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Paper

CO_2 concentration and pH alters subsurface microbial ecology at reservoir temperature and pressure

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Abstract

The security of long-term CO_2 storage following geologic carbon sequestration may be impacted by biogeochemical reactions in the formation; yet little understanding exists about the impact of CO_2 gradients on microorganisms that drive biogeochemistry in the deep subsurface. The effect of CO_2 gradients on the microbial community from a brine aquifer was examined at reservoir pressure (14 MPa) and temperature (40˚C). The community was exposed to pCO_2 at 0, 0.1, 1.4 and 14 MPa for up to 56 days and was examined using 16S rRNA gene clone libraries and qPCR. Diversity indices (equitability) were also determined. In addition, the effect of lowered pH without CO_2 exposure was examined. Exposure to CO_2 resulted in a decrease in microbial diversity and a decrease in 16S rRNA gene concentrations. After 56 days, no 16S genes were recovered following exposure to 1.4 MPa pCO_2 or greater. Exposure to 0.1 MPa pCO_2 resulted in 16S gene concentrations an order of magnitude less than 0 MPa pCO_2. The equitability of 0 MPa exposure (0.54) decreased with 0.1 MPa exposure to 0.29. Microbial community with the lowered pH (pH=4.4) and 0 MPa pCO_2 resulted in 5 orders of magnitude increased 16S gene concentration than reactors with CO_2 exposure, indicating that CO_2 was detrimental independent of pH. This suggests that even buffered reservoirs may have a decreased microbial population due to injected CO_2. Halotolerant strains *Halomonas* and *Marinobacter* appeared to be the most tolerant to CO_2 exposure and decreased pH. This is the first study to examine the initial microbial community response to a gradient of CO_2 that would follow geologic carbon sequestration.

Introduction

Subsurface geologic carbon sequestration is a proposed component of a comprehensive solution to the accumulation of CO_2 in the atmosphere. Behaviour of CO_2 injected into potential subsurface sites, such as saline aquifers and petroleum reservoirs is currently being examined [1-5]. During this process, CO_2 is injected as supercritical CO_2 ($SC\text{-}CO_2$). However, due to heterogeneity of the formation's permeability, injected CO_2 would lead to a gradient of $CO_{2(aq)}$ concentrations within the reservoir[1, 2, 6] (Figure 1). Residual subsurface gas may further add to heterogeneous distribution of injected CO_2 in the subsurface[5]. The scale of resulting heterogeneities will likely depend on geology, lithology, and injection rate. Unexposed formation liquids and solids adjacent to injected CO_2 will result in gradients of dissolved CO_2, both at the macro and pore scale, which may take up to thousands of years to reach full equilibrium[7].

Since CO_2 is an acid, its dissolution into porewater will decrease pH[3, 8, 9]. This mineral dissolution will result in the release of previously precipitated ions in the porewater. Although mineral dissolution can mitigate this decrease in pH via buffering, a gradient of CO_2 concentration and pH will occur, moving from the $SC\text{-}CO_2$ into the unimpacted formation (Figure 1). While exposure to the $SC\text{-}CO_2$ phase may sterilize the subsurface[10, 11], heterogeneous distribution and dissolution of $SC\text{-}CO_2$ may create niches in the formation brines and solids where microbes may survive and impact the fate, transport, and storage capacity for $SC\text{-}CO_2$.

Figure 1 Conceptual model of heterogeneous flow of $SC\text{-}CO_2$ and resulting CO_2 concentration and pH gradients.

Exposure to CO_2 can hinder cell growth[10-12] and at high pressures terminate growth altogether[13]. In aqueous systems with a high pCO_2 exposure, the pH may be as low as ~3.5 to 4.5[3], and much of the dissolved CO_2 is in the form of $CO_{2(aq)}$ rather than a carbonic acid[14, 15]. This small, nonpolar molecule may then diffuse through cell membranes, disrupting cell growth by changing the cell fluidity, inhibiting intercellular enzyme performance, and affecting the intercellular pH[11]. However, while laboratory cultures have been found to decrease cell viability with CO_2 exposure,[10, 11, 13, 16, 17] other studies suggest native subsurface species may be adaptable to CO_2 environments[18-21]. Bacterial survival and growth will likely differ

along the gradient of $CO_{2(aq)}$ as the microorganisms are exposed to varying carbonic acid speciation, pH, and ion concentrations. In addition, the response to CO_2, in its various forms is also likely to be species dependent, giving rise to changing microbial communities along the gradients. Along these gradients within the storage reservoir, microbial processes are poorly understood, and may be important for CO_2 storage, security, and capacity.

Microorganisms exist in subsurface formations that are analogous to ones in which geologic carbon sequestration has been proposed, such as deep sea sediments, deep continental aquifers, and petroleum reservoirs[22-24]. Interactions of these microbial populations with the geochemically and physically altered subsurface may drive geochemical processes that are a benefit or detriment to long-term storage of CO_2. For example, biomineralization of carbonate[25, 26] may increase the injectable mass of CO_2 and produce non-mobile carbon phases or may decrease storage capacity if carbonate mineral formation leads to pore plugging[27]. On the other hand, bacteria that produce acids as a by-product of metabolism may dissolve carbonate minerals as well as increase metal ion mobility. In order to best model the capacity and security of injected CO_2 during geologic carbon sequestration, there is a need for better understanding of the microbial populations that survive and perhaps thrive in formations receiving SC-CO_2.

The change of a subsurface microbial community under the conditions that simulate heterogeneous SC-CO_2 flow was examined. Formation water and suspended solids from a future carbon sequestration site, the Arbuckle saline aquifer in Southwestern Kansas, were exposed to varying CO_2 partial pressures at formation temperature (40 ˚C) and pressure (14 MPa). The relative impact of pH changes (as a result of CO_2 concentration) versus dissolved CO_2 was also examined by comparing microbial community changes due to a decrease in pH without CO_2 exposure. This is the first study to examine the initial ecological response of a subsurface microbial community to a gradient of CO_2 that may be expected following geologic carbon sequestration.

