# Effects of Global Change on Soil Methane Dynamics

**Dissertation**

zur

**Erlangung der naturwissenschaftlichen Doktorwürde**

**(Dr. sc. nat.)**

vorgelegt der

**Mathematisch-naturwissenschaftlichen Fakultät**

der

**Universität Zürich**

von

**Saeed Karbin**

aus dem

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**Zürich 2015**

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# Summary

Atmospheric methane ($CH_4$) concentration has increased since pre-industrial times. The $CH_4$ concentration in the atmosphere is determined by the balance of sources and sinks. Methanotrophic bacteria are the main biological sink for $CH_4$ and widely distributed in upland soils. The ecology of methanotrophic bacteria can not be understood without studying their niches in the soil profile. The spatial distribution of methanotrophic bacteria in the soil profile can be affected by different factors such as soil moisture and nitrogen content. These changes in micro-distribution of methanotrophic bacteria can lead to changes in ecosystem-level $CH_4$ fluxes. In this dissertation, I focus on soil methanotrophic activity and its spatial distribution in soils under anthropogenic changes in different ecosystems.

In Chapter 1, I studied soil warming effects on methanotrophic activity in the last two years of a six-year long field experiment in an alpine treeline ecosystem. I measured $CH_4$ fluxes using static chambers, and characterized N cycling by quantifying soil $N_2O$ emissions, $NH_4^+$ and $NO_3^-$ concentrations. To study changes in the micro-distribution of methanotrophic bacteria within the soil profile, I labeled intact soil cores with $^{14}CH_4$ and traced the labeled methanotrophic bacteria using an auto-radiographic imaging technique.

In Chapter 2, I investigated $CH_4$ dynamics in a permanent grassland exposed to elevated $CO_2$ for 14 years. We measured $CH_4$ fluxes using static chambers in the field and potential methanotrophic activity in soil layers in the laboratory. I labeled soil cores with $^{14}CH_4$ and produced auto-radiographic images.

In addition, we carried out an experiment in an afforestation chronosequence in a subalpine forest (Chapters 3 & 4) to study the micro-distribution of methanotrophic bacteria and soil $CH_4$ uptake. I measured soil–atmosphere $CH_4$ fluxes in soil cores and sieved soils in laboratory conditions. I produced auto-radiographic images for soil cores using the same method as in the previous chapters. In addition, in Chapter 3, I used this technique to study the distribution of methanotrophic bacteria in relation to soil aggregate structures.

Generally, the soil in the alpine treeline ecosystem was a net sink for $CH_4$. Soil warming

reduced soil moisture in the litter layer, but not deeper in the soil profile. As a result, gas transport into deeper layers, where methanotrophic bacteria were located, remained unchanged. Soil warming did not affect mineral N concentration. Soils under European larch (*Larix decidua*) showed higher $CH_4$ assimilation than soils under mountain pine (*Pinus mugo* ssp.)(*uncinata*). This difference may be due to differences in the structure of the organic surface layer. Auto-radiographic image analysis of soil cores revealed a shift of methanotrophic bacteria toward the soil surface.

In the elevated $CO_2$ experiment, elevated $CO_2$ concentration did not affect $CH_4$ flux rates. Potential methanotrophic activity, determined by incubation of fresh sieved soil under standardized conditions, also did not reveal any effect of $CO_2$ treatment. Moreover, auto-radiographic image analysis showed that the spatial niche of $CH_4$ oxidation does not shift in response to $CO_2$ enrichment or $CH_4$ concentration, and that the same type of methanotrophs may oxidize $CH_4$ from atmospheric and soil-internal sources.

In the afforestation chronosequence, in situ soil–atmosphere $CH_4$ fluxes were highest in the oldest forest stands and lowest in pasture. In contrast, in soil cores and sieved soils $CH_4$ fluxes did not follow the field $CH_4$ uptake patterns. Soil mineral N content and $CH_4$ uptake were not correlated. In forests, because of lower soil moisture in deeper soil layers methanotrophic activity extended to lower layers in auto-radiographic images. Higher organic matter content and evapotranspiration in forests may contribute to drier mineral soils in deeper layers. As a result, increased air diffusion into these layers facilitates higher methanotrophic activity in deeper soil layers in forest stands. Auto-radiographic images of soil aggregates in this experiment revealed that methanotrophic bacteria are not homogeneously distributed but more active around soil aggregates than in the center.

# Zusammenfassung

Die atmosphärische Methankonzentration ($CH_4$) hat seit Beginn der Industrialisierung zugenommen. Die atmosphärische $CH_4$-Konzentration wird durch das Gleichgewicht von Quellen und Senken bestimmt. Die grösste biologische $CH_4$-Senke sind methanotrophe Bakterien, die in Böden weit verbreitet sind. Eine zentrale Rolle in der Ökologie von methanotrophen Bakterien spielen ihre Nischen im Bodenprofil. Die räumliche Verteilung methanotropher Bakterien im Bodenprofil hängt von verschiedenen Faktoren wie Bodenfeuchte und Stickstoffgehalt ab. Solche Unterschiede in der Mikroverteilung methanotropher Bakterien können ökosystemweite Methanflüsse beeinflussen. In dieser Dissertation untersuchte ich methanotrophe Aktivität und deren räumliche Verteilung in Böden dreier unterschiedlicher Ökosysteme unter anthropogenen Einflüssen.

Im 1. Kapitel untersuchte ich während der letzten zwei Jahre eines sechsjährigen Feldexperiments die Auswirkung von Bodenerwärmung auf methanotrophe Bakterien an der subalpinen Baumgrenze. Ich mass $CH_4$-Flüsse mit statischen Kammern und quantifizierte sowohl die $N_2O$-Emissionen als auch die $NH_4^+$ und $NO_3^-$-Konzentrationen um den Stickstoffkreislauf zu charakterisieren.

Im 2. Kapitel untersuchten ich die $CH_4$-Dynamik in einer Wiese, die 14 Jahre erhöhten $CO_2$-Konzentrationen ausgesetzt war. Ich mass $CH_4$ Flüsse mit statischen Kammern im Feld und potentielle methanotrophe Aktivität in mehreren Bodenschichten im Labor. Ich markierte Bodenkerne mit $^{14}CH_4$ und machte autoradiographische Bilder.

Zudem führten wir ein Experiment in Aufforstungen unterschiedlichen Alters in einem subalpinen Wald durch (Kapitel 3 & 4) um die kleinräumige Verteilung von methanotrophen Bakterien und die $CH_4$-Aufnahme in den Boden bei Landnutzungsänderungen zu untersuchen. Ich mass die Boden-Atmosphäre-Flüsse von $CH_4$ in Bodenkernen und gesiebtem Boden unter Laborbedingungen. Ich machte autoradiographische Bilder der Bodenkerne wie in den vorangegangenen Kapiteln.

In Kapitel 3 untersuchte ich mit dieser Technik auch die Verteilung methanotropher Bakterien innerhalb von Bodenaggregaten. Ich machte autoradiographische Bilder der

Bodenkerne wie in den vorangegangenen Kapiteln.

Generell war der Boden an der subalpinen Baumgrenze eine Nettosenke für $CH_4$. Die Bodenerwärmung verringerte die Bodenfeuchte in der Streuschicht leicht, jedoch nicht in tieferen Bodenschichten. Daher veränderte sich auch der Gastransport in tiefere Bodenschichten nicht, in denen sich die methanotrophen Bakterien befinden. Die Bodenerwärmung veränderte die Konzentration von mineralischem Stickstoff nicht. Böden unter Bäumen der Gattung Europäische Lärche (*Larix decidua*) zeigten höhere $CH_4$-Assimilation als Böden unter Bergkiefer (*Pinus mugo* ssp.)(*uncinata*). Dies könnte durch Unterschiede in der Struktur des organischen Bodenhorizontes bedingt sein. Autoradiographische Bildanalysen der Bodenkerne zeigten eine Verschiebung der methanotrophen Bakterien Richtung Bodenoberfläche.

Im $CO_2$-Anreicherungsexperiment beeinflussten erhöhte $CO_2$-Konzentrationen die $CH_4$-Flüsse nicht. Die potentielle methanotrophische Aktivität, welche durch Inkubation von frischer, gesiebter Erde unter standardisierten Bedingungen bestimmt wurde, zeigte ebenfalls keinen Einfluss der $CO_2$-Erhöhung. Die autoradiographische Bildanalyse zeigte, dass sich die räumliche Nische der $CH_4$-Oxidation weder als Reaktion auf die $CO_2$-Anreicherung noch auf die $CH_4$-Konzentration veränderte, was darauf hinweist dass womöglich derselbe Typ methanotropher Bakterien das $CH_4$ aus der Atmosphäre und aus bodeneigenen Quellen oxidiert.

Im Experiment in den Aufforstungsgebieten war der *in situ* Boden-Atmosphäre-Fluss von $CH_4$ im ältesten Wald am grössten und auf Wiesenflächen am niedrigsten. Im Gegensatz dazu zeigten die $CH_4$-Flüsse Bodenkernen und der gesiebte Böden im Labor kein eindeutiges Muster. Unsere Ergebnisse zeigen keinen Zusammenhang zwischen Bodenstickstoffgehalt und $CH_4$-Aufnahme. Wegen der geringeren Bodenfeuchte im Waldboden zeigten autoradiographische Bilder methanotrophe Aktivität bis in tiefere Bodenschichten. Die erhöhte Menge an organischem Material und die Evapotranspiration traga zu den trockeneren mineralischen Böden in tieferen Schichten bei. Die dadurch erhöhte Diffusion

von Luft ermöglicht höhere methanotrophe Aktivität bis in die tieferen Bodenschichten des Waldes. Die autoradiographischen Bilder der Bodenaggregate zeigten, dass die methanotrophen Bakterien in den Aggregaten nicht gleichmässig verteilt sind. Sie sind aktiver an der Oberfläche der Aggregate als in deren Zentrum. In allen unseren Experimenten war Bodenfeuchte der wichtigste Einflussfaktor für Methanaufnahme und die Verteilung methanotropher Aktivität im Boden.

# General introduction

## Methane cycle

Methane ($CH_4$) is the second most important anthropogenic greenhouse gas after $CO_2$ (Conrad, 2009). Compared to $CO_2$, $CH_4$ absorbs more infrared radiation per molecule (IPCC, 2013). Atmospheric $CH_4$ concentration was 1803 ppb in 2011, which is 150% times the pre-industrial level (IPCC, 2013). $CH_4$ concentration is about 5% higher in the northern hemisphere, which may be due to either weaker sink activity or strong source strength (IPCC, 2007).

The source of $CH_4$ can be thermogenic including natural emissions of fossil $CH_4$ from geological sources (e.g. geothermal vents and mud volcanoes) and anthropogenic emissions caused by fossil fuel extraction. Pyrogenic sources occur due to incomplete combustion of organic matter (e.g. biomass and biofuel burning, IPCC (2013)). The biogenic source of $CH_4$ emissions are methanogenic archaea which exist in wetlands, oceans and termites (Conrad, 2009). Anthropogenic biogenic sources include rice paddies, ruminant livestock, landfills, man-made lakes and wetlands (IPCC, 2007). Biogenic, thermogenic and pyrogenic $CH_4$ sources contribute 55–70 %, 25–45 % and 13–25 % to $CH_4$ emissions, respectively (IPCC, 2013). Natural geological sources are estimated between 42 and 64 Tg $CH_4$ $yr^{-1}$. This contribution equals about 30% of the global $CH_4$ budget and was under-estimated (20%) in IPCC (2007) (IPCC, 2013).

Atmospheric $CH_4$ is removed mainly by oxidation with OH radicals in the stratosphere. OH removes about 90% of total $CH_4$ emission. Other removal processes in the stratosphere are the reactions with chlorine and oxygen radicals (IPCC, 2013). In the marine boundary layer, chemical reaction of $CH_4$ with chlorine is suspected to be a small sink (Allan et al., 2007). Methanotrophic bacteria have the ability to utilize $CH_4$ as their energy source (Semrau et al., 2010) and have been found in many terrestrial ecosystems (Conrad, 1996, 2007, 2009). Methanotrophic bacteria can oxidize about 9 to 47 Tg $CH_4$ $yr^{-1}$ (IPCC, 2013).

## Methanotrophic bacteria

Methanotrophic bacteria exist in all soil types, sediments, lakes, oceans and mud volcanoes (Conrad, 2007; Dunfield, 2007; Shukla et al., 2013). They can oxidize $CH_4$ and were isolated for the first time in 1906 (Söhngen, 1906). Growth of these bacteria is limited to $CH_4$ and they may utilize methanol, formate, formaldehyde and methylamine (Kolb, 2009).

In rice paddies, methanotrophs are usually found at the oxic-anoxic interface (Shukla et al., 2013). $CH_4$ that originates from lower anoxic microsites diffuses to upper soil layers where it partly is consumed by methanotrophic bacteria located there (Conrad, 2009). Some of the unprocessed $CH_4$ is emitted into the atmosphere (Conrad, 2009). In rice paddies, methanotrophs can consume about 20% of gross fluxes (Bodelier and Frenzel, 1999; Conrad, 2009; Lüke et al., 2010).

In upland soils, methanotrophic bacteria located in the top aerobic layer are not only active in consuming atmospheric $CH_4$, but can also utilize the $CH_4$ that originates from anoxic microsites in deeper layers (Conrad, 2007) . Kammann et al. (2001) showed that methanotrophic bacteria in the top soil layer of a grassland with a high potential for $CH_4$ production can modify $CH_4$ flux and act as a biofilter layer to reduce $CH_4$ emissions. In lower soil layers, $CH_4$ production can occur in anaerobic microsites (von Fischer and Hedin, 2002; Kammann et al., 2009). $CH_4$ originating form soil internal sources can be oxidized by low affinity and high affinity methanotrophic bacteria.

There are two systems of $CH_4$ oxidation by methanotrophic bacteria in soils including high affinity and low affinity systems. The high affinity system utilizes atmospheric $CH_4$ whereas the low affinity system utilizes $CH_4$ in high mixing ratios (Singh et al., 2010). The kinetic parameters of $CH_4$ oxidation (half saturate constants ($K_m$), maximum oxidation rates ($V_{max}$) and apparent threshold (Th)) in low affinity methanotrophic bacteria are higher than high affinity methanotrophic bacteria (Bender and Conrad, 1992).

Low affinity methanotrophic bacteria utilize $CH_4$ close to anaerobic microsites before

it escapes to the atmosphere. Low affinity methanotrophic bacteria has been isolated and cultured in the laboratory (Costello and Lidstrom, 1999; Henckel et al., 1999; Auman et al., 2000; McDonald et al., 2008).

The half saturate constant ($K_m$) in high affinity methanotrophs are low and range from 0.8 to 280 nM in upland soils (Shukla et al., 2013). High affinity methanotrophic bacteria in soils exist in the top aerobic layer and has larger populations compared to low affinity methanotrophic bacteria (Degelmann et al., 2010). These group of methanotrophic bacteria has not been isolated and attempts to culture them were not successful to date.

## Spatial distribution

Methanotrophic bacteria are distributed both vertically and horizontally in soils. The vertical distribution in the soil profile can be regulated by various factors including soil moisture, $O_2$ and $CH_4$ concentration, soil disturbance (e.g. tillage), nitrogenous fertilizers and soil pH (Shukla et al., 2013). $CH_4$ transport in the soil occurs in the gas phase. Soil moisture controls air diffusion into the soil and thus regulates the activity of methanotrophic bacteria in the soil profile (Czepiel et al., 1995). In deeper soil layers, $CH_4$ concentrations are lower and thus methanotrophic bacteria are unable to grow. Therefore, methanotrophic activity should be the highest in top soil layers and decrease with increasing soil depth (Shukla et al., 2013). Maximum $CH_4$ oxidation zone varies in soils ( 0-10 cm depth; Hütsch (1998), 4-20 cm depth; Schnell and King (1994)).

The vertical distribution of methanotrophic bacteria may shift in response to changes in environmental factors and soil physical and chemical properties. For example, Stiehl-Braun et al. (2011a) labeled methanotrophic bacteria with $^{14}C$ and visualized their activity in the soil profile under drought stress and N fertilizers. These authors showed that the $CH_4$–assimilating zone extended further down the soil profile under drought stress and N application. Moreover, soil surface organic layer may act as a barrier for air diffusion into soils and affect the distribution of methanotrophic bacteria in the soil profile (Borken

and Brumme, 1997). Brumme and Borken (1999) reported that physical characteristics of the organic layer can be a reason for low methanotrophic activity in soils. These authors showed that removing the top organic layer increased methanotrophic activity. In addition, chemical compounds in the organic layer can limit methanotrophic activity. For example, Amaral et al. (1998) reported that monoterpenes inhibit $CH_4$ oxidation in soils.

Methanotrophic bacteria are distributed in upland soils, rice paddies and wetlands. In wetland and rice paddies plants can transport $O_2$ into the peat or sediment (Dunfield, 2007), supporting methanotrophic activity in the rhizosphere (Conrad, 2007).

There is limited information about the small-scale distribution of methanotrophic bacteria in the soil profile. Culturing methanotrophic bacteria with high affinity system was not successful to date and there is a considerable obstacle in the study of the spatial distribution of methanotrophic bacteria. New methods for these types of studies need to be developed for further investigations.

## Factors affecting methanotrophic bacteria

Many studies illustrated how the activity of methanotrophic bacteria is affected by natural and anthropogenic factors (Schnell and King, 1996; Whalen and Reeburgh, 1996; Mosier et al., 2003; Kolb et al., 2005). Natural factors such as soil moisture, temperature, nutrient availability, pH and biological factors like vegetation can regulate methanotrophic bacteria (Adamsen and King, 1993; Castro et al., 1995; Dunfield et al., 1995; Whalen and Reeburgh, 1996; Mosier et al., 2003). Moreover, anthropogenic changes (e.g. fertilizers, elevated temperature and atmospheric $CO_2$ concentration, land use changes) can strongly affect methanotrophic activity in soils (Dunfield et al., 1995; Priemé et al., 1997; Ball et al., 1999; Dubbs and Whalen, 2010).

## Natural factors

### Soil moisture

Soil moisture is the main driver of methanotrophic activity (Czepiel et al., 1995; Menyailo, 2003; Luo et al., 2013). Soil moisture controls air diffusion into the soil. The optimal range of water content depends on land use. In grassland soils, maximum $CH_4$ oxidation occurred in a range from 18 to 33% of gravimetric moisture content and in forest soils optimal soil moisture was between 30 and 51% (Czepiel et al., 1995). Czepiel et al. (1995) suggested that organic matter can explain this variability in optimal water content. Federer et al. (1993) showed that bulk density and pore volume are closely related with organic matter content of soils. With increasing soil organic matter, bulk density decreases while pore volume increases and soil aggregates form. Changes in pore volume can alter $CH_4$ transport in soils (Czepiel et al., 1995).

Water stress can restrict the activity of methanotrophic bacteria. The effects of water stress on methanotrophs have received little attention (Schnell and King, 1996; Stiehl-Braun et al., 2011b). Decreasing water potential until about $-0.5$ MPa increases $CH_4$ consumption (Schnell and King, 1996). King (1997) showed that water potentials much lower than $-0.5$ MPa occur in surface soils during dry periods between precipitation events. He reported that the ability of methanotrophs to recover from water stress is very limited. The author reported that as periodic drying of surface soils is the main limiting factor for methanotrophic bacteria, extreme water potentials in surface soils are the most important factor for predicting methanotrophic activities.

### Temperature

Elevation in soil temperature may increase methanotrophic activity by facilitated air diffusibility (Shukla et al., 2013). There are some reports regarding the optimal temperature range for methanotrophic activity. Price et al. (2004) reported that this range is

12-30°C and also in pure culture of methanotrophs the optimum range was around 19-38°C (Whalen et al., 1990; King and Adamsen, 1992). When water content is constant, $CH_4$ oxidation can increase 1.8–fold when temperature increases from 5 to 12°C (Price et al., 2004). The upper limit for methanotrophic activity is in the range of 40 to 45°C (King and Adamsen, 1992). The lower temperature limit varies in different ecosystem types ( 0°C in permafrost soils, 5°C in temperate soils (Reay et al., 2001) and 15°C in rice paddies (Mohanty et al., 2007))

## Anthropogenic factors

### Fertilizers

Ammonium ($NH_4^+$) inhibition of methanotrophic activity in the field was first reported by Steudler et al. (1989) in temperate forests. Since then there have been reports of inhibition in many studies (Hütsch et al., 1993; Castro et al., 1995; Dunfield et al., 1995; Whalen and Reeburgh, 1996; Mosier et al., 2003). Physical similarities between $CH_4$ and $NH_3$ permit both compounds to compete for the methane monooxygenase enzyme (MMO) (Bedard and Knowles, 1989; Dunfield and Knowles, 1995). However, nitrogen fertilization can affect $CH_4$ fluxes in different ways. In some cases, $CH_4$ oxidation rates decrease after fertilizer application. This short-term inhibition can be explained by competition for MMO and $NO_2^-$toxicity. The oxidation of $NH_4^+$ by MMO leads to accumulation of hydroxylamine and $NO_2^-$, which is toxic to methanotrophs (Bedard and Knowles, 1989). King and Schnell (1994) suggested that one of several possible explanations for decreasing methanotrophic activity by $NH_4^+$ is $NO_2^-$toxicity. Price et al. (2004) showed that $KNO_2$ application at a rate of 44.2 µmol $g^{-1}$ soil can completely inhibit $CH_4$ oxidation compared to with $NH_3Cl$ at equivalent concentration. Biochemically, $NO_2^-$can inhibit formate dehydrogenase, the enzyme responsible for the final step in the $CH_4$ oxidation pathway, and hence can reduce the supply of NADH in the cells (King and Schnell, 1994). Moreover,

Kightley et al. (1995) suggested another explanation and stated that immediate inhibition may result simply from non-specific ionic effects due to salt addition, rather than from a selective $NH_4^+$ effect. Inhibition is not always immediate after fertilizer application. In some studies, it may be delayed or develop over several years, or never occur (Hütsch, 1998; Bradford et al., 2001).

Some other studies did not show any N-application effect on $CH_4$ oxidation even after several years (Hütsch et al., 1993). These results suggest that either the methanotrophic bacteria in these soil are tolerant to excess $NH_4^+$, or other soil properties like N immobilization and pH protect them. Gulledge et al. (1997) hypothesized that the inhibition pattern could be driven by immobilization or nitrification that initially buffered the $CH_4$ oxidizers from exposure to $NH_4^+$. However, continued fertilization causes N–saturation of the soil and thus exposes methanotrophic bacteria to $NH_4^+$. Another possible explanation is a change in the methanotroph community composition, either by a shift between $NH_4^+$–tolerant and $NH_4^+$–intolerant methanotrophs or by an increase in $NH_4^+$ oxidizers capable of $CH_4$ oxidation (Bodelier and Laanbroek, 2004).

In a study conducted by Hütsch et al. (1994), soils receiving organic N fertilizer forms had similar $CH_4$ oxidation rates than soils receiving no N-fertilizer. The effect of manure on methanotrophic bacteria has been related to the C:N ratio of manure. Application of crop residues with high C:N ratio like wheat straw did not affect $CH_4$ oxidation rate while residues with small C:N ratio like sugar beet leaves strongly inhibited $CH_4$ oxidation (Boeckx et al., 1996).

### Elevated atmospheric $CO_2$ concentration

The atmospheric $CO_2$ concentration increased about 35% since 1750 (IPCC, 2013). McLain and Ahmann (2008) showed that the atmospheric $CH_4$ oxidation by soils decreased by about 70% under elevated $CO_2$ and that the driving factor was the increased soil moisture. They concluded that elevated $CO_2$ on one hand increased soil moisture and on the other

hand increased anoxia in deep soils due to higher soil respiration. These two factors led to lower $CH_4$ uptake in the elevated $CO_2$ treatment. Reduced stomatal conductance and increased water use efficiency under elevated $CO_2$ lead to higher soil moisture.

Additionally, elevated $CO_2$ can affect the abundance of methanotrophic bacteria (Kolb et al., 2005). Kammann et al. (2001) showed that after 3 years of $CO_2$ enrichment, there was a decrease in the $CH_4$ consumption rate by about 14.5%. Other scientists tried to understand the effects of elevated $CO_2$ on $CH_4$ uptake by using various molecular methods (Horz et al., 2002; Kolb et al., 2005). These authors found similar structure of methanotrophic community in soils under elevated $CO_2$ treatment. They reported that the reduction in methanotrophic bacteria biomass under elevated $CO_2$ can explain why the consumption of atmospheric $CH_4$ was reduced. Kolb et al. (2005) reported that increased abundances of bacterial–feeding protozoa can be responsible for the reduction of methanotroph abundance in the elevated $CO_2$ treatment. Moreover, Barnard et al. (2005) suggested that reduced mineral N through increased plant uptake in elevated $CO_2$ might affect $CH_4$ uptake. Also, there are some studies showing no relation between elevated $CO_2$ and $CH_4$ uptake in soils (Mosier et al., 2002; Smith et al., 2010).

### Land use changes

Several recent studies investigated effects of land use changes on soil $CH_4$ uptake (Priemé et al., 1997; Reay et al., 2001; Menyailo et al., 2008). Smith et al. (2000) found that the conversion of forests to agricultural fields can decrease the strength of the soil $CH_4$ sink by up to 60%. Soil compaction by agricultural and forestry equipment can also reduce the strength of the soil $CH_4$ sink (Hansen et al., 1993; Frey et al., 2011). Furthermore, fertilization and irrigation can reduce the rate of $CH_4$ uptake (Mosier et al., 1991). Goulding et al. (1995) argued that $CH_4$ uptake can be reduced by all forms of agriculture and probably by forestry through increased N inputs and soil acidification.

Tillage reduces soil $CH_4$ oxidation substantially compared to natural undisturbed soils

(Hütsch et al., 1994). The destruction of soil structure and the removal of the organic layer that develops at the top of the uncultivated soils may be responsible for reduction of $CH_4$ uptake rates in cultivated soils (Hütsch, 1998) . Maxfield et al. (2011) focused on changes in methanotrophe community structure and suggested that tillage can significantly reduce methanotrophic biomass and activity. In addition, these authors described that tillage shifted community structure of active high affinity methanotrophs in the soil.

## Global change and atmospheric $CH_4$

Global change in last decades is the main reason for the increased atmospheric $CH_4$ concentration (IPCC, 2013). Increases in number of livestock, the emission from fossil fuel extraction and use, the expansion of rice fields and landfills are main anthropogenic sources for elevated $CH_4$ in recent years. Today, total anthropogenic sources are approximately equal or slightly larger than natural sources (IPCC, 2013).

