PLOS ONE

RESEARCH ARTICLE

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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OPEN ACCESS

Citation: Karbin S, Guillet C, Kammann CI, Niklaus PA (2015) Effects of Long-Term CO_2 Enrichment on Soil-Atmosphere CH_4 Fluxes and the Spatial Micro-Distribution of Methanotrophic Bacteria. PLoS ONE 10(7): e0131665. doi:10.1371/journal.pone.0131665

Editor: Riikka Rinnan, University of Copenhagen, DENMARK

Received: April 7, 2015

Accepted: June 4, 2015

Published: July 6, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: SK has been funded by a stipend of Aga Khan Foundation. PAN supported the study from regular funds from the University of Zurich. The Hessian Agency for the Environment and Geology (HLUG) contributed to the running costs of the Giessen Free Air CO2 Enrichment. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

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.

Methods and Results

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 less than 5× 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.

Conclusions

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 autoradiographic 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.

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 [1] (but see [2, 3]). 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 [4], which then in turn will oxidize larger amounts of atmospheric CH_4 once the soil-internal sources cease [5]. 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 [5].

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 [6]. However, moisture can also limit methanotrophic activity due to physiological stress [7]. 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 [8]. Finally, inhibition of methanotrophic activity does not necessarily translate into reduced soil CH_4 uptake. [9] 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 [10, 11]. 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 [12–15], 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 [16]. 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 [17], some organic acids [18], and terpenes [19].

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 [20]. 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?

Methods

Study site and experimental design

We studied effects of long-term elevated atmospheric CO_2 on soil-atmosphere CH_4 fluxes and the micro-distribution of methanotrophic bacteria in a permanent grassland near Giessen, Germany (50°32' N and 8°41.3' E, 172 m a.s.l.). For at least the past 50 years, the site has been permanent grassland fertilized with 50–80 kg N $ha^{-1.}$ From 1995 onwards, fertilization was reduced to 40 kg N ha^{-1} a^{-1} (see [20] 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. [21] and contains about 60 vascular plant species [20]. 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 [20].

In situ soil-atmosphere CH_4 fluxes

From 2009 to 2012, we measured soil-atmosphere CH_4 fluxes on a total of 191 days *in situ* with large static chambers (94 cm inner diameter, ca. 160L volume; modified according to [22]; for further details see [7]). 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 fulfil 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 CO2 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 x 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. The jars were ventilated under ambient conditions, and headspace CH_4 concentration determined 0, 2, and 4 h after closing of the lids. CH_4 uptake rates were calculated by linear regression against sampling time.

Radiolabelling 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 labelled with $^{14}CH_4$. Two soil cores per plot and sampling date were labelled at slightly above-ambient CH_4 concentrations (max. 10 ppm). Two additional soil cores from the July 6, 2011 sampling were labelled 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. 100kBq was applied over a period of 6 days. Plastic tubes with 100 mL 1M NaOH were placed in each jar to trap CO_2 (incl. $^{14}CO_2$) 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 [9]. 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 levelled 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 levelled 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 (S1 Dataset) 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±4.7 and 28.6±5.2 μmol CH_4 m^{-2} d^{-1} in ambient and elevated CO_2, respectively). During periods in which "bubble emissions" occurred (Fig 2A, grey 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 and 2C). 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.

