

## ORIGINAL ARTICLE

# Effects of cattle husbandry on abundance and activity of methanogenic archaea in upland soils

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**In the present study, we tested the hypothesis that animal treading associated with a high input of organic matter would favour methanogenesis in soils used as overwintering pasture. Hence, methane emissions and methanogen populations were examined at sections with different degree of cattle impact in a Farm in South Bohemia, Czech Republic. In spring, methane emission positively corresponded to the gradient of animal impact. Applying phospholipid etherlipid analysis, the highest archaeal biomass was found in section severe impact (SI), followed by moderate impact (MI) and no impact. The same trend was observed for the methanogens as showed by real-time quantitative PCR analyses of methyl coenzyme M reductase (*mcrA*) genes. The detection of monounsaturated isoprenoid side chain hydrocarbons (i20:1) indicated the presence of acetoclastic methanogens in the cattle-impacted sites. This result was corroborated by the phylogenetic analysis of *mcrA* gene sequences obtained from section SI, which showed that 33% of the analysed clones belonged to the genus *Methanosarcina*. The majority of the sequenced clones (41%) showed close affiliations with uncultured rumen archaeons. This leads to the assumption that a substantial part of the methanogenic community in plot SI derived from the grazing cattle itself. Compared to the spring sampling, in autumn, a significant reduction in archaeal biomass and number of copies of *mcrA* genes was observed mainly for section MI. It can be concluded that after 5 months without cattle impact, the severely impact section maintained its methane production potential, whereas the methane production potential under moderate impact returned to background values.**

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## Introduction

Agriculture offers a great potential for green house gas mitigation and for a systematic sequestration of carbon dioxide in soils and in plant biomass (OCA, 2006). In this respect, upland grasslands in low-input farming systems are considered as sinks for the green house gas methane (CH<sub>4</sub>) (Hütsch *et al.*, 1994) and only weak sources for nitrous oxide (N<sub>2</sub>O) (Mosier *et al.*, 1991). However, these mitigation potential can change when grasslands are used as pastures by grazing

cattle. For example, Clayton *et al.* (1994) found that N<sub>2</sub>O emissions from a grazed pasture were three times higher than those from the pasture without grazing and speculated that interaction between treading and excretal returns could have stimulated this process. Animal treading causes reduction in soil aeration through soil compaction, which may enhance rates of denitrification (Menneer *et al.*, 2005). Moreover, treading can also mix organic carbon-rich material originated from excrement into soil, stimulating microbial metabolism and thus increasing oxygen demand also at lower soil depths (Šimek *et al.*, 2006). Both, anaerobic environment and availability of organic carbon may favour methanogenesis. Indeed, some studies have shown a temporal increase in methane emissions immediately after application of manure for soil fertilization (Sommer *et al.*, 1996; Sherlock *et al.*, 2002).

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Although changes in methane fluxes are expected in soils strongly impacted by animal husbandry, data about the structure and function of methanogenic archaeal community present in this environment are missing. Therefore, the objective of this study was to determine the effect of outdoor overwintering cattle husbandry on methane emissions in coherency to the abundance and diversity of soil methanogenic communities. Particularly, the following hypothesis were tested: (i) cattle overwintering pasture leads to changes in soil function, towards methane emission instead of methane sink, (ii) this emissions are correlated with the degree of animal impact and (iii) cattle excrements serve as an inoculum for rumen methanogens and stimulate growth and activity of soilborne methanogens.

To characterize total and methanogenic archaeal biomass, we used the phospholipid etherlipid (PLEL) approach (Gattinger *et al.*, 2002, 2003). The taxonomic resolution of this method is relatively low, but its robustness towards varying soil matrices has been demonstrated (Bai *et al.*, 2000; Wachinger *et al.*, 2000; Wagner *et al.*, 2005). Moreover, real-time quantitative PCR and clone library analyses of the methyl coenzyme M reductase (*mcrA*) gene, which codes for methyl-coenzyme M reductase  $\alpha$ -subunit, were conducted for more detailed investigation of the abundance and structure of methanogenic communities. This enzyme catalyses the reduction of methyl-coenzyme M leading to the release of methane (Ellermann *et al.*, 1988). The presence of the *mcrA* gene is restricted to methanogenic archaea (Thauer, 1998); hence its quantity serves as an estimate for methanogenic biomass in soil.

In the present study, we demonstrated that use of grasslands for cattle overwintering drastically changed soil properties, which in turn affected the abundance and activity of soil methanogens.

