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Assessment of microbial methane oxidation above a petroleum-contaminated aquifer using a combination of in situ techniques

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[1] Emissions of the greenhouse gas CH_4, which is often produced in contaminated aquifers, are reduced or eliminated by microbial CH_4 oxidation in the overlying vadose zone. The aim of this field study was to estimate kinetic parameters and isotope fractionation factors for CH_4 oxidation in situ in the vadose zone above a methanogenic aquifer in Studen, Switzerland, and to characterize the involved methanotrophic communities. To quantify kinetic parameters, several field tests, so-called gas push-pull tests (GPPTs), with CH_4 injection concentrations ranging from 17 to 80 mL L^{-1} were performed. An apparent V_{max} of 0.70 ± 0.15 mmol CH_4 (L soil air)$^{-1}$ h^{-1} and an apparent K_m of 0.28 ± 0.09 mmol CH_4 (L soil air)$^{-1}$ was estimated for CH_4 oxidation at 2.7 m depth, close to the groundwater table. At 1.1 m depth, K_m (0.13 ± 0.02 mmol CH_4 (L soil air)$^{-1}$) was in a similar range, but V_{max} (0.076 ± 0.006 mmol CH_4 (L soil air)$^{-1}$ h^{-1}) was an order of magnitude lower. At 2.7 m, apparent first-order rate constants determined from a CH_4 gas profile (1.9 h^{-1}) and from a single GPPT (2.0 ± 0.03 h^{-1}) were in good agreement. Above the groundwater table, a V_{max} much higher than the in situ CH_4 oxidation rate prior to GPPTs indicated a high buffer capacity for CH_4. At both depths, known methanotrophic species affiliated with *Methylosarcina* and *Methylocystis* were detected by cloning and sequencing. Apparent stable carbon isotope fractionation factors α for CH_4 oxidation determined during GPPTs ranged from 1.006 to 1.032. Variability was likely due to differences in methanotrophic activity and CH_4 availability leading to different degrees of mass transfer limitation. This complicates the use of stable isotopes as an independent quantification method.

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1. Introduction

[2] Methane (CH_4) is the second most important greenhouse gas after CO_2 [*Ehhalt et al.*, 2001]. In many anaerobic environments, such as wetlands, rice fields, landfills and ocean and lake sediments, CH_4 is microbially produced during the degradation of organic material [*Reeburgh*, 2003]. The amount of CH_4 that is emitted from these environments into the atmosphere is estimated to be reduced by 20 to 90% through the microbial processes of aerobic and anaerobic CH_4 oxidation [*Reeburgh*, 2003]. Therefore, methanotrophic zones in the subsurface form an important biofilter for CH_4, with a dominant role of aerobic CH_4 oxidation in terrestrial environments [*Hanson and Hanson*, 1996].

[3] Contaminated aquifers are a further potential CH_4 source as methanogenesis is an important contaminant degradation pathway [*Chapelle et al.*, 2002; *Cozzarelli et al.*, 2001]. Degradation under methanogenic conditions can lead to high CH_4 concentrations of up to 80% v/v in the deeper vadose zone above an aquifer [*Bhupathiraju et al.*, 2002; *Conrad et al.*, 1999; *Revesz et al.*, 1995]. To our knowledge, actual or potential CH_4 emissions from anaerobic, contaminated aquifers into the atmosphere have not been quantified and are usually not included in estimates of CH_4 budgets. Actual CH_4 emission rates above contaminated aquifers may often be low because of CH_4 oxidation in the vadose zone as indicated by transport modeling [*Molins and Mayer*, 2007] and low CH_4 concentrations observed near the soil surface [*Chaplin et al.*, 2002; *Conrad et al.*, 1999; *Revesz et al.*, 1995]. However, without CH_4 removal through CH_4 oxidation, anaerobic aquifers may be considerable CH_4 sources as aquifer contamination with

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hydrocarbons is a widespread problem [*Riser-Roberts*, 1992]. Furthermore, CH_4 could constitute an explosion hazard for buildings at contaminated sites [*Conrad et al.*, 1999]. Therefore, quantitative data on the activity and capacity, i.e., maximum activity V_{max}, of methanotrophs in this environment are needed to understand CH_4 turnover and predict effects of changing conditions on the fate of CH_4. For example, formation of bubbles leading to outgassing [*Revesz et al.*, 1995] or water table fluctuations [*Conrad et al.*, 1999] can release high and variable amounts of CH_4 into the vadose zone. This could lead to CH_4 emissions if the methanotrophic population cannot respond quickly to increased substrate availability.

[4] Laboratory incubation studies to quantify microbial processes allow the control of individual parameters, but potential biases like changes in temperature and in the physical structure of samples prevent the direct extrapolation of obtained data to the field scale [e.g., *Istok et al.*, 1997; *Madsen*, 1998]. Therefore, it is important to quantify rates and rate constants for microbial processes in situ [*Madsen*, 1998; *Scow and Hicks*, 2005]. A new approach for in situ quantification of microbial processes in the vadose zone, the gas push-pull test (GPPT), has been developed and applied to quantify CH_4 oxidation [*Urmann et al.*, 2005]. The GPPT consists of the injection of a gas mixture containing the reactants CH_4 and O_2 and the nonreactive tracers, neon (Ne) and argon (Ar) into the vadose zone. While the injected mixture migrates away from the injection point, the reactants are consumed by indigenous microorganisms. The gas mixture is subsequently pumped back, i.e., extracted together with soil air from the same location. First-order rate constants of CH_4 oxidation can be calculated from CH_4 and tracer concentration data using simplified methods that do not require knowledge of physical parameters of the system [*Schroth and Istok*, 2006; *Urmann et al.*, 2005]. Michaelis-Menten parameters V_{max} and K_m describing the capacity of the methanotrophic community and its affinity to CH_4 could be inferred from GPPT data by inverse modeling in homogeneous systems [*Gonzalez-Gil et al.*, 2007]. However, modeling the physical transport of tracer and reactant gases during GPPTs in heterogeneous systems is challenging. Therefore, a simplified approach to estimate Michaelis-Menten parameters is needed.

