RESEARCH ARTICLE

# Long-term $CO_2$ injection and its impact on near-surface soil microbiology

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One sentence summary: Impacts of elevated $CO_2$ on soil microbiology.

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## ABSTRACT

Impacts of long-term $CO_2$ exposure on environmental processes and microbial populations of near-surface soils are poorly understood. This near-surface long-term $CO_2$ injection study demonstrated that soil microbiology and geochemistry is influenced more by seasonal parameters than elevated $CO_2$. Soil samples were taken during a 3-year field experiment including sampling campaigns before, during and after 24 months of continuous $CO_2$ injection. $CO_2$ concentrations within $CO_2$-injected plots increased up to 23% during the injection period. No $CO_2$ impacts on geochemistry were detected over time. In addition, $CO_2$-exposed samples did not show significant changes in microbial $CO_2$ and $CH_4$ turnover rates compared to reference samples. Likewise, no significant $CO_2$-induced variations were detected for the abundance of Bacteria, Archaea (16S rDNA) and gene copy numbers of the mcrA gene, Crenarchaeota and amoA gene. The majority (75%–95%) of the bacterial sequences were assigned to five phyla: Firmicutes, Proteobacteria, Actinobacteria, Acidobacteria and Bacteroidetes. The majority of the archaeal sequences (85%–100%) were assigned to the thaumarchaeotal cluster I.1b (soil group). Univariate and multivariate statistical as well as principal component analyses showed no significant $CO_2$-induced variation. Instead, seasonal impacts especially temperature and precipitation were detected.

Keywords: CCS; $CO_2$-leakage; Bacteria; Archaea; qPCR; pyrosequencing

## INTRODUCTION

Rising anthropogenic greenhouse gas emissions into the atmosphere will lead to negative climate impacts and enormous environmental, economic and social consequences (Solomon et al. 2007). Geological sequestration of captured and compressed $CO_2$ (carbon capture and storage; CCS) may be one option to reduce the atmospheric concentration of this important greenhouse gas. The major risk associated with geologic CCS is the possibility of $CO_2$ leakage from deep storage reservoirs. To implement CCS, investigation of possible consequences of $CO_2$ leakage is needed. Thus, artificial $CO_2$ injection sites were

established for deep monitoring of geochemical reactions within storage sites and possible impacts of $CO_2$ leakages (Class *et al.* 2009; Jenkins, Chadwick and Hovorka 2015). Near-surface monitoring has focused mainly on soil gas and geochemical alterations (Little and Jackson 2010; Beaubien *et al.* 2013). Few studies have monitored environmental responses in near-surface soils, e.g. the ZERT facility in MT, USA (Morales and Holben 2013, 2014) or the CIUDEN facilities at Cubillos del Sil (Spain) (Fernández-Montiel *et al.* 2015). Leakage of $CO_2$ could conceivably cause changes in soil microbial communities or plant–microbe interactions. So far, most of the information about near-surface $CO_2$–soil–plant–microbe interactions has been gained with natural $CO_2$ vents (e.g. Beaubien *et al.* 2008; Oppermann *et al.* 2010; Sáenz de Miera *et al.* 2014; Beulig *et al.* 2015; Fernández-Montiel *et al.* 2015, 2016; Fernández-Montiel, Pedescoll and Bécares 2016).

With our project at the ASGARD (Artificial Soil Gassing and Response Detection) field site at the University of Nottingham, we aimed to understand the environmental effects of long-term $CO_2$ injection. $CO_2$-injected and control plots were monitored for 3 years with 24 months of continuous $CO_2$ injection and a subsequent recovery phase of 5 months. This first, near-surface long-term $CO_2$ injection study presents geochemical and microbiological results from sampling campaigns before, during and after 24 months of $CO_2$ injection. In contrast to previous short-term studies of $CO_2$-injected sites or investigations at natural $CO_2$ vents, we link our microbiological findings to geochemistry and seasonal conditions during long-term $CO_2$ injection and monitoring.

## MATERIALS AND METHODS

### Site description

The ASGARD site is located in the University of Nottingham's Sutton Bonington Campus, south of central Nottingham (Fig. S1a, Supporting Information). Previously it was used as sheep pasture and had remained grassland for more than 10 years. The ASGARD area is geologically characterised by up to 1.5 m of head deposit overlying mudstones of the Mercia Mudstone Group. Previous analyses of the mineralogical composition showed that quartz was the main component of the soil (>90% of the dry weight) followed by K-feldspar and albite along with trace amounts of mica, kaolinite, chlorite and hematite. The top soil layer (~0.1 m) contains 8.9% clay, 22.9% silt and 68.2% sand, with no differences between the A horizon (0.15–0.30 m depth) and B horizon (0.45–0.50 m depth) (West *et al.* 2009). For a detailed geological description of the ASGARD facility, see also Ford (2006).

The facility consisted of 30 $CO_2$-injected and reference plots (15 and 15), each with an area of 2.5 × 2.5 m (Fig. S1b and c, Supporting Information). Ten plots were kept as pasture for different experiments. The other plots were planted with different agricultural crops. $CO_2$ was injected into the centre of the plots through permanently installed pipework at a depth of 50–60 cm below ground level. Non-gassed but otherwise identically treated plots were used as references. Previous short-term, intermittent $CO_2$-injection periods took place between 2006 and 2008 with both 1 and 3 L $CO_2$ min$^{-1}$ (Pierce and Sjögersten 2009; West *et al.* 2009).

For the present study, $CO_2$ was continuously injected into one pasture plot at a constant flow rate of 1 L min$^{-1}$ (~1 ton y$^{-1}$) from October 2010 to May 2012 (Smith *et al.* 2013). One ungassed plot was used as reference.

### Sampling procedure

For our study, samples were taken before, during and after $CO_2$ injection from 2010 to 2012 twice a year (May and October) at 15–30 cm depth, in the middle of the investigated $CO_2$-injected and reference pasture plot. The samples were stored at 8°C and transported to the laboratory as fast as possible. Collected soil samples from both plots in each sampling campaign were homogenised, and subsamples were taken for geochemical (stored at 4°C), biomolecular (stored at –20°C) and microbial activity analyses (incubated at 20°C).

### Meteorology

Climate data including averages of temperature, rainfall and irradiance each month for the years 2010–2012 were recorded at Sutton Bonington weather-monitoring station located at the Nottingham University (http://www.metoffice.gov.uk/climate/uk/summaries).

