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Grazing affects methanotroph activity and diversity in an alpine meadow soil

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Summary

The role of methane-oxidizing bacteria (MOB) in alpine environments is poorly understood, but is of importance given the abundance of alpine environments and the role of MOB in the global carbon cycle. Using a combination of approaches we examined both seasonal and land usage effects on the ecology of microbial methane oxidation in an alpine meadow soil. Analysis of the abundance and diversity of MOB demonstrated that the abundance and diversity of the dominant type II MOB, predominantly *Metylocystis* and relatives, was only influenced by season. Conversely type Ia MOB abundance was significantly affected by season and land usage, while diversity changes were effected predominantly by land use. Assessment of methane oxidation potential and soil physical properties demonstrated a strong link between type Ia MOB abundance and methane oxidation potential as well as a complex series of relationships between soil moisture, pH and MOB abundance, changing with season. The results of this study suggest that, while type II MOB, unaffected by land use, represent the dominant MOB, *Methylobacter*-related type Ia MOB appear to be responsible for the majority of methane oxidation and are strongly affected by the grazing of cattle.

Introduction

Methane-oxidizing bacteria (MOB) or methanotrophs are a physiologically distinct group of bacteria utilizing methane as their sole source of carbon and energy. Methane-oxidizing bacteria are ubiquitous in nature and represent an important link in the global carbon cycle, being the single oxic biogenic sink for the greenhouse gas methane (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008). They oxidize methane via methanol and formaldehyde to carbon dioxide or incorporate carbon from methane into cell biomass at the oxidation level of formaldehyde. The first step in the pathway is catalysed by one of the two types of the enzyme methane monooxygenase (MMO). The soluble, cytoplasmic MMO (sMMO) is found in only some of these bacteria while the particulate, membrane-bound MMO (pMMO) is present in all known methanotrophs (Hanson and Hanson, 1996) except for *Methylocella palustris* (Dedysh *et al*., 2000). The sequence of *pmoA* encoding the 27 kDa subunit of pMMO reflects evolutionary relationships among *pmoA*-containing bacteria and is evolutionarily related to the ammonia monooxygenase (AMO) of autotrophic ammonia-oxidizing bacteria (AOB) (Holmes *et al*., 1995a).

Methanotrophs are traditionally classified into two taxonomical groups, type I and type II, based on their 16S rRNA phylogeny, carbon assimilation pathways, PLFA profiles and the architecture of their intracellular membrane system (Hanson and Hanson, 1996). Type I methanotrophs (the family *Methylococcaceae*; Bowman, 1999) belong to the γ-*Proteobacteria* and comprise the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, *Methylosphaera*, *Methylosoma*, *Crenothrix*, *Clonothrix*, *Methylococcus*, *Methylocaldum*, *Methylohalobius* and *Methylothermus* (Bodrossy *et al*., 1997; Bowman *et al*., 1997; Bodrossy *et al*., 1999; Wise *et al*., 2001; Heyer *et al*., 2005; Tsubota *et al*., 2005; Stoecker *et al*., 2006; Rahalkar *et al*., 2007; Vigliotta *et al*., 2007). Type I methanotrophs are further divided into type Ia and type Ib (sometimes also referred to as type X), the latter being typically thermotolerant to thermophilic bacteria, consisting of the genera *Methylococcus*, *Methylocaldum*, *Methylohalobius* and *Methylothermus*. Type II (the family *Methylocystaceae*; Bowman, 1999) includes the genera *Methylosinus*, *Methylocystis*, *Methylocapsa* and *Methylocella* (Dedysh *et al*., 2000; Dedysh *et al*., 2002). Recent studies have demonstrated nitrate reduction linked to anaerobic methane oxidation is possible (Raghoebarsing *et al*., 2006) and that methane oxidation may also be carried out by members of the *Verrucomicrobium* genus (Pol *et al*., 2007; Dunfield *et al*., 2007).

