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Traditional cattle manure application determines abundance, diversity and activity of methanogenic *Archaea* in arable European soil

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Abstract

Based on lipid analyses, 16S rRNA/rRNA gene single strand conformation polymorphism fingerprints and methane flux measurements, influences of the fertilisation regime on abundance and diversity of archaeal communities were investigated in soil samples from the long-term (103 years) field trial in Bad Lauchstädt, Germany. The investigated plots followed a gradient of increasing fertilisation beginning at no fertilisation and ending at the 'cattle manure' itself. The archaeal phospholipid etherlipid (PLEL) concentration was used as an indicator for archaeal biomass and increased with the gradient of increasing fertilisation, whereby the concentrations determined for organically fertilized soils were well above previously reported values. Methane emission, although at a low level, were occasionally only observed in organically fertilized soils, whereas the other treatments showed significant methane uptake. Euryarchaeotal organisms were abundant in all investigated samples but 16S rRNA analysis also demonstrated the presence of *Crenarchaeota* in fertilised soils. Lowest molecular archaeal diversity was found in highest fertilized treatments. *Archaea* phylogenetically most closely related to cultured methanogens were abundant in these fertilized soils, whereas *Archaea* with low relatedness to cultured microorganisms dominated in non-fertilized soils. Relatives of *Methanoculleus* spp. were found almost exclusively in organically fertilised soils or cattle manure. *Methanosarcina* related microorganisms were detected in all soils as well as in the cattle manure, but soils with highest organic application rate were specifically dominated by a close phylogenetic relative of *Methanosarcina thermophila*. Our findings suggest that regular application of cattle manure increased archaeal biomass, but reduced archaeal diversity and selected for methanogenic *Methanoculleus* and *Methanosarcina* strains, leading to the circumstance that high organic fertilised soils did not function as a methane sink at the investigated site anymore.

Introduction

The application of farmyard or cattle manure to agricultural land to improve soil fertility has a long, worldwide tradition, with records dating back to prehistoric times (Troels-Smith, 1984). The use of farmyard manure is a core principle in organic and low-input farming systems and enables management of soil organic matter (SOM) (Lampkin, 1992). SOM is crucial to maintain and to improve physical, chemical and biological properties of agricultural soils and is essential for the storage of energy and nutrients, as a structural stabilizer and as a chemical buffer for introduced xenobiotics (von Lützow et al., 2002).

Microbial communities play a key role in SOM transformation. Their contribution to nutrient cycling in agricultural soils is significant. Soil ecosystems are colonized by members of all three domains of life, i.e. *Bacteria*, *Eukarya* and *Archaea* (Liesack et al., 1997). Up to now representatives of the archaeal kingdoms *Euryarchaeota* and *Crenarchaeota* have been found in a variety of agricultural soils, especially rice field soils (e.g. Bai et al. 2000, Großkopf et al. 1998, Ramakrishnan et al. 2001). In this system, abundance of methanogenic *Archaea* generally leads to high methane emissions, but for arable soils this has not been described yet. In contrast, these have been considered to be a methane sink (Hütsch, 1998). However, it has also been demonstrated that under special conditions this capacity can significantly be reduced (Kammann et al., 2001). In this context, little is known about the interactions between archaeal communities and SOM under different soil management regimes. One approach in the elucidation of the ecological role of uncultivated *Archaea* is to study changes in their occurrence in various habitats. This may help to identify environmental factors promoting growth of *Archaea*, and, vice versa, may help to develop new isolation and cultivation strategies.

Based on a long-term field trial situated in Bad Lauchstädt, Germany, and using a phospholipid approach we investigated the archaeal abundance in soil samples getting no fertilisation, nitrogen-phosphorous-potassium (NPK) fertilizer or NPK plus organic

fertilisation during a three year lasting period from 2000 to 2002. The phospholipid analysis is based on the composition of polar membrane lipids from living microbial cells (Zelles and Bai, 1993). The so called archaeal phospholipid etherlipids (PLEL) are unique and readily distinguished from the acyl phospholipids of bacteria and eukaryotes containing phosphospholipid fatty acids (PLFA). PLEL are composed of di- and tetraethers of glycerol (archaeols and caldarchaeols, respectively) or more complex polyols with chains consisting of C_{15}, C_{20}, C_{25} or C_{40} isoprenoids (Kates, 1993, Mancuso et al., 1985, Schouten et al., 2000). Identification and quantification of archaeal lipids in soils provide estimates for archaeal community structure and archaeal biomass (Gattinger et al., 2002a, Gattinger et al., 2003). The taxonomic resolution of this method is relatively low, but it could be demonstrated that the archaeal abundance increased significantly with increasing fertilisation in the 2000 and 2001 samples. Thus, we decided to extend the analyses for the April 2002 sampling date to a 10 times higher organic fertilisation plot as well as to the original cattle manure. Moreover, a second approach, single strand conformation polymorphism (SSCP) fingerprinting, was used as a genotyping method for a more detailed description of archaeal communities. The analysis of 16S rRNA genes described the structure of archaeal communities, whereas the 16S rRNA provided information about its potential active members. Based on these complementary approaches we were able to study the effect of long-term organic and mineral fertilisation on the archaeal biomass, methane fluxes and community composition in an arable soil.

Results

Archaeal PLEL concentrations from October 2000 until July 2002. Statistical analyses of five samplings during a three year lasting monitoring period highlighted an increase in total archaeal PLEL concentration with increasing fertilisation intensity (Fig. 1) and followed the statistically significant ranking order L-l $\leq$ L-n $<$ L-h $<$ L-10h. The same was true for the proportion of archaeal PLEL of total concentration of phospholipid chains

giving an estimate for the relative abundance of *Archaea* in soil. Median values of PLEL concentrations for L-l, L-n, L-h and L-10h were 0.07, 0.27, 2.2 and 8.0 nmol g^{-1}, respectively, which accounted for 0.40, 1.0, 4.2 and 14.9% of the concentration of all detected phospholipid chains (Fig. 2A). A seasonal trend on archaeal PLEL concentration could not be found during the five year lasting observation period (data not shown).

Two-way ANOVA attributed a significant influence on absolute and relative archaeal PLEL concentration in soil to the factor "fertilisation". The factor "soil depth" and the interaction of "fertilisation x depth" did not affect either of these two criteria.

April 2002 sampling: soil properties. The more detailed study of the research site in Bad Lauchstädt in April 2002 underlined the distinct chemical differences among soil treatments. Organic C, total N and WEOC contents were lowest in non-fertilised plots and followed the ranking order L-l < L-n < L-h < L-10h < cattle manure (Table 1). Organic C content decreased with increasing depth in all soils whereas this was not the case for total N or WEOC.

April 2002 sampling: archaeal PLEL concentrations. In general, microbial biomass – defined as the sum of PLFA and PLEL concentrations – also followed the already mentioned ranking order of L-l ≤ L-n < L-h < L-10h < cattle manure (Table 1, statistical results not shown). Analogously, this was found for archaeal PLEL concentrations (Fig. 2A). The lowest value of 0.96 nmol PLEL isoprenoids g^{-1} dry soil was detected in the non-fertilised plot L-l and the highest with 239 nmol g^{-1} dry matter was detected in cattle manure (arithmetic means). PLEL chains, previously detected in acetoclastic methanogens such as *Methanosarcina* spp. (i20:1, Gattinger et al., 2002b) were detected in significant amounts in L-10h and cattle manure. PLEL chains containing cyclic C_{40} isoprenoids (i40:0-2cy) occurred only in L-10h samples (Fig. 2A).

April 2002 sampling: archaeal diversity and identification. Archaeal 16S rRNA and archaeal 16S rRNA genes were detected in all soil samples (Fig. 3). The total number of

different partial 16S rcDNA bands and 16S rRNA gene bands varied between 4 and 15 (Table 2). However, considering all soil samples soil depth did not influence the number of bands (statistical results not shown). With a Shannon index of around 0.5 and an Eveness of 0.6 16S rcDNA diversity of potentially active Archaea was significantly lowest in plot L-10h (Fig. 2B).