Results and Discussion

In order to understand the impact of pCO_2 on the microbial community from a future carbon sequestration site, samples of drill stem test water from the Arbuckle saline aquifer (1220 to 1460 m depth) was exposed to different pCO_2 (0, 0.1, 1.4 and 14 MPa of CO_2) at formation temperature and pressure (40 °C and 14 MPa). Reactors at each pCO_2 concentration were sacrificed after 1 day, 7 days and 56 days of exposure. The microbial community was examined using a 16S rRNA gene clone library approach and gene copies were examined using quantitative PCR. Some general trends in total recoverable 16S DNA are apparent upon exposure to different pCO_2 and over time. The 16s gene copies of the 0 MPa and 0.1 MPa reactors suggest microbial growth when compared to 16S gene copies of the initial water (Figure 2). In contrast, the 1.4 MPa and 14 MPa reactors had decreased 16S gene copies compared to the initial water, suggesting cell decay. In general, increasing pCO_2 and length of exposure accompanied a decrease in 16S rRNA gene copies recovered by qPCR (Figure 3). The effect of CO_2 can been seen in concentrations as low as 0.1 MPa; after 56 days of exposure to 0.1 MPa of CO_2, the concentration of 16S rRNA genes had decreased by approximately an order of magnitude to 10^5 copies/mL compared to the 0 MPa CO_2 reactor. At higher pCO_2 exposure, the decrease was more severe and occurred in a shorter time. Only 7 days of 1.4 or 14 MPa pCO_2 exposure decreased the concentration of genes recovered to 10^2 and 10^1 copies/ml, respectively. After 56 days of exposure at 1.4 or 14 MPa, no 16S rRNA genes were detected using qPCR. Nested PCR was able to recover DNA from the 1.4 MPa reactor at very low concentrations (less than 1 copy/mL). At 1.4 MPa and 14 MPa pCO_2, the decreasing concentration of 16S rRNA genes recovered over both exposure time and pCO_2 suggest that the number of cells in the reactors was declining.

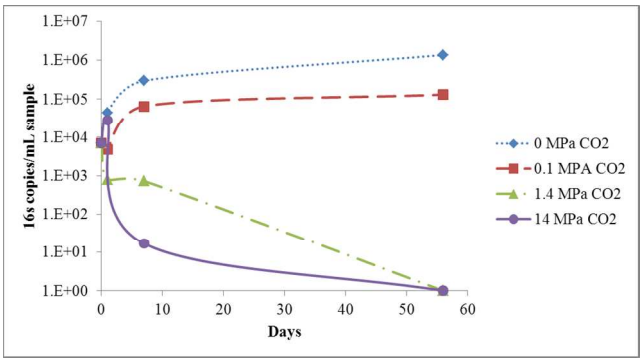

Figure 2 Concentration of 16S rRNA gene copies recovered from reactors exposing unfiltered saline aquifer samples to increasing pCO_2 as revealed by qPCR for 0 days, 1 day, 7 days, and 56 days of exposure. Concentrations of genes are reported as gene copies/mL of drill stem test sample.

With one exception, previous work with pure cultures shows that increasing pressures of CO_2 inhibits microbial growth. *Shewanella oneidensis* growth was inhibited with pCO_2 as low as 0.01MPa and terminated with pCO_2 of 0.1 MPa[13]. Other work showed that suspended cell cultures of *Bacillus mojavanesis* had a 3 log reduction of cell numbers and biofilm cultures had a 1 log reduction of cell numbers when exposed to SC-CO_2 for 17 hours[28]. Cultures of *Pseudomonas putida*, *Bacillus subtilis*, *Thauera aromatica*, and *Desulfovibrio vulgaris* were all found to have decreased growth rates after exposure to pCO_2 less than 1 atm (0.1 MPa) for 30-70 hrs[12]. Declining cell numbers following increasing CO_2 exposure with time suggest that biological processes may only be relevant at a distance from the SC-CO_2 front where pCO_2 has attenuated. The one exception has been the identification of a SC-CO_2 tolerant strain, MIT 0214 (Peet, 2011).

