Doctoral Thesis

Field techniques for the quantification of microbial methane oxidation in the subsurface

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Publication Date:
2006

Permanent Link:
https://doi.org/10.3929/ethz-a-005318787

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Field Techniques for the Quantification of Microbial Methane Oxidation in the Subsurface

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of

Doctor of Natural Sciences

presented by

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Dr. Rolf Siegwolf, co-examiner

Zurich 2006
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Summary

Methane (CH₄) is the second most important greenhouse gas after carbon dioxide (CO₂). To understand the factors that influence CH₄ emissions, it is important to quantify microbial processes involved in the turnover of CH₄ in the subsurface. Microbial CH₄ oxidation under aerobic conditions is a key process decreasing CH₄ emissions from different environments such as wetlands, peat bogs and contaminated aquifers. Quantification of microbial processes in situ is important to overcome biases inherent in laboratory studies and therefore to obtain rates or rate constants that may be more relevant under field conditions.

The objective of this thesis was to develop a new in-situ method, the gas push-pull test (GPPT), for the quantification of microbial CH₄ oxidation in the vadose zone. This new method was combined with concentration profile and stable carbon isotope analysis and molecular tools. It was applied in the vadose zone above a contaminated aquifer in Studen, Switzerland and in an alpine peat bog in Eigenthal, Switzerland. To complement the field studies, a laboratory model system was designed to study CH₄ oxidation in a CH₄/O₂ counter-gradient under controlled conditions.

In a field-scale feasibility study, the GPPT was successfully applied to quantify microbial CH₄ oxidation in the vadose zone above the contaminated aquifer. The GPPT consists of the injection of a gas mixture of reactants (e.g. CH₄, O₂) and non-reactive tracer gases (e.g. Ne, Ar) into the vadose zone and the subsequent extraction of the injected gas mixture together with soil air from the same location. Rate constants of CH₄ oxidation are calculated from breakthrough curves of extracted reactants and tracers. Co-injection of a specific inhibitor for methanotrophic bacteria (acetylene) showed that Ne was a suitable tracer for CH₄ under the restricted gas transport conditions at this site and that the observed CH₄ oxidation was microbially mediated. The latter was also confirmed by a large shift in CH₄ stable carbon isotope ratios of up to 42.6‰.

At the same site, Michaelis-Menten parameters for CH₄ oxidation were estimated using GPPTs with a range of CH₄ injection concentrations. The apparent maximum activity $V_{max}$ was an order of magnitude higher at 2.7 m below soil surface, close to the groundwater table ($0.70 \pm 0.15 \text{ mmol CH}_4 \ (\text{L soil air})^{-1} \ \text{h}^{-1}$) than at 1.1 m depth ($0.076 \pm 0.006 \text{ mmol CH}_4 \ (\text{L soil air})^{-1} \ \text{h}^{-1}$). Conversely, values for the apparent affinity constant $K_m$ were in a similar range at both depths. At 2.7 m, the apparent first-order rate constant $k$ ($2.0 \pm 0.03 \ \text{h}^{-1}$) calculated from GPPTs agreed well with the $k$ of 1.9 h⁻¹ estimated from CH₄ gas concentration profiles. At both depths, known methanotrophic species that may contribute to the observed CH₄
oxidation were detected by cloning and sequencing. Stable carbon isotope fractionation factors for CH₄ oxidation determined during GPPTs ranged from 1.006 to 1.032. The observed variability may complicate the use of stable isotopes as an independent quantification method.

For application in the peat bog, the GPPT was adapted as noble gases are not suitable as tracers for CH₄ under diffusion-dominated transport conditions due to differing diffusion coefficients. We showed that the reactant CH₄ can be used as a substitute tracer by performing two consecutive GPPTs and co-injecting acetylene (C₂H₂) as an inhibitor in the second test. Applying this procedure, apparent first-order rate constants for CH₄ oxidation from 0.38 to 0.82 h⁻¹ were quantified in the unsaturated zone of the peat bog. In this highly porous system, stable carbon isotopes were suitable only to a limited extent as independent indicators of microbial CH₄ oxidation.

To complement the field studies, we designed a laboratory column to study CH₄ oxidation in diffusional CH₄/O₂ counter-gradients in unsaturated porous media. Analysis and simulations of the steady-state CH₄, CO₂ and O₂ gas profiles showed that in a 15-cm-deep active zone, CH₄ oxidation followed first-order kinetics with respect to CH₄ with a high apparent first-order rate constant of 30 h⁻¹. The methanotrophic community responded rapidly to changing substrate availability induced by changes in O₂ concentration at the top of the column. A ~7% enrichment in CH₄ stable carbon isotope ratios along profiles confirmed microbial CH₄ oxidation. A high fractionation factor of 1.025 ± 0.0005 for microbial oxidation estimated from this shift was corroborated by simulations.

In conclusion, in this thesis we demonstrated that the gas push-pull test is a valuable field method for the quantification of in-situ and potential microbial CH₄ oxidation in the vadose zone. Quantification of this process was successful in two environments with very different physical characteristics. In addition to field quantification, the designed laboratory model system was valuable for investigating response of methanotrophs to environmental parameters under controlled conditions.
Zusammenfassung


Am gleichen Standort wurden Michaelis-Menten Parameter für Methanoxidation aus einer Serie von GPPTs mit verschiedenen Methaninjektionskonzentrationen abgeschätzt. In 2.7 m Tiefe, nahe dem Grundwasserspiegel, lag die maximale Aktivität, $V_{\text{max}}$ (0.70 ± 0.15 mmol CH$_4$ (L Bodenluft)$^{-1}$ h$^{-1}$), eine Größenordnung über $V_{\text{max}}$ in 1.1 m Tiefe (0.076 ± 0.006 mmol...
CH₄ (L Bodenluft)⁻¹ h⁻¹. Umgekehrt lagen die Werte für die Affinitätskonstante $K_m$ in beiden Tiefen in einem ähnlichen Bereich. In 2.7 m Tiefe stimmten der aus GPPTs berechnete (2.0 ± 0.03 h⁻¹) und der aus Konzentrationsprofilen abgeschätzte (1.9 h⁻¹) Ratenkoeffizient erster Ordnung gut überein. In beiden Tiefen wurden durch Klonierung und Sequenzierung bekannte methanotrophe Spezies gefunden, die zur beobachteten Methanoxidation beitragen könnten. Aus GPPTs bestimmte Fraktionierungsfaktoren für stabile Kohlenstoffisotope während der Methanoxidation reichten von 1.006 bis 1.032. Diese hohe Variabilität könnte die Verwendung von Isotopenfraktionierung als unabhängige Quantifizierungsmethode erschweren.

Für die Anwendung an dem Moorstandort, wurde der GPPT angepasst, da Edelgase unter durch Diffusion dominierten Transportbedingungen keine geeigneten Tracer für Methan sind. Wir konnten zeigen dass durch die Durchführung zweier aufeinanderfolgender Tests und die gleichzeitige Injektion von Acetylen als Inhibitor im zweiten Test, das Substrat Methan als Ersatztracer verwendet werden kann. Mit dieser Methode wurden Ratenkoeffizienten erster Ordnung im Bereich von 0.38 bis 0.82 h⁻¹ in der ungesättigten Zone des Moors quantifiziert. In diesem hochporösen System waren stabile Kohlenstoffisotope nur in begrenztem Maß als unabhängige Indikatoren für Methanoxidation geeignet.


1

Introduction

Karina Urmann
1.1 The methane cycle

1.1.1 The global methane budget

Methane (CH$_4$) is the most important greenhouse gas after carbon dioxide (CO$_2$) (Ehhalt et al. 2001). Its contribution to increased radiative forcing is greater due to its higher radiative efficiency per molecule. It has a larger global warming potential than CO$_2$, which decreases over time due to its short lifetime in the atmosphere (Table 1-1). Nitrous oxide has a yet higher radiative efficiency and global warming potential but its contribution to increased radiative forcing since 1750 is only 1/3 that of CH$_4$ due to a lower increase in its atmospheric concentration.

### Table 1-1: Properties of important greenhouse gases

<table>
<thead>
<tr>
<th>Gas</th>
<th>Current tropospheric concentration$^a$</th>
<th>Increased radiative forcing$^b$</th>
<th>Atmospheric lifetime$^c$</th>
<th>Global warming potential$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>W m$^{-2}$</td>
<td>years</td>
<td>20 years</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>375</td>
<td>+1.46</td>
<td>5-200</td>
<td>1</td>
</tr>
<tr>
<td>CH$_4$</td>
<td>1.85</td>
<td>+0.48</td>
<td>12</td>
<td>62</td>
</tr>
<tr>
<td>N$_2$O</td>
<td>0.32</td>
<td>+0.15</td>
<td>114</td>
<td>275</td>
</tr>
</tbody>
</table>


$^b$ Increased radiative forcing due to each gas since 1750 (Ramaswamy et al. 2001)

$^c$ The average atmospheric lifetime includes chemical feedbacks that change the duration of the atmospheric response for CH$_4$ and N$_2$O (Ehhalt et al. 2001). For CO$_2$, an estimated range is given as the different sinks have very different turnover times (Prentice et al. 2001).

$^d$ Global warming potentials (GWP) of CH$_4$ and N$_2$O are given relative to CO$_2$ for different time horizons (Ramaswamy et al. 2001). The GWP has been defined as the ratio of the time-integrated radiative forcing from the instantaneous release of 1 kg of trace substance relative to that of one kg of reference gas. Therefore, the GWP changes depending on the time horizon due to different lifetimes of different gases.

In order to effectively mitigate CH$_4$ emissions, it is important to study the sources and sinks of CH$_4$ in order to estimate their contribution to the global budget. A number of biogenic and abiogenic sources of CH$_4$ emissions to the atmosphere have been identified with the most important biogenic sources being natural wetlands, rice agriculture and ruminants and the most important abiogenic source being energy production (Table 1-2). Before CH$_4$ reaches the atmosphere, the microbial processes of aerobic and anaerobic CH$_4$ oxidation reduce the strength of these CH$_4$ sources by an estimated 58% (Reeburgh et al. 1993). Anaerobic oxidation of CH$_4$ has long been known to occur coupled to sulfate reduction especially in marine sediments and waters (Valentine 2002). Recently, anaerobic CH$_4$ oxidation coupled to denitrification was shown in a freshwater sediment (Raghoebarsing et al.
2006). In terrestrial ecosystems, aerobic CH$_4$ oxidation is the key process lowering CH$_4$
emissions (Hanson and Hanson 1996). The major sink for removal of CH$_4$ from the
atmosphere is chemical oxidation in the troposphere and a minor but important sink is aerobic
microbial oxidation in oxic soils. There is a large uncertainty associated with the global CH$_4$
budget as evidenced by the variety of net emission and consumption estimates for different
sources and sinks (Table 1-2). Recently, it was proposed that plants might produce CH$_4$ under
aerobic conditions by a yet unknown, possibly chemical process (Keppler et al. 2006). If this
turns out to be correct, the global CH$_4$ budget may have to be re-evaluated. For the well-
established biogenic sources such as wetlands (Table 1-2), the origin of CH$_4$ is microbial
production. In this work the focus is on terrestrial ecosystems with microbial production and
aerobic oxidation of CH$_4$.

Table 1-2: Estimates for global net CH$_4$ emission and net CH$_4$ consumption for different sources
and sinks.

<table>
<thead>
<tr>
<th>Range of estimates$^*$</th>
<th>[Tg CH$_4$ year$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sources</strong></td>
<td></td>
</tr>
<tr>
<td>Biogenic</td>
<td></td>
</tr>
<tr>
<td>Natural wetlands</td>
<td>92 - 237</td>
</tr>
<tr>
<td>Rice agriculture</td>
<td>25 - 100</td>
</tr>
<tr>
<td>Landfills</td>
<td>35 - 73</td>
</tr>
<tr>
<td>Ocean</td>
<td>10 - 15</td>
</tr>
<tr>
<td>Ruminants</td>
<td>80 - 115</td>
</tr>
<tr>
<td>Termites</td>
<td>20</td>
</tr>
<tr>
<td>Abiogenic</td>
<td></td>
</tr>
<tr>
<td>Hydrates</td>
<td>5 - 10</td>
</tr>
<tr>
<td>Energy production</td>
<td>75 - 110</td>
</tr>
<tr>
<td>Biomass burning</td>
<td>23 - 55</td>
</tr>
<tr>
<td><strong>Total source</strong></td>
<td>500 - 600</td>
</tr>
<tr>
<td><strong>Sinks</strong></td>
<td></td>
</tr>
<tr>
<td>Soils</td>
<td>10 - 44</td>
</tr>
<tr>
<td>Tropospheric ·OH</td>
<td>450 - 510</td>
</tr>
<tr>
<td>Stratospheric loss</td>
<td>40 - 46</td>
</tr>
<tr>
<td><strong>Total sinks</strong></td>
<td>460 - 580</td>
</tr>
</tbody>
</table>

$^*$Ranges of estimates as compiled from seven studies in the 2001 IPCC report (Ehhalt et al. 2001). Note that total estimates are taken from individual studies and therefore do not equal the sum of lowest and highest estimates.

1.1.2 Methane in terrestrial ecosystems

Methanogenic organisms belong to the domain *Archaea* and produce CH$_4$ under anaerobic
conditions from methylated compounds, acetate and CO$_2$ + H$_2$, with the latter two being the
major substrates in most environments (Le Mer and Roger 2001). In terrestrial ecosystems,
aerobic conditions frequently occur in water-saturated soils or contaminated aquifers but
can also be found in micro-niches in oxic soils (Andersen et al. 1998). The produced CH₄ is transported from the methanogenic zone to the atmosphere via different pathways. The main pathways are advective (Czepiel et al. 2003) and diffusive transport through the soil, bubble formation and ebullition and transport through vascular plants (Walter and Heimann 2000). Under aerobic conditions, CH₄ can be oxidized by methanotrophic bacteria prior to reaching the atmosphere. During this process, CH₄ can be completely oxidized to CO₂ (Equation 1-1) or partially oxidized and partially assimilated into microbial biomass (Equation 1-2).

\[ \text{CH}_4 + 2\text{O}_2 \rightarrow \text{CO}_2 + \text{H}_2\text{O} \]  
\[ \text{CH}_4 + (2 - x)\text{O}_2 \rightarrow (1 - x)\text{CO}_2 + x\text{CH}_2\text{O} + (2 - x)\text{H}_2\text{O} \]  

(Equation 1-1)  
(Equation 1-2)

The O₂ necessary for aerobic oxidation generally originates either from diffusion from the atmosphere into the soil or release into the root zone by plants. Consequently, methanotrophic zones are found in the unsaturated zone, in the rhizosphere and just below the water table depending on simultaneous availability of CH₄ and O₂ (Le Mer and Roger 2001).

Methane emissions into the atmosphere are the net result of methanogenesis and CH₄ oxidation. Therefore, factors influencing one or the other process or influencing both processes to a different degree will influence net emissions (Le Mer and Roger 2001). Methanogenesis is more strongly dependent on temperature than CH₄ oxidation (Pearce and Clymo 2001 and references therein) and is dependent on substrate availability e.g. through plant exudation or organic matter degradation by other organisms (Le Mer and Roger 2001). Furthermore, availability of other electron acceptors can inhibit methanogenesis. Availability of O₂ for CH₄ oxidation, on the other hand, can depend on the effective gas diffusivity in the soil and therefore on soil water content (Smith et al. 2003), diurnal cycles of O₂ release by roots (Damgaard et al. 1998) or competition with heterotrophic bacteria (van Bodegom et al. 2001). Furthermore, the position of the water table determines the extent of the unsaturated zone where O₂ is more easily available. An example of a methanotrophic ecosystem with a large unsaturated zone and no influence of plants is shown in figure 1-1.
Chapter 1: General introduction

Figure 1-1: Simplified scheme of CH₄ production and oxidation in a terrestrial ecosystem with a large vadose zone and without the influence of plants.

### 1.2 Methanotrophic bacteria

#### 1.2.1 Characteristics of methanotrophic bacteria

Methylotrophs are a diverse group of bacteria that utilize one-carbon compounds as their source of carbon and energy. Methanotrophs, a subgroup of the methylotrophs, use CH₄ as their main source of carbon and energy. Traditionally, methanotrophs have been divided into two major groups, type I with the subgroup type X and type II, belonging to the γ- and α-Proteobacteria, respectively (Hanson and Hanson 1996). Apart from phylogeny, these types are distinguished by several cell characteristics such as internal membrane structure, predominant phospholipid fatty acids (PLFA), assimilation pathway and type of enzyme (Table 1-3). Currently, there are 13 recognized genera of methanotrophs (Dumont and Murrell 2005). With the more recently detected genera the classic distinction between type I, II and X has become less applicable. For example, the recently described genera *Methylocella* contains a different internal membrane system from type I or type II methanotrophs (Dedysh et al. 2000). Furthermore, it does not contain the particulate methane monooxygenase (pMMO), the enzyme mediating the first oxidation step in the oxidation from CH₄ to methanol present in all other known methanotrophs. Instead, it contains the soluble form of
methane monooxygenase (sMMO) otherwise found mainly in type II and type X methanotrophs in addition to pMMO. Similar to the classic division of methanotrophs, the hypothesis that type I and type II methanotrophs occupy niches with differing nutrient availability (Hanson and Hanson 1996) has recently been questioned (Knief et al. 2006).

In addition to the distinction of methanotrophs based on phylogeny and cell characteristics, two different types of kinetics have been found for CH₄ oxidation: low affinity kinetics with a high maximum conversion rate $V_{\text{max}}$ and a high half-saturation constant $K_m$ (concentrations in the water in the mM range) and high affinity kinetics with low $V_{\text{max}}$ and low $K_m$ (concentrations in the water in the nM range) (Bender and Conrad 1992). All cultivated (type I, II and X) methanotrophs are known to show low affinity kinetics. High affinity kinetics have been found in oxic soils consuming atmospheric CH₄, however the organisms involved have not been cultivated or identified. There is evidence that they might belong to distinct clusters related to known type I and type II methanotrophs (Holmes et al. 1999; Knief et al. 2003). Recently, it was proposed that known methanotrophs might also play a role in this process (Knief and Dunfield 2005). Increasing knowledge on the role of different methanotrophs in the environment is largely based on studies using culture-independent techniques.

### Table 1-3: Characteristics of different types of methanotrophs, adapted from Murrell et al. (1998) and Hanson and Hanson (1996).

<table>
<thead>
<tr>
<th>Type</th>
<th>I</th>
<th>X</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylogeny</td>
<td>γ-Proteobacteria</td>
<td>γ-Proteobacteria</td>
<td>α-Proteobacteria</td>
</tr>
<tr>
<td>Type of internal</td>
<td>Bundles distributed</td>
<td>Bundles distributed</td>
<td>Arranged along the</td>
</tr>
<tr>
<td>membranes</td>
<td>throughout the cell</td>
<td>throughout the cell</td>
<td>periphery of the cell</td>
</tr>
<tr>
<td>Major PLFA</td>
<td>16:1</td>
<td>16:1</td>
<td>18:1</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Ribulose monophosphate</td>
<td>Ribulose monophosphate</td>
<td>Serine</td>
</tr>
<tr>
<td>assimilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pathway</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme type</td>
<td>pMMO</td>
<td>pMMO/sMMO</td>
<td>pMMO/sMMO</td>
</tr>
</tbody>
</table>

### 1.2.2 Molecular methods to analyze methanotrophic communities

Several molecular methods have been adapted for the analysis of methanotrophic bacteria in environmental samples (Murrell and Radajewski 2000) (Table 1-4). A number of primers for polymerase chain reaction (PCR) have been developed to amplify methanotrophic
functional genes (Dumont and Murrell 2005) or 16S rDNA (Murrell and Radajewski 2000), e.g. from DNA extracted from environmental samples. The most widely used functional gene for methanotrophic community analysis is the α-subunit of the particulate methane monooxygenase (pmoA). The advantages of using this gene are that a) methanotrophic phylogeny based on pmoA and on 16S rDNA is similar (Holmes et al. 1995), b) a direct link to the function of detected organisms can be made and c) even uncultured methanotrophs not closely related to known species can potentially be detected (e.g. Holmes et al. 1999). Identification of species or strains present in an environment can be achieved by cloning and sequencing of PCR products (e.g. McDonald et al. 1999; Knief et al. 2003). A relatively quick fingerprinting method to study community structure is denaturing gel electrophoresis (DGGE) (Muyzer et al. 1993; Henckel et al. 1999). This method is based on the separation of amplified DNA strands on a polyacrylamide gel with a gradient of denaturants. To identify different species separated on the gel, sequencing of bands is required. Using quantitative PCR, the amount of target DNA originally present in a sample can be quantified by comparison with a standard. This method has been adapted to quantify five different groups of methanotrophic bacteria in environmental samples (Kolb et al. 2003). An alternative method, used to quantify active methanotrophs, is fluorescence in situ hybridization (FISH). This method is based on microscopic cell counts after labeling of cells with fluorescent probes that target ribosomal RNA. Probes targeting type I and type II methanotrophs or specific genera have been developed and applied in environmental samples (Eller and Frenzel 2001; Eller et al. 2001). However, this method can be difficult to apply in soil and sediment samples, especially with low cell numbers, due to autofluorescence of particles and attachment of cells to particles (Zarda et al. 1997). A problem inherent in all the mentioned methods is the specificity of primers and probes and the rapid changes in the available sequence data making frequent adaptations of primers and probes necessary. An alternative method that is not DNA or RNA based is phospholipid fatty acid (PLFA) analysis (e.g. Crossman et al. 2004). The cytoplasmic membranes of type I and type II methanotrophs contain typical and unusual PLFA that can be used as specific biomarkers (Hanson and Hanson 1996). However, biomarker assignment is not always unequivocal and it is not possible to obtain a higher resolution, e.g. to identify genera, with this approach.
## Table 1-4: Some molecular methods available to analyze methanotrophic communities

<table>
<thead>
<tr>
<th>Method</th>
<th>FISH</th>
<th>DGGE</th>
<th>Quantitative PCR</th>
<th>Cloning - Sequencing</th>
<th>PLFA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target molecule</strong></td>
<td>16S rRNA</td>
<td>functional gene DNA, 16S rDNA</td>
<td>functional gene DNA, 16S rDNA</td>
<td>functional gene DNA, 16S rDNA</td>
<td>PLFA</td>
</tr>
<tr>
<td><strong>Quantitative information</strong></td>
<td>quantitative</td>
<td>semi-quantitative at best</td>
<td>quantitative</td>
<td>not quantitative</td>
<td>semi-quantitative</td>
</tr>
<tr>
<td><strong>Information on phylogeny</strong></td>
<td>type I / II or genus level</td>
<td>species level, identification with sequencing</td>
<td>group or genus level</td>
<td>identification of species and strains</td>
<td>type I / II</td>
</tr>
<tr>
<td><strong>Potential biases/limitations</strong></td>
<td>- Specificity of probes</td>
<td>- PCR bias</td>
<td>- PCR bias</td>
<td>- PCR bias</td>
<td>- Limited biomarker specificity</td>
</tr>
<tr>
<td></td>
<td>- Permeabilization of cells</td>
<td>- Specificity of primers</td>
<td>- Specificity of primers</td>
<td>- Specificity of primers</td>
<td>- Limited resolution</td>
</tr>
<tr>
<td></td>
<td>- Interference of soil particles</td>
<td>- double banding</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3 Concentration-based methods to quantify microbial methane oxidation in situ

Common problems inherent in the quantification of microbial processes such as CH₄ oxidation in laboratory incubation studies are contamination during sampling, disruption of the physical structure of samples, changes in microbial communities and the influence of physical conditions (e.g. temperature) in the laboratory (Madsen 1998). To overcome some of these biases and obtain information that may be more relevant under field conditions, it is therefore important to complement laboratory methods with in-situ quantification methods (Madsen 1991; Reeburgh 1996; Madsen 1998). Comparison of field and laboratory measurements have indeed shown that results do not necessarily agree (e.g. Moore and Dalva 1993).

However, in-situ methods are often associated with large uncertainties and the influence of individual environmental factors on the studied processes often cannot be separated (Madsen 1998). Therefore, in addition to field studies, it is important to study processes under controlled conditions in the laboratory while mimicking some important characteristics of field environments. For example, in chemostats, constant low substrate concentrations that are often found in the environment (Koch 1990) can be maintained. In soil or sediment columns the physical structure of an environment can be essentially preserved and chemical gradients can be applied (e.g. De Visscher et al. 1999; Scheutz and Kjeldsen 2003). This is important as physical conditions have a large influence on microbial processes in unsaturated environments (Smith et al. 2003).

Methane oxidation or CH₄ fluxes have been quantified in situ in unsaturated environments using several methods based on CH₄ concentration and transport analysis such as gas concentration profiles, emission chamber techniques, the eddy covariance method, and, in this study, gas push-pull tests. Furthermore, analysis of stable carbon isotope fractionation has been used for this purpose. Some characteristics of these methods are summarized in table 1-5 and discussed in more detail in this and the following section.
Table 1-5: Methods for the in-situ quantification of CH₄ emissions and CH₄ oxidation

<table>
<thead>
<tr>
<th>Method</th>
<th>Parameter measured</th>
<th>Important pre-requisite</th>
<th>Reference (e.g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas push-pull test</td>
<td>Rate constants</td>
<td>Possibility to inject gaseous substrates</td>
<td>This study</td>
</tr>
<tr>
<td>Gas concentration</td>
<td>Oxidation rate per area or volume</td>
<td>Knowledge of physical characteristics of the environment, diffusive steady-state</td>
<td>Fechner and Hemond (1992)</td>
</tr>
<tr>
<td>profiles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chamber technique</td>
<td>Flux per area (≤ 1m²)</td>
<td>Minimization of influence of the chamber on physical conditions</td>
<td>Kruger et al. (2001), Frenzel and Karofeld (2000)</td>
</tr>
<tr>
<td>technique with</td>
<td>Fraction of total CH₄ oxidized or oxidation rate per area</td>
<td>Processes close to the surface</td>
<td>as above</td>
</tr>
<tr>
<td>inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eddy covariance</td>
<td>Flux per area (m² - km²)</td>
<td>Wind speed above threshold, uniform upwind fetch</td>
<td>Hargreaves et al. (2001), Norman et al. (1997)</td>
</tr>
<tr>
<td>method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable isotope mass</td>
<td>Fraction of total CH₄ oxidized</td>
<td>Knowledge of the fractionation factor and the gas transport regime</td>
<td>Liptay et al. (1998), De Visscher et al. (2004)</td>
</tr>
<tr>
<td>balance in an open</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>system</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.3.1 Gas push-pull tests

Tracer tests are commonly used to assess physical properties (e.g. Werner and Höhener 2002; Keller and Brusseau 2003) or microbial processes (e.g. Thierrin et al. 1995; Amerson and Johnson 2002) in the subsurface. A single well test, the push-pull test (PPT) was developed for the quantification of microbial processes in groundwater (Istok et al. 1997). This method has been successfully applied for the quantification of sulfate reduction (Schroth et al. 2001), nitrate reduction, aerobic respiration (Schroth et al. 1998) and reduction of halogenated compounds (Hageman et al. 2001). The PPT consists of the injection of an aqueous test solution containing reactants and a conservative tracer. After an incubation time, the mixture of injection solution and background groundwater is extracted from the same well and first-order rate constants are calculated from reactant and tracer breakthrough curves. To develop the gas push-pull test (GPPT) in this study, this principle was transferred to the vadose zone using a gas mixture instead of an aqueous solution as test solution. Important
questions during the development of the GPPT were the selection of suitable non-reactive tracers, and the length of contact time between substrates and organisms to allow for consumption of the reactive gas while ensuring sufficient recovery of the injected gases for measurement purposes. The physical side of these issues was addressed in a laboratory model system consisting of a 1 m-diameter tank filled with dry sand (Gonzalez-Gil et al. submitted- b). For field testing of the GPPT, a site was chosen where the vadose zone consisted of sand and gravel allowing easy gas injection and extraction. An unsaturated porous medium that allows injection and extraction of gases is a pre-requisite for the application of this method.

1.3.2 Gas concentration profiles

As shown in table 1-5, pre-requisites to calculate rates of CH₄ oxidation from CH₄ gas concentration profiles are diffusive steady-state conditions and knowledge of the physical parameters of the vadose zone. Under diffusive steady-state conditions in a homogeneous medium, a concave CH₄ profile indicates CH₄ conversion (Figure 1-2). Methane fluxes into and out of an active zone can then be calculated from gradients in the profile (Figure 1-2) and converted into a conversion rate. Alternatively, simple diffusion-consumption models can be fitted to a profile to obtain rate constants (e.g. Reeburgh et al. 1997). All rate calculations require the knowledge of the effective diffusion constant in the gas phase of the vadose zone. For a relatively homogeneous medium, effective diffusivity can be measured (e.g. Johnson et al. 1998) or estimated from physical parameters of the vadose zone, such as porosity and water content (Millington and Quirk 1961). In the environment, physical conditions of gas transport in the vadose zone are often more complex. Obtaining conversion rates from profiles can be complicated by heterogeneous soil layers with changing effective diffusivity as observed for example in a peat bog (Fechner and Hemon 1992). Furthermore, advective gas transport can be temporarily induced by atmospheric pressure pumping (Czepiel et al. 2003) or wind (Hargreaves et al. 2001) and is common in landfill cover soils (De Visscher et al. 1999). When concentration profiles are influenced by advective gas transport interpretation becomes difficult (De Visscher et al. 1999). However, in the subsurface, e.g. above aquifers, diffusive profiles at quasi steady-state are frequently found (e.g. Chaplin et al. 2002). Numerical models may extend the possibilities to interpret complex profiles (Baehr and Baker 1995; De Visscher and Van Cleemput 2003), but the necessity to obtain sufficiently accurate physical parameters as input parameters remains.
1.3.3 Chamber methods

Methane emissions from different ecosystems have frequently been measured using surface flux chambers (e.g. Frenzel and Karofeld 2000; Kruger et al. 2001). A chamber with an area in the dm²-range is placed over the soil and sealed from the atmosphere. Concentrations in the chamber are then followed over time and subsequently a flux is calculated. Due to several sources of error associated with this method, such as disturbance of the gas concentration gradient in the soil (Norman et al. 1997) this method was modified in several ways. For example, in the dynamic chamber method a gas flow is passed through the chamber so that the gas of interest does not accumulate (Norman et al. 1997). However, the chamber still influences the physical conditions of the system. To quantify CH₄ oxidation using the chamber method, CH₄ oxidation can be quantified by comparison of CH₄ fluxes with and without the addition of an inhibitor of CH₄ oxidation to the chamber (Frenzel and Karofeld 2000; Kruger et al. 2001; Ding et al. 2004). With this approach only CH₄ oxidation taking place relatively close to the surface can be assessed as the inhibitor has to reach the zone of activity.
1.3.4 Eddy covariance method

An alternative method to measure CH₄ emissions is the eddy covariance method, which has been applied for example above peatlands (Verma et al. 1992; Hargreaves et al. 2001) or landfills (Laurila et al. 2005). Using this approach, CH₄ emissions are calculated from wind speed and gas concentration data that are usually measured continuously with an anemometer and an infrared analyzer attached to a mast (Hargreaves et al. 2001). Pre-requisites for this method are wind speeds above a minimum threshold and wind from a direction with a uniform fetch 100 times the height of the mast (Norman et al. 1997). Therefore, emission data can be obtained continuously and are integrated over a larger scale (m²-km²) as opposed to chamber measurements (Verma et al. 1992). However, days with certain meteorological conditions are excluded. This method disturbs in-situ conditions to a much lower degree than chamber measurements, but it is usually not possible to infer the attenuation of CH₄ emissions by CH₄ oxidation.

