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Long-term CO_2 injection and its impact on near-surface 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 three-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 into 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 (PCA) showed no significant CO_2-induced variation. Instead, seasonal impacts especially temperature and precipitation were detected.

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 *et al.* 2015). Near-surface monitoring has focussed 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 Montana (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 information about near-surface CO_2-soil-plant-microbe interactions have 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).

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. A CO_2-injected and control plot were monitored for three years with 24 months of continuous CO_2 injection and a subsequent recovery phase of five 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 on the University of Nottingham's Sutton Bonington Campus, south of central Nottingham (Supplementary Figure 1a). Previously it was used as sheep pasture and had remained grassland for more than 10 years. The ASGARD area is geologically characterized 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 & 15) each with an area of 2.5 m x 2.5 m (Supplementary Figure 1b and 1c). 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} (approx. 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 2010-2012 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 each sampling campaign were homogenized and subsamples were taken for geochemical (stored at 4°C), bio molecular (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 mineralization rates, glass bottles flushed with sterile filtered, synthetically 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 GC-FID gas chromatographer (SRI 8610C, SRI Instruments, USA) equipped with a methanizer as described in (Nauhaus *et al.* 2002). The rates were calculated in µmol gdw^{-1} soil d^{-1}.

DNA Extraction

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

Quantitative PCR analyses

Quantitative PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction contained 5 µl TaqMan 2x Universal PCR Master Mix (NoAmpEraseUNG, Applied Biosystems), 0.4 µl PCR primers and probe for *Bacteria* (2.5 µM each) and *Archaea* (20 µM primers and 5 µM probe) , 2.3 µl sterile water, 0.5 µl BSA (20 mg/ml) and 1 µl 16S rDNA template added to a final volume of 10 µl. Samples were analysed for total numbers of *Bacteria*, *Archaea* and *Crenarchaeota* as well as several functional groups including ammonia-oxidizing *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 Supplementary Table 1. Overall, nine replicates (3 replicates x 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 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, D.J. 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 *et al.* 1991) were used. The PCR reactions contained 25 µl DreamTaq PCR Master Mix (2x) (Thermo Scientific), 0.3 µM of each primer, 4 µl template DNA and nuclease-free water up to 50 µl. 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 was 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 *et al.* 2012). Sequences with mismatches or gaps were removed using MOTHUR (Schloss *et al.* 2009). For clustering the sequences into 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 *et al.* 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 ANOVA was used. Nonmetric multidimensional scaling (MDS) plots, based on distance matrixes were generated for a 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 *et al.* 2001). For the archaeal clone libraries, the calculation of diversity (Shannon) and richness (Chao) indices were 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. Before and after the first four 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 (Figure 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 (Oct 2012), the CO_2 and O_2 values were comparable to those before CO_2 injection (CO_2 conc.: 0.2-4%) (Figure 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 three-year observation period. Previous soil gas measurements within the CCS project CLEAN in Germany and at the ZERT field site in Montana (Lewicki *et al.* 2010; Schlömer *et al.* 2014) observed highly variable CO_2 concentrations between 0.2-19% 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, $P > 0.05$; Supplementary Table 3). The soil pH measurements 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 *et al.* 2011; Harvey *et al.* 2012). Alterations over time without CO_2 impacts have also been detected for total carbon (TC) and total organic carbon (TOC) contents (ANOVA, $P > 0.05$; Supplementary Table 3). 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, $P > 0.05$; Supplementary Table 4). Instead, significant differences between the sampling years and the May and October samples were observed (ANOVA, $P < 0.05$; Supplementary Table 4). As to be expected, lower microbial metabolic rates under

anaerobic conditions were detected (up to 1 500 times lower) (Figure 2a- 2d). Potential CO_2 production rates were slightly higher under aerobic conditions than under anaerobic conditions (Figure 2a and 2b). The values for the aerobic CO_2 production ranged between 0.5 and 2.9 µmol CO_2 d^{-1} gdw^{-1} for the CO_2-affected samples and between 0.5 and 3.2 µmol CO_2 d^{-1} gdw^{-1} for the reference samples. Under anaerobic conditions, CO_2 production ranged between 0.1 and 1.6 µmol CO_2 d^{-1} gdw^{-1} for the CO_2-affected samples and between 0.1 and 1.5 µmol CO_2 d^{-1} gdw^{-1} for the reference samples. A maximum peak for the CO_2 production was detected in May 2011 (Figure 2a and 2b). Unfortunately, because of technical problems no data were collected for the October 2011 samples.

