The soil microbiome as a driver of methane fluxes in temperate grassland and forest soils

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Abstract

Methane (CH_4) is a potent greenhouse gas with rising atmospheric concentrations. Microorganisms are essential players in the global methane cycle. In fact, the largest part of methane emissions derives from microbial production by methanogenic Archaea (methanogens). Microorganisms do not only produce methane: methanotrophs can also oxidize the methane produced by methanogens. In addition, soil methanotrophs are the only biological methane sink, oxidizing up to 30-40 Tg of this potent greenhouse gas per year worldwide. However, intensified management of grasslands and forests may reduce the methane sink capacity of soils.

In general, the interaction of methanogens and methanotrophs determines whether a soil is a source or a sink for methane. It is, therefore, crucial to understand the microbial part of the methane cycle and which factors influence the abundance and activity of methane-cycling microbes. However, capturing the soil microbiome's abundances, activity, and identity is challenging. There are numerous target molecules and myriad methods, each with certain limitations. Linking microbial markers to methane fluxes is therefore challenging. This thesis aimed to understand how methane-cycling microbes in the soil are related to soil methane fluxes and how soil characteristics and human activity influence them.

The first publication investigated the biotic and abiotic drivers of the atmospheric methane sink of soils. It assessed the influence of grassland land-use intensity (150 sites) and forest management type (149 sites) on potential atmospheric methane oxidation rates (PMORs) and the abundance and diversity of CH_4-oxidizing bacteria (MOB) with qPCR in topsoils of three temperate regions in Germany. PMORs measured in microcosms under defined conditions were approximately twice as high in forest than in grassland soils. High land-use intensity of grasslands negatively affected PMORs (−40%) in almost all regions. Among the different aspects of land-use intensity, fertilization had the most adverse effect reducing PMORs by 20%. In contrast, forest management did not affect PMORs in forest soils. Upland soil cluster (USC)α was the dominant group of MOBs in the forests. In contrast, USCγ was absent in more than half of the forest soils but present in almost all grassland soils. USCα abundance had a direct positive effect on PMOR in forests, while in grasslands, USCα and USCγ abundance affected PMOR positively with a more pronounced contribution of USCγ than USCα.

In the second publication, we used quantitative metatranscriptomics to link methane-cycling microbiomes to net surface methane fluxes throughout a year in two grassland soils. Methane

fluxes were highly dynamic: both soils were net methane sources in autumn and winter and net methane sinks in spring and summer. Correspondingly, methanogen mRNA abundances per gram soil correlated well with methane fluxes. Methanotroph to methanogen mRNA ratios were higher in spring and summer when the soils acted as net methane sinks. Furthermore, methane uptake was associated with an increased proportion of USCα and γ *pmoA* and *pmoA2* transcripts. High methanotroph to methanogen ratios would indicate methane sink properties. Our study links the seasonal transcriptional dynamics of methane-cycling soil microbiomes for the first time to gas fluxes *in situ*. It suggests mRNA transcript abundances as promising indicators of dynamic ecosystem-level processes.

We conclude that reduction in grassland land-use intensity and afforestation can potentially increase the methane sink function of soils and that different parameters determine the microbial methane sink in forest and grassland soils. Furthermore, this thesis suggests mRNA transcript abundances as promising indicators of dynamic ecosystem-level processes. Methanogen transcript abundance may be used as a proxy for changes in net surface methane emissions from grassland soils.

Zusammenfassung

Methan (CH_4) ist ein starkes Treibhausgas mit steigenden atmosphärischen Konzentrationen. Mikroorganismen sind wesentliche Akteure im globalen Methankreislauf. Tatsächlich stammt der größte Teil der Methanemissionen aus der mikrobiellen Produktion durch methanogene Archaeen (Methanogene). Mikroorganismen produzieren nicht nur Methan: Methanotrophe Organismen können das von Methanogenen produzierte Methan auch oxidieren. Darüber hinaus sind Methanotrophe im Boden die einzige biologische Methansenke, die bis zu 30-40 Tg dieses starken Treibhausgases pro Jahr oxidiert. Eine intensivere Bewirtschaftung von Grünland und Wäldern kann jedoch die Kapazität der Böden als Methansenke verringern.

Im Allgemeinen bestimmt das Zusammenspiel von Methanogenen und Methanotrophen, ob ein Boden eine Quelle oder eine Senke für Methan ist. Daher ist es wichtig den mikrobiellen Teil des Methankreislaufs zu kennen um zu verstehen, welche Faktoren die Häufigkeit und Aktivität der methanbildenden Mikroben beeinflussen. Die Abundanz, Aktivität und Identität des Bodenmikrobioms zu bestimmen ist jedoch sehr anspruchsvoll. Es gibt zahlreiche Zielmoleküle und eine Vielzahl von Methoden, die jeweils bestimmte Einschränkungen aufweisen. Die Verknüpfung von mikrobiellen Markern mit Methanflüssen ist daher eine Herausforderung. Ziel dieser Arbeit war es, zu verstehen, wie Bodenmikroben mit Methanflüsse von Böden zusammenhängen und wie sich Bodeneigenschaften und menschliche Aktivitäten auf die Methanflüsse von Böden auswirken.

In der ersten Veröffentlichung wurden die biotischen und abiotischen Faktoren untersucht, die die atmosphärische Methansenke der Böden beeinflussen. Dabei wurden die Auswirkungen der Intensität der Grünlandnutzung (150 Standorte) und der Art der Waldbewirtschaftung (149 Standorte) auf die potenziellen atmosphärischen Methanoxidationsraten (PMORs) sowie die Häufigkeit und Vielfalt von methanoxidierenden Bakterien (MOB) mit qPCR in Proben von Oberböden aus drei gemäßigten Regionen in Deutschland untersucht. Die in Mikrokosmen unter definierten Bedingungen gemessenen PMORs waren in Waldböden etwa doppelt so hoch wie in Grünlandböden. Die hohe Landnutzungsintensität von Grünland wirkte sich in fast allen Regionen negativ auf die PMOR-Werte aus (-40 %). Von den verschiedenen Aspekten der Landnutzungsintensität wirkte sich die Düngung am stärksten negativ aus und verringerte die PMORs um 20 %. Im Gegensatz dazu hatte die Waldbewirtschaftung keinen Einfluss auf die PMORs in Waldböden. Das Upland Soil Cluster (USC)α war die dominierende Gruppe von MOBs in den Wäldern. Im Gegensatz dazu fehlte USCγ in mehr als der Hälfte der Waldböden, war aber in fast allen Grünlandböden vorhanden. Die Abundanz von USCα wirkte sich in

Wäldern direkt positiv auf PMOR aus, während sich im Grasland die Abundanz von USCα und USCγ positiv auf PMOR auswirkte, wobei der Beitrag von USCγ stärker war als der von USCα.

In der zweiten Veröffentlichung setzten wir quantitative Metatranskriptomik ein, um die Methan-zyklierenden Mikrobiome mit den Nettomethanflüssen zweier Graslandböden über ein Jahr hinweg zu verbinden. Die Methanflüsse waren sehr dynamisch: Beide Böden waren im Herbst und Winter Nettomethanquellen und im Frühjahr und Sommer Nettomethansenken. Dementsprechend korrelierten die mRNA-Häufigkeiten der Methanogene pro Gramm Boden gut mit den Methanflüssen. Das Verhältnis von Methanotrophen zu Methanogenen mRNA war im Frühjahr und Sommer höher, wenn die Böden eine Nettomethansenken waren. Außerdem war die Methanaufnahme mit einem erhöhten Anteil an USCα- und γ pmoA- und pmoA2-Transkripten verbunden. Die Methanaufnahme der Böden war von einem hohen Verhältnis von Methanotrophen zu Methanogenen begleitet. Diese Thesis stellt erstmals einen Zusammenhang zwischen der saisonalen Transkriptionsdynamik von Methan-zyklierenden Bodenmikrobiomen und den Gasflüssen *in situ* her.

Wir kommen zu dem Schluss, dass eine Verringerung der Intensität der Grünlandnutzung und Aufforstung die Methansenkenfunktion von Böden potenziell erhöhen kann und dass unterschiedliche Parameter die mikrobielle Methansenke in Wald- und Grünlandböden bestimmen. Darüber hinaus zeigt diese Arbeit, dass die Abundanz von mRNA Transkripten ein vielversprechender Indikator für dynamische Prozesse auf Ökosystemebene sein können. Die Abundanz von Methanogen-Transkripten kann als Indikator für Veränderungen der Netto-Oberflächen-Methanemissionen von Grünlandböden verwendet werden.

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Abbreviations

ANME	anaerobic methane-oxidizing archaea
BExIS	Biodiversity exploratories information system
CoM	methyl-coenzyme M
DW	dry weight
ForMI	forest management index
LUI	land-use intensity index
LSU rRNA	large subunit ribosomal ribonucleic acid
MMO	methane monooxygenase
MOB	methane-oxidizing bacteria
mRNA	messenger RNA
NGS	next generation sequencing
NOAA	National Oceanic and Atmospheric Administration
PCR	polymerase chain reaction
PLFA	phospholipid-derived fatty acid
pMMO	particulate methane monooxygenase
PMOR	potential atmospheric methane oxidation rates
ppb	parts per billion
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
sMMO	soluble methane monooxygenase
SSU rRNA	small subunit ribosomal ribonucleic acid
USC	upland soil cluster
WHC	water holding capacity
yr	year

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1 Introduction

There is a vast diversity and variety of microorganisms, with 1.2 x 10^{30} microbial cells living on Earth [1]. Microorganisms are of uttermost importance for greenhouse gas cycles, including the production and consumption of methane (CH_4) [2].

1.1 The global CH_4 cycle

CH_4 is a very potent greenhouse gas [3]. Even though the atmospheric CH_4 concentration is about 220 times lower than the carbon dioxide (CO_2) concentration, its radiative efficiency is much higher, making it the 2nd most important greenhouse gas [4]. The atmospheric CH_4 concentration has more than doubled since the beginning of the industrial era, from 722 ppb in 1750 to 1866 ppb in 2019. It is now thus higher than at any time during the last 800,000 years [5]. Also, its concentration is increasing faster than ever: for 2021, the NOAA reported the highest annual increase in atmospheric CH_4 concentrations since starting systematic measurements in 1983 [6].

About 60% of total CH_4 emissions derive from anthropogenic activity [7], which unequivocally caused the increase in atmospheric CH_4 concentration [5]. Thus, decreasing CH_4 emissions is a major goal: more than 100 countries have recently supported the global CH_4 pledge, aiming to reduce CH_4 emissions by at least 30% by 2030 [8]. Anthropogenic CH_4 sources include agriculture (rice cultivation and livestock), fossil fuel extraction and use, and landfills and waste treatment. Natural CH_4 emissions originate from wetlands and freshwater environments, termites, geological sources, and gas hydrates [5]. Several sinks remove atmospheric CH_4. The largest one is the abiotic oxidation by OH radicals, mainly in the troposphere [7].

Microorganisms are essential players in the global CH_4 cycle; about 69% of the total CH_4 emissions derive from microbial production by methanogens in anoxic environments [9]. They produce up to 1000 Tg CH_4 per year [10]. However, most of the produced CH_4 does not reach the atmosphere because methanotrophs oxidize about 80% of the CH_4 produced by methanogens or released by gas hydrates [10]. In addition, aerated soils are the only biotic sink for atmospheric CH_4; about 5% of the CH_4 loss occurs through CH_4 oxidation by methanotrophic bacteria [5].

1.2 CH_4-cycling microorganisms in soils

CH_4-cycling microorganisms have conquered many different environments in all climatic zones of Earth. They live in aquatic and terrestrial habitats and occupy different ecological niches based on their preferences for CH_4 and oxygen (O_2).

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 CH_4 oxidation. Presumably, methanogens and methanotrophs' interactions determine whether soils act as sources or sinks for CH_4 [11].

This thesis investigated CH_4-cycling microorganisms in three different systems: upland soils, drained wetland soils, and intermittent streams.

1.2.1 Methanogenic Archaea

Methanogenic Archaea produce CH_4 as an end-product of their energy metabolism. They inhabit various anaerobic ecosystems, e.g., (natural and artificial wetland) soils, freshwater and marine sediments, and digestive tracts of animals, such as ruminants and termites [9]. They can grow within various conditions, including extreme environments like submarine hydrothermal vents or hot springs [12, 13]. Generally, methanogenic Archaea are obligately anaerobic. However, they can tolerate oxygen to a certain degree and were already detected in oxygenated soils [14]. Methanogens were grouped into class I and II methanogens based on their potential to express antioxidant enzymes and small redox proteins [15].

There are currently eight validly-described orders of methanogens: Methanococcales, Methanopyrales, and Methanobacteriales within the Euryarchaeota, Halobacterota Methanomicrobiales, Methanocellales, Methanonatronarchaeales, Methanosarcinales and Methanomassiliicoccales within the Thermoplasmatota. Additionally, there are four candidate orders containing methanogens: Candidatus (Ca.) "Methanofastidiosales," Ca. "Methanoflorentaceae," Ca. "Methanomethylicia" (also known as "Verstraetearchaeota"), and Ca. "Bathyarchaeia" [16].

Four types of methanogens can be characterized according to their substrate specificity. Acetoclastic methanogens utilize acetate, hydrogenotrophic methanogens utilize H_2/CO_2 and formate, and methylotrophic methanogens utilize methylated compounds, such as methanol or methylamines, to form CH_4 [16]. In addition, methoxydotrophic methanogens that utilize methoxylated aromatic compounds were recently proposed as a novel methanogenic group [15, 17]. Acetoclastic and hydrogenotrophic methanogenesis are considered the predominant CH_4

sources in most environments, including soils [16, 18–20]. However, recent research indicates that methanogenesis from methylated compounds may contribute more to global CH_4 emissions than previously thought [21–23].

Methanogenesis is catalyzed by a complex enzyme system [16]. For instance, the hydrogenotrophic CH_4 formation can require more than 200 genes [24]. The key enzyme for methanogenesis is the methyl-coenzyme M (CoM) reductase [16, 25]. It catalyzes the reductive cleavage of methyl-CoM, which produces CH_4 and is the final step of methanogenesis [26]. Due to its universal distribution in all methanogenic pathways, molecular studies often use the methyl-CoM reductase genes as a molecular marker for detecting methanogens. Specifically, the *mcrA* gene encoding its alpha subunit is often targeted [27–29] (see chapter 1.5.1).

1.2.2 Methanotrophic bacteria and Archaea

1.2.2.1 Well-known aerobic methanotrophs

Methanotrophs oxidize CH_4 to CO_2, which has a lower global warming potential than CH_4 [4]. They thus reduce CH_4 emissions from CH_4-producing environments and mitigate global warming. Methanotrophs inhabit diverse habitats such as wetlands, upland soils, landfills, and marine and freshwater environments. They typically live at oxic/anoxic interfaces where CH_4 and O_2 are present. Many isolates of aerobic CH_4-oxidizing bacteria (MOB) exist within the Alphaproteobacteria, Gammaproteobacteria, and Verrucomicrobia [30]. However, culture-independent studies indicate that the isolated strains only cover a small part of the existing methanotrophs [31].

Most methanotrophic isolates within the Alpha- and Gammaproteobacteria are mesophilic and neutrophilic. In addition, many strains are moderately acidophilic, psychro- and thermophilic, or halophilic [32]. Verrucomicrobial methanotrophs are adapted to acidic geothermal environments [33]. Some of them can even grow at pH 0.5 [34].

The MOB diversity is highest within the Gammaproteobacteria. They contain two methanotrophic families: *Methylococcaceae* and *Methylothermaceae*. Therein, common genera include *Methylomonas*, *Methylobacter*, *Methylosarcina*, *Methylomicrobium*, *Methylococcus*, *Methylocaldum*, *Methylosoma*, and *Methyloparacoccus* [30, 31]. The filamentous *Crenothrix polyspora* is likely also a member of the *Methylococcaceae* but was not isolated in pure culture yet. However, it was identified as the major CH_4 consumer in stratified lakes [35]. The families *Methylocystaceae* and *Beijerinckiaceae* (Alphaproteobacteria) additionally contain non-

methanotrophic species. Common methanotrophic genera within the Alphaproteobacteria are *Methylocystis* and *Methylosinus* [31].

The methane monooxygenase (MMO) is the key enzyme for aerobic methanotrophy. All known aerobic methanotrophs utilize this enzyme to oxidize CH_4 to methanol [36]. It exists in two different forms: the particulate, membrane-bound MMO (pMMO) and the soluble MMO (sMMO) [37]. The pMMO is widely present within methanotrophs and therefore commonly used as a molecular marker. For example, all gammaproteobacterial and verrucomicrobial methanotrophs and the alphaproteobacterial *Methylocystaceae* possess the pMMO. Still, some methanotrophs lack the pMMO: within the *Beijerinckiaceae*, the genera *Methylocella* and *Methyloferula* have only the sMMO [38–41]. However, the sMMO is not consistently present in methanotrophs, even at the species level [42].

1.2.2.2 CH_4 oxidation at atmospheric CH_4 concentrations

Soils are the only known biological sink for atmospheric CH_4 [7, 43]. This microbial sink is provided by aerobic MOB that oxidize CH_4 at atmospheric concentrations. Even though researchers isolated many MOB growing at high CH_4 concentrations, MOB growing at atmospheric CH_4 concentrations resisted cultivation until recently [44]. It is, therefore, still not fully understood which microorganisms are responsible for the atmospheric CH_4 sink of soils. Molecular studies identified possible candidates: *pmoA* gene sequencing or PLFA analysis frequently detected uncultured MOB in upland soils with atmospheric CH_4 uptake [45–47]. These unknown bacteria were grouped into Upland soil cluster (USC) α and γ [48]. Furthermore, a *Methylocystis* isolate was proven to use atmospheric CH_4 for over three months [49]. However, only in 2019 the first bacterium growing at atmospheric CH_4 levels was obtained in pure culture: the USC-α bacterium *Methylocapsa gorgona* [44]. While the USCs likely contribute to atmospheric CH_4 oxidation in aerated soils, conventional CH_4 oxidizers were recently linked to atmospheric CH_4 uptake in paddy soils rather than USC [50]. In that study, the soils lost the atmospheric CH_4 uptake after two weeks unless they were flush-fed with higher CH_4 concentrations. It is thus exciting to explore the atmospheric MOB in soils. This thesis explored the atmospheric CH_4 uptake of aerated upland soils and drained wetland soils.

1.2.2.3 Anaerobic methanotrophic archaea and bacteria

Long time, CH_4 oxidation was attributed exclusively to aerobic microorganisms. First indications of anaerobic CH_4 oxidation came from geological observation in marine, anoxic, sulfate-reducing seawater and sediments [51]. The organisms responsible for the CH_4

consumption in these habitats were later identified as anaerobic methanotrophic archaea (ANME) and were syntrophically associated with sulfate-reducing bacteria. Later, anaerobic CH_4 oxidation was also found coupled with denitrification [52]. The archaeon *Ca.* Methanoperedens nitroreducens (belonging to the ANME-2d clade) uses nitrate as a terminal electron acceptor in a reversed methanogenesis pathway [53]. The bacterium *Ca.* Methylomirabilis oxyfera, belonging to the NC10 candidate phylum, reduces nitrite to dinitrogen and oxygen [54]. In addition, anaerobic CH_4 oxidation can be coupled with reducing other electron acceptors such as metal ions or humic substances [55–57]. The anaerobic archaeal CH_4 oxidizers belong to the clades ANME-1, ANME-2a/b, ANME-2c, and ANME-3 within Euryarchaeaota [55]. They use reversed methanogenesis pathway for CH_4 oxidation and contain the *mcrA* gene like methanogens. In contrast, *Ca.* M. oxyfera uses a pMMO like aerobic methanotrophs. Still, it does not rely on external O_2 because it utilizes an intra-aerobic denitrification pathway to disproportionate NO to O_2 and N_2 [54, 58]. The sulfate-dependent ANME live in sulfate-rich habitats such as marine sediments, cold seeps, or lake sediments, whereas the nitrate and nitrite-dependent anaerobic methanotrophs live in freshwater environments [59].

1.3 CH_4 fluxes of soils and intermittent streams

1.3.1 CH_4 emission of (drained) wetland soils

Wetland soils are permanently or temporarily flooded. The reduced diffusibility of oxygen in water leads to anoxic conditions in these water-saturated soils. Hence anoxic microorganisms and processes such as methanogens and methanogenesis are prevailing. Wetlands contribute 22 - 30% to global CH_4 emissions, with mean emissions of 149 Tg CH_4 yr^{-1} (bottom-up estimates) and 194 Tg CH_4 yr^{-1} (top-down estimates) [7].

Despite their high CH_4 emissions, wetlands have an overall positive influence on GHG budgets. The anoxic conditions prevent the decomposition of organic matter to CO_2; wetlands thus store large amounts of carbon in the form of peat [60, 61]. Many wetlands were drained to harness agricultural and building land during the last centuries [62, 63]. Drainage increases O_2 availability in the soil, affecting especially O_2-sensitive anaerobic microbes, such as methanogens. Hence, drained peatlands produce less CH_4 than wet peatlands [64]. However, oxygen generally stimulates aerobic degradation of the peat, emitting considerable amounts of CO_2 [65, 66]. Aerated soil conditions thus result in fewer CH_4 emissions but massively

increased CO_2 emissions [67]. This thesis investigated the CH_4-cycling microorganisms in two former wetland soils drained several decades ago.

1.3.2 Upland soils as a sink for atmospheric CH_4

In contrast to wetland soils, upland soils are typically well-drained and aerated. They, therefore, do not offer optimal living conditions for anaerobic methanogens and hence methanotrophs that rely on high CH_4 supplies. Nevertheless, aerated soils can harbor anoxic zones where anaerobic processes occur [68]. A study investigating aerated soils detected the anaerobic *Methanosarcina* and *Methanocella* in many upland soils [14]. Even though upland soils usually lack internal CH_4 production, they can contain CH_4 at atmospheric and sub-atmospheric concentrations [69], originating from air diffusion into the soils [70]. Indeed, soils are the only known biological sink for atmospheric CH_4, capable of consuming 30-40 Tg CH_4 per year [7, 43]. Atmospheric CH_4 uptake is widespread in different ecosystems, for instance, forest, grassland, or savannah soils [71]. This CH_4 sink results from the oxidation of CH_4 by MOB [72]. Many factors influence the soil's CH_4 uptake, for instance, ecosystem type and soil texture [71]. Also, land-use change and management practices can impact soil environmental parameters and alter soil CH_4 fluxes [73] (see 1.3.4).

1.3.3 Intermittent streams

Rivers and streams are usually CH_4 sources with global emissions of about 27 Tg CH_4 yr^{-1}. However, CH_4 emissions from rivers are poorly constrained [7]. Especially the influence of intermitted streams on the global carbon cycle is not well understood [74]. However, many streams are intermittent: about 34% and 69% of fifth-order and first-order streams, respectively [75]. Dry stages alter the structure of microbial communities and soil physicochemical conditions of the sediments [76, 77]. The absence of water exposes them to air and oxygen, affecting particularly CH_4-cycling microbes. Higher redox status in the soil likely hampers methanogen abundances and activity and, therefore, methanotrophs that rely on higher CH_4 concentrations.

1.3.4 Effect of anthropogenic land use on soil CH_4 fluxes

As the number of humans on earth increases, the implications of anthropogenic activities have intensified. Anthropogenic activities probably affect all aspects of soil ecosystems, such as vegetation cover, soil properties, and soil microbiomes [78, 79]. In addition, they can impact soil environmental parameters and alter soil CH_4 fluxes [73, 80, 81]. For example, converting forests to cropland or grassland reduces CH_4 oxidation of soils [82]. In support of these results,

a recent global meta-analysis concluded that transformation from natural to anthropogenic land use increases CH_4 emissions [80].

Several aspects of anthropogenic land use can cause these increased CH_4 emissions. For instance, in grassland soils, fertilization and heavy livestock grazing can negatively impact the CH_4 uptake of soils [83–85]. The adverse effect of nitrogen input may be caused by competitive inhibition of the CH_4 monooxygenase [72, 86, 87]. The negative impact of grazing may be due to nitrogen input and soil compaction, resulting in reduced soil permeability. In addition to lowering the CH_4 uptake of grasslands, grazing may even turn soils into net CH_4 sources; a study found non-grazed soils to be a CH_4 sink and grazed soils to be a CH_4 source [88].

Generally, forests usually have higher CH_4 uptake than grassland soils. However, forest management also influences atmospheric CH_4 uptake, similar to grassland management. For example, converting natural hardwood forests to spruce and pine forests reduced their CH_4 sink potential by about two-thirds [89]. In addition, soil disturbance, compaction during clear-cutting and thinning, or nitrogen deposition, can negatively affect the CH_4 sink function of forest soils [90–92]. However, a general negative effect of nitrogen fertilization on CH_4 oxidation in forest and upland grassland soils has also been questioned as it seems to depend on the amount of nitrogen present in soil [93, 94].

1.4 The biodiversity exploratories

This thesis is part of the DFG-funded Infrastructure Priority Program SPP 1374 “biodiversity exploratories” (http://www.biodiversity-exploratories.de/en/) [95]. This program aims to explore biodiversity and how it influences ecosystem processes. It emphasizes the influence of different forms and intensities of land use on biodiversity and ecosystem processes. The program encompasses sites in three climatically and geographically different areas of Germany (so-called “Exploratories”, i.e., Schwäbische Alb, Hainich-Dün and Schorfheide Chorin [95, 96]. Each exploratory contains forest and grassland sites differing in management type and land-use intensity. Samples for publications I and II were taken from biodiversity exploratory sites.

1.5 Molecular methods for characterization of CH_4-cycling microbes *in situ*

The majority of microorganisms are currently not cultivable [97]. For instance, most methanotrophic diversity is not represented by cultivated strains, and many validly described methanotrophic species are rarely detected in environmental samples [31]. Furthermore, the isolation of microorganisms is time-consuming. Due to the shortcomings of culture-based methods, soil microbial communities are often characterized by culture-independent techniques such as phospholipid fatty acid analysis or nucleic-acid-based methods [98]. Especially the use of next-generation sequencing (NGS) gained new insights into microbial communities in different environments [99]. This thesis applied quantitative (q)PCR and nucleic acid sequencing to analyze CH_4-cycling microbes in soils and sediments.

1.5.1 PCR and quantitative PCR

The PCR is one of the most widespread tools in microbial ecology to identify microorganisms in different habitats [100, 101]. In a PCR, small and specific DNA probes (called primers) hybridize to complementary sites in environmental DNA. The DNA segment within these binding sites is amplified and can be detected with nucleic acid-binding dyes. The detection of the product allows concluding the presence or absence of particular sequences in the sample. However, it does not yield information about its abundance.

With quantitative PCR (qPCR), one can go beyond stating the mere presence or absence of the target DNA and make quantitative statements. In one approach of qPCR, a fluorescent dye, such as SybrGreenI, is added to the PCR reaction. The amount of incorporated dye is detected after each PCR cycle and compared to the fluorescence of standards with known DNA concentrations. Primer pairs can be more or less specific for detecting certain groups of (micro-)organisms. For example, several primer pairs were developed for identifying methanotrophic and methanogenic microorganisms [27, 102].

For methanogens, many primer pairs exist targeting a broad range of methanogens or specific groups [27, 103]. Some of these were also optimized for qPCR [104]. The gene encoding the alpha subunit of methyl-CoM reductase (*mcrA*) is used commonly for detecting and characterizing methanogens in environmental samples [27–29]. Primer pairs to tackle a broad range of methanogens include mlas-mod (and mlas) and mcrA-rev [19, 104].

Most methanotroph-specific primer pairs target the functional gene *pmoA*, which encodes the alpha subunit of the particulate methane monooxygenase (pMMO) [105]. The pMMO is

widespread in methanotrophs, including the anaerobic CH_4-oxidizing NC10 bacterium *Ca.* Methylomirabilis oxyfera [54]. However, some *Beijerinckiaceae* like *Methylocella* and *Methyloferula* lack the pMMO [102] and possess only the soluble methane monooxygenase (sMMO). Often, primers that detect these methanotrophs target the *mmoX* gene, which encodes the α-subunit of the sMMO hydroxylase component [105]. However, since the pMMO is more widespread in methanotrophs, it is more frequently used for detecting methanotrophs in environmental samples. The most common primer pair for detecting *pmoA* is A189f/A682r. This primer pair cover the broadest diversity of methanotrophs and is the gold standard for methanotroph detection [102]. However, the A189f/A682r primer pair does not amplify *pmoA* from Verrucomicrobia or *Ca.* M. oxyfera-like methanotrophs [106, 107]. There are specific primers for detecting these groups, e.g., "Ca. Methylomirabilis oxyfera" can be targeted using a nested PCR approach with A189_b/cmo682 and cmo182 and cmo568 [106]. Also, the A189f/A682r primer pair co-amplifies *amoA* sequences that encode the alpha subunit of the ammonia monooxygenase [102, 108]. The ammonia monooxygenase and pMMO proteins are related [109] and, therefore, hard to distinguish using molecular diagnostics. A189f/mb661r primer combination is more specific towards *pmoA* and amplifies fewer *amoA* genes. However, the A189f/mb661r primer combination discriminates genes belonging to putative atmospheric CH_4 oxidizers like USCα, USCγ, and JR1 [102]. Thus, group-specific primer pairs were developed for the detection of these groups. USCα and USCγ can be targeted with the primer pairs A189f/forest675r and USCα-346f/A682r [110, 111]. USCγ is detectable with A189f/Gam634r [112]. These PCR assays were optimized for qPCR also [110–112]. Covering all methanotrophic diversity with only one primer pair is not possible. Therefore, when choosing a PCR assay and interpreting its results, it is crucial to consider that no primer covers all methanotrophic organisms

1.5.2 Detangling microbial diversity with sequencing and meta-omics approaches

Sequencing techniques have developed rapidly over the last decades, continually reducing the costs and time required for sequencing projects. The Sanger dideoxy method was one of the first DNA sequencing methods [113, 114]. Even though already developed in the 1970s, Sanger sequencing is still widely used because it is fast and inexpensive when only a few sequences need to be identified. However, larger sequencing projects require methods that facilitate high-throughput sequencing, also called next-generation sequencing (NGS) [115]. Illumina sequencing is the most widespread NGS technology [115, 116] and offers various platforms with different capabilities in terms of read length, data output, and maximum reads per run. The NextSeq 550 platform, used in this thesis, outputs up to 400 Mio paired-end reads with a length

of 2 x 150 bp [117]. High throughput sequencing technologies are rapidly advancing. For example, sequencing platforms of the third and fourth generation can produce read lengths up to 30 to 50 kb bp and longer [115, 118].