[5] For in situ quantification of microbial processes, it is advisable to use different methods to obtain a more complete picture of the investigated process [*Madsen*, 1991; *National Research Council*, 1993]. Further methods used to estimate in situ CH_4 oxidation include evaluation of CH_4 concentration profiles [*Fechner and Hemond*, 1992] and evaluation of CH_4 stable carbon isotope ratios [*Liptay et al.*, 1998]. The first method can be applied in soils with diffusive transport at steady state if the physical properties of the soil are reasonably well known [*Damgaard et al.*, 1998; *Fechner and Hemond*, 1992]. For the use of stable carbon isotope fractionation, it is critical to know the fractionation factor α for microbial CH_4 oxidation that applies under the prevalent conditions in the field [*Chanton and Liptay*, 2000]. A wide range of α from 1.003 to 1.039 (references given by *Snover and Quay* [2000] and *Templeton et al.* [2006]) has been determined for methanotrophs in the laboratory and in some field studies. Several factors have been proposed to influence the extent of fractionation, including temperature [*Chanton and Liptay*, 2000] and mass transfer limitation [*Templeton et al.*, 2006]. The latter depends on CH_4 availability and methanotrophic activity. To assess the applicability of isotope-based methods in the field, it is important to determine the dependency of the fractionation factor on different parameters in situ, which is difficult to achieve. Using GPPTs, the influence of CH_4 availability on fractionation may be assessed in the field as several tests can be performed at the same site with varying CH_4 injection concentrations.

[6] Quantification of CH_4 oxidation activity in combination with community analysis has shown that differences in CH_4 oxidation kinetics may correspond to differences in methanotrophic community structure. For example, in upland soils, novel clusters of methanotrophic bacteria were proposed to be responsible for methanotrophic activity with a high affinity for CH_4 and therefore a low-affinity constant [*Holmes et al.*, 1999; *Knief et al.*, 2003]. Although methanotrophic communities have been frequently studied in wetland or upland soils [e.g., *Jaatinen et al.*, 2005; *Knief et al.*, 2003], to our knowledge, diversity of methanotrophic bacteria in the vadose zone above contaminated aquifers has not been investigated.

[7] Thus, the aim of this study was to apply different in situ methods to quantify kinetic parameters for CH_4 oxidation and characterize the methanotrophic community structure above a petroleum hydrocarbon-contaminated aquifer in Studen, Switzerland. Specific objectives were (1) to estimate Michaelis-Menten and first-order kinetic parameters using GPPTs with different CH_4 injection concentrations and compare them with calculations from gas profiles, (2) to compare the methanotrophic community structure with obtained kinetic parameters and (3) to assess the feasibility of using stable carbon isotope fractionation for in situ quantification by investigating whether the fractionation factor α was constant during GPPTs with a range of CH_4 injection concentrations.

2. Materials and Methods

2.1. Field Site

[8] Microbial CH_4 oxidation was quantified in the vadose zone above a petroleum hydrocarbon-contaminated, anaerobic aquifer in Studen, Switzerland using GPPTs. In a previous study to assess the feasibility of the GPPT [*Urmann et al.*, 2005], tests were performed near monitoring well PS4, which is located in the center of the methanogenic zone of the aquifer [*Bolliger et al.*, 1999]. Specifically, experiments were conducted in the annular space between well PS4 and the surrounding 70-cm-diameter concrete casing, which was refilled in 1996 with calcareous coarse sand and gravel with an estimated porosity of 0.39. Prior to GPPTs, maximal CH_4 concentrations of 28 $\mu L\ L^{-1}$ were measured close to the groundwater table. From GPPTs, a higher apparent first-order rate constant was determined above the groundwater table (at 2.7 m depth) than closer to the soil surface (at 1.1 m depth).

[9] In this study, field tests to determine Michaelis-Menten parameters and stable carbon isotope fractionation factors were performed at the same location within a 3-week period in summer 2003. After the experiments, a core of the

Table 1. Operational Parameters for GPPTs

GPPT	Depth (m)[a]	Injection Concentrations[b] ($mL\ L^{-1}$) Ne	CH_4	O_2	Injection Volume (L)	Injection Pump Rate ($L\ min^{-1}$)	Extraction Volume (L)	Extraction Pump Rate ($L\ min^{-1}$)	Total Time (h)
A	1.1	17.9	17.1	217.4	34	0.68	90	0.55	3.6
B	1.1	83.3	79.8	209.5	33	0.66	90	0.55	3.6
C	2.7	19.4	17.7	216.4	32	0.62	78	0.50	3.5
D	2.7	81.3	79.6	213.0	32	0.62	87	0.53	3.6
E	2.7	18.9	17.7	61.1	32	0.59	78	0.51	3.5

[a]Refers to the depth of the tip of the injection rod measured from the soil surface.
[b]Injection mixtures additionally contained 200 $mL\ L^{-1}$ Ar and were prepared in N_2.

filling material was collected to a depth of 2.7 m. The water content was 0.12 and 0.06 ($cm^3\ cm^{-3}$ soil) at 1.1 m and 2.7 m depth, respectively (calculated from gravimetric water content and an estimated bulk density of 1.51 $g\ cm^{-3}$). The groundwater table in well PS4 increased from 3.01 m below surface to 2.95 m during the time of the experiments. The CH_4 concentration in the groundwater remained stable at 0.10 mM, while groundwater temperature increased from 16.4 to 17.2°C.

2.2. Gas Push-Pull Tests

[10] A total of five GPPTs were performed at 1.1 m (GPPT A and B) and 2.7 m (GPPT C, D and E) below soil surface (Table 1). The depth refers to the depth of the tip of the injection rod. The injection gas mixtures contained the reactants CH_4 and O_2, and the nonreactive tracer gases Ar and Ne. At each depth, a test with a low (GPPT A and C) and a high (GPPT B and D) CH_4 injection concentration was performed. At 2.7 m, an additional test (GPPT E) was conducted similar to GPPT C but with a low O_2 concentration (Table 1). GPPTs were performed as described earlier [*Urmann et al.*, 2005] with slight modifications. Briefly, a background sample of soil air was collected at the respective depth prior to each test. Between 32 and 34 L of gas mixture (Table 1) was injected with an average flow rate of 0.63 $L\ min^{-1}$ at the desired depth through an injection rod. Within 2 to 3 min from the end of injection, flow was reversed and between 78 and 90 L were extracted from the same location with an average flow rate of 0.53 $L\ min^{-1}$. For injection and extraction, a gas flow controller (GFC) was used. The core equipment of the GFC was a diaphragm pump and a mass flow meter. Note that units of L and mL of gas in this paper all refer to volumes normalized to 0°C. Deviating from previous procedures, injection and extraction samples were collected in clamped serum bottles with butyl rubber stoppers and all sample bottles were preevacuated (−0.6 bar). Using methods described previously, samples were analyzed for CH_4 and CO_2 [*Urmann et al.*, 2005] and for Ne, Ar and O_2 [*Gonzalez-Gil et al.*, 2007]. Carbon dioxide data are not shown as little information was gained from these data similar to a previous study [*Urmann et al.*, 2005].

