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1 **Dung application increases CH₄ production potential and alters the**
2 **composition and abundance of methanogen community in restored**
3 **peatland soils from Europe**

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15

16

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24

25 **Abstract**

26 Peatland restoration via rewetting aims to recover biological communities and biogeochemical processes typical
27 to pristine peatlands. While rewetting promotes recovery of C accumulation favorable for climate mitigation, it
28 also promotes methane (CH₄) emissions. The potential for exceptionally high emissions after rewetting has been
29 measured for Central European peatland sites previously grazed by cattle. We addressed the hypothesis that
30 these exceptionally high CH₄ emissions result from the previous land use. We analyzed the effects of cattle dung
31 application to peat soils in a short- (2 weeks), a medium- (1 year) and a long-term (grazing) approach. We
32 measured the CH₄ production potentials, determined the numbers of methanogens by *mcrA* qPCR and analyzed
33 the methanogen community by *mcrA* T-RFLP-cloning-sequencing. Dung application significantly increased the
34 CH₄ production potential in the short- and the medium-term approach and non-significantly at the cattle-grazed
35 site. The number of methanogens correlated with the CH₄ production in the short- and the long-term approach.
36 At all three time horizons, we found a shift in methanogen community due to dung application and a transfer of
37 rumen methanogen sequences (*Methanobrevibacter* spp.) to the peatland soil that seemed related to increased
38 CH₄ production potential. Our findings indicate that cattle grazing of drained peatlands changes their
39 methanogenic microbial community, may introduce rumen-associated methanogens and leads to increased CH₄
40 production. Consequently, rewetting of previously cattle grazed peatlands has the potential to lead to increased
41 CH₄ emissions. Careful consideration of land use history is crucial for successful climate mitigation with
42 peatland rewetting.

43

44 **Keywords**

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

46

47 **Introduction**

48 Peatlands are wetland ecosystems characterized by water saturated soil and thereby accumulation of
49 organic matter as peat due to incomplete decomposition. In their natural state, peatlands are a sink of
50 atmospheric carbon dioxide (CO₂) and a source of 20 – 30 % of global annual methane (CH₄)
51 emissions (Gorham 1991; Turunen et al. 2002; Lafleur et al. 2003; Nilsson et al. 2008). In addition to
52 global C dynamics, peatlands play a key role in maintaining high biodiversity at all scales from local
53 to global (Parish et al. 2008).

54 Many peatlands have been drained for the utilization as agriculture and forestry. Drainage
55 changes a peatland ecosystem dramatically and disrupts its ecological functions. Consequences
56 include altered vegetation and microbial communities (Laine et al. 1995; Jaatinen et al. 2007), loss of
57 C through increased decomposition of peat and decreased emissions of CH₄ (Jaatinen et al. 2008;
58 Mäkiranta et al. 2009; Yrjälä et al. 2011). Pasture on drained peatlands adds further disruptions,
59 namely the input of nutrients with urine and dung (Haynes and Williams 1993) and compaction of the
60 peat with trampling (Hamza and Anderson 2005). Fresh dung pats have been shown to turn a boreal
61 sward from a weak sink to a small source of CH₄ (Maljanen et al. 2012), and reindeer droppings have
62 been shown to increase peat CH₄ production potential (Laiho et al. 2017). Rumen methanogens can be
63 introduced into soil via cattle feces and detected in grazed soils (Gattinger et al. 2007). After enteric
64 fermentation (32 – 40% of total agriculture emissions), manure deposited on pasture is the second
65 largest CH₄ emitting category (15% of total) with cattle contributing the largest share (Smith et al.
66 2014). Indeed, agricultural emissions represent the greatest source of CH₄ in the EU with 10.2 million
67 tons per year. Of these, approximately one-third comes from livestock manure (Moss et al. 2000).

68 The ecological restoration of drained peatlands aims to recover communities and hydrological
69 and biogeochemical processes typical to pristine peatlands (Nellemann and Corcoran 2010). In
70 Europe, large areas of drained peatlands have already been restored (Aapala et al. 2008; Joosten and
71 Tanneberger 2017) for climate mitigation (Pfadenhauer and Grootjans 1999). Here, rewetting, i.e.,
72 raising the water table to re-establish saturated conditions, decreases the loss of C and leads to
73 recovery of the CO₂ 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 CH₄ (e.g., Waddington and Day 2007) - a 28-times stronger GHG than CO₂ (Myhre et al.
76 2013).

77 In northern peatlands, however, several studies have shown lower CH₄ 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 CH₄ 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 CH₄ 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 CH₄
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 CH₄ 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 CH₄ 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 CH₄
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?