Progress in high throughput sequencing technologies is steadily advancing the possibilities in molecular ecology, empowering the analysis of complex microbiota. Microbes previously not detectable with culture-based methods can now be analyzed with techniques such as metabarcoding, metagenomics, and metatranscriptomics.

Metabarcoding, the sequencing of marker genes, has been a widespread tool to unravel soil microbial communities [119]. The DNA is extracted from the soil, and the desired marker gene is amplified by PCR and sequenced. These sequences can be searched against designated databases. The 16S and 18S rRNA genes are most commonly targeted to classify prokaryotic and eukaryotic communities in soils. These genes are often analyzed because they are relatively conserved and well-characterized. In addition, it is also possible to access particular groups of soil microorganisms with specific functional markers. For example, soil methanotrophic and methanogenic communities are often characterized by sequencing the *pmoA* and *mcrA* genes [29, 50, 120, 121] (as described in 1.5.1). Even though metabarcoding has become a widespread tool in molecular ecology, it has some downsides. For instance, certain biases may be introduced during PCR amplification due to different primer binding site efficiencies or chimeric amplification products [122, 123]. Also, the gene copies of the rRNA in a cell vary between species: the 16S rRNA gene can have 1 – 15 copies per cell [124]. The different gene copies per cell may bias the portion of relative abundances between microbes.

Furthermore, accessing the diversity of Archaea, Bacteria, and Eukaryotes with only one primer pair is impossible. As a possible solution, metagenomics enables the analysis of the total genomic content of microbial communities, including all three domains of life and viruses [99, 125]. It facilitates the exploration of the soil's potential to accomplish specific pathways.

Another approach to characterize complex microbial communities is metatranscriptomics, via the sequencing of RNA (RNA-seq). The RNA extracted from soils contains ribosomal RNA from prokaryotes and eukaryotes. This rRNA can be filtered out and aligned against a database containing ribosomal sequence data. Thus, with RNA-seq, Archaea, Bacteria, and Eukaryotes can be characterized within the same sequence library. Furthermore, with RNA-seq, it is possible to analyze the mRNA fraction. This so-called double RNA approach facilitates the taxonomical classification of the communities (SSU rRNA) and, in addition, the characterization of gene expression in the soil (mRNA fraction) [126].

In summary, there are numerous methods that can be applied to characterize soil microbiomes and methane-cycling microbes.

1.6 Determining CH_4 fluxes

Measuring CH_4 emissions *in situ* is challenging, and numerous methods exist spanning different space and time scales. Practices reach from chamber measurements typically covering < 1 m^2 to satellite measurements of absorption spectra covering a global scale [127]. Each method has advantages and limitations, and choosing the proper method for the scientific question is crucial.

The chamber techniques are well suited for measuring CH_4 at a small spatial resolution [128]. In closed chambers, one can determine CH_4 fluxes from the change in CH_4 concentration in the chamber over time. These chamber methods are relatively cheap and easy to operate in small areas. However, they are labor-intensive when more extensive time and spatial scales must be covered [127, 129]. There are automated chambers, allowing for close sampling over an extended time period [128]. However, other techniques are preferred for measuring CH_4 for covering larger areas. For instance, eddy covariance towers are commonly applied to measure CH_4 of soils [129–132], or satellite measurements are especially suited to detecting emissions from unexpected sources, e.g., CH_4 leakages [133]. In conclusion, combining different techniques aids in getting realistic assumptions about the global, regional and site-specific CH_4 budget [127, 132].

1.7 Linking microbes and ecosystem processes

Soils are an extremely heterogeneous habitat, harboring myriads of microorganisms. The soils on Earth hold $3*10^{29}$ microorganisms belonging to many different taxa [1]. This versatile microbiome facilitates numerous processes impacting nutrient cycles and ecosystem processes. However, not all of the microbes in the soil are active: in fact, the portion of active microbes in soils is usually below 2% [134]. Also, some ecological relevant functions, such as CH_4 oxidation, are carried out by a small fraction of the soil microbiota [135]. Hence, linking microbes to ecosystem processes remains challenging [101, 136–139].

One widespread problem is that sequencing techniques usually yield relative abundances. Even though this gives valuable insights into the composition of microbial communities, one cannot infer their absolute changes in the environment [140–142]. For instance, an increase in the

relative abundance (RA) may be caused by its actual growth but also by a decrease in the absolute abundance of other organisms. [142]. To get from relative to absolute quantification, one can combine sequencing data with methods such as flow cytometry, qPCR [143], total DNA content [144], or spiking a known amount of nucleic acid to the sample or DNA extract [145–147]. Some metatranscriptomics studies have applied absolute quantification approaches, too [148, 149]. For instance, artificial mRNA standards have been used in metatranscriptomics studies with seawater samples [148, 150]. Furthermore, one can also use the total RNA content to infer absolute abundance and estimate transcript abundances per gram of soil [149]. For example, in a metatranscriptomics study in cow rumen, the mRNA abundances of methanogens per gram of rumen fluid, but not the relative abundances, were related to CH_4 emissions from the rumen [149].

Absolute quantification of genes with qPCR can yield gene copies per gram of soil. However, also these have to be interpreted with care. For instance, gene copies per cell vary heavily between species: the 16S rRNA gene can have 1 – 15 copies per chromosome [124]. In methanotrophs, one to three *pmoA* and 16S rRNA gene copies were detected [151]. Moreover, type II MOB likely possess more *pmoA* copies than type I MOB [151]. Not accounting for these differential abundances may lead to erroneous conclusions about the microbiome's community composition.

Furthermore, the choice of marker molecule influences how well one can link molecular data to ecosystem processes. Many Metagenomics studies use DNA because its handling is relatively easy. However, DNA is long-term stable and may originate from inactive or even dead microbes [152, 153]. Still, DNA-based studies give valuable insights into the environment's potential to perform specific processes. However, it may not reflect well whether these processes occur at a particular moment, *in situ*. Microbes adapt their activity and gene expression according to changes in environmental conditions [134].

Investigating the RNA is an alternative to DNA studies. Ribosomal RNA is a proxy for ribosomes in a cell and thus represent the cells' protein synthesis potential [154]. However, rRNA may not reflect well on activity. For instance, the relationship between rRNA and cell growth activity is inconsistent within different bacteria and likely depends on the growth phase [154]. Also, although approximately 94% of microbial taxa in soil are active and synthesize new rRNA, dormant cells can contain high loads of ribosomes [154], sometimes even more than cells in a vegetative state [155]. Hence, although the rRNA composition can resolve potentially active microbes, connecting rRNA to soil processes is not straightforward.

In contrast to rRNA, mRNA reflects gene expression. Several studies have shown mRNA to be more responsive to environmental factors than rRNA [126, 156]. For instance, methanogen-related mRNA, but not SSU rRNA, decreased in soil microcosms exposed to drought [157]. While DNA and rRNA-based studies can yield information about the environmental potential, mRNA may be the better link to ecosystem processes.

Another approach to investigate the active microbial community is to use stable or radioisotope probing. For instance, microbes can be incubated with ^{18}O-labeled water [158] or ^{13}C-labeled organic substrates [159], which are incorporated into the nucleic acids. Analysis of the labeled fraction gives insights into the active microbiome.

In summary, there are numerous approaches to investigating microorganisms' activity, abundance, and community composition. However, they come with advantages and shortcomings that one needs to consider when relating the data to ecosystem measurements. In this thesis, we aimed to link DNA-based qPCR assays and metatranscriptomics to gas flux measurements.

2 Aims of this thesis

Understanding the soil CH_4 cycle is crucial since CH_4 is an important greenhouse gas contributing to global warming. Global warming will have a massive impact on the earth's climate. It likely will, for instance, increase the frequency and intensity of droughts and heatwaves in some regions [160]. Thus, mitigating global warming is one of the most important tasks of our time, and it is essential to understand greenhouse gas sinks and sources.

Microorganisms are crucial players in greenhouse gas production and consumption, impacting climate change. On the other hand, microorganisms are also affected by climate change and anthropogenic activity [161]. Soils can be sinks and sources for the potent greenhouse gas CH_4, depending on the activity of CH_4-cycling microorganisms. Therefore, understanding their ecophysiology, their interactions with each other, and how anthropogenic activity influences them is environmentally and societally relevant. This thesis aimed to link CH_4-cycling microorganisms to CH_4 fluxes of soils.

I was particularly interested in two aspects; this thesis aimed to explore:

1) the **soil CH_4 sink function** and **putative atmospheric MOB** and how they are influenced by **land use, land-use intensity** and soil properties.

2) the **seasonal dynamics of CH_4-cycling microbes** and their link to net CH_4 emissions in soils and sediments with microbial CH_4 production and consumption

3) how microbial markers relate to soil CH_4 fluxes

1) MOB are the only known biological sink for atmospheric CH_4. The first project focused on atmospheric CH_4 oxidation in soils and the putative atmospheric MOB. We investigated the potential of soil microbiomes to oxidize CH_4 at atmospheric concentrations and wanted to find out what drives the atmospheric CH_4 sink (Figure 1). We especially wanted to characterize the influence of land-use intensity on the atmospheric CH_4 sink. Therefore, data for the land-use intensity index (LUI) and forest management index (ForMI) were retrieved for each site in the grassland and forest, respectively [162, 163]. In grasslands we were interested in mowing frequency, grazing, and fertilization intensity (Figure 1). Another important aspect of this project was to study the MOB in the soils that generate the soil's CH_4 sink. Furthermore, I analyzed influential soil parameters such as bulk density, pH, carbon, and nitrogen content. These parameters were measured by co-authors of the study.

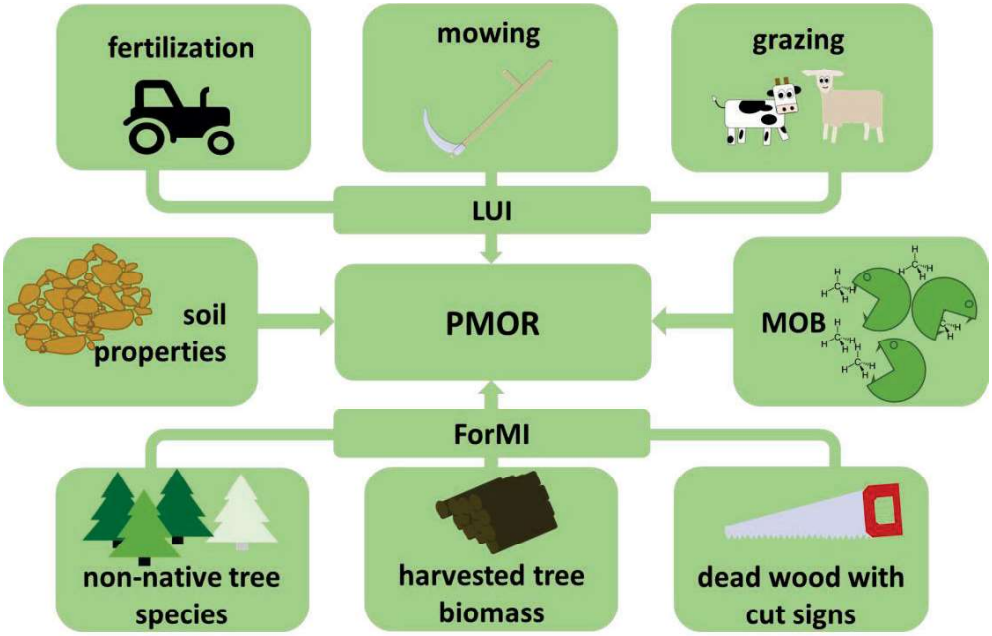

Figure 1 Identifying the drivers of the soil CH_4 sink. We aimed to identify the drivers of the potential atmospheric CH_4 oxidation rates (PMORs) of soils. We investigated almost 300 soils from forest and grassland sites and their forest and grassland management intensity, the abundance and composition of two groups of CH_4-oxidizing bacteria (MOB), and soil properties (bulk density, water holding capacity, organic carbon and nitrogen content). Abbreviations: PMOR: potential CH_4 oxidation rates, LUI: land-use intensity index, ForMI: forest management index, MOB: CH_4 -oxidizing bacteria.

We hypothesized that high management intensity reduces potential CH_4 oxidation rates in grasslands. High land-use intensity (fertilization or frequent grazing and mowing) will increase the nitrogen input in soil and compact the soil by machinery use or livestock trampling. In forests, intense management might reduce CH_4 oxidation rates due to soil compaction from forest machinery. Further, we wanted to learn how the abundance of the putative atmospheric CH_4 oxidizers USCα and USCγ relates to potential CH_4 uptake rates. In addition, we assumed that soil edaphic properties drive CH_4 uptake and the abundance of MOB in soils.

2) Publication II aimed to understand the seasonal and spatial dynamics of CH_4-cycling microorganisms in two grassland soils with internal CH_4 production and consumption. The weather conditions and, with them, the soil's physicochemical properties profoundly changed throughout the year, influencing the occurrence and activity of microorganisms. Methanogens and methanotrophs have opposing oxygen preferences. Therefore, especially soil water content may differentially affect their activity, resulting in different net CH_4 fluxes of the soils throughout the year. Linking CH_4 fluxes and the CH_4-cycling community with metatranscriptomics has hardly been achieved *in situ* [164].

With quantitative metatranscriptomics, this thesis wanted to unravel seasonal and depth variations in the microbiomes of two drained wetland soils. We sequenced rRNA and mRNA of 60 soil samples and measured CH_4 and CO_2 net surface fluxes in autumn, winter, spring, and summer. We aimed to link the abundances of the CH_4-cycling microbes to CH_4 fluxes throughout the year.

Furthermore, I was involved as a co-author in a third study investigating CH_4 and CO_2 fluxes of intermitted stream sediments that were drained over a few weeks (publication III). The frequency of droughts and, therefore, the intermittency of rivers will increase; so it is essential to understand how this affects greenhouse gas fluxes. Publication III aimed to understand CH_4 fluxes after desiccation of intermittent streams and how the microbial community changes with time.

3) Soil microbes determine soil net CH_4 fluxes. However, linking soil microbiomes to the soil gas fluxes is not straightforward. Therefore, this thesis aimed to find out which microbial markers may suit this purpose. Especially, the relationship between rRNA and mRNA abundances of CH_4-cycling microbes and CH_4 fluxes has not been studied *in situ*. Thus, this thesis aimed to explore the differences between small subunit (SSU) rRNA and mRNA transcripts of the CH_4-cycling microbiomes and their links to gas fluxes (Figure 2). With RNA-seq, we can evaluate the microbial community composition in the soils and, simultaneously, get a glimpse into transcriptional activity by analyzing the SSU rRNA and mRNA, respectively. Comparing these two RNA fractions allows us to ascertain whether SSU rRNA abundances of CH_4-cycling microbes or the mRNA abundances of relevant genes can be related to the net CH_4 gas fluxes. Furthermore, in publication I, we used two different *pmoA*-specific qPCR assays to learn if the *pmoA* abundance can indicate potential atmospheric methane uptake of soils.

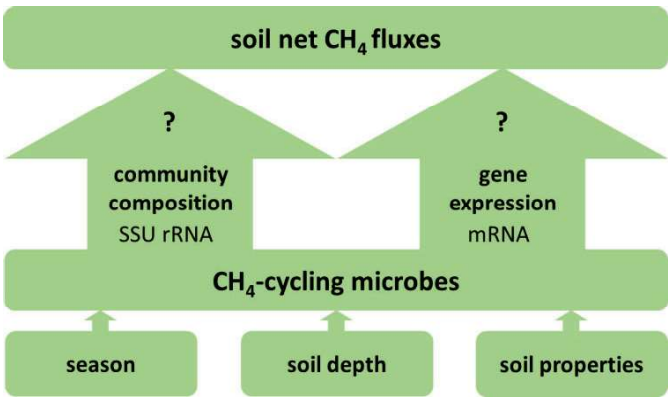

Figure 2 How to link CH_4-cycling microbes to soil net CH_4 fluxes? First, we aimed to investigate if it is possible to relate the abundances and community composition of CH_4-cycling microbes to net CH_4 fluxes of soils. Furthermore, we aimed to assess which microbial marker relates better to the measured fluxes: the abundance and composition of SSU rRNA of CH_4-cycling organisms or the abundance or composition of relevant mRNA transcripts.

2.1 Field work

Sampling for the first publication was part of a coordinated soil sampling campaign organized by the biodiversity exploratories. During this sampling campaign, soil samples were taken from all 300 sites and distributed among the collaborating groups. Later, sample data were shared in a data management system (co-called BExIS) to facilitate data exchange between groups.

For publication II, two grassland sites in the biosphere reserve Schorfheide Chorin were sampled four times during one year (from November 2017 to September 2018). We only investigated two sites to allow an in-depth analysis of the soils, with proper replication across seasons. The sites were preselected based on soil type and the presence of methanogens, detected by PCR with mlas-mod and mcrA-rev [19, 104]. Both sites have an histosolic soil type and have been drained for several decades. Due to the drainage, the site's peat layer was highly degraded.

Samples for publication III were taken by Maria Isabel Arce from a temperate, intermittent, lowland stream located in Brandenburg at the end of May 2015 before surface flow declined.

3 Main findings and conclusions

3.1 Main findings

3.1.1 Publication I: Divergent drivers of the microbial CH_4 sink in temperate forest and grassland soils

We wanted to constrain biotic and abiotic drivers of the soil sink for atmospheric CH_4. Therefore, we investigated atmospheric CH_4 oxidation and abundances of methanotrophs in soil samples from almost 300 sites in three different regions in Germany in microcosms. These sites varied in management type (forest and grassland), land-use intensity, and soil properties. Furthermore, through the BExIS system, we had access to information about soil physicochemical properties, soil types and land-use intensity. As a result, we have created and analyzed the so far largest single dataset that combines data for potential CH_4 uptake rates (PMOR) with gene abundances of two groups of putative atmospheric MOB and physicochemical soil parameters.

Influence of management type and soil parameters. The PMORs measured under defined conditions were about twice as high in forest soils than in grassland soils. This finding accords with the literature on ecosystem-level measurements [71, 80]. Interestingly, some soil properties had differential effects in grassland and forest soils. In grasslands, the water holding capacity (WHC) positively influenced PMORs, while there was no effect of WHC in forests. Also, soil textures with low PMORs in grassland had higher PMORs in forests. For instance, in grassland and forests, PMORs of loamy sand soils were 0.05 and 0.75 ng CH_4 g^{-1} DW h^{-1}, respectively. It may be possible that the higher PMORs in forest soils were partially due to the canopy cover in forests. After rainfall events, the canopy cover mitigates a high increase in soil water content in the upper mineral soil layer [165], potentially favoring MOB activity. Also, the relationship of PMOR and pH differed between grasslands and forests. Albeit the PMORs increased with higher pH in grasslands, the PMORs in the forest had an optimum at around 4. The differential effect of pH may be caused by different microbial communities in the two ecosystem types. Bulk density negatively affected PMORs in forest and grassland soils. High bulk density indicates high soil compaction and lower diffusibility for gasses. In conclusion, the PMORs were generally higher in forest soils, and there was minor variation between forest regions compared to the grassland regions. Our results indicate that forest soils are potent sinks for CH_4 across wide ranges of physicochemical soil.

Influence of land use. Generally, land-use intensity negatively influenced PMORs in grasslands. PMORs were about 40% reduced in sites with high LUI compared with low LUI.

In particular, fertilization lowered PMORs, possibly due to the ammonium ions in the fertilizer since ammonium ions can inhibit the MMO enzyme [87]. Additionally, grazing and mowing added to the negative effect of high LUI. The compaction of the soils may cause this adverse effect due to the trampling of animals or mowing machinery. Furthermore, heavy grazing reduces water infiltration into the soil [166]. Our results support an adverse effect of high land-use intensity on soil CH_4 oxidation, reported by previous studies.

In contrast, forest management practices were not influencing PMORs. However, adverse effects may be present in logging trails that we excluded from sampling. Even though forest management practices had no effect, tree species slightly affected PMORs. They were lower in oak compared to beech-dominated forests. Our results suggest that commonly applied forest management practices have no adverse effect on CH_4 oxidation in forests.

Putative atmospheric MOB. We quantified the abundances of two groups of the putative MOB, USCα and USCγ, with qPCR to ascertain their influence on PMORs. We detected an ecosystem-specific distribution of these two groups. USCα was present in all forest soils but not detectable in 56% of the grassland soils. In contrast, USCγ was present in nearly all grassland soils but absent in more than two-thirds of the forest soils. The abundances of USCα and USCγ were correlated with PMORs in all forest and grassland soils, respectively. Also, structural equation modeling identified USCα and USCγ gene abundance as direct drivers of PMORs. In forests, only USCα had a significant effect, while in grasslands, USCα and USCγ both positively affected PMORs, with USCγ having a slightly stronger impact on PMORs. Soil pH was the most influential predictor of MOB abundances. USCα abundance was influenced by pH: they were generally higher abundant in acidic soils. In contrast, pH positively influenced USCγ, which was mostly absent in acidic soils. Our results underscore that these two groups are involved in atmospheric CH_4 oxidation in soils.

3.1.2 Publication II: Linking transcriptional dynamics of CH_4-cycling grassland soil microbiomes to seasonal gas fluxes

In publication II, we investigated net CH_4 fluxes and the CH_4-cycling communities in two grassland soils with internal CH_4 production. We detected seasonal variations in CH_4 fluxes. Despite relatively high PMORs in both soils (as presented in publication I), both soils emitted CH_4 in autumn and winter. In contrast, they took up CH_4 in spring and summer with relatively high uptake rates. This difference highlights that soil gas fluxes are highly dynamic and dependent on environmental conditions. The soils emitted CH_4 when the soil water content was high and took up CH_4 when low.

Next to net gas fluxes, we investigated the CH_4-cycling community. It is challenging to link microbial abundances assessed by molecular methods to ecosystem processes. Therefore, we used quantitative metatranscriptomics to investigate mRNA and SSU rRNA abundances and methanogen and MOB composition. We aimed to evaluate if SSU rRNA or mRNA abundances of CH_4-cycling microbes better reflected the seasonal changes in soil net CH_4 fluxes:

1) The net CH_4 fluxes correlated positively with abundances of mRNA of methanogenesis pathways ($p < 0.01$). In contrast, they did not correlate significantly with SSU rRNA abundances of methanogens.
2) At the mRNA transcript level, the ratio of pMMO to methanogenesis was higher in spring and summer than in autumn and winter. Hence a higher ratio was linked to net CH_4 uptake. However, the methanotroph to methanogen SSU rRNA transcripts ratio had no clear trend across seasons. It was even highest in autumn when the soils emitted CH_4.
3) The community composition was slightly different between SSU rRNA and mRNA. We used a *pmoA* database to annotate putative mRNA sequences of MOB. The community composition of the *pmoA* transcripts slightly differed from the composition of the SSU rRNA. The differential community composition may indicate differences between microorganisms' ribosomal abundances and gene expression.
4) Despite higher methanogen SSU rRNA abundances in one site, the abundances of methanogenesis mRNA transcripts were similar on both sites. This discrepancy may indicate lower transcriptional activity in one grassland. Since the community composition differed between the sites, it may be that the transcriptional activities vary between methanogenic groups.

Thus, our results underscore that mRNA is a better indicator for soil CH_4 fluxes than SSU rRNA. Generally, these results may also indicate that mRNA may be better representing ecosystem processes than SSU rRNA.

3.1.2.1 Do metatranscriptomics markers reflect net soil CH_4 fluxes?

Publication II shows the high variability of CH_4 fluxes and the CH_4-cycling community across seasons. The abundance, composition, and activity of the CH_4-cycling community rely on soil physicochemical properties that change throughout the year. The CH_4 production by methanogens and CH_4 consumption by methanotrophs determine whether soils are a sink or a source of CH_4 at a given time.

Likely, soil water content and redox status largely determine the activity of methanogens and methanotrophs. Generally, a high water content leads to lower redox potential favoring methanogen activity. However, it also hampers methanotroph activity.

In autumn and winter, a high soil water content caused low redox potentials, which favored methanogen activity and gene expression of mRNA related to methanogenesis pathways (Figure 3). In turn, the produced CH_4 fueled the methanotrophs' activity and growth and the expression of genes encoding the pMMO. The methanotrophs acted as a filter mitigating large parts of the CH_4 emissions. However, the soils were still net emitters of CH_4. The ratio of pMMO to methanogenesis mRNA transcript was low during autumn and winter. Hence, such a low ratio was a further indicator of the soils being a net source of CH_4.

In spring and summer, the situation in the soil was different: the water content was low, favoring high redox potential in the soils. These conditions hampered methanogen activity, resulting in low abundance and gene expression of methanogens and lower CH_4 production (Figure 3). The CH_4 availability was likely smaller than in wet soils; hence there was less methanotroph activity. The methanotrophic CH_4 oxidation was high enough to prevent CH_4 emissions from the soil. On the contrary, the soils were even a sink for atmospheric CH_4. A higher pMMO to methanogenesis-related mRNA ratio also indicated this CH_4 uptake. In spring and summer, the low water content favors aeration of the soils, and the methanotrophs can oxidize the CH_4 at atmospheric concentration.

Generally, there were more aerobic than anaerobic methanotrophs. 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. Anaerobic methanotrophs were present mainly in the deeper soil layer, which was likely due to their sensitivity to oxygen [167]. Still, redox potential alone cannot explain the distribution of anaerobic methanotrophs in our study. The relative abundance of anaerobic methanotrophs in one site 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 due to grazing and subsequent mineralization

and N release from feces. Our results suggest that NC10 is a relevant methanotrophic group that mitigates CH_4 emissions from drained peatlands and that oxygen and substrate availability influence their abundance.

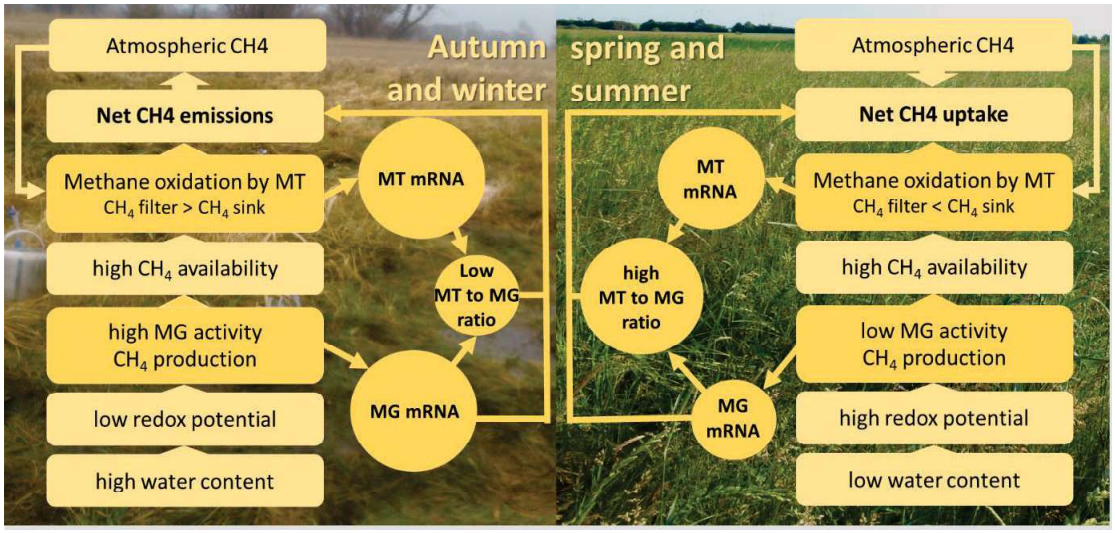

Figure 3 The activity of CH_4-cycling microorganisms and CH_4 emissions across seasons. Overview of soil physicochemical conditions, microbial activity, and their molecular markers in autumn/winter and spring/summer. Dark yellow boxes refer to biotic microbial processes and microbial markers. Light yellow boxes and circles refer to abiotic ones. The circles and size of the circles represent microbial activity.

3.1.3 Publication III: Desiccation time and rainfall control gaseous carbon fluxes in an intermittent stream

In publication III, CH_4 fluxes of drained sediments were monitored across 62 days. There was an early peak of CH_4 emissions on day three (30 mg C m^{-2} h^{-1}), followed by soil CH_4 uptake. After that, the sediments were minor sources of CH_4 until there were no detectable fluxes during the last weeks of the experiment.