## Material and methods

### *Experimental site*

Investigations were carried out at a cattle overwintering area located at Borová Farm near Český Krumlov in South Bohemia, Czech Republic (latitude 48°52' N, longitude 14°13' E). The approximately 4-ha area has been used for overwintering (from October/November until April/May) of about 90 cows since 1995 (for more details see Šimek *et al.*, 2006). The soil on the site is a sandy loam, classified as Cambisol, and containing 60–80% sand, 14–32% silt and 6–14% clay (according to USDA classification system). The plant cover is a perennial mixture of grasses, clovers and other dicotyledonous plants. The long-term mean annual precipitation in the area is 650 mm and the annual average temperature is 7°C (data from meteorological station located at 7 km distance from the experimental farm). At the end of the winter season (from April to May), there is a visible gradient of impact due to the cattle husbandry. The degree of impact on overwintering sites is

not identical on the whole pasture. We investigated three sections of this gradient: the most affected area situated near the barn (section SI = severe impact), a less impacted area in the middle of the gradient (section MI = moderate impact) and unaffected area at the opposite side of the overwintering area (section NI = no impact). In spring, the studied sections were characterized as follows: SI by completely destroyed vegetation, damaged soil structure and oversaturation by the excrements; MI by partly destroyed vegetation and remains of excrements on the soil surface; NI by undisturbed grass cover and no visible remains of excrements.

### *Methane flux measurements and soil sampling*

Field works were performed twice, in spring (11 May 2005), after removing the animals from overwintering area and in fall (18 October 2005), before they were brought back. Different locations at each section were randomly selected from each section for gas flux measurements and soil sampling and used as independent field replicates in this study. Methane fluxes were determined using medium-sized (basal area, 0.076 m<sup>2</sup>; volume, 15 dm<sup>3</sup>) non-vented manual closed chambers. The chambers were pushed ca 3 cm into the soil immediately before the measurement. Each chamber had a rubber septum for gas sampling inserted immediately after chamber installation. Headspace gas samples were collected at the time of chamber deployment and after 60 min. Preliminary investigations on the site showed that the increases in gas concentration within the closed chambers were linear during chamber deployment. Nine gas samples were taken at each section, stored in pre-evacuated 3.5-ml glass vials, transported to the laboratory and analysed immediately. The amount of CH<sub>4</sub> was quantified using an HP 5890 Series II gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with a 2 m Porapak N column at 750°C, and a flame ionization detector. For instrument calibrations standard mixtures of CH<sub>4</sub> were used, and the results were corrected for gas losses in connection with transfer to and from glass vials.

Parallel to the gas sampling, true replicate soil samples were obtained from the area of four chambers (0–20 cm depth), by pooling seven subsamples, which were randomly taken using a soil auger of 3-cm diameter. The soil was immediately homogenized by sieving through a 5-mm mesh sieve. Samples for nucleic acid and phospholipid analyses were immediately stored at –80°C and 4°C, respectively.

A higher number of replicate was used for the gas measurements due to the great heterogeneity observed for those areas in preview analysis.

### *Soil analyses*

Gravimetric moisture content was determined after drying at 105°C. Soil pH was measured using a glass

electrode in 1:2.5 (w/w) soil/water suspension. Soil mineral N ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ) was measured colourimetrically from 1 M KCl extracts using a soil (fresh field-moist) solution ratio of 40 g:200 ml (Zbiral *et al.*, 1997). Total organic carbon was determined by wet oxidation with acid dichromate (Jackson, 1958), and total nitrogen content by Kjeldahl digestion (Zbiral, 1995).

#### Determination of total and archaeal biomass by PLFA and PLEL

Lipids were extracted from fresh soil samples equivalent to 10 g dry weight (d.w.), according to the Bligh–Dyer method as described elsewhere (Zelles and Bai, 1993). The resulting lipid material was fractionated into neutral lipids, glycolipids and phospholipids on a silica-bonded phase column (SPE-SI; Bond Elute, Analytical Chem International, USA) by elution with chloroform, acetone and methanol, respectively. An aliquot of the phospholipid fraction equivalent to 2.5 g d.w. was taken for phospholipid fatty acid (PLFA) analysis. After mild alkaline hydrolysis, described in detail by Oravec *et al.* (2004), the resulting fatty acid methyl esters were separated on a capillary gas chromatograph and identified using the MIS automatic identification system (Agilent 6850, FID, TSBA50, MIDI Inc., Newark, DE, USA).

Another aliquot of the phospholipid fraction equivalent to 7.5 g soil d.w. was used for PLEL analysis according to Gattinger *et al.* (2003). After the formation of ether core lipids, ether-linked isoprenoids were released following cleavage of ether bonds with HI and reductive dehalogenation with Zn in glacial acetic acid. The resulting isoprenoid hydrocarbons were dissolved in 100  $\mu\text{l}$  internal standard solution (nonadecanoic methyl ester) and subjected to gas chromatography-mass spectrometry analysis at operating conditions described elsewhere (Gattinger *et al.*, 2003).