The majority of the bands identified from SSCP gels belonged to representatives of the archaeal kingdom *Euryarchaeota* (Fig. 3; Table 2) with close phylogenetic relationships to the cultured methanogens *Methanoculleus* spp., *Methanosarcina* spp. and *Methanobacterium thermoautotrophicum*. Two other euryarchaeotal clusters (cluster 1 and 2) were detected with a distant relationship to *Thermoplasma acidophilum* (approx. 87% sequence similarity). Soils L-l and L-n showed a consistent presence of 16S rRNA gene and 16S rcDNA sequences of clusters 1 and 2, but these were rarely detected in plot L-10h (Table 2). 16S rcDNA sequences belonging to the SCA cluster, indicative of *Crenarchaeota*, were found in L-n, L-h and L10-h plots. Their respective rRNA genes could not be detected. Both DNA- and RNA-targeted approaches detected sequences of the *Methanoculleus* cluster almost exclusively in organically fertilised soils and cattle manure. *Methanosarcina* spp. 16S rRNA genes were found in all treatments. However, using the RNA-targeted approach they were almost exclusively detected in the organically fertilised plot L-10h (99 % 16S rRNA sequence similarity with *Methanosarcina thermophila*) or cattle manure (98 % sequence similarity with *Methanosarcina mazei* [Table 2; Fig. 3, 4]) with relative abundances of approximately 85 and 33 %, respectively (Fig. 2C; Table 2). These findings could be confirmed by the PLEL approach: PLEL biomarkers indicative for *Methanosarcinaceae* (i20:1 isoprenoids) were only detected in L-10h and cattle manure accounting for 20.4 and 1.4% of total archaeal PLEL concentrations. *Methanobacterium thermoautotrophicum* 16S rRNA gene sequences were solely found in the cattle manure pile.

Methane fluxes. Significantly highest methane flux values were determined in the plot L-h (mean: +0.35, median: -0.35) followed by L-l (mean: -3.12, median: -3.08) and L-n (mean: -5.81, median: -5.75) (Fig. 5). In contrast to plot L-h, methane fluxes in plots L-l and L-n never reached positive values. The observation of positive methane flux values is considered as methane emission, whereas negative values indicate methane uptake. No data were available for plot L-10h; however, in order to estimate potential methane fluxes from this treatment, for each experimental plot corresponding biomarker ratios were calculated from the lipid data set. According to the DNA/RNA sequence data, it can be assumed that at least in soils L-h, L-10h and cattle manure, archaeal PLEL chains derived mostly from methanogenic archaea. These biomarkers were related to those mononunsaturated fatty acids known to occur at significant concentrations in type I and type II methanotrophs. The molar ratios of biomarkers from methanogenic to methanotrophic microorganisms increased significantly with fertilisation intensity, but stayed below 1 for the four soils and above 1 in cattle manure (Fig. 6).

Discussion

General soil properties and archaeal biomass estimates. The experiment at Bad Lauchstädt provided strong evidence that *Archaea* responded to the organic C and total N content in soil and were influenced by agricultural management practices. Regular application of mineral fertiliser and farmyard manure led to an increase in organic C, total N and WEOC content and total microbial biomass in Bad Lauchstädt, as reported earlier (Körschens et al., 1998). This was in line with findings from other long-term field trials (Jenkinson and Johnston [1977], Mäder et al. [2002], Parham et al. [2002]) and resulted from direct effects, such as addition of nutrients and energetic equivalents as well as through indirect effects caused by higher above- and below-ground biomass production of fertilised soils.

177 Archaeal PLEL concentrations of 0.27 and 2.2 nmol g^{-1} dry matter in the fertilised plots
178 L-n and L-h, respectively, were comparable to the values from other arable soils (Gattinger et
179 al., 2002b, Fließbach and Gattinger, unpublished data). PLEL values of treatment L-10h and
180 cattle manure of 8.0 and 240 nmol g^{-1} dry matter were well above reported values for
181 agricultural and wetland soils (Bai et al., 2000; Reichardt et al., 1997). Moreover, the relative
182 proportions of PLEL of total phospholipid chains were, with 14.9 and 16.1% for L-10h and
183 cattle manure, respectively, the highest values reported so far for such habitats. Even
184 compared with organic household and garden waste (Gattinger, unpublished data), the PLEL
185 concentration in cattle manure was around 20 times higher. Neither in April 2002 nor at the
186 other sampling dates soil depths had a significant effect on total and relative PLEL
187 concentration (Table 1). Presumably, regular ploughing and other tillage practices provide a
188 homogeneously structured soil environment for *Archaea* in the upper horizon (0-40 cm) of
189 this arable soil.

190 **Archaeal diversity in April 2002.** Archaeal 16S rRNA gene and 16S rcDNA
191 fingerprinting revealed that long-term fertilisation selected archaeal communities according to
192 the fertilisation treatment of the soil. High organic fertilisation resulted in decreasing archaeal
193 diversity (Fig. 2B). The response of archaeal communities to agricultural practices was also
194 shown by Nicol et al. (2003) who determined different archaeal community structures in
195 improved (N-fertilised) and unimproved grassland soils.

196 Soil depth did not influence archaeal diversity as revealed by molecular methods. This is
197 in contrast to findings in a forest soil, where a change in archaeal community structure was
198 observed with increased soil depth (Pesaro and Widmer, 2002). As already mentioned for
199 absolute and relative PLEL concentrations, regular ploughing might have led to a more
200 homogenous horizontal organisation of *Archaea* in soil.

201 In order to determine the variation of our molecular approach, cattle manure was
202 representatively analysed in four replicates. In general, resulting number of bands was

reproducible, but relative band concentrations could vary (Table 2; Fig. 3). This was especially apparent for DNA based analyses. RNA based quantification of the *Methanoculleus* or *Methanosarcina* cluster was characterized by standard deviations of less than 20 %. This seemed to be appropriate for quantifications influenced by the well known PCR bias and could be an indicator for an overall activity of these organisms in the cattle manure. Subsequently, due to the potential lower PCR bias of the RNA based approach (23 cycles used for RT-PCR compared to 45 cycles for PCR, see Material & Methods), only 16S rcDNA quantifications were used for Shannon Indices and Eveness calculations.

Identification and quantification of archaeal taxa. The majority of archaeal 16S rRNA sequences identified in the soil samples and cattle manure investigated belonged to the kingdom *Euryarchaeota*. A number of these euryarchaeotal sequences showed close phylogenetic relationships (96 to 99 % 16S rRNA sequence similarity [Fig. 4; supplementary data: Table 1]) to known methanogenic species. The occurrence of methanogens was also reported for other oxic soils (Gattinger et al., 2002b, Peters and Conrad, 1995, Wachinger et al., 2000). Additionally, two euryarchaeotal clusters (Cluster 1 and 2) were obtained from the Bad Lauchstädt research site with *Thermoplasma acidophilum* as its closest, but still very distant, cultivated representative. *Thermoplasmales* and related organisms were also detected by molecular analyses in forest and rice field soils (Pesaro and Widmer, 2002, Ramakrishnan et al., 2001).