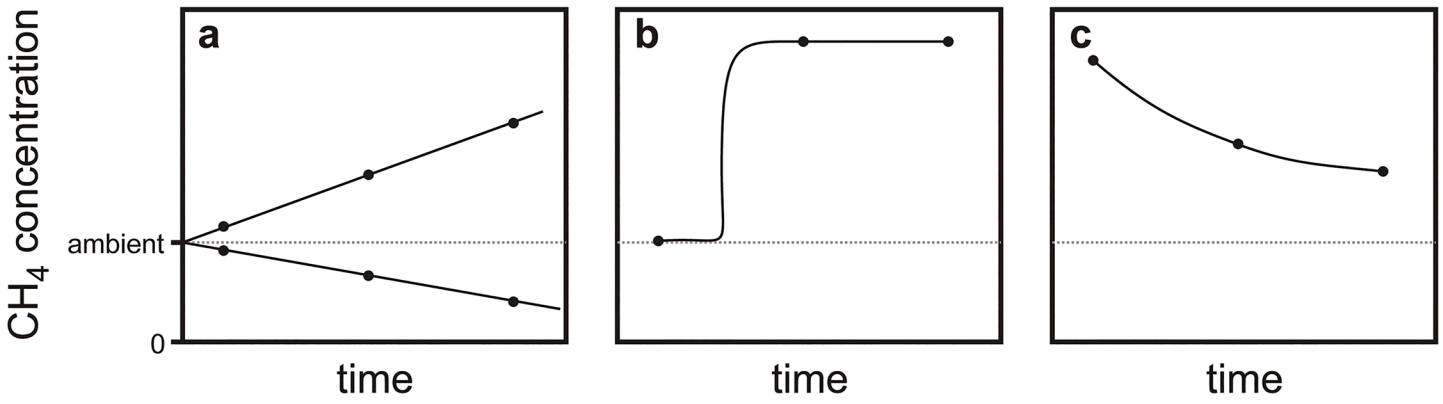

Fig 1. Typical time-courses of CH_4 concentrations during static chamber sampling. (a) Linear concentration changes with time, indicating continuous soil CH_4 uptake or release. (b) Step-increase in CH_4 concentration, likely caused by emission bursts that could originate from ebullition from the underlying 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.

doi:10.1371/journal.pone.0131665.g001

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).

$^{14}CH_4$ labelling of soil cores

Visual inspection of autoradiographies revealed heterogeneous label assimilation, with distinct zones of enhanced net CH_4 assimilation (Figs 5,6 and 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 labelling of the uppermost layer on October 25 when labelled at high CH_4 concentration. Since the analysis of depth x CO_2 treatment includes some degree of autocorrelation of residuals between soil layers, we calculated mean oxidation depth per soil core as

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

i.e. as activity-weighted mean depth of net CH_4 assimilation (Table 1); figuratively, this is the depth of the center of gravity an activity depth profile. Mean assimilation depth averaged 3.8 cm, irrespective of CO_2 treatment and labelling concentration. 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