Data of the individual PLFA/PLEL compounds (expressed in  $\text{nmol g}^{-1}$  soil d.w.) were added to obtain the concentration of total phospholipid chains, a measure for total microbial biomass in soil (Zelles, 1999). The proportion of individual PLFA/PLEL compounds to the concentration of total phospholipid chains is expressed in % and gives an estimate of the relative abundance of certain microbial groups (Zelles, 1999).

The following abbreviations were used for the different PLEL-derived isoprenoid hydrocarbons: i20:0 indicates a saturated and i20:1 a monounsaturated isoprenoid chain with 20 C atoms; i40:0 denotes an isoprenoid chain with 40 C atoms.

#### DNA extraction from soil

DNA was extracted from 500 mg soil as described by Griffiths *et al.* (2000). The quality and quantity of

the extracts was evaluated using a spectrophotometer (Nanodrop, PeqLab, Erlangen, Germany).

#### Real-time quantitative PCR of *mcrA* genes

The real-time quantitative PCR (qPCR) was performed in an ABI Prism 7700 sequence detection system (Perkin Elmer, Foster City, CA, USA). Reaction mixtures contained 5  $\mu\text{l}$  of qPCR ROX & Go Green (qBiogene, Illkirch, France), 1.5  $\mu\text{g}$  bovine serum albumin (Sigma-Aldrich, Germany), 5 pmol of each primer (Luton *et al.*, 2002), 5% dimethyl sulphoxide (Sigma-Aldrich, Steinheim, Germany), 0.5  $\mu\text{l}$  DNA template and water to a final volume of 25  $\mu\text{l}$ . The amplification was carried out as follows: initial denaturation at 95°C for 15 min and 40 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. Standard curves were constructed using 10-fold serial dilutions of plasmids containing a partial sequence of *Methanosarcina mazei mcrA* gene. Amplification efficiencies of the PCRs were calculated using data from the standard curves with the following formula: efficiency  $[10^{-(1/\text{slope})}] - 1$ . To discard inhibitory effects of substances co-extracted with the DNA, amplification of serial dilutions were performed for samples of each plot. Four independent assays were run per sample. The quality of the amplification was evaluated by the generation of melting curves of the PCR products and confirmed in 1.5% agarose gels stained with ethidium bromide.

#### Cloning and sequencing of *mcrA* genes

Fifty nanograms of DNA were pooled from four replicate extracts of section SI and used as template for the amplification of *mcrA* genes. The reaction mixture and amplification cycle were performed as described above, excepting the polymerase (*Taq* polymerase, Invitrogen, Karlsruhe, Germany).

Purified PCR products from the four replicates were pooled and cloned using the TA cloning kit (Invitrogen), following manufacturer's instructions. The reaction contained ca 50 ng of PCR product, 1  $\mu\text{l}$  of ligation buffer, 2.5  $\mu\text{l}$  of sterile  $\text{dH}_2\text{O}$ , 2  $\mu\text{l}$  of the vector (pCR 2.1) and 1  $\mu\text{l}$  of T4 ligase. White colonies, containing the insert, were inoculated in Luria–Bertani medium with 50  $\mu\text{l ml}^{-1}$  kanamycin, grown overnight and used for plasmid isolation (Qiagen Plasmid mini kit, Qiagen, Hilden, Germany). Plasmids holding inserts of correct size were selected after digestion with *EcoRI* (MBI Fermentas, Vilnius, Lithuania).

Fifty-four clones were randomly selected and sequenced in a ABI Prism 3730 DNA sequencer using the BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Deduced amino-acid sequences of MCR  $\alpha$ -subunit (141 positions) were obtained using the program expase and aligned by clustalW. Distance matrix was calculated using 'Protdist' (PHYLIP: <http://evolution.genetics>).

washington.edu/phylip.html) and Dayhoff PAM 001 matrix as amino-acid replacement model. Phylogenetic dendrograms deduced MCR sequences from reconstructed distance matrix using neighbour-joining analysis and randomization of species input. Bootstrap values were calculated of 1000 sets.

To estimate the completeness of sampling of a community, rarefaction curves were generated using the software Analytic Rarefaction (<http://www.uga.edu/~strata/software/Software.html>). Deduced amino-acid sequences showing more than 98% similarity were considered as one operational taxonomic unit (OTU) (Juottonen *et al.*, 2006). The obtained sequences were submitted to National Center for Biotechnology Information database and can be found under the following accession number: DQ994833–DQ994886.

### Statistical analysis

All statistical analyses were performed with SPSS software package release 12.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to determine the significance between treatments followed by all pairwise multiple comparison procedure (Student–Newman–Keuls test). Kruskal–Wallis one-way ANOVA was applied for cases where normality test of data distribution failed.