102 **Materials and Methods**

103 **Peat samples**

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

108 **Sampling sites**

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

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

127

128 **Peat sampling and sample processing**

129 The four Finnish sites were sampled after one year of in-situ incubation of the transplanted dung on
130 12th and 13th of May 2014 and the grazed and ungrazed sites in Germany were sampled on 4th of
131 February 2014. The German sites were flooded (water level 2 ± 3 cm, n=6) at the time of sampling and
132 the uppermost 10 cm of soil were frozen. At the Finnish sites the water levels at the time of sampling
133 were at 17 ± 3 cm (n=18) in Aitoneva, -3 ± 1 cm (n=18) in Jokivarsisuo, -11 ± 2 cm (n=18) in
134 Konilamminsuo and 7 ± 2 cm (n=18) in Vanneskorpi.

135 The peat samples were taken with a peat corer - a round one in Germany (8 cm diameter,
136 adapted from Buttler et al. 1998) and a box corer in Finland (10×10 cm²). We took three peat cores
137 per treatment from each site. Depending on the degree of soil compaction we sampled the upper 30
138 cm. At the German sites the frozen uppermost layer (10 cm) was removed before sampling (details in
139 supplementary section Table S.3). We sealed the peat cores instantly after sampling with wrapping
140 film and plastic bags to reduce oxygen exposure. The sealed samples were transported to the
141 laboratory of the Natural Resources Institute Finland, Vantaa, overnight where samples were stored at
142 +4°C until processing.

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

147 **Peat characteristics**

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

153

154 **Dung-application treatments**

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

157 Dung samples originated from three different farms (see below) and were stored at +4 °C in
158 sealed in plastic bags before addition to the peat. Subsamples of the dung were stored at -20°C for
159 methanogen community analysis. N-content in the dung samples was analyzed using Kjeldahl method
160 (Blume et al. 2011). All used dung samples had comparable qualities, namely a dry matter content of
161 $11 \pm 1.5\%$, a $85 \pm 3.6\%$ content of organic matter and a pH of 7.2 ± 0.4 (n=7). The N-content was
162 $2.7 \pm 0.5\%$ (n=4).

163 **Short-term effects of DA**

164 For the short-term effect of DA, a suspension of fresh dung was added to control peat samples from
165 the Finnish sites and from the ungrazed German site (**Table S.3**). A dung-water suspension was mixed
166 with 15 ml of fresh peat and suspended in 30 ml autoclaved purified water in 125 ml glass flasks
167 (dung : peat : water $0.83\bar{3} : 15 : 31.66\bar{6}$, v/v/v). Moreover, we wanted to estimate the effect of rewetting
168 on the CH₄ production potential of dung-treated peat. For this purpose the CH₄ production was
169 compared between pure peat and peat suspended in water before and after DA; peat samples from the
170 Finnish site Jokivarsisuo (n=18) were used. The dung used in the experiments for short-term DA
171 originated from cattle at Haltiala farm near Helsinki, Finland (60° 16' N, 24° 57' E). The cattle were
172 fed on a mixed diet (straw and concentrated feed once a week) and had the opportunity of grazing. On
173 average the dung's content of dry matter was 9%, the content of organic matter $80 \pm 1\%$, the N-
174 content 3.1% and the pH 7.1 (n=2).

175 Additionally, fresh dung was added to peat samples from the cattle grazed grassland site to
176 determine its effect in contrast to the effects of a long-term field exposure (n=5, **Table S.3**). The dung
177 for this experiment was collected from cows grazing the peatland site in Germany (**Table 1**). Besides
178 grazing the cows were fed on straw and concentrated feed. The content of dry matter of the dung was
179 11%, the content of organic matter $86 \pm 1\%$ and the pH $7.6 \pm 1.5\%$ (n=3). The N content was not
180 determined.

181 **Medium-term effects of DA**

182 In May 2013, dung was transplanted to three plots at each of the four sites in Finland and incubated
183 there for one year. The vegetation cover was removed aside, together with peat to a depth of 35 cm.
184 Two buckets of cow dung (approx. 20 l in total) were then spread into the resulting hole ($0.5 \times 0.5 \times$
185 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
186 were placed back to cover the hole. The dung originated from the farm Kaupintila, Simuna,
187 Hämeenkyrö, Finland ($61^\circ 36' \text{ N}$, $23^\circ 14' \text{ E}$) from cows fed on silage, grains (oat and barley) and
188 crushed grains of field mustard. On average the dung's content of dry matter was 13%, the content of
189 organic matter 89%, the N-content 2.3% and the pH-value 7.0 (n=2). The properties of this dung were
190 not determined from the fresh dung spread in 2013. Instead, they were measured from dung lumps in
191 peat samples 2014 in which the dung did not successfully mix with the peat.