### Soil geochemistry

Concentrations of $CO_2$, $CH_4$ and $O_2$ were determined using portable infrared landfill gas analysers GA2000 and GA5000 (Geotech, Warwickshire, UK). In addition, soil $CO_2$ flux measurements were taken during the sampling campaigns using a portable flux meter equipped with an infrared $CO_2$ sensor connected via Bluetooth to a palm-top computer (West Systems, Firenze, Italy). Soil moisture was determined after each sampling campaign prior to the biogeochemical analyses by drying 1 g of wet soil sample at 60°C to constant weight. In addition, soil carbon content (total carbon; TC) was analysed using a LECO CS 230 analyser (LECO Corporation, St. Joseph, USA). To obtain the total organic carbon (TOC), inorganic carbon was removed by adding a 10% solution of hydrochloric acid to the soil samples. Trace elements were analysed by X-ray fluorescence spectrometry as previously described by Shibata *et al.* (2009). All samples were analysed in triplicates.

### Microbial $CO_2$ and $CH_4$ turnover

To study the influence of $CO_2$ on microbial activity, aerobic and anaerobic microcosms were prepared using $CO_2$-induced and reference soil samples of each sampling campaign. For the investigation of aerobic mineralisation rates, glass bottles flushed with sterile-filtered, synthetic air were filled with 3 g soil. For the determination of $CH_4$ oxidation rates, 5 vol.% $CH_4$ was added to the microcosms and its consumption was measured over the course of the experiment. In addition, anaerobic respiration and $CH_4$ production rates were measured. Microcosms were prepared with 5 g soil and flushed with sterile-filtered nitrogen to remove $O_2$. Microcosms were prepared in triplicates and incubated at 20°C. Microbial activities were detected measuring $CO_2$ and $CH_4$ using a gas chromatography including a flame-ionisation detector (GC-FID) gas chromatographer (SRI 8610C, SRI Instruments, Torrance, CA, USA) equipped with a methanizer as described in Nauhaus *et al.* (2002). The rates were calculated in $\mu$mol gdw$^{-1}$ soil d$^{-1}$.

### DNA extraction

Total DNA was extracted from frozen soil samples (–20°C) in duplicates following the manufacturer's manual of the FastDNA Spin Kit for soil (Bio 101; MP Biomedicals).

## Quantitative PCR analyses

Quantitative PCR (qPCR) was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction contained 5 $\mu$l TaqMan 2$\times$ Universal PCR Master Mix (NoAmpEraseUNG, Applied Biosystems), 0.4 $\mu$l PCR primers and probe for *Bacteria* (2.5 $\mu$M each) and *Archaea* (20 $\mu$M primers and 5 $\mu$M probe), 2.3 $\mu$l sterile water, 0.5 $\mu$l bovine serum albumin (BSA) (20 mg/ml) and 1 $\mu$l 16S rDNA template added to a final volume of 10 $\mu$l. Samples were analysed for total numbers of *Bacteria*, *Archaea* and *Crenarchaeota* as well as several functional groups including ammonia-oxidising *Archaea* (AOA) and *Bacteria* (AOB) and methanogens (methyl coenzyme-M reductase; *mcrA*). The qPCR assays were calibrated by 10-fold serial dilutions of PCR-amplified pure standards and PCR-amplified, non-target 16S rDNA gene amplicons (environmental archaeal and bacterial *amoA* sequence mixtures) of the relevant target gene. Microbial primers, standards and description of the qPCR specifications can be found in Table S1 (Supporting Information). Overall, nine replicates (3 replicates $\times$ 3 dilutions; 10-, 100- and 1000-fold) of each sample were analysed. Amplification efficiencies were calculated from the StepOne v2.1 software on the basis of threshold cycle versus gene abundance of standards according to the relationship E = 10(–1/slope). Amplification efficiencies ranged between 80% and 100%. The qPCR amplifications were analysed with the StepOne v2.1 software, and the results are expressed in gene copies per gram dry weight.

## Clone library construction

PCR amplicons of bacterial and archaeal 16S rDNA genes were generated using 1:10 or 1:100 dilutions of each soil DNA extract. Bacterial 16S rDNA genes were amplified by using primer sets 27f (5′-AGRGTTYGATYMTGGCTCAG-3′)–1392r (5′-ACGGGCGGTGTGTRC-3′) and 109f (5′-ACKGCTCAGTAACACGT-3′)–915r (5′-GTGCTCCCCCGCCAATTCCT-3′), respectively (Lane 1991). For the Archaea, the universal archaeal primers 109f (5′-ACK GCT CAG TAA CAC GT-3′) (Whitehead and Cotta 1999) and 915r (5′-GTG CTC CCC CGC CAA TTC CT-3′) (Stahl and Amann 1991) were used. The PCR reactions contained 25 $\mu$l DreamTaq PCR Master Mix (2$\times$) (Thermo Scientific), 0.3 $\mu$M of each primer, 4 $\mu$l template DNA and nuclease-free water up to 50 $\mu$l. The PCR conditions for the bacterial primer pair were as follows: initial denaturation for 5 min at 95°C followed by 25 cycles of 30 s at 94°C, 30 s at 52°C and 75 s at 72°C, and a final extension at 72°C for 20 min. The PCR conditions for the archaeal primer pair were as follows: initial denaturation for 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 52°C and 90 s at 72°C, and a final extension at 72°C for 10 min. Triplicate PCRs were performed for each soil DNA extract. Purification, cloning and sequencing of the 16S rDNA PCR products were conducted at LGC Genomics GmbH (LGC Genomics GmbH, Berlin, Germany) and Microsynth AG (Microsynth AG, Balgach, Switzerland).

## Phylogenetic analyses

Chimeras were identified and removed using Geneious R7 software and DECIPHER (Wright, Yilmaz and Noguera 2012). Sequences with mismatches or gaps were removed using MOTHUR (Schloss *et al.* 2009). For clustering the sequences into operational taxonomic unit (OTUs) and additional analysis of community structure, MOTHUR was used (Schloss *et al.* 2009). Representatives of each OTU at the 0.03 distance level were aligned and classified with the ARB-SILVA aligner, and assembled in the existing parsimony tree of the SILVA reference database SSURef 118 (Pruesse, Peplies and Glöckner 2012). The phylogenetic trees for *Archaea* and *Bacteria* were calculated using ARB software tools (Ludwig *et al.* 2004).

