Supplement of

Interferences caused by the microbial methane cycle during the assessment of abandoned oil and gas wells

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S1 Lipids after aerobic methane oxidation in the laboratory

It is well known that many methanotrophic bacteria of the Type I (γ-Proteobacteria) and Type II (α-Proteobacteria) cluster consist of specific phospholipid fatty acids (PLFA) with Type I mainly consisting of 14 and 16 carbon atoms and Type II of 18 carbon atom PLFA (e.g., Bodelier et al. (2009) and references therein). Furthermore, many of the methanotrophic bacteria in these Type clusters contain signature PLFA with C16:1Δ8c and C16:1Δ11t (or C16:1ω8c and C16:1ω5t, respectively) specific for Type I and C18:1Δ10c or C18:1ω8c for type II (Bowman et al., 1991; Nichols et al., 1985). This classification was, however, challenged by findings of the Type I specific 16:1ω8 (together with an abundant 18:1ω8) in the Type II methanotroph *Methylocystis heyeri* (Dedysh et al., 2007). *Methylocystis* are demonstrated to be abundant at Northern Europe, acidic peatlands (Dedysh, 2009) like e.g. Steimbke. In concordance to these studies, *Methylocystis* were indeed prominent in our phylogenetic data of the peat organic matter used for this study from Steimbke (sample 7468) prior and after the lab experiment. Furthermore, all Type II methanotrophic *Beijerinckia* species lack the classical 18:1ω8 PLFA (Knief, 2015 and reference therein). The differentiation between Type I and II methanotrophs is therefore complicated, while the presence of ω8 unsaturated PLFA in general remain a strong hint to methanotrophic bacteria.

Material & Methods

Peat soil samples taken in 2022 at Steimbke, two samples from the Meadow and Peat sites both with underlying peat, and two other sites for comparison were incubated with about 1–2% methane in air for a duration of 22 hours up to 58 days (see Table S1). Four experiments were analyzed for changes in the lipid composition in the course of the experiments. The following samples were processed:

Table S1: Experiments analyzed for lipid changes during the course of methane addition (prior and after the experiments). Samples originate from the Peat site near Steimbke and the Nienhagen oil field

ID	origin	time [h]	MOx activity	MOx rate [µmol g^{-1} d^{-1}]
7468	Rodewald WA-272 (Steimbke peat extraction site), 10–15 cm	3	high	200
7470	Rodewald WA-275 (Steimbke meadow/peats) , 10–15 cm	22.5	low	5
7732	Carolinenhall, Nienhagen oil field, 10–15 cm	~1400	None	0
7734	Carolinenhall, Nienhagen oil field, 10–15 cm	~1400	none	0

All samples were subjected to alkaline hydrolysis to cleave intact phospho- and glycolipids using a method slightly modified after e.g., Oppermann et al. (2010). Briefly, 5 g soil samples were subjected to 6% KOH in methanol (MeOH) in excess for 2 h at 80°C in a screwed glass centrifuge tube. The resulting neutral lipids (NL) were extracted with *n*-hexane and were further separated into fractions of increasing polarity (hydrocarbons, ketones/aldehydes, alcohols, polar rest). Prior of the analysis, the alcohols were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Acids extracted from the acidified residue after KOH-methanol hydrolysis, were transferred into their fatty acid methyl esters (FAME) in a screwed vial using trimethylchlorosilane (TMCS in MeOH 1:9; v:v; 2 h; 80 °C) and the combined extracted *n*-hexane phases (3x) were then, in addition to hydrocarbon, ketone/aldehyde and alcohol (BSTFA treated) fractions, analyzed with a GC-MS. The GC-MS comprised of an Agilent-7890 gas chromatograph equipped with a Programmable Temperature Vaporizing inlet splitting on two 50 m DB-1 columns (Agilent; 0.2 mm inner diameter; 0.11 µm film thickness), coupled to an FID and to an Agilent 7000 QQQ MS, each. Helium was used as carrier gas. MS-measurements were carried out in full scan mode. Compounds were identified by mass spectra and retention times in comparison with measurements of standard compounds. Here, only the acid fractions are presented and for simplification are named phospholipid fatty acids or "PLFA", which make up the main compounds in fresh soil samples after alkaline hydrolysis. Position of double bonds were determined after GC-MS analyses of DMDS derivatives of FAME using a method modified after Buser et al. (1983) and Gatellier et al. (1993). Details can be found elsewhere (Berndmeyer et al., 2013).