Wetlands are a substantial but highly variable source of $CH_4$ emissions (177 to 284 Tg $CH_4$ $yr^{-1}$). The inter-annual variability in wetland environments is due to environmental factors, which means that $CH_4$ emissions are sensitive to climate change (IPCC, 2013; Dlugokencky et al., 2009). Furthermore, elevated $CO_2$ can increase $CH_4$ emissions from rice paddies (Cheng et al., 2006). Changes in soil conditions simulated by elevated $CO_2$ concentration (e.g. soil organic matter input, soil moisture) can increase the $CH_4$ production (Cheng et al., 2006; McLain and Ahmann, 2008). Moreover, increased precipitation under global change can elevate $CH_4$ emissions from wetlands. This $CH_4$ emission increase is related to higher water table position and the reduced oxic portion of the soil column (IPCC, 2013).

The soil $CH_4$ sink strength can be affected by global change. Elevated $CO_2$ and air temperature, higher precipitation and land use changes can affect methanotrophic activity in soils (McLain and Ahmann, 2008; Shukla et al., 2013; Smith et al., 2000). Even though the soil $CH_4$ sink is small, it can alter atmospheric $CH_4$ concentrations in the future.

## Auto-radiographic imaging technique

Interactions between different factors that influence methanotrophic bacteria and their spatial variability constitute a complex network of process which requires novel investigation methods. We used a unique technique in this study to reveal how the spatial location of methanotrophic bacteria in the soil profile is affected by anthropogenic factors.

The ecological niche of methanotrophic bacteria in soils is unknown yet. Soils have been sieved and incubated in laboratory studies to investigate methanotrophic activity in soil layers (Bradford et al., 2001; Adamsen and King, 1993; Reay et al., 2005). However, soil structure effects on $CH_4$ uptake did not receive sufficient attention to date (Stiehl-Braun et al., 2011a). We applied a novel technique to study the spatial distribution of methanotrophic bacteria in intact soil structure. We labeled methanotrophic bacteria in soil cores with $^{14}CH_4$ and used an auto-radiographic imaging technique to produce a profile image. We determined the vertical distribution of $^{14}C$ in the soil profile.

## Thesis outlook

In this thesis, I investigated changes of methanotrophic activity under global change drivers (elevated temperature and $CO_2$ concentration) and land use changes. It is important to study niches of methanotrophic bacteria to understand their ecology. Previous studies investigated either changes in $CH_4$ fluxes at the ecosystem-level or at the bulk-soil level (in the laboratory) (White et al., 2008; Price et al., 2004; Shukla et al., 2013). The micro-distribution of methanotrophic bacteria in the soil profile has been ignored in most of $CH_4$ uptake studies. I focused on methanotrophic activity in three different ecosystems and quantified changes in the micro-distribution of methanotrophic activity in the soil profile.

In Chapter 1, I studied effects of elevated soil temperature on soil $CH_4$ uptake in the final 2 years of a 6-year soil warming experiment near the alpine treeline in Davos,

Switzerland. I had two alternative hypotheses: elevated temperature can either increase or decrease $CH_4$ oxidation. The decrease of $CH_4$ oxidation rates could be driven by an increase of N mineralization due to increased soil temperature. Increases in soil $NH_4^+$ concentration often lead to a reduction in $CH_4$ oxidation rates by competitive enzyme inhibition (Dunfield and Knowles, 1995; Gulledge and Schimel, 1998). Alternatively, elevated temperature can reduce soil moisture promoting $CH_4$ diffusion into the soil, thus increasing soil $CH_4$ oxidation. I measured $CH_4$ fluxes and soil N concentrations in the field. Furthermore, I studied the soil micro-distribution of methanotrophic bacteria in soil the profile.

It is not clear to date how elevated $CO_2$ concentration will affect methanotrophic bacteria in different ecosystems. Environmental factors (e.g. soil moisture, N availability) interact with the effect of elevated $CO_2$ concentration and make this process complicated to predict. To quantify how $CH_4$ fluxes, methanotrophic activity and the micro-distribution of methanotrophic bacteria in soil cores can be affected by the long-term application of elevated $CO_2$ I investigated soils from the University of Giessen Free-Air Carbon Dioxide Enrichment. This study site is currently the oldest running long-term FACE experiment on a semi-natural grassland ecosystem in Europe (Chapter 2). We measured soil–atmosphere $CH_4$ fluxes in the study site over 4 years and quantified the micro-distribution of active methanotrophic bacteria in the soil profile using auto-radiographic technique.

Afforestation can affect the activity of methanotrophic bacteria (Singh et al., 2007), but the mechanism driving these changes is not well-understood to date. In forest soils, higher organic matter input can increase macro-aggregate formation (Tisdall and Oades, 1982). There is a direct relation between soil macro-aggregate and water-filled pore space. Higher water-filled pore space increases air diffusion into soils and can increase methanotrophic activity. To explore how the micro-distribution of methanotrophic bacteria is affected by soil structural changes and afforestation, I carried out an experiment (Chapter 3) in an afforestation chronosequence in a sub-alpine region. I labeled methanotrophic bacteria

in soil cores with $^{14}C$ and studied the micro-distribution of methanotrophic bacteria. In addition, I labeled soil aggregate structure to compare methanotrophic activity in exterior and interior fractions of soil aggregates.

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# Chapter 1:

## Treeline soil warming does not affect soil methane fluxes and the spatial micro-distribution of methanotrophic bacteria

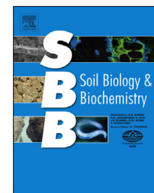
**Saeed Karbin**, Frank Hagedorn, Melissa A. Dawes and Pascal A. Niklaus, (2015), Soil Biology and Biochemistry, 86, 164-171

Soil Biology & Biochemistry 86 (2015) 164–171

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio



# Treeline soil warming does not affect soil methane fluxes and the spatial micro-distribution of methanotrophic bacteria

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A R T I C L E I N F O

*Article history:*
Received 26 January 2015
Received in revised form
19 March 2015
Accepted 23 March 2015
Available online 14 April 2015

*Keywords:*
Auto-radiography
$^{14}C$ labeling
Methanotrophic bacteria
Soil warming
Spatial distribution

A B S T R A C T

The impact of a warmer climate on $CH_4$ fluxes from soils is highly uncertain, because soil warming may affect methanotrophic bacteria in two opposed ways: $CH_4$ assimilation in soils might be increased by the decreasing soil moisture often associated with soil warming. In contrast, $CH_4$ oxidation might be suppressed by higher $NH_4^+$ concentrations in warmed soils resulting from an accelerated nitrogen mineralization. We investigated effects of soil warming on soil-atmosphere $CH_4$ fluxes in the last two years of a six-year long field experiment at a Swiss alpine treeline. Specifically, we measured $CH_4$ fluxes using static chambers, and characterized N cycling by quantifying soil $N_2O$ emissions and $NH_4^+$ and $NO_3^-$ concentrations. We further labeled intact soil cores with $^{14}CH_4$ and traced the labeled bacteria using an auto-radiographic technique to study the potential warming-related changes in the micro-distribution of methanotrophic bacteria within the soils. Our results did not show a significant effect of soil warming on net $CH_4$ fluxes after five and six years of soil warming. In general, soils were a net sink for $CH_4$ but $CH_4$ emissions were observed occasionally. One reason for the unaltered $CH_4$ fluxes might be the negligible warming effects on soil water contents in the treeline environment with frequent rainfalls. In the warmed soils, soil moisture was lower in the litter layer, but not deeper in the soils. Therefore, soil warming did not affect gas transport rates into deeper soil layers where methanotrophic bacteria were located. Another reason might be the general absence of substantial warming effects on mineral N, with $NH_4^+$ concentrations being marginally significantly higher in warmed soils only in ion exchange resin bags ($P < 0.1$) but not in soil extracts. Auto-radiographic image analysis of soil cores revealed an overall heterogeneous $^{14}C$ distribution and a warming-induced shift of methanotrophic bacteria toward the soil surface. The absence of responses of $CH_4$ fluxes to warming in this alpine treeline ecosystem is likely related to the rather minimal changes in the putative drivers soil moisture and $NH_4^+$ concentration.

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## 1. Introduction

Net land-atmosphere fluxes of methane ($CH_4$) are determined by the balance of $CH_4$ sources and sinks, which are both almost exclusively driven by soil microbial processes (Conrad, 2007; Shukla et al., 2013; but see Wang et al., 2013 for abiotic $CH_4$ sources). Methanogenic archaea produce $CH_4$ under conditions that are generally anaerobic (Mer and Roger, 2001; Conrad, 2007), although emissions are to a lesser degree also observed from some upland soils (Angel et al., 2012). On the other hand, soil $CH_4$ sinks are driven by assimilation of $CH_4$ by methanotrophic bacteria, yielding organic carbon (C) that eventually is respired by methanotrophs, yielding $CO_2$, or that enters the soil C cycle as their biomass turns over (Hanson and Hanson, 1996; Semrau et al., 2010).

Methanogens and methanotrophs often co-occur in the same soils. $CH_4$ produced by methanogens in anoxic soil domains will diffuse through more oxic soil areas where part of it can be oxidized by methanotrophs before it reaches the atmosphere. Under these conditions, methanotrophs thrive on soil-internal $CH_4$ sources, functionally acting as a "biofilter" that reduces net $CH_4$ emissions to the atmosphere (Horz et al., 2001; Kammann et al., 2001; Urmann et al., 2009). However, some groups of methanotrophs can also assimilate atmospheric $CH_4$, turning soils into a net $CH_4$ sink. In many soils, methanotrophs in fact oxidize $CH_4$ both from internal sources and from the atmosphere, with one or the other process

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http://dx.doi.org/10.1016/j.soilbio.2015.03.022
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dominating depending on environmental conditions. The nature of the methanotrophic organisms consuming $CH_4$ at atmospheric and lower concentrations remains elusive since all isolation attempts have been unsuccessful to date. There is evidence from genetic markers that these organisms are distinct from the methanotrophic populations dominating $CH_4$ consumption in high-methane environments (Dunfield et al., 1999; Henckel et al., 2000; McDonald et al., 2008). On the other hand, some isolated methanotrophic strains are capable of oxidizing $CH_4$ over a wide range of concentrations (Knief and Dunfield, 2005), challenging this view (Dunfield, 2007). $CH_4$ fluxes are of interest because this greenhouse gas substantially contributes to anthropogenic radiative forcing and climate change (currently ≈30%; IPCC, 2013). A related important question is whether and how $CH_4$ dynamics in terrestrial ecosystems are altered under climate change, in particular warming. These effects are complicated to predict, on the one hand because $CH_4$ fluxes are controlled by a multitude of proximal (e.g. C supply and redox potential) and more distal factors (e.g. soil structure, climate), with only some of them affected by climate change. On the other hand, the net effect on fluxes manifest at the ecosystem level is the result of different responses of $CH_4$ fluxes.

Many previous studies have shown that methanogenesis increases with temperature (e.g. Moore and Dalva, 1993; Wang et al., 1999; Mer and Roger, 2001). This effect is driven by an accelerated metabolism of soil methanogens at higher temperatures. Also, the increase in general soil heterotrophic activity at higher temperatures increases the size of anaerobic domains with a redox potential sufficiently low for methanogenesis. However, experimental warming in field studies often not only leads to warmer but also drier soils (White et al., 2008; Luo et al., 2013). While one could consider this an undesired side effect of the experimental treatment, climate models in fact predict that warming will be accompanied by lower soil moisture in many regions, at least for part of the year (Luo et al., 2013). This potential drying effect could counteract positive warming effects on methanogenesis.

Direct temperature effects on $CH_4$ oxidation are comparably small (Hanson and Hanson, 1996; Price et al., 2004). However, soil $CH_4$ oxidation can be affected indirectly by warming via changes in plant and soil processes (Blankinship et al., 2011). $CH_4$ assimilation is generally substrate-limited, especially when $CH_4$ concentrations are low. In upland soils, soil $CH_4$ uptake rates therefore are often controlled by a diffusive supply from the atmosphere to the microsites where methanotrophic bacteria exist. Across sites, soil diffusivity is related to soil porosity. However, on a diurnal to seasonal basis, diffusivity is related to variation in soil moisture, which controls $CH_4$ transport rate by filling of pore networks (Dunfield et al., 1995; Billings et al., 2000; Luo et al., 2013). Drier soils under warming should therefore show higher soil $CH_4$ uptake, unless moisture is so low that methanotrophic activity is restricted due to physiological stress (e.g. Price et al., 2004).

A second important factor controlling soil $CH_4$ uptake is nitrogen (N) status, in particular ammonium ($NH_4^+$) concentrations (e.g. Dunfield et al., 1995; Hartmann et al., 2010; Shukla et al., 2013). In laboratory settings, $CH_4$ oxidation is inhibited by $NH_4^+$ (Dunfield and Knowles, 1995; Hanson and Hanson, 1996; Duan et al., 2013), most likely through an enzymatic effect (inhibition of methane mono-oxygenase by $NH_3$). In line with this finding, the soil $CH_4$ sink is often reduced under $NH_4^+$ fertilizer application (Hütsch, 1996; Stiehl-Braun et al., 2011a). However, more complex ecological mechanisms are often at play in natural ecosystems, and soil $CH_4$ uptake is also sometimes correlated positively with N supply (Bodelier and Laanbroek, 2004). In grassland, Stiehl-Braun et al. (2011b) found that $NH_4^+$ application inhibited $CH_4$ oxidation in some soil layers, but this effect did not translate into a smaller soil $CH_4$ sink because the reduced methanotrophic activity was compensated by an increased sink activity in deeper soil layers. N-related effects on $CH_4$ oxidation are relevant in a climate change context because warming can accelerate organic matter mineralization and $NH_4^+$ production (Rustad et al., 2001; Curtin et al., 2012; Bai et al., 2013). While this phenomenon may be transient (Butler et al., 2012), it nevertheless has the potential to reduce soil $CH_4$ uptake during this period. However, whether such an effect occurs also depends on whether $NH_4^+$ can accumulate in the soil, or whether it is continuously removed by nitrification, plant uptake, or microbial immobilization. For example, Hartmann et al. (2010) reported increased $NH_4^+$ concentrations after fertilization only under concomitant drought. Warming also may not affect mineralization if microbial activity drops because of reduced soil moisture (Carrillo et al., 2012).

Here, we present a study of simulated warming effects on soil $CH_4$ uptake in an afforestation located near the alpine treeline. We hypothesized that soil $CH_4$ uptake would increase due to reduced soil moisture, since these conditions would facilitate $CH_4$ diffusion into soils and thus $CH_4$ oxidation. We additionally expected that drier soils would decrease rates of methanogenesis if this process was important for the $CH_4$ balance in the soil. Alternatively, if higher soil temperatures were more influential on $CH_4$ fluxes than drying, we expected to find reduced soil $CH_4$ oxidation due to increased organic matter mineralization and consequently soil $NH_4^+$ concentrations. We thus measured soil-atmosphere $CH_4$ fluxes, soil moisture and soil mineral N concentrations during the final two summers of a six year warming experiment. To disentangle mechanisms that involve changes in the spatial distribution of methanotrophic activity, we additionally labeled intact soil cores with $^{14}CH_4$ and studied the spatial distribution of the label using an auto-radiographic technique (Stiehl-Braun et al., 2011b).

## 2. Materials and methods

### 2.1. Study site and experimental design

We investigated soil warming effects on $CH_4$ transformations in a long-term experiment near the alpine treeline at Stillberg (2180 m a.s.l) in the Central Alps near Davos, Switzerland. The warming experiment studied here was set up as an extra factorial treatment added in the course of a long-term elevated $CO_2$ study (Hagedorn et al., 2010).

In 2001, the original elevated $CO_2$ experiment was set up encompassing 20 plots with *Larix decidua* (European larch) and 20 plots with *Pinus mugo* ssp. *uncinata* (mountain pine). Each hexagonal 1.1 $m^2$ plot had a single tree in the center. Trees were surrounded by a dense cover of understory vegetation (for details, see Dawes et al., 2011).

These trees were part of an afforestation planted in 1975 on a steep north-east facing slope with 25–30° inclination). Plots were organized in five blocks, with each block consisting of two groups of four plots (two with *L. decidua* and two with *P. uncinata*). One group of plots per block was exposed to atmospheric concentrations of 550 μmol $CO_2$ $mol^{-1}$, while the other group was exposed to ambient $CO_2$. Details of the experimental set up with $CO_2$ enrichment were reported in Hättenschwiler et al. (2002). The $CO_2$ treatment was discontinued at the end of 2009 after 9 years of enrichment.

Starting in 2006, a soil warming treatment was established, using the same plots. The plots in which soils were warmed were chosen so that the former $CO_2$ exposure and the new warming treatment were orthogonal (Hagedorn et al., 2010). Warming was implemented with heating cables laid out on the ground surface of the plots in spirals with a 5 cm distance between cable loops. The soil warming treatment increased the soil temperature at 5 cm depth by 3–4 K. Each year, experimental warming began after snow

melt and lasted until the site was covered with snow (≈June to October; warming treatment was stopped in early August in 2012 due to the final harvest). Further details of the warming treatment were given in Hagedorn et al. (2010) and Dawes et al. (in press).

Soils are classified as Ranker and weakly developed Podzols on paragneiss parent material (Bednorz et al., 2000). The Humimor organic layer is dominated by a 5–20 cm thick $O_a$ horizon with a pH of ≈ 3.5 and C:N ≈ 27. Long-term (1975–2012) annual precipitation averaged 1155 mm, with a mean maximum snow depth of 1.5 m, and the mean annual air temperature was 2.1 °C. The main growing season months (June–August) had a mean precipitation of 444 mm and a mean air temperature of 9.2 °C (Dawes et al., in press).

### 2.2. Soil moisture and temperature

Volumetric soil moisture was measured regularly at 0–6 cm depth in all plots by frequency domain reflectometry (Theta ML2x probe, Delta-T, Cambridge, UK), using a soil-specific calibration (for details, see Hagedorn et al., 2010). In addition, gravimetric soil moisture was measured for each individual soil horizon on August 8, 2012. Soil temperature was measured automatically at 5 cm depth (1 h resolution, Hobo Pro v2 temperature logger, Onset Computer Corporation, Bourne, USA). Soil temperature also was measured manually at 5 cm depth with each $CH_4$ flux measurement.

### 2.3. Soil $NH_4^+$ and $NO_3^-$

Soil samples were collected using a 2 cm diameter corer. On July 19, 2011, samples were split into 0–5, 5–10 and 10–15 cm depth layers. On June 18, July 10 and August 10, 2012, only the top 0–5 cm layer was sampled. The samples were immediately stored at 4 °C and transported to the laboratory, where plant roots and stones were removed by sieving 2 mm mesh size). Sieved subsamples of 5 g equivalent dry weight were extracted with 50 mL KCl for 1 h, the suspension filtered (Whatman, Sigma–Aldrich, USA), and the extract analyzed for $NH_4^+$ and $NO_3^-$ concentrations (SAN$^{++}$ segmented flow analyzer, Skalar, Germany). Ion exchange resins were used to obtain an integrated measure of mineral N availability (Giblin et al., 1994; Schleppi et al., 2012). Four to five resin bags were incubated in each experimental plot for 2 months (June and July 2012). The 1 cm diameter × 9 cm length bags were inserted vertically into the soil so that they reached 10 cm depth and were covered by 1 cm of soil. Bags were made of 0.3 mm-mesh nylon cloth and filled with 3.5 g of a 1:1 mixture of cation (Dowex HCR-W2, 16–40 mesh, Sigma–Aldrich, Switzerland) and anion exchange resins (Dowex 1 × 4, 20–50 mesh). After removing the resin bags from the soil, they were rinsed with deionized water and stored frozen until extraction for 24 h with 100 mL 1 M KCl. Extracts were analyzed for nitrate by UV absorption using $CuSO_4$-coated Zn granules as a reductant (Norman and Stucki, 1981; Schleppi et al., 2012). $NH_4^+$ concentrations were measured by flow injection analysis.

### 2.4. Soil-atmosphere $CH_4$ and $N_2O$ fluxes

Static chambers (20 cm diameter × 20 cm height) were fixed to the soil surface of each plot using tent pegs. The chambers were placed at a minimum distance of 50 cm from the tree to avoid direct effects of the tree trunk and the surrounding coarse roots. These chambers could not be lowered into the ground due to the presence of the heating cables. Instead, the soil-chamber interface was sealed with moist clay. The chambers were re-sealed before every flux measurement to minimize leakage.

Chamber lids were closed and four 25 mL headspace samples collected in 7 min intervals. These samples were stored in pre-evacuated exetainers until they were analyzed for $CH_4$ and $N_2O$ (Agilent 7890N gas chromatograph equipped with a flame ionization detector for $CH_4$ and an electron-capture detector for $N_2O$; Agilent, Wilmington, Delaware, USA). Soil-atmosphere flux rates were calculated by linear regression of headspace concentrations against sampling time. Additionally the residual standard error of the fit was calculated and used to give poor fits a lower weight in the statistical analysis (see below).

### 2.5. Radiolabeling of methanotrophs

Intact soil cores were collected, radiolabeled with $^{14}CH_4$, and the micro-scale distribution of methanotrophic bacteria analyzed by auto-radiography. On August 8–9, 2012, plots were first probed with a metal pin to find locations that were free of large stones. Then, intact soil cores were collected with a corer fitted with an inner plastic sleeve of 5 cm diameter and 30 cm length. The harvested soil cores were kept in their sleeves and stored upright to minimize disturbance of the soil structure. In the laboratory, each soil core was placed into a gas-tight 3 L jar, the jar lids closed, and soil $CH_4$ consumption determined by analyzing headspace $CH_4$ and $N_2O$ concentrations after 0, 1.5, 3, 4.5 and 6 h as described above for the field flux measurements.

In a next step, jars were ventilated, closed again, and labeled with a total of 100 kBq $^{14}CH_4$ per soil core during a 7 d incubation. Headspace $CH_4$ concentrations were monitored during the incubation and kept in the range of 5–8 μL $CH_4$ $L^{-1}$ by re-supplying the soil cores with $^{14}CH_4$, and, towards the end of the incubation, unlabeled $CH_4$. $CO_2$ released during the incubation was trapped in plastic tubes containing 60 mL 1.5 M NaOH. $O_2$ was regularly injected into the jars to maintain aerobic conditions (15–20% $O_2$).

Subsequently, soil cores were frozen and impregnated with epoxy resin (Laromin C 260, BASF, Ludwigshafen, Germany, mixed at a ratio of 2:3 with Araldite DY026SP hardener, Astorit AG, Einsiedeln, Switzerland). To improve resin penetration, the soil cores were placed in a dessicator and evacuated to an end pressure of 25 kPa. The cores were kept at this pressure for 3 min before pressure was slowly restored to atmospheric levels. After about 2–3 days at room temperature, the impregnated soil cores were incubated overnight at 60 °C to completely harden the resin. Then, the cores each were cut twice length-wise with a diamond saw, creating a flat section of ≈8 mm thickness. This section was further divided cross-wise into three parts which were mounted onto a 5 × 5 cm glass carrier. The surface of the sections was leveled with a diamond cup mill (Discoplan, Struers GmbH, Birmensdorf, Switzerland).

The flat soil sections were used to expose phosphor imaging plates (BAS III S, Fuji Photo Film Ltd., Tokyo, Japan) for 3 d. Finally, the imaging plates were digitized by red-excited fluorescence scanning at a resolution of 200 μm (Fujix, BAS-1000, Tokyo, Japan). $^{14}C$ $\beta$-particles have a low energy and therefore travel only an estimated 10 μm in the resin matrix, so blurring did not occur.

The three parts of each section were recomposed to a single image covering a vertical cross-section through the soil core. After correcting for background exposure unrelated to the $^{14}C$ label, the vertical distribution of $^{14}C$ in the soil profile was determined by averaging the pixel intensities per horizontal pixel line. Areas with big stones were excluded during this procedure.

### 2.6. Data analysis

All data were analyzed using mixed-effects models fit by REML (ASReml, VSN International Ltd., Hemel Hempstead, UK). Models

included block, soil warming treatment, past-elevated $CO_2$ treatment, and tree species as fixed effects. Plot was fit as a random effect, together with plot × warming and plot × species, which ensured correct replication of the significance tests. For the analysis of the depth distribution of $^{14}C$ in auto-radiographies, subplot and plot × warming × layer were fit as additional random effects (these define the level of replication for the test of warming × depth).

Field-measured trace gas fluxes sometimes suffered from poor chamber sealing. Therefore, flux data were weighed based on the residual standard error (RSE) of the linear regression fitted to estimate the flux. Note that RSE is preferable to quality filtering based on $R^2$ because the latter necessarily is zero when fluxes are zero. Weights were scaled linearly with the inverse of RSE, so that the best 10% of the measurements had a weight of one while the poorest 10% had a weight of zero. This procedure appears less arbitrary than choosing a single threshold defining invalid measurements and likely reflects the true information content of the data more adequately. Results, however, did not critically depend on the exact procedure chosen.

## 3. Results

### 3.1. Soil temperature and moisture

Soil temperature at 6 cm depth (13 manual measurements made in May–September, 2011 and June–August, 2012) averaged 13.7 °C in warmed and 10.7 °C in control plots. Over the two growing seasons, automatically logged data showed similar warming effects at 5 cm depth (12.4 ± 1.9 and 9.2 ± 2 °C in warmed and control plots, respectively).

Warming did not affect volumetric soil moisture in the top 6 cm significantly (frequency domain reflectometry, 10 measurements made during the 2011 and 2012 growing seasons, warmed: 56 ± 15% and control: 60 ± 12%). In contrast, the gravimetric measurements made when the experiment was destructively harvested showed decreased soil moisture under warming in the litter and F layer, but not in deeper soil layers (Fig. 1, P < 0.001 for warming × depth).

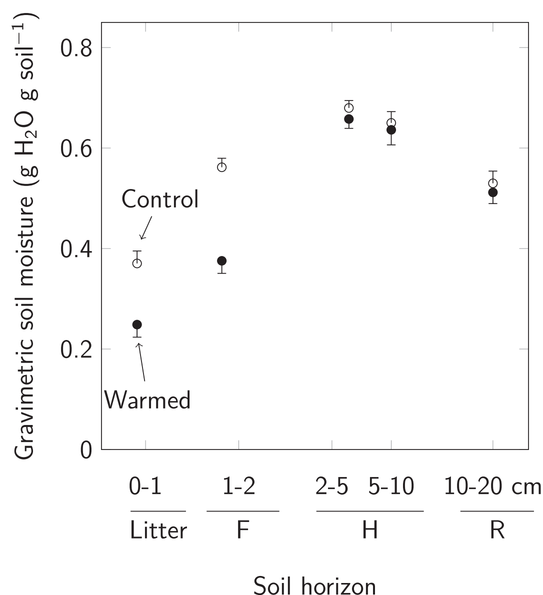


**Fig. 1.** Gravimetric soil moisture in individual soil layers. Mean values ± 1 SE are shown for each soil warming treatment (control and warmed), pooled across past $CO_2$ treatments and plot tree species (n = 20). Data are shown from samples collected on August 8, 2012, when the experiment was destructively harvested. Litter: plant structures are still identifiable, F: plant structures are partially decomposed by soil fauna and/or fungi, H: well-humified material, R: consolidated bedrock.