1.4 Stable isotope-based methods to quantify microbial methane oxidation in situ

As stable isotope analysis was important in all studies in this thesis, a separate section is dedicated to basic principles of isotope fractionation during physical processes and during microbial CH₄ oxidation. Measurement of natural abundances of stable isotopes of carbon, hydrogen, oxygen and nitrogen have been used extensively to assess biogeochemical processes in the environment (Hoefs 1997; Meckenstock et al. 2004). Stable isotopes of an element have the same number of protons and electrons, but a different number of neutrons and therefore different masses. This influences their chemical and physical behavior resulting in a fractionation between molecules containing the lighter and the heavier isotope of an element (Hoefs 1997). The natural abundance of the heavier isotopes is usually low. The absolute abundance of $^{13}$C in the environment is around 1.1% of total carbon (Hoefs 1997). Therefore, isotope ratios are given in the $\delta$-notation in per mil relative to a standard (Equation 1-3) to make small absolute differences or changes in $^{13}$C-content discernible.

$$
\delta^{13}C(\%o) = \left( \frac{\frac{^{13}C}{^{12}C}_{\text{sample}}}{\frac{^{13}C}{^{12}C}_{\text{PDB}}} \right) - 1000
$$

(Equation 1-3)
PDB is Peedee Belemnite, the standard for carbon isotope ratios with \((^{13}\text{C}/^{12}\text{C})_{\text{PDB}} = 0.011237\) (Craig 1957).

Isotope fractionation can occur during equilibrium and kinetic processes. The focus here is on kinetic fractionation which occurs during unidirectional processes, such as diffusion along a gradient and microbial, i.e. enzymatic reactions (Hoefs 1997). Stable isotope fractionation during chemical or enzymatic reactions is due to a difference in bond energy of the different stable isotopes at the reactive site of the molecule. As bond energy is dependent mainly on the masses of the neighboring atoms (Meckenstock et al. 2004), fractionation during chemical or enzymatic reactions is independent of the overall mass of the molecule. In contrast, the gas-phase diffusion coefficient of a molecule is mass-dependent, i.e. heavier molecules diffuse slower than lighter molecules. Hence, for molecules with a low mass like CH\(_4\), there is a relatively large difference in diffusion coefficients for different isotopic species. Consequently, in addition to enzymatic reactions, physical processes need to be considered when inferring information about microbial CH\(_4\) conversion in the environment from stable carbon isotope data (Table 1-6). The extent of fractionation by different processes will be discussed in more detail in the following paragraphs.
Table 1-6: Simplified scheme of processes associated with CH₄ transport and oxidation in an unsaturated porous medium with the respective isotope fractionation factor \( \alpha \).

<table>
<thead>
<tr>
<th>Process</th>
<th>Isotope Fractionation Factor</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore water [^{13}\text{CH}_4, ^{12}\text{CH}_4]</td>
<td>Water-phase diffusion ( \alpha = 1.0012^a )</td>
<td>[^a\text{Knox et al.}(1992), \alpha \text{ for water-phase diffusion calculated from measured } \alpha \text{ for air-water interface transfer.}]</td>
</tr>
<tr>
<td>Soil air [^{13}\text{CH}_4, ^{12}\text{CH}_4]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gas-phase diffusion ( \alpha = 1.0178, 1.0195^b )</td>
<td>[^b\text{De Visscher et al.}(2004), \text{the first value is measured, the second one is calculated based on theory.}]</td>
<td></td>
</tr>
<tr>
<td>Air-water interface transfer ( \alpha = 1.0008^c \text{ - } 1.0009^c )</td>
<td>[^c\text{Happell et al.}(1995), \text{measured in the laboratory.}]</td>
<td></td>
</tr>
<tr>
<td>Air-water equilibrium partitioning ( \alpha = 1.0003^d )</td>
<td>[^d\text{Fuex (1980), measured in the laboratory.}]</td>
<td></td>
</tr>
<tr>
<td>Microbial oxidation ( \alpha = 1.003\text{-}1.039^e )</td>
<td>[^e\text{g. Templeton et al. (2006), Snover and Quay (2000); Note that the range of isotope fractionation factors for microbial oxidation includes apparent fractionation factors that may be influenced by physical processes like transport to the cell.}]</td>
<td></td>
</tr>
</tbody>
</table>

1.4.1 Stable carbon isotope fractionation of methane by physical processes

The physical processes potentially affecting isotope fractionation of CH₄ during transport in unsaturated porous media are diffusion in the gas phase, transfer across the air-water interface, diffusion in the water phase and equilibrium partitioning between gas and water. The stable carbon isotope fractionation factor for molecular diffusion \( \alpha_{\text{diff}}(a) \) of CH₄ in air is defined as the ratio of the diffusion coefficients in air \( D_a \) for \(^{12}\text{CH}_4\) and \(^{13}\text{CH}_4\), respectively:

\[
\alpha_{\text{diff}} = \frac{^{12}D_a}{^{13}D_a}
\]  

(Equation 1-4)
According to ideal gas law, diffusivities are inversely proportional to the square root of the reduced masses (De Visscher et al. 2004). Therefore, $\alpha_{\text{diff}(o)}$ theoretically equals 1.0195, which means that $^{12}\text{CH}_4$ diffuses faster than $^{13}\text{CH}_4$ by a factor of 1.0195. Experimentally, $\alpha_{\text{diff}(o)}$ was determined to be 1.0178 in a column with glass beads (De Visscher et al. 2004), which is slightly lower than the theoretical value.

In contrast to gas-phase diffusion, fractionation due to transfer across the air-water interface and due to diffusion in the water phase cannot be easily predicted (Knox et al. 1992). The flux $F$ of a gas across the air-water interface is defined as (Happell et al. 1995):

$$F = K(C_w - C_o) \quad \text{(Equation 1-5)}$$

where $K$ is the exchange coefficient, $C_w$ is the measured dissolved concentration of the gas in water and $C_o$ is the calculated equilibrium concentration in the water with respect to the measured concentration in the gas phase. $K$ is a function of the physical conditions affecting transfer such as turbulence, the kinematic viscosity of water and the diffusion coefficient of the gas in water. Therefore, different water-phase diffusion coefficients determine the fractionation factor for transfer across the air-water interface. The latter is defined as:

$$\alpha_K = \frac{^{12}K}{^{13}K} \quad \text{(Equation 1-6)}$$

Similarly, the fractionation factor for diffusion in water $\alpha_{\text{diff}(w)}$ is defined as:

$$\alpha_{\text{diff}(w)} = \frac{^{12}D_w}{^{13}D_w} \quad \text{(Equation 1-7)}$$

where $D_w$ is the diffusion coefficient in water for $^{12}\text{CH}_4$ and $^{13}\text{CH}_4$, respectively. The relationship between $\alpha_K$ and $\alpha_{\text{diff}(w)}$ can be described as (Knox et al. 1992):

$$\frac{1}{\alpha_K} = \left(\alpha_{\text{diff}(w)}\right)^n \quad \text{(Equation 1-8)}$$

where $n = -2/3$ for a still water surface without turbulence. Similar low values of $1.0009 \pm 0.0004$ (Happell et al. 1995) and $1.0008 \pm 0.0002$ (Knox et al. 1992) were determined for $\alpha_K$ in two laboratory studies. (Note that Happell et al. (1995) give values for $\alpha_K$ according to
equation 1-6, but erroneously define $\alpha_K$ as the reverse.) Using equation 1-8, a value for $\alpha_{\text{diff}(w)}$ of 1.0012 was calculated from the experimentally measured $\alpha_K$ (Knox et al. 1992). Overall, this shows that fractionation due to transfer across the air-water interface or diffusion in water is small, e.g. compared to diffusion in the gas phase. However, it has to be noted that higher fractionation due to transfer across the air-water interface can occur in the environment. For example, $\alpha_K$ values of $1.0067 \pm 0.0044$ and $1.0028 \pm 0.0009$ were determined in situ in wooded swamps (Happell et al. 1995). The authors hypothesized that an insoluble organic surfactant film on the water surface inhibited gas transfer and caused stronger isotope fractionation.

Equilibrium fractionation due to partitioning between water and gas phase, or in other words the ratio of Henry's law constants (in (mol (L gas)$^{-1}$/(mol (L water)$^{-1}$)) of $^{12}$CH$_4$ and $^{13}$CH$_4$, was experimentally found to be very small (1.00033) (FueX 1980), i.e. only a slightly higher amount of $^{13}$CH$_4$ than $^{12}$CH$_4$ is dissolved in water at equilibrium. Other physical processes that are not dependent on the properties of the molecule, such as advective transport, do not fractionate. Note that due to mass balance requirements, no fractionation will be apparent for gas-phase diffusion if a system is at steady state and CH$_4$ is not removed by other processes, such as CH$_4$ oxidation (Levin et al. 1993).

1.4.2 Stable carbon isotope fractionation of methane by microbial oxidation

The stable carbon isotope fractionation factor for microbial conversion following first-order kinetics is defined as:

$$\alpha_{\text{ox}} = \frac{k}{k}$$

(Equation 1-9)

with $^{12}k$ and $^{13}k$ being the first-order rate constant for CH$_4$ containing $^{12}$C and CH$_4$ containing $^{13}$C respectively. For Michaelis-Menten kinetics $\alpha_{\text{ox}}$ is defined as (Hunkeler and Aravena 2000):

$$\alpha_{\text{ox}} = \frac{12V_{\text{max}}/12K_m}{13V_{\text{max}}/13K_m}$$

(Equation 1-10)

with $^{12}V_{\text{max}}/^{12}K_m$ and $^{13}V_{\text{max}}/^{13}K_m$ being the ratio of maximum rate $V_{\text{max}}$ and affinity constant $K_m$ for CH$_4$ containing $^{12}$C and CH$_4$ containing $^{13}$C respectively. The first enzymatic reaction during CH$_4$ oxidation is the oxidation of CH$_4$ to methanol, mediated by the enzyme methane
monooxygenase (Hanson and Hanson 1996). This is the fractionating step in the enzymatic pathway that may influence the isotope ratio of residual CH$_4$ observed in the environment (Templeton et al. 2006). Note that further fractionation can take place at the branch point between carbon assimilation and complete oxidation to CO$_2$. Therefore, the fractionation factor discussed here is not necessarily the same as a fractionation factor between CH$_4$ and CO$_2$ or CH$_4$ and biomass.

A wide range of fractionation factors from 1.003 to 1.039 has been determined in cultures or environmental samples in several laboratory studies (Table 1-7) and in some field studies (Table 1-8). On a single occasion an even higher factor of 1.049 (Chanton and Liptay 2000) was determined. It has to be noted that, when determining fractionation factors in the field or in environmental samples, obtained values may be apparent fractionation factors as the rate-limiting step is not necessarily the enzymatic reaction. The apparent fractionation factor due to microbial oxidation could be determined by different processes: a) by physical processes that are induced by the oxidation of CH$_4$, such as transport to the cell, b) the enzymatic reaction itself or a mixture of both. Environmental conditions that influence either process and/or determine which is rate-limiting may determine the magnitude of fractionation. Several factors have been proposed to influence the magnitude of the fractionation factor observed in the environment (Table 1-9) however few studies have looked into related mechanisms. For example, an inverse as well as a direct proportional relationship of the fractionation factor with temperature has been found (Table 1-9), but the reasons are not well understood. In one of the few studies looking at the mechanism of fractionation, the latter was found to be independent of the type of methanotroph or the type of enzyme (Templeton et al. 2006). During growth of mixed or pure methanotrophic cultures in that study, the main factor governing fractionation was found to be increasing rate-limitation due to CH$_4$ mass transfer. Observations of decreasing fractionation with decreasing CH$_4$ concentrations and increasing CH$_4$ oxidation rates in samples of marine sediments and tropical forest soils were attributed to the same mechanism (Teh et al. 2006; Kinnaman et al. in press).
Table 1-7: (Apparent) fractionation factors for microbial CH$_4$ oxidation $\alpha_{ox}$ determined in batch incubations of cultures or environmental samples. The $\alpha_{ox}$ value refers to the oxidation from CH$_4$ to methanol unless otherwise noted.

<table>
<thead>
<tr>
<th>Ecosystem and method to determine $\alpha_{ox}$</th>
<th>$\alpha_{ox}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pure cultures or enrichment cultures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two enrichment cultures</td>
<td>1.013-1.025</td>
<td>Coleman et al. (1981)</td>
</tr>
<tr>
<td>Enrichment culture</td>
<td>1.005-1.031</td>
<td>Barker and Fritz (1981)</td>
</tr>
<tr>
<td>((\alpha_{CH_4-CO_2}))</td>
<td>1.002-1.035</td>
<td>Temperley et al. (2006)</td>
</tr>
<tr>
<td>Summary of several pure culture studies</td>
<td>1.010-1.034</td>
<td>Zykun and Zakharchenko (1998)</td>
</tr>
<tr>
<td><strong>Methylosinus trichosporium (type II)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Methylomonas methanica (type I)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed culture</td>
<td>1.024 – 1.025</td>
<td>this study</td>
</tr>
<tr>
<td>Enrichment culture in a unsaturated porous medium column with a CH$_4$ / O$_2$ counter-gradient</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Landfill cover soils</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>two different sites</td>
<td>1.018 ± 0.001</td>
<td>De Visscher et al. (2004)</td>
</tr>
<tr>
<td>different soil types (mulch and clay)</td>
<td>1.022 ± 0.008</td>
<td>Liptay et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>1.023 – 1.038</td>
<td>Borjesson et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>1.027 – 1.039</td>
<td>Chanton and Liptay (2000)</td>
</tr>
<tr>
<td></td>
<td>(extremes 1.025, 1.049)</td>
<td></td>
</tr>
<tr>
<td><strong>Other soils and sediments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slurry of rice field soil</td>
<td>1.038</td>
<td>Kruger et al. (2002)</td>
</tr>
<tr>
<td>Forest soil (at atmospheric concentrations)</td>
<td>1.012 – 1.018</td>
<td>Tyler et al. (1994)</td>
</tr>
<tr>
<td>Humid tropical forest soil</td>
<td>1.010 – 1.023</td>
<td>Teh et al. (2006)</td>
</tr>
<tr>
<td>Hydraulic reservoirs in boreal forest, three sites</td>
<td>1.013 – 1.021</td>
<td>Venkiteswaran and Schiff (2005)</td>
</tr>
<tr>
<td>Slurries of marine sediment</td>
<td>1.012 – 1.026</td>
<td>Kinnaman et al. (in press)</td>
</tr>
</tbody>
</table>
Table 1-8: Apparent fractionation factor for microbial CH4 oxidation $\alpha_{ox}$ determined in field experiments. The $\alpha_{ox}$-value refers to the oxidation from CH4 to methanol.

<table>
<thead>
<tr>
<th>Ecosystem and method to determine $\alpha_{ox}$</th>
<th>$\alpha_{ox}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Landfills</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Landfill cover soils, comparison of CH4 with $^{222}$Rn profiles</td>
<td>$1.008 \pm 0.004$</td>
<td>Bergamaschi et al. (1998)</td>
</tr>
<tr>
<td>Landfill gas from gas collection system, estimation from Rayleigh equation</td>
<td>$1.008 \pm 0.003$</td>
<td>Bergamaschi and Harris (1995)</td>
</tr>
<tr>
<td><strong>Uptake of ambient CH4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tundra soil, uptake in chambers (combined $\alpha$ for diffusion and oxidation)</td>
<td>$1.016 - 1.027$</td>
<td>King and Smith (1987), King et al. (1989)</td>
</tr>
<tr>
<td>Forest soil, uptake in chambers (combined $\alpha$ for diffusion and oxidation)</td>
<td>$1.017 - 1.029$</td>
<td>Tyler et al. (1994)</td>
</tr>
<tr>
<td>Grassland and forest oxic soils, uptake in chambers</td>
<td>$1.014 - 1.017$</td>
<td>Snover and Quay (2000)</td>
</tr>
<tr>
<td>Boreal forest soil, estimation from profiles</td>
<td>$1.022 - 1.025$</td>
<td>Reeburgh et al. (1997)</td>
</tr>
<tr>
<td><strong>Wetlands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swamp forest, estimation from open system mass balance</td>
<td>$1.003-1.021$ (best estimate 1.020)</td>
<td>Happell et al. (1994)</td>
</tr>
<tr>
<td><strong>Contaminated sites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vadose zone above a methanogenic aquifer, estimation from GPPTs</td>
<td>$1.006 - 1.032$</td>
<td>this study</td>
</tr>
</tbody>
</table>
Table 1-9: Factors influencing the apparent fractionation factor for microbial CH$_4$ oxidation

<table>
<thead>
<tr>
<th>Influencing factor</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>Higher temperature $\implies$ higher fractionation</td>
<td>Coleman et al. (1981), King et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Lower temperature $\implies$ higher fractionation</td>
<td>Borjesson et al. (2001), Chanton and Liptay (2000), Tyler et al. (1994), Venkiteswaran and Schiff (2005)</td>
</tr>
<tr>
<td>Methanotrophnic community</td>
<td>Speculative attribution of observed differences to this factor</td>
<td>Kruger et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>No differences between a type I and a type II culture or sMMO and pMMO</td>
<td>Templeton et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Higher fractionation by pMMO than sMMO</td>
<td>Jahnke et al. (1999)</td>
</tr>
<tr>
<td>CH$_4$ concentration</td>
<td>Starting from atmospheric concentrations: the lower the concentration, the lower the fractionation</td>
<td>Tyler et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>The lower the concentration, the lower the fractionation$^a$</td>
<td>Kinnaman et al. (in press), Teh et al. (2006)</td>
</tr>
<tr>
<td>Methanotrophic activity</td>
<td>The higher the activity, the lower the fractionation$^a$</td>
<td>Teh et al. (2006)</td>
</tr>
<tr>
<td>Fraction of CH$_4$ oxidized</td>
<td>The higher the fraction of CH$_4$ oxidized, the lower the fractionation$^a$</td>
<td>Templeton et al. (2006), this study</td>
</tr>
</tbody>
</table>

$^a$ Effect attributed to mass transfer limitation.

1.4.3 Determination of the fractionation factor in a closed system

In a closed system, isotope fractionation can be treated as a Rayleigh distillation process (Mariotti et al. 1981). This means that the remaining substrate becomes increasingly enriched in the heavy isotope, while the instantaneous product at a certain time is always lighter than the substrate by an the enrichment factor $\varepsilon$ which is constant and is approximately $(1-\alpha)\times1000$. As the system is closed, the product has the isotopic signature of the initial substrate when the substrate is totally consumed. The fractionation factor $\alpha_{oa}$ is determined by plotting the natural logarithm of $(\delta^{13}C_{CH_4}+1000)$ at time $t$ over the natural logarithm of the fraction of
substrate remaining \( f \) at this time according to a simplified Rayleigh distillation equation (Snover and Quay 2000). The approximation in this equation, that total CH\(_4\) equals \(^{12}\)CH\(_4\) was found to be valid when natural abundances of isotopes in CH\(_4\) are investigated (Mahieu et al. 2006):

\[
\ln\left(1000 + \delta ^{13}C_{CH_4}(t)\right) = \left(\frac{1}{\alpha_{ax}} - 1\right) \ln f(t) + \ln\left(1000 + \delta ^{13}C_{CH_4}(t_0)\right)
\]

(Equation 1-11)

If the fractionation is constant, \( \alpha_{ax} \) can be calculated after linear regression by:

\[
\alpha_{ax} = \frac{1}{\text{slope} + 1}
\]

(Equation 1-12)

This approach is normally used to determine the fractionation factor in batch cultures or batch incubations of environmental samples. With the assumption that the CH\(_4\) in the background has a constant isotope ratio, diffusion is not an important fractionating process and dilution can be accounted for using a tracer, this approach can also be applied to push-pull tests or gas push-pull tests (Kleikemper et al. 2002). Furthermore, if the fractionation factor is known, this method can be used to determine the oxidized fraction in a closed system.

1.4.4 Quantification of methane oxidation

For the interpretation of isotope ratios observed in the environment, a closed system approach is often not applicable as CH\(_4\) is continuously produced in or transported into the CH\(_4\) oxidation zone. Isotope ratios in an open, flow-through system can be interpreted for example using the mass balance-based approach presented by Liptay et al. (1998). This method is based on the assumption that transport of CH\(_4\) through a porous medium can be represented by a first-order reaction in competition with CH\(_4\) oxidation (De Visscher et al. 2004) and that the system is at steady-state (Blair et al. 1985). In other words, if we imagine a parcel of gas in a porous medium, \(^{12}\)CH\(_4\) could be preferentially removed from this parcel either by diffusion along a concentration gradient along the major transport pathway or by uptake by methanotrophs. This method has been used for the in-situ determination of the fraction of CH\(_4\) oxidized (Liptay et al. 1998) and the fractionation factor (Reeburgh et al. 1997). To calculate the fraction of CH\(_4\) oxidized, the difference between \( \delta ^{13}C \) in produced and emitted CH\(_4\) is related to the difference between the fractionation factor for transport and for microbial oxidation using the following equation (Blair et al. 1985; Liptay et al. 1998):
\[ f_0 = \frac{\delta_e - \delta_a}{(\alpha_{ox} - \alpha_{trans}) \times 1000} \]  

(Equation 1-13)

where \( \delta_e \) is the \( \delta^{13}C \) of emitted CH\(_4\) and \( \delta_a \) is the \( \delta^{13}C \) of produced CH\(_4\) or CH\(_4\) in the anoxic zone, \( f_0 \) is the fraction of CH\(_4\) oxidized, \( \alpha_{ox} \) is the fractionation factor for microbial oxidation and \( \alpha_{trans} \) is the fractionation factor for gas transport. The latter is 1 if transport is exclusively by advection and equals \( \alpha_{diff} \) in the gas phase if transport is only by diffusion. If both transport mechanisms play a role it depends on the importance of diffusion (De Visscher et al. 2004). This may be difficult to assess in an ecosystem. In addition, it is difficult to obtain a fractionation factor \( \alpha_{ox} \) that is valid for an ecosystem under the conditions prevalent at the time of investigation (as discussed above). Therefore, this method can be associated with large uncertainties.

The presented mass balance approach assumes a constant \( \delta^{13}C \) value for CH\(_4\) produced in an anoxic zone that is separate from the oxidation zone. However, methanogenesis and CH\(_4\) oxidation can occur in close proximity and the \( \delta^{13}C \) in produced CH\(_4\) will vary depending on the \( \delta^{13}C \) of the substrate of methanogenesis and on the pathway (Conrad 2005). Therefore, carbon stable isotope patterns observed in the environment may result from complex interactions not only of CH\(_4\) oxidation and transport but also methanogenesis and may be difficult to interpret. One approach to differentiate between processes is to look at the fractionation of stable hydrogen isotopes (i.e. \( \deltaD \) values) in addition to stable carbon isotopes (Bergamaschi and Harris 1995). Another approach to infer information from isotope data for example in a system with complex interactions of CH\(_4\) oxidation and transport is numerical modeling (Gonzalez-Gil et al. submitted-a).
1.5 Scope and outline of the thesis

The main aim of this thesis was to develop a new in-situ method to quantify microbial CH₄ oxidation in the vadose zone. The new method was based on a technique developed to quantify microbial activities in aquifers, the “push-pull test” (Istok et al. 1997). In this work the focus was on the field scale testing and application of the method. Microbial CH₄ oxidation was chosen as a model process for method development as it is a relatively fast process and has two gaseous substrates. The zone above a contaminated aquifer in Studen, Switzerland, an aquifer known from previous studies to produce CH₄ (Bolliger et al. 1999; Kleikemper et al. 2005) was chosen to test the gas push-pull test (GPPT) at the field scale (chapter 2). Experiments were performed at monitoring well PS4 where highest CH₄ concentrations had been found previously. In this first study, apparent first-order rate constants were quantified. In a next step, GPPTs were performed at the same site to extend quantification to Michaelis-Menten parameters, compare results with isotope and profile analysis and characterization of the methanotrophic community (chapter 3). Subsequently, the GPPT was adapted for application in a peat bog, an ecosystem with very different physical properties (chapter 4 and 5). Finally, a CH₄/O₂ counter-gradient column was designed as a model system to test different in-situ methods and investigate the dependency of methanotrophic activity on environmental parameters under controlled conditions (chapter 6). Specifically, the objectives of the individual studies were:

Chapter 2: to assemble the equipment for a gas push-pull test and perform a feasibility study at the field scale to quantify apparent first-order rate constants for CH₄ oxidation above a contaminated aquifer. The study included use of an inhibitor and measurement of stable carbon isotope ratios for verification of microbial CH₄ conversion.

Chapter 3: to quantify Michaelis-Menten parameters at two different depths at the same site as in chapter 2 using several GPPTs with different CH₄ injection concentrations. Furthermore, to compare results with profile calculations and assessment of methanotrophic community structure and to assess the feasibility of using stable carbon isotopes as an independent quantification method.
Chapter 4: to develop a procedure to use CH₄ from a GPPT with the co-injection of an inhibitor as a substitute tracer for quantification of CH₄ oxidation in a peat bog, a system where gas transport during GPPTs is diffusion-dominated. Furthermore, we wanted to assess if, under these conditions, stable carbon isotope fractionation can serve as an indicator for microbial CH₄ oxidation.

Chapter 5: to better understand the extent of observed variability in CH₄ concentrations in the peat bog and possible causes for it. For this purpose permanent samplers, temperature probes and a weather station were installed.

Chapter 6: to design a column with a CH₄/O₂ counter-gradient as a model system mimicking important physical parameters of a methanotrophic environment. In this model system, we wanted to assess methanotrophic activity, its response to changing substrate gradients and associated stable carbon isotope fractionation.

Chapter 7: This chapter comprises a final discussion on some of the key issues of this thesis, especially on the potential and limitations of the gas push-pull test and other in-situ quantification methods.
Chapter 1: General introduction

1.6 References


Chapter 1: General introduction


New Field Method: Gas Push-Pull Test for the In-Situ Quantification of Microbial Activities in the Vadose Zone

Karina Urmann, Graciela Gonzalez-Gil, Martin H. Schroth, Markus Hofer and Josef Zeyer

Published in Environmental Science & Technology (2005), 39, 304-310.
2.1 Abstract

Quantitative information on microbial processes in the field is important. Here we propose a new field method, the "gas push-pull test" (GPPT) for the in-situ quantification of microbial activities in the vadose zone. To evaluate the new method, we studied microbial methane oxidation above an anaerobic, petroleum-contaminated aquifer. A GPPT consists of the injection of a gas mixture of reactants (e.g. methane, oxygen) and non-reactive tracer gases (e.g. Neon, Argon) into the vadose zone and the subsequent extraction of the injection gas mixture together with soil air from the same location. Rate constants of gas conversion are calculated from breakthrough curves of extracted reactants and tracers. In agreement with expectations from previously measured gas profiles, we determined first-order rate constants of 0.68 h⁻¹ at 1.1 m below soil surface and 2.19 h⁻¹ at 2.7 m, close to the groundwater table. Co-injection of a specific inhibitor (acetylene) for methanotrophs showed that the observed methane consumption was microbially mediated. This was confirmed by increases of stable carbon isotope ratios in methane by up to 42.6‰. In the future, GPPTs should provide useful quantitative information on a range of microbial processes in the vadose zone.
2.2 Introduction

Microorganisms play a major role in the turnover of a number of gases in the vadose zone. Examples for important microbial activities involving gaseous compounds are aerobic respiration (Hanson et al. 2000), nitrogen fixation (Vitousek et al. 2002), denitrification, methanogenesis and methane oxidation (Conrad 1996). To better understand the dynamics of these processes it is important to quantify microbial activities in the subsurface. Microbial activities have been frequently studied in laboratory incubation experiments (e.g. Segers 1998). However, laboratory-based methods for quantification of microbial activities have several biases which hamper the extrapolation of the data to the field. Potential biases are for example: a) incubation under conditions that differ from those in the environment, b) changes in soil structure, c) shifts in microbial populations during sampling and incubation and d) typically small sample volumes that are potentially unrepresentative for the system investigated (National Research Council et al. 1993; Istok et al. 1997; Madsen 1998). Therefore, there is a need to complement laboratory-based methods with field-scale in-situ quantification methods (Madsen 1991; Reeburgh 1996; Madsen 1998). Among the methods used so far to assess in-situ microbial activities involved in gas turnover at the field scale are chemical profile measurements, emission measurements using chambers, and investigations of stable isotope fractionation patterns (Levin et al. 1993; Happell et al. 1994; Liptay et al. 1998; Madsen 1998; Kruger et al. 2001).

