process led to 16 clean T-RFs, and all of them were included in the statistical analyses. Analysis of data matrices used the additive main effect and multiplicative interaction (AMMI) model based on analysis of variance (ANOVA), as discussed by Culman et al. (34). Interaction principal component (IPC) scores were obtained and used for further statistical analyses (see below). Only the T-RFs that were considered "true" by the *T-REX* analysis were used for subsequent analysis. The relative abundance of a detected T-RF within a given T-RFLP profile was calculated as the respective signal height of each peak divided by the total peak height of all the peaks of the T-RFLP profile.

(ii) Statistical tests. The significance of differences in soil characteristics, oxidation rates, and community structures (IPC scores) with different land use types and during different seasons was determined by nested ANOVA (followed by Tukey's test for multiple pairwise comparisons [$\alpha = 0.05$]) and multivariate ANOVA (MANOVA). Regression analysis was used to explore the linear relationship ($\alpha = 0.05$) between methanotroph diversity (community structure and operational taxonomic unit [OTU] richness) in each habitat and the corresponding net CH_4 fluxes and ecosystem stability. The latter was calculated for each habitat as the mean net CH_4 flux (between-season average) divided by the corresponding standard error (35). Data were arcsine (proportion of upland soil cluster alpha [USCα] members) and square root (methanotroph richness and ecosystem stability) transformed to obtain normal distributions. To examine the effect of soil abiotic properties on methanotrophs, we performed detrended correspondence analysis (DCA) followed by redundancy analysis (RDA) on T-RLP data to understand the influence of different environmental variables on individual T-RFs. First, DCA gave gradient lengths of 0.975 (Bad à Cheo), 1.241 (Glensaugh), 1.663 (Craggan), and 1.633 (Tulchan) on the first axis, which suggested that RDA was suitable for the analysis of relationships of T-RFs with environmental variables. Finally, following the square-root transformation of the PLFA data (percentage of total enriched PLFA content), a cluster analysis, based on a Bray-Curtis similarity matrix, was performed using the group average linking method to affiliate the active methanotroph population present in soils with published methanotrophs. The statistical analyses were carried out using GenStat 14th edition software (VSN International Limited, United Kingdom).

RESULTS

Land use change affects soil function. We used four sites across Scotland, which included three land use categories per site (nonforested, young forest, and old forest) except for Glensaugh, which had no old forest, resulting in a total of five different habitats (bog, grassland, moorland, conifer forest, and birch woodland). We sampled each site four times over the period of a year, as a sampling campaign occurred once during each season: autumn (October/November 2008), winter (February 2009), spring (April 2009), and summer (July 2009). There was no significant seasonal effect on the CH_4 fluxes measured at the four sites investigated (Fig. 1A to D) except at Tulchan in summer. Additionally, the age of the (pine/birch) forest did not influence the net CH_4 flux (Fig. 1A, C, and D). The CH_4 flux data from similar land uses (as well as from young/old forests) and from each seasonal sampling (total $n = 176$) were combined and upscaled to a yearly estimate. The seasonal estimates (total $n = 44$) were then averaged (final flux unit in kg CH_4-C · ha^{-1} · $year^{-1}$) as shown in Fig. 1E. Tree growth (afforestation of the bog and grassland with pine trees as well as heathland colonization by birch trees) significantly improved the CH_4 sink in soils ($P < 0.001$). On average, tree growth induced 2.6-fold (conversion of heathland to birch woodland) and 4.5-fold (conversion of bog/grassland to pine forest) increases in CH_4 sink.

Characterization of functional microbial communities. The T-RFLP analysis of the *pmoA* genes to characterize the methanotrophic community demonstrated that the T-RFs Hha-32, Hha-81, and Hha-129 were the most abundantly found at all sites (64 to 98% of all T-RFs detected) (Table 1). Based on our previous work involving cloning and sequencing (25, 36), these operational taxonomic units (OTUs) were related to type II methanotrophs of the *Beijerinckiaceae* and *Methylocystacae* families (Table 1). The identity of these OTUs was confirmed by *pmoA* microarray analysis (see next section). In particular, the T-RF Hha-32 was closely related to clones of the upland soil cluster alpha (USCα; first referred to as RA14 clade [37]) and the T-RF Hha-129 was associated with microorganisms of a clade called cluster 5 (38). Both USCα and cluster 5 are distant relatives of *Methylocapsa* sp., while the T-RF Hha-81 was related to members of the *Methylocystaceae* family (Table 1). At the Bad à Cheo and Glensaugh sites, the T-RFs Hha-32, Hha-81, and Hha-129 were equally present in nonforested (peatland, grass) and pine forest soils, although there was a trend toward an increase of the relative abundance of the T-RF Hha-32 in pine forests. Similarly, a dominant and significant trend was observed at both heathland sites, while the relative abundance of T-RF Hha-81 decreased in the birch woodland (Table 1). Birch invasion had a strong effect on specific OTUs. In particular, microorganisms distantly related to the *Methylocystaceae* family (T-RF Hha-81; $P = 0.006$ at Craggan and $P < 0.001$ at Tulchan) were found mostly in soils under moorland whereas bacteria related to *Methylocapsa* sp./USCα clones (T-RF Hha-32; $P < 0.001$) were dominant in soils under birch forest. Birch invasion also decreased the relative abundance of the T-RF Hha-129 ($P = 0.038$ at Craggan; $P = 0.001$ at Tulchan) (Table 1). Compared to cluster 5, USCα constituted the dominant microbial population in soils from forest except at Bad à Cheo (Table 1). No significant seasonal effect on the T-RF relative abundance was detected at any of the sites except a slight increase in the abundance of T-RF Hha-81 ($P = 0.014$) in spring in the old birch forest at Tulchan (see Table S4 in the supplemental material).