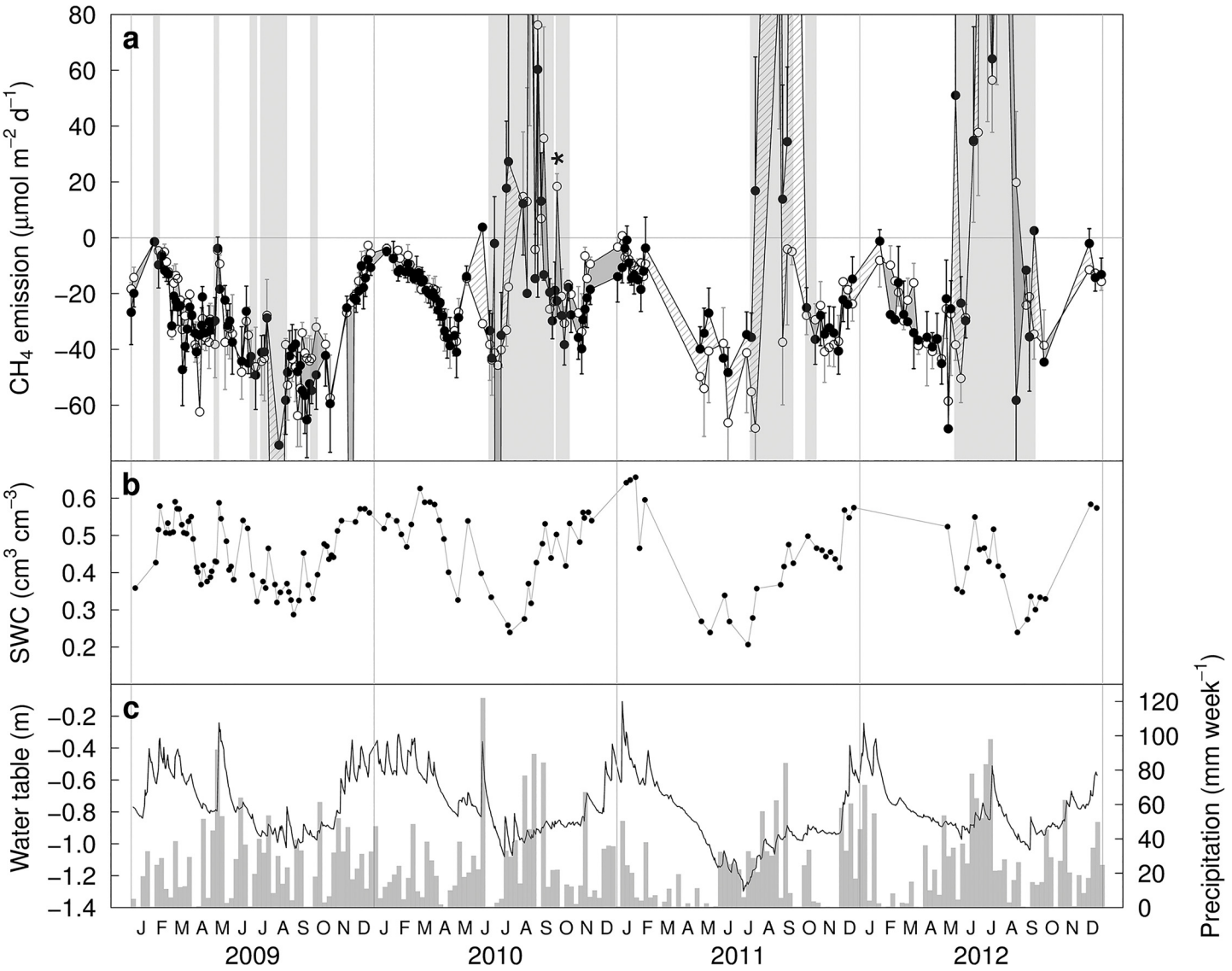

Fig 2. CH_4 fluxes and related environmental data. (a) CH_4 emission rates in ambient (○) and elevated CO_2 (●) plots, calculated when concentration changes were linear (mean ± s.e., n≤3 per CO_2, depending on the number of plots with emissions following the pattern of Fig 1A). Effects of elevated CO_2 were not statistically significant. Periods during which emissions occurred (Fig 1B and 1C) 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.

doi:10.1371/journal.pone.0131665.g002

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 [23, 24] 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 [25]. 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 [26]. Interestingly, in semi-arid grassland, opposite effects of elevated CO_2 were found when soils were dry