Studies of the ecology of methanotrophs, using molecular methods (reviewed in McDonald *et al*., 2008), from a range of environments suggest that these organisms

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show a cosmopolitan distribution, being detected in a broad range of environments, including soil (Saari *et al.*, 1997; Benstead and King, 2001; Knief *et al.*, 2006), rhizosphere (Gilbert and Frenzel, 1995; Calhoun and King, 1998; Bodelier *et al.*, 2000), marine (Holmes *et al.*, 1995b; Bowman *et al.*, 1997; Kimura *et al.*, 1999; Fujiwara *et al.*, 2000; Inagaki *et al.*, 2004) and freshwater (Auman and Lidstrom, 2002; Bussmann *et al.*, 2004; Lin *et al.*, 2004) environments.

The role of the different methanotroph taxa in the environment is unclear; however, experimental evidence suggests that they occupy different niches (Hanson and Hanson, 1996; Bodelier *et al.*, 2000; Noll *et al.*, 2008). There are indications that type I methanotroph populations react faster to changes in methane availability while type II populations appear to be minimally affected (Henckel *et al.*, 2000). Some experimental evidence indicates that type II MOB may be responsible for high-affinity methane oxidation (i.e. oxidation at atmospheric levels) in well-drained soils (Bull *et al.*, 2000).

The role of MOB in alpine environments is poorly understood, and represents an important consideration given the significant proportion of alpine environments in Europe (Mosier *et al.*, 1993; West and Schmidt, 2004; Margesin *et al.*, 2009; Zinger *et al.*, 2009).

The impact of agricultural practices, particularly grazing, on the methanotrophic communities in alpine meadows has not been studied; however, it is expected that long-term addition of manure to the soil would result in alterations to the microbial community (Hartmann *et al.*, 2006). Such a change may likely be related to the increased nitrogen inputs into the soil; however, whether this would have a positive or negative effect on methane oxidation is unclear (see review by Bodelier and Laanbroek, 2004).

The aim of this study was to determine the abundance, diversity and activity of MOB in an alpine environment and to examine the seasonal dynamics of these. In addition, we aimed to investigate if long-term land use (grazing) had a significant effect on methane oxidation potential and the corresponding MOB community structure, and to relate MOB ecology to edaphic parameters.

Microarray and quantitative PCR (QPCR)-based molecular analyses were carried out to determine the abundance, diversity, community structure and temporal dynamics of MOB. Methane oxidation potential was determined and related to community structure, soil pH and soil moisture.

Results and discussion

Field site

The field site was located at Kirchberg am wechsel, 70 km southwest of Vienna, Austria (47.4°N, 15.6°E) at 1340 m above sea level; the area comprised two adjacent sites, one ungrazed (unperturbed) and one subject to long-term grazing (Perturbed) (Fig. 1). The perturbed site comprised the same soil type and plant diversity as the unperturbed site; however, long-term cattle grazing had resulted in a reduced plant cover and significant additions of manure to the soil.

Climatic variability

There were large fluctuations in both temperature and precipitation over the four sampling time points (during winter, spring, summer and autumn) (Table 1). The lowest average daily temperatures occurred in the 3 weeks preceding the winter sampling, corresponding with snow cover and frozen surface soil, while summer and spring temperatures were the highest. Precipitation was the highest in summer and the lowest in winter. During winter and spring, snow cover and snow melting further modulated the effect of precipitation on soil water content.

Changes in soil pH, moisture content, methane oxidation potential and gene abundance

Moisture content and pH of the soil samples from the two sites were compared for all seasons (Table 1). Soil was generally acidic at both sites (mean pH 5.45 $\pm$ 0.42), with significantly lower pH in the unperturbed site for all seasons except winter. Soil moisture was the highest in winter, followed by spring, while the lowest water content was found during summer and autumn. Previous studies suggest that pH and moisture differences between the sites may result from manure inputs associated with cattle grazing (Whalen *et al.*, 2000).

Methane oxidation potential (MOx, measured using a 1% v/v headspace concentration according to the method of Börjesson and Svensson, 1997) in both sites was the highest in autumn but was not detectable in either site during winter (Fig. 2). Methane oxidation potential was consistently higher in the perturbed site, with significant differences between the two sites during summer and spring ($F = 14.2$, $P < 0.01$; $F = 10.5$, $P < 0.01$ respectively).