In contrast to the highly variable CH_4 fluxes, the community composition of the CH_4-cycling community was relatively stable throughout the experiment. Despite initial high CH_4 emissions, we did not detect an increase of methanogens in the sediments. Physical causes might thus explain the release of CH_4. Before the drainage, the water acted as a physical barrier preventing gas evasion from the sediments. With the water gone, the previously trapped CH_4 could diffuse into the atmosphere.

3.1.4 Can microcosm measurements resemble in situ CH_4 uptake?

Publications II and III have shown that CH_4 fluxes of soils can vary heavily across time or seasons, depending on the activity of CH_4-cycling microorganisms and physicochemical soil conditions. It is thus challenging to compare *in situ* CH_4 fluxes measured at different sites and time points.

For this reason, we investigated soils and the methanotrophs living in them in a microcosms experiment (publication I). We adjusted the water content to the optimal range for CH_4 oxidation. Ensuring these standardized conditions between the different soils enabled us to measure PMORs independent of *in situ* conditions. The abundances of *pmoA* genes in the soil positively correlated with the PMORs indicating that DNA-based *pmoA* gene abundance measurements can point to potential soil CH_4 uptake. However, to what extent the soil methanotrophs can ultimately use this potential depends on the soil's physicochemical properties such as soil water content, temperature, water table, O_2, and CH_4 availability. These undergo seasonal changes, and the question is how well these laboratory measurements reflect CH_4 uptake *in situ*.

Therefore, in publication II, we investigated net CH_4 fluxes and the CH_4-cycling microbes in two grasslands with internal CH_4 production across seasons. Despite relatively high PMORs (as presented in publication I), both grasslands emitted CH_4 in autumn and winter. Whether soils act as net sources or sinks for CH_4 thus depends on the interactions of methanogens and methanotrophs and the ecophysiology of these two groups. The soil water content used in the microcosms was too low to expect much methanogenic activity. In contrast to autumn and winter, the soils took up CH_4 in spring and summer. The net methane uptake rates in summer were within 10% of the mean PMORs measured in publication I (Table 1). The comparison between the PMORs (publication I) and the CH_4 uptake rates in summer (publication II) supports that the PMORs measured under defined conditions can resemble ecosystem-scale measurements.

This thesis highlights that the same grassland can be a sink and a source of CH_4 during the year, depending on the dynamic interplay of methanogens and MOB, affected by soil water content and temperature. It furthermore underscores the necessity for temporarily repeated ecosystem-scale measurements to estimate CH_4 fluxes from soils.

Table 1 Comparison of potential CH_4 oxidation rates (PMOR) measured in publication I and the CH_4 fluxes measured in publication II in LI (SEG15) and HI (SEG9). The thickness of soils in the microcosms was only 1.9 cm. Therefore, the PMORs (per m_2) were extrapolated to a thickness of 10 cm to resemble the thickness of the most active zone for CH_4 oxidation in soils.

	publication I	**publication II**	
		CH_4 uptake	CH_4 uptake
	PMOR	spring	summer
	mg C $m^{2\text{-}1}$ d^1	mg C $m^{2\text{-}1}$ d^1	mg C $m^{2\text{-}1}$ d^1
LI (SEG15)	1.09 ± 0.08	0.41 ± 0.22	0.98 ± 0.10
HI (SEG9)	0.74 ± 0.13	0.62 ± 0.12	0.75 ± 0.08

3.1.5 Who oxidizes CH_4 at atmospheric concentrations in the soils?

To find a link between soil CH_4 uptake and soil MOB, this thesis investigated the community composition of MOB with qPCR targeting the *pmoA* gene and quantitative metatranscriptomics. In publication I, the abundance of USCα and γ *pmoA* genes correlated with atmospheric CH_4 oxidation rates. This correlation supports that USCα and γ are involved in the atmospheric CH_4 uptake of soils and are likely the most important groups for this process. Furthermore, in publication II, a high proportion of atmospheric CH_4 oxidizers (USCα, USCγ, and *pmoA2*) in the *pmoA* transcripts was linked to net CH_4 uptake of the soils in spring and summer. Still, the largest fraction of *pmoA* transcripts was assigned to canonical MOB. Recently, atmospheric CH_4 oxidation in paddy soils was attributed to canonical CH_4 oxidizers rather than USCα and USCγ [50]. However, despite the wide distribution of USCα and γ, *pmoA* genes of canonical MOB, detectable with the A189f/mb661 primer pair, were absent in most soils investigated (as presented in publication I). Only the grasslands in the Schorfheide with its many temporarily wet soils were an exception. Possibly, the canonical MOB contribute to the soil CH_4 sink in such soils with (temporal) internal CH_4 production. In soils without such internal CH_4 oxidation, USCα and γ MOB probably outcompete canonical CH_4 oxidizers. This thesis supports that USCα and γ methanotrophs constitute the atmospheric CH_4 sink in soils without internal CH_4 production. In soils with internal CH_4 production, canonical CH_4 oxidizers may also contribute to the atmospheric CH_4 sink.

3.2 Conclusions and perspectives

In publication I, we investigated the microbial CH_4 sink of soils. Methanotrophic soil bacteria oxidize up to 30-40 Tg of this potent greenhouse gas per year [7, 43]. These microorganisms hence catalyze the only biological process of removing CH_4 from the atmosphere. Only if we understand which factors influence the soil CH_4 uptake can we find strategies to enhance it. Thus, this study can give valuable insight into how to augment the soil's CH_4 sink.

Publication I suggests that reducing land-use intensity, especially fertilization, may increase the soil CH_4 sink of grasslands. Additionally, reducing the grazing and mowing intensity can further assist in enlarging the soil CH_4 oxidation. Furthermore, since PMORs were approximately two times higher in forest soils compared to grassland soils, afforestation may be a powerful strategy to increase the CH_4 sink. However, one limitation of this study was that we measured CH_4 oxidation rates under defined conditions in microcosms. It thus represents possible rates that may not resemble *in situ* rates. Still, the PMORs accord with measurements in summer, as determined in publication II. This indicates that the PMORs well represent the soil microbiome's potential for oxidizing at atmospheric concentrations *in situ*. Nevertheless, more research is needed to study the CH_4 uptake rates with on-site measurements across different seasons in soils with different land-use intensities. Furthermore, it may be interesting to investigate PMORs at elevated CH_4 concentrations to determine their potential to mitigate CH_4 emissions from soils.

In addition to negatively affecting CH_4 oxidation in soils, high land-use intensity may also influence methanogenesis and soil CH_4 emissions. For instance, one study found higher CH_4 emissions and archaeal gene abundances in a grazed compared with a non-grazed site [88]. However, we investigated only two sites in publication II and cannot make statistically assured statements about this effect. To investigate this link further, one could measure CH_4 emissions and CH_4-cycling communities, including more sites. Another approach may be to determine potential CH_4 production rates in microcosms at higher soil water contents or under an O_2-depleted atmosphere.

Furthermore, this thesis sheds light on the microorganisms that oxidize CH_4 at atmospheric concentrations. Publication I and II underline that USCα and USCγ are involved in atmospheric CH_4 oxidation in soils. Moreover, publication II adds to the growing body of literature (e.g. [50]) that suggests that the canonical MOB contribute to atmospheric CH_4 oxidation in soils with internal CH_4 formation. Still, in upland soils that are permanent net sinks of CH_4, USCα

and USCγ are likely more influential, as shown in publication I. Stable isotope probing may be well suited to investigate this further.

In publication II, we could link the mRNA abundances of methanogens to soil CH_4 net fluxes. It may thus be feasible to estimate soil CH_4 emissions using mRNA transcript abundances of methanogens. However, to find out if this holds for annual rates based on temporarily highly resolved real-time data requires more research. Therefore, large-scale studies, such as the one presented here, are encouraged to investigate the link between methanogens and methanotrophs and CH_4 fluxes across different soil types, seasons, and land-use intensities. The ever-decreasing sequencing costs and further automatization in bioinformatics workflows may make this feasible in the future. Then, having more data, one could ultimately try to use metatranscriptomics data to model soil CH_4 fluxes. Next to quantitative metatranscriptomics, parallel RT qPCRs of *mcrA* and *pmoA* transcripts may be viable tools to estimate CH_4 fluxes of soils from many samples and sites.

4 Publications

4.1 Statement of authorship

This thesis consists of three published manuscripts with multiple authors. The contribution of each author was as follows:

Publication I: Täumer, Jana, Steffen Kolb, Runa S. Boeddinghaus, Haitao Wang, Ingo Schöning, Marion Schrumpf, Tim Urich, and Sven Marhan. 2021. "Divergent drivers of the microbial methane sink in temperate forest and grassland soils." Glob Change Biol 27 (4): 929–40. https://doi.org/10.1111/gcb.15430.

Author contributions: TU, SK, and SM designed the study. Sampling was coordinated by MS and IS and the biodiversity exploratory office. JT joined the sampling in the Schorfheide region. SM and JT performed microcosms incubation, gas measurements, and determination of soil water content. DNA was extracted by the members of the biodiversity exploratories and sent to JT. JT executed qPCR analysis. Determination of water holding capacity, bulk density, organic carbon, total nitrogen, soil type, and texture were performed by MS and IS. SM and RB determined ammonium and nitrate content of the soil. Data analysis was performed by JT, SM, TU, and SK. The manuscript was written by JT and SM, assisted by all co-authors.

Publication II: Täumer, Jana, Sven Marhan, Verena Groß, Corinna Jensen, Andreas W Kuss, Steffen Kolb, and Tim Urich. 2022. "Linking transcriptional dynamics of CH_4-cycling grassland soil microbiomes to seasonal gas fluxes." ISME J 16 (7): 1788–97. https://doi.org/10.1038/s41396-022-01229-4.

Author contributions: The study was designed by TU, SK, and SM. Sampling was coordinated by JT assisted by TU, SM, SK. Gas flux and C_{mic}, N_{mic} analysis were performed by SM. RNA extractions and amplifications were performed by JT and VG. Sequencing and library preparation was performed by CJ and AK assisted by JT, VG, and TU. Data analysis was performed by JT, VG, TU, SM, and SK. The manuscript was written by JT and TU, assisted by all co-authors.

Publication III: Arce, Maria Isabel, Mia M. Bengtsson, Daniel von Schiller, Dominik Zak, Jana Täumer, Tim Urich, and Gabriel Singer. 2021. "Desiccation time and rainfall control gaseous carbon fluxes in an intermittent stream." Biogeochemistry 155 (3): 381–400. https://doi.org/10.1007/s10533-021-00831-6.

Author contributions: MIA, DvS and GS conceived and designed the experiment. Material preparation and data collection were performed by MIA and DZ. MMB, TU and JT analysed 16sRNA sequences. The first draft of the manuscript was written by MIA. All authors contributed to the development of the manuscript and read and approved the final version. All data, materials and software application supporting our published claims comply with field standards. Illumina MiSeq 16 S rRNA amplicon sequence data is submitted to the NCBI Short Read Archive (Accession No. SRP137655). Other datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

In agreement for publications I – III,

Greifswald, ----------	Greifswald, ----------
Prof. Dr. Tim Urich	Jana Täumer
Supervisor	PhD Student

4.2 Publication I

Divergent drivers of the microbial methane sink in temperate forest and grassland soils

authors: Jana Täumer, Steffen Kolb, Runa S. Boeddinghaus, Haitao Wang, Ingo Schöning, Marion Schrumpf, Tim Urich, and Sven Marhan

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PRIMARY RESEARCH ARTICLE

Global Change Biology WILEY

Divergent drivers of the microbial methane sink in temperate forest and grassland soils

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Abstract

Aerated topsoils are important sinks for atmospheric methane (CH_4) via oxidation by CH_4-oxidizing bacteria (MOB). However, intensified management of grasslands and forests may reduce the CH_4 sink capacity of soils. We investigated the influence of grassland land-use intensity (150 sites) and forest management type (149 sites) on potential atmospheric CH_4 oxidation rates (PMORs) and the abundance and diversity of MOB (with qPCR) in topsoils of three temperate regions in Germany. PMORs measurements in microcosms under defined conditions yielded approximately twice as much CH_4 oxidation in forest than in grassland soils. High land-use intensity of grasslands had a negative effect on PMORs (−40%) in almost all regions and fertilization was the predominant factor of grassland land-use intensity leading to PMOR reduction by 20%. In contrast, forest management did not affect PMORs in forest soils. Upland soil cluster (USC)-α was the dominant group of MOBs in the forests. In contrast, USC-γ was absent in more than half of the forest soils but present in almost all grassland soils. USC-α abundance had a direct positive effect on PMOR in forest, while in grasslands USC-α and USC-γ abundance affected PMOR positively with a more pronounced contribution of USC-γ than USC-α. Soil bulk density negatively influenced PMOR in both forests and grasslands. We further found that the response of the PMORs to pH, soil texture, soil water holding capacity and organic carbon and nitrogen content differ between temperate forest and grassland soils. pH had no direct effects on PMOR, but indirect ones via the MOB abundances, showing a negative effect on USC-α, and a positive on USC-γ abundance. We conclude that reduction in grassland land-use intensity and afforestation has the potential to increase the CH_4 sink function of soils and that different parameters determine the microbial methane sink in forest and grassland soils.

KEYWORDS

greenhouse gas, land-use intensity, methane, methanotrophs, potential methane oxidation rates, soil, Upland soil cluster

1 | INTRODUCTION

The tropospheric concentration of methane (CH_4) has increased by 150% since the beginning of the industrial era and its warming potential is 28 times higher than that of CO_2 (Ciais et al., 2013). More than one-third of global CH_4 emissions derive from methanogenesis in soils under anoxic conditions, which occur, for example, in wet rice cultivation and permanent or temporary wetlands (Ciais et al., 2013; Conrad, 2009). In contrast, well-aerated soils typically function as net sinks for atmospheric CH_4 due to the consumption of CH_4 by methanotrophic bacteria (Kolb, 2009; Le Mer & Roger, 2001; Tate, 2015). CH_4 oxidation is primarily considered to be aerobic and is catalysed by bacteria within the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* but also the anaerobic candidate phylum NC10 (Knief, 2015). The key enzyme for atmospheric methanotrophy is the particulate CH_4 monooxygenase (pMMO; Baani & Liesack, 2008; Knief, 2015). Studies targeting the gene encoding the alpha subunit of pMMO (*pmoA*) as a functional marker have found that CH_4-oxidizing bacteria (MOB) are highly diverse; additionally, several major soil lineages are currently poorly characterized or even missing cultured representatives, such as the Upland soil cluster (USC)-γ (Knief, 2015). Methanotrophs solely dependent on atmospheric CH_4, however, have resisted cultivation until very recently, when the atmospheric CH_4 oxidizer *Methylocapsa gorgona* was isolated (Tveit et al., 2019). *M. gorgona* is a member of USC-α that has been detected in many different soils, such as forest and permafrost soils with mostly neutral to acidic pH (Degelmann et al., 2010; Kolb, 2009; Kolb et al., 2005; Pratscher et al., 2018; Tveit et al., 2019). Other MOB assumed to be involved in atmospheric CH_4 oxidation are members of USC-γ, which was detected in neutral to alkaline upland soils and have recently been identified as the main methanotrophs in alpine grassland soils (Deng et al., 2019; Knief, 2015).

Whether a soil acts as source or sink for CH_4 is strongly controlled by soil environmental parameters such as oxygen, substrate availability, temperature, and N status, all of which are known to change the habitat and living conditions for methanogens as well as for MOB (Bodelier, 2011; Lyu et al., 2018).

Land-use change and management practices influence these soil environmental parameters and may therefore alter soil CH_4 fluxes (Tate, 2015). A recent global meta-analysis revealed that the conversion from a natural to any anthropogenic land use increases CH_4 emissions (McDaniel et al., 2019). However, the effects of land-use intensity and its mediating drivers on CH_4 emissions have not yet been resolved. It is generally assumed that fertilizers, especially ammonium-based fertilizers, decrease CH_4 oxidation rates due to competitive inhibition of the methane monooxygenase. In grassland soils, different management practices and intensities have been shown to influence atmospheric CH_4 uptake. For example, heavy livestock grazing reduces CH_4 uptake by 24%–31% (Chen et al., 2011) and N fertilization can negatively affect CH_4 oxidation in cultivated soils (Mosier et al., 1991). In a more recent study on three Swiss grassland sites with different management intensities and elevations, highest CH_4 uptake was found at the least intensively and lowest CH_4 uptake at the most intensively managed site (Imer et al., 2013). A meta-analysis by Liu and Greaver (2009), which found CH_4 uptake reduced when upland grassland soils were N-fertilized, further indicates that CH_4 uptake by grassland soils can be influenced by land-use intensity.

CH_4 uptake rates by forest soils were typically more pronounced than those of grassland soils with deciduous forests the strongest sinks for atmospheric CH_4 (Degelmann et al., 2009; Liu & Greaver, 2009). Similar to grassland management, forest management also influences atmospheric CH_4 uptake. The conversion of natural hardwood forests to spruce and pine forests reduced its CH_4 sink potential by about two-thirds (Borken et al., 2003; Maurer et al., 2008). Other forest management effects, such as soil disturbance, compaction during clear-cutting and thinning, or N-deposition, have also been found to negatively affect the CH_4 sink function of forest soils (Frey et al., 2011; Steudler et al., 1989; Teepe et al., 2014). However, a general negative effect of N fertilization on CH_4 oxidation in both forest and upland grassland soils has also been questioned as it seems to depend on the amount of N present in soil (Bodelier, 2011; Bodelier & Laanbroek, 2004).

To date, few studies have linked atmospheric CH_4 oxidation to the abundances of the methanotrophic groups and the environmental factors influencing their abundances. It has been found for different soils that the proportion of USC-α was positively correlated with CH_4 uptake (Nazaries et al., 2013) and thus might be a key group of MOB contributing to the global atmospheric CH_4 sink. Malghani et al. (2016) also linked the abundance of USC-α methanotrophs to CH_4 oxidation rates. However, environmental factors can differentially influence CH_4 oxidation and the methanotrophic community. For example, increasing soil moisture has been shown to lower CH_4 oxidation while stimulating MOB abundance in forest soils (Shrestha et al., 2012). Recently, USC-γ has been identified as a dominant group in grassland soils (Zhao et al., 2018), but it is not clear how the abundances of different MOB groups relate to CH_4 oxidation in soils or how they respond to land use and land-use intensity.

To investigate the relationship between MOB abundance, CH_4 oxidation, land-use type (grassland and forest) and intensity of land use in more detail, we sampled topsoils of 150 grassland and 150 forest sites that differ in their grassland land-use intensity and in the type of forest management, respectively, in three temperate regions in Germany (Schwäbische Alb [ALB], Hainich-Dün [HAI], and Schorfheide-Chorin [SCH] region). We measured potential CH_4 oxidation rates, soil physicochemical properties, and determined the abundances of the methanotrophic bacterial groups USC-α and USC-γ, which are assumed to be involved in CH_4 oxidation at atmospheric concentrations. We hypothesized that in grasslands, high management intensity (fertilization and/or frequent grazing and mowing) will reduce CH_4 oxidation rates due to higher availability of ammonium in soils and to greater soil compaction by machinery use and/or livestock trampling. In forests, intense management will reduce CH_4 oxidation rates due to soil compaction resulting from forest machinery. Furthermore, soils with a higher abundance of MOB will have higher potential CH_4 uptake rates. In addition, we assume that soil environmental properties drive both CH_4 uptake and the abundance of MOB in soils.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The study was conducted within the framework of the Biodiversity Exploratories project for long-term functional ecosystem research (Fischer et al., 2010; www.biodiversity-exploratories.de). The Biodiversity Exploratories are located in three different climate regions of Germany: Schwäbische Alb (southwest, annual mean precipitation: 700–1,000 mm, annual mean temperature 6–7°C, abbreviated as ALB), Hainich-Dün (central Germany, annual mean precipitation: 500–800 mm, annual mean temperature 6.5–8°C, abbreviated as HAI), and Schorfheide-Chorin (northeast, annual mean precipitation: 500–600 mm, annual mean temperature 8–8.5°C, abbreviated as SCH). In each region, 50 grassland (50 m × 50 m) and 50 forest sites (100 m × 100 m) were selected (Table S1). Soil types varied between sites and were classified according to WRB (IUSS Working Group WRB, 2015). The grasslands were managed as meadows, pastures, or mown pastures. Grazing intensity, fertilization, and mowing frequency were monitored annually and a land-use intensity index (LUI) was calculated for each site for 2016 (Blüthgen et al., 2012). The LUI was calculated for the year 2016 for each plot as the square root of the sum of the standardized grazing intensity (livestock units days of grazing ha^{-1} $year^{-1}$), mowing frequency per year and the amount of nitrogen applied on the plot per year (kg nitrogen ha^{-1} $year^{-1}$). The values were standardized according to its mean within all plots.

In the forest sites, dominant tree species were beech, spruce, pine, or oak. A forest management index (ForMI) was calculated based on the proportion of non-native tree species, the proportion of harvested tree biomass, and the proportion of dead wood showing signs of saw cuts (Kahl & Bauhus, 2014).

2.2 | Soil sampling and soil properties

All 299 sites were sampled in May 2017. In each plot, one composite soil sample was prepared consisting of 14 soil cores (upper 10 cm of mineral soil) that were taken along two intersecting transects (20 m in grasslands; 40 m in forest). The organic layer (forests) and vegetation above the soil (grasslands) had been removed before sampling. Samples were sieved (<5 mm) and stored at 4°C for measurements of potential CH_4 oxidation and at −20°C for DNA extraction and measurements of soil properties.

Gravimetric soil water content was determined by drying 3–6 g of soil at 105°C to constant weight. Soil pH was measured by mixing 10 g of air-dried sieved soil with 25 ml 0.01 M $CaCl_2$ solution and measuring the pH of the suspension with a glass electrode (pH meter 538 and pH glass electrode SenTix 61; WTW). An aliquot of the soil sample was dried at 105°C to determine the bulk density based on the sample volume and mass. The proportion of sand (2–0.063 mm), silt (0.063–0.002 mm), and clay (<0.002 mm) in the soil samples was determined by sieving and sedimentation (DIN-ISO 11277). Samples for the determination of soil texture were taken in May 2011 as described above. Soil texture was classified according to the German 'Standortserkundungsanweisung' (SEA 1974) with the R package 'soiltexture' (Moeys, 2018). For total carbon and total nitrogen measurements, samples were sieved (<2 mm) and air-dried, ground in a ball mill (RETSCH MM200; Retsch) and analysed in an elemental analyzer (VarioMax) at 1,100°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 hr. Organic carbon concentrations were calculated as the difference between total carbon and inorganic carbon. Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 0.5 M K_2SO_4 (soil to extractant ratio [w/v] of 1:4), shaken on a horizontal shaker for 30 min at 250 r.p.m., and centrifuged for 30 min at 4,400 *g*. The concentrations of NH_4^+-N and NO_3^--N were measured on an autoanalyzer using UV spectroscopy (Bran & Luebbe).

2.3 | Potential methane oxidation rates

Potential atmospheric CH_4 oxidation rate (PMOR) was measured under atmospheric mixing ratios (2 ppm CH_4) in microcosms of all 299 soil samples in triplicate. For this, an equivalent to 40 g soil dry weight (organic soils) and 70 g (mineral soils) fresh soil was weighed into plastic vessels (average diameter 6.8 cm). The water content was adjusted to 34% of the maximum water holding capacity of the respective soil, since 34% of maximum water holding capacity has been previously identified as the mean optimum for CH_4 oxidation in different soils (Gulledge & Schimel, 1998). The water content was adjusted by gently air-drying the soil at 4°C (for 3–72 hr) or adding deionized water to the soil. The soil was compacted in the plastic vessels to a bulk density of 0.7–0.8 g/cm^3 and pre-incubated at 20°C for 5 days. The plastic vessels with the soils were put into glass jars (500 ml Weck Gläser; J. Weck GmbH u. Co. KG) which were closed with airtight lids and incubated the soil samples at 20°C in the dark. After airtight closing of the microcosms the headspace was over-pressurized by adding 50 ml of ambient air. Gas samples (12 ml) were taken from the headspace immediately, 1, 2 and 6 hr after closing with an airtight syringe through a three-way stopcock and transferred into pre-evacuated exetainers (5.9 ml; Labco Lt). Gas concentrations were measured with an Agilent 7890 gas chromatograph equipped with a flame ionization detector (for CH_4) coupled with a methanizer (for CO_2; Agilent Technologies Inc.). Gas flux rates were calculated by the slope of the regression line of a linear regression of the gas concentration against time.

2.4 | DNA extraction and qPCR

DNA was extracted from the soil (stored at −20°C) with the Qiagen DNeasy PowerSoil Kit according to the manufacturer's instructions, and stored at −20°C until further use. DNA concentrations were measured on a NanoDrop™ 8000 (Thermo Fischer Scientific). A preselection of 30 soils from all regions and land-use types were screened for the presence of *pmoA/mmoX* genes of specific methanotrophic taxa (general *pmoA*, USC-α, USC-γ, Verrucomicrobia, and *Methylocella*; Costello & Lidstrom, 1999; Kolb et al., 2003,

2005; Sharp et al., 2012; Rahman et al., 2011; Table S2). For the preselection, five samples with different PMOR (highest, lowest and from each region and land-use type) were chosen. Three groups of methanotrophic bacteria were quantified with three different quantitative PCR assays in a 7500 Fast Real-Time PCR System (Applied Biosystems). A general *pmoA* assay was used to detect a broad spectrum of MOB (Costello & Lidstrom, 1999), the FOREST assay (Kolb et al., 2003) to quantify USC-α specific *pmoA*, and the GAM assay to amplify a USC-γ specific *pmoA* (Kolb et al., 2005). The qPCRs (20 μl) were performed in 96-well plates with SensiFAST™Sybr Lo-ROX master mix (Bioline [Meridian Life Science], Inc.) using a three-step thermal profile with denaturation at 95°C for 25 s, annealing at assay specific temperature (Table S2) for 20 s, and elongation at 72°C for 45 s. Bovine serum albumin was added to the master mix (final concentration 2 ng/μl).

2.5 | Statistics

All statistical analyses were carried out in R (version 3.5.1; R Core Team, 2018). Data were checked for normal distribution and homogeneity of variance and transformed if necessary. Significant differences between groups were tested with a two-sample *t* test for normally distributed data and a Mann–Whitney test for non-normally distributed data. Linear regression analysis was used to assess the relationship between PMORs and physicochemical and land-use parameters. The significance levels reported were based on Pearson's coefficient. Grasslands were grouped into high and low LUI and into heavily and weakly grazed using the k-means algorithm (Hartigan & Wong, 1979). Since PMORs were region-specific (especially in grasslands), PMORs were normalized to be able to compare the effects of land use among all regions. The PMOR norm was calculated by dividing the PMOR of each plot by the mean PMOR of the respective region. qPCR measurements that were below detection limit were set to 100 for correlation analyses and structural equation modelling (SEM). SEM was used to unravel direct and indirect effects on PMORs. For this, an a priori model was set up. It was hypothesized that soil parameters (bulk density, pH, and sand content) and land-use intensity in forest and grassland have a direct influence on PMORs and also an indirect effect via MOB abundances. Bulk density was chosen as a representative for other soil factors (water holding capacity, organic carbon, and total nitrogen content) with which it covariates strongly. pH was chosen since it is an important factor for microbial activity (Lauber et al., 2009). Also sand content was included in the model to represent the soil texture. The variables were transformed to normal distribution according to Templeton (2011). The model was fit with maximum-likelihood estimation ('sem' function in lavaan; Rosseel, 2012). Since multivariate normality was not met in every model, we used Satorra–Bentler correction to obtain robust fitting statistics (estimator = 'MLM'). In the forest model, the path coefficient of pH to PMOR was constrained to zero since this improved model fit. In the forest models of the single regions, the path coefficient of USC-γ to PMOR was constrained to zero since USC-γ was absent in many forest soils.

3 | RESULTS

3.1 | Influence of land use, soil type, and soil texture on PMORs

Uptake of atmospheric CH_4 was detected in all 299 topsoils. PMORs varied between 0.006 and 1.695 ng CH_4 g^{-1} DW hr^{-1} and were significantly higher in forest than in grassland soils ($mean_{forest} = 0.60$ ng CH_4 g^{-1} DW hr^{-1}, $mean_{grassland} = 0.31$ ng CH_4 g^{-1} DW hr^{-1}, $p < .001$; Figure 1a). This difference between forest and grassland soils was significant in all regions ($p_{ALB} < .001$, $p_{HAI} < .001$, $p_{SCH} < .01$; Figure 1b). In the forest soils, PMORs did not vary among regions, but in grassland soils PMORs were highest in ALB, lowest in HAI, and highly variable in SCH, presumably due to the high diversity of soil types and textures in this region.

In the forest soils, PMORs did not differ with respect to soil texture (Figure S1a), but in grasslands PMORs were highest in loamy clay and loamy silt and lowest in loamy sand, silty clay, and sandy loam soils (Figure S1b). However, in the silty clay and loamy sand textures of the forest, soils' PMORs were higher than in in grassland soils of similar texture. High clay content appeared to have a generally negative effect in the ALB region (both forest and grassland sites) but a positive effect in the SCH region (grasslands only, Figure S2). Sand content therefore resulted in opposite trends in these two regions. Sand content was mostly high in SCH and typically low in the ALB region grasslands (Figure S2c,f).

