Dung application increases CH_4 production potential and alters the composition and abundance of methanogen community in restored peatland soils from Europe

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Abstract

Peatland restoration via rewetting aims to recover biological communities and biogeochemical processes typical to pristine peatlands. While rewetting promotes recovery of C accumulation favorable for climate mitigation, it also promotes methane (CH_4) emissions. The potential for exceptionally high emissions after rewetting has been measured for Central European peatland sites previously grazed by cattle. We addressed the hypothesis that these exceptionally high CH_4 emissions result from the previous land use. We analyzed the effects of cattle dung application to peat soils in a short- (2 weeks), a medium- (1 year) and a long-term (grazing) approach. We measured the CH_4 production potentials, determined the numbers of methanogens by *mcrA* qPCR and analyzed the methanogen community by *mcrA* T-RFLP-cloning-sequencing. Dung application significantly increased the CH_4 production potential in the short- and the medium-term approach and non-significantly at the cattle-grazed site. The number of methanogens correlated with the CH_4 production in the short- and the long-term approach. At all three time horizons, we found a shift in methanogen community due to dung application and a transfer of rumen methanogen sequences (*Methanobrevibacter* spp.) to the peatland soil that seemed related to increased CH_4 production potential. Our findings indicate that cattle grazing of drained peatlands changes their methanogenic microbial community, may introduce rumen-associated methanogens and leads to increased CH_4 production. Consequently, rewetting of previously cattle grazed peatlands has the potential to lead to increased CH_4 emissions. Careful consideration of land use history is crucial for successful climate mitigation with peatland rewetting.

Keywords

climate mitigation; rewetting; methane; cattle grazing; methanogen; land use

Introduction

Peatlands are wetland ecosystems characterized by water saturated soil and thereby accumulation of organic matter as peat due to incomplete decomposition. In their natural state, peatlands are a sink of atmospheric carbon dioxide (CO_2) and a source of 20 – 30 % of global annual methane (CH_4) emissions (Gorham 1991; Turunen et al. 2002; Lafleur et al. 2003; Nilsson et al. 2008). In addition to global C dynamics, peatlands play a key role in maintaining high biodiversity at all scales from local to global (Parish et al. 2008).

Many peatlands have been drained for the utilization as agriculture and forestry. Drainage changes a peatland ecosystem dramatically and disrupts its ecological functions. Consequences include altered vegetation and microbial communities (Laine et al. 1995; Jaatinen et al. 2007), loss of C through increased decomposition of peat and decreased emissions of CH_4 (Jaatinen et al. 2008; Mäkiranta et al. 2009; Yrjälä et al. 2011). Pasture on drained peatlands adds further disruptions, namely the input of nutrients with urine and dung (Haynes and Williams 1993) and compaction of the peat with trampling (Hamza and Anderson 2005). Fresh dung pats have been shown to turn a boreal sward from a weak sink to a small source of CH_4 (Maljanen et al. 2012), and reindeer droppings have been shown to increase peat CH_4 production potential (Laiho et al. 2017). Rumen methanogens can be introduced into soil via cattle feces and detected in grazed soils (Gattinger et al. 2007). After enteric fermentation (32 – 40% of total agriculture emissions), manure deposited on pasture is the second largest CH_4 emitting category (15% of total) with cattle contributing the largest share (Smith et al. 2014). Indeed, agricultural emissions represent the greatest source of CH_4 in the EU with 10.2 million tons per year. Of these, approximately one-third comes from livestock manure (Moss et al. 2000).

The ecological restoration of drained peatlands aims to recover communities and hydrological and biogeochemical processes typical to pristine peatlands (Nellemann and Corcoran 2010). In Europe, large areas of drained peatlands have already been restored (Aapala et al. 2008; Joosten and Tanneberger 2017) for climate mitigation (Pfadenhauer and Grootjans 1999). Here, rewetting, i.e., raising the water table to re-establish saturated conditions, decreases the loss of C and leads to recovery of the CO_2 sink function (Komulainen et al. 1999; Tuittila et al. 1999; Wilson et al. 2007;

74 Waddington et al. 2010; Wilson et al. 2016). Concomitantly peatland rewetting increases the
75 emissions of CH_4 (e.g., Waddington and Day 2007) - a 28-times stronger GHG than CO_2 (Myhre et al.
76 2013).

77 In northern peatlands, however, several studies have shown lower CH_4 emissions from
78 restored than from pristine sites (Komulainen et al. 1998; Tuittila et al. 2000; Vasander et al. 2003;
79 Marinier 2004; Jauhiainen et al. 2008; Juottonen et al. 2012). There is indication that after restoration
80 CH_4 emissions might be limited by the presence of methanogenic microbes. Juottonen et al. (2012)
81 linked the low emissions from successfully restored, forested peatlands after rewetting to low
82 methanogen density and a changed community composition. The recovery of CH_4 turnover can take
83 over 50 years (Putkinen et al. 2018).

84 In the context of these findings the question arises whether the potential for very high CH_4
85 emissions measured in some restored peatland sites in Central Europe (Hendriks et al. 2007; Augustin
86 and Chojnicki 2008; Freibauer 2008) is fueled by their previous agricultural use, mainly cattle grazing.
87 It is possible that the increased CH_4 emissions are at least partly due to the earlier transfer of rumen
88 methanogens via the dung of grazing cattle or just due to the dung fertilization effect. If the hypothesis
89 holds, high CH_4 emissions from rewetted peatlands could be avoided by out-selection of sites for
90 restoration with grazing history.

91 In this study the effects of dung application (DA) on the methanogenic potential and
92 community were analyzed in pristine and restored peatland soils on three time horizons with differing
93 control of the experimental conditions: The short-term effect of DA (maximum two weeks) was
94 examined by artificial DA to peat soil under laboratory conditions. The medium-term effect (1 year)
95 was assessed by dung-transplantation in a field experiment and for the long-term effect a restored
96 peatland area influenced by cattle grazing was investigated. With this approach we aimed to provide
97 answers to the following questions regarding DA to restored peat soils: (a) Does DA increase the CH_4
98 production potential of restored peat soils?, (b) Does DA increase the number of methanogens in
99 restored peat soils?, (c) Does DA change the methanogen community composition in restored peat
100 soils?, (d) Can rumen-associated methanogens be transferred to restored peat soils via dung?, (e) How
101 persistent are the changes due to DA in restored peat soils?

Materials and Methods

Peat samples

Peat samples originated from peatland sites in Finland and Germany (**Table 1**). For the estimation of short-term effects (2 weeks) of dung application (DA) untreated peat samples from all sites were used. For medium-term DA (1 year) a field experiment was conducted in Finland, and for the long-term effect (approx. 20 years) a restored, grazed peatland site in Germany was sampled.

Sampling sites

The effect of medium-term DA was assessed at four peatland sites in southern Finland in vicinity of the Helsinki University field station Hyytiälä (61° 85' N, 24° 29' E) (**Table 1**). The long term annual mean temperature in that region is 3.5 °C and mean annual precipitation is approx. 700 mm (Tuittila et al. 2000 and references therein). Three of the sites had been rewetted after drainage, and we used a pristine peatland (Jokivarsisuo) as a reference site. The vegetation of the rewetted sites was dominated by tussock cottongrass (*Eriopohorum vaginatum* L.) and fine bogmoss (*Sphagnum angustifolium* (Russow) C.E.O. Jensen). At the pristine site a mosaic of *E. vaginatum*, bottle sedge (*Carex rostrata* Stokes) and baltic bogmoss (*Sphagnum balticum* (Russow) C.E.O. Jensen) occurred. None of the sites has ever been grazed by cattle. In May 2013, dung was transplanted and mixed with the peat at three plots at each of the four sites and incubated there for one year (for details see DA treatments).