Quantitative PCR analysis of *pmoA* from type Ia and type II MOB, performed based on the method published previously (Kolb *et al.*, 2003), demonstrated the presence of both type Ia and type II MOB in all samples analysed (Fig. 2). Type II MOB were significantly more abundant (as determined by QPCR of *pmoA* genes) than type Ia MOB across all sites and seasons ($P < 0.001$). The lowest abundance of both type Ia and type II MOB occurred in winter, while the highest abundance of type Ia MOB occurred in autumn and the highest type II MOB abundance occurred in summer. The abundance of both type

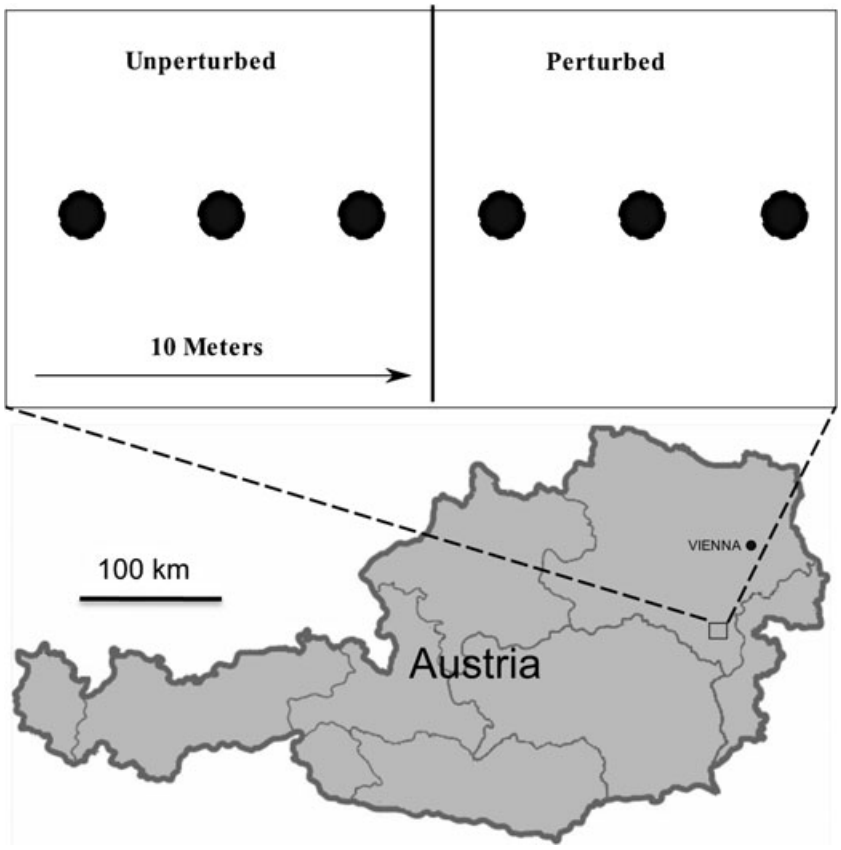

Fig. 1. Location and layout of the sampling site, showing perturbed (cattle grazing) and unperturbed (no cattle) sites. Samples were taken at four different time points corresponding to winter, spring, summer and autumn. Sampling comprising six 30 mm diameter soil cores with five 100 mm depth layers, with three cores taken from each site (total $n = 120$). DNA was extracted from each of the soil samples using a method described by Yeates and colleagues (1998) and modified by Stralis-Pavese *et al.* (2004).

Ia and type II methanotrophs appeared to be affected by changing weather conditions (rainfall and soil moisture, temperature etc.) as indicated by the seasonal changes in their abundance (Fig. 2). It is possible that *Methylocella* are present in this environment and, as they lack the *pmoA* gene, would be unaccounted for in this study.