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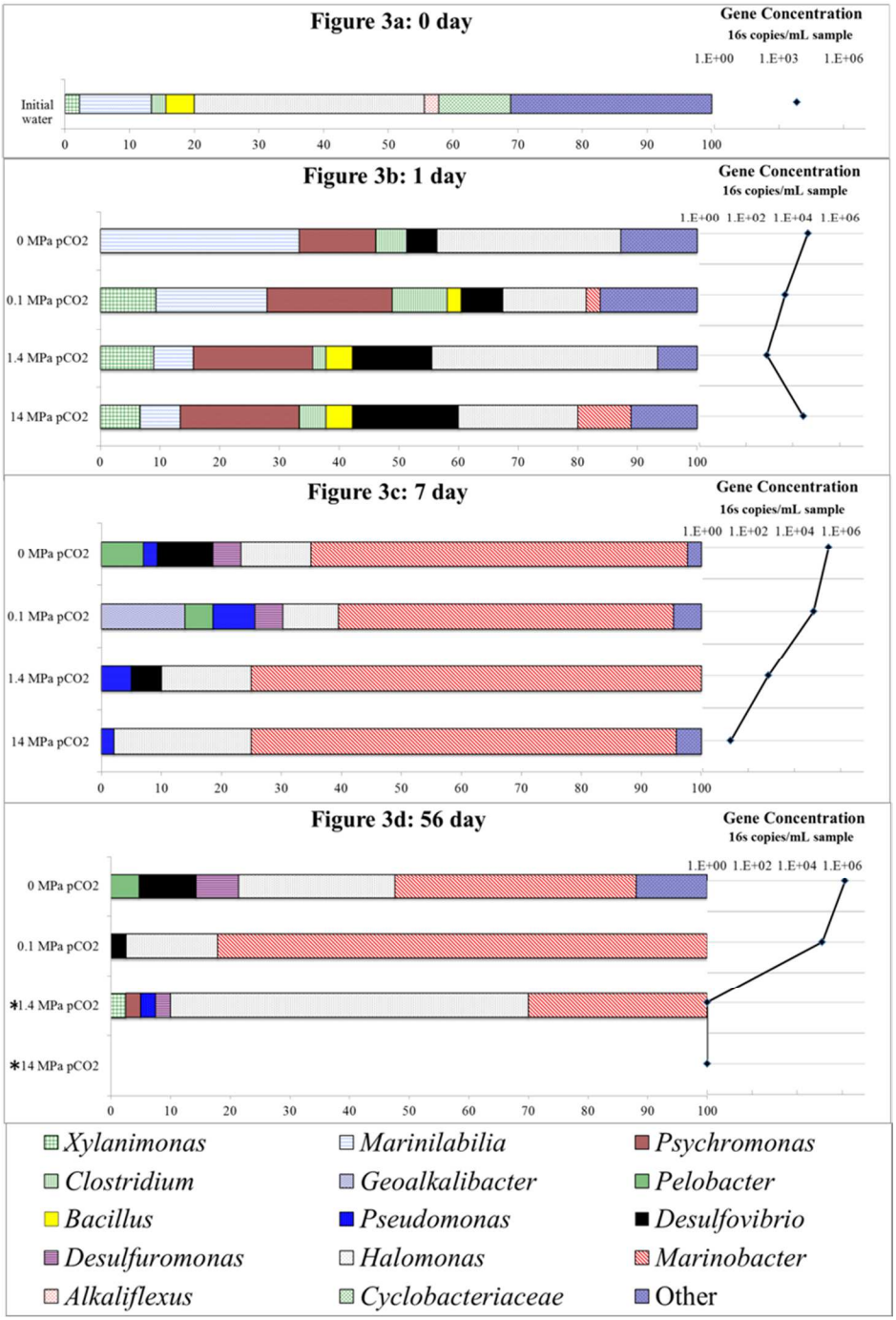

Figure 3 Relative proportions of phylotypes recovered from reactors exposing unfiltered saline aquifer samples to increasing pCO_2 as revealed by 16S rRNA gene clone libraries and qPCR for a) initial drill stem test sample, and following b) 1 day of exposure, c) 7 days of exposure, and d) 56 days of exposure. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other". The inset shows the concentration of 16S rRNA genes recovered by qPCR for each sample for a given pCO_2 exposure. Concentrations of genes are reported as gene copies/mL of drill stem test sample. * = required nested PCR.

Results reveal that increasing pCO_2 decreases microbial

diversity (Figure 4). Overall, fewer unique 16S rRNA genes were recovered over both increasing exposure times and increasing pCO_2 greater than 0.1 MPa. Equitability decreased from 0.74 in the initial water to as low as 0.28 after 56 days of exposure to 1.4 MPa CO_2 (Figure 4). After 56 days of exposure to any pCO_2, the equitability decreased by more than 1.4 times over the initial water sample for all pCO_2 concentrations. It should be noted that the diversity percentage after 56 days of exposure to 1.4 MPa was estimated based on a nested PCR procedure, and may not directly compared to the other samples; the reported diversity was higher than expected by a single PCR amplification (as utilized for the other samples) and may be artefact from the nested PCR.

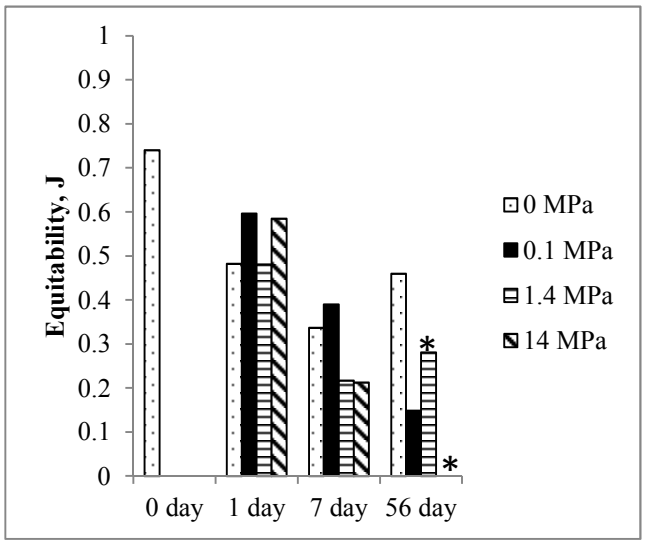

Figure 4 Impact of exposure time and pCO_2 on microbial diversity of unfiltered saline aquifer samples. Results show the comparison of Equitability values (J) for each pCO_2 concentration examined with time. *sample required nested PCR. A J = 0 is a pure culture, whereas J = 1 if each clone is a unique phylotype.