Results and Discussion

Fatty acids including hydrolyzed acids from phospho- and glycolipids remained relatively similar in all experiments (Figure S1). For better comparison the relative changes were visualized from normalizing the (short chain) fatty acid composition to the eicosanoic acid methyl ester, which was assumed to remain unchanged as this likely originates from plant litter (and not microbial lipids). However, some peaks (three specific unsaturated C16 and C18 fatty acids marked with an arrow in (Figure S1) increased in the course of one experiment, where methane oxidation rate was high (7468). These compounds were mostly

absent in other experiments and did also not change in those experiments after methane-addition. Further analyses of the double bond positions revealed C16:1ω8c and C18:1ω8 (and, less specific, 16:1ω7), which are specific to methanotrophic bacteria. Normalized to 100% of FAMEs shown in Fig. S1 (without C20:0) C16:1ω8 increased in the experiment 7468 (peat site) from 0.69 to 3.37% and C18:1ω8 from 0.95 to 4.67%. Also C16:1ω7 increased slightly in abundance from 0.57 to 1.26%. This compound is abundant in the other experiment samples as well, but remained unchanged there. Thus, it appears less specific. C16:1ω8 and C18:1ω8 are the most abundant PLFA in *Methylocystis heyeri* (Dedysh et al., 2007) and a *Methylocystis*-origin is in line with our phylogenetic data.

Although changes are specific to methanotrophic bacteria, changes in the PLFA (and free fatty acids) are relatively minor. This, however, is not uncommon and even occurs in highly active samples and can be explained by the high (non-methanotrophic) load of microbial and plant organic matter in the original soil (peat) samples Knoblauch et al. (2008) and the likely generally relatively low abundance of methanotrophs among the microbial community in peat (Kaupper et al., 2021). PLFA data are indicative of the presence of methanotrophic stemming PLFA in the original sample 7468, while respective biomarkers are lacking at the other sites. This evidences the presence of methanotrophic bacteria in the studied peats and the presence of PLFA known from *Methylocystis*-relatives is in line with phylogenetic proofs of this group of MOB.

In sample 7470, only very low methane oxidation was observed during the lab experiments compared to rates in samples 7468. In addition, the methane concentrations in the original surrounding soil gases were much lower in 7470 than in 7468 (on average 9 ppm CH_4 and 24% CH_4, respectively). PLFA data were lacking proof of MOB in 7470 from Steimbke (prior and after the experiment) as well as in other studied samples from another area in Lower Saxony (7732 and 7734).

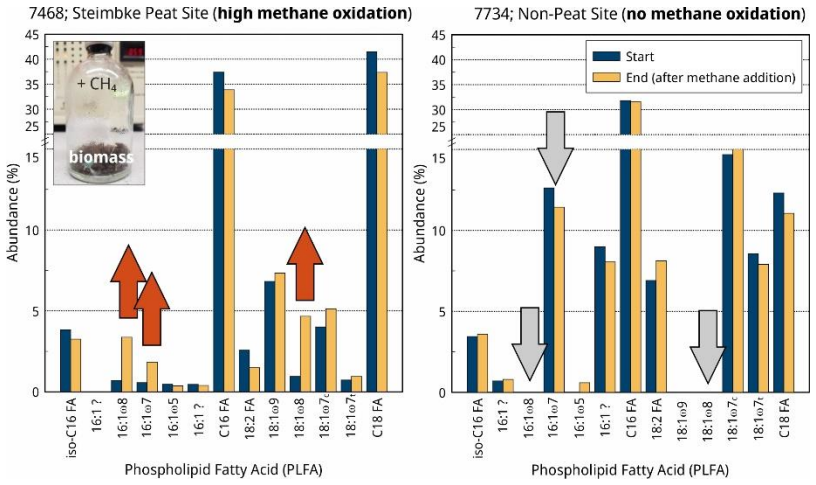

Figure S1: Relative changes of fatty acids of selected samples after KOH-hydrolysis, indicating changes in the microbial community (and/or heterogeneities in the samples). The samples originating from the peat site near Steimbke (7468) with high methane oxidation and the Nienhagen oil field (7734) where no methane oxidation activity was observed in the laboratory for comparison.