Land use (grazing and associated effects on plant abundance, soil organic N and C content) had a positive effect on the abundance of type Ia methanotrophs for all seasons with type Ia MOB more abundant in the perturbed site compared with the unperturbed site across the whole year ($F = 5.7–18.9$, $P < 0.05–0.001$) (Fig. 2). Type II MOB abundance was not significantly different between perturbed and unperturbed sites for any season (Fig. 2). This, and the strong correlation between type Ia abundance and methane oxidation potential (Fig. 2B and C), suggest that the type Ia methanotroph population was primarily responsible for the observed methane oxidation potential. This is further supported by the finding that the abundance of type II methanotrophs appears to be unrelated to changes in land use and to the corresponding increased methane oxidation potential. The reason for the high abundance of type II MOB in this soil apparently unrelated to MOx potential is unclear. There are several potential explanations for this phenomenon. They may represent inactive or resting cells as suggested previously (Noll *et al.*, 2008); Baani and Liesack (2008) demonstrated the potential for *Methylocystis* to oxidize methane

Table 1. Seasonal sample characteristics.

			Soil pH		Moisture (%)	
Season	Temperature (°C)[a]	Rainfall (mm)[a]	U	P	U	P
Summer	13	139.7	5.35*	5.52	31.2*	36.8
Autumn	4	31.75	5.20*	5.46	40.6*	28.1
Winter	–0.4	27.94	5.07	5.79	58.3	50.0
Spring	13	95.26	5.49*	6.61	44.8	42.7

a. Temperature and rainfall data (mean of 3 weeks prior to sampling) taken from Hirschenkogel weather station (http://www.tutiempo.net/en/Climate). For soil pH and moisture content significant differences in means between sites are indicated by an asterisk (*) (one-way ANOVA). U, unperturbed site; P, perturbed site.

