

Functional Response of a Near-Surface Soil Microbial Community to a Simulated Underground CO₂ Storage Leak

Sergio E. Morales^{1,2*}, William E. Holben^{2,3}

1 Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand, **2** Cellular, Molecular and Microbial Biology Program and Systems Ecology Program, Division of Biological Sciences, The University of Montana, Missoula, Montana, United States of America, **3** Montana—Ecology of Infectious Diseases Program, The University of Montana, Missoula, Montana, United States of America

Abstract

Understanding the impacts of leaks from geologic carbon sequestration, also known as carbon capture and storage, is key to developing effective strategies for carbon dioxide (CO₂) emissions management and mitigation of potential negative effects. Here, we provide the first report on the potential effects of leaks from carbon capture and storage sites on microbial functional groups in surface and near-surface soils. Using a simulated subsurface CO₂ storage leak scenario, we demonstrate how CO₂ flow upward through the soil column altered both the abundance (DNA) and activity (mRNA) of microbial functional groups mediating carbon and nitrogen transformations. These microbial responses were found to be seasonally dependent and correlated to shifts in atmospheric conditions. While both DNA and mRNA levels were affected by elevated CO₂, they did not react equally, suggesting two separate mechanisms for soil microbial community response to high CO₂ levels. The results did not always agree with previous studies on elevated atmospheric (rather than subsurface) CO₂ using FACE (Free-Air CO₂ Enrichment) systems, suggesting that microbial community response to CO₂ seepage from the subsurface might differ from its response to atmospheric CO₂ increases.

Citation: Morales SE, Holben WE (2013) Functional Response of a Near-Surface Soil Microbial Community to a Simulated Underground CO₂ Storage Leak. PLoS ONE 8(11): e81742. doi:10.1371/journal.pone.0081742

Editor: Melanie R. Mormile, Missouri University of Science and Technology, United States of America

Received: July 3, 2013; **Accepted:** October 24, 2013; **Published:** November 26, 2013

Copyright: © 2013 Morales, Holben. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding for this project was provided by the United States Department of Energy—Experimental Program to Stimulate Competitive Research (DOE-EPSCoR Implementation Grant DE-FG02-08ER46527). 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.

* E-mail: sergio.morales@otago.ac.nz

Introduction

Carbon capture and storage (CCS) technology is being explored as an option for reducing carbon dioxide (CO₂) emissions to the atmosphere [1–3]. For this option to achieve significant reductions in CO₂ emissions, approximately 1 teraton (one trillion tons) of atmospheric CO₂ will have to be removed and/or prevented from entering the atmosphere [1]. Under current CCS scenarios geologic carbon sequestration requires injecting supercritical CO₂ deep in the subsurface (>1 km) into porous sedimentary formations in natural sites such as deep geological cavities, saline aquifers, spent oil or gas fields, coal mines or on the ocean floor [1–3]. However, supercritical CO₂ expands and tends to be buoyant, continuously moving upwards until a “cap rock” (e.g. a barrier of non-porous rock) is encountered. This fact, and the ability of a CO₂ plume to spread out over a large area suggest that, over time, pathways for CO₂ to move upward and escape could lead to stored gas

reaching surface soils or the atmosphere [3]. This has led to research aimed at developing monitoring techniques for the detection of CO₂ leaks from geologic formations, and to studies on the effects such leaks could have on surface ecosystems [4–6].

Thus far, examples of the effects underground CO₂ leaks can have on surface ecosystems are primarily limited to large natural magmatic CO₂ emissions as reported at Mammoth Mountain, California [7,8] and a volcanic vent in Germany [9]. One controlled-release CO₂ research site is the ASGAR site located on a grassland at the University of Nottingham's Sutton Bonington Campus, which comprises permanently installed underground pipework emitting CO₂ at a rate of 3 liters per minute at a depth of between 50–60 cm below ground surface [10]. This flow rate represents the equivalent of 3 tons y⁻¹, or 0.0003% of what is injected into an active storage site [11]. A 19-week (May to September 2006) gas release at this site resulted in changes in plant coverage and a significant drop in

bacterial abundance in areas of high CO₂ concentration, with microbial activity (measured as ATP concentration) also decreasing (in some cases to below detection) in response to high (87%) CO₂ levels [10]. To date, though, the majority of studies of elevated CO₂ effects on microbial, plant and animal communities have focused on the effect of elevated atmospheric CO₂ as conducted in FACE (free-air CO₂ enrichment) sites or similar scenarios. Thus biological response in CCS leak scenarios is lacking.

Here, we report the effect of a simulated underground CO₂ leak (e.g. as in escape from a deep subsurface CO₂ sequestration reservoir) on microbial populations at a shallow subsurface controlled-release facility in Bozeman, Montana, USA. During 2009, two separate controlled releases (spring and summer) of CO₂ were carried out at the Zero Emission Research and Technology (ZERT) field site. Our objectives were to determine: (1) the effect of CO₂ seepage on microbial communities in near-surface soils; (2) how microbial response was affected by seasonal variations; and (3) the suitability of monitoring the microbial community and its constituent guilds as sentinels for elevated CO₂ levels.

Materials and Methods

Study Site, CO₂ Release Regime and Sample Collection

The ZERT field site (both control and CO₂ exposed areas) and its shallow subsurface controlled release facility have been previously described in detail [12–15]. The field studies did not involve endangered or protected species and no specific permissions were required for sample collection this location as it is part of the university facilities. In brief, the site is an agricultural plot located at the Montana Agricultural Experiment Research Center, at Montana State University in Bozeman MT, dominated by mixed grasses (70%) with the remainder being alfalfa (15%), clover (8%), dandelion (5%) and various forbs (2%) [6]. The whole field site consists of plants that were there previously (or descendants of those plants) when the field was used as a pasture, and vegetation is consistent between the control and CO₂ sparged areas (M. Apple, personal communication). The soil structure throughout the field site (control and treatment sites) is characterized by a ~30 cm-thick clay topsoil overlying a ~20 cm-thick clayey silt layer, which overlies an alluvial sandy cobble with 10–25 cm diameter cobbles [15].

The infrastructure (i.e. ‘plumbing’) for subsurface CO₂ release is described in full elsewhere [13,14]. In brief, a 98 m long horizontal well (304L stainless steel pipe) is located 2 m below the ground surface. The central 70 m are slotted to allow CO₂ to vent into the soil. Six separate zones, spaced 10 m apart, are plumbed separately allowing for flow rates to individual zones to be varied independently.

Previous to the experiment described herein, three prior separate releases had been carried out in 2007 and 2008. From 9–18 July 2007 (Release 1) and from 3–10 August 2008 (Release 2), 0.1 t CO₂ day⁻¹ and 0.3 t CO₂ day⁻¹ were released, respectively. From 9 July to 7 August 2008 (Release 3) 0.3 t CO₂ day⁻¹ were released. For the current experiment, two separate releases were carried out. The spring release

(hereafter ‘June release’) began June 7, 2009 and lasted until June 14. The sustained release rate was 0.3 t CO₂ day⁻¹. A second summer release (hereafter ‘July release’) of 0.4 t CO₂ day⁻¹ occurred from July 17 through August 4, 2009.