Investigations of specific microbial metabolic rates have so far mainly been 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 (Figure 2c and 2d).

The potential rates of CH_4 production ranged between 0.01 and 0.3 µmol d^{-1} gdw^{-1} within CO_2-exposed samples and 0.02-0.1 µmol d^{-1} gdw^{-1} for reference samples (Figure 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 µmol 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 µmol CH_4 d^{-1} gdw^{-1} followed by a decrease in October 2012 to 0.4 µmol CH_4 d^{-1} gdw^{-1} (Figure 2c). Similar to our results, during a one 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 (Figure 3a and 3b) without significant CO_2-induced differences between CO_2-exposed and reference samples (ANOVA, $P > 0.05$; Supplementary Table 6a). 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). Similar results were detected during a previous 19 weeks 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 to 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 et *al.* 2012; Frerichs *et al.* 2013; Dubey *et al.* 2014). Similar to the ASGARD results, a previous study during about 20 days of CO_2 injection at the

ZERT field site showed no significant CO_2 impact on *mcr*A abundane (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 ammonia-oxidizing *Bacteria* (AOB) in May samples ranged between 10^6-10^8 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 10^4-10^9 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).

CO_2 impacts on soil microbial communities

Bacteria

Independent of the soil treatment, the majority of the bacterial sequences were assigned into five phyla: *Firmicutes* (9-65%), *Proteobacteria* (10-30%), *Actinobacteria* (2-44%), *Acidobacteria* (1-25%) and *Bacteroidetes* (0-12%) (Figure 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 (Figure 4). No significant differences were observed between bacterial community compositions from CO_2-injected and reference samples (ANOSIM, R = -0.15, P = 0.91; Figure 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; Supplementary Table 6b). The most dominant phylum within CO_2-exposed and reference samples at 15-30 cm depth was the *Firmicutes* phylum (Figure 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%) (Supplementary Table 7). Several previous studies from CO_2-affected and unaffected sites reported only low abundances of 0-8% *Firmicutes* in soils (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 remains 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* (Figure 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 *et al.* 2011; Spang *et al.* 2012). The archaeal 16S rRNA clone libraries cover 75-94% of the estimated archaeal representatives (Supplementary Table 5) without significant differences between archaeal community compositions from CO_2-injected and reference samples (ANOSIM, R = -0.10, P = 0.89; Figure 5). Similar results were detected for the Shannon diversity index and Chao1 richness index (Supplementary Figure 1) 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; Supplementary Table 6b). 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%) (Supplementary Table 7). 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 principal component analysis (PCA) have been applied. The results showed no significant CO_2 impact on soil properties (ANOVA, $P > 0.05$; Supplementary Table 3), microbial activity (ANOVA, $P > 0.05$; Supplementary Table 4), microbial abundance or community composition (PERMANOVA, $P > 0.05$; Supplementary Table 5a and 5b) but alterations over time. The PCA biplot explained 72% of the variance (Figure 6). The PC 1 (x-axis) comprises the climate factors including e.g. 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 which 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 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, $P > 0.05$; Supplementary Table 3). 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 *et al.* 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 e.g. 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 three years had no detectable impact on soil microbiology. 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 which 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. In consequence, 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.

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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 (Oct 2012).

Samples/Time point	**CO_2Flux** [g/mq/day]	**Temp.** [°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*) (± 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)	$3.9 \times 10^7 \pm 4.8 \times 10^5$	$6.5 \times 10^9 \pm 6.3 \times 10^8$	$5.4 \times 10^9 \pm 1.7 \times 10^8$	3.8×10^7 1.1×10^6
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	$4.0 \times 10^6 \pm 1.5 \times 10^6$	$3.7 \times 10^8 \pm 1.4 \times 10^7$	$5.8 \times 10^7 \pm 1.2 \times 10^7$	$2.0 \times 10^5 \pm 3.4 \times 10^4$

19

20

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.