The additive main effect and multiplicative interaction (AMMI) model (34, 39) was used to identify an interaction between the pattern of the T-RFs (or OTUs) and the different habitats investigated (Fig. 2). This was confirmed by the multivariate analysis of variance (MANOVA) on the four interaction principal component (IPC) scores of the AMMI analysis, which gave an overall significant effect ($P < 0.001$) of land use change at all sites (see Table S5 in the supplemental material). Afforestation with pine trees had a consistent effect on the methanotrophic community structure at all sites, with a more dominant effect at Bad à Cheo than at Glensaugh (Fig. 2A; see also Table S5 in the supplemental material). Birch tree invasion and the age of the birch forest appeared to have had a significant effect on the community structure and resulted in the detection of a different methanotrophic community structure in each habitat (Fig. 2C and D). No significant seasonal effect on the community structure was detected at any of the sites except in summer in soils from grassland at Glensaugh (IPC = 2) (see Table S5 in the supplemental material) and in spring in soils from moorland at Craggan (IPC = 4) (see Table S5 in the supplemental material), which accounted for only 23.2% and 7.2% of variation, respectively, in community data. Redundancy analysis of T-RFs and environmental variables indicated that only a few environmental variables were found to significantly influence the T-RFs. The variables were porosity (at Craggan only) and soil moisture (at Tulchan only). At Craggan, soil porosity was found to significantly influence the T-RFs ($P = 0.020$) by having a positive impact on the T-RFs related to *Methylocystaceae* (T-RF Hha-81) and a negative impact on the T-RFs related to *Methylocapsa* sp. (T-RF Hha-32). At Tulchan, soil moisture and the age of the birch forest significantly influenced the T-RFs ($P = 0.010$). Soil moisture had a positive impact on the T-RFs related to *Methylocystis*/*Methylosinus* spp. (Hha-81), while it had a negative impact on the T-RFs related to *Methylocapsa* sp. (Hha-32). However, the C/N ratio and porosity showed weak influences at Bad à Cheo ($P = 0.066$) and Glensaugh ($P = 0.060$),

