Transcriptional dynamics of methane-cycling microbiomes are linked to seasonal CH₄ fluxes in two hydromorphic and organic-rich grassland soils

Running title: Transcriptional dynamics of methane-cycling microbiomes

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Abstract

Soil CH₄ fluxes are driven by CH₄-producing and -consuming microorganisms that determine whether soils are sources or sinks of this potent greenhouse gas. Using quantitative metatranscriptomics, we linked CH₄-cycling microbiomes to net surface CH₄ fluxes throughout a year in two drained peatland soils differing in grassland land-use intensity and physicochemical properties. CH₄ fluxes were highly dynamic; both soils were net CH₄ sources in autumn and winter and sinks in spring and summer. Despite similar net CH₄ emissions, methanogen and methanotroph loads, as determined by small subunit rRNA transcripts per gram soil, differed strongly between sites. In contrast, mRNA transcript abundances were similar in both soils and correlated well with CH₄ fluxes. The methane monooxygenase to methanogenesis mRNA ratio was higher in spring and summer, when the soils were net CH₄ sinks. CH₄ uptake was linked to an increased proportion of USC α and γ and pmoA2 *pmoA* transcripts. We assume that methanogen transcript abundance may be useful to approximate changes in net surface CH₄ emissions from drained peat soils; high methanotroph to methanogen ratios would indicate CH₄ sink properties. Our study shows the strength of quantitative metatranscriptomics; mRNA transcript abundance holds promising indicator to link soil microbiome functions to ecosystem-level processes.

Introduction

CH₄ is the second most important greenhouse gas, its global warming potential is 34 times higher than CO₂ [1]. More than two-thirds of global CH₄ emissions derive from microbial production in anoxic environments, such as wetland soils [2]. In past centuries, wetlands were commonly drained to gain agricultural land [3]. This lowers the water table, however, altering the physical properties of soils such as water content and oxygen availability. These altered soil properties, in turn, substantially affect the soil microbiota and activity and thus the soils' greenhouse gas fluxes [4].

CH₄-producing microbes (i.e., methanogens) are mostly anaerobic archaea that inhabit anoxic environments, e.g., wet rice paddies and wetland soils [2, 5]. Four types of methanogens can be characterized according to their substrate specificity. Acetoclastic methanogens utilize acetate, hydrogenotrophic methanogens utilize H₂/CO₂ and formate, and methylotrophic methanogens utilize methanol/methylamines to form CH₄ [5]. Recently, methoxydotrophic methanogens that utilize methoxylated aromatic compounds were proposed as a novel methanogenic group [6, 7]. In wetlands, acetoclastic and hydrogenotrophic methanogens are considered the predominant sources of CH₄ [5, 8]. However, recent research indicates that methanogenesis from methylated compounds also contributes to CH₄ emissions from wetlands [9, 10].

Up to 90% of CH₄ produced in oxygen-limited soils can be mitigated through oxidation by aerobic methanotrophs [11]. These methanotrophs are bacteria within the lineages *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* [12]. CH₄ oxidation can also be conducted anaerobically by bacteria in the NC10 phylum and archaea in the ANME group. Anaerobic oxidation of CH₄ couples the reduction of other electron acceptors such as nitrite (NC10) or nitrate (ANME-2d) or ferric iron to the oxidation of CH₄ [13–15]. Aerobic methanotrophs are considered the main oxidizers in wetland soils since alternative electron acceptors favoring anaerobic methanotrophs are often scarce in wetland soils. Besides their CH₄

filter function, methanotrophs provide the only known biological sink for atmospheric CH₄ [16]. However, it is not fully understood which microorganisms oxidize CH₄ at atmospheric concentrations in soils. Bacteria of upland soil clusters (USC- α) and USC- γ have been identified as likely important atmospheric CH₄ oxidizers in upland soils [12, 17, 18], while well-known methanotrophic lineages also account for CH₄ oxidation in anoxic paddy soils [19].

Methanogens and aerobic methanotrophs differ substantially in their oxygen and substrate preferences. Generally, wet and anoxic conditions favor methanogenesis, whereas dry and oxic conditions favor aerobic oxidation of CH₄. Drained peatland grasslands are highly dynamic across seasons in terms of water content and oxygen availability. Presumably, the interaction of methanogens and methanotrophs determines whether a wetland soil acts as a source or a sink for CH₄ [20]. Linking CH₄-cycling microbiome dynamics to CH₄ fluxes, especially at the transcriptional level, has seldom been achieved [21].

DNA- and RNA-based meta-omics techniques have provided insight into the microbiome compositions of soils. However, DNA is stable over the long-term; extracted soil DNA may therefore partially originate from persistent extracellular DNA of dead organisms [22, 23]. In contrast, ribosomal RNA (rRNA) acts as a proxy for ribosomes and can provide a more accurate picture of the active microorganism community composition in soils. Still, the validity of rRNA as an indicator for the microbial activity of soils is questionable. For instance, dormant cells can contain high loads of ribosomes [24, 25]. Hence, although rRNA is a good proxy for potential soil microbiome composition, it may not relate well to microbial activity and ecosystem processes. The simultaneous sequencing of mRNA and rRNA can overcome this issue [26] because messenger RNA (mRNA), can serve as a proxy for potential transcriptional activity. Other metatranscriptomic studies indicate that mRNA is more responsive to environmental factors than rRNA [27, 28]. We thus aim to explore differences between small subunit (SSU) rRNA and mRNA transcripts of the CH₄-cycling microbiomes and their links to

CH₄ fluxes. Furthermore, utilizing double RNA metatranscriptomics, it is possible to assign sequences to all three domains of life (bacteria, archaea, and eukaryotes) and to simultaneously assess their transcriptional activity [26].

Another drawback of meta-omics techniques is that they yield only relative abundances. However, the relationship between absolute abundances and relative abundances is not predictable [29]. It is thus hard to relate ecosystem processes to relative abundances. Studies have applied absolute quantification for metatranscriptomes in marine microbiomes [30, 31]. Recently, a quantification approach that uses total RNA to infer absolute from relative abundance was developed for metatranscriptomics [32]. This approach uses total RNA to infer absolute from relative abundance.

In this study, we aimed to link transcriptional dynamics of methane-cycling microbiomes to CH₄ fluxes in two drained peatland soils. We used quantitative metatranscriptomics to analyze ribosomal rRNA and mRNA [26, 32] of 60 soil samples from drained peatland taken from different soil depths during autumn, winter, spring and summer. In addition, we measured CH₄ and CO₂ net surface fluxes from the two sites. We aimed to (a) evaluate the RNA content of the soils as a marker for microbial activity, (b) examine the CH₄ fluxes of the two soils throughout a year, (c) study the composition and abundance of rRNA SSU and mRNA transcripts of CH₄-cycling microbes, (d) link microbiome composition of CH₄-cycling organisms to CH₄ fluxes in drained peatland soils across seasons.