2.3. Estimation of Kinetic Parameters

[11] Relative concentrations (C^*) were calculated from injection concentrations (Table 1) and extraction concentrations, corrected for their background concentrations measured in soil air (CH_4 0.8–117 $\mu L\ L^{-1}$, Ar 10–11 $mL\ L^{-1}$, Ne below detection) [*Urmann et al.*, 2005]. Averages of the last extraction samples were used for background corrections if they were lower than background concentrations prior to experiments. From here on corrected values will be referred to as Ar and CH_4, respectively. Oxygen data were not corrected for background concentrations as no further data analysis was performed.

[12] A simplified method was used to evaluate GPPTs [*Schroth and Istok*, 2006]. This method allows accounting for reaction during injection even when only parts, i.e., segments of a GPPT, are evaluated. It is based on the assumption that no mixing occurs between "parcels" of gas injected at different times of the injection phase. Accordingly, a residence time t_R was calculated for each "parcel" of gas, which is the time from its injection until its extraction:

$$t_R = t^* + \frac{\int_{t_{ext}=0}^{t_{ext}} Q_{ext} C_{Ne}(t)dt}{M_{Ne}} T_{inj} \quad (1)$$

where t^* is time since end of injection, Q_{ext} is the extraction pump rate, t_{ext} is time since extraction began, M_{Ne} is the total mass of the tracer Ne injected and T_{inj} is the injection time. Subsequently, the natural logarithm of the ratio of relative reactant and tracer concentration was plotted against residence time (equation (2)). In case of a linear relationship, the slope k gives a first-order rate constant. Note that the nonreactive tracer Ne thereby accounts for dilution of the injected gas with soil air.

$$\ln\left(\frac{C^*_{CH_4}}{C^*_{Ne}}\right) = -kt_R \quad (2)$$

To estimate Michaelis-Menten parameters, data plotted according to equation (2) were evaluated in segments. Extraction samples in which the influence of the CH_4 background concentration became too high were excluded from the analysis. For each test, two to three segments were defined that appeared linear over 10 to 44 L of extraction. The slope for each segment and its standard error was determined by linear regression. To estimate CH_4 oxidation rates for each segment, slopes were multiplied with the CH_4 concentration averaged over the segment. Parameters K_m and V_{max} were estimated from obtained rates and average concentrations by linear regression using Eadie-Hofstee plots [*Bender and Conrad*, 1993]. Apparent first-order rate constants were obtained (1) directly from the slope of the tests with the lowest concentrations and (2) from the ratio of V_{max} over K_m. Standard errors for K_m, V_{max} and k were derived from standard errors of the corresponding slope and intercept.

2.4. Gas Profiles

[13] Prior to the first GPPT, CH_4, O_2 and CO_2 concentration profiles in soil air were measured as described earlier [*Urmann et al.*, 2005]. Briefly, a sampling rod was gradually pushed down to a maximum depth of 2.95 m below soil surface and the GFC was used to extract gas samples. To obtain a higher resolution than previously, only 1.5 L of gas was extracted, sampling bottles were preevacuated (−0.6 bar) and, close to the groundwater table, samples were taken every 15 cm.

[14] To estimate an apparent first-order rate constant from measured CH_4 profiles, an analytical solution for a simple steady state diffusion-consumption model [*Reeburgh et al.*, 1997] was fitted to the data using the CH_4 concentration above the groundwater table as boundary concentration. As an approximation, CH_4 concentration was assumed to approach zero toward the soil surface. The effective diffusion constant for CH_4 was calculated from porosity, water content and diffusivity in air (0.215 $cm^2\ s^{-1}$) at 15°C [*Massman*, 1998] using the Millington and Quirk equation [*Millington and Quirk*, 1961].

[15] The in situ CH_4 oxidation rate prior to GPPTs was calculated by multiplying the median of CH_4 concentrations between 2.45 and 2.95 m with the apparent first-order rate constant estimated from GPPT E and from the profile. Estimated porosity and measured water content at 2.7 m were used to transfer the CH_4 oxidation rate into total oxidation per area.

2.5. Molecular Analysis

[16] After the last GPPT, soil samples were collected from the core at 1.1 m and 2.7 m depth and immediately used for total cell counts. Subsamples for DNA extraction were stored at −80°C in DNA extraction buffer [*Kleikemper et al.*, 2002] at pH 9.5.

2.5.1. Total Cell Counts

[17] Total cell numbers were estimated by 4′,6′-diamidino-2-phenylindole (DAPI) staining. For this purpose, a protocol originally developed for fluorescence in situ hybridization (FISH) [*Zarda et al.*, 1997] was used with the following modifications: (1) 2 g of each sample were fixed in triplicate at 4°C for 16 h in 1 mL of 4% (wt vol^{-1}) paraformaldehyde in phosphate-buffered saline. (2) Samples were vortexed for 1 min, diluted 1:10 (soil sample derived from 1.1 m) or 1:20 (soil sample derived from 2.7 m) with 0.1% (wt vol^{-1}) sodium pyrophosphate and dispersed for 10 min in a sonication bath before spotting onto ethanol-washed slides. (3) Samples were incubated at 46°C for 2.5 h with 1 μL DAPI (500 ng μL^{-1}) in 9 μL hybridization buffer with 2% (wt vol^{-1}) blocking reagent (Roche, Germany) [*Sekar et al.*, 2003]. Six wells and 40 fields per well were counted for the sample derived at 1.1 m. For the sample derived at 2.7 m, six wells and 20 fields per well were sufficient because of higher cell numbers [*Maruyama and Sunamura*, 2000]. Using this modified protocol led to both the reduction of background fluorescence and increase of cell brightness. With the same protocol, quantification of methanotrophs by applying methanotroph-specific FISH probes [*Eller et al.*, 2001] was not successful. Possibly, the available probes did not target the methanotrophs present in the samples or methanotrophic cell numbers were too low.