192

193 **CH₄ production potentials**

194 The CH₄ production potentials of all samples were measured by anoxic incubation experiments
195 according to Juottonen et al. (2008). Glass flasks (125 ml) were filled with 30 ml deionised water, then
196 autoclaved, flushed with N₂ for 2 min and closed airtight. To start the incubation 15 ml of peat were
197 added to the flasks, flushed with N₂ to remove oxygen, and closed airtight with a rubber stopper. To
198 allow the methanogens from the dung to adapt to the new substrate prior to the measurements, samples
199 were stored at 4°C in the dark for 5 days. Thereafter the flasks were again flushed with N₂ and
200 incubated at 16°C in the dark. The CH₄ concentration in the headspace was measured with a gas
201 chromatograph (Hewlett Packard, G1530A, USA) at five times during the incubation period of 6 to 15
202 days depending on the rate of accumulation of the respective experiment. To obtain rates of the CH₄
203 production potential ($\text{nmol g}^{-1} \text{ dw h}^{-1}$) linear regression of the CH₄ concentrations over the
204 measurement time was applied.

205

206 **Community of methanogenic archaea**

207 **Extraction of DNA**

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

214 **Quantification of methanogenic archaea**

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

222 The gene for methyl coenzyme M reductase subunit A (*mcrA*) was used for quantitative
223 analysis of methanogenic archaea. We used the primer pair *mlas/mcrA-rev* (Steinberg and Regan,
224 2008) with an amplicon length of ca. 465-490 bp (Luton et al. 2002). For total bacteria we targeted
225 bacterial 16S rRNA genes (*b16S*) with the primer pair 1055F/1392R (Harms et al. 2003). The
226 amplicon length was expected to be 337-352 bp (Harms et al. 2003; Toes et al. 2008). For total
227 archaea we targeted archaeal 16S rRNA genes (*a16S*) with the primer pair Arch967f/Arch1060r
228 (Cadillo-Quiroz et al. 2006 and references therein). The thermal cycling conditions for *mcrA* were:
229 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
230 72°C, and the final extension 7 min at 72°C. The thermal cycling conditions targeting *b16S* were:
231 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
232 72°C. The thermal cycling conditions for *a16S* were: initial denaturation 10 min at 95°C, followed by

233 40 cycles of 15 sec at 95°C, 30 sec at 55°C, 20 sec at 72°C. Fluorescence was measured after each
234 extension step. A melting curve analysis was performed for quality verification of the PCR products
235 (*mcrA* from 72 to 99°C, b16S from 60 to 95°C, a16S from 72 to 95°C). Standard curves were obtained
236 with serial dilutions (10^1 – 10^9 gene copies per reaction) of recombinant plasmids containing a fragment
237 of the *mcrA*, bacterial or archaeal 16S gene targets, respectively. The gene copies μl^{-1} in the samples
238 were calculated for each target using linear regression parameters fit to a plot of cycle threshold (C_T)
239 versus log of the concentration of gene copies for the standards runs. The effectiveness of the qPCR
240 reactions as well as the limits of detection and quantification are given in the supplementary material
241 (section S1.2).

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

243 A terminal restriction fragment length polymorphism (T-RFLP) approach was used to detect whether
244 methanogenic archaea from the cattle rumen were transferred to the peat. For the effect of long-term
245 field exposure on the community of rumen methanogens, the samples from the cattle-grazed and from
246 the ungrazed site (n(C)=6, n(DA)=5) were analyzed. For the short-term effects of DA in contrast to
247 long-term exposure the samples from the cattle grazed site before and after the addition of fresh dung
248 under laboratory conditions (n(C)= n(DA)=5) were used. Thus, the short- and the long-term approach
249 shared the same control samples in the T-RFLP analyses. For the medium-term effects we selected
250 samples from the dung-transplantation experiment in Finland (n(C)= n(DA)=12). Here, from each DA
251 sample the T-RF pattern of the 10cm-section (i.e., 0-10cm, 10-20cm, or 20-30cm) with the highest
252 CH₄ production potential was determined together with the corresponding 10-cm section from the
253 control sample. The dung samples that were used for DA were included as a reference.

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

260 495 bp length. Terminal restriction fragment (T-RF) peaks with <200 relative fluorescence units
261 (background noise) and peaks with <2% of the total peak area were excluded. Results are presented
262 based on relative peak area.