## Statistical analyses

To determine whether $CO_2$, seasonal or interannual alterations affected soil properties and soil microbial activities, two-way analysis of variance (ANOVA) was used. Non-metric multidimensional scaling (MDS) plots, based on distance matrixes, were generated for visualisation and interpretation of $CO_2$ impacts for bacterial and archaeal communities. Analysis of similarity (ANOSIM) was performed to detect differences of the bacterial and archaeal communities between the $CO_2$-injected and reference samples. Permutational multivariate analysis of variance (PERMANOVA) was used to determine whether $CO_2$, seasonal or interannual alterations affected bacterial and archaeal communities with 9999 permutations. Similarity percentage (SIMPER) analysis was performed to identify the taxa that contributed most of the variance among $CO_2$-injected and reference samples. MDS as well as analyses of similarity and variance base on the Bray–Curtis dissimilarity index. Analyses of MDS, ANOVA and multivariate statistics were performed in PAST (Hammer, Harper and Ryan 2001). For the archaeal clone libraries, the calculation of diversity (Shannon) and richness (Chao) indices was generated using MOTHUR (Schloss *et al.* 2009). For the illustration of correlations between climate or geochemical data, archaeal sequences and $CO_2/O_2$ concentrations, principal component analysis (PCA) was conducted using reshape2 and ade4 packages in R (Dray and Dufour 2007; Wickham 2007; Team 2012).

# RESULTS AND DISCUSSION

## Soil gas measurements and $CO_2$ impacts on soil geochemistry

During the 24 months of $CO_2$ injection at the ASGARD site, soil geochemical investigations were conducted to determine the concentration and flux of soil gases as well as soil pH, mineralogy and C content (Table 1). Before and after the first 4 months of $CO_2$ injection, no significant differences in soil gas concentrations and flux rates between $CO_2$-exposed and reference plots were detected (Table 2). The subsequent measurements at 10, 16 and 24 months of $CO_2$ injection showed increasing $CO_2$ and decreasing $O_2$ concentrations within the upper 15–30 cm of the soil (Fig. 1). A $CO_2$ maximum of 23% in soil gas was detected after 24 months of gassing (May 2012). Five months after the stop of $CO_2$ injection (October 2012), the $CO_2$ and $O_2$ values were comparable to those before $CO_2$ injection ($CO_2$ concentration: 0.2%–4%) (Fig. 1). Within the reference plots, $CO_2$ and $O_2$ concentrations remained relatively stable between 0.2%–1.3% $CO_2$ and 19.7%–22% $O_2$ over the 3-year observation period. Previous soil gas measurements within the CCS project CLEAN in Germany and at the ZERT field site in MT, USA (Lewicki *et al.* 2010; Schlömer, Möller and Furche 2014) observed highly variable $CO_2$ concentrations between 0.2% and 19% $CO_2$, quite similar to the $CO_2$ concentrations detected at the ASGARD site for both $CO_2$-injected and reference plots. A significant relationship between $CO_2$ injection at ASGARD and alterations in geochemistry was not detected (ANOVA, $P > 0.05$; Table S3, Supporting Information). The soil pH measurements

**Table 1.** Soil $CO_2$ flux, atmospheric conditions (temperature, irradiance, rainfall), soil moisture and soil carbon content for $CO_2$-exposed and reference samples over time ($n = 1$ for each sampling campaign). Soil samples of the $CO_2$-exposed plot were taken before $CO_2$ injection (May 2010), after 4, 10, 16 and 24 months of $CO_2$ injection and after $CO_2$ injection (October 2012).

| Samples/time point | $CO_2$ flux [g/mq/day] | Temperature [°C] | Irradiance [kJ/m$^2$] | Rainfall [mm$^1$] | Moisture [%] | TC [%] | TOC [%] |
|---|---|---|---|---|---|---|---|
| $CO_2$-exposed site | | | | | | | |
| Pre-gassing, May 2010 | 21.26 | 15.84 | 17959.73 | 0.59 | 8.09 | 2.38 | 2.30 |
| Oct 2010 (4 months) | 15.29 | 14.06 | 6136.18 | 1.60 | 11.92 | 1.97 | 1.64 |
| May 2011 (10 months) | 195.59 | 17.05 | 17904.09 | 1.25 | 10.71 | 1.87 | 1.76 |
| Oct 2011 (16 months) | 748.28 | 16.85 | 6685.11 | 1.35 | 6.53 | 2.38 | 2.20 |
| May 2012 (24 months) | 630.74 | 16.34 | 17720.01 | 0.92 | 8.12 | 2.00 | 1.79 |
| Post-gassing, Oct 2012 | 29.24 | 12.94 | 6252.36 | 1.61 | 15.57 | 2.04 | 1.86 |
| Reference site | | | | | | | |
| May 2010 | 17.64 | 15.84 | 17959.73 | 0.59 | 7.59 | 2.54 | 2.39 |
| Oct 2010 | 11.57 | 14.06 | 6136.18 | 1.60 | 13.90 | 2.03 | 1.96 |
| May 2011 | 18.75 | 17.05 | 17904.09 | 1.25 | 8.74 | 2.33 | 2.20 |
| Oct 2011 | 27.78 | 16.85 | 6685.11 | 1.35 | 8.21 | 2.13 | 2.01 |
| May 2012 | 33.68 | 16.34 | 17720.01 | 0.92 | 9.31 | 2.22 | 2.13 |
| Oct 2012 | 34.00 | 12.94 | 6252.36 | 1.61 | 15.92 | 1.88 | 1.68 |

**Table 2.** qPCR analysis of the microbial community composition in $CO_2$-exposed and reference samples over time using 16S rRNA genes and functional genes (*mcrA*, *amoA*) ($\pm$SD; $n = 3$ samples; 9 measurements per sample; nd: not detected). Soil samples of $CO_2$-exposed plots were taken before $CO_2$ injection (May 2010), after 4, 10, 16 and 24 months of $CO_2$ injection and after $CO_2$ injection (Oct 2012). Results are expressed in gene copies per gram dry weight (gene copies gdw$^{-1}$).