The site under long-term impact of DA was located in a fen area of the northern German lowlands and belongs to the research station Paulinenaue of the Leibniz Centre for Agricultural Landscape Research (**Table 1**). The long term annual mean temperature is 8.9°C and the mean annual precipitation is 552 mm (Climate-Data). Due to the degradation of the drainage system, unscheduled rewetting took place causing flooding during each winter since 2007 (Drösler et al. 2013). The vegetation was dominated by reed canarygrass (*Phalaris arundinacea* L.). While one part of the grassland sites has been grazed since the 1990s (i.e., since approx. 20 years before sampling) the other part has never been grazed by cattle.

Peat sampling and sample processing

The four Finnish sites were sampled after one year of in-situ incubation of the transplanted dung on 12th and 13th of May 2014 and the grazed and ungrazed sites in Germany were sampled on 4th of February 2014. The German sites were flooded (water level 2 ± 3cm, n=6) at the time of sampling and the uppermost 10 cm of soil were frozen. At the Finnish sites the water levels at the time of sampling were at 17 ± 3cm (n=18) in Aitoneva, -3 ± 1cm (n=18) in Jokivarsisuo, -11 ± 2cm (n=18) in Konilamminsuo and 7 ± 2cm (n=18) in Vanneskorpi.

The peat samples were taken with a peat corer - a round one in Germany (8 cm diameter, adapted from Buttler et al. 1998) and a box corer in Finland (10 × 10 cm^2). We took three peat cores per treatment from each site. Depending on the degree of soil compaction we sampled the upper 30 cm. At the German sites the frozen uppermost layer (10 cm) was removed before sampling (details in supplementary section Table S.3). We sealed the peat cores instantly after sampling with wrapping film and plastic bags to reduce oxygen exposure. The sealed samples were transported to the laboratory of the Natural Resources Institute Finland, Vantaa, overnight where samples were stored at +4°C until processing.

For processing, the peat cores were cut into 10 cm layers. Each 10-cm layer was vertically cut in half and subsamples for all following analyses were taken from the innermost, oxygen-free part of the section. Subsamples were homogenized. If not used immediately, the processed samples were stored at -20°C.

Peat characteristics

One subsample from each sample was used to determine the peat characteristics of the site at sampling day (**Table 1**). For dry bulk density, 5 ml of fresh peat were dried at 105 °C for 48 h. For loss-on-ignition an average of 1 g of dry peat were incinerated in a muffle furnace at 550 °C for 4 h. The pH values were determined from suspended peat (1:3 (v/v)) (pH-Fix, Macherey-Nagel GmbH & Co KG, Germany).

Dung-application treatments

The effects of DA treatments were measured in terms of methane production potential, abundance of methanogens, community composition of methanogens and taxonomic affiliation of methanogens.

Dung samples originated from three different farms (see below) and were stored at +4 °C in sealed in plastic bags before addition to the peat. Subsamples of the dung were stored at -20°C for methanogen community analysis. N-content in the dung samples was analyzed using Kjeldahl method (Blume et al. 2011). All used dung samples had comparable qualities, namely a dry matter content of 11±1.5%, a 85 ±3.6% content of organic matter and a pH of 7.2±0.4% (n=7). The N-content was 2.7±0.5% (n=4).

Short-term effects of DA

For the short-term effect of DA, a suspension of fresh dung was added to control peat samples from the Finnish sites and from the ungrazed German site (**Table S.3**). A dung-water suspension was mixed with 15 ml of fresh peat and suspended in 30 ml autoclaved purified water in 125 ml glass flasks (dung : peat : water $0.8\overline{3}$: 15 : $31.6\overline{6}$, v/v/v). Moreover, we wanted to estimate the effect of rewetting on the CH_4 production potential of dung-treated peat. For this purpose the CH_4 production was compared between pure peat and peat suspended in water before and after DA; peat samples from the Finnish site Jokivarsisuo (n=18) were used. The dung used in the experiments for short-term DA originated from cattle at Haltiala farm near Helsinki, Finland (60° 16' N, 24° 57' E). The cattle were fed on a mixed diet (straw and concentrated feed once a week) and had the opportunity of grazing. On average the dung`s content of dry matter was 9%, the content of organic matter 80 ± 1%, the N-content 3.1% and the pH 7.1 (n=2).

Additionally, fresh dung was added to peat samples from the cattle grazed grassland site to determine its effect in contrast to the effects of a long-term field exposure (n=5, **Table S.3**). The dung for this experiment was collected from cows grazing the peatland site in Germany (**Table 1**). Besides grazing the cows were fed on straw and concentrated feed .The content of dry matter of the dung was 11%, the content of organic matter 86 ± 1% and the pH 7.6 ± 1.5% (n=3). The N content was not determined.

Medium-term effects of DA

In May 2013, dung was transplanted to three plots at each of the four sites in Finland and incubated there for one year. The vegetation cover was removed aside, together with peat to a depth of 35 cm. Two buckets of cow dung (approx. 20 l in total) were then spread into the resulting hole (0.5 × 0.5 × 0.35 m^3, i.e., 87.5 l) with a mixing ratio of 1:4.4 (dung:peat (v/v)). Afterwards, peat and vegetation were placed back to cover the hole. The dung originated from the farm Kaupintila, Simuna, Hämeenkyrö, Finland (61° 36' N, 23° 14' E) from cows fed on silage, grains (oat and barley) and crushed grains of field mustard. On average the dung`s content of dry matter was 13%, the content of organic matter 89%, the N-content 2.3% and the pH-value 7.0 (n=2). The properties of this dung were not determined from the fresh dung spread in 2013. Instead, they were measured from dung lumps in peat samples 2014 in which the dung did not successfully mix with the peat.

CH_4 production potentials

The CH_4 production potentials of all samples were measured by anoxic incubation experiments according to Juottonen et al. (2008). Glass flasks (125 ml) were filled with 30 ml deionised water, then autoclaved, flushed with N_2 for 2 min and closed airtight. To start the incubation 15 ml of peat were added to the flasks, flushed with N_2 to remove oxygen, and closed airtight with a rubber stopper. To allow the methanogens from the dung to adapt to the new substrate prior to the measurements, samples were stored at 4°C in the dark for 5 days. Thereafter the flasks were again flushed with N_2 and incubated at 16°C in the dark. The CH_4 concentration in the headspace was measured with a gas chromatograph (Hewlett Packard, G1530A, USA) at five times during the incubation period of 6 to 15 days depending on the rate of accumulation of the respective experiment. To obtain rates of the CH_4 production potential (nmol g^{-1} dw h^{-1}) linear regression of the CH_4 concentrations over the measurement time was applied.

Community of methanogenic archaea

Extraction of DNA

DNA was extracted from freeze dried (-50°C for 48-60 h) samples of dung (50 mg), control peat or dung treated peat (100 mg). The NucleoSpin®Soil kit (Macherey-Nagel GmbH & Co KG, Germany) was used for isolation of genomic DNA according to manufacturer's instructions with the following modification: Lysis buffer SL1 was used without Enhancer SX. Success of the DNA extraction was checked by endpoint PCR of bacterial 16S rDNA according to Harms et al. (2003). The extracted DNA was stored in elution buffer at -20°C until analyses.

Quantification of methanogenic archaea

Quantitative PCR (qPCR) was performed to investigate the abundance of methanogenic archaea, total bacteria and total archaea. Amplifications were carried out in duplicate on the Rotor-Gene 6000 PCR system (Corbett Research, Australia) by using SYBR green as the detection system in a reaction mixture of 20 µl containing 1× Maxima SYBR Green qPCR Master reaction mixture (Fermentas, USA), 8.4 µl nuclease-free water (Fermentas, USA), 375 nM primers, and 1 µl DNA template. The DNA template was diluted as inhibitory compounds may be present in environmental samples (Bessetti 2007; Hargreaves et al. 2013) (**Supplement, S1.1**).