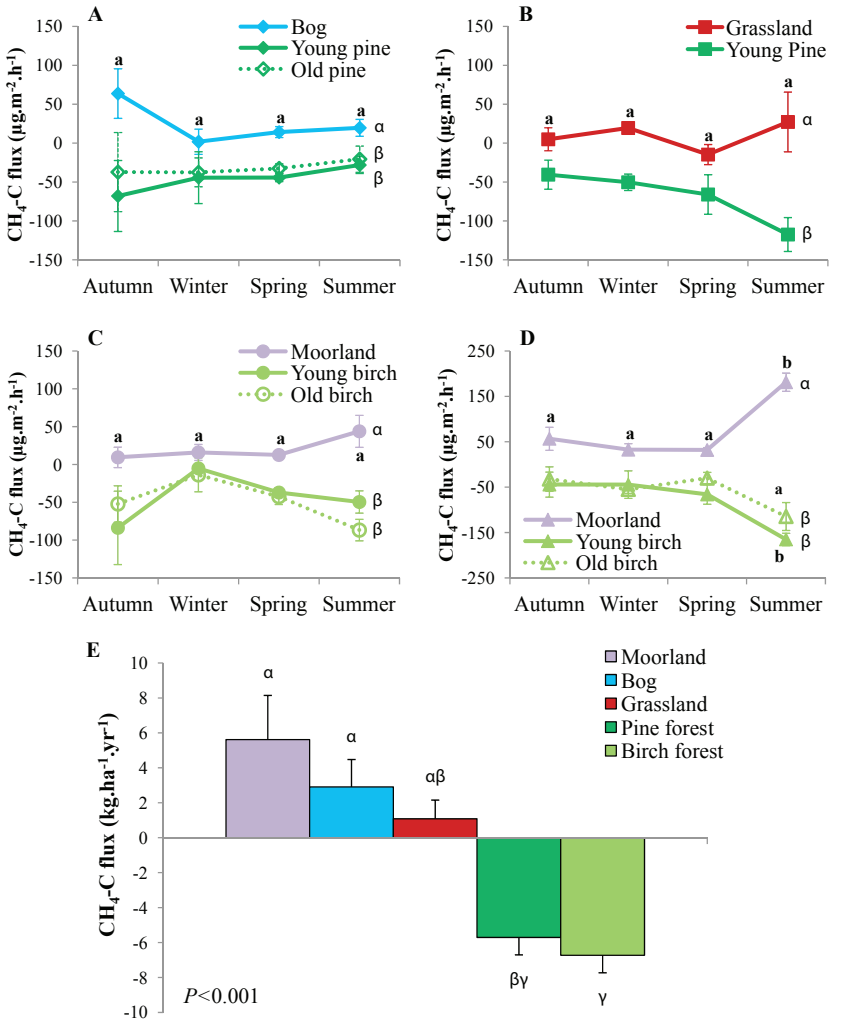

FIG 1 (A to D) Seasonal net CH_4-C fluxes from soils at Bad à Cheo (A), Glensaugh (B), Craggan (C), and Tulchan (D); (E) yearly CH_4 flux estimates from the nonforested and forested habitats. A positive value means that a production of CH_4 occurs, whereas a negative flux denotes a sink of CH_4. (A to D) The curves represent the flux means (error bars are standard errors of the means [SEM]; $n = 4$) of each season for each habitat from the closed-chamber experiment. Significance was analyzed through a nested ANOVA (land use by season). (E) Each histogram represents the yearly net CH_4 flux average ± the SEM ($n = 4$ replicates) based on the upscaling of seasonal net CH_4-C flux measurements. Data from the four sampling sites (Bad à Cheo, Glensaugh, Craggan, and Tulchan) which had similar land uses (bog, grassland, heathland, pine forest, or birch forest) were combined during upscaling. The method used for upscaling can be found within the text. For each site, statistical differences between seasons within each habitat are indicated by different Roman letters (a, b), while Greek letters (α, β, γ) indicate statistical differences between land uses according to multiple pairwise comparisons ($\alpha = 0.05$).

respectively, on the T-RFs related to *Methylocapsa* sp. (T-RFs Hha-32 and Hha-129). Due to that general lack of pattern, we concluded that the abiotic properties investigated were not strong drivers of the changes observed.

We applied a high-throughput diagnostic *pmoA* microarray to further characterize the methanotrophic community. Type I methanotrophs were not detected at any sites (data not shown), a finding which confirms the *pmoA*-based T-RFLP findings (Table 1). Neither nitrifiers nor any *Methylocapsa* sp. was detected at either site (Fig. 3).

Type II methanotrophs of different clades were detected in the nonforested and forested habitats. In soils from peatland and heathland (Bad à Cheo, Craggan, and Tulchan), the type II methanotroph community consisted of members of the *Methylocystaceae* family (probes McyM309, Mcy413, Mcy459, Mcy522, and Msi233), peat-related type II methanotrophs (probe Peat264), and watershed 1 clade (probe Wsh1-566) organisms (40) (Fig. 3; see also Table S6 in the supplemental material). Conversely, the type II methanotroph community consisted of organisms of the RA14/USCα (probes RA14-299, RA14-594, and RA14-591) and watershed 2 (probe Wsh2-491) clades (40) in soils from pine and birch forests (Bad à Cheo, Craggan, and Tulchan) (Fig. 3; see also Table S6 in the supplemental material). Overall, principal component analysis (PCA) and subsequent MANOVA on the first five principal components (PCs) indicated that tree growth (afforestation/tree invasion) changed the soil methanotrophic community at Bad à Cheo ($P = 0.003$), Craggan ($P = 0.002$), and Tulchan ($P < 0.001$) (see Table S7 in the supplemental material). The *pmoA* microarray data from the Bad à Cheo, Craggan, and Tulchan sites were congruent with the *pmoA*-based T-RFLP data presented in Fig. 2A, C, and D. Similarly, MANOVA did not detect a significant difference in the soil

TABLE 1 Relative annual abundance and phylogenetic affiliation of the most abundant T-RFs found in some Scottish soils[a]

Site and habitat	Relative annual abundance ± SEM (%) of indicated T-RF			T-RF relative total (%)
	Hha-32	Hha-129	Hha-81	
Bad à Cheo				
Bog	17 ± 2	29 ± 2	31 ± 3	77
Young pine	21 ± 4	29 ± 3	38 ± 4	88
Glensaugh				
Grassland	61 ± 7	9.0 ± 2.3	3.8 ± 2.7	74
Young pine	71 ± 6	11 ± 1	0.8 ± 0.78	83
Craggan				
Moorland	18 ± 2^{α}	10 ± 3^{α}	36 ± $6^{\alpha\beta}$	64
Young birch	45 ± 8^{β}	2.5 ± 1.2^{β}	46 ± 7^{α}	94
Old birch	58 ± 9^{β}	7.8 ± $2.7^{\alpha\beta}$	17 ± 6^{β}	81
Tulchan				
Moorland	13 ± 3^{α}	14 ± 2^{α}	55 ± 6^{α}	82
Young birch	82 ± 8^{β}	2.7 ± 1.0^{β}	13 ± 7^{β}	98
Old birch	61 ± 9^{β}	10 ± 3^{α}	13 ± 8^{β}	84
Associated organism	Distant relative of *Methylocapsa* sp./USCα	Distant relative of *Methylocapsa* sp./cluster 5	Distant relative of *Methylocystaceae*	

[a] For each T-RF, Greek letters (α, β) indicate statistical differences between habitats within each site according to multiple pairwise comparisons (α = 0.05). $n = 16$ replicates for each habitat. Digestion of *pmoA* was performed with the restriction enzyme HhaI. Information for associated organisms was obtained from Nazaries et al. (25) and Singh et al. (36).

methanotrophic communities between habitats at Glensaugh ($P = 0.299$) (see Table S7 in the supplemental material), a finding which reflects the weak community shift observed with the *pmoA*-based T-RFLP analysis (Fig. 2B).