### 3.2. Soil $NH_4^+$ and $NO_3^-$

Soil $NH_4^+$ concentrations in extracts were not affected by warming (July 19, 2011: soil 0–5, 5–10 and 10–15 cm; June 18, July 7, and August 8, 2012: 0–5 cm layer only). Soil $NO_3^-$ concentrations were below or close to the detection limit of our analytical procedure (1 mg N $L^{-1}$) and therefore were not analyzed (Fig. 2).

Soil $NH_4^+$ and $NO_3^-$ concentrations collected on ion exchange resins in 2012 averaged 21% and 13% higher in the warming than in control soils, but this difference was only marginally statistically significant for $NH_4^+$ (P < 0.1, Fig. 3).

### 3.3. Soil-atmosphere $CH_4$ and $N_2O$ fluxes

We observed net soil $CH_4$ emissions in 5–10% of all measurements. However, on average soils were a net sink for $CH_4$, with no significant difference between warming treatments (1.8 ± 0.2 μmol $CH_4$ $m^{-2}$ $h^{-1}$ in both treatments). Soils with *Larix* trees showed a marginally significantly higher net $CH_4$ uptake than soils with *Pinus* trees (2.2 ± 0.4 and 1.4 ± 0.2 μmol $CH_4$ $m^{-2}$ $h^{-1}$; P = 0.07). Net soil $CH_4$ uptake was negatively related to soil moisture, even after first accounting for differences between sampling dates and position on the slope using linear models (P < 0.001). There was no statistically significant relationship between soil-atmosphere $CH_4$ flux and $NH_4^+$ concentration collected on ion exchange resins or $NH_4^+$ concentrations in soil extracts.

The laboratory incubation of soil cores showed similar effects on $CH_4$ fluxes as the static chamber measurements in the field, i.e. no effects of warming but a negative correlation between $CH_4$ uptake and soil water content (data not shown).

Soils were net $N_2O$ sources, with emission rates averaging around 55 nmol $N_2O$ $m^{-2}$ $h^{-1}$, with no statistically significant effect of warming and species.

### 3.4. Distribution of assimilated $^{14}CH_4$

The auto-radiographies of soil sections revealed a heterogeneous $^{14}C$ distribution, with domains of high $^{14}C$ incorporation and areas with little labeling (Fig. 4). Most $^{14}C$ was found at 4–7 cm depth. The organic layer at shallower depths and the deeper soil layers incorporated almost no $^{14}C$ label. When considering the relative depth distribution of $^{14}C$, warmed soils showed more label in the 2–4 cm depth range but less from 5 to 7 cm than control soils (P < 0.01 for depth × warming) (Figs. 5 and 6).

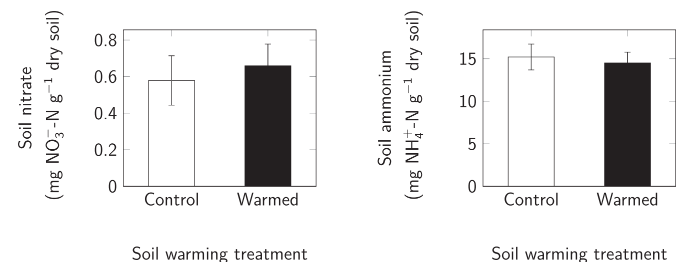


**Fig. 2.** $NH_4^+$ and $NO_3^-$ concentrations in the top 5 cm of the soil. Data are means ± 1 SE of measurements for each soil warming treatment made on July 19, 2011, June 18, 2012, July 7, 2012 and August 8, 2012. Estimates pooled across past $CO_2$ treatments and plot tree species are shown (n = 20).

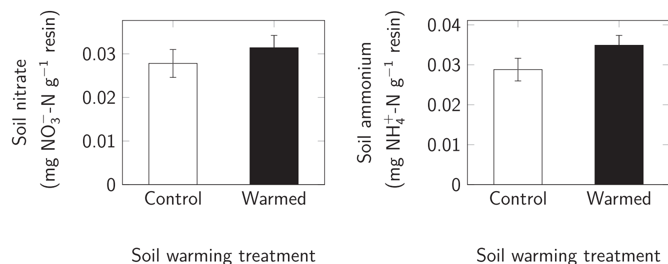


**Fig. 3.** Soil $NH_4^+$ and $NO_3^-$ collected on ion exchange resins incubated in situ in the soil in June–July 2012. Estimates pooled across past $CO_2$ treatments and plot tree species are shown (n = 20).

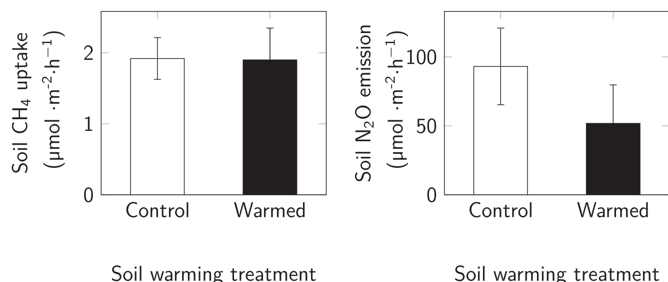


**Fig. 4.** Average soil-atmosphere $CH_4$ and $N_2O$ flux recorded during the study period. Estimates pooled across past $CO_2$ treatments and plot tree species are shown (n = 20).

## 4. Discussion

We hypothesized that two contrasting effects of soil warming on soil-atmosphere fluxes of $CH_4$ might occur. First, soil $CH_4$ uptake could increase if warming led to soil drying because soil diffusivity would facilitate access to $CH_4$ and $O_2$, promoting atmospheric $CH_4$ oxidation; at the same time, methanogenesis (if important) might decrease. Second, accelerated organic matter decomposition in warmed soils could release extra mineral nitrogen, which could inhibit $CH_4$ oxidation via a range of mechanisms (e.g. Dunfield and Knowles, 1995; Rustad et al., 2001; Bai et al., 2013). Our analysis, however, did not reveal any significant effects of soil warming on net soil $CH_4$ fluxes after six years of treatment. While we observed considerable within-treatment variability, power analysis by Monte Carlo-simulation reflecting the variance structure of our data indicated that effects of ≈35–40% would have been detected at a significance level of $\alpha = 0.05$ with a power of ≈50%. Effects of ≈30% would have been detected as marginally significant ($\alpha = 0.1$). We thus are confident that there were at least no large effects of warming on $CH_4$ fluxes. In support of these results, laboratory measurement showed unaltered $CH_4$ oxidation in samples from warmed soils.

On average, soils were a net sink for $CH_4$, with a $CH_4$ consumption rate at the lower end of the typical range for forests (Smith et al., 2000). The occasional $CH_4$ emissions we detected indicate soil-internal anaerobic domains harboring active methanogens. These $CH_4$ sources might also have been active during periods of net $CH_4$ uptake but with a magnitude smaller than gross $CH_4$ oxidation. Indeed, there is evidence that episodic methanogenesis can stimulate the sink activity of soils for atmospheric methane (e.g. West and Schmidt, 2002), possibly by supporting the growth and survival of otherwise energy-limited methanotrophic bacteria. $CH_4$ transport in soils occurs almost exclusively in the gas phase. Water filled pore space, which depends on soil moisture, thus controls uptake rates of atmospheric $CH_4$ in many soils (Castro et al., 1994; Hartmann et al., 2010; Hiltbrunner et al., 2012; Bai et al., 2013). In our study, soil moisture was negatively correlated with $CH_4$ fluxes over time, indicating diffusion limitations. However, warming had no effect on soil moisture except for a slight reduction in the top-most soil layer. In particular, the $CH_4$-assimilating soil horizon at 4–7 cm depth remained unaffected by experimental warming. We thus conclude that direct physiological effects of soil moisture on methanotrophs could not develop under the typical frequent rainfalls in alpine regions. Further, it seems that minor soil moisture differences in the top soil either did not affect gas transport rates, because this layer dries relatively quickly and is unlikely to limit diffusion, or that any changes in transport rates were negligible. Interestingly, soils under *Larix* showed higher $CH_4$ assimilation than soils under *Pinus*; this difference may be due to differences in the structure of the organic surface layer that developed during the 35 years since establishment of the afforestation. Plots with deciduous *Larix* had more litter than those with evergreen *Pinus*. The mass of organic layer in plots with *Larix* in the center was 40% larger than in plots with *Pinus*. In addition to the mass and thickness, the organic compounds may be different in litter derived from these two conifer species. While Khan and Salenko (1990) reported that *Pinus* needles contain more monoterpenes than those of *Larix*, Amaral et al. (1998) showed that terpenes can decrease the activity of methanotrophic bacteria.

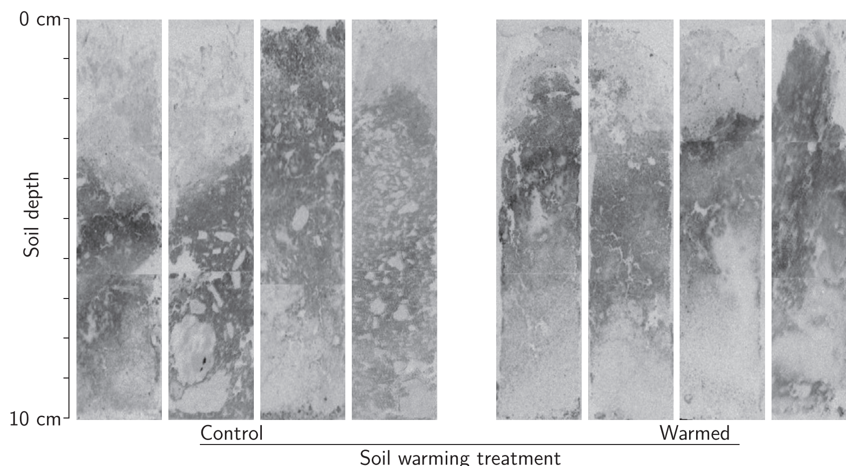


**Fig. 5.** Selected representative auto-radiographic images showing the spatial distribution of net $^{14}C$ assimilation. These scans have a resolution of 200 μm. Darker pixels indicate higher net $^{14}C$ assimilation.

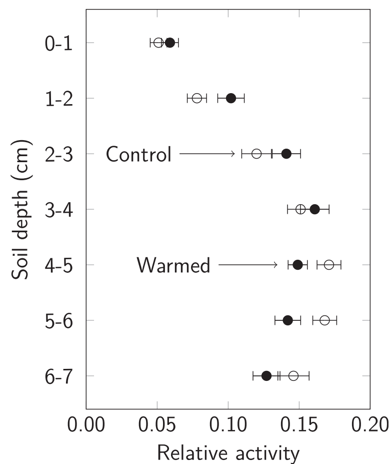


**Fig. 6.** Vertical distribution of assimilated $^{14}C$ in soils of control and warmed plots (mean ± 1 SE). Note that activities are normalized, i.e. the total activity in the profile equals one for each soil core. The figure thus shows the relative spatial distribution but not treatment effects on total amounts. Estimates pooled across past $CO_2$ treatments and plot tree species are shown (n = 20).

Soil $NH_4^+$ concentrations have been shown to inhibit $CH_4$ assimilation in laboratory studies, possibly by competitive inhibition of the enzyme methane mono-oxygenase (King and Schnell, 1994; Dunfield and Knowles, 1995). Experimental warming often stimulates N mineralization rates. For example, Shaw and Harte (2001) reported large increases in gross N mineralization rates in a sagebrush ecosystem warmed in situ, with effects differing between mesic and xeric conditions. In our experiment, at least in the initial years of treatment, warming increased soil respiration (Hagedorn et al., 2010), which likely indicates higher organic matter mineralization rates and associated increases in ammonification. In support of this argument, $NH_4^+$ concentrations increased by 140% in warmed plots in 2009, the third treatment year (Dawes et al., 2011). However, in our sampling period during 2011, we did not find increased $NH_4^+$ concentrations in soil extracts and it was only marginally higher in ion exchange resins incubated in 2012, suggesting that there was primarily only a transient increase in $NH_4^+$ in initial years. Further, we did not find increased $NO_3^-$ concentrations during our sampling period. $N_2O$ emissions originate from nitrification and denitrification. Although controls of $N_2O$ fluxes involve many factors, both proximal and distal, and denitrification also results in $N_2$, higher $N_2O$ fluxes would have suggested increased N cycling rates. However, we did not find such a pattern. Further, we did not find evidence of a $NH_4^+$-mediated effect of warming on $CH_4$ fluxes, regardless of whether ammonification increased or not.

Previously published evidence of temperature effects on soil $CH_4$ fluxes is equivocal. A particular difficulty in analyzing effects seems to be the separation of direct warming from indirect soil moisture effects. In the study of Blankinship et al. (2010), mesocosms of four ecosystems were warmed by transplantation to lower altitudes; in two of the four ecosystems, soil $CH_4$ uptake decreased. However, the low elevation site also had lower precipitation. The authors therefore argued that lower diffusive limitations would have increased rather than decreased the soil $CH_4$ sink and attributed the observed effects on $CH_4$ fluxes to soil temperature. In contrast, Carter et al. (2011) reported increases in soil $CH_4$ sink strength under warming and speculated that this effect was caused by a combination of direct temperature effects and increased $CH_4$ transport rates in drier soils; however, in a parallel drought study they found that reducing soil moisture to levels lower than those observed in the field warming treatment inhibited soil $CH_4$ uptake. Dijkstra et al. (2013) found a net decrease in cumulative seasonal soil $CH_4$ uptake but observed a bell-shaped dependency of $CH_4$ fluxes on soil moisture when analyzing the whole time series, with a maximum $CH_4$ sink at intermediate soil moisture. Overall, findings from these studies suggest that warming effects on soil $CH_4$ fluxes are determined by a complex, non-linear interaction between direct and indirect effects of temperature, in particular via soil moisture changes. At very low soil moisture, physiological limitations of methanotrophs might outweigh positive effects of increased diffusive transport rates. However, other studies indicated idiosyncratic responses unrelated to moisture, with positive and negative effects alternating over time, suggesting that other temperature-mediated factors also play a role (e.g. Rustad and Fernandez, 1998). In any case, inferring true warming effects from the analysis of temporal correlations between temperature and $CH_4$ fluxes (e.g. Castro et al., 1995) is problematic because other factors covary with season (e.g. plant activity), and because the condition of a system does not simply depend on the current temperature but is co-determined by legacy effects.

It has recently been suggested that dissolved organic carbon (DOC) compounds might stimulate soil $CH_4$ oxidation (Sullivan et al., 2013). This hypothesis is interesting in the context of soil warming, because accelerated organic matter decomposition could increase DOC concentrations. However, this was not the case in our treeline experiment (<10% DOC concentration change; Hagedorn et al., 2010). Perhaps any stimulation of microbial DOC production in warmed soils was balanced by a higher DOC consumption in our experiment. However, $CH_4$ oxidation is affected by many chemical compounds, including ethylene (Jackel et al., 2004), organic acids (Wieczorek et al., 2011), and terpenes (Amaral et al., 1998). Further, the nature of the organisms driving atmospheric $CH_4$ uptake in natural systems remains enigmatic (Dunfield et al., 2007), and the ecological controls on their activity are only partly understood. Most analyses to date focus on temperature, moisture, and mineral nitrogen, but many other factors might also be at play. Experimental manipulations of a single driver (e.g. temperature) can safely attribute any observed effects to this driver (although the mechanisms may be indirect and unknown). In contrast, conclusions based on temporal covariation of putative drivers (e.g. temperature or moisture) with $CH_4$ fluxes have to be treated with caution, since many potentially important factors (many of which likely are unrecognized) covary. This problem is particularly aggravated when studying gradients across ecosystems (Sullivan et al., 2013; Zhao et al., 2013). These studies may lead to important discoveries but specific conclusions based on such data require a more stringent verification.

Our auto-radiographic image analysis indicated a warming-induced shift of methanotrophic activity towards the soil surface. While statistically significant, we think that this effect must be treated with caution for several reasons. First, the observed shift was rather small. Second, the depth distribution was expressed relative to the soil surface; this point of reference, however, was difficult to identify precisely, in particular given the low density of the organic surface layer and uneven soil surface. Finally, the statistical test for depth × warming may involve some degree of pseudoreplication, even if no strong residual correlation between the potentially non-independent adjacent soil layers was evident. Assuming the reported shift represented a true effect, soil moisture could not explain the observed pattern since drier soils would rather lead to a downward shift of methanotrophic activity within the soil profile (c.f. Stiehl-Braun et al., 2011a). We thus can only speculate that some of the other factors discussed above might have been responsible for this change. In any case, the small magnitude of the shift makes important ecological consequences unlikely.

Effects of elevated atmospheric $CO_2$ on soil $CH_4$ uptake have been reported from several field studies. McLain and Ahmann (2008), Dubbs and Whalen (2010) and Carter et al. (2011) reported a reduced soil sink activity under experimentally $CO_2$-enriched vegetation, perhaps representing increased soil moisture due to reduced stomatal conductance by plants (Morgan et al., 2004). Other authors related reduced methanotrophy under elevated $CO_2$ to increased protozoan grazing of these bacterial communities (Ronn et al., 2003); increases in some protozoan groups under elevated $CO_2$ have previously been reported (Hungate et al., 2000) and attributed to soil moisture increases. We therefore argue that it may be difficult to separate the two effects if they occur simultaneously. In our study, $CO_2$ enrichment had been discontinued two years prior to our $CH_4$ flux measurement and we did not detect any legacy effects of elevated $CO_2$. Although we are unable to draw any conclusions about potential past effects during the time when elevated $CO_2$ was applied, our results indicated that any such effects did not have long-lasting impacts.

In conclusion, no effects of experimental warming on soil $CH_4$ fluxes were detected at the alpine treeline. These findings are in contrast to the large warming responses of some other processes that were found in this ecosystem, including increased soil respiration implying accelerated organic matter decomposition and possibly nitrogen mineralization. The unresponsiveness in $CH_4$ fluxes may be related to the small (or lack of) changes in soil moisture and $NH_4^+$ concentrations. However, the ecological controls of methanotrophy are currently too poorly understood to relate $CH_4$ flux rates to these two factors alone.

## Acknowledgments

This work was funded in part by a fellowship of Aga Khan Foundation (AKF) to SK and the University of Zurich. We gratefully acknowledge Rene Husi for assistance in mineral nitrogen measurements.

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# Chapter 2:

## Effects of long-term $CO_2$ enrichment on soil-atmosphere $CH_4$ fluxes and the spatial micro-distribution of methanotrophic bacteria

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# Abstract

Effects of elevated atmospheric $CO_2$ concentrations on plant growth and associated C cycling have intensively been studied, but less is known about effects on the fluxes of radiatively active trace gases other than $CO_2$. Net soil–atmosphere $CH_4$ fluxes are determined by the balance of soil microbially-driven methane ($CH_4$) oxidation and methanogenesis, and both might change under elevated $CO_2$. Here, we studied $CH_4$ dynamics in a permanent grassland exposed to elevated $CO_2$ for 14 years. Soil-atmosphere fluxes of $CH_4$ were measured using large static chambers, over a period of four years. The ecosystem was a net sink for atmospheric $CH_4$ for most of the time except summer to fall when net $CH_4$ emissions occurred. We did not detect any elevated $CO_2$ effects on $CH_4$ fluxes, but emissions were difficult to quantify due to their discontinuous nature, most likely because of ebullition from the saturated zone. Potential methanotrophic activity, determined by incubation of fresh sieved soil under standardized conditions, also did not reveal any effect of the $CO_2$ treatment. Finally, we determined the spatial micro-distribution of methanotrophic activity at near-atmospheric (10 ppm) and elevated (10000 ppm) $CH_4$ concentrations, using a novel auto-radiographic technique. These analyses indicated that domains of net $CH_4$ assimilation were distributed throughout the analyzed top 15 cm of soils, with no dependence on $CH_4$ concentration or $CO_2$ treatment. Our investigations suggest that elevated $CO_2$ exerts no or only minor effects on $CH_4$ fluxes in the type of ecosystem we studied, at least as long as soil moisture differences are small or absent as was the case here. The auto-radiographic analyses further indicate that the spatial niche of $CH_4$ oxidation does not shift in response to $CO_2$ enrichment or $CH_4$ concentration, and that the same type of methanotrophs may oxidize $CH_4$ from atmospheric and soil-internal sources.

**Keywords:** autoradiography, long-term $CO_2$ enrichment, $^{14}C$ labeling, ecological niche, methanogenesis, methanotrophic bacteria, spatial distribution.

# Introduction

The atmospheric concentrations of greenhouse gases including carbon dioxide ($CO_2$) and methane ($CH_4$) have increased since pre-industrial times due to anthropogenic activities. A question of particular concern is how elevated atmospheric $CO_2$ concentrations affect terrestrial ecosystems and their functioning. Studies of plant growth responses and of effects on the carbon balance of ecosystems have dominated elevated $CO_2$ research to date. However, although $CO_2$-effects are solely mediated by the plant's photosynthetic apparatus, elevated $CO_2$ can influence virtually every plant or microbial process through alterations of the ecosystem's carbon, nitrogen or water dynamics. An intriguing question is whether these effects will affect the ecosystem's balance of trace gases other than $CO_2$ such as $CH_4$. Such a mechanism would interact with global climatic change, similar to effects on carbon sequestration.

The $CH_4$ balance of an ecosystem is determined by the sum of sources and sinks, both of which are almost exclusively driven by soil microbial processes (Conrad, 1996) (but see Keppler et al., 2006; Wang et al., 2013). Whether sources or sinks dominate is often determined by oxygen availability, with $CH_4$ oxidizing micro-organisms driving soil $CH_4$ uptake under aerobic conditions whereas methanogenesis by archaea dominates under anaerobic conditions, e.g. in waterlogged soils. Methanogenesis and $CH_4$ oxidation often co-occur, with a substantial fraction of the $CH_4$ produced in anoxic soil domains being consumed by methanotrophs before it diffuses to the atmosphere. Under these conditions, methanotrophs functionally act as a "biofilter" for endogenous $CH_4$. Conversely, methanogenesis can prime the activity of methanotrophs (West and Schmidt, 2002), which then in turn will oxidize larger amounts of atmospheric $CH_4$ once the soil-internal sources cease (Dunfield, 2007). Oxidation of atmospheric $CH_4$ (low concentrations) or soil-internal $CH_4$ (high concentrations) requires enzymes with vastly different kinetic properties. Methanotrophic organisms growing at atmospheric $CH_4$ concentrations have not been isolated to date, and

it therefore remains unclear whether different groups of methanotrophs are responsible for these two sinks or whether the same organisms exhibit different $CH_4$ oxidation kinetics by physiological adjustment (Dunfield, 2007).

The ecology of atmospheric $CH_4$ oxidation is not well understood to date. Many studies have shown that gas phase diffusive $CH_4$ transport limitations often control soil $CH_4$ uptake, at least at moderate to high soil moisture (Doerr et al., 1993). However, moisture can also limit methanotrophic activity due to physiological stress (Kammann et al., 2001). A second important factor is nitrogen availability. High mineral nitrogen levels, in particular $NH_4^+$, can inhibit $CH_4$ oxidation. Laboratory studies have attributed this effect to inhibition of methane mono-oxygenase, the enzyme catalyzing the first step of $CH_4$ assimilation. However, mineral N also is an essential nutrient and the relationship between $CH_4$ oxidation and N levels therefore is more complicated (Bodelier and Laanbroek, 2004). Finally, inhibition of methanotrophic activity does not necessarily translate into reduced soil $CH_4$ uptake. Stiehl-Braun et al. (2011a) have demonstrated that mineral fertilizer N that accumulates under drought (because plant uptake is reduced) can inhibit methanotrophs in the top soil layers, but that methanotrophs in deeper soil layers can compensate for this loss of function (because diffusion is facilitated by low soil moisture), so that no effect manifests in soil surface $CH_4$ fluxes.

Elevated $CO_2$ concentrations have the potential to affect soil $CH_4$ transformations by various mechanisms. First, $CO_2$-enrichment is often found to increase soil moisture due to increased photosynthetic water use efficiency (Eamus, 1991; Niklaus et al., 1998). Since soil moisture is an important controller of $CH_4$ diffusion rates, $CH_4$ oxidation could be reduced by this mechanism. Second, elevated $CO_2$ can reduce mineral N availability through increased plant and microbial N uptake and through effects on microbial N transformation rates (Diaz et al., 1993; Niklaus et al., 2001; Billings et al., 2004; Barnard et al., 2005), which in turn might alter $CH_4$ oxidation. Third, plants exposed to elevated $CO_2$ can produce larger amounts of organic compounds that enter the soil via rhizodeposition and

litterfall (Rogers et al., 1994). These could fuel methanogenesis through higher substrate availability and lower redox potential caused by higher respiration rates. Some of these compounds could also directly inhibit methanotrophs, since inhibitory effects have been demonstrated for ethylene (Jackel et al., 2004), some organic acids (Wieczorek et al., 2011), and terpenes (Amaral et al., 1998).

We studied soil-atmosphere $CH_4$ fluxes in a grassland that had been exposed to elevated $CO_2$ using free-air $CO_2$ enrichment (FACE) for 14 years (Jäger et al., 2003). Fluxes were assessed with large static chambers. We further determined the spatial micro-distribution of methanotrophs that actively assimilated $CH_4$ under low and high $CH_4$ concentrations, using a novel auto-radiographic technique. These investigations addressed the following questions: (1) does elevated $CO_2$ affect soil-atmosphere $CH_4$ fluxes? (2) Does the spatial micro-distribution of active methanotrophs change under elevated $CO_2$, and can such effects be related to the observed system-level fluxes? (3) Is the spatial niche of active methanotrophs oxidizing $CH_4$ originating from the atmosphere or from soil-internal sources different?

# Materials and Methods

## Study site and experimental design

We studied elevated atmospheric $CO_2$ concentration effects on $CH_4$ uptake by methanotrophic bacteria at a study site near Giessen, Germany (50°32′ N and 8°41.3′ E at an elevation of 172 m a.s.l.). For at least the past 50 years, the site has been permanent grassland fertilized with 50-80 kg N $ha^{-1}.a^{-1}$. From 1995 onwards, fertilization was reduced to 40 kg N $ha^{-1}.a^{-1}$ (see Jäger et al. (2003) for further details).