Phylogenetic analysis revealed the presence of 16S rRNA gene and 16S rRNA sequences related to the two methanogenic taxa *Methanoculleus* and *Methanosarcina* in the soils investigated. In particular, a relative of *Methanosarcina thermophila* (99 % 16S rRNA sequence similarity) was detected in relatively water-rich depths of less than 85 % dry matter content of treatment L-10h, indicating anoxic metabolism (Table 1). SSCP analyses indicated that potentially active *M. thermophila* dominated the archaeal population of treatment L-10h (Fig. 2C). It has to be kept in mind that these molecular analyses are semiquantitative and

229 susceptible to primer selectivity and PCR bias. However, the molecular data were supported
230 by the detection of PLEL chain i20:1, previously found in acetoclastic methanogens such as
231 *Methanosarcina* ssp. (Gattinger et al., 2002a), which was observed in significant amounts in
232 organically fertilised plot L-10h, but not in plots L-l, L-n or L-h (Fig. 2C). Based on 16S
233 rRNA analyses, *M. thermophila* was not found in the cattle manure, indicating the
234 establishment of a soil specific archaeal population in plot L-10h. The thermophilic
235 methanogen *M. thermophila* was originally isolated from a sewage digester (Zinder et al.,
236 1979). However, it could not just survive in many environments, including permafrost
237 sediments (Rivkina et al., 1998) or hot springs (Stetter et al., 1990), but *Methanosarcina* spp.
238 could also be enriched from different dry soils with oxic conditions *in situ* (Peters & Conrad,
239 1995). *Methanosarcina*- and *Methanoculleus*-related archaeal sequences have also been
240 detected in anoxic rice field soils from Italy, China and the Philippines, with pronounced
241 methane emissions (Ramakrishnan et al., 2001). 16S rRNA genes and 16S rRNA sequences
242 affiliated with the genus *Methanobacterium* were only found in cattle manure and related
243 sequences for this genus have also obtained from cattle rumen and rice field soils
244 (Ramakrishnan et al., 2001, Tajima et al., 2001).

245 Only a minor proportion of the archaeal 16S rcDNA SSCP bands could be attributed to
246 the kingdom *Crenarchaeota*, in contrast to the widespread occurrence of *Crenarcheaota* in
247 arable soils from geographically different locations (Bintrim et al., 1997, Buckley et al., 1998,
248 Ueda et al., 1995). Moreover, *Crenarchaeota* were only detected in RNA-based fingerprints,
249 possibly because only organisms with high rRNA level were present at the time of sampling.
250 This could be due to their activity status or they may maintain in general a high rRNA level.

251 **Methane fluxes.** With a mean of 0.35 and a median of –0.35 methane flux rates were
252 highest in fertilized treated soil L-h. Unfortunately, fluxes were not determined for the L-10h
253 plot. However, for this site lipid data (Fig. 2) demonstrated the increase of the archaeal
254 biomass, combined with an increase of methanogenic Archaea (Fig. 6). Moreover, it has

255 already been demonstrated earlier, that this extremely large organically treated site inhibited
256 CH_4 oxidation significantly, again resulting in higher methane fluxes (Hütsch, 1996). Thus, it
257 can be assumed that both organically treated soils, L-h and L-10h, nearly lost their methane
258 sink potential, which was probably due to the high abundance and activity of methanogenic
259 Archaea, especially of *Methansarcina* species. However, vice versa it can also be assumed
260 that biological methane oxidation based on the activity of methane oxidizing bacteria (Fig. 6)
261 still acted as a kind of "biofilter", as proposed by Kammann et al. (2001) and Hütsch (1998).
262 Despite a higher archaeal biomass in soil L-n, its methane fluxes were significantly lower
263 than in L-l. This might be explained by a reduced methanotrophic activity due to a higher
264 level of ammonia as a competing substrate in the mineral fertilised L-n (Hütsch et al., 1996).
265 **Consequences of long-term organic fertilisation.** Archaeal DNA- and RNA-based
266 fingerprints revealed that long-term organic fertilisation stimulated *Archaea* and also favoured
267 population size and activity of archaeal communities, dominated by a close relative of *M.*
268 *thermophila* (Fig. 2C). It could be assumed, that the archaeal communities detected in the
269 manure pile originated from cattle rumen and entered the manure heap via excretion, because
270 absolute and relative archaeal biomarker concentration increased with increased cattle manure
271 application and by far the highest values were determined in cattle manure itself. Moreover,
272 sequences of the *Methanoculleus* cluster were detected almost exclusively in DNA and RNA
273 from organically fertilised soils and cattle manure (Table 2). The genus *Methanoculleus*
274 belongs to the family *Methanomicrobiacaea* and close relatives such as *Methanogenium*
275 *mobile* have been detected in cattle rumen (Tajima et al., 2001). Furthermore, *M. mazei* have
276 also been previously isolated from cattle rumen (Stewart et al., 1997) and sequences affiliated
277 with *Methanosarcina mazei* were determined in all organically fertilised soils and cattle
278 manure. From these results it could be concluded that regular application of cattle manure
279 served as an inoculum for *Methanoculleus* and *Methanosarcina* species.

On the other hand, although in organically fertilised soil *M. thermophila*-like taxa accounted for a higher proportion of total archaeal DNA and rcDNA SSCP bands (Table 2), *Methanosarcina*-related 16S rRNA sequences were also detected in mineral and unfertilised soils. Analogous to results of Peters and Conrad (1995) this could indicate a permanent presence of these archaea in all soils investigated, which were just stimulated by fertilisation. Moreover, potentially active *Methanosarcina* species of plot L-10h were closer related to *M. thermophila* and those of the cattle manure closer to *M. mazei* (Figs. 3, 4).

In contrast, archaeal communities belonging to the phylogenetic cluster 1 and 2 (Fig. 4) seemed to be unaffected by organic fertilisation. In soil treatments L-l and L-n corresponding archaeal DNA- and RNA-based SSCP bands accounted on average for more than 50% of the total, which is much higher than the percentage in treatments L-h and L-10h (Table 2). Presumably, humus rich soil properties are rather unsuitable for these *Archaea* but suitable and stimulatory for several methanogenic groups.

Currently, the *in situ* functions of the various archaeal groups in the Chernozem horizons at Bad Lauchstädt research site are unknown. Apart from methanogenesis, several functions in the N cycle have been reported for *Archaea* (Philippot, 2002). However, organically fertilised soils such as L-h and L-10h with high archaeal biomass and a substantial percentage of methanogens in the ploughed horizon could show methane emission under water saturated conditions. This was generally not the case during sampling period 2002 (Fig. 5) and might not be a problem for the site Bad Lauchstädt due to its rather dry, continental climate. But the data presented here indicated that under more rainy conditions methane production, especially due to *Methanosarcina* relatives, is likely to occur in highly organically fertilized arable soils.

Experimental procedures

Field site and experimental design. Soil samples from a long-term field trial situated in Bad Lauchstädt, Germany, were investigated. Bad Lauchstädt (11°53'E, 51°24'N) is located

approximately 15 km southwest of Halle (Saxony-Anhalt) at 110 m above sea level. The annual precipitation is 484 mm and the annual temperature is 8.7°C (average 1896-1995). The soil type is a Haplic Chernozem (FAO) with 6% sand, 73% silt, 21% clay and with a pH ranging between 6.3 and 6.6. Samples were taken from the following experimental plots, which had a size of 26.5 m x 10 m = 265 m^2:

(I) 'L-l', without mineral and organic fertilisation, = low fertilisation intensity.

(II) 'L-n', receiving only NPK fertilisation, = normal fertilisation intensity. The annually applied mineral nutrients were 140 kg N ha^{-1}, 60 kg P ha^{-1}, 230 kg K ha^{-1}.

(III) 'L-h', receiving NPK fertiliser and organic fertilisation, by using cattle manure at an application rate of 20 t ha^{-1} a^{-1}, = high fertilisation intensity. The applied mineral nutrients were 120 kg N ha^{-1}, 12 kg P ha^{-1}, 50 kg K ha^{-1}.

(IV) 'L-10h', receiving only organic fertilisation, by using cattle manure at an application rate of 200 t ha^{-1} a^{-1}.

(V) Cattle manure pile, sampling only on April 23th, 2002.

