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Anaerobic oxidation of methane in grassland soils used for cattle husbandry

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Abstract

While the importance of anaerobic methane oxidation has been reported for marine ecosystems, the role of this process in soils is still questionable. Grasslands used as pastures for cattle-overwintering show an increase in anaerobic soil micro-sites caused by animal treading and excrement deposition. Therefore anaerobic potential methane oxidation activity of severely impacted soil from a cattle winter pasture was investigated in an incubation experiment under anaerobic conditions using ^{13}C-labeled methane. We were able to detect a high microbial activity utilizing CH_4 as nutrient source shown by the respiration of $^{13}CO_2$. Measurements of possible terminal electron acceptors for anaerobic oxidation of methane were carried out. Soil sulfate concentrations were too low to explain the oxidation of the amount of methane added, but enough nitrate and iron(III) were detected. However, only nitrate was consumed during the experiment. ^{13}C-PLFA analyses clearly showed the utilization of CH_4 as nutrient source mainly by organisms harbouring 16:1ω7 PLFAs. These lipids were found in Gram-negative microorganisms and anaerobes. The fact that these lipids are also typical for type I methanotrophs, known as aerobic methane oxidizers, might indicate a link between aerobic and anaerobic methane oxidation.

1 Introduction

Methane is well known as a carbon and energy source for a specific group of methanotrophic microorganisms under aerobic conditions (Mancinelli, 1995). Today, however, a new role of methane has attracted the focus of research: the importance for the growth of microorganisms under anaerobic conditions (Thauer and Shima, 2008). Though the anaerobic oxidation of methane (AOM) was thought to be biochemically impossible for a long time and thus absent in nature (reviewed by Strous and Jetten, 2004), there has been recent evidence that this microbial mediated process could be

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a major factor in global carbon cycling (Shima and Thauer, 2005; Strous and Jetten, 2004; Valentine and Reeburgh, 2000; Smemo and Yavitt, 2011).

Different terminal electron acceptors have been described so far for AOM (Thauer and Shima, 2008; Smemo and Yavitt, 2011). Studies on marine sediments indicated the use of sulphate by a consortium of anaerobic methanogenic archaea (so-called ANME) and sulphate-reducing bacteria (Strous and Jetten, 2004; Valentine and Reeburgh, 2000; Boetius et al., 2000; Hinrichs et al., 1999). This process is considered to consume most of the methane produced in marine sediments (Valentine, 2002). The reaction mechanism, its biochemistry and physiology, are still under discussion and apparently involve reverse methanogenesis (Thauer and Shima, 2008).

The second electron acceptor described so far is nitrate/nitrite (Ettwig et al., 2009, 2010; Raghoebarsing et al., 2006). In contrast to the microbial consortium using sulphate as electron acceptor under anaerobic conditions, which requires the close interaction of bacteria and archaea, it has been postulated that AOM in the presence of nitrate/nitrite can be catalyzed by one single bacterium which belongs to the so far uncultured phylum NC10 (Ettwig et al., 2009; Thauer and Shima, 2008).

AOM coupled to nitrate reduction has been reported for various nutrient-rich aquatic habitats, including contaminated groundwater (Smith et al., 1991), sewage sludge (Islas-Lima et al., 2004), eutrophic canals and ditches (Ettwig et al., 2009), but also sediments of an oligotrophic freshwater lake (Deutzmann and Schink, 2011). Next to these aquatic habitats, the AOM was also recently described in drained peat and automorphic sod-podzol soils. The addition of oxidized compounds (e.g. nitrate) had a stimulatory effect to methane consumption in these soils (Pozdnyakov et al., 2011) under anaerobic conditions. The latter finding indicates that AOM might not be limited to aquatic systems only, but might also occur in other (soil) ecosystems, especially in soils with prevailing or frequently occuring larger anaerobic compartments like, e.g. submerged soils, subsoils or mechanically compacted soils.

Pasture sites used for cattle overwintering in Central Europe are highly influenced by the animals. Compared to ordinary pastures, which are used for grazing during summer

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time, a much higher number of animals is present on a small area for a relatively long period. This causes a reduction in soil aeration through soil compaction (Menneer et al., 2005). The presence of animals is accompanied by a high input of organic material through excrements which consequently stimulates microbial metabolism, increases oxygen demand and supports the occurence of anoxic (micro)sites in the soil (Šimek et al., 2006). Not surprisingly, Radl et al. (2007) could therefore demonstrate high abundance of methanogens in these soils during winter time as well as high methane production rates. Due to the prevailing anaerobic conditions and the high concentrations of ammonia from excrements in the soil, abundances of aerobic methane oxidizers carrying the *pmoA* gene were low (Radl et al., 2007). Nevertheless, in situ methane oxidation rates in those soils were high; therefore it has been postulated that AOM might play an important role in the investigated site for methane consumption.

To test this hypothesis we incubated severely impacted soils from a cattle winter pasture under anaerobic conditions using ^{13}C-labeled methane. Soil from the same area, which was not used for cattle overwintering, served as control. Measurements of gases were performed using isotope ratio mass spectrometry (IRMS). Subsequent phospholipid fatty acid (PLFA) analysis provided insights into the microorganisms possibly involved in anaerobic methane oxidation.