For this study we chose methane (CH\textsubscript{4}) oxidation as an example activity. The greenhouse gas CH\textsubscript{4} is produced in several ecosystems such as paddy fields, wetlands, landfills (Reeburgh 1996; Milich 1999) and in contaminated aquifers (Bolliger et al. 1999; Cozzarelli et al. 2000; Chapelle et al. 2002). It was estimated that globally more than half of the produced CH\textsubscript{4} is microbially converted to the less effective greenhouse gas carbon dioxide (CO\textsubscript{2}) before it reaches the soil surface (Reeburgh et al. 1993). Percentages of CH\textsubscript{4} oxidized have been quantified in several field studies using the methods mentioned earlier. These studies included profile measurements of CH\textsubscript{4} with 222\textsuperscript{Rn} as a natural tracer (Levin et al. 1993), emission chamber techniques together with inhibition of CH\textsubscript{4} oxidation using either chemical inhibitors (Kruger et al. 2001) or oxygen replacement by nitrogen (Happell et al. 1994), and stable carbon isotope measurements of produced and emitted CH\textsubscript{4} (Liptay et al. 1998). The latter method relies on the fact that methanotrophs preferentially oxidize \textsuperscript{12}CH\textsubscript{4} over \textsuperscript{13}CH\textsubscript{4} so that remaining, unconsumed CH\textsubscript{4} becomes increasingly enriched in \textsuperscript{13}C (Whiticar 1999). Despite their advantages the above described methods have certain limitations. For example,
Chapter 2: Gas push-pull test for the in-situ quantification of microbial activities

calculations of rates from profiles often require fitting of a transport model and knowledge about physical properties of the vadose zone (Lahvis et al. 1999). Chamber methods are limited to assess processes close to the soil surface and are associated with a number of potential measurement errors (Norman et al. 1997). Finally, stable isotope methods are sensitive to the precise knowledge of the microbial isotope fractionation factor (Chanton and Liptay 2000), which in field systems is difficult to obtain.

Here we propose a new in-situ method to quantify microbial activities in the vadose zone that complements the above mentioned approaches. The new method is based on a technique that was developed to quantify microbial activities in aquifers (1997), a so-called “push-pull test”. During an aquifer push-pull test an aqueous test solution containing at least one reactant and a conservative tracer is injected into the groundwater. After a desired incubation period, the mixture of test solution and background groundwater is extracted from the same well. Reaction rate constants and mass balances can then be calculated from breakthrough curves of reactant(s) and tracer(s) (Haggerty et al. 1998; Snodgrass and Kitanidis 1998). Push-pull tests have been successfully applied to investigate a number of microbial activities in contaminated aquifers including aerobic respiration, denitrification, sulfate reduction and reductive dehalogenation (Schroth et al. 1998; Hageman et al. 2001; Kleikemper et al. 2002; Pombo et al. 2002). In the vadose zone, gaseous tracers have mainly been used to determine different physical properties (Johnson et al. 1998; Werner and Höhener 2002). In addition, they have been used to assess oxygen consumption associated with bioventing (Hinchee and Ong 1992; National Research Council et al. 1993). However, to our knowledge, no attempt has been made so far to modify the above described push-pull test for use above the groundwater table, i.e. to develop a gas push-pull test (GPPT) to quantify in-situ microbial activities in the vadose zone.

Thus, the aim of this study was first to assemble adequate equipment for GPPTs that allows the controlled injection and extraction of defined volumes of gas mixtures into and from the vadose zone. Secondly, we wanted to demonstrate the feasibility of the GPPT method by quantifying methanotrophic activity in the vadose zone above a petroleum-contaminated aquifer using noble gases as tracers. Thirdly, stable carbon isotope measurements and acetylene (C2H2) as an inhibitor for methanotrophic bacteria were used in combination with GPPTs to verify microbial activity.
2.3 Gas push-pull test methodology

2.3.1 Test principle

In a GPPT a defined volume of a gas mixture is injected at a certain flow rate into the vadose zone at a location of interest (Figure 2-1a). The injection gas mixture contains one or more reactive gases depending on the microbial activity to be investigated and one or more non-reactive gases that serve as tracers. The injection gas mixture subsequently flows away from the injection point and reactive gases can be consumed by indigenous microorganisms. After injection and a potential additional incubation period without pumping (if further consumption of reactive gases is desired), flow is reversed and the blend of injection gas mixture and background soil air is extracted from the same location (Figure 2-1b). During extraction, the gas blend is sampled periodically and analyzed to obtain breakthrough curves of tracers, reactants and potential metabolic products. The non-reactive tracer can be used to account for any loss of the reactive gas due to dilution and transport if both gases exhibit similar transport behavior. Differences between their relative concentrations can then be attributed to microbial consumption or production and reaction rate constants can be calculated using established protocols (Haggerty et al. 1998; Snodgrass and Kitanidis 1998). In addition, mass balances of all gases can be computed as their injected masses are known.
Chapter 2: Gas push-pull test for the in-situ quantification of microbial activities

a) Injection

Gas bag GFC
Ar Ne CH₄ O₂

Vadose zone

Groundwater

b) Extraction

Serum bottle GFC

Vadose zone

Groundwater

Figure 2-1: Scheme of a) injection and b) extraction during a gas push-pull test (GPPT). GFC: Gas flow controller. An idealized shape of the injected gas mixture (theoretically spherical in a homogenous, isotropic medium) is depleted. Vadose zone depth is not drawn to scale.
2.3.2 Gas flow controller

For injection and extraction we use a gas flow controller (GFC) (Figure 2-2). The core equipment of the GFC is a) a diaphragm pump with a brushless motor (KNF Neuberger, Balterwil, CH), which allows pump rate regulation via a direct-current voltage regulator and b) a mass flow meter (0-2.5 L air/min, M+W Instruments GmbH, Bronkhorst, Reinach, CH) to measure gas flow. The mass flow meter converts the mass of gases in the gas stream to cumulative volume normalized to 0 °C and 1013 mbar. Therefore units of L and mL of gas in this paper all refer to normalized volumes. Mass is converted to volume based on calibration with air. Therefore measured sample concentrations and the corresponding conversion factors for gas mixtures (Bronkhorst, www.fluidat.com) are used to correct the measured volumes. The mass flow meter is protected against backflow with a one-way valve. Additionally, the GFC contains a drying tube, i.e. a coiled acrylic glass tube (tube ID 12 mm, coil OD 12 cm, 3.5 coils) filled with silica gel, a mechanical vacuum gauge (0 to -1 bar), a mechanical pressure gauge (0 to +1 bar) and a 100 mL gas sampling tube with two polytetrafluorethene (PTFE) stopcocks. This sampling tube is connected to the main line by two stainless steel three-way-valves creating a bypass and is placed as close to the outlet of the GFC as possible. The GFC is equipped with two quick connects for easy switching between injection and extraction.

![Figure 2-2: Schematic detail of the gas flow controller (GFC).](image)

2.3.3 Injection and extraction

During the injection phase of a GPPT the injection gas mixture is pumped from a gas bag into the vadose zone using the GFC. To reach the required depth a steel rod (7mm ID, 20mm OD) with a perforated tip is driven into the vadose zone (Figure 2-1a) using equipment similar to that commercially available for soil gas sampling (Eijkelkamp, Giesbeek, The Netherlands). The pressure gauge in the GFC is used to monitor gas pressure relative to
Chapter 2: Gas push-pull test for the in-situ quantification of microbial activities

atmospheric pressure. Samples of the injection gas mixture are taken by temporarily directing the gas flow through the sampling tube.

Gas extraction is achieved by reversing the GFC. The gas mixture that is pumped out of the soil is dried in the drying tube. The vacuum gauge is used to monitor vacuum relative to atmospheric pressure. Samples are collected with pressure at the outlet end of the GFC by flushing scrum bottles closed with butyl rubber stoppers with at least 15 times their volume of extracted gas using two needles with luer connections (Figure 2-1b).

The GFC components, the rod, and the gas bag are connected with PTFE tubing (ID 4.35 mm, OD 6.35 mm) and nylon or stainless steel compressed fittings. The total inner volume of the system from rod tip to the inlet of the gas bag or the sampling outlet is about 350 mL (without sampling tubes).

2.4 Example application

2.4.1 Field site description

We applied the described GPPT method to quantify microbial CH₄ oxidation in the vadose zone above a petroleum-contaminated, anaerobic aquifer in Studen, Switzerland. The site was characterized in detail by Bolliger et al. (1999). We conducted three GPPTs near monitoring well PS4, which is located in the methanogenic zone of the aquifer (Bolliger et al. 1999). Specifically, well PS4 is contained within a 70 cm-diameter concrete casing, and the GPPTs were performed in the annular space between the well and the casing. The annular space was backfilled in 1996 mainly with calcareous coarse sand and gravel with an estimated porosity of 0.39 and with loam from ca. 1.5 to 1.8 m depth. The depth of the groundwater table in well PS4 ranged from 2.88 to 2.90 m during the time of the tests. The CH₄ concentration in groundwater was 0.18 mM, and groundwater temperature was 12.2 °C. One day prior to the first test, we measured a depth profile of CH₄, O₂ and CO₂ in soil air to identify potential zones of methanotrophic activity. We used the GPPT equipment as described for extraction and the analytical methods described later. The sampling rod was gradually pushed downward and soil air was sampled every 10 to 50 cm. To take samples 60 mL clamped scrum bottles closed with butyl rubber stoppers were flushed with at least 2 L of soil air. Between 2.85 and 2.60 m depth, that is just above the groundwater table, we detected a steep gradient of CH₄ with transition to nearly atmospheric concentrations above 2.60 m (Figure 2-3). Assuming diffusion as the major gas transport mechanism and steady-state conditions, this gradient transition indicates an active methanotrophic community that oxidizes the CH₄ presumably
Chapter 2: Gas push-pull test for the in-situ quantification of microbial activities

originating from the groundwater. Conversely, O$_2$ concentrations decreased to values below 10 mL/L near the groundwater table (Figure 2-3), whereas CO$_2$ concentrations increased to over 150 mL/L at that location (data not shown).

![Figure 2-3: Depth profiles of gaseous CH$_4$ and O$_2$ concentrations in the vadose zone above a petroleum-contaminated aquifer near monitoring well PS4 in Studen, Switzerland. Note the different scales of the concentration-axes for CH$_4$ and O$_2$. The arrows indicate the depths at which three gas push-pull tests (GPPTs) were conducted.](image)

2.4.2 Gas push-pull tests

We conducted a first test (GPPT1) at 2.7 m depth (Figure 2-3), a second test (GPPT2) at 1.1 m depth and a third test (GPPT3) again at 2.7 m depth. The depth refers to the position of the rod tip. In all three tests similar injection concentrations and test parameters were used (Table 2-1). All injection gas mixtures contained two non-reactive tracer gases, Argon (Ar, grade 4.8) and Neon (Ne, grade 4.5), and the two reactants of CH$_4$ oxidation, CH$_4$ (grade 2.5) and O$_2$. The mixture in GPPT3 additionally contained C$_2$H$_2$ (grade 2.6), a known gaseous inhibitor for methanotrophs (Chan and Parkin 2000). All mixtures were prepared in air (used as O$_2$ source). Prior to each test, a background sample of soil air was collected at the respective depth by pumping at least 3 L of soil air.
To start each test, around 31 L of gas mixture were injected as described earlier in 51 to 54 min with a pressure of +0.02 to +0.1 bar. Thereafter, about 90 L were extracted in 172 to 174 min with a vacuum ranging from -0.14 to -0.02 bar. Thus, the total duration of each test was around 4 h. Measured gas volumes were corrected for the gas composition of injected and extracted gas mixtures. Corrected volumes were larger than uncorrected volumes by a maximum of 6%. Assuming a range of volumetric water contents of 0.06 - 0.25 cm³/cm³ (calculated from gravimetric water contents that were measured in core samples of the coarse sand and gravel during a dry and a wet period assuming a bulk density of 1.51 g/cm³) and no mixing with soil air, the 31 L of injected gas penetrated a bulk volume of 100 to 240 L in the vadose zone. During the injection period three samples of the gas mixture were collected in pre-evacuated (-0.6 bar) gas sampling tubes (Figure 2-2). During extraction, samples were collected every 2 L in 60 mL clamped serum bottles closed with butyl rubber stoppers with +0.6 bar pressure. Samples 1 to 8 and every second sample from 10 to 45 were analyzed for CH₄, Ne, Ar and O₂, as measured concentrations changed less in later samples. Carbon dioxide was measured only in approximately every fourth sample. The δ¹³C-value of CH₄ was measured in two to three samples of each test, in one sample of an injection gas mixture and in a sample of the CH₄ that was used to prepare the injection gas mixtures. All samples were stored upside down in water at 10 °C and re-equilibrated to room temperature (23 - 25 °C) before analysis.

Table 2-1: Operational parameters for three gas push-pull tests (GPPTs).

<table>
<thead>
<tr>
<th>Test</th>
<th>Depth of rod tip [m]</th>
<th>Injection gas mixture</th>
<th>Injection</th>
<th>Extraction</th>
<th>Total test duration [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPPT1</td>
<td>2.7</td>
<td>Ne 31.5, Ar 194.1, CH₄ 34.8, C₂H₂ -</td>
<td>0.61</td>
<td>31.0</td>
<td>0.52</td>
</tr>
<tr>
<td>GPPT2</td>
<td>1.1</td>
<td>Ne 31.7, Ar 201.6, CH₄ 34.7, C₂H₂ -</td>
<td>0.60</td>
<td>31.8</td>
<td>0.52</td>
</tr>
<tr>
<td>GPPT3</td>
<td>2.7</td>
<td>Ne 32.2, Ar 208.1, CH₄ 32.6, C₂H₂ 20.4</td>
<td>0.58</td>
<td>31.4</td>
<td>0.52</td>
</tr>
</tbody>
</table>

All mixtures were prepared in air and contained 156 to 169 mL/L O₂.

2.4.3 Analytical methods

Methane was measured by gas chromatography (Carlo Erba, GC8000series, Rodano, Italy) with a HayeScp N column and a FID detector. Neon, Ar and O₂ were measured on a gas chromatograph (Shimadzu GC-14 A, Kyoto, Japan) with a TCD detector. This GC was equipped with a molecular sieves column and a valve system to obtain peaks of Ar plus O₂ or
only Ar by reducing the O₂ over heated activated charcoal (Hofer and Imboden 1998). The O₂ concentration was obtained by subtracting the two peaks. At least duplicate measurements of each sample were performed on the GC-FID and at least one with O₂ and one without on the GC-TCD. Carbon dioxide was measured by gas chromatography (Carlo Erba, GC8000series, Rodano, Italy) with a Haysep D column and a TCD detector. To measure CH₄ concentration in the groundwater we used the headspace method described by Bolliger et al. (1999).

The carbon isotope composition of CH₄ was determined using a Precon preparation device (Finnigan, Bremen, Germany): After injecting the sample through a septum into a helium stream, the injected gas passed a liquid-nitrogen trap and a chemical trap (based on NaOH) to remove all CO₂; then the CH₄ from the sample was combusted to CO₂ with 100% efficiency in an oven at 1000 °C. The sample gas was collected in a liquid-nitrogen trap, and later injected into a GC column (Poraplot Q) for separation of interfering gases such as N₂O. The gas was then passed to an isotope-ratio mass-spectrometer (Delta Plus XL, Finnigan, Bremen, Germany) for determination of the masses 44, 45, 46. The isotope ratio was determined by reference to the standard PDB (Peedee Belemnite) (Hoefs 1997) in the δ-notation (Mariotti et al. 1981) in per mil:

\[
\delta^{13}C(\%o) = \left( \frac{^{13}C}{^{12}C} \right)_{\text{sample}} - 1 \times 1000 \quad \text{(Equation 2-1)}
\]

The standard deviation for repeated injection of 0.2 mL of 10 mL/L CH₄ standard gas (in N₂) was 0.4‰.

2.4.4 Data analysis

Concentrations of Ne, Ar and CH₄ in the injection gas mixtures of each of the three GPPTs varied by less than 2% (relative standard deviation). Therefore, average injection concentrations (Table 2-1) were used to calculate relative concentrations \( C^* \) (concentrations in extraction samples divided by the concentration in the injection gas mixture). Before calculating \( C^* \), measured extraction concentrations of CH₄ in GPPT1 and Ar in all tests were corrected for their background concentrations measured in soil air (CH₄ 10 µL/L, Ar 9.6-12.2 mL/L), as these gases approached background concentrations towards the end of the respective tests. To perform these corrections, we used relative Ne concentrations as a
measure for mixing between injection gas mixtures and soil air assuming constant background concentrations (Schroth et al. 1998). From here on corrected values will be referred to as Ar and CH$_4$, respectively, unless otherwise noted.

From relative tracer and reactant concentrations we calculated first-order rate constants $k$ for CH$_4$ consumption using the method of Haggerty (1998) with some modifications. The method was developed to determine first-order rate constants from push-pull tests in aquifers assuming a) that the injection solution is well-mixed within the assayed volume of the aquifer and b) that tracer and reactant transport behavior, i.e. diffusion, advection, sorption and partitioning is similar. It is based on a first-order type reaction and accounts for an injection phase of finite duration, during which reaction occurs. However, as our data suggested little reaction during early stages of the GPPTs (shown below), we simplified the method of Haggerty et al. (1998) and used the following equation:

$$\ln \left( \frac{C^*_t(t^*)}{C^*_o(t^*)} \right) = -kt^* + c \quad \text{(Equation 2-2)}$$

where $C^*$ is relative concentration of tracer (tr) and reactant (r), $t^*$ is time since the end of injection and $c$ is an arbitrary constant. To obtain estimates of $k$, linear regression analysis was applied to the segment of experimental breakthrough curve data that showed a ln-linear relationship.

2.5 Results

2.5.1 Gas push-pull tests

To obtain breakthrough curves of Ne, Ar and CH$_4$, relative concentrations $C^*$ were plotted against the ratio of extracted over injected volume (Figure 2-4). Relative concentrations $C^*$ showed an initially rapid and then slower decrease as the injection gas mixtures became increasingly diluted with soil air during the tests. Relative CH$_4$ concentrations were smaller than Ne concentrations in GPPT1 (Figure 2-4a). This indicated consumption of CH$_4$ in the zone above the groundwater table, presumably due to microbial CH$_4$ oxidation. In GPPT2, CH$_4$ consumption was much smaller and appeared to begin later in the test than in GPPT1 (Figure 2-4b). In GPPT3, the test with C$_2$H$_2$ as inhibitor for methanotrophic bacteria, breakthrough curves of Ne, CH$_4$, Ar (Figure 2-4c) and C$_2$H$_2$ (data not shown) nearly coincided. The absolute C$_2$H$_2$ concentration decreased to 0.9 mL/L at the end of extraction.
Breakthrough curves of Ar corrected for its background in soil air as shown in figure 2-4 were similar to Ne curves in all tests. However, uncorrected Ar curves were higher due to the notable concentration of Ar in soil air (data not shown). Similar to the other gases, O₂ became quickly diluted at the beginning of GPPT1. Subsequently, concentrations fluctuated between 28 mL/L and 91 mL/L during this test. In GPPT2, O₂ concentrations fluctuated in a narrow range between 146 mL/L and 176 mL/L throughout the test (data not shown).

Figure 2-4: Breakthrough curves for the three gas push-pull tests (GPPTs): a) at 2.7m depth with high activity, b) at 1.1m depth with low activity, c) at 2.7m depth with C₂H₂ as inhibitor (C₂H₂ not shown). The curves show relative concentrations C*, that is measured concentrations in extraction samples divided by respective injection concentrations of Ar, Ne and CH₄.
We integrated breakthrough curves (Figure 2-4) to obtain percentages of the injected mass of gases recovered during extraction (Figure 2-5). In all tests between 63 and 72% of Ne and Ar were recovered. Recoveries of CH$_4$ ranged from 41 to 67% with lowest recovery in GPPT1 and highest recovery in GPPT3. In the latter, CH$_4$ recovery was 3.7% higher than Ne recovery, whereas in GPPT1 CH$_4$ recovery was 25.2% and in GPPT2 5.5% lower. The latter values correspond to an overall consumption of 12.2 mmol CH$_4$ (GPPT1) and 2.7 mmol CH$_4$ (GPPT2) during the tests. Recovery of C$_2$H$_2$ in GPPT3 (59.9%) was similar to the recovery of tracers in this test (data not shown).

2.5.2 Rate constants

We used Ne as a tracer to account for dilution of injection gas mixtures with soil air when calculating first-order rate constants $k$ for CH$_4$ consumption from breakthrough curves. For GPPT1 and GPPT2 a lag phase without apparent CH$_4$ consumption was followed by a linear decrease of $\ln \left[ C_{CH_4}(t^*) / C_{Ne}(t^*) \right]$ versus $t^*$, indicating a first-order type reaction (Figure 2-6). The computed first-order rate constant for CH$_4$ consumption was more than three times higher for GPPT1 than for GPPT2 (Table 2-2). Relative confidence intervals (95%) were ± 11.9% (GPPT1) and ± 7.4% (GPPT2) of the respective $k$-value. In contrast, data plotted for GPPT3 showed a slight increase corresponding to a small negative $k$-value, associated with a large relative confidence interval of ± 54.4% (Table 2-2). Oxygen consumption or CO$_2$ production
could not be quantified during the tests due to high and variable background concentrations of these gases (O₂ and CO₂ data not shown).

Figure 2-6: Plot for the determination of first-order rate constants \( k \) for CH₄ oxidation during three gas push-pull tests (GPPTs). The \( k \)-values were calculated as the slopes of the lines fitted by linear regression to the segment of experimental breakthrough curve data where the \( \ln \left| \frac{C_{CH_4}^i(t^*)}{C_{CH_4}^o(t^*)} \right| \) vs. \( t^* \) showed a linear relationship. The calculation procedure was adapted from the method of Haggerty et al. (1998).

Table 2-2: First-order rate constants and stable carbon isotope \((\delta^{13}C)\) values of CH₄ for three gas push-pull tests (GPPTs).

<table>
<thead>
<tr>
<th>Test</th>
<th>First-order rate constant ( k \pm 2\sigma_k ) ([h^{-1}])</th>
<th>( \delta^{13}C ) in CH₄ (^b) ([%])</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPPT1</td>
<td>2.19 ± 0.26</td>
<td>-3.6</td>
</tr>
<tr>
<td>GPPT2</td>
<td>0.68 ± 0.05</td>
<td>-30.7</td>
</tr>
<tr>
<td>GPPT3</td>
<td>-0.07 ± 0.04</td>
<td>-41.8</td>
</tr>
</tbody>
</table>

\(^a\) \( k \)-values are given with their 95\% confidence interval.

\(^b\) measured after extraction of 50 L; \( \delta^{13}C(CH_4) \) in injection gas mixtures was -46.2\%.
2.5.3 Stable carbon isotope ratios

The CH₄ we used to prepare injection gas mixtures had a δ¹³C-value of -46.2‰, which remained unchanged during preparation and handling. In GPPT1 and GPPT2 the extracted CH₄ became enriched in ¹³C over time with a stronger effect in GPPT1 (Table 2-2). Specifically, between injection and extraction of 50 L, the δ¹³C-value increased by 42.6‰ in GPPT1 and by 15.5‰ in GPPT2. In the test with the inhibitor (GPPT3) δ¹³C-values changed only slightly. They initially increased to a value of -41.8‰, but decreased subsequently to -44.2‰ at the end of the extraction.

2.6 Discussion

2.6.1 Test performance

The assembled hardware for GPPTs allowed controlled and reproducible injection and extraction of gas mixtures at a desired, constant rate. The pressure differences induced by pumping were small in the coarse textured material at the site. Reproducibility was indicated by similar extraction concentrations of the tracer gases, i.e. similar shapes of their breakthrough curves (Figure 2-4) and similar mass recoveries (Figure 2-5) in all three tests. The mass recoveries of the tracers (63 to 72%) were in the lower half of a range of 54 to 99% usually reported for groundwater push-pull tests of similar injection volume and test duration (Istok et al. 1997; Schroth et al. 1998; Istok et al. 2001; Schroth et al. 2001). Lower mass recoveries in GPPTs in the vadose zone than in PPTs in groundwater are expected as molecular diffusion in the gas phase is by a factor of ~10⁴ faster than in the aqueous phase (Schwarzenbach et al. 1993). The finding that recoveries were still within the same range may have been partly due to the fact that we performed the tests within a 70 cm-diameter concrete casing, which might have partially confined gas transport. In 2.7 m depth (GPPT1 and GPPT3) gas transport was additionally limited by the groundwater table and a loam layer from ca. 1.5 to 1.8 m depth. However, a similar mass recovery was obtained in GPPT2 where upward gas transport was not restricted. A final conclusion on the effect of this particular site on mass recovery is not possible, but recoveries for GPPTs in locations without artificial boundaries might be lower than in this study. However, it has to be noted that for accurate rate calculations during a GPPT or a PPT high or even complete mass recovery is not necessary (Haggerty et al. 1998).
2.6.2 Quantification of microbial methane oxidation

From GPPT breakthrough curves we calculated \(k\)-values for CH\(_4\) consumption with small 95% confidence intervals. The \(k\)-value at 2.7 m depth was more than three times higher than at 1.1 m depth (Figure 2-6, Table 2-2). Thus, the GPPTs confirmed high methanotrophic activity at 2.7 m depth, close to the groundwater table, and lower activity at 1.1 m depth, which corroborated our qualitative interpretation of the CH\(_4\) gas profile (Figure 2-3).

There is a lack of field data on CH\(_4\) oxidation rate constants and on methanotrophic activity above contaminated aquifers. Therefore, we can mainly compare our data with the rather few laboratory studies where first-order rate constants were measured directly in landfill cover and upland soils. First-order rate constants of 2.57 h\(^{-1}\) and 0.54 h\(^{-1}\) were measured in the core of a landfill topsoil with initial CH\(_4\) concentrations of 77 mL/L and 1.7 \(\mu\)L/L and ambient O\(_2\) concentrations in the jar headspace respectively (Whalen et al. 1990). Despite the differences in methodology and ecosystem, these values correspond well to our values of 2.19 h\(^{-1}\) (GPPT1) and 0.68 h\(^{-1}\) (GPPT2). In contrast, in a laboratory batch study with a different landfill topsoil a maximum \(k\) of 0.3 h\(^{-1}\) was measured under elevated CH\(_4\) concentrations (Chan and Parkin 2000). Additionally, a wide range of \(k\)-values from 0.007 h\(^{-1}\) to 1.17 h\(^{-1}\) was measured in soils exposed to (nearly) atmospheric CH\(_4\) concentrations. These are maximum or average values from several laboratory and one field chamber study (Whalen and Reeburgh 1990; Adamsen and King 1993; Koschorreck and Conrad 1993; Schnell and King 1994; Kruse et al. 1996; Gulledge and Schimel 1998; Wang et al. 1999). The range comprises the \(k\)-value we measured at 1.1 m depth, where the CH\(_4\) background concentration was close to atmospheric. In GPPT3, we computed a small negative \(k\)-value (Figure 2-6, Table 2-2). This could have been caused by an increase in background concentration due to the inhibition of CH\(_4\) oxidation.

During our tests, CH\(_4\) oxidation was not O\(_2\)-limited as we injected O\(_2\) and CH\(_4\) at a ratio of more than 4:1 (Table 2-1), but only a ratio of 2:1 is stochiometrically required for complete oxidation. Similarly, CH\(_4\) oxidation was also not O\(_2\)-limited under in-situ conditions (Figure 2-3). Thus, while we altered the O\(_2\) background concentration during the tests, we did not change the system with respect to the limiting substrate. At the beginning of GPPT1 and GPPT2 we observed a lag phase with no apparent CH\(_4\) consumption for which the reason is not clear. Following this lag phase, first-order rate constants were determined over a range of concentrations from the onset of apparent consumption (at < 20% of the injection concentration) down to the last extraction sample. Even though overall turnover of CH\(_4\) during the tests was presumably higher than in-situ turnover (due to the addition of CH\(_4\)) our
data suggested that the CH₄ concentrations in this range were low enough so that the assumption of a first-order type reaction was reasonable. In addition, for GPPT1 this range comprised in-situ CH₄ concentrations as measured in the depth profile. Conversely, it is possible that e.g. for higher test concentrations and/or slower processes different kinetic models such as zero-order kinetics (Snodgrass and Kitanidis 1998) or Michaelis-Menten kinetics (Istok et al. 2001) would apply. In these cases other computation methods (Snodgrass and Kitanidis 1998; Istok et al. 2001) could probably be adapted to the GPPT, or gas transport modeling could be used to estimate kinetic parameters from GPPT breakthrough curves.

2.6.3 Verification of microbial methane oxidation

We used two approaches to verify that the measured consumption of CH₄ was microbially mediated. First, comparing the breakthrough curves of the tests with and without the specific inhibitor C₂H₂ for methanotrophs suggested that apparent CH₄ consumption in GPPT1 and GPPT2 was due to microbial CH₄ oxidation (Figure 2-4). Moreover, the following findings indicated that inhibition of CH₄ oxidation was reasonably efficient: (a) In GPPT3 breakthrough curves of CH₄ and Ne were similar (Figure 2-4c) and (b) at the end of extraction of this test the concentration of the inhibitor C₂H₂ was still nine times more than the concentration needed for effective inhibition of CH₄ oxidation in laboratory tests (Chan and Parkin 2000). Second, in GPPT1 and GPPT2 the remaining CH₄ became substantially enriched in ¹³C with a larger effect in GPPT1 (Table 2-2). Conversely, in the test with C₂H₂ as inhibitor changes in δ¹³C were small and without an apparent trend. This clearly indicated that CH₄ consumption in GPPT1 and GPPT2 was microbially mediated.

Due to the lack of robust and easy-to-use field methods, quantitative verification of the measured rate constants in the field is difficult. An independent method, e.g. quantitative interpretation of the measured CH₄ profile (Figure 2-3), would require estimation of vadose zone physical parameters. But the latter are typically associated with relatively high uncertainties and therefore would probably yield only rough estimates for CH₄ oxidation activity. Further work is under way to assess the feasibility of using stable carbon isotope fractionation as a means to independently quantify rates of CH₄ oxidation.