Plots 'L-l' and 'L-h' are part of the 'Static Fertilisation Experiment Bad Lauchstädt', started in 1902. Plots 'L-n' and 'L-10h' were introduced in 1978 to discern the influence of highly different SOM contents on crop yield and C and N dynamics. To avoid inadvertent spread of cattle manure to neighbouring (unfertilised) plots, field workers dispersed the dung manually using forks. Subsequent soil cultivation consisted of ploughing (0-20 cm depth) to enable a homogenous incorporation of the manure in the top soil. Detailed information on the Lauchstädt field trials and on the site can be found elsewhere (Körschens et al., 1998).

Soil sampling and preparation. Phospholipid profiling was performed on samples from three different soil depths of the plots L-l, L-n and L-h in October 2000 (winter wheat), July 2001 (winter wheat) and October 2001 (bare soil), April 2002 (potato) and July 2002 (potato).

Soil samples for a detailed analysis were taken four weeks after NPK fertilisation on 30th April 2002 from 0-15 (depth 1), 15-30 (depth 2) and 30-40 cm depth (depth 3). All plots were

planted with potatoes. Previous investigations showed little spatial heterogeneity in microbial and organic C characteristics (Böhme et al., 2005; Embacher et al., 2005), hence from each soil depth, 5 randomly taken soil cores were pooled, homogenised and sieved (< 2 mm particle size). Subsamples for phospholipid analysis were immediately stored on dry ice, whereas subsamples for nucleic acid analysis were frozen in liquid nitrogen prior to storage on dry ice.

Soil analytical procedures. Organic C and total N contents were measured in dried, milled samples using a CN analyser with high catalytic combustion. All soil profiles were free of carbonates; hence, HCl treatment to exclude inorganic carbon was not necessary. Water extractable organic carbon (WEOC) was determined as an indicator for C availability by shaking 50 g of fresh soil with 100 ml 0.01 M $CaCl_2$ solution for 10 minutes (Zsolnay et al., 1999). The suspension was then passed through a 0.45 µm polycarbonate filter (Whatman) and WEOC concentration was measured using a total organic C analyzer (Shimadzu TOC-5050).

Determination of methane fluxes. Methane fluxes were recorded for April 2002 when detailed molecular analyses were performed. The quantification of the methane fluxes was done on basis of the closed chamber method using the Automatic Trace gas Chamber (ATC) system as described elsewhere (Russow et al., 2000). Briefly, the ATC system consists of a movable plastic chamber, a gas sampling unit and a programmable control unit. For the methane measurements three ATC's per fertilisation treatment – altogether 9 systems (L-l, L-n and L-h) – were installed to ensure a threefold replication. The observation period lasted from 26th until the 30th of April 2002, resulting in a total of 15 measurements per treatment. Therefore the stainless steel frame of each system was embedded in the soil surface. The plastic chamber for covering a defined surface of 0.225 m^2 was moved by a microprocessor controlled electric motor. At the beginning of each sampling, the chamber was automatically lowered into the water seal, than the start sample was taken by pumping the gas from the

covered volume for 5 minutes in cycle to ensure a homogenous trace gas distribution over the system and the first sample vial. After one hour of covering the soil surface by the chamber the same procedure was repeated with the next sample vial. After 2 hours the third gas sample was taken into another vial and the chamber was lifted by the electric motor. After sampling all gas-vials were promptly transported into the lab.

Methane concentrations of each sample were analyzed using a Shimadzu GC-14BPFE with a flame ionization detector (FID). Based on the constant dimensions of the chamber as well as the covered area and under consideration of temperature and air pressure, the flux of CH_4 was calculated for the corresponding time interval for all 9 ATC-systems. Flux data were calculated from linear change of CH_4 with time (during 1h, see above) using linear regression.

Lipid extraction of soil samples. Lipids were extracted from a fresh soil sample equivalent to a dry weight of 50 g, according to the Bligh-Dyer method as described elsewhere (Zelles and Bai, 1993). The resulting lipid material was fractionated into neutral lipids, glycolipids and phospho-(polar) lipids on a silica-bonded phase column (SPE-SI; Bond Elute, Analytical Chem International, CA, USA) by elution with chloroform, acetone and methanol, respectively.

Determination of phospholipid fatty acids (PLFA) and phospholipid etherlipids (PLEL). Both lipid analyses are based on the determination of phospholipid chains. An aliquot of the phospholipid fraction equivalent to 12.5 g soil dry matter (dm) was taken for PLFA analysis following the procedures described elsewhere (Zelles and Bai, 1993). Extraction, separation, hydrolysis and derivatization generated four esterlinked (EL-PLFA) and two non-esterlinked PLFA (NEL-PLFA) fractions which were further separated and identified as individual fatty acid methyl esters (FAME) by GC/MS. In this study only signature PLFA for methanotrophic bacteria (16:1wcis8 and 18:1wcis8) are reported. Therefore, the fraction of esterlinked mono-unsaturated fatty acids was derivatized with

382 dimethyl disulfide (DMDS) to determine the position of double bonds. Quantification was
383 based on the abundance of characteristic ions of their DMDS adducts (Zelles and Bai, 1993).

384 Another aliquot of the phospholipid fraction equivalent to 25.0 g soil dry matter (dm) was
385 used for PLEL analysis according to Gattinger et al. (2003). After formation of ether core
386 lipids, ether-linked isoprenoids were released following cleavage of ether bonds with HI and
387 reductive dehalogenation with Zn in glacial acetic acid. The resulting isoprenoid
388 hydrocarbons were dissolved in 100 µl internal standard solution (0.36 nMol
389 Nonadecylacidmethylester) and transferred into a GC vial.

390 PLEL derived hydrocarbons and PLFA were analysed by gas chromatography mass
391 spectrometry (GCMS) (Hewlett Packard 5971A MSD, combined with a GC 5890 series II) at
392 operating conditions described elsewhere (Gattinger et al. 2003, Zelles and Bai, 1993).
393 Identification of individual compounds was based on comparison of retention time and mass
394 spectral data obtained from standard compounds, pure cultures and environmental samples.
395 Quantification was achieved using chromatography software (HP ChemStation, SOVLVIT,
396 CH) based on response factors for the individual phospholipid chains.

397 The absolute concentration of archaeal PLEL isoprenoids serves as an estimate for
398 archaeal biomass in soil, whereas the relative PLEL concentration (in % of total phospholipid
399 chains) describes the proportion of archaeal markers in relation to total microbial
400 phospholipid chains. The following abbreviations for the different PLEL derived isoprenoid
401 hydrocarbons were used: i: isoprenoid-branched; i20:0 indicates a saturated i20:1
402 monounsaturated hydrocarbon chain; i40:0-cy is a C_{40} isoprenoid with one to three
403 cyclopentane rings.

404 **Methane production potential.** Ratios of PLEL biomarkers from (methanogenic)
405 archaea and PLFA from methanotrophic bacteria were calculated according to Gattinger et al.
406 (2002b) in order to compare abundances of both taxonomic groups among various soils. This
407 serves as an estimate for the methane production potential.

Nucleic acid extraction of soil samples. The FastDNA spin kit for soil and the FastRNA BLUE kit (both BIO101, Vista, Ca.) were used to extract DNA and RNA from soil. Soil (0.5 g) was bead-beated using the FastDNA spin kit according to the manufacturer's instructions. Raw extract (200 µl) was collected, added to the FastRNA BLUE kit and RNA was isolated according to the manufacturer's instructions. In parallel, extraction of DNA continued using the FastDNA spin kit for soil as recommended by the manufacturer. In order to determine variations in nucleic acid treatments, cattle manure RNA and DNA was separately isolated in four replicates. Nucleic acid concentrations were determined fluorometrically as described previously (Weinbauer and Höfle, 2001).

16S rcDNA generation. Prior to RT-PCR, RNA extracts were treated with DNAse I (Roche Diagnostics, Mannheim, Germany) for 60 min at 37°C. To obtain 16S ribosomal complementary DNA (rcDNA), 10 ng of template RNA was reverse transcribed at 50°C using the Thermoscript RT-PCR System (Invitrogen) and the universal reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991) following the manufacturer's instructions. In each reverse transcription reaction, some RNA-samples were not supplemented with reverse transcriptase to assess DNA-contamination.