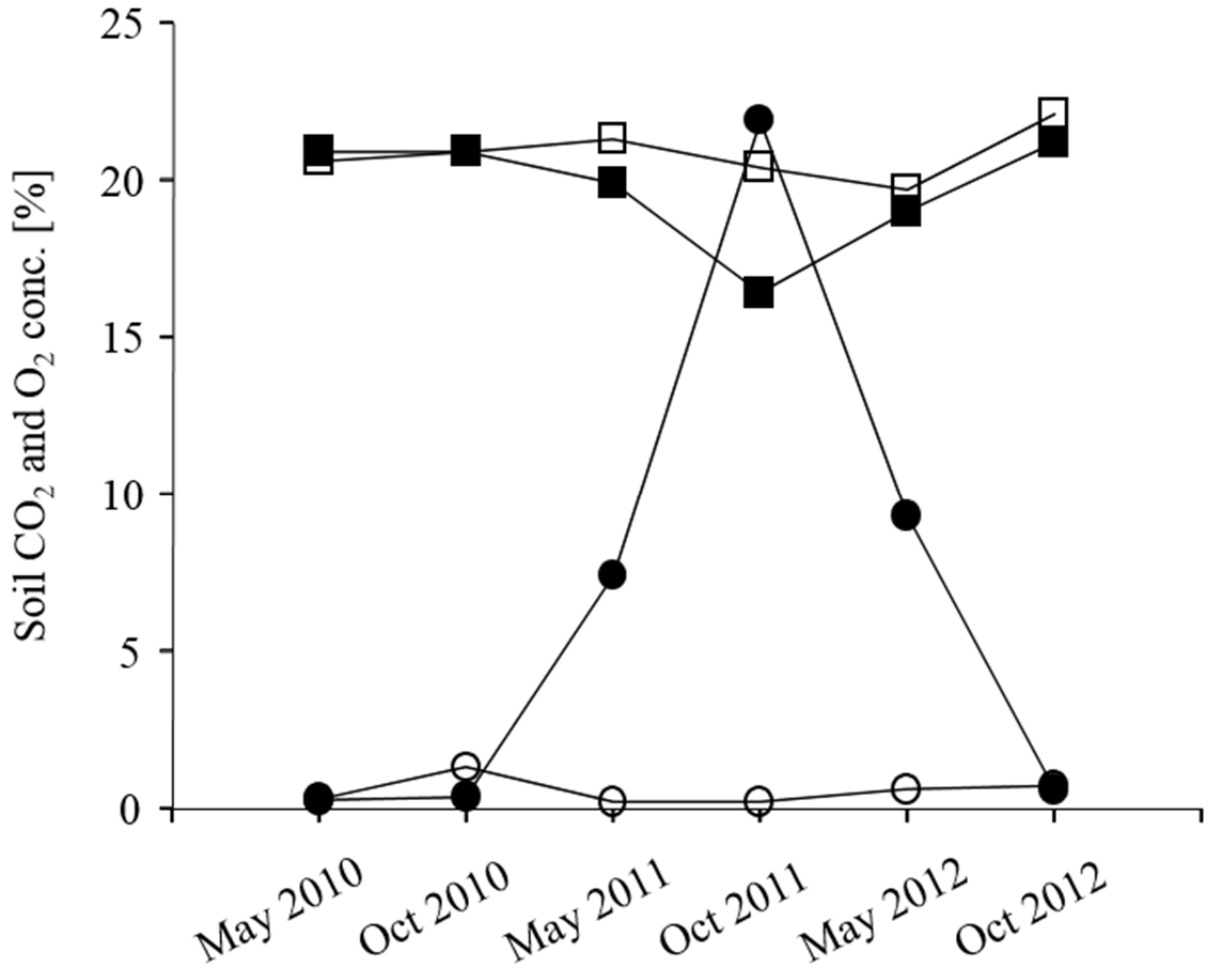
Figure 2. Potential microbial metabolic rates over time including a) aerobic CO_2 production, b) anaerobic CO_2 production, c) aerobic CH_4 oxidation and d) anaerobic CH_4 production. Closed symbols indicate CO_2-exposed samples. Open symbols indicate reference samples. CO_2 injection from October 2010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