In 1997, three circular plot pairs (FACE rings with 8 m inner diameter) were established. One plot per pair was selected randomly and atmospheric $CO_2$ enriched to 20% above ambient conditions during daylight hours since May 1998, using free-air $CO_2$ enrichment (FACE). The other plot of the pair served as ambient $CO_2$ control.

Vegetation at the site is classified as Arrhenatheretum elatioris Br.-Bl. (Weber et al., 2000) and contains about 60 vascular plant species (Jäger et al., 2003). The soil is a Fluvic Gleysol with sandy loam texture over clay. The top soil is slightly acidic (pH of 6.0) and has an organic C content of 4.6% and 3.6% in 0-5 and 5-15 cm depth (Jäger et al., 2003).

## In situ soil-atmosphere $CH_4$ fluxes

From 2009 to 2012, we measured soil-atmosphere $CH_4$ fluxes *in situ* with large static chambers (94 cm inner diameter, ca. 160L volume; modified according to Jäger et al. (2003); for further details see Kammann et al. (2001)). We collected three 25 mL headspace samples at 30 minute intervals and analyzed these by gas chromatography. $CH_4$ fluxes were estimated by linear regression of concentrations against time. We accepted all measurements with a residual standard error (RSE) of less than 15 ppb $CH_4$, plus the measurements where the ratio of RSE to calculated flux indicated that omission of any of the three points would have changed the result by less than 20%. Measurements that did not fulfill

these criteria were analyzed separately, using other methods, as is discussed in the results section.

## Soil moisture and water table depth

Soil moisture was recorded automatically at 4 locations per plot using TDR-probes (P2G, 0–15 cm depth, Imko, Ettlingen, Germany). Water table depth was recorded manually on each weekday, using three custom-built water-level gauges that were placed between pairs of ambient and elevated $CO_2$ plots.

## Soil sampling

On July 6 and October 25, 2011, we harvested two intact soil cores per plot. Cores were sampled with PVC tubes (20 cm depth $\times$ 6.5 cm internal diameter) that were driven 15 cm into the soil. In order to minimize soil compaction, the top soil had first been pre-cut along the tube's circumference with a knife. Cores were then capped at both ends to prevent water loss.

On July 6, 2011, we further collected soil at two random locations per plot. These samples were divided by five centimeter depth interval, down to a depth of 20 cm. The two replicate samples per plot were combined per depth layer and transported to the laboratory for further analysis.

## $CH_4$ oxidation of sieved soil samples

We sieved the soil samples (2 mm mesh) and determined soil moisture gravimetrically (5 g fresh soil, 105°C, 24 h). Fresh soil equivalent to 100 g dry weight per plot and depth layer was incubated at 20°C in 1 L air-tight glass jars. Headspace $CH_4$ concentration were determined after 0, 2, and 4 h and $CH_4$ uptake rates calculated by linear regression against sampling time.

## Radiolabeling of intact soil cores

The intact soil cores collected at the field site were placed in gas-tight 3 L jars (with the bottom end of the tube still capped). The jars were closed and headspace samples analyzed for $CH_4$ after 0, 2, 4 and 6 h to determine the core's net $CH_4$ uptake rates.

The jars were then ventilated and the soil cores labeled with $^{14}CH_4$. Two soil cores per plot and sampling date were labeled at slightly above-ambient $CH_4$ concentrations (max. 10 ppm). Two additional soil cores from the July 6, 2011 sampling were labeled at high $CH_4$ concentrations (ca. 10000 ppm). The rationale of this procedure was to test for differences in spatial activity distribution under these contrasting conditions. A total $^{14}C$ activity of ca. 100 kBq was applied over a period of 6 days. Plastic tubes with 100 mL 1 M NaOH were placed in each jar to trap $CO_2$ produced by microbial respiration. We regularly injected $O_2$ into the jars to maintain $O_2$ concentrations around 20%.

Then, the soil cores were freeze-dried and impregnated with epoxy resin (Laromin C 260, BASF, Ludwigshafen, Germany, mixed at a ratio of 2:3 with Araldite DY 026SP hardener, Astorit AG, Einsiedeln, Switzerland) as described in (Stiehl-Braun et al., 2011a). The resin was left curing at room temperature for 3 days, followed by an overnight incubation at 60 °C for final hardening. The soil cores were then cut twice vertically using a diamond saw, creating a section of ca. 8 mm thickness. This section was cut into three equal pieces which were glued onto 5 × 5 cm glass carriers and leveled with a diamond cup mill (Discoplan, Struers GmbH, Birmensdorf, Switzerland).

We exposed phosphor imaging plates (BAS III S, Fuji Photo Film Ltd., Tokyo, Japan) to leveled soil sections for 3 days. The imaging plates were then digitized by red-excited fluorescence scanning at a resolution of 200 µm (BAS-1000, Fujix corp., Tokyo, Japan). We corrected the scans for background exposure and recombined the three image sections to a single image of the cross-sectional area of the original soil cores, using custom Matlab scripts (Image processing toolbox, Matlab, Mathworks, Natick, MA). The sections were inspected visually, and the vertical distribution of the label calculated by averaging pixel

values by horizontal pixel line (excluding large stones).

## Statistical analysis

The unit of replication for the elevated $CO_2$ treatment is the field plot. We therefore analyzed the data using one-way ANOVA with $CO_2$ treatment as fixed effect and field plot (n=6) as replicate. We considered pairs of plots ("block" factor) and the geographical northing and easting to account for spatial variation, but these terms consumed excessive degrees of freedom given the small sample size, and did not change the results, so that we did not include them in the final model. Effects with $P \leq 0.05$ are referred to as significant, effects with $P \leq 0.1$ as marginally significant.

# Results

## In situ soil-atmosphere $CH_4$ fluxes

Our static chamber measurements revealed three characteristic patterns in which $CH_4$ concentrations evolved over the three headspace samplings (Fig. 1). During the major part of the measurements, concentrations progressed linearly with time (Fig. 1a), either decreasing from ambient to sub-ambient $CH_4$ concentrations (net soil $CH_4$ uptake), or increasing to a few hundred to thousand ppb above ambient concentrations (net soil $CH_4$ emission). However, in other cases, episodic emissions resulted in a sudden increase of concentrations between some of the headspace samplings (Fig. 1b, here shown for emission between 1st and 2nd headspace sampling). We refer to these cases as "bubble emission" since they are likely caused by ebullition from deeper soil layers or the water table. Finally, we also observed $CH_4$ concentrations that were markedly above ambient at the first sampling and decreased thereafter (Fig. 1c). We termed this pattern "redistribution" since it is likely caused by a localized "bubble emission" prior to the first sampling, followed by redistribution of $CH_4$ in the chamber and soil pore volume. There were also cases suggesting a combination of "bubble emission" and "redistribution", but these were more difficult to classify.

Meaningful emission rates can only be calculated for the linear case (Fig. 1a). In the absence of non-linear emissions, soils were net sinks for $CH_4$ (Fig. 2a, white background). Soil $CH_4$ uptake during these periods did not differ significantly between $CO_2$ treatments ($26.2 \pm 4.7$ and $28.6 \pm 5.2$ µmol $m^{-2}d^{-1}$ in ambient and elevated $CO_2$, respectively). During periods in which "bubble emissions" occurred (Fig. 2a, gray background), average rates determined from the remaining chambers showing linear emissions were generally positive, i.e. indicated net soil $CH_4$ emissions. These emissions likely are lower bounds of the real fluxes because they do not include the supposedly higher emission rates when "bubbles" are formed.

Soil $CH_4$ fluxes (excl. periods with "bubble" emissions) were correlated to soil moisture and water table depth, which explained 37% and 57% of the temporal variation in soil-atmosphere $CH_4$ exchange ($P<0.001$, two extreme flux values excluded, sampling day as replicate, Fig. 2b,c). Soil moisture and water table depth were highly correlated ($r=0.74$). $CH_4$ fluxes did not significantly depend on daily precipitation.

Bubble emissions occurred in 14.3 (average of 6 plots) out of 168 samplings, with no significant difference between $CO_2$ treatments ($P=0.9$, generalized linear model with binomial distribution). Virtually identical results were obtained when the number of static chambers per plot showing such emissions (0 to 3 per plot) was considered instead of simply discriminating between occurrence and absence on a plot basis.

## $CH_4$ uptake of incubated soil samples

The sieved 5-cm soil layers did not reveal any effect of $CO_2$ enrichment when incubated at 20 °C and field moisture (Fig. 3). Intact soil cores incubated in the laboratory at 20 °C also did not show any effect of elevated $CO_2$ on net $CH_4$ uptake (Fig. 4, volumetric soil moisture content of 23% and 46% on July 6 and October 25, respectively).

## $^{14}CH_4$ labeling of soil cores

Visual inspection of autoradiographies revealed heterogeneous label assimilation, with distinct zones of enhanced net $CH_4$ assimilation (Figs. 5,6,7). These appeared to be along cracks and around aggregate structures (e.g. Fig. 6). On both July 6 and October 25, net $^{14}CH_4$ assimilation was reduced in the top 1-2 centimeters relative to the rest of the soil profile which showed relatively little variation in label intensity with depth.

$CO_2$ enrichment did not affect the vertical distribution of the label except for an interaction with depth ($P<0.05$) that originated from lower labeling of the uppermost layer on October 25 when labeled at high $CH_4$ concentration. Since the analysis of depth ×

$CO_2$ treatment includes some degree of autocorrelation between soil layers, we calculated mean oxidation depth per soil core as

$$\int_y y \cdot a\,(d)\,dy / \int_y a\,(d)\,dy,$$

i.e. activity-weighted mean depth of net $CH_4$ assimilation. Mean assimilation depth averaged 3.8 cm, irrespective of $CO_2$ treatment and labeling concentration (Table 1). There was a marginally significant shift of 0.5 cm towards the soil surface in October relative to July 2011 (P=0.06).

# Discussion

In the grassland investigated, soil-atmosphere $CH_4$ fluxes were characterized by alternating phases of soil net $CH_4$ uptake and emission. On an annual basis, the studied ecosystem was a net source of $CH_4$, with emissions peaking during the summer months and oxidation prevailing during most of the remaining time. However, the annual $CH_4$ balance is difficult to constrain due to the "burst" character of emissions which is not amenable to the static chamber technique we adopted. We did not detect any effects of elevated $CO_2$ on fluxes or micro-distribution of $CH_4$ assimilation, but this also may be related to the relatively low power originating from the low replication typical of FACE studies.

Evidence regarding effects of elevated $CO_2$ on $CH_4$ fluxes is equivocal. In a study in Loblolly pine plantation (McLain and Ahmann, 2008; Dubbs and Whalen, 2010) reductions in soil $CH_4$ sink were found under $CO_2$ enrichment, which were related to increased soil moisture due to reduced stomatal conductance and increased water use efficiency (Morgan et al., 2004). The authors argued that this effect on $CH_4$ uptake originated from diffusive $CH_4$ transport limitation in the top soil but possibly also from increased anoxia in deeper soil layers due to higher plant and heterotrophic soil microbial activity, which could promote methanogenesis. Similar effects were found in trembling aspen stands (Ambus and Robertson, 1999). Interestingly, in semi-arid grassland, opposite effects of elevated $CO_2$ were found when soils were dry (Dijkstra et al., 2011); the authors attributed these effects to a reduction of drought stress due to moister soils under elevated $CO_2$. This conclusion was supported by soil $CH_4$ uptake rates decreasing when soil moisture was above or below some intermediate optimum. However, Ineson et al. (1998) found reduced $CH_4$ uptake under elevated $CO_2$ in a mixed *Lolium*/*Trifolium* sward, and this effect was unrelated to soil moisture. Finally, $CH_4$ uptake and $CO_2$ concentration were unrelated in a number of other studies (wheat: Lam et al. (2011), Sorghum and soybean: Smith et al. (2010); shortgrass steppe: Mosier et al. (2002)). We observed a median net soil

CH4 uptake of 23 µmol m-2 d-1 during periods without emissions. These soil uptake rates are in the upper range of the ones reported in these elevated CO2 studies, but not atypical when compared to temperate grassland fluxes reported in an European (Smith et al., 2000) or global analysis (Dutaur and Verchot, 2007). Elevated $CO_2$ did not induce significant changes in soil moisture in our study during the time studied, and it is well possible that $CH_4$ fluxes remained unaltered for this reason.

The different character of $CH_4$ sources and sinks that contribute to the net balance of the present grassland makes it very difficult to constrain the true annual $CH_4$ balance of this ecosystem, for several reasons. First, sink rates due to methanotrophic activity are generally smaller than emissions rates from methanogenesis (Le Mer and Roger, 2001). Second, while sinks are largely controlled by diffusion and continuous in time, emissions tend to be episodic because they are often mediated by ebullition, which is –on a short time scale– a discontinuous process (Shrestha et al., 2012). In the grassland investigated, the water table was relatively close to the soil surface, and it is well conceivable that the emission bursts occurred from $CH_4$ bubbles originating from the saturated zone. A substantial fraction of these bubbles likely traveled relatively quickly to the soil surface via preferential diffusion paths, so that this flux was not buffered. Third, the static chambers trapped localized emissions, resulting in an apparent uptake kinetic due to the re-distribution of $CH_4$ in the surrounding soil and possibly also an associated increase in oxidation due to the elevated $CH_4$ concentrations. This phenomenon is artificial and would not occur without the chamber. Finally, it is well possible that chamber handling and soil disturbance from human weight triggered the release of bubbles that would otherwise have occurred later (although the static chambers were placed carefully on the pre-installed base rings, and the weight of the person handling the chambers was distributed by a walking grid). Temporary soil compression could also have pushed high-methane air out of parts of the soil pore network where it would have stayed longer otherwise. Indeed, an indication of disturbance-triggered "burst" $CH_4$ release could be that the step-increase in

concentrations associated with bubble emission often occurred before or just after the first headspace sampling, but rarely after the second sampling. Generally, handling-induced $CH_4$ release appears especially critical, since pressure variation can flush near-surface pore volumes ($CH_4$ fluxes: Czepiel et al. (2003); $CO_2$ fluxes: Davidson et al. (2002)), disturbing diffusion gradients that take long to re-equilibrate. Overall, we thus conclude that it probably is not possible to accurately assess the true $CH_4$ balance using static chambers in such a system, at least for periods in which net $CH_4$ emissions occur. One strategy may be to analyze different processes or different parts of the season independently, using different techniques (e.g. assess continuous fluxes with standard techniques and separately count the occurrence of "burst"-type events).

$CH_4$ fluxes exhibited marked seasonal dynamics, with emissions peaking in summer and early fall. While water table depth, soil moisture, and heavy precipitation are likely drivers of these $CH_4$ emissions due to their effect on oxygen supply, other factors also may have been at play. High plant activity during peak season could have supplied heterotrophic soil organisms with organic substrate, which would have lowered oxygen partial pressures when consumed- soil $CH_4$ oxidation, however, is generally rather limited by $CH_4$ concentrations unless $O_2$ is nearly depleted, so that seasonal dynamics are unlikely to have been affected by this mechanism. Some organic compounds can also inhibit $CH_4$ oxidation directly (Amaral et al., 1998; Wieczorek et al., 2011). Methanogenesis also is strongly temperature-dependent, and it may be that – depending on the zone in which methanogenesis occurred – sufficiently high temperatures were only reached in late summer. Finally, large numbers of Scarabidae larvae are active at the site studied, and incubation of soil cores taken from the site have previously shown that these larvae can release large amounts of $CH_4$ (Kammann et al., 2009), a phenomenon that has not received much attention to date for temperate ecosystems.

The nature of methanotrophs capable of growing at atmospheric or sub-atmospheric $CH_4$ concentrations remains enigmatic, despite many years of research. Early studies

have suggested that methanotrophs predominantly consuming $CH_4$ at low or high concentrations differ in nature (Bender and Conrad, 1992), but it has also been argued that these organisms may be less distinct than previously thought (Dunfield et al., 1999). Indeed, methanotrophs capable to adapt physiologically to environments differing in $CH_4$ supply have been found (Dunfield, 2007), and some possess of isoenzymes differing in kinetic properties (Baani and Liesack, 2008). Methanotrophs are alternatingly exposed to low and high $CH_4$ concentrations in the studied grassland, depending on whether the atmospheric or soil-internal sources dominate. Our labeling experiments suggest that the methanotrophs actively consuming $CH_4$ under these contrasting conditions occupy the same spatial niche. Typically, high $CH_4$ concentrations would be supplied from the bottom of the soil column, but our experiments showed that assimilation was nevertheless possible throughout the soil profile, so that this likely did not bias our results. The most abundant $CH_4$ oxidizer at our site is a *Methylocystis* strain closely related to a cultured type (LR1) capable of displaying high-affinity kinetics when starved (Horz et al., 2002). In this light, it appears well possible that the radiolabel assimilation we observed not only occurred at the same spatial location but that it also was driven by the same type of organisms.

The autoradiographic technique we have developed has not been applied to many sites so far. The patterns we observed, however, were similar to the ones found in the Rothamsted "Park Grass" experiment (Stiehl-Braun et al., 2011b) and in two drought studies (Stiehl-Braun et al., 2011a). Labeled $CH_4$ assimilation concentrated in the periphery of soil features such as aggregates, probably reflecting the ease of diffusive transport to these sites. In October, when soils were wetter, $CH_4$ assimilating zones were more concentrated towards the soil surface, and in a smaller part of the pore network (probably macro-pores).

In conclusion, no effects of elevated $CO_2$ on net $CH_4$ fluxes and the spatial microdistribution of methanotrophic bacteria were found in the present study. Net $CH_4$ fluxes were the result of $CH_4$ oxidation and production, with the latter dominating. There are

also indications that emissions are mediated by the activity of ground-dwelling arthropods (Kammann et al., 2009) and possibly fungi (Lenhart et al., 2012), but the mechanisms involved remain unclear. The range of sources and sinks involved, together with their different dynamic and ecological characteristics, indicate the challenges in estimating a system-level $CH_4$ balance and highlight the need to develop a framework in which these fluxes can be constrained; this might include analyzing periods with uptake and emissions separately, constraining these parts of the balance separately.

## Acknowledgements

This work was funded by Aga Khan Foundation (AKF) and the University of Zurich. We gratefully acknowledge Gerald Moser for assistance in soil sampling, Ludger Grunhage for long-term data management, and the Giessen FACE staff for long-term site management. The continued financial support of the Hessian Agency for the Environment and Geology (HLUG) is gratefully acknowledged.

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# Tables

Table 1: Methane oxidation depth. Activity-weighted depth of labelling (mean ± s.e.) in soil cores from ambient and elevated $CO_2$ plots, incubated under low and high $CH_4$ concentrations. Effects of elevated $CO_2$ were not statistically significant.

| **Date** | **$CH_4$ concentration (ppm)** | **$CO_2$ treatment** | **Oxidation depth (cm)** |
|---|---|---|---|
| 6 July 2011 | 10 | Ambient $CO_2$ | $3.88 \pm 0.07$ |
| 6 July 2011 | 10 | Elevated $CO_2$ | $4.00 \pm 0.06$ |
| 25 October 2011 | 10000 | Ambient $CO_2$ | $3.81 \pm 0.08$ |
| 25 October 2011 | 10000 | Elevated $CO_2$ | $4.24 \pm 0.42$ |
| 25 October 2011 | 10 | Ambient $CO_2$ | $3.40 \pm 0.19$ |
| 25 October 2011 | 10 | Elevated $CO_2$ | $3.45 \pm 0.17$ |

# Figure legends

Fig. 1. Typical time-courses of $CH_4$ concentrations during static chamber sampling. (a) Linear concentration changes with time, indicating continuous soil $CH_4$ uptake or release. (b) Step-increase in $CH_4$ concentration, likely caused by emission bursts that could originate from ebullition from the underlying saturated zone. (c) Decrease in $CH_4$ concentrations, starting at substantially above-ambient $CH_4$ concentrations; this pattern is likely caused by a re-distribution of localized $CH_4$ emissions trapped in the static chamber.

Fig. 2. $CH_4$ fluxes and related environmental data. (a) $CH_4$ emission rates in ambient (○) and elevated $CO_2$ (●) plots, calculated when concentration changes were linear (Fig. 1a, all other data excluded). Periods during which emissions occurred (Fig. 1b,c) are shaded in gray, indicating that emission rates likely are underestimates. (b) Volumetric soil moisture, averaged across $CO_2$ treatments. (c) Weekly precipitation and water table depth.

Fig. 3. Potential $CH_4$ oxidation activity. Net $CH_4$ uptake rates of sieved field-moist soil incubated at 20°C in the laboratory (mean ± s.e., by 5 cm soil layer).

Fig. 4. Net $CH_4$ uptake rates of intact soil cores. These cores have been collected in ambient and elevated $CO_2$ plots and were incubated in the laboratory at 20°C.

Fig. 5. Soil micro-autoradiography of typical soil sections collected on June 6, 2011, and incubated under near-ambient $CH_4$ concentrations. Darker pixels indicate higher labeling. Vertical profiles of labeling (right panel), aggregated by 1 cm depth intervals.

Fig. 6. Soil micro-autoradiography of typical soil sections collected on October 25, 2011, and incubated under near-ambient $CH_4$ concentrations. Darker pixels indicate higher labeling. Vertical profiles of labeling (right panel), aggregated by 1 cm depth intervals.

Fig. 7. Soil micro-autoradiography of typical soil sections collected on October 25, 2011, and incubated under $CH_4$ concentrations around 10000 ppm. Darker pixels indicate higher labeling. Vertical profiles of labeling (right panel), aggregated by 1 cm depth intervals.