PCR, SSCP community fingerprinting and sequencing. Archaeal primers w036F (5'-TCCAGGCCCTACGGGG-3') / w039RpH (5'-CTCCCCCGCCAATTCCT-3') (333F/915R, *E. coli* numbering) used for 16S rRNA gene or 16S rcDNA amplification are described by Leclerc et al. (2001). The forward primer w036F was biotinylated. Each PCR was carried out in a final volume of 50 µl. PCR mixtures contained 1x PCR buffer, 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 400 nM of the forward and reverse primers and 1.25 U Hot star Taq polymerase (Qiagen). Thermocycling started with an initial denaturation step for 15 min at 95°C. A total of 23 (for cDNA) or 45 (for DNA) cycles, 30 s at 94°C, 30 s at 62°C, 2 min at 72°C, were followed by a final elongation step of 10 min at 72°C. The PCR products were purified with Streptavidin magnetic beads using the manufacturer's instructions (Promega,

Madison, WI) and the protocol of Lee et al. (2000) was used to generate and purify ssDNA. The ssDNA content of each sample was quantified on an agarose gel using a quantitative 1 kb ladder (Peqlab) for standardization. Approximately 100 ng ssDNA were mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) in a final volume of 8 µl. After incubation for 3 min at 95°C, the ssDNA samples were stored on ice, loaded onto a non-denaturing acrylamide gel, separated by electrophoresis at 20°C and 400V for 18h and silver stained according to Schwieger and Tebbe (1998). Re-amplification of individual bands excised from the SSCP gels was performed as described by Pöhler et al. (2002) followed by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA, USA) including the primers applied before. After purification using the BigDyeEx Purification Kit (Qiagen), products were sequenced on an ABI Prism 3100 Genetic Analyzer.

Phylogenetic analyses. Forward and reverse sequences of all samples were checked for accuracy using the Sequencer software package (www.genecodes.com). Phylogenetic affiliations of the partial sequences were initially estimated using the program BLAST (basic local alignment search tool) (Altschul et al., 1997) and available nucleotide databases. Single sequences were aligned against close relatives in ARB (a software environment for sequence data) (Ludwig et al., 2004). Similarity of partial sequences was determined using ARB, and those with greater than 98% similarity were grouped. Representatives from each group were used in the phylogenetic analyses. Sequences for analysis were managed in ARB and reduced to unambiguously alignable positions using group-specific filters. Evolutionary distance dendrograms were constructed using the Jukes-Cantor correction and neighbour joining in ARB. Bootstrap analyses (1,000 resamplings) were performed for the neighbour joining method. Sequences were labelled as Bo-, corresponding to Bo = soil.

Relative quantification of SSCP bands. SSCP gels were scanned into Adobe Photoshop 5.0 and documented as a tif-file. In order to determine 16S rRNA gene and 16S rcDNA

fingerprint similarities, the tif-files were analyzed using the program *Gelcompare II* version 3.0 (Applied Maths, Belgium). Band search parameters were: Minimum profiling 5.00, "Gray zone" 0, Minimum area 1.00 and Shoulder sensitivity 1. Relative fraction of bands was calculated by the determination of band areas (in % of total band area above 1 % minimum area per lane).

Statistical analyses. All statistical analyses were performed with SPSS software package release 11.0 (SPSS Inc., Chicago, USA). Box plots within figures represent quartiles and limits for outliers and extreme values as shown in Fig. 1B. Two-way analysis of variance (ANOVA) was used to determine the F values (variance ratio) and the statistical significance ($P < 0.05$) of fertilisation, sampling depth and their interaction. The size of the F value was used to quantify the contribution of the factors to the observed variance.

The Shannon diversity index (H) was used to characterize species diversity based on SSCP fingerprinting. Shannon's index accounted for both abundance and evenness of the species detected. The proportion of species i relative to the total number of species (p_i) was calculated, and then multiplied by the natural logarithm of this proportion ($\ln p_i$). The resulting product was summed across species, and multiplied by -1:

$$H = -\sum_{i=1}^{S} p_i \ln(p_i)$$

Shannon's equitability (E_H) was calculated by dividing H by H_{max}. Equitability assumed a value between 0 and 1 with 1 being complete evenness:

$$E_H = H / H_{max}$$

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in this study have been deposited in the GenBank Database under accession numbers DQ004700 to DQ004733.

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628 **Figure legends**

629 **Figure 1. A.** Box plots of the archaeal PLEL biomarker concentration in soil profiles (0-40
630 cm) of Bad Lauchstädt from 5 samplings during 2000-2002 (n = 15). Different letters (a, b, c)
631 indicate significance at the P = 0.05 level. **B.** The statistical principle of the box plot
632 illustration.

633

634 **Figure 2. A.** Archaeal PLEL concentration in soil samples from different plots of Bad
635 Lauchstädt and original cattle manure during April 2002 sampling. Each bar of the four
636 different soil treatments represents the mean value of the three soil depths as well as the cattle
637 manure. Percent values indicate the proportion of PLEL from total phospholipid chains. **B.**
638 Molecular diversity based on archaeal 16S rcDNA SSCP fingerprint bands and expressed as
639 Shannon Index and Eveness. **C.** Relative abundance of the *Methanosarcina* cluster based on
640 quantification of 16S rcDNA SSCP fingerprint bands as well as on PLEL data.

641

642 **Figure 3.** 16S rRNA gene (A) and 16S rcDNA (B) SSCP fingerprints of soil samples from
643 April 2002. Representative partial archaeal sequences are marked as follows: 1, Bo69-06-07;
644 2, Bo55-21-04; 3, Bo69-11-08; 4, Bo64-05-01; 5, Bo55-15-13; 6, Bo64-17-04; 7, Bo64-16-
645 04; 8, Bo64-09-02; 9, Bo69-11-12; 10, Bo64-06-08; St, standard. More detailed information
646 about sequences is given in Fig. 4 and Table 1 (supplementary data).

647

648 **Figure 4.** 16S rRNA gene sequence-based phylogenetic tree (based on approx. 450
649 nucleotides) indicating phylogenetic relationships between representative sequences from the
650 studied soil samples and members of the domain *Archaea*. 16S rRNA gene sequence of
651 *Escherichia coli* was used as an outgroup (not shown).). Accession numbers of the new
652 sequences are given in brackets. More detailed information about investigated sequences is
653 given at the supplementary data (Table 1).

654 **Figure 5.** Methane fluxes of three differently fertilised soils from the field trial in Bad
655 Lauchstädt during April 2002. Different letters indicate significance at the P=0.05 level.
656
657 **Figure 6.** Molar ratios of lipid biomarkers from methanogenic and methanotrophic
658 microorganisms in four differently fertilised soils and cattle manure. Bars denote standard
659 deviation. Different letters indicate significance at the P = 0.05 level.

Table 1. Chemical properties and phospholipid composition in the investigated soil and cattle manure samples (April 2002).

		L-l			L-n			L-h			L-10h			Cattle manure
Depth[1]		1	2	3	1	2	3	1	2	3	1	2	3	
Dry matter content (%)		86	85	83	85	84	84	85	84	86	81	77	83	22.5
Organic C (%)		1.72	1.71	1.63	2.00	1.96	1.42	2.47	2.31	1.80	3.29	3.83	2.74	32.9
	SD	0.02	0.01	0.04	0.02	0.07	0.05	0.16	0.18	0.06	0.15	0.28	0.98	4.92
Total N (%)		0.14	0.14	0.13	0.17	0.16	0.13	0.21	0.20	0.15	0.29	0.31	0.24	2.4
	SD	0.01	0.01	0.04	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.21
WEOC[3] ($\mu g\ g^{-1}$)		37.7	39.0	39.1	57.1	53.3	57.5	85.1	85.6	78.2	146	149	120	13278
	SD	3.1	5.4	10.6	10.1	7.8	10.5	2.5	3.6	4.2	52.6	50.8	74.9	2530
Total phospholipid chains[4] (nmol g^{-1})		24.8	25.5	19.4	33.9	32.2	23.0	46.5	49.8	37.7	91.6	98.6	51.9	1476
	SD	3.0	2.1	2.4	3.6	3.1	3.7	6.5	4.5	4.9	14.7	11.8	7.8	591

Values are expressed as arithmetic means from triplicate samples.