We also used T-RFLP analysis of the *mcrA* genes to characterize the methanogenic community. Most of the samples in afforested plots did not produce any PCR products for this gene despite several attempts to optimize DNA concentrations and PCR conditions. Only the bog, moorland, and young forest at Bad à Cheo and Tulchan produced PCR products and T-RFLP profiles. High aerobic conditions in the top 10 cm of soils in the afforested plots may explain this failure, as methanogens are anaerobes. Data analysis from available samples again suggested that season had no impact on the community composition, which was driven mainly by land use treatment ($P < 0.001$ [ANOVA] on PC axis 1, which explained 51% of total variation). Because the data on methanogens were not available for a significant number of land uses, sites, and times, these data were not used for regression analysis (to link community with function) or statistical treatment.

Linking community structure and biodiversity with soil function and evidence for ecological theory. This was first investigated by a simple linear regression analysis (Fig. 2) between the *pmoA* IPC scores of each habitat and the corresponding net CH_4 flux values. At all sites, tree growth and change in CH_4 flux were significantly related to a shift in the community structure ($P = 0.022$ at Bad à Cheo, $P = 0.027$ at Glensaugh, $P = 0.027$ at Craggan, and $P = 0.015$ at Tulchan). However, the relationship was stronger at Bad à Cheo and Tulchan than at Glensaugh and was the lowest at Craggan (Fig. 2).

Stable-isotope probing of phospholipid fatty acid (PLFA-SIP) analysis was used to link methanotroph communities with their activity, i.e., oxidation of atmospheric CH_4. Based on the enriched phospholipid fatty acid (PLFA) content (percentage of ^{13}C incorporation) (see Fig. S1 in the supplemental material) extracted from the incubated soils, a cluster analysis was performed (see Fig. S2 in the supplemental material). It suggested that the active methanotrophs in the soils from the different habitats were all distant relatives (<90%

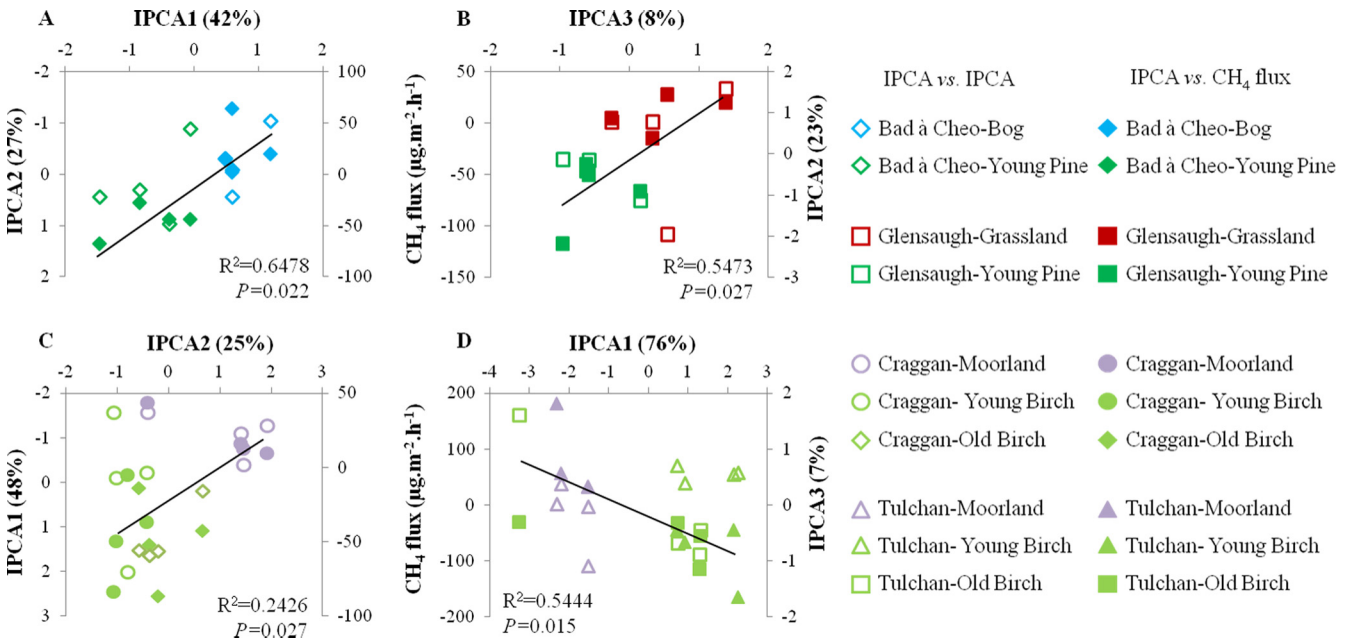

FIG 2 Relationship between CH_4 flux and methanotrophic community structure at Bad à Cheo (A), Glensaugh (B), Craggan (C), and Tulchan (D). The empty shapes (IPCA versus IPCA) are data points representing the IPC scores after analysis of the *pmoA*-based T-RFLP profiles with the AMMI model. The filled shapes (IPCA versus CH_4 flux) are data points corresponding to the strongest linear regression of the net seasonal CH_4 fluxes (Fig. 1A to D) with one of the IPC scores. The data points within each habitat represent the averages over replicates ($n = 4$) of the IPC scores of each season.