# Figures

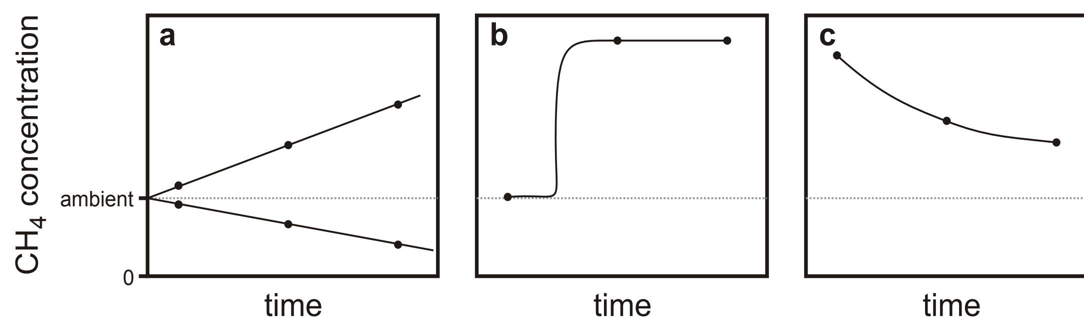


Figure 1

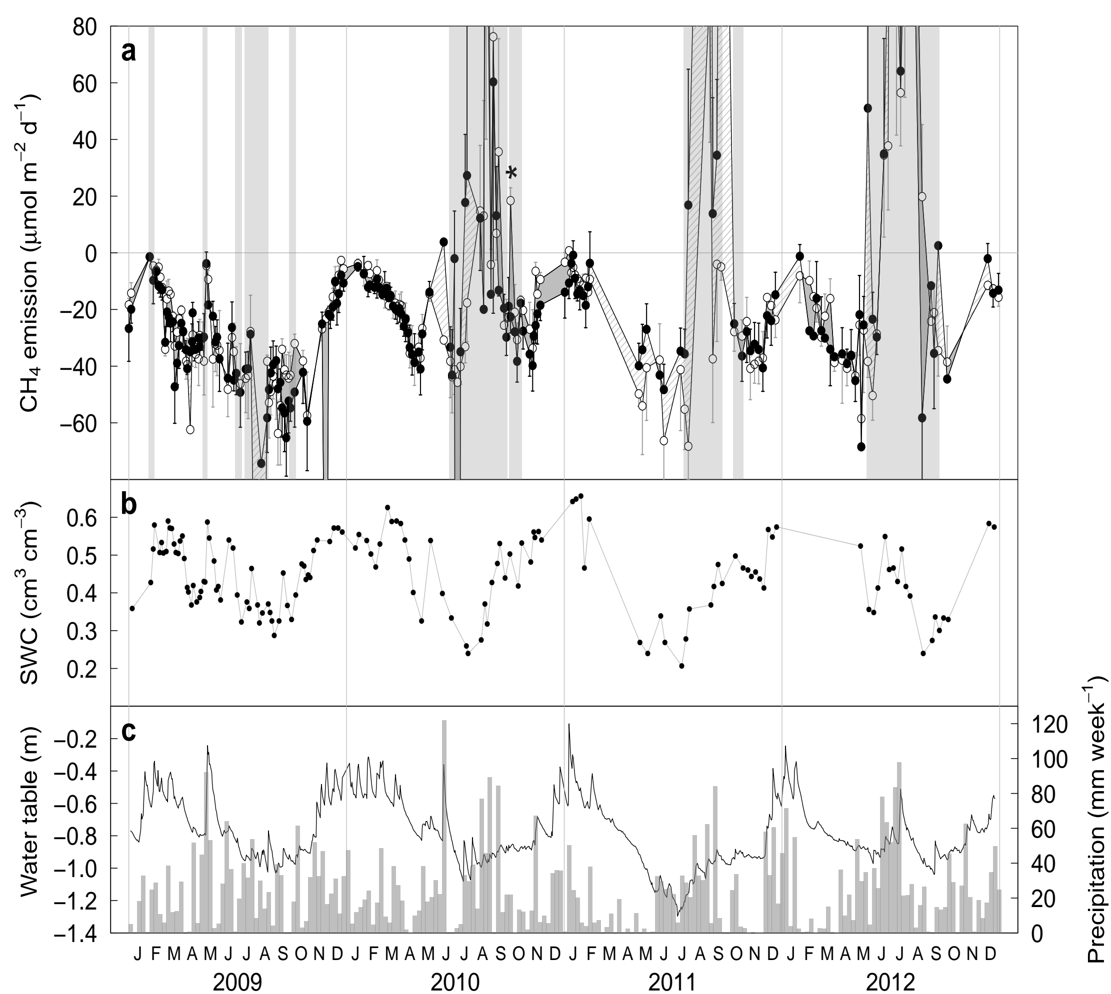


Figure 2

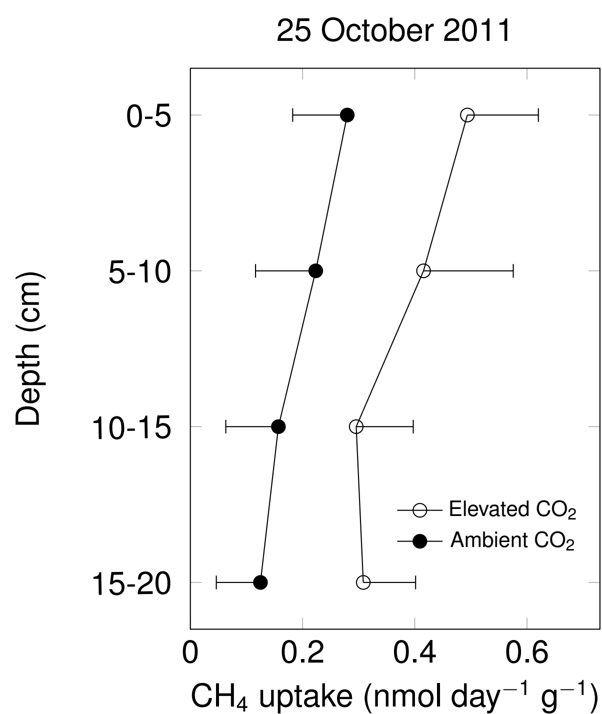


Figure 3

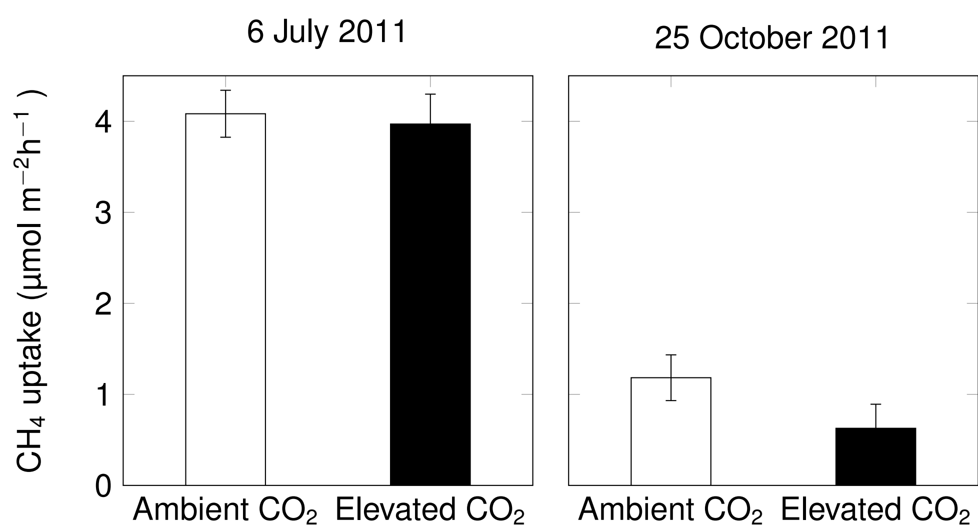


Figure 4

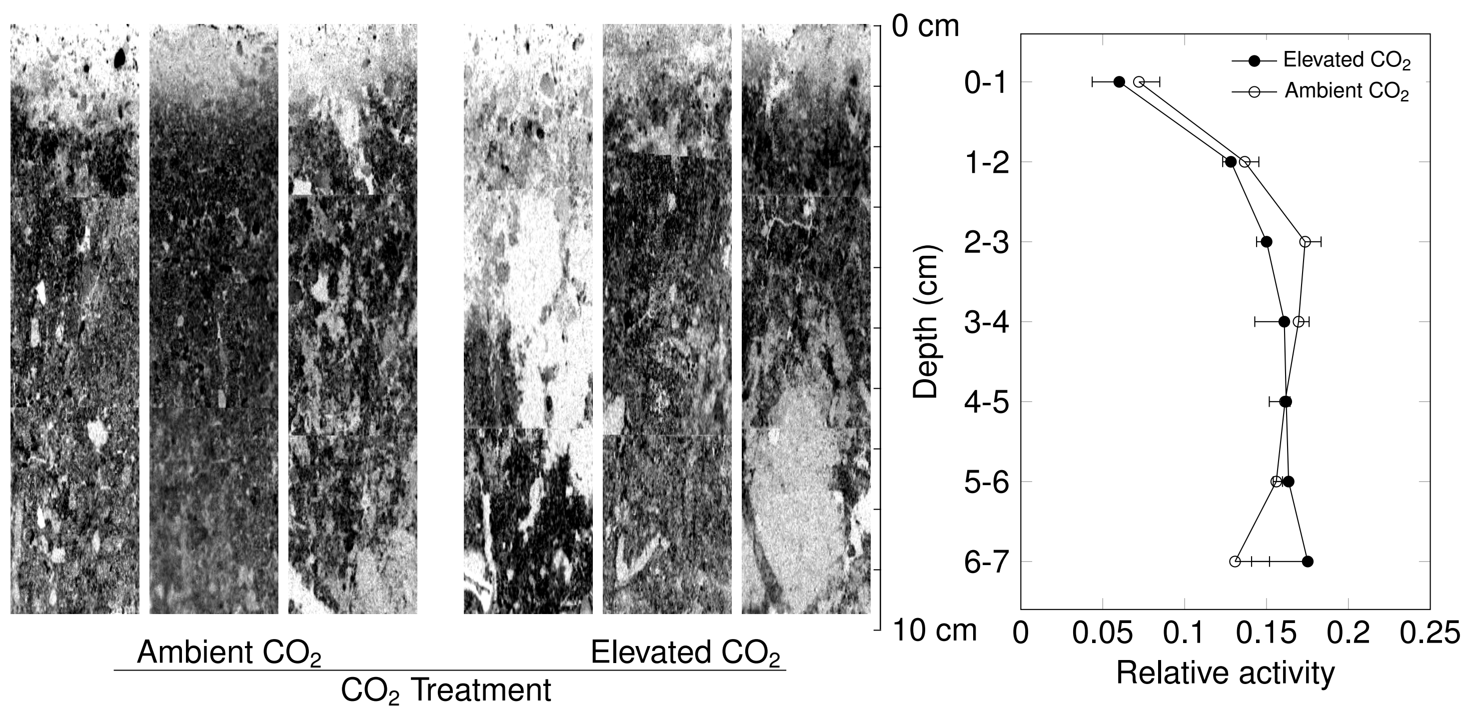


Figure 5

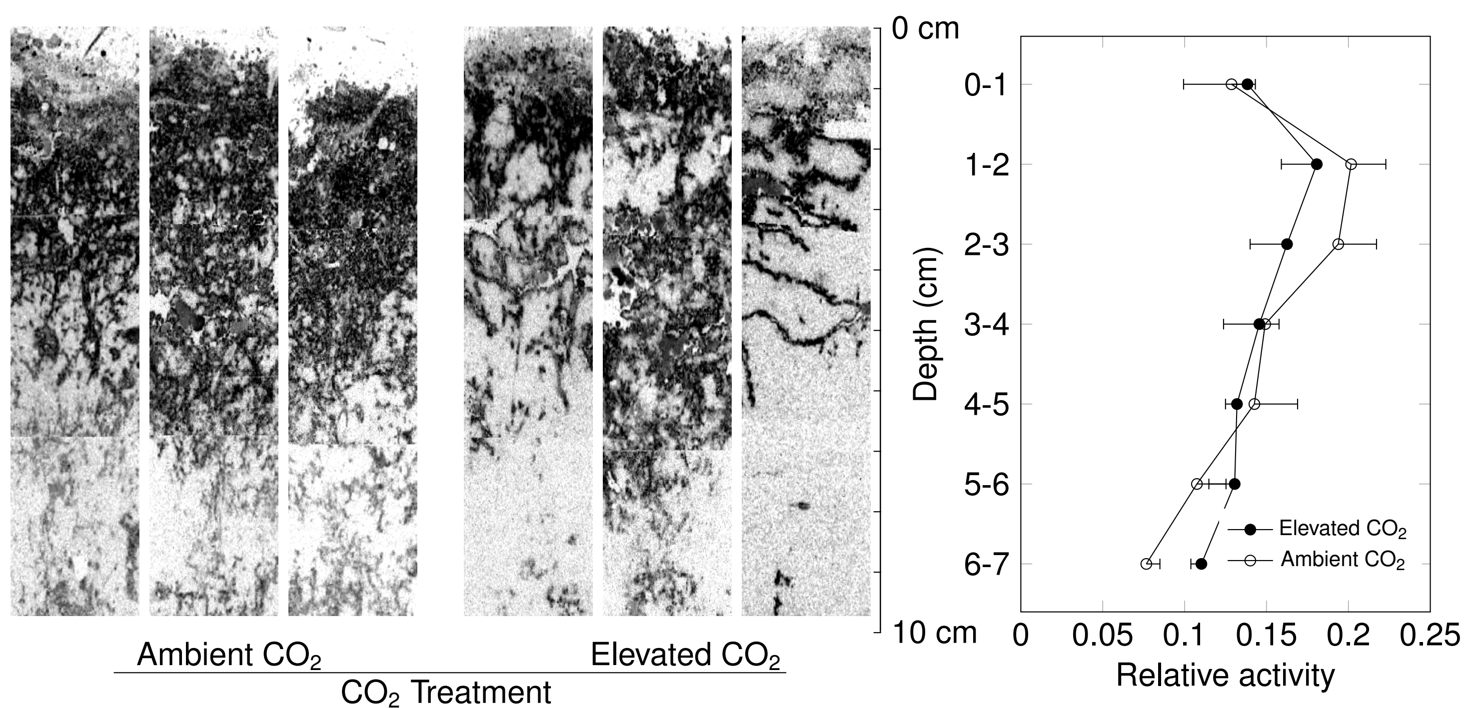


Figure 6

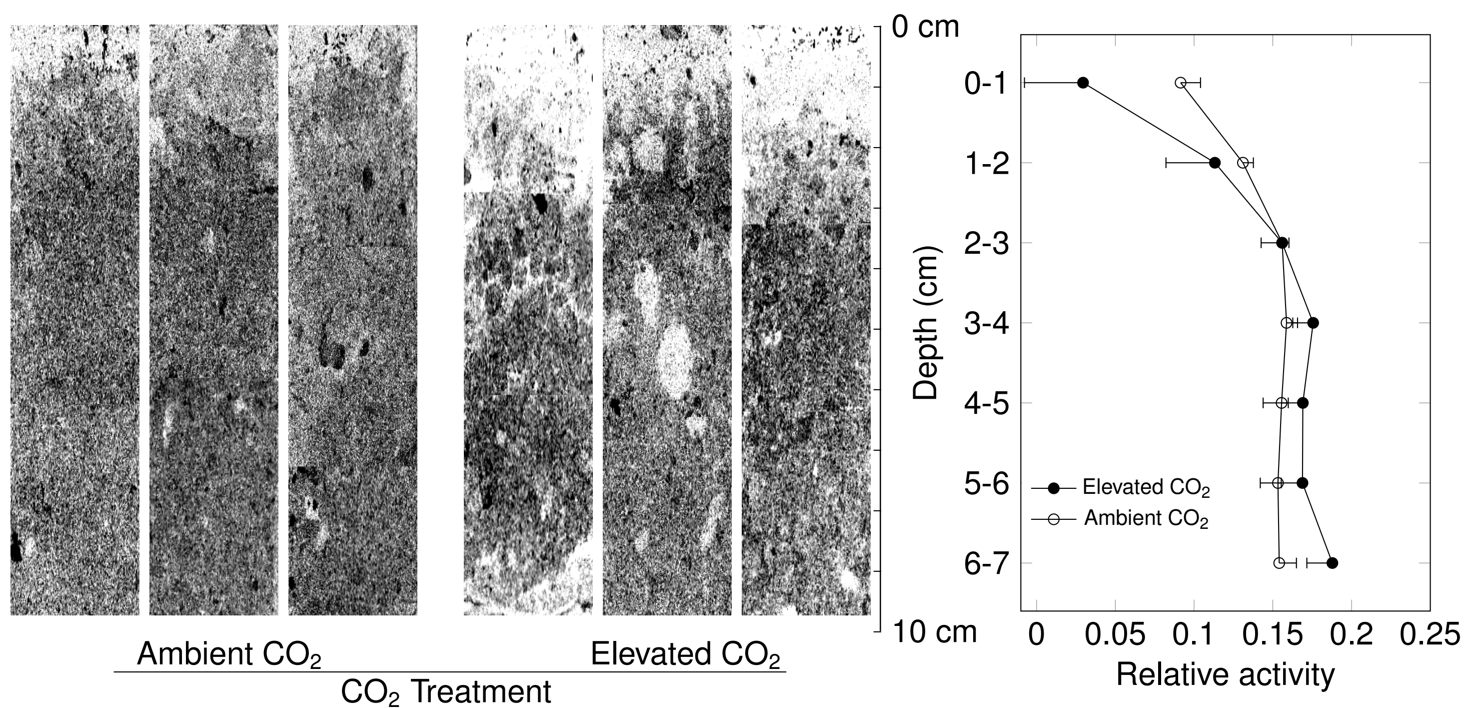


Figure 7

# Chapter 3:

## Spatial micro-distribution of methanotrophic activity along a 120-year afforestation chronosequence

**Saeed Karbin**, Frank Hagedorn, David Hiltbrunner, Stefan Zimmermann and Pascal A. Niklaus

# Summary

Methane ($CH_4$) uptake by methanotrophic bacteria in upland soil can be affected by land-use changes. Afforestation can increase $CH_4$ uptake in soils but the mechanism underlying this change is currently not well understood. Here, we studied soil methane uptake in an afforestation chronosequence in subalpine Norway spruce stands that developed on a former pasture. We have previously reported that soil $CH_4$ uptake increased with stand age. Here, we studied the spatial distribution of soil methanotrophic bacteria in relation to aggregate structure. This was achieved by labeling undisturbed soil cores with $^{14}CH_4$. Then, the spatial micro-distribution of the active methanotrophs was analyzed in intact soil sections using an autoradiographic technique. We further physically fractionated soil and tested for effects of forest stand age on aggregate size distribution and association of active methanotrophs with these size fractions. These aggregates were then subjected to an erosion treatment separating material located at the periphery from material located more towards the center of aggregates. Methanotrophic activity shifted down the soil profile as forest stands developed, which was related to the development of an organic top soil layer. This downward shift had no effect on ecosystem-level fluxes, most likely due to the low diffusive resistance of the top soil. Net $^{14}CH_4$ assimilation was largest in aggregates 2-4 mm in diameter, and was concentrated on soil aggregate surfaces regardless of their size class. Older forest stands showed decreased numbers of large aggregates. Overall, our analyzes suggest that the increased soil uptake in older forest stands was mostly related to lower soil moisture due to increased interception by taller canopies and possibly also due to a larger share of aggregates showing a higher than average methanotrophic activity.


**Keywords:** methanotrophic bacteria, afforestation, $^{14}$Clabeling, land-use changes, spatial distribution

# Introduction

Terrestrial soils contribute to both $CH_4$ sources and sinks that ultimately affect the atmospheric $CH_4$ inventory (IPCC, 2013). Methanogenic archaea produce $CH_4$ under anoxic conditions as they are often found in water-logged soils. In contrast, methanotrophic bacteria oxidize methane in oxic soil domains, with $CH_4$ originating either from soil-internal or atmospheric sources. In the course of this process, $CH_4$-carbon is converted to organic forms or $CO_2$. The latter is environmentally beneficial because $CH_4$ has a much larger greenhouse warming potential than $CO_2$.

$CH_4$ uptake by upland soils significantly depends on land-use. Forests generally oxidize larger amounts of atmospheric $CH_4$ than grassland (Boeckx et al., 1997; Tate et al., 2007), and $CH_4$ uptake by arable land often is even lower (Smith et al., 2000). As a general rule, conversion of forest to agricultural land reduces the soil $CH_4$ sink, whereas reverting arable or pasture systems back to forest increases soil $CH_4$ uptake.

Several mechanisms have been proposed to explain the dependence of the soil $CH_4$ sink on land use. First, the decline in $CH_4$ uptake in agricultural land may be related to the application of mineral nitrogen fertilizers. In particular ammonium-based fertilizer have been shown to decrease soil $CH_4$ uptake (Hütsch et al., 1993), possibly by direct inhibition of $CH_4$ assimilation at the enzymatic level (Dalton, 1977; Carlsen et al., 1991). While this mechanism has been demonstrated under laboratory settings (Bedard and Knowles, 1989), it is less clear whether it also is responsible for such effects under field conditions (e.g. Bodelier and Laanbroek, 2004). Second, soil $CH_4$ sinks may also decrease in response to soil structural disturbance (Ball et al., 1999; Abichou et al., 2011). Soil aggregates are a key feature of soil structure and functioning, affecting water, air, heat and nutrient availability, the size and numbers of pores, and therefore also water movement and gas diffusive transport (Kasper et al., 2009). Boeckx and Van Cleemput (2001) reported that $CH_4$ oxidation occurred in certain soil niches and that these may be changed by

alterations of the original soil structure. In general, macro-aggregates are more sensitive to soil disturbance than micro-aggregates. Often, $CH_4$ uptake declines when aggregation is lost (Abichou et al., 2011), suggesting that the ecological niches of methanotrophic bacteria are somewhat more dependent on macro-aggregates. Soil structural changes can affect methanotrophs by several mechanisms. First, soil aggregate size distribution can regulate $CH_4$ availability in the soil atmosphere and thereby affect methanotrophic bacteria. Given the comparably low concentrations of $CH_4$, $O_2$ concentrations, even if reduced, are rarely limiting. When soil structure is disturbed, e.g. by mechanical soil treatment, the number of macro-pores decreases and methane transport may be hindered. At the same time, water filled pore space may increase, which would further limit $CH_4$ transport rates. Indeed, a number of studies demonstrated changes in soil $CH_4$ fluxes under soil disturbance. Ball et al. (1999) found a reduction of soil $CH_4$ uptake in croplands that were ploughed compared to no-till management. At least in the long term, soil structural changes also have been shown to affect methanotrophic community composition (Singh et al., 2007; McNamara et al., 2008; Kumaresan et al., 2011).

When a grassland or cropland is abandoned and reverts to forest, the soil $CH_4$ sink often takes very long to recover, with $CH_4$ uptake rates often continuing to increased decades or even a century after land use change (Priemé et al., 1997; Smith et al., 2000). The mechanism involved are not fully clear but may be related to the low growth rates of methanotrophs thriving on atmospheric $CH_4$. Second, soil structure may take many years to change (King, 1997; Hütsch, 1998; Smith et al., 2000). Soil organic matter generally increases when a pasture is converted to forest, and this often is paralleled by an increased fraction of macro-aggregates (Wang et al., 2013; Wei et al., 2013).

Investigating the ecological mechanisms that drive $CH_4$ uptake during forest establishment on former grassland or agricultural land is challenging, in part because most methanotrophs thriving in upland soils cannot be isolated and cultivated. Second, and related to that, the spatial niche of methanotrophs in relation to soil structure is poorly

known. We have studied soil-atmosphere $CH_4$ flux rates in an afforestation chronosequence in which Norway spruce (*Picea abies* L.) established on a former subalpine pasture (Hiltbrunner et al., 2012). Field-measured soil $CH_4$ uptake increased with stand age, and we speculated that these fluxes were driven by soil moisture changes. Here, we present further investigations in which we analyzed soil structural changes and determined the spatial micro-distribution of soil $CH_4$ uptake. This was achieved by $^{14}CH_4$ labeling of soil cores, followed by autoradiographic analysis of intact soil sections. We further physically fractionated these soil cores and eroded aggregates to determine the spatial niche of the active methanotrophs in relation to aggregate structure. We hypothesized that larger soil carbon inputs in older forest stands would alter soil structure by increasing aggregation, which could possibly also contribute to the increasing soil $CH_4$ sink.

# Materials and Methods

## Site description

We studied the spatial distribution of methanotrophic activity along a sub-alpine afforestation chronosequence in Switzerland (Jaun pass area, Canton of Fribourg, 46°37'17 N; 7°15'54 E), on a south-facing slope extending from 1450 m a.s.l to 1700 m a.s.l. that has been used as pasture for at least the past 150 years. After severe avalanches in 1956, an area of about 15 ha on the eastern part of the slope was gradually afforested with Norway spruce (*Picea abies* L.), while the western part remained as a pasture. Separate patches of forest were planted in different years, resulting in stands 25, 30, 40, 45, and ≥120 years old (see Hiltbrunner et al. (2012) for more details).

Mean summer and winter temperature at the pasture site are 11.4 °C and 0.6 °C receptively (Hiltbrunner et al., 2012). Mean annual precipitation averages is1250 mm with a maximum in summer. Soils are Cambisols on calcareous bedrock.

## Soil sampling and $^{14}CH_4$ labeling

In September 2011 and again in September 2012, intact soil cores (20 cm depth × 6.5 cm internal diameter) were sampled in PVC tubes. In order to minimize soil compaction, we pre-cut the top soil along the tube's circumference with a knife before it was driven 15 cm into the ground. Then, the tubes were excavated, capped at both ends to prevent water loss, and transported upright to the laboratory.

We then incubated the soil cores (still capped at the bottom end) at in air-tight 3 L jars. Three headspace samples were collected at hourly intervals to determine the sample's net $CH_4$ uptake (Agilent 7890N gas chromatograph equipped with a flame ionisation detector, Wilmington, Delaware, USA). Soil-atmosphere flux rates were determined by linear regression of headspace concentrations vs. sampling time.

Then, the jars were ventilated for about 15 min before they were radio-labeled by

injecting a total of ≈100 kBq $^{14}CH_4$ per jar over a period of 7 d Headspace $CH_4$ concentrations were kept in the range of 5–8 µL$CH_4$ $L^{-1}$ by re-supplying the soil cores with $^{14}CH_4$ and, towards the end of the incubation, unlabeled $CH_4$. The incubation jars also contained plastic tubes with 100 mL 1.5 M NaOH to prevent secondary fixation of $^{14}CO_2$ released during the incubation. O2 was regularly injected into the jars to maintain aerobic conditions (15–20 %$O_2$).

## Autoradiographic imaging of intact soil cores

Two labeled soil cores per sampling date and plot were freeze-dried and impregnated with epoxy resin (Laromin C 260, BASF, Ludwigshafen, Germany, mixed at a ratio of 2:3 with Araldite DY 026SP hardener, Astorit AG, Einsiedeln, Switzerland). Resin penetration was improved by evacuating the soil cores with resin to an end pressure of 25 kPa, and slowly bringing the core back to atmospheric pressure slowly. After about 2–3 d of curing at room temperature, the resin was fully hardened overnight at 60°C. Then, the soil cores was cut twice length-wise and divided in three sections that were each mounted on 5×5 cm glass slides. The section's surfaces were leveled with a diamond cup mill (Discoplan, Struers GmbH, Birmensdorf, Switzerland) and used to expose phosphor imaging plates (BAS III S, Fuji Photo Film Ltd., Tokyo, Japan) for 3 d The imaging plates were scanned by red-excited blue fluorescence at a resolution of 200 µm and the three slides representing a soil core recomposed. The label distribution was explored visually and the vertical distribution of $^{14}C$ determined by averaging pixel intensities per horizontal pixel line. Areas with large stones were excluded for this procedure.

## Autoradiographic imaging of isolated soil aggregates

We isolated soil aggregates 2 to 12 mm in size by manually pulling apart a labeled soil core per plot (September 28, 2011 sampling). These aggregates were placed in petri

dishes and impregnated with epoxy resin as described previously for intact soil cores. Autoradiographies were obtained similarly.

## Aggregate fractionation

We eroded labeled soil aggregates mechanically to separate exterior and interior fractions using the method described by Wilcke et al. (1999) with some modification. First, labeled soil cores were divided into (0–6, 6–12 and 12–18 cm) depth segments. Then, fractionated soil were separated into size fractions ($\geq$16, 16-8, 8-4, 2-4, and $\leq$ 2 mm) by sieving through a stack of sieves, exercising only minimal mechanical force. Each size fraction was weighed and water content determined gravimetrically (5 g subsample, 105°C). Approximately 300 g of soil aggregates were then frozen by immersion in liquid nitrogen before they were eroded by shaking them on a sieve that was repeatedly immersed in distilled water. To erode approximately equal amounts of soil in each aggregate size fraction, the sieve was shaken 75, 60, 50 and 50 times for the $\geq$16, 16–8, 8–4 and 2–4 mm fraction, respectively. In the course of this procedure, aggregates dispersed from the exterior to the interior, with the eroded fractions collected in a water bowl. We placed turbid water in a long glass tube to separate water from solid material. To increase colloid sediment we added a flocculation agent (2 mL of 1M $MgSO_4$) and pipetted water above the settled clay layer after 24 hours. All soil fractions were dried at 105°C. A subsample was ground in a mortar, oxidized (200 mg material, A307 sample oxidizer, Perklin Elmer, Waltham, MA), and $^{14}C$ content determined by liquid scintillation counting (TRI-2900TR, Perklin Elmer, Waltham,MA, USA). Soluble $^{14}C$ remaining in the supernatant of the solution used for aggregate erosion was determined similarly (Ultima Gold scintillation cocktail, Perklin Elmer).

## Statistical analysis

Data were analysed by fitting linear models reflecting the design of the study. We fitted effects of land use type (forest vs. grassland), forest stand age (log-transformed), and altitude. Since altitude and stand age were not orthogonal (Hiltbrunner et al., 2012), we fitted models correcting for altitude-effects before and after stand age to estimate the degree of confounding. When several measures per plot were analysed (e.g. repeated measures or analyses including several soil layers), linear mixed effects models were fitted (ASReml, VSN International, Hempel-Hempstead, UK) which included the additional random effects plot, and, where appropriate, plot $\times$ year. These terms are necessary to ensure proper replication of significance tests. For the analysis of activity by soil layer, data were averaged by 1 cm soil layers and a first order autoregressive spatial correlation structure between soil layers included in the analysis to account for non-independence of residuals from the same soil core and plot.

# Results

## $CH_4$ uptake by soil cores

Net $CH_4$ uptake of soil cores did not differ significantly between grassland and forest, but decreased with forest stand age (Fig. 1, linear model correcting for altitude; $P=0.06$ for joint analysis of both years; $P=0.03$ when 2012 data analyzed separately).

Volumetric soil moisture was significantly higher in pasture than forest (Fig. 2, $P<0.01$, joint analysis for both years), but no effect of forest age was detected on a volumetric basis.

## Soil aggregate fractions

The soils investigated were well-aggregated, with 8% of the soil material recovered in the ≤2 mm fraction, 18% in both the 2–4 and 4–8 mm fraction, 36% in the 8–16 mm fraction, and 21% in the ≥16 mm fraction (Fig. 3). The size of the largest fraction was sensitive to soil handling during the sieving process and more variable; the statistical analysis were therefore restricted to the small fractions. Average aggregate size increased with depth, both in grassland and forest ($P<0.001$ for size × depth), and decreased with forest stand age ($P=0.02$ for size × age), and more so in deeper soil layers ($P<0.01$ for size × age × depth).

The concentrations of $^{14}C$ were generally higher in small than in large aggregates in sites of the young soils and in deeper soil layers; exceptions were the top soils (0–6 cm) of the two oldest forest sites (45 and 120 years) where this relationship reversed. The eroded exterior of the aggregates contained approximately twice as much $^{14}C$ per unit mass than the stable interior (Fig. 4).