Although the 16S clone library method only allowed observation trends in abundant microorganisms, utilized methods still reveal changes in the microbial community with increasing CO_2 concentration and exposure time. In all cases, the decreased microbial diversity with time and increasing pCO_2 was due to the selection of organisms with phylotypes most similar to cultured members of the genera, *Marinobacter* and *Halomonas* (Figure 3). Initially, the drill stem test water had 35% of the 16S rRNA genes that could be assigned to *Halomonas* and 0% of the 16S rRNA genes that could be assigned to *Marinobacter*. The lack of phylotypes associated with *Marinobacter* in the initial sample was likely due to a low cell population number that could not be detected utilizing the clone library method. After 7 days of exposure, the percentage of *Halomonas* and *Marinobacter* had increased above 65% of the community at each pCO_2 examined (Figure 3). After 56 days of exposure, the 0.1 and 1.4 MPa had 97% and 90 % of their communities (respectively) similar to *Marinobacter* and *Halomonas*. After 56 days, the control reactor (0 MPa CO_2 exposure), was still enriched with phylotypes having the greatest similarity to *Halomonas* and *Marionbacter* genera (65%) and to a lesser degree *Desulfuromonas* (7%), *Desulfovibrio* (10%), and *Pelobacter* (5%). Organisms related to *Halomonas* and *Marinobacter* are known to be adaptive to high pressure, high temperature marine environments, as described later [29, 30]

While the reactors exhibited an increasing proportion of *Marinobacter* and *Halomonas* like phylotypes, there was also a general trend towards lower concentrations of 16S rRNA genes in the samples with higher pCO_2 and longer exposure times (Figure 3). Under conditions where the concentration of rRNA genes was decreasing, the increased proportions of the community assignable to *Marinobacter* and *Halomonas* may be due to their survival under the conditions examined rather than enrichment and growth of organisms. While both *Marinobacter* and *Halomonas* appear to be the most dominant taxa under all pressure vessel conditions, the decreased DNA concentration at 1.4 MPa and 14 MPa demonstrate these taxa were equally inhibited by 7 days and 56 days of CO_2 exposure. However, since the 0.1 MPa exposure contained a similar concentration of 16S rRNA genes after 56 days as after 7 days, the increased proportion of *Halomonas* and *Marinobacter* at 56 days compared to 7 days, may not merely be survival, but potentially growth under these conditions. *Halomonas* and *Marinobacter* appeared to be the most tolerant to low concentration CO_2 exposure.

Concomitant with the decrease in diversity and 16S rRNA gene concentrations in the exposed water, a decrease in the relative proportions of initial sample phylotypes *Marinilabilia*, *Pelobacter*, *Xylanimonas*, and *Clostridia*, among others was observed. *Clostridia*[31], *Bacillus*[32, 33], *Marinilabilia*[34, 35] and *Psychromonas*[36] were previously isolated from saline subsurface environments and were observed to survive the initial 1 day exposure. Since the decrease of these phylotypes occurred after 7 days of incubation at all pCO_2 exposures, it is likely that temperature and pressure rapidly selected against these organisms, rather than CO_2 (Figure 3).

The microbial communities of each pressure vessel were compared on a community tree (Figure 5). The community tree demonstrates the microbial communities were most similar amongst reactors after 1 day of exposure. After 7 days and 56 days, community tree demonstrates both CO_2 concentration and exposure time resulted in a change in the microbial community.

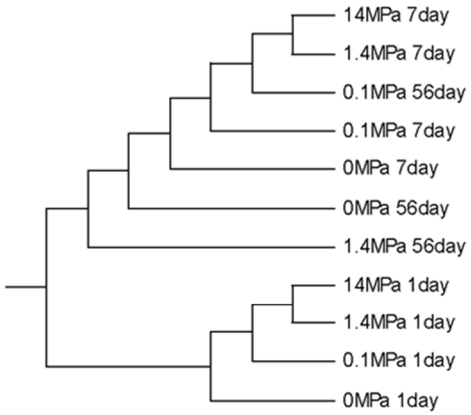

Figure 5 In order to compare microbial communities amongst reactors, a community tree was constructed utilizing Mothur and TreeViewerX. The distance matrix was calculated with the Yue and Clayton measure.

The survival and perhaps growth of halotolerant genera *Halomonas* and *Marinobacter*, and sulfate reducing genera

Desulfovibrio and *Desulfomonas* in later incubation periods without CO_2 exposure is not surprising. These microbial populations have been identified in similar high saline, high pressure and temperature conditions. *Halomonas* are resistant to lysing in highly saline and extreme environments[37]. Cultured strains of both *Halomonas* and *Marinobacter* are known to have a versatile metabolism, capable of degrading a variety of hydrocarbons in high salinity environments[29, 30, 38]. *Halomonas* were found in oil field samples[24] and marine and hypersaline environments[39-42] and are generally known for an ability to grow across a broad range of temperature and pH conditions[43, 44]. Similarly, *Marinobacter* have been found in a variety of marine environments[45, 46] as well as oil fields[24, 47]. Strains of *Desulfovibrio* have been isolated from ground water of deep granitic formations[48], as well as marine sediments[49] and saline waters of a natural gas reserve[50]. *Desulfomonas* has been found in saline subsurface environments such as hydrothermal vents[32] and oil fields[23]. Indicating a potential linkage with the observed geochemistry, a higher population of sulfate reducers was observed in the reactor with lowest concentration of sulfate; 0 MPa pCO_2 exposure (Table 1).

The adaptability of *Halomonas* and *Marinobacter* to temperature, pressure, and the salinity of the formation water is not surprising, however their adaptation to CO_2 contradicts findings in a review of high-pressure CO_2 for sterilization in the food industry suggesting that gram negative species are less tolerant of CO_2 exposure than gram positive organisms[16]. In the 0 MPa, 0.1 MPa, and 1.4 MPa reactors the phylotypes of the *Halomonas* genera were most closely related to the species, *Halomonas alimentaria*, (accession numbers HM583971.1, GU397400.1) representing 15% to 47% of the diversity. The phylotypes of the *Marinobacter* genera were closely related to *Marinobacter sp. M9(2010)* (accession number HQ433441.2) and *Marinobacter sp. TBZ126* (accession number HQ845769.1), representing 30% to 82% of the diversity. These *Marinobacter* and *Halomonas* species were isolated from hypersaline lakes in Iran. While it is uncertain whether these particular strains of *Halomonas* and *Marinobacter* will affect reservoir capacity and integrity, species of these genera have been previously found capable of biofilm formation and biologically inducing carbonate mineralization[51, 52] and warrant further study.