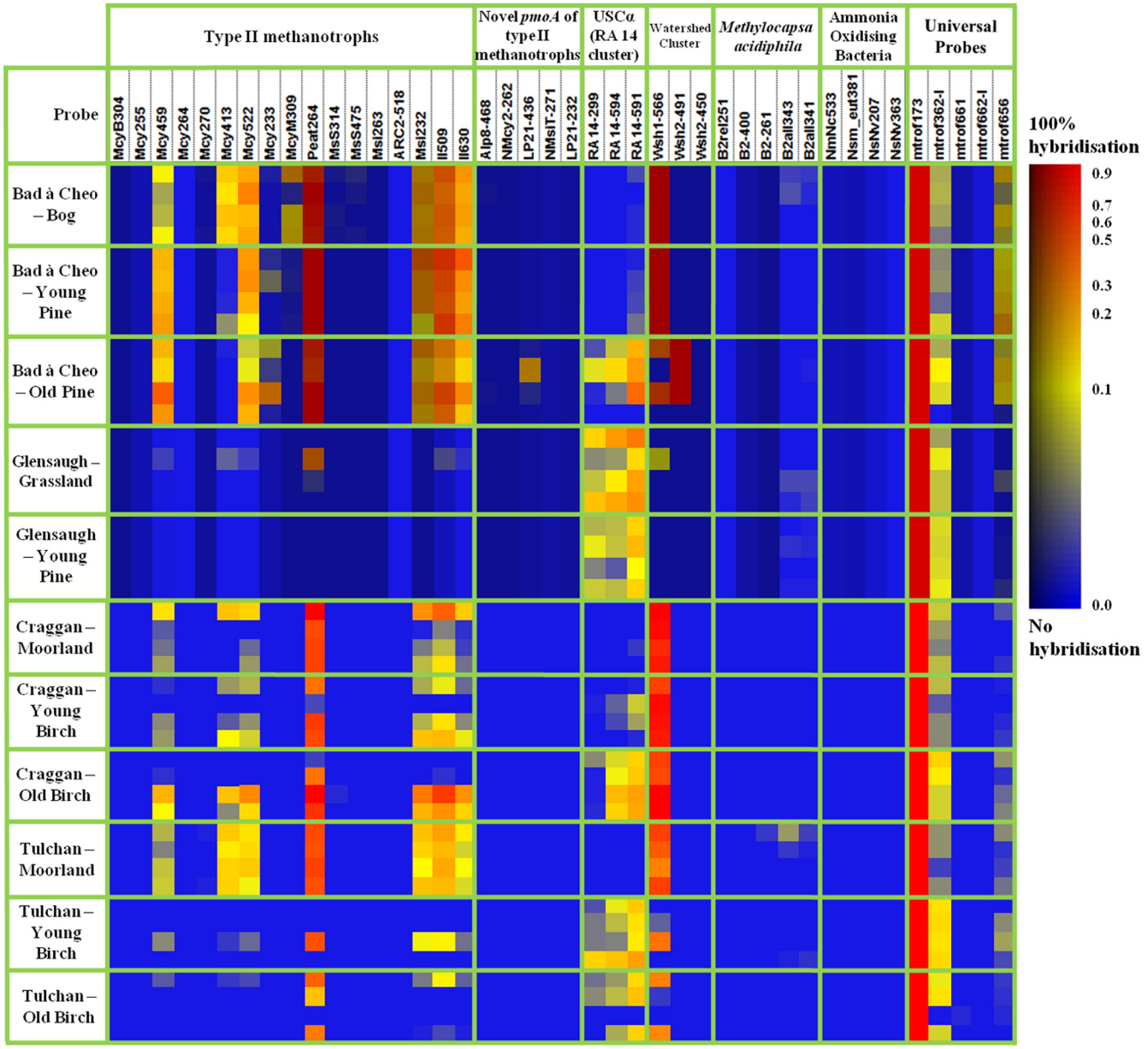

FIG 3 Community analysis of type II methanotrophs and related organisms using the *pmoA* microarray ($n = 44$). Within each habitat, each row represents a replicate. The results were normalized first to the positive-control probe mtrof173 and then to the reference values determined individually for each probe (29). Color coding is as follows: red color indicates the maximum achievable signal for an individual probe, while blue color indicates that no detectable PCR product hybridized to that probe. The color gradient between blue and red reflects the proportion of hybridization.

similarity) of *Methylosinus sporium* and the *Beijerinckiaceae* family (containing *Methylocapsa* spp.) due to a high incorporation of ^{13}C in the PLFA 18:1ω7 (41, 42) (see Fig. S1 in the supplemental material), which confirms the findings from the *pmoA*-based T-RFLP and *pmoA* microarray data (see previous section). However, it should be emphasized that PLFA-SIP analysis does not provide a high level of resolution (42) and was used to identify which organisms are responsible for the oxidation of CH_4.