S2 Fractionation factor of aerobic methane oxidation in the laboratory

Materials & Methods

A peat soil sample (7644) taken in 2023 at Steimbke (depth: 10–25 cm) was incubated with about 3% methane in air. This sample was taken at the industrial peat site, where methane in the soil gas was highly concentrated. The sample, however, does not resemble that taken for the incubation experiments for PLFA analyses (7468). Three incubations were run in parallel and stopped after 24 h, 48 h, and 72 h (Table S2). Gases in the headspace of the vials were sampled and analyzed for gas composition and stable carbon isotopic signatures (for CH_4 and CO_2). Gas samples were analyzed with a refinery gas analyzer and carbon isotopes with a GC-IRMS. More details can be found in the main paper (sections 2.4).

Table S2: Experimental isotope ($\delta^{13}C$) and concentration data of gases in the headspace after addition of methane (sample 7644; Steimbke peat). f = fraction of remaining methane after addition.

ID	time [h]	N_2 [%]	CH_4 [ppm]	CO_2 [ppm]	O_2 [ppm]	CH_4 [$\delta^{13}C$]	CO_2 [$\delta^{13}C$]	*f*	*ε [‰]*
7644	0	75.7	32,729	414	195,500	–44.58	–28.44	1.00	
7644	24	77.7	23,717	7811	170,001	–31.08	–68.84	0.72	
7644	48	79.9	10,974	14,513	156,917	–8.01	–59.18	0.34	
7644	72	81.1	2967	19,006	147,561	31.41	–51.05	0.09	
Enrichment factor									**–31.3**

Results & Discussion

Gas composition changed during the experiment with methane strongly and oxygen slightly de- and CO_2 increasing, typical for an experiment where methane is aerobically oxidized. At the same time, $\delta^{13}C$ values of CH_4 increased strongly, whereas $\delta^{13}C$ values of CO_2 decreased.

Figure S2 depicts the isotopic composition of the remaining methane, instantaneous and accumulating carbon dioxide as function of the fraction of the remaining methane (solid lines) assuming an initial zero CO_2 concentration. According to the mass and isotope balances at the time of the first experimentally measured $\delta^{13}C$-CO_2 value (at $f = 0.72$) the large amount of accumulating CO_2 (28% of ~3% added methane oxidized) with a cumulative $\delta^{13}C$-value of –71‰ easily shifts the original soil-derived, low concentrated (~414 ppm) and isotopically slightly depleted CO_2 ($\delta^{13}C$-CO_2 –28.4‰) towards very negative values (red dashed line).