## Results

### General soil characteristics and CH<sub>4</sub> fluxes

Table 1 compiles the soil properties of the three sections studied. All variables showed significant differences among sections. The increasing levels of organic C (C<sub>org</sub>), total N (N<sub>t</sub>), ammonium (NH<sub>4</sub><sup>+</sup>) and

pH along the gradient from section NI over MI to SI clearly reflected the impact of cattle due to excretal returns. Site SI also showed much higher concentrations of total phospholipids compared to the other sites. The observed differences among the three sections were more pronounced at spring (after cattle left the overwintering area) than at fall sampling (before cattle entered the area).

Table 2 compiles the *in situ* measurements of CH<sub>4</sub> emissions. At both samplings, CH<sub>4</sub> emissions at the three sections reflected the different degrees of animal impact on the pasture, that is the highest values in SI and the lowest in NI. Section NI appeared even as a sink for methane. However, the differences among the sites were only statistically significant at spring sampling. CH<sub>4</sub> emissions measured for sections SI and MI in fall were much lower than in spring.

### Archaeal biomass

The concentration of archaeal PLEL decreased in accordance with degree of animal impact from section SI to NI (Figure 1a). In spring, PLEL concentration decreased from 28 to 0.5 nmol PLEL isoprenoids g<sup>-1</sup> soil d.w., in fall from 28 to 0.7 nmol g<sup>-1</sup> soil d.w. The same was true for the relative archaeal abundance (% values), for which the highest values were observed in SI (5.3% and 4.7%) and the lowest in NI (0.4% and 0.7%). Absolute and relative archaeal abundance in section SI remained rather constant, whereas a significant decrease was observed in section MI from spring to fall sampling. For all samples, i20:0 was the most abundant etherlipid, which is a general marker for organism of the phylum *Euryarchaeota* (Gattinger, 2001). i20:1 isoprenoid chains, detected

**Table 1** Properties of the soils collected along a gradient of animal impact of the study site Borova (*n* = 9)

	Plots	pH [H <sub>2</sub> O]	C <sub>org</sub> (g 100 g <sup>-1</sup> )*	NH <sub>4</sub> <sup>+</sup> (µg N g <sup>-1</sup> )*	N <sub>t</sub> (g 100 g <sup>-1</sup> )n*	Microbial biomass (nmol phospholipid compounds g <sup>-1</sup> )n*
<i>Spring</i>						
Mean	SI	8.96 <sup>a</sup>	5.79 <sup>a</sup>	25.98 <sup>a</sup>	1.94 <sup>a</sup>	576.58 <sup>a</sup>
S.d.		±0.16	±0.31	±71.3	±0.29	±124.02
Mean	MI	7.25 <sup>b</sup>	4.05 <sup>b</sup>	8.39 <sup>b</sup>	1.21 <sup>b</sup>	303.97 <sup>b</sup>
S.d.		±0.22	±0.84	±6.3	±0.45	±96.55
Mean	NI	5.91 <sup>c</sup>	2.32 <sup>c</sup>	2.22 <sup>a</sup>	0.65 <sup>c</sup>	147.33 <sup>c</sup>
S.d.		±0.23	±0.27	±0.46	±0.24	±30.4
<i>Fall</i>						
Mean	SI	8.77 <sup>a</sup>	5.80 <sup>a</sup>	5.54 <sup>a</sup>	1.69 <sup>a</sup>	584.05 <sup>a</sup>
S.d.		±0.19	±0.56	±13.45	±0.31	±100.18
Mean	MI	6.91 <sup>b</sup>	1.33 <sup>b</sup>	0.67 <sup>a</sup>	0.54 <sup>b</sup>	108.53 <sup>b</sup>
S.d.		±0.27	±0.13	±0.48	±0.3	±29.79
Mean	NI	6.57 <sup>c</sup>	1.79 <sup>c</sup>	0.86 <sup>a</sup>	0.51 <sup>b</sup>	133.77 <sup>b</sup>
S.d.		±0.32	±0.27	±0.16	±0.19	±27.93

Abbreviations: ANOVA, analysis of variance; SI, severe impact; MI, moderate impact; NI, no impact.

Different letters indicate significant differences between sites at specific sampling time at the 0.05 level. Differences between means were evaluated by one-way ANOVA followed with all pairwise multiple comparison procedure (Student–Newman–Keuls test). Kruskal–Wallis one-way ANOVA was applied for cases where normality test of data distribution failed (for NH<sub>4</sub><sup>+</sup> and in C<sub>org</sub> values of spring 2005 and fall 2005 samples).

\*Related to soil dry weight (d.w.).