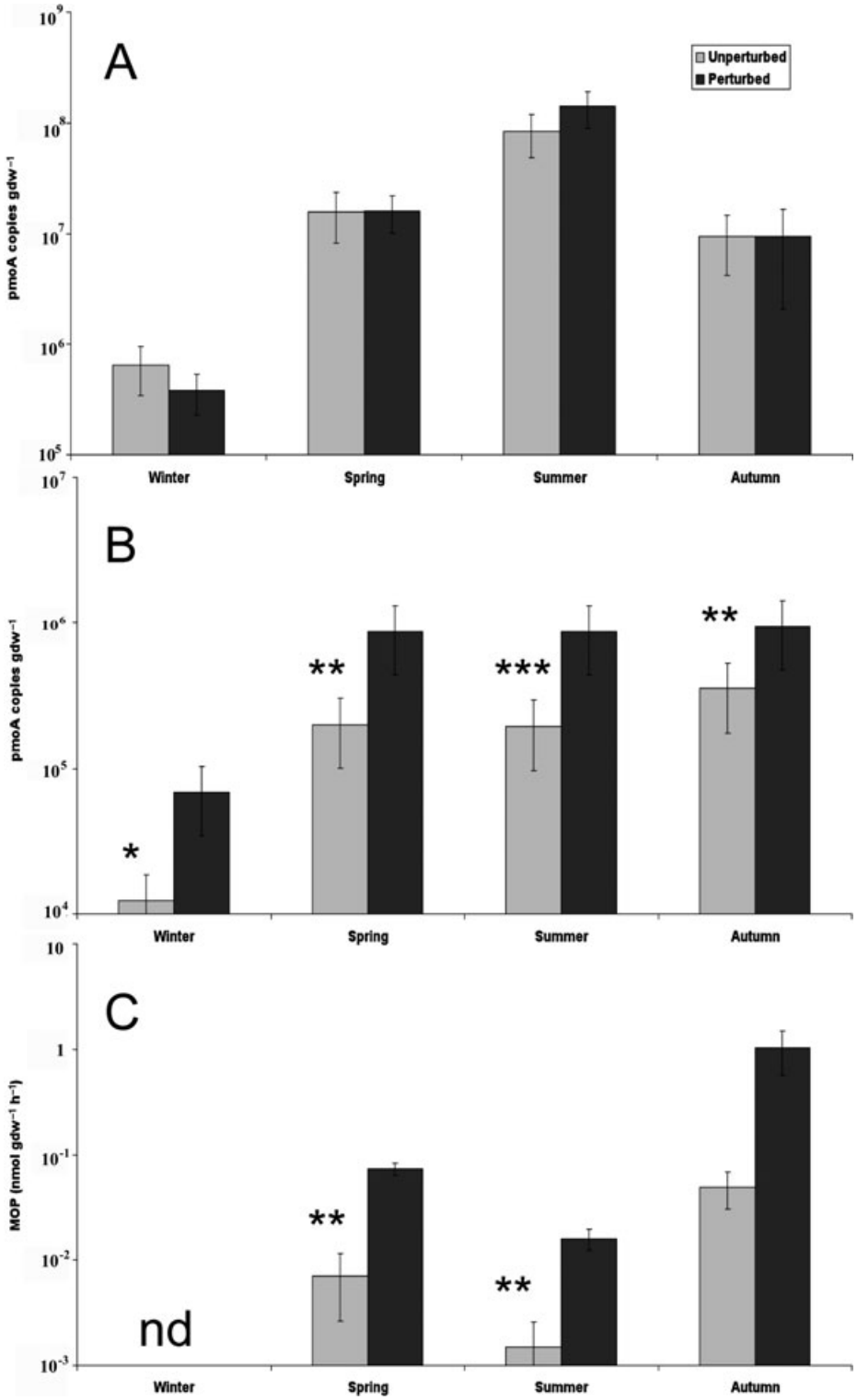

Fig. 2. Temporal changes in samples from the perturbed and unperturbed sites across four seasons. Variation was assessed using ANOVA (SPSS, SPSS, USA); significant factors were then compared using Tukey *post hoc* test. Significant differences between sites are shown (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

A. Assessment of the temporal change in abundance of type II MOB using QPCR (assessed using the QPCR method as per Kolb *et al.*, 2003)

B. Assessment of the temporal change in abundance of type Ia MOB (assessed using the QPCR method as per Kolb *et al.*, 2003)

C. Assessment of the temporal change in methane oxidation potential (assessed using the method of Börjesson and Svensson, 1997).

Table 2. Relationships between physical parameters and gene abundance.

	Summer	Autumn	Winter	Spring
pH vs. type Ia	0.444*	0.324	0.389*	0.556**
pH vs. type II	–0.373*	0.096	–0.100	0.052
pH vs. MOx	0.516**	0.217	–	0.716**
Moist vs. Ia	0.560**	0.267	0.380*	0.580**
Moist vs. II	0.737**	0.324	–0.228	0.730**
Moist vs. MOx	0.123	0.062	–	0.336
Ia vs. II	0.600**	0.261	0.06	0.673**
Ia vs. MOx	0.821**	0.435*	–	0.806**
II vs. MOx	0.117	–0.446*	–	0.196

Pearsons product moment correlations between environmental parameters and gene abundance for each season. Data that were not normally distributed were transformed (square root or Log), prior to analysis. Level of significance is shown: *$P < 0.05$, **$P < 0.01$.

at low mixing ratios helping to explain their dominance in soils with low Km values for methane oxidation. While it is possible that the *Methylocystis* species detected may oxidize methane at atmospheric concentrations, with their succession being related to physicochemical soil variables such as pH, NH_4 and Cu^{2+} (Bender and Conrad, 1995), the strong seasonal dynamics of type II MOB suggests that they are more likely to be utilizing compounds other than methane for growth. Murase and Frenzel (2008) demonstrated differences in the preference for protozoa to graze on type I rather than type II MOB; this may also have a fundamental effect on the dynamics and abundance of the two groups in soil.

Statistical analyses revealed a number of potential relationships between MOB abundance and soil physical parameters, varying with sampling season (Table 2). Abundance of type Ia MOB was significantly related to soil moisture content and to pH in all samples except autumn, while type II MOB abundance was significantly related to moisture in summer and spring and negatively related to pH in summer. Type Ia MOB were significantly related to MOx in all seasons where MOx was detected and MOx was also related to pH in spring. Type II MOB were negatively related to MOx in autumn. Abundances of type Ia and type II MOB were related in summer and spring.