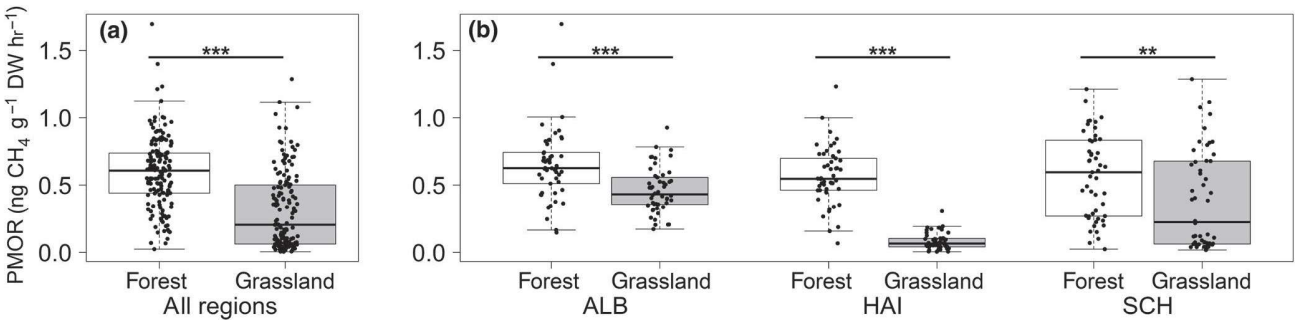

FIGURE 1 Potential methane oxidation rates (PMORs) in forests and grasslands. PMORs (a) including all regions separated into forests and grasslands and (b) in the regions Schwäbische Alb (ALB), Hainich (HAI) and Schorfheide (SCH) in forests and grasslands, significance codes: $p < .01$ (**), $p < .001$ (***), $n = 299$

3.2 | Influence of forest management, tree species, and grassland land-use intensity on PMORs

PMORs in forest soils were neither correlated to the ForMI nor to its components (proportion of non-native tree species, harvested tree biomass, and proportion of dead wood showing signs of cut, Figure S3). However, the dominant tree species did significantly affect PMORs, with lowest CH_4 oxidation in oak, and highest in beech and spruce forests (Figure 2). Oak were only in slightly loamy sand soils in SCH region. However, when only this soil texture and region were considered, PMORs were still significantly lower in oak than beech forests ($p < .05$).

In contrast to forests, where management showed no influence, the LUI in grasslands was negatively correlated with PMORs when all regions were included ($r_{LUI} = -.27$, $p < .001$; Figure S4a). When grasslands of all regions taken together were categorized into low and high LUI, PMORs were reduced by about 40% in high as compared to low LUI grassland soils (Figure 3a). With respect to the single components of LUI, fertilization decreased PMORs by about 20% (Figure 3b). Considering all grassland sites, grazing intensity and mowing frequency had no significant effect on PMORs (Figure 3c,d). Grassland management also affected the concentrations of NH_4^+ in soil, which were higher in non-fertilized compared to fertilized grasslands (Figure S5b), while NO_3^- concentrations were higher in fertilized than in non-fertilized soils (Figure S5f).

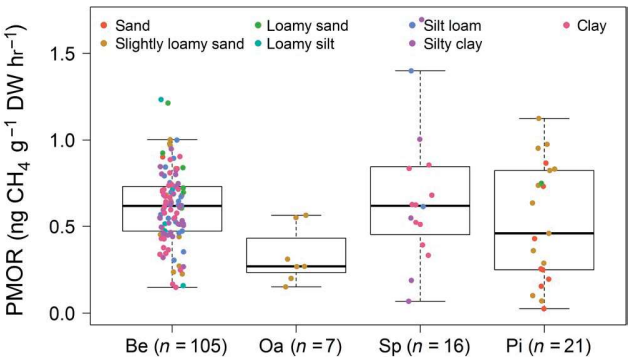

FIGURE 2 Effect of tree species (Be = beech, Oa = oak, Sp = spruce and Pi = pine) on potential methane oxidation rates (PMORs) in the forests including all regions. Coloured points indicate soil texture, $n = 149$

3.3 | Correlations of soil properties with PMORs

Considering all forest soils, PMORs were neither correlated with water holding capacity nor with organic carbon and total nitrogen content (Figure S6a–c). PMORs were, however, negatively correlated with bulk density across all forest soils ($r_{bd} = -.17$, $p < .05$; Figure S6e). In ALB and HAI, pH was negatively correlated with PMORs, while in SCH pH was positively correlated with PMORs (Figure S6d). However, pH was generally lower in SCH than in ALB or HAI (pH_{ALB}: 3.3–6.9, pH_{HAI}: 3.85–7.15, pH_{SCH}: 3.2–3.77). The thickness of the organic layer measured in the natural habitat, but which was not included in the PMOR measurements in the microcosms, had an effect on PMORs only in the HAI region, with a positive correlation between PMORs and the thickness of the organic layer ($r_{bd} = 0.34$, < 0.05, Figure S6g).

In contrast to the forest soils, PMORs in grassland soils were positively correlated with soil organic carbon and total soil nitrogen content ($r_{OC} = .60$, $r_{Ntot} = .67$, $p < .001$; Figure S7a,b). PMORs increased with increasing soil water holding capacity, but decreased with increasing bulk density ($r_{whc} = .80$, $r_{bd} = -.77$, $p < .001$; Figure S7c,e) when all grasslands were considered together, but not for the HAI region alone. Concentrations of both, NH_4^+ and NO_3^- were positively correlated with PMORs in the grasslands ($r_{NH_4} = .38$, $r_{NO_3} = .40$, $p < .001$; Figure S7g,h) and this was most pronounced in the SCH region. The effects of the mentioned soil physicochemical conditions were usually most pronounced in the SCH region.

3.4 | Influence of land use, soil type, and soil texture on MOB

In a preselection of 30 topsoil samples, no methanotrophs belonging to *Verrucomicrobia* or *Methylocella* (*Alphaproteobacteria*)

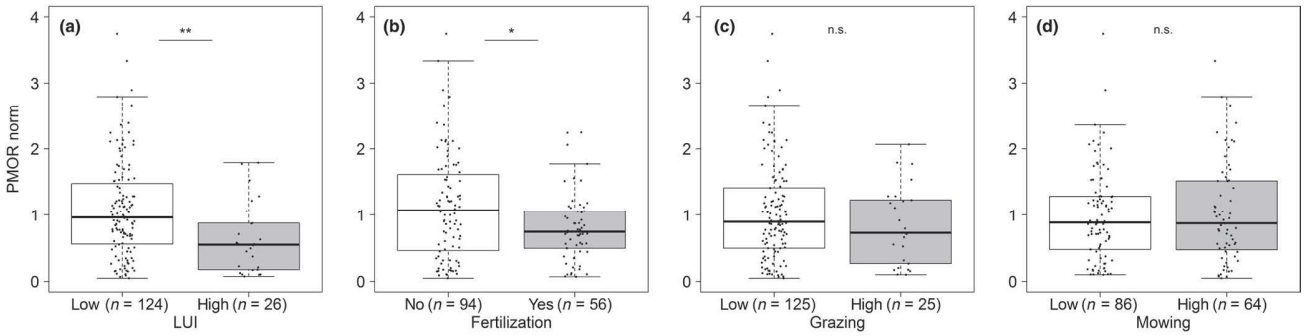

FIGURE 3 Effects of (a) land-use intensity index (LUI), (b) without (no) and with (yes) fertilization, and low and high intensity of (c) grazing and (d) mowing on normalized potential methane oxidation rates (PMORs norm) in the grassland soils. PMORs norm was calculated by dividing the PMORs of each plot by the mean PMORs of the respective region; significance codes: $p < .05$ (*), $p < .01$ (**), $n = 150$

were detected with specific PCR assays; hence, these assays were not performed for all 299 soils (data not shown). The primer pair A189f/mb661 (which targets a broad range of proteobacterial methanotrophs) yielded specific PCR products only in grassland soils from SCH while no specific products were detected in the other regions (data not shown). In contrast, we detected methanotrophs belonging to USC-α and USC-γ clades in most soils but with land-use type (forests vs. grasslands) and region-specific abundance distributions (Figure 4). USC-α abundance varied widely, from 2.8×10^4 to 8.7×10^8 *pmoA* gene copies per gram dry soil and occurred in all forest soils, but in only 56% of the grassland soils (Figure 4). USC-γ abundance ranged from 2.8×10^3 to 3.8×10^6 *pmoA* gene copies per gram dry soil and was detected in almost all grassland soils, but present only in approximately 30% of the forest soils (Figure 4). The median abundance of USC-α *pmoA* gene was almost 100 times higher in the forest than in the grassland soils in all regions ($p < .001$). In forest soils, USC-α *pmoA* gene abundance was about 50-fold higher in SCH than in either HAI or ALB. In contrast to USC-α *pmoA*, gene abundance of USC-γ was about 100 times higher in grassland than in forest soils. However, trends differed between the exploratories. In the ALB region, for example, USC-γ abundance was only twice as high in forest than in grassland sites.

3.5 | Influence of forest management, tree species, and grassland land-use intensity on MOB

USC-α gene abundance was higher in oak- and pine-dominated forests compared to spruce and beech forests while USC-γ gene abundance was higher in beech and spruce forests (Figure S8). USC-α did not correlate with ForMI, but there was a negative correlation between harvested tree biomass and USC-α (Figure S9c). USC-γ positively correlated with ForMI, non-native tree species and harvested tree biomass (Figure S9a–c). In the grasslands, there was no correlation between abundances of USC-α or USC-γ and LUI (Figure S10) and there was also no difference in USC-α and-γ copy numbers between high and low LUI or its components (Figure S11).

3.6 | Correlations of MOB abundance with soil properties and with PMORs

Upland soil cluster-α and USC-γ *pmoA* gene copy numbers responded differently to abiotic soil properties (Figures S12–S15). In forests for instance, USC-α gene copy numbers per gram soil were negatively correlated to organic carbon ($r_{Corg} = -.70$, $p < .01$; Figure S12a), whereas USC-γ abundance was positively correlated with organic carbon and nitrogen content ($r_{Corg} = .70$, $p < .001$; Figure S14a). Overall, USC-α and USC-γ abundances were differentially correlated with pH, whereas USC-α abundance was negatively correlated with pH ($r_{for} = -.70$, $r_{gras} = -.32$, $p < .001$; Figures S12d and S13d), USC-γ abundance was positively correlated with pH ($r_{for} = .54$, $r_{gras} = .39$, $p < .001$; Figures S14d and S15d).

Upland soil cluster-α abundance was negatively correlated with NH_4^+ content, whereas USC-γ abundance was positively correlated in the SCH region only ($r_{USC\alpha} = -.32$, $r_{USC\gamma} = .47$, $p < .05$; Figures S13g and S15g). USC-α and USC-γ *pmoA* gene abundances were not correlated with NO_3^- content in the grassland soils (Figures S13h and S15h).

In the forests, USC-α *pmoA* abundance correlated positively with PMORs including all regions, as well as in each of the three regions ($r_{for} = .18$, $p < .05$; $r_{ALB} = .57$, $r_{HAI} = .51$, $r_{SCH} = .68$, $p < .001$; Figure 5a). In the forests, there were no positive correlations between USC-γ *pmoA* abundance and PMORs (Figure S16a) and in the grasslands there were no positive correlations between PMORs and USC-α abundance (Figure S16b). However, in grasslands USC-γ *pmoA* copy numbers were positively correlated with PMORs when all grasslands were taken together, and also in each of the three regions (soils $r_{gra} = .44$, $r_{ALB} = .53$, $r_{HAI} = .53$, $r_{SCH} = .59$, $p < .001$; Figure 5b). When related to MOB abundance, PMOR was lower in forest than in grassland soils (Figure S17a). Within the forest soils, PMOR related to MOB was lowest in SCH region while in grasslands it was highest in SCH region (Figure S17b).

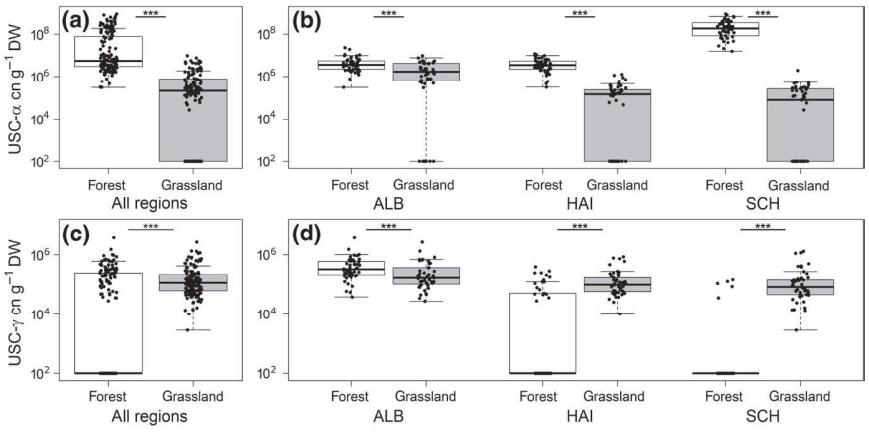

FIGURE 4 Abundance of *pmoA* copies of Upland soil cluster-α (USC-α) in (a) all regions (b) in the regions Schwäbische Alb (ALB), Hainich (HAI) and Schorfheide (SCH) in forest (white) and grassland (grey) soils. Abundance of Upland soil cluster-γ (USC-γ) in (c) all regions (d) in the regions Schwäbische Alb (ALB), Hainich (HAI) and Schorfheide (SCH). Abundances below detection limit were set to 100. $p < .001$ (***), $n = 295$

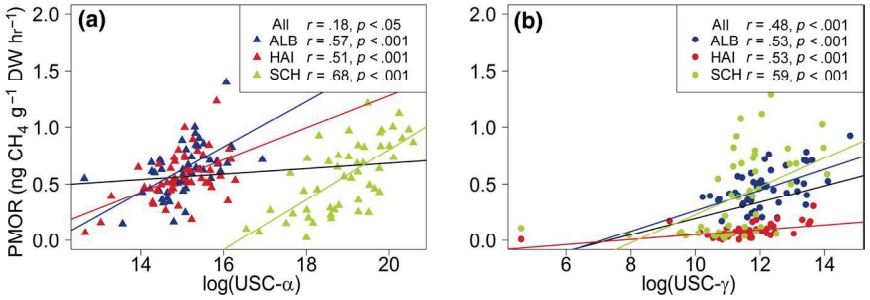

FIGURE 5 Correlation of potential methane oxidation rates (PMORs) with (a) Upland soil cluster-α (USC-α) abundance in forest soils and with (b) Upland soil cluster-γ (USC-γ) abundance in grassland soils. The colours represent the different regions Schwäbische Alb (ALB), Hainich (HAI) and Schorfheide (SCH). The significance levels reported are based on Pearson's coefficient

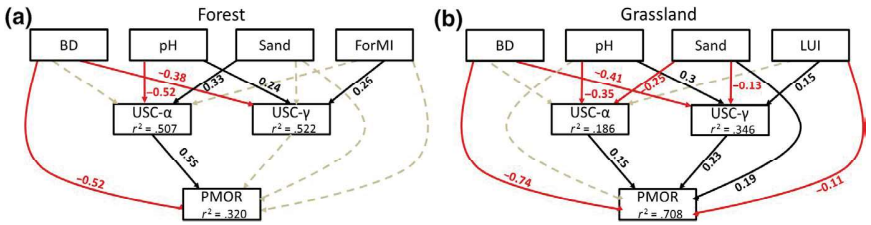

FIGURE 6 Results of structural equation modelling showing the direct and indirect effect of soil properties and land-use intensity on PMOR for (a) all forest and (b) all grassland soils. The numbers at the lines show the standardized path coefficients. Significant ($p < .05$) paths are shown as black (positive effect) or red (negative effect) lines. Non-significant ($p > .05$) paths are shown in dashed grey lines. Amount of variance explained by the model (r^2) is listed for the response variables. BD, bulk density; sand, sand content (%); ForMI, forest management index; LUI, land-use intensity index; PMOR, potential methane oxidation rate; USC-α, abundance of Upland soil cluster-α copy numbers per soil; USC-γ, abundance of Upland soil cluster-γ copy numbers per soil. $\chi^2 = 1.079; 0.255$, $df = 2;1$; p (χ^2) = .583; .628, CFI = 1;1, RMSEA = 0; 0, SRMR = 0.011; 0.006 in (a) and (b) respectively

3.7 | Direct and indirect effects of soil parameters and land-use intensity on PMOR

Generally, a larger part of PMOR variance could be explained in grasslands compared to forests (Figure 6). Bulk density had strong direct negative effects on PMOR in both, forests and grasslands (Figure 6a,b). pH had no direct effects but indirect effects on PMOR via the MOB abundances, with a negative effect on USC-α abundance and a positive effect on USC-γ abundance. Sand content had an overall positive effect on PMOR in grasslands and an indirect positive effect via abundance of USC-α in forest. However, when looking at the regions separately, the soil sand content had only a positive effect on PMOR in the ALB forest region (Figure S18a). While the forest management had no effect on PMOR, land-use intensity of grasslands had a direct negative effect on PMOR but an indirect positive effect via USC-γ abundance. However, the overall effect was negative and when looking at the regions separately there was only a direct negative effect (Figure S19a,b). Only in SCH grasslands, LUI had no direct effect on PMOR. MOB abundance had a direct effect on PMOR almost all regions. In forests, USC-α abundance had a strong direct positive effect on PMOR, whereas USC-γ showed no direct effect on PMOR (Figure 6a; Figure S19a–c). In grasslands, both USC-α and USC-γ had direct positive effects on PMOR and here the effect of USC-γ was stronger than that of USC-α (Figure 6b; Figure S19a–c). Only in grasslands of the SCH region, MOB abundance had no influence on PMOR.

4 | DISCUSSION

4.1 | Potential methane oxidation rates and soil parameters

Potential atmospheric CH_4 oxidation rates (PMORs) were generally about two times higher in forest than in grassland soils. This accords with meta-analyses of CH_4 oxidation rates in different habitats (ecosystem-level measurements) that identified 2.5-fold higher CH_4 oxidation rates in forests than in other ecosystems and about two times higher in forest than in herbaceous ecosystems (Dutaur & Verchot, 2007; McDaniel et al., 2019). Compared to these studies, our PMORs were 1.5–2 times higher than the in situ CH_4 fluxes. This may be due to the fact that we analysed soil only from the layer with the highest potential for CH_4 oxidation (Kolb, 2009) and adjusted the water content to its optimal value for CH_4 oxidation (Gulledge & Schimel, 1998). Our measurements did not include deeper soil layers, which may be a source of CH_4. However, by standardizing moisture, we reduced variation found in the field, permitting better

analyses of the influence of drivers such as soil properties or MOB on PMORs. We consider the measured abundances of MOB and the standardized PMORs as proxies that integrate CH_4 uptake and MOB activity dynamics over time.

Temperature and precipitation can influence methane oxidation. For instance, Van Den Pol-van Dasselaar et al. (1998) found highest methane uptake in soils with high temperature and intermediate soil moisture content. In our study, PMORs vary between the different regions in grassland but not in forest soils. As the overall climate is rather similar between grassland and forest sites within the same region this indicates that climatic differences cannot explain much of the differences of PMOR in grasslands between the three regions. In addition, the differences in mean temperature between the regions are relatively small. In a meta study, mean annual temperature and annual rainfall had only a weak correlation with atmospheric methane oxidation (Dutaur & Verchot, 2007). So, the differences in temperature and precipitation might be too small between the regions to induce large differences in PMORs. The variation of PMOR in grasslands was likely caused by other factors, as for example differences in soil properties between the three regions. In SCH, soil texture is sandier than in the other two regions where silty, loamy, and clayey soil textures dominate. In HAI grasslands, PMORs were 4.5-fold to 5.5-fold lower than in the other grasslands. The soils of this region are generally denser, which may be a limiting factor for PMORs. The SEM indicated a general negative effect of bulk density in forests and grasslands. The high variability in SCH region might be explained by high variability in OC content of the soils.

Interestingly, many factors that correlated with PMORs in grasslands did not correlate with PMORs in forests. Within the grasslands, the PMORs increased with increasing water holding capacity but in the forest soils, water holding capacity did not have a significant effect on PMORs. Also, soil textures that were associated with low PMORs in grasslands were associated with higher PMORs in forests. These findings suggest that ecosystem type is an important driver of PMORs and that a response to soil physicochemical conditions is specific to the type of ecosystem. The high PMORs in loamy grassland soils of the SCH, however, could also be a result of their high OC concentrations. The organic layer of forest soils has been reported to reduce CH_4 oxidation in soils, probably acting as a diffusion barrier for CH_4 (Saari et al., 1998). However, we found no negative effect of the thickness of the organic layer (determined at each forest site during sampling, but the organic layer material itself was not included in the PMOR measurement) on PMORs. To the contrary, in the HAI region, the organic layer thickness had a slight positive effect on PMORs. It may be that canopy cover and the presence of an O horizon is responsible for the different responses of PMORs to soil factors in forests and grasslands. Canopy cover and O-horizon inhibit the increase in water content of the upper mineral soil layers after rainfall events (Li et al., 2014). Lower water content could, in turn, hamper gas diffusive transport in soils.

Bulk density and pH had an influence on PMORs in almost all grassland and forest soils of all three regions. PMOR generally decreased with increasing bulk density. Higher bulk density indicates low soil porosity and pronounced soil compaction which, considered together, may result in lower diffusion capacity of atmospheric gases into the soils. This, in turn, could lead to lower CH_4 availability in the soil and thus lower the CH_4 oxidation rates (Malghani et al., 2016). It is worth noting that the original bulk density in the field had an effect on CH_4 oxidation even after sieving and re-compaction of the soil, indicating a legacy effect of the former natural conditions. Also Sitaula et al. (2000) reported that soil compaction led to decreased CH_4 uptake even after compaction was removed by sieving of the soil samples. The response of PMORs to pH differed between forests and grasslands. While in forest soils, PMORs had an optimum at around pH 4, in grasslands PMORs increased with increasing pH in two out of three regions. Soils have been shown CH_4 oxidation over a wide range of pH values and incubation of forest soils demonstrated CH_4 oxidation from pH 3–7.5 even though the optimal pH for CH_4 oxidation ranged from 4 to 7.5 (Amaral et al., 1998; Benstead & King, 2001; Saari et al., 2004). Sitaula et al. (1995) observed an increase in CH_4 oxidation when soil from a pine forest was irrigated with acidic water. In contrast, CH_4 oxidation has been reported to decrease with lower pH in grasslands (Hütsch et al., 1994), which is in accordance with our results. Also in arable soils, strong inhibition of CH_4 oxidation was reported when the soil pH was lowered from 8 to 7.1 (Hütsch, 2001). Thus, our findings underline that pH has a substantial impact on CH_4 oxidation; however, its influence differs between different ecosystem types. While in forests CH_4 oxidation is favoured by slightly acidic conditions, in grasslands CH_4 oxidation is higher in neutral soils. We found that the effect of pH was direct only in forest sites in the ALB region, while in the other cases the observed effects of pH were indirect via the abundances of the two types of methane-oxidizing bacteria. This indicates that there are different MOB communities with different pH optima in forests and grasslands.

We note that the variation in PMORs was far greater within the grasslands than in the forests and was region-dependent within the grasslands. PMORs were generally higher in forest than in grassland soils, indicating that forest soils act as robust sinks for CH_4 over a wide range of different physicochemical soil conditions.

4.2 | Drivers of MOB abundances and relationship with PMOR

We measured MOB abundances in nearly 299 different soils, thus yielding a comprehensive dataset to connect MOB with soil physicochemical soil properties and PMORs. The composition and importance of the MOBs seem to be ecosystem type- and region-specific. USC-α *pmoA* abundances were positively correlated with PMORs in forests of all regions, but USC-α was absent in many grasslands. In contrast, USC-γ *pmoA* were consistently present in the grasslands and were positively correlated with PMORs in all of the grasslands but in none of the forest regions. This indicates the far greater importance of USC-α MOBs for CH_4 oxidation in forest soils and that of USC-γ MOBs for CH_4 oxidation in grasslands. In some grasslands,

USC-α abundance might be an additional driver of CH_4 oxidation, even though it has a smaller effect on CH_4 oxidation than USC-γ abundance. USC-α MOBs have been previously detected in forest soils and 16S rRNA gene amplicon datasets demonstrate that they occur in forest soils (Tveit et al., 2019). A recent study found that USC-γ was dominant in upland grassland soils from a region in China (Deng et al., 2019). In combination with their wide occurrence also in our samples provides evidence that USC-γ is an important MOB in grassland soils in different regions of the world.

Soil pH was the most important predictor of USC-α and -γ gene abundances, with USC-α preferring more acidic and USC-γ preferring neutral soils. The lower pH of the forests and more neutral pH in the grasslands may therefore explain, in part, the distribution patterns of the two USC groups. However, USC-α were also present in neutral soils. This confirms results of former studies (Kolb, 2009; Kolb et al., 2003). However, the negative correlation of USC-α abundance and pH is also surprising, given the latest findings on the physiology of atmospheric MOB belonging to the USC-α *M. gorgona*. The optimal pH for growth of *M. gorgona* is at an almost neutral pH of 6.5–7, but other *Methylocapsa* strains that were able to grow at atmospheric CH_4 concentrations had a lower pH optimum of 5–6.2 (Tveit et al., 2019).

PMOR per unit biomass was generally lower in forest than in grassland soils. This might be due to the different microbial communities in these land-use type which might have a different specific activity. In forests, PMOR per unit biomass was lowest in SCH region that was also the region with the highest bulk density among forest soils. The PMOR per unit biomass might thus be influenced by gas diffusive transport which is lower in soils with high bulk density and thus a higher abundance of MOBs might be needed to oxidize similar amounts of CH_4.

4.3 | Effects of grassland land-use intensity and forest management

We found that grassland land-use intensity had a negative effect on PMORs, which supports our initial hypothesis. Structural equation modelling showed a direct negative effect of LUI across all regions. Only in SCH region, no effect on PMOR was detectable. This region is less intensively managed in terms of fertilization compared to the other two regions. This may explain why there was no effect of LUI on PMORs in SCH region since fertilization in particular negatively influenced PMORs, with 20% lower rates in fertilized compared to non-fertilized soils. Ammonium ions, that are a component of fertilizers, are known to inhibit methane monooxygenase (Schnell & King, 1994). However, we could not detect higher ammonium concentrations in the fertilized soils. Since we do not know the exact date of fertilization, and as the ammonium concentration in soils is highly dynamic, the concentration at our sampling date may not have reflected the mean ammonium concentrations over the year. There may also be a legacy effect of formerly high ammonium concentrations from fertilization that negatively influences the MOB community over the long term. Interestingly, fertilization had no effect on the abundances of MOBs but it did have an effect on PMORs. With respect to the other two components of grassland land-use intensity, we could not detect any significant effect of either grazing or mowing on PMORs. However, a high LUI, which integrates fertilization, grazing, and mowing, reduced PMORs by 40% in comparison to grasslands with low land-use intensity. Hence, the latter two factors did have an additive negative effect on PMORs. The reduction of PMORs by grazing and mowing may have been due to soil compaction as caused to animal trampling and mowing machines. However, only in combination with N-fertilization did soil compaction lead to a reduction in CH_4 oxidation in these soils. Heavy grazing reduces water infiltration into soil (Abdel-Magid et al., 1987) and thus also alters gas diffusive transport into soil.

Our study investigated PMORs over many different grasslands and land-use intensities and we can confirm that fertilization has a negative effect on PMORs over different soil types over a regional gradient of more than 800 km, in contrast to previous studies reporting somewhat contradictory effects of fertilization on CH_4 oxidation (Imer et al., 2013; Liu & Greaver, 2009). We thus conclude that by a reduction of land-use intensity, especially N-fertilization, the CH_4 sink function of temperate grasslands could be improved or the other way around, an intensification of grassland land use bears the risk of the reduction of methane uptake in grassland soils.

Within the investigated 149 forest soils, we did not observe any effect of forest management on PMORs. This suggests that the ability of temperate forest soils to serve as CH_4 sinks is not substantially affected by commonly applied forest management practices. Homogenization of the soils prior to measuring PMOR may have partly removed negative effects that were consequences of forest management practices, such as soil compaction due to forest machinery. However, we still see a legacy effect of the natural bulk density. Hence, it is unlikely that forest management effects were completely eliminated by the treatment of the soil before PMOR measurements. It is likely that inhibition of PMORs is most prevalent in the logging trails, which were excluded from soils sampling in our study. A closer sampling of the forest soils may be necessary to better understand the influence of management in forests. However, based on our data, we must reject our initial hypothesis of a negative effect of forest management on PMORs.

We found that the dominant tree species had some effect on PMORs. Even though the literature indicates that spruce forest soils exhibit a lower capacity to oxidize CH_4 than beech forest soils (Borken & Beese, 2006; Degelmann et al., 2009), we could not detect significant differences between beech-dominated and coniferous forests (pine or spruce) across all forest sites. However, PMORs were lower in oak than in beech dominated forests. In oak-dominated forests, USC-α abundance and soil respiration rates were also reduced, indicating the presence of inhibitory substances in the soil that hamper microbial activity. Bárcena et al. (2014) also found that CH_4 oxidation rates were higher in spruce than in young oak forests. However, others have reported that oak forests have higher

rates than spruce and pine forests (Reay et al., 2005). It is likely that tree species alone is not the most important factor impairing PMORs. Soil physicochemical conditions can differentially influence CH_4 oxidation with respect to different tree species. For example, while higher water content increased CH_4 oxidation in spruce soils, it decreased CH_4 oxidation in scots pine and larch soils (Menyailo & Hungate, 2003). Possibly, there are optimal soil types and textures for a certain tree species and thus, a specific main tree species could maximize CH_4 oxidation in a particular soil.