**Table 2** CH<sub>4</sub> emissions from the differently impacted plots of the study site Borova (n = 9)

Plots		CH <sub>4</sub> emission µg C-CH <sub>4</sub> m <sup>-2</sup> h <sup>-1</sup>
<i>Spring</i>		
Mean	SI	30554.0 <sup>a</sup>
S.d.		± 63659.3
Mean	MI	309.7 <sup>b</sup>
S.d.		± 599.0
Mean	NI	-37.6 <sup>c</sup>
S.d.		± 23.9
<i>Fall</i>		
Mean	SI	56.5 <sup>a</sup>
S.d.		± 46.5
Mean	MI	28.8 <sup>a</sup>
S.d.		± 15.4
Mean	NI	3.8 <sup>a</sup>
S.d.		± 19.5

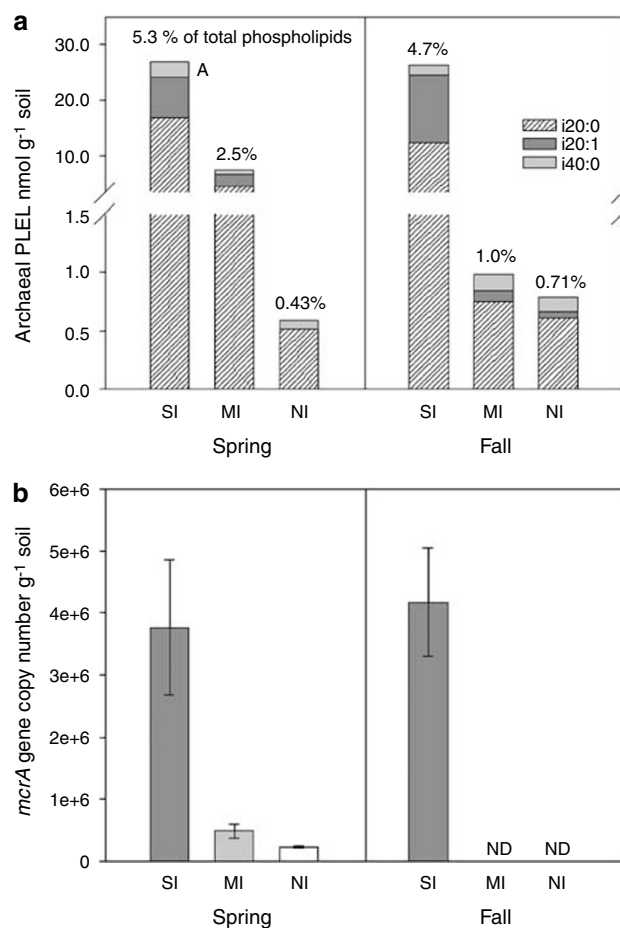
Abbreviations: ANOVA, analysis of variance; SI, severe impact; MI, moderate impact; NI, no impact. Different letters indicate significant differences between sections at specific sampling time at the 0.01 level. Differences between means were evaluated by one-way ANOVA followed by all pairwise multiple comparison procedure (Student–Newman–Keuls test). Kruskal–Wallis one-way ANOVA was applied for cases where normality test of data distribution failed (fall 2005 measurements).

previously in acetoclastic methanogens from the genus *Methanosarcina* (Gattinger *et al.*, 2002), were detected in significant amounts in dung (data not shown), SI samples (spring and fall) and in MI samples (only spring).

#### Abundance and diversity of methanogens

The quantification of *mcrA* genes revealed a similar pattern as archaeal PLEL concentrations (Figure 1b). In plot SI, the number of target molecules per gram of d.w. soil ranged between 3.8 and 4.2 × 10<sup>6</sup> copies and was significantly higher than that observed in the other sites at both sampling dates. For plots MI and NI, 4.9 and 2.3 × 10<sup>5</sup> molecules per gram d.w. soil were detected in spring, respectively. In fall, the values decreased and were below detection limit of approximately 1000 target molecules per gram of d.w. soil. Subsequent qPCR with stepwise dilution of soil DNA revealed no inhibitory substances for PCR in the original nucleic acid extracts (data not shown).

Following the qPCR and PLEL results, only *mcrA* gene fragments from section SI, with distinct methanogenic characteristics, were subjected to cloning and sequencing. Fifty-four clones were randomly selected for the analysis. The rarefaction's analysis showed that number of clones was sufficient to cover the diversity of *mcrA* OTUs. Phylogenetic analysis of *mcrA* clones revealed the presence of two main clusters (Figure 2a). Cluster II comprises sequences affiliated with cultured methanogens. The majority of the sequences within this cluster show