2 Materials and methods

2.1 Site description and soil sampling

Soil samples were taken from a cattle grazed farmland in Borová, South Bohemia, Czech Republic (48°52′ N; 14°13′ E), characterized by a mean annual temperature of 7 °C and a sum of precipitation of 650 mm. The soil is characterized as sandy loam and classified as Cambisol containing 60–80 % sand, 14–32 % silt and 6–14 % clay (for other details on the site and soil see Hynšt et al., 2007, and Radl et al., 2007).

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For the present study we sampled soils in April 2009, at the end of the overwintering period 2008/2009. Soils from two sites were sampled as follows: a severely impacted site (SI) located in close proximity to the stables and being the overwintering area for the cattle, and a control plot (CO) which has not been used for cattle grazing and overwintering since 1999. The control had an intact plant cover consisting of a perennial mixture of grasses and dicotyledonous plants as clovers (Jirout et al., 2009), while the plant cover of the severely impacted site was completely destroyed at the time of sampling, exposing the surface soil. Five independent field replicates (200 g each) from each site were randomely taken with a soil auger from 0–20 cm depth pooled for further analysis to reduce field heterogeneity, and stored at 4 °C for 2 weeks before the incubation experiment started.

2.2 ^{13}C labeling experiment

To generate anaerobic conditions, 10 g of soil were filled into a serum bottle (100 ml), followed by the adjustment of the water content to 100 % of the maximum water holding capacity using distilled water. Overall 13 bottles of SI and CO soil were prepared. Finally the bottles were sealed with a butyl rubber septum. To ensure the absence of oxygen also in soil pores, bottles were purged with nitrogen (N_2, 5.0 grade) twice within 25 h. At each time the added nitrogen replaced the headspace atmosphere two times. Finally, 500 µl of labeled methane (20.2 % ^{13}C-enriched) were injected into 8 serum bottles with SI soil (SI_{CH_4}) and 8 bottles with CO soil (CO_{CH_4}). As a control 5 replicates of SI and 5 replicates of CO soil were left without methane amendments (SI, CO).

The samples were subsequently maintained at a constant temperature of 14 °C on a CombiPAL Autosampler (CTC, Zwingen, Switzerland) for 7 days.

During the incubation, CO_2 and CH_4 concentrations, as well as their corresponding ^{13}C:^{12}C ratios in headspaces were determined on-line using a gas chromatography/isotope ratio mass spectrometer system (GC/IRMS; Finnigan MAT, delta plus, Germany). Part of the assembly was a modified PreCon Interface (Finnigan MAT)

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which enables also measurements of high CO_2 and CH_4 concentrations by varying the injection volumes. The fraction of CO_2 originating from oxidized CH_4 (f_{CH_4}) was calculated from:

$$f_{CH_4} = \frac{^{13}C_{\text{emissions treatment}} - ^{13}C_{\text{emission control}}}{^{13}C_{CH_4} - ^{13}C_{\text{emission control}}} \quad (1)$$

where $^{13}C_{CH_4}$ is the ^{13}C amount of the added CH_4 and $^{13}C_{\text{emission}}$ is the ^{13}C amount in the CO_2 emitted during a certain time period.

$^{13}C_{\text{emission}}$ was calculated as follows:

$$^{13}C_{\text{emission}} = \frac{C_{n+1} \cdot ^{13}C_{n+1} - C_n \cdot ^{13}C_n}{C_{n+1} - C_n} \quad (2)$$

in wich $C_{n/n+1}$ are the CO_2 concentration at points in time $n/n+1$ and $^{13}C_{n/n+1}$ are the ^{13}C amounts at points in time $n/n+1$.

At the end of the incubation experiment, soil subsamples for PLFA analysis were stored at −80 °C, while subsamples subjected to measurements of microbial biomass as well as abiotic soil properties were analyzed immediately.

2.3 Soil physical and chemical properties

Soils from both the incubation experiment (SI, CO, SI_{CH_4}, CO_{CH_4}) as well as from the time before the incubation experiment started (SI_{T0}, CO_{T0}) were extracted with 0.01 M $CaCl_2$ at a soil to liquid ratio of 1:2. For the determination of water extractable organic carbon (WEOC) and nitrogen (WEON), an analyzer DIMATOC 100 (DIMATEC Analysentechnik GmbH, Essen, Germany) was used. Determination of ammonium (NH_4^+-N) and nitrate (NO_3^--N) soil concentrations was done by continuous flow analysis with a photometric autoanalyzer (CFA-SAN Plus/Skalar Analytik, Germany). The content of reducible iron [Fe(III)] was determined using the hydroxylamine extraction method and

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spectrophotometric analysis (Lovley and Phillips, 1987). Sulphate was measured with the Nanocolor Sulphate 200® kit (Macherey-Nagel, Germany) at a soil to liquid ratio of 1:9.

Microbial biomass was determined by the chloroform-fumigation extraction method (Vance et al., 1987) with 1 g of soil in 16 ml 0.5 M K_2SO_4 solution. For the determination of microbial biomass carbon (C_{mic}) aliquots of the soils were fumigated with chloroform for 24 h prior to $CaCl_2$extraction (Jörgensen and Brookes, 1991). C concentration and ^{13}C abundance were measured by a liquid chromatography/isotope ratio mass spectrometer system (LC/IRMS) (LC IsoLink coupled with MAT 253, both Thermo Finnigan, Bremen) as described by Krummen et al. (2004) and Marx et al. (2007). ^{13}C abundance was calculated by allegation alternate (Marx et al., 2007).