The gene for methyl coenzyme M reductase subunit A (*mcrA*) was used for quantitative analysis of methanogenic archaea. We used the primer pair mlas/mcrA-rev (Steinberg and Regan. 2008) with an amplicon length of ca. 465-490 bp (Luton et al. 2002). For total bacteria we targeted bacterial 16S rRNA genes (b16S) with the primer pair 1055F/1392R (Harms et al. 2003). The amplicon length was expected to be 337-352 bp (Harms et al. 2003; Toes et al. 2008). For total archaea we targeted archaeal 16S rRNA genes (a16S) with the primer pair Arch967f/Arch1060r (Cadillo-Quiroz et al. 2006 and references therein). The thermal cycling conditions for *mcrA* were: initial denaturation 10 min at 95°C, followed by 45 cycles of 30 sec at 95°C, 45 sec at 55°C, 30 sec at 72°C, and the final extension 7 min at 72°C. The thermal cycling conditions targeting b16S were: initial denaturation 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 50°C, 30 sec at 72°C. The thermal cycling conditions for a16S were: initial denaturation 10 min at 95°C, followed by

40 cycles of 15 sec at 95°C, 30 sec at 55°C, 20 sec at 72°C. Fluorescence was measured after each extension step. A melting curve analysis was performed for quality verification of the PCR products (*mcrA* from 72 to 99°C, b16S from 60 to 95°C, a16S from 72 to 95°C). Standard curves were obtained with serial dilutions (10^1–10^9 gene copies per reaction) of recombinant plasmids containing a fragment of the *mcrA*, bacterial or archaeal 16S gene targets, respectively. The gene copies μl^{-1} in the samples were calculated for each target using linear regression parameters fit to a plot of cycle threshold (C_T) versus log of the concentration of gene copies for the standards runs. The effectiveness of the qPCR reactions as well as the limits of detection and quantification are given in the supplementary material (**section S1.2**).

Community composition of methanogenic archaea (PCR- T-RFLP analyses)

A terminal restriction fragment length polymorphism (T-RFLP) approach was used to detect whether methanogenic archaea from the cattle rumen were transferred to the peat. For the effect of long-term field exposure on the community of rumen methanogens, the samples from the cattle-grazed and from the ungrazed site (n(C)=6, n(DA)=5) were analyzed. For the short-term effects of DA in contrast to long-term exposure the samples from the cattle grazed site before and after the addition of fresh dung under laboratory conditions (n(C)= n(DA)=5) were used. Thus, the short- and the long-term approach shared the same control samples in the T-RFLP analyses. For the medium-term effects we selected samples from the dung-transplantation experiment in Finland (n(C)= n(DA)=12). Here, from each DA sample the T-RF pattern of the 10cm-section (i.e., 0-10cm, 10-20cm, or 20-30cm) with the highest CH_4 production potential was determined together with the corresponding 10-cm section from the control sample. The dung samples that were used for DA were included as a reference.

The methyl coenzyme M reductase gene (*mcrA*) fragments were amplified with the primers mlas and mcrA-rev (Steinberg and Regan 2008). The 50 µl PCR reactions contained 0.5 µM of each primer, 200 µM of dNTPs and 2.5 U of DNA polymerase (DreamTaq, Thermo Fisher Scientific, USA) in 1 × reaction buffer and 1 µl of template DNA. We used a hot start version of the cycling conditions described in Steinberg and Regan (2008). The products were analyzed by T-RFLP with restriction enzymes *Hha*I and *Mbo*I as in Juottonen et al. (2015). In the analyses we included fragments from 79-

495 bp length. Terminal restriction fragment (T-RF) peaks with <200 relative fluorescence units (background noise) and peaks with <2% of the total peak area were excluded. Results are presented based on relative peak area.

Cloning, DNA sequencing and phylogenetic analysis for identification of methanogenic archaea

From the medium-term approach, clone libraries from selected *mcrA* PCR products were constructed to identify methanogenic archaea that might have been transferred from the transplanted dung to the peat soils in Finland. We selected two DA sites that showed T-RFs also found in dung samples. Two replicate samples were pooled into each library. Corresponding libraries were constructed for control samples of the same sites. In addition, one library was constructed for a third DA site (one sample only) and for dung that was used for DA.

An endpoint PCR targeting *mcrA* using HiFi-PCR reaction mix (Fermentas, USA) and the primers mlas and mcrA-rev of Steinberg and Regan (2008) was conducted. Apart from the use of the HiFi-PCR reaction mix (Fermentas, USA) the PCR reaction composition and the cycling conditions were the same as described for the T-RFLP analyses. The PCR products were purified with the GeneJET™ Gel Extraction Kit (Fermentas, USA), ligated into Topo-TA vector (Invitrogen, USA) and transformed into *Escherichia coli* competent cells (Invitrogen, USA). Depending on the number of T-RFs, inserts from 20-40 blue–white-screened clones from each library were amplified with primers M13f and M13r. Inserts from two clone colonies per library were reamplified with *mcrA* primers to check for the correct insert. The M13-PCR-products were purified (GeneJET PCR Purification kit, Fermentas, USA). In total 177 clones were sequenced with vector primer M13F (Macrogen, South Korea).

The *mcrA* sequences (469-493 bp) were compared to database sequences by BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast). Potential chimeric sequences were identified with Uchime in mothur (v. 1.33, Schloss et al. 2009) and removed. Deduced *mcrA* amino acid sequences were aligned with Clustal Omega (Sievers et al. 2011, http://www.ebi.ac.uk/Tools/msa/clustalo/). Evolutionary models were selected with ProtTest (Abascal et al. 2005,

287 http://darwin.uvigo.es/software/prottest2_server.html), and a maximum likelihood tree was
288 constructed of 129 aligned amino acid positions with PhyML (Guindon et al. 2010) with model
289 LG+I+G+F. Bootstrap values were generated from 100 replicates in PhyML. Nucleotide sequences
290 have been deposited in the EBML database under the accession numbers LT632436-LT632531.

291 Finally, we aimed to identify the T-RFs based on *in silico* digestion of the sequences with the
292 enzymes *Hha*I and *Mbo*I (http://www.nrbsc.org/gfx/genedoc/) and by analyzing a selection of clones
293 by T-RFLP. In addition, previous sequence data from Finnish peatlands was used as an additional
294 guide for identification (**Supplement Table S2**; Peltoniemi et al. 2016).

295

296 ## Statistical evaluation

297 One-sided (pairwise) Wilcoxon tests were used-to check for the effects of DA on the CH_4 production
298 potentials and on the numbers of *mcrA*, b16S and a16S copies per gram dry weight (gDW^{-1}) of peat.
299 Additionally, the one-sided (pairwise) Wilcoxon test was applied to test the effect of suspending peat
300 in water on CH_4 production potentials. Correlations between the CH_4 production potentials and the
301 *mcrA* copy numbers were assessed by linear or polynomial regression. The distribution of the data for
302 the CH_4 potentials was not suitable to set up linear models. The level of significance was set to $\alpha =$
303 0.05. All statistical analyses were conducted using R version 3.2.2 (R Core Team 2015).

304 We used detrended correspondence analysis (DCA) to explore the variation in the T-RF
305 patterns and assess the main gradients and their length in the methanogen communities found in dung,
306 DA and control samples. Canonical correlation analysis (CCA) was used to assess how much of the
307 compositional variation in the T-RF patterns was explained by the three treatments (dung, DA,
308 control). The T-RF patterns were analyzed using continuous data of peak areas with the program
309 package Canoco ver. 5.0 (TerBraak and Smilauer 2012). Results based on a binary matrix (presence or
310 absence of a certain T-RF) are given in the supplementary material (**section S2.3**).

Results

Dung application and CH_4 production potentials

CH_4 production of peat soils after DA

Dung addition significantly increased the CH_4 production potentials in the short- and in the medium-term approach - on average by the factor 8 and 19, respectively (**Fig. 1a, b).** Likewise, the mean CH_4 production potential of the cattle-grazed site was 6-times higher than that of the ungrazed site but the effect was not significant (**Fig. 1c**). In contrast to the long-term field exposure, however, the addition of fresh dung to peat from the grazed site significantly increased the CH_4 production potential (n=5, p=0.0313).The increased CH_4 production was observed at all samples depths.