Soil sampling for our experiments was performed between June 6 and September 9, 2009 (spanning the spring to late summer seasons) by collecting soil cores consisting of the top 5 cm of soils (2 cm dia. X 5 cm deep) directly above the horizontal well, at adjacent points roughly in the center of Zone 2 [13,14]. Care was taken to collect cores between plants and to minimize inadvertent collection of roots or other tissues, with any visible contaminating plant materials removed from the soil with forceps prior to nucleic acid recovery. At each time point, a CO₂-negative control sample was taken from an adjacent plot approximately 75 m northeast of the experimental site. This location was chosen as it is predominantly upwind from the CO₂ release zone in an unused and unimpacted area of the site. Both sampling locations (control and treatment) are within a pasture site actively managed for feed hay, with harvesting occurring once a year. An aerial view of the site and detailed description of soil and plant communities can be found here: [6,16]. Samples were collected every 6 hours for 24 hours prior to CO₂ releases, and continued at 6-hour intervals for 24 hours post-release. Multiple additional samplings were performed in the weeks following and between CO₂ releases. All soil samples were stored in Whirl-Pak bags on dry ice in the field and kept at -70°C until processed for microbial community nucleic acid extraction.

Environmental Conditions

Atmospheric temperature, relative humidity and barometric pressure were monitored continuously on-site by the ZERT Weather Station (<http://orsl.eps.montana.edu/weather/zert/>).

DNA & RNA extraction and real-time quantitative PCR assays

Total community DNA and RNA were extracted from 1 g of each manually homogenized soil sample, in triplicate using the MoBio PowerSoil™ Total RNA Isolation Kit (MoBio, Solana Beach, CA) in conjunction with the MoBio RNA PowerSoil® DNA Elution Accessory Kit according to the manufacturer’s instructions. All purified DNA samples were stored at -20°C until used in downstream analyses. All RNA samples were immediately treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and assessed for integrity using denaturing agarose gels and standard protocols. A subset of DNase treated samples were used as negative controls for RT reactions to confirm complete degradation of DNA. All purified RNA samples were stored at -70°C until used in downstream analyses.

Real-time qPCR was performed using modified conditions from [17]. Reactions were performed in an iCycler iQ thermocycler (Bio-Rad, Hercules, CA) with an Absolute Blue qPCR Sybr green ROX mix (ABgene, Rochester, NY) using primers and conditions summarized in Table S1. Each qPCR run included relevant known template standards made from cloned PCR products as described previously [17] and summarized in Table S1. Reaction mixtures (25µl) included 1µl

of template (see concentration below), 10 pmol of each primer, 20µg bovine serum albumin, and 12.5 µl of Absolute Blue QPCR Sybr green ROX mix. Specific genes and their respective transcripts targeted in this study were: *nifH*, nitrogenase gene; *nosZ*, nitrous oxide reductase gene; *nirS*, nitrite reductase gene; *rbcL*, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) gene; *mxoF*, methanol dehydrogenase gene; and *mcrA*, methyl-coenzyme M reductase gene. In detail, the *nifH* gene was amplified using an initial denaturation step of 15 min at 95°C; followed by 10 cycles of touchdown consisting of denaturation (1 min at 96°C), primer annealing (1 min starting at 65°C and lowering 1.5°C per cycle), and primer extension (1 min at 72°C); followed by 30 cycles of amplification consisting of denaturation (1 min at 96°C), primer annealing (1 min at 50°C), and primer extension (1 min at 72°C), with a final extension step of 10 min at 72°C. The *nirS* and *nosZ* genes were amplified using an initial denaturation step of 15 min at 95°C; followed by 6 cycles of touchdown consisting of denaturation (15 s at 96°C), primer annealing (30 s starting at 63°C and lowering 1°C per cycle), and primer extension (15 s at 72°C); followed by 35 cycles of amplification consisting of denaturation (15 s at 96°C), primer annealing (30 s at 58°C), and primer extension (15 s at 72°C). The *mxoF* gene was amplified following the conditions for the *nifH* gene with a touchdown temperature starting at 65°C and lowering 1°C per cycle, and primer annealing for the remaining 35 cycles carried out at 55°C. The *mcrA* gene was amplified using an initial denaturation step of 15 min at 95°C; followed by 45 cycles of amplification consisting of denaturation (40 s at 96°C), primer annealing (1.5 min at 55°C), and primer extension (2 min at 72°C), and a final extension step of 10 min at 72°C. The *rbcL* gene was amplified using the same conditions as the *mxoF* gene but with a touchdown temperature starting at 65°C and lowering 1°C per cycle, and primer annealing for the remaining 35 cycles carried out at 55°C. Standards were constructed, and target specificity was assessed for each target by PCR amplifying DNA extracted from the field site. PCR products were then cloned and sequenced as described previously [18] to confirm specificity of each assay.

Gene abundance measurements were determined by quantifying specific gene copy numbers in 5 ng of total community DNA from individual replicate extractions. For measurement of specific gene transcript abundances, 0.525µg of RNA was reverse transcribed in a 35µl reaction using random hexamer primers (Promega) and the Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) according to the manufacturer's recommended protocol. Subsequently, 1µl of RT reaction product was used directly for real-time qPCR of gene transcripts. All qPCR reactions for any single sample were run at least in triplicate (for each individual extraction) as described above.

Statistical analysis

Relationships between ambient atmospheric conditions (i.e. weather parameters) and microbial gene and transcript abundances were determined by principal components analysis (PCA) with data matrices composed of environmental

data and mean values for qPCR data. Data was organized with rows representing different sampling dates and columns representing individual variables. Principal component scores were plotted for the first two principal components. Parameters driving the ordination of the PCA plots were determined by querying all variables against the first two principal components.

To account for variance associated with replicate sampling and technical replication, all qPCR data was analyzed independently. Individual variables were independently queried to each other by conducting pair-wise correlations and the non-parametric Spearman's ρ . One-way analysis of variance (ANOVA) and the non-parametric Wilcoxon method were used to test for significant CO₂ effects on the various microbial parameters.