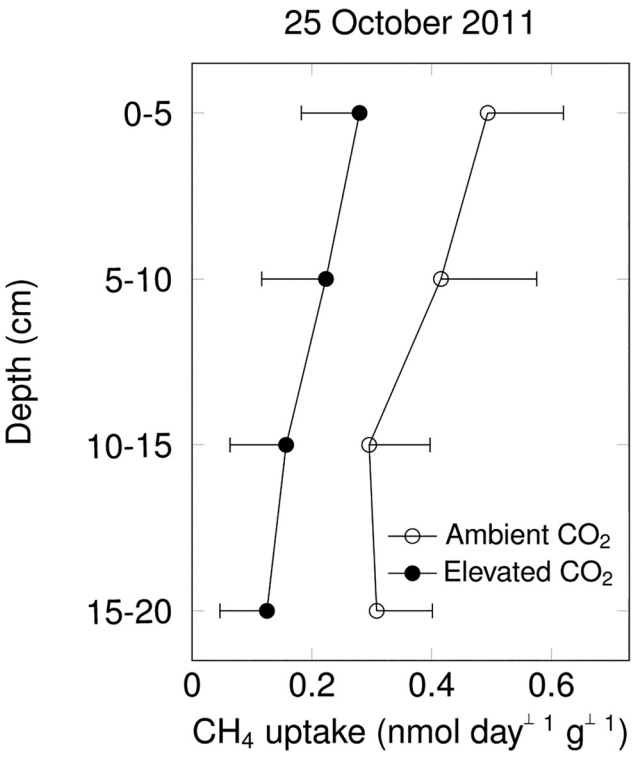

Fig 3. Net CH_4 uptake rates of sieved field-moist soil incubated at 20°C in the laboratory (mean ± s.e., by 5cm soil layer; n = 3 per CO_2 treatment; effects of elevated CO_2 were not statistically significant).

doi:10.1371/journal.pone.0131665.g003

[27]; 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, [28] found reduced CH_4 uptake under elevated CO_2 in a mixed *Lolium/Trifolium* sward, and this effect was

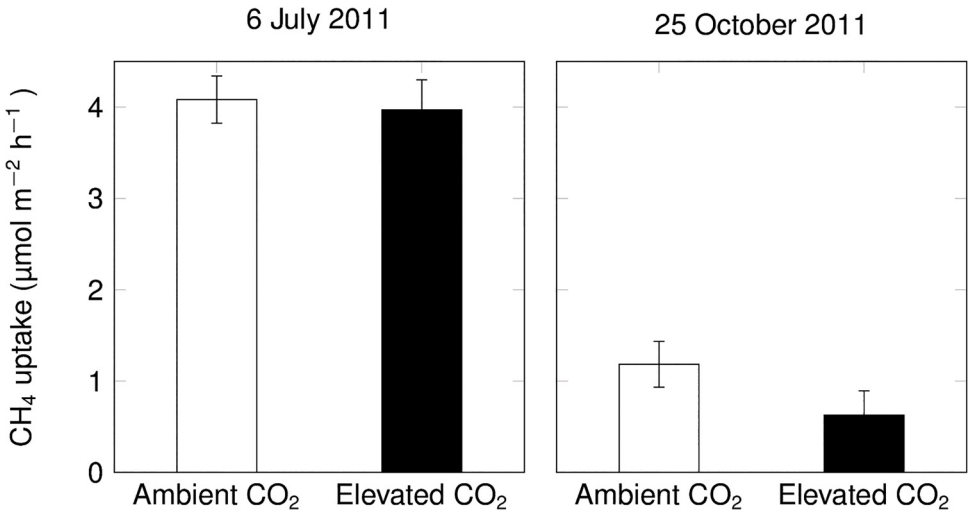

Fig 4. Net CH_4 uptake rates of intact soil cores collected in ambient and elevated CO_2 plots and incubated in the laboratory at 20°C (mean ± s.e., n = 3 per CO_2 treatment; effects of elevated CO_2 were not statistically significant).