Materials and Methods

Site description

The experiment was conducted in the framework of the Biodiversity Exploratories project for long-term functional ecosystem research [33]. Samples were taken at two grassland sites (LI and HI) located in the Biosphere Reserve „Schorfheide-Chorin“(Table S1). Both sites are drained peatlands with a histosolic soil type (according to WRB 2015 [34]). The upper 30 cm of the peat soils was highly degraded. The two sites differ in the intensity of grassland management; the low land-use intensity site (LI) was mowed once or twice a year, while the high land-use intensity site (HI) was grazed by cows and additionally mowed sometimes once a year. Vegetation on LI was dominated by *Poa trivialis* (60%) and *Alopecurus pratensis* (25%); vegetation on HI was dominated by *Poa pratensis* aggr. (32 %), *Trifolium repens* (15%) and *Agrostis stolonifera* (10%).

Soil Sampling

On each site, an area of 1 m x 7 m was sampled at all four seasons: autumn (11/09/2017), winter (03/08/2018), spring (05/30/2018), summer (09/13/2018). At each sampling date, three samples were taken between 12:00 and 13:00 at each site from the upper 10 cm and from the 20-30 cm layer. Each sample was a composite of soil cores from three locations. In spring, additional samples were taken at sunrise (05:00) and sunset (21:30). Samples for RNA, ammonium (NH_4^+), and nitrate (NO_3^-) extraction were immediately frozen at -80°C and subsequently stored as follows: RNA: -80°C , NH_4^+ , and NO_3^- -20°C . Samples for determination of C_{mic} , N_{mic} , pH, and soil water content were transported on ice and subsequently stored at -20°C . Redox potentials were measured with Mansfeld redox electrodes with an Ag/AgCl-reference electrode and a handheld ORP-meter GMH3531 (ecoTech, Bonn, Germany). For equilibration. the electrodes were placed in the soil 24 hours before sampling. Redox potentials were measured at soil depths of 5 cm and 25 cm.

Determination of soil properties

Gravimetric soil water content was determined by drying 3–6 g soil at 65 °C to constant weight. Soil pH was determined by mixing 10 g dried sieved soil with 25 ml 0.01 M CaCl₂ solution; pH of the suspension was then measured with a glass electrode (pH Electrode LE438, Mettler Toledo, Columbus, OH, USA). For total carbon and total nitrogen, samples were sieved (< 2 mm) and air-dried, ground in a ball mill (RETSCH MM200, Retsch, Haan, Germany), and analyzed in an elemental analyzer (VarioMax, Hanau, Germany) at 1100 °C. Inorganic carbon was determined with the same elemental analyzer after the organic carbon had been removed by combustion of soil samples at 450 °C for 16 h. Organic carbon concentration was calculated as the difference between total carbon and inorganic carbon. Microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) were determined by the chloroform-fumigation-extraction method (CFE), [35]. For this, frozen soils were thawed (at 4°C for 10 h), then 5 g field moist soils were fumigated with ethanol-free CHCl₃ for 24 hours in a desiccator. C and N were extracted with 40 ml 0.5 M K₂SO₄, shaken horizontally (30 Min, 150 rpm), and centrifuged (30 Min, 4400 g) to separate extract from the soil. Non-fumigated soil samples were treated identically. Aliquots of the extracts were dissolved (1:4 extract:deionized. H₂O) and measured on a TOC/TN analyzer (Multi N/C 2100S, Analytik Jena AG, Jena, Germany). A kEC factor [36] and a kEN factor [37] were used to calculate C_{mic} and N_{mic}, respectively. Extractable organic carbon (EOC) and extractable nitrogen (EN) concentrations were determined on the non-fumigated samples. Mineral nitrogen in the forms of ammonium (NH₄⁺) and nitrate (NO₃⁻) was determined in the non-fumigated, non-diluted extracts with an AutoAnalyzer 3 (Bran & Luebbe, Norderstedt, Germany).

Gas Fluxes

On each sampling date, gas emissions were measured 15 – 24 times throughout the day using the closed chamber method. Excessive vegetation was removed before pressing the stainless steel chambers (A = 150 cm², V = 1800 ml) into the soil [38]. Four chambers per sampling per

site were used. Gas samples (12 ml) were taken with syringes from the headspace immediately, 20, 40, and 60 minutes after closing the chambers via a three-way stopcock, and transferred into pre-evacuated exetainers (5.9 ml, Labco Lt, UK). Gas concentrations were measured on an Agilent 7890 gas chromatograph equipped with a flame ionization detector (for CH₄) coupled with a methanizer (for CO₂) (Agilent Technologies Inc., Santa Clara, CA, USA). Gas flux rates were calculated by the slope of the regression line of a linear regression of the gas concentration against time [17].

RNA extraction, library preparation, and sequencing

Total nucleic acids were extracted using a phenol/chloroform/isoamylalcohol protocol [26]. The extracts were subsequently treated with DNase to remove DNA (DNase I, Zymo Research, Freiburg, Germany). RNA concentrations were measured with the Qubit® RNA HS Assay Kit (Qubit®3.0 Fluorometer, Invitrogen, Waltham, MA, USA.). RNA extracts were cleaned with the MEGAclean kit (Thermo Fisher Scientific, Waltham, MA, USA); quality of the RNA was verified by agarose gel electrophoresis and bioanalyzer (2100 Bioanalyzer, Agilent, Santa Clara CA, USA). We enriched the mRNA fraction and diluted inhibitory substances in the RNA extracts using the MessageAmp™ II-Bacteria RNA Amplification Kit (Thermo Fisher Scientific, MA, USA, input: 12.5 ng RNA). This method was previously validated for the preparation of metatranscriptomes [39]. Sequencing libraries were prepared with NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA; input 60 ng). Manufacturer's instructions were followed except for Step 4, where fragmentation time was adjusted to 3 min and a size selection step with HighPrep™ PCR beads (MagBio Genomics Inc., Gaithersburg, USA) was introduced (desired insert size 250 bp). Libraries were paired-end sequenced with an Illumina Next Seq 550 System using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles) (Illumina, San Diego, CA, USA).

Bioinformatic processing and Statistics

Reverse and forward sequences were overlapped with a minimum overlap of 10 or 5 bp with FLASH [40]. The sequences were filtered to a minimum mean quality score of 25 with PrinseqLite [41]. Sequences were then sorted into SSU, LSU, and non-rRNA fractions with SortMeRNA [42]. The SSU fraction was randomly subsampled to 200000 sequences with USEARCH [43]. Sequences were taxonomically classified against the SilvaMod128 databases [44] with BlastN [45] using a lowest common ancestor (LCA) algorithm in MEGAN (min score 155; top percent 2.0; min support 1, [46]). The non-rRNA fraction was aligned against the NC_nr database (retrieved 12/03/2020) with Diamond [47]. The sequences were taxonomically and functionally aligned with LCA in MEGAN (2011, min score 155; top percent 4; min support 1, [46]). Absolute abundances were calculated from read counts according to Söllinger *et al.* [32]. At the mRNA level, methanogenesis transcripts refer to sequences assigned to the SEED category “methanogenesis” (with subcategory “methanogenesis from methylated compounds”). Methanotrophy transcripts refer to sequences assigned to the SEED category “Particulate methane monooxygenase (pMMO)”. To classify *pmoA* sequences, the non-rRNA fraction was blasted against a *pmoA* database [48] and taxonomically classified with MEGAN as described in reference [48].