| Sample | *mcrA* gene | *Crenarchaeota* | Archaeal *amoA* | Bacterial *amoA* |
|---|---|---|---|---|
| $CO_2$-exposed site | | | | |
| Pre-gassing, May 2010 | nd | nd | $2.4 \times 10^6 \pm 5.6 \times 10^4$ | $3.3 \times 10^8 \pm 1.3 \times 10^7$ |
| Oct 2010 (4 months) | $2.0 \times 10^7 \pm 1.7 \times 10^6$ | $1.8 \times 10^9 \pm 3.7 \times 10^7$ | nd | nd |
| May 2011 (10 months) | $8.9 \times 10^7 \pm 4 \times 10^7$ | nd | $2.2 \times 10^6 \pm 1.3 \times 10^5$ | $1.4 \times 10^8 \pm 6.4 \times 10^6$ |
| Oct 2011 (16 months) | $3.2 \times 10^7 \pm 6.9 \times 10^6$ | $1.9 \times 10^8 \pm 1.5 \times 10^7$ | nd | nd |
| May 2012 (24 months) | $3.9 \times 10^7 \pm 4.8 \times 10^5$ | $6.5 \times 10^9 \pm 6.3 \times 10^8$ | $5.4 \times 10^9 \pm 1.7 \times 10^8$ | $3.8 \times 10^7\ 1.1 \times 10^6$ |
| Post-gassing, Oct 2012 | nd | $1.5 \times 10^7 \pm 4.4 \times 10^6$ | $2.4 \times 10^7 \pm 3 \times 10^6$ | $3.5 \times 10^5 \pm 8.8 \times 10^4$ |
| Reference site | | | | |
| May 2010 | $2.9 \times 10^6 \pm 2 \times 10^5$ | $3.2 \times 10^8 \pm 3.6 \times 10^7$ | $1.9 \times 10^6 \pm 7.2 \times 10^4$ | $2.5 \times 10^8 \pm 1.1 \times 10^7$ |
| Oct 2010 | $4.4 \times 10^8 \pm 1.4 \times 10^8$ | $1.9 \times 10^9 \pm 5.5 \times 10^7$ | nd | nd |
| May 2011 | $1.7 \times 10^8 \pm 1.3 \times 10^8$ | nd | $1.9 \times 10^6 \pm 8.4 \times 10^4$ | $1.4 \times 10^8 \pm 7.8 \times 10^6$ |
| Oct 2011 | $5.2 \times 10^7 \pm 1.9 \times 10^7$ | $4.3 \times 10^8 \pm 7.7 \times 10^7$ | nd | nd |
| May 2012 | $4.3 \times 10^7 \pm 9.6 \times 10^5$ | $6.4 \times 10^9 \pm 2.4 \times 10^8$ | $3.0 \times 10^9 \pm 1 \times 10^8$ | $7.8 \times 10^6 \pm 4.1 \times 10^5$ |
| Oct 2012 | $4.0 \times 10^6 \pm 1.5 \times 10^6$ | $3.7 \times 10^8 \pm 1.4 \times 10^7$ | $5.8 \times 10^7 \pm 1.2 \times 10^7$ | $2.0 \times 10^5 \pm 3.4 \times 10^4$ |

were conducted once per season in the beginning of the growing season and again following harvest. The pH values ranged between 5 and 6.5 and did not show significant alterations during $CO_2$ injection. Similar results were detected during a previous injection period in 2006 (West *et al.* 2009). The results of both sampling campaigns at the ASGARD site demonstrated the soil buffering capacities and the necessity of long-term observations. In contrast to our results, previous studies of $CO_2$-injection sites reported the potential of $CO_2$-induced weathering of soils, groundwater and aquifer minerals including increasing dissolution of metals and trace elements (Altevogt and Jaffe 2005; Wei, Maroto-Valer and Steven 2011; Harvey *et al.* 2012). Alterations over time without $CO_2$ impacts have also been detected for TC and TOC contents (ANOVA, $P > 0.05$; Table S3, Supporting Information). For $CO_2$-affected samples in May, a mean TC content of 2.1% was observed and only slightly higher TC values of 2.4% were detected within the reference samples. For October, a mean TC content of 2.1% in $CO_2$-injected samples and 2% in reference samples was detected. Similar results were observed with TOC values.

## $CO_2$ impacts on soil microbial activity

During $CO_2$ injection, not only alterations in the degradation of soil organic matter have to be considered but also the influence of the rising supply of $CO_2$ as a direct microbial electron acceptor. Thus, in addition to $CO_2$ production as a result of the degradation of soil organic matter, $CH_4$ production and $CH_4$ oxidation are of potential interest. For the investigation of microbial activity within the soil samples, cultivation experiments focusing on microbial $CO_2$ and $CH_4$ production, and $CH_4$ oxidation were conducted.

For the ASGARD site, no significant $CO_2$-induced differences in microbial metabolic rates were detected (ANOVA, $P > 0.05$; Table S4, Supporting Information). Instead, significant differences between the sampling years and the May and October samples were observed (ANOVA, $P < 0.05$; Table S4, Supporting Information). As to be expected, lower microbial metabolic rates under anaerobic conditions were detected (up to 1500 times lower) (Fig. 2a–d). Potential $CO_2$ production rates were slightly higher under aerobic conditions than under anaerobic conditions (Fig. 2a and b). The values for the aerobic $CO_2$
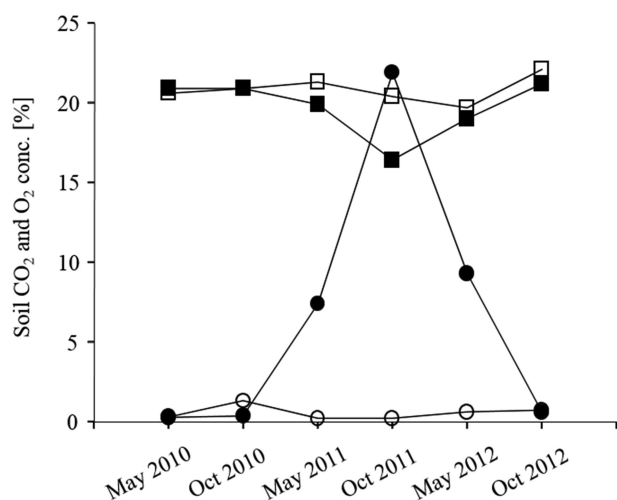
**Figure 1.** Soil $CO_2$ (circles) and $O_2$ (squares) concentrations (%) over time at 15–30 cm depth ($n = 1$ for each sampling campaign). Closed symbols indicate $CO_2$-exposed samples. Open symbols indicate reference samples. $CO_2$ injection from October 2010 to May 2012.
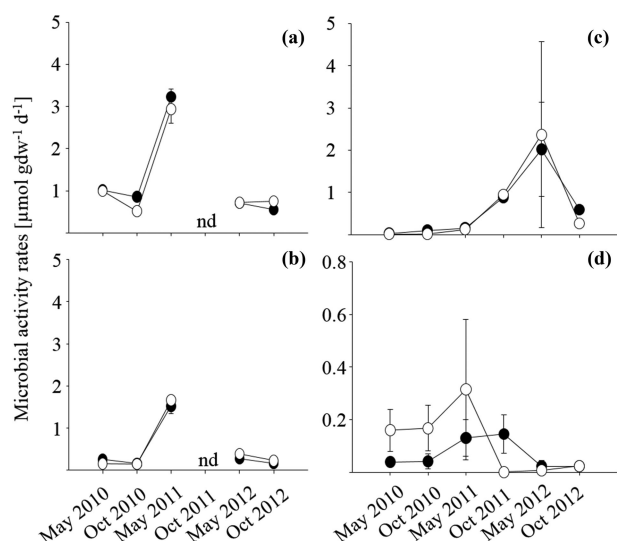


**Figure 2.** Potential microbial metabolic rates over time including (**a**) aerobic $CO_2$ production, (**b**) anaerobic $CO_2$ production, (**c**) aerobic $CH_4$ oxidation and (**d**) anaerobic $CH_4$ production. Closed symbols indicate $CO_2$-exposed samples. Open symbols indicate reference samples. $CO_2$ injection from October 2010 to May 2012. The error bars show $\pm 1 \times$ standard error ($n = 3$).