Our results clearly demonstrate that forest soils are an important sink for atmospheric CH_4 and that this is largely stable over different physicochemical conditions and forest management practices. Since PMORs were higher in forests than in grasslands, afforestation has the potential to enlarge the global CH_4 sink of soils and thus, help mitigate global warming by decreasing atmospheric CH_4 concentrations.

5 | CONCLUSIONS

PMORs are differentially controlled in forest and grassland soils. Our survey demonstrates that forests are an important and robust sink for CH_4 over a wide range of different physicochemical soil conditions while in grasslands PMORs are clearly more influenced by site-specific soil properties. Additionally, we detected a negative effect of grassland land-use intensity, especially fertilization, while the different forest management practices did not affect PMORs. Thus, reduction in grassland management intensity as well as afforestation may increase the capacity of soils to serve as CH_4 sinks.

Furthermore, our results strongly suggest that USC-α and USC-γ have land-use type specific distributions, with USC-α the dominant group in forests and USC-γ the dominant group in grasslands. Also, the direct positive correlations between PMORs and USC-α in forests and between PMORs and mainly USC-γ in grasslands indicate that USC-α is the major microbial group responsible for the CH_4 sink capacity in forests and USC-γ is the major group responsible for the CH_4 sink capacity of grasslands. Finally, the study also revealed that different sets of site parameters control the microbial methane capacity sink in forests and grasslands.

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DATA AVAILABILITY STATEMENT

Data are stored in BExIS and available on request according to the rules of BExIS (https://www.bexis.uni-jena.de/).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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4.2.1 Supplementary figures and tables

Supplementary figures and tables are provided on the CD.

4.3 Publication II

Linking transcriptional dynamics of CH_4-cycling grassland soil microbiomes to seasonal gas fluxes

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ARTICLE OPEN

Linking transcriptional dynamics of CH_4-cycling grassland soil microbiomes to seasonal gas fluxes

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Soil CH_4 fluxes are driven by CH_4-producing and -consuming microorganisms that determine whether soils are sources or sinks of this potent greenhouse gas. To date, a comprehensive understanding of underlying microbiome dynamics has rarely been obtained in situ. Using quantitative metatranscriptomics, we aimed to link CH_4-cycling microbiomes to net surface CH_4 fluxes throughout a year in two grassland soils. CH_4 fluxes were highly dynamic: both soils were net CH_4 sources in autumn and winter and sinks in spring and summer, respectively. Correspondingly, methanogen mRNA abundances per gram soil correlated well with CH_4 fluxes. Methanotroph to methanogen mRNA ratios were higher in spring and summer, when the soils acted as net CH_4 sinks. CH_4 uptake was associated with an increased proportion of USCα and γ *pmoA* and *pmoA*2 transcripts. We assume that methanogen transcript abundance may be useful to approximate changes in net surface CH_4 emissions from grassland soils. High methanotroph to methanogen ratios would indicate CH_4 sink properties. Our study links for the first time the seasonal transcriptional dynamics of CH_4-cycling soil microbiomes to gas fluxes in situ. It suggests mRNA transcript abundances as promising indicators of dynamic ecosystem-level processes.

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INTRODUCTION

CH_4 is a powerful greenhouse gas [1]. Between 41% and 53% of global CH_4 emissions derive from aquatic systems. Therein freshwater wetlands are the largest single source, emitting about 138–165 Tg CH_4 yr^{-1} [2, 3]. Since 1700, between 54% and 57% of the wetlands were lost due to drainage to gain agricultural land, such as grasslands [4, 5]. Drainage lowers the water table, altering water content and oxygen availability. These altered soil physical conditions, in turn, substantially affect the soil microbiota and activity and thus the soils' greenhouse gas fluxes [6, 7]. Drained former wetlands are a large source of CO_2 but can also emit substantial amounts of CH_4, depending on their dynamic hydrological status throughout the year [4, 5].

More than two-thirds of global CH_4 emissions derive from microbial production [8]. CH_4-producing microbes (i.e., methanogens) are mostly anaerobic Archaea that inhabit anoxic environments [8, 9]. Four types of methanogens can be characterized according to their substrate specificity. Acetoclastic methanogens utilize acetate, hydrogenotrophic methanogens utilize H_2/CO_2 and formate, and methylotrophic methanogens utilize methanol/methylamines to form CH_4 [9]. Recently, methoxydotrophic methanogens that utilize methoxylated aromatic compounds were proposed as a novel methanogenic group [10, 11]. In soils, acetoclastic and hydrogenotrophic methanogens are considered the predominant sources of CH_4 [9, 12]. However, recent research indicates that methanogenesis from methylated compounds also contributes to CH_4 emissions from soils and wetlands [13, 14].

Up to 90% of CH_4 produced in oxygen-limited soils can be mitigated through oxidation by aerobic methane-oxidizing Bacteria (MOB) within the lineages *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* [15–17]. CH_4 oxidation can also be conducted anaerobically by Bacteria of the NC10 phylum and Archaea in the ANME group that couple oxidation of CH_4 to the reduction of other electron acceptors such as nitrite (NC10), nitrate (ANME-2d), or ferric iron [18–20]. Aerobic methanotrophs are considered the main oxidizers in wetland soils since alternative electron acceptors favoring anaerobic methanotrophs are often scarce in wetland soils. Tracing stable isotopes and radioisotopes can link CH_4 consumption to active methanotrophs [21–25]. For instance, incubating soil cores with ^{13}C-CH_4 identified γ-proteobacterial subgroups as the main active methanotrophs in a riparian floodplain [22]. Additionally, methanotrophs provide the only known biological sink for atmospheric CH_4 [26]. However, it is not fully understood which microorganisms oxidize CH_4 at atmospheric concentrations in soils. Bacteria of upland soil clusters (USC)α and USCγ have been identified as likely important atmospheric MOBs in upland soils [15, 27–29], while well-known methanotrophic lineages may also oxidize atmospheric CH_4 in anoxic paddy soils [30]. A study using stable-isotope labeled CH_4 identified type II methanotrophs related to *Methylocapsa acidophila* active in grassland and forest soils at low CH_4 concentrations [25].

Presumably, the combined net activities of methanogens and methanotrophs determine whether wetland soils act as net sources

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or sinks for CH_4 [31]. However, linking CH_4-cycling microbiome dynamics of soils in situ to CH_4 fluxes, especially at the transcriptional level, has rarely been achieved [32]. DNA- and RNA-based meta-omics techniques have provided insight into the microbiome compositions of soils. However, DNA is long-term stable; extracted soil DNA may therefore partially originate from persistent extracellular DNA of dead organisms [33, 34]. In contrast, ribosomal RNA (rRNA) acts as a proxy for ribosomes. Even though dormant cells can contain high loads of ribosomes [35, 36], RNA-SIP studies [37, 38] indicate that approximately 94% of microbial taxa in soil are active and synthesize new rRNA [39]. Still, rRNA content does not necessarily reflect the gene expression. Hence, although rRNA is a good proxy for potential active soil microbiome, it may not relate well to ecosystem processes. The simultaneous sequencing of mRNA and rRNA potentially can overcome this issue [40] because messenger RNA (mRNA), can serve as a proxy for transcriptional activity. Other metatranscriptome studies indicate that mRNA is more responsive to environmental factors than rRNA [41, 42]. For instance, methanogen-related mRNA, but not SSU rRNA, decreased in soil microcosms exposed to drought [43]. The relationship between the abundances of rRNA and mRNA of CH_4-cycling microbes and CH_4 fluxes has not been studied in situ. We thus aim to explore differences between small subunit (SSU) rRNA and mRNA transcripts of the CH_4-cycling microbiomes and their links to gas fluxes.

Another drawback of meta-omics techniques is that they usually yield only relative abundances. However, the relationship between absolute abundances and relative abundances is not predictable [44]. It is thus challenging to relate ecosystem processes to relative abundances. Studies have applied absolute quantification for metatranscriptomes in marine microbiomes [45, 46]. Recently, a quantification approach that uses total RNA to infer absolute from relative abundance has been developed for metatranscriptomics [47].

In this study, we aimed to link transcriptional dynamics of CH_4-cycling microbiomes to CH_4 fluxes in two grassland soils. These soils were wetlands in the past but have been drained for agricultural use several decades ago. We used quantitative metatranscriptomics to analyze ribosomal rRNA and mRNA [40, 47] of 60 soil samples taken from different soil depths during autumn, winter, spring, and summer. In addition, we measured CH_4 and CO_2 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 CH_4 fluxes of the two in grasslands throughout a year, (c) study the composition and abundance of SSU rRNA and mRNA transcripts of CH_4-cycling microbes, and (d) link microbiome composition of CH_4-cycling organisms to net surface CH_4 fluxes 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 [48]. Samples were taken at two grassland sites (LI and HI) located in the Biosphere Reserve „Schorfheide-Chorin" (Supplementary Table S1). Both sites are drained peatlands with a histosolic soil type (according to WRB 2015 [49]). 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 (400–700 livestock units * grazed days ha^{-1} y^{-1}) 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 × 7 m was sampled at all four seasons: autumn (11/09/2017), winter (03/08/2018), spring (05/30/2018), and summer (09/13/2018). At each sampling date, three spatial replicate samples were taken between 12:00 and 13:00 at each site from the upper 10 cm and the 20–30 cm layer. Each soil sample was a mixture of the respective soil layer from three soil cores, taken close to each other (5–10 cm). The replicates were located at least 1 m apart from each other. At each seasonal sampling, the replicates were taken at least 1 m apart from replicates taken during the previous sampling campaigns. In spring, additional samples were taken at sunrise (05:00) and sunset (21:30), but only at the HI site. 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 h 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 $CaCl_2$ 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) [50]. For this, frozen soils were thawed (at 4 °C for 10 h), then 5 g field moist soils were fumigated with ethanol-free $CHCl_3$ for 24 h in a desiccator. C and N were extracted with 40 ml 0.5 M K_2SO_4, 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. H_2O) and measured on a TOC/TN analyzer (Multi N/C 2100S, Analytik Jena AG, Jena, Germany). A kEC factor [51] and a kEN factor [52] were used to calculate C_{mic} and N_{mic}, respectively. The organic C and N content determined from non-fumigated samples were used as a measure for the extractable C (EOC) and N (EN) which can be considered as microbially available resource in soil [53]. Mineral nitrogen in the forms of ammonium (NH_4^+) and nitrate (NO_3^-) was determined in the non-fumigated, non-diluted extracts with an Auto-Analyzer 3 (Bran & Luebbe, Norderstedt, Germany).

Gas fluxes

On each sampling date, gas emissions were measured with four closed chambers per site. With each chamber, the measurements were repeated four to six times per day and site, resulting in 15–24 net surface rate measurements. Excessive vegetation was removed before pressing the stainless steel chambers ($A = 150\ cm^2$, $V = 1800$ ml) into the soil [54]. The chambers had a sharp-edged bottom, which allowed the installation in the organic soils without compacting the soil. Gas samples (12 ml) were taken with syringes from the headspace immediately, 20, 40, and 60 min 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 CH_4) coupled with a methanizer (for CO_2) (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 [27].

RNA extraction, library preparation, and sequencing

Total nucleic acids were extracted using a phenol/chloroform/isoamylalcohol protocol [40]. 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 (Qubit3.0 Fluorometer, Invitrogen, Waltham, MA, USA.). RNA extracts were cleaned with the MEGAclear kit (Thermo Fisher Scientific, Waltham, MA, USA); the 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 [55]. 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 a NextSeq 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 [56]. The sequences were filtered to a minimum mean quality score of 25 with PrinseqLite [57]. Sequences were then sorted into SSU rRNA, LSU rRNA, and non-rRNA fractions with SortMeRNA [58]. The SSU rRNA fraction was randomly subsampled to 200000 sequences with USEARCH [59]. Sequences were taxonomically classified against the SilvaMod128 databases [60] with BlastN [61] using a lowest common ancestor (LCA) algorithm in MEGAN (min score 155; top percent 2.0; min support 1 [62]). The non-rRNA fraction was aligned against the NCBI_nr database (retrieved 12/03/2020) with Diamond [63]. The sequences were taxonomically and functionally aligned with LCA in MEGAN (2011, min score 155; top percent 4; min support 1 [62]). Absolute abundances were calculated from read counts according to Söllinger et al. [47]. This calculation integrates the relative read abundance obtained from metatranscriptomics with the amount of mRNA and SSU rRNA extracted from the soil, respectively, and the average number of transcripts per µg RNA. At mRNA level, methanogenesis transcripts refer to sequences assigned to the SEED category "methanogenesis". Methanotrophy transcripts refer to sequences assigned to the SEED category "Particulate methane monooxygenase (pMMO)". To classify *pmoA* sequences, the non-rRNA fraction was searched against a *pmoA* database [64] and taxonomically classified with MEGAN as described in reference [64]. To assess the transcriptional activity of CH_4-cycling microbes throughout the years, we binned mRNAs taxonomically classified as methanogens (Euryarchaeota) and alpha and gammaproteobacterial methanotrophs, respectively to then analyze the functionally assigned mRNAs using SEED and KEGG.

Statistical analyses were performed in R [65]. Distance-based redundancy analysis was performed on the Bray–Curtis dissimilarity matrix read counts of the 60 samples (function "dbrda" in the vegan package [66]). 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. The difference of transcript abundances and the ratio of methanotrophs to methanogens between seasons at one and the same site was assessed by ANOVA and subsequent post-hoc Tukey's test, resulting in adjusted *p*-values. We used the arithmetic mean of methanogenesis and methanotroph transcript abundances from the upper (0–10 cm) and the lower (20–30 cm) of one sample. Significant differences between seasons were identified with the R package "multcompView" with *p*-adjusted <0.05 [67].

RESULTS AND DISCUSSION

Highly dynamic CH_4 fluxes across the year

We measured net surface fluxes from two grasslands across one day during autumn, winter, spring, and summer to assess their seasonal variation, in particular CH_4. Daytime did not affect CH_4 emissions (Supplementary Fig. 1A). In contrast, CH_4 and CO_2 fluxes were highly dynamic throughout the year (Fig. 1). While the soils emitted CH_4 in autumn and winter (7.0 and 6.9 mg C $m^{-2}d^{-2}$, in autumn in LI and HI, respectively), they took up CH_4 in spring and summer (−0.9 and −0.8 mg C $m^{-2}d^{-2}$, in summer in LI and HI, respectively) (Fig. 1A). CO_2 fluxes showed an opposite trend, with higher CO_2 emissions in spring and summer than in autumn and winter (Fig. 1B). The opposing trends of CO_2 and CH_4 fluxes reflected the changes in soil physicochemical properties across the year (Fig. 1C, D, Supplementary Table S2). Especially water content and temperature were likely the key factors in regulating gas turnover. High water content and low redox potentials in autumn and winter (Fig. 1C, Supplementary Table S2) likely favored anaerobic microbial processes, such as methanogenesis, while at the same time hampering aerobic microbial processes such as respiration (Supplementary Fig. 2). Low temperatures in winter likely resulted in smaller CH_4 fluxes compared to autumn (Fig. 1A, D). In spring and summer, soils had lower water content and positive redox potential favoring aerobic over anaerobic degradation processes. Generally, mean CO_2 net surface emissions were about 1.5 times higher than IPCC default emission factors [68, 69]. Our observed higher emissions may have been due to the degraded peat at the studied site. Soils with highly disturbed peat have been reported to have higher CO_2 emissions than less degraded peat soils [70]. Next to soil water content, also temperature may have caused relatively high CO_2 emissions as spring and summer 2018 were dry and hot compared to the long-term average. High temperatures increase organic matter decomposition and CO_2 emissions [71, 72].

Net surface CH_4 emissions rates in autumn and winter were lower compared to IPCC default emission factors [68]. However, we measured emissions at only four days and may have not accounted for high emissions after heavy rainfall events. Net CH_4 uptake rates in spring and summer were in the range of other herbaceous and temperate ecosystems (0.36 and 0.47 ± 0.63 mg C $m^{-2}d^{-1}$) [73, 74] and higher than in pastures (mean 0.05 mg C $m^{-2}d^{-1}$) [74]. The beginning drought in 2018 caused low soil water content (Supplementary Table S2), favoring CH_4 oxidation. The soil water content of the upper layer was mostly within the optimal range for atmospheric CH_4 oxidation [75].

Our results underscore the high temporal variability of greenhouse gas emissions from temperate drained peatlands and their dependence on dynamic soil physicochemical properties, like temperature and soil moisture, which are themselves linked to seasons. Moreover, depending on the time of the year and conditions in the soil such sites can be net sinks for CH_4 as well as net sources. This versatility regarding CH_4 sink and source functions requires further long-term monitoring of such groundwater-impacted and organic-rich drained grassland soils in postglacial landscapes to ensure proper consideration in global budgets.

Linking metatranscriptomics and microbial biomass

We quantified soil total RNA content to examine if it reflects microbial biomass in the soils. Total RNA and N_{mic} and C_{mic} were determined from 60 top- and subsoil samples. They exhibited similar dynamics across seasons. Overall, total RNA per gram soil was positively correlated with both N_{mic} and C_{mic} ($r_{Nmic} = 0.68$, $r_{Cmic} = 0.54$, $p < 0.001$, Fig. 2, Supplementary Fig. 3). The RNA content correlated better with N_{mic}, than with C_{mic}, likely due to the high nitrogen content of the RNA. This finding supports the validity of RNA as a proxy for living microorganisms and the use of RNA content to infer transcript abundances per gram soil from relative transcript abundances obtained in metatranscriptomics [47]. Through this quantitative approach, one can overcome challenges typically associated with the interpretation of relative abundance data in 'meta-omics' datasets. A recent study used this quantitative approach and found that absolute transcript abundance correlated better to ecosystem processes than relative transcript frequencies [47].

Spatial and seasonal dynamics in CH_4-cycling (micro-)biomes

High-throughput sequencing of metatranscriptomes yielded approximately 20 million paired-end reads per sample [76]. Three-domain analysis based on SSU rRNA reads revealed that the (micro-)biomes of the 60 samples were dominated by Bacteria, followed by eukaryotes and Archaea (Supplementary Tables S3 and S4, Supplementary Fig. 4). The community composition of all taxa in the soil samples exhibited a clear site- and depth-specific pattern (Fig. 3A), with site and depth explaining 20.0% and 19.6%

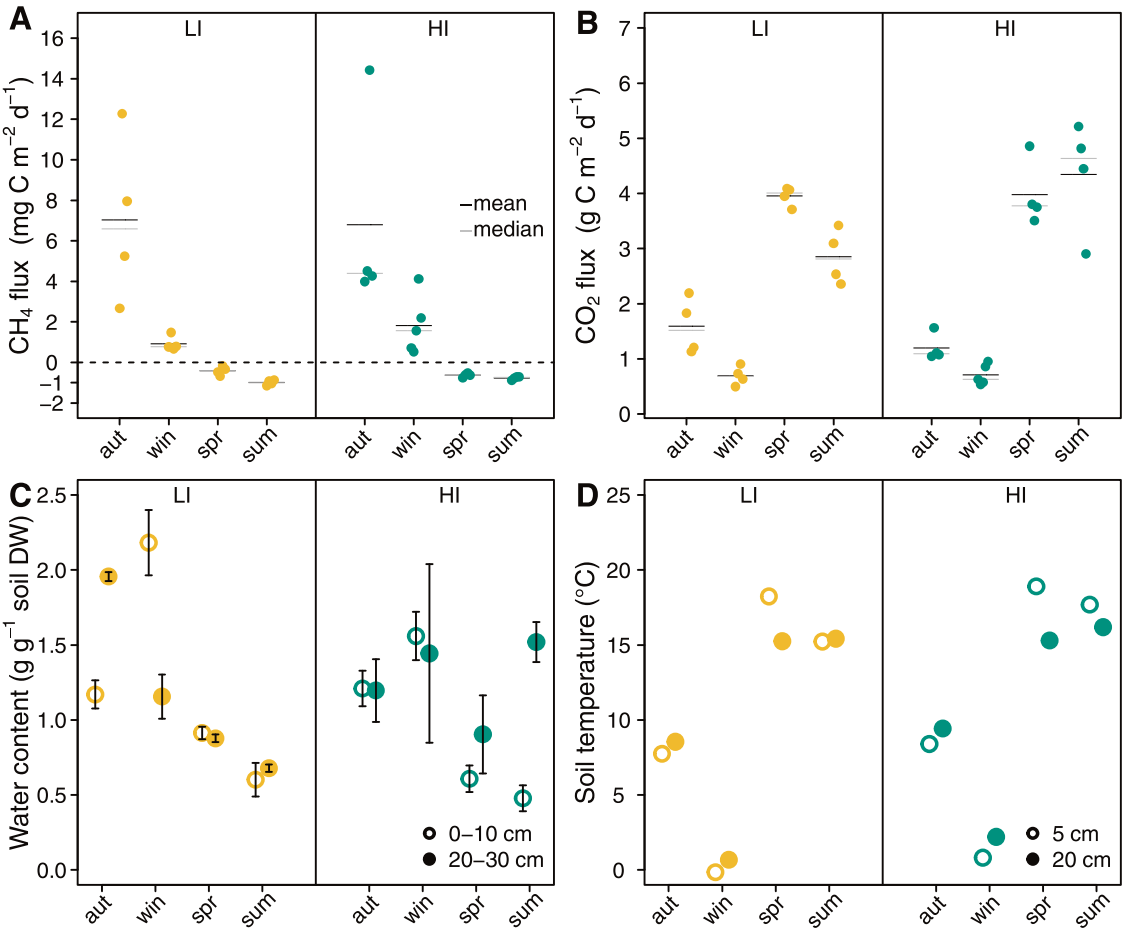

Fig. 1 Net surface gas fluxes, soil temperature, and water content. Gas fluxes of CH_4 (**A**), CO_2 (**B**), gravimetric soil water content (**C**), and temperature (**D**) in the soils of the grassland site with low (yellow, LI) and high (turquoise, HI) land-use intensity in autumn (aut) 2017 and winter (win), spring (spr), and summer (sum) 2018. In **A** and **B**, one point shows the average of 4–6 repeated measurements of one chamber across one day; the mean and median are indicated with a black and gray line, respectively. In **C**, one point represents the mean and standard deviation of three replicates taken at noon, $n = 3$. In **D**, points represent the temperature measured at 12:00 in 5 cm and 20 cm soil depth, respectively.

of the variance, respectively ($p < 0.001$, Supplementary Table S5). Site-specific differences are likely attributed to site-specific soil properties, such as pH, texture, organic carbon, and nitrogen content, and land-use intensity (Supplementary Table S1). Depth is generally considered to be associated with differences in oxygen and nutrient availability. Eukaryotes were usually higher abundant in the upper soil layer, compared with the lower soil layer (Supplementary Fig. 4).

The composition of CH_4-cycling microbes was also influenced by site, season, and depth (Fig. 3B). Site had the most explanatory power (14.0%, $p < 0.001$), but season, depth, and water content accounted for 6.5%, 5.7%, and 5.3% ($p < 0.001$) of the variance, respectively (Supplementary Table S6). Thus, the seasonal variability of the CH_4 fluxes was accompanied by seasonal changes in CH_4-cycling community composition. The seasonal effect likely resulted from varying precipitation, water table depth, and plant growth activity throughout the year. Especially the drought in spring and summer may have strongly affected the CH_4-cycling microorganisms by lowering the soil water content. Oxygen diffusion into dry soils is much faster than into water-saturated soils, resulting in a higher O_2 availability, which, in turn, is a fundamental factor shaping CH_4-cycling community composition [77].

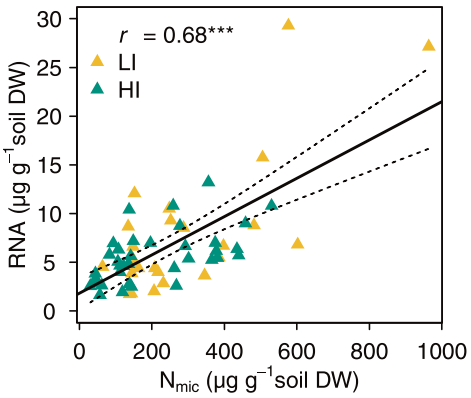

Fig. 2 RNA and microbial biomass nitrogen content. Correlation between RNA content and microbial nitrogen content (N_{mic}) per g soil dry weight (DW) in the soils of the grassland sites with low (LI, yellow) and high (HI, turquoise) land-use intensity. Linear regression RNA = 1.8182 + 0.0197 N_{mic}, df = 58 (dashed lines show 95% CI). The "*r*" denotes the Pearson correlation coefficient. Significance codes: ***$p < 0.001$, $n = 60$.

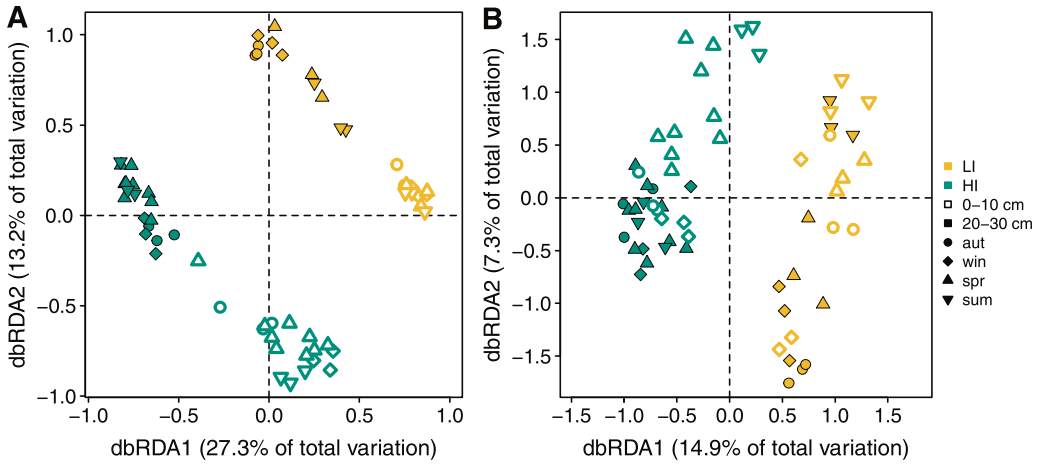

Fig. 3 Soil (micro-)biome composition at the two grassland sites. Distance-based redundancy analysis (dbRDA) of the Bray–Curtis dissimilarity matrix of all 39,854 bacterial, archaeal and eukaryotic taxa (**A**) and the 287 CH_4-cycling Archaea and Bacteria (**B**) in the soils of the grassland sites with low (LI, yellow) and high (HI, turquoise) land-use intensity from the upper (0–10 cm) and the deeper soil layer (20–30 cm) taken in autumn (aut) 2017 and winter (win), spring (spr) and summer (sum) 2018. Samples from autumn, winter, spring, and summer are depicted as circles, diamonds, upward-pointing triangles, and downward-pointing triangles, respectively.

Methanogen community composition and transcriptional activity

We aimed to evaluate if SSU rRNA and mRNA abundances of CH_4-cycling microbes reflected the seasonal changes in CH_4 fluxes of the soils. For this purpose, we integrated the total RNA content and metatranscriptomes [47] to infer methanogen SSU rRNA and mRNA transcript abundances per gram soil (Fig. 4A, C). Generally, methanogen SSU rRNA abundances were higher in autumn and winter and the deeper soil layer, with abundances up to 1.4×10^{10} transcripts g^{-1} soil (Fig. 4A). Most methanogen families in the soils were class II methanogens, e.g., *Methanosarcinaceae*, *Methanosaetaceae* (now *Methanotrichaceae*) (Fig. 4B) which generally possess more antioxidant features than class I methanogens [78]. The predominance of class II methanogens likely reflected the dynamic water and redox status across seasons (Fig. 1C, Supplementary Table S2).

Methanogenesis mRNA transcripts were generally less abundant in spring and summer (0.21 and 0.43 * 10^7 transcripts g^{-1} in summer in LI and HI, respectively) than in autumn and winter (5.6 and 3.6 * 10^7 transcripts g^{-1}in winter in LI and HI, respectively) (Fig. 4C). According to Tukey's HSD test, methanogenesis transcript abundances were significantly lower ($p < 0.05$) in spring and summer compared to autumn and winter, in both LI and HI (Supplementary Tables S7 and 8). This drop in methanogenesis mRNA agrees with the cessation of CH_4 emissions from the soils in spring in summer; both correlated significantly with each other ($r = 0.87$, $p < 0.01$, Fig. 4D). In contrast, the abundances of methanogen SSU rRNA transcripts and CH_4 fluxes did not correlate significantly (Supplementary Fig. 7). Our results indicate that methanogenesis mRNA transcripts are better indicators of net CH_4 fluxes than methanogen SSU rRNA transcripts (Fig. 4D, Supplementary Fig. 7). We thus underscore studies that have found mRNA more responsive to environmental factors than rRNA [41, 42].

We only sampled two sites and cannot make statistically assured statements about the influence of land-use intensity. Nevertheless, we observed some site-specific patterns. Methanogen SSU rRNA transcript abundances were higher in HI than in LI soils (Fig. 4A) despite similar methanogenesis mRNA transcript abundances (Fig. 4C). The taxonomic composition may influence the transcriptional activity of methanogenesis transcripts (Fig. 4B). The strictly acetoclastic *Methanosaetaceae* (*Methanothrix*) were more pronounced in HI than in LI (Fig. 4B). *Methanosaeta* have lower growth rates and can grow at lower acetate concentrations than the metabolically diverse *Methanosarcina* [79]. In turn, 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 [9, 80]. The varying proportions of acetoclastic and hydrogenotrophic methanogens and lower acetate concentrations may explain lower transcriptional activity at HI compared to LI. Messenger RNA transcripts that were unambiguously attributed to a certain methanogenesis pathway, support that the share of acetoclastic mRNAs was lower in LI than in HI (Supplementary Fig. 8). However, large-scale studies, that include more sites would be needed to explore this effect further.