2.5.2. DNA Extraction, PCR and DGGE

[18] Total DNA was extracted from samples from both depths (1.1 m and 2.7 m) in triplicate according to *Kleikemper et al.* [2002] with the following minor modifications: Disruption of the microbial cells was performed by bead beating for 30 s at 5.5 ms^{-1} instead of 15 s at 4.5 ms^{-1} to increase efficiency, and supernatant was transferred to a new tube after digestion with lysozyme and proteinase K. After extraction, total community DNA was resuspended in 30 μL TE buffer and quantified photometrically using the PicoGreen method as described by *Burgmann et al.* [2001].

[19] A fragment of the functional gene particulate methane monooxygenase (*pmoA*) was amplified by polymerase chain reaction (PCR) from extracted DNA using the primer pair A189f and mb661 [*Costello and Lidstrom*, 1999]. A GC clamp [*Dunfield et al.*, 1999] was attached to A189f for fingerprinting of the methanotrophic community by denaturing gradient gel electrophoresis (DGGE).

[20] The PCR reaction mix (50 μL) was prepared and PCR conditions were employed as given by *Henckel et al.* [1999] for *pmoA* primers with minor modifications: the reaction mix contained 1 μL (2.7 m) or 1–4 μL (1.1 m) of template DNA and annealing temperatures in the touchdown program were decreased from 62°C to 56°C.

[21] DGGE was performed in a denaturing gradient of 30 to 65% at 200V for 5h as described previously [*Muyzer et al.*, 1993]. Gels were stained for 30 min with GelStar® (Cambrex Bio Science, Baltimore, US) and photographed using the GelDoc 2000 system and Quantity One software (Bio-Rad Laboratories, Hercules, Calif.).

2.5.3. Cloning and Sequencing

[22] PCR products obtained as described above without GC clamp were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to manufacturer's instructions. Clones were sequenced commercially (GATC Biotech AG, Konstanz, Germany). Cloned *pmoA* sequences were analyzed for secondary anomalies and submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) to detect the presence of possible chimeric artifacts. Nonchimeric *pmoA* sequences were integrated into a self-created *pmoA*-database, which was constructed from all available public *pmoA* sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov, last updated October 2006). For phylogenetic reconstructions, sequences were translated into their corresponding amino acid sequences and aligned with the alignment tools of the ARB software package (http://www.arb-home.de). Similarities of cloned amino acid sequences were calculated to the closest affiliation by the similarity matrix function using the neighbor-joining method (Kimura 2-parameter model) with a deduced 112 amino acid position consensus filter [*Kimura*, 1980]. The nucleotide sequences data obtained in this study have

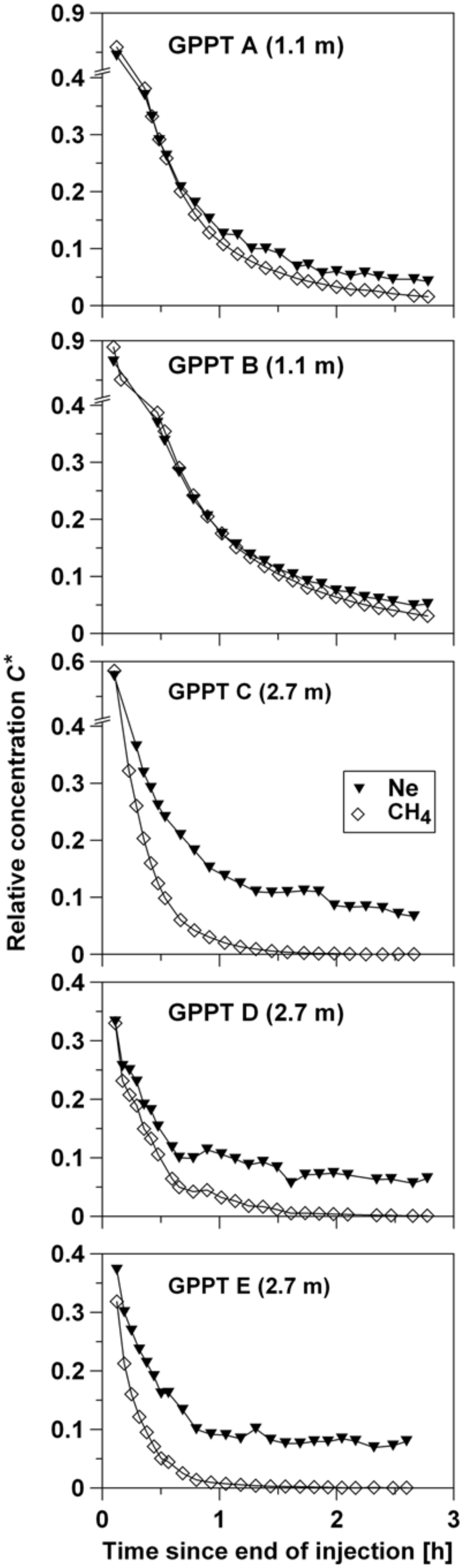

Figure 1. Methane and Ne breakthrough curves for GPPTs with low (GPPT A, GPPT C, and GPPT E) and high (GPPT B and GPPT D) CH_4 injection concentrations. Note the discontinuous *y* axis for GPPT A, GPPT B, and GPPT C.

been deposited in the EMBL nucleotide sequence database under the accession nos. AM410172 to AM410182.