2.6.4 Operational considerations

To calculate first-order rate constants from GPPT breakthrough curves based on the simplified method of Haggerty et al. (1998), it is important that the physical transport
behavior of the tracer and the reactant are similar. The properties of the noble gases Ne and Ar that we used as tracers are relatively close to CH₄ closer than for example He. Their diffusion coefficients in air (at 15 °C in cm²/s) are 0.215 for CH₄ (Massman 1998), 0.289 for Ne and 0.179 for Ar (both calculated according to Fuller et al. (1966)). Their Henry constants (at 15 °C in (mol/L gas) / (mol/L water)) are 25.7 (CH₄) (Perry and Green 1997), 87.7 (Ne), 25.1, (Ar) (both calculated according to Shan and Pruess (2003)). Despite some differences in properties, breakthrough curves of Ne and CH₄ in the test with the inhibitor (GPPT3) indicated similar transport behavior under our test conditions (Figure 2-4c). This allowed us to use Ne as a conservative tracer for calculations of rate constants of CH₄ consumption. Uncorrected Ar was not suitable as a tracer due to its notable background concentration in soil air. However, after correcting for Ar contained in the background (using Ne as a tracer), Ar showed a transport behavior similar to the other gases (Figure 2-4c). For future applications of GPPTs it will be important to further evaluate the physical transport behavior of gases during GPPTs under different environmental and test conditions, and to identify suitable tracers for other reactants. Laboratory studies and numerical simulations are being conducted to address these issues.

Additionally, a compromise has to be found between a) sufficient mass recovery of tracer(s) and reactant(s) and b) a sufficiently long contact time between reactant(s) and microorganisms to be able to quantify the activity of interest at a given site. This contact time is determined by factors such as overall duration of a test and the speed of dilution. The test conditions applied in this study were suitable to quantify CH₄ oxidation even in a zone where activity was relatively low (GPPT2). However, test conditions with respect to contact time and mass recovery may have to be optimized for different environmental conditions, especially in zones with low activities and when small injection volumes are desired to allow high spatial resolution.

In summary, we demonstrated that GPPTs are technically feasible and can be performed reproducibly in the vadose zone. We were able to quantify first-order rate constants for CH₄ oxidation above a petroleum-contaminated aquifer that confirmed expectations from CH₄ profile measurements. We think that this method has the potential for further applications. With some modifications other processes in the vadose zone such as soil respiration or N₂-fixation may be investigated. Further research is under way to investigate the capabilities and limitations of this new in-situ method.
2.7 Acknowledgements

We thank Matthias Saurer and Rolf Siegwolf (PSI, Villigen, CH) for help with stable carbon isotope measurements, Rolf Kipfer (EAWAG, Dübendorf, CH) for help with noble gas and oxygen measurements, and Patrick Höhener (EPFL, Lausanne, CH) for help with initial profile measurements. MHS would like to acknowledge Jonathan D. Istok (Oregon State University, Corvallis, USA) for many discussions on the subject of this work. Helpful comments by two anonymous reviewers were greatly appreciated. Financial support was provided in part by ETH Zürich and by the Swiss National Science Foundation (SNF).
Chapter 2: Gas push-pull test for the in-situ quantification of microbial activities

2.8 References


Pombo, S. A., O. Pelz, M. H. Schroth and J. Zeyer (2002). Field-scale ¹³C-labeling of phospholipid fatty acids (PLFA) and dissolved inorganic carbon: tracing acetate


Assessment of Microbial Methane Oxidation Above a Petroleum-Contaminated Aquifer Using a Combination of In-Situ Techniques

Karina Urmann, Martin H. Schroth, Matthias Noll, Graciela Gonzalez-Gil and Josef Zeyer

Submitted to Journal of Geophysical Research-Biogeosciences
3.1 Abstract

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 $V_{\text{max}}$ of $0.70 \pm 0.15$ mmol CH$_4$ (L soil air)$^{-1}$ h$^{-1}$ and an apparent $K_m$ of $0.28 \pm 0.09$ 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, $K_m$ ($0.13 \pm 0.02$ mmol CH$_4$ (L soil air)$^{-1}$) was in a similar range, but $V_{\text{max}}$ ($0.076 \pm 0.006$ 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 ($2.0 \pm 0.03$ h$^{-1}$) were in good agreement. Above the groundwater table, a $V_{\text{max}}$ 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 $\alpha$ 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.
3.2 Introduction

Microbial methane (CH₄) oxidation is a major sink for CH₄ in the subsurface. It reduces or eliminates emissions of this greenhouse gas from different methanogenic environments such as landfills, rice paddies, wetlands and contaminated aquifers (Reeburgh et al. 1993; Reeburgh 1996; Conrad et al. 1999). Even large amounts of CH₄ that can occur in the vadose zone above contaminated aquifers are generally fully oxidized to CO₂ (Revesz et al. 1995; Conrad et al. 1999). However, there is little quantitative information about the kinetics of CH₄ oxidation in such subsurface environments. Quantitative data on the activity and capacity, i.e. maximum activity $V_{\text{max}}$, of methanotrophs are important to understand CH₄ turnover and effects of changing conditions on potential CH₄ emissions. 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 CH₄ into the vadose zone. This could lead to CH₄ emissions if the methanotrophic population cannot respond quickly to increased substrate availability.

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 CH₄ oxidation (Urmann et al. 2005). The GPPT consists of the injection of a gas mixture containing the reactants CH₄ and O₂ and the non-reactive 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 CH₄ oxidation can be calculated from CH₄ and tracer concentration data using simplified methods that do not require knowledge of physical parameters of the system (Urmann et al. 2005; Schroth and Istok 2006). Michaelis-Menten parameters $V_{\text{max}}$ and $K_m$ describing the capacity of the methanotrophic community and its affinity to CH₄ have been inferred from GPPTs by inverse modeling (Gonzalez-Gil et al. submitted-a). However, modeling the physical transport of tracer and reactant gases during a GPPT in a heterogeneous system is challenging. Therefore a simplified approach to estimate Michaelis-Menten parameters is needed.
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 et al. 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 when the physical properties of the soil are reasonably well known (Fechner and Hemond 1992; Damgaard et al. 1998). For the use of stable carbon isotope fractionation it is critical to know the fractionation factor $\alpha$ for microbial CH$_4$ oxidation that applies under the prevalent conditions in the field (Chanton and Liptay 2000). A wide range of $\alpha$ from 1.003 to 1.039 (references in Snover and Quay 2000; 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.

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 (Knief et al. 2003; Jaatinen et al. 2005), to our knowledge, diversity of methanotrophic bacteria in the vadose zone above contaminated aquifers has not been investigated.

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 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 \( \alpha \) was constant during GPPTs with a range of
\( \text{CH}_4 \) injection concentrations.

Estimates of parameters for microbial \( \text{CH}_4 \) oxidation from GPPTs and profile calculations
were in good agreement. Low affinity \( \text{CH}_4 \) oxidation was observed at two different depths
with a high \( \text{CH}_4 \) oxidizing capacity and therefore high buffer capacity for \( \text{CH}_4 \) close to the
groundwater table. In agreement with low affinity kinetics, the detected microorganisms were
closely related to known methanotrophs. High variability of the isotope fractionation factor
for \( \text{CH}_4 \) oxidation was observed, which complicates the use of stable carbon isotope analysis
for in-situ quantification.

3.3 Materials and methods

3.3.1 Field site

Microbial \( \text{CH}_4 \) oxidation was quantified in the vadose zone above a petroleum-
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 \( \text{CH}_4 \)
concentrations of 28 \( \mu \text{L} \text{ L}^{-1} \) 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).

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 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 \( \text{CH}_4 \) concentration in the groundwater remained
stable at 0.10 mM, while groundwater temperature increased from 16.4 to 17.2 °C.
3.3.2 Gas push-pull tests

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 3-1). The depth refers to the depth of the tip of the injection rod. The injection gas mixtures contained the reactants CH₄ and O₂, and the non-reactive tracer gases Ar and Ne. At each depth, a test with a low (GPPT A and C) and a high (GPPT B and D) CH₄ injection concentration was performed. At 2.7 m, an additional test (GPPT E) was conducted similar to GPPT C but with a low O₂ concentration (Table 3-1). 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 3-1) 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 pre-evacuated ( -0.6 bar). Using methods described previously, samples were analyzed for CH₄ and CO₂ (Urmann et al. 2005) and for Ne, Ar and O₂ (Gonzalez-Gil et al. submitted-b). Carbon dioxide data are not shown as little information was gained from these data similar to a previous study (Urmann et al. 2005).

Table 3-1: Operational parameters for GPPTs

<table>
<thead>
<tr>
<th>GPPT</th>
<th>Depth [m]</th>
<th>Injection concentrations [mL L⁻¹]</th>
<th>Ne</th>
<th>CH₄</th>
<th>O₂</th>
<th>Injection Volume [L]</th>
<th>Pump rate [L min⁻¹]</th>
<th>Extraction Volume [L]</th>
<th>Pump rate [L min⁻¹]</th>
<th>Total time [h]</th>
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<tr>
<td>A</td>
<td>1.1</td>
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<td>34</td>
<td>0.68</td>
<td>90</td>
<td>0.55</td>
<td>3.6</td>
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<tr>
<td>B</td>
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<td>0.66</td>
<td>90</td>
<td>0.55</td>
<td>3.6</td>
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<tr>
<td>C</td>
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<td>32</td>
<td>0.62</td>
<td>78</td>
<td>0.50</td>
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<td>D</td>
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<td>81.3, 79.6, 213.0</td>
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<td>E</td>
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<td>78</td>
<td>0.51</td>
<td>3.5</td>
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</tr>
</tbody>
</table>

*Refers to the depth of the tip of the injection rod measured from the soil surface.

*Injection mixtures additionally contained 200 mL L⁻¹ Ar and were prepared in N₂.
3.3.3 Estimation of kinetic parameters

Relative concentrations ($C^*$) were calculated from injection concentrations (Table 3-1) and extraction concentrations, corrected for their background concentrations measured in soil air ($\text{CH}_4$ 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.

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 reaction time $t_R$ was calculated for each “parcel” of gas which is the time from its injection until its extraction:

$$t_R = t^* + \frac{\int_0^{t_{\text{ext}}} Q_{\text{ext}} C_{\text{Ne}}(t) dt}{M_{\text{Ne}} T_{\text{inj}}}$$

(Equation 3-1)

where $t^*$ is time since end of injection, $Q_{\text{ext}}$ is the extraction pump rate, $t_{\text{ext}}$ is time since extraction began, $M_{\text{Ne}}$ is the total mass of the tracer Ne injected and $T_{\text{inj}}$ is the injection time. Subsequently, the natural logarithm of the ratio of relative reactant and tracer concentration was plotted against reaction time (Equation 3-2). In case of a linear relationship, the slope $k$ gives a first-order rate constant. Note that the non-reactive tracer Ne thereby accounts for dilution of the injected gas with soil air.

$$\ln\left( \frac{C_{\text{CH}_4}^*}{C_{\text{Ne}}^*} \right) = -kt_R$$

(Equation 3-2)

To estimate Michaelis-Menten parameters, data plotted according to equation 3-2 were evaluated in segments. Extraction samples where 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...
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over the segment. Parameters $K_m$ and $V_{\text{max}}$ 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 $V_{\text{max}}$ over $K_m$. Standard errors for $K_m$, $V_{\text{max}}$ and $k$ were derived from standard errors of the corresponding slope and intercept.

3.3.4 Gas profiles

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 pre-evacuated (-0.6 bar) and, close to the groundwater table, samples were taken every 15 cm.

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 towards the soil surface. The effective diffusion coefficient 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).

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.

3.3.5 Molecular analysis

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.

3.3.5.1 Total cell counts

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: i) 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. ii) 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. iii) 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 due to 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.

3.3.5.2 DNA extraction, PCR and DGGE

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).

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).

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.

DGGE was performed in a denaturing gradient of 30 to 65% at 200 V for 5 h as described previously (Muyzer et al. 1993). Gels were stained for 30 min with GelStar® (Cambrex Bio
3.3.5.3 Cloning and sequencing

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. Non-chimeric pmoA sequences were integrated into a self-created pmoA-database, which was constructed from all available public pmoA sequences retrieved from GenBank (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 been deposited in the EMBL nucleotide sequence database under the accession nos. ABxxx to ABxxx.

3.3.6 Stable carbon isotope fractionation factor

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 δ¹³C-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 (Snover and Quay 2000; Mahieu et al. 2006):

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

(Equation 3-3)

where \( f \) is the fraction of CH₄ remaining corrected for dilution using the Ne breakthrough curves and \( \alpha_{ox} \) is the fractionation factor defined for first-order kinetics as:
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 $\alpha_{ox}$ is defined as (Hunkeler and Aravena 2000):

$$\alpha_{ox} = \frac{^{12}V_{\text{max}} / ^{12}K_m}{^{13}V_{\text{max}} / ^{13}K_m}$$  \hspace{1cm} (Equation 3-5)
Figure 3-1: Methane and Ne breakthrough curves for GPPTs with low (A,C,E) and high (B,D) CH₄ injection concentrations. Note the discontinuous y-axis for GPPT A, B and C.
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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 reaction time (Figure 3-2). 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 3-2). Only at the very beginning of extraction of GPPT C a curvature of the graph was observed.

![Figure 3-2: Plots for rate calculations from GPPTs at 1.1 m and 2.7 m. Only data included in the analysis are shown. Segments indicated by double-arrows were used to estimate Michaelis-Menten parameters.](image)

The dependency of subsequently computed CH$_4$ oxidation rates on CH$_4$ concentrations was well described by the Michaelis-Menten equation (Figure 3-3). 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 3-2). The apparent first-order rate constant was a factor of four higher at 2.7 m than at 1.1 m depth (Table 3-2). The $k$-values calculated from the later segment of GPPT A and from GPPT E (Table 3-2) agreed well with $k$-values computed from $K_m$ and $V_{max}$ at 1.1 m ($0.60 \pm 0.14$ h$^{-1}$) and at 2.7 m depth ($2.5 \pm 1.28$ h$^{-1}$). Errors associated with the latter values were much higher due to 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 3-2) due to more scatter (Figure 3-3). At 1.1 m, background concentrations were too high to observe O$_2$ consumption. At 2.7 m it was apparent in all tests, but not quantified.
Figure 3-3: 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 3-2: 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 aquifer.

<table>
<thead>
<tr>
<th>Depth</th>
<th>Data set used</th>
<th>$V_{max} \pm \sigma^a$ [mmol (L soil air)$^{-1}$ h$^{-1}$]</th>
<th>$K_m \pm \sigma$ [mmol (L soil air)$^{-1}$]</th>
<th>$k \pm \sigma$ [h$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 m</td>
<td>GPPT A,B</td>
<td>0.076 $\pm$ 0.006</td>
<td>0.13 $\pm$ 0.02</td>
<td>0.53 $\pm$ 0.05</td>
</tr>
<tr>
<td></td>
<td>GPPT A$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.7 m</td>
<td>GPPT C,D,E</td>
<td>0.70 $\pm$ 0.15</td>
<td>0.28 $\pm$ 0.09</td>
<td>2.0 $\pm$ 0.03</td>
</tr>
<tr>
<td></td>
<td>GPPT E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Profile$^c$</td>
<td></td>
<td></td>
<td>1.9</td>
</tr>
</tbody>
</table>

$^a$ 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.

The CH$_4$, O$_2$ and CO$_2$ gas profiles were similar to previous observations at this site, indicating similar activity (Figure 3-4, 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 3-4). An apparent first-order rate constant computed from the profile (1.9 h$^{-1}$) agreed well with the results from GPPTs (Table 3-2). From apparent first-order rate constants and in-situ CH$_4$ concentrations we estimated that an average of 1.1 $\mu$mol 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$^{-1}$.
3.4.2 Methanotrophic community

At 2.7 m depth, the number of DAPI-stained cells per g dry weight \((3.4 \times 10^7 \pm 1.6 \times 10^6, \pm 1\) standard error for 6 wells) were 115 fold higher than at 1.1 m depth \((3.0 \times 10^5 \pm 4.0 \times 10^4)\). 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 3-3). Based on the retrieved sequences, there were no discernible differences between the clone libraries at both depths. Obtaining sufficient PCR product for DGGE analysis was difficult due to 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).

Figure 3-4: Methane and \(O_2\) gas profiles above the contaminated aquifer.
3.4.3 Stable carbon isotope fractionation factor

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%o 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 3-5). 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 CH4 oxidized (Figure 3-5). 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 CH4 extraction concentrations, i.e. lowest CH4 availability, high activity and the highest fraction of CH4 oxidized. In contrast, the highest apparent α of 1.032 was found in GPPT B, the test with the highest CH4 extraction concentrations, i.e. highest CH4 availability, low activity and the lowest fraction of CH4 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.
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3.5 Discussion

3.5.1 Methane oxidation activity and kinetic parameters

In this study, we presented a novel method to estimate Michaelis-Menten kinetic parameters from a series of GPPTs with different CH$_4$ injection concentrations. 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 two. Good agreement between apparent first-order rate constants obtained at 2.7 m using two independent methods, confirmed that parameter estimates were reasonable.

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 - 58 μM reported for landfill cover soils (Kightley et al. 1995; Gebert et al. 2003 and references therein) 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 CH₄ 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 CH₄ oxidation at ambient CH₄ 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 CH₄ oxidation in immediate response to increased CH₄ concentrations. Similar to our study, an immediate response of CH₄ oxidation to increased CH₄ concentrations was observed in an upland forest soil (Mohanty et al. 2006).

In agreement with elevated CH₄ 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 CH₄ 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 CH₄ (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 CH₄ concentrations (Whalen and Reeburgh 1996).

Based on activity per area, in-situ CH₄ oxidation in the zone above the groundwater table prior to GPPTs was similar to CH₄ oxidation observed in peat bogs (Whalen and Reeburgh 2000; Pearce and Clymo 2001) but three orders of magnitude lower than in landfill cover soils (Kightley et al. 1995; De Visscher et al. 1999).

Comparing the in-situ rate prior to GPPTs with $V_{max}$, we estimated that the CH₄ 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 in De Visscher and Van Cleemput 2003) and in a forest soil incubated under elevated CH₄ concentrations doubling times of 20 - 60 d were reported (Mohanty et al.
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 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).

3.5.2 Methanotrophic communities

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). Based on our results, the methanotrophic communities above the contaminated aquifer appear to be similar to those in other ecosystems. 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.

3.5.3 Isotope fractionation factors

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. Changes in stable isotope signatures during a GPPT where 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 from GPPT1 of a previous study (Gonzalez-Gil et al. submitted-a). 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. The reason for the small effect of diffusional fractionation was likely that GPPTs were
performed within a 70 cm-diameter concrete casing, which partially restricted gas transport (Urmann et al. 2005).

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 in 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 due to 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 due to lower activity.

3.6 Conclusions and implications

We studied microbial CH$_4$ oxidation above a methanogenic aquifer in Studen, Switzerland using gas push-pull tests (GPPTs), gas profile calculations, stable isotope measurements and molecular methods. We presented a novel method to estimate Michaelis-Menten parameters from GPPTs covering a range of CH$_4$ injection concentrations, which can be applied in future studies. Good agreement between gas profile calculations and GPPTs confirmed that reasonable rate constant estimates were obtained.

Estimated $K_m$-values indicating "low affinity" CH$_4$ oxidation both above the groundwater table and higher in the profile coincided with the detection of clones affiliated to cultured methanotrophic species known to perform low affinity oxidation. 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 ~600 times higher than in-situ oxidation prior to GPPTs. Therefore, much higher CH$_4$ fluxes 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 are not expected at this site unless large water table fluctuations occur or the methanotrophic zone is bypassed physically, e.g. due to strong advective fluxes or preferential flow paths. 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.

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 pre-requisite for the quantification of CH$_4$ oxidation from stable isotope ratios. Therefore high variability of the fractionation factor even at a single location complicates the use of this approach as an independent quantification method.

### 3.7 Acknowledgements

We thank Matthias Saurer and Rolf Siegwolf (PSI, Villigen, Switzerland) for help with stable carbon isotope measurements and Elena Norina (ETHZ) for help with molecular assays in the laboratory.
3.8 References


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Quantification of Microbial Methane Oxidation in an Alpine Peat Bog

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and Josef Zeyer

Submitted to Vadose Zone Journal
4.1 Abstract

Methane (CH₄) is an important greenhouse gas that is produced in different subsurface environments. Its main biological sink, microbial CH₄ oxidation, can be quantified in situ in the vadose zone using gas push-pull tests (GPPTs). This field method is based on the comparison of breakthrough curves of the reactant CH₄ and a non-reactive tracer. Under diffusion-dominated transport conditions, previously employed noble gases are unsuitable as tracers to calculate rate constants for CH₄ oxidation due to differing diffusion coefficients. Here, we show that by performing two consecutive GPPTs and co-injecting acetylene (C₂H₂) as an inhibitor of CH₄ oxidation in the second test, the reactant CH₄ can be used as a substitute tracer. Applying this procedure, apparent first-order rate constants for CH₄ oxidation ranging from 0.38 to 0.82 h⁻¹ were obtained in the vadose zone of three hummocks in an alpine peat bog near Lucerne, Switzerland. Corresponding estimated in-situ rates ranged from 4 - 299 ng CH₄ [gram dry weight]⁻¹ h⁻¹. In all but one GPPT, strong stable carbon isotope fractionation due to diffusion masked isotope fractionation due to microbial oxidation. Therefore, stable carbon isotope fractionation is suitable only to a limited extent as an indicator of microbial oxidation during a GPPT with diffusion-dominated gas transport. In contrast, the presented procedure for the quantification of microbial CH₄ oxidation using GPPTs can be applied without restrictions even in systems with high porosity. Furthermore, the presented method may be useful for the quantification of other processes for which suitable inhibitors but no suitable tracers are available.
4.2 Introduction

Methane (CH₄) is the second most important greenhouse gas after carbon dioxide (Ehhalt et al. 2001). To understand the factors that influence CH₄ emissions from different subsurface environments such as wetlands or peat bogs (Segers 1998; Whalen and Reeburgh 2000), it is essential to quantify microbial CH₄ oxidation, a process which was estimated to reduce overall CH₄ emissions by approximately 58% (Reeburgh et al. 1993). In addition to controlled laboratory experiments, it is important to quantify microbial processes in situ at the field scale to obtain rates or rate constants that may be more representative for the studied environment (Reeburgh 1996; Madsen 1998).

A new method, the gas push-pull test (GPPT), was developed and applied to quantify methanotrophic activity in situ in the vadose zone above a petroleum-contaminated aquifer (Urmann et al. 2005). The GPPT consists of the injection of a gas mixture of the reactants CH₄ and O₂ and non-reactive tracers, for example neon (Ne) and argon (Ar), into the vadose zone at a specific location. While the injected mixture is transported away from the injection point, the reactants are consumed by indigenous microorganisms. Subsequently, the gas mixture is extracted together with soil air from the same location. First-order rate constants for CH₄ oxidation can be calculated from breakthrough curves of extracted CH₄ and a tracer using simplified methods that do not require knowledge of physical parameters of the investigated environment (Haggerty et al. 1998; Schroth and Istok 2006). An important prerequisite for the application of these methods is that CH₄ and tracer exhibit similar transport behavior. During a GPPT, transport occurs via advection, induced by pumping, and gas-phase diffusion. The relative importance of both processes depends on the applied test conditions and the properties of the investigated subsurface environment.

In a first application, GPPTs were performed above a contaminated aquifer within a 70 cm-diameter concrete well-casing (Urmann et al. 2005). Under these apparently restricted transport conditions, Ne was a suitable non-reactive tracer for CH₄. However, in an open system with diffusion-dominated transport, noble gases or other tracer gases may be unsuitable as tracers for CH₄ due to differing diffusion coefficients (Fuller et al. 1966). When simplified methods cannot be applied due to the lack of a suitable tracer, GPPTs can be evaluated by inverse modeling (Haggerty et al. 1998). However, this is challenging in heterogeneous environments as the required physical parameters are spatially variable and therefore difficult to determine.
Inhibitors for CH$_4$ oxidation can be used to directly compare the transport of CH$_4$ and a tracer during a GPPT without the influence of microbial conversion and to verify that CH$_4$ oxidation is microbially-mediated. This is particularly important when applying the GPPT in a subsurface environment that has previously not been studied with this method. Acetylene (C$_2$H$_2$), a frequently applied gaseous inhibitor for methanotrophic activity (e.g. McDonald et al. 1996; Frenzel and Karofeld 2000) has been shown to effectively inhibit CH$_4$ oxidation during GPPTs (Urmann et al. 2005).

A different approach to verify microbial CH$_4$ oxidation is stable carbon isotope analysis (Liptay et al. 1998; Conrad et al. 1999), which is based on the preferential oxidation of $^{12}$CH$_4$ over $^{13}$CH$_4$ by methanotrophic bacteria causing isotope fractionation (Whiticar 1999). However, the use of this approach is complicated by fractionation due to gas-phase diffusion of CH$_4$ with a fractionation factor of similar magnitude as CH$_4$ oxidation (De Visscher et al. 2004). During GPPTs in which the importance of diffusion was reduced due to restricted transport conditions, stable carbon isotope fractionation served as an additional indicator for microbial CH$_4$ oxidation (Urmann et al. 2005). However, the usefulness of this approach during GPPTs with diffusion-dominated transport conditions remains to be investigated in the field.

The vadose zone of a peat bog represents an extreme case for an ecosystem with unrestricted gas transport as peat bogs have a high porosity of up to 0.98 (Kellner and Lundin 2001). Furthermore, the vadose zone is generally limited in vertical extent due to a high water table, so that losses of injected gases to the atmosphere may occur. Peat bogs are an important source of CH$_4$ emissions (Bartlett and Harriss 1993). In a number of studies CH$_4$ oxidation has been quantified in peat material in laboratory incubations (e.g. Sundh et al. 1994; Whalen and Reeburgh 2000). Furthermore, CH$_4$ oxidation has been quantified in peat cores under close to in-situ conditions (Pearce and Clymo 2001) and rates have been inferred from CH$_4$ profiles (Fechner and Hemond 1992). However, to our knowledge, no attempts have been made to measure rate constants of CH$_4$ oxidation in situ in a peat bog.

Here we present a method to quantify CH$_4$ oxidation in a highly porous system such as a peat bog by using the reactant CH$_4$ as a substitute tracer during GPPTs. This was achieved by performing two consecutive GPPTs at the same location with the co-injection of C$_2$H$_2$ in the second test. Specifically, the aims of this study were to (1) show the feasibility to quantify CH$_4$ oxidation using CH$_4$ as a substitute tracer in the vadose zone of a peat bog as an example application and to (2) assess if stable isotope fractionation can be used as an indicator of microbial CH$_4$ oxidation during a GPPT under diffusion-dominated transport conditions.
4.3 Materials and methods

4.3.1 Field site

GPPTs were performed at a drained but partially regenerated elevated peat bog located at 960 m above sea level in Eigenthal, above the city of Lucerne, Switzerland. GPPTs were performed in three different hummocks during 2005 (H1, H2, GPPT 1 and 2) and 2004 (H3, GPPT 3). The top 20 to 30 cm of hummocks consisted of living and sparsely degraded Sphagnum mosses with increasing humification below 30 cm depth.

To determine CH₄ gas concentration profiles prior to GPPTs, samples were collected through permanently installed stainless steel needles (1 mm ID) with 3-way valves at 10 cm depth intervals. Samples were taken slowly with a syringe that was flushed twice with 1 mL sample gas before injection of 4 mL sample into 8 or 13 mL N₂-flushed vials. Methane concentrations were measured by GC-FID as described previously (Urmann et al. 2005). Dilution was determined by measuring pressure in the vials in the laboratory before and after sampling using a manometer (Keller AG, Winterthur, Switzerland) with a needle attached to it to account for pressure differences between the site and the laboratory.

At the lowest measured depth, high CH₄ gas concentrations occurred at H1 while CH₄ concentrations were only slightly elevated at H2, and around ambient at H3 (Figure 4-1). Water table levels in the hummocks were similar in H1 and H2, but lower in H3 (Figure 4-1). Methane concentrations in the water were 0.47 mM at 82.5 cm depth at H1 and 0.2 mM at 50 cm at H2 (in a saturated layer above the water table). Volumetric water contents, estimated from TDR measurements in the vicinity, were 0.48 and 0.75 cm³ cm⁻³ at 40 and 50 cm depth (H1), 0.65 cm³ cm⁻³ at 40 cm depth (H2) and 0.57 cm³ cm⁻³ at 50 cm depth (H3). Temperatures between 20 and 60 cm depth were on average 12 °C at H1 and 9 °C at H2. Water-phase CH₄ concentrations and temperature were not determined in H3 as installations were not yet completed.
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4.3.2 Gas push-pull tests

At each of the three hummocks, an “active” GPPT (designated “a”) to determine CH$_4$ oxidation activity was followed by an “inactive” GPPT (designated “i”) as a reference (Table 4-1). All injection gas mixtures contained the substrates of CH$_4$ oxidation, CH$_4$ and O$_2$, and the non-reactive tracer gases He and Ne. The injection gas mixtures of the inactive tests additionally contained C$_2$H$_2$ as an inhibitor of CH$_4$ oxidation. Methane concentrations were adapted according to observed background CH$_4$ concentrations and expected activity. GPPTs were performed as close to the water table as injection and extraction of gas was possible (Table 4-1). GPPTs were performed as described earlier (Urmann et al. 2005) with slight modifications. Briefly, background soil air was sampled through the respective needle at the injection/extraction depth by pumping 1 L of gas. Subsequently, 13 to 16 L of injection gas mixture (Table 4-1) was injected with an average flow rate of 0.20 L min$^{-1}$. After a transition phase of 0.7 to 2 min, flow was reversed and 20 to 29 L was extracted with an average flow rate of 0.18 L min$^{-1}$ from the same location. In the case of GPPT 1, extraction of gas was not possible at the injection depth due to clogging and the extraction point was moved up by 5 cm. After each active GPPT, the procedure was repeated using an injection gas mixture of the same composition but additionally containing C$_2$H$_2$ (GPPT “i”).

![Figure 4-1: Gas-phase CH$_4$ concentration profiles at three hummocks prior to GPPTs. Note the logarithmic scale of the x-axis. The depth of the water table measured from the peat surface in each hummock is indicated by a dashed line.](image)
For injection and extraction, a gas flow controller (GFC) was used (Urmann et al. 2005). The core equipment of the GFC was a diaphragm pump and a mass flow meter. Injection samples were taken in the same way as extraction samples in clamped, pre-evacuated (-0.6 bar) serum bottles with butyl rubber stoppers.