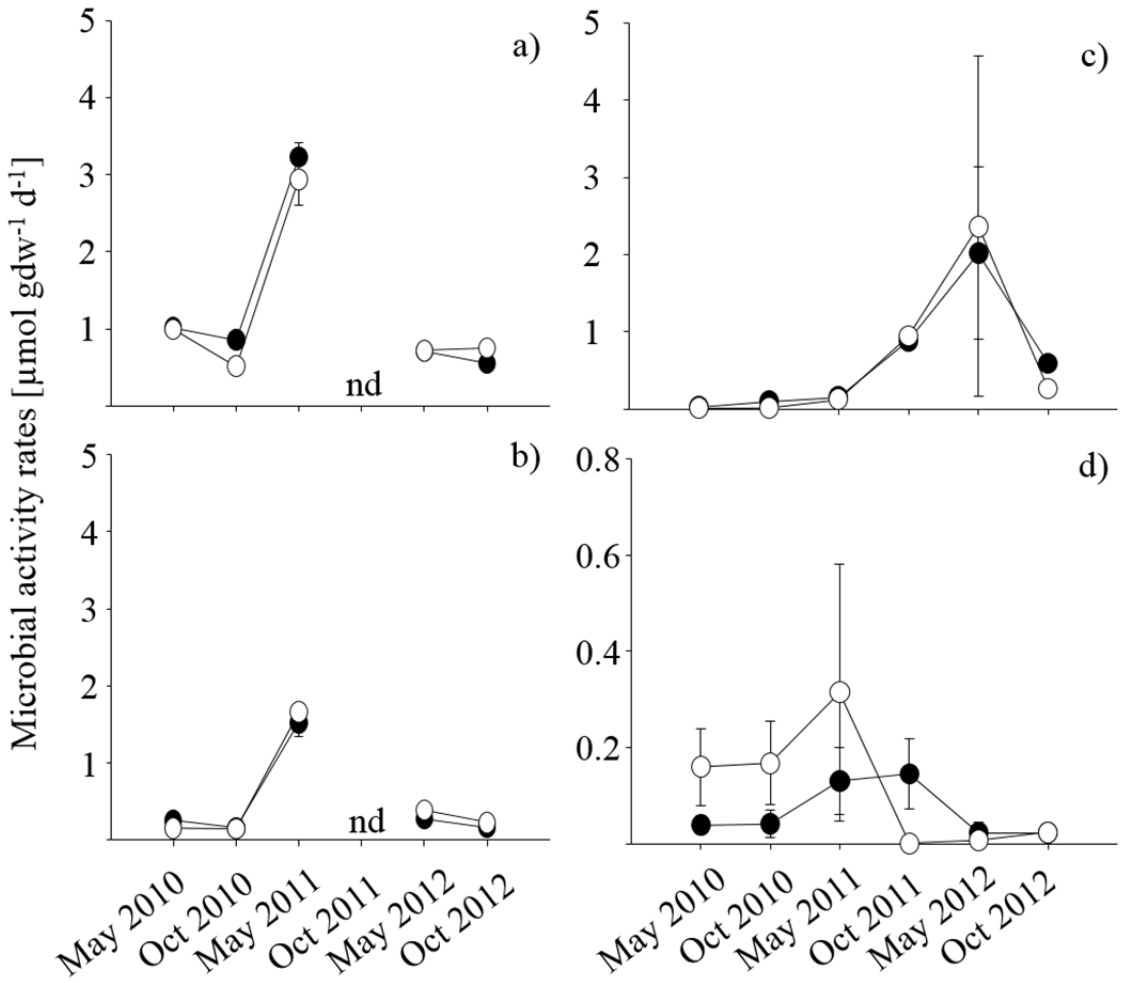
Figure 3. Comparison of qPCR quantification of a) bacterial and b) archaeal 16S rRNA genes from CO_2-exposed (black bars) and reference (white bars) samples. CO_2 injection from October 2010 to May 2012. Averages for replicates are shown with standard deviations indicated.