2.6. Stable Carbon Isotope Fractionation Factor

[23] From all GPPTs except GPPT C and from two GPPTs performed previously (GPPT1 (2.7 m) and GPPT2 (1.1 m)) [*Urmann et al.*, 2005] the apparent stable carbon isotope fractionation factor α was quantified. The $\delta^{13}C$ value was measured in 5 to 8 extraction samples of each test, 5 injection samples and the two lowest samples of the profile to obtain a background value using a GC-IRMS as described previously [*Urmann et al.*, 2005]. Apparent stable carbon isotope fractionation factors α were determined by using the following simplified Rayleigh equation [*Mahieu et al.*, 2006; *Snover and Quay*, 2000]:

$$\ln\left(1000 + \delta^{13}C_{CH_4}\right) = \left(\frac{1}{\alpha_{ox}} - 1\right)\ln f + \ln\left(1000 + \delta^{13}C_{CH_4}(t_0)\right) \tag{3}$$

where f is the fraction of CH_4 remaining, corrected for dilution using the Ne breakthrough curves and α_{ox} is the fractionation factor defined for first-order kinetics as:

$$\alpha_{ox} = \frac{^{12}k}{^{13}k} \tag{4}$$

with ^{12}k and ^{13}k being the first-order rate constant for $^{12}CH_4$ and $^{13}CH_4$ respectively. For Michaelis-Menten kinetics α_{ox} is defined as [*Hunkeler and Aravena*, 2000]:

$$\alpha_{ox} = \frac{^{12}V_{\max}/^{12}K_m}{^{13}V_{\max}/^{13}K_m} \tag{5}$$

with $^{12}V_{\max}/^{12}K_m$ and $^{13}V_{\max}/^{13}K_m$ being the ratio of Michaelis-Menten parameters for $^{12}CH_4$ and $^{13}CH_4$ respectively. The isotopic signature in the background (measured in profile samples) was accounted for by using Ne as a measure for mixing of injection gas and soil air [*Kleikemper et al.*, 2002]. Fractionation factors and their standard errors were calculated by linear regression.

3. Results

3.1. Methane Oxidation Activity

[25] To obtain breakthrough curves of CH_4 and Ne, relative concentrations C^* were plotted against time since end of injection (Figure 1). In all GPPTs, extraction breakthrough curves with lower relative CH_4 concentrations than relative Ne concentrations indicated microbial consumption of CH_4. Consumption was apparent from 40 min to 1 h after the end of injection at 1.1 m depth. Conversely, at 2.7 m depth, CH_4 consumption was apparent from the beginning of extraction. Relative CH_4 consumption, indicated by the

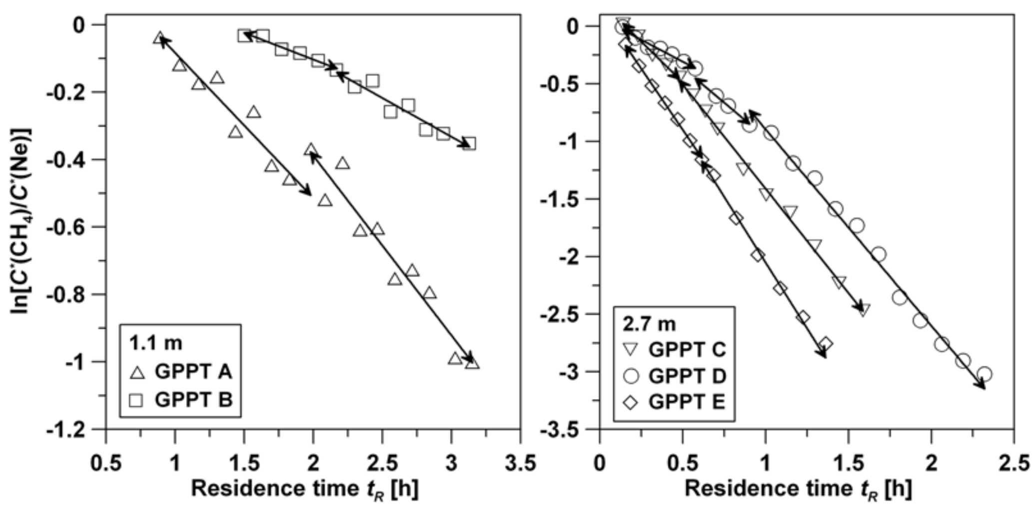

Figure 2. Plots for rate calculations from GPPTs at 1.1 m and 2.7 m depth. Only data included in the analysis are shown. Segments indicated by double arrows were used to estimate Michaelis-Menten parameters.

gap between the CH_4 and Ne breakthrough curves, was higher in the tests with the lower injection concentrations (GPPT A, C and E) and was higher at 2.7 m depth than at 1.1 m depth (Figure 1).

[26] Neon has previously been identified as a suitable nonreactive tracer for CH_4 under the applied test conditions at this site, as both gases were transported similarly during a GPPT with the coinjection of acetylene (C_2H_2) to inhibit CH_4 oxidation [*Urmann et al.*, 2005]. Similar transport behavior of gases with different diffusion coefficients was confirmed in this study as breakthrough curves of Ar (data not shown) were similar to Ne curves in all tests. Oxygen concentrations in all tests were always more than twice as high as CH_4 concentrations (data not shown).

[27] To estimate apparent first-order rate constants and Michaelis-Menten parameters for CH_4 oxidation, the natural logarithm of the ratio of relative CH_4 and Ne concentrations was plotted against residence time (Figure 2). In GPPT A, B and D curvatures in the graphs indicated Michaelis-Menten kinetics. Conversely, a linear relationship indicated a first-order type reaction in GPPT C and E (Figure 2). Only at the very beginning of extraction of GPPT C a curvature of the graph was observed.

[28] The dependency of subsequently computed CH_4 oxidation rates on CH_4 concentrations was well described by the Michaelis-Menten equation (Figure 3). Apparent K_m values for both depths agreed within a factor of two while the apparent $V_{\max}$ was an order of magnitude higher at 2.7 m than at 1.1 m depth (Table 2). The apparent first-order rate constant k was a factor of 4 higher at 2.7 m than at 1.1 m depth (Table 2). The k values calculated from the later segment of GPPT A and from GPPT E (Table 2) agreed well with k values computed from K_m and $V_{\max}$ at 1.1 m (0.60 ± 0.14 h^{-1}) and at 2.7 m depth (2.5 ± 1.28 h^{-1}). Errors associated with the latter values were much higher because of the propagation of errors from K_m and $V_{\max}$. Standard errors for parameters estimated at 2.7 m were higher than at 1.1 m (Table 2) because of more scatter (Figure 3). At 1.1 m, background concentrations were too high to observe O_2 consumption while at 2.7 m O_2 consumption was apparent in all tests, but not quantified.