### Table 4-1: Operational parameters for three pairs of gas push-pull tests (GPPTs).

<table>
<thead>
<tr>
<th>GPPT</th>
<th>Site</th>
<th>Test depth[cm]</th>
<th>Injection concentrations</th>
<th>Injection</th>
<th>Extraction</th>
<th>Total time [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH₄ [mL L⁻¹]</td>
<td>C₂H₂ [mL L⁻¹]</td>
<td>Volume [L]</td>
<td>Pump rate [L min⁻¹]</td>
</tr>
<tr>
<td>1a</td>
<td>H₁</td>
<td>45/40</td>
<td>297</td>
<td>-</td>
<td>13.3</td>
<td>0.19</td>
</tr>
<tr>
<td>1i</td>
<td>H₁</td>
<td>45/40</td>
<td>303</td>
<td>40</td>
<td>12.6</td>
<td>0.19</td>
</tr>
<tr>
<td>2a</td>
<td>H₂</td>
<td>42</td>
<td>8.1</td>
<td>-</td>
<td>14.5</td>
<td>0.21</td>
</tr>
<tr>
<td>2i</td>
<td>H₂</td>
<td>42</td>
<td>8.1</td>
<td>40</td>
<td>13.6</td>
<td>0.20</td>
</tr>
<tr>
<td>3a</td>
<td>H₃</td>
<td>50</td>
<td>32</td>
<td>-</td>
<td>15.5</td>
<td>0.20</td>
</tr>
<tr>
<td>3i</td>
<td>H₃</td>
<td>50</td>
<td>31</td>
<td>79</td>
<td>15.3</td>
<td>0.21</td>
</tr>
</tbody>
</table>

*“a” designates active test, “i” inactive test;

b Refers to the depth of the injection/extraction needle below peat surface. For GPPT 1 the needle was lifted by 5 cm for extraction.

c Injection mixtures all additionally contained 190 - 240 mL L⁻¹ He, 220 - 260 mL L⁻¹ Ne, 60 - 170 mL L⁻¹ O₂ and were prepared in N₂.

#### 4.3.3 Analytical methods

Samples were analyzed for CH₄ and C₂H₂ by GC-FID and stable carbon isotope ratios in CH₄ by GC-IRMS as described previously (Urmann et al. 2005). The isotope ratios were determined by reference to the standard PDB (Peedee Belemnite) (Hoefs 1997) in the δ-notation (Mariotti et al. 1981) in per mil:

\[
δ^{13}C(\%) = \left( \frac{^{13}C}{^{12}C} \right)_{\text{sample}} - 1 \times 1000
\]  

(Equation 4-1)

In GPPT 3, He, Ne and O₂ were analyzed using the method described in Gonzalez-Gil et al. (submitted-b). In GPPT 1 and 2, the latter gases were measured using a Trace GC Ultra (Thermo Electron, Rodano, Italy) with a TCD detector and a capillary Molsieve 5A column (50 m x 0.53 mm ID, 50 μm) at 30 °C with a micropacked ShinCarbon ST pre-column at 50 °C using H₂ as carrier gas. To prevent adsorption of CO₂ and H₂O to the molecular sieve...
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column, the pre-column was back-flushed before the elution of CO₂ and H₂O. During back-flush carrier gas flow on the main column was maintained.

4.3.4 Data analysis

To obtain breakthrough curves of the different gases, relative concentrations (C*) were calculated from injection (Table 4-1) and extraction concentrations and plotted versus time since end of injection. A simplified method was used to evaluate GPPTs (Schroth and Istok 2006). This method allows accounting for reaction during injection even when only a segment of a GPPT is evaluated. It is based on the assumption that no mixing occurs between “parcels” of gas injected at different times of the injection phase. To apply this method, a reaction time tR was calculated for each parcel j, which is the time from its injection until its extraction (Equation 4-2).

\[ t'_R = t^{*} + \frac{\int Q_{ext} C_{CH_4} (t) dt}{M_{CH_4}} T_{inj} \]  

(Equation 4-2)

where t* is time since end of injection, Q_{ext} is the extraction pump rate, t_{ext} is time since extraction began, M_{CH_4} is the total mass of CH₄ in the inactive test “i,” C_{CH_4} is the CH₄ concentration from test “i” at time t_{ext} and T_{inj} is the injection time. Subsequently, the natural logarithm of the ratio of relative CH₄ concentration C* in the active test “a” and relative CH₄ concentration in the inactive test “i” was plotted versus reaction time tR (Equation 4-3). The substitute tracer CH₄, i.e. CH₄ from the inactive test, thereby accounts for dilution of the injected gas with soil air.

\[ \ln \left( \frac{C_{CH_4}^* (t')} {C_{CH_4}^* (t')} \right) = -kt_R + c \]  

(Equation 4-3)

Apparent first-order rate constants k were calculated by linear regression from the segment of the data that showed a ln-linear relationship according to equation 4-3 with c as an arbitrary constant.

In-situ CH₄ oxidation rates per g dry peat (in ng CH₄ [gdw h]⁻¹) prior to GPPTs were calculated by multiplying apparent first-order rate constants k with measured in-situ CH₄ gas-phase concentrations using the estimated volumetric water content and assuming a porosity of
0.96 and a peat bulk density of 0.05 g cm\(^{-3}\) as measured by Kellner and Lundin (2001) at 30 - 40 cm depth in hummocks of a \textit{Sphagnum} peat bog.

### 4.4 Results

#### 4.4.1 Gas push-pull test performance

During the extraction phase of all GPPTs relative concentrations \(C^*\) of \(\text{CH}_4\), \(\text{Ne}\) and \(\text{He}\) decreased rapidly due to diffusion and dilution with soil air (Figure 4-2). Background \(\text{CH}_4\) concentrations prior to active GPPTs were low with 13 \(\mu\text{L L}^{-1}\) (H1), 8.9 \(\mu\text{L L}^{-1}\) (H2) and 1.9 \(\mu\text{L L}^{-1}\) (H3) (see also Figure 4-1). At the end of active tests, \(\text{CH}_4\) concentrations again approached background values. Breakthrough curves were clearly different for the different gases in all GPPTs with highest relative concentration for \(\text{CH}_4\) and lowest relative concentrations for \(\text{He}\) (Figure 4-2). Neon and \(\text{He}\) breakthrough curves of active and inactive tests were in good agreement for all GPPT test pairs. Conversely, \(\text{CH}_4\) breakthrough curves from active GPPTs were slightly lower than those from inactive GPPTs with the largest difference in GPPT 3 (Figure 4-2-Details). Lower \(\text{CH}_4\) curves in active tests indicated consumption of \(\text{CH}_4\). Neon, \(\text{He}\) and \(\text{CH}_4\) breakthrough curves (Figure 4-2) were integrated to obtain percentages of the injected mass of gases recovered during extraction. In all tests between 2.8 and 10.4\% of \(\text{Ne}\), 0.8 and 5.4\% of \(\text{He}\) and 5.8 and 15.5\% of \(\text{CH}_4\) were recovered. Relative \(\text{C}_2\text{H}_2\) concentrations decreased during inactive GPPTs similar to the other gases (data not shown) with absolute concentrations dropping to 0.2 (GPPT 1i), 3.4 (GPPT 2i) and 4.6 mL L\(^{-1}\) (GPPT 3i) at the end of extraction. Oxygen concentrations fluctuated between 105 mL L\(^{-1}\) and 210 mL L\(^{-1}\) during the extraction of all tests.
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Figure 4-2: Breakthrough curves of He, Ne and CH$_4$ from three pairs of GPPTs with “a” designating curves from active GPPTs and “i” designating curves from inactive tests. The part of each GPPT test pair where CH$_4$ consumption was discernible is shown in more detail. Note the different scales of both axes. Noble gases could not be measured for the entire extraction period in GPPT 3 as the analytical method available at the time was less sensitive. In GPPT 1, He dropped below the quantification limit after 1.4 h, Ne after 2.1 h.

4.4.2 First-order rate constants and methane oxidation activity

First-order rate constants were determined using CH$_4$ from inactive GPPTs as a substitute tracer to account for decrease in concentration due to diffusion and dilution with soil air. In all GPPTs, a ln-linear relationship between corrected relative CH$_4$ concentrations and reaction time indicated CH$_4$ oxidation that followed approximately first-order kinetics (Figure 4-3).
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This was apparent later during extraction in GPPT 1 and 2 than in GPPT 3. In GPPT 1 and 2, apparent first-order rate constants $k$ were similar while the $k$-value determined in GPPT 3 was by a factor of two lower (Figure 4-3). The 95% confidence interval was small for GPPT 1 and 3, but larger for GPPT 2 due to fewer, more scattered data points.

Estimated in-situ CH$_4$ oxidation rates prior to GPPTs at H1 ranged from 48 to 299 ng CH$_4$ [gdw h]$^{-1}$ between 40 and 50 cm depth. At H2, 42 cm depth, the estimated CH$_4$ oxidation rate was with 45 ng CH$_4$ [gdw h]$^{-1}$ similar to the rate at H1, 40 cm depth. At H3, the estimated in-situ CH$_4$ oxidation rate was one to two orders of magnitude smaller (4.0 ng [gdw h]$^{-1}$).

![Figure 4-3: Plot for the determination of apparent first-order rate constants $k$ for CH$_4$ oxidation from three pairs of GPPTs. Slopes from linear regressions (solid lines) are apparent first-order rate constants. $C'(CH_4)$ "a" and "i" are relative CH$_4$ concentrations from an active and the corresponding inactive GPPT. Early data from GPPT 1 and 2 are not shown for better readability. First-order rate constants $k$ are given with 95% confidence intervals.](image)

4.4.3 Isotope fractionation during gas push-pull tests

The $\delta^{13}$C-value of CH$_4$ in the injection mixture of all GPPTs was -45.9‰. During the first 0.2 h of extraction, CH$_4$ became strongly enriched in $\delta^{13}$C with an average isotope shift of 19.6‰ (Figure 4-4). Subsequently, $\delta^{13}$C-values increased further in all tests with a maximum value of +20.5‰ in GPPT 1a. Towards the end of extraction in GPPT 1a and 1i, $\delta^{13}$C-values decreased again by 8.7-9.8‰. In GPPT 2, the isotope shift in the active and the inactive test
were similar. However, due to low CH$_4$ concentrations, isotope data could only be obtained until just before the part of extraction where CH$_4$ oxidation followed first-order kinetics. Conversely, in GPPT 1 and 3, there was a difference in isotope shifts with time between the active and inactive test, with a maximum difference of 3.5% in GPPT 3 and a maximum difference of 15.5% in GPPT 1. A larger isotope shift in GPPT 1a than 1i was observed already before CH$_4$ oxidation followed first-order kinetics, i.e. after 1.04 h since end of injection (Fig. 4-4) which corresponds to 1.14 h reaction time (Fig. 4-3). The $\delta^{13}$C-value of CH$_4$ in background soil air was -45.5%, measured two days prior to GPPTs in H1.

![Graph showing isotope ratios in CH$_4$ over time during three pairs of GPPTs](image)

Figure 4-4: Isotope ratios in CH$_4$ over time during three pairs of GPPTs; “a” designates active GPPTs, “i” designates inactive GPPTs.

4.5 Discussion and conclusions

4.5.1 Methane as a substitute tracer to quantify methane oxidation

Different diffusion coefficients resulted in clearly different transport behavior of noble gases and CH$_4$ during GPPTs with diffusion-dominated gas transport, similar to previous laboratory experiments and simulations (Gonzalez-Gil et al. submitted-b). Consequently, noble gases were unsuitable as tracers to quantify CH$_4$ oxidation from GPPTs using simplified methods. Instead, we presented an approach to use the reactant CH$_4$ as a substitute
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tracer. We showed that during GPPTs in a peat bog, the three prerequisites to apply this approach were met: a) reproducibility of two consecutive GPPTs with respect to gas transport was confirmed by coinciding Ne and He breakthrough curves during all three test pairs; b) methane background concentrations did not interfere with breakthrough curve analysis as CH4 background concentrations before active and inactive tests were low and similar; c) the presence of sufficient inhibitor was confirmed by C2H2 concentrations at least 20 times higher than C2H2 concentrations required for 94% (Chan and Parkin 2000) or 100% (Bodelier and Frenzel 1999) inhibition in laboratory experiments. Effectiveness of C2H2 as an inhibitor of CH4 oxidation during GPPTs has been shown previously (Urmann et al. 2005).

The described procedure should be applicable for the quantification of CH4 oxidation in a range of ecosystems. A disadvantage of the use of C2H2 is that inhibition, i.e. binding of C2H2 to the enzyme, is non-reversible (Prior and Dalton 1985). This may complicate time-dependent studies of CH4 oxidation at the same location using GPPTs. Recovery of methanotrophic activity after C2H2 application in the field is currently under investigation. Alternatively, a reversible gaseous inhibitor such as difluoromethane (Miller et al. 1998) could be employed.

4.5.2 Quantification of rate constants

In all GPPTs, dilution and diffusive transport led to a fast decrease in relative gas concentrations and low recoveries of injected gases. This in itself does not impede accurate quantification of rate constants (Haggerty et al. 1998). However, a fast decrease in concentrations limits the available test time, which may complicate quantification of low activity. Despite fast decrease in CH4 concentrations, we were able to quantify apparent first-order rate constants for CH4 oxidation in the unsaturated zone of three different hummocks using CH4 as a substitute tracer. Moreover, the sensitivity of the GPPT was sufficient to detect activity, even though a) apparent first-order rate constants were at the lower end of those previously determined above a contaminated aquifer and b) CH4 oxidation followed apparent first-order kinetics at lower CH4 concentrations than in previous GPPTs (Urmann et al. 2005). During GPPTs at all sites, CH4 oxidation following first-order kinetics was only observed in the later part of extraction. Most likely, in the earlier part, oxidation followed Michaelis-Menten kinetics as indicated by a curvature in the rate plots (Figure 4-3, not well visible for GPPT 2 due to scale). Michaelis-Menten parameters were not quantified as this is difficult to achieve from a single GPPT (Urmann et al. submitted) and quantification of these parameters was not the purpose of this study.
4.5.3 Quantification of methane oxidation rates

At H3, the in-situ CH$_4$ oxidation rate prior to GPPTs was one to two orders of magnitude lower than at H1 and H2. As the $k$-value at H3 was only a factor of two lower, this difference was due to the low in-situ concentration at H3 which was similar to ambient CH$_4$ concentrations. At ambient concentrations, different kinetic parameters, i.e. higher affinity for CH$_4$, are usually observed (e.g. Bender and Conrad 1992; Gulledge et al. 2004). Therefore, the obtained in-situ rate at H3 has to be interpreted with care as the first-order rate constant may not apply for measured in-situ CH$_4$ concentrations. However, the observation of apparent first-order kinetics at high CH$_4$ concentrations during GPPT 3 suggested that higher in-situ concentrations have been occurring at this site inducing high activity.

Due to high variability in CH$_4$ gas-phase concentrations observed at all sites in the peat bog (data not shown), CH$_4$ oxidation rates could not be inferred from CH$_4$ concentration gradients for comparison with those calculated from GPPT results. However, comparison with values reported in the literature showed that, at H1 and H2, estimated in-situ CH$_4$ oxidation rates prior to GPPTs were two orders of magnitude lower than in-situ rates calculated from profiles in a northern peat bog in summer, but one order of magnitude higher than rates determined at the same site in autumn (Fechner and Hemond 1992). As reports of in-situ oxidation rates are limited, we further compared our results with potential oxidation rates measured in laboratory incubations. In-situ rates in our study were three orders of magnitude lower than potential oxidation rates at a temperate bog ranging from 1.3 - 37.9 µg CH$_4$ [gdw h]$^{-1}$ (McDonald et al. 1996) and similar to a range of potential oxidation rates of 32 – 650 ng CH$_4$ [gdw h]$^{-1}$ observed in a boreal bog (Whalen and Reeburgh 2000). Considering that oxidation followed first-order kinetics in our study, potential activity might have been in a similar range as in the temperate but higher than in the boreal bog.

4.5.4 Isotope fractionation during gas push-pull tests

Methane oxidation during GPPTs with restricted gas transport has previously been confirmed by a shift in CH$_4$ stable carbon isotope ratios towards heavier values (Urmann et al. 2005). This shift was induced by preferential oxidation of $^{12}$CH$_4$ over $^{13}$CH$_4$ (Coleman et al. 1981; Whiticar 1999). At the same site, isotope shifts during a GPPT with the co-injection of C$_2$H$_2$ as inhibitor of CH$_4$ oxidation were small. In contrast, in the highly porous peat bog in this study, large isotopic shifts towards heavier δ$^{13}$C-values were observed during GPPTs with the co-injection of C$_2$H$_2$ indicating strong isotope fractionation due to gas-phase diffusion.
(Figure 4-4). This is in accordance with theory and experimental observations of gas-phase diffusion of CH$_4$ (De Visscher et al. 2004) and with previous laboratory GPPT experiments and simulations (Gonzalez-Gil et al. submitted-a). The reversal of the observed isotope trend towards more negative ("lighter") values at the end of GPPT 1 may be explained by mixing with CH$_4$ in background soil air which was comparatively light.

Isotope ratios in active GPPTs generally showed a similar trend with time as those in inactive tests, underlining the strong influence of diffusion on isotope fractionation under the test conditions in this study. Despite this strong influence, a clearly discernible difference in isotope ratios between active and inactive test was observed throughout a large part of extraction in GPPT 1. Consequently, isotopic shifts provided a valuable independent verification of microbially mediated CH$_4$ oxidation that followed Michaelis-Menten and first-order kinetics. In GPPT 3, on the other hand, differences between active and inactive tests were small. In this case, the observed differences in $\delta^{13}$C with time could only be attributed to microbial oxidation because similar transport behavior of gases in both tests was verified by noble gas breakthrough curves. In GPPT 2, no difference in $\delta^{13}$C time trends was apparent between active and inactive test although CH$_4$ oxidation was observed in the active test. Consequently, similar shifts in isotope ratios do not necessarily indicate absence of CH$_4$ oxidation.

The degree of fractionation due to CH$_4$ oxidation, described by the fractionation factor, is likely an important parameter determining whether isotope fractionation is a suitable indicator for CH$_4$ oxidation during GPPTs. The fractionation factor is highly variable, with one possible determinant being the degree of mass transfer limitation of CH$_4$ oxidation (Templeton et al. 2006). At high CH$_4$ concentrations combined with the consumption of a small fraction of CH$_4$ and therefore no mass transfer limitation, high fractionation was observed as enzymatic fractionation was probably fully expressed (Templeton et al. 2006; Urmann et al. submitted). This could explain why in GPPT 1 with a very high CH$_4$ injection concentration, isotope fractionation due to microbial oxidation could be clearly distinguished from isotope fractionation due to diffusion.

In this study, we presented an approach to quantify CH$_4$ oxidation in situ based on GPPTs combined with inhibitors that can be applied in a range of ecosystems. Stable carbon isotope data obtained during GPPTs may provide independent verification of CH$_4$ oxidation under certain conditions but have to be interpreted with care. The presented procedure may be useful for the quantification of other microbial processes for which suitable inhibitors but no suitable tracers are available.
4.6 Acknowledgements

We thank Matthias Saurer and Rolf Siegwolf (PSI, Villigen, Switzerland) for help with stable carbon isotope measurements, Elena Norina (ETH Zurich) for help with field work and ProNatura for providing access to the site.
4.7 References


Chapter 5: Environmental parameters in a peat bog

Environmental Parameters and Methane Concentrations in an Alpine Peat Bog

Karina Urmann
5.1 Introduction

First gas push-pull tests (GPPTs) in Forrenmoos, a peat bog in Eigenthal, Switzerland, were performed in 2004. Concomitantly, samples for gas-phase concentration profiles were taken through temporarily inserted needles. Measured CH$_4$ gas concentration profiles showed high spatial and temporal variability. Short term temporal variability in CH$_4$ emissions or profiles has been explained previously by atmospheric pressure changes (Czepiel et al. 2003) or wind (Hargreaves et al. 2001). Long term temporal variability might depend on peat temperature as methanogenesis is more strongly dependent on temperature than CH$_4$ oxidation (Pearce and Clymo 2001). To assess spatial and temporal variability, permanent samplers to measure CH$_4$ concentrations in the saturated and unsaturated zone were installed in the peat bog. Additionally, temperature sensors and a small weather station were installed to elucidate possible reasons for observed variability.

5.2 Materials and methods

5.2.1 Field site and measurement scheme

Measurements were performed in Forrenmoos, a raised peat bog covering around 0.05 km$^2$ which is located at 960 m above sea level in Eigenthal above the city of Lucerne, Switzerland. The peat bog was naturally and artificially drained, but partially regenerated since the 1980s. Vegetation in the eastern, drier part of the bog, where experiments were conducted, is dominated by *Sphagnum* mosses and dwarf-shrubs. The bog is sparsely forested mainly with birches. The peat topography is characterized by hummocks and hollows. Even in hollows, the water table is never permanently above the peat surface (Breitenbach 1991; Schneebeli 1991). The top 20 to 30 cm of hummocks consist of living and sparsely degraded *Sphagnum* mosses with subsequent increasing but very heterogeneous humification with increasing depth.

The three hummocks chosen for measurement of CH$_4$ concentrations and for GPPTs (see chapter 4) were maximum 30 m apart (Figure 5-1). At each site, the peat surface (that is the surface of the living *Sphagnum* moss layer) in the middle of the hummock was defined as the 0 cm reference level. This was the location where needles for gas sampling at different depths were installed (Figure 5-2). Depths were measured referring to this level using a thread and a water balance. Gas and water samplers were installed on 2 May 2005 and samples were collected on 13 days until 24 November 2005.
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Figure 5-1: Schematic map of the sampling area in the peat bog (Forrenmoos) with the three hummocks H1 - H3. Only distances between the hummocks are drawn to scale.

Figure 5-2: Schematic representation of a hummock with samplers and probes. The 0 cm line shows the reference level for depth measurements. In two out of three hummocks the water table appeared to follow peat topography, which is not depicted. Note that exact sampling depths are not shown.
5.2.2 Meteorological parameters

Air temperature, relative air humidity, atmospheric pressure and wind speed were recorded in the peat bog between 19 May and 24 November 2005 with two interruptions using a weather station as described in Vonlanthen et al. (2006). The weather station was installed next to H1 (Figure 5-1) and consisted of a 1.3 m aluminum mast with the following sensors attached to it: an air temperature and a relative air humidity sensor (TRH 100, Pace Scientific Inc., Mooresville, N. C., USA, installed 15 cm above ground) an anemometer (type f.555.1.18, Schiltknecht, Gossau, Switzerland, installed 100 cm above ground) and a pressure sensor (P300, Pace Scientific Inc., protected from insects and wind). 10 min readings were recorded using a data logger (XR 440, Pace Scientific Inc.).

5.2.3 Temperature

Profiles of temperature in the peat were recorded at hourly intervals using iButtons® containing a temperature sensor and a data logger (Maxim Integrated Products, Sunnyvale, CA, USA). Temperature probes were prepared by submerging several iButtons® in a wooden probe that was inserted into the peat at the desired depth. A first probe was installed from 3 March until 23 April 2005 with a sensor to measure air temperature at 22 cm above the peat surface and temperature sensors at 3 cm, 28 cm and 53 cm depth below the surface. Between 13 May and 21 October 2005 peat temperatures were recorded at the three hummocks. Once within this period the data was retrieved and the probes were re-installed. Probes contained sensors at 0, 20, 40, 50, 60, 70 and 90 cm depth. Depths refer to the reference level at each hummock. As temperature probes were installed at the edge of each hummock 0 cm sensors were just above the peat surface and recorded air temperature.

5.2.4 Water content

Time domain reflectrometry (TDR) was used to measure water content at one reference hummock next to H3. Depths refer to the reference level at H3. As the TDR hummock was slightly higher than H3, the 0 cm reading was 8.5 cm below the peat surface at the highest part of the hummock. Due to a drop of the peat surface towards a drainage ditch, the side of the hummock was accessible down to 50 cm depth and TDR probes could be inserted horizontally into the hummock. By using 38 cm-long TDR probes, an average water content for each depth was obtained. For comparison, 38 and 48 cm-long probes were inserted vertically from the top in the reference hummock and in H1. In this way averages over the
respective depths of the probes were obtained. Water content was mainly measured at the reference hummock in order not to disturb the sites where GPPTs were performed.

TDR probes were calibrated with an empirical third-order relationship between the dielectric number and volumetric water content as presented by Topp et al. (1980). To account for the specific properties of peat, the coefficients derived for pooled data from peat of different degrees of humification were used in this equation (Kellner and Lundin 2001).

5.2.5 Water table levels

Two piezometers were installed at each of the three sampling sites to measure water table levels. One was installed at the edge of the hummock (designated “a”) and the other one in a flat area next to the hummock (designated “b”). Piezometers consisted of 2 cm-diameter PVC tubes, perforated along a depth interval of 40 cm and with an aluminum tip glued into the lower end for easy installation. Piezometers were closed with rubber stoppers and water table levels were determined by sound (submerging a tube until air entry into the water was audible). Water table levels were referred to the reference level at each hummock. The peat surface was 6 - 13 cm lower than reference level at the sites of the piezometers in the hummocks and 18 - 47 cm beside the hummocks (Table 5-1).

Table 5-1: Level of peat surface at the locations of six piezometers

<table>
<thead>
<tr>
<th>Piezometer</th>
<th>P1a</th>
<th>P1b</th>
<th>P2a</th>
<th>P2b</th>
<th>P3a</th>
<th>P3b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of peat surface below reference level [cm]</td>
<td>8</td>
<td>18.5</td>
<td>6.5</td>
<td>28.5</td>
<td>13.5</td>
<td>47</td>
</tr>
</tbody>
</table>

5.2.6 Gas and water sampling and analysis

Gas sampling procedures and analysis of gas samples at the three hummocks were described in chapter 4 (4.3.1). Water samples were taken through metal rods (maximum dead volume 32 mL) with a plastic tip with a 5 cm interval screened with 0.6 mm diameter holes using a syringe or through the gas samplers if water saturation above the water table occurred. After flushing with at least one dead volume, 5 - 6 mL water samples were injected in N2-flushed and pre-evacuated vials and CH4 in the water determined using a headspace method as described previously (Bolliger et al. 1999). Only in few layers water could be sampled, probably due to low and differing hydraulic conductivities of peat layers. Additionally, pH was measured in water samples using pH indicator stripes with a range from pH 2.5 - 4.5, which covered the range of observed values.
5.3 Results and discussion

5.3.1 Meteorological parameters

The average wind speed recorded at 1 m height next to H1 (Figure 5-3a) decreased between 19 May and 24 November 2005 while maximum hourly averages remained similarly high (4 to 5 km h\(^{-1}\)) (Figure 5-3a). The maximum 10 min value recorded was 16.5 km h\(^{-1}\). Atmospheric pressure fluctuated between 891.9 and 922.8 mbar (Figure 5-3b). Relative humidity ranged from 29 and 100% (Figure 5-3c). Temperatures recorded at the weather station (data not shown) were very similar to the ones measured at the peat surface using the temperature probes.

![Figure 5-3a: Hourly averages of wind speed at 1 m height, measured near hummock H1 between 19 May and 24 November 2005 (Day 0 is 2 May).](image)
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Figure 5-3b: Hourly averages of atmospheric pressure measured in the peat bog between 19 May and 24 November 2005 (Day 0 is 2 May).

Figure 5-3c: Hourly averages of relative humidity measured at 15 cm above peat surface between 19 May and 24 November 2005 (Day 0 is 2 May).
5.3.2 Air and peat temperature

Until end of March, when the peat was under snow cover, the highest temperature, 3.5 °C, was found at 53 cm, i.e. at the lowest measured depth (Figure 5-4). Subsequently, the peat became warmer close to the surface (3 cm depth). In contrast, the peat at 28 cm depth remained slightly colder than at 53 cm until the end of April. Temperatures measured in the three hummocks from 13 May until 21 October 2005 were similar. Therefore, examples from single sites are shown. During the measurement period, air temperatures at the peat surface at H1 ranged from -1.5 to 44.4 °C (Figure 5-5a). Strong day and night fluctuations could be observed in the peat down to 20 cm, while peat temperatures were not influenced by diurnal cycles at 40 cm depth or below (Figure 5-5b). Below 40 cm depth, temperatures decreased with increasing depth from May till September, while from October onwards temperatures started to decrease from the top (Figure 5-5c). A similar temperature distribution to the one found at the end of spring/beginning of summer in this study was found in summer in a boreal bog but with a few °C lower temperatures (Whalen and Reeburgh 2000). Average temperatures for each hummock between May and October ranged from a maximum of 12.7 °C at 20 cm depth to a minimum of 8.2 °C at 90 cm depth. Fluctuations within one depth were maximum 15.5 °C at 20 cm and 7.5 °C from 40 cm downwards.

![Figure 5-4: Hourly temperature values in air and at different depths in the peat from 3 March until 23 April 2005.](image-url)
Figure 5-5a: Example for hourly air temperature values measured at the peat surface at H1 between 13 May until 21 October 2005 (Day 0 is May 2).