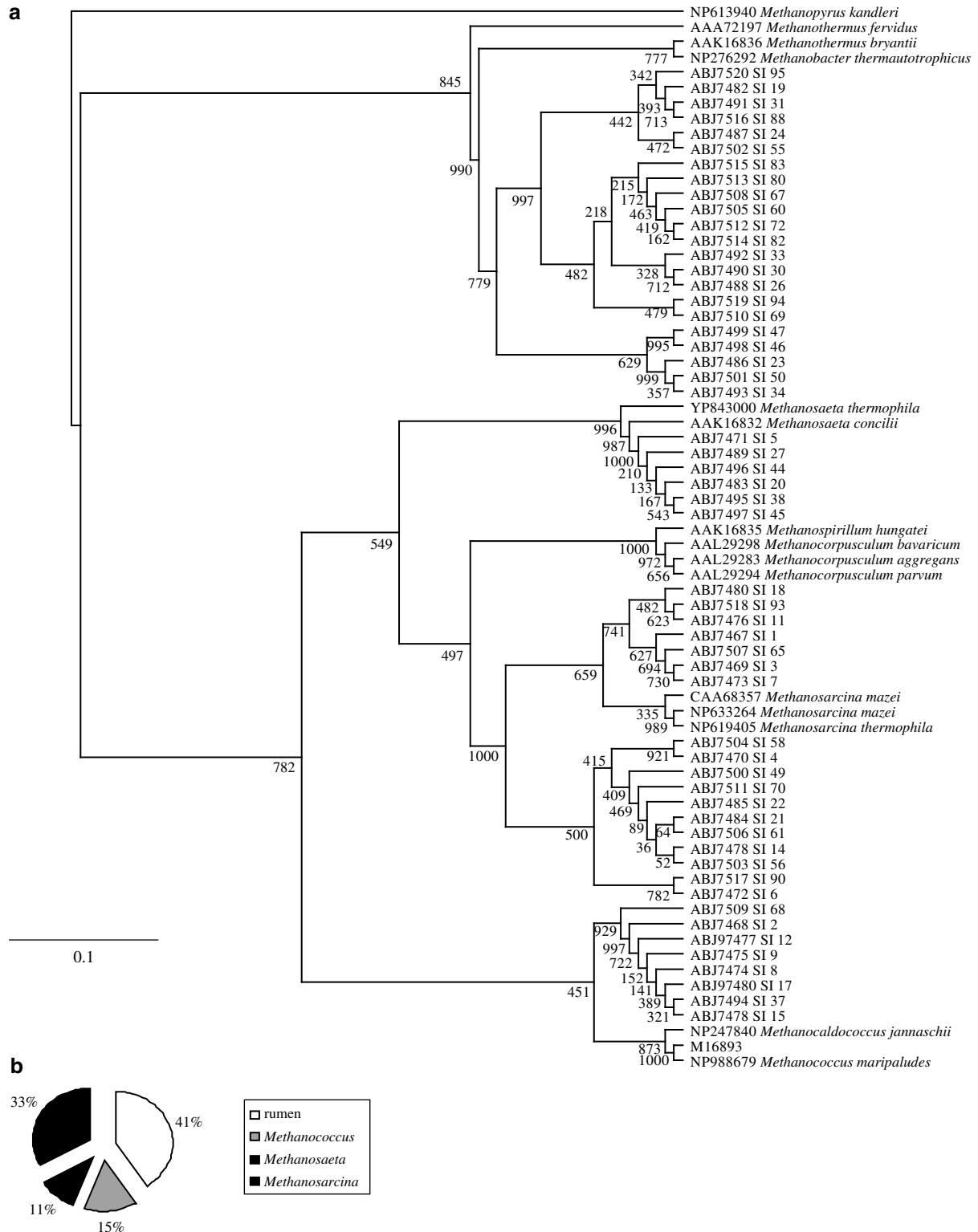


**Figure 1** (a) PLEL composition in soil samples along a gradient of animal impact. (b) Numbers of *mcrA* gene copies along the same gradient of animal impact of the study site Borova. Bars denote s.d. Values for MI and NI from fall sampling were below detection limit. SI, severe impact; MI, moderate impact; NI, no impact. Per cent values indicate the percentage of PLEL in relation to the concentration of total phospholipid chains. The following abbreviations for the different PLEL-derived isoprenoid hydrocarbons were used: i = isoprenoid branched; 20 and 40 = size of the hydrocarbon chain; i20:0 and i20:1 = saturated and unsaturated hydrocarbon chains, respectively. Different letters indicate significance of absolute PLEL concentration at the  $P = 0.05$  level. PLEL, phospholipid etherlipid.

close similarities to *Methanothermus*, *Methanosaeta* and *Methanosarcina* and represents 15%, 11% and 33% of the analysed sequences, respectively (Figure 2b). Cluster I contains sequences only from uncultured rumen methanogens. Twenty-two sequences from soil section SI (41% of the total, Figure 2b) and three sequences from uncultured rumen archaeons are forming this cluster.