[1]1, 0-15 cm; 2, 15-30 cm; 3, 30-40 cm.

[2]SD: standard deviation.

[3]WEOC: water extractable organic carbon

[4]Phospholipid chains: sum of PLFA and PLEL concentration

Table 2. Diversity (as number of bands) and molecular abundance of prominent archeal clusters in Bad Lauchstädt soil based on 16S rRNA gene (rDNA) and 16S rcDNA (in *italic*) SSCP fingerprints (April 2002). Only bands with relative amounts of >1% were investigated. Number of bands are displayed before the backslash and their corresponding fraction [%] behind it. Representative standard deviations for cattle manure replicates are given in parentheses.

Soil sample (treatment/depth)	16S	Total number of bands	Cluster I	Cluster II	*Methanoculleus* cluster	*Methanosarcina* cluster	*Methano-bacterium* cluster	SCA cluster	No specific cluster	Not identified
L-l 0-15	rDNA	9		3/34.2		3/30.8				3/34.2
	rRNA	*13*	*3/26.1*	*6/61.3*						*4/12.6*
L-l 15-30	rDNA	7	3/12.1			1/27.4				3/60.5
	rRNA	*15*	*9/74.4*	*2/14.6*						*4/11.0*
L-l 30-40	rDNA	11	10/88.3							1/11.7
	rRNA	*11*	*5/61.7*	*2/18.1*						*4/20.2*
L-n 0-15	rDNA	12	2/31.1	3/27.4	2/3.8	4/32.3				1/5.4
	rRNA	*10*	*1/10.4*	*4/54.8*				*3/14.5*		*2/20.3*
L-n 15-30	rDNA	12	2/28.2	3/34.3		3/26.5				4/10.2
	rRNA	*9*	*4/40.1*	*3/37.0*				*1/2.9*		*1/20.0*
L-n 30-40	rDNA	9	5/56.3			3/32.0				1/10.4
	rRNA	*17*	*5/35.0*	*2/23.3*				*4/9.1*		*7/32.7*
L-h 0-15	rDNA	10	1/23.4		1/1.7	3/53.7				5/21.2
	rRNA	*14*	*1/5.3*	*4/59.6*	*1/1.9*			*4/15.3*		*4/17.9*
L-h 15-30	rDNA	11	1/20.7		1/8.7	3/26.0				6/44.6
	rRNA	*17*	*2/6.4*	*5/39.7*	*1/3.0*			*2/15.5*		*7/35.4*
L-h 30-40	rDNA	10	1/18.1		1/1.2	4/44.4				4/36.3
	rRNA	*14*	*2/11.1*	*7/55.9*				*3/6.7*		*2/26.3*
L-10h 0-15	rDNA	7			1/1.1	3/73.3				3/25.6
	rRNA	*4*				*2/97.2*		*2/2.8*		*0/0.0*
L-10h 15-30	rDNA	7			1/3.4	3/81.1			1/1.7	2/12.8
	rRNA	*8*			*2/8.9*	*1/82.2*		*3/4.9*		*2/4.0*
L-10h 30-40	rDNA	9			1/1.2	3/78.2			2/3.7	3/19.0
	rRNA	*6*			*1/6.0*	*1/80.3*		*3/8.3*		*1/5.4*
Cattle manure	rDNA	11 (±1)			3 (±0)/48.5 (±24.4)	2 (±1)/16.4 (±17.3)	3 (±0)/15.7 (±14.0)			3 (±2)/15.8 (±7.4)
	rRNA	*8 (±2)*			*5 (±2)/56.4 (±10.5)*	*2 (±1 /32.9 (±5.3)*			*0 (±1)/2.2 (±4.4)*	*1 (±2)/8.5 (±10.8)*

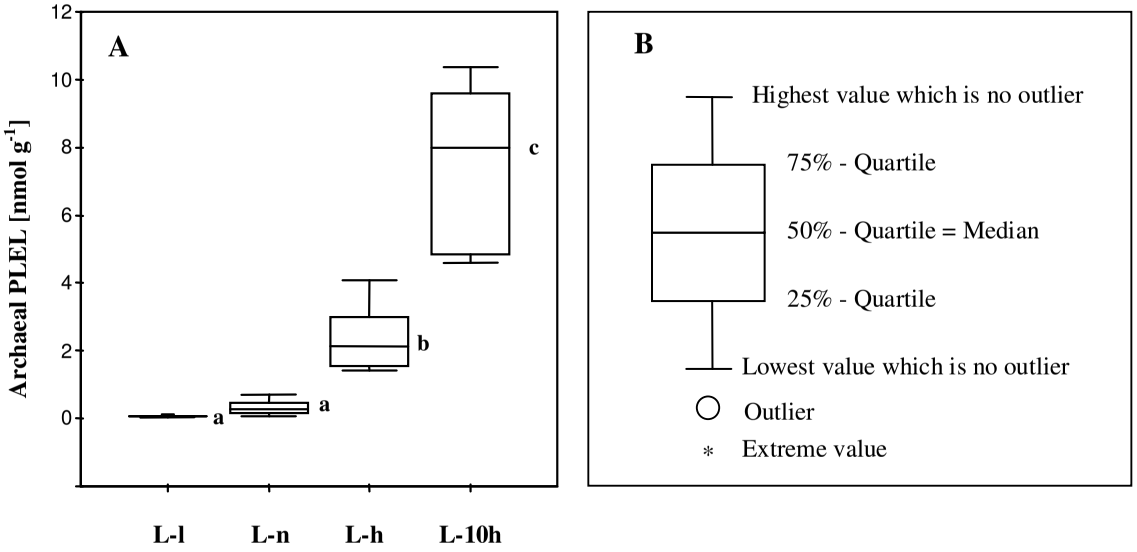
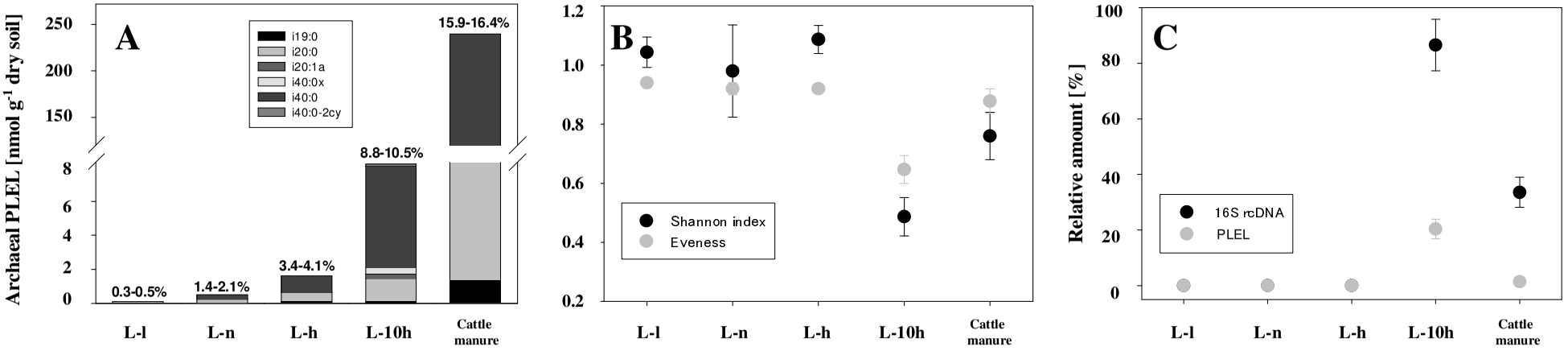