doi:10.1371/journal.pone.0131665.g004

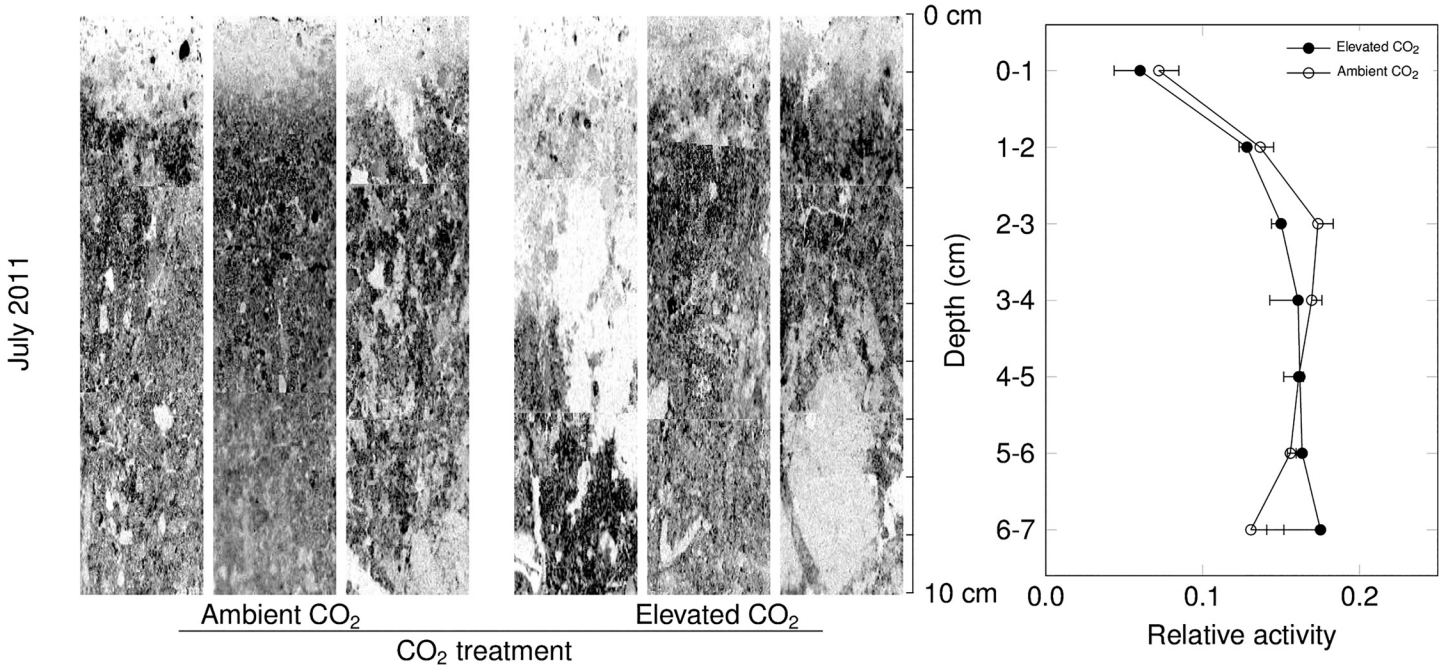

Fig 5. Soil micro-autoradiography of typical soil sections collected on June 6, 2011, and incubated under near-ambient CH_4 concentrations. Darker pixels indicate higher labelling. Vertical profiles of labelling (right panel), aggregated by 1cm depth intervals (mean ± s.e., n = 3 per CO_2 treatment). Effects of elevated CO_2 were not statistically significant.