Because the above-mentioned results described an overall lack of temporal and spatial variation, the data from the different sites (and seasons) were grouped into the main habitats explored: heathland, peatland, grassland, pine forest, and (young/old) birch forest. Further linear regressions were performed on the proportions of members of the USCα and cluster 5 clades, methanotroph richness, and CH_4 flux (Fig. 4). There was a strong relationship ($R^2 = 0.4755$, $P < 0.001$) between the increase in CH_4 oxidation rates and the loss of OTU richness due to tree growth (Fig. 4A and Table 2). In particular, the land use change revealed an increase in the proportions of USCα and cluster 5 *pmoA* sequences in soils while net CH_4 sinks increased ($R^2 = 0.3032$, $P < 0.001$) (Fig. 4B and Table 2). In other words, there was a negative relationship between the loss of methanotroph diversity (number of OTUs) and the increased dominance of members of the USCα and cluster 5 clades ($R^2 = 0.2019$, $P = 0.004$) (see Fig. S3 in the supplemental

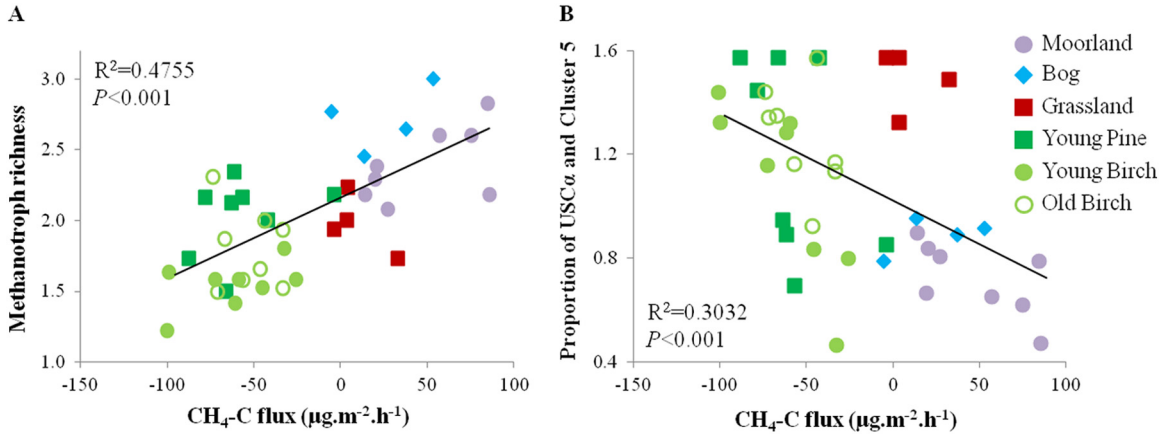

FIG 4 Relationships between methanotroph diversity and changes in CH_4 flux associated with land use treatment ($n = 4$ replicates). (A) The methanotroph richness (or OTU richness) was calculated as the square root transformation of the number of T-RFs present in each sample (among the 15 most abundant T-RFs of the T-RFLP profiles, which constituted >94% coverage). (B) Proportions of USCα and cluster 5 microorganisms were 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/cluster 5 and the *Methylocystaceae* family (T-RF Hha-81). Refer to Table 1 for T-RF reference values.

material). The "ecosystem stability" was defined as a component of the amplitude of variation in plant respiration by Tilman et al. (35). We used the same logic for CH_4 flux, which indicated that the standard deviation of net CH_4 fluxes decreased as the CH_4 sink rates increased in the forests, suggesting that the functional (ecosystem) stability improved with tree growth ($P = 0.038$) (Table 2).

Land use treatment did not consistently affect the abiotic properties of the soil (see Table S2 in the supplemental material). However, linear regression showed that some soil characteristics had a significant relationship with net CH_4 fluxes (see Table S3 in the supplemental material). These abiotic factors were related mainly to soil structure (particle size, water-filled pore space [WFPS], and/or moisture), which overall helped with gas diffusivity. However, these changes were not consistently observed between sites (see Table S3 in the supplemental material).

DISCUSSION

Soil function and functional microflora are affected by land use treatment but not by temporal and spatial variations. This study provides important correlative evidence that both soil function and associated functional microflora are stable over time and space. All sites showed increased CH_4 consumption rates with afforestation, suggesting a tree growth-induced effect, which were directly linked to a shift in the methanotrophic communities. In addition, both rate of CH_4 flux and community structure remained relatively stable throughout time and space. Our data demonstrate that a robust and consistent sampling procedure can overcome some variation in data which was previously assigned to temporal and spatial variations. We are confident in our data because previous studies demonstrated that our (laboratory-based) approach to CH_4 flux measurements provides results that are very similar to *in situ* (field-based) measurements (25, 30). Our evidence is supported by previous findings which report a lack of effect of temperature on CH_4 oxidation but an influence of soil physical structure (WFPS, soil moisture) (43, 44). Consistent and significant increases in CH_4 sinks in forest soils indicate that afforestation can be effectively used to mitigate net CH_4 fluxes. Upscaling of our data suggests that afforestation of 50% of all bogs and heathland in Scotland may offset about 5% of CH_4 emissions from the farming sector (based on 2006 CH_4 emission estimates [45]).