Figure 5-5b: Examples for hourly temperature values at four different depths in the peat at H1 between May 13 and October 21 2005 (Day 0 is May 2). Only selected depths are shown for better readability.
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5.3.3 Water content

Calibration of TDR measurements for a peat soil is difficult. However, using a pooled calibration previously obtained for peat samples with a different degree of humification (Kellner and Lundin 2001), measured values should be a reasonable estimate. Water content distribution at the reference hummock remained the same throughout the season with a large increase in water content below 30 cm depth (Figure 5-6). This most likely coincided with a large increase in the degree of humification increasing the water holding capacity of the peat. The wettest conditions were found at the beginning of the measuring period, driest conditions were found at the end of June. Assuming a porosity of 0.96 (Kellner and Lundin 2001), maximum measured saturation was 64%. Vertical insertion of TDR probes gave an average water content over 38 – 48 cm depth from the peat surface. Comparison of these average water contents between the reference site and H1 showed that water contents were on average 50% higher at H1. The lower water contents in the reference hummock were probably caused by stronger drainage due to its location close to a drainage ditch. Therefore, water contents were multiplied with a factor of 1.5 when estimating water contents for H1 and H2.
5.3.4 Water table levels

Water table levels were relatively stable throughout the season at all three sites with maximum fluctuations of 10 cm (Figure 5-7). The difference in water table depth between the piezometers in and beside the hummock was on average 9.8 cm at H1 and 11.0 cm at H3. As the peat surface in the hummocks is higher than beside the hummocks, this may indicate that the water table followed, to some degree, the topography of the peat. This also means that the water table within the hummocks might have varied with the peat surface level of the hummocks. At H2 on the other hand, no difference was observed between average values measured in the two piezometers. Possibly this was due to a larger hydraulic conductivity at this site.
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5.3.5 Methane concentrations and relation to environmental parameters

Between the three hummocks, clear differences in CH\textsubscript{4} concentrations were observed, that remained throughout the season. The pH in the groundwater at the three hummocks was similar with an average value of 3.9. At H1, CH\textsubscript{4} concentrations in the water at 82.5 cm depth ranged from 0.41 to 0.67 mM between May and end of June. Afterwards it became more difficult to obtain water samples so that obtained values were not reliable. A (nearly) water-saturated layer was observed at 40 cm depth at this site at the beginning of the sampling season which became drier and subsequently became wetter again towards the end of the sampling season. Gas sampling became impossible at 50 cm depth from July on while a gas sample could still be obtained at 55 cm. This could have also been due to increased water content at 50 cm depth. Overall, this indicates large differences in physical properties of different peat layers. Gas-phase CH\textsubscript{4} concentrations observed at H1 were very high, but also very variable. Changes in concentrations at a specific depth were occurring fast, for example at 50 cm, concentrations dropped by a factor of 100 from 11 mL L\textsuperscript{-1} to 0.12 mL L\textsuperscript{-1} within two days. At 55 cm, CH\textsubscript{4} concentrations ranged from 28 mL L\textsuperscript{-1} to 244 mL L\textsuperscript{-1} while at 40 cm depth only 2 to 50 \( \mu \)L L\textsuperscript{-1} were observed. This indicates that a large methanotrophic activity must have occurred between those two depths which was partially quantified in late June using GPPTs (see chapter 4, GPPT 1). Further quantifications at the same site later in the year were impossible, as water was entering the pumping system due to increased water content.

Figure 5-7: Water table depth below reference level in two piezometers at each of the three hummocks. Piezometers “a” were installed in the hummocks, while piezometers “b” were installed in flat areas next to the hummocks.
At H2, a layer at 50 cm depth (i.e. a layer above the water table) remained water-saturated until mid June with CH₄ concentrations of 0.17 to 0.20 mM. At the same depth, gas-phase CH₄ concentrations subsequently ranged from 0.11 to 32.6 mL L⁻¹. Above this depth, CH₄ gas-phase concentrations were maximum 22 µL L⁻¹. This again was an indication of CH₄ oxidation which was quantified using GPPTs (see chapter 4, GPPT 2).

At H3, CH₄ concentrations in the water at 83.5 cm depth ranged from 0.10 to 0.27 mM. Only occasionally CH₄ concentrations slightly above atmospheric were observed in the gas phase at this site. In a GPPT in 2004 (see chapter 4, GPPT 3), CH₄ conversion was quantified in the same hummock, but in a GPPT in 2005 (test data not shown) no CH₄ conversion was apparent. However, one week after that test, the highest gas-phase CH₄ concentration (1.3 mL L⁻¹) at this site was observed. This is an indication that, even though CH₄ oxidation was not observed during the test, CH₄ oxidation may have been inhibited by the injected acetylene.

Methane concentrations in the water at all sites did not appear to be related to peat temperatures. Temperature changes at the relevant depths were relatively small over the season and occurred gradually. In contrast, water-phase CH₄ concentrations at H1, for example, increased by over 50% within a relatively short time of 17 days at the beginning of the season. Therefore, it is more likely that other factors, such as availability of substrates were responsible for observed variability.

At all sites, enhanced CH₄ concentrations were occasionally observed higher up in the profile (see for example Figure 5-8). Based on the available wind and pressure data, wind and pressure fluctuations could not explain these enhanced CH₄ concentrations or in general the observed variability in CH₄ gas concentrations profiles. However, for a more in-depth analysis more data would be needed.
In summary, the peat bog in Eigenthal is a heterogeneous environment with large spatial and temporal variability in CH$_4$ concentrations. Based on the available data, temporal variability could not be explained by peat temperature or meteorological parameters. Non-destructive sampling to obtain CH$_4$ concentrations in the peat was difficult. Water samples could only be obtained in certain layers of the peat and, even below the water table, sampling became increasingly difficult over the season. Gas sampling through needles became difficult between 40 cm depth and the water table. To obtain information on a relationship between gas-phase concentrations and meteorological parameters, more profile samples and a higher time resolution in meteorological data may have been necessary. This was not possible to obtain due to time constraints.
5.4 References


Methanotrophic Activity in a Diffusive Methane / Oxygen Counter-Gradient in an Unsaturated Porous Medium

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Submitted to Journal of Contaminant Hydrology
6.1 Abstract

Microbial methane (CH$_4$) oxidation is a main control on emissions of this important greenhouse gas from ecosystems such as contaminated aquifers or wetlands under aerobic conditions. Due to a lack of suitable model systems, we designed a laboratory column to study this process in diffusional CH$_4$/O$_2$ counter-gradients in unsaturated porous media. Analysis and simulations of the steady-state CH$_4$, CO$_2$ and O$_2$ gas profiles showed that in a 15-cm-deep active zone, CH$_4$ oxidation followed first-order kinetics with respect to CH$_4$ with a high apparent first-order rate constant of $\approx$30 h$^{-1}$. Total cell counts obtained using DAPI-staining suggested growth of methanotrophic bacteria, resulting in a high capacity for CH$_4$ oxidation. This together with apparent tolerance to anoxic conditions enabled a rapid response of the methanotrophic community to changing substrate availability, which was induced by changes in O$_2$ concentrations at the top of the column. Microbial oxidation was confirmed by a $\approx$7% enrichment in CH$_4$ stable carbon isotope ratios along profiles. Using a fractionation factor of 1.025 ± 0.0005 for microbial oxidation estimated from this shift and the fractionation factor for diffusion, simulations of isotope profiles agreed well with measured data confirming large fractionation associated with microbial oxidation. The designed column should be valuable for investigating response of methanotrophs to environmental parameters in future studies.
6.2 Introduction

Methane (CH₄) is ~60 times more efficient as a greenhouse gas than carbon dioxide (CO₂) with respect to a 20 year time horizon (Ramaswamy et al. 2001). Microbial CH₄ oxidation under aerobic conditions is the key process decreasing CH₄ emissions from environments such as wetlands, peat bogs (Segers 1998; Wagner et al. 2003) and contaminated aquifers (Revesz et al. 1995; Conrad et al. 1999). In the latter environment this process is additionally important as the final step in contaminant degradation. The conversion of CH₄ and O₂ to CO₂ and water is mediated by methanotrophic bacteria that use CH₄ as their main source of carbon and energy (Hanson and Hanson 1996). The reaction can be described by the following stoichiometry:

$$CH_4 + (2-x)O_2 \rightarrow (1-x)CO_2 + xCH_2O + (2-x)H_2O$$  \hspace{1cm} (Equation 6-1)

where x depends on the fraction of carbon that is incorporated into biomass (CH₂O). In subsurface environments, CH₄ usually migrates towards the soil surface from an anoxic, methanogenic zone, while O₂ migrates downwards into the soil from the atmosphere. Under diffusive transport conditions, the CH₄ and O₂ gradients determine the zone of CH₄ oxidation (Koch 1990), provided there is no vertical gradient of other limiting factors such as nutrients. The zone of CH₄ oxidation is generally characterized by a concentration of methanotrophic biomass (Amaral and Knowles 1995) and often low substrate concentrations as a result of the interaction between substrate fluxes and conversion (Koch 1990). Under diffusive steady-state conditions, depth profiles combined with knowledge of physical parameters of the system have been used to identify zones of microbial activity and calculate rates of CH₄ oxidation in the field (Fechner and Hemond 1992; Damgaard et al. 1998). Diffusive transport is by a factor of ~10⁴ faster in the gas than in the aqueous phase (Schwarzenbach et al. 2003). Therefore, O₂ can penetrate much deeper into the soil under unsaturated than under saturated condition making the unsaturated zone a more effective buffer for CH₄ emissions (Whalen and Reeburgh 2000). Diffusion-dominated, gas-phase transport of CH₄ and O₂ occurs for example in the unsaturated zone above methanogenic aquifers (Revesz et al. 1995; Conrad et al. 1999; Urmann et al. 2005) or in the unsaturated zones of wetlands or peat bogs (Fechner and Hemond 1992; Walter and Heimann 2000). In contrast to diffusion-dominated systems, gas profiles in systems with advection-dominated transport are determined by the interaction of advective gas flow, diffusion and consumption (De Visscher et al. 1999). Consequently, activity does not concentrate at the oxic-anoxic interface but is distributed over a wider zone.
In methanotrophic environments changes in substrate gradients and therefore substrate availability are common, e.g. due to fluctuating water tables (King 1992). A main factor determining the response of the methanotrophic community to these changes is their tolerance to anoxic conditions or low CH₄ availability (Carini et al. 2005). It is important to better understand the ability of methanotrophs to adapt to changing substrate availability due to its potential effect on CH₄ emissions.

In addition to substrate gradients, stable carbon isotope fractionation has been used to qualitatively or quantitatively assess CH₄ oxidation. The percentage of produced CH₄ that was oxidized in an open, flow-through system, has been calculated using CH₄ stable carbon isotope ratios in the zone of production, emitted CH₄ and the fractionation factor for CH₄ oxidation at the specific site (e.g. Liptay et al. 1998; Chanton and Liptay 2000). Knowledge of the fractionation factor for CH₄ oxidation is critical for the use of this method (Chanton and Liptay 2000). Recently, it has been suggested that estimating this factor in batch incubations and extrapolating it to the field as done in most studies may be inappropriate (Templeton et al. 2006). An indication of the role of physical conditions for isotope fractionation may be obtained if fractionation factors could be quantified under well-defined conditions that physically resemble in-situ conditions.

Physical characteristics of an environment are not preserved in batch experiments. Similarly, well-mixed conditions in chemostats may not be representative of in-situ conditions, even though constant, low substrate concentrations can be maintained in such a system. Conversely, laboratory columns allow the physical characteristics of a habitat in an unsaturated porous medium to be mimicked while controlled experiments can be performed. In a number of studies, columns supplied with CH₄ via advective gas flux, have been used to investigate CH₄ oxidation in landfill cover soils (Kightley et al. 1995; De Visscher et al. 1999; Scheutz and Kjeldsen 2003; Wilshusen et al. 2004). To our knowledge a model system of an unsaturated zone with a CH₄/O₂ counter-gradient dominated by diffusive transport has not yet been developed.

Therefore, the aims of this study were threefold: first, to design an unsaturated, methanotrophic laboratory column with diffusive and vertically separated supply of CH₄ and O₂ and to assess methanotrophic activity using measured and simulated gas concentration and biomass profiles. Second, to follow the response of methanotrophic activity to changes in substrate gradients, induced by applying different O₂ concentrations at the top of the column. Third, to determine the fractionation factor for microbial oxidation in the column under diffusive steady-state conditions.
6.3 Materials and methods

6.3.1 Column design and operation

An acrylic glass column (ID 10.9 cm, length 80 cm) was equipped with 16 gas sampling ports with 3-way-valves (Figure 6-1). At the bottom end, a supporting frit with 2 mm slits separated the main part of the column from a 2.5 cm-high chamber with two inlets at the sides and one outlet at the bottom and an additional headspace sampling port. A similar chamber with an inlet and outlet at each side, but no frit, was used to close the top of the column, creating 5 cm of headspace. Gas tightness of the column was verified by performing a pressure leak test. The column was filled from 80 cm to 66 cm depth with gravel pebbles (3 – 5.6 mm diameter, porosity 0.40) to facilitate drainage and from 66 cm to 0 cm depth with coarse sand (0.7 – 1.2 mm diameter, porosity 0.43). Constant boundary conditions were maintained at the top and the bottom of the column. Methane-saturated water (~1.4 mM CH₄) was pumped from a reservoir with a CH₄ headspace through the bottom chamber at an average flow rate of 1.0 mL min⁻¹ using a peristaltic pump. The solution was buffered to pH 9 with a carbonate buffer (10 mM NaHCO₃, 1 mM Na₂CO₃) and kept anoxic by addition of 5 mM Na₂SO₃ (all chemicals Sigma-Aldrich, Buchs, Switzerland). The chamber at the top of the column was flushed with different O₂/N₂ gas mixtures at an average flow rate of 35 mL min⁻¹ (50 - 120 mL min⁻¹ for abiotic conditions). To avoid dehydration of the sand, the gas was passed through a wash bottle with distilled water. References to locations in the column in this paper are given as depth below the sand surface.
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6.3.2 Column experiments

Initially, the column was run under abiotic conditions (with dry sand) to test a) the stability of CH₄ supply, b) whether diffusive, steady-state conditions with a linear CH₄ concentration profile were obtained and c) if any isotope fractionation occurred during steady-state CH₄ diffusion. Subsequently, the sand was inoculated with a mixed methanotrophic culture. The column was then operated in three phases (I-III) for a total of 126 days with different O₂ concentrations at the top (Table 6-1) to investigate a) microbial CH₄ oxidation in the counter-gradient, b) response to changes in O₂ concentration and c) the resulting stable isotope profiles.

The methanotrophic culture was enriched from different environmental samples in nitrate minimum salts medium (NMS) (NMS as described in (Bowman 2000) except that Ferric ammonium EDTA was replaced by chelated iron solution (http://www.lgcpromochem-atcc.com (atccNum=33009), last accessed March 06). Cultures were grown for 10 days (initial headspace concentrations: CH₄ 90 mL L⁻¹, CO₂ 10 mL L⁻¹, O₂ 190 mL L⁻¹) and filtered
to remove soil particles. For inoculation, 1650 mL of culture were combined with 2500 mL NMS diluted 1:10 with demineralized water. This inoculum was pumped into the column up to 10 cm below the sand surface, left overnight and then drained. The water content (0.039 cm$^{-3}$ cm$^{-3}$ sand) in the coarse sand was measured in sand samples collected at the end of the experiment. As this value is close to the irreducible water content (0.036) experimentally determined for this sand, we assumed that the water content was constant throughout the experiment. The water content in the gravel pebbles was estimated to be 0.02 cm$^{-3}$ cm$^{-3}$.

During the experiment, the temperature in the laboratory was 23 – 26 °C. A temperature of 25 °C was assumed for calculations and simulations. At the end of phase III, 100% N$_2$ was applied to the top of the column and some CH$_4$ oxidation was still observed, probably due to O$_2$ diffusing into the gas supply line. Actual O$_2$ concentrations supplied to the top of the column were therefore slightly higher than the gas cylinder concentrations analyzed by the supplier as given in Table 6-1.

Table 6-1: Column operational parameters, sampling frequency and CH$_4$ fluxes and oxidation rates.

<table>
<thead>
<tr>
<th>Duration [days]</th>
<th>Abiotic</th>
<th>Phase I</th>
<th>Phase II</th>
<th>Phase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$ [mL L$^{-1}$]$^a$</td>
<td>24</td>
<td>77</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>Sampling frequency$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_4$</td>
<td>11</td>
<td>24</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>$\delta^{13}$C</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Cells (DAPI)</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CH$_4$ fluxes and oxidation rates [μmol CH$_4$ h$^{-1}$]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$J_{in}$$^c$</td>
<td>47.9 ± 7.3</td>
<td>47.5 ± 6.2</td>
<td>44.9 ± 3.2</td>
<td>41.5 ± 2.3</td>
</tr>
<tr>
<td>$J_{out}$$^c$</td>
<td>47.9 ± 7.3</td>
<td>0.18 ± 0.12</td>
<td>0.50 ± 0.65</td>
<td>0.04 ± 0.06</td>
</tr>
<tr>
<td>r$^d$</td>
<td>-</td>
<td>47.3 ± 6.2</td>
<td>44.4 ± 2.8</td>
<td>41.5 ± 2.3</td>
</tr>
</tbody>
</table>

$^a$ Oxygen concentrations applied at the top of the column; nominal values as given for the used gas cylinders.

$^b$ The number of sampling occasions for CH$_4$, CO$_2$, $\delta^{13}$C in CH$_4$ and cell counts using DAPI is given for each phase.

$^c$ $J_{in}$ is the average CH$_4$ flux into the column; $J_{out}$ the average CH$_4$ flux out of the column (equals emissions). All fluxes are given for steady-state conditions.

$^d$ r is the average total CH$_4$ oxidation rate in the column.

6.3.3 Sampling and analytical methods

Methane concentrations were frequently measured in the column, at the column inlets and outlets and in the reservoir. Carbon dioxide and stable carbon isotope ratios in CH$_4$ were measured on selected days (Table 6-1). Samples (0.5 - 1 mL) for CH$_4$ and CO$_2$ were taken
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from gas sampling ports of the column with a gas-tight glass syringe after flushing twice with 0.2 mL sample gas and measured directly by gas chromatography. For stable carbon isotope analysis of CH₄, 7 - 14 mL samples were collected in 1 mL increments distributed over one day to avoid disturbance of steady-state conditions, injected into N₂-flushed, partially-evacuated vials and analyzed on a GC-IRMS. The gas outlet at the top of the column was sampled for isotope analysis by flushing 100 mL gas sampling vessels for at least 1 hour. The full content of these sampling vessels was directly transferred to the GC-IRMS so that isotope measurements were possible at atmospheric CH₄ concentrations. Water samples of the supply system were injected into N₂-flushed, partially-evacuated vials and CH₄ or δ¹³C in CH₄ was measured in the headspace. All samples were analyzed as described previously (Urmann et al. 2005). We attempted to measure O₂ using a GC-TCD system, but the obtained data were not utilisable due to the low O₂ concentrations encountered.

6.3.4 Concentration and isotope profile analysis

Methane flux into the column \( J_{in} (\mu\text{mol h}^{-1}) \) was calculated from measured CH₄ data using Fick’s first law of diffusion:

\[
J_{in} = -D_s \frac{dc}{dz} A
\]  

(Equation 6-2)

The CH₄ gas-phase concentration gradient with depth, \( dc/dz \) was obtained by regression from the linear part of the profiles. The flux was converted to \( \mu\text{mol h}^{-1} \) using the cross-sectional area, \( A \) of the column (93.3 cm²). For the biotic phases of the experiment, effective diffusivity for CH₄ in the sand (\( D_s \)) was computed from diffusivity in air \( D_a \) of 0.2287 cm² s⁻¹ (25 °C) (Massman 1998) and average measured porosity and water content using the Millington and Quirk equation (Millington and Quirk 1961). In the dry sand during the abiotic phase, effective diffusivity was calculated in the same way from average measured porosity. Total CH₄ oxidation, \( r (\mu\text{mol h}^{-1}) \) in the column was calculated by a mass balance approach similar to (Chaplin et al. 2002):

\[
r = J_{in} - J_{out}
\]  

(Equation 6-3)

The flux out of the column, \( J_{out} \) was either calculated from CH₄ concentrations at the outlet of the column and the gas flow rate or, when these data were not available, from the top linear part of the CH₄ profile (analogous to equation 6-2). Total production of CO₂ was computed by
adding together the fluxes out of the top and bottom of the column, calculated from gradients of the linear parts of the CO₂ profiles using a $D_a$ of 0.161 cm² s⁻¹ (25 °C) (Perry and Green 1997; Massman 1998). To calculate a first-order rate constant from measured CH₄ profiles, an analytical solution for a simple steady-state diffusion-consumption model (Reeburgh et al. 1997) was fitted to the data using the CH₄ concentration at the bottom of the active zone (see results) and, as an approximation, a concentration of zero at the top of the column as boundary concentrations. The effective diffusion coefficient was calculated as given above.

The fractionation factor $\alpha_{ox}$ for CH₄ oxidation in the column was calculated from measured isotope ratios, similar to Reeburgh et al. (1997) using equation 6-4. The latter describes isotope fractionation in an open, flow-through system based on a mass balance (Liptay et al. 1998).

$$\alpha_{ox} = \frac{(\delta_c - \delta_a)}{1000 f_o} + \alpha_{trans} \quad \text{(Equation 6-4)}$$

In our experiments, $\delta_c$ is the $\delta^{13}$C of emitted CH₄ and $\delta_a$ is the average $\delta^{13}$C of CH₄ in the lower part of the profile where little isotope shifts occurred. The fraction of CH₄ oxidized $f_o$ was obtained from concentration profile analysis. In our experiments, the isotope fractionation factor for transport $\alpha_{trans}$ equaled $\alpha_{diff}$ as transport in the column occurred only by diffusion. For $\alpha_{diff}$, we used a value of 1.0178 as determined experimentally by De Visscher et al. (2004), which is close to the theoretical value of 1.0195.

### 6.3.5 Concentration and isotope profile simulations

We used the Subsurface Transport Over Multiple Phases (STOMP) simulator (White and Oostrom 2000) to numerically simulate CH₄, CO₂ and O₂ concentration profiles and profiles of $\delta^{13}$C in CH₄. Using the air-water operational mode, STOMP simulates transport by solving the water and air mass conservation equation. Microbial oxidation was simulated as a first-order type reaction with respect to CH₄ with a correction factor if O₂ was almost totally consumed, thus preventing reaction. In this way conditions without CH₄ oxidation were simulated in the lower part of the column while a first-order rate constant for CH₄ was defined for the entire domain. The simulated domain was 1-dimensional with 160 nodes, 0.5 cm apart. The maximum time step was 0.007 - 0.015 min and simulated time was 12 h. The Millington and Quirk model was used to calculate effective diffusion coefficients. Input parameters were measured porosity and water content, diffusion coefficients in air at 25 °C for CH₄ and CO₂.
(given previously) and 0.2086 cm$^2$ s$^{-1}$ for O$_2$ (Perry and Green 1997; Massman 1998), dimensionless Henry constants at 25 °C: 30.43 for CH$_4$, 32.27 for O$_2$ (Perry and Green 1997) and 1.21 for CO$_2$ (Letterman 1999).

The following boundary conditions were employed: gas boundary conditions were constant pressure at the top and no flow at the bottom, water boundary conditions were constant pressure at the bottom and no flow at the top. Solute boundary conditions were constant concentration for all gases. Methane and CO$_2$ concentrations on the days when both gases were measured were used as boundary concentrations. Oxygen boundary concentrations at the top were adjusted to accurately simulate the observed depth where activity occurred and were zero at the bottom. Measured carbon assimilation ratios were used to calculate stochiometries. Initially, an apparent first-order rate constant of 25 h$^{-1}$ as estimated from measured data was employed. The $k$-value was then adjusted using increments of 5 h$^{-1}$ and the goodness of the fit tested by minimizing the sum of calculated square residuals between measured and simulated CH$_4$ and CO$_2$ data.

Methane oxidation rates as a function of depth were calculated by multiplying the first-order rate constant $k$ obtained from simulations with the simulated concentration at each depth (Jones and Nedwell 1993). Subsequently, total CH$_4$ oxidation was calculated for 0.5 cm depth intervals.

To simulate profiles of isotope ratios, simulations were performed with $^{13}$CH$_4$ and $^{12}$CH$_4$ as separate species. The diffusion coefficient and first-order rate constant for $^{13}$CH$_4$ were calculated from the parameters for $^{12}$CH$_4$ using the fractionation factors for diffusion $\alpha_{\text{diff}}$ (given previously) and for oxidation ($\alpha_{\text{ox}}$), respectively. The boundary concentrations for $^{13}$CH$_4$ were calculated from measured $\delta^{13}$C-values, total CH$_4$ concentrations (measured on the same days as the isotopes) and the $^{13}$C/$^{12}$C ratio for the PDB (Peedee Belemnite) reference standard of 0.011237 (Craig 1957).

### 6.3.6 Total cell counts

Sand (0.1 - 0.5 g) was collected with a sterile spatula out of 3 (phase I and II) or 7 (phase III) sampling ports of the column towards the end of each phase. No samples were taken out of the column at the beginning of the experiment and only few samples were taken in phase I and II to minimize disturbance. Total cell numbers in the inoculum and in the sand were estimated using 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. The sand samples and 1 mL of the inoculum were fixed at 4 °C for at least 3 hours in 1 mL of 4% [wt vol⁻¹] paraformaldehyde in phosphate-buffered saline. Samples were vortexted for 1 min, diluted 1:1 with 0.1% [wt vol⁻¹] sodium pyrophosphate and dispersed for 10 min in a sonication bath before spotting onto ethanol-washed slides. Samples were incubated at 46 °C for 2.5 h with 1 μL DAPI (500 ng μL⁻¹) in 9 μL hybridization buffer containing 2% [wt vol⁻¹] blocking reagent (Roche, Germany) (Sekar et al. 2003). At least 20 fields and 400 cells per well and 3 wells per sample were counted. Using this modified protocol reduced background fluorescence of sand particles and increased cell brightness. With the described protocol, no signal was obtained with the methanotrophic FISH probes developed by Eller et al. (2001). Possibly, the available probes did not target the species present in the column.

6.4 Results

6.4.1 Gas concentration profiles

After an initial equilibration time of 5 days a linear CH₄ profile was obtained in the column under abiotic conditions (Figure 6-2A). This indicated that diffusive gas transport and steady-state conditions were achieved with the column design. Under biotic conditions, steady-state was reached after ~ 16 days. Days when the example CH₄ and CO₂ profiles for phases I-III (Figure 6-2 B,C,D) were measured are indicated with black arrows in Figure 6-3A. On the day when CO₂ was measured in phase III profiles where not typical for this phase. Therefore, CO₂ data were excluded from further analysis and are not shown. In all three phases, CH₄ profiles were linear in the lower part of the column. Transition from linear to non-linear profiles indicated CH₄ oxidation. Around 15 cm above the transition point, CH₄ concentrations dropped to atmospheric levels. This 15 cm-deep zone will be designated "active zone" throughout this paper. The shape of CH₄ profiles indicated a decrease of CH₄ oxidation from the bottom towards the top of this zone. In the upper and lower part of the column, CO₂ profiles were also linear (Figure 6-2B and C). The maximum CO₂ concentrations coincided with the transition point in CH₄ profiles, indicating that maximum activity occurred at this depth. This depth will be designated "depth of maximum activity" throughout this paper.

The depth of maximum activity increased from 70 cm to 55 cm until steady-state was reached in phase I. Subsequently, the predominant depth of maximum activity shifted from 55 cm to 50 cm depth (Figure 6-3B). At the beginning of phase II, the depth of maximum
activity shifted upwards from 55 cm to 40 cm within 1 day after lowering the (nominal) O₂ concentration at the top of the column from 4.1 mL L⁻¹ to 2.5 mL L⁻¹ (Figure 6-3A and B). During phase II, profiles were more stable than in phase I. At the beginning of phase III, after increasing the (nominal) O₂ concentration at the top of the column back to 4.1 mL L⁻¹, the depth of maximum activity shifted back down to 55 cm depth within 1 day. However, it subsequently moved downwards and appeared to stabilize at 65 cm depth, 10 cm lower than in phase I under similar conditions (Figure 6-3B).

Figure 6-2: Measured CH₄ and CO₂ gas-phase concentrations and simulated CH₄, CO₂ and O₂ concentration profiles in the column. An example profile is shown for each phase. Note that simulated O₂ boundary concentrations at the top of the column had to be increased with respect to nominal values (given for gas cylinders) to accurately simulate the depth of the active zone. Carbon dioxide was not measured on the day shown for phase III and no simulations were performed.
6.4.2 Fluxes and activity

Under abiotic conditions, CH₄ flux into the column was similar to the CH₄ flux during the biotic phases of the experiment (data not shown). Throughout the three phases of the experiment, CH₄ flux into the column was relatively stable with fluctuations especially during phase I and a slight decrease with time (Figure 6-3C, Table 6-1). Occasionally, problems with the CH₄ supply occurred after changing the reservoir, but interruptions were never longer than one day. Emissions out of the column were on average 0.3 ± 0.46 μmol h⁻¹ (± one standard deviation), that is 0.7 ± 1.0% of supplied CH₄ (Figure 6-3D, Table 6-1). At the beginning of phase I, before steady-state was reached, CH₄ emissions reached a maximum of 3.6% of supplied CH₄ and dropped subsequently to around average values (Figure 6-3D). At the beginning of phase II, CH₄ emissions increased for 8 days with a maximum of 2.5 μmol h⁻¹ or 5.0% of supplied CH₄. In contrast, at the transition from phase II to III, emissions remained low. In- and outgoing CH₄ fluxes showed that, on average 45.3 ± 5.0 μmol h⁻¹ (± one standard deviation), that is 99.3 ± 1.0% of supplied CH₄ were converted in the column with a slight decrease with time parallel to the decrease in supplied CH₄ (Table 6-1). Fitting a simple diffusion-consumption model (Reeburgh et al. 1997) to the data of phase I and II yielded an average apparent first-order rate constant k of 26 ± 6 h⁻¹ (± range).

Carbon dioxide production was assessed by adding together the CO₂ fluxes out of the top and bottom of the column. Based on the assumption that all CO₂ production originated from CH₄ oxidation, comparison of CH₄ oxidation and CO₂ production showed that on the days when CO₂ was measured 34% (phase I) and 46 – 51% (phase II) of CH₄ were assimilated. As only one instead of two O₂ molecules is required for one CH₄ assimilated, this translates into a stochiometric ratio of CH₄:O₂ of ca. 1:1.7 (phase I) and 1:1.5 (phase II).
Figure 6-3: (A) Experimental phases and nominal O₂ concentrations at the top of the column. Large (black) arrows indicate the days when the profiles shown in Figure 6-2 were measured, small (white) arrows indicate days when isotope samples were taken (averages shown in Figure 6-5). (B) Depth of maximum activity, i.e. depth of the bottom of the active zone. (C) Total CH₄ flux (Jₘᵢ) into the column. (D) CH₄ emissions (Jₘₒ) out of the column.
6.4.3 Profiles of DAPI cell counts

The methanotrophic enrichment culture used to inoculate the column contained $1.1 \times 10^7$ cells mL$^{-1}$ counted by DAPI-staining. From this cell concentration and the water content in the column, an initial cell number in the column of $2.8 \times 10^5$ cells (g dry weight)$^{-1}$ was estimated. Towards the end of phase I, high cell numbers of $1.2 \times 10^7$ cells (g dry weight)$^{-1}$ were found in the column at 50 cm depth that is in the lower part of the active zone (Figure 6-4). Cell numbers decreased towards the top, but at 40 cm depth, cell numbers were still more than half of those found at 50 cm depth. With $9.5 \times 10^5$ cells (g dry weight)$^{-1}$, cell numbers above the active zone were in the same order of magnitude as initial cell numbers. Towards the end of phase II, measured cell numbers in the active zone (40 and 25 cm depth) were lower than in phase I but the pattern was similar. Furthermore, cell numbers remained high in the active zone of the previous phase (at 45 cm). At the end of phase III, high and similar cell numbers occurred between 60 and 40 cm depth. A high cell number at 60 cm agreed with the observation that the active zone had extended below 55 cm depth in phase III.