263 **Cloning, DNA sequencing and phylogenetic analysis for identification of** 264 **methanogenic archaea**

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

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

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

287 http://darwin.uvigo.es/software/protest2_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 *HhaI* and *MboI* (<http://www.nrbcs.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 CH₄ 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 CH₄ production potentials. Correlations between the CH₄ production potentials and the
301 *mcrA* copy numbers were assessed by linear or polynomial regression. The distribution of the data for
302 the CH₄ 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**).

311 **Results**

312 **Dung application and CH₄ production potentials**

313 **CH₄ production of peat soils after DA**

314 Dung addition significantly increased the CH₄ production potentials in the short- and in the medium-
315 term approach - on average by the factor 8 and 19, respectively (**Fig. 1a, b**). Likewise, the mean CH₄
316 production potential of the cattle-grazed site was 6-times higher than that of the ungrazed site but the
317 effect was not significant (**Fig. 1c**). In contrast to the long-term field exposure, however, the addition
318 of fresh dung to peat from the grazed site significantly increased the CH₄ production potential (n=5,
319 p=0.0313). The increased CH₄ production was observed at all samples depths.

320 The levels of increased CH₄ production potential with dung addition greatly differed between
321 the examined time-horizons. While the dung-treated samples from the short- and medium-term
322 approach produced at maximum 4 – 15 mmol gdw⁻¹ h⁻¹ CH₄, the production from the cattle grazed
323 grassland site was as low as 0.06 – 0.3 mmol gdw⁻¹ h⁻¹ CH₄ (**Fig. 1**). The highest CH₄ production
324 potentials were measured in the medium-term approach. We want to note that three out of the four top
325 values (> 10 mmol gdw⁻¹ h⁻¹ CH₄, **Fig. 1b**) were produced in samples where dung and peat were not
326 mixed perfectly homogeneously and visible dung lumps were found when sampled after one year of
327 field exposure.

328

329 **The role of rewetting**

330 The CH₄ production was significantly higher when water was added to peat samples from site
331 Jokivarsisuo than in field fresh peat (n=9; one-sided paired Wilcoxon test, p= 0.0020). In the field
332 fresh peat the addition of dung increased the CH₄ production potential from zero to 1.7±1.8 mmol
333 gdw⁻¹ h⁻¹ CH₄ (n=9; one-sided paired Wilcoxon test; p=0.0071). In suspension the peat produced
334 0.9±1.3 mmol gdw⁻¹ h⁻¹ CH₄ before and 6.4±5.6 mmol gdw⁻¹ h⁻¹ CH₄ after DA (n=9; one-sided paired
335 Wilcoxon test; p=0.0020).

336

337 **Dung application and abundance of methanogenic archaea**

338 Dung application increased the abundances of methanogenic archaea, total bacteria and total archaea
339 (copy numbers of *mcrA*, b16S and a16S) in the short-term approach (n=10, one sided paired Wilcoxon
340 tests, $p(\text{mcrA})=p(\text{b16S})=p(\text{a16S}) < 0.0001$). These numbers were also higher at the cattle-grazed
341 compared to the ungrazed site (n(C)=6, n(DA)=5, one-sided Wilcoxon test, $p(\text{mcrA})=0.0476$,
342 $p(\text{b16S})= 0.0022$, $p(\text{a16S})= 0.0260$). In the field experiment of the medium-term approach, however,
343 the copy numbers did not differ between control and DA-treated peat (n(C)=103, N(DA)=104, $p > 0.2$).

344 The number of *mcrA* copies correlated positively with the CH₄ production potentials in the
345 short-term approach (**Fig. 2a**). The best fit of the correlation was found after the addition of fresh dung
346 to peat from the cattle grazed site (black, solid squares in **Fig. 2a**, n=5, polynomial regression,
347 $p=0.0042$, $R^2=0.9544$). In the samples from the grassland sites themselves, a positive correlation was
348 found at the cattle-grazed site, but not at the ungrazed (control) site (**Fig. 2c**). In contrast, the CH₄
349 production potentials from the field experiment did not correlate with copy numbers of *mcrA* (**Fig.**
350 **2b**).