Results

Microbial functional response to CO₂ exposure

To determine community dynamics in response to CO₂, we compared functional gene (DNA) and transcript (mRNA) abundance for all six functional gene targets over time for both control and experimental samples (Figure 1). These targets were chosen as they provide a good overview of the prokaryotic organisms controlling net inputs and outputs of C and N from soils, as well as the chemical state of any losses (e.g. whether carbon was lost as CO₂ or CH₄, and nitrogen as N₂O or N₂). Results are summarized in Table 1, with details of the statistical analyses presented in Table S2. No consistent pattern emerged across all six targets, but both gene and transcript abundances for each target were affected in some way. The number of nitrogen fixers, as determined by abundance of the nitrogenase gene (*nifH*), decreased in response to elevated CO₂. This decrease was temperature dependent with 1.3 to 1.6-fold mean differences during and following the July release. However, the activity of nitrogen fixers, as determined by the abundance of *nifH* gene transcripts did not respond similarly. The *nifH* transcript abundance values showed up to a 1.7-fold mean increase in response to CO₂ release, with a subsequent 1.3-fold decrease upon cessation of CO₂ release.

Nitrite reducers, as determined by abundance of the nitrite reductase gene (*nirS*), also displayed a temperature dependent decrease. This decrease was restricted to the active release period in June when a short drop in temperature was recorded; however no statistically significant change in total mean was detected for that period. A similar pattern was seen with nitrite reducing activity, where a 1.9-fold decrease in transcript abundance was observed during the June release. As the temperature dropped again late into the second release, an effect was once again observed, with a statistically significant difference detected between the total control and experimental means.

Nitrous oxide reducers, as indicated by abundance of the nitrous oxide reductase gene (*nosZ*), were slightly increased after the first release, but the effect was not sustained. In contrast, the activity of the nitrous oxide reducers showed a similar response to that of the nitrite reducers, with an early

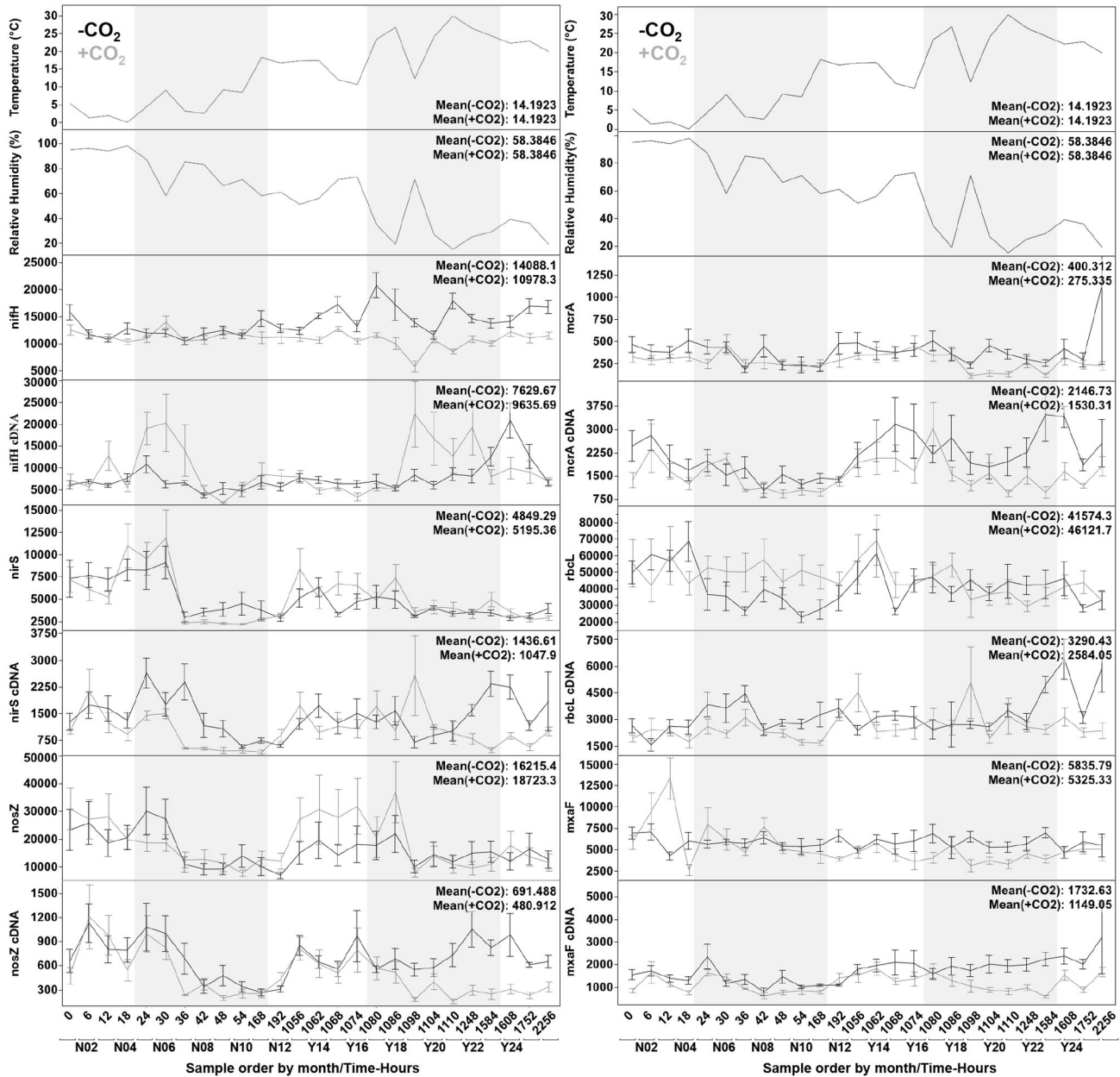


Figure 1. Community response to CO₂ exposure over time. Plot of atmospheric temperature, relative humidity, gene abundance and transcript abundance at time of sampling for six functional genes involved in nitrogen (left panel) and carbon (right panel) cycling under simulated CO₂ leakage (grey lines) or background (black lines) conditions. Data is presented sequentially by month (N=June, Y=July-Sept) and by hours from start of the experiment. Grey-shaded bars indicate active CO₂ flux (injections) for June and July. Values indicate copy numbers per 5 ng of nucleic acid based on averaged measurements of separate triplicate extractions and at least triplicate qPCR reactions for each extraction (n≥9). Error bars are one standard error of the mean (SE). *nifH*: nitrogenase gene; *nosZ*: nitrous oxide reductase gene; *nirS*: nitrite reductase gene; *rbcL*: ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO); *mxnF*: methanol dehydrogenase; *mcrA*: methyl-coenzyme M reductase.

doi: 10.1371/journal.pone.0081742.g001

decrease in transcript abundance during the June release. However, nitrous oxide reducer activity showed a strong response during the July release, with a 1.4 to 2.2-fold

decrease in transcript abundance sustained until 28 days after cessation of CO₂ release.