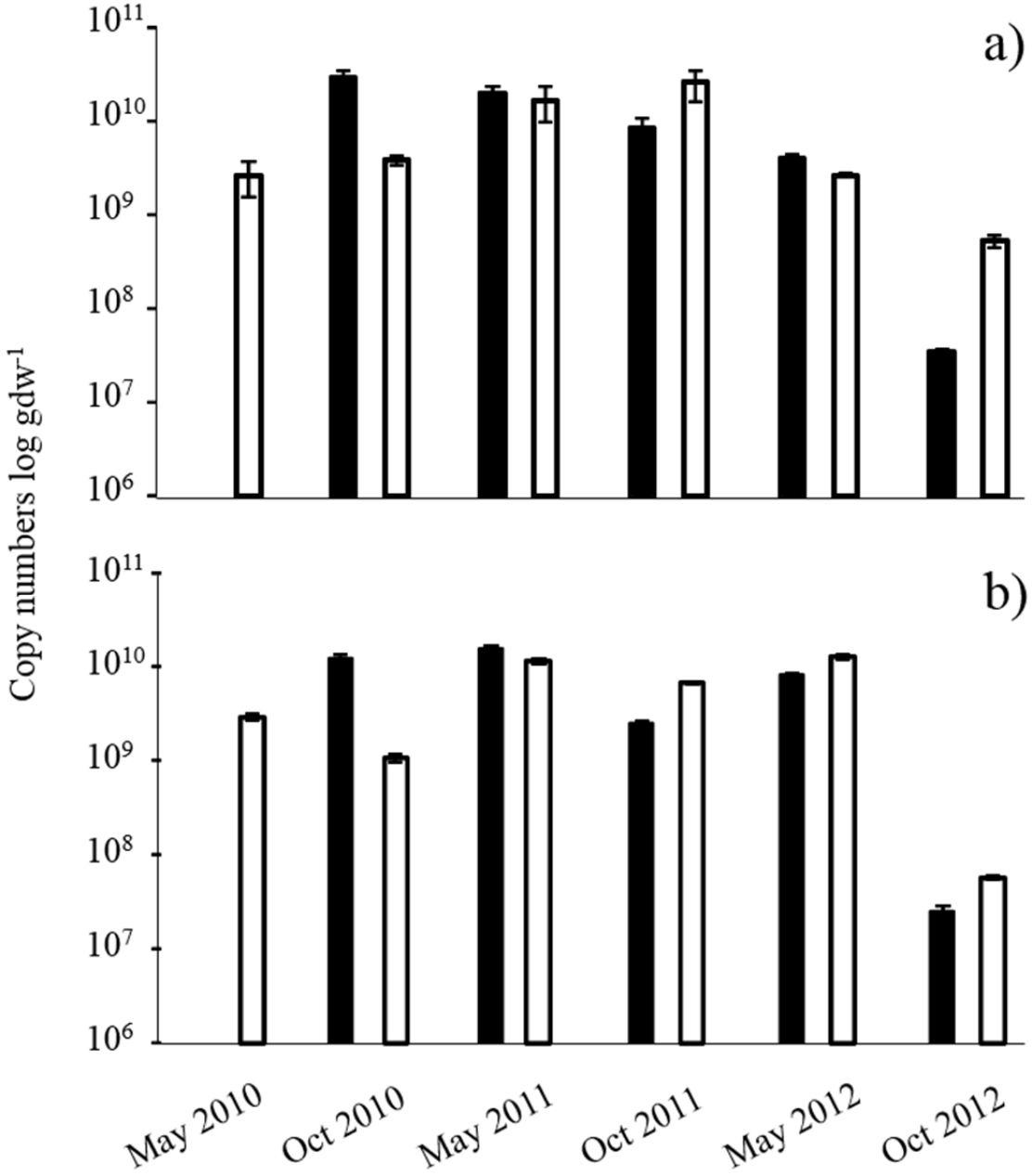
Figure 4. Bacterial community composition of CO_2-exposed ($+CO_2$) and reference ($-CO_2$) samples over time without significant differences (ANOSIM: R = -0.15; P = 0.91). CO_2 injection from October 2010 to May 2012.

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.

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.