The consistent presence throughout the year of the obligate methylotrophic *Methanomassiliicoccales* (up to 14% of the methanogen SSU rRNA in the topsoils, Fig. 4B) points to methylated compounds as additional substrates for methanogenesis in both sites. The contribution of methanogenesis from methylated compounds to terrestrial CH_4 emissions is considered to be small [9]. However, recent research suggests it to be more important [10, 13, 81, 82]. For instance, the methylotrophic *Methanomassiliicoccales* were the second most abundant methanogenic group in Zoige peatlands [83] and also highly abundant in wetlands in northeast Germany [84].

Furthermore, we wanted to know if methanogens exhibited a differential gene expression across seasons. For this purpose, we assessed broad functional categories of mRNA transcripts taxonomically binned to Euryarchaeota. Methanogen transcript profiles had similar seasonal patterns in both soils. For instance, protein biosynthesis and transcription were upregulated in methanogens during winter (Supplementary Fig. 9). The upregulation of the protein biosynthesis machinery in soil microbiomes was recently attributed to diminished enzymatic reaction rates of metabolic enzymes at colder temperatures [85]. Likewise, our results point to a temperature-dependent regulation of central cellular processes in the here studied methanogens.

High spatio-temporal dynamics of methanotrophs

The aerobic methanotrophs in the soils, assessed by SSU rRNAs, mostly belonged to canonical MOBs, i.e., *Methylococcaceae*, *Crenotrichaceae*, *Methylocystaceae* (Fig. 5A, B). They were generally higher abundant in topsoils, as compared to subsoils, except of summer (Fig. 5A). Anaerobic methanotrophic bacteria (*Ca.* Methylomirabilis)

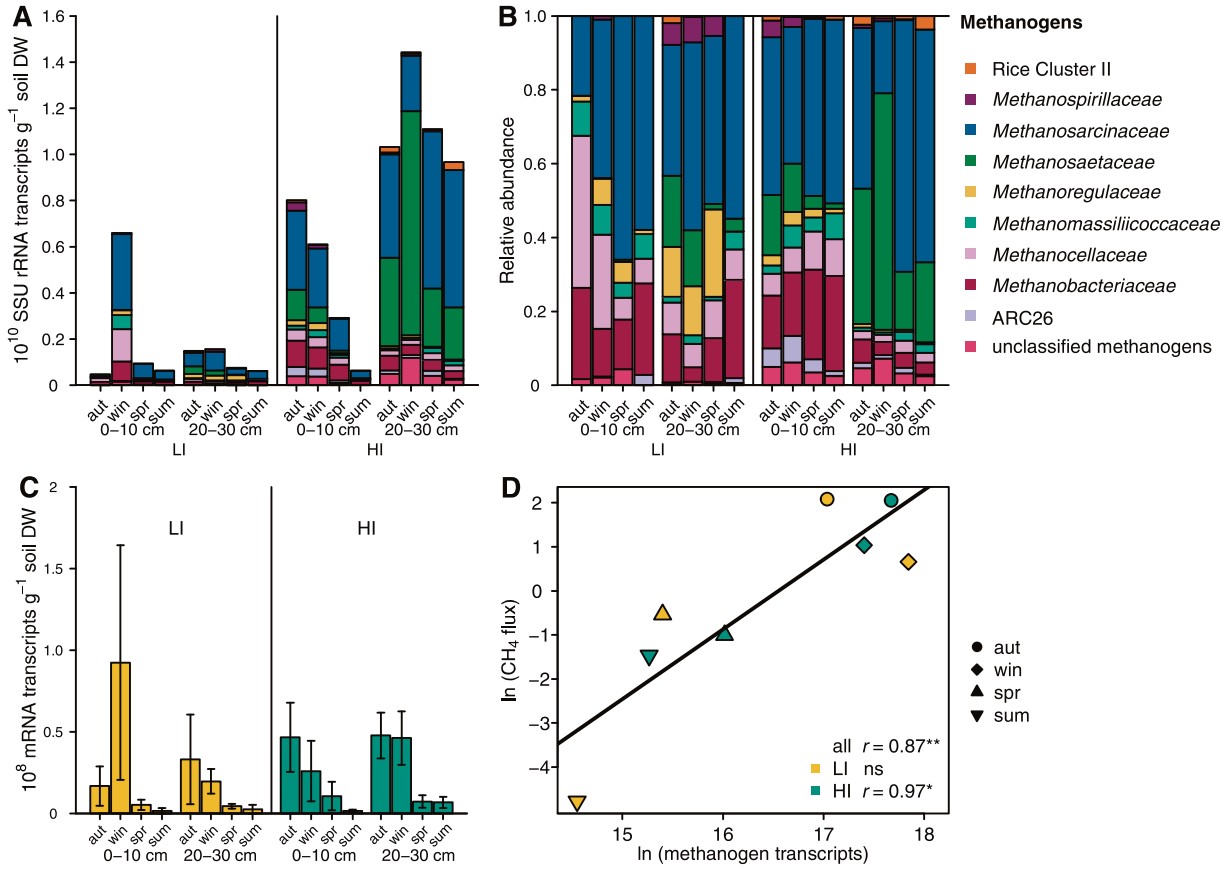

Fig. 4 Methanogen SSU rRNA and mRNA abundances across seasons and depths. Absolute abundances (SSU rRNA transcripts g^{-1} soil DW) of methanogenic Archaea (**A**), the relative abundance of SSU rRNA transcripts belonging to methanogenic Archaea normalized to the total amount of SSU rRNA transcripts belonging to methanogenic Archaea (**B**), and transcript abundances (mRNA transcripts g^{-1} soil DW) of mRNA of methanogenesis pathways (**C**) in soils from the upper (0–10 cm) and the deeper soil layer (20–30 cm) of the grassland sites with low (LI, yellow) and high (HI, turquoise) land-use intensity taken in autumn (aut) 2017 and winter (win), spring (spr) and summer (sum) 2018. In **A**, **B**, and **C**, columns show means per season and depth of the upper (0–10 cm) and the deeper soil layer (20–30 cm) in LI and HI. "unclassified methanogens" contain methanogens unclassified at the class level and low abundance methanogenic groups. Bars represent the means of three replicates. In **C**, error bars represent the means and the standard deviations of three replicates. Linear correlation of absolute abundances of methanogenesis mRNA transcripts with CH_4 fluxes (**D**). In **D**, points represent seasonal means across both depths; samples from autumn, winter, spring, and summer are depicted as circles, diamonds, upward-pointing triangles, and downward-pointing triangles, respectively. The "*r*" denotes the Pearson correlation coefficient. Significance codes: $*p < 0.05$, $**p < 0.01$, ns not significant. DW dry weight. We refer to Supplementary Fig. 5 and 6 showing the absolute and relative abundances of methanogen SSU rRNA in the individual samples, respectively.

and Archaea (ANME-2d) comprised a substantial part of the methanotroph community (up to 20% of all methanotrophs in subsoil) (Fig. 5B). They were present mainly in the deeper soil layer (20–30 cm), which was likely due to their sensitivity to oxygen [86]. Across seasons, methanotroph abundance (aerobic and anaerobic) was highest in autumn and winter (Fig. 5A), resembling seasonal dynamics of methanogens.

In addition to SSU rRNA, we assessed the active MOBs using transcripts of the most widespread functional marker, the alpha subunit of the pMMO [64]. While the same clades were detected, their relative abundance was sometimes different to the SSU rRNA derived MOB profiles (Fig. 5C). For instance, Methylococcales SSU rRNA transcripts (type I) clearly dominated in LI (up to 96% of all methanotroph SSU rRNA) but comprised less than 50% of *pmoA* transcripts (Fig. 5C). Generally, type II methanotrophs were more abundant in the *pmoA* than in the SSU rRNA transcripts. Studies assessing SSU rRNA composition might generally underrepresent this group. Especially in autumn and winter, the *pmoA* transcripts were dominated by canonical MOBs that probably feed on the CH_4 produced by the methanogens. Remarkably, the proportions of *pmoA* transcripts classified as USCα, USCγ, and *pmoA2* increased in spring and summer in both sites (Fig. 5C). These *pmoA*s are assumed to be associated with atmospheric MOBs [15, 28, 87, 88]. Their increase matched the net CH_4 uptake of the soils in spring and summer (Fig. 1A). The relative abundance of USCα and γ *pmoA* and *pmoA2* transcripts was up to 34%. Still, other type I and type II *pmoA* sequences dominated the soils. Recently, atmospheric CH_4 oxidation in paddy soils was attributed to canonical CH_4 oxidizers rather than USCα and USCγ [30]. Thus, also the detected type I and type II methanotrophs might be involved in atmospheric CH_4 oxidation in spring and summer. However, it is also possible that CH_4 is still produced in deeper soil layers and that the canonical CH_4 oxidizers feed on this CH_4. To complicate matters even more, the, yet only isolate of USCα methanotrophs, *Methylocapsa gorgona*, can grow at both atmospheric and elevated CH_4 concentrations [89].

Similar as with methanogens, we wanted to explore differences in expression of general functions of methanotrophs across seasons. Transcripts taxonomically binned to gamma and alphaproteobacterial methanotrophs showed an upregulation of protein synthesis and processing as well as transcription and RNA processing in autumn and winter (Supplementary Fig. 13). This is

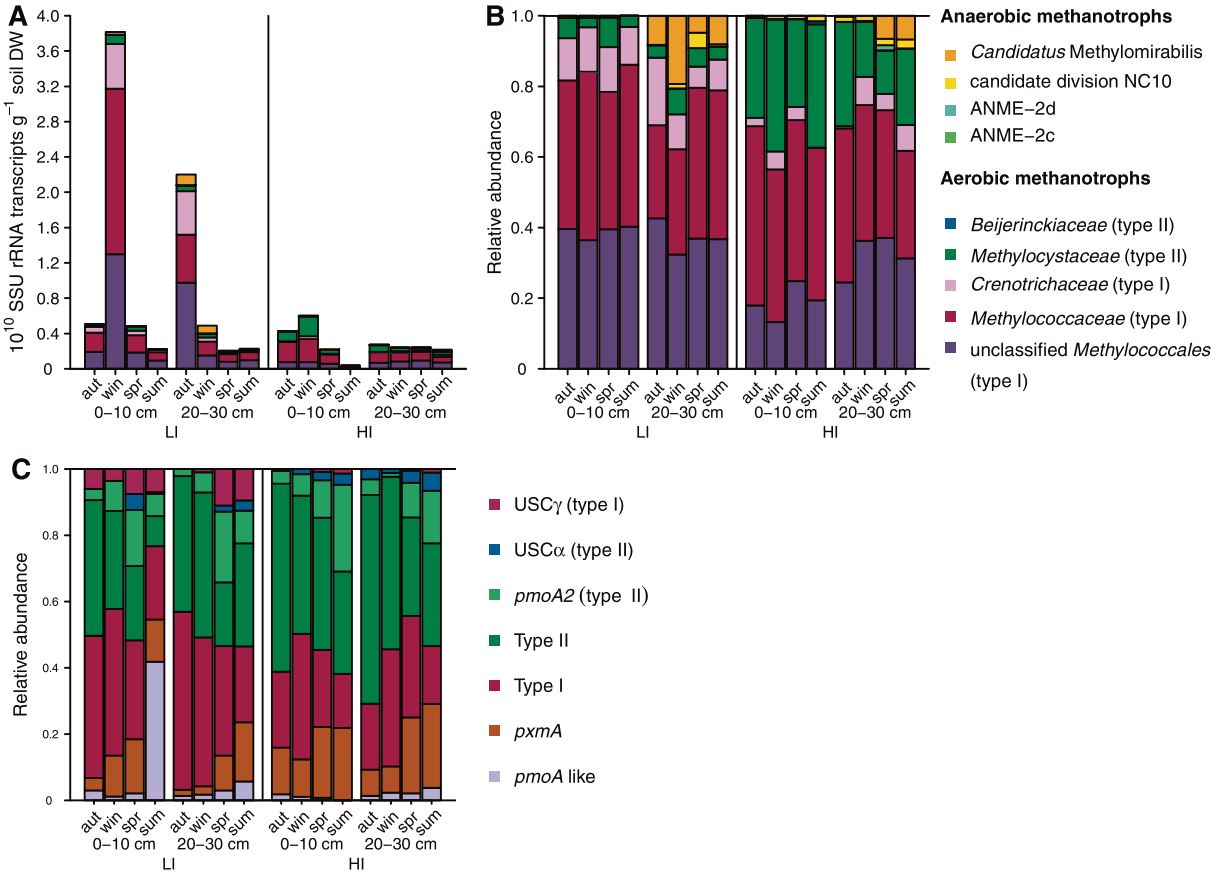

Fig. 5 Absolute and relative methanotroph SSU rRNA abundances and composition of *pmoA* transcripts. Absolute abundances (SSU rRNA transcripts g^{-1} soil DW) of methanotrophic microorganisms (Archaea and Bacteria) (**A**), proportion of SSU rRNA transcripts belonging to methanotrophic microorganisms normalized to the total amount of SSU rRNA transcripts belonging to methanogenic Archaea and methanotrophs (**B**), and the proportion of *pmoA* groups normalized to the total amount of *pmoA* transcripts (**C**). Columns show means per seasons and depth in soils from the upper (0–10 cm) and the deeper soil layer (20–30 cm) of the grassland sites with low (LI) and high (HI) land-use intensity taken in autumn (aut) 2017 and winter (win), spring (spr) and summer (sum) 2018. "unclassified *Methylococcales*" contain *Methylococcales* unclassified at the family level and low abundance *Methylococcales* families. "pmoA like" = unclassified *pmoA*-like sequences. Bars represent the means of three replicates. Abbreviations: DW dry weight. We refer to Supplementary Figs. 10–12 showing the absolute and relative abundances of methanotroph SSU rRNA and the *pmoA* composition in the individual samples, respectively.

strikingly similar to the gene expression in methanogens, providing further support that protein biosynthesis apparatus might be larger at lower temperatures [85]. In some samples only a few mRNAs could be functionally assigned. This must be considered when interpreting these results.

Functional transcript abundances as a proxy for soil net surface CH_4 fluxes

We have shown above that the abundance of methanogenesis-related mRNAs was a good estimator of CH_4 fluxes in the studied soils (Fig. 4D). We now aimed to integrate methanotroph and methanogen markers to assess if a comprehensive understanding of soil CH_4 fluxes can be derived from quantitative metatranscriptomics.

The pMMO mRNA transcripts of both sites correlated positively with methanogenesis transcripts ($r = 0.62$, $p < 0.001$) but there was no significant correlation in SSU rRNA transcripts across sites (Supplementary Fig. 14). The correlation of mRNA transcripts suggests that methanotrophs predominantly use CH_4 derived from methanogenesis in the soil. They thus act as a filter mitigating CH_4 emission to the atmosphere [31]. However, there is seasonal variation; the pMMO to methanogenesis mRNA ratio was higher in spring and summer than in autumn and winter (4.8 and 3.6 in winter, and 30.0 and 12.1 in summer, in LI and HI, respectively) (Fig. 6B). Such a ratio may thus indicate whether soils are CH_4 sources or sinks. A high methanotroph to methanogen ratio may hint at a soil being a CH_4 sink, while a low ratio may hint at a soil being a net CH_4 source. Yet, it is necessary to consider transcriptional activity since the ratio of methanotroph to methanogen SSU rRNA was not indicative of soils' CH_4 fluxes (Fig. 6A). Furthermore, the MOB community composition could be an additional indicator for soil CH_4 uptake since a high proportion of atmospheric CH_4 oxidizers in the *pmoA* transcripts was linked to net CH_4 uptake of the soils.

CONCLUSIONS

This study is, to our knowledge, the first that uses quantitative metatranscriptomics to link CH_4 fluxes from grasslands with CH_4-cycling microbiomes through all seasons of the year. We validated mRNA transcripts rather than SSU rRNA transcripts to be necessary for linking microbial activity to soil net surface CH_4 fluxes in the two studied soils measured on a daily time scale. If this holds for annual rates based on temporarily highly resolved real-time data, requires more research. Still, since the abundance of mRNA of methanogenesis pathways correlated well with the net CH_4 fluxes, it may thus be feasible to estimate soil CH_4 fluxes using *mcr* transcript abundances when additionally considering the

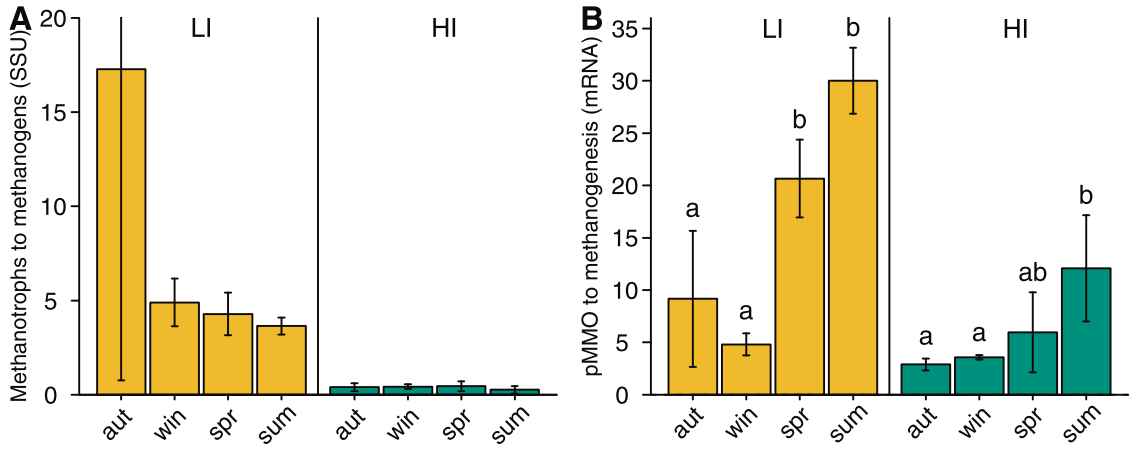

Fig. 6 Methanotroph to methanogen ratio across seasons. The ratio of methanotroph to methanogen SSU rRNA transcripts (**A**) and pMMO to methanogenesis mRNA transcripts (**B**). The ratio was calculated with mean transcript abundances of the upper (0–10 cm) and the deeper soil layer (20–30 cm) of one soil sample. of the grassland sites with low (LI, yellow) and high (HI, turquoise) land-use intensity taken in autumn (aut) 2017 and winter (win), spring (spr) and summer (sum) 2018. Statistically significant categories of the ratios between seasons were tested with an ANOVA and subsequent post-hoc Tukey's test at *p-adjusted* < 0.05 level.

transcript ratio of methanotroph and methanogen key enzymes. The latter is suggested by the different ratios between the seasons in both grasslands.

Soils are the largest biological sink for atmospheric CH_4, an important ecosystem function given the increasing concentration of atmospheric CH_4 [1]. However, its magnitude and controlling factors are currently poorly constrained [3, 27]. Our study adds to the growing body of literature (e.g., [30]) that suggests that in soils with internal CH_4 formation, such as the drained peatlands investigated here, many methanotroph groups contribute to atmospheric CH_4 oxidation as compared to upland soils that are permanent net sinks of CH_4. Stable isotope probing may be well suited to investigate this further [21–25].

We investigated 60 samples by RNAseq, a technique currently still restricted in terms of throughput and costs. Two RT qPCR studies found a relationship between *mcrA* transcript abundances and CH_4 fluxes in a paddy soil and a peat bog, respectively [90, 91]. Parallel RT qPCRs of *mcrA* and *pmoA* transcripts might thus currently be also viable tools to estimate CH_4 fluxes of soils from many samples and sites, respectively. Nevertheless, more large-scale studies, such as the one presented here, are encouraged to further investigate the link between methanogens and methanotrophs and CH_4 fluxes across different soil types and seasons, especially when considering the ever decreasing costs of sequencing and further automatization in bioinformatics workflows.

DATA AVAILABILITY

All raw sequencing data have been deposited in NCBI sequence read archive under BioProject ID PRJNA741868.

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AUTHOR CONTRIBUTIONS

The study was designed by TU, SK, and SM. Sampling was performed and coordinated by JT assisted by TU, SM, SK. Gas flux and C_{mic}, N_{mic} analysis were performed by SM. RNA extractions and amplifications were performed by JT and VG. Sequencing and library preparation was performed by CJ and AK assisted by JT, VG, and TU. Data analysis was performed by JT, VG, TU, SM, and SK. The manuscript was written by JT and TU, assisted by all co-authors.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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4.3.1 Supplementary figures and tables

Supplementary figures and tables are provided on the CD.

4.4 Publication III

Desiccation time and rainfall control gaseous carbon fluxes in an intermittent stream

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Desiccation time and rainfall control gaseous carbon fluxes in an intermittent stream

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Abstract Droughts are recognized to impact global biogeochemical cycles. However, the implication of desiccation on in-stream carbon (C) cycling is not well understood yet. We subjected sediments from a lowland, organic rich intermittent stream to experimental desiccation over a 9-week-period to investigate temporal changes in microbial functional traits in relation to their redox requirements, carbon dioxide (CO_2) and methane (CH_4) fluxes and water-soluble organic carbon (WSOC). Concurrently, the implications of rewetting by simulated short rainfalls (4 and 21 mm) on gaseous C fluxes were tested. Early desiccation triggered dynamic fluxes of CO_2 and CH_4 with peak values of 383 and 30 mg C m^{-2} h^{-1} (mean ± SD), respectively, likely in response to enhanced aerobic mineralization and accelerated evasion. At longer desiccation, CH_4 dropped abruptly, likely because of reduced abundance of anaerobic microbial traits. The CO_2 fluxes ceased later, suggesting aerobic activity was constrained only by extended desiccation over time. We found that rainfall boosted fluxes of CO_2, which were modulated by rainfall size and the preceding desiccation time. Desiccation also reduced the amount of WSOC and the proportion of labile compounds leaching from sediment. It remains questionable to which extent changes of the sediment C pool are influenced by respiration processes, microbial C uptake and cell lysis due to drying-rewetting cycles. We highlight that the severity of the dry period, which is controlled by its duration and the presence of precipitation events, needs detailed consideration to estimate the impact of intermittent flow on global riverine C fluxes.

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Introduction

Streams play a substantial role in global carbon (C) cycling by releasing carbon dioxide (CO_2) and methane (CH_4) to the atmosphere and by shaping the quantity and quality of dissolved organic C through organic matter processing during its transit towards the ocean (Raymond et al. 2016). Uncertainties still exist on C fluxes in streams that suffer from discontinuities in flow. Over 50% of the global stream network is intermittent, experiencing flow cessation and drying at some points in space and time (Acuña et al. 2014). The frequency, duration and magnitude of intermittency are expected to dramatically increase in response to climate change and growing water use (Messager et al. 2021). In arid and semiarid regions, intermittent streams are the dominant surface water type (Datry et al. 2014). Regions that are more humid are no exception, however, and numerous temperate lowland streams are increasingly experiencing hydrological drought (Andersen et al. 2006; Nützmann and Mey 2007); yet, the impact of flow intermittency on biogeochemical cycles has been poorly addressed (Dewey et al. 2020).

Recent research suggests that the dry reaches of intermittent streams, which are typically excluded from global C budgets, can contribute significantly to global gaseous CO_2 fluxes from stream networks to the atmosphere (Marcé et al. 2019; Keller et al. 2020). Beyond the dry phase, intermittent streams can supply C to other downstream ecosystems through leachates from dry substrates upon rewetting (Shumilova et al. 2019). Compared with streams in arid and semiarid areas, lowland streams in temperate climate regions tend to accumulate larger amounts of organic matter; thus, understanding how dry-wet cycles alter C turnover is essential to anticipate environmental consequences of drying in stream networks.

The duration of the dry period, seen as a proxy of desiccation severity, is recognized to greatly shape biogeochemical cycling in intermittent streams (Muñoz et al. 2018; Arce et al. 2019; Vidal-Abarca et al. 2020). Desiccation induces changes in the structure and functioning of microbial communities (Febria et al. 2012; Romaní et al. 2017) and in the physico-chemical conditions of sediments (von Schiller et al. 2017; Casas-Ruiz et al. 2016; Harjung et al. 2019a). As redox requirements are fundamental in defining microbial niches, turnover in the microbial community may be expected, with ensuing implications for C cycling and gaseous C fluxes. Besides, biogeochemical implications of desiccation can be locally noted at vertical scale in sediments, with surface sediments being generally more susceptible to drying than deeper, more desiccation-protected layers (Arce et al. 2019). Exposure to air diminishes the activity of anaerobic functional groups, including CH_4-producing methanogens, especially in surface sediments. Conversely, the activity of aerobic microbes like CH_4-oxidizers (i.e., methanotrophs) may remain unchanged as long as CH_4 is available. Correspondingly, low CH_4 but high CO_2 fluxes have been reported from dry reaches of intermittent streams (von Schiller et al. 2014; Gómez-Gener et al. 2015, 2016).

Increased CO_2 fluxes are attributed to favored growth of aerobic heterotrophic microbes during oxygen-rich desiccation periods (Fromin et al. 2010). However, persisting desiccation both in aquatic sediments and soils is known to impact microbial community structure and activity and to limit diffusion and supply of dissolved nutrients (Romaní et al. 2017; Schimel 2018). This may result in the cessation of evasion of biogenic (i.e. microbially produced) gases (Arce et al. 2019; Marcé et al. 2019). In soils, nutrient and C processing, specifically CO_2 fluxes, tend to rapidly increase in response to rewetting, a phenomenon known as "Birch effect" (Birch 1958; Kim et al. 2012); relatively less studied in dry riverbed sediments (Arce et al. 2019; Marcé et al. 2019; von Schiller et al. 2019). Rain-induced rewetting during the dry period without generating surface flow may trigger temporarily high C fluxes to the atmosphere despite streams appearing as seemingly quiescent because of the lack of surface water. For instance, Gallo et al. (2014) observed increased fluxes of CO_2, CH_4 and N_2O following simulated rainfalls in several dry ephemeral streams in Arizona, USA. Furthermore, the size of the rainfall and the duration of the precedent desiccation period can shape the magnitude of rainfall-induced gas fluxes (Arce et al. 2018; Gebremichael et al. 2019).

Since a surface water compartment providing nutrients and C is not available during desiccation, streambed sediments gain importance as a source of substrates fueling the short-term C fluxes to atmosphere. Furthermore, while short rainfall can impact C fluxes to atmosphere, organic C stocks in sediments can also be mobilized and exported to downstream reaches only upon a more intense rewetting.

Yet, there are large uncertainties regarding the effects of desiccation on water-soluble organic C (WSOC), an important fraction of C stored in sediments (Tao and Lin 2000). Understanding changes in the quantity and quality of WSOC leaching from dry streambed sediments is fundamental to appraise the quality of stream water once water flow recovers (Shumilova et al. 2019). Biotic and abiotic transformations, such as respiration and microbial leaching due to dry-wet osmotic shifts (Fellman et al. 2011; Vázquez et al. 2011; Ylla et al. 2011) can alter the quantity and composition of WSOC (Dahm 1981; McMaster and Bond 2008; Sabater and Tockner 2010). In intermittent stream networks, fluxes of the released elements upon flow resumption may exceed baseflow values by several orders of magnitude. Thus, these fluxes can substantially contribute to annual catchment export (Skoulikidis and Amaxidis 2009; Bernal et al. 2013).

This study addresses two main objectives. First, we investigate changes of in-stream C gaseous fluxes (CO_2 and CH_4 emissions to atmosphere) and WSOC leached from sediments, as desiccation progresses in time. Second, we explore the implications of short rainfall events of variable size (4 and 21 mm) on gaseous C fluxes to the atmosphere in dependence of the precedent desiccation time. We predict that temporal progression of desiccation will drive rapid and substantial changes in the fluxes of CO_2 and CH_4 from sediments to the atmosphere primarily because of shifts in water and oxygen availability. More specifically, we expect an increase in the flux of CO_2 and a decrease in the flux of CH_4 over desiccation time. Furthermore, physicochemical changes in sediments will shape the abundance of microbial functional traits based on redox requirements (aerobic vs. anaerobic taxa), especially in surface sediments, and will impact microbial C production due to water stress. Over progressing desiccation time, altered C-transformations will imprint on the quantity and quality of WSOC leached from sediments. Considering that longer desiccation induces microbial stress but water can alleviate this stress, we also predict that rainfall-induced rewetting will boost gaseous C fluxes. However, this response will be stronger the higher the amount of rewetting water, and weaker the longer the precedent desiccation time.

Methods

Experimental design

Water and submerged sediments were collected from the Fredersdorfer Mühlenfließ, a temperate, intermittent, lowland stream located in Brandenburg, southeast of Berlin (North Germany, 52°26' 27.51''N; 13°41'00.85'' E, 38 m asl). The catchment size of the 32.6-km long stream is 230 km^2. The stream channel is typically colonized by *Ranunculion fluitantis* and *Callitricho-Batrachion* alliances and floating aquatic mosses (Natura 2000 habitat type 3260). The riparian zone consists of a mosaic of calcareous open peatlands, grasslands and natural floodplain forests with *Alnus glutinosa* und *Fraxinus excelsior*. Sampling was done at the end of May 2015 before surface flow declined. Nutrient and C concentrations in surface water (mean ± SD, n = 3) were 230 ± 25 µg N L^{-1} for NO_3^-, 360 ± 50 µg N L^{-1} for NH_4^+, 31 ± 2 µg P L^{-1} for soluble reactive phosphorus, 10.6 ± 1.5 mg C L^{-1} for dissolved organic carbon (DOC), and 0.53 ± 0.21 mg N L^{-1} for dissolved organic nitrogen (DON).