The levels of increased CH_4 production potential with dung addition greatly differed between the examined time-horizons. While the dung-treated samples from the short- and medium-term approach produced at maximum 4 – 15 mmol gdw^{-1} h^{-1} CH_4, the production from the cattle grazed grassland site was as low as 0.06 – 0.3 mmol gdw^{-1} h^{-1} CH_4 (**Fig. 1**). The highest CH_4 production potentials were measured in the medium-term approach. We want to note that three out of the four top values (> 10 mmol gdw^{-1} h^{-1} CH_4, **Fig. 1b**) were produced in samples where dung and peat were not mixed perfectly homogeneously and visible dung lumps were found when sampled after one year of field exposure.

The role of rewetting

The CH_4 production was significantly higher when water was added to peat samples from site Jokivarsisuo than in field fresh peat (n=9; one-sided paired Wilcoxon test, p= 0.0020). In the field fresh peat the addition of dung increased the CH_4 production potential from zero to 1.7±1.8 mmol gdw^{-1} h^{-1} CH_4 (n=9; one-sided paired Wilcoxon test; p=0.0071). In suspension the peat produced 0.9±1.3 mmol gdw^{-1} h^{-1} CH_4 before and 6.4±5.6 mmol gdw^{-1} h^{-1} CH_4 after DA (n=9; one-sided paired Wilcoxon test; p=0.0020).

Dung application and abundance of methanogenic archaea

Dung application increased the abundances of methanogenic archaea, total bacteria and total archaea (copy numbers of *mcrA*, b16S and a16S) in the short-term approach (n=10, one sided paired Wilcoxon tests, p(mcrA)=p(b16S)=p(a16S) <0.0001). These numbers were also higher at the cattle-grazed compared to the ungrazed site (n(C)=6, n(DA)=5, one-sided Wilcoxon test, p(mcrA)=0.0476, p(b16S)= 0.0022, p(a16S)= 0.0260). In the field experiment of the medium-term approach, however, the copy numbers did not differ between control and DA-treated peat (n(C)=103, N(DA)=104, p>0.2).

The number of *mcrA* copies correlated positively with the CH_4 production potentials in the short-term approach (**Fig. 2a**). The best fit of the correlation was found after the addition of fresh dung to peat from the cattle grazed site (black, solid squares in **Fig. 2a**, n=5, polynomial regression, p=0.0042, R^2=0.9544). In the samples from the grassland sites themselves, a positive correlation was found at the cattle-grazed site, but not at the ungrazed (control) site (**Fig. 2c**). In contrast, the CH_4 production potentials from the field experiment did not correlate with copy numbers of *mcrA* (**Fig. 2b**).

Dung application and methanogen community composition

Community change of methanogenic archaea

On all three time-horizons the main variation in the *mcrA* T-RF patterns was related to the three treatments (dung, DA, control; **Fig. 3 a-c**, first DCA axis). Methanogen communities of dung and control peat formed the opposite ends of the compositional gradient and DA was located in between but closer to the control (**Fig. 3 a-c**). In the medium-term approach (**Fig. 3b**) DA samples were closer to the control than in the short- and long-term approach. Based on CCA, the three treatments significantly explained community variation (short-term: pseudo-F=2.0, p=0.006; medium-term: pseudo-F=1.7, p=0.026; long-term: pseudo-F=3.3, p=0.002) (**Supplement, Fig. S1**). The three treatments together explained 14.9% of the compositional variation in the short-, 5.5% in the medium-

362 and 26.3% in the long-term approach. In total, we found 27 different T-RFs, and the lowest number of
363 T-RFs per sample (two to five) was consistently found in the dung (**Fig. 3**).

364 The shift in the composition of the community was accompanied by an increase in both the
365 number of *mcrA* copies and the CH_4 production potentials in the short- and long-term approach (**Fig.**
366 **3a, c**). In the medium-term approach, only the CH_4 production potentials increased (**Fig. 3b**).

367

368 **Dung application and transfer of methanogens**

369 The control peat samples from Finland used in the medium-term approach included mainly
370 methanogens from Methanoregulaceae (T-RFs 406 bp and 473 bp), Methanocellales,
371 Methanomassilliicoccales and some Methanosarcinaceae (T-RF 220 bp) (**Fig. 4, Fig. S1**).
372 Additionally, a member of Methanoregulaceae (T-RF 214 bp) was present in ¾ of all control samples
373 with up to 89% of the total peak area. At the ungrazed control site in Germany the peat was dominated
374 by the T-RFs 86 bp (unidentified), 232 bp (Methanosarcinaceae) and 492 bp (Methanomicrobiaceae).

375 Compared to the control peat we found a reduced diversity of T-RFs in the dung samples (23
376 T-RFs vs. 7 T-RFs). In all dung samples, the T-RFs 106 bp and 214 bp were dominant (**Fig. 3a-c**),
377 accounting for 24±9% and 70±10% of the total peak area, respectively (n=5). Both T-RFs were
378 identified as *Methanobrevibacter* sp. in the dung samples. Accordingly, the methanogens in the dung
379 used for DA in the medium-term approach mainly stemmed from the genus *Methanobrevibacter* (70%
380 of *mcrA*-sequences) followed by *Methanosarcina* (13%), *Methanocorpusculum* (13%) and
381 *Methanoregula* (4%) (**Fig. 4**, samples DFs1-DFs30).

382 The T-RF 106 bp assigned to the genus *Methanobrevibacter* was found exclusively in dung
383 and in dung-treated peat indicating a transfer of this methanogen from dung to peat soil. It was present
384 in at least one DA sample from each site of the medium- and in one DA sample from the short-term
385 approach. Likewise, the detection of T-RF 371 bp (unidentified) only in dung and in DA peat samples
386 of the short-term approach, and the T-RF 237 bp (unidentified) only in dung and at the grazed site
387 points to a transfer between dung and peat as well. However, T-RF 237 bp also occurred in one control
388 sample of the medium-term approach. The T-RF 214 bp occurred in both dung-treated and control

389 peat, but it apparently represented two very close T-RFs that we could not differentiate:
390 *Methanobrevibacter* from dung and Methanoregulaceae from peat. All clone sequences from dung and
391 dung-treated peat with this T-RF were identified as the known rumen methanogen
392 *Methanobrevibacter* (Janssen and Kirs 2008). No *Methanobrevibacter* sequences were detected in
393 control peat, but Methanoregulaceae with a 1-bp T-RF length difference has earlier been detected at
394 one of our sites (Konilamminsuo; Juottonen et al.2012). As much as 60% of the sequences from the
395 DA samples of the medium-term approach belonged to *Methanobrevibacter, Methanosarcina* or
396 *Methanocorpusculum* sequence types that occurred only in dung and dung-treated peat but not in any
397 control sample (**Fig. 4**, samples Ad, Jd, Kd). Generally, the T-RFs 214 bp
398 (*Methanobrevibacter*/Methanoregulaceae), 220 bp and 232 bp (Methanosarcinaceae), 106 bp
399 (*Methanobrevibacter*) and 101 bp (Methanocorpusculaceae) were dominant in DA samples of the
400 medium-term approach. The DA samples in the short- and long-term approach were dominated by T-
401 RF 395 bp (unidentified) and 492 bp (Methanomicrobiaceae) as well as by T-RF 232 bp
402 (Methanosarcinaceae). Additionally, T-RF 101 bp (Methanocorpusculaceae) and 141 bp
403 (Methanobacteriaceae) occurred at the grazed site, only.

404 A summary of the transfer between dung and peat as well as the taxonomic affiliation of the
405 individual T-RFs is given in the supplementary material (**Fig. S1, Table S2**).