2.4 Phospholipid fatty acid analysis

Phospholipid fatty acid extraction was performed according to Zelles et al. (1995). Replicates from incubated SI soils with as well as respectively without methane (SI_{CH_4}, SI) were pooled and an equivalent of 10 g dry soil was extracted with a mixture of 125 ml methanol, 63 ml chloroform and 50 ml phosphate buffer (0.05 M, pH 7).

After 2 h of horizontal shaking, 63 ml water and 63 ml chloroform were added to promote phase separation. After 24 h the water phase was removed and discarded. The total lipid extract was separated into neutral lipids, glycolipids and phospholipids on a silica-bonded phase column (SPE-SI 2 g/12 ml; Bond Elut, Analytical Chem International, CA, USA). The phospholipid fraction was further separated into saturated (SATFA), monounsaturated (MUFA), polyunsaturated (PUFA), unsaponifiable non-ester linked (NEL-UNSFA) and hydroxy substituted (NEL-UNOH) fatty acids (see Zelles et al., 1995 and Zelles, 1999 for details). PLFA were analyzed as fatty acid methyl esters (FAME) on a gas chromatograph/mass spectrometry system (5973MSD GC/MS Agilent Technologies, Palo Alto, USA) linked via a combustion unit to an isotope ratio mass spectrometer (GC/MS-C-IRMS, DeltaPlus^{Advantage}, Thermo Finnigan,

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Bremen, Germany). Separation and detection of FAME was performed via GC/MS, while the isotopic composition of fatty acids was detected after combustion (GC Combustion III, Thermo Finnigan, Bremen, Germany) in the IRMS. Columns and temperature programs were used according to Esperschuetz et al. (2009). The mass spectra of the individual FAMEs were identified by comparison with established fatty acid libraries (Solvit, Luzern, Switzerland) using MSD Chemstation (Version D.02.00.237).

Standard nomenclature for PLFA was used according to Frostegard et al. (1993), at which the number before the colon represents the number of C-atoms, the number after the colon gives the number of double bonds and their location from the aliphatic end (ω). The prefixes "cy", "iso" and "ant" indicate cyclopropyl-groups, and iso- and anteiso-branching, respectively. Saturated straight-chained fatty acids were indicated by "n". The "br" and the number before a fatty acid indicate methyl-branching at the individual C-atom. The prefixes α and β indicate that the OH groups of an unsaponifiable OH fatty acid are located at positions 2 and 3, respectively, while numbers preceded by ω indicate the position of OH groups from the aliphatic end. Non ester linked (unsaponifiable) fatty acids were indicated by NEL (Zelles, 1999).

2.5 Statistical analysis

Prior to analysis, data were tested for normal distribution by Q-Q plots and the Kolmogorov-Smirnoff test. Homogeneity of variances was checked by the Levene test. Soil chemical parameters were subjected to analysis of variance (two-factor ANOVA). Means were considered significantly different at $P \leq 0.05$. A linear mixed-effects model was fit to time series of CO_2 concentration originating from methane (Fig. 1). The model considered the fixed effects soil, time, and their interaction; repeated measures were considered as a random effect. Temporal autocorrelation of residuals was not significant and, consequently, was not modeled. All statistical calculations were carried out using SPSS software version 13.0 (SPSS Inc, Chicago, IL), except the linear mixed-effects model for which R 2.14.1 (www.R-project.org) with library *nlme* was used.

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3 Results

3.1 Consumption of methane

In contrast to CO, SI showed a significant consumption of the added methane and production of carbon dioxide under anaerobic conditions (Fig. 1). Whereas in CO a maximum of 0.83 % of C incorporation after 6.4 days of methane addition was measured, the incorporation of carbon derived from CH_4 into CO_2 in SI samples was observed already after 2.8 days (2.4 %). The incorporation reached the maximum of 8.5 % after 4.4 days and then declined to 4.0 % after 6.2 days. The high standard deviations of mean C incorporated from CH_4 into CO_2 might be related to the fact, that methane consumption in some replicates started later than in others.

Concentration increase of CO_2 originating from CH_4 ,measured by ^{13}C incorporation, with time was significantly ($p < 0.0001$) higher for SI soil than for CO soil. In total, the percentage of C in CO_2 originating from CH_4 oxidation was 22 % for the SI soil and 3.7 % for the CO soil. The methane added to SI soil samples incubated under anaerobic conditions was almost completely consumed (91.3 %). In the control treatments of SI soil without methane addition, the share of CH_4-C incorporated into CO_2 was below 0.03 % over the whole experimental period.

3.2 Soil chemical properties

Content of water extractable organic carbon (WEOC) was about ten times higher in SI soils compared to CO soils and was neither affected by the addition of methane nor by the incubation (Table 1). Content of water extractable organic nitrogen (WEON) significantly decreased in SI soil during anaerobic incubation, from 13 to 8.2 and 9.7 $\mu g\,N\,g^{-1}$ dw in the treatment without methane and with methane, respectively. Nitrate concentrations in SI and CO soils were under or near the detection limit in the anaerobic treatments, however values in the original soils were significantly higher and counted for 23 $\mu g\,NO_3^-$-N g^{-1} of soil in SI and 9.0 $\mu g\,NO_3^-$-N g^{-1} in CO. Ammonium

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concentration increased significantly in SI soil samples from 1.0 µg N g^{-1} dw before anaerobic incubation to 13 and 12 µg N g^{-1} dw in treatments without and with methane amendment. Ammonium concentration in CO soils, however, did not change during the incubation (Table 1). Reducible iron was significantly higher in CO than in SI soils, but no significant difference between the treatments was found. The content of sulphate was below the detection limit (0.13 mg S g^{-1} dw) in all soil samples.