351

352 **Dung application and methanogen community composition**

353 **Community change of methanogenic archaea**

354 On all three time-horizons the main variation in the *mcrA* T-RF patterns was related to the three
355 treatments (dung, DA, control; **Fig. 3 a-c**, first DCA axis). Methanogen communities of dung and
356 control peat formed the opposite ends of the compositional gradient and DA was located in between
357 but closer to the control (**Fig. 3 a-c**). In the medium-term approach (**Fig. 3b**) DA samples were closer
358 to the control than in the short- and long-term approach. Based on CCA, the three treatments
359 significantly explained community variation (short-term: pseudo-F=2.0, $p=0.006$; medium-term:
360 pseudo-F=1.7, $p=0.026$; long-term: pseudo-F=3.3, $p=0.002$) (**Supplement, Fig. S1**). The three
361 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 CH₄ production potentials in the short- and long-term approach (**Fig.**
366 **3a, c**). In the medium-term approach, only the CH₄ 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 Methanomassiliicoccales 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

407 Discussion

408 The addition of cow dung (DA) increased the CH₄ production potential of soil samples from restored
409 peatlands at all three time horizons of our study. This supported our hypothesis that dung is likely to
410 play a role in the exceptionally high CH₄ emissions from rewetted peatlands with grazing history. We
411 found indication that the increased methanogenic potential is linked to changes in the composition of
412 the microbial community.

413 After DA we found higher numbers of methanogenic archaea and total bacteria and archaea in
414 the laboratory experiment and at the cattle-grazed grassland, similarly to the increased microbial
415 biomass found in a severely cattle impacted pasture in the Czech Republic (Elhottova et al. 2012).
416 Generally, in our study the abundance of methanogens was positively correlated with CH₄ production
417 potential with the exception of the field experiment. Both patterns have been found earlier. Positive
418 correlation has been found between the abundance of *mcrA* gene copies or the *mcrA* transcript/gene
419 ratio and CH₄ production rates (Morris et al. 2014, 2016; Freitag and Prosser 2009; Putkinen et al.
420 2018). No relationship in the field experiment agrees with findings from a peat rewetting laboratory
421 experiment (Urbanová et al. 2011) and a cattle rumen and emission study (Carberry et al. 2014a). The
422 production of CH₄ has been reported to correlate with methanogenic and bacterial communities in the
423 rumen of dairy cows (Danielsson et al. 2017). It might be that in soil a direct relation between the total
424 number of methanogens and CH₄ production may not be observed because a large part of the
425 methanogen community can be present in an inactive state (Yavitt et al. 2005; Basiliko et al. 2007) as
426 suggested by Urbanová et al. (2011).

427 In addition to the higher numbers of methanogens the DA led to a change of the composition
428 of the methanogen community towards that of the applied dung although sites differed heavily from
429 each other regarding land-use management and soil properties. The majority of methanogens in the
430 dung-treated peat belonged to the genera *Methanobrevibacter* (Methanobacteriales), *Methanosarcina*
431 (Methanosarcinales), and *Methanocorpusculum* (Methanomicrobiales). These *mcrA* sequence types
432 were not detected in control peat and were identical or highly similar to sequences from the dung, and
433 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 Methanomassiliococcales 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ production. For
472 instance, the CH₄ 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 CH₄
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 CH₄ 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

490 consistent at all three approaches. We still detected a higher CH₄ production and some dung-associated
491 methanogens after approx. 20 years of grazing compared to an ungrazed site. Thus, it appears that the
492 effects of dung remain even if its composition changes with time – and its impact may not be highly
493 significant anymore.

494 With regard to peatland restoration it seems likely that rewetting will trigger the increased CH₄
495 emissions from the previously grazed sites. Rewetting itself has been reported to increase CH₄
496 emissions in drained peat soils (e.g., Urbanová et al. 2011; Hahn et al. 2015) by promoting the growth
497 and activity of methanogens (Putkinen et al. 2018; Turetsky et al. 2014). In accordance with Aguilar et
498 al. (2014) we measured a marked increase in CH₄ production in samples with dung addition when
499 water was added. Even short-term bursts of CH₄ are problematic as its global warming potential is 34-
500 times that of CO₂ on a 100-year time horizon and even 86-times on a 20-year time horizon (Myhre et
501 al. 2013).

502 In Europe, the need for restoration is strongest in Central Europe where the peatlands are
503 highly impacted by agriculture; for example in Germany and the Netherlands as much as 85% of the
504 organic soils are under agricultural use, compared to 3.5% in Finland and Sweden (Oleszczuk et al.
505 2008). Unfortunately, the risk of a “dung-induced” burst in CH₄ emissions after rewetting is high in
506 this region as well because the peatlands are often used as grassland (i.e., pasture + meadow), e.g., in
507 Austria (85%), the Netherlands (79%), Germany (40%), Ukraine (31%), Ireland (20%), the United
508 Kingdom (15%) and Poland (13%) (calculated from the “Global Peatland Database”, 30.11.2016,
509 International Mire Conservation Group (IMCG)). These data, however, are estimates as peatlands are
510 still often not mapped completely or in appropriate quality (personal communication A. Barthelmes,
511 International Mire Conservation Group (IMCG)). Further, it is even unknown which share of the
512 already restored 108,000 ha of peatlands in the EU has been under agricultural use before rewetting -
513 although there might be some previously grazed hotspots in northeastern Germany and the UK
514 (Joosten and Tanneberger 2017). Moreover, it is unknown whether the dung of other ruminants
515 frequently held on peatlands, e.g. sheep, has the same effect on CH₄ production as cattle dung. Thus, at
516 the moment the risk assessment on a peatland rewetting- induced burst in CH₄ emissions due to
517 previous cattle grazing is limited to an estimate, only.