The absence of detectable MOx in winter may relate to the low temperatures and subsequent decreased biomass of MOB demonstrated, as indicated by the decrease in *pmoA* abundance. Low temperature and its indirect consequences (decreased overall metabolic activity, limited methane and/or oxygen availability), resulting in a strongly limited methanotroph activity, may be one of the major reasons behind the overall decrease in methanotroph abundance in winter (Börjesson and Svensson, 1997).

The higher MOx and type Ia abundance in the perturbed site may both relate to the increased pH. Both of these parameters were significantly related to pH in summer and spring, and may well be linked to increased nitrogen load, as would be expected as a result of long-term manure inputs, in the perturbed site. It is therefore likely that the effect of increased nitrogen deposition from long-term grazing of the perturbed site has led to higher pH and subsequent increased N availability, through nitrification. This may, in turn, lead to stimulation of MOx capacity in the soil through increased abundance of low-affinity type Ia MOB. Previous studies have demonstrated that methane oxidation rates related to type I MOB were increased significantly by nitrogen fertilization (Noll *et al.*, 2008), while inorganic nitrogen (nitrate and ammonia) has also been suggested to select for type I MOB (Hanson and Hanson, 1996).

Microarray analysis

Microarray analysis demonstrated the MOB community (as assessed using the *pmoA* gene) to comprise predominantly type II phylotypes, while type Ia and RA14-related phylotypes (Holmes *et al.*, 1999) were also detected. Type II phylotypes were shown to comprise predominantly *Methylocystis* relatives including the 'Peat group' (McDonald and Murrell, 1997), while type Ia phylotypes were mostly *Methylomonas* and *Methylobacter* related (Fig. 3). *Methylocystis* belonged predominantly to the *Methylocystis* A group, while the *Methylocystis* B group (targeted by probes Mcy255, McyM309 and McyB304) was also detected but was comparatively less dominant with more variation in signal intensity.

Multivariate statistical analysis (Primer 6, Appendix S1) was used to determine the effects of depth, site and season on MOB community composition. PERMANOVA analysis demonstrated no significant effect of soil depth on MOB community composition, suggesting that the MOB community is homogenous across depth and soil layers. There was however a significant effect of site and season on community composition, while there was no statistical interaction between site and season, suggesting that seasonal changes are not related to land use.

ANOSIM analysis was used to test the difference in MOB community composition across site and season (Table 3). Methane-oxidizing bacteria community composition was significantly different between sites ($R = 0.23$, $P < 0.01$). There was a significant difference in MOB community composition between all seasons ($R = 0.142$–0.351, $P < 0.001$). The greatest change in community composition was observed from spring to summer and the smallest from winter to spring.

Analysis of the difference between sites using SIMPER analysis (Primer 6) demonstrated that probes corresponding to the RA14 group (RA14_594/591); to an uncultivated group, related to RA14, found in watershed and flooded forest soils (Wsh1-566; Uz *et al.*, 2003; Knief *et al.*, 2006);