Autotrophic bacterial numbers, as indicated by abundance of the ribulose-1, 5-bisphosphate carboxylase oxygenase

Table 1. Fold changes in abundance (means) in response to CO₂ release based on sampling time.

| Target | mRNA | | | | | | | | | | |
|-------------|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | DNA | Pre-injection | Injection (June) | Injection (July) | Injection (All) | Post-injection | Pre-injection | Injection (June) | Injection (July) | Injection (All) | Post-injection |
| <i>nifH</i> | -1.1(0.03;0.10) | 1.0(0.28;0.39) | -1.6(0.00;0.00)* | -1.6(0.00;0.00)* | -1.3(0.00;0.00)* | -1.3(0.00;0.00)* | +1.2(0.30;0.16) | +1.7(0.01;1.00) | +1.6(0.01;0.86) | +1.6(0.00;0.90) | -1.3(0.04;0.04)* |
| <i>nifS</i> | 1.0(0.87;0.29) | -1.1(0.68;0.35) | +1.2(0.12;0.41) | +1.2(0.12;0.41) | 1.0(0.73;0.21) | +1.2(0.09;0.86) | -1.1(0.49;0.20) | -1.9(0.00;0.00)* | -1.1(0.49;0.03) | -1.5(0.00;0.00)* | -1.4(0.01;0.07) |
| <i>nosZ</i> | +1.2(0.31;0.18) | -1.2(0.34;0.07) | 1.0(0.86;0.30) | 1.0(0.86;0.30) | -1.1(0.64;0.72) | +1.5(0.03;0.04)* | 1.0(0.05;0.10) | -1.4(0.06;0.31) | -2.2(0.00;0.00)* | -1.7(0.00;0.00)* | -1.4(0.02;0.00)* |
| <i>mcrA</i> | -1.4(0.02;0.13) | -1.2(0.24;0.30) | -1.7(0.00;0.00)* | -1.7(0.00;0.00)* | -1.4(0.00;0.00)* | -1.5(0.13;0.18) | -1.4(0.05;0.06) | -1.2(0.11;0.11) | -1.5(0.00;0.00)* | -1.4(0.00;0.00)* | -1.5(0.00;0.01)* |
| <i>rbcL</i> | -1.2(0.27;0.09) | +1.6(0.00;0.00)* | -1.1(0.49;0.38) | -1.1(0.49;0.38) | +1.2(0.01;0.01)* | +1.1(0.22;0.08) | -1.1(0.33;0.21) | -1.5(0.00;0.00)* | -1.1(0.60;0.08) | -1.2(0.01;0.00)* | -1.4(0.00;0.00)* |
| <i>mxoF</i> | +1.3(0.07;0.47) | 1.0(0.74;0.59) | -1.5(0.00;0.00)* | -1.5(0.00;0.00)* | -1.2(0.00;0.00)* | -1.2(0.01;0.01)* | -1.4(0.03;0.00)* | -1.3(0.01;0.04)* | -1.9(0.00;0.00)* | -1.6(0.00;0.00)* | -1.4(0.01;0.01)* |

P-values based on ANOVA and non-parametric Wilcoxon methods are shown in parenthesis respectively. Bold cells indicate significantly different results (P-value ≤ 0.05) for at least one of the two methods. Significantly different results using both methods are denoted with an asterisk (*).
doi: 10.1371/journal.pone.0081742.t001

(RuBisCO) gene (*rbcL*), showed a 1.6-fold increase during the June release, but no significant effects were noted at any other time. RuBisCO gene transcript abundance, however, indicated a 1.5-fold decrease in response to the June release and also a sustained 2-fold decrease starting late during the July release, which continued until the end of the experiment.

Methanogen numbers, as determined by abundance of the methyl-coenzyme M reductase gene (*mcrA*), showed a 1.4-fold decrease prior to the June CO₂ exposure. Although numbers were generally lower in treatment plots, a significant decrease (1.7-fold) was not seen until the July release, and those numbers recovered after cessation of the release. Methanogen activity response closely followed methanogen abundance, with a 1.5-fold decrease in transcript abundance in response to the July release and recovery not observed until 28 days later.

Methanotroph numbers, as determined by abundance of the methanol dehydrogenase gene (*mxoF*), decreased 1.5-fold, with post injection numbers following the June injection driving the significant decrease observed. A decrease in transcript abundance was also detected, with 1.3 to 1.9-fold decreases throughout the entire experiment.

Multivariate analysis of microbial response to CO₂ exposure

Principal components analysis (PCA) on mean values revealed both a seasonal response as well as a CO₂ effect on gene and transcript abundance (Figure 2 and Figure S1). Both mRNA and DNA showed marked responses, although clustering based on DNA was more defined. PCA based solely on gene abundance, transcript abundance, or all variables combined showed clustering based on seasons, with the CO₂ effect being strongest during active CO₂ release. Post-injection samples (between the June and July release, and after the July release) displayed some recovery, with CO₂-exposed samples shifting away from active release samples and toward background controls. In this analysis, 75.8% of the variance could be explained by the first two components in a PCA with only transcript data, with an additional 10.5% explained by the third component (86.3% total). The percent of variance accounted for in the first three components decreased when PCAs were performed with just the gene abundances (79.6%), with gene and transcript abundance combined (68.4%), or for all abundance data and atmospheric data (64.1%). A factor analysis (Table S3) for each individual PCA suggested that clustering was driven on one axis by the CO₂ effect and on the other by seasons.

To account for changes in microbial community structure and activity relationships in response to CO₂, pairwise correlations were performed on all data for background controls and CO₂-exposed samples separately (summary of significantly affected correlations presented in Figure 3 and Table S4, with full statistical results in Tables S5 and S6 respectively). CO₂ exposure resulted in loss of correlations naturally found in the background samples, while it created others not detected under normal conditions. However, these shifts were only found to occur in weakly correlated variables, and the new correlations that evolved as a result of CO₂ exposure tended to