406

Discussion

The addition of cow dung (DA) increased the CH_4 production potential of soil samples from restored peatlands at all three time horizons of our study. This supported our hypothesis that dung is likely to play a role in the exceptionally high CH_4 emissions from rewetted peatlands with grazing history. We found indication that the increased methanogenic potential is linked to changes in the composition of the microbial community.

After DA we found higher numbers of methanogenic archaea and total bacteria and archaea in the laboratory experiment and at the cattle-grazed grassland, similarly to the increased microbial biomass found in a severely cattle impacted pasture in the Czech Republic (Elhottova et al. 2012). Generally, in our study the abundance of methanogens was positively correlated with CH_4 production potential with the exception of the field experiment. Both patterns have been found earlier. Positive correlation has been found between the abundance of *mcrA* gene copies or the *mcrA* transcript/gene ratio and CH_4 production rates (Morris et al. 2014, 2016; Freitag and Prosser 2009; Putkinen et al. 2018). No relationship in the field experiment agrees with findings from a peat rewetting laboratory experiment (Urbanová et al. 2011) and a cattle rumen and emission study (Carberry et al. 2014a). The production of CH_4 has been reported to correlate with methanogenic and bacterial communities in the rumen of dairy cows (Danielsson et al. 2017). It might be that in soil a direct relation between the total number of methanogens and CH_4 production may not be observed because a large part of the methanogen community can be present in an inactive state (Yavitt et al. 2005; Basiliko et al. 2007) as suggested by Urbanová et al. (2011).

In addition to the higher numbers of methanogens the DA led to a change of the composition of the methanogen community towards that of the applied dung although sites differed heavily from each other regarding land-use management and soil properties. The majority of methanogens in the dung-treated peat belonged to the genera *Methanobrevibacter* (Methanobacteriales), *Methanosarcina* (Methanosarcinales), and *Methanocorpusculum* (Methanomicrobiales). These *mcrA* sequence types were not detected in control peat and were identical or highly similar to sequences from the dung, and represented methanogens known to occur in cattle rumen (e.g., Shin et al. 2004; Wright et al. 2007;

434 Janssen and Kirs 2008; Sirohi et al. 2010; Carberry et al. 2014b). This suggests that rumen-associated
435 methanogens were transferred to the peat with the dung. Similarly, in other environments cattle
436 manure has been found to serve as inoculum for the establishment of a new soil microbial community
437 derived from cattle intestine, including *Methanoculleus* and *Methanosarcina* species (Radl et al. 2007;
438 Gattinger et al. 2007; Elhottova et al. 2012).

439 Although our results show that a transfer of methanogen species from rumen to rewetted peat
440 is possible under certain conditions, our study did not address how persistent the rumen methanogens
441 are in the restored peat soils. Generally, methanogenic archaea grow in nearly every anaerobic
442 environment with a temperature range between 5 and 110°C and pH-values from 3 up to 9.2 (Ferry
443 2012 and references therein). Rumen methanogens, however, prosper in a narrow niche with a
444 temperature optimum between 37 and 45°C and neutral pH-values (5.9-7.7) (Sirohi et al. 2010). In
445 addition, *Methanobrevibacter ruminantium*, a species isolated from rumen, requires co-enzyme M for
446 the growth (Taylor et al. 1974). Nevertheless, there are methanogens such as *Methanobacterium*
447 *formicicum* and Methanomassiliicoccales that occur in both marshy soils and cattle rumen (Sirohi et
448 al. 2010, Söllinger et al. 2015). Thus, these methanogens could survive and grow in the peat soils of
449 the temperate climate zone despite they are adapted to the cattle rumen. Growth will most likely be
450 very slow so that they cause only weak or short-term effects in the soil ecosystem. That kind of short-
451 term impact of DA might explain the rapid increase of CH_4 emissions after cattle slurry addition
452 (Flessa and Beese 2000) and the relatively rapid decrease of emissions (months to years) after
453 stopping cattle impact (Radl et al. 2007; Prem et al. 2014). Furthermore, we found only few potentially
454 transferred rumen methanogen T-RFs at our cattle grazed grassland site (receiving a varying amount
455 of dung for years) in contrast to the laboratory (fresh dung instantly before the measurements) and
456 field experiment (large amount of dung). The dung lumps found in some peat cores from our field
457 experiment might have prolonged the short-term effect of DA by creating a more rumen-like micro-
458 environment that promoted CH_4 production by rumen specific methanogens. This brings up the
459 question whether rumen-methanogens can become dormant and may be reactivated once fresh dung is
460 added to the peat soil again or if temperature and other environmental conditions become suitable.
461 Vigorous CH_4 production and an increasing number of methanogenic archaeal 16S rRNA after

462 artificial rewetting of a paddy soil that had been air-dried for fifteen years (Watanabe et al. 2007)
463 supports the idea of potential reactivation after dormancy.

464 Instead of methanogen transfer, the increase of metabolic activity caused by dung addition has
465 also been related to the nutrients provided by the dung and the activation of dormant native microbes
466 with the nutrient increase (Lovell and Jarvis 1996; Elhottova et al. 2012). Under constant temperature
467 and moisture, the quality of the substrate together with the microbial community becomes the main
468 determinant of CH_4 production (Basiliko et al. 2007). Peat itself is a rather recalcitrant substrate with a
469 wide C/N-ratio (up to 60 in bogs (Scheffer et al. 2002)) and high shares of humic acids, lignins and
470 waxes (Dierßen and Dierßen 2008). Thus, the sole addition of a far more readily-available substrate
471 like dung (average C/N 15 in Lovell and Jarvis 1996) could lead to higher rates of CH_4 production. For
472 instance, the CH_4 production of peat samples from our (previously drained and degraded) grassland
473 site was very low initially but increased significantly after the addition of fresh dung. In these samples
474 we could not detect transferred rumen-methanogens possibly indicating the stimulation of soil-borne
475 methanogens as reported by Ho et al. (2015) and Gattinger et al. (2007). Further, Yang et al. (2017)
476 reported that the addition of manure can significantly affect the composition of soil microbial
477 communities. In addition, the added rumen-associated methanogens have to compete with the
478 established native soil microflora. Consequently, it remained unclear which share of the increased CH_4
479 production in our study was due to a dung-caused activation of peat-borne methanogens with nutrient
480 increase and which to rumen-methanogens.

481 Another debatable point is that the results from the short-term approach cannot be directly
482 extrapolated into long-term. First, during grazing the dung was added repeatedly but only once in the
483 other approaches. Second, the composition of the dung changes with time (aeration, decomposition).
484 Liu et al. (2018) have examined the physicochemical and microbial characteristics of cattle manure
485 during storage. They found a significant change from the dominance of *Methanobrevibacter* and
486 *Methocorpusculum* (fresh dung) to *Methanocorpusculum* and *Methanobacterium* after 20 days that
487 was driven by different physicochemical characteristics, mainly moisture and P content. Changes in
488 CH_4 emission during dung storage were related to these alterations in dominant methanogen type and
489 correlated bacterial taxa (Liu et al. 2018). The effects of DA observed in our study, however, were

consistent at all three approaches. We still detected a higher CH_4 production and some dung-associated methanogens after approx. 20 years of grazing compared to an ungrazed site. Thus, it appears that the effects of dung remain even if its composition changes with time – and its impact may not be highly significant anymore.

With regard to peatland restoration it seems likely that rewetting will trigger the increased CH_4 emissions from the previously grazed sites. Rewetting itself has been reported to increase CH_4 emissions in drained peat soils (e.g., Urbanová et al. 2011; Hahn et al. 2015) by promoting the growth and activity of methanogens (Putkinen et al. 2018; Turetsky et al. 2014). In accordance with Aguilar et al. (2014) we measured a marked increase in CH_4 production in samples with dung addition when water was added. Even short-term bursts of CH_4 are problematic as its global warming potential is 34-times that of CO_2 on a 100-year time horizon and even 86-times on a 20-year time horizon (Myhre et al. 2013).