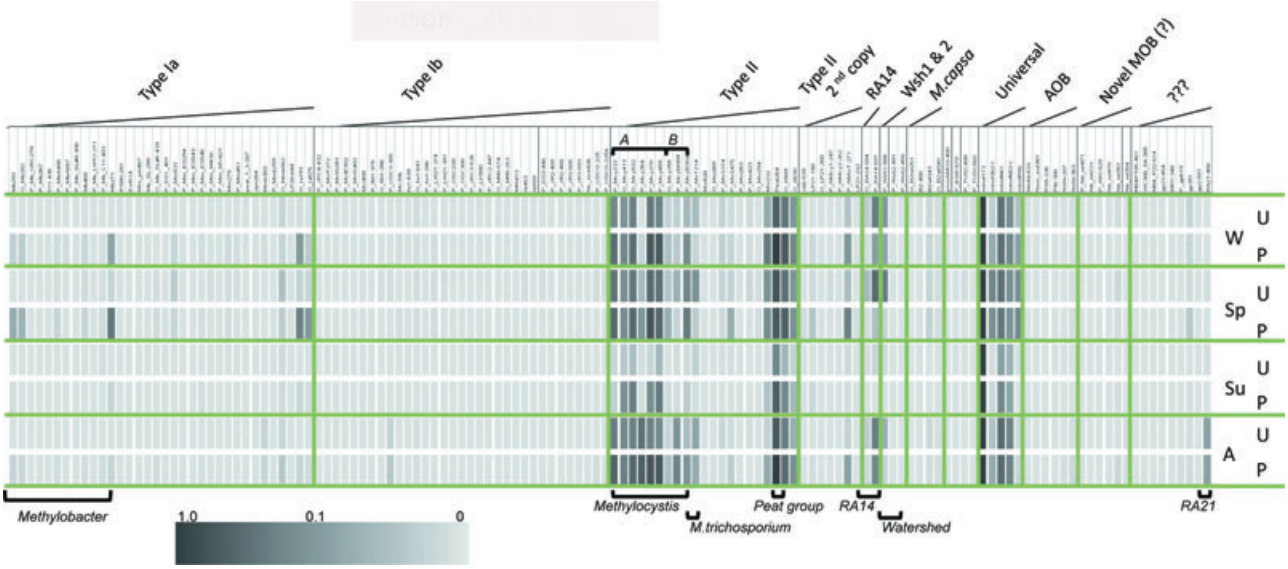

Fig. 3. Microarray results showing the composition of MOB communities by season across the 0–10 cm layer of replicate cores in the unperturbed (U) and perturbed (P) sites: *pmoA* gene fragments (amplified using a nested PCR with primers 189f–682R, then 189f–T7661R; Holmes *et al*., 1999; Stralis-Pavese *et al*., 2004) were analysed using a *pmoA* microarray as published elsewhere (Stralis-Pavese *et al*., 2004). Important probes are highlighted in brackets; full details of the applied probe set (Chen *et al*., 2008) are provided in Table S2.

and to *Methylosinus trichosporium* (MsT214) showed higher signals in the unperturbed site. Probes corresponding to the *Methylobacter* cluster (Mb271/460 and Mb282/Mb292) showed much higher signals, while probes targeting the *Methylocystis* cluster (Mcy264, Mcy270, Mcy522 and Mcy459) displayed slightly stronger signals in the perturbed site (Table S1).

Seasonal variation across both sites, assessed using CAP (canonical analysis of principal coordinates) (Fig. 4), demonstrated that autumn and spring samples all showed increased signals in probes corresponding to the *Methylocystis* group phylotypes (Mcy264, Mcy233, Mcy270, McyM309 and McyB304, Mcy-255, Mcy413 respectively), compared with the other months, while summer showed an overall increased dominance of *Methylocystis* probes, particularly Mcy413 and decreased signals for probes corresponding to the other groups of phylotypes. Autumn samples showed an increase in dominance of the probe signal associated with the RA14 group (RA14-591) as well as a probe associated with forest soil clones of unknown function (RA21-486; Holmes *et al*., 1999), compared with the other groups. Spring and winter samples differed from the other seasons due to probes targeting *Methylobacter* phylotypes (Mb271) and the watershed I group (Wsh1-566; Uz *et al*., 2003; Knief *et al*., 2006).

Table 3. Results of ANOSIM analysis of *pmoA* microarray data, comparing seasons and sites.

	Summer	Autumn	Winter	Perturbed
Autumn	0.289	–		
Winter	0.224	0.252	–	
Spring	0.351	0.305	0.142	–
Unperturbed	–	–	–	0.23

All $P < 0.01$.