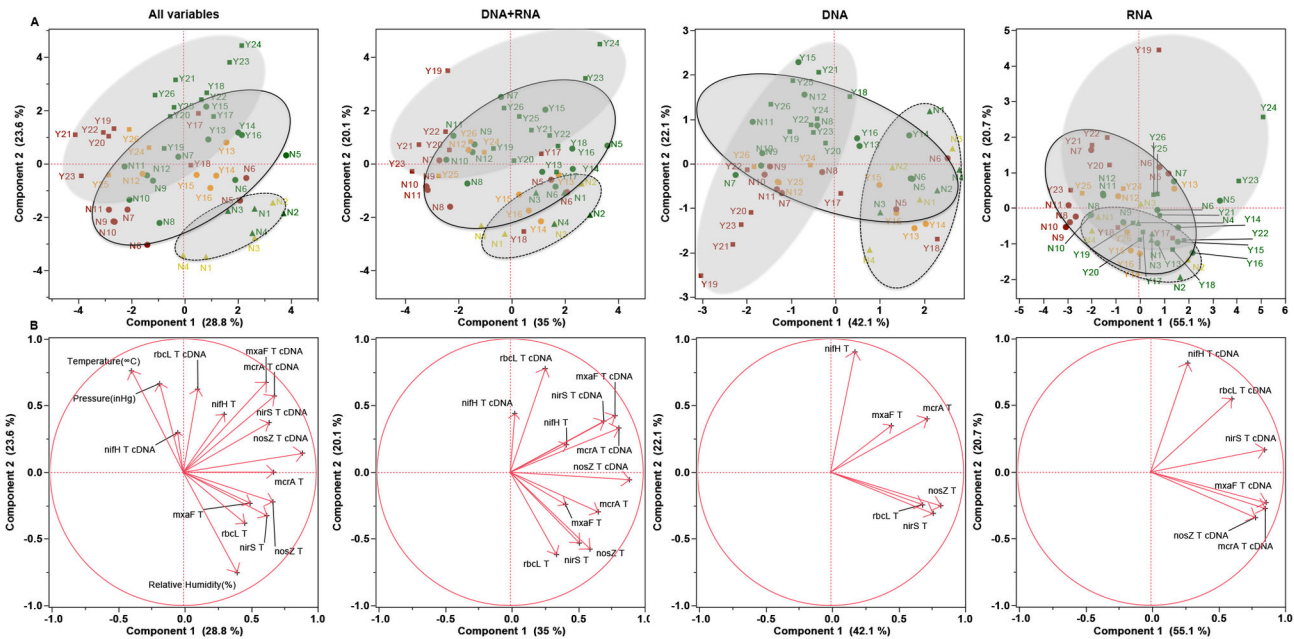


Figure 2. Principal component analysis (A) and factor loadings (B) for (from right to left) transcript (mRNA) abundances, gene abundances, gene and transcript abundance combined, and gene and transcript abundances combined with atmospheric data. Samples are labeled sequentially by treatment (green=background site; red=treatment site active CO₂ injection; orange=treatment site post-CO₂ injection; and yellow=treatment site pre-CO₂ injection), and by month (N=June, Y=July-Sept). Shaded areas represent pre-injection period (N1 - 4, ▲), June injection period (N5 - 12 and Y1 3- 16, ●) and July injection period (Y17 - 26, ■). The percentage of the variation in the samples described by the plotted principle components is indicated on the axis. Gene names in Panel B are as for Figure 1.

doi: 10.1371/journal.pone.0081742.g002

be weak (<0.35 correlated) independent of the analytical methods used.

Discussion

In recent decades, CO₂ has led the trend of increasing greenhouse gases in the atmosphere with a 28% increase in CO₂ emissions between 1990-2004 [19]. In 2004, CO₂ emissions were estimated at 30 Gt CO₂ equivalents/year, with emissions projected to increase 40% to 110% by 2030 [19]. The Intergovernmental Panel on Climate Change has identified CCS as a potential mitigation strategy. In this scenario, storage methods could include injection of CO₂ into underground geological formations, into the deep-ocean, or even industrial fixation as inorganic carbonates. In the case of underground storage, the continuous storage of such large amounts of CO₂ will require assessment of the potential environmental impacts from accidental release of stored gases. Early studies have identified potential lethal effects on plants and subsoil animals and contamination of groundwater from unintended releases [3,4,6,20]. However, the effect of accidental CO₂ release on the microbial populations and guilds driving biogeochemical cycles has not been studied. Here, we show that a short-term, low-rate, simulated underground leak significantly altered both the abundance and activity of key microbial populations responsible for carbon and nitrogen cycling in soils.

The current study shows that seasonal patterns affect soil microbial communities at both DNA and RNA levels, even when exposed to elevated CO₂ conditions, but that the specific seasonal response patterns were altered. This suggests that two different microbial community responses might be simultaneously at play in this system, namely ecological (i.e. environmental) selection on population/guild abundance and regulation of biogeochemical activities at the level of transcription. Elevated CO₂ resulted in both positive and negative shifts of both DNA and mRNA levels of nitrogen and carbon cycling genes (Figure 1 and Table 1). In the case of the nitrogen fixer population, a 1.3 to 1.6-fold decrease in abundance was seen in response to elevated CO₂, while mRNA levels were increased up to 1.7-fold. This suggests that only a portion of the original population was capable of surviving under high levels of CO₂, but those that did persist tended to respond rapidly by increasing expression (via up-regulated gene transcription) of nitrogen fixation pathways. The opposite effect was observed for denitrifiers carrying out nitrous oxide reduction; in this case there was an increase in the population both pre- and post-injection. In contrast, although no difference was detected during injections in relationship to background samples, the abundance of *nosZ* genes was lower during CO₂ injections in exposed samples compared to other CO₂-exposed samples pre- and post-injection. This suggests that although no effect was seen between the treatments