3.3 Microbial biomass and microbial community structure

Microbial biomass (C_{mic}) was 3.7 times higher in SI soil compared to CO soil (Table 1). After methane addition and anaerobic incubation, C_{mic} was significantly enriched in ^{13}C (+19 ‰ VPDB) compared to the treatment without methane (−28 ‰ VPDB) in SI soil, while no enrichment was found in soil CO (Fig. 2).

In total, 69 different PLFAs were detected in SI_{CH_4} and SI treatments. The absolute content (nmol g^{-1} dw soil) of individual PLFAs in CH_4 untreated SI soil is shown against the difference resulting from the CH_4 treatment (Δ SI_{CH_4}; SI) in Fig. 3, illustrating the response of the microbial community structure to CH_4. In general, most lipids showed increased concentrations in CH_4-treatments. Highest increases were observed in saturated iso15:0, ant15:0, n16:0, monounsaturated 16:1ω7, 17:1ω8, 18:1ω7c, 18:1ω9 and unsaponifiable, hydroxy substituted α23:0, αβ22:0 and αβ24:0. Since CH_4 was enriched with ^{13}C, PLFAs of methane oxidizing microbial communities showed increased $\delta^{13}C$ values compared to the controls. In Fig. 4, a ranking is given of PLFAs with high ^{13}C incorporation. Highest ^{13}C enrichment was detected in monounsaturated 16:1ω7 (+330 ‰ $\delta^{13}C$) and two unsaponifiable, non-ester linked PLFAs of unknown branching (ubr16:0, +284 ‰ and +288 ‰ $\delta^{13}C$).

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4 Discussion

Anaerobic methane oxidation (AOM) has been reported as important methane consuming process in aquatic habitats, mainly marine ecosystems (Valentine, 2002). For soils the importance of this process is still questionable. Anaerobic conditions in soils might not only be caused by flooding, but also by soil compaction and aggregation. Grasslands used as pastures for cattle overwintering show an increase in anaerobic soil micro-sites caused by animal treading and excrement deposition. Not only the high content of carbon itself, but also the resulting increases in microbial biomass (72 % higher in cattle highly-impacted SI soil compared to unimpacted CO soil) and activity may stimulate soil aggregation (Martens and Frankenberger, 1992). Our stable isotope experiment, using ^{13}C-labeled methane, allowed us to separate methane production and consumption processes occurring simultaneously. The percentage of CH_4-C incorporated into CO_2 was significantly increased in anaerobically incubated SI soil compared to CO soil (Fig. 1). This gives clear evidence that AOM occurs in the severely impacted soil under the conditions of the incubation experiment.

No oxygen is supposed to be left in soil pores, as a slurry was used and flushed in addition repeatedly using O_2-free nitrogen. However, the possible amount of oxygen in the soil pores was calculated theoretically. At a conservatively estimated dry bulk density of 1.0 g cm^{-3}, if all water-free pores were filled with air containing 21 % oxygen, 6.2 mmol O_2 would be available as oxidizing reactant. However, 22.4 mmol O_2 are necessary to fully oxidize 500 µl of CH_4. Thus, oxidation of 73 % of the 500 µl CH_4 added cannot be fully explained by aerobic oxidation, even if one assumes that free oxygen was present in soil and that it would not be consumed by oxidation of other substrates. Both of these assumptions are extremely unlikely. Furthermore, decreasing amounts of nitrate and increasing amounts of ammonium (Table 1) indicate absence of nitrification and prevalence of denitrification or other anaerobic processes during incubation, which also confirms that the soil was actually anaerobic.

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The presence of other possible electron acceptors for AOM was checked. Sulphate concentrations in SI and CO soils were below the detection limit of 0.2 µg S g^{-1} dw soil. Theoretical calculations showed that at least 126 µg $[SO_4]^{2-}$ g^{-1} soil would have been necessary to oxidize the added 500 µl CH_4 (for reaction equation see Scheller et al., 2010). Therefore, it is very likely that sulphate was not the terminal electron acceptor for AOM in the investigated grassland soil. Furthermore, so far AOM coupled to sulphate reduction was only described for marine systems (Hinrichs et al., 1999; Boetius et al., 2000; Strous and Jetten, 2004; Valentine and Reeburgh, 2000).

It was also proposed that Fe(III) could serve as electron acceptor for AOM in freshwater wetlands (Murase and Kimura, 1994; Daniel et al., 1999). The reaction is energetically favourable (Smemo and Yavitt, 2011). In the present experiment iron concentrations ranged between 63 and 83 µg g^{-1} in the different treatments of the SI soil and between 120 and 140 µg g^{-1} in the CO soil. The theoretical amount of Fe(III) necessary for the oxidation of 500 µl methane is ca. 73 µg g^{-1} soil (for reaction equation see Daniel et al., 1999). Thus, enough Fe(III) has been in SI soil to serve as terminal electron acceptor for anaerobic oxidation of methane. But only 20 µg g^{-1} dw were consumed in SI_{CH_4} during the experiment.