25
20
15
10
5
0
Soil CO_2 and O_2 conc. [%]
May 2010
Oct 2010
May 2011
Oct 2011
May 2012
Oct 2012


Microbial activity rates [µmol gdw-1 d-1]
a)
b)
c)
d)
nd
nd
May 2010
Oct 2010
May 2011
Oct 2011
May 2012
Oct 2012


a)
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Copy numbers log gdw-1
10^11
10^10
10^9
10^8
10^7
10^6
May 2010
Oct 2010
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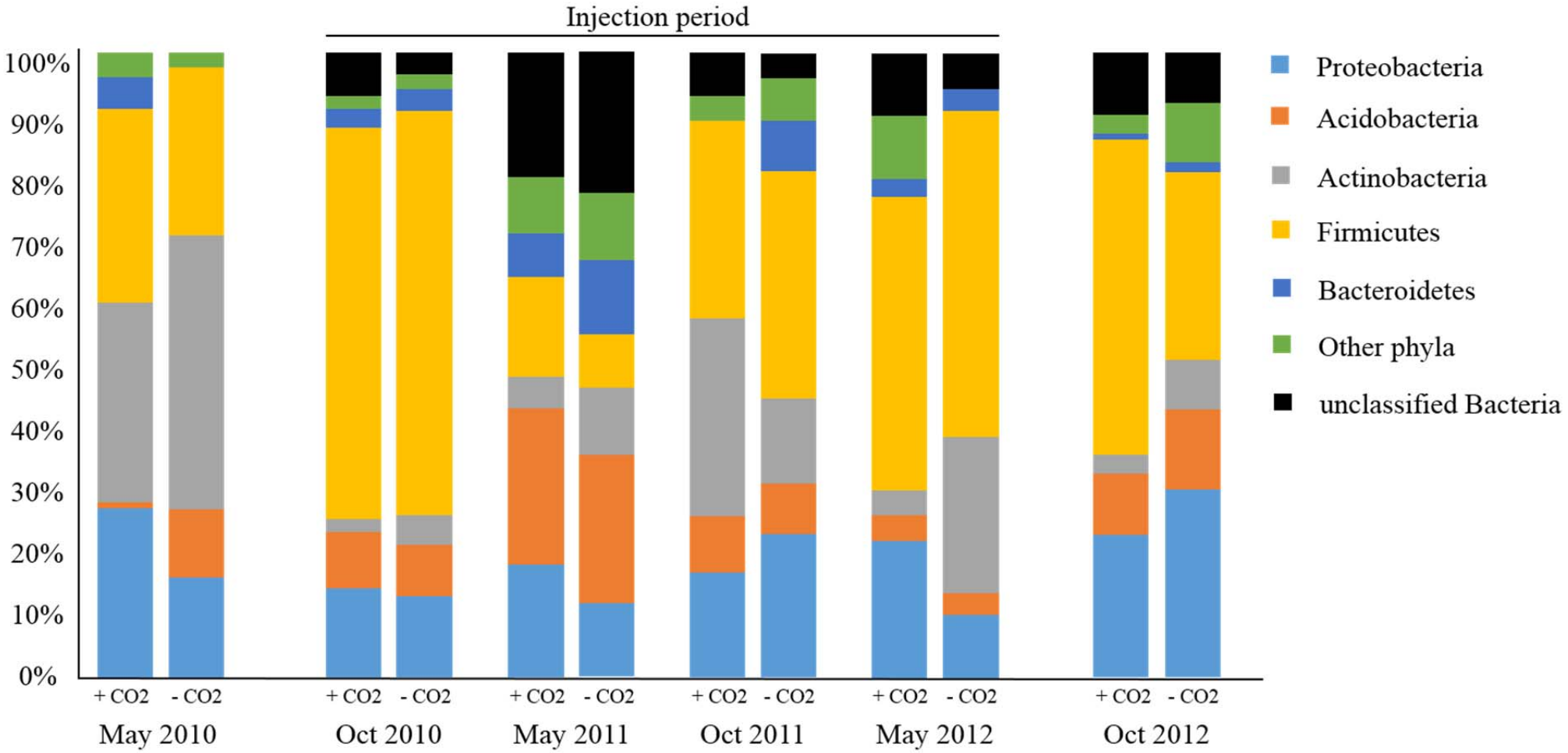
May 2012
Oct 2012