![Figure 6-4: Depth profiles of DAPI-stained cells at the end of each of the three phases of the experiment (phase I and III with 4.1 mL L$^{-1}$ O$_2$, phase II with 2.5 mL L$^{-1}$ O$_2$ at the top of the column). Horizontal lines depict the active zone of each phase. Only three samples were taken at the end of phase I and II to minimize disturbance.](image-url)
6.4.4 Isotope fractionation along profiles

Methane in the headspace above the water at the bottom of the column was on average enriched by 20% in relation to CH$_4$ in the water. Under abiotic conditions the isotopic composition of the CH$_4$ diffusing upwards in the column remained constant up to 25 cm depth. Between 25 and 5 cm depth, isotope ratios then shifted by up to 7.4% ± 1.4 (± range) towards lighter values (Figure 6-5). Reasons for these effects are not clear. Theoretically isotope ratios are constant with respect to depth under diffusive steady-state conditions (Levin et al. 1993). In phase I, the isotopic composition of CH$_4$ stayed nearly constant up to 60 cm depth and then got heavier by 6.6% up to 35 cm. Similarly, in phase II, the isotopic composition got heavier by 7.6% between 55 cm and 30 cm depth. Measurements above 35 cm and 30 cm respectively were not possible due to low CH$_4$ concentrations. Isotope analysis at low concentrations was only possible at the gas outlet at the top of the column due to larger sample volumes obtained by flushing. The CH$_4$ emitted out of the column showed similar δ$^{13}$C values to CH$_4$ at 35 cm and 30 cm depth. Fractionation factors $\alpha_{ox}$, calculated from observed isotope shifts (Equation 6-3) were similar in the two phases with values of 1.0244 (phase I) and 1.0254 (phase II).

![Figure 6-5: Measured and simulated profiles of δ$^{13}$C in CH$_4$ in the column with dry sand (abiotic) and in the column inoculated with a methanotrophic enrichment culture under 4.1 mL L$^{-1}$ O$_2$ (Phase I) and 2.5 mL L$^{-1}$ O$_2$ (Phase II). Note that the increasing enrichment shown in simulations (in the depth interval where no data could be obtained) was likely a result of a too high rate constant in this depth interval. The "model" abiotic profile is a theoretical profile based on the δ$^{13}$C value at the bottom of the column.](image-url)
6.4.5 Simulation of concentration and isotope profiles

Example gas concentration profiles under abiotic conditions and in phase I and II (Figure 6-2 A,B,C) were simulated using the numerical simulator STOMP. Good agreement was achieved between measured and simulated CH₄ profiles under abiotic conditions (Figure 6-2A). For profile simulations in phase I and II, carbon assimilation rates calculated from measured profiles were used (see 6.4.2). Two parameters were adjusted to fit the measured data a) the O₂ boundary concentration at the top of the column and b) the apparent first-order rate constant for CH₄. In both phases the nominal O₂ concentration at the top boundary had to be increased by 0.9 mL L⁻¹ to match the observed depth of the active zone. Likely the O₂ concentration being supplied to the top of the column was higher by ~0.9 mL L⁻¹ than the nominal concentration in the gas cylinder due to diffusion of O₂ into the supply line. The shape of the CH₄ and CO₂ profiles was best fitted by an apparent first-order rate constant of 30 h⁻¹ for CH₄ oxidation in both phases (Figure 6-2B and C). Only at very low CH₄ concentrations, CH₄ oxidation was overestimated with this rate constant (not discernible in Figure 6-2 due to scale). Note that the shape of the profiles was not influenced by the O₂ boundary concentrations (not shown). Simulated O₂ profiles in all phases were linear in the upper part of the column followed by a non-linear transition zone to anoxic conditions. This zone was in agreement with the observed transition zones of CH₄ and CO₂ profiles.

Simulated profiles allowed a more detailed analysis of CH₄ oxidation as a function of depth due to higher resolution. Assessment of CH₄ oxidation as a function of depth confirmed that highest CH₄ oxidation occurred at the bottom of the active zone and decreased towards the top. In both phases, ~50 % of CH₄ was converted within a 3 cm depth-interval, while ~97 % of CH₄ was converted within the entire active zone. Simulated CH₄ and O₂ concentrations within the active zone were similar in the two phases and ranged on average (given from top to bottom) from 0.01 to 0.20 mL L⁻¹ for CH₄ and 1.11 to below 0.01 mL L⁻¹ for O₂.

For simulations of isotope profiles in phase I and II, information on carbon assimilation was not available as CO₂ concentrations and isotopes were not measured on the same days. To accurately simulate the CH₄ concentration data on the days when isotopes were measured in phase I, the carbon assimilation rate had to be adjusted from 30 to 50%. The αₐᵦₐ-values calculated from measured isotope data were used to calculate first-order rate constants for ¹³CH₄. Simulations agreed well with the measured δ¹³C profiles for both phases, showing a trend towards heavier isotope ratios in and below the active zone (Figure 6-5). In the simulations, CH₄ got even more strongly enriched above the active zone where isotope data
could not be obtained. Simulated isotope ratios reached a maximum at 12 and 15 cm depth and then got lighter again towards the top of the column.

6.5 Discussion

6.5.1 Methanotrophic activity and kinetics

We designed a laboratory column with CH$_4$ diffusing out of CH$_4$-saturated water into an unsaturated zone from the bottom and O$_2$ diffusing into the unsaturated zone from the top. This design allowed mimicking the physical conditions in a methanotrophic ecosystem with diffusive substrate supply, such as the unsaturated zone of wetlands and peat bogs or the unsaturated zone above a methanogenic aquifer. The CH$_4$ flux into the column was relatively constant throughout the different phases of the experiment indicating that it was limited by mass transfer across the air-water interface and not determined by processes in the unsaturated zone. The concurrence of the depth of highest CO$_2$ production and CH$_4$ oxidation suggested that CH$_4$ oxidation was the main microbial process in the system. The CH$_4$ flux into the column ($J_{in}$) was relatively constant and the flux out of the column $J_{out}$ was small. Therefore, the assimilation rate, which determines the stoichiometric ratio of O$_2$:CH$_4$, and the O$_2$ concentration at the top of the column determined the depth where highest CH$_4$ oxidation activity developed. Due to the described dependency, variations in assimilation rates may have caused the observed fluctuations of the depth of maximum activity in phase I and the differences between phase I and III.

Methane concentration profiles indicated that methanotrophic activity was concentrated in a 15 cm-deep zone with maximum activity at the bottom and decreasing activity towards the top. Concentration of methanotrophic activity in a distinct zone is common in environments with substrate supply through diffusive counter-gradients (Amaral and Knowles 1995; Damgaard et al. 1998; Wagner et al. 2003). Comparison of rates of CH$_4$ oxidation showed that total CH$_4$ oxidation per area in the column was one to two orders of magnitude higher than in peat bogs (Whalen and Reeburgh 2000; Pearce and Clymo 2001) or above a contaminated aquifer (Urmann et al. submitted). Therefore, total CH$_4$ oxidation activity in the column was at the high end for a system with diffusive CH$_4$ supply. Using a simple diffusion-consumption model, a high value of 26 h$^{-1}$ was obtained for the average apparent first-order rate constant for CH$_4$ in phase I and II.

Numerical simulations of concentration profiles confirmed that CH$_4$ oxidation followed first-order kinetics in the column with an apparent first-order rate constant of 30 h$^{-1}$, similar to
the one obtained from the analytical solution. The $k$-value likely resulted from low CH$_4$
concentrations in the column being maintained by (relatively fast) CH$_4$ fluxes in the gas phase
and high CH$_4$ oxidation by methanotrophic bacteria. Therefore $k$ is affected by both, the
microbial community and the rate of substrate supply by gas flux. This shows that physical
characteristics of a system have to be taken into account when interpreting first-order rate
constants.

Oxygen profiles could not be determined experimentally, but measured CH$_4$ and CO$_2$
fluxes defined the O$_2$ fluxes stochiometrically required for the observed CH$_4$ oxidation
activities. Therefore, the simulated O$_2$ profiles probably represented actual profiles in the
column. At the depth of maximum activity, simulations suggested that in-situ O$_2$
concentrations were approximately 10 µL L$^{-1}$. Despite low O$_2$ concentrations, first-order
kinetics with respect to CH$_4$ implied no O$_2$ limitation. It has been found previously that CH$_4$
oxidation was CH$_4$-limited in lake sediments even at low O$_2$ concentrations (Kuivila et al.
1988).

6.5.2 Biomass and response to changing gradients

To complement information on CH$_4$ oxidation activity, total biomass at different depths in
the column was assessed by DAPI-staining. As CH$_4$ oxidation was the dominant process in
the column (see above), numbers of DAPI-stained cells can give an indication of
methanotrophic biomass. Cell numbers in the active zones of the different phases were 10 - 40
times higher than estimated initial cell numbers, suggesting growth in the column. Initial cell
numbers were at the low end of a range of methanotrophic cell numbers observed in soils
exposed to ambient CH$_4$ concentrations (Bender and Conrad 1994). Growth in the column is
in agreement with the relatively high carbon assimilation rates of 34 and 51% that fall within
the wide range of 2-73% previously reported for methanotrophs (references in Whalen et al.
1990). Apparently, more growth had occurred in zones of higher CH$_4$ oxidation, as in phase I
and II zones with higher oxidation coincided with zones with higher total cell numbers
(compare Figures 6-2 and 6-4). A correlation between activity and biomass or cell number has
been found previously in a number of studies in different environments (e.g. Bender and
Conrad 1994). However, different amounts of biomass combined with constant CH$_4$
oxidation in the column suggested that activity was limited by CH$_4$ gas fluxes and not by (total)
biomass. This is in agreement with the observation of first-order kinetics in the column.

As outlined above, the O$_2$ concentration at the top of the column determined the depth of
the methanotrophically active zone in the column. Changing the O$_2$ concentration therefore
exposed a different zone to the substrates \( \text{CH}_4 \) and \( \text{O}_2 \). At both transitions, from phase I to II and II to III a rapid response of the methanotrophic community to changing substrate availability was observed. At the transition from phase I to II, \( \text{CH}_4 \) emissions out of the column increased slightly for 8 days after the applied change (Figure 6-3D). This suggests that activation of resting stages, growth or increase in cell specific capacity (Bender and Conrad 1995) were necessary for the newly developing active zone to reach its full oxidation capacity. At the transition from phase II to III no increase in emissions was observed. The methanotrophic community present below the active zone of phase II had likely survived under anoxic conditions and recovered rapidly as observed in other studies in different environments (Nedwell and Watson 1995; Whalen and Reeburgh 2000; Carini et al. 2005). In general, methanotrophically active zones shifting rapidly according to substrate supply have been observed previously for example in the water column of a lake or a rice paddy (Damgaard et al. 1998; Carini et al. 2005).

### 6.5.3 Isotope profiles and fractionation factor

The column design allowed quantification of the fractionation factor for microbial \( \text{CH}_4 \) oxidation in an environment physically imitating in-situ conditions. Observed shifts in isotope ratios were a combined effect of fractionation due to microbial activity and due to diffusive gas transport through the sand (Levin et al. 1993; Reeburgh et al. 1997). This was accounted for when quantifying the stable carbon isotope fractionation factor for microbial \( \text{CH}_4 \) oxidation in the column. The obtained fractionation factors of 1.024 and 1.025 were in the upper half of the range of 1.003 - 1.039 reported in several field and laboratory studies (references in Snover and Quay 2000; Templeton et al. 2006). This large variation in the reported fractionation factors for microbial oxidation could be partially due to a shift of the rate limiting step from transport of the substrate to the cell to the enzymatic reaction. For methanotrophic cultures in a chemostat, it was observed that the fractionation factor became small when a large fraction of \( \text{CH}_4 \) was oxidized (Templeton et al. 2006). It was proposed that this was due to rate-limitation by the transfer across the air-water interface, which fractionates only slightly (Knox et al. 1992). Under steady-state conditions with a high gas flux and low water content as in the column of this study, mass transfer is likely not the main rate-limiting step and transport of \( ^{13}\text{CH}_4 \) from the aqueous phase back into the gas phase can occur even if a high fraction of \( \text{CH}_4 \) is consumed. Consequently, enzymatic fractionation may be mainly responsible for the high isotope fractionation observed in this study. Previously, a similarly
high fractionation factor for oxidation of atmospheric CH₄ along CH₄ profiles was determined in upland soils (Reeburgh et al. 1997).

Simulations of isotope profiles verified that the obtained \( \alpha_{\text{ox}} \)-values of 1.024 and 1.025 were reasonable estimates for fractionation due to microbial oxidation. When \( \alpha_{\text{ox}} \) was changed to bracketing values of 1.020 and 1.030 (data not shown) the obtained profiles were clearly different from measured data in phase II, while in phase I the profiles simulated with the original \( \alpha_{\text{ox}} \) of 1.0244 and 1.03 bracketed the measured data. Above the active zone, where we could not obtain isotope data, isotope profiles were probably not simulated correctly as the rate constant in this zone was too high causing large isotope shifts. Unfortunately, this could not be changed in the simulator. However, the trend in isotope ratios towards heavier and then lighter values seems reasonable as a similar trend has been found in situ in several landfill cover soils (De Visscher et al. 2004). Our results support the hypothesis that, in an unsaturated system, the fractionation factor for microbial CH₄ oxidation is influenced by physical conditions. However, further investigations to elucidate this relationship are needed.

### 6.6 Conclusions

The column designed in this work allowed studying microbial CH₄ oxidation in an unsaturated porous medium with a CH₄/O₂ counter-gradient under diffusive steady-state conditions. Methane oxidation occurred with a high apparent first-order rate constant and was not limited by O₂. The methanotrophically-active zone formed an effective buffer to remove CH₄ from the system. Induced changes in substrate gradients led to rapid shifts in the location of the active zone combined with only minor CH₄ emissions. The stable carbon isotope fractionation factor for CH₄ oxidation was relatively high, probably due to enzymatic fractionation being (fully) expressed under steady-state conditions. Simulated and measured CH₄, CO₂ and \( \delta^{13}\text{C} \) profiles were in good agreement, showing that the system was well described by simulations. In future studies, the developed column design should be valuable for investigating the influence of environmental parameters on CH₄ oxidation and other processes with gaseous substrates under physical conditions resembling those in the environment.
6.7 Acknowledgements

We thank Matthias Saurer and Rolf Siegwolf (PSI, Villigen, Switzerland) for help with stable carbon isotope measurements, M. Oostrom and M. White (Pacific Northwest National Laboratory) for continued support with the STOMP simulator and Hanspeter Läser (Institute of Terrestrial Ecosystems, Soil physics group, ETH Zurich, Switzerland) for manufacturing the column.
6.8 References


Chapter 6: Methanotrophic activity in a methane/oxygen counter-gradient


Discussion

Karina Urmann
7.1 Potential and limitations of in-situ methods to assess microbial activity

7.1.1 Gas push-pull tests

In this thesis we developed a new method, the gas push-pull test (GPPT), for the in-situ quantification of microbial processes in the vadose zone (chapter 2). We used CH₄ oxidation as a model process and applied the method at two different sites, a) the vadose zone above a contaminated aquifer in Studen (chapter 2 and 3) and b) an alpine peat bog in Eigenthal (chapter 4). These two sites had very different properties which enabled us to evaluate the GPPT under different conditions. In Studen, injection and extraction was performed at 1 m to 3 m depth within a 70 cm-diameter concrete casing that partially restricted gas transport. The concrete casing was filled with a sand and gravel mixture with a porosity of 0.39 which is at the lower end of the porosity typical for soils (Schachtschabel et al. 1998). In the peat bog, on the other hand, the maximum injection/extraction depth was 50 cm, the peat had a high estimated porosity of 0.96 and gas transport was unrestricted. Gas exchange with the atmosphere was probably even enhanced in peat bog experiments because GPPTs were performed in hummocks which have a large contact area with the atmosphere.

The main issues concerning development and application of the GPPT are a) the choice of a suitable tracer, b) the choice of a suitable inhibitor, c) the choice of the injection concentration and distinction of injected gases from their background in soil air d) sufficient contact time between substrates and organisms for consumption and at the same time sufficient recovery of the injected gases for quantification and e) the test volume.

a) Choice of tracer

A perfect tracer for rate quantification during GPPTs would have exactly the same physical properties as the reactant. However, this is not the case for any two gases. For example, gas-phase diffusion coefficients differ widely between gases (Table 7-1) as they are mass dependent (Fuller et al. 1966). Furthermore, the tracer has to be recalcitrant with respect to chemical or microbial reactions, not only with respect to the process of interest but also other processes that might occur within the test zone. Due to their inert behavior, noble gases or halogenated compounds such as SF₆ or chlorofluorocarbons are therefore possible tracers. For the initial development of the GPPT, we chose to test the noble gases He, Ne and Ar as tracers for the reactant CH₄ due to their relatively similar properties (Table 7-1). Furthermore, unlike halogenated compounds, noble gases do not contribute to the greenhouse effect. An advantage of using halogenated compounds would be their easy analytical detection with a much lower
detection limit than noble gases. However, care has to be taken as some halogenated compounds can be degraded by methanotrophic bacteria or inhibit their activity (Hanson and Hanson 1996; Matheson et al. 1997). Among halogenated compounds CF₄, SF₆ or CCl₂F₂ may be suitable tracers in future studies as they are inert, have a similar or lower solubility in water than CH₄ and diffusion coefficients that are similar to the diffusion coefficient of CH₄ within a factor of two to three (Table 7-1). Trifluoromethane (CHF₃) is much more soluble and therefore less suitable even though it is neither degradable nor inhibitory to CH₄ oxidation (King 1997).

In the vadose zone above the contaminated aquifer in Studen, Ne and Ar were tested as possible tracers. Neon was found to be a suitable tracer for the quantification of rate constants for CH₄ oxidation under the conditions at this site, i.e. with partially restricted gas transport (chapter 2 and 3). Neon could be used as a tracer for rate calculations in future studies under similar conditions. However, similarity of physical behavior of Ne and CH₄ should be re-evaluated for each site. For example, water content at the site in Studen was low (0.06 – 0.12 cm³ cm⁻³). The effect of higher water content on transport behavior is not clear as CH₄ is about three times more soluble than Ne. This is currently under investigation.

Investigations of gas transport during GPPTs in a 1 m-diameter laboratory model system with dry sand (Gonzalez-Gil et al. submitted-b) and first trials to apply the GPPT in the peat bog showed that none of the employed noble gases, He, Ne or Ar could be used directly as tracers for rate quantification. However, we demonstrated that the reactant CH₄ can be used as a tracer if a) two consecutive tests can be performed reproducibly with respect to gas transport and b) a breakthrough curve for CH₄ determined only by physical processes can be obtained in the second test by co-injecting an inhibitor for CH₄ oxidation (chapter 4). It should be possible to apply this approach at sites with a range of physical conditions. Application is limited by the possibility to inject and extract gases, which may be difficult due to low permeability for example in clay or in systems with high water content. Furthermore, performing two tests reproducibly with respect to gas transport could in some cases be impeded by changing conditions even throughout one day. For example, we performed two GPPTs at 30 cm depth in a small hummock in Gross Moos (Schwendital, Oberurnen, Switzerland), an open, unwooded peat bog. Tracer recoveries during the second GPPT were much lower than during the first one which was probably due to a higher wind velocity later in the day. The wind apparently influenced gas transport especially due to the shallow injection depth and the large surface of the hummock in relation to its volume.
Table 7-1: Diffusion coefficients in air ($D_{\text{air}}$) and Henry’s law constants ($H$) for CH$_4$ and (potential) tracer gases for GPPTs at 15°C.

<table>
<thead>
<tr>
<th>Gas</th>
<th>$D_{\text{air}}$ [cm$^2$ s$^{-1}$]</th>
<th>$H$ [(mol L$^{-1}$$<em>{\text{gas}}$)/(mol L$^{-1}$$</em>{\text{water}}$)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_4$</td>
<td>0.215$^a$</td>
<td>25.7$^b$</td>
</tr>
<tr>
<td>He</td>
<td>0.627$^c$</td>
<td>110.2$^d$</td>
</tr>
<tr>
<td>Ne</td>
<td>0.289$^c$</td>
<td>87.7$^e$</td>
</tr>
<tr>
<td>Ar</td>
<td>0.179$^c$</td>
<td>25.1$^e$</td>
</tr>
<tr>
<td>SF$_6$</td>
<td>0.088$^c$</td>
<td>133.4$^d$</td>
</tr>
<tr>
<td>CF$_2$Cl$_2$</td>
<td>0.095$^f$</td>
<td>16.3$^d$</td>
</tr>
<tr>
<td>CHF$_3$</td>
<td>0.098$^g$</td>
<td>2.2$^d$</td>
</tr>
<tr>
<td>CF$_4$</td>
<td>0.117$^f$</td>
<td>163.4$^d$</td>
</tr>
</tbody>
</table>

$^a$ Massman (1998)
$^b$ Perry and Green (1997)
$^c$ Calculated according to Fuller et al. (1966).
$^e$ Calculated according to Shan and Pruess (2003)
$^f$ Measured by Raw and Raw (1976), adjusted for temperature according to Fuller et al. (1966).
$^g$ Calculated according to Fuller et al. (1966) with an estimated contribution of 15.5 cm$^3$ mol$^{-1}$ per Fluor atom to the total molar volume (Schwarzenbach et al. 2003).
b) Choice of inhibitor

For both approaches, a) using Ne as a tracer under restricted transport conditions and b) using CH₄ from an inactive test as a substitute tracer under diffusion-dominated transport conditions, the choice of an inhibitor for CH₄ oxidation was important. Acetylene (C₂H₂), the inhibitor that was used for both approaches, is an irreversible inhibitor (Prior and Dalton 1985) that has been shown to be effective at concentrations as low as 0.001% (Bodelier and Frenzel 1999; Chan and Parkin 2000) and that has a high water solubility (Table 7-2). High water solubility contributes to the effectiveness of the inhibitor at low headspace concentrations. However, higher water solubility of the inhibitor than CH₄ could also lead to chromatographic separation of the two gases during GPPTs. This would cause the inhibitor to be transported less far than the substrate. Acetylene breakthrough curves indicated that this was not the case during GPPTs in this study. Generally, complete inhibition during GPPTs is difficult to verify. However, reasonable efficiency was shown by similar breakthrough curves of CH₄ and noble gases and little isotope fractionation during the inactive test above the contaminated aquifer in Studen (chapter 2). The fact that C₂H₂ is an irreversible inhibitor may be a disadvantage in studies investigating temporal variability of CH₄ oxidation at the same location. Recovery of methanotrophic activity after C₂H₂ inhibition is currently under investigation. Alternative inhibitors that have been used in several studies to inhibit CH₄ oxidation and that could potentially be used during GPPTs are methyl fluoride (CH₃F) (Frenzel and Bosse 1996) or difluoromethane (CH₂F₂) (Miller et al. 1998) (Table 7-2). Physical properties of these inhibitors are similar to C₂H₂, however, higher concentrations may have to be injected in inactive tests. Furthermore, effectiveness of reversible inhibitors during GPPTs would have to be investigated as concentrations in the water phase have to remain high enough in relation to CH₄ concentrations during the entire test (Matheson et al. 1997) and inhibitors may be consumed by methanotrophic bacteria at low concentrations (Oremland and Culbertson 1992; Miller et al. 1998). At sites where methanogenesis is expected to occur in the same zone as CH₄ oxidation, concurrent inhibition of methanogenesis by the inhibitor may also be an issue. Methane breakthrough curves might be influenced to a different degree if methanogenesis is inhibited during the inactive test but not during the active test. All three inhibitors also inhibit methanogenesis, but CH₂F₂ and CH₃F inhibit mainly acetoclastic methanogenesis and inhibition by CH₂F₂ is effective only at higher concentrations than for the other two compounds.
Chapter 7: General discussion

Table 7-2: Properties of different gaseous inhibitors for CH\textsubscript{4} oxidation.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( \text{C}_2\text{H}_2 \text{a} )</th>
<th>( \text{CH}_3\text{F} \text{b} )</th>
<th>( \text{CH}_2\text{F}_2 \text{e} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of inhibition</td>
<td>irreversible</td>
<td>competitive/reversible</td>
<td>competitive/reversible</td>
</tr>
<tr>
<td>Concentration for (near) complete inhibition of CH\textsubscript{4} oxidation</td>
<td>0.001%</td>
<td>0.01% - 0.1%</td>
<td>0.03%</td>
</tr>
<tr>
<td></td>
<td>CH\textsubscript{4}: CH\textsubscript{3}F (in water) &gt; ~2:1</td>
<td>(Not effective at 0.001%)</td>
<td></td>
</tr>
<tr>
<td>Concentration for partial inhibition of methanogenesis</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.1%</td>
</tr>
<tr>
<td></td>
<td>(mainly acetoclastic methanogenesis)</td>
<td>(mainly acetoclastic methanogenesis)</td>
<td></td>
</tr>
<tr>
<td>( D_{\text{air}} \ [\text{cm}^2 \text{ s}^{-1}] ) at 15 °C</td>
<td>0.149\text{d}</td>
<td>0.138\text{f}</td>
<td>0.113\text{f}</td>
</tr>
<tr>
<td>( H [(\text{mol L}^{-1}<em>{\text{gas}})/ (\text{mol L}^{-1}</em>{\text{water}})] ) at 15 °C</td>
<td>0.9\text{e}</td>
<td>0.6\text{e}</td>
<td>0.6\text{e}</td>
</tr>
</tbody>
</table>

\text{a} Required concentrations determined in a landfill cover soil (Chan and Parkin 2000), in the rhizosphere of rice plants (Bodelier and Frenzel 1999) and in the rhizosphere of wetland plants (King 1996).

\text{b} Required concentrations determined in a landfill cover soil (Chan and Parkin 2000) and in the rhizosphere of wetland plants (King 1996). CH\textsubscript{4}: CH\textsubscript{3}F ratio given in Bodelier and Frenzel (1999) and Matheson et al. (1997).

\text{c} All information on CH\textsubscript{2}F\textsubscript{2} from Miller et al. (1998); concentration for inhibition of CH\textsubscript{4} oxidation was determined at 5% CH\textsubscript{4}.

\text{d} Calculated according to Fuller et al. (1966)

\text{e} Wilhelm et al. (1977), correction for temperature as described by R. Sander: Henry’s law constants (http://www.mpch-mainz.mpg.de/~sander/res/henry.html, last accessed 19 May 2006)

\text{f} Calculated according to Fuller et al. (1966) with an estimated contribution of 15.5 cm\textsuperscript{3} mol\textsuperscript{-1} per Fluor atom to the total molar volume (Schwarzenbach et al. 2003).

\text{g} Bunsen coefficient given by Miller et al. (1998) corrected for temperature as described by R. Sander: Henry’s law constants (http://www.mpch-mainz.mpg.de/~sander/res/henry.html, last accessed 19 May 2006) with an average correction term from CH\textsubscript{3}F and CHF\textsubscript{3}. 

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c) Injection and background concentration

One feature inherent in the GPPT method is that CH₄ or another substrate has to be added in concentrations above background in order to observe its consumption. This changes the in-situ conditions, but enables investigation of activity as a function of substrate concentrations. We estimated Michaelis-Menten parameters from several GPPTs with a range of CH₄ injection concentrations above the contaminated aquifer in Studen (chapter 3). This yielded information on the maximum capacity \( V_{\text{max}} \) of the methanotrophic community and therefore indirectly on the active biomass present and on the affinity of methanotrophic bacteria for CH₄ \( (K_m) \). On the other hand, if CH₄ oxidation follows (near) first-order kinetics during GPPTs, determined apparent first-order rate constants are valid for in-situ concentrations observed before GPPTs and in-situ oxidation rates can be calculated (chapter 3,4). Dilution of the injection mixture during GPPTs is usually fast and at least during part of extraction, apparent first-order kinetics were observed in most GPPTs (chapter 2,3,4). To calculate first-order rate constants from GPPTs, methods developed for groundwater push-pull tests were applied (Haggerty et al. 1998; Schroth and Istok 2006).

Application of GPPTs for the quantification of CH₄ oxidation may be complicated by high CH₄ background concentrations. Due to fast dilution of the injected gases it may be difficult to keep CH₄ above background concentration for a time long enough to allow quantification of CH₄ oxidation. Another problem can arise if background concentrations are not stable. This introduces uncertainty into corrections for background concentrations when evaluating GPPTs, especially if extraction concentrations approach background concentrations. Methane background concentrations in all tests performed in this study were low enough to allow quantification of CH₄ oxidation. However, O₂ background concentrations complicated the concurrent quantification of O₂ consumption. Even though quantification of O₂ consumption was not a major aim of this study, we tried to reduce CH₄ and O₂ background concentrations above the aquifer in Studen. The applied procedure consisted of flushing the test zone with 120 to 131 L of water-saturated N₂ gas at a pump rate of 1.4 to 1.8 L min⁻¹. Unexpectedly, this procedure increased CH₄ background concentrations at 2.7 m possibly due to stripping of CH₄ from the saturated zone. At 2.7 m, where the O₂ background concentration was between 10 mL L⁻¹ and 27 mL L⁻¹, the flushing procedure reduced O₂ background concentrations below the detection limit of the analytical method (~ 1 mL L⁻¹), but after 1 - 1.5 h of extraction, mixing in of background O₂ became apparent (chapter 3). At 1.1 m, where O₂ background concentrations were between 170 and 180 mL L⁻¹, the flushing procedure reduced the background concentration by a factor of 10 - 20, but was not effective in keeping
background concentrations low during the test. Injecting $^{13}$C-labeled CH$_4$ could be a possibility if injected and background CH$_4$ cannot be distinguished during GPPTs. However, this would increase the cost and analysis time of a GPPT significantly.

d) Contact time and mass recovery

An important issue for the successful application of the GPPT is sufficient contact time between the injected substrate and the indigenous microorganisms. Contact time is limited by dilution of the injected gases and diffusion of the injected gases away from the injection point. Extracted gas concentrations have to remain high enough for analytical measurement and, as discussed previously, above background concentrations to distinguish injected gas from background. However, for accurate determination of rate constants from GPPTs, high or even complete mass recovery is not required (Haggerty et al. 1998). At the site in Studen, contact time was sufficient as activity was high enough to cause a discernible difference between CH$_4$ and the tracer Ne during the 4 h of the test. This was the case even at 1.1 m where a relatively low CH$_4$ oxidation capacity was observed (chapter 3). With a high enough injection concentration, it would have been possible to conduct longer GPPTs under the conditions in Studen, so that even lower activities could possibly have been quantified.