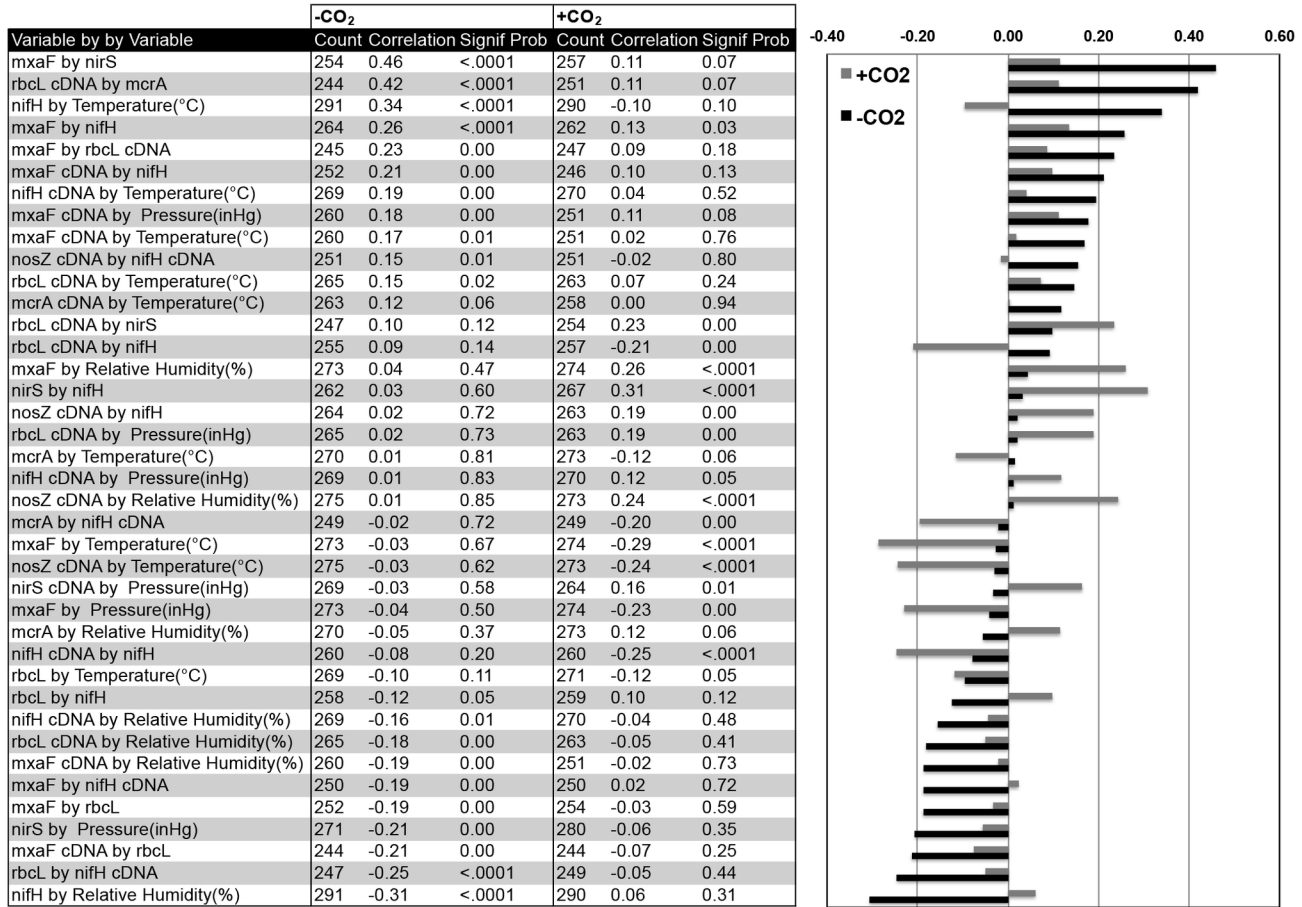


Figure 3. Changes in pairwise correlation between different variables in response to CO₂. All data points for all variables were compared independently for background and CO₂ exposed samples. Only variables displaying shifts in correlations in response to CO₂ are presented. Non-parametric version of this figure can be found in Table S4, with full statistical results in Tables S5 and S6.

doi: 10.1371/journal.pone.0081742.g003

during the injection period, there was variance within the same treatment over time, which is not detected unless time series are examined. *nosZ* transcriptional activity during injections decreased up to 2.2-fold compared to background samples. Further, at this transcriptional level, the negative effect continued well past the July injection. The clearest example of these two response mechanisms in action is seen during the June injection for the *rbcl* (RuBisCO) targets. Although the CO₂ pulse led to an increase in the number of organisms harboring this gene, the total level of transcription was equally reduced, indicating that although organisms capable of carrying out this reaction were present, they were not all up-regulating the transcription of the gene, and thus either a lower percentage of the autotrophic population was actively carrying out the function of fixing CO₂ via RuBisCO or there was a generalized suppression of RuBisCO gene expression. The observed increase in abundance coincided with the plant-growing season.

While studies on the effects of elevated CO₂ in soils are not uncommon, most studies used ppm ranges of CO₂ ranging between atmospheric (year-dependent, approximately 350 ppm) and a maximum of 1200 ppm to assess the environmental effects of elevated atmospheric CO₂. In contrast, the current study used a below-ground delivery system that creates “hot zones” above leak points, indicating that gases tended to escape directly upwards [16,21] leading to concentrations that would be equivalent to 2,500-30,000 ppm within a “hot zone” [12,14] to emulate and assess impacts of escaped CO₂ from a CCS site. Also, most studies have relied on elevated CO₂ treatments which primarily affect the top 10 cm of soils as in FACE facilities [22], while our approach saturated the entire soil column above the injection zone and the proximal aboveground area. Plant stress at the site could be detected up to 7.5 m away from the release zone, with the most distinguishable plant stress signatures within 2.5 m [6,23]. This suggests that any response seen in previous studies using lower concentrations of CO₂ could be magnified in a CCS

escape scenario due to the much higher concentrations of CO₂ involved. However, given that the pulses in our experiments were short-lived (1 - 4 weeks), maximal impacts from CO₂ exposure may have not yet been manifested.

We observed that, although exposure to CO₂ altered the microbial community and its activity, it did not eliminate the seasonal response. In a previous study, Marhan et al. (2011) reported temporal variations, with decreases in denitrifiers linked to lower moisture levels during June - August, similar to what was observed in the current study, but they did not detect the CO₂ effect that we describe [24]. Here, we observed that the first component of a PCA mostly accounted for the effect of CO₂, while the second component accounted mostly for seasonal effects when all variables were examined (Figure 2 and Table S3). This, and the time-dependent (seasonally-dependent) response observed for many targets, indicates that CO₂ effects are dependent on other factors. These other factors are not known for this site, but could include N and P levels in soils as suggested in prior studies with elevated atmospheric CO₂ [22,25,26]. Although termination of subsurface CO₂ injection apparently led to a start in recovery toward background levels of gene and transcript abundance, the speed of recovery was target-dependent and differences in pre-injection measures of these parameters suggest a legacy effect of elevated CO₂ observable even 9 months after the prior injection. However a second possible interpretation is that high soil heterogeneity led to a simple discrepancy due to spatial variability, a factor that we cannot account for in this experiment.

We also observed temperature/moisture-dependent responses to elevated CO₂ as has been previously reported [22,27]. It has also been suggested that changes in precipitation tended to have a greater effect on microbial community composition than factors related to climate change [28], a phenomenon that was observed in a prior study during sharp temperature declines (data not shown). The ZERT field site also showed a plant-level response, with legumes and forb numbers decreasing under elevated CO₂ and being replaced by short grasses [6]. This response differs from prior FACE experiments that have shown an increase in legumes, forbs, legume-bacterial symbiosis, nitrogen fixation genes and nitrogen fixation [29-31]. However other researchers have indicated that a positive response in nitrogen fixation is not universal, and is dependent on plant species and N and P availability [32-36].

In the current study the decline in nitrogen fixers was also accompanied by an increase in *nifH* transcriptional activity, suggesting that high levels of CO₂ in soil might be selecting against symbiosis-based N fixation given that legume numbers were reduced, and towards a smaller, more CO₂ tolerant, free living-nitrogen fixer community as suggested by Tissue et al [32]. Other microbial responses in the current study agree with previous work monitoring DNA-level changes, including increases in *rbcL* gene numbers and a marginal increase in *mcrA* gene numbers [31], although the variance accounted for in the current ZERT dataset is larger. The increase observed in *rbcL* (RuBisCO) gene abundance here was concomitant with decreased transcription. It has been noted that elevated levels

of CO₂ increase efficiency of RuBisCO in plants and algae [37]. If the same response is applicable to all carbon fixing microbes, it could mean that the excess CO₂ is allowing a larger population of autotrophs to prosper, but due to a higher efficiency in CO₂ assimilation they have reduced need for high-level expression of the *rbcL* gene.