Sediment samples were collected at 3 different sites along a 100 m-reach, sieved (4 mm mesh size), and transported to the laboratory. In the laboratory, 30 transparent acrylic glass columns (length 30 cm, diameter 5.5 cm) were filled with sediments to a depth of 15 cm and stream water to completely wet them with a 2–3 cm surface water layer. Ten microcosms were each filled with sediment from one of the three field sites so that the 3 lab replicates sampled destructively at later time points always represented field replicates. All 30 microcosms were closed at the bottom with a gas- and water-tight plug fitted with a drainage and a stopcock, and placed in a ventilated climate chamber (25 °C, dark). After 1 week of acclimation, 3 replicate microcosms were destructively sampled for initial, wet conditions (pre-desiccation conditions or t = 0 weeks) (Fig. 1). Such pre-

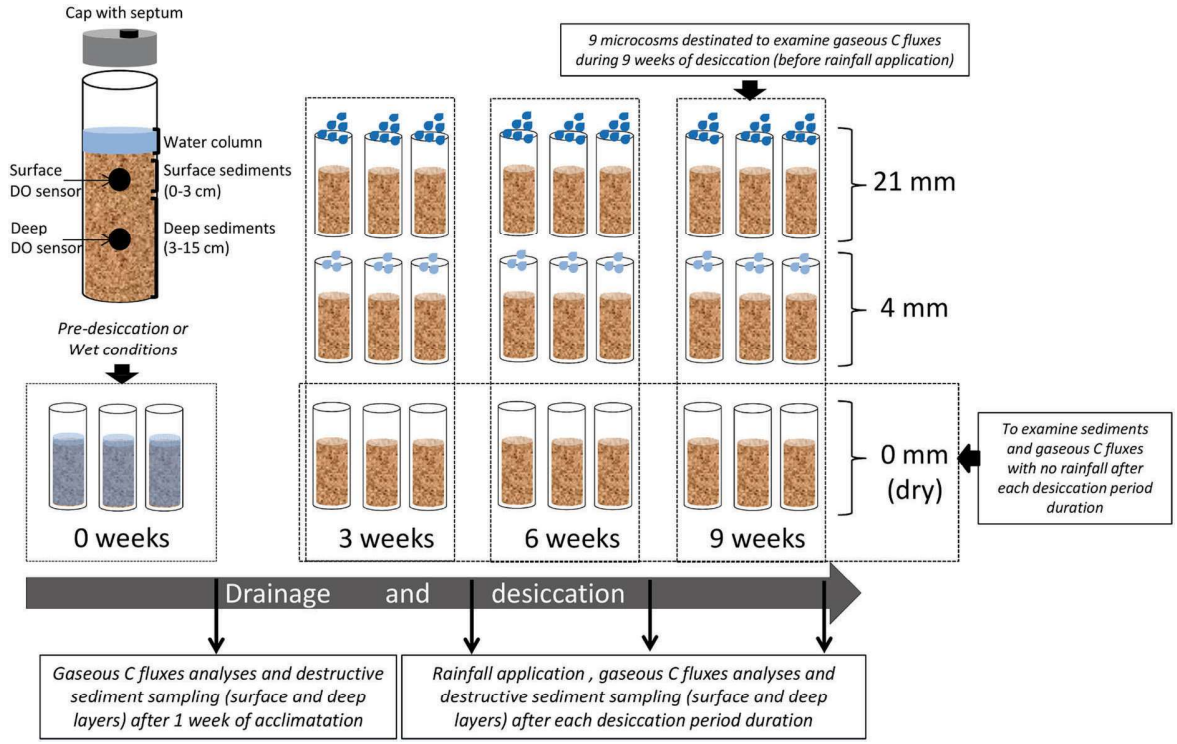

Fig. 1 Experimental design

desiccation conditions hydrologically simulate a pool environment, which typically occurs during stream fragmentation before desiccation (von Schiller et al. 2017). The remaining 27 microcosms were grouped in 3 sets of 9 microcosms desiccated by drainage over 3, 6 or 9 weeks. Desiccation was achieved by draining water through the bottom outlets until the whole overlaying water in the microcosms vanished (approx. 24 h) and by evaporation in the ventilated climate chamber; this simulated a losing intermittent reach, which is subjected to desiccation by loss of water through the hyporheic zone to groundwater (Boulton et al. 2017). After each desiccation period (3, 6 and 9 weeks), different rainfall size was simulated, namely 0 (dry), 4 and 21 mm (Fig. 1), by spraying artificial rainwater mimicking local rain chemistry (1.4 mg L^{-1} of Cl^{-}, 2.2 mg L^{-1} of SO_4^{2-}, 0.4 mg L^{-1} of K^{+} and 1.6 mg L^{-1} of Ca^{2+}). The different rainfalls just differed in the amount of water added over the microcosms, being duration and intensity of application the same. After gas collection, the dry treatment (i.e. 0 mm) set was destructively sampled with the purpose of studying biogeochemical and microbial parameters in two depth layers over desiccation time. The 4 and 21 mm- treatment sets were similarly sampled after the simulated rainfalls to characterize the percentage of water content (WC%) in two sediment depth layers. Gaseous C fluxes were determined throughout the whole experiment from microcosms set aside for sampling after 9 weeks of desiccation. This set was also employed to monitor the variation in WC% in the whole sediment column via change in microcosm weight, and the dissolved oxygen (DO) in the interstitial surface and deep sediments (Fig. 1). For that, we used optode sensors (circular, diameter 0.5 cm) attached on the inner walls at depths of 2 and 9 cm, respectively, and a Fiber Optic Oxygen Meter Microx4 (PreSens, Regensburg, Germany).

Gaseous C fluxes

To study CO_2 and CH_4 fluxes, we collected 2–3 gas samples from microcosms closed by a gas-tight plug fitted with a septum over 1 h to compute concentration changes. To study desiccation effects, this was done in

9 microcosms before eventual rainfall-treatment throughout 9 weeks of desiccation (Fig. 1): before drainage, when microcosms had a water table (t = − 3 days), immediately after (t = 0 days) and several days after drainage (t = 3, 6, 11, 17, 27, 39, 52 and 62 days).

To study short-term effects of rainfall events, 5 monitoring times were established: before (t = -2 h), immediately (t = 0 h) and three times after rainfall treatment (t = 2, 6 and 24 h). In all cases, headspace gas samples (1 mL) were directly injected into a Los Gatos ultraportable GHG analyzer (San José, CA, USA) for CH_4 and CO_2 measurements (Wilkinson et al. 2018). All measured concentrations were converted to mass units through the application of the Ideal Gas Law:

$$n = (P \cdot V)/(R \cdot T) \quad (1)$$

where n is the number of moles/volume concentration (e.g. moles C-CO_2 L^{-1} enclosure), P is the barometric pressure, V is the volume/volume concentration (trace gas concentration expressed in ppmv or µL C-CO_2 L^{-1}), R is the universal gas constant (0.0820575 L atm K^{-1} mol^{-1}), and T is the air temperature expressed in K. By using the molecular weight of the trace gas species (e.g. 12 µg C-CO_2 per µmol CO_2) the number of moles were converted to concentration values (C_m). They were then used to calculate gaseous fluxes via linear regression of headspace gas concentration versus time elapsed since the plug placement and considering microcosm headspace and the sediment area by means of the following equation:

$$f = (\Delta C_m/\Delta t \cdot V_{hs})/A, \quad (2)$$

where f is gas flux as mass of C as CO_2 or CH_4 per area and time expressed as mg C m^{-2} h^{-1}, $\Delta C_m/\Delta t$ is the change in concentration of gas (C_m) over the enclosure period time expressed as mg C m^{-3} h^{-1}, V_{hs} is internal volume of the headspace expressed as m^3, and A is the sediment area expressed as m^2. Additionally, the total or cumulative gas flux post rainfall (mg C m^{-2}) was calculated by integrating the fluxes over 24 h following the moment of simulated rainfall.

Sediment sampling, water content and organic matter properties

Subsequent to gas sampling, sediments of the dry microcosms (i.e. rainfall = 0 mm) were destructively sampled at the surface (0–3 cm) and deep layers (3–15 cm) to determine water content. Immediately after sampling, 1 mL of sediment was frozen with liquid N for microbial analyses. Remaining material was stored in plastic bags at 5° C pending further processing within 24 h.

The WC% per sediment layer was calculated after drying sediments at 60 °C for 72 h. To quantify the percent of organic matter (OM%), dried sediments were further combusted at 500 °C for 4 h.

Water-soluble organic matter was extracted from sediments (volume ratio 1:5) using 2 M KCl (Gabor et al. 2015). The salt solution mimics ionic strength of the stream water and thus avoids extreme osmotic stress on microorganisms' cells upon rewetting when using pure water (McNamara and Leff 2004). Besides, unlike pure water, salty leaching solutions appear preferable if spectrometric dissolved organic matter measures are targetted (Gabor et al. 2015). Extracted samples were centrifuged (3400 rpm, 10 min) and filtered through pre-ashed (450 °C, 4 h) Whatman GF/F filters (Maidstone, England, UK, 0.7 µm nominal pore size). We then used a liquid chromatography-organic carbon-organic nitrogen detection system (LC-OCD-OND, DOC-Labor Huber, Karlsruhe, Germany) (Huber et al. 2011; Graeber et al. 2012) to measure total dissolved organic C and N concentrations, and molecular size distributions including UV-absorbance of size fractions. Bulk specific ultraviolet absorbance at 254 nm ($SUVA_{254}$; L mg C^{-1} m^{-1}), a surrogate of average aromaticity (Weishaar et al. 2003), was calculated by normalizing decadal absorbance at 254 nm to DOC concentration. Molecular size distributions allowed to define three fractions of WSOC (von Schiller et al. 2015): (i) 'biopolymers', i.e. non-humic high molecular weight substances (HMWS > 10 kDa) of hydrophilic character and no unsaturated structures like polysaccharides and proteins, (ii) aromatic 'humic or humic-like substances' (HS) including building blocks, and (iii) 'low molecular-weight substances' (LMWS) including acidic and neutral substances. Fractions were assigned based on standards of the International Humic Substances Society. Before analysis, all samples were stored at 5°C for less than 2 weeks to avoid changes of dissolved organic matter composition (Heinz and Zak 2018). The same system also provides water-soluble organic nitrogen (WSON) concentrations. For the size fractions with detected N (HMWS and HS) we also computed molar C:N ratios. Leaching yields of

WSOC and WSON were calculated as a fraction of dry mass (mg g DM^{-1}). Besides sediment leachates, we also used LC-OCD-OND to analyze the surface and drainage water of the microcosms for DOC and DON concentrations.

Microbial community composition

We assessed microbial community composition in functional terms of oxygen requirements (aerobic vs. anaerobic taxa) and CH_4 cycling (methanogens vs. methanotrophs) by amplicon sequencing of the 16 S rRNA gene and using FAPROTAX ("functional annotation of prokaryotic taxa", Louca et al. 2016) to assign microbial functions to taxa data. Total community DNA was extracted from 0.25-g sediment samples stored at -80 °C using the PowerSoil DNA isolation kit (MoBio Laboratories, CA, USA) according to the manufacturer´s instructions with exception of a bead beating step performed in a MP FastPrep-24 5G High Speed Homogenizer (MP Biomedical, CA, USA) during 30 s at a speed of 5 m s^{-1}. The concentration and purity of DNA was examined using a NanoDrop spectrophotometer (Fisher Scientific, Schwerte, Germany). The measured concentrations ranged between 20 and 63 ng μL^{-1} and were expressed in ng per g of dry mass (DM). The DNA was amplified with a primer pairs targeting the V4 region of the 16 S ribosomal RNA (rRNA) gene for archaea and bacteria (515 F and 806R; Walters et al. 2016, conforming with the Earth Microbiome Project, Thompson et al. 2017). The PCR amplification, Illumina MiSeq library preparation (including equimolar pooling of amplicons from different samples) and paired-end sequencing (V3 chemistry) was carried out by LGC Genomics (Berlin, Germany). Sequence reads (clipped from adaptor and primer sequence remains) were processed using the DADA2 package in R (version 1.2.0) (Callahan et al. 2016). Forward and reverse reads were filtered (maxN = 0, maxEE = 2, truncQ = 2) and truncated to 180 bp, corresponding to a minimum quality score of 30 along the reads. An average of 94% of the reads were retained after quality filtering. Chimeric sequences (< 5% of unique sequences) were removed using the *removeBimeraDenovo* function. The resulting amplicon sequence variants (ASVs, analogous to operational taxonomic units) were used to construct a table containing relative abundances of ASVs across all samples. ASVs were taxonomically classified with BlastN using a lowest common ancestor (LCA) approach on a manually curated version (silvamod, Lanzén et al. 2012) of the Silva SSURef database (version 128, Pruesse et al. 2007) in MEGAN5 (Huson et al. 2007) with the following LCA parameters: top percent 2, minimum bit score 155, minimum number of hits 1. Illumina MiSeq 16 S rRNA amplicon sequence data was submitted to the NCBI Short Read Archive (accession number SRP137655). Functional trait estimation of the taxa was performed using FAPROTAX (Louca et al. 2016) on the ASVs. Using the current literature on cultured strains, FAPROTAX maps microbial taxa to established metabolic or other ecologically relevant functional traits. The ASV Table (9404 ASVs in total, average ASV richness per sample 1401 ± 535, SE), including taxonomic classification of ASVs, was normalized to the total sum of reads (TSS normalization) in each sample before functional trait estimation. From this functional table, we selected the relative abundance of methanotrophs and methanogens to examine their variation. We also selected main aerobic and anaerobic functions to sum their relative abundances. The aerobic processes included *methanotrophy*, *aerobic ammonia oxidation*, *aerobic nitrite oxidation*, *nitrification* and *aerobic chemoheterotrophy*. The anaerobic processes encompassed *methanogenesis*, *respiration of sulfur compounds*, *denitrification*, *fermentation*, *iron respiration* and *chlorate reducers.*

Comparative field sampling

In August 2015, after approximately 6 weeks of desiccation period, we conducted a comparative field sampling for gaseous C fluxes and chemical characteristics of sediments. The aim of this sampling was to obtain results under in situ desiccation conditions that could be used to complement the outcomes concerning C fluxes using the microcosm set up and to discuss the potential variables that can shape experimental findings under field conditions. Gaseous C fluxes were measured using 1.2-L soil chambers (n = 3) provided with a septum-cap and a collar that was inserted 5 cm into the sediments. After 1 h of collar insertion, chambers were capped and four gas tight gas vials (10 mL) were collected from headspace over 1 h by using 20-mL syringes and pre-evacuated gas vials (Machery-Nagel GmbH & Co., Berlin, Germany). Surface

and deep sediments were collected with a hand shovel and transported in plastic bags to the laboratory to determine WC%, OM%, and the quantity and quality of water-soluble organic matter within 24 h after collection. The CO_2 and CH_4 fluxes were determined on the same day of sampling as described for microcosms experiments.

Statistical analysis

Changes of chemical and microbial variables through desiccation time and per sediment layer were examined by means of general linear models (GLM). A factoral design including desiccation time (0, 3, 6 and 9 weeks) and sediment layer (surface and deep) as fixed factors as well as their interaction (time x sediment) were included in the models. The temporal factor desiccation time could be treated as fixed because measurements were done in different microcosms (Fig. 1), Holm´s post hoc test were used for planned comparisons between pre-desiccation wet conditions (i.e., 0 weeks) and the 3 desiccation dates (3, 6 and 9 weeks). The effects of rainfall and duration of precedent desiccation on cumulative gas fluxes were examined using a similar GLM that included the factors rainfall size (dry or 0 mm, 4 and 21 mm) and desiccation time (3, 6 and 9 weeks) as well as their interaction (time x rainfall). Holm´s post hoc tests were used for comparisons among rainfall sizes by each desiccation period. When significant differences were found for cumulative fluxes, we graphically examined the gaseous flux trajectories over the monitoring time (−2, 0, 2, 6 and 24 h) in response to the different rainfall treatments (0, 4 and 21 mm).

Statistical analyses were performed using SPSS software vs. 24 (Chicago, IL, USA). Results were considered significant at $P < 0.05$.

Results

Sediment water content and dissolved oxygen

Sediment water content (WC%) of the whole microcosms decreased from 42% on average at the beginning to 9% after 9 weeks of desiccation (i.e. 62 days) (Fig. 2a). In parallel, DO increased faster in surface than in deep sediments and approached saturation at approx. 9 mg L^{-1} after 10 days of desiccation (Fig. 2a). After 24 days, DO in both sediment layers matched and remained so until the end of the experiment (Fig. 2a). WC% measured in surface and deep sediments in dry microcosms (i.e., 0 mm) decreased significantly after 6 and 9 weeks if compared with initial conditions (Holm´s post hoc test, $P < 0.05$) (Fig. 2b, c). Simulated rainfall after each desiccation time increased WC% in both sediment layers with the treatment of 21mm generally inducing the largest values (Fig. 2b, c).

Gaseous C fluxes

Over the 9 weeks of desiccation, we found significant temporal variation in the fluxes of CO_2 and CH_4 (Fig. 3). The CO_2 fluxes reflected a change in the direction of the emission with increasing fluxes at the beginning of desiccation followed by a gradually decreasing pattern. Average CO_2 fluxes increased from 5.3 mg C m^{-2} h^{-1} (t = − 3 days) to 38 mg C m^{-2} h^{-1} after drainage (t = 0 days) and peaked with an average of 383 mg C m^{-2} h^{-1} after 11 days of desiccation (Fig. 3). Afterwards, CO_2 fluxes gradually decreased with a minimum average value of 1.2 mg C m^{-2} h^{-1} at the end of the experiment. CH_4 fluxes were higher than CO_2 fluxes in the beginning of the experiment, and the fluctuations of CH_4 fluxes through desiccation were more dynamic than those of CO_2 fluxes (Fig. 3). Average CH_4 fluxes increased from − 5 mg C m^{-2} h^{-1} (t = − 3 days) to 28 mg C m^{-2} h^{-1} after drainage (t = 0 days), with the highest fluxes after 3 days of desiccation (30 mg C m^{-2} h^{-1}). At t = 6 days, fluxes exhibited a sharp drop to − 10 mg C m^{-2} h^{-1}, remained low with an average value of 0.03 mg C m^{-2} h^{-1} after 27 days of desiccation, and were not detectable afterwards (Fig. 3).

During the simulated rainfall events following desiccation periods of variable duration, we detected fluxes only for CO_2 but not for CH_4. Overall, the cumulative CO_2 fluxes 24 h after rainfall decreased with longer preceding desiccation. However, the response to the different rainfall sizes varied depending on the desiccation time; differences appearing only after 3 and 9 weeks (interaction term *time x rainfall*: $P < 0.05$, Holm´s post hoc test, $P< 0.05$) (Fig. 4a). Furthermore, the trajectories of CO_2 fluxes examined over 24 h varied differently in response to the applied rainfall pulses, yet such responses were not equal after 3 and 9 weeks (Fig. 4b,c). After 3 weeks, while the

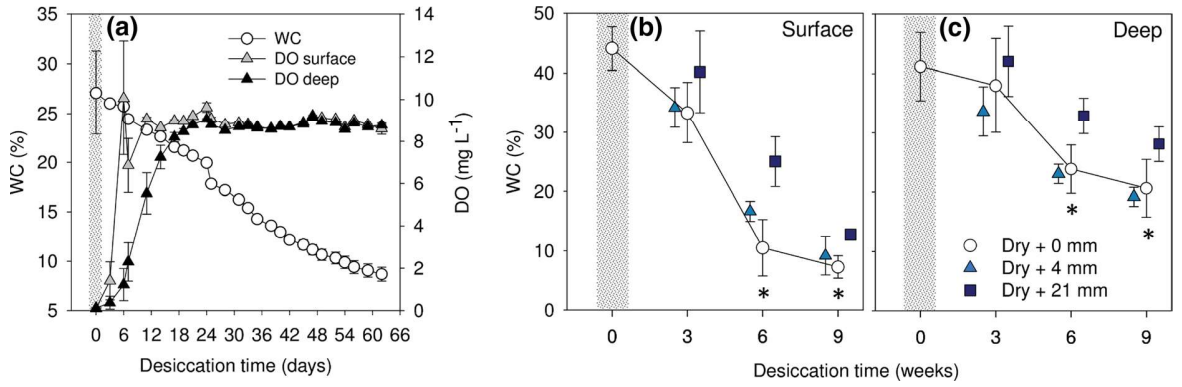

Fig. 2 **a** Variation of water content (WC%) in the whole microcosms and dissolved oxygen (DO) percentage in surface and deep sediments of microcosms that kept dry over the entire desiccation period (mean ± SE, n = 9). Mean WC% (± SE, n = 3) calculated in surface (**b**) and deep (**c**) sediments over desiccation time and after the different rainfall sizes (0, 4 and 21 mm). Asterisks (*) denote significant ($P < 0.05$) differences of dry treatments (i.e., dry + 0 mm) after each desiccation period with pre-desiccation conditions (0 weeks) after Holm's post hoc test for multiple comparisons. The dashed bar illustrates the wet conditions of the pre-desiccation. The continuous line connects values of WC% in microcosms that received no rainfall (dry or 0 mm) for a better comparison

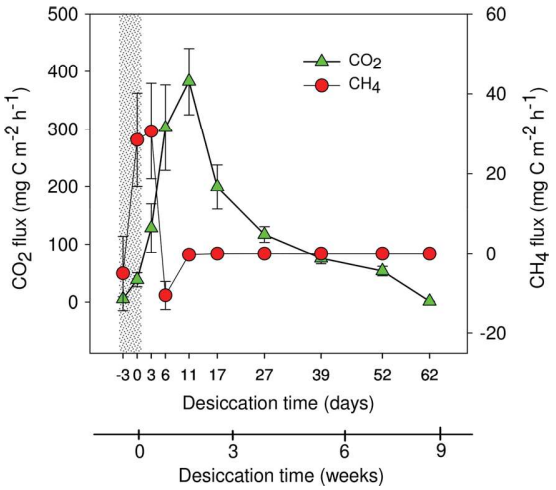

Fig. 3 Variation of mean (± SE, n = 9) fluxes of CO_2 and CH_4 from sediments before microcosms drainage (microcosms with a overlaying water column) (t = − 3 days), immediately after drainage (t = 0 days) and several times after drainage and desiccation. The dashed bar illustrates the wet conditions of the pre-desiccation

CO_2 fluxes in the dry microcosms (i.e. 0 mm) hardly changed over 24 h, the 4-mm and 21-mm rainfall treatments induced considerable dynamics, yet with contrasting patterns (Fig. 4b). For example, an instantaneous (t = 0) peak in the average flux of 157 mg C m^{-2} h^{-1} was found after 4 mm rainfall when compared with the flux before (20 mg C m^{-2} h^{-1} at t = − 3) (Fig. 4b). Conversely, the 21-mm rainfall first caused an instantaneous drop in CO_2 flux (19 mg C m^{-2} h^{-1}) to levels below those in dry conditions (i.e., 0 mm: 81 mg C m^{-2} h^{-1}), but then followed with a peak value of 274 mg C m^{-2} h^{-1} after 2 h. After 24 h, CO_2 fluxes slightly increased under the 4-mm rainfall, while a drop was detected under the 21-mm rainfall (Fig. 4b). After 9 weeks of desiccation, rainfall also induced dynamics of CO_2 fluxes; yet, patterns were more similar between the two rainfall sizes and fluxes in the dry microcosms (i.e. 0 mm) were generally lower (Fig. 4c).

Organic C and N in surface water and sediments

By considering the water volume moving through microcosms, the mean amount (± SD) of DOC and DON of surface water in the microcosms at the start of the experiment were 0.5 ± 0.02 mg C and 0.03 ± 0.01 mg N L^{-1}, respectively. Amounts were higher in the drained water, especially for DOC, with an average of 1 ± 0.2 mg C while the amount of DON was 0.04 ± 0.01 mg N. The average initial sediment OM% was 8.0% ± 2.4 and 11.6% ± 3.5 in surface and deep sediments, respectively. The average OM% did not show significant variation as desiccation progressed as well as between sediment layers (data not shown).

In surface and deep sediments, concentrations of WSOC after 3 and 9 weeks significantly differed from initial conditions (Holm´s post hoc test, $P < 0.05$,

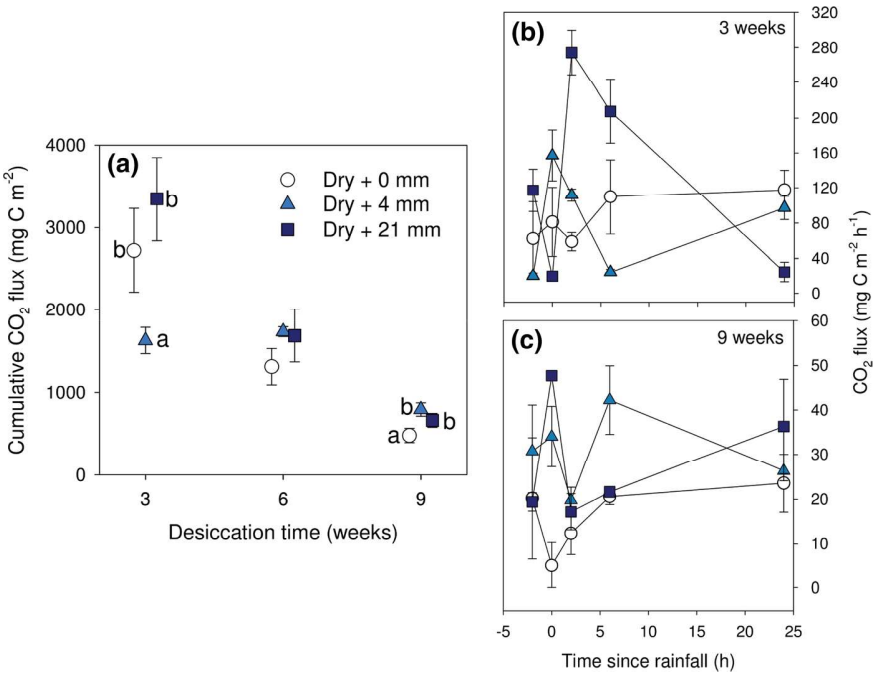

Fig. 4 **a** Variation of mean (± SE, n = 3) cumulative fluxes of CO_2 following 24 h in response to desiccation (dry + 0 mm) and to variable rainfall size (dry + 4 and dry + 21 mm) after the different desiccation weeks. Different letters denote significant differences between rainfall treatments after Holm's post hoc test for multiple comparisons. Variation of mean (± SE, n = 3) fluxes of CO_2 before (t = -2 h), immediately (t = 0 h) and following rainfall events (t = 2, 6 and 24 h) after 3 (**b**) and 9 (**c**) weeks of desiccation

Fig. 5a). For example, average WSOC in the microcosms dropped more than half during the first 3 weeks, from 0.93 to 0.36 mg C g^{-1} DM in surface and from 0.86 to 0.33 mg C g^{-1} DM in deep layers, respectively (Fig. 5a). In the case of WSON, significant differences with respect to initial conditions were found for all desiccation periods (Holm´s post hoc test, $P < 0.05$, Fig. 5b). No significant differences between sediment layers were detected for both variables. In parallel, $SUVA_{254}$, tended to increase over desiccation time in both surface and deep sediments (Fig. 5c), yet we only found significant differences after 3 and 9 weeks when compared with initial conditions (Holm´s post hoc test, $P < 0.05$, Fig. 5c). Again, no differences between sediment layers were detected.

Before desiccation, the largest fraction of WSOC in surface and deep sediments was, respectively, in form of HS (54 and 52%) followed by LMWS (37 and 36%) and HMWS (9.4 and 12%). These relative differences were maintained during the whole experiment (Fig. 6a, b). There were no significant differences between the two sediment layers for any fraction. The only changes over desiccation were detected for %HMWS in both sediment layers, which dropped significantly after 3 and 6 weeks when compared with pre-desiccation conditions (Holm´s post hoc test, $P < 0.05$), although an increasing trend after 9 weeks of desiccation appeared (Fig. 6a, b).

The two analyzed high-molecular fractions for WSON, HS and HMWS, varied similarly in both sediment layers showing significant differences after desiccation (Holm´s post hoc test, $P < 0.05$) (Fig. 6c, d). During the whole experiment, HS was the dominant fraction of WSON. During initial conditions %HS and % HMWS were, respectively, 60 and 40% in surface (Fig. 6c) and 43 and 57% in deep sediments (Fig. 6d). Because of the contrasting temporal patterns, these relative differences amplified as desiccation progressed. Thus, after 9 weeks of desiccation, %HMWS and %HS were, respectively, 22 and 78% in surface (Fig. 6c) and 20 and 80% in deep sediments (Fig. 6d).

Before desiccation, C:N molar ratios were about 4-times higher for HS than for HMWS in surface (mean ± SD, 18 ± 2 and 4.3 ± 0.4, respectively) and deep (16 ± 1 and 5 ± 1.5, respectively) layers (Fig. 6e, f). Overall, in both sediment layers, ratios for HMWS increased as desiccation progressed, showing significant differences after 9 weeks (Holm´s post hoc test, $P < 0.05$). Such increase led HMWS to have similar C:N to HS at the end of the experiment (Fig. 6e, f).