Statistical analyses were performed in R [49]. Distance based redundancy analysis was performed on the Bray Curtis dissimilarity matrix read counts of the 60 samples (function “dbrda” in the vegan package). Counts were Hellinger-transformed beforehand. We tested the following parameters: site (HI; LI), depth (“0-10 cm”, “20-30 cm”), season (“autumn”, “winter”, “spring”, “summer”), temperature, water content, nitrite, and nitrate. Continuous variables were z-scaled. Statistically significant categories between seasons and depths (within sites) were assessed by ANOVA and subsequent post-hoc Tukey test or multiple comparison test after Kruskal-Wallis at $p < 0.05$ level.

Results and discussion

Net surface gas fluxes have similar patterns across the year at both grassland sites

CH₄ and CO₂ fluxes were highly dynamic across the year (Figure 1). While the soils emitted CH₄ in autumn and winter, they took up CH₄ in spring and summer (Figure 1a). CO₂ fluxes showed an opposite trend, with higher CO₂ emissions in spring and summer than in autumn and winter (Figure 1b). The opposing trends of CO₂ and CH₄ fluxes reflected the changes in soil physicochemical properties across the year (Table S2). High water content and low redox potentials in autumn and winter favored anaerobic microbial processes, such as methanogenesis, while at the same time hampering aerobic microbial processes such as respiration. In contrast, soils had lower water content and positive redox potential in spring and summer, favoring aerobic respiration over anaerobic degradation processes. Generally, mean CO₂ net surface emissions were about 1.5 times higher than IPCC default emission factors [50, 51]. Relatively high CO₂ emissions have also been reported in other drained, nutrient-rich grasslands in Germany [52]. Our observed higher emissions may have been due to the highly degraded peat at the studied site. Soils with highly disturbed peat have been reported to have higher CO₂ emissions than less degraded peat soils [52].

In contrast, net surface CH₄ emissions rates in autumn and winter were lower compared to IPCC default emission factors [50, 51]. However, we measured emissions at only four sampling times and may have not accounted for high emissions after heavy rainfall events. Net CH₄ uptake rates in spring and summer were in the range of other grasslands and temperate ecosystems [53] and even slightly higher than in pastures [54]. In 2018, weather in spring and summer was dry and hot compared to the long-term average, leading to low soil water content (Table S2), favoring CH₄ oxidation. The soil water content of the upper layer was mostly within the optimal range for atmospheric CH₄ oxidation [55].

Our results underscore the highly variable greenhouse gas emissions from temperate drained peatlands and their dependence on dynamic soil physicochemical properties, clearly linked to seasons. Moreover, such sites can be net sinks for CH₄ as well as net sources. This versatility regarding CH₄ and source functions needs to be accounted for when estimating global carbon budgets.

Linking metatranscriptomics and microbial biomass

We quantified total RNA content in the soils as a proxy for microbial biomass (Figure 2a). As expected, in most seasons the topsoils contained more RNA than the subsoils, especially the LI soil. The RNA content varied between seasons and was highest in winter and autumn in the topsoil and subsoil, respectively. Generally, RNA content was positively correlated with water content ($r = 0.52$, $p < 0.001$). Total RNA per gram soil was positively correlated with microbial biomass carbon and nitrogen ($r_{\text{Cmic}}^2 = 0.29$, $r_{\text{Nmic}}^2 = 0.46$, $p < 0.001$, figure 2). Interestingly, RNA content correlated better with N_{mic}, likely due to the high N content of the RNA. This finding supports the validity of RNA as a proxy for living microorganisms and enables the use of RNA content to infer absolute transcript abundances from relative abundances [32]. Through this quantitative approach, we overcame challenges typically associated with the interpretation of relative abundance in ‘meta-omics’ datasets. A recent study used this quantitative approach and found that absolute transcript abundance correlated better to processes than relative transcript frequencies [32].

Spatio-temporal dynamics in methane-cycling (micro-)biome

The relative abundances (RAs) of SSU rRNA transcripts of the three domains ranged from 54.6% to 95.4% for Bacteria, 0.7% to 10.1% for archaea, and 1.7% to 41.3% for Eukaryotes (Table S3 and Table S4). The community composition of all taxa in the soil samples exhibited a clear site- and depth-specific pattern (Figure 3a). The tested parameters explained 52.7% of the variance in the community composition of all taxa (Table S2). Site and depth had the

greatest explanatory power (20.0% and 19.6% respectively, $p < 0.001$, Table S4). Site-specific differences could likely be attributed to site-specific soil properties, such as pH, texture, organic carbon, and nitrogen content (Table S1). Depth is generally considered to be associated with differences in oxygen and nutrient availability. Season accounted for 6.0% of the variation ($p < 0.01$). This effect likely resulted from varying precipitation, water table depth, and plant growth activity throughout the year.

We further identified and quantified three different groups of CH₄-cycling microorganisms in the SSU sequences: (i) methanogenic archaea (RA: 0.01% to 1.99%), (ii) aerobic methanotrophic bacteria (RA: 0.02% to 1.39%), and (iii) anaerobic methanotrophic archaea and bacteria (RA: 0% to 0.12%, Table S3 and Table S4). When considering only CH₄-cycling taxa (total of 287 identified taxa), the tested parameters explained 36.1% of the variance in community composition (Table S5). Site had the most explanatory power (14.0%, $p < 0.001$), whereas depth, season, and water content accounted for 5.7% ($p < 0.001$), 6.5% ($p < 0.001$), and 5.3% ($p < 0.001$) of the variance, respectively. The water content of soils directly affects O₂ availability, which, in turn, is a fundamental factor shaping CH₄-cycling community composition. O₂ diffuses more slowly in water-saturated soils, which thus have lower O₂ availability [56].

Methanogen community composition influences transcriptional activity

Methane formation is a complex process involving several functional guilds that metabolically interact closely with each other [20]. We integrated the total RNA content and metatranscriptomes to infer SSU rRNA and mRNA transcript abundances per gram soil [32]. Most methanogen families in the soils were class II methanogens, e.g., *Methanosarcinaceae*, *Methanosaetaceae* (now *Methanotrichaceae*; Figure 4). Generally, these families possess more antioxidant features than class I methanogens [57]. The predominance of class II methanogens reflected the dynamic water and redox status in the studied soils (Table S2). Despite comparable