Two major findings in this study are that microbial community response is also elicited by environmental factors (moisture, temperature, nutrient, etc.) other than CO₂, and that microbial response can be detected at both the population (DNA) level and the transcriptional level (mRNA), although these parameters do not always respond in the same way. Accounting for seasonally-dependent responses and contradicting responses at the DNA and mRNA level are important if we are to understand the effects of large CO₂ leaks from geologic underground storage sites. Shifts in both functional populations and their activity, as reported herein, could create localized disruptions in ecosystem processes affecting soil biogeochemical cycles. These effects might be similar to those observed in previous studies using FACE systems, but due to the different CO₂ delivery systems and gas concentrations used, additional assessments will be needed to determine the overall effect of leaks from underground storage. It is also important to note that the simulated leaks in these experiments represented low leakage rates for relatively short periods of time. Sustained exposure or higher CO₂ levels in real-world storage leak scenarios might well result in different or more drastic responses. Since we cannot directly identify the mechanisms leading to the observed shifts, we can only speculate. Elevated levels of CO₂ could be directly affecting these communities, or indirectly doing so by altering factors such as pH. However, soil pH measurements taken 5cm below the surface along a transect in 2009 (pre- and post-injections) showed no indication of a pH shift with an average of 6.65 (std. dev. = 0.38) (M. Apple, personal communication) so other factors including micro-scale variation could be involved [38].

Considering the current work in a broader context, a number of recent studies by our group and others show that microbial communities in general are highly dynamic, even under entirely ambient conditions. Thus, microbial responses to other perturbations such as elevated CO₂ may be masked by, embedded within, or quenched by natural responses to ambient environmental change. This means that more intense short-term temporal and seasonal sampling of microbial communities under investigation should be performed in order to fully delineate, define and understand their response to perturbation. Further, in environments exhibiting heterogeneous distribution of microbial populations and their corresponding functions, sampling should be replicated commensurately to allow investigators to measure natural variance in microbial community responses, rather than averaging that variance through homogenization of large samples or bulking of multiple small samples. Finally, with increasing knowledge of microbial ecology, it becomes clear that microbes are not just responding to, but in many cases driving, changes that alter ecosystems [31,39]. Thus, continued studies that attempt to observe, understand and explain the

integral role of microbes in both local and global biogeochemical cycles are necessary.

Supporting Information

Figure S1. Principal component analysis for (from right to left) transcript (mRNA) abundances, gene (DNA) abundances, transcript and gene abundance combined, and gene and transcript abundances combined with atmospheric data. Panel (A) shows June sample data only. Panel (B) shows only the July - Sept sample data. A compiled view for all samples can be seen in Figure 2. Samples are labeled sequentially by treatment (green=background site; red=treatment site active CO₂ injection; orange=treatment site post-CO₂ injection; and yellow=treatment site pre-CO₂ injection), and by month (N=June, Y=July-Sept). The percentage of the variation in the samples described by the plotted principle components is indicated on the axis.

(TIF)

Table S1. Primers and PCR conditions used in this study.

(TIF)

Table S2. Details for statistical analyses based on comparison of target abundance in response to CO₂ release based on sampling period. Summary provided in Table 1.

(XLSX)

Table S3. Factor analysis.

References

- Mikkelsen M, Jørgensen M, Krebs FC (2010) The teraton challenge. A review of fixation and transformation of carbon dioxide. *Energy–Journal of Environmental Sciences* 3: 43. doi:10.3390/en3010043.
- White CM, Strazisar BR, Granite EJ, Hoffman JS, Penline HW (2003) Separation and Capture of CO₂ from Large Stationary Sources and Sequestration in Geological Formations—Coalbeds and Deep Saline Aquifers. *Journal of the Air and Waste Management Association* 53: 645-715.
- IPCC (2005) IPCC Special Report on Carbon Dioxide Capture and Storage. Cambridge, United Kingdom and New York, NY, USA 442 p.
- Stenhouse M, Arthur R, Zhou W (2009) Assessing environmental impacts from geological CO₂ storage. *Energy Procedia* 1: 1895-1902. doi:10.1016/j.egypro.2009.01.247.
- Wielopolski L (2011) Geological carbon sequestration: a new approach for near-surface assurance monitoring. *Int J Environ Res Public Health* 8: 818-829. doi:10.3390/ijerph8030818. PubMed: 21556180.
- Male EJ, Pickles WL, Silver EA, Hoffmann GD, Lewicki J et al. (2010) Using hyperspectral plant signatures for CO₂ leak detection during the 2008 ZERT CO₂ sequestration field experiment in Bozeman, Montana. *Environmental Earth Sciences* 60: 251-261. doi:10.1007/s12665-009-0372-2.
- Farrar CD, Neil JM, Howle JF (1999) Magmatic Carbon Dioxide Emissions at Mammoth Mountain. California. U.S. Geological Survey. pp. 4217-98
- Farrar CD, Sorey ML, Evans WC, Howle JF, Kerr BD et al. (1995) Forest-killing diffuse CO₂ emission at Mammoth Mountain as a sign of magmatic unrest. *Nature* 376: 675-678. doi:10.1038/376675a0.
- Frerichs J, Oppermann BI, Gwosdz S, Möller I, Herrmann M et al. (2012) Microbial community changes at a terrestrial volcanic CO₂ vent induced by soil acidification and anaerobic microhabitats within the soil column. *FEMS Microbiology Ecology*.
- West JM, Pearce JM, Coombs P, Ford JR, Scheib C et al. (2009) The impact of controlled injection of CO₂ on the soil ecosystem and chemistry of an English lowland pasture. *Energy Procedia* 1: 1863-1870. doi:10.1016/j.egypro.2009.01.243.
- Torp TA, Gale J (2004) Demonstrating storage of CO₂ in geological reservoirs: The Sleipner and SACS projects. *Energy* 29: 1361-1369. doi:10.1016/j.energy.2004.03.104.
- Spangler LH, Dobeck LM, Repasky KS, Nehrir AR, Humphries SD et al. (2010) A shallow subsurface controlled release facility in Bozeman, Montana, USA, for testing near surface CO₂ detection techniques and transport models. *Environmental Earth Sciences* 60: 227-239. doi:10.1007/s12665-009-0400-2.
- Spangler LH, Dobeck LM, Repasky K, Nehrir A, Humphries S et al. (2009) A controlled field pilot for testing near surface CO₂ detection techniques and transport models. *Energy Procedia* 1: 2143-2150. doi:10.1016/j.egypro.2009.01.279.
- Lewicki JL, Hillel GE, Dobeck L, Spangler L (2010) Dynamics of CO₂ fluxes and concentrations during a shallow subsurface CO₂ release. *Environmental Earth Sciences* 60: 285-297. doi:10.1007/s12665-009-0396-7.
- Lewicki JL, Oldenburg CM, Dobeck L, Spangler L (2007) Surface CO₂ leakage during two shallow subsurface CO₂ releases. *Geophysical Research Letters* 34.
- Strazisar BR, Wells AW, Diehl JR, Hammack RW, Veloski GA (2009) Near-surface monitoring for the ZERT shallow CO₂ injection project. *International Journal Greenhouse Gas Control* 3: 736-744. doi:10.1016/j.jggc.2009.07.005.
- Morales SE, Cosart TF, Holben WE (2010) Bacterial gene abundances as indicators of greenhouse gas emission in soils. *ISME J* 4: 799-808. doi:10.1038/ismej.2010.8. PubMed: 20182521.
- Morales SE, Holben WE (2009) Empirical testing of 16S rRNA gene PCR primer pairs reveals variance in target specificity and efficacy not suggested by in silico analysis. *Appl Environ Microbiol* 75: 2677-2683. doi:10.1128/AEM.02166-08. PubMed: 19251890.