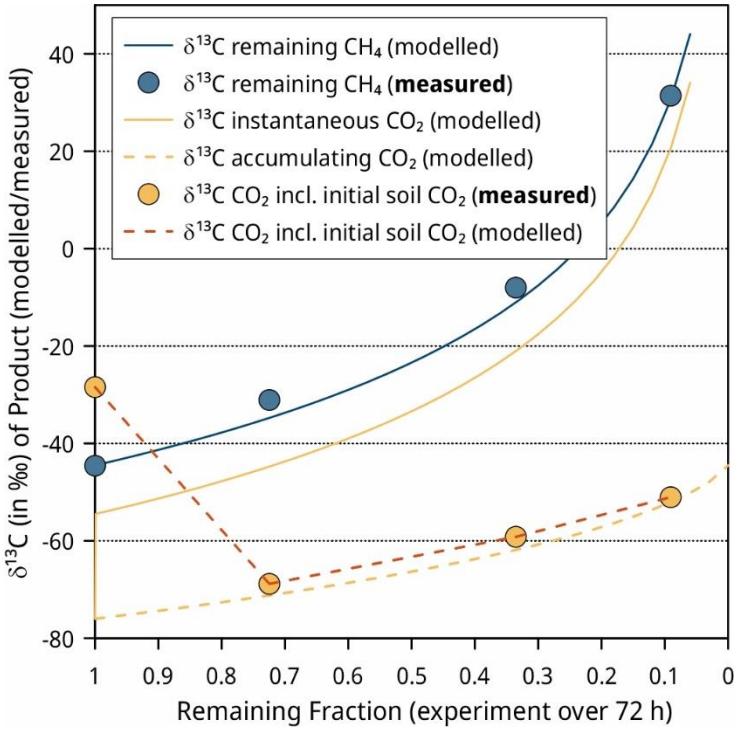

Figure S2: Closed System Rayleigh fractionation (modelled (lines) and data (dots) of the experiment) of the incubation with methane and air.

After Elsner et al. (2007) and Feisthauer et al. (2011) the slope of plotting $\ln(\frac{(\delta_t+1000)}{\delta_0+1000})$ *versus* $\ln(\frac{C_t}{C_0})$ gives the enrichment factor ε (in‰; see Table S2 and Figure S3).

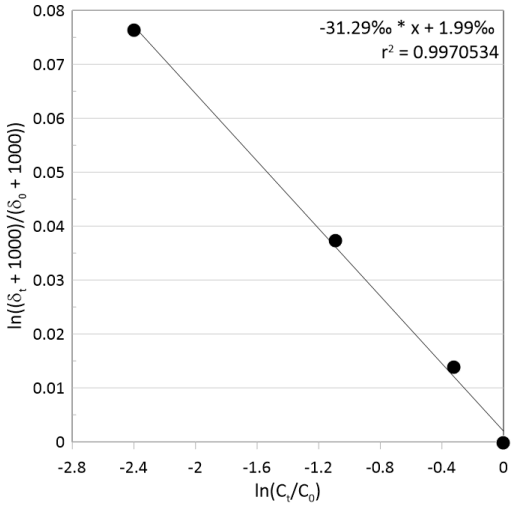

Figure S3: Methane concentration and $\delta^{13}C$ change during the lab experiment 7644 (Steimbke peat). The slope (in ‰) corresponds to the enrichment factor ε (after Elsner et al. (2007) and Feisthauer et al. (2011)).

The calculated enrichment factor ε is with –31.3‰ (Figure S3) in the upper known range of data from laboratory experiments and cultures for aerobic methanotrophs (–3 to –39‰; Templeton et al. (2006) and references therein). It has to be considered that respective data are still limited, but the so far reported data demonstrate that a differentiation between the alpha- and gamma-proteobacteria or between the carbon assimilation pathway (Type I: assimilation of formaldehyde via the ribulose-monophosphate pathway; Type II: serine pathway) cannot be achieved from isotopic enrichment factors (Feisthauer et al., 2011; Rasigraf et al., 2012). In a comparative study highest fractionation factors were with –27.9 and –27.7‰, however, reported for the Type I methanotrophs *Methylococcus capsulatus* and *Methylomonas methanica* (Feisthauer et al., 2011). Our data and our lab enrichment culture dominated by members of the genera *Methylocystis* and *Methylobacter* add, however, further data on this still limited data set. Obviously, the isotopic fractionation of methane during aerobic methanotrophy at Steimbke results in a relatively strong fractionation. Reasons for this observation are, however, unclear. Previous studies speculated on the growth rates and physiological factors, and/or substrate limitation. Low oxygen concentrations may, theoretically, slow down methane oxidation by that favoring fractionation (Rasigraf et al., 2012; Templeton et al., 2006).