In Europe, the need for restoration is strongest in Central Europe where the peatlands are highly impacted by agriculture; for example in Germany and the Netherlands as much as 85% of the organic soils are under agricultural use, compared to 3.5% in Finland and Sweden (Oleszczuk et al. 2008). Unfortunately, the risk of a "dung-induced" burst in CH_4 emissions after rewetting is high in this region as well because the peatlands are often used as grassland (i.e., pasture + meadow), e.g., in Austria (85%), the Netherlands (79%), Germany (40%), Ukraine (31%), Ireland (20%), the United Kingdom (15%) and Poland (13%) (calculated from the "Global Peatland Database", 30.11.2016, International Mire Conservation Group (IMCG)). These data, however, are estimates as peatlands are still often not mapped completely or in appropriate quality (personal communication A. Barthelmes, International Mire Conservation Group (IMCG)). Further, it is even unknown which share of the already restored 108,000 ha of peatlands in the EU has been under agricultural use before rewetting - although there might be some previously grazed hotspots in northeastern Germany and the UK (Joosten and Tanneberger 2017). Moreover, it is unknown whether the dung of other ruminants frequently held on peatlands, e.g. sheep, has the same effect on CH_4 production as cattle dung. Thus, at the moment the risk assessment on a peatland rewetting- induced burst in CH_4 emissions due to previous cattle grazing is limited to an estimate, only.

Conclusion

The application of cattle dung to pristine and restored peatland soils increased the CH_4 production potential and the abundance of methanogenic archaea in three different approaches with decreasing control of environmental conditions. The increase was driven either by a change in the composition of the methanogen community or by a fertilization effect of the dung itself. Further, the composition of the methanogen community changed towards that of dung and a transfer of rumen methanogens to peat soils seems likely. Therefore, the rewetting of peatlands with a history of cattle-grazing poses the risk of increased CH_4 emissions compared to non-grazed sites. Alarmingly, in Europe the need for restoration and the risk of a burst in CH_4 emissions after rewetting meet in same region. Globally, the largest share of drained peatlands is found in Central Europe where peatlands are additionally highly impacted by agriculture. Consequently, the careful selection of sites that have no history as pasture is crucial for a peatland restoration that aims to climate mitigation.

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Tables

Table 1 Characteristics and management of the sampling sites in Finland (FIN) and Germany (GER) and characteristics of the peat at sampling day

Site (country)	Location	Type	Previous management	Rewetting year	Long term WL (cm)	CH_4 emission (mg m^{-2} d^{-1})	Peat		
							pH	DBD	OM
Aitoneva (FIN)	62°12'N, 23°18'E	oligotrophic fen	peat extraction	2008	14±7[a]	5.0±9.2[a]	5.7	0.15±0.1	82±23
Jokivarsisuo (FIN)	61°50'N, 24°17'E	oligotrophic fen	-	-	nd	nd	5.6	0.06±0.2	96±4
Konilamminsuo (FIN)	61°48'N, 24°17'E	oligotrophic pine fen	forestry	1995	15±2[b]	3.0±1.3[b]	5.6	0.10±0	96±1
Vanneskorpi (FIN)	61°51'N, 23°42'E	spruce mire	forestry	1997	9±1[b]	10.8±3.2[b]	5.6	0.22±0.3	73±35
Paulinenaue (GER)	52°40'N, 12°42'E	minerotrophic fen	grassland	2007	-60 to -5 (0 in winter)[c]	0	7.0	nd	nd

WL = water level referred to soil surface; DBD = dry bulk density in g cm^{-3}; OM = content of organic matter in %; nd = not determined

[a] Putkinen, personal communication December 2014, measurements 2009-2011

[b] Juottonen et al. (2012)

[c] Drösler et al. (2013)

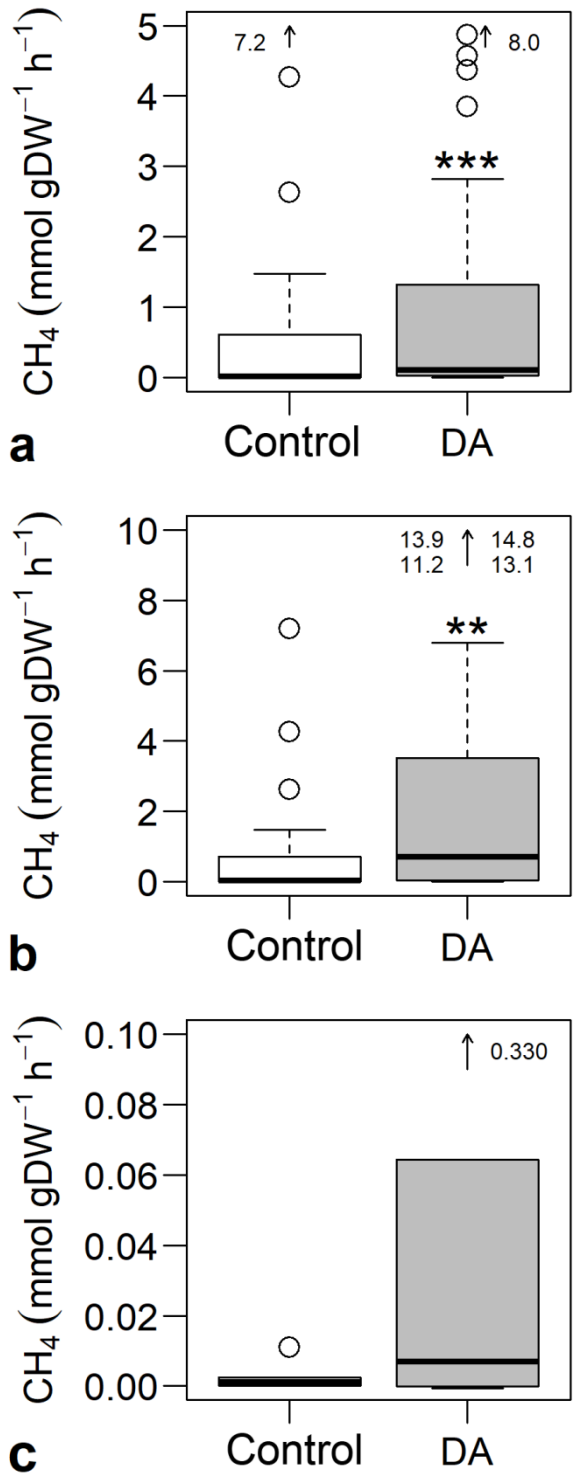
Figure Legends

Fig. 1 CH_4 production potentials of peat (Control) and of peat with dung application (DA) in the short- (a), medium- (b), and long-term approach (c). Significance was tested by paired, one-sided Wilcox rank sum tests and the level of significance is indicated by *** ($p<0.001$) and ** ($p<0.01$). Sample sizes were 42 and 36 for a and b, respectively. For the long-term approach 6 Control and 5 DA samples were examined. Outliers that are greater than the y-axis are indicated by arrows

Fig. 2 Correlation of CH_4 production potentials with *mcrA* copy numbers in control peat and peat samples with dung application (DA) in the short- (a), medium- (b), and the long-term approach (c). For the short-term approach only samples from the German site were used and dung was added to peat from both the ungrazed (circles) and the grazed (squares) site. Sample sizes (C+DA) were 21, 72 and 11 for a, b and c, respectively. Levels of significance of linear or polynomial regressions are indicated by *** ($p<0.001$), * ($p<0.05$) and . ($p<0.1$). Outliers that exceed the y-axis are indicated by arrows

Fig. 3 Methanogenic archaeal community based on *mcrA* T-RFs in dung (D), control peat (C) and dung treated peat (DA) as determined in the short- (a), medium- (b) and long-term approach (c). The ordination is based on DCA. In (a) the DCA axis 1 explained 27% and axis 2 13% of the variation. In (b) 17% and 11% and in (c) 24% and 15% of the variation was explained by the first two DCA axes. The closer a T-RF (smaller triangles) is located to the centroid of a treatment (larger triangles) the more typical it is to the respective treatment. The arrows display direction and magnitude of increasing CH_4 production potential, number of *mcrA* copies and the number of T-RFs. Peat from the grazed site was used as control samples for the short term DA-treatment

Fig. 4 Maximum likelihood phylogenetic tree of *mcrA* sequences from clones from dung (DFs) and from peat samples without (c=control) and with dung application (d) in the medium-term approach at the sites Aitoneva (A), Jokivarsisuo (J) and Konilamminsuo (K). The sequences were obtained from dung-treated peat samples in which the T-RF 106 bp occurred and from the corresponding controls (n=6 each). The filled circles are bootstrap values over 75%, and the open circles are values over 50%.