doi:10.1371/journal.pone.0131665.g005

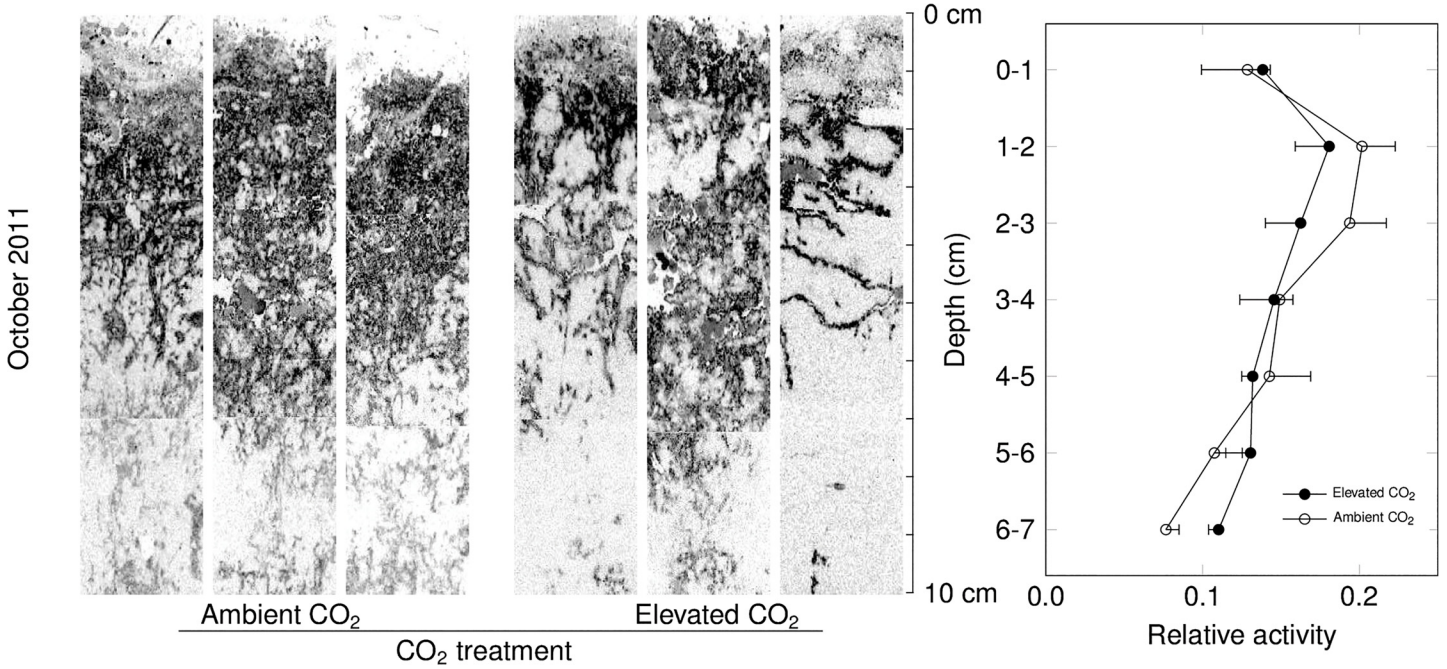

Fig 6. Soil micro-autoradiography of typical soil sections collected on October 25, 2011, and incubated under near-ambient CH_4 concentrations. Darker pixels indicate higher labelling. Vertical profiles of labelling (right panel), aggregated by 1 cm depth intervals (mean ± s.e., n = 3 per CO_2 treatment). Effects of elevated CO_2 were not statistically significant.