CH₄ emissions at both sites, the abundance of methanogens was 4.6 times higher in the soils of high land use intensity (HI) compared with that of low land use intensity (LI) (Figure 4a,b, $p < .001$). Interestingly, methanogenesis-related transcript abundances were not statistically different between sites (Figure 5a), suggesting lower transcriptional activity of the methanogens in the low land-use intensity (LI) grassland soil. The incongruity between methanogen SSU rRNA abundances and CH₄ fluxes at the SSU level may also be attributable to differences in taxonomic compositions of methanogens. The strictly acetoclastic *Methanosaetaceae* (*Methanothrix*) were about 30 times more abundant in HI than in LI (Figure 4a, $p < 0.01$), especially in the deeper soil layer. *Methanosaeta* have lower growth rates but can grow at lower acetate concentrations than the metabolically diverse *Methanosarcina* [58]. Lower growth rates may lead to lower transcriptional activity and lower CH₄ emissions. *Methanosaeta* often outcompete other acetoclastic methanogens at low acetate concentrations [58, 59]. Thus, the higher proportion of *Methanosaetaceae* may have indicated lower acetate availability in HI. The lower acetate availability in HI could thus have resulted in observed lower CH₄ emissions despite the higher methanogen abundances compared with LI. Interestingly, the share of hydrogenotrophic methanogens, such as *Methanocellaceae*, *Methanoregulaceae* and *Methanobacteriaceae*, was higher in LI than in HI. The energy yield of hydrogenotrophic methanogenesis is larger than that of acetoclastic methanogenesis, which has the lowest energy yield of all methanogenic metabolisms [5, 60]. The varying proportions of acetoclastic and hydrogenotrophic methanogens and lower substrate availabilities at the two sites may explain our observed differences between CH₄ emissions and methanogen abundances at the SSU level. Our results suggest that not solely the abundances but also the composition of methanogens influence net surface CH₄ fluxes of soils.

Methanogenesis from methylated compounds is the predominant source of CH₄ in marine environments and its contribution to the global CH₄ budget is considered to be small [5]. However, recent research suggests that it might be more important than previously assumed [9,

61–63]. The presence of *Methanomassiliicoccales* (up to 14% of the total methanogen community in a winter topsoil sample), and especially *Methanosarcinaceae*, points to methylated compounds as a substrate for methanogenesis at our sites (Figure 4a,b [62]). In support of this, the portion of mRNAs for methanogenesis from methylated compounds was up to 50% of all methanogenesis transcripts in some datasets (Figure 5a). Methanogenesis from methylated compounds in wetlands may thus be more important than previously thought [6, 9, 61–63].

High methanotrophic diversity in the studied grassland soils

We detected aerobic and anaerobic methanotrophs belonging to *Methylococcaceae*, *Crenotrichaceae*, *Methylocystaceae* (aerobic), and NC10 and ANME (anaerobic) in all microbiomes (Figure 4c,d). Our studied sites had highly dynamic soil properties and fluctuating water levels throughout the year. Therefore, the soil provided versatile niches for different kinds of methanotrophs with distinct demands on CH₄ and O₂ levels. The majority of the detected methanotrophs belonged to the obligate methanotrophic *Methylococcaceae*. Normalized to all methanotrophic taxa, *Methylocystaceae* was more abundant in HI than in LI, while *Crenotrichaceae* were more abundant in LI ($p < 0.05$).

Anaerobic methanotrophs

Anaerobic methanotrophs were present mainly in the deeper soil layer, which was likely due to their sensitivity to oxygen [64]. Generally, there were more aerobic than anaerobic methanotrophs (Figure 4c,d). However, anaerobic methanotrophs accounted for up to 20% of all methanotrophs and consequently comprised a substantial part of the methanotroph community at the studied sites. Another study found a similar proportion of anaerobic methanotrophs (5.7–25.8%) in the upper 10 cm of urban wetlands [65]. Zhong *et al.* [66] found that the relative abundance of anaerobic methanotrophs in wetland soils was highest at the 50–60 cm depth. We investigated only to a depth of 30 cm. The proportion of NC10 with respect to all methanotrophs may have been even higher in the less oxygen-influenced deeper soil layer.

However, redox potential alone cannot explain the distribution of NC10 in our study since abundances of NC10 showed divergent trends at the two sites. In HI, their abundance was highest in summer, despite rising redox potentials. This increase may have been due to the increase in soil nitrate content in spring and summer, probably a result of grazing and subsequent mineralization and N release from feces.

Predominant anaerobic methanotrophs were the nitrite-dependent NC10 bacteria, especially *Candidatus* “Methyloirabilis” (Figure 4c,d). They have also been identified as the most relevant anaerobic CH₄ oxidizer group in freshwater marsh sediments [67]. Our results indicate *Ca. Methyloirabilis* is also the main anaerobic methanotroph in drained peatlands. We found archaeal CH₄ oxidizers to be absent at most seasons and highest in abundance in spring in HI (Figure 4c,d), which coincides with a nitrate increase in this soil (Table S2). Another study found archaeal CH₄ oxidizers more abundant than NC10 bacteria in a fertilized paddy soil [68]. Thus, high nitrate content or nitrogen input may favor archaeal over bacterial anaerobic CH₄ oxidizers. Our results suggest that NC10 is a relevant methanotrophic group mitigating CH₄ emissions from drained peatlands and that oxygen and substrate availability are important factors influencing their abundance.

Methanotrophs as CH₄ filter and CH₄ sink

Methanotrophs can oxidize CH₄ at atmospheric and elevated CH₄ concentrations [12]. In our study, mRNA methanogenesis transcripts correlated with PMO transcripts ($r = 0.62$, $p < 0.05$, Figure 6d). This relationship suggests that methanotrophs use CH₄ derived from methanogenesis in the soil. They thus act as a filter mitigating CH₄ emission to the atmosphere. In spring and summer, the studied sites were a CH₄ sink, suggesting that the detected methanotrophs metabolized CH₄ at atmospheric concentrations and below. Which organisms are responsible for atmospheric CH₄ oxidation in soils remains unresolved since until recently atmospheric CH₄ oxidizers resisted cultivation. However, atmospheric CH₄ consumption in upland soils is assumed to be performed by bacteria of Upland soil clusters (USC) α and γ [12,

18]. The first isolated USC α methanotroph, *Methylocapsa gorgona*, can grow at both atmospheric and elevated CH₄ concentrations [69]. Furthermore, *Methylocystis* strains can also oxidize atmospheric CH₄ [70]. We did not detect an increase in known atmospheric CH₄ oxidizers in the SSU transcripts. However, the proportion of mRNA transcripts belonging to USC α and γ increased in spring and summer (Figure 6). This supports the proposal that both groups are involved in atmospheric CH₄ oxidation in temporarily wet grassland soils. Additionally, the relative abundance of *pmoA2* transcripts increased towards summer. *pmoA2* is part of the *pmoCAB2* operon, which encodes a pMMO capable of oxidizing CH₄ at atmospheric levels [70]. Many type II methanotrophs contain *pmoA2* [71]. These groups could therefore also be responsible for atmospheric CH₄ oxidation in the studied soils. The relative abundance of USC α and γ *pmoA* transcripts and *pmoA2* was up to 34%. Still, other type-I and type-II *pmoA* sequences dominated in the soils. Thus, also type I and type II methanotrophs may be involved in atmospheric CH₄ oxidation. Recently, high-affinity CH₄ oxidation in paddy soils was attributed to well-known CH₄ oxidizers rather than USC α and γ [19]. In a former study, we found USC α and γ *pmoA* gene abundances to be explanatory factors for atmospheric CH₄ oxidation in grassland soils of three different regions of Germany, including the Schorfheide region investigated in this study [17]. Despite the wide distribution of USC α and γ sequences, *pmoA* sequences detectable with A189f/mb661 primers were absent in most regions except for the Schorfheide grasslands. The A189f/mb661 primer pair amplifies a broad range of well-known *pmoA* sequences but discriminates USC α and γ sequences [72]. The presence of these well-known sequences in the Schorfheide was likely due to the hydromorphic character of the soils. This region contains many drained peatland soils with internal CH₄ formation by methanogens. It is possible that different groups contribute to atmospheric CH₄ oxidations in soils with internal CH₄ formation (such as the drained peatlands investigated here), as compared to upland soils that are permanent net sinks of CH₄. Possibly, USC α and γ methanotrophs are the predominant atmospheric CH₄ oxidizers in such soils. In contrast, in soils

with internal CH₄ formation, even only in some seasons, other methanotrophs could also contribute to atmospheric CH₄ oxidation, as suggested in [19].