In addition to exposure to CO_2, microbial communities will likely have to adapt to increased acidity in the formation. The introduction of CO_2 into saline aquifer will decrease the overall pH of the reservoir fluids[3]. Following CO_2 injection into the Cranfield sequestration aquifer, pH=5 and lower were reported[4]. However, the magnitude of the pH change will depend on the mineral composition of the formation solids and the presence of carbonate cements and accessory minerals that may buffer the system. The Arbuckle Aquifer did not have core available for the exposure experiments; addition of aquifer rock would be expected to buffer the system replicated in the reactors. The effect of a lowered pH versus CO_2 concentration therefore had to be characterized. The expected drop in pH is likely to inhibit growth of populations that are sensitive to pH and enhance growth of those that can adapt to the lower pH. In order to separate the impact of pH and CO_2 on the microbial communities, two additional reactors were established in which the pH of the formation water was modified to represent the hydrogen ion concentration created by addition of 1.4 MPa CO_2, but without CO_2 addition.

The Arbuckle formation water obtained for these studies was poorly buffered by minimal amounts of suspended solids in the water (less than 0.5 g/L). The expected pH for a given pCO_2 was calculated using Geochemist Workbench with ICP-OES data (Table 1) and measured initial sample alkalinity (of 388 mg/L HCO_3^- [53](Scheffer, 2012). The calculations revealed that the expected pH decrease was from pH=7.8 in the initial unreacted water to pH=4.4 at pCO_2=1.4 MPa. Therefore, formation water was acidified to pH=4.4 with HCl and maintained at a have an increased pH to 5.0 and 5.1 respectively. The microbial community was examined using the 16S rRNA genes approaches as before. The results from this pH-only control were compared to results obtained from the exposures to 1.4 MPa CO_2 (Figure 6). The addition of HCl to the vessel resulted in decreased rRNA gene copy numbers in a shorter period than the CO_2-exposed vessels. The reasons for this are not clear. However, after a longer exposure time of 56 days, the vessels with only a pH change and no CO_2 exposure were found to contain five orders of magnitude more gene copies than the 1.4 MPa CO_2 vessel.

The diversity of the pH-only samples (0.34-0.37) is only slightly larger than the pCO_2 exposed samples (0.22-0.28) (Figure 7). This is still greatly reduced from the equitability of the initial water (0.74). The finding of *Halothiobacillus* and *Pseudoaltermonas* in the pH adjusted reactors suggests that they may be more resilient to lowered pH than CO_2 exposure. Similar to the CO_2 exposed water, the bacterial community shifted towards large populations of *Halomonas* and *Marinobacter*. The much greater recovery of 16S rRNA genes in samples with only lowered pH than samples with CO_2-lowered pH is consistent with previous studies with pure cultures[11, 13]. Wu et al. found that 1 hour of exposure to 150 psi (1.03 MPa) CO_2 resulted in extracellular protein release by *Shewanella oneidensis* whereas cultures exposed to only a lowered pH did not[13]. The results from previous pure culture studies and our studies show that CO_2 is inhibitory to cell growth.

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Table 1: Elements and selected anions measured in the reactor water after 56 days of exposure to 0 MPa, 0.1 MPa, 1.4 MPa, and 14 MPa pCO_2. pH was modelled with the initial alkalinity of 388 mg/L as HCO_3^-. <DL = below detection limit

	Units	Initial	0 MPa CO_2	0.1 MPa CO_2	1.4 MPa CO_2	14 MPa CO_2
Ca	mg/L	2502	2071	2186	2186	2104
Fe	mg/L	< DL	< DL	< DL	0.5	2.5
K	mg/L	259	252	256	253	257
Mg	mg/L	702	568	578	577	591
Mn	mg/L	0.9	0.7	0.9	0.9	0.9
Na	mg/L	22690	18280	18440	17530	17640
P	mg/L	1.5	3.4	1.4	2.4	2.0
S	mg/L	631	594	618	601	613
Si	mg/L	10.0	9.4	10.1	10.8	11.0
Zn	mg/L	0.2	0.1	0.8	1.8	2.4
SO_4^{2-}	mg/L	1190	370	1007	954	1096
Cl^-	mg/L	31528	31992	32397	31639	37757
Modeled pH		7.80	7.50	5.50	4.38	2.88