## Discussion

Upland land soils, including grassland soils, are normally described as sink for the green house gas CH<sub>4</sub> (Hütsch *et al.*, 1994; Castaldi *et al.*, 2007).



**Figure 2** (a) Dendrogram showing phylogenetic relationships of *mcrA* sequences of soil samples collected in spring at the severe animal impacted (SI) area. The tree was constructed from inferred amino-acid sequences (141 positions) using Dayhoff PAM 001 distance matrix and neighbour-joining analysis. Numbers at the nodes represent the percentages of bootstrap resamplings based on 1000 replicates. *Methanopyrus kandleri* was used as outgroup reference. (b) Distribution of partial sequences from *mcrA* genes retrieved from SI.

Nevertheless, as shown in the present study, keeping cattle in overwintering pastures may cause enormous changes in soil properties, mainly due to

excretal returns and treading (Oenema *et al.*, 2001; White *et al.*, 2001), which in turn stimulate CH<sub>4</sub> production.

In spring, after the animals have been removed from the pasture, microbial biomass, organic C and total N values were higher in section SI followed by MI and NI, showing a clear relationship between the degree of impact and changes in soil properties and soil microbial community. Although, in fall (5 months after the animals have been removed) biomass values remained high in section SI, a clear reduction was observed in MI. Hernández *et al.* (2007) and collaborators postulated that animal excrements stimulate growth of soil microorganism, however high inputs are necessary to maintain an elevated microbial biomass content.

Archaeal biomass measurements showed the same trend as observed for total microbial biomass. In section NI, the concentration of PLEL was 0.6 in spring and  $0.8 \text{ nmol g}^{-1}$  dry matter in fall and contributed to 0.4% and 0.7% of total phospholipid chains of that section. These values are in the range of previously reported data from light or unfertilized arable soils (Gattinger *et al.*, 2002). In section SI, values were well above the range determined so far for any upland (Gattinger *et al.*, 2007) or wetland soil (Bai *et al.*, 2000; Wachinger *et al.*, 2000). Wachinger *et al.* (2000) suggested that archaeal abundance reflects *in situ* and *in vitro*  $\text{CH}_4$  production/emission potential, even though a linear correlation could not be established. This result is not surprising as archaeal biomass is probably not an ideal indicator for methanogenesis, as only a fraction of the whole community is able to carry out this process.

The determination of the *mcrA* gene gives further insights into the functional role of the detected archaea. As until now, no quantitative data on *mcrA* gene copies have been reported for a similar terrestrial environment, comparisons in this respect can hardly be made. However, it must be taken into account that the measured values might be biased by the used primers and the nucleic acid extraction efficiency (Sharma *et al.*, 2007), although several protocols were tested using soil samples from the corresponding farm, indicating that the chosen protocol for this study gave the highest and most reproducible values. Therefore, it was of great importance to have quantitative data from a second, PCR independent marker (etherlipids), which show similar trends compared to the molecular data. Overall, the number of *mcrA* gene copies showed the same trend as has been described for the archaeal phospholipids: highest values in section SI and lowest in NI. Also the decrease in PLEL concentration in MI from spring to fall sampling went parallel with the decrease in the number of *mcrA* gene copies from spring to fall in MI. Moreover, although qPCR can provide accurate measurements of gene numbers per unit of soil, it is more difficult to convert these values into cell densities and biomass or to set them in direct relation to soil mass or soil volume. A conversion to cell densities requires the knowledge of the copy

number per genome and the genome size (Sharma *et al.*, 2007).

Quantification of *mcrA* genes may be better indicator for methanogenesis than the total archaea biomass; nevertheless, it only represents the potential of the community, as it is based on the DNA level. This assertion can be confirmed by the analysis of fall data for section SI, in which methanogenic biomass values remained high even though a harsh decrease in methane emissions was observed.

Phylogenetic analysis revealed the presence of a diverse methanogenic community in section SI. Most of the detected methanogenic taxa were also found in arable soils fertilized with cattle manure for more than 50 years (Gattinger *et al.*, 2007). Approximately 41% of the sequences formed a separate cluster and showed close relationships (97% based on DNA sequences) to uncultured rumen archaeons (Tatsuoka *et al.*, 2004). Moreover, 33% were similar to *Methanosarcinaceae* representatives, which are known to occur in cattle rumen (Jarvis *et al.*, 2000). Hence, a substantial part of the retrieved clones from section SI can be considered as typical rumen colonizers. Lipids normally present in the membrane of *Methanosarcina* (i20:1 side chain; Gattinger *et al.*, 2002) were also detected in significant amounts in fresh excrements sampled from the overwintering area (data not shown). PLEL profiling revealed at both samplings the highest concentration of *Methanosarcinaceae*-derived lipids in section SI. These findings support the hypothesis that with the cattle excrements 'rumenborne' methanogens are transferred to the grassland soil. On the other hand, *Methanosarcinaceae* are generally considered as somewhat ubiquitous methanogens occurring in oxic and anoxic soils of the temperate zone (Lueders *et al.*, 2001; Ramakrishnan *et al.*, 2001). Signature lipids of this group have also been detected, although in low amounts, in the non-impacted section NI.