Comparatively slow dilution of injected gas mixtures in Studen was indicated by high Ne recoveries of 26 to 72%. Conversely, only 2.8 - 10.4% of Ne were recovered during GPPTs of similar duration in the peat bog, indicating much faster dilution of injection mixtures due to mixing with soil air and diffusion. Fast dilution limited the possible duration of a test and therefore its sensitivity. For example, in GPPT 2a and GPPT 3a (chapter 4), test times could not have been extended beyond 3 - 5 h. Estimated in-situ activities prior to GPPTs in the peat bog (0.03 - 4.4 μmol CH$_4$ (L soil air)$^{-1}$ h$^{-1}$) and in Studen (1.2 - 2.0 μmol CH$_4$ (L soil air)$^{-1}$ h$^{-1}$) were in a similar range per volume of soil air and up to two orders of magnitude higher in the peat on a dry matter basis. However, it was more difficult to quantify apparent first-order rate constants in the peat. In addition to faster dilution of gases in the peat bog, this was due to the fact that a) first-order rate constants were at the lower end of the range of first-order rate constants measured in Studen and b) CH$_4$ oxidation followed apparent first-order kinetics only at lower CH$_4$ concentrations in the peat. The influence of the latter factor can also be seen when comparing GPPT 2 and GPPT 3 in the peat bog (chapter 4). The determined apparent first-order rate constant in GPPT 3 was a factor of two lower than in GPPT 2, but oxidation followed apparent first-order kinetics at ~30 times higher CH$_4$ concentration and was therefore much easier to detect.
By changing test parameters, dilution can be decreased to a certain degree and recovery can be increased. For example, to optimize the parameters for GPPTs in the peat bog, we performed pairs of GPPTs as described in chapter 4 with different parameters. Injecting 15 L instead of only 6 L and pumping at double the pump rate led to slower dilution (Figure 7-1). A further idea to increase recovery was to decrease the diffusive gradient for gases during GPPTs by injecting a large volume of injection mixture at a high speed prior to the actual GPPT. However, injection of 18 L at a high pump rate of 0.7 L min\(^{-1}\) followed by injection of 10 L at 0.2 L min\(^{-1}\) led initially to faster dilution than with 15 L injection at 0.2 L min\(^{-1}\). After 30 min of extraction, breakthrough curves reached the same level as for the test with 15 L injection. Therefore, the injection of additional gas mixture at a high speed prior to a GPPT did not increase recovery. Overall, the injection of 15 L at 0.2 L min\(^{-1}\) appeared to be a good compromise between injection volume, injection/extraction speed and GPPT test time. Employing these parameters, quantification of rate constants for CH\(_4\) oxidation was successful in three different hummocks in the peat bog (chapter 4). During GPPTs in dry sand in the laboratory model system, injection/extraction speed was also identified as a main factor influencing recovery (Gonzalez-Gil et al. submitted-b).

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**Figure 7-1**: Methane breakthrough curves from four different GPPTs performed with the co-injection of C\(_2\)H\(_2\). The approximate injection volume and injection/extraction pump rate are given for each GPPT. Test times were ca. 3 h for all tests, but only the first 50 min of extraction are shown. The tests in H3 and H2 correspond to GPPT 3i and GPPT 2i (chapter 4). In the test listed third, injection of 18 L at 0.7 L min\(^{-1}\) was followed by injection of 10 L at 0.2 L min\(^{-1}\).
f) Test volume

For the GPPTs presented in this thesis, optimization of contact time resulted in relatively large injection volumes of 15 to 30 L. Therefore, rate constants obtained from GPPTs were integrated over a relatively large volume, for example, a zone extending approximately 50 – 70 cm upwards from the groundwater table in GPPTs at 2.7 m in Studen. This has the advantage that values are integrated over potential heterogeneities and do not represent only single point measurements. However, in several applications it may be desirable to be able to obtain a higher spatial resolution. Therefore, miniaturization of the test is currently being investigated. One problem is that at very low injection volumes and fast pump speeds (to counteract diffusion), test times become very short. This problem could be solved by temporarily encasing a test zone to prevent fast dilution and diffusion or to re-circulate the injection mixture.

7.1.2 Concentration profiles

Besides GPPTs, the second in-situ method applied in the different studies in this thesis was the interpretation of CH₄ gas concentration profiles. Interpretability of CH₄ profiles varied greatly between the two different sites and the laboratory column depending on the characteristics of the system and the available knowledge about physical parameters. Knowledge about the physical properties of the porous medium and steady-state conditions are pre-requisites for quantitative evaluation of profiles as outlined in chapter 1 (1.3.2).

The laboratory column was a well-defined system at steady-state which allowed detailed interpretation of profiles (Figure 6-2), e.g. to obtain a depth distribution of CH₄ oxidation (chapter 6). An assimilation rate for CH₄ oxidation could be inferred from CO₂ and CH₄ profiles as CH₄ oxidation was the main process in the column which was therefore responsible for observed CO₂ production.

Above the contaminated aquifer in Studen, we were also able to estimate a CH₄ oxidation rate and first-order rate constant from CH₄ gas profiles (Figure 3-4). At this site, porosity, air-filled porosity and subsequently diffusivity could be estimated relatively easily from a core of the in-situ material. The assumption of a constant effective diffusion coefficient with depth was reasonable given that the material did not change within the zone of activity. The assumption of steady-state was supported by similar CH₄ concentration profiles obtained in April (chapter 2, Figure 2-3) and in August (chapter 3, Figure 3-4). In contrast to the column, CO₂ and O₂ profiles above the aquifer indicated that CH₄ oxidation observed in the vadose
zone above the aquifer accounted for less than 1% of the O₂ consumption and CO₂ production (additional data).

In contrast to the site in Studen, CH₄ gas-phase concentrations only qualitatively indicated that CH₄ oxidation was occurring in the peat bog. Quantitative interpretation was complicated by several factors: a) at times CH₄ gas profiles did not show a clear gradient or indicate a zone of CH₄ oxidation, but showed enhanced CH₄ concentrations at a shallow depth within the profile (chapter 5, Figure 5-8), b) the high temporal variability observed in profiles indicated that the system was not at steady-state, c) the peat was very heterogeneous and consisted of different layers with different physical properties, therefore effective diffusivity varied with depth and would have been difficult to estimate. Consequently, CH₄ oxidation rates were not estimated from profiles even in cases where concave profiles were observed (chapter 4, Figure 4-1).

In a study to quantify CH₄ oxidation in the unsaturated zone of a northern Sphagnum bog, Fechner and Hemond (1992) estimated CH₄ oxidation rates from CH₄ profiles. Effective diffusivity in this study was estimated in two ways: a) from total porosity measured in four peat cores and b) from several in-situ propane tracer tests at one location. For interpretation of CH₄ profiles the peat of the unsaturated zone was divided into two layers with different physical properties for the entire site. Therefore, the variation in local vertical heterogeneity of the peat material and the horizontal heterogeneity of the site were not accounted for. Even though the authors do not give information on the heterogeneity of the site, this may have introduced large uncertainty into the obtained results. For the applied in-situ tracer test to assess diffusivity, knowledge of peat porosity and insertion of silicon tubing into the peat was required. Therefore it was partially destructive and could not easily be applied for example to estimate effective diffusivity at the different sampling sites in our study. An alternative method is a tracer test developed by Johnson et al. (1998) that is based on the injection of a tracer as a point source and retrieval of finite volumes of gas after different time periods to determine diffusivity. In most cases, this approach does not require an independent measurement of the air-filled porosity and therefore does not require sampling of material. However, spatial heterogeneity of vadose zone properties over relatively small scales as for example observed in the peat bog in our study also complicates the application of this method. A third approach to determine effective diffusivity of CH₄ in soils in-situ is measurement of emission fluxes and profiles of ²²²Rn naturally developing from radioactive decay of ²²⁶Ra (Dorr and Munnich 1990; Levin et al. 1993). However, this method is also based on the assumption of constant diffusivity with depth.
In summary, interpretation of concentration profiles is a valuable tool in a well-defined system, but the method becomes increasingly difficult with increasing heterogeneity and variability. However, even in a complex system, depth-distribution of substrate concentrations is an important indicator for the location of activity.

7.1.3 Stable carbon isotope fractionation

In this thesis a third in-situ method, stable carbon isotope fractionation, was used as a qualitative indicator for CH₄ oxidation. Furthermore, the potential of this method as a tool to quantify CH₄ oxidation was assessed. There are two main characteristics of a system that have to be known when quantifying CH₄ oxidation from CH₄ stable carbon isotope ratios in an unsaturated porous medium (chapter 1, 1.4.4) a) the fractionation factor for CH₄ oxidation and b) the transport regime.

An example for the quantitative application of this tool is the estimation of the fraction of CH₄ oxidized in landfill cover soils (Liptay et al. 1998; Chanton and Liptay 2000; Borjesson et al. 2001; Abichou et al. 2006). Interpretation of isotope ratios in these studies was based on a mass balance in an open system (see equation 1-13). In these studies, fractionation factors for CH₄ oxidation were measured in batch incubations of landfill cover soil sampled at the investigated sites. In most cases, temperature dependence was taken into account (Chanton and Liptay 2000; Borjesson et al. 2001; Abichou et al. 2006) and in some cases samples with a different soil type were investigated (Liptay et al. 1998; Borjesson et al. 2001). In one study, the fractionation factor determined from samples at one site was used for the quantification of CH₄ oxidation at six different sites (Liptay et al. 1998). This may be problematic as sites have different characteristics, and different fractionation factors from 1.018 to 1.039 were found in different landfill cover materials (see Table 1-7). As discussed in chapter 1, temperature is not the only factor influencing isotope fractionation during CH₄ oxidation. The physical conditions of the environment are important and might not be represented appropriately in well-mixed batch experiments (Templeton et al. 2006). So far, evidence is still lacking that batch incubations are suitable to accurately determine in-situ fractionation in landfill cover soils or other environmental systems. In two studies in this thesis, fractionation factors were determined under (close to) in-situ conditions. During GPPTs above the contaminated aquifer in Studen, we observed that the measured fractionation factor mainly depended on the CH₄ injection concentration and the fraction of CH₄ oxidized. This was likely due to rate limitation by the enzymatic reaction at high CH₄ injection concentrations with a low fraction of CH₄ being oxidized leading to large fractionation and rate limitation by mass transfer at low
injection concentrations with a high fraction of CH$_4$ being oxidized leading to low fractionation (chapter 3). On the other hand, during steady-state conditions in the laboratory column (chapter 6) we observed a relatively high fractionation factor. This indicated that, at steady-state, the enzymatic reaction was mainly responsible for fractionation despite low CH$_4$ concentrations and almost complete oxidation of available CH$_4$. If enzymatic fractionation also dominated in situ in the landfill cover soils in the mentioned studies, the fractionation factors determined in batch incubations may have represented field conditions reasonably well. In summary, uncertainty may be introduced into estimation of CH$_4$ oxidation at the field scale if the fractionation factor was not determined under conditions that were similar to in-situ conditions. One problem is that for the determination of the fractionation factor in situ, knowledge about CH$_4$ oxidation is required a priori. For example, in a study to quantify CH$_4$ oxidation in swamp forests, the associated stable carbon isotope fractionation factor was determined in situ (Happell et al. 1994). However, to determine the fractionation factor, CH$_4$ oxidation had to be quantified independently with an inhibitor experiment. Similarly, the fractionation factor for microbial CH$_4$ oxidation in a landfill cover soil was determined using $^{222}$Rn as a tracer (Bergamaschi et al. 1998). The tracer was thereby used to quantify the fraction of CH$_4$ oxidized. Also in our study above the contaminated aquifer in Studen, knowledge about CH$_4$ oxidation from GPPTs was used to quantify the fractionation factor (chapter 3).

The second factor that is important when using stable isotopes to quantify CH$_4$ oxidation is knowledge about the transport regime. In the above mentioned studies of landfill cover soils, fractionation due to diffusion was usually neglected as transport was assumed to be dominated by advection. However, it was shown in a laboratory column study that diffusion plays a role even under advection-dominated transport conditions and that the fractions of CH$_4$ oxidized may have been underestimated by a maximum factor of two to four due to neglect of diffusion (De Visscher et al. 2004). Note that one potential problem associated with the latter study is that the analysis is based on a fractionation factor for microbial oxidation that was measured in a batch incubation of material from the studied system. Accounting for fractionation due to diffusion under advection-dominated transport conditions in future studies will be complicated as the degree of diffusion is difficult to assess. In summary, these example applications of the stable isotope method in an unsaturated system illustrate that the quantitative use of this tool is complicated by uncertainties about the fractionation factor and the transport regime.
Apart from using stable isotopes as a quantitative tool, shifts in stable isotope ratios can serve as qualitative indicators for microbial CH₄ oxidation. However, as in quantitative studies, the interaction of fractionation due to diffusion and fractionation due to microbial CH₄ oxidation may complicate data interpretation. During GPPTs above the contaminated aquifer in Studen, stable isotopes were a clear qualitative indicator of microbial CH₄ oxidation. During a GPPT with the co-injection of C₂H₂ as inhibitor, comparatively little fractionation due to diffusion occurred between ¹²CH₄ and ¹³CH₄ due to partially restricted transport conditions. In contrast, strong enrichment in the heavier isotope was observed during active GPPTs confirming microbial CH₄ oxidation (chapter 2). This enrichment was variable depending on the CH₄ injection concentration and on the fraction of CH₄ that was oxidized (chapter 3). Conversely, during a number of GPPTs in the peat bog, i.e. under diffusion-dominated transport conditions, diffusional fractionation masked fractionation due to CH₄ oxidation. However, when high fractionation due to microbial oxidation was expressed, it could be distinguished from diffusional fractionation (chapter 4). Thus, stable isotopes provided an independent line of evidence that the observed CH₄ conversion was microbially mediated. Even if only observed in one GPPT, this was valuable especially as the GPPT was applied in an environment not previously studied with this method.

Isotope fractionation along depth profiles in the laboratory column (chapter 6) qualitatively corroborated the observed microbial CH₄ oxidation as CH₄ became increasingly enriched in ¹³C (Figure 6-5). However, similar to the observations during GPPTs in the peat bog, simulations of isotope profiles in the column showed that microbial oxidation may not always lead to isotope enrichment. Isotope ratios became heavier along profiles when fractionation due to oxidation was larger than due to diffusion as observed in measured data (Figure 6-5, 7-2). Conversely, isotope ratios became lighter along the profile if a larger fractionation factor due to diffusion than due to oxidation was employed in simulations (Figure 7-2). Additionally, if both fractionation factors had the same magnitude, no shifts in isotope ratios were observed.

The possibility of lighter or heavier isotope ratios along profiles due to interaction of diffusion and microbial oxidation has previously been suggested based on a brief mathematical description of isotope fractionation along steady-state diffusional profiles (Levin et al. 1993). However, in most studies an enrichment in δ¹³C values was observed concomitantly with microbial oxidation (e.g. Reeburgh et al. 1997; Bergamaschi et al. 1998; Conrad et al. 1999). We measured δ¹³C in the two lowest samples from a CH₄ profile above
the contaminated aquifer in Studen (chapter 3, additional data) and found similar $\delta^{13}$C values of -46.0 and -46.6‰ at 2.95 and 2.80 m depth respectively. Therefore, no fractionation occurred even though the profile indicated microbial CH$_4$ oxidation. This may be due to the above described effect.

![Figure 7-2: Profiles of $\delta^{13}$C simulated as described in chapter 6 with $\alpha_{\text{diff}} = 1.018$ and $\alpha_{\text{ox}} = 1.030$, 1.018 and 1.007. Note that the simulations are influenced by the top boundary condition for $\delta^{13}$C given by values measured at the top of the column during phase I (chapter 6).](image)

Interpretation of profiles may be further complicated by the influence of changing concentration gradients of $^{12}$CH$_4$ and $^{13}$CH$_4$ on the relative fluxes of the two isotopic species. For example, an enrichment in $\delta^{13}$C along the profile followed by a shift towards lighter values was observed in the simulation of isotope ratios measured in the column (Figure 6-5 and Figure 7-2 for $\alpha_{\text{ox}} > \alpha_{\text{diff}}$). A similar effect has been observed in several landfill cover soils (De Visscher et al. 2004). The authors attributed this effect to an increasing gradient for $^{13}$CH$_4$ due to accumulation and a subsequently increased flux causing a shift towards lighter values. This effect may further complicate the interpretation of isotope ratios and is not well understood (Chanton 2005).

In summary, stable isotopes are an important tool to detect and, under specific circumstances, quantify microbial CH$_4$ oxidation in the field. Furthermore, stable isotopes are
important as an independent indicator for microbially mediated CH₄ oxidation during GPPTs. However, it has to be emphasized that especially in the case of CH₄ in the unsaturated zone, data have to be interpreted with great care due to complex interactions of physical and microbial processes. Isotope shifts occurring due to CH₄ oxidation in the aqueous phase, e.g. in lake sediments (Bastviken et al. 2002) may be easier to interpret. In that case, transport processes fractionate only to a small degree, but uncertainties with respect to the magnitude of microbial fractionation still have to be considered.

7.1.4 Combination of in-situ methods

Due to uncertainties inherent in field quantification methods, it is advisable to combine several methods in order to obtain several lines of evidence in the assessment of microbial processes (Madsen 1991). We showed that, when used in combination, the methods presented in this study gave interesting insights about microbial CH₄ oxidation above a contaminated aquifer (chapter 3). Methane gas concentration profiles indicated the major zone of CH₄ oxidizing activity and could be used to quantify an in-situ CH₄ oxidation rate. GPPTs allowed estimation of the CH₄ oxidizing capacity of two different zones above the aquifer, the affinity of methanotrophic bacteria for CH₄ in those zones and also the in-situ CH₄ oxidation rate. The latter agreed well with the rate obtained from profiles. Finally, stable isotope fractionation during GPPTs confirmed that CH₄ oxidation was microbially mediated. In addition to quantification of activity, information about the methanotrophic community was obtained using molecular tools.

7.2 Methods to assess microbial community structure

7.2.1 Problems encountered with the use of molecular methods

We applied different methods to characterize the methanotrophic community at the two different depths investigated above the contaminated aquifer in Studen (chapter 3) and in the column inoculated with a methanotrophic enrichment culture (chapter 6). However, several problems were encountered. When applying the FISH probes My84/My705 for type I and Ma450 for type II methanotrophic bacteria (Eller et al. 2001) to samples from Studen, only few cells were stained and numbers were too low for counting. Possibly this was due to two problems. a) The number of methanotrophic bacteria in relation to total biomass may have been low. Furthermore, samples had to be diluted due to interference of sediment particles with counting. For comparison, in rice field microcosms, total methanotrophic cell numbers
were only 0.3 – 5% of total cell numbers (Eller and Frenzel 2001). b) A significant part of the methanotrophic community may not have been targeted by the available FISH probes. Analyzing the sequences of the probes using the probe match function of the Ribosomal Database Project II (http://rdp.cme.msu.edu, accessed on 17 Nov 2005) showed that each of the two type I probes theoretically only bind to ca. 1/3 of known type I strains and the type II probe theoretically only binds to 2/3 of known type II strains in the database. More up to date probes are currently not available for methanotrophic bacteria and it was beyond the scope of this project to design new probes. Similarly, cells of the enrichment culture in the column (chapter 6) were not stained with the available methanotrophic probes. Again it is possible that the species present were not targeted by the probes. This possibility is supported by a low diversity in the column as indicated by the same pattern of up to 5 bands in a DGGE gel obtained with pmoA primers A189f-GC and mb661r (Henckel et al. 1999; Knief et al. 2003) (data not shown) in samples from seven depths in the column.

Samples from Studen were also analyzed using DGGE with the same primers. Patterns indicated a similar community structure at both depths, 1.1 m and 2.7 m, but only weak bands were obtained due to low DNA content especially at 1.1 m. Attempts to clone and sequence bands were not successful. This was probably also due to a low DNA content. Alternatively, DNA fragments amplified with the same primer pair were directly cloned and sequenced. Despite several attempts, only two clones at 2.7 m and nine clones at 1.1 m were obtained (chapter 3). Nevertheless, this was the first time the methanotrophic community was analyzed in this type of environment.

7.2.2 Linking microbial structure and function

To better understand a microbial process, it is important to link the analysis of the structure of a microbial community with the analysis of its function. For example, in forest and rice field soils, the methanotrophic community structure was shown to directly affect CH₄ fluxes (Mohanty et al. 2006). We combined a field technique, the GPPT, to measure kinetic parameters for CH₄ oxidation with the analysis of the methanotrophic community in field samples (chapter 3). Even though the above mentioned problems occurred when analyzing the methanotrophic community, obtained sequences of fragments of the pmoA gene, the gene encoding the particulate CH₄ monoxygenase, clearly indicated the presence of methanotrophic bacteria that were related to known species (chapter 3). This was in agreement with the low affinity kinetics observed during GPPTs which is typical for known, cultured methanotrophic bacteria (Conrad 1996). The analysis of functional genes is
beneficial in this case as detected organisms can be assigned the ability to perform a certain function even if similarities with known species are relatively low as observed for some sequences above the contaminated aquifer (Table 3-3). A useful tool to elucidate which organisms are performing a certain function under specific conditions is stable isotope probing. By labeling the substrate, in this case CH$_4$, with $^{13}$C, incorporation of substrate into biomarkers such as PLFA (Knief et al. 2003; Crossman et al. 2004; Mohanty et al. 2006) or into DNA and RNA (Lueders et al. 2004) of the active bacteria can be traced. The advantage of the use of DNA or RNA is that the labeled organisms can subsequently be identified e.g. by cloning and sequencing. However, incorporation into DNA usually takes a long time (Lueders et al. 2004) while RNA is difficult to extract from environmental samples (McDonald et al. 2005). Interpretation of PLFA profiles is often difficult. However some PLFA in methanotrophic bacteria are unusual and consequently suitable biomarkers (Hanson and Hanson 1996). Therefore, this approach has given interesting insights into the active methanotrophic population e.g. in samples from upland soils and landfill cover soils (Knief et al. 2003; Crossman et al. 2004; Mohanty et al. 2006). All studies of methanotrophic bacteria using stable isotope probing have been performed in laboratory incubations, at times even with the addition of medium to the samples (Lin et al. 2004). Especially the addition of medium makes the relevance of obtained results to field situations questionable. A combination of the GPPT technique with stable isotope probing may be a promising field-scale approach if long enough incubation times can be achieved for sufficient label incorporation. The latter is challenging as typical incubation times for laboratory studies are in the range of several weeks (Knief et al. 2003; Lueders et al. 2004). Field-scale stable isotope probing has been performed previously to assess nitrate and sulphate reduction in the contaminated aquifer in Studen using push-pull tests and $^{13}$C-labeling of PLFA (Pombo et al. 2002; Pombo et al. 2005).
7.3 Questions and approaches for future research

The major tools developed in this study were a new field method for the quantification of microbial processes in the vadose zone, the gas push-pull test (GPPT) and a model system to study microbial CH$_4$ oxidation in CH$_4$/O$_2$ counter gradients under controlled conditions in the laboratory.

7.3.1 Further developments and applications of the GPPT

As outlined in 7.1.1, a main issue that should be addressed for the further development of the GPPT technique is miniaturization of the test. Some interesting processes, for example soil respiration or N$_2$ fixation (Table 7-3), mainly take place in surface soil layers. Therefore, miniaturization would be of major importance when adapting the GPPT for the assessment of these processes. Furthermore, in the case of soil respiration, distinguishing consumption of a substrate or formation of a product against background concentrations may be especially challenging. One possibility to address this issue may be to apply labeled substrate. In the case of N$_2$ fixation, the commonly used acetylene reduction assay (Table 7-3) may be applied in conjunction with GPPTs.

Table 7-3: Possible processes that could be quantified using GPPTs

<table>
<thead>
<tr>
<th>Process</th>
<th>Gaseous substrates and products</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil respiration</td>
<td>C$_{org}$ + O$_2$ $\rightarrow$ CO$_2$</td>
<td></td>
</tr>
<tr>
<td>Nitrogen fixation</td>
<td>C$_{org}$ + N$_2$ $\rightarrow$ 2NH$_3$</td>
<td>C$_{org}$ + C$_2$H$_2$ $\rightarrow$ C$_2$H$_4$</td>
</tr>
</tbody>
</table>

For the study of CH$_4$ oxidation it would be interesting to combine the GPPT with field techniques (e.g. push-pull tests) to assess methanogenesis and possibly CH$_4$ oxidation in the saturated zone at the same site, for example in the peat bog in Eigenthal or a similar ecosystem. Furthermore, the GPPT could be applied to investigate temporal and spatial variability of CH$_4$ oxidation e.g. in a landfill cover soil or a peat bog. Combined with assessment of environmental parameters, this may give insight into the main factors influencing CH$_4$ oxidation in situ.

We observed that CH$_4$ oxidation could be quantified in a zone that was mainly exposed to atmospheric CH$_4$ concentrations closer to the soil surface (at 1.1 m) above the contaminated aquifer in Studen (chapter 2 and 3). As outlined in chapter 3, the obtained kinetic parameters may be characteristic for a “low affinity” CH$_4$ oxidation that occurred in response to the injection of elevated CH$_4$ concentrations. A next step would be to test if, at much lower CH$_4$
injection concentrations, “high affinity” CH₄ oxidation could be quantified. Furthermore, as outlined in 7.2, a combination of the GPPT with stable isotope probing may yield interesting results concerning the methanotrophic community active under field conditions.

7.3.2 Further applications of the laboratory model system

The laboratory model system designed to investigate CH₄ oxidation in a CH₄/O₂ counter-gradient (chapter 6) raises a number of possibilities. An interesting next step would be to investigate the influence of water content on CH₄ oxidation activity and its distribution within the CH₄/O₂ counter-gradient. Water content has been proposed as an important factor governing CH₄ oxidation due to its influence on soil diffusivity (Smith et al. 2003). Furthermore, it would be interesting to study the influence of temperature on CH₄ oxidation as this has only been done in batch incubations (e.g. Whalen et al. 1990). Especially for the study of temperature dependence, it would be important to include methanogenesis as a process in the system to study the differential effect of temperature on the two processes and resulting interactions. Methanogenesis has been found to be more strongly temperature dependent than CH₄ oxidation (Pearce and Clymo 2001 and references therein). Along with including a saturated, methanogenic zone in the design, a valuable addition would be the possibility to collect water samples in the saturated zone and in the capillary fringe. This would enable the investigation of profiles at the transition from saturated to unsaturated conditions.

7.3.3 Further research on isotope fractionation

The analysis of stable carbon isotope fractionation factors due to microbial CH₄ oxidation could be included in the study of environmental parameters in the column. In this way, further indications about the influence of physical conditions (such as different water contents) on fractionation could be obtained. To better understand the mechanisms regulating isotope fractionation and to investigate whether these could be predicted for a specific system, more controlled experiments looking at single influencing factors such as mass transfer, oxidation rate and type of enzyme would be needed.
Chapter 7: General discussion

7.4 References


Chapter 7: General discussion


Chapter 7: General discussion


Massman, W. J. (1998). A review of the molecular diffusivities of $\text{H}_2\text{O}$, $\text{CO}_2$, $\text{CH}_4$, $\text{CO}$, $\text{O}_3$, $\text{SO}_2$, $\text{NH}_3$, $\text{N}_2\text{O}$, $\text{NO}$, and $\text{NO}_2$ in air, $\text{O}_2$ and $\text{N}_2$ near STP. *Atmospheric Environment* 32(6), 1111-1127.


Curriculum Vitae

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Thanks

I would like to thank Prof. Josef Zeyer for giving me the opportunity to work in his group, for his support throughout my PhD and the interest he has taken in my work.

I would like to thank Prof. Michael Kreuzer and Dr. Rolf Siegwolf for accepting to co-examine my thesis and Prof. Ruben Kretzschmar for chairing the thesis exam.

Dr. Martin Schroth was an excellent supervisor. Thanks for the support with field and lab work, constructive criticism on my manuscripts, talks and posters and helpful discussions.

I would like to thank Rolf Kipfer and Markus Hofer from EAWAG for their help and for adjusting their equipment so that we could do noble gas measurements during the first part of the project. I would also like to thank Rolf Siegwolf and Matthias Saurer from PSI and the people in their lab for their help with the methane stable isotope measurements.

Thanks a lot to the people who have worked with me on the “Methane” project. I would like to especially thank Graciela Gonzalez-Gil for the shared work on putting the GPPT equipment together, help with numerous field experiments and all the discussions. Thanks also to her husband Romel for helping out in the field. Many thanks to Elena Norina for “running” the column experiment, numerous trials to get molecular tools to work and help in the field. Thanks also to the whole “Methane” group and especially Kate Gomez for correcting my manuscripts and this thesis.

Thanks to all the people working in the soil biology group for support, for the good work atmosphere and for the shared social occasions. Thanks in particular to Helmut Bürgmann, Jutta Kleikemper, Michael Bunge and Matthias Noll for showing me the world of molecular tools and to Richard Dobson for help with computer problems.

Thanks to everybody at the ITOe for always being helpful and thanks for the good time. My special thanks go to Hanspi Läser for making the column, making all sorts of field equipment, helping out with technical questions and always having time for a joke.

Finally, I would like to thank my friends, Ian, my parents and my sister for their great support.