Taking the recent results into account, it is most likely that nitrate/nitrite functioned as electron acceptor for AOM in the SI soil. So far, investigated enrichment cultures preferred nitrite over nitrate. But it remains to be shown whether nitrate can actually be used by "*Candidatus Methylomirabilis oxyfera*" (Wu et al., 2011). The concentrations of nitrate in the severely impacted soil declined from 23 µg NO_3^--N g^{-1} dw soil below detection limit after anaerobic conditions were established, but a significant decline was also observed for the control soil and no difference was found between the treatments with and without methane. This might be due to a high activity of denitrifiying organisms previously found in the soil SI (Chronáková et al., 2009). The amount of NO_3^--N necessary to oxidize 500 µl of methane would theoretically be 29 µg g^{-1} soil (for reaction equation see Raghoebarsing et al., 2006). Taking methodological biases into account,

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there might have been enough nitrate for the oxidation of the added methane in our incubation experiment.

Significantly increased amounts of ammonium concentrations in the anaerobically incubated SI soils (13 and 12 $\mu g\,g^{-1}$ soil) compared to SI soil after aerobic preincubation (1 $\mu g\,g^{-1}$ soil) might indicate that dissimilatory nitrate reduction to ammonia (DNRA) occurred. This process takes place under low oxygen conditions like denitrification and might be favoured under high C/NO_3^- ratios when the electron acceptor (NO_3^-) becomes limiting (Tiedje et al., 1983), as it has been reported for SI. If nitrate/nitrite is the electron acceptor for AOM in the system, there might be a competition for electron acceptors between the two processes. Another possible explanation for the increasing amount of ammonia points towards the mineralization of organic nitrogen and its accumulation resulting from the lack of oxygen (Ghosh and Bhat, 1998).

PLFAs 16:1ω7, 17:1ω8, 18:1ω7, 18:1ω9 and $\alpha\beta$22:0 showed substantially higher abundance in CH_4-treatments compared to control treatments incubated without methane under anaerobic conditions. These PLFAs have been frequently detected in Gram-negative organisms and methanotrophs of type I (16:1ω7) and II (18:1ω9) (Bowman et al., 1991; Boschker et al., 1998; Holmes et al., 1999; Nichols et al., 1987; White, 1994), as well as methane oxidizing bacteria (17:1ω8, Bowman et al., 1993). PLFA 16:1ω7 was also found in strictly and syntrophic anaerobes (Leckie, 2005; Evershed et al., 2006; Zelles, 1997; Henson et al., 1988) and was, e.g. detected as the major PLFA in different *Methylococcus* and *Methylomonas* strains (Bowman et al., 1991). However, also some Gram-positive bacteria (iso15:0, ant15:0, Zelles et al., 1999) and markers for fungi, Sphingomonas and *Archaea* (α23:0, $\alpha\beta$24:0, Zelles, 1997; Gattinger et al., 2002) seem to profit from the additional C-source, although these were already present in high concentrations in SI treatments without methane addition.

The finding that the ^{13}C-label of methane finally appeared in the microbial biomass (Fig. 2) in soils from SI under anaerobic conditions confirms that CH_4-C was not only oxidized, but also incorporated by the respective organisms. This finding provided the opportunity to analyze ^{13}C-marked phospholipid fatty acids. High label incorporation

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was only detected in PLFA 16:1ω7. This indicates that organisms harbouring 16:1ω7 are highly active in degrading methane under anaerobic conditions. This assumption is supported by the ratio of ω7 monounsaturated fatty acids (ω7) to cyclopropylic fatty acids (cy). The latter named cyclic derivates of ω7 MUFAs operate as membrane stabilizing fatty acids. They are reported to be formed under environmental stress conditions such as starvation (Findlay and Dobbs, 1993; Frostegard et al., 1993). While in the SI soil without methane ω7/cy ratio of 1.7 was observed, it increased to values between 3.1 and 5.3 in the methane treated SI soil. This indicates that methane was used as nutrient source by these organisms and hence the nutritional stress decreased.

We also observed a high degree of label incorporation in PLFAs n14:0 and 16:1ω5. PLFA 16:1ω5 was found to be a major component in type I methanotrophs (Bowman et al., 1991). Both lipids were found in *Methylomonas* (Bowman et al., 1991), whereas the unknown branched, unsaponifiable 16:0 PLFAs highly ^{13}C-enriched might indicate anaerobe organisms (Ratledge and Wilkinson, 1988; Henson et al., 1988). It cannot be excluded that the incorporation could also be due to the consumption of ^{13}C-CH_4-derived ^{13}C-CO_2 or feeding on intermediate products. The intensity of the labeling, however, suggests that the lipids in question must be at least partially of methanotrophic origin.

PLFA 18:1ω9 showed no significant label incorporation, but high abundances were found in methane treated soils. Probably microorganisms harboring this PLFA oxidize CH_4 directly to CO_2 and do not incorporate it into their biomass. Another explanation could be that they are no direct degraders of methane, but are apparently activated by the addition of CH_4.