S3 Methane flux data

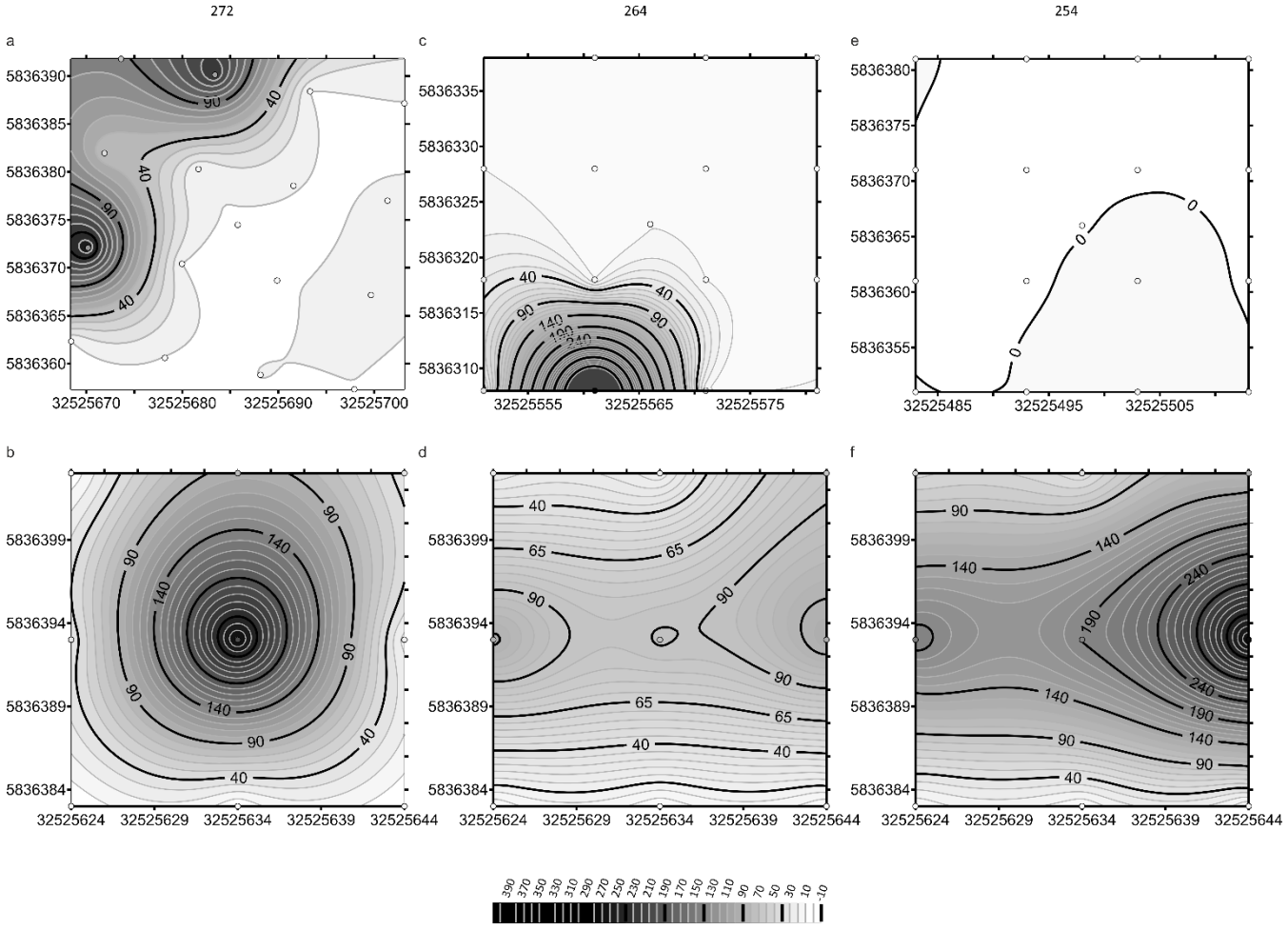

Figure S4: Gridding of methane fluxes from soil to the atmosphere at three well sites (a, c, e) and one reference site (b, d, f), which was measured at different time points in the active peat cutting area. The reference measurement is displayed below the corresponding well measurement. The measurements in b) was conducted one week prior to d), and the emissions in f were determined one day after d) (see Table 1). The grey scale is depicted on the bottom right.

7 References

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