## Distribution of $^{14}C$ in soil sections

Net assimilation of $^{14}C$ in soil was heterogeneous (Figs. 5 and 6), with increased activities on the surface of aggregate structures. While aggregate surfaces are difficult to identify in intact sections, this finding was confirmed by the autoradiographies of isolated aggregates (Fig. 7). The depth-profiles differed between grassland and forest (Fig. 8, $P<0.001$), with mean oxidation depths about 1 cm closer to the soil surface in grassland ($P<0.01$). Withing the forest plots, oxidation activity in the layers near the soil decreased with stand age ($P<0.05$ for layer $\times$ age), and mean oxidation depth therefore increased with age ($P<0.05$).

# Discussion

In a previous study, we have measured soil-atmosphere fluxes of $CH_4$ along the afforestation chronosequence investigated here (Hiltbrunner et al., 2012) and found that soil $CH_4$ uptake increased with forest stand age and that this effect correlated positively with rainfall interception by forest canopies and soil moisture. However, soil $CH_4$ fluxes are also affected by a multitude of other drivers (Hütsch, 1998; Shukla et al., 2013). Here, we studied the micro-scale distribution of methanotrophic activity and found that activity was heterogeneously distributed in soils, with higher activities concentrated at the periphery of soil aggregates. Furthermore, the active $CH_4$-assimilating zone shifted downwards the soil profile when stand age increased, i.e. when forest soils developed.

Soil-atmosphere fluxes of trace gases are difficult to measure under laboratory conditions, and often deviate from in-situ assessments (Nedwell et al., 2003; Abichou et al., 2011; Hiltbrunner et al., 2012). Reasons include altered environmental conditions (e.g. temperature profiles and gas transport), effects of disturbance (e.g. compaction, removal of live plant roots), and in the case of soil cores also edge effects (e.g. facilitated diffusion along the cores edges). Our laboratory incubations were therefore not intended as substitute for field measurements; nevertheless, they roughly reflected the patterns found previously (Hiltbrunner et al., 2012), suggesting that the previously published effects on fluxes remained stable.

Methanotrophic activity shifted downwards with forest soil development, an effect that is most likely related to the buildup of an organic layer which showed no substantial $CH_4$ oxidation. In many forest systems, $CH_4$ oxidation is low or absent in the organic layer and concentrates in the top layers of the mineral soils (e.g. Adamsen and King, 1993; Bradford et al., 2001). Oxidation rates of atmospheric $CH_4$ generally are limited by diffusion rates due to the low substrate concentrations; a downward shift of activity thus would tend to decrease soil $CH_4$ uptake rates. In the present study, however, such an effect was not

evident, probably because the diffusive resistance of the top organic layer was low. This situation is comparable to the findings of Stiehl-Braun et al. (2011a) who reported that fertilizer application inhibited methanotrophic activity in top soil layers of a grassland but that this had no effect on soil-atmosphere $CH_4$ exchange at least when soils were dry. In the afforestation chronosequence we studied, soils in older forest stands also were drier because the taller forest canopies intercepted more rainfall. If there was a limiting effect of the downward shift in methanotrophic activity, it might have been compensated by lower soil moisture.

Reay et al. (2005) reported that $CH_4$ uptake in the top organic layer in forests can be decreased by higher nitrification rate in this layer. Potential nitrification rate can be greatest at the near soil surface in forests and it decreases with depth (Laverman et al., 2000). However, higher presence of $NH_4^+$ can lower $CH_4$ uptake in the top organic layer in forests.

Small-scale heterogeneity in soil methanotrophic activity has been reported in previous studies that adopted autoradiographic techniques (Stiehl-Braun et al., 2011a,b; Karbin et al., 2015b,a). While we have argued that this heterogeneity was related to aggregate structure, soil structure was not unambiguously identifiable once the soil cores were embedded in resin. The enhanced activities in eroded aggregate surfaces now demonstrate that indeed methanotrophic activity is concentrated in the aggregate's periphery and largely absent from their center. This can be understood in the light of diffusion limitations. On the other hand, locations more towards the aggregate center would protect methanotrophs from adverse biotic and abiotic effects such as protozoan grazing, drought stress, freeze-thaw events, and possibly also chemical inhibition by organic compounds such as terpenes. Such adverse effects could be important given the oligotrophic nature of methanotrophs in low-$CH_4$ environments which implies a low resilience after disturbance.

We found the largest labeling in aggregates 2–4 mm in size, irrespective of forest age. As soil aggregate volume increases, air diffusion into the central part of soil aggregate

decreases (Sexstone et al., 1985), and $CH_4$ availability will be restricted. Small aggregates have a larger surface to volume ratio, which may explain why $^{14}$Clabeling decreased with size when aggregates were larger than 2 mm in diameter. In our study, aggregation decreased with forest stand age, which also might have contributed to higher soil $CH_4$ uptake rates in older forest stands. In other studies, increasing fractions of macro-aggregates were found under afforestation (Wei et al., 2013; Wu et al., 2013), but macro-aggregates were by a size $\geq$0.25 mm which is different from our study.

Interestingly, aggregates smaller than 2 mm showed lower labeling. We did not further subdivide this fraction by size, so that it presumably also contained very fine material. Aggregates generally form hierarchic structures (Oades, 1993; Tisdall and Oades, 1982), and it is well possible that some of the fine material collected in the $\leq$2 mm fraction originated from the center of larger aggregates that disintegrated during the fractionation procedure. Alternatively, smaller aggregates may have been packed more densely, resulting in a surrounding pore network that restricted gas diffusion to these soil domains (Sierra and Renault, 1996; Blagodatsky and Smith, 2012). Finally, these small aggregates simply may have had different physic-chemical properties that make them less suitable as habitat for methanotrophs.

In conclusion, our results indicate that increased soil $CH_4$ uptake in older forest stands is related to lower soil moisture due to interception and possibly also due to a larger share of aggregates showing a higher than average methanotrophic activity. Our erosion analysis indicates a small-scale heterogeneity in the distribution of microbial activity that follows a radial gradient. It would be interesting to analyze, using molecular methods, whether this gradient in activity coincides with a gradient in methanotroph abundance. More generally, the small-scale (sub-aggregate) spatial heterogeneity of soil microbial activity and diversity appears underexplored, although it may have important implications for our understanding of the ecological mechanisms that control microbially-mediated processes at the ecosystem-level.

## Acknowledgements

We gratefully acknowledge Patrick Schleppi for field and laboratory assistance. This study was funded by the University of Zurich and a stipend of the Aga Khan Foundation to SK.

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# Figure legends

Fig. 1: Net $CH_4$ uptake of soil cores collected on September 28, 2011 (left) and September 28, 2012 (right).

Fig. 2: Volumetric soil moisture in soil cores collected on September 28, 2011 (left) and September 28 2012 (right).

Fig. 3: Soil aggregate size distribution in pasture and forest plots.

Fig. 4: Ratio of $^{14}C$ concentration in the eroded exterior part of aggregates relative to the concentration in the remaining interior part, in dependence of land use, forest stand age, and aggregate size class. Means ± s.e., using plots as replicate.

Fig. 5: Auto-radiographic image of soil cores harvested on September 28 2011. Darker pixels indicated stronger $^{14}C$ labeling.

Fig. 6: Auto-radiographic image of soil cores harvested on September 28 2012. Darker pixels indicated stronger $^{14}C$ labeling.

Fig. 7: Auto-radiographic images of soil aggregates. Darker pixels indicate higher amounts of assimilated $^{14}C$.

Fig. 8: Depth distribution of net $^{14}CH_4$assimilation in soil cores harvested on September 28 2011 (left) and September 28 2012 (right). Activities were standardized core-wise to unity sum of all 1 cm-layers. Data shown are means ± 1 s.e., using plots as replicates.

# Figures

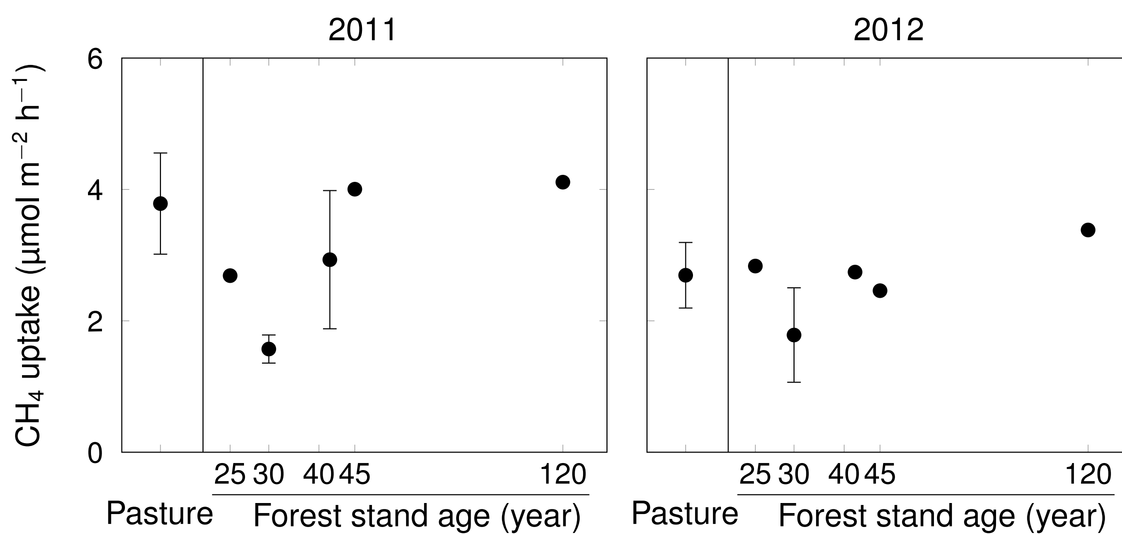


Figure 1

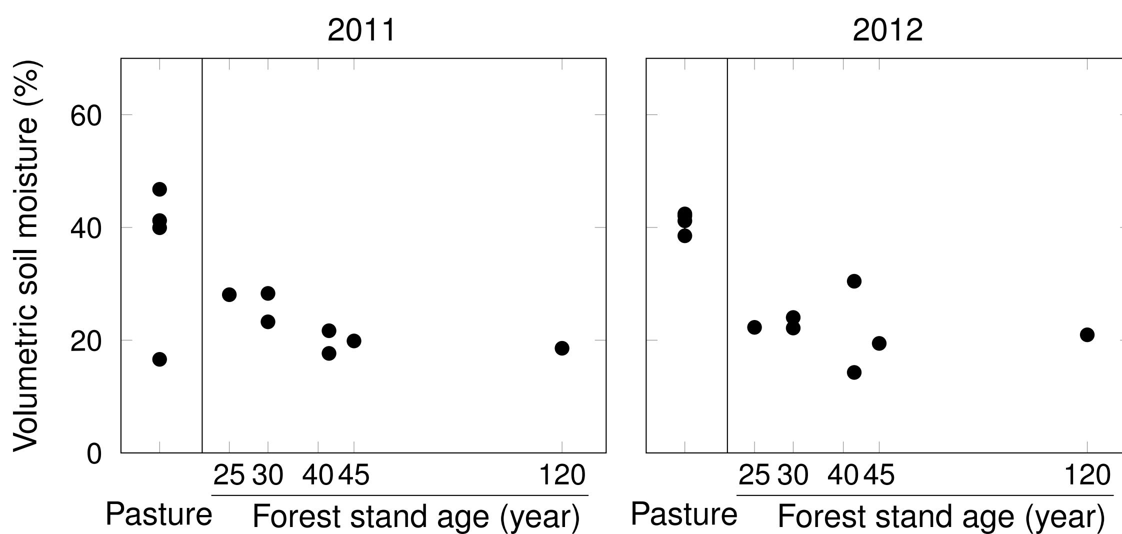


Figure 2

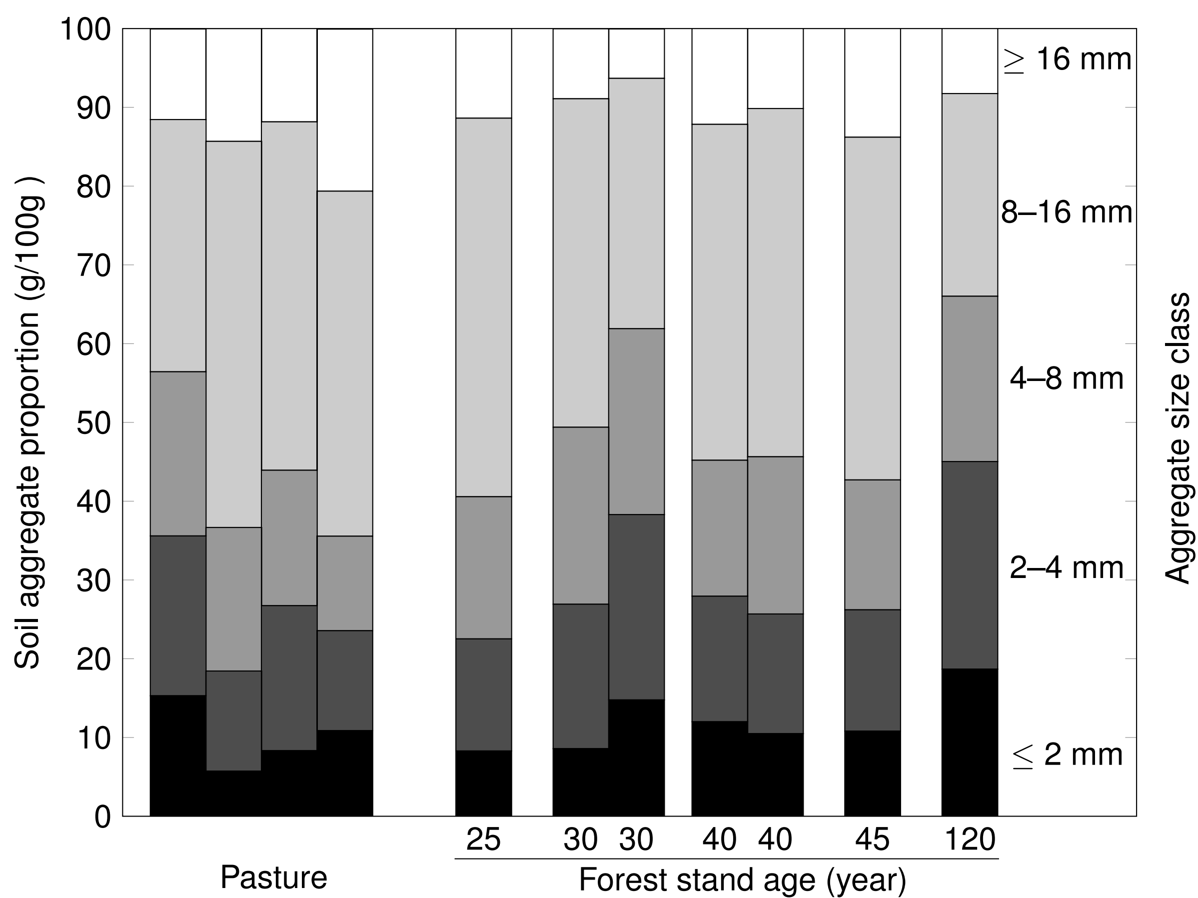


Figure 3

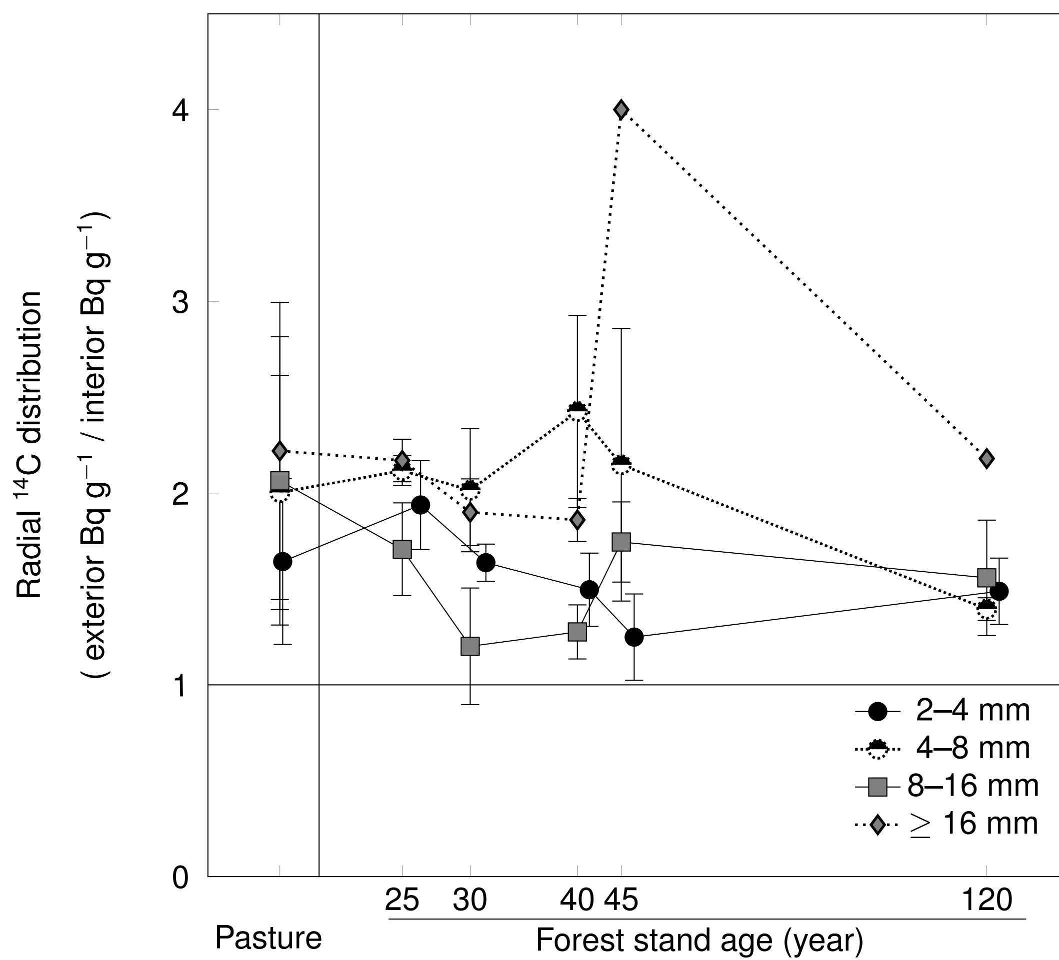


Figure 4

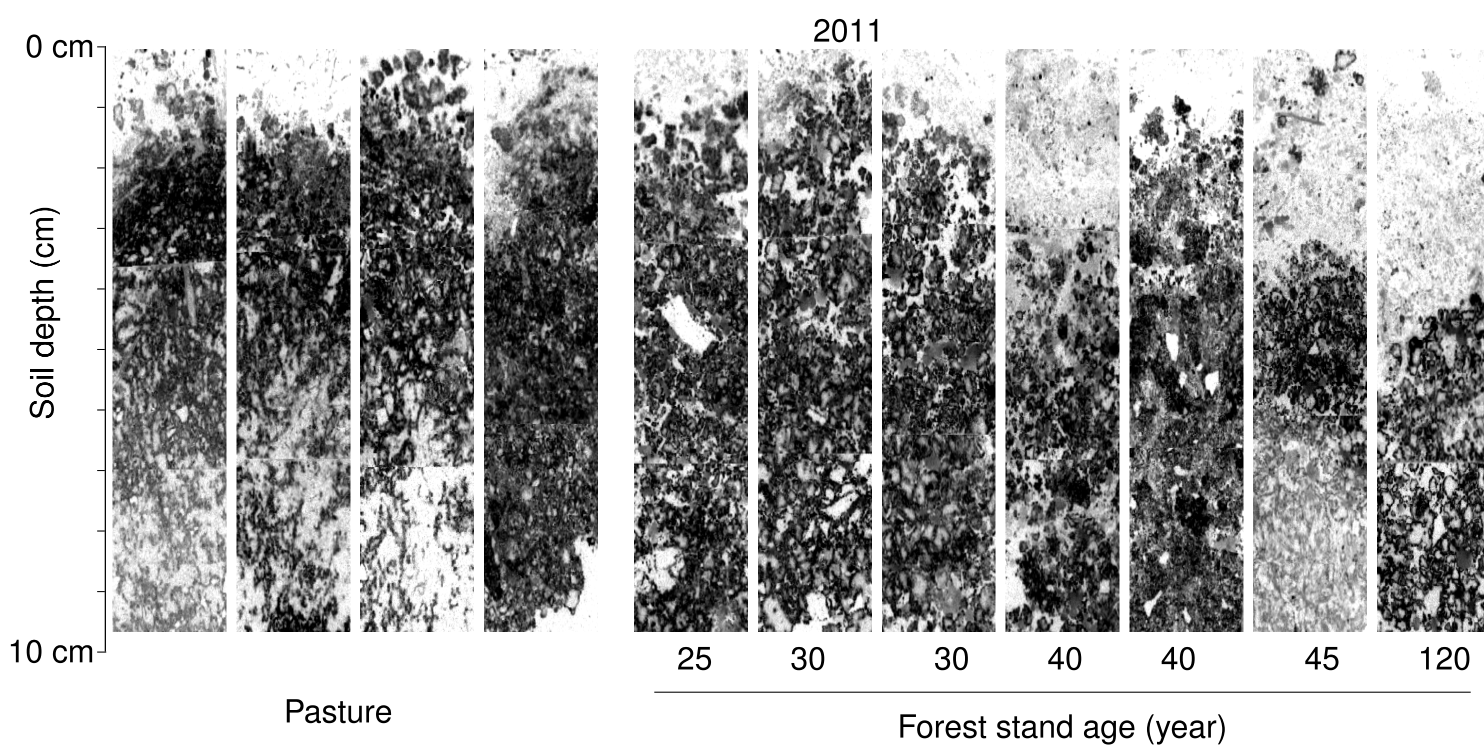


Figure 5

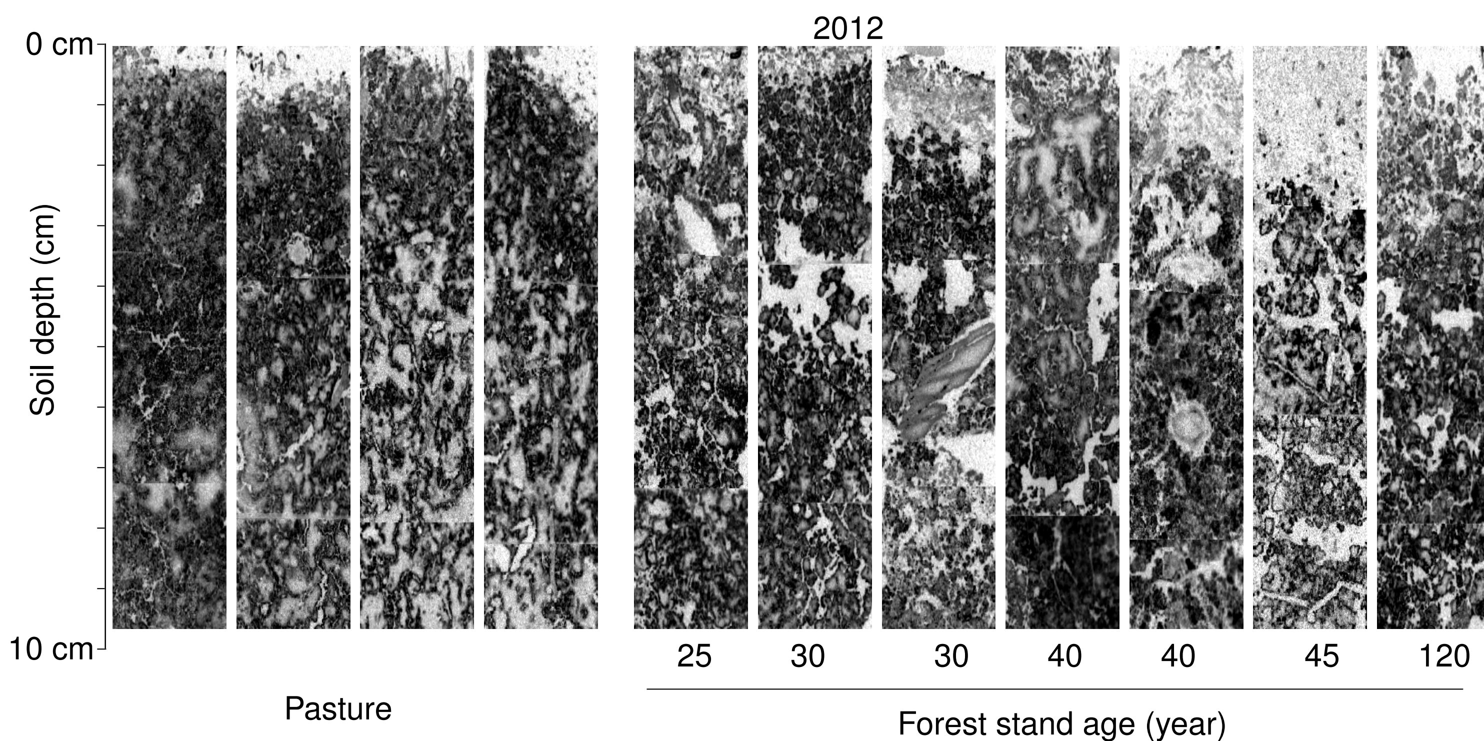


Figure 6

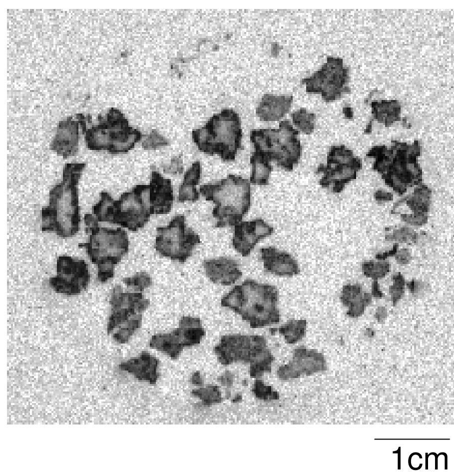


Figure 7

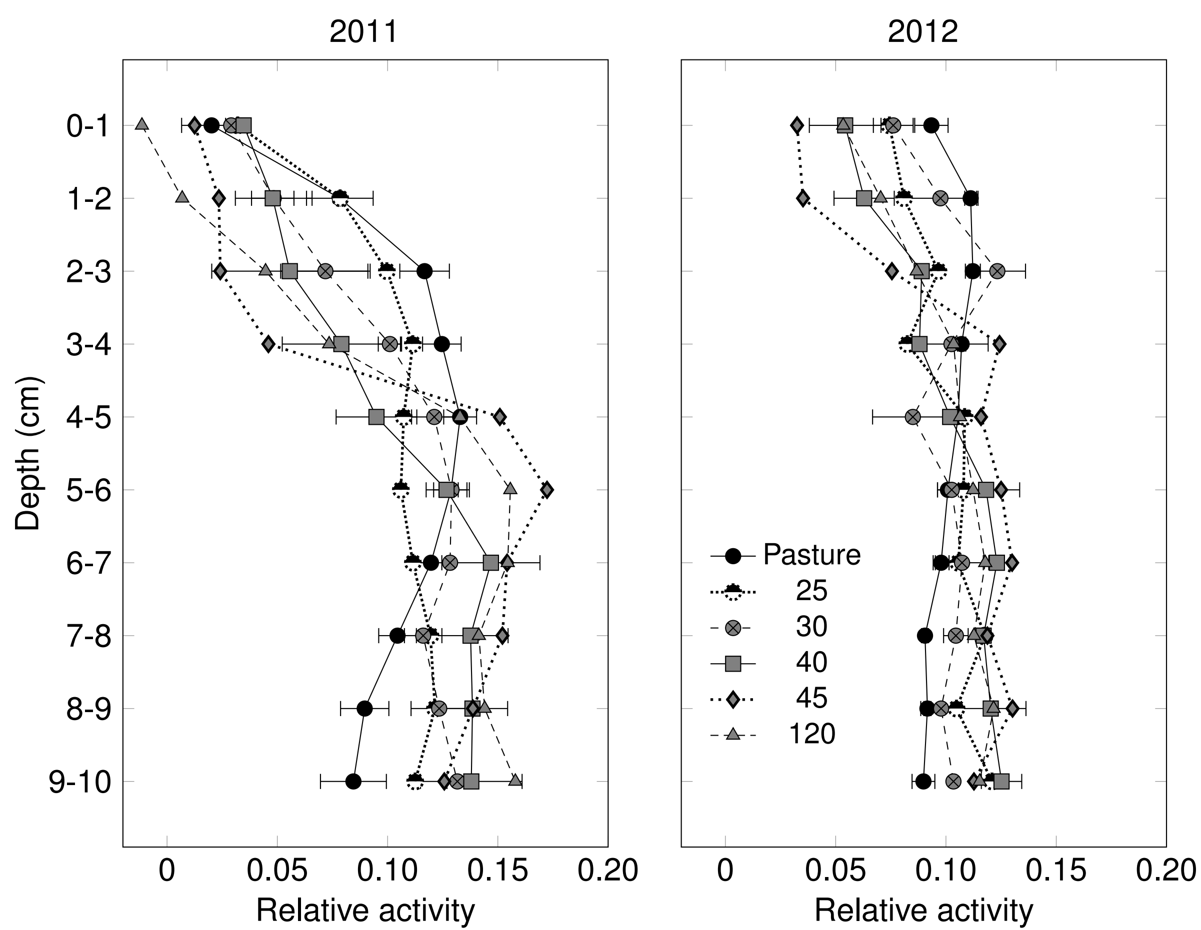


Figure 8

# Chapter 4:

## Increasing soil methane sink along a 120-year afforestation chronosequence is driven by soil moisture

David Hiltbrunner, Stefan Zimmermann, **Saeed Karbin**, Frank Hagedorn and Pascal A. Niklaus, (2012), Global Change Biology 18, 3664–3671.