5 Conclusions

Results from the present study give clear evidence for an anaerobic activity of methane oxidizing microorganisms in severely cattle-impacted grassland soil. In a stable isotope experiment using ^{13}C-enriched CH_4 we were able to detect a high microbial activity

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utilizing CH_4 as nutrient source shown by respiration of $^{13}CO_2$. Thus, there is evidence that soil microorganisms have the potential to perform anaerobic oxidation of methane. Moreover, ^{13}C-PLFA analyses clearly showed the utilization of CH_4 as nutrient source mainly by organisms harbouring 16:1ω7 PLFAs. The fact that these lipids are also typical for type I methanotrophs, known as aerobic methane oxidizers, might indicate a link between aerobic and anaerobic methane oxidation. A biochemical relation between these two processes was shown for "*Candidatus Methylomirabilis oxyfera*" which oxidizes methane via the classical aerobic methane oxidation pathway under anaerobic conditions by metabolizing nitrite into oxygen and dinitrogen gas. However, process dynamics under field conditions remain to be shown. Further molecular analyses, including sequencing approaches, are necessary to characterize anaerobic methane oxidizers in different soils.

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Table 1. Soil characteristics of SI and CO before the incubation experiment started (CO_{T0}, SI_{T0}) and after major anaerobic experiment without (CO, SI) and with methane (CO_{CH_4}, SI_{CH_4}). Different parameters were measured: pH value ($CaCl_2$), water extractable organic carbon (WEOC) and nitrogen (WEON), nitrate and ammonium concentrations, reducible iron [Fe(III)], microbial biomass (C_{mic}) and $\delta^{13}C_{mic}$. Means and standard deviations, significant differences among treatments at $p < 0.05$ are indicated by different letters (a, b, c).

Soil parameters	CO_{T0}	CO	CO_{CH_4}	SI_{T0}	SI	SI_{CH_4}
pH	5.3	5.8	5.8	7.3	7.1	7.1
WEOC ($\mu g\,g^{-1}$ dw)	27^a (2.6)	23^a (8.2)	23^a (4.6)	250^b (39)	220^b (13)	230^b (23)
WEON ($\mu g\,g^{-1}$ dw)	2.6^a (0.89)	b.d.l.*	0.35^b (0.63)	13^c (1.2)	8.2^d (0.67)	9.7^e (0.55)
Nitrate ($\mu g\,N\,g^{-1}$ dw)	9.0^a (0.0)	0.25^b (0.43)	b.d.l.*	23^c (2.1)	b.d.l.	b.d.l.
Ammonium ($\mu g\,N\,g^{-1}$ dw)	1.2^a (0.24)	2.4^a (0.56)	1.7^a (0.38)	1.0^a (0.82)	13^b (1.5)	12^b (1.5)
Iron ($\mu g\,g^{-1}$ dw)	140^a (15)	130^a (8.6)	120^a (21)	83^b (20)	69^b (7.7)	63^b (6.6)
C_{mic} ($\mu g\,g^{-1}$ dw)	150^a (16)	140^a (16)	140^a (16)	560^b (58)	490^b (35)	460^b (63)
$\delta^{13}C_{mic}$ (‰ VPDB**)	-26^a (0.21)	-26^a (0.25)	-24^a (1.8)	-28^b (0.19)	-28^b (0.27)	19^c (31)

* Below detection limit, meaning below 0.2 $\mu g\,N\,g^{-1}$ soil dry weight.
** Vienna Pee Dee Belemnite.

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Table 2. References and indicator microbial groups of important PLFA detected in context with anaerobic methane oxidation.

PLFA	Indicatorvalue	Reference
UNOH mix $\alpha\beta$22:0	Gram-negative	A
UNOH α23:0	Fungi	B
UNOH mix $\alpha\beta$24:0	*Sphingomonas*	B
	Bacteroides/Flavobacterium	B
	(Archaea)	G
MUFA 16:1ω7	Gram-negative aerobes	B, I
	Methanotrophs type I	D, E, F
	Strictly anaerobes	B
	Syntrophic anaerobes	J
	Methylococcus	D
MUFA 17:1ω8	Gram-negative	B
	Methanotrophs unknown type	F
	Methane oxidizing bacteria	D
MUFA 18:1ω7cis	Gram-negative	B
MUFA 18:1ω9	Methanotrophs type II	C, D, F
	Eukaryotes	B
SATFA ant15:0	Gram-positive	B
	Flavobacterium	H
	Nonthermophilic organisms	I
SATFA iso15:0	Gram-positive	B
	Flavobacterium	H
	Thermophilic organisms	I
SATFA n16:0	Ubiquitous	B

A – White (1994); B – Zelles (1997); C – Nichols et al. (1987); D – Bowman et al. (1991); E – Boschker et al. (1998); F – Holmes et al. (1999); G – Gattinger et al. (2002); H – Brennan (1988), Haack et al. (1994); I – Carpenter-Boggs et al. (1998); J – Henson et al. (1988)

