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# Interplay between Microorganisms and Geochemistry in Geological Carbon Storage

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**Abstract (200 words maximum)**

Researchers at the Center for Frontiers of Subsurface Energy Security (CFSES) have conducted field, laboratory, and modeling activities to better understand the interplay between microorganisms and geochemistry for geological carbon storage (GCS). We provide evidence of microorganisms adapting to high $P_{CO2}$ conditions and identify factors that may influence survival of cells to $CO_2$ stress. Factors that influenced the ability of cells to survive exposure to high-pressure $CO_2$ in our experiments include mineralogy, the permeability of cell walls and/or membranes, intracellular buffering capacity, and whether cells live planktonically or within biofilm. Column experiments show that, following exposure to acidic water, biomass can remain intact in porous media and continue to alter hydraulic conductivity. Our research also shows that geochemical changes triggered by $CO_2$ injection can alter energy available to populations of subsurface anaerobes and that microbial feedbacks on this effect can influence carbon storage. Our research documents the impact of $CO_2$ on microorganisms on multiple scales, ranging from pores to field sites. We conclude that microbial presence and activities can have important implications on carbon storage and that their presence should not be overlooked in further GCS research.

## 1. INTRODUCTION

Geologic carbon storage (GCS) involves the capture, compression, injection and storage of anthropogenic carbon dioxide ($CO_2$) in order to mitigate carbon emissions to the atmosphere. Deep (approximately greater than 1000 m below the ground surface) sedimentary formations are one of the largest sets of likely injection targets. The pore waters in these formations are brines with ionic strengths ranging from that of seawater to levels near those of fluids saturated with halite. The injected $CO_2$ will exist as a supercritical phase given the ranges of pressures and temperatures at these depths (10 to 30 MPa and 310 to 380 K). High concentrations of dissolved $CO_2$ will create an aggressive fluid that will change brine pH and dissolved inorganic carbon (DIC), accelerate mineral diagenetic reactions, and elevate the concentration of dissolved ions while creating new secondary mineral phases (Kaszuba and Janecky, 2009; Lu et al., 2010).

Benson et al. (2005) describes the four trapping mechanisms for GCS: structural, residual, solubility and mineral. It is well recognized that these mechanisms are driven by geochemical and hydrological processes. Microbial processes are also important, however, because microorganisms can influence the hydrological and geochemical processes in subsurface environments (Baker et al., 2010; Banks et al., 2010; Davidson et al., 2011; Fredrickson et al., 1998; Gorbushina, 2007; Onstott et al., 1998; Pedersen et al., 1996; Sahl et al., 2008). For example, microbial biomass can enhance precipitation of carbonate minerals (Cunningham et al., 2009; Kandianis et al., 2008; Mitchell et al., 2010), clog porous media (Baveye et al., 1998), impact storage-formation capacity and well injectivity (Zettlitzer et al., 2010), and alter water chemistry on a regional scale (Flynn et al., 2013). Understanding the interplay between microbiology, geochemistry, and the hydraulic properties of GCS reservoirs may be a key to ensuring safe, economically feasible and secure carbon storage. Moreover, this knowledge can

provide a basis for developing biological strategies to enhance GCS reservoir performance (Mitchell et al., 2010).

Microbial life is widespread in the subsurface and accounts for a significant portion of Earth's biomass (Whitman et al., 1998). The depth limit of microbial life in the subsurface is somewhat uncertain. However, active microorganisms have been observed at depths greater than 3 km (Kieft et al., 2005) indicating that they exist over much of the range of depths of interest for GCS. Within these depths, microorganisms are capable of adapting to the wide range of conditions in salinity, temperature, pressure, and energy availability (Pikuta et al., 2007), allowing them to play a major role in mediating global-scale biogeochemical processes (Colwell and D'Hondt, 2013; Orcutt et al., 2013).

When $CO_2$ is injected in saline reservoirs, a tremendous stress is placed on the system, potentially triggering changes in community composition (Mu et al., 2014a; Peet et al., 2015; Wilkins et al., 2014). At supercritical $CO_2$ pressures, $CO_2$ may dissolve microbial membranes and sterilize microbial communities (Dillow et al., 1999; White et al., 2006). At lower pressures, where $CO_2$ dissolves in the aqueous phase, $CO_2$ is also toxic to microbes as $CO_2$ is liposoluble. As a result, $CO_2$ enters cells, acidifies the cytoplasm, and disrupts cellular functions (Ballestra et al., 1996). However, the details of the microbial physiological responses to this stress are still vague, and many unanswered questions remain, such as, which microbes are most resistant to $CO_2$ stress, how resistant are microbial membranes to $CO_2$, and how will microbial communities change as a result of $CO_2$ perturbation.