Global Change Biology

Global Change Biology (2012) 18, 3664–3671, doi: 10.1111/j.1365-2486.2012.02798.x

# Increasing soil methane sink along a 120-year afforestation chronosequence is driven by soil moisture

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## Abstract

Upland soils are important sinks for atmospheric methane ($CH_4$), a process essentially driven by methanotrophic bacteria. Soil $CH_4$ uptake often depends on land use, with afforestation generally increasing the soil $CH_4$ sink. However, the mechanisms driving these changes are not well understood to date. We measured soil $CH_4$ and $N_2O$ fluxes along an afforestation chronosequence with Norway spruce *(Picea abies L.)* established on an extensively grazed subalpine pasture. Our experimental design included forest stands with ages ranging from 25 to >120 years and included a factorial cattle urine addition treatment to test for the sensitivity of soil $CH_4$ uptake to N application. Mean $CH_4$ uptake significantly increased with stand age on all sampling dates. In contrast, $CH_4$ oxidation by sieved soils incubated in the laboratory did not show a similar age dependency. Soil $CH_4$ uptake was unrelated to soil N status (but cattle urine additions stimulated $N_2O$ emission). Our data indicated that soil $CH_4$ uptake in older forest stands was driven by reduced soil water content, which resulted in a facilitated diffusion of atmospheric $CH_4$ into soils. The lower soil moisture likely resulted from increased interception and/or evapotranspiration in the older forest stands. This mechanism contrasts alternative explanations focusing on nitrogen dynamics or the composition of methanotrophic communities, although these factors also might be at play. Our findings further imply that the current dramatic increase in forested area increases $CH_4$ uptake in alpine regions.

*Keywords:* afforestation, alpine regions, chronosequence, fertilization, methane oxidation, nitrous oxide, Norway spruce, soil moisture regime

*Received 7 June 2012; revised version received 7 June 2012 and accepted 2 July 2012*

## Introduction

Methane ($CH_4$) is produced in water-logged soils by methanogenic archaea (Boone *et al.*, 1993). In contrast, well-aerated upland soils are the most important biological sink for atmospheric $CH_4$ (IPCC, 2007). Soil $CH_4$ uptake is essentially driven by the oxidation of $CH_4$ by soil methanotrophic bacteria. In many soils, both processes – methanogenesis and $CH_4$ oxidation – take place concurrently, with the soil acting as a net source or sink depending on which process dominates.

The largest terrestrial sinks for atmospheric $CH_4$ are generally found in forest soils. When forests are converted into grassland or arable fields, soil $CH_4$ uptake generally decreases (Hütsch *et al.*, 1994; Willison *et al.*, 1995; Smith *et al.*, 2000). Many investigations have attributed this decrease in methanotrophic activity to the disturbance of soil physical structure associated with such land-use changes, and to the application of mineral nitrogen fertilizers. Physical disturbances of the soils through ploughing disrupts aggregates, which might affect the ecological niche of methanotrophs (Boeckx & Cleemput, 2001), especially in coarse-textured soils (Hütsch, 1998). The use of heavy machinery on cultivated land also compacts soils, thereby restricting diffusive transport of atmospheric $CH_4$ into soils (Ball *et al.*, 1997b; Smith *et al.*, 2003). Fertilization of agricultural fields, in particular with ammonium-based fertilizers, has been shown to inhibit $CH_4$ oxidation (King & Schnell, 1994; Gulledge *et al.*, 1997; Whalen, 2000; Jassal *et al.*, 2011); however, positive effects of N fertilization also have been reported (Bodelier & Laanbroek, 2004).

Interestingly, when cultivated land is abandoned, $CH_4$ oxidation reverts only very slowly to precultivation levels. Paired-site studies have demonstrated that this process can take many years (Priemé *et al.*, 1997; Smith *et al.*, 2000), but it is not well understood to date why the increase in soil $CH_4$ uptake is so slow. One factor involved might be the very low-growth rates of methanotrophic bacteria thriving on atmospheric $CH_4$ (Priemé *et al.*, 1996; King, 1997; Menyailo *et al.*, 2008). Another reason may be that the original soil structure is

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restored only after many years (Priemé *et al*., 1997; Hütsch, 1998; Smith *et al*., 2000; Regina *et al*., 2007). However, not many studies on the recovery of the soil $CH_4$ sink are available, presumably because not many such chronosequences have been established.

Across Europe and North America, large areas of land have been abandoned for socioeconomic reasons. In the European mountains, woody plant encroachment in abandoned grasslands is widespread (FAO, 2001). In Switzerland, the forest cover in the Alps increased by 900 km$^2$ between 1984 and 2005, which corresponds to a 15% increase in total forested area in this region (Brändli, 2010). Whether and to what extent soil $CH_4$ uptake increases under these conditions is unclear. Subalpine pastures have, generally, only moderately been grazed with little nutrient inputs and they have never been tilled. Thus, the loss of methanotrophic activity when these pastures have been established has probably been smaller than in intensified low-land pastures and arable fields (Priemé *et al*., 1997; Peichl *et al*., 2010; Christiansen & Gundersen, 2011). As a consequence, the increase in soil $CH_4$ uptake after afforestation might also be smaller.

In our study, we have measured soil $CH_4$ uptake and potential $CH_4$ oxidation along a chronosequence of Norway spruce afforestations spanning more than 120 years. All forest plots are located in an extensively grazed subalpine pasture. To test the sensitivity of soil $CH_4$ uptake to nitrogen additions, we further established a N-fertilizer treatment (cattle urine) in all chronosequence plots. Our aims were (i) to test for effects of afforestation on the soil $CH_4$ sink, focusing in particular on the temporal dynamics of these changes; and (ii) to test how these changes were related to changes in soil physical properties and nitrogen status.

## Materials and methods

### *Study site and experimental design*

The present study was conducted in a subalpine region in the Canton of Fribourg, Switzerland (7°15′54 E; 46°37′17 N), on a south-facing slope extending from 1450 m a.s.l to 1700 m a.s.l. This slope has been used as pasture for the last 150 years; no land-use records are available prior to this period, but it seems likely that the slope has been under pasture for several centuries. Mean summer and winter air temperatures are 11.4 °C and 0.6 °C, respectively; mean annual precipitation averages 1250 mm with a maximum in summer. Soils are Cambisols on calcareous bedrock.

After severe avalanches in 1956, an area of about 15 ha on the eastern part of the slope was gradually afforested with Norway spruce *(Picea abies L.)*, while the western part remained as a pasture (Fig. 1). Separate patches of forest were planted on different dates, resulting in stands 25, 30, 40, 45 and >120 years old. We established one 15 × 15 m plot in each forest patch, plus an additional four similarly sized plots in the adjacent pasture. Within each plot, four pairs of subplots were established. One randomly selected subplot per pair was treated with synthetic cattle urine, while the other one served as unfertilized reference. The synthetic cattle urine was prepared according to Fraser *et al.* (1994) and contained urea as the main N source plus glycine representing the amino acid fraction in the cattle urine, potassium bicarbonate, potassium bromide, potassium chloride and potassium sulphate. The synthetic urine solution was applied to the subplots at a rate of 20 g N m$^{-2}$ (as 3.35 L m$^{-2}$ aqueous solution) on August 12, 2010. The same amount of water was added to the unfertilized control plots.

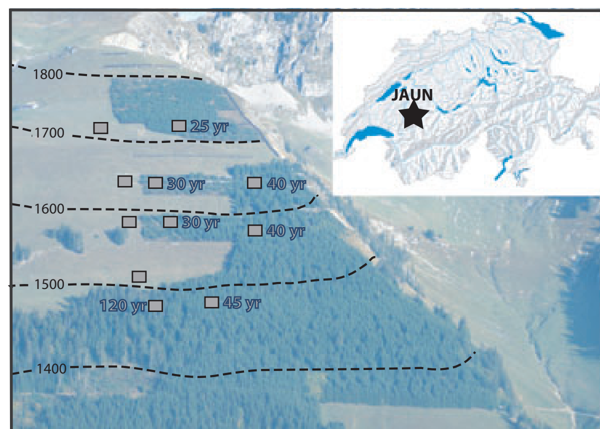


**Fig. 1** Photograph of the study site showing the plots where the $CH_4$ and $N_2O$ fluxes have been measured (grey squares) and the age of the respective forest stands.

### *Soil-atmosphere $CH_4$ and $N_2O$ fluxes*

Soil-atmosphere fluxes of $CH_4$ and $N_2O$ were measured using static chambers. On May 17, 2010, a 32 cm diameter × 30 cm tall static chamber was lowered 20 cm into the soil of each subplot and remained there until the end of the growing season. The chambers were placed at some distance from the tree stems to avoid coarse roots. The remaining headspace volume of each chamber was determined by measuring its height aboveground at several locations within the chamber. Soil-atmosphere trace gas fluxes were determined on July 17, August 10, 13 and 20, September 3 and October 1, 2010, by closing the chamber with a gas-tight lid and sampling the headspace through a septum after 5, 20 and 35 min. The headspace samples were injected into pre-evacuated exetainers and analysed for $CH_4$ and $N_2O$ concentrations using a gas chromatograph (Agilent 7890 fitted with a flame ionization ($CH_4$) and an electron-capture detector ($N_2O$), Agilent Technologies Inc., Santa Clara, CA, USA). $CH_4$ and $N_2O$ flux rates were calculated by linear regression of measured concentrations against sampling time. Estimates with regression coefficients $r^2$ < 0.8 were excluded except when fluxes were close to zero.

Soil surface temperature (0–2 cm) and volumetric water content (0–15 cm) were measured concomitantly with the gas

measurement using a thermometer and time domain reflectometry (TDR) probes (TRIME-FM, IMKO, Ettlingen, Germany).

We further measured potential $CH_4$ oxidation rates of sieved soil under standardized laboratory conditions. On September 28, 2011, two soil cores were taken from each subplot and divided into the 0–5, 5–10, 10–15 and 15–20 cm depth layer of the mineral soil horizon. The soil fractions were sieved (2 mm mesh size), and fresh soil equivalent to 100 g dry weight placed into gas-tight jars fitted with a septum. The soils were equilibrated at 20 °C overnight; then, the jars were aerated for 30 min, closed again; and $CH_4$ oxidation rates were determined by measuring headspace $CH_4$ concentrations after 5, 125 and 425 min. These incubations were conducted under atmospheric $CH_4$ concentrations, i.e. no extra $CH_4$ was injected into the headspace.

### *Soil bulk density and porosity*

On November 8, 2011, three soil cylinders of 10.8 cm diameter × 11 cm depth were collected per plot. Bulk soil density was estimated by dividing the mass of the dried soil (105 °C) by the volume of the cylinder. Particle density was determined by the pycnometer method (Blake & Hartge, 1986). Total porosity was calculated as 1-(bulk density/particle density). Soil texture was determined with the pipette method according to Gee & Bauder (1986).

### *Soil acidity and mineral N concentrations*

Three weeks after the application of synthetic cattle urine, four soil samples (2 cm diameter × 5 cm depth) were collected in each subplot. The soils were sieved, roots removed and soil pH measured potentiometrically in a dried (60 °C) aliquote suspended in 0.01 M CaCl2 at a soil:extractant ratio of 1 : 2.

Ammonium ($NH_4^+$) and nitrate ($NO_3^-$) were extracted from 10 g fresh soil with 100 mL 1M KCl in an overhead shaker (1.5 h). Extracts were filtered (0790½, Whatman International, Maidstone, UK) and $NH_4^+$ concentrations were measured colorimetrically by automated flow injection analysis (Perkin Elmer UV/VIS Spectrometer Lambda 2S, Waltham, MA, USA). Nitrate was determined colorimetrically at 210 nm (Varian Cary 50, Palo Alto CA, USA) as difference in absorbance between nonreduced and reduced (using $H_2SO_4$ and copperized zinc) extracts (Navone, 1964).

### *Potential nitrification and denitrification*

Potential nitrification (PN) was determined by the shaken slurry method (Hart *et al.*, 1994). Briefly, 10 g sieved fresh soil was suspended in 90 mL 1 mM phosphate buffer adjusted to pH 7.0. Ammonium sulphate (140 mg N $kg^{-1}$ soil) was added and the slurry incubated at 25 °C on an orbital shaker. Aliquots of 10 mL were taken after 1, 4, 18 and 22 h. These aliquots were immediately mixed with 15 mL 2.5 M KCl to stop nitrification, centrifuged, and the supernatant analysed for $NO_3^-$ as described above. Potential nitrification rates were calculated by linear regression of $NO_3^-$ concentration against time.

Denitrifying enzyme activity (DEA), which shows the denitrification potential under excess substrate availability, was determined by the application of the acetylene inhibition assay (Smith & Tiedje, 1979; Patra *et al.*, 2005). Fresh sieved soil samples equivalent to 5 g dry weight were placed in 125 mL plasma flasks and the headspace replaced by a 90 : 10 mixture of helium:acetylene. The flasks were incubated at 26 °C and, after 1 h, an aqueous solution containing $KNO_3$, glucose and glutamic acid was added. $N_2O$ concentrations in the headspace were analysed after 60, 90 and 120 min as described above. $N_2O$ production per unit time (DEA) was estimated by linear regression.

### *Tree aboveground biomass*

Tree aboveground biomass in each plot was calculated using allometric relations depending on stem diameter at breast height, tree height (Kaufmann, 2001) and basal area per ground area. The diameter of all trees was measured in two areas, 25–100 $m^2$ in size in stands up to 30 years old. In the older afforestations, trees were measured in a single large area of 250–600 $m^2$ to account for the bigger size and lower density of trees found there. In addition, the heights of 5–10 single trees per area were measured.

### *Statistical analysis*

We analysed our data by fitting mixed-effects models by maximum likelihood (ASReml 3.0, VSN International, UK; Gilmour *et al.*, 2009). The model included the sequential fixed effects altitude (elevation in m a.s.l.), land use (forest vs. meadow), forest stand age, fertilization and the interactions of fertilization with land use and stand age. The effect of stand age was fitted as a log-linear contrast [1 df, testing for effects of log(age)] followed by a term testing for the deviation from log-linearity (3 df, age fitted as categorical term). The significance of the fixed effects was determined using Wald statistics. Reflecting the structure of the experiment, the model included the nested random effects plot, subplot and static chamber. Altitude and stand age were partly confounded in our study, with higher average forest stand age at the bottom of the slope, and younger forest patches dominating the top of the slope. We therefore fitted a second model in which the terms for altitude and stand age were interchanged; testing for effects of age after accounting for altitude underestimates the age effect, whereas age effects potentially include an altitude component when fitted first. Effects with $P < 0.05$ were considered statistically significant, effects with $0.05 < P < 0.1$ as marginally significant.

## Results

### *Soil bulk density, porosity and water content*

Average bulk density of the soils (0–10 cm) showed no consistent trend with land use and stand age. The

densities varied between 0.7 and 0.9 g $cm^{-3}$, with the highest values in the 40 years old afforestations (0.91 g $cm^{-3}$) and the lowest ones in the old forest (0.70 g $cm^{-3}$) (Table 1). These findings were confirmed by measuring an additional 65 soil cores sampled across the whole site; these did not show a statistically significant effect of stand age or land use on bulk density (data not shown). In accordance, soil porosities were in a rather narrow range (63–69%) and also did not depend on land use or stand age (Table 1).

Microclimate greatly differed between the two land-use types. During the growing season, surface soils of the forest stands were on average 5 °C cooler than the pasture soils, which exceeds the temperature lapse rate across 250 m in altitude of 1.5 °C. Soil moisture varied within the pasture, but this variation was not related to altitude. In fact, pasture soils at the top and the bottom of the slope had approximately equal soil water contents of 0.40 $m^3$ $m^{-3}$ when averaged over the six sampling dates (Fig. 2c). A general trend, however, was that soil moisture significantly decreased with stand age on all except one date, with variable levels of significance ($P < 0.05$ to $P < 0.001$). The measurement period encompassed a wide range of climatic conditions resulting with rather dry (14–26% volumetric water content; July 17, 2010) and wet soils (24–57%; October 3, 2010). Forest soils were drier than pasture soils on all dates. Reflecting soil moisture, water-filled pore space (WFPS) also decreased along the chronosequence (Fig. 3).

### Soil methane uptake

Soil $CH_4$ uptake was higher under forest than under pasture ($P < 0.001$ for effects of land use). Soil $CH_4$ uptake significantly increased with stand age, with the log-linear component ($P < 0.001$) explaining twice as much variance as the term testing for deviations from log-linearity ($P < 0.05$). Stand age explained less variance when fitted after accounting for altitude ($P = 0.01$ for log(age) and $P = 0.08$ for the deviation from log-linearity). Reflecting the partially confounding influence of age and altitude, the effect of altitude was significant at $P < 0.001$ when fitted before age, but explained ~8 times less variance and was at the border to significance ($P = 0.05$) when fitted after age.

Water-filled pore space was significantly negatively related to soil $CH_4$ uptake ($P < 0.001$; Fig. 3), explaining more than 70% of the variance accounted for by the fixed effects contained in the model. Effects of log(age) explained only half as much variance and were less significant ($P < 0.05$) when fitted after WFPS, suggesting that at least part of the observed age effect was due to altered soil moisture. Soil $NH_4^+$ and $NO_3^-$ (fitted as log([$NH_4^+$]) and √[$NO_3^-$]) did not explain significant fractions of the variation in $CH_4$ fluxes.

Cattle urine addition exerted only little effect on $CH_4$ oxidation; when data for the different sampling dates were tested individually, a decrease in soil $CH_4$ uptake of 20% was found 1 day after fertilizer addition ($P = 0.02$). Averaged over all sampling dates, effects of cattle urine addition were no longer statistically significant (−11%, n.s.).

Interestingly, the $CH_4$ uptake of sieved soils incubated in the laboratory did not reveal any systematic effect of age (Fig. 4), but effects of soil moisture remained significant at $P < 0.001$.

### Soil $N_2O$ emissions and N cycling

In the absence of cattle urine, $N_2O$ fluxes did not change with age. Cattle urine increased $N_2O$ emissions from soils in the younger forest stands. This resulted in a significant overall effects of log(age) ($P < 0.001$) and a significant interaction between log(age) and cattle urine application ($P < 0.05$, respectively, Fig. 2b). Soil extractable $NH_4^+$ increased with stand age ($P < 0.05$), whereas $NO_3^-$ did not show such an effect (Fig. 5a, b). Potential nitrification did not depend on stand age, but increased with cattle urine addition ($P < 0.01$) (Fig. 5c). Denitrification enzyme activity did not respond either to stand age or to cattle urine addition (Fig. 5d). Soil acidity

**Table 1** Soil properties and aboveground tree biomass in the pasture and the different afforestations with (standard errors) representing the different plots per age

| Land use | pH ($CaCl_2$) | | Bulk density (g $cm^{-3}$) | | Porosity (%) | | Clay content (%) | | Tree biomass (t $ha^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Pasture | 4.9 | (0.1) | 0.83 | (0.04) | 65 | (1.4) | 55 | (1) | - | - |
| Afforestation 25 years | 4.8 | - | 0.73 | - | 69 | - | 57 | - | 157 | - |
| Afforestation 30 years | 4.9 | (0.3) | 0.79 | (0.06) | 67 | (1.5) | 55 | (4) | 140 | (3) |
| Afforestation 40 years | 4.2 | (0.0) | 0.91 | (0.05) | 63 | (1.4) | 36 | (12) | 277 | (38) |
| Afforestation 45 years | 3.9 | - | 0.76 | - | 68 | - | 51 | - | 263 | - |
| Afforestation 120 years | 4.8 | - | 0.70 | - | 69 | - | 49 | - | 579 | - |

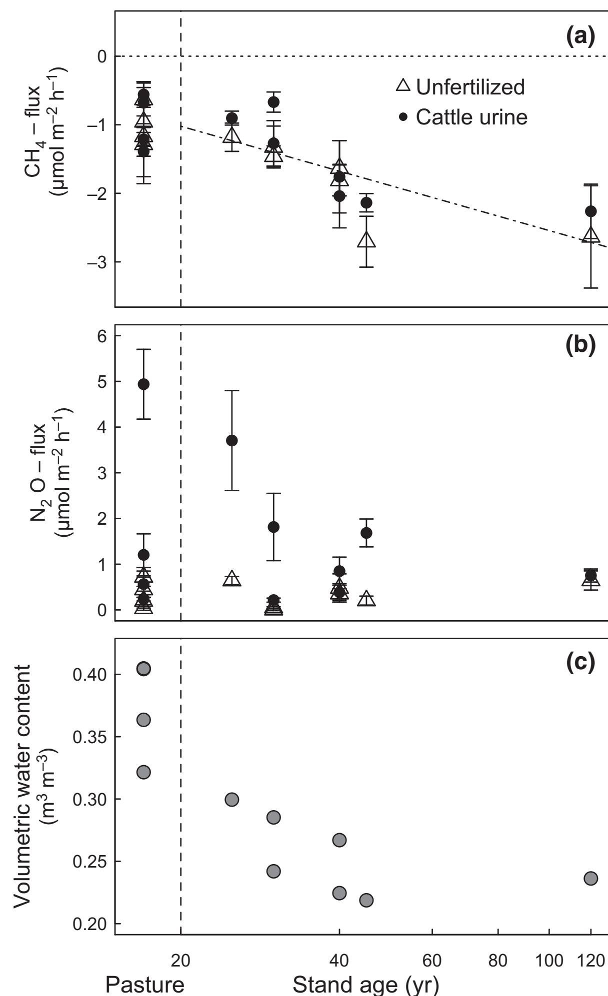


**Fig. 2** Fluxes of $CH_4$ (a) and $N_2O$ (b) in dependence of forest stand age and cattle urine application (negative values indicate uptake from the atmosphere). Error bars are standard errors based on $n$ = 4 subplots per forest or pasture plot. Cattle urine addition did not affect volumetric soil water content (c), so that data of fertilized and unfertilized subplots were combined.

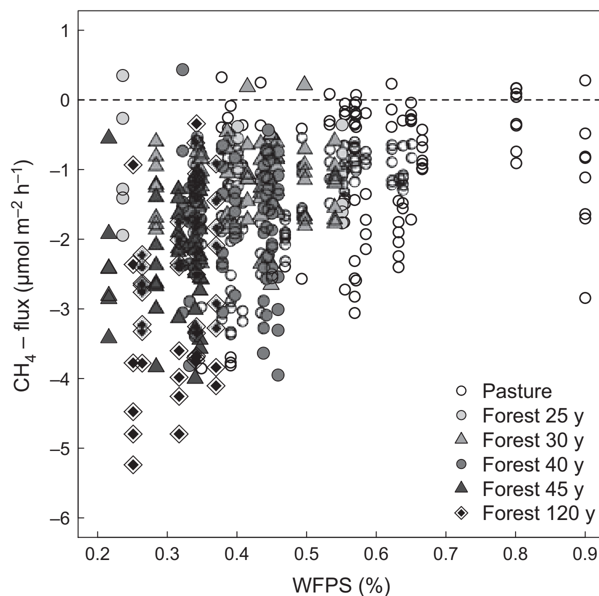


**Fig. 3** Soil $CH_4$ fluxes in dependence of water-filled pore space (WFPS) and stand age (negative values indicate uptake from the atmosphere).