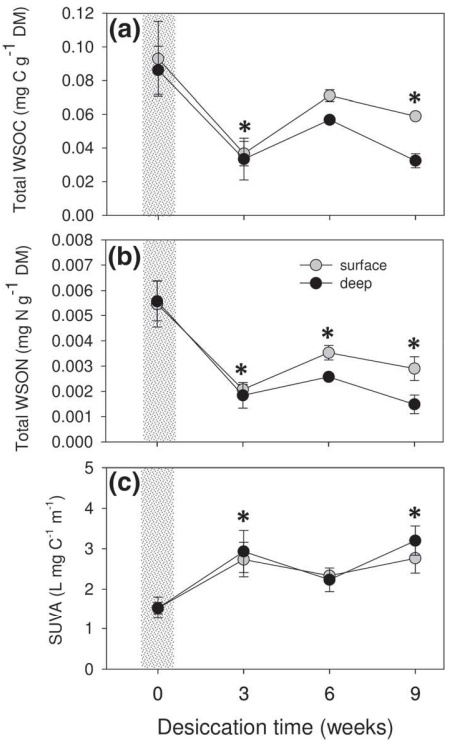

Fig. 5 Variation of mean (± SE, n = 3) concentrations of water-soluble organic carbon (WSOC) (**a**), water-soluble organic nitrogen (WSON) (**b**), and specific UV absorbance ($SUVA_{254}$) (**c**) in surface and deep sediment leachates over desiccation time. Asterisks (*) denote significant ($P < 0.05$) differences to pre-desiccation conditions (0 weeks) for both sediment layers after Holm's post hoc test for multiple comparisons. The dashed bar illustrates the wet conditions of the pre-desiccation

Microbial community functions

During the whole experiment, the relative abundance of summed aerobic functional groups was larger than that from anaerobic groups both in surface and deep sediment (Fig. 7a). Indeed, we found no significantly different abundances of these functional groups between sediment layers. When compared with initial conditions (0 weeks) only a significant reduction was found for anaerobic functions in both sediment layers (Holm´s post hoc test, $P < 0.05$, Fig. 7a).

When we focused on CH_4-related functional groups, i.e., aerobic methanotrophs and anaerobic methanogens (Fig. 7b, c), we found that their average relative abundance varied within the same range of magnitude over the experiment. The relative abundance of methanogens was stable over the experiment. Only the abundance of methanotrophs in deep sediments seemed to show a marked increase after 3 weeks of desiccation when compared with pre-desiccation conditions (0 weeks). It was at this time when the largest variation between sediment layers was also observed, yet these results were not statistically significant (interaction term *time x sediment layer* $P = 0.057$) (Fig. 7b).

Discussion

This study underpins that the duration of desiccation, a proxy of drying severity, is an important driver of biogeochemical processing in intermittent streams. As predicted, drying modulated microbial functions and the evasion of CO_2 and CH_4 from streambed sediments to the atmosphere, both during progressing desiccation as well as in later rainfall-induced evasion peaks. Moreover, desiccation altered the quantity and quality of water-soluble organic matter, with implications for biogenic gas production and downstream organic matter transport upon rewetting.

Gaseous C fluxes in response to desiccation

In agreement with other studies (von Schiller et al. 2014; Gómez-Gener et al. 2015; 2016; Obrador et al. 2018), we found that CO_2 fluxes to the atmosphere boosted in response to desiccation both in laboratory and field conditions. The CO_2 flux we observed in situ after 6 weeks of non-flow conditions (234 ± 21 mg C m^{-2} h^{-1}, Table 1) tended to be higher but overlapped with estimates reported from a field survey at global scale for other temperate streams under dry conditions (89 ± 154 mg C m^{-2} h^{-1}, median = 50) (Keller et al. 2020). This finding supports the importance of considering drying aquatic ecosystems in global upscaling and modeling studies on C cycle (Marcé et al. 2019).

Contrary to CO_2, CH_4 fluxes were low (5.5 ± 9.6 mg C m^{-2} h^{-1}) in our microcosms and not detectable in situ, which agrees with previous work reporting low CH_4 emissions in exposed sediments of inland waters, typically falling below 0.5 mg C m^{-2} h^{-1} (Marcé et al. 2019).

As predicted, temporal progression of desiccation impacted CO_2 and CH_4 fluxes to atmosphere. Fluxes of both gases peaked during early desiccation and then decreased. This pattern was especially quick for CH_4.

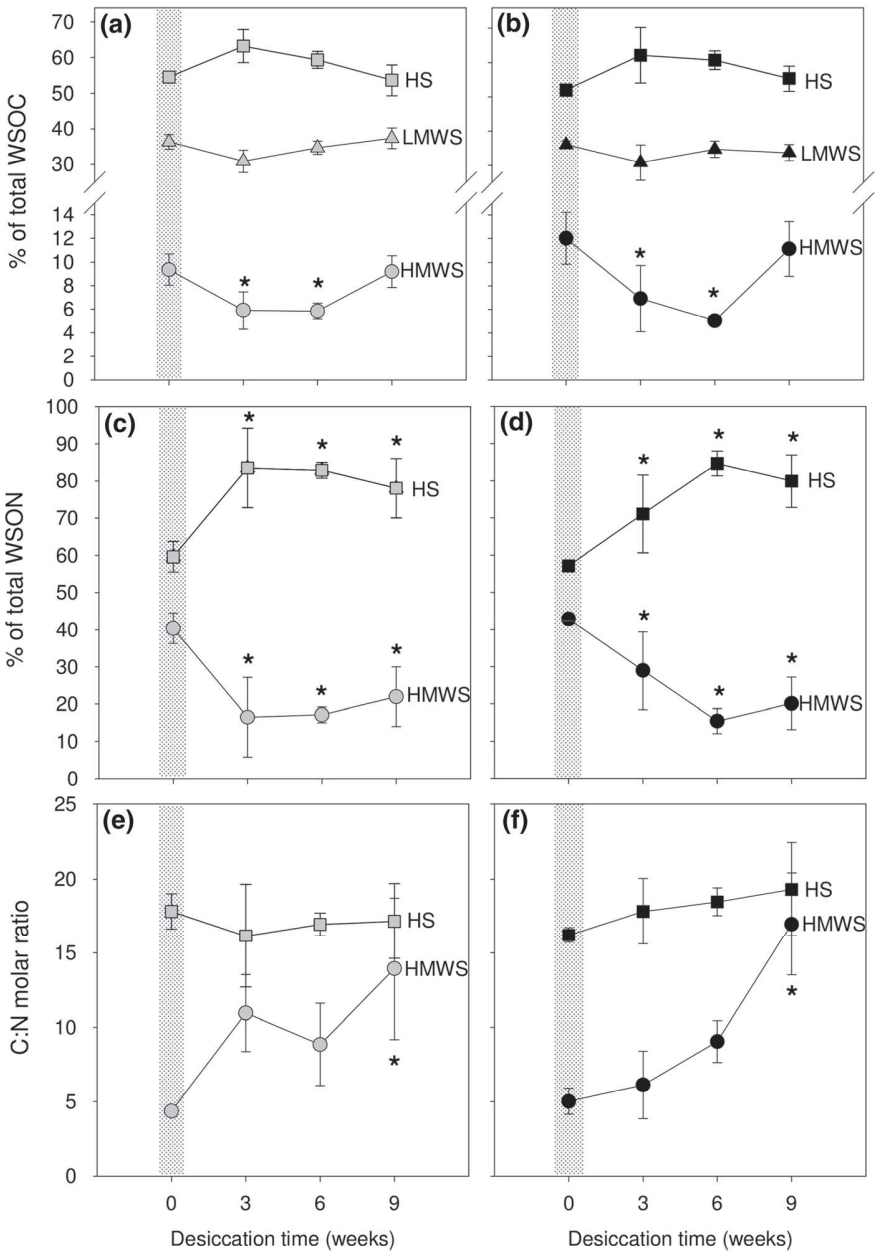

Fig. 6 Variation of the percentage contribution of the different fractions to the total water-soluble organic carbon (WSOC) and nitrogen (WSON) and the C:N molar ratio of each fraction in surface (**a, c, e**) and deep (**b, d, f**) sediment leachates, respectively, over desiccation time. Asterisks (*) denote significant ($P < 0.05$) differences to pre-desiccation conditions (0 weeks) after Holm's post hoc test for multiple comparisons. The dashed bar illustrates the wet conditions of the pre-desiccation. HMWS = high molecular weight substances, LMWS = low molecular weight substances and HS = humic or humic-like substances. Values are means ± SE (n = 3)

Gaseous fluxes are influenced by multiple factors, including physical forcing and microbial production (Looman et al. 2017). Due to low gas diffusion, water acts as a physical barrier curbing gas evasion from wet sediments, its absence in dry streambeds in turn enhances gaseous C emissions (Gallo et al. 2014;

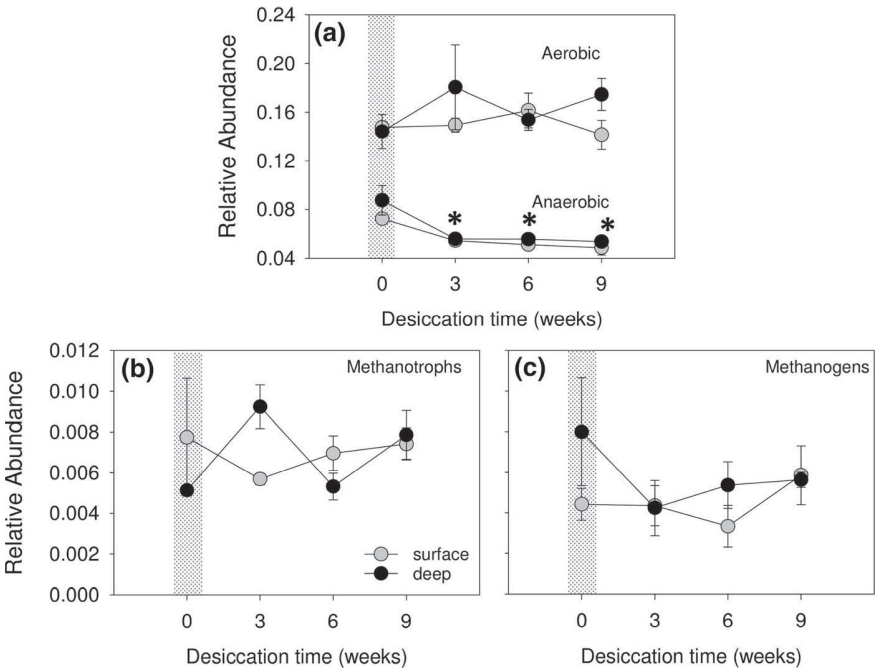

Fig. 7 Variation in relative abundances (%) of 16 S rRNA sequence tags from aerobic and anaerobic functional groups (**a**), methanotrophs (**b**) and methanogens (**c**) identified by using FAPROTAX in surface and deep layers over desiccation time. Asterisks (*) denote significant ($P < 0.05$) differences to pre-desiccation conditions (0 weeks) after Holm's post hoc test for multiple comparisons for both sediment layers. The dashed bar illustrates the wet conditions of the pre-desiccation. Values are means ± SE (n = 3)

Gómez-Gener et al. 2015; 2016). This physical mechanism may explain the rapid peak of CH_4 observed 3 days after drying of microcosms. Later, we observed low CH_4 fluxes from sediments exposed to air, likely due to lower production in increasingly oxic sediment and oxidation of CH_4 prior to evasion to atmosphere (Koschorreck 2000; Wang et al. 2006; Jin et al. 2016; Kosten et al. 2018). Unlike CH_4, CO_2 emissions progressively increased during the first 11 days of desiccation. Besides improved physical gas exchange, oxygenation of exposed sediments favors aerobic transformation processes (Gómez-Gener et al. 2015; von Schiller et al. 2017), microbial growth and associated biogenic CO_2 production (Fromin et al. 2010). Biogenic CO_2 and CH_4 dynamics are prone to show opposite behavior due to different redox requirements of involved microorganisms (Marcé et al. 2019). Accordingly, we hypothesized contrasting patterns in the relative abundance of putatively anaerobic vs. aerobic microbial functional traits in sediments as desiccation progressed. Over 9-weeks of drying, relative abundance of aerobic microbial traits did not vary, but anaerobic functional traits decreased. The slight decrease in the relative abundance of methanogens in deep sediments after 3 weeks of desiccation is consistent with the low CH_4 emissions found over desiccation. Besides, this drop corresponded with the significant increase of methanotrophs, which suggests oxygenation reached deep sediments. In fact, we detected negative CH_4 fluxes in some microcosms within the first 27 d of desiccation, which indicates that methanotrophy could be active in this timeframe.

These results illustrate a microbial functional response to drying, yet should be interpreted with caution as they are based on functional trait estimation, rather than direct detection of functional gene expression. In addition, relative abundance data does not capture microbial responses that lead to an overall biomass increase, rather than changes in the relative proportions of organisms.

The decrease of CO_2 emission as sediment became drier was also observable in cumulative fluxes during 24 h. These findings indicate that after 17 d of

Table 1 Mean (SE) values, n = 3, of variables studied after 6 weeks of desiccation in the study stream under in situ conditions

Variable (units)	Sediment layer	
	Surface	Deep
WC (%)	56 (4)	42 (5)
OM (%)	29 (6)	12 (2)
WSOC (mg C g^{-1} DM)	0.18 (0.05)	0.05 (0.01)
HMWS (%)	9.4 (0.6)	12 (1)
LMWS (%)	59 (2)	53 (2)
HS (%)	31 (1)	34 (2)
WSON (mg N g^{-1} DM)	0.014 (0.01)	0.004 (0.001)
HMWS (%)	30 (12)	28 (5)
HS (%)	70 (12)	71 (5)
Molar C:N ratio HMWS	9.4 (2.5)	13.7 (0.7)
Molar C:N ratio HS	10 (3.4)	15 (4)
$SUVA_{254}$ (L mg C^{-1} m^{-1})	2.3 (0.1)	2.4 (0.3)
Flux of CO_2 (mg C m^{-2} h^{-1})	234.52 (21)	
Flux of CH_4 (mg C m^{-2} h^{-1})	nd	

WC water content, *OM* organic matter, *WSOC* water soluble organic carbon, *WSON* water soluble organic nitrogen, *HMWS* high molecular weight substances, *LMWS* low molecular weight substances, *HS* humic substances. $SUVA_{254}$ spectroscopic index indicative of aromaticity of organic matter, *nd* no detected

desiccation, sediment water becomes a limiting factor for microbial activity, probably through the direct impact on the survival of microbial communities (Amalfitano et al. 2008) and by limiting resource supply in a dry matrix (Schimel 2018). This plausible loss of activity did not translate into substantial changes in the relative abundance of aerobic microbial groups, which remained constant during the experiment. Finding synchronisms between microbial community and activity patterns is not easy (Prosser 2012). We must note that we are examining relative abundances of microbial traits (instead of absolute ones), and DNA-based community analysis may in addition suffer from the persistence of DNA material from dead and therefore inactive members of the community. Furthermore, the persistence of extracellular enzymatic activity during severe desiccation (Zoppini and Marxsen 2011; Pohlon et al. 2018) has been proposed to contribute to CO_2 emissions in dry riverbed sediments at certain levels (Gómez-Gener et al. 2016).

We acknowledge that methods targeting microbial gene expression, such as metatranscriptomics, would have revealed possibly more substantial functional shifts in the microbial community as RNA can be less persistent in the environment than DNA and proteins (Lanzén et al. 2011). In addition, the functional trait estimation used here cannot detect and resolve the effect of facultative anaerobes, which may perform anaerobic processes during anoxia and aerobic processes during oxic conditions.

Gaseous C fluxes in response to rainfall

It is well known that transitions from dry to wet conditions can give rise to hot moments with accelerated C and nutrient cycling (McClain et al. 2003; Pinto et al. 2020). In our experiment, sudden water availability by punctuated rainfall had a rapid positive effect on CO_2 fluxes to the atmosphere. The recently formulated *Ecosystem Control Points* concept (Bernhardt et al. 2017) suggests places and moments of disproportionate biogeochemical relevance. Early studies have demonstrated that rewetting events represent such *Ecosystem Control Points* for respiration and CO_2 emissions from intermittent streams (von Schiller et al. 2019), equivalent to the "Birch effect" described in soils. However, as observed in our experiment, anaerobic pathways producing CH_4 emissions may not recover rapidly upon rewetting after a severe desiccation (Conrad et al. 2014). Enhanced CO_2 fluxes upon water pulse support the importance of WSOC stocks, as a proxy of sediment organic matter, to fuel respiration processes once sediment humidity is no longer limiting microbial activity in water-stressed ecosystems (Sponseller 2007). Yet, as expected, our results demonstrate that the duration of the precedent desiccation period has implications for rewetting-induced CO_2 fluxes, as previously observed in soils (Fierer and Schimel 2002) or temporary ponds (Fromin et al. 2010). The magnitude of the response to rewetting decreased with the duration of the preceding desiccation period, highlighting a legacy impact of water stress on microbial communities (Schimel et al. 2017; Schimel 2018).

Water pulses do not always drive positive net CO_2 fluxes, and when they occur, biogenic but also physical sources can be involved. For instance, Gallo et al. (2014) found large fluxes of CO_2 in response to substantial increases in sediment water content ($\sim$ to

50%) by rainfalls in extremely dry (~ 0% sediment moisture) ephemeral streams of Arizona, USA. They proposed that the increased CO_2 fluxes immediately after rainfall (t = 0 h), between 6 and 30 times larger than pre-rainfall, could result from a rapid increase in microbial activity, but especially from a physical displacement immediately after rainfall impact. On the contrary, CO_2 may also dissolve in additional interstitial sediment water after rainfall and thus experience dilution in the gas space, which could buffer transitory CO_2 exchange to the atmosphere (Looman et al. 2017; Arce et al. 2018). Thus, rewetting effects may likely result from a combination of mechanisms representing sinks and sources, whose relative importance ultimately translate into higher or lower CO_2 net fluxes to the atmosphere. After 3 weeks of desiccation, the reduced flux found in our experiment upon the 21-mm rainfall could be attributed to CO_2 dissolution into interstitial sediment water. On the contrary, 2 h after, the substantial flux detected could be indicative of active microbial contribution, including the production of extracellular enzymes (Zoppini and Marxsen 2011; Pohlon et al. 2018). Indeed, narrow peaks of CO_2 upon rewetting have been attributed to resuscitation strategies of some microbial groups that become activated within hours of water arrival (Placella et al. 2012). As initially expected, after 9 weeks, when desiccation was more severe, rainfalls of 4 and 21 mm appeared insufficient to enhance biogenic gaseous C emissions. In fact, the unique substantial pulse seen after the 21-mm rainfall immediately after water addition was presumably the consequence of dislocation of accumulated gas from already dry and increasingly less active sediments (Gallo et al. 2014) rather than a rapid microbial activation.

Implications of desiccation for leaching of sediment organic matter

Understanding how desiccation affects the dynamics of organic matter is critical in streams with organic matter-rich sediments since excess, unprocessed C can affect downstream perennial reaches, reservoirs and coastal areas (Datry et al. 2016). The study stream exhibited high DOC concentration in surface water and exfiltrates of incubated sediments before desiccation, indicative of the high organic matter content of the ecosystem. Alongside the gaseous C fluxes, desiccation also drove changes in the quantity and quality of WSOC. The notable reduction in the quantity of WSOC after 3 weeks to levels 2.5 times lower than pre-desiccation supports the role of microbial respiration in using the WSOC stored in streambed sediments (Schimel 2018) when alternative C sources like stream water are not available during non-flow periods. Thus, biogenic CO_2 and CH_4 fluxes observed during the first days of desiccation can translate to substantial sediment organic C mineralization. While rapid anaerobic decomposition of organic C could occur at early stages, including methanogenesis, aerobic respiration maintained during desiccation for prolonged time could additionally contribute to the decline of WSOC in dry sediments after 3 weeks. Enhanced respiration of sediment organic C associated with desiccation was also supported by the WSON results. The reduction of WSON (both in surface and deep sediments) as desiccation progressed indicates enhanced N mineralization pathways associated to the increase in O_2, as recently outlined for dry riverbeds (Arce et al. 2018, 2019).

After 3 weeks of desiccation, the progressively decreasing C respiration led to relatively stable WSOC. A moderate increase of WSOC and WSON was detected after 6 weeks followed by a modest drop after 9 weeks. Certain imbalances in the relative importance of mechanisms acting as sink or source of the organic C sediment pool over prolonged desiccation could explain such fluctuations. There is evidence that drying-rewetting phenomena may drive the release of organic substrates from microbes through osmolysis (Fellman et al. 2011; Vázquez et al. 2011; Ylla et al. 2011). In combination with slow microbial activity, this would explain the increase in WSOC (and WSON) after 6 weeks. Conversely, the subsequent drop in WSOC after 9 weeks of desiccation could be associated to the fact that resistant microbes can quickly use this labile C fraction. The persistence of functional extracellular enzymes in desiccated sediments (Zoppini and Marxen 2011) may also have implications on WSOC quantity either by reducing concentrations through C mineralization (and CO_2 production) or by contributing to the organic C pool themselves.

Changes of organic matter quality provide evidence of the proposed mechanisms behind changes in WSOC. For instance, the contrasting developments of $SUVA_{254}$ and WSOC over desiccation time revealed that organic matter in leachates became more aromatic at lower concentrations. This suggests microbial metabolism of labile compounds leading to accumulation of more recalcitrant, aromatic WSOC and WSON. Conversely, microbial contributions to the WSOC and WSON pool, presumably due to cellular lysis coupled to drying-rewetting, led to lower $SUVA_{254}$ values (Mavi and Marschner 2012). Insights that are more detailed are provided by size exclusion chromatography results, which identified dynamics of various organic matter fractions over desiccation time. While proportions of HS and LMWS appeared almost steady over desiccation, the proportion of HMWS in WSOC showed a drop that was significant after 6 weeks but further increased after 9 weeks. In parallel, a consistent decay was observed for WSON in this fraction. This finding confirms previous research in intermittent streams, where this fraction was found to be largely reactive in water (von Schiller et al. 2015; Catalán et al. 2017). The large HMWS fraction is mainly composed of biopolymers, but supramolecular assemblies formed by several individual compounds or dissolved organic matter bonded through weak forces may also contribute to this fraction (Piccolo et al. 2002; Kellerman 2015). The activity of exoenzymes (Zoppini and Marxen 2011) may particularly mediate biopolymer cleavage and loss of this organic matter fraction (Sinsabaugh and FollstadShah 2012). HMWS also exhibited a low C:N molar ratio, indicative of abundant nitrogen-containing proteins or amino sugars (Huber et al. 2011) and suggesting higher reactivity (von Schiller et al. 2015). Indeed, the observed progressively increasing C:N of HMWS may result from either a decrease in N (while C does not substantially vary) or otherwise, a supply of C (while N does not substantially vary). The proposed progressive cycles of microbial processing, including exoenzyme activity, and release of cellular compounds associated to water stress over desiccation, would explain such a trend of increasing C:N ratio for HMWS. Together, the increased C:N ratio and the decline of HMWS strongly suggest preferential protein depolymerization (Reuter et al. 2020).

Comparison of experimental and field results

In-situ CO_2 fluxes after 6 weeks of non-flow period (234 ± 21 mg C m^{-2} h^{-1}, Table 1) were 3 times higher than measured in microcosms at comparable duration of desiccation (75 ± 16 mg C m^{-2} h^{-1}). The high sediment humidity measured in the field at that time (WC% = 56 and 42% in surface and deep sediments, respectively, Table 1) probably favored microbial respiration and CO_2 production. In fact, microbial activity in dry streambeds may be maintained thanks to sporadic rainfalls, dew formation and the groundwater table buffering surface water loss (Harjung et al. 2019b; Gionchetta et al. 2019). Yet, anaerobic pathways (like CH_4 production) are deactivated due to desiccation (Conrad et al. 2014), and although rainfall brings back inundated habitats in streambeds, the required low redox potential needed for methanogenic activity may require time to develop. Furthermore, unlike in our microcosms, terrestrial or airborne microbes may quickly colonize dry streambeds and compensate the loss of aquatic microbial diversity and activity caused by desiccation (Romaní et al. 2017).

Results from our in-situ assessment also suggest that as desiccation advances in time, there are substantial shifts in the quantity and quality of sediment C caused by sequential mineralization and release that comprise, respectively, a sink and a source of C stored in sediments. Nonetheless, we must consider that under natural conditions, accumulation of in-stream organic stocks (e.g. macrophytes, algae) and external inputs of leaf litter (Datry et al. 2018) can shape the quantity of C in streambed sediments. Such input explains that the OM% in the surface sediment of the stream after 6 weeks of natural desiccation (mean = 29%) was clearly higher than that observed in the microcosms (8%) (Table 1). Consequently, WSOC in surface sediments was also larger in situ (0.18 mg C g^{-1} DM, Table 1) than in the microcosms at that time (0.07 mg C g^{-1} DM).

Changes in C quality were also detected in the field after 6 weeks in comparison with the microcosms. While in microcosms the dominant WSOC fractions were HS, LMWS and HMWS respectively, LMWS was the dominant fraction in the field (Table 1), showing shifts in the bulk composition of WSOC towards a higher proportion of non-humic substances such as low molecular weight alcohols, aldehydes,

ketones, sugars and amino acids (Huber et al. 2011). $SUVA_{254}$ showed values comparable to 6 weeks of experimental microcosms; yet, without reference values in the field before desiccation we cannot accurately evaluate the contribution of microbial processes to WSOC (and WSON) concentrations and composition. There is strong evidence of the importance of photodegradation during dry periods in intermittent streams (Dieter et al. 2013), which seems to enhance the decomposability of leaf litter (Datry et al. 2018; del Campo et al. 2021). Thus, this process, which did not happen in our experiment, may impose key changes in quality of C over desiccation beyond those we observed. Likewise, macroinvertebrates and terrestrial fungal decomposers on particulate organic matter may affect WSOC levels in the long run (Romaní et al. 2017). Yet, drying tends to reduce presence of macroinvertebrates (Schlief and Mutz 2009) and inhibit lignocellulolytic activity (Mora-Gómez et al. 2016). Thus, within a relatively short desiccation period, most changes in sediment organic matter rely on the WSOC fraction, with aquatic microbial communities still exhibiting C processing capacities.

Finally, we must note that differences between our field and laboratory results may also be at least partially due to the disturbance of the physical structure of the sediments used in the laboratory experiment during sampling.

Conclusions

Desiccation in intermittent streams boosts emissions of CH_4 and mainly CO_2 in the short term by favoring both physical evasion and aerobic mineralization. As drying progresses, these gaseous C fluxes decrease due to increasingly oxic conditions and water limitation. Rewetting in dry riverbeds does not always directly lead to flow recovery. Especially in temperate climates, a non-flow period can be frequently interrupted by sporadic rainfalls that can similarly trigger C cycling as described by the Birch effect in soils. Thus, rainfalls can rapidly trigger CO_2 emissions fueled by WSOC from sediments and temporarily revert the desiccation impacts on biogenic CO_2 production. The enhanced mineralization coupled to desiccation consequently shapes the role of sediments as a source and sink of organic C with a net tendency towards reducing in-stream organic loads as we found by the general drop in WSOC.

In intermittent streams, concentrations of the released substances may exceed baseflow values by several orders of magnitude and can thus substantially contribute to annual fluxes (Skoulikidis and Amaxidis 2009; Corti and Datry 2012; Bernal et al. 2013). We showed that desiccation can alter the quantity and quality of water-soluble organic matter, with important consequences for the organic matter released to the stream water upon rewetting. In the crucial moment of flow reestablishment (the so-called "first flush event"), material accumulated on the dry streambed is mobilized (Obermann et al. 2009; Corti and Datry 2012), and its reactivity may depend on the precedent preconditioning during the desiccation phase. Desiccation not only brings a cessation of stream water but also opens the door to other environmental drivers to operate within the dry streambed with more intensity than during flowing conditions. Processes such as photodegradation, terrestrial inputs of micro- and macro-biota to streambed as well as organic matter accumulation amplify with the lack of surface water, and their opportunity to impact C budgets will be clearly favored under long non-flow periods.

Collectively, our results support intermittent stream reaches as *Ecosystem Control Points* for C cycling within the stream network, with the duration of the dry phase exerting a strong control on this role. Further research that considers all mechanisms that operate in situ as desiccation progresses in time will surely complement our findings and improve our understanding of the biogeochemistry of intermittent streams.

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Author contributions MIA, DvS and GS conceived and designed the experiment. Material preparation and data collection were performed by MIA and DZ. MMB, TU and JT analysed 16sRNA sequences. The first draft of the manuscript was written by MIA. All authors contributed to the development of the manuscript and read and approved the final version. All data, materials and software application supporting our published claims comply with field standards. Illumina MiSeq

16 S rRNA amplicon sequence data is submitted to the NCBI Short Read Archive (Accession No. SRP137655). Other datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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4.4.1 Supplementary figures and tables

Supplementary figures and tables are provided on the CD.

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6 Appendix

6.1 List of Figures

6.2 List of Tables

7 Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Unterschrift des*der Promovend*in

9 Erklärung zur Abgabe einer elektronischen Kopie der Dissertation/Declaration on the submission of an electronic copy of the PhD thesis

Mathematisch-Naturwissenschaftliche Fakultät
Einverständniserklärung nach § 4 Abs. 1 Nr. c Promotionsordnung

Hiermit erkläre ich, dass von der Arbeit eine elektronische Kopie gefertigt und gespeichert werden darf, um unter Beachtung der datenschutzrechtlichen Vorschriften eine elektronische Überprüfung der Einhaltung der wissenschaftlichen Standards zu ermöglichen.

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