819

820 Fig. 1

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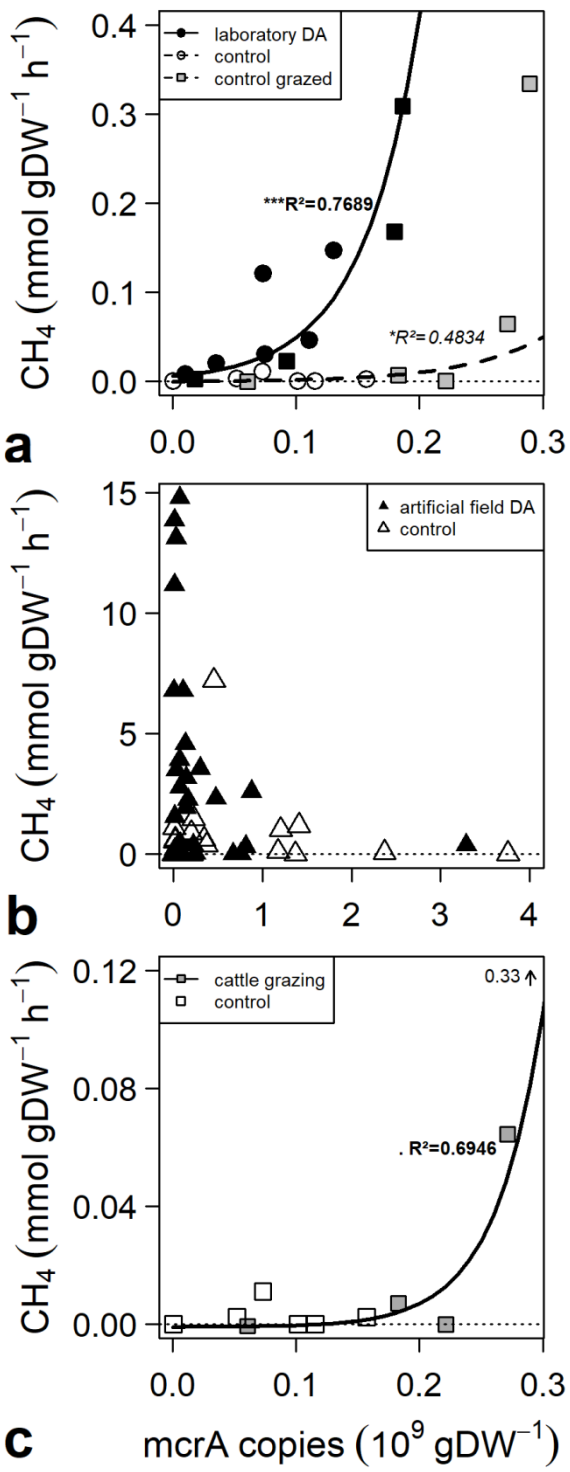

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825 Fig. 2

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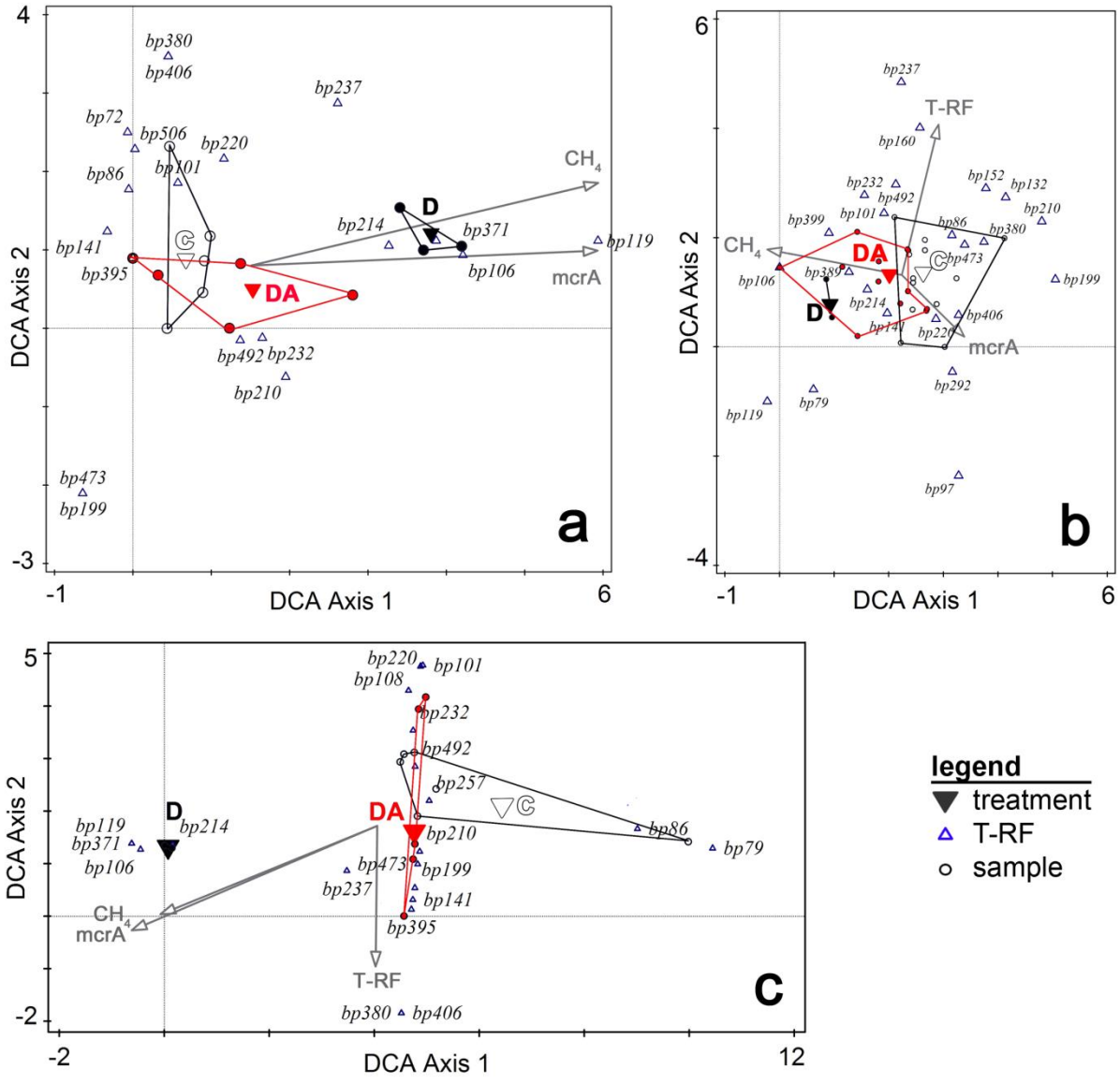
a
DCA Axis 2
DCA Axis 1
4
-3
-1
6
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bp406
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bp72
bp506
bp220
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CH4
D
bp214
bp371
bp141
bp119
bp106
bp395
mcrA
DA
bp492
bp232
bp210
bp473
bp199
b
DCA Axis 2
DCA Axis 1
6
-4
-1
6
bp237
T-RF
bp160
bp152
bp132
bp232
bp492
bp210
bp399
bp101
bp86
bp380
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DA
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bp106
bp389
bp199
D
bp214
bp406
bp140
bp220
mcrA
bp292
bp119
bp79
bp97
c
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DCA Axis 1
5
-2
-2
12
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bp257
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bp119
bp214
bp210
bp86
bp79
bp371
bp106
bp473
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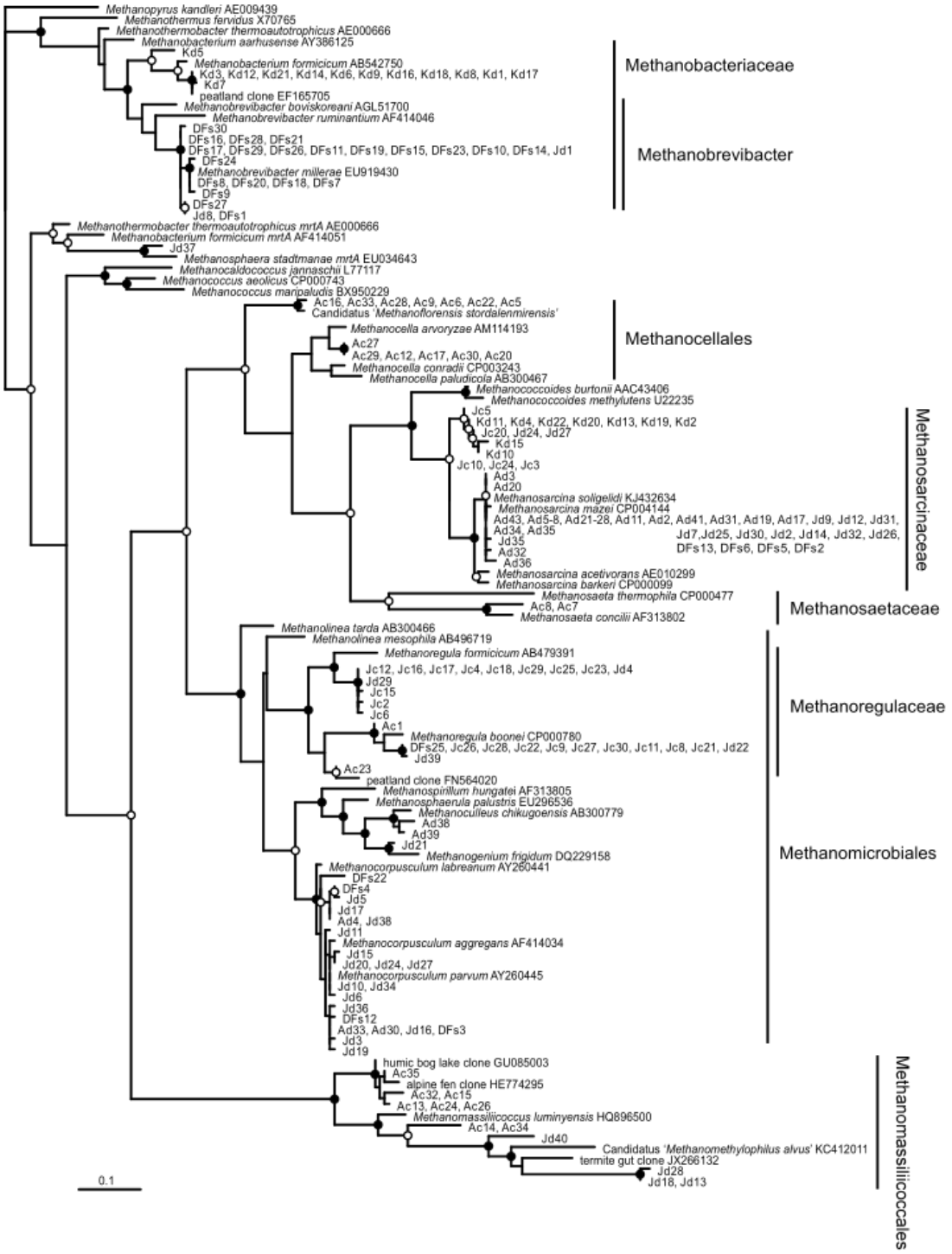
CH4
mcrA
bp395
T-RF
bp380
bp406
legend
treatment
T-RF
sample