doi:10.1371/journal.pone.0131665.g006

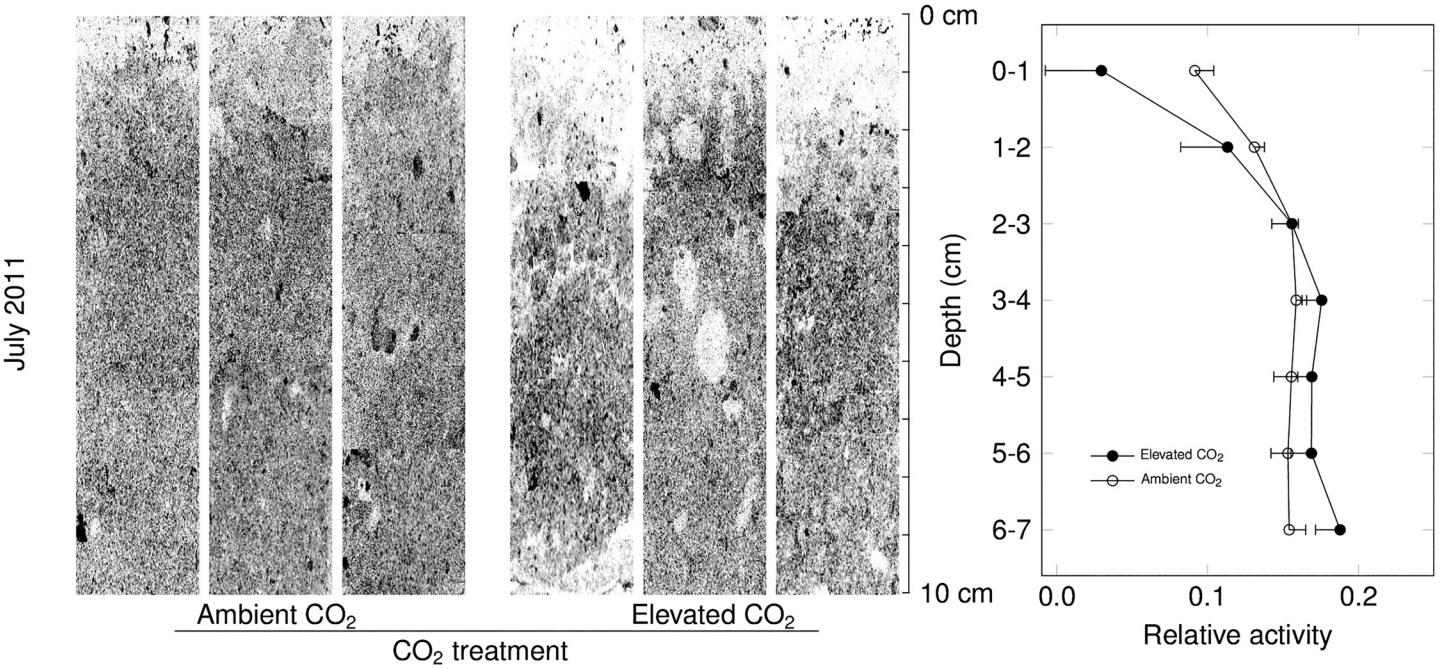

Fig 7. Soil micro-autoradiography of typical soil sections collected on October 25, 2011, and incubated under CH_4 concentrations around 10000 ppm. Darker pixels indicate higher labelling. Vertical profiles of labelling (right panel), aggregated by 1cm depth intervals (mean ± s.e., n = 3 per CO_2 treatment). Elevated CO_2 marginally significantly affected the depth distribution of methanotrophic activity (P = 0.06 for depth × CO_2).

doi:10.1371/journal.pone.0131665.g007

unrelated to soil moisture. Finally, CH_4 uptake and CO_2 concentration were unrelated in a number of other studies (wheat: [29], Sorghum and soybean: [30]; shortgrass steppe: [31]). We observed a median net soil CH_4 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 CO_2 studies, but not atypical when compared to temperate grassland fluxes reported in an European [32] or global analysis [33]. 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 [34]. 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 [35]. In

Table 1. 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 CH4 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 ± 0.07
	10	elevated CO_2	4.00 ± 0.06
25 Oct 2011	10000	ambient CO_2	3.81 ± 0.08
	10000	elevated CO_2	4.24 ± 0.42
	10	ambient CO_2	3.40 ± 0.19
	10	elevated CO_2	3.45 ± 0.17

doi:10.1371/journal.pone.0131665.t001

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 travelled 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: [36]; CO_2 fluxes: [37]), 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 [18, 19]. 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 incubations of soil cores taken from the site have previously shown that these larvae can release large amounts of CH_4 [38], 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 [39], but it has also been argued that these organisms may be less distinct than previously thought [40]. Indeed, methanotrophs capable to adapt physiologically to environments differing in CH_4 supply have been found [5], and some possess of isoenzymes differing in kinetic properties [41]. 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 labelling 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 [42]. 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 [43] and in two drought studies [9]. Labelled 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 micro-distribution 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 [38] and possibly fungi [44], 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

Supporting Information

S1 Dataset. Methane flux data presented in this article. A detailed description of the data is contained in the file.
(ZIP)

Acknowledgments

This work was funded by a personal stipend of the Aga Khan Foundation (AKF) to SK and the University of Zürich. We gratefully acknowledge Gerald Moser for assistance in soil sampling, Ludger Grünhage 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) to the running costs of the FACE experiment is gratefully acknowledged.

Author Contributions

Conceived and designed the experiments: PAN CIK. Performed the experiments: SK CG. Analyzed the data: SK CG PAN. Wrote the paper: SK CG CIK PAN.

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