[29] The CH_4, O_2 and CO_2 gas profiles were similar to previous observations at this site, indicating similar activity (Figure 4, CO_2 not shown). The curvature in the CH_4 profile indicated that CH_4 was oxidized mainly between 2.45 and 2.95 m depth (Figure 4). An apparent first-order rate constant computed from the profile (1.9 h^{-1}) agreed well with the results from GPPTs (Table 2). From apparent first-order rate constants and in situ CH_4 concentrations we estimated that an average of 1.1 μmol CH_4 (L soil air)$^{-1}$ h^{-1} was oxidized in the 0.5 m deep active zone. This translates into a total oxidation per area of 0.2 mmol CH_4 m^{-2} h^{-1}.

3.2. Methanotrophic Community

[30] At 2.7 m depth, the number of DAPI-stained cells per g dry weight ($3.4 \times 10^7 \pm 1.6 \times 10^6$, ± one standard error for 6 wells) were 115 fold higher than at 1.1 m depth (3.0 ×

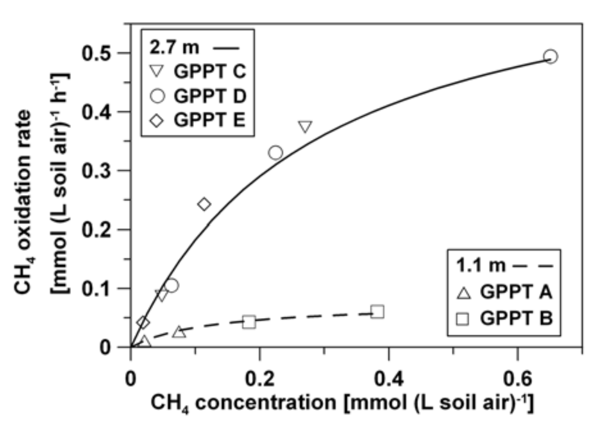

Figure 3. Methane oxidation rates computed from several GPPTs plotted against CH_4 concentrations and Michaelis-Menten curves derived from subsequently estimated K_m and $V_{\max}$ values.

Table 2. Apparent Michaelis-Menten Parameters (K_m, V_{max}) and First-Order Rate Constants (k) for CH_4 Oxidation at Two Different Depths in the Vadose Zone Above the Contaminated Aquifer

Depth	Data Set Used	$V_{max} \pm \sigma$[a]	$K_m \pm \sigma$	$k \pm \sigma$
		(mmol (L soil air)$^{-1}$ h^{-1})	(mmol (L soil air)$^{-1}$ h^{-1})	(h^{-1})
1.1 m	GPPT A, B	0.076 ± 0.006	0.13 ± 0.02	
	GPPT A[b]			0.53 ± 0.05
2.7 m	GPPT C, D, E	0.70 ± 0.15	0.28 ± 0.09	
	GPPT E			2.0 ± 0.03
	Profile[c]			1.9

[a]Errors are one standard error.
[b]Data from the later part of extraction used.
[c]Obtained by fitting an analytical solution to measured profile data.

$10^5 \pm 4.0 \times 10^4$). Total amount of cells counted at 1.1 m were low, but variation between wells was reasonably small. Furthermore, similar differences were observed for DNA extract concentrations at the two depths. Several attempts to clone and sequence amplified *pmoA* fragments yielded nine sequences at 1.1 m and two sequences at 2.7 m. Eight sequences clustered with type I genera *Methylobacter* and *Methylosarcina* and three with the type II genera *Methylocystis* (Table 3). On the basis of the retrieved sequences, there were no discernible differences between the clone libraries at both depths. Obtaining sufficient PCR product for DGGE analysis was difficult because of low DNA content in 1.1 m samples. However, even if weak, PCR products from 1.1 m and 2.7 m depth showed 5–6 bands with similar patterns (data not shown).

3.3. Stable Carbon Isotope Fractionation Factor

[31] Apparent fractionation factors were calculated from all GPPTs in this study except GPPT C. Furthermore, GPPT1 and 2 from a previous study were included in the analysis [*Urmann et al.*, 2005]. Methane became enriched in δ^{13}C by 15 to 43‰ during 1.2 to 2.5 h of extraction of all GPPTs. Apparent fractionation factors calculated from GPPTs using the Rayleigh distillation approach ranged widely from 1.006 to 1.032 (Figure 5). Fractionation was constant throughout each test, apart from GPPT B, where no fractionation occurred during the first 37 min of extraction (data not shown). Comparing individual GPPTs, the fractionation factor decreased with increasing fraction of CH_4 oxidized (Figure 5). The latter was determined for each test after extraction of 78 L as a reference point. The lowest α value of 1.006, that is the least fractionation, was found in GPPT E, the test with the lowest CH_4 extraction concentrations, i.e., lowest CH_4 availability, high activity and the highest fraction of CH_4 oxidized. In contrast, the highest apparent α of 1.032 was found in GPPT B, the test with the highest CH_4 extraction concentrations, i.e., highest CH_4 availability, low activity and the lowest fraction of CH_4 oxidized. The apparent α values of the remaining tests (GPPT 1, 2, A and D) were within a much narrower range of 1.013–1.019.

4. Discussion

4.1. Methane Oxidation Activity and Kinetic Parameters

[32] In this study we investigated the activity and capacity of the methanotrophic community above a contaminated aquifer in situ to assess the efficiency of the methanotrophic zone as a biofilter for CH_4. For this purpose, we presented a novel method to estimate Michaelis-Menten kinetic parameters from a series of GPPTs with different CH_4 injection concentrations, which could be applied at other sites and in other ecosystems in future studies. Michaelis-Menten parameters have been previously estimated from push-pull tests for enzyme activity in groundwater, however using an approach that is not transferable to the quantification of CH_4 oxidation [*Istok et al.*, 2001]. In the method presented here, the uncertainty associated with Michaelis-Menten parameters could likely be reduced by increasing the number of GPPTs and therefore the number of data points defining the Michaelis-Menten curve. All kinetic parameters were determined under the presence of sufficient O_2 as indicated by O_2:CH_4 ratios higher than 2. Good agreement between apparent first-order rate constants obtained at 2.7 m using two independent methods, confirmed that parameter estimates were reasonable.