Fig. 3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Methanothermobacter thermoautotrophicus AE000666
Methanobacterium aarhusense AY386125
Kd5
Methanobacterium formicicum AB542750
Kd3, Kd12, Kd21, Kd14, Kd6, Kd9, Kd16, Kd18, Kd8, Kd1, Kd17
Kd7
peatland clone EF165705
Methanobrevibacter boviskoreani AGL51700
Methanobrevibacter ruminantium AF414046
DFs30
DFs16, DFs28, DFs21
DFs17, DFs29, DFs26, DFs11, DFs19, DFs15, DFs23, DFs10, DFs14, Jd1
DFs24
Methanobrevibacter millerae EU919430
DFs8, DFs20, DFs18, DFs7
DFs9
DFs27
Jd8, DFs1
Methanothermobacter thermoautotrophicus mrtA AE000666
Methanobacterium formicicum mrtA AF414051
Jd37
Methanosphaera stadtmanae mrtA EU034643
Methanocaldococcus jannaschii L77117
Methanococcus aeolicus CP000743
Methanococcus maripaludis BX950229
Ac16, Ac33, Ac28, Ac9, Ac6, Ac22, Ac5
Candidatus 'Methanoflorensis stordalenmirensis'
Methanocella arvoryzae AM114193
Ac27
Ac29, Ac12, Ac17, Ac30, Ac20
Methanocella conradii CP003243
Methanocella paludicola AB300467
Methanococcoides burtonii AAC43406
Methanococcoides methylutens U22235
Jc5
Kd11, Kd4, Kd22, Kd20, Kd13, Kd19, Kd2
Jc20, Jd24, Jd27
Kd15
Kd10
Jc10, Jc24, Jc3
Ad3
Ad20
Methanosarcina soligelidi KJ432634
Methanosarcina mazei CP004144
Ad43, Ad5-8, Ad21-28, Ad11, Ad2, Ad41, Ad31, Ad19, Ad17, Jd9, Jd12, Jd31,
Ad34, Ad35
Jd7,Jd25, Jd30, Jd2, Jd14, Jd32, Jd26,
Jd35
DFs13, DFs6, DFs5, DFs2
Ad32
Ad36
Methanosarcina acetivorans AE010299
Methanosarcina barkeri CP000099
Methanosaeta thermophila CP000477
Ac8, Ac7
Methanosaeta concilii AF313802
Methanolinea tarda AB300466
Methanolinea mesophila AB496719
Methanoregula formicicum AB479391
Jc12, Jc16, Jc17, Jc4, Jc18, Jc29, Jc25, Jc23, Jd4
Jd29
Jc15
Jc2
Jc6
Ac1
Methanoregula boonei CP000780
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Jd39
Ac23
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Methanoculleus chikugoensis AB300779
Ad38
Ad39
Jd21
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Methanocorpusculum labreanum AY260441
DFs22
DFs4
Jd5
Jd17
Ad4, Jd38
Jd11
Methanocorpusculum aggregans AF414034
Jd15
Jd20, Jd24, Jd27
Methanocorpusculum parvum AY260445
Jd10, Jd34
Jd6
Jd36
DFs12
Ad33, Ad30, Jd16, DFs3
Jd3
Jd19
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Ac35
alpine fen clone HE774295
Ac32, Ac15
Ac13, Ac24, Ac26
Methanomassiliicoccus luminyensis HQ896500
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Jd40
Candidatus 'Methanomethylophilus alvus' KC412011
termite gut clone JX266132
Jd28
Jd18, Jd13
0.1
Methanobacteriaceae
Methanobrevibacter
Methanocellales
Methanosarcinaceae
Methanosaetaceae
Methanoregulaceae
Methanomicrobiales
Methanomassiliicoccales

838

839 Fig. 4