Our study provides correlative evidence that tree growth was associated with a change in CH_4 flux which was related to a shift in the methanotrophic community structure. This finding was consistent with the different analytical techniques used in this study (T-RFLP, microarray, and PLFA-SIP). A strong effect of land use treatment on the methanotrophic community structure was detected. In particular, the site and type of land use treatment influenced the type of methanotrophs present. The soils under grassland and pine trees at Glensaugh were dominated mainly by members of USCα, which are type II methanotrophs involved in the oxidation of CH_4 at atmospheric concentrations (37, 46). However, at the other sites (Bad à Cheo, Craggan, and Tulchan), members of the *Methylocystaceae* were relatively more dominant in the nonforested soils while USCα cells were characteristic of the forest soils. Methanotrophs of the USCα and cluster 5 clades were the main CH_4 oxidizers, but members of cluster 5 had a low abun-

TABLE 2 Significance between land uses for net CH_4 fluxes, methanotroph richness, proportions of USCα and cluster 5 members, and ecosystem stability[a]

Habitat	CH_4-C flux ($\mu g \cdot m^{-2} \cdot h^{-1}$)	Methanotroph richness (no. of OTUs)	Proportion of USCα/ cluster 5	Stability
Moorland	48.10^{α}	$2.39^{\alpha\beta}$	0.57^{α}	$1.29^{\alpha\beta}$
Bog	24.89^{α}	2.72^{α}	$0.63^{\alpha\beta}$	$1.24^{\alpha\beta}$
Grassland	9.26^{α}	$1.98^{\beta\gamma\delta}$	1.48^{γ}	0.73^{α}
Young pine	-57.33^{β}	$2.02^{\beta\gamma}$	$1.06^{\beta\gamma}$	1.59^{β}
Young birch	-61.87^{β}	1.54^{δ}	$1.06^{\alpha\beta\gamma}$	$1.52^{\alpha\beta}$
Old birch	-53.38^{β}	$1.80^{\gamma\delta}$	$1.24^{\beta\gamma}$	1.65^{β}
P value	<0.001	<0.001	<0.001	0.038

[a] Ecosystem stability was calculated as the square root transformation of the ratio of CH_4 flux to its corresponding standard error (35). For each variable, land uses followed by different Greek letters (α, β, γ, δ) are statistically different according to multiple pairwise comparisons ($\alpha = 0.05$). Tree growth was related to increased net CH_4 sink, decreased methanotroph richness, increased abundance of USCα, and increased ecosystem stability.

TABLE 3 Spatial stability of the methanotroph communities between sites with similar land uses[a]

Site	Habitat	Methanotroph richness	Proportion of USCα and cluster 5
Bad à Cheo	Bog	α	αβ
	Young pine	βγ	αβ
Glensaugh	Grassland	γδ	γ
	Young pine	γδε	γ
Craggan	Moorland	αβγ	α
	Young birch	δε	α
	Old birch	δε	βγ
Tulchan	Moorland	αβ	α
	Young birch	ε	γ
	Old birch	γδε	γ

[a] For each variable, land uses followed by different Greek letters (α, β, γ, δ, ε) are statistically different according to multiple pairwise comparisons ($\alpha = 0.05$).

dance in soils from forests. We found that tree growth, while improving CH_4 sink, always favored USCα cells over type I methanotrophs (in New Zealand [25]) or over members of the *Methylocystaceae* family (in Scotland) (this study). Further analysis of the literature suggests that USCα-related species dominate forest ecosystems (25, 36, 38, 47, 48), which may explain the enhanced CH_4 sink observed in forest soils worldwide. This further strengthens the link between change in functional microflora and improvement of soil function when tree growth occurred (see next section).

Overall, we did not detect any temporal variation due to seasonal change with regard to the methanotrophic community structure. There was some influence of the age of the birch forest at Craggan or, rather, of the methanotrophic community being slower to adapt to land use treatment at this site. Despite the comparatively weak effect of afforestation on community structure at Glensaugh, there was no spatial variation between sites with similar habitats following land use treatment (Table 3). In particular, our replicated moorland/birch woodland sites (Craggan/Tulchan) displayed a similar effect of tree invasion on methanotroph richness and relative abundance of USCα/cluster 5 cells (Table 3), thus suggesting a weak temporal and spatial variation. These results are supported by findings that methanotrophs lack spatial heterogeneity in agroecosystems (49, 50), which indicates that the effect of land use treatment on the soil microbial community is stronger and can be distinguished from temporal and spatial heterogeneity.