A
B
Archaeal PLEL [nmol g-1]
12
10
8
6
4
2
0
a
a
b
c
L-l
L-n
L-h
L-10h
Highest value which is no outlier
75% - Quartile
50% - Quartile = Median
25% - Quartile
Lowest value which is no outlier
Outlier
Extreme value

Fig. 1

Fig. 2


A
B
C
Archaeal PLEL [nmol g-1 dry soil]
i19:0
i20:0
i20:1a
i40:0x
i40:0
i40:0-2cy
0.3-0.5%
1.4-2.1%
3.4-4.1%
8.8-10.5%
15.9-16.4%
Shannon index
Eveness
Relative amount [%]
16S rcDNA
PLEL
L-l
L-n
L-h
L-10h
Cattle manure

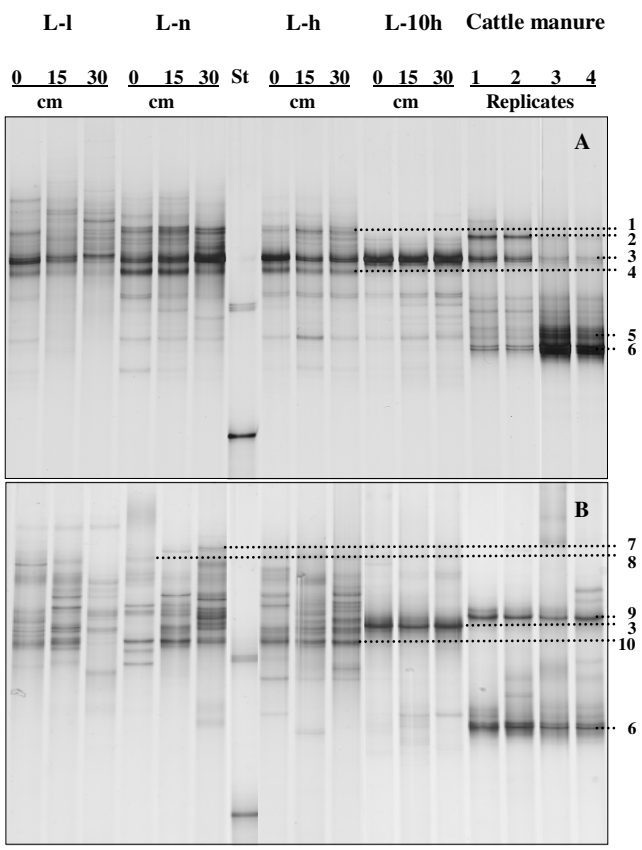

L-l
L-n
L-h
L-10h
Cattle manure
0 15 30
0 15 30
St
0 15 30
0 15 30
1 2 3 4
cm
cm
cm
cm
Replicates
A
1
2
3
4
5
6
B
7
8
9
3
10
6

Fig. 3

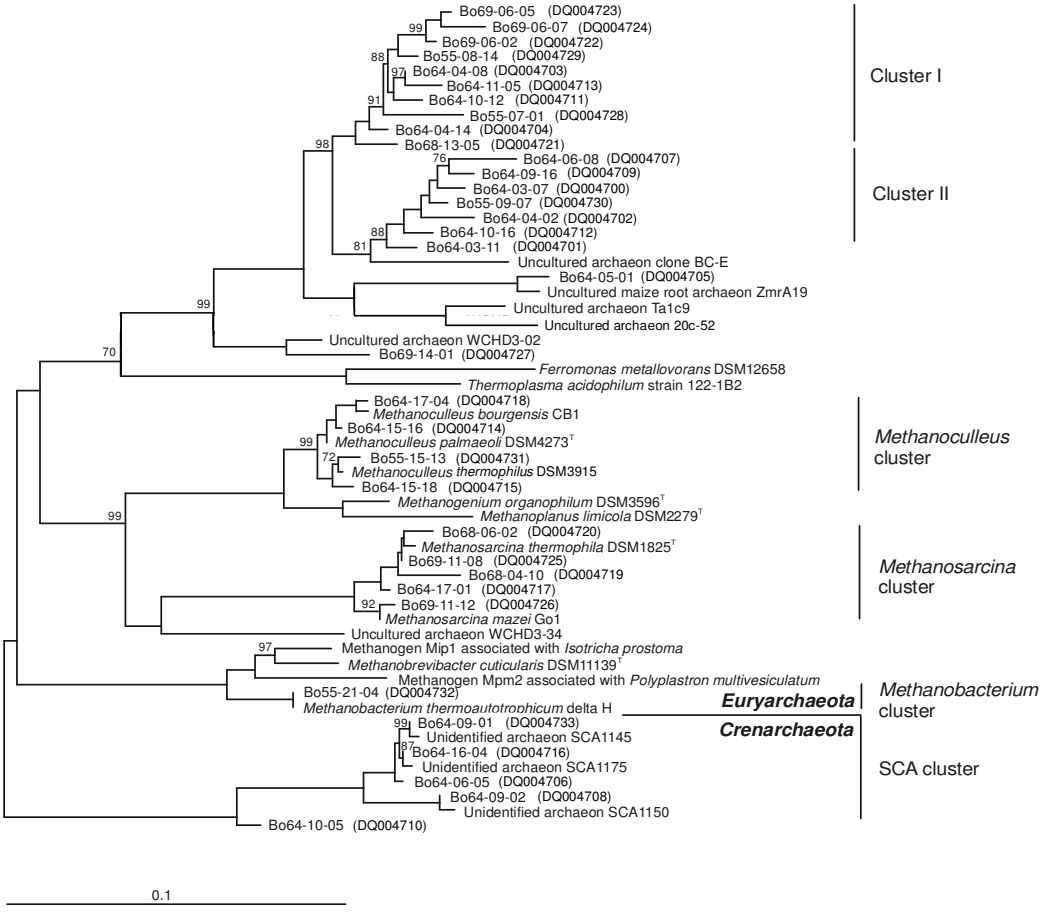

Bo69-06-05 (DQ004723)
Bo69-06-07 (DQ004724)
Bo69-06-02 (DQ004722)
Bo55-08-14 (DQ004729)
Bo64-04-08 (DQ004703)
Bo64-11-05 (DQ004713)
Bo64-10-12 (DQ004711)
Bo55-07-01 (DQ004728)
Bo64-04-14 (DQ004704)
Bo68-13-05 (DQ004721)
Bo64-06-08 (DQ004707)
Bo64-09-16 (DQ004709)
Bo64-03-07 (DQ004700)
Bo55-09-07 (DQ004730)
Bo64-04-02 (DQ004702)
Bo64-10-16 (DQ004712)
Bo64-03-11 (DQ004701)
Uncultured archaeon clone BC-E
Bo64-05-01 (DQ004705)
Uncultured maize root archaeon ZmrA19
Uncultured archaeon Ta1c9
Uncultured archaeon 20c-52
Uncultured archaeon WCHD3-02
Bo69-14-01 (DQ004727)
Ferromonas metallovorans DSM12658
Thermoplasma acidophilum strain 122-1B2
Bo64-17-04 (DQ004718)
Methanoculleus bourgensis CB1
Bo64-15-16 (DQ004714)
Methanoculleus palmaeoli DSM4273T
Bo55-15-13 (DQ004731)
Methanoculleus thermophilus DSM3915
Bo64-15-18 (DQ004715)
Methanogenium organophilum DSM3596T
Methanoplanus limicola DSM2279T
Bo68-06-02 (DQ004720)
Methanosarcina thermophila DSM1825T
Bo69-11-08 (DQ004725)
Bo68-04-10 (DQ004719
Bo64-17-01 (DQ004717)
Bo69-11-12 (DQ004726)
Methanosarcina mazei Go1
Uncultured archaeon WCHD3-34
Methanogen Mip1 associated with Isotricha prostoma
Methanobrevibacter cuticularis DSM11139T
Methanogen Mpm2 associated with Polyplastron multivesiculatum
Bo55-21-04 (DQ004732)
Methanobacterium thermoautotrophicum delta H
Bo64-09-01 (DQ004733)
Unidentified archaeon SCA1145
Bo64-16-04 (DQ004716)
Unidentified archaeon SCA1175
Bo64-06-05 (DQ004706)
Bo64-09-02 (DQ004708)
Unidentified archaeon SCA1150
Bo64-10-05 (DQ004710)
Cluster I
Cluster II
Methanoculleus cluster
Methanosarcina cluster
Euryarchaeota
Methanobacterium cluster
Crenarchaeota
SCA cluster
0.1