On the population and community scales, an increase in $CO_2$ abundance can shift community composition and alter the dominant geochemical reactions occurring within saline

reservoirs. When $CO_2$ dissolves into water, carbonic acid is produced, which can then dissociate into protons and dissolved inorganic carbon species:

$$CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

Because many of the redox reactions used as a source of energy by microbes include dissolved inorganic carbon species as well as hydrogen ions, changes in $CO_2$ abundance affects the extent to which those reactions are out of equilibrium (Harvey et al., 2013; Kirk, 2011).

Such changes can significantly affect microbial activity because the free energy yield of a microbial metabolic reaction affects the rates of microbial metabolism and the ability of microorganisms to compete with one another. Microbial reaction rates become limited directly by thermodynamics where the energy yield of a reaction decreases and approaches that stored by a cell (Jin and Bethke, 2007). Moreover, microorganisms that conserve energy from more energetically favorable reactions also have physiological advantages over those using less favorable reactions (e.g., Lovley and Goodwin, 1988; Roden and Jin, 2011). Organisms capable of detoxifying against $CO_2$ may be able to take advantage of the large energy available as a result of $CO_2$ dissolution in the subsurface.

In this paper we summarize the geomicrobiological work conducted at the Center for Frontiers of Subsurface Energy Security (CFSES) and discuss the implications of the results on GCS. CFSES is an Energy Frontier Research Center established by the Office of Science, Basic Energy Sciences program in the U.S. Department of Energy in 2009 and chosen for renewal until 2018. Researchers at CFSES have taken many different approaches to better understand the interplay between microorganisms and geochemistry for GCS. We identified and characterized an isolate from Crystal Geyser, Utah, U.S.A., a $CO_2$-rich spring. We tested four model organisms

representing microbial morphologies potentially present in the subsurface as well as representative subsurface metabolisms for their ability to survive high pressures of $CO_2$ exposure in batch cultures. We considered how decreasing pH, a geochemical change caused by $CO_2$ injection, will affect the stability of bioclogging in porous media. And, we used bioenergetics and mixed-community bioreactor experiments to assess potential changes in the relative significance of different microbial processes in response to increasing $CO_2$ abundance.

## 2. METHODS

### 1.1. Field

Water and microbe samples were collected from six sites where groundwater discharges near the surface: four sites with $CO_2$-rich water and two sites where water with low-$CO_2$ content discharges (Table 1). These sites varied in their lithologies and compositions, but were similar in that they were all $CO_2$-rich environments with the exception of the low-$CO_2$ springs which acted as comparison sites. The sample at Bravo Dome was collected at a central container that collects condensed water from the gas from multiple wells. For the other sites, sterile tubing was placed into the discharge sites and pumped. Filtered samples were analyzed for pH, temperature, conductivity, dissolved $O_2$ (DO), ferrous and total Fe, $PO_4^{2-}$, $NO_3^-$, and $NH_4^+$ in the field. Analyses for cations, anions, and alkalinity, total nitrogen, and dissolved inorganic and organic carbon were conducted in the laboratory. Mineral saturation indices and $P_{CO_2}$ were calculated based on measured water chemistry data using Geochemist's Workbench (Bethke, 2009). More details on the field experiment methods can be found in Santillan (2014).

To determine what organisms may survive at high $P_{CO2}$, Next Generation community sequencing was performed utilizing universal bacterial and archaeal primers 27F and 349F, respectively (Molecular Research LP, Shallowater, TX). DNA collected from filtering spring

water was preserved in a liquid nitrogen dewar and extracted in the laboratory. Operational taxonomic units were defined at 97% similarity and were classified using BLASTn against a curated GreenGenes database through the program QIIME (Caporaso et al., 2010). Calculations for alpha and beta diversity were performed at the genus level. Richness and diversity are assessed with number of observed taxa, Shannon Index, and Reciprocal Simpson's Index. The Shannon index provides a diversity calculation showing both the richness and evenness of the organisms present while the Reciprocal Simpson's Index does a similar calculation but with an emphasis on rare taxa present.

Culturing was performed to isolate microorganisms adapted to elevated $CO_2$ levels. Cultures were incubated in1 MPa of $CO_2$ for approximately 1 month and recultured multiple times in Tryptic soy broth (TSB) with 15 g/L NaCl. Successive transfers were then diluted to extinction to obtain an isolate. The isolate discussed in this paper, designated CG-1, was assessed for growth under various conditions that focused on $CO_2$, temperature, salinity, pH, carbon substrates, electron acceptors, and fermentation capability. Cloning was performed on GC-1 to determine its 16S gene identity through the Basic Local Alignment Search Tool search (BLASTn) search (http://blast.ncbi.nlm.nih.gov/). A phylogenetic tree relating the isolate to related sequences was made using CLUSTALX (Chenna et al., 2003). Samples were also analyzed for morphology using transmission electron microscopy (TEM) following methods outlined by (Romanovicz, 2011). Details of the isolation can be found in Santillan et al. (2015).