Injection period
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
+ CO2
- CO2
May 2010
Oct 2010
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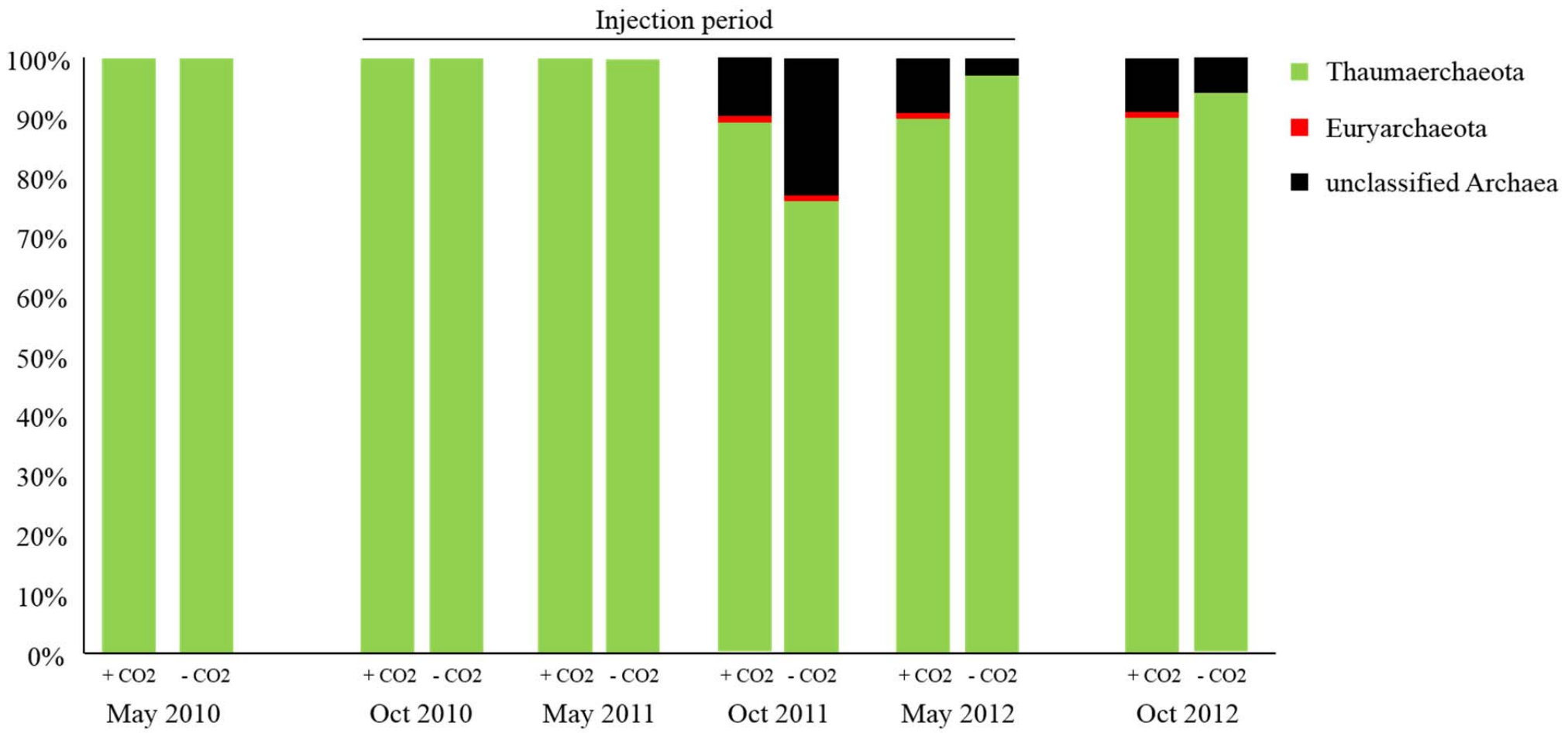
Actinobacteria
Firmicutes
Bacteroidetes
Other phyla
unclassified Bacteria