[33] The apparent K_m values obtained from GPPTs translate into water-phase concentrations of 4.4 μM at 1.1 m and 12.9 μM at 2.7 m assuming equilibrium between gas and water phase. K_m values in the μM range indicated low-affinity CH_4 oxidation at both depths [*Bender and Conrad*, 1992] and were within a wide range of K_m values of 0.3–

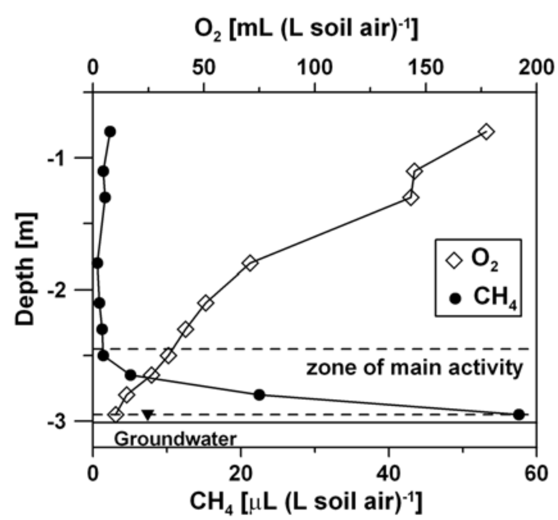

Figure 4. Methane and O_2 gas profiles above the contaminated aquifer.

Table 3. Phylogenetic Affiliation of Cloned *pmoA*-Based Sequences Retrieved From Samples at 1.1 m and 2.7 m

Depth	Number of Clones	Next Relative[a]	Taxonomic Assignment[b]	Similarity[c]
1.1 m	5	Freshwater sediment clone [DQ067081]	*Methylosarcina* (I)	0.88–0.92
	2	*Methylobacter sp.* LW14 [AY007286]	*Methylobacter* (I)	0.95
	2	soil clone [AJ868259]	*Methylocystis* (II)	0.80–0.82
2.7 m	1	Freshwater sediment clone [DQ067081]	*Methylosarcina* (I)	0.90
	1	*Methylocystis sp.* 494 [AJ459012]	*Methylocystis* (II)	0.98

[a]Accession numbers of the next relative are given in brackets.
[b]Methanotrophic type classifications of the genera are given in parentheses.
[c]Similarity values indicate levels of homology to sequences in the database; values of 0.9 and greater indicate a close match to sequences in the database.

58 μM reported for landfill cover soils [*Gebert et al.*, 2003, and references therein; *Kightley et al.*, 1995] and peat soils [*Roslev and King*, 1996; *Watson et al.*, 1997]. Apparent K_m values in the water phase represent maximum values as equilibrium concentrations may not have been fully reached in soil water during GPPTs. Partitioning of gases between water and gas phase during GPPTs is currently under investigation. Although CH_4 background concentrations at 1.1 m were similar to ambient concentrations, the estimated apparent K_m value was much higher than K_m values for CH_4 oxidation at ambient CH_4 concentrations usually reported in the nM range [e.g., *Bender and Conrad*, 1992; *Gulledge et al.*, 2004]. The determined K_m is likely characteristic for CH_4 oxidation in immediate response to increased CH_4 concentrations. Similar to our study, an immediate response of CH_4 oxidation to increased CH_4 concentrations was observed in an upland forest soil [*Mohanty et al.*, 2006].

[34] In agreement with elevated CH_4 background concentrations above the groundwater table, a higher V_{max} or higher capacity was found at 2.7 m than at 1.1 m. The apparent V_{max} at 2.7 m, corresponding to 0.17 μmol (g dry soil)$^{-1}$ h^{-1}, was at the lower end of a range of V_{max} from 0.04 to 2.6 μmol (g dry soil)$^{-1}$ h^{-1} measured in landfill cover soils [*Gebert et al.*, 2003, and references therein]. The V_{max} at 1.1 m corresponding to 0.013 μmol (g dry soil)$^{-1}$ h^{-1} was lower than this range, but was within a range of CH_4 oxidation rates of 0.007–0.036 μmol (g dry soil)$^{-1}$ h^{-1} determined in a forest soil incubated under 1000 or 10000 ppmv CH_4 [*Mohanty et al.*, 2006] and slightly higher than the range of V_{max} of 0.001–0.009 μmol (g dry soil)$^{-1}$ h^{-1} determined in batch incubations of a boreal upland soil under ambient CH_4 concentrations [*Whalen and Reeburgh*, 1996].

[35] On the basis of activity per area, in situ CH_4 oxidation in the zone above the groundwater table prior to GPPTs was slightly lower than the range of CH_4 oxidation rates from 0.64–1.56 mmol CH_4 m^{-2} h^{-1} calculated from CH_4 fluxes above a crude oil–contaminated aquifer [*Chaplin et al.*, 2002], similar to CH_4 oxidation observed in peat bogs [*Pearce and Clymo*, 2001; *Whalen and Reeburgh*, 2000] but 3 orders of magnitude lower than in landfill cover soils [*De Visscher et al.*, 1999; *Kightley et al.*, 1995].

[36] Comparing the in situ rate prior to GPPTs with V_{max}, we estimated that the CH_4 oxidation rate of the methanotrophic community in the zone above the groundwater table could be ~600 times higher than the in situ oxidation rate at the time of our study. The estimated V_{max} was probably characteristic for the in situ population and not influenced by growth during the GPPTs as test times were less than 4 h. Doubling times reported for methanotrophs in culture are 4–30 h (references given by *De Visscher and Van Cleemput* [2003]) and in a forest soil incubated under elevated CH_4 concentrations doubling times of 20–60 d were reported [*Mohanty et al.*, 2006]. Therefore, the in situ population could react rapidly to increased CH_4 availability and prevent CH_4 from migrating further up into the vadose zone and possibly being emitted to the atmosphere without the necessity of adaptation by growth. Fast response to increased CH_4 availability is common, for example, in methanotrophic ecosystems where substrate availability shifts with depth according to annual [*Carini et al.*, 2005] or diurnal cycles [*Damgaard et al.*, 1998]. The lower CH_4 oxidation capacity detected in the upper part of the vadose zone is in accordance with little exposure of this zone to elevated CH_4 concentrations.