Functional transcript abundances as a proxy for soil net surface CH₄ fluxes

Messenger RNAs of methanogenesis pathways were less abundant per gram soil in spring and summer than in autumn and winter at both sites and depths (Figure 5a). This drop in mRNA agrees with the cessation of CH₄ emissions from the soils in spring in summer; both correlated significantly with each other ($r = 0.82$, $p < 0.05$, Figure 7b). In contrast, abundances of methanogen SSU transcripts and CH₄ fluxes did not significantly correlate within either plot (Figure 7a). However, there was a site-specific trend. Our results indicate that quantified methanogenesis mRNA transcripts were better indicators of net CH₄ fluxes than methanogen SSU rRNA transcripts. Our findings thus underscore studies that have found mRNA more responsive to environmental factors than rRNA [27, 28].

Methanotrophs are important CH₄ filters, mitigating soil CH₄ emissions. The pMMO to methanogenesis mRNA ratio was higher in spring and summer than in autumn and winter (Figure 7f). However, the ratio of methanotroph to methanogen SSU transcripts did not permit conclusions about the soils' CH₄ fluxes (Figure 7e). The mRNA ratio may thus indicate whether soils are a CH₄ source or sink. A high methanotroph to methanogen ratio indicates the soil serves as a CH₄ sink, while a low ratio indicates the soil serves as a net CH₄ source. Yet it is necessary to consider not only SSU rRNA transcripts but also mRNA transcripts. Furthermore, community composition could be an additional indicator, since a high proportion of atmospheric CH₄ oxidizers were linked to net CH₄ uptake of the soils.

Additionally, our results may suggest that abundance of methanogenesis related mRNAs is an estimator of soil CH₄ fluxes. in soils with a low methanotroph to methanogen ratio. We used RNA sequencing, which is still restricted in terms of throughput. Two RT-qPCR studies found a relationship between *mcrA* transcript abundances and CH₄ fluxes in a paddy soil and a peat

bog [73, 74]. RT qPCR might thus be a tool to estimate CH₄ fluxes from *mcrA* transcript abundances in combination with *pmoA* transcript abundances. However, large-scale studies are needed to further investigate the link between methanogen and methanotroph transcripts and CH₄ fluxes across different soil types and seasons.

Conclusions

CH₄-cycling microbes interact with each other on a process level. These interactions among anaerobic archaea and aerobic and anaerobic bacteria determine the net surface CH₄ fluxes from soils. This study is, to our knowledge, the first to use quantitative metatranscriptomics to link CH₄ fluxes from grasslands with CH₄-cycling microbiomes through all seasons of the year. We investigated two drained peatland soils with highly variable soil physicochemical properties, an associated dynamic microbial community composition, and net surface CH₄ fluxes in both directions, at the same sites through the year. It is crucial to account for the observed ambiguous behavior of such drained wetlands when estimating the CH₄ budgets of grassland ecosystems in regional and global surveys. We further validated that mRNA transcripts rather than SSU transcripts are necessary to link microbial activity to soil net CH₄ fluxes. Particulate MMO transcripts were correlated to mRNA of methanogenesis pathways, indicating that methanotrophs acted as CH₄ filters. Net CH₄ uptake was linked to an increase in proportions of USCa and γ and *pmoA2* transcripts, supporting evidence that these groups are involved in atmospheric CH₄ oxidation. Messenger RNA of methanogenesis pathways correlated with net CH₄ fluxes. It may thus be feasible to estimate soil CH₄ fluxes using *mcrA* transcript abundances, additionally considering *pmoA* transcripts. Our results suggest that the ratio of *pmoA* to *mcrA* transcripts is an easily applicable estimator of whether a soil acts as a sink or source of CH₄. However, large-scale and long-term studies are needed to evaluate and to elaborate this topic further.

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Conflict of interest

All authors declare no conflict of interest.

References

1. Myhre, Shindell, G. D, Breon F-M, Collins W, Fuglestedt J, Huang J, et al. Anthropogenic and natural radiative forcing. In: Stocker TF, Qin D, Plattner G-K, Tignor M, Allen SK, Boschung J, et al. (eds). *Climate Change 2013 the Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. 2013. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA., pp 659–740.
2. Conrad R. Minireview The global methane cycle : recent advances in understanding the microbial processes involved. *Environ Microbiol Rep* 2009; **1**: 285–292.
3. Lamentowicz M, Gałka M, Pawlyta J, Lamentowicz Ł, Goslar T, Miotk-Szpiganowicz G. Climate change and human impact in the southern Baltic during the last millennium reconstructed from an ombrotrophic bog archive. *Stud Quat* 2011; **28**: 3–16.
4. Oertel C, Matschullat J, Zurba K, Zimmermann F, Erasmí S. Greenhouse gas emissions from soils—A review. *Chemie der Erde* 2016; **76**: 327–352.
5. Lyu Z, Shao N, Akinyemi T, Whitman WB. Methanogenesis. *Curr Biol* 2018; **28**: R727–R732.
6. Kurth JM, Nobu MK, Tamaki H, de Jonge N, Berger S, Jetten MSM, et al. Methanogenic archaea use a bacteria-like methyltransferase system to demethoxylate aromatic compounds. *ISME J* 2021.
7. Mayumi D, Mochimaru H, Tamaki H, Yamamoto K, Yoshioka H, Suzuki Y, et al. Methane production from coal by a single methanogen. *Science (80-)* 2016; **354**: 222–226.
8. Bridgham SD, Cadillo-Quiroz H, Keller JK, Zhuang Q. Methane emissions from wetlands: Biogeochemical, microbial, and modeling perspectives from local to global scales. *Glob Chang Biol* 2013; **19**: 1325–1346.
9. Narroze AB, Borton MA, Hoyt DW, Smith GJ, Daly RA, Angle JC, et al. Uncovering the Diversity and Activity of Methylophilic Methanogens in Freshwater Wetland Soils. *mSystems* 2019; **4**: 1–15.
10. Zalman CA, Meade N, Chanton J, Kostka JE, Bridgham SD, Keller JK. Methylophilic methanogenesis in Sphagnum-dominated peatland soils. *Soil Biol Biochem* 2018; **118**: 156–160.
11. Le Mer J, Roger P. Production, oxidation, emission and consumption of methane by soils: A review. *Eur J Soil Biol* 2001; **37**: 25–50.
12. Knief C. Diversity and habitat preferences of cultivated and uncultivated aerobic methanophilic bacteria evaluated based on pmoA as molecular marker. *Front Microbiol* 2015; **6**, 1346.
13. Welte CU, Rasigraf O, Vaksmaa A, Versantvoort W, Arshad A, Op den Camp HJM, et al. Nitrate- and nitrite-dependent anaerobic oxidation of methane. *Environ Microbiol Rep* 2016; **8**: 941–955.
14. Cui M, Ma A, Qi H, Zhuang X, Zhuang G. Anaerobic oxidation of methane: An ‘active’ microbial process. *Microbiologyopen* 2015; **4**: 1–11.
15. Ettwig KF, Zhu B, Speth D, Keltjens JT, Jetten MSM, Kartal B. Archaea catalyze iron-dependent anaerobic oxidation of methane. *Proc Natl Acad Sci U S A* 2016; **113**: 12792–12796.
16. Ciais P, Sabine C, Bala G, Bopp L, Brovkin V, Canadell J, et al. Carbon and Other Biogeochemical Cycles. In: Stocker TF, Qin D, Plattner G-K, Tignor M, Allen SK, Boschung J, et