Fig. 4

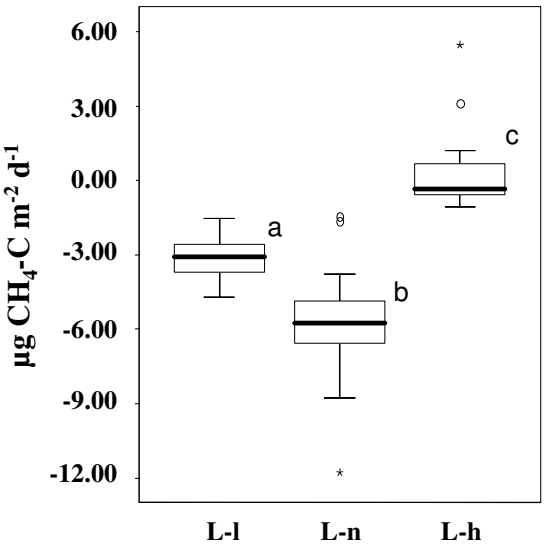


Fig. 5

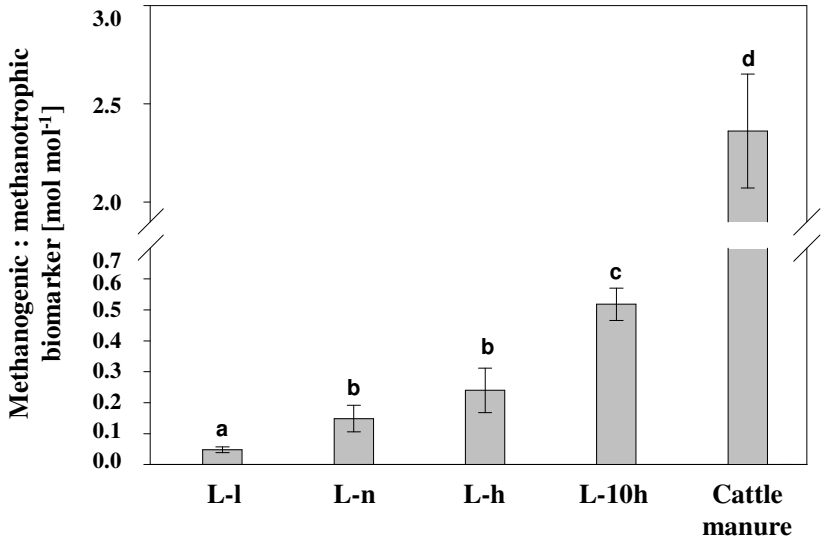

Methanogenic : methanotrophic biomarker [mol mol-1]
3.0
2.5
2.0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
c
d
L-l
L-n
L-h
L-10h
Cattle manure

Fig. 6

Supplementary data

Table 1. 16S rRNA or 16S rRNA gene sequence (indicated by an asterisk*) designation and origin, nucleotide accession number, sequence length as well as closest phylogenetic neighbour of investigated soil sequences.

Sequence designation	Sequence origin	Nucleotide Accession number	Closest phylogenetic neighbour (Accession number in parentheses)	Sequence similarity [%][1]
Bo64-03-07	L-l 0-15	DQ004700	Uncultured archaeon WCHD3-02 (AF050616)	83
Bo64-03-11	L-l 0-15	DQ004701	Uncultured archaeon WCHD3-02 (AF050616)	85
Bo64-04-02	L-l 15-30	DQ004702	Uncultured archaeon TA1f2 (AF134390)	87
Bo64-04-08	L-l 15-30	DQ004703	Uncultured archaeon WCHD3-02 (AF050616)	85
Bo64-04-14	L-l 15-30	DQ004704	Uncultured archaeon WCHD3-02 (AF050616)	85
Bo64-05-01	L-l 30-40	DQ004705	Uncultured maize root archaeon ZmrA19 (AF226268)	97
Bo64-06-05	L-n 0-15	DQ004706	Unidentified archaeon SCA1150 (U62812)	96
Bo64-06-08	L-n 0-15	DQ004707	Uncultured archaeon 20c-52 (AJ299201)	89
Bo64-09-02	L-h 0-15	DQ004708	Unidentified archaeon SCA1150 (U62812)	99
Bo64-09-16	L-h 0-15	DQ004709	Uncultured archaeon TA1c9 (AF134388)	88
Bo64-10-05	L-h 15-30	DQ004710	Unidentified archaeon SCA1175 (U62819)	93
Bo64-10-12	L-h 15-30	DQ004711	Uncultured archaeon 33-1 (AF424770)	85
Bo64-10-16	L-h 15-30	DQ004712	Uncultured archaeon WCHD3-02 (AF050616)	83
Bo64-11-05	L-h 30-40	DQ004713	Uncultured archaeon 33-1 (AF424770)	86
Bo64-15-16	L-10h 15-30	DQ004714	*Methanoculleus thermophilum* (M59129)	97

Bo64-15-18	L-10h 15-30	DQ004715	*Methanoculleus palmaeoli* (Y16382)	96
Bo64-16-04	L-10h 30-40	DQ004716	Unidentified archaeon SCA1175 (U62819)	98
Bo64-17-01	Cattle manure	DQ004717	*Methanosarcina* sp. FR (AF020341)	99
Bo64-17-04	Cattle manure	DQ004718	*Methanoculleus oldenburgensis* (AB065298)	99
Bo68-04-10	L-l 0-15	DQ004719	*Methanosarcina thermophila* (M59140)	98
Bo68-06-02	L-l 15-30	DQ004720	*Methanosarcina thermophila* (M59140)	97
Bo68-13-05*	L-n 15-30	DQ004721	Uncultured archaeon WCHD3-16 (AF418940)	84
Bo69-06-02	L-h 15-30	DQ004722	Uncultured archaeon 63-A23 (AJ305083)	87
Bo69-06-05	L-h 15-30	DQ004723	Uncultured archaeon WCHD3-02 (AF050616)	85
Bo69-06-07	L-h 15-30	DQ004724	Uncultured archaeon 63-A23 (AJ305083)	84
Bo69-11-08*	L-10h 0-15	DQ004725	*Methanosarcina thermophila* (M59140)	97
Bo69-11-12*	L-10h 0-15	DQ004726	*Methanosarcina mazei* (AJ012095)	99
Bo69-14-01	L-10h 15-30	DQ004727	Uncultured archaeon 61D (AF268643)	97
Bo55-07-01*	L-l 15-30	DQ004728	Uncultured maize root archaeon ZmrA19 (AF226268)	86
Bo55-08-14*	L-l 30-40	DQ004729	Uncultured archaeon 63-A23 (AJ305083)	86
Bo55-09-07*	L-n 15-30	DQ004730	Uncultured archaeon 20c-52 (AJ299201)	86
Bo55-15-13*	L-h 15-30	DQ004731	*Methanoculleus thermophilum* (M59129)	99
Bo55-21-04*	Cattle manure	DQ004732	*Methanobacterium thermoautotrophicum* (AB020530)	97
Bo64-09-01	L-h 0-15	DQ004733	Unidentified archaeon SCA1145 (U62811)	99

[1]Determined by the program BLAST