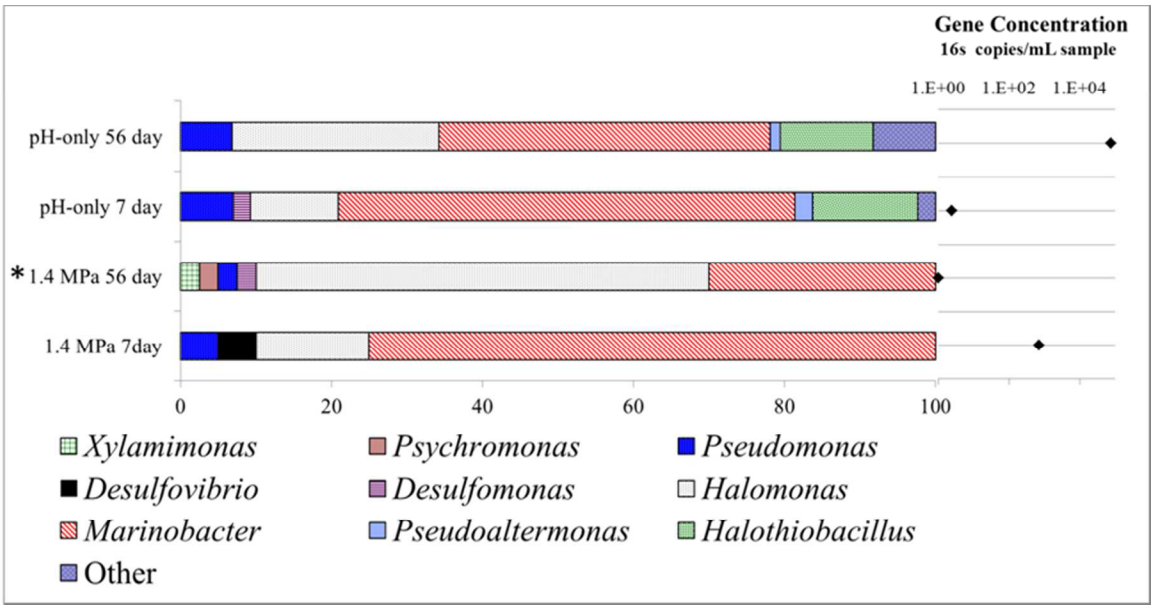

Figure 6 Microbial ecology characterized by 16S rRNA gene clone libraries and microbial population quantified by qPCR methods for 7 day and 56 day exposures to 1.4 MPa pCO_2 or the equivalent pH-only adjustment to 4.4. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other". * = required nested PCR.

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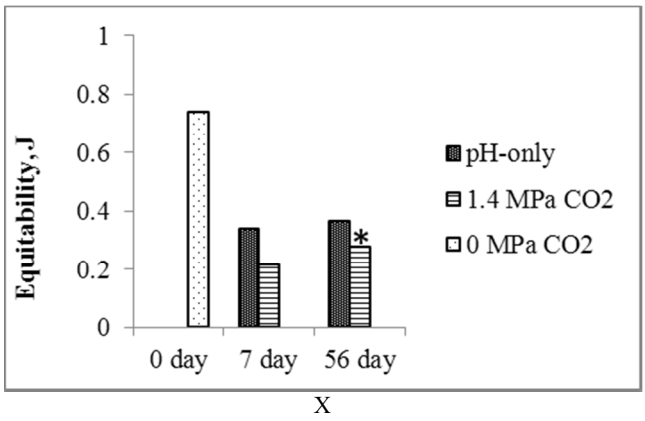

Figure 7 Impact of pCO_2 versus pH-only on microbial diversity of unfiltered saline aquifer samples. Results show the comparison of Equitability values (J) for each pCO_2 concentration examined with time. *sample required nested PCR. A J = 0 is a pure culture, whereas J = 1 if each clone is a unique phylotype.

It is noteworthy that high CO_2 concentrations increased the iron and zinc content in the reactor water; similar to previous studies[54] (Table 1). Elevated iron and or zinc concentrations may have a stimulatory or toxic impact on individual populations of bacteria and select for a community that is adapted to these concentrations. More research is needed to fully understand what mechanisms cause cell growth inhibition.

One limitation of this study was a lack of significant amounts formation solids that would act as a buffer and be present in most CO_2 sequestration sites. To address this limitation, an additional vessel was pressurized with 1.4 MPa CO_2 and calcite as a buffer for 7 days. This vessel was modelled to have a pH of 5.0. The diversity from the fluid sampled from this vessel was found to decrease and the microbial community was similar to the 7 day vessel with 1.4 MPa pCO_2 and no buffer. The loss of diversity was similar in the absence and presence of calcite to buffer the system (Figure SI.1). These findings suggest that injected CO_2 will affect microbial communities, even when the rock matrix buffers the decrease in pH.

Experimental

Site Description

The Wellington oil field, Sumner County, Kansas, contains 47 production wells and 15 injection wells. The Arbuckle saline aquifer (about 1220 to 1460 m depth) is part of the Ozark Plateau aquifer system, and has not yet been injected with CO_2, but has been estimated by the Kansas Geologic Survey to have a CO_2 sequestration capacity of 1.1 to 3.8 billion metric tonnes. The aquifer consists of mostly dolomite containing lenses of shale[55] and is isolated from freshwater aquifers by the 15 m thick Chattanooga shale. For a detailed description on the geology of the Arbuckle aquifer, please refer to Franseen et al., 2004[55]. For a detailed description on the geochemistry and mineralogy of the Arbuckle Aquifer, please refer to Barker et al., 2012[56]. The total dissolved solids of this reservoir ranges from 10,000 to 250,000 ppm[56].

Well 1-32 (latitude 37.3154639, longitude 97.4423481), in the Arbuckle formation, was drilled in January of 2011. Formation samples were obtained during the drill stem test. Drill stem testing is a well-established sampling procedure in the oil/gas industry for obtaining unfiltered formation water and testing reservoir flow parameters[57, 58]. The well was packed above and below the screened intervals to prevent contamination by well water. Drill stem test water was drawn for samples after 4800 L of purging. The samples were collected at the two depth intervals of the reservoir, at 1305 m to 1340 m depth and 1270 m to 1280 m depth. At this depth, salinity ranged from 55,100 ppm to 57,800 ppm and DOC ranged from 110 ppm C to 170 ppm C[53] . Gas composition was tested for CO_2 and CH_4 for at these depth intervals. CO_2 ranged from 0.39 ppm to 4.03 ppm, and CH_4 ranged from 280 ppm to 4370 ppm at this depth[53]. Samples were collected in sterile 1 L Teflon bottles and immediately shipped overnight on ice prior to initiating experiments. A total of 3 L of sample water was available for this study.

Exposure Experiments

Formation water was exposed to CO_2 in 1-L, Teflon-lined stainless steel static pressure vessels capable of maintaining pressures up to 27.5 MPa and constant temperatures to 90 ˚C. These vessels were manufactures by Thar Technologies Inc, and details of these vessels can be found in Kutchko et al., 2007[59]. A volume of 200 ml of reservoir water was added to each vessel. ISCO syringe pumps pressurized vessels with pure CO_2 gas first to achieve the desired pCO_2 and 99.5% N_2/0.5% H_2 gas second to maintain a fixed total pressure of 14 MPa. The experimental pCO_2 of the reactors was 0, 0.1, 1.4 and 14 MPa which represented 0%, 1%, 10% and 100% of the total pressure respectively. All reactors were maintained at a constant temperature of 40 ˚C. Sampling was performed by sacrificing vessels after 1, 7, and 56 days of exposure to CO_2.