518 **Conclusion**

519 The application of cattle dung to pristine and restored peatland soils increased the CH₄ production
520 potential and the abundance of methanogenic archaea in three different approaches with decreasing
521 control of environmental conditions. The increase was driven either by a change in the composition of
522 the methanogen community or by a fertilization effect of the dung itself. Further, the composition of
523 the methanogen community changed towards that of dung and a transfer of rumen methanogens to
524 peat soils seems likely. Therefore, the rewetting of peatlands with a history of cattle-grazing poses the
525 risk of increased CH₄ emissions compared to non-grazed sites. Alarming, in Europe the need for
526 restoration and the risk of a burst in CH₄ emissions after rewetting meet in same region. Globally, the
527 largest share of drained peatlands is found in Central Europe where peatlands are additionally highly
528 impacted by agriculture. Consequently, the careful selection of sites that have no history as pasture is
529 crucial for a peatland restoration that aims to climate mitigation.

530

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777 **Tables**

778

779 **Table 1** Characteristics and management of the sampling sites in Finland (FIN) and Germany (GER)
 780 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

781 WL = water level referred to soil surface; DBD = dry bulk density in g cm⁻³; OM = content of organic matter in %;
 782 nd = not determined

783 ^a Putkinen, personal communication December 2014, measurements 2009-2011

784 ^b Juottonen et al. (2012)

785 ^c Drösler et al. (2013)

786 **Figure Legends**

787

788 **Fig. 1** CH₄ production potentials of peat (Control) and of peat with dung application (DA) in the short-
789 (a), medium- (b), and long-term approach (c). Significance was tested by paired, one-sided Wilcoxon
790 rank sum tests and the level of significance is indicated by *** (p<0.001) and ** (p<0.01). Sample sizes
791 were 42 and 36 for a and b, respectively. For the long-term approach 6 Control and 5 DA samples
792 were examined. Outliers that are greater than the y-axis are indicated by arrows

793

794 **Fig. 2** Correlation of CH₄ production potentials with *mcrA* copy numbers in control peat and peat
795 samples with dung application (DA) in the short- (a), medium- (b), and the long-term approach (c). For
796 the short-term approach only samples from the German site were used and dung was added to peat
797 from both the ungrazed (circles) and the grazed (squares) site. Sample sizes (C+DA) were 21, 72 and
798 11 for a, b and c, respectively. Levels of significance of linear or polynomial regressions are indicated
799 by *** (p<0.001), * (p<0.05) and . (p<0.1). Outliers that exceed the y-axis are indicated by arrows

800

801 **Fig. 3** Methanogenic archaeal community based on *mcrA* T-RFs in dung (D), control peat (C) and
802 dung treated peat (DA) as determined in the short- (a), medium- (b) and long-term approach (c). The
803 ordination is based on DCA. In (a) the DCA axis 1 explained 27% and axis 2 13% of the variation. In
804 (b) 17% and 11% and in (c) 24% and 15% of the variation was explained by the first two DCA axes.
805 The closer a T-RF (smaller triangles) is located to the centroid of a treatment (larger triangles) the
806 more typical it is to the respective treatment. The arrows display direction and magnitude of increasing
807 CH₄ production potential, number of *mcrA* copies and the number of T-RFs. Peat from the grazed site
808 was used as control samples for the short term DA-treatment