(TIF)

Table S4. Changes in non-parametric Spearman's ρ between different variables in response to CO₂.

(TIF)

Table S5. Pairwise correlations for all variables with and without CO₂ exposure.

(XLSX)

Table S6. Non-parametric Spearman's correlations for all variables with and without CO₂ exposure.

(XLSX)

Acknowledgements

We thank Philip Drummond, Maria Rosario and Laura Dobeck for help with sampling, and Joseph Shaw for sharing atmospheric data with us. We also thank Martha Apple for useful discussions and field site information.

Author Contributions

Conceived and designed the experiments: SEM WEH. Performed the experiments: SEM WEH. Analyzed the data: SEM WEH. Contributed reagents/materials/analysis tools: SEM WEH. Wrote the manuscript: SEM WEH.

19. IPCC (2007) Climate Change 2007: Mitigation of Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA: Intergovernmental Panel on Climate Change.
20. Little MG, Jackson RB (2010) Potential impacts of leakage from deep CO₂ geosequestration on overlying freshwater aquifers. *Environ Sci Technol* 44: 9225-9232. doi:10.1021/es102235w. PubMed: 20977267.
21. Gouveia FJ, Johnson MR, Leif RN, Friedmann SJ (2005) Aerometric measurement and modeling of the mass of CO₂ emissions from Crystal Geysir, UT
22. Rice CW, Garcia FO, Hampton CO, Owensby CE (1994) Soil microbial response in tallgrass prairie to elevated CO₂. *Plant and Soil* 165: 67-74. doi:10.1007/BF00009963.
23. Keith CJ, Repasky KS, Lawrence RL, Jay SC, Carlsten JL (2009) Monitoring effects of a controlled subsurface carbon dioxide release on vegetation using a hyperspectral imager. *International Journal Greenhouse Gas Control* 3: 626-632. doi:10.1016/j.ijggc.2009.03.003.
24. Marhan S, Philippot L, Bru D, Rudolph S, Franzaring J et al. (2011) Abundance and activity of nitrate reducers in an arable soil are more affected by temporal variation and soil depth than by elevated atmospheric [CO₂]. *FEMS Microbiol Ecol* 76: 209-219. doi:10.1111/j.1574-6941.2011.01048.x. PubMed: 21223340.
25. Mayr C, Miller M, Insam H (1999) Elevated CO₂ alters community-level physiological profiles and enzyme activities in alpine grassland. *J Microbiol Methods* 36: 35-43. doi:10.1016/S0167-7012(99)00009-3. PubMed: 10353798.
26. Edwards EJ, McCaffery S, Evans JR (2006) Phosphorus availability and elevated CO₂ affect biological nitrogen fixation and nutrient fluxes in a clover-dominated sward. *New Phytol* 169: 157-167. doi:10.1111/j.1469-8137.2005.01568.x. PubMed: 16390427.
27. Horz HP, Barbrook A, Field CB, Bohannan BJ (2004) Ammonia-oxidizing bacteria respond to multifactorial global change. *Proc Natl Acad Sci U S A* 101: 15136-15141. doi:10.1073/pnas.0406616101. PubMed: 15469911.
28. Castro HF, Classen AT, Austin EE, Norby RJ, Schadt CW (2010) Soil microbial community responses to multiple experimental climate change drivers. *Appl Environ Microbiol* 76: 999-1007. doi:10.1128/AEM.02874-09. PubMed: 20023089.
29. Hu S, Firestone MK, Chapin FS III (1999) Soil microbial feedbacks to atmospheric CO₂ enrichment. *Trends Ecol Evol* 14: 433-437. doi:10.1016/S0169-5347(99)01682-1. PubMed: 10511719.
30. Ross DJ, Newton PCD, Tate KR (2004) Elevated [CO₂] effects on herbage production and soil carbon and nitrogen pools and mineralization in a species-rich. *Plant and Soil* 260: 183-196. doi:10.1023/B:PLSO.0000030188.77365.46.
31. He Z, Xu M, Deng Y, Kang S, Kellogg L et al. (2010) Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO₂. *Ecol Lett* 13: 564-575. doi:10.1111/j.1461-0248.2010.01453.x. PubMed: 20337697.
32. Tissue DT, Megonigal JP, Thomas RB (1997) Nitrogenase activity and N₂ fixation are stimulated by elevated CO₂ in a tropical N₂-fixing tree. *Oecologia* 109: 28-33. doi:10.1007/s004420050054.
33. Cheng W, Inubushi K, Yagi K, Sakai H, Kobayashi K (2001) Effects of elevated carbon dioxide concentration on biological nitrogen fixation, nitrogen mineralization and carbon decomposition in submerged rice soil. *Biology and Fertility of Soils* 34: 7-13. doi:10.1007/s003740100345.
34. Hungate BA, Dijkstra P, Johnson DW, Hinkle CR, Drake BG (1999) Elevated CO₂ increases nitrogen fixation and decreases soil nitrogen mineralization in Florida scrub oak. *Global Change Biology* 5: 781-789. doi:10.1046/j.1365-2486.1999.00275.x.
35. Soussana JE, Hartwig UA (1996) The effects of elevated CO₂ on symbiotic N₂ fixation: a link between the carbon and nitrogen cycles in grassland ecosystems. *Plant and Soil* 187.
36. Zanetti S, Hartwig UA, Luscher A, Hebeisen T, Frehner M et al. (1996) Stimulation of Symbiotic N₂ Fixation in *Trifolium repens* L. under Elevated Atmospheric pCO₂ in a Grassland. *Ecosyst - Plant Physiology* 112: 575-583.
37. Bowes G (1991) Growth at elevated CO₂: photosynthetic responses mediated through Rubisco. *Plant, Cell and Environment* 14: 795-806. doi:10.1111/j.1365-3040.1991.tb01443.x.
38. Vos M, Wolf AB, Jennings SJ, Kowalchuk GA (2013) Micro-scale determinants of bacterial diversity in soil. *FEMS Microbiol Rev* 37: 936-954. PubMed: 23550883.
39. van der Heijden MG, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol Lett* 11: 296-310. doi:10.1111/j.1461-0248.2007.01139.x. PubMed: 18047587.