In order to better isolate the impact of pCO_2 from pH changes due to the added CO_2, identical reactors were studied with pH adjustment using 1M HCl and no addition of CO_2. The pH examined in this control reactor was 4.4, the value that resulted from exposure to 1.4 MPa CO_2. The pH-only vessels were pressurized with 99.5% N_2/0.5% H_2 gas to 14 MPa and were maintained at 40 ˚C; sampling was performed by sacrificing vessels after 7 and 56 days of exposure to CO_2. The pH was measured after sacrificing each vessel. The 7 day vessel was found to have a pH of 5.0 and the 56 day vessel was found to have a pH of 5.1.

Headspace samples of gas were collected in 500 ml Tedlar bags for immediate analysis by gas chromatography as described

below. Water samples containing suspended solids were centrifuged at 5,000 g for 1 hr. The entire pellet was collected for microbial analysis and the supernatant was collected for chemical analysis as described below. Each pellet weighed approximately 0.5 g per reactor.

In order to determine the baseline geochemistry and microbial community, prior to experimentation in the pressure vessels, a 200 ml aliquot of the initial water was centrifuged at 5,000 g for 1 h. The pellet was approximately 0.5 g and was collected for microbiological analysis. The supernatant was used for chemical analysis described below.

Microbial Community Analysis

DNA was extracted using a modified method described previously in Holmes et al, 2004[60]. Briefly, the DNA was first suspended in TE-sucrose buffer, and followed by lysing with lysozyme, sodium dodecyl sulfate (SDS), and proteinase K. After incubation at 37 ˚C, samples were bead-beated for 30 sec. Extraction with a 1.2:1 ratio of 5M NaCl and 10% CTAB followed with incubation at 65 ˚C. Finally, DNA was purified with 24:1 chloroform:isoamyl alcohol and 25:24:1 phenol:chloroform:isoamyl alcohol. The DNA was precipitated with isopropanol and washed with 70% ethanol. After resuspending the DNA in water, samples were incubated overnight in a 4 ˚C fridge. The samples were then stored at -20 ˚C until further analysis.

The bacteria 16S rRNA gene fragments were amplified using the 8 forward primer 5'-AGAGTTTGATCMTGGCTCAG-3'[61] with the 519 reverse primer 5'-GTATTACCGCGGCTGCTGG-3'[61] and the 338 forward primer 5'-ACTCCTACGGGAGGCAGC-3'[62] with the 907 reverse primer 5'-CCGTCAATTCMTTTRAGTTT-3'[63]. The archaeal 16S rRNA fragments were amplified using the 344 forward primer 5'-ACGGGGCGCAGCAGGCGCGA-3'[64] with the 915 reverse primer 5'-GTGCTCCCCCGCCAATTCCT-3'[65]. Each primer set was run in a PCR mixture with a total volume of 20 µl containing Qiagen Q-solution, 10x buffer, MgCl, and BSA along with the DNA template. The thermocycler was run with a taq initiation step at 95 ˚C for 3 min, followed by 30 cycles of a denaturing step of 94 ˚C for 1 min, an annealing step at 47 ˚C for 45 sec, and an elongation step at 72 ˚C for 45 sec. After the 30 cycles, a final extension occurred at 72 ˚C for 7 min. Efficacy of the PCR reaction was tested via electrophoresis gel, using *E. coli* DNA as a positive bacteria control and methanogenic sediment as a positive archaea control (Holmes et al)[60].

For samples that did not amplify with this method, nested PCR was additionally used, first using the 8 forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' with the 1114 reverse primer 5'-GGGTTGCGCTCGTTGC-3'[66], followed by the amplification using the 338 forward primer 5'-ACTCCTACGGGAGGCAGC-3' with the 907 reverse primer 5'-CCGTCAATTCMTTTRAGTTT-3'. The thermocycler procedure was identical to the single PCR amplification method, but with 30 cycles followed by 35 cycles. Between the amplifications, the Qiagen clean-up kit was utilized. Nested PCR was only performed on the 1.4 MPa 56 day vessel and the 14 MPa 56 day vessel, with amplification only occurring in the 1.4 MPa 56 day vessel.

The PCR products of the two bacterial primer sets were mixed, and cloning followed using the Invitrogen TOPO TA cloning kit according to the manufacturer's instructions. Using the universal archaeal primers listed, no archaeal DNA was detected in any of the samples. 48 clones were sequenced from each reactor at Functional Biosciences (Madison, WI). Because the sample size was 48 clones for each reactor, Good's coverage was calculated using Mothur[67] (Schloss, 2004) to ensure sufficient coverage for the given diversity. Good's coverage values are reported in Table SI.1, Supplementary Information. Sequences were trimmed using FinchTV and chimeras were detected using Bellophon[68]. Sequences with 97% similarity were grouped into OTU's on the genus level using Mothur and NCBI Blast[67, 69]. In cases where OTU's were less than 97% similar to a phylotype of a cultured organism, the microorganisms were described as "other". All sequences were entered into the NCBI Blast database, and are assigned accession numbers JX439644-439761.

16S rRNA gene concentration were determined by quantitative real-time polymerase chain reaction (qPCR), using the 1369 forward primer 5'-CGGTGAATACGTTCYCGG-3' with the 1492 reverse primer 5'-GGWTACCTTGTTACGACTT-3' and the TAMRA 6 FAM 1389 forward probe CTTGTACACACCGCCCGTC[70]. The PCR mixture for the primer set and probe consisted of a total volume of 20 µl containing Applied Biosystems TaqMan Master mix along with the DNA template. DNA concentration was amplified using an initiation step at 50 ˚C for 2 min and denaturing of 95 ˚C for 10 min, followed by 40 cycles of 95 ˚C for 15 sec and 56 ˚C for 1 min. Diluted samples of known concentrations of *E. coli* were used as standards, where the *E. coli* was quantified with pico green procedures described in Invitrogen Quanti-iT kit.