The combination of the obtained data from physicochemical, methane emission, phospholipid and nucleic acid analyses gives strong indication of the following phenomenon: during the overwintering period, cattle stay most of the time in section SI, where they excrete faeces and urine. As a result of the urine input, the soil pH rises from slightly acidic to alkaline. A pH of 8 is known to be optimal for the activity of acetoclastic methanogens like *Methanosarcinaceae* (Beeman and Suflita, 1990). Supplied with easily available organic substrates from the cow dung, soilborne methanogens like *Methanosarcina* spp are stimulated in growth and activity. In addition, hitherto unknown, 'rumenborne' archaea along with representatives from known methanogenic groups including *Methanosarcina* species are transferred from the cattle intestinal tract to the soil. In this terrestrial habitat, these microorganisms will find suitable conditions for growth and methanogenic activity. A similar phenomenon was also described for arable soils receiving high amounts

of cattle manure (Gattinger *et al.*, 2007). However, in this case, methane emissions were by far less pronounced, presumably because of significant methanotrophic activities. The reasons for the low activity of methane oxidizers are not clear. On the one hand, it is known that high concentration of ammonia, originated in our experiment from cattle urine, could inhibit methane oxidation (Hütsch *et al.*, 1993; Schnell and King, 1994). On the other hand, it could be argued that the ratio between methane in the soil and ammonium is so high that the methane oxidizers are not inhibited by the high ammonium concentrations but even suffer from nitrogen limitation, which reduces their activity (Chan and Parkin, 2001). Moreover, in section SI, an increase in anaerobic micro-sites caused by animal treading and excrements, could also affect this microbial group. In any case, methane oxidation by low-affinity methane oxidizers or anaerobic methane oxidation may play an important role on site SI. However, this aspect is not addressed in the present study.

For the moderate impacted section MI, we assumed that the same phenomenon occurred, but to a far lesser extent. Only at spring, after the cattle left the overwintering area, a significant methanogenic biomass was observed, as indicated by the high abundance of *mcrA* genes. This presumably occurred because of suitable soil ecological properties (pH and organic C), leading to a distinct methane production. Nevertheless, after 6 months of no cattle impact, methanogenic biomass declined sharply and *mcrA* genes were not detectable along with a clear reduction in pH and organic C content, which were almost the same as in section NI. Owing to these changes in soil characteristics, a far lesser methane emission in section MI was observed at fall than at spring sampling.

In contrast, in SI, the methanogenic community sustained its biomass, as indicated by the abundance of the *mcrA* genes, during a 6 month cattle-free period. Also organic C and pH remained at a high level providing an appropriate habitat for methanogenic archaea. Nevertheless, as postulated by Plaza (Plaza *et al.*, 2007) and collaborators, high amounts of easily degradable organic matter are introduced in soil via animal excrement, which in turn increases microbial activity. Once this nutrient source is no longer available, the community adapts to the new environmental condition and reduces its metabolic activity. Moreover, the abundance of phospholipid biomarkers for anaerobic bacteria was higher in spring compared to fall, indicating changes in overall oxygen availability probably due to a recovery of the highly compacted soils in spring. However, SI plots showed strong aggregate formation during summer (data not shown). This is not surprising as soils of high pH and high carbon concentrations are known to aggregate easily (Boix-Fayos *et al.*, 2001). Also the influence of cattle manure on soil aggregate formation is well known

(Pare *et al.*, 1999). Not only the high content of carbon itself but also the resulting increases in microbial biomass and activity may stimulate soil aggregation (Martens and Frankenberger, 1992). The aggregate interior, with low oxygen concentrations may be a good habitat for methanogenic microbes to survive the unfavourable summer conditions. In MI, the recovery of vegetation during the cattle-free period was very much pronounced. Obviously, the methanogenic Archaea are not highly adapted to grassland rhizosphere and were outcompeted by other microbes. To clarify more the relation between microbial activity and methane production, mRNA-based studies indicating the induction of gene expression of *mcrA* are necessary. The results of this study are based on DNA studies which are reflecting more a genetic potential than an actual activity.

It can be concluded that after a period of at least 6 months of no cattle impact, the formerly, severely impacted section maintained its methane production potential, whereas the methane production potential under moderate impact returned to background values.

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