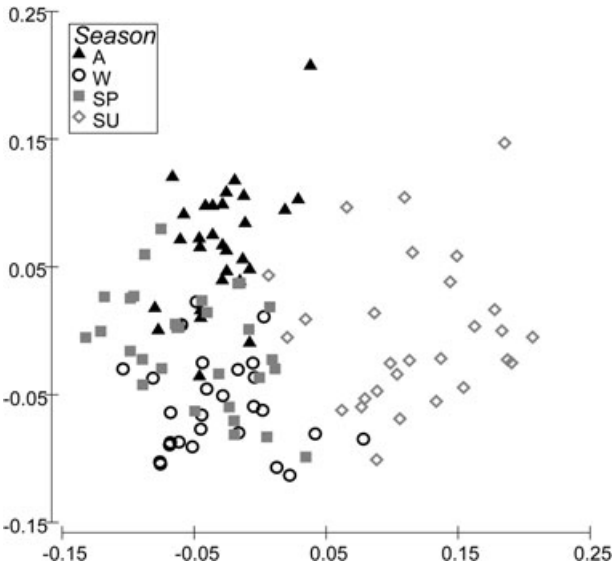

Fig. 4. Canonical analysis of principal coordinates (CAP) analysis of *pmoA* microarray data performed using the Primer 6 package (Primer-E, UK) on standardized array data after removal of values for universal and control probes, highlighting the difference in MOB community structure between seasons autumn (A), winter (W), spring (SP) and summer (SU). Leave one out Cross Validation (Anderson *et al*., 2008) gave 75% correct assignment to seasonal groups.

While the difference in MOB community structure between summer and the other seasons was large, samples from spring and winter were not easily distinguished suggesting they had a similar MOB community structure.

Type Ia methanotrophs, in particular *Methylobacter*, appeared to be associated with potentially oxygen-limited, methane-rich conditions. They were predominantly found in perturbed sites (except for summer when the soil was completely dry and thus presumably well aerated) and in unperturbed sites in spring when the soil was water saturated from the slow melting of the snow cover and thus presumably poorly aerated. A similar tendency was seen in individual samples for *Methylomonas*, which appeared only sporadically, but only in the same samples mentioned above (data not shown).

Atmospheric methane oxidizers belonging to the RA14 clade (USC-α, belonging to the α-*Proteobacteria*) and to a related clade, Wsh1, also predominantly found in soils showing atmospheric methane uptake (Knief *et al.*, 2003), were significantly reduced under grazing conditions. Interestingly, probes targeting the other clade associated with atmospheric methane oxidation, USC-γ (Knief *et al.*, 2003) (USCG-255 and USC-255b), were rarely positive. This may be due to the more acidic nature of the soils since USC-γ are predominantly detected in upland soils with $pH > 6.0$ (Knief *et al.*, 2003).

Type Ib methanotrophs were rarely detected (sporadic probe signals were seen in a few individual samples). This finding is in line with the general observation that type Ib methanotrophs are typically associated with mud/sludge/sediment environments (as indicated by details from GenBank entries of type Ib *pmoA* sequences).

Conclusions

Grazing of an alpine meadow resulted in increased (low-affinity) methane oxidation potential as well as an increase in type Ia MOB abundance.

Type II MOB, in particularly members of the *Methylocystis* genus and the so far uncultivated 'peat' clade, were the predominant methanotrophs in both perturbed and unperturbed sites, and while they appeared to be unaffected by land use their abundance changed seasonally possibly in response to environmental factors such as temperature, pH and moisture that subsequently affected micronutrient availability as has been suggested previously (Harmsen and Vlek, 1985).

Type Ia MOB abundance, however, varied significantly with both season and land usage and was strongly related to MOx.

The apparent differences in the ecology of the different groups of MOB in the two alpine meadow sites suggest a number of strategists occupying specific niches, responding independently to the effects of seasonal change and land use (grazing).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Results of SIMPER analysis of *pmoA* microarray data, showing the differences in probe contribution to total signal (%) between perturbed (P) and unperturbed (U) sites. Values are average dominance of the probe for the respective site.

Table S2. *pmoA* microarray probes used in this study, indicating the probe names, intended specificity, sequence, length, GC content and T_m values for each probe.

Appendix S1. Methods: multivariate statistical analysis.

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