**Figure 3.** Comparison of qPCR quantification of (**a**) bacterial and (**b**) archaeal 16S rRNA genes from $CO_2$-exposed (black bars) and reference (white bars) samples. $CO_2$ injection from October 2010 to May 2012. Averages for replicates are shown with standard deviations indicated.

production ranged between 0.5 and 2.9 $\mu$mol $CO_2$ d$^{-1}$ gdw$^{-1}$ for the $CO_2$-affected samples and between 0.5 and 3.2 $\mu$mol $CO_2$ d$^{-1}$ gdw$^{-1}$ for the reference samples. Under anaerobic conditions, $CO_2$ production ranged between 0.1 and 1.6 $\mu$mol $CO_2$ d$^{-1}$ gdw$^{-1}$ for the $CO_2$-affected samples and between 0.1 and 1.5 $\mu$mol $CO_2$ d$^{-1}$ gdw$^{-1}$ for the reference samples. A maximum peak for the $CO_2$ production was detected in May 2011 (Fig. 2a and b). Unfortunately, because of technical problems no data was collected for the October 2011 samples.

Investigations of specific microbial metabolic rates have been so far mainly conducted for natural $CO_2$ vents (e.g. Oppermann *et al.* 2010; Frerichs *et al.* 2013; Fernández-Montiel *et al.* 2015). However, $CO_2$-induced differences in aerobic and anaerobic $CO_2$ production rates as described in these studies could not be detected for the ASGARD site (Oppermann *et al.* 2010; Frerichs *et al.*
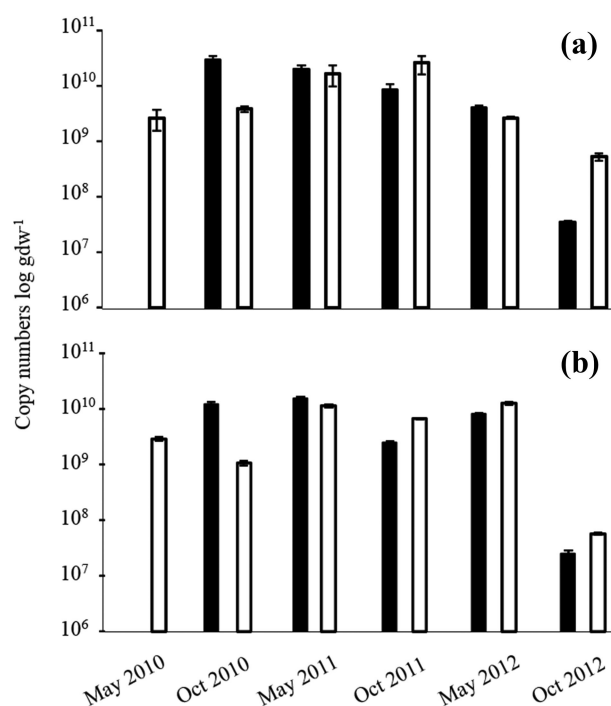
2013). In addition, no significant $CO_2$ impact over time was detected for the $CH_4$ production and oxidation (Fig. 2c and d).

The potential rates of $CH_4$ production ranged between 0.01 and 0.3 $\mu$mol d$^{-1}$ gdw$^{-1}$ within $CO_2$-exposed samples and 0.02–0.1 $\mu$mol d$^{-1}$ gdw$^{-1}$ for reference samples (Fig. 2d). Similar $CH_4$ production rates have been detected in previous studies at natural $CO_2$ vents with values between 0.001 and 0.1 $\mu$mol d$^{-1}$ gdw$^{-1}$ (Oppermann *et al.* 2010; Beulig *et al.* 2015). However, distinct increasing $CH_4$ production rates with increasing $CO_2$ concentration as described at natural sites could not be detected at the ASGARD site.

For both $CO_2$-exposed and reference samples, $CH_4$ oxidation rates slightly increased over time with a peak in May 2012 of 2.2 $\mu$mol $CH_4$ d$^{-1}$ gdw$^{-1}$ followed by a decrease in October 2012 to 0.4 $\mu$mol $CH_4$ d$^{-1}$ gdw$^{-1}$ (Fig. 2c). Similar to our results, during a 1-year $CO_2$ injection experiment at the PISCO$_2$ experimental site, in the CIUDEN facilities at Cubillos del Sil (León, Spain), no $CO_2$ impacts on microbial activity were detected (Fernández-Montiel *et al.* 2015).

## $CO_2$ impacts on soil microbial abundance

For the investigation of $CO_2$ impacts on microbial abundance, bacterial and archaeal 16S rDNA gene copy numbers were determined. Both bacterial and archaeal 16S rDNA gene copy numbers ranged from $10^7$ to $10^{10}$ gdw$^{-1}$ of soil (Fig. 3a and b) without significant $CO_2$-induced differences between $CO_2$-exposed and reference samples (ANOVA, $P > 0.05$; Table S6a, Supporting Information). Similar results were detected for the $CO_2$ injection site PISCO$_2$ as well as for some natural $CO_2$ vents (Beaubien *et al.* 2008; Frerichs *et al.* 2013; Beulig *et al.* 2015; Fernández-Montiel *et al.* 2015). In contrast, decreasing microbial gene copy numbers by up to two orders of magnitude with increasing $CO_2$ at

natural $CO_2$ vents were previously detected (Oppermann *et al.* 2010; Fernández-Montiel *et al.* 2016; Fernández-Montiel, Pedescoll and Bécares 2016). Similar results were detected during a previous 19-week gas release experiment at the ASGARD site in 2009 with decreasing total microbial numbers detected with, however, different methodological approaches (West *et al.* 2009, 2015).