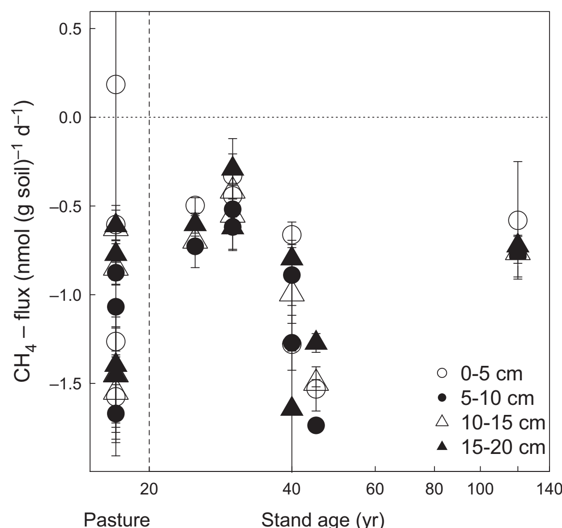


**Fig. 4** $CH_4$ fluxes of sieved soils incubated in the laboratory, in dependence of stand age and soil depth (negative values indicate uptake from the atmosphere). Error bars show standard errors of means.

generally decreased with stand age, but the oldest stand had pH similar to the youngest stand; this resulted in no effect of log(age), but a significant deviation from linearity ($P < 0.001$).

## Discussion

Our results show that $CH_4$ oxidation in subalpine soils increased by a factor of two to three after conversion from pasture to forest. $CH_4$ oxidation increased with stand age on all sampling dates, spanning a wide range of climatic conditions, emphasizing that this effect is robust.

In contrast to $CH_4$ fluxes, $N_2O$ emissions showed no similar change with stand age, at least as long as no cattle urine was added (Fig. 2b). The main driver of $N_2O$ emissions was the mineral N status of the soils, but particularly concentrations of $NO_3^-$ were not related to stand age. The primary objective of the $N_2O$ flux measurements was not to assess $N_2O$ fluxes in detail (which would require far more measurements), but to obtain an indicator of the ecosystem's N status and its dependency on age and fertilizer application.

In the 120-year-old subalpine forest, soil $CH_4$ oxidation had reached rates comparable to the range

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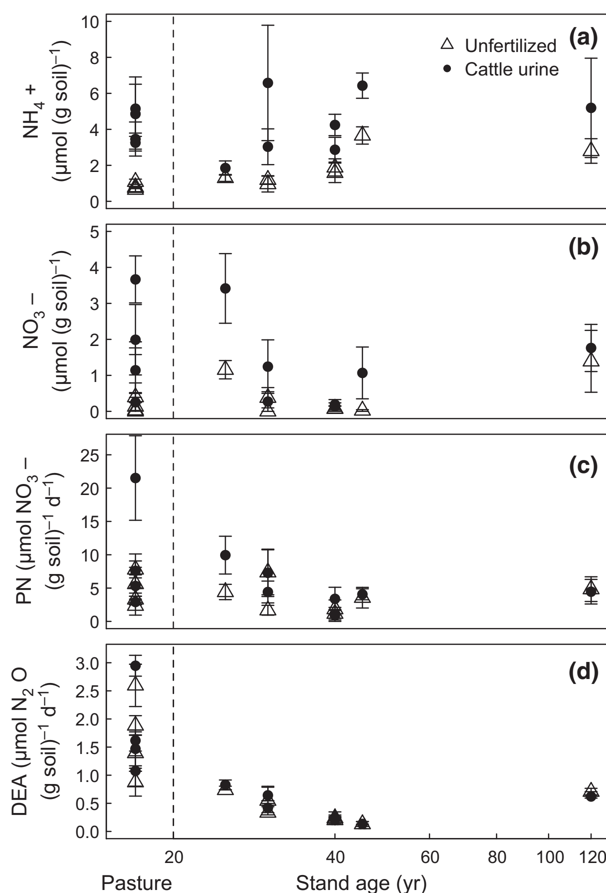


**Fig. 5** Soil extractable $NH_4^+$ (a) and $NO_3^-$ (b), potential nitrification rates (PN) (c) and denitrification enzyme activity (DEA) (d) in dependence of forest stand age and cattle urine application. All data refer to the 5 cm of mineral soil. Error bars represent standard errors.

published for temperate coniferous forests (Smith *et al.*, 2000; Jang *et al.*, 2006; Peichl *et al.*, 2010), although much higher rates have been reported for some forests (Ishizuka *et al.*, 2000; Price *et al.*, 2004). However, it remains unclear whether further increases in $CH_4$ oxidation can be expected in the future when stand age exceeds 120 years.

Stand age and altitude were not orthogonal in our study. The oldest and the 45 years old forest were at the lower end of the slope (1450 m a.s.l.), whereas the youngest (25 years old) stand was at the upper end at 1700 m a.s.l. $CH_4$ uptake is relatively insensitive to temperature (Smith *et al.*, 2003), and therefore not likely to be affected by the relatively short altitudinal gradient of 250 m. Nevertheless, vegetation period, plant growth and the biological activity might be higher at the lower end of the slope and effects of altitude could therefore be confounded with effects of stand age. We argue, however, that this is unlikely in our study, for several reasons. First, and most importantly, the effect of stand age remained statistically significant after adjusting for altitude. Second, the effect of altitude was not statistically significant in the reference grassland ($P = 0.09$), although there was a slight trend towards increased $CH_4$ oxidation rates at lower elevation. Third, if the effect of altitude was to increase productivity and the length of the growing season, then one might argue that the forest stands at the bottom of the slope are even older on a biomass or 'degree-days' scale. In this case, altitude would increase the 'effective age' of the older stands more than the one of the younger stands, and thus do not alter the conclusions. Indeed, when we analysed $CH_4$ oxidation as function of stand biomass (which is a proxy for biomass), we obtained similar results ($P < 0.001$).

Stand-age effects found in the few afforestation studies available to date are ambiguous. Whereas the majority of the studies observed a slow increase of $CH_4$ uptake after tree establishment (Priemé *et al.*, 1997; Singh *et al.*, 2007; McNamara *et al.*, 2008; Peichl *et al.*, 2010), some found no age effect (Ball *et al.*, 2007), or an age effect which depended on tree species (Christiansen & Gundersen, 2011). However, the mechanisms driving this change remain uncertain. One possibility might be that the populations of methanotrophic bacteria require decades to increase due to slow growth rates under atmospheric $CH_4$ concentrations (Priemé *et al.*, 1996; King, 1997; Menyailo *et al.*, 2008). In addition, the change from herbaceous to tree cover may induce shifts in methanotrophic community structure. In New Zealand, Singh *et al.* (2009) related higher soil $CH_4$ uptake in pine afforestation compared with pastures to a higher activity of type II methanotrophs which are thought to oxidize atmospheric $CH_4$ in soils (Knief *et al.*, 2006). We did not measure methanotrophic community structure; however, the systematic effect of stand age was lost when $CH_4$ oxidation rates were measured on sieved soils, i.e. when diffusive limitations by soil horizons were eliminated. This suggests that the potential to oxidize $CH_4$ was similar at all sites, independent of age, although our comparison of incubation and field measurements clearly has some limitations. In particular, laboratory experiments only reflect the oxidation potential of the incubated soil layer, excluding processes lower in the soil column, e.g. methanogenesis. Discrepancies between laboratory incubations and *in situ* measurements were also reported, for example by (Reay *et al.*, 2005), who measured considerable $CH_4$ uptake in sieved grassland soils incubated in the laboratory while the same soils were net sources of $CH_4$ under field conditions (Nedwell *et al.*, 2003). We argue that different diffusive limitations are the most likely explanation for the discrepancy between $CH_4$ uptake in laboratory incubations and *in situ*. Soil gas diffusivity is

controlled by pore network structure and water-filled pore space. In our study, $CH_4$ uptake decreased with water-filled pores space, a phenomenon commonly found (Dörr *et al.*, 1993; Ball *et al.*, 1997a; Bowden *et al.*, 1998). Soil bulk density and porosity varied only little (60–70%) among plots, with no systematic effect of stand age. Soil bulk density was fairly low even in the pasture plots, mainly because the cattle moved on specific tracks, leaving the major part of the pastures unaffected by trampling (Hiltbrunner *et al.*, 2012).

Why did soil moisture decrease with forest stand age? Evapotranspiration and interception often increase with forest age (Farley *et al.*, 2005). Moreover, the organic layer under spruce trees shelters the underlying mineral soil from rainfall (Borken & Beese, 2006). In our study, a gradual accumulation of the organic layer, reaching a thickness of 4–10 cm in the two oldest stands, might have contributed to drier mineral soils in the older forest plots. In conjunction with increased water retention by the forest stand, this might have promoted $CH_4$ diffusion into soils, which in turn enhanced $CH_4$ uptake with forest development.

High $NH_4^+$ concentrations can inhibit soil $CH_4$ oxidation in many ecosystems (Gulledge *et al.*, 1997; King & Schnell, 1994; Le Mer & Roger, 2001; Smith *et al.*, 2000; Steudler *et al.*, 1989). In our study, soil extractable $NH_4^+$ increased with stand age, with no evidence of an inhibition of $CH_4$ uptake. Similarly, Tate *et al.* (2007) also did not find a significant relationship between extractable $NH_4^+$ and soil $CH_4$ oxidation in a land-use change study in New Zealand, despite relatively high soil $NH_4^+$ concentrations. The cattle urine application in our study also did not substantially suppress soil $CH_4$ uptake, despite relatively large amounts added and resulting in increased contents of extractable $NH_4^+$ and increased associated $N_2O$ emissions in the following 2 months. However, the fertilization effects were largest in the younger stands, raising the possibility that rapid N uptake by more N-limited old forest stands and their soils protected methanotrophs against effects of $NH_4^+$.

$CH_4$ oxidation often decreases with soils acidification, either due to direct effects of soil pH, or due to reduced nitrification rates and therefore increased soil $NH_4^+$ concentrations (Weslien *et al.*, 2009; Stiehl-Braun *et al.*, 2011). Although soil pH differed between forest plots in our study, these changes did not explain the patterns observed in soil $CH_4$ uptake. Furthermore, soil pH changes spanned only one single pH unit.

Currently, forest cover is increasing rapidly in the European Alps. Our data can be combined with estimates of land-use change to arrive at an educated guess of the order of magnitude by which soil $CH_4$ uptake may increase as consequence of land abandonment. We base our calculation on Switzerland, but expect similar changes in other European alpine areas. Forest cover increased by more than 90 000 ha between 1984 and 2005 in the Swiss Alps (Brändli, 2010), which is equivalent to as much as 8% per decade. The Swiss alpine forests are dominated by conifers, covering 75–85% of the total forested area, with Norway spruce being by far the most abundant species (Brändli, 2010). We assume that (i) the investigated forest stands are reasonably representative of the new forest area, (ii) our flux measurements are a good estimate of soil $CH_4$ uptake for the snow-free period (May to October) and (iii) the difference in soil $CH_4$ uptake between pasture and 45 years old stands reflects the anticipated changes ($\Delta = 1.0$–$1.5$ $\mu$mol $CH_4$ $m^{-2}$ $h^{-1}$). Combining these data yields an increase in soil $CH_4$ uptake in the order of ~0.5–0.8 kg $CH_4$–C $ha^{-1}$ $yr^{-1}$ or ~50–70 t $CH_4$–C for the entire 90 000 ha area. Soil $CH_4$ uptake has been estimated at ~6000 t $CH_4$-C $yr^{-1}$ for Switzerland (Minonzio *et al.*, 1998). However, this figure is associated with a large uncertainty (minimum ~1000 t, maximum ~18 000 t $CH_4$ $yr^{-1}$) mainly due to a lack of data for forest soil $CH_4$ uptake. Our data thus suggest that the ongoing forest expansion in alpine areas increases the Swiss soil $CH_4$ sink by up to a few percent per decade.

In summary, our study shows increases in soil $CH_4$ uptake by a factor of two to three after conversion from subalpine pasture to forest. Our data indicate that the most likely reason for this change was shifts in the soil moisture balance due to increased interception and higher evapotranspiration in older forest stands. As a consequence, water-filled pore space decreased and the diffusion of atmospheric $CH_4$ into soils was facilitated. This mechanism contrasts alternative mechanisms suggested, including altered soil N status, altered soil structure or shifts in the methanotrophic community structure (Priemé *et al.*, 1997; Singh *et al.*, 2007; Christiansen & Gundersen, 2011).

## Acknowledgements

We gratefully thank R. Köchli, S. Fuchs and O. Schramm for field assistance and G.D. Lieberherr and D. Christen for assistance in the laboratory. We also thank four anonymous reviewers for their comments, which helped to improve this manuscript. This study was funded by the COST Action 639 (BurnOut) and the Swiss Federal Office for the Environment (FOEN).

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# General discussion

# Anthropogenic changes in soil $CH_4$ uptake

Any significant change in $CH_4$ sinks will affect the net accumulation of this greenhouse gas in the atmosphere (IPCC, 2013). Some studies have investigated effects of anthropogenic changes on the activity of methanotrophic bacteria in recent years (Zak et al., 1993; White et al., 2008; Pendall et al., 2011; Butler et al., 2012). The results they reported vary most likely due to different soil properties and conditions in different ecosystem types (Bai et al., 2013).

Soil $CH_4$ uptake can be affected by anthropogenic changes such as elevated air and soil temperatures and atmospheric $CO_2$ concentration and by changes in precipitation patterns and N deposition (Hütsch et al., 1994; Castro et al., 1995; Dunfield et al., 1995; King, 1997). Compared to $CH_4$ production, soil $CH_4$ uptake is relatively insensitive to temperature changes (Ding and Cai, 2003). Thus, $CH_4$ emission may increase faster than $CH_4$ uptake under climate change. In addition, elevated $CO_2$ can affect soil $CH_4$ uptake (in the range of -1 to +3 Tg $CH_4$ $yr^{-1}$). Ridgwell et al. (1999)) reported that results vary among studies (Angel et al., 2012; Dijkstra et al., 2011). Often, methanotrophic activity in upland soils is substrate limited and any change in $CH_4$ diffusion rate thus has the potential to influence the soil $CH_4$ sink strength (Czepiel et al., 1995; Menyailo, 2003; Luo et al., 2013). Consequently, changes in climate that alter precipitation have the potential to affect soil $CH_4$ uptake by soils (IPCC, 2007). Moreover, land use patterns explain some variations in soil $CH_4$ uptake among ecosystems. Natural ecosystems exhibited higher $CH_4$ uptake compared to agricultural fields (Powlson et al., 1997). However, deforestation and agricultural practices could decrease the soil $CH_4$ sink in the future (Young and Ritz, 2000). In summary, it is not well-understood to date how the future global $CH_4$ budget will be affected by changes in the activity of soil methanotrophic bacteria induced by anthropogenic disturbances.

I my thesis, I focused on effects of elevated temperature and atmospheric $CO_2$ concen-

tration on the activity and micro-distribution of methanotrophic bacteria. Furthermore, I studied changes in methanotrophic activity along an afforestation chronosequence in a sub-alpine region. Elevated soil temperatures in the alpine treeline ecosystem in the last 2 years of a 6-year soil warming treatment did not alter soil–atmosphere $CH_4$ fluxes (Chapter 1). Soil moisture and N content also were not affected by the soil warming treatment. In a grassland exposed to elevated $CO_2$ soil–atmosphere $CH_4$ fluxes remained unaffected, probably because soil moisture did not change (Chapter 2). In contrast, soil moisture was the main driver for soil $CH_4$ uptake in the afforestation chronosequence (Chapter 3), leading to higher soil $CH_4$ uptake in older forest stands, whereas soil N concentrations were not related to soil $CH_4$ uptake rate (Chapter 4).

## Elevated soil temperatures

Soil warming effects on methanotrophic activity vary among ecosystem types. Bai et al. (2013) showed in a meta-analysis that soil moisture was reduced by experimental soil warming in forests, grasslands and croplands but was unaffected in shrublands. Czepiel et al. (1995) suggested that organic matter explains variability in water content among different soils. Soil warming increased $CH_4$ uptake in soils (Hart, 2006) but had no effect in others (Rustad and Fernandez, 1998). Soil moisture regulates $CH_4$ diffusion into soil layers and affects $CH_4$ uptake rates. However, reduced soil moisture under elevated temperature increases $CH_4$ uptake rates. Our study in the alpine treeline (Chapter 1) showed that the soil warming treatment did not affect soil moisture and thus $CH_4$ fluxes remained unaffected. Soil warming reduced soil moisture slightly in the top-most soil layer, while soil moisture remained unaffected in the $CH_4$–assimilating soil horizon.

The importance of the increased soil temperature on $CH_4$ uptake mainly has been studied in ecosystem- levels. Process-based biogeochemistry studies modeled increased temperature effects in various ecosystem types to estimate global soil $CH_4$ uptake under global change. Estimates of warming effects on the global soil $CH_4$ sink vary among mod-

els. For example, Zhuang et al. (2013) used a process-based model to quantify soil $CH_4$ consumption during the 20th and 21st centuries. He attributed that a 3°C air temperature increase will lead to a 21-32% increase in global soil $CH_4$ uptake. In contrast, Ridgwell et al. (1999) used a biogeochemistry model and reported that $CH_4$ uptake by methanotrophic bacteria in soils is less sensitive to higher temperature compared to methanogenesis and suggested that global warming will not change the global soil $CH_4$ sink.

Soil moisture correlates with soil $CH_4$ consumption and controls $CH_4$ diffusion into soils. The magnitude of changes in soil moisture depends on range of precipitation changes in global change. Zhuang et al. (2013) factored a 15% change in annual precipitation in his model and showed that this amount of change in precipitation pattens did not affect soil moisture significantly and consequently the soil $CH_4$ consumption remained unaffected at the global scale. Changes in precipitation pattens is not a major factor in global soil $CH_4$ uptake during global change (Ridgwell et al., 1999; Zhuang et al., 2013).

Biogenic $CH_4$ sources (wetlands, rice paddies, landfills) are probably more sensitive to temperature changes compared to methanotrophic activity in upland soils. Any changes in climate variables like increased soil temperatures can increase $CH_4$ emissions from these sources (IPCC, 2007). Several studies show that $CH_4$ production is sensitive to increased temperature in wetlands (Dise et al., 1993; Wang et al., 1999; Bazhin, 2010; Das and Adhya, 2012). Chapman and Thurlow (1996) modeled responses of $CH_4$ emission from two wetland sites in Scotland under elevated temperature. These authors showed that $CH_4$ emission increased by 17, 30 and 60% if temperature increased by 1.5°C, 2.5°C and 4.5°C, respectively. Moreover, Christensen and Christensen (2003) reported that emissions increase if increased temperature is associated with increased net ecosystem production and precipitation, but that emissions decrease if elevated temperature leads to reduced precipitation or reduced net ecosystem production. In conclusion, these studies indicate that there are some uncertainties in effects of elevated air temperature on global $CH_4$ source and sink strength in future.

## Atmospheric $CO_2$

Elevated $CO_2$ reduced soil $CH_4$ uptake in some studies (McLain and Ahmann, 2008; Dubbs and Whalen, 2010). This effect was related to increased soil moisture due to reduced stomatal conductance and increased water use efficiency under elevated $CO_2$ concentration. In contrast, other studies did not revealed a relation between $CO_2$ concentration and soil $CH_4$ uptake (Smith et al., 2010; Lam et al., 2011). In our experiment, elevated $CO_2$ did not affect soil moisture and consequently soil $CH_4$ flux remained unaffected (Chapter 2). The water table in our study site was relatively close to the soil surface and there was $CH_4$ emissions that probably originated from the saturated zone. Soil moisture remained constant because plants could access water from deeper horizons.

Effects of elevated $CO_2$ concentrations on soil $CH_4$ uptake are not factored into many biogeochemical models. Consequently, effects of elevated $CO_2$ concentrations on changes in soil $CH_4$ uptake at the global scale are not estimated yet.

Elevated atmospheric $CO_2$ concentrations increases $CH_4$ emissions by 78% from wetlands (IPCC, 2007). Higher atmospheric $CO_2$ can reduce plant demand for water and increases soil moisture in wetlands. Moreover, it can lead to higher substrate availability for methanogens (IPCC, 2007). In other ecosystems, elevated $CO_2$ may not have an effect on $CH_4$ emissions. For example, Baggs and Blum (2004) reported that elevated $CO_2$ did not influence net $CH_4$ emission from a grassland. Generally, elevated $CO_2$ causes stronger effects on soil $CH_4$ emission in wetlands than in other ecosystems.

## Land use change

Plant cultivation decreases the rate of $CH_4$ consumption in soils (Arif et al., 1996; Boeckx et al., 1997). There is a range of mechanisms that can explain inhibitory effects of land use change on soil methanotrophic activity (Dunfield et al., 1995; Ball et al., 1999; Aronson et al., 2013). Some studies have shown that fertilizers applied to agricultural fields

decrease soil $CH_4$ oxidation through the competition of $NH_4^+$ and $CH_4$ for the active site of methane mono-oxygenase (MMO) or by changing the community structure of methanotrophic bacteria (Hütsch et al., 1994; Dunfield et al., 1995; Bodelier and Laanbroek, 2004). In Chapter 3 and 4, I investigated an afforestation chronosequence located in a sub-alpine region in the Canton of Fribourg, Switzerland, to understand how land use changes affect soil $CH_4$ uptake and the micro-distribution of methanotrophic bacteria. As it is shown in Chapter 4, in situ $CH_4$ uptake was higher in forest stands than in pasture soils and methanotrophic activity increased with forest stand age. I did not find a correlation between soil N content and methanotrophic activity in soils, but there was a direct relation between soil moisture and soil $CH_4$ uptake. Since soil $CH_4$ uptake was higher in forests than in pasture, afforestation could increase soil $CH_4$ uptake. In Switzerland, forest cover increased by about 8% per decade from 1984 to 2005 (Brändli, 2010), and consequently increased soil $CH_4$ uptake by 0.5-0.7 kg $CH_4$ $ha^{-1}$ $yr^{-1}$.

It is not clear yet how land use changes will affect global $CH_4$ sink in the future. In most process-based biogeochemistry models land use patterns (e.g. forests, agricultural fields, wetlands, deserts) are included in models but land use change (e.g. afforestation, agricultural abandonment, urbanization) effects in future are ignored to be included (Zhuang et al., 2004, 2013; Ghosh et al., 2015). For example, Zhuang et al. (2013) used land use patterns in their model and estimated that agricultural soils oxidized 5.13 Tg $CH_4$ $yr^{-1}$ in 20th century. These authors showed that the global soil consumption varied among ecosystem types and that more than 80% of the global soil $CH_4$ uptakes occurred in natural ecosystems. Temperate forests and grassland were moderate sink and took up 5.47 and 0.81 Tg $CH_4$ $yr^{-1}$ respectively. Woodlands and shrublands accounted for more than 25% of the global soil $CH_4$ uptake. Deserts consumed 1.5 Tg $CH_4$ $yr^{-1}$.

In addition, land use changes such as the expansion of rice paddies, man-made lakes and wetlands and landfills have the potential to increase $CH_4$ emissions and affect global $CH_4$ budget. These land use changes should be included in future biogeochemical models

for more accurate quantification in earth system modeling (IPCC, 2013).

## Projections of future soil $CH_4$ uptake

Biogeochemistry models have been used to quantify global sources and sinks of atmospheric $CH_4$ (Zhuang et al., 2004, 2013; Ghosh et al., 2015). Oxidation by hydroxyl radicals (OH), loss to the stratosphere, and biological $CH_4$ oxidation by soil methanotrophic bacteria are major $CH_4$ sinks (IPCC, 2013). Effects of environmental changes on consumption of atmospheric $CH_4$ by methanotrophic bacteria were analyzed in some studies (Hütsch et al., 1993; King and Schnell, 1998; Conrad, 2009; Bai et al., 2013).

Biogeochemistry models do not factor in all major drivers of soil $CH_4$ uptake. For example, Ridgwell et al. (1999) included effects of changes in atmospheric $CH_4$ concentration, land use, and climate in their model but did not include N deposition. In contrast, Zhuang et al. (2013) included N deposition in their model and showed that it had a significant effect on global soil $CH_4$ uptake. Ghosh et al. (2015) showed that changes in $CH_4$ sink due to increased temperature and losses by reaction with Cl in the stratosphere are important source of uncertainty in estimation of global $CH_4$ budget.

In a biogeochemistry model by Zhuang et al. (2013), some factors such as nitrogen deposition, rising atmospheric concentration and agricultural land use were modeled. The authors indicated that N deposition changes played a minor effect in determining $CH_4$ consumption at the global scale but that land use was a moderate important. They estimated that natural ecosystems were a major sink during the 1990s (32-36 Tg $CH_4$ $yr^{-1}$) while agricultural ecosystems oxidized only 5.13 Tg $CH_4$ $yr^{-1}$. They predicted that during the 21st century global soil $CH_4$ consumption would increase. In that model arid areas (deserts, shrublands and woodlands) consistently took up $CH_4$ and boreal ecosystems became stronger sinks due to increasing soil temperatures. Nitrogen deposition marginally reduced the future sink strength at the global scale.

The ecological niche of soil methanotrophic bacteria can be affected by global change

and thus alter soil $CH_4$ fluxes at ecosystem-levels. It is important to study changes in the spatial niche of methanotrophic bacteria in soils to find the relation between micro-distribution of methanotrophic bacteria and soil $CH_4$ uptake. Understanding the mechanism of changes in micro-distribution of methanotrophic bacteria in soils might help to resolve conflicting published results about methanotrophic activity among different ecosystems. Methanotrophic activity in soils mainly have been investigated at ecosystem-levels or at bulk soil-levels (in the laboratory). However, effects of soil structure and spatial niche of methanotrophic bacteria have not received sufficient attention to date. Developing a detailed understanding of the spatial distribution of methanotrophic bacteria in global change is needed in the future investigations.

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# Acknowledgments

Thanks to my supervisor, Pascal A. Niklaus, for giving me the opportunity to work in this project and for his patient instructions in writing my papers and analysing data. Thank you to my PhD committee members, Bernhard Schmid, Michael Schmidt and Martin Schroth for their advice and time investments. To Frank Hagdedorn for his advices and supports in field works. To Gerald Moser and David Hiltbrunner for all advices and practical helps in soil samplings. To Isabel Schöchli and Shelina Momin for their administrative supports in University of Zürich and Aga Khan Foundation, respectively. Thanks to my fellow PhD students for their good company and encouragements. All the people in IEU, thank you.

I want to thank my parents for their moral support and encouragement. My sister and brothers, thanks for their supports to achieve my goal. And last but not least, Maryam for her support and practical help, my most gratitude.

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- **Karbin,S**., Guillet, C., Kammann I.K., and Niklaus P.A., (2015), Effects of long-term $CO_2$ enrichment on soil-atmosphere $CH_4$ fluxes and the spatial micro-distribution of methanotrophic bacteria, PLoS ONE, in press

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