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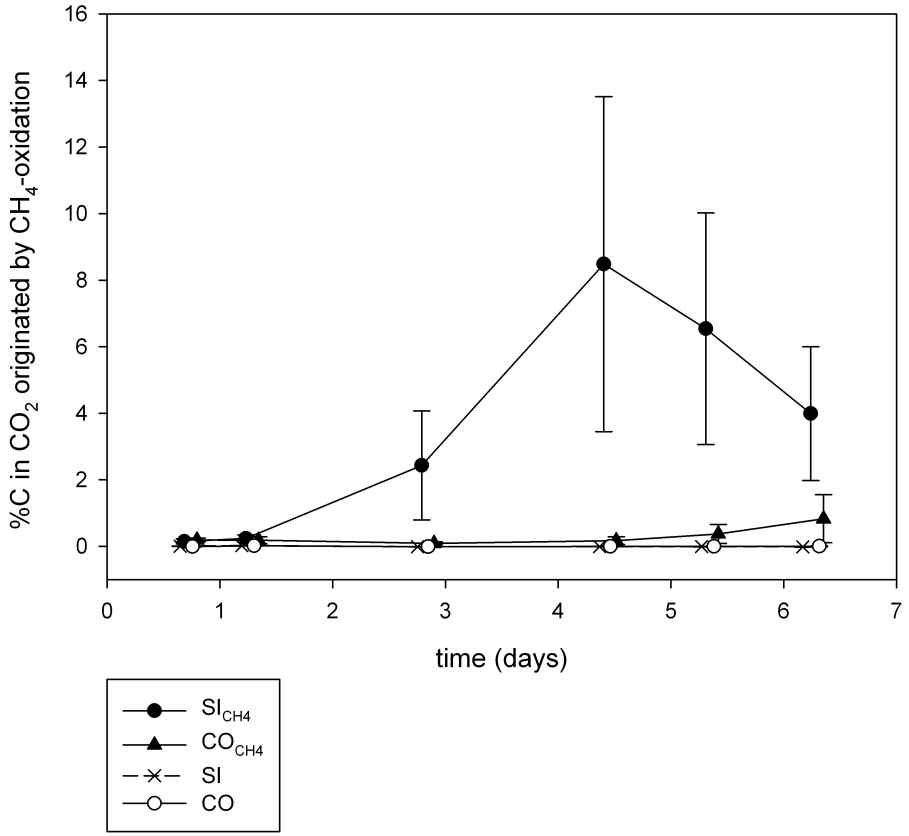

Fig. 1. Percentage of C incorporated into CO_2 originated from CH_4 at different time points ($f_{CH_4} \cdot 100\,\%$) for the severely impacted soil with methane (SI_{CH_4}), control soil with methane (CO_{CH_4}) and treatments without methane (SI, CO). Results are presented as means ± standard deviation ($n = 8$ for SI_{CH_4} and CO_{CH_4}; $n = 5$ for SI and CO).

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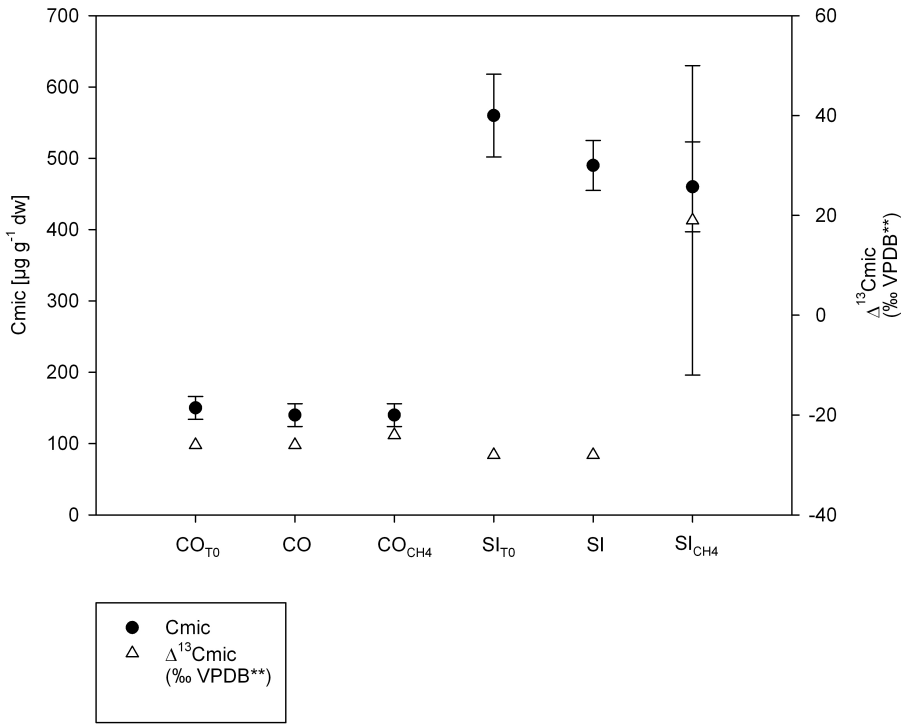

Fig. 2. Microbial Biomass (C_{mic}) and $\delta^{13}C$ content of microbial biomass ($\delta^{13}C_{mic}$) before (CO_{T0}, SI_{T0}) and after the incubation experiment without (CO, SI) and with methane (CO_{CH_4}, SI_{CH_4}). Results are presented as means ± standard deviation ($n = 8$ for SI_{CH_4} and CO_{CH_4}; $n = 5$ for SI_{T0}, CO_{T0} and SI, CO).
** Vienna Pee Dee Belemnite