In addition to general bacterial and archaeal abundances, *Crenarchaeota* as well as several functional genes with potential relevance for $CO_2$ turnover were quantitatively analysed. Similar to the bacterial and archaeal abundances, no $CO_2$ impacts were detected (Table 2). The *mcr*A gene was detected with $10^7$ gene copy numbers gdw$^{-1}$ in $CO_2$-exposed samples and between $10^6$ and $10^8$ gene copy numbers gdw$^{-1}$ in reference samples (Table 2). Higher values were detected for *Crenarchaeota* with $10^7$–$10^9$ 16S rDNA gene copy numbers gdw$^{-1}$ in $CO_2$-exposed and $10^8$–$10^9$ gene copy numbers gdw$^{-1}$ in reference samples (Table 2). Similar values for both *mcr*A and crenarchaeal gene copies were detected in previous studies for different soil types (Oppermann *et al.* 2010; Angel, Claus and Conrad 2012; Frerichs *et al.* 2013; Dubey *et al.* 2014). Similar to the ASGARD results, a previous study during $\sim$20 days of $CO_2$ injection at the ZERT field site showed no significant $CO_2$ impact on *mcr*A abundance (Morales and Holben 2013). However, contrary results were detected especially for natural $CO_2$ vents with both increasing and decreasing numbers of *mcr*A genes with increasing $CO_2$ concentration (Oppermann *et al.* 2010; Frerichs *et al.* 2013). Gene copy numbers of AOB in May samples ranged between $10^6$ and $10^8$ for both $CO_2$-exposed and reference samples. In October 2012, AOB gene copy numbers for both $CO_2$-exposed and reference samples were one to three orders of magnitude lower compared with May samples (Table 2). Similar results were observed for AOA in both $CO_2$-exposed and reference samples with $10^6$–$10^9$ gene copy numbers in May samples and $10^7$ gene copy numbers in October 2012 (Table 2). Previous investigations of AOB and AOA gene copy numbers in $CO_2$-unaffected agricultural soil and pasture showed similar numbers between $10^4$ and $10^9$ gene copies gdw$^{-1}$ (Nicol *et al.* 2008; Daebeler *et al.* 2014). Unfortunately, the

potential stimulation or inhibition of AOA and AOB by increasing $CO_2$ concentrations remains unclear (Frerichs *et al.* 2013; Morales and Holben 2013, 2014).

Altogether, possible $CO_2$ impacts on soil microbial abundance remain uncertain and seem to vary. In contrast to rather inconsistent results from $CO_2$-injection studies (Morales and Holben 2013; Fernández-Montiel *et al.* 2015), most previous results from natural $CO_2$ vents suggest a $CO_2$-induced decrease in microbial abundance (e.g. Fernández-Montiel *et al.* 2016; Fernández-Montiel, Pedescoll and Bécares 2016).

## $CO_2$ impacts on soil microbial communities

### *Bacteria*
Independent of the soil treatment, the majority of the bacterial sequences were assigned to five phyla: *Firmicutes* (9%–65%), *Proteobacteria* (10%–30%), *Actinobacteria* (2%–44%), *Acidobacteria* (1%–25%) and *Bacteroidetes* (0%–12%) (Fig. 4). They represent 75%–95% of the sequences within all 16S rRNA clone libraries. Lower abundances (5%–15%) were detected for *Planctomycetes*, *Nitrospirae*, *Chloroflexi*, *Verrucomicrobia*, *Gemmatimonadetes* and *Candidate* divisions (Fig. 4). No significant differences were observed between bacterial community compositions from $CO_2$-injected and reference samples (ANOSIM, R = $-$0.15, P = 0.91; Fig. 4). Instead, common soil *Bacteria* were detected (Janssen 2006; Lauber *et al.* 2009; Sáenz de Miera *et al.* 2014) with significant seasonal variations over time (PERMANOVA, F = 4.23, P = 0.01, permutation 9999; Table S6b, Supporting Information). The most dominant phylum within $CO_2$-exposed and reference samples at 15–30 cm depth was the *Firmicutes* phylum (Fig. 4) with sequences assigned to the families *Bacillaceae* and *Planococcaceae*. SIMPER analysis at phylum level indicated that *Firmicutes* contributed most of the differences in the relative abundance between $CO_2$-injected and reference sites (30.1%), followed by *Actinobacteria* (22.5%), unclassified *Bacteria* (11.6%), *Acidobacteria* (11.5%), *Proteobacteria* (7.3%) and *Bacteroidetes* (5.6%) (Table S7, Supporting Information). Several previous studies from $CO_2$-affected and unaffected sites reported only low abundances of 0%–8% *Firmicutes* in soils
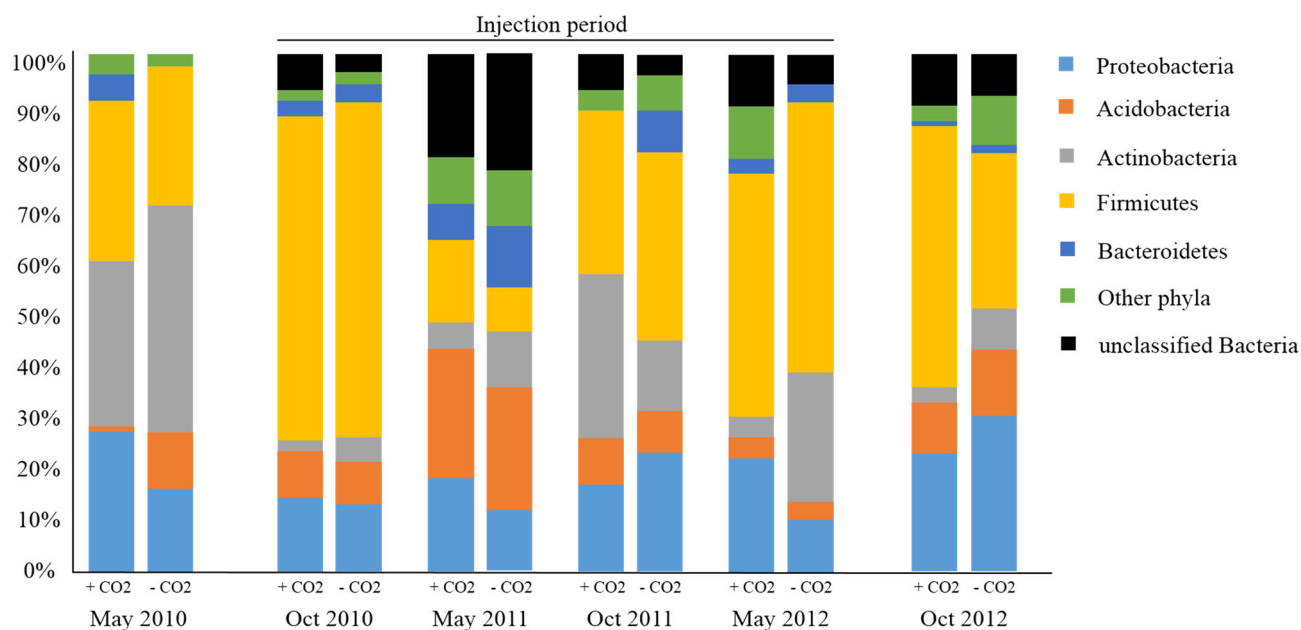