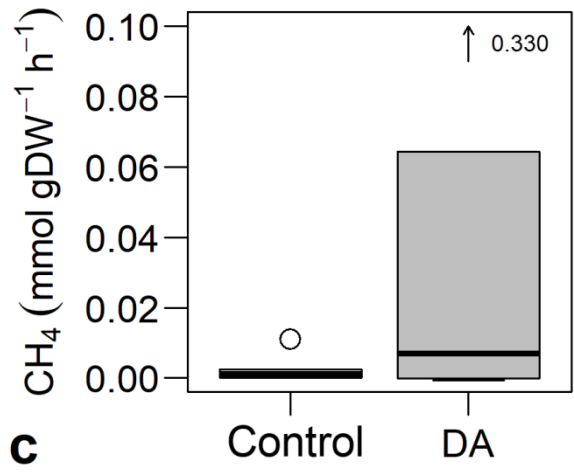
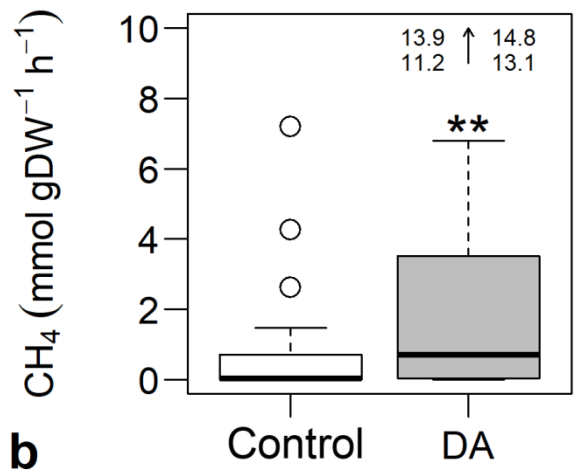
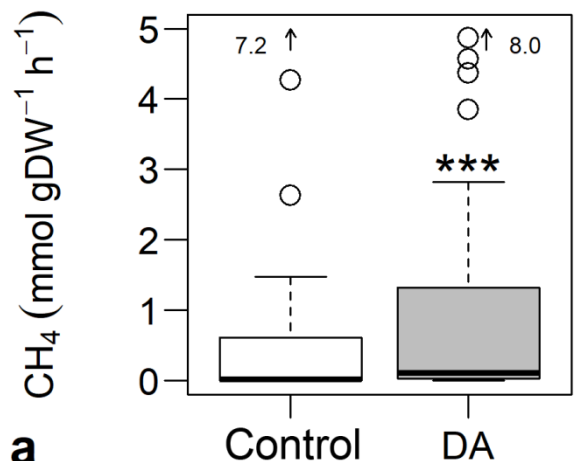
809

810 **Fig. 4** Maximum likelihood phylogenetic tree of *mcrA* sequences from clones from dung (DFs) and
811 from peat samples without (c=control) and with dung application (d) in the medium-term approach at
812 the sites Aitoneva (A), Jokivarsisuo (J) and Konilamminsuo (K). The sequences were obtained from
813 dung-treated peat samples in which the T-RF 106 bp occurred and from the corresponding controls
814 (n=6 each). The filled circles are bootstrap values over 75%, and the open circles are values over
815 50%.

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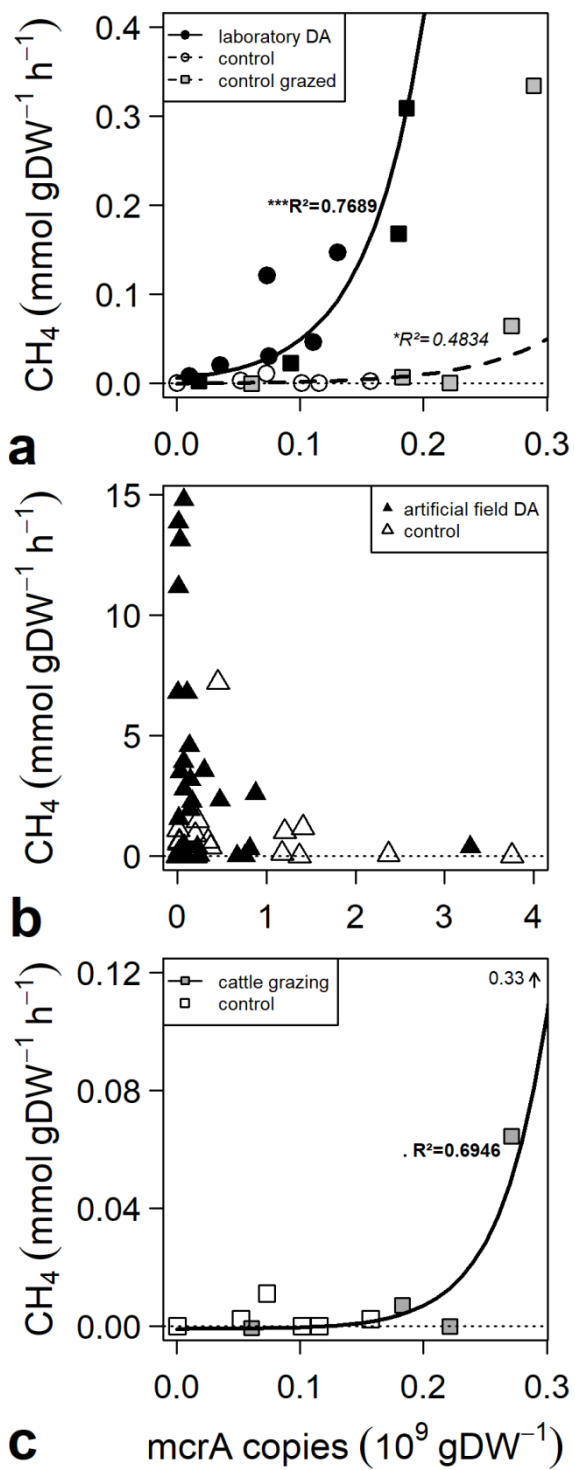
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820 Fig. 1