Injection period
100%
90%
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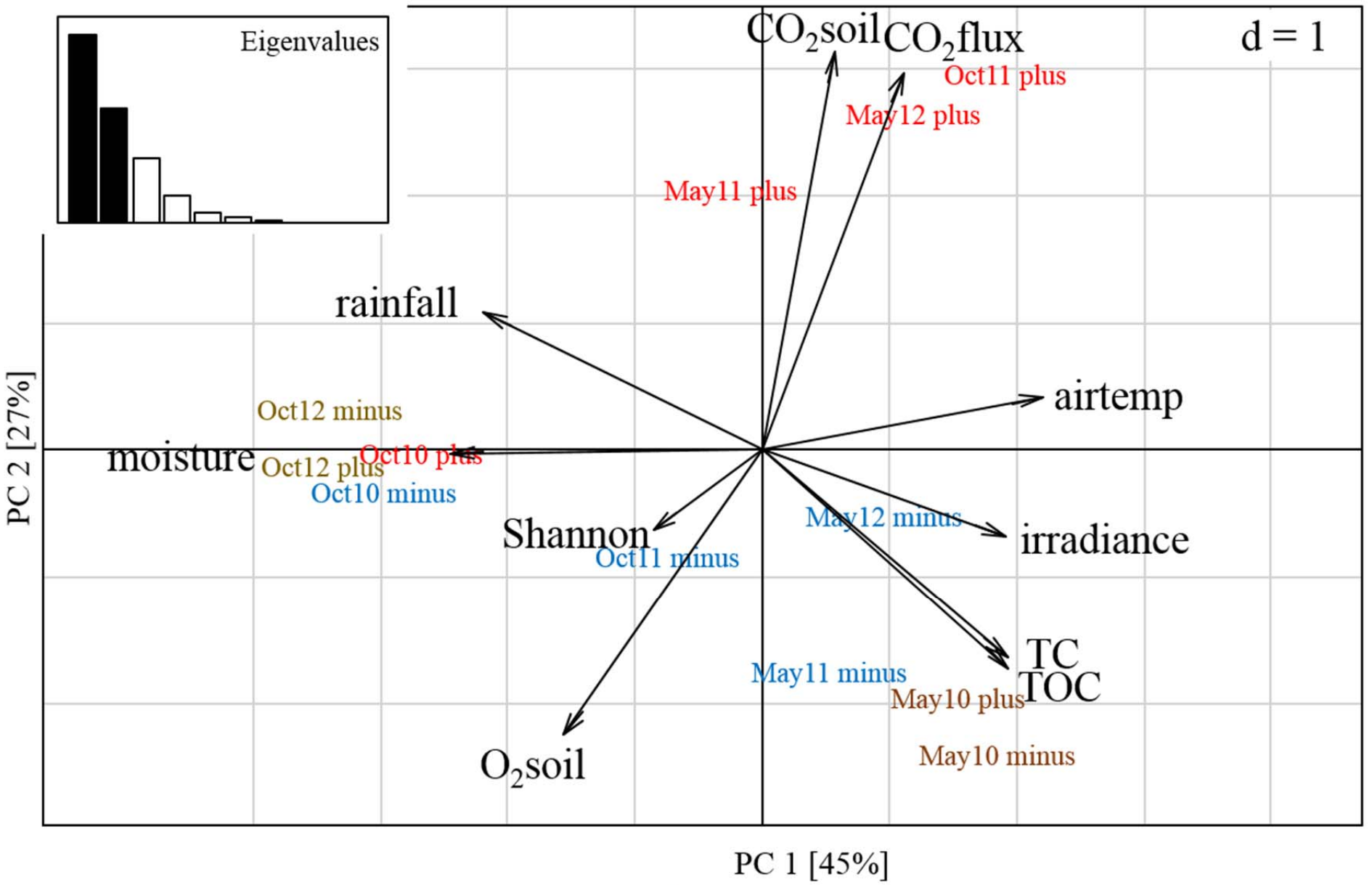
Thaumaerchaeota
Euryarchaeota
unclassified Archaea


Eigenvalues
CO_2soil
CO_2flux
d = 1
Oct11 plus
May12 plus
May11 plus
rainfall
airtemp
Oct12 minus
moisture
Oct10 plus
Oct12 plus
Oct10 minus
Shannon
May12 minus
irradiance
Oct11 minus
TC
TOC
May11 minus
May10 plus
May10 minus
O_2soil
PC 2 [27%]
PC 1 [45%]

1