**Figure 4.** Bacterial community composition of $CO_2$-exposed (+$CO_2$) and reference ($-CO_2$) samples over time without significant differences (ANOSIM: R = $-$0.15; P = 0.91). $CO_2$ injection from October 2010 to May 2012.
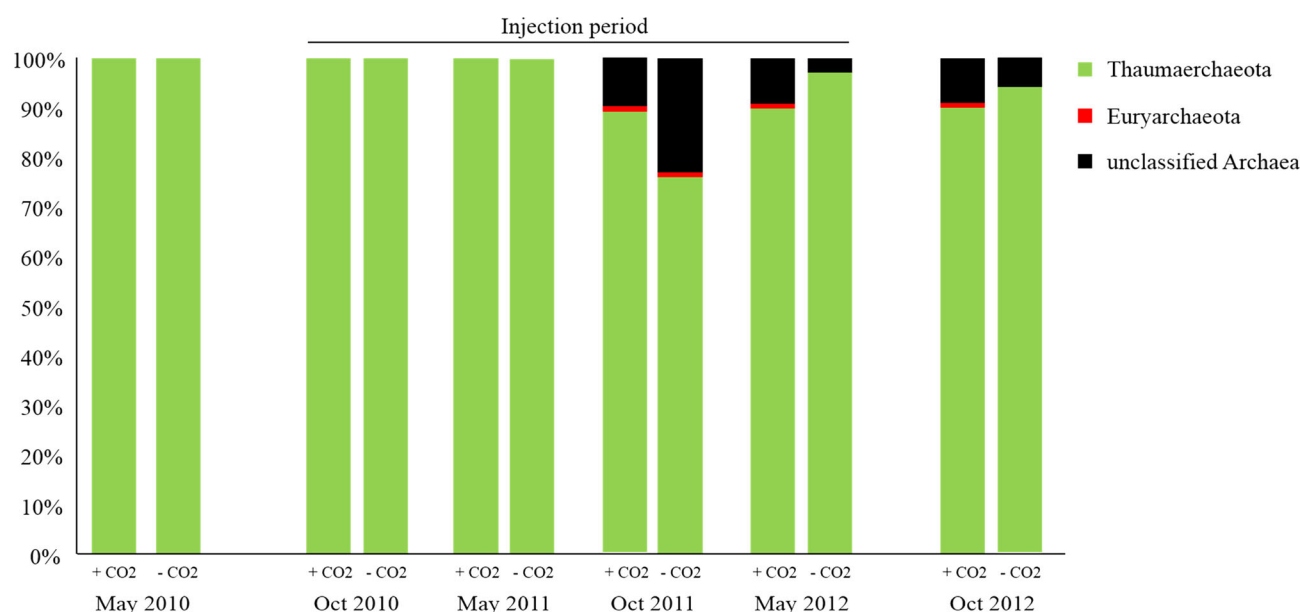
**Figure 5.** Archaeal community composition of $CO_2$-exposed (+$CO_2$) and reference (–$CO_2$) samples over time without significant differences (ANOSIM: R = –0.10; P = 0.89). $CO_2$ injection from October 2010 to May 2012.

(Janssen 2006; Sáenz de Miera *et al.* 2014). Other studies detected higher abundances of *Firmicutes* representatives of up to 50% in grassland soil (Felske *et al.* 1998) or rhizosphere (Teixeira *et al.* 2010). In addition, analyses of soil samples from the Stavesinci $CO_2$ vent area (Slovenia) showed increasing numbers of Firmicutes sequences with increasing $CO_2$ (Šibanc *et al.* 2014). So far, analyses of soil samples from natural $CO_2$ vents suggest that high $CO_2$ concentrations might result in increasing numbers of *Acidobacteria*, *Chloroflexi* and anaerobic acetogens (Sáenz de Miera *et al.* 2014; Šibanc *et al.* 2014; Beulig *et al.* 2015). However, the microbial interactions and metabolic activity of soil *Bacteria* under high $CO_2$ concentrations remain unclear and need further research.

*Archaea*
For both $CO_2$-exposed and reference samples, the majority of the archaeal sequences (85%–100%) were assigned to *Candidatus Nitrososphaera*, the first cultivated representative of the thaumarchaeotal 16S rRNA sequence cluster I.1b (soil group), followed by 0%–7% of unclassified Archaea and 0%–1% *Euryarchaeota* (Fig. 5). The cultivation of *Candidatus Nitrososphaera gargensis* has proven its ability for aerobic, chemolithoautotrophic nitrification and the potential for a mixotrophic life (Hatzenpichler *et al.* 2008; Pratscher, Dumont and Conrad 2011; Spang *et al.* 2012). The archaeal 16S rRNA clone libraries cover 75%–94% of the estimated archaeal representatives (Table S5, Supporting Information) without significant differences between archaeal community compositions from $CO_2$-injected and reference samples (ANOSIM, R = –0.10, P = 0.89; Fig. 5). Similar results were detected for the Shannon diversity index and Chao1 richness index (Fig. S1, Supporting Information) without significant $CO_2$-induced alterations (t-test, P > 0.05).