4.2. Methanotrophic Communities

[37] Despite few clones, sequencing results suggested similar methanotrophic communities at 1.1 m and 2.7 m depth. This was supported by similar DGGE patterns. Communities consisting of clones affiliated to known methanotrophic species were in agreement with the observed high apparent affinity constants that are typical for cultured methanotrophs [*Conrad*, 1996]. On the basis of our results, the methanotrophic communities above the contaminated aquifer appear to be similar to those in other ecosystems.

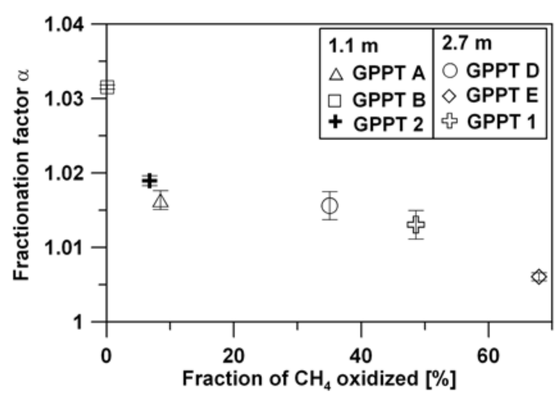

Figure 5. Fractionation factors α for CH_4 oxidation, calculated at 1.1 m and 2.7 m from four GPPTs from this study (GPPT A, GPPT B, GPPT D, and GPPT E) and two GPPTs (1, 2) from a previous study [*Urmann et al.*, 2005]. Fractionation factors are plotted against the fraction of the injected CH_4 that was oxidized after 78 L of extraction. Error bars represent one standard error.

For example, *Methylobacter* and *Methylosarcina*, the two genera most frequently detected, were among the genera enriched in an agricultural soil under elevated CH_4 concentrations [*Seghers et al.*, 2003]. Furthermore, methanotrophs of the *Methylobacter*/*Methylosarcina* group were most abundant in a wet meadow soil with slightly elevated CH_4 concentrations [*Kolb et al.*, 2005]. Although the community structure appeared to be similar at both depths, the higher V_{max} at 2.7 m depth may suggest a higher methanotrophic population density at that location. While methanotrophic biomass was not quantified specifically, a far greater total biomass at 2.7 m than at 1.1 m is in agreement with this suggestion.

4.3. Isotope Fractionation Factors

[38] When calculating apparent isotope fractionation factors from GPPTs, fractionation due to diffusion was not taken into account as previous experiments showed that its influence was small in our experiments. Changes in stable isotope signatures during a GPPT in which CH_4 oxidation was inhibited were small in comparison to isotope enrichment during active tests at our field site [*Urmann et al.*, 2005]. Little influence of diffusional fractionation was furthermore supported by an inverse modeling approach used to estimate the fractionation factor associated with CH_4 oxidation during a GPPT of a previous study. In this approach, fractionation due to diffusion was accounted for, yet the obtained value was in very good agreement with the value estimated for the same test using the simplified approach employed here [*Gonzalez-Gil et al.*, 2008]. The reason for the small effect of diffusional fractionation was likely that GPPTs were performed within a 70-cm-diameter concrete casing, which partially confined gas transport [*Urmann et al.*, 2005].

[39] The range of apparent fractionation factors calculated from GPPTs spanned a large fraction of the wide range from 1.003 to 1.039 measured in previous studies (references given by *Snover and Quay* [2000] and *Templeton et al.* [2006]). We found an inverse relationship between the apparent fractionation factor and the fraction of CH_4 oxidized. A similar relationship between fractionation factor and oxidized fraction was found in a chemostat study [*Templeton et al.*, 2006]. In accordance with this study, we may attribute the observation that the fractionation factor was lowest in the field test where the highest fraction of CH_4 was consumed to limitation of CH_4 oxidation by mass transfer of CH_4 across the air-water interface. Fractionation due to the latter process is low [*Knox et al.*, 1992]. Conversely, the highest fractionation factor was observed during a GPPT where CH_4 oxidation was probably not mass transfer limited because of high CH_4 availability, low activity and a small fraction of CH_4 oxidized. Therefore, this high factor may be attributed to enzymatic fractionation [*Templeton et al.*, 2006]. The finding that fractionation factors were different in GPPTs at the same depth suggests that the CH_4 availability and the resulting fraction of CH_4 oxidized highly influenced the degree of mass transfer limitation and therefore the fractionation factor. Differences between the two depths were likely due to the fact that for the same CH_4 injection concentrations, a lower fraction of CH_4 was oxidized at 1.1 m because of lower activity.

5. Conclusions and Implications

[40] We studied microbial CH_4 oxidation above a methanogenic aquifer in Studen, Switzerland using a combination of gas push-pull tests (GPPTs), gas profile calculations, stable isotope measurements and molecular methods to assess the characteristics of the vadose zone as a biofilter to oxidize CH_4. We presented a novel method to estimate Michaelis-Menten parameters from GPPTs covering a range of CH_4 injection concentrations, which could be applied in future studies in different ecosystems.

[41] Estimated K_m values indicating "low-affinity" CH_4 oxidation coincided with the detection of clones affiliated to cultured methanotrophic species known to perform low-affinity oxidation. The methanotrophic community above the contaminated aquifer was therefore similar to other ecosystems with elevated CH_4 concentrations. Comparison of an estimate of the maximum CH_4 oxidation capacity above the groundwater table and in situ turnover at the time of this study indicated that CH_4 oxidation rates could be up to $\sim$600 times higher than in situ oxidation prior to GPPTs. Therefore, much higher CH_4 fluxes from the groundwater could be intercepted in the methanotrophic zone above the groundwater table without the need for growth of the microbial community. Consequently, higher CH_4 emissions into the atmosphere or the upper parts of the vadose zone following increased CH_4 release from the groundwater are not expected at this site, unless large water table fluctuations occur or the methanotrophic zone is bypassed physically, e.g., because of strong advective fluxes or preferential flow paths.

[42] Stable carbon isotope fractionation factors determined in situ using GPPTs varied greatly. Variability was likely depending on different degrees of mass transfer limitation caused by different CH_4 availability and methanotrophic activity. Precise knowledge of the fractionation factor is a prerequisite for the quantification of CH_4 oxidation from stable isotope ratios. Therefore, high variability of the fractionation factor, observed even at a single location, complicates the use of this approach as an independent quantification method and careful verification of its applicability is needed in each individual case.

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