- 505 al. (eds). *Climate Change 2013 the Physical Science Basis: Working Group I Contribution to the*
506 *Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. 2013. Cambridge
507 University Press, Cambridge, United Kingdom and New York, NY, USA, Cambridge, United
508 Kingdom and New York, NY, USA., pp 465–570.
- 509 17. Täumer J, Kolb S, Boeddinghaus RS, Wang H, Schöning I, Schrumpf M, et al. Divergent drivers
510 of the microbial methane sink in temperate forest and grassland soils. *Glob Chang Biol* 2021;
511 **27**: 929–940.
- 512 18. Kolb S. The quest for atmospheric methane oxidizers in forest soils. *Environ Microbiol Rep*
513 2009; **1**: 336–346.
- 514 19. Cai Y, Zheng Y, Bodelier PLE, Conrad R, Jia Z. Conventional methanotrophs are responsible for
515 atmospheric methane oxidation in paddy soils. *Nat Commun* 2016; **7**: 1–10.
- 516 20. Dean JF, Middelburg JJ, Röckmann T, Aerts R, Blauw LG, Egger M, et al. Methane Feedbacks to
517 the Global Climate System in a Warmer World. *Rev Geophys* 2018; **56**: 207–250.
- 518 21. Levy-Booth DJ, Giesbrecht IJW, Kellogg CTE, Heger TJ, D’Amore D V., Keeling PJ, et al. Seasonal
519 and ecohydrological regulation of active microbial populations involved in DOC, CO₂, and CH₄
520 fluxes in temperate rainforest soil. *ISME J* 2019; **13**: 950–963.
- 521 22. Lombard N, Prestat E, van Elsas JD, Simonet P. Soil-specific limitations for access and analysis
522 of soil microbial communities by metagenomics. *FEMS Microbiol Ecol* 2011; **78**: 31–49.
- 523 23. Carini P, Marsden PJ, Leff JW, Morgan EE, Strickland MS, Fierer N. Relic DNA is abundant in
524 soil and obscures estimates of soil microbial diversity. *Nat Microbiol* 2016; **2**:
525 <https://doi.org/10.1038/nmicrobiol.2016.242>.
- 526 24. Blazewicz SJ, Barnard RL, Daly RA, Firestone MK. Evaluating rRNA as an indicator of microbial
527 activity in environmental communities: Limitations and uses. *ISME J* 2013; **7**: 2061–2068.
- 528 25. Sukenik A, Kaplan-Levy RN, Welch JM, Post AF. Massive multiplication of genome and
529 ribosomes in dormant cells (akinetes) of *Aphanizomenon ovalisporum* (Cyanobacteria). *ISME J*
530 2012; **6**: 670–679.
- 531 26. Urich T, Lanzén A, Qi J, Huson DH, Schleper C, Schuster SC. Simultaneous assessment of soil
532 microbial community structure and function through analysis of the meta-transcriptome. *PLoS*
533 *One* 2008; **3**.
- 534 27. Peng J, Wegner CE, Liesack W. Short-term exposure of paddy soil microbial communities to
535 salt stress triggers different transcriptional responses of key taxonomic groups. *Front*
536 *Microbiol* 2017; **8**: 1–12.
- 537 28. Peng J, Wegner CE, Bei Q, Liu P, Liesack W. Metatranscriptomics reveals a differential
538 temperature effect on the structural and functional organization of the anaerobic food web in
539 rice field soil. *Microbiome* 2018; **6**: 1–16.
- 540 29. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome datasets are
541 compositional: And this is not optional. *Front Microbiol* 2017; **8**: 1–6.
- 542 30. Moran MA, Satinsky B, Gifford SM, Luo H, Rivers A, Chan LK, et al. Sizing up
543 metatranscriptomics. *ISME J* 2013; **7**: 237–243.
- 544 31. Gifford SM, Sharma S, Rinta-Kanto JM, Moran MA. Quantitative analysis of a deeply
545 sequenced marine microbial metatranscriptome. *ISME J* 2011; **5**: 461–472.
- 546 32. Söllinger A, Tveit AT, Poulsen M, Noel SJ, Bengtsson M, Bernhardt J, et al. Holistic Assessment
547 of Rumen Microbiome Dynamics through Quantitative Metatranscriptomics Reveals

548 Multifunctional Redundancy during Key Steps of Anaerobic Feed Degradation. *mSystems*
549 2018; **3**: 1–19.

550 33. Fischer M, Bossdorf O, Gockel S, Hänsel F, Hemp A, Hessenmöller D, et al. Implementing large-
551 scale and long-term functional biodiversity research: The Biodiversity Exploratories. *Basic Appl*
552 *Ecol* 2010; **11**: 473–485.

553 34. FAO. World reference base for soil resources 2014. International soil classification system for
554 naming soils and creating legends for soil maps. *World Soil Resour Reports No 106* . 2015.
555 FAO, Rome. , **106**

556 35. Vance ED, Brookes PC, Jenkinson DS. An extraction method for measuring soil microbial
557 biomass C. *Soil Biol Biochem* 1987; **19**: 703–707.

558 36. Joergensen RG, Mueller T. The fumigation-extraction method to estimate soil microbial
559 biomass: calibration of the kEN value. *Soil Biol Biochem* 1996; **28**: 33–37.

560 37. Brookes PC, Landman A, Pruden G, Jenkinson DS. Chloroform fumigation and the release of
561 soil nitrogen: A rapid direct extraction method to measure microbial biomass nitrogen in soil.
562 *Soil Biol Biochem* 1985; **17**: 837–842.