Also in the archaeal community composition, significant seasonal variation over time was observed (PERMANOVA, F = 10.89, P = 0.01, permutation 9999; Table S6b, Supporting Information). SIMPER analysis at phylum level indicated that *Thaumarchaeota* contributed most of the differences in the relative abundance between $CO_2$-injected and reference sites (78.1%) (Table S7, Sup-

porting Information). The predominance and importance of thaumarchaeotal I.1b soil group within both $CO_2$-injected and reference soil samples agrees with previous studies in different soil ecosystems (Hansel *et al.* 2008; Bates *et al.* 2011; Eilers *et al.* 2012). Nevertheless, previous analyses of $CO_2$-exposed and reference soil samples from natural $CO_2$ vents showed contrary results with $CO_2$-induced alterations in archaeal community composition. Within $CO_2$ vents, thaumarchaeotal I.1b soil group members were dominating in contrast to the predominant euryarchaeotal *Methanomicrobia* members at the reference sites (Frerichs *et al.* 2013; Šibanc *et al.* 2014). In contrast to these findings, in another study Beulig *et al.* (2015) detected *Nitrososphaerales* of the *Thaumarchaeota* only within the reference samples of the investigated mofette field in the Cheb Basin (Czech Republic). Here, the $CO_2$ vent samples were dominated by the euryarchaeotal classes *Methanosarcinales* and *Methanomicrobiales* (50%–90%).

## Importance of seasonal conditions

In order to determine whether $CO_2$ exposure or seasonal factors have more influence on the investigated soil samples, univariate and multivariate analyses of variance (ANOVA; PERMANOVA) and PCA have been applied. The results showed no significant $CO_2$ impact on soil properties (ANOVA, P > 0.05; Table S3, Supporting Information), microbial activity (ANOVA, P > 0.05; Table S4, Supporting Information), microbial abundance or community composition (PERMANOVA, P > 0.05; Table S5a and b, Supporting Information) but alterations over time. The PCA biplot explained 72% of the variance (Fig. 6). The PC 1 (*x*-axis) comprises the climate factors including moisture and temperature. The PC 2 (*y*-axis) comprises the measured soil gases $CO_2$ and $O_2$. Interestingly, most samples cluster close to PC 1, climate factors. Therefore, most October samples cluster together with rainfall in contrast to most of the May samples that correlate with temperature and high carbon availability (TC, TOC), due to high rainfall in autumn and the growing season in spring. The majority of both May and October samples cluster in the lower half of
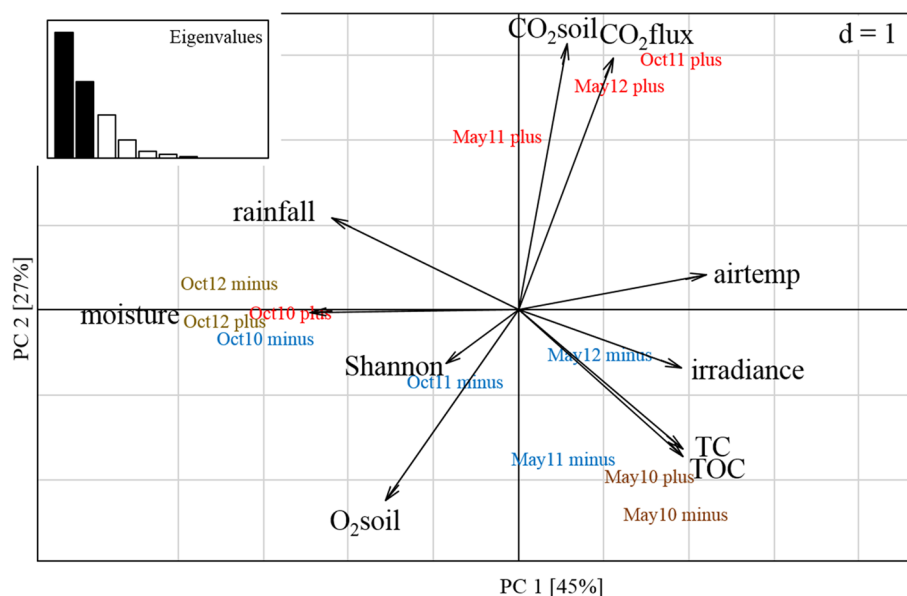
**Figure 6.** Principal component analysis (PCA) biplot showed correlations of atmospheric and geochemical data as well as archaeal clone libraries. Variables are symbolized by arrows; eigenvalues are presented in the bar chart drawn in the upper left corner. The correlation matrix explains 72% of the variance. $CO_2$-exposed samples are indicated in red, reference samples in blue, pre- and post-gassing samples in brown. Scale d = 1.

PC 2, with oxygen as the major influencing parameter without or less $CO_2$ impact. In contrast, samples collected in May and October 2011 as well as in May 2012 are clustering close to PC 2, with $CO_2$ as the most influencing parameter. These samples were collected after 10, 16 and 24 months of $CO_2$ injection with, however, no significant $CO_2$ impacts (ANOVA, $P > 0.05$; Table S3, Supporting Information). The impact of climate parameters on near-surface ecosystems as detected within our study was confirmed in previous near-surface studies (Kreyling *et al.* 2008; Lauber *et al.* 2013; Schlömer, Möller and Furche 2014). However, less data are available for microbial responses on changing climate parameters during $CO_2$ injection. Morales and Holben (2013), for example, described $CO_2$-induced alterations in soil microbial community related to seasonal conditions during a $CO_2$-injection period in summer 2009. They also assume a strong probability that $CO_2$ impacts might be masked by seasonal variations.

## CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH STUDIES

Rising $CO_2$ concentrations in near-surface soils can induce multiple ecological responses. To date, results of the various studies available are inconsistent as a consequence of a broad range of soil types, $CO_2$ elevation methods/$CO_2$ concentrations, exposure time and detection techniques of potential $CO_2$-induced microbiological alterations. In addition, previous studies often focused on short-term $CO_2$-injection periods or natural $CO_2$ vents. So far, no experimental setup has focused on $CO_2$-dependent biogeochemical alterations in near-surface soils before, during and after long-term $CO_2$ injection. With our project, we aimed to fill this gap of research. The results presented in our study were the first obtained from a long-term, near-surface $CO_2$ injection experiment with 24 months of continuous $CO_2$ gassing. This included geochemical and microbiological analyses before, during and after $CO_2$ injection. Our results from the ASGARD facility showed that soil $CO_2$ concentrations between 0.3% and 23% over a time period of 3 years had no detectable impact on soil micro-

biology. Still, the potential and maybe site-specific links between $CO_2$ exposure time, $CO_2$ concentration/injection rate, soil buffering capacities and ecological responses have to be clarified. Furthermore, the impacts of climate variations have to be investigated. For the ASGARD site, impacts of climate parameters, especially temperature and precipitation, have been detected that possibly mask $CO_2$ effects. Therefore, a detailed evaluation of the ecosystem baseline is of fundamental importance to clarify how $CO_2$ may influence near-surface microbial communities. Consequently, future research studies need extended sampling campaigns to classify seasonal impacts and to identify short-term effects after $CO_2$ injection (increase statistical reliability). In addition, further comprehensive microbial community analyses are needed.

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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