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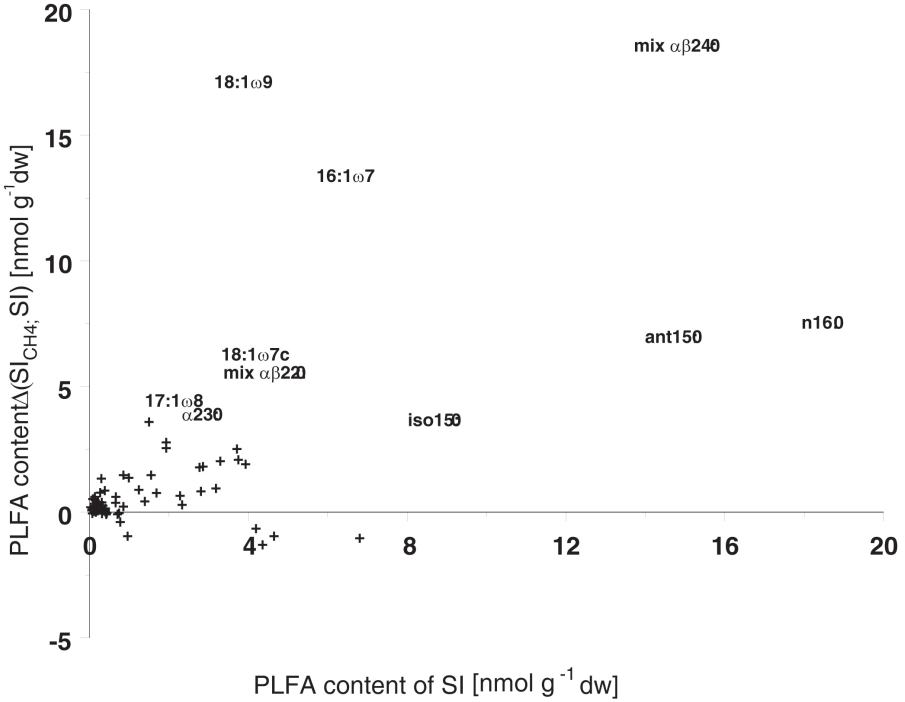

Fig. 3. Soil PLFA content [nmol g^{-1} dw] of SI soil without methane (SI) against the difference (Δ) between SI and SI$_{CH_4}$ treatments. PLFA with highest differences are indicated in the figure. PLFA analyses based on pooled samples of $n = 4$ (SI) and $n = 8$ (SI$_{CH_4}$). All PLFAs with positive Y-value show increased values in CH_4-treatments. PLFAs with highest differences are indicated in the figure.

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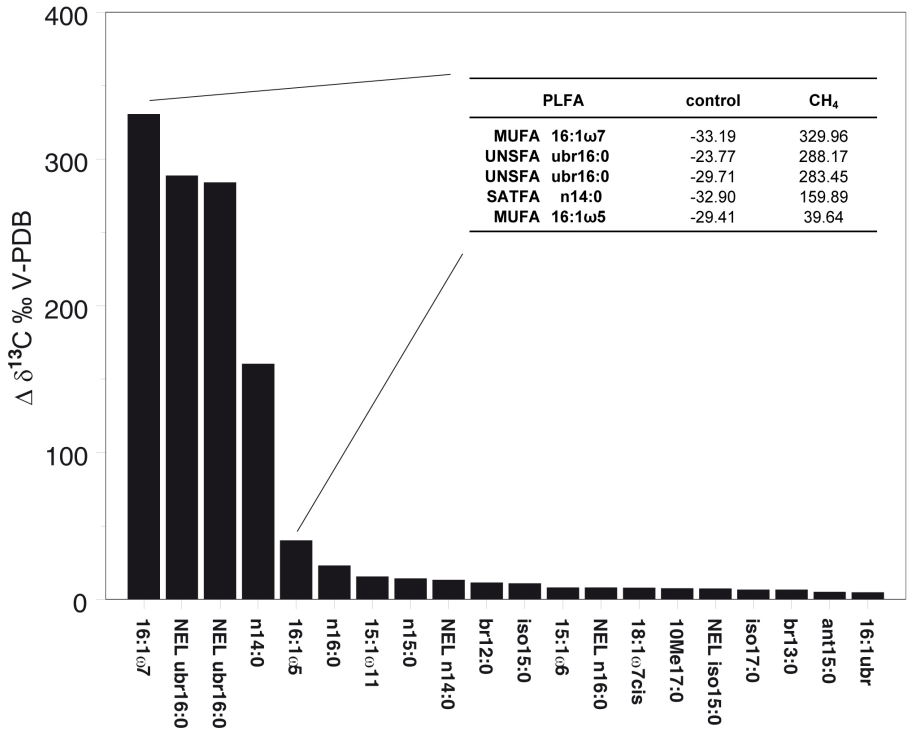

PLFA		control	CH_4
MUFA	16:1ω7	-33.19	329.96
UNSFA	ubr16:0	-23.77	288.17
UNSFA	ubr16:0	-29.71	283.45
SATFA	n14:0	-32.90	159.89
MUFA	16:1ω5	-29.41	39.64

Fig. 4. Ranking of the $\delta^{13}C$ enrichment in PLFA of SI_{CH_4} against SI. Values of the five most ^{13}C enriched lipids are given in the inset table.

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