The microbial diversity represented in each clone library was determined by grouping clones into phylotypes based on 16S rRNA gene similarity of > 97%. Because the number of clones examined was relatively small (48 clones), Equitability (J) was calculated from Shannon-Weaver indices to remove artifact from the sample size. Equitability was calculated at the species-level phylotypes for each sample using the equation $J=H/H_{max}$, where H_{max} is the maximum Shannon-Weaver index and H is the sample Shannon-Weaver index. Shannon-Weaver indices were calculated using the equation $-\Sigma[(n_i/N) \ln (n_i/N)]$, where N is the total number of phylotypes and n_i is the number of "i" phylotypes[60]. A community tree was produced on Mothur utilizing the Yue Clayton measure, and visualized using TreeView X.

Chemical Analyses (Gas and Liquid)

Gas samples from the pressure vessels were analyzed for H_2, CO_2, CH_4, and N_2 using a gas chromatograph (PerkinElmer Clarus 600) immediately after sampling. Gases were separated by a 1/8 in diameter Carboxin column, 15 m in length and 60/80 µm particle size. The oven was programmed to begin at 36 °C and ramp at 20 °C/min to 225 °C and hold for 1.3 min. No gases other than CO_2 and N_2 were detected, and GC data was used as a method of ensuring the desired pCO_2 was maintained and no unwanted oxygen had leaked into the reactors.

Supernatant sample water was filtered through a 0.2 µm filter, but was not acidified or otherwise preserved before chemical analysis. Sample water was analyzed for selected elements by ICP-OES (PerkinElmer Optima 7300 DV) using EPA method

6010C. Supernatant anions were also analyzed via a Dionex Ion Chromatograph using EPA method 300.1. Supernatant samples were stored at -4 °C until analysis but were otherwise unpreserved.

Water Chemistry Model

The pH could not be directly measured in the pressurized reactors. pH was estimated using Geochemist Workbench. The initial system was defined using the measured alkalinity and initial pH of the initial water. In addition, cation concentrations above 10^{-2} M (from the ICP-OES data) were used to define the initial system, with chloride as a counter ion to ensure electroneutrality. Conversion of pCO_2 to moles of CO_2 reacted in each system was calculated from the gas law using the pCO_2, the remaining 800 mL vessel volume, and 40 ˚C. The gas compressibility factor was assumed to be 1 for all reactors except the 14 MPa CO_2 reactor, which was calculated to be 0.27.

Conclusions

The results demonstrate that exposure of subsurface microbial communities to CO_2 will reduce both cell numbers and diversity. After 56 days, 16S rRNA gene copies decreased by one order of magnitude at CO_2 exposures as low as 0.1 MPa, and cell population was undetectable at CO_2 exposures above 1.4 MPa. Cell growth was not hindered by a drop in pH to pH=4.4 without exposure to CO_2 implying that cell death was due to the increase in pCO_2, and not the corresponding decrease in pH. In regions of high CO_2 concentration, biological processes (e.g. biomineralization of carbonate minerals) may be significantly hindered or absent. However, further from the CO_2 front, where cells will be exposed to CO_2 concentrations of 0.1 MPa or less, microorganisms may still thrive; within these niches left by the heterogeneous flow of SC-CO_2, biological processes will likely be retained. The organisms that survive and thrive may be targets for development of engineered processes to enhance beneficial outcomes (or minimize detrimental ones) for long-term CO_2 storage.

The decrease in microbial diversity that was observed in these exposure experiments is in agreement with previous results at a single CO_2 concentration[71]. Wandrey et al showed that the diversity of subsurface microorganisms decreased from 5 species to 3 species after 24.5 months of 5.5 MPa CO_2 exposure. Halotolerant genera, such as *Halomonas* and *Marinobacter*, were found to be the most resilient microorganisms to both lowered pH and CO_2 exposure. This decrease in diversity may lead to a shift in any bioprocesses that are currently playing a role in storage capacity and reservoir security, or may result in novel bioprocesses.

While the drill stem test sampling is widely accepted for its ability to obtain formation fluids, it is no guarantee that only native organisms from the formation were present in the initial samples as drilling fluids may be a source of contamination [72].

The research presented here is the first to characterize a gradient of CO_2 on microbial ecology in formation water from a future CO_2 sequestration site. Ideally, the experiments would have been performed in triplicates, but small quantities of formation water available and limited number of high-pressure and temperature reactors enabled only single runs at each pCO_2. However, patterns of diversity decrease and population number decrease emerge from the data across the exposure conditions and durations.

The findings are most relevant to the Arbuckle formation and its pressure and temperature conditions. Nevertheless, some of the microbial populations appeared to thrive in the subsurface saline aquifer conditions with CO_2 present, suggesting that certain species may persist post-injection. There are many other potential sequestration sites and each has its own native microbial population. CO_2 exposures may give rise to different ecological responses at different sites. Other sites should be studied to obtain a better understanding of how microbial communities will be impacted by exposure to CO_2. Generally, subsurface microbes have slower growth rates than their counterparts at the surface[73]. Longer-term experiments (years or more) may be necessary to thoroughly describe the microbial processes that may occur in the deep subsurface during the long term of carbon sequestration. Identification of the phylotypes that are likely to persist after injection can guide future studies to determine biogeochemical reactions that impact CO_2 storage and security.

Disclaimer

Reference in this report to any specific commercial process, product, or service is to facilitate understanding and does not necessarily imply its endorsement or favoring by the United States Department of Energy.

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Notes and references

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