Macroecological theory can explain microbial control of net methane fluxes. The lack of ecological theory to explain the microbial regulation of ecosystem functions hampers the interpretation of the consequences of microbial shifts at the functional level and is hindered further by the lack of microbial data in predictive models. Here we provide correlative evidence that ecological theories developed for macroorganisms, such as plants and animals (macroecology), can be used to explain microbial regulation of biogeochemical cycles. Our data suggest that the microbial regulation of the CH_4 flux may be explained by the selection theory and illustrate that members of USCα are key species in atmospheric CH_4 sink globally (25, 46–48). Contrastingly, previous studies reported that microbial diversity (initial community evenness and increased species richness) enhances functional predictability and stability of ecosystems (35, 51–53). However, our results indicate a decrease in the methanotroph diversity (OTU richness) when CH_4 consumption increased (Fig. 4A). This discrepancy was able to be explained because we studied systems with large-scale land use changes whereas other studies either manipulated species richness and abundance in the laboratory (52, 53) or used agricultural fields (35) which are highly disturbed due to management practices such as plowing, irrigation, and fertilizer application (51). Additionally, the focus of our study was CH_4 consumption, which is restricted to a selected group of microbial communities. Biodiversity offers a buffer (insurance hypothesis) to maintain an ecosystem process when environmental changes occur and is due to the functional redundancy of the organisms involved (54, 55). Although this principle can be applied theoretically to any organisms (6), the situation for specific physiological groups of microbes, such as methanotrophs, is unusual because functional redundancy may not exist as such and because methanotrophy is a process limited to selected microbial taxa. Thus, a change in environmental conditions, such as land use treatment, can affect only the competition between certain methanotrophs, which will then affect species richness and evenness. Our data support the importance of the soil methanotrophic community structure as a regulator of CH_4 flux during land use change. Specifically, the proportions of USCα and cluster 5 microorganisms were associated with the net CH_4 oxidation rates of a habitat. Moreover, the decrease in methanotroph diversity was associated with an increase of the dominance of the USCα/cluster 5 cluster (see Fig. S3A in the supplemental material). This suggests that, in the systems we investigated, there was a niche-specific distribution of USCα/cluster 5 cells induced by land use change. Nonetheless, we argue that even though it is possible that ecosystem functioning can be explained by the selection theory, maintaining biodiversity should remain a key goal in conservation policy, as natural or anthropogenic disturbance may create new niches, and if these new niches remain unoccupied due to a lack of physiological diversity, it may have serious consequences for ecosystem functioning.

Although other studies investigated the relationship between land use change and CH_4 oxidation rates (56) or between land use change and the methanotrophic communities involved (26), this is the first time that a relationship linking land use change with a change in CH_4 oxidation rates and community structure and activity has been characterized over time and space. Our data suggest that the proportions of USCα and cluster 5 methanotrophs in the habitats investigated influence whether a soil is a source or sink for CH_4. Especially in the moorland/birch forest sites (Craggan and Tulchan) (Fig. 4B and Table 1), the switch between net CH_4 emissions and sinks occurred when the relative abundance of USCα/cluster 5 was about the same as that of *Methylocystaceae* (50/50), although the transition was slower in the young birch in Craggan. Data analysis from previous works suggests that a similar threshold was observed in New Zealand (25) except that USCα/cluster 5 cells had to constitute ~80% of the total methanotrophs to observe significantly higher CH_4 consumption rates. Nevertheless, the improvement of CH_4 sinks was concomitant with a change in the methanotrophic community structure in all sites, therefore indicating that microbes were driving the ecosystem function rather than being a consequence of changes in soil properties.

Herein we provide important evidence of regulation of CH_4 flux by methanotrophic bacteria. Some of our results should be

interpreted with caution because this study has some limitations. (i) We provide correlative evidence that macroecology theory may explain CH_4 flux in soils. However, unlike plants and animals, the species concept is not well defined for microbial communities. Consequently, OTUs obtained from T-RFLP and microarray analyses may be at the taxonomic level, which is not exactly comparable to plant and animal species. Additionally, both molecular approaches used here are based on PCR amplification, which is well known to introduce bias in data and have some implications in data interpretations. (ii) We measured ecosystem stability by variance of CH_4 flux without distinguishing rates of CH_4 production and consumption, and therefore, our data are a proxy to actual ecosystem stability. (iii) The nature of our study and conclusions that are drawn are correlative only. For achieving a mechanistic link between diversity and CH_4 flux, manipulative experiments, such as diversity dilution (52), may be needed. (iv) There are some other processes (e.g., anaerobic CH_4 consumption) and microbial groups (e.g., verrumicrobial methanotrophs) which were not accounted for in this work. These factors may also have played a role in the total CH_4 flux. (v) Our spatial and temporal data are limited. To confirm the conclusions from this study, future works should address all the above-mentioned issues by explicitly considering many more land use types, management practices, more-frequent flux measurements, and statistically designed spatial measurements.

In conclusion, this study provides experimental evidence for the increase and stability of CH_4 sink after afforestation, which was correlated with a shift in methanotrophic communities. The establishment of forest was accompanied with a decrease in methanotroph richness correlated to an increase in dominance of members of the USCα, while ecosystem stability improved. This study illustrates the microbial regulation of biogeochemical cycles by using CH_4 flux as an example. This supports the growing demand for explicit consideration of the inclusion of microbial data in predictive models studying the effects of global changes.

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