563 38. Koch O, Tschirko D, Kandeler E. Seasonal and diurnal net methane emissions from organic
564 soils of the Eastern Alps, Austria: Effects of soil temperature, water balance, and plant
565 biomass. *Arctic, Antarct Alp Res* 2007; **39**: 438–448.

566 39. Tveit AT, Urich T, Svenning MM. Metatranscriptomic analysis of arctic peat soil microbiota.
567 *Appl Environ Microbiol* 2014; **80**: 5761–5772.

568 40. Magoč T, Salzberg SL. FLASH: Fast length adjustment of short reads to improve genome
569 assemblies. *Bioinformatics* 2011; **27**: 2957–2963.

570 41. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets.
571 *Bioinformatics* 2011; **27**: 863–864.

572 42. Kopylova E, Noé L, Touzet H. SortMeRNA: Fast and accurate filtering of ribosomal RNAs in
573 metatranscriptomic data. *Bioinformatics* 2012; **28**: 3211–3217.

574 43. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;
575 **26**: 2460–2461.

576 44. Lanzén A, Jørgensen SL, Huson DH, Gorfer M, Grindhaug SH, Jonassen I, et al. CREST -
577 Classification Resources for Environmental Sequence Tags. *PLoS One* 2012; **7**.

578 45. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol*
579 *Biol* 1990; **215**: 403–410.

580 46. Huson DH, Beier S, Flade I, Górski A, El-Hadidi M, Mitra S, et al. MEGAN Community Edition -
581 Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. *PLoS*
582 *Comput Biol* 2016; **12**: 1–12.

583 47. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat*
584 *Methods* 2014; **12**: 59–60.

585 48. Dumont MG, Lüke C, Deng Y, Frenzel P. Classification of pmoA amplicon pyrosequences using
586 BLAST and the lowest common ancestor method in MEGAN. *Front Microbiol* 2014; **5**: 1–11.

587 49. R Core Team. R: A Language and Environment for Statistical Computing. 2018. R Foundation
588 for Statistical Computing, Vienna, Austria.

589 50. Günther A, Barthelme A, Huth V, Joosten H, Jurasinski G, Koebisch F, et al. Prompt rewetting

590 of drained peatlands reduces climate warming despite methane emissions. *bioRxiv* 2019; 1–
591 14.

592 51. IPCC Task Force on National Greenhouse Gas Inventories. Methodological Guidance on Lands
593 with Wet and Drained Soils, and Constructed Wetlands for Wastewater Treatment. *2013*
594 *Supplement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories: Wetlands* .
595 2014.

596 52. Tiemeyer B, Albiac Borraz E, Augustin J, Bechtold M, Beetz S, Beyer C, et al. High emissions of
597 greenhouse gases from grasslands on peat and other organic soils. *Glob Chang Biol* 2016; **22**:
598 4134–4149.

599 53. Dutaur L, Verchot L V. A global inventory of the soil CH₄ sink. *Global Biogeochem Cycles* 2007;
600 **21**: n/a-n/a.

601 54. McDaniel MD, Saha D, Dumont MG, Hernández M, Adams MA. The Effect of Land-Use Change
602 on Soil CH₄ and N₂O Fluxes: A Global Meta-Analysis. *Ecosystems* 2019; **22**: 1424–1443.

603 55. Gulledge J, Schimel JP. Moisture control over atmospheric CH₄ consumption and CO₂
604 production in diverse Alaskan soils. *Soil Biol Biochem* 1998; **30**: 1127–1132.

605 56. Conrad R. Methane Production in Soil Environments—Anaerobic Biogeochemistry and
606 Microbial Life between Flooding and Desiccation. *Microorganisms* 2020; **8**: 881.

607 57. Lyu Z, Lu Y. Metabolic shift at the class level sheds light on adaptation of methanogens to
608 oxidative environments. *ISME J* 2018; **12**: 411–423.

609 58. Smith KS, Ingram-Smith C. Methanosaeta, the forgotten methanogen? *Trends Microbiol* 2007;
610 **15**: 150–155.

611 59. Angle JC, Morin TH, Solden LM, Narrowe AB, Smith GJ, Borton MA, et al. Methanogenesis in
612 oxygenated soils is a substantial fraction of wetland methane emissions. *Nat Commun* 2017;
613 **8**: 1567.

614 60. Whitman WB, Bowen TL, Boone DR. The Methanogenic Bacteria. In: Rosenberg E, DeLong EF,
615 Lory S, Stackebrandt E, Thompson F (eds). *The Prokaryotes: Other Major Lineages of Bacteria*
616 *and The Archaea*. 2014. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 123–163.

617 61. Conrad R. Importance of hydrogenotrophic, acetoclastic and methylotrophic methanogenesis
618 for methane production in terrestrial, aquatic and other anoxic environments: A mini review.
619 *Pedosphere* 2020; **30**: 25–39.

620 62. Söllinger A, Urich T. Methylotrophic methanogens everywhere — physiology and ecology of
621 novel players in global methane cycling. *Biochem Soc Trans* 2019; **47**: 1895–1907.

622 63. Tveit AT, Urich T, Frenzel P, Svenning MM. Metabolic and trophic interactions modulate
623 methane production by Arctic peat microbiota in response to warming. *Proc Natl Acad Sci U S*
624 *A* 2015; **112**: E2507–E2516.

625 64. Luesken FA, Wu ML, Op den Camp HJM, Keltjens JT, Stunnenberg H, Francoijs KJ, et al. Effect
626 of oxygen on the anaerobic methanotroph ‘*Candidatus Methyloirabilis oxyfera*’: Kinetic and
627 transcriptional analysis. *Environ Microbiol* 2012; **14**: 1024–1034.

628 65. Chen S, Chen J, Chang S, Yi H, Huang D, Xie S, et al. Aerobic and anaerobic methanotrophic
629 communities in urban landscape wetland. *Appl Microbiol Biotechnol* 2018; **102**: 433–445.

630 66. Zhong Q, Xue D, Chen H, Liu L, He Y, Zhu D, et al. Structure and distribution of nitrite-
631 dependent anaerobic methane oxidation bacteria vary with water tables in Zoige peatlands.
632 *FEMS Microbiol Ecol* 2020; **96**: 1–12.