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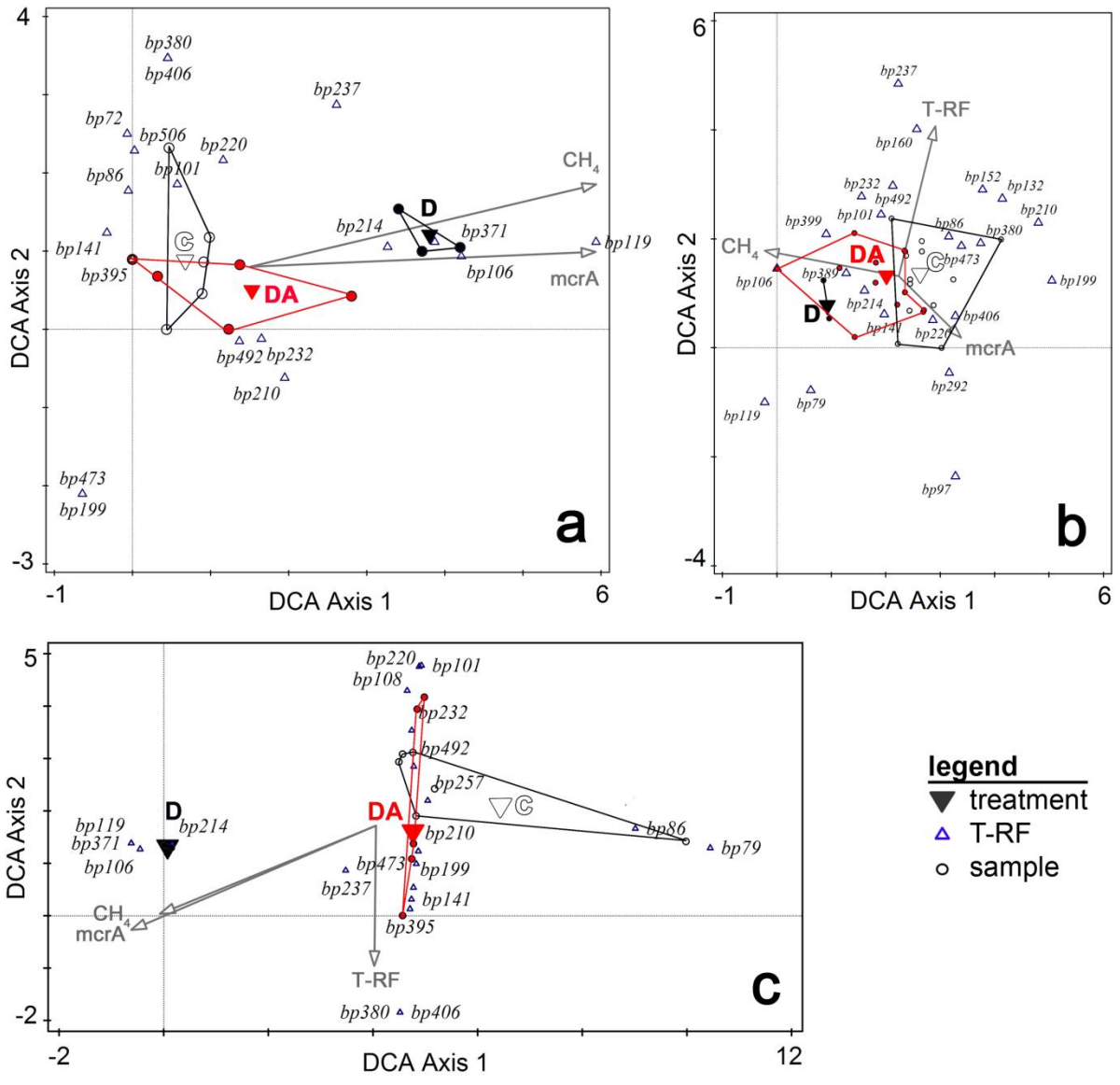
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825 Fig. 2

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831 Fig. 3

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