- 633 67. Shen L dong, Wu H sheng, Liu X, Li J. Cooccurrence and potential role of nitrite- and nitrate-
634 dependent methanotrophs in freshwater marsh sediments. *Water Res* 2017; **123**: 162–172.
- 635 68. Vaksmaa A, Lüke C, Van Alen T, Valè G, Lupotto E, Jetten MSM, et al. Distribution and activity
636 of the anaerobic methanotrophic community in a nitrogen-fertilized Italian paddy soil. *FEMS*
637 *Microbiol Ecol* 2016; **92**: 1–11.
- 638 69. Tveit AT, Hestnes AG, Robinson SL, Schintlmeister A, Dedysh SN, Jehmlich N, et al. Widespread
639 soil bacterium that oxidizes atmospheric methane. *Proc Natl Acad Sci* 2019; **116**: 8515–8524.
- 640 70. Baani M, Liesack W. Two isozymes of particulate methane monooxygenase with different
641 methane oxidation kinetics are found in *Methylocystis* sp. strain SC2. *Proc Natl Acad Sci* 2008;
642 **105**: 10203–10208.
- 643 71. Yimga MT, Dunfield PF, Ricke P, Heyer J, Liesack W. Wide distribution of a novel pmoA-like
644 gene copy among type II methanotrophs, and its expression in *Methylocystis* strain SC2. *Appl*
645 *Environ Microbiol* 2003; **69**: 5593–5602.
- 646 72. Dumont MG. Primers: Functional Marker Genes for Methylootrophs and Methanotrophs. In:
647 McGenity TJ, Timmis KN, Nogales B (eds). *Hydrocarbon and Lipid Microbiology Protocols -*
648 *Springer Protocols Handbooks*. 2015. Springer Verlag Berlin Heidelberg, pp 1–21.
- 649 73. Freitag TE, Toet S, Ineson P, Prosser JI. Links between methane flux and transcriptional
650 activities of methanogens and methane oxidizers in a blanket peat bog. *FEMS Microbiol Ecol*
651 2010; **73**: 157–165.
- 652 74. Qin H, Tang Y, Shen J, Wang C, Chen C, Yang J, et al. Abundance of transcripts of functional
653 gene reflects the inverse relationship between CH₄ and N₂O emissions during mid-season
654 drainage in acidic paddy soil. *Biol Fertil Soils* 2018; **54**: 885–895.

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Figure captions

Figure 1. Net surface gas fluxes over the year. Gas fluxes of CH₄ (a) and CO₂ (b) in mg C m² d⁻¹ at the grassland site with low (yellow, LI) and high (turquoise, HI) land-use intensity in autumn (aut), winter (win), spring (spr), and summer (sum). Boxes show the 25th and 75th percentiles and the lines inside the boxes indicate the median. Dots denote the individual measurements. Statistically significant categories (denoted with letters a,b and c) between seasons (per site) were tested with multiple comparison test after Kruskal-Wallis at p < 0.05 level. LI = low land-use intensity site, HI = high land-use intensity site.

Figure 2. RNA and microbial nitrogen content. RNA content (μg g⁻¹ soil DW) across seasons in the upper (0-10 cm) and the deeper soil layer (20-30 cm) at the site with low (yellow) and high (turquoise) land-use intensity (a), and correlation between RNA and microbial nitrogen content (N_{mic}) per g soil (b). Linear regression RNA = 1.81824 + 0.01968 N_{mic}, df = 58. LI = low land-use intensity site, HI = high land-use intensity site. R denotes the Pearson correlation coefficient. Significance codes: *** = p < 0.001, n = 60. Abbreviations: aut = autumn, win = winter, spr = spring, sum = summer, LI = low land-use intensity site, HI = high land-use intensity site, DW = dry weight.

Figure 3. Soil (micro-)biome composition across sites and soil depths. Distance-based redundancy analysis (dbRDA) of the Bray-Curtis dissimilarity matrix of all 39854 bacterial, archaeal and eukaryotic taxa (a) and the 287 CH₄-cycling archaea and bacteria (b) in the grassland sites with low (yellow) and high (turquoise) land-use intensity. Samples from the upper (0-10 cm) and the deeper soil layer (20-30 cm) are depicted with hollow and filled circles, respectively. Samples from autumn, winter, spring and summer are depicted as circles, diamonds, upward-pointing triangles and downward-pointing triangles, respectively. Abbreviations: aut = autumn, win = winter, spr = spring, sum = summer, LI = low land-use intensity site, HI = high land-use intensity site.

Figure 4. Absolute and relative abundances of CH₄-cycling organisms across seasons and depths at small subunit (SSU) rRNA level. Absolute abundances (SSU transcripts g⁻¹ soil DW) of methanogenic Archaea (a) and methanotrophic microorganisms (Archaea and Bacteria) (c) and the proportion of transcripts belonging to methanogenic Archaea (b) and methanotrophic microorganisms (d) normalized to the total amount of transcripts belonging to methanogenic Archaea and methanotrophic organisms. Columns show means across seasons of the upper (0-10 cm) and the deeper soil layer (20-30 cm) in the grassland site with low (LI) and high land-use intensity (HI). “unclassified methanogens” contains methanogens unclassified at the class level and low abundance methanogenic groups. “unclassified *Methylococcales*” contains *Methylococcales* unclassified at the family level and low abundance *Methylococcales* families. Bars represent the means of three replicates. Abbreviations: aut = autumn, win = winter, spr = spring, sum = summer, LI = low land-use intensity site, HI = high land-use intensity site, DW = dry weight. n = 60

Figure 5. Abundances of CH₄-cycling mRNA transcripts across seasons and depths. Transcript abundances (transcripts g⁻¹ soil DW) of methanogenesis pathways (a) and pMMO (b) across seasons in the upper (0-10 cm) and the deeper soil layer (20-30 cm) the grassland sites with low and high land-use intensities. Columns and error bars represent the means and the standard deviations of three replicates, respectively. Statistically significant categories between seasons and depths (per site) were tested with ANOVA and subsequent post-hoc Tukey

test at $p < 0.05$ level, ns = not significant, $n = 60$. Abbreviations: aut = autumn, win = winter, spr = spring, sum: summer, LI = low land-use intensity site, HI = high land-use intensity site, methylated compounds = methanogenesis from methylated compounds, DW = dry weight.

Figure 6. Composition of *pmoA* transcripts. Proportion of *pmoA* groups normalized to the total amount of *pmoA* transcripts. Columns show means across seasons of the upper (0-10 cm) and the deeper soil layer (20-30 cm) in the grassland sites with low (LI) and high land-use intensity (HI), $n = 60$. “*pmoA*_like” = unclassified *pmoA*-like sequences. Abbreviations: aut = autumn, win = winter, spr = spring, sum: summer, LI = low land-use intensity site, HI = high land-use intensity site.

Figure 7 The link between methanogenesis gene transcripts, net surface CH₄ fluxes, and methanotrophy gene transcripts. Linear correlation of absolute abundances of methanogen transcripts and CH₄ flux at SSU level (a) and mRNA level (b). Linear correlation of absolute abundances of methanogen and methanotroph transcripts at SSU level (c) and mRNA level (d). Ratio of methanotroph to methanogen transcripts across the year at SSU rRNA level (e) and mRNA level (f) across seasons at the grassland sites with low and high land-use intensity. R denotes the Pearson correlation coefficient. Significance codes: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant, $n = 60$. Statistically significant categories were tested with ANOVA and subsequent post-hoc Tukey test at $p < 0.05$ level. In (a) and (b) points represent seasonal means across both depths. In (c) and (d) points represent mean values per core (means of both depths). The different colors represent the site with low (yellow) and high (turquoise) land-use intensity. Samples from autumn, winter, spring and summer are depicted as circles, diamonds, upward-pointing triangles and downward-pointing triangles, respectively. Abbreviations: aut = autumn, win = winter, spr = spring, sum: summer, LI = low land-use intensity site, HI = high land-use intensity site.