### 1.2. Laboratory

*Pure-Culture Experiments*

In addition to testing $CO_2$ growth and survival in an environmental isolate, we also examined the impact of $CO_2$ exposure on growth and survival of four model organisms:

*Shewanella oneidensis* strain MR-1 (ATCC BA-1096), *Geobacillus stearothermophilus* (ATCC 7953), *Bacillus subtilis* Δsp01 (BGSC 1S53, Columbus, OH), and *Methanothermobacter thermoautitrophicus* (ATCC 29096). *S. oneidensis* is a Gram negative bacterium that was used to represent both the response of Fe reducing bacteria as well as Gram negative bacteria to $CO_2$ sequestration. *G. stearothermophilus* and *B. subtilis* are aerobic Gram positive, bacteria both used to understand the response of organisms with thick cell walls to $CO_2$. *G. stearothermophilus* is capable of forming endospores whereas the strain of *B. subtilis* used has a deletion in its genome preventing it from sporulating (Table 2). *M. thermoautitrophicus*, a methanogen, is an Archaeaon.

To investigate $CO_2$ toxicity, organisms were grown in batch cultures under three different conditions: $P_{CO_2}$ below 1 MPa, $P_{CO_2}$ above 1 MPa, and HCl acidified media to determine pH survival in the absence of $CO_2$ (Santillan et al., 2013). Cultures grown in glass test tubes were placed in pressure reactors (Parr instruments, Moline, IL) and the pressure slowly increased to the final experimental conditions. Pressure and temperature were held constant until the end of the experiment at which time they were slowly outgassed over approximately 2 min. Next, cultures were removed from the reactors and sonicated to remove biofilms and separate attached cells. Cell survival was assessed based on the quantification of culturable cells. *S. oneidensis, G. stearothermophilus,* and *B. subtilis* cells were quantified using the pour plate method. Survival for *M. thermoautitrophicus* was evaluated by growing samples in liquid anaerobic cultures with low $CO_2$ content. Fe reducing activity was evaluated by measuring ferrous iron concentration using the ferrozine method (Stookey, 1970). The activity of methanogen cells was evaluated by measuring $CH_4$ partial pressure using gas chromatography.

*S. oneidensis*, the model organism most susceptible to $CO_2$ exposure of those tested, was selected to examine the effects of mineral solid phases on $CO_2$ toxicity (Santillan et al., 2013). Minerals and rock samples (Ward's Natural Science, Rochester, NY) were crushed to coarse sand size, cleaned of residual iron with a hand magnet, and sterilized at 121 °C for 30 min. Test tubes with 10 mL of growth medium and 1 g of autoclaved mineral were inoculated with *S. oneidensis* and anaerobically incubated at 30 °C for 3 days. Cultures were then exposed to 2.5 MPa $CO_2$ for up to 8 h and the cell suspensions tested for survival using plating methods. To determine whether biofilm formation provided protection against $CO_2$ toxicity, selected mineral culture experiments were repeated.

*Bioclogging Experiments*

Column experiments were run in 10 cm long square capillary tubes with a 1 $mm^2$ cross-sectional area packed with 105–150 μm diameter glass beads (Kirk et al., 2012). Each experiment had three phases: pre-growth, growth at pH 7.2, and acidic pH, which started four days after inoculation. The acidic phases of six biologically-active experiments received medium with an average pH of 4.0 and six additional experiments received medium with an average pH of 5.7. Abiological-control experiments were also performed at pH 4 (two) and pH 5.7 (one). Experiments were terminated after hydraulic conductivity was stable for at least 24 h.

Artificial Na-Cl type groundwater with glucose and bicarbonate was used as the aqueous medium. Rhodamine was included for pore-space imaging. pH was adjusted using HCl. Medium was pumped through the columns at 0.015 mL $min^{-1}$ (specific discharge of 22 m $day^{-1}$) using syringe pump. After the hydraulic properties were allowed to stabilize for at least three days the system was inoculated with an average of 8.4 log CFU (st. dev. 0.3) of *P. fluorescens* tagged with a green fluorescent protein (GFP). Following inoculation, flow was stopped for 2 h to allow

initial cell attachment and growth to occur. Cells injected into the control experiments were heat-sterilized before injection.

Hydraulic conductivity was calculated for each of the three phases of an experiment. The average saturated hydraulic conductivity over the entire length of each column was evaluated using pressure measurements. We also estimated hydraulic conductivity within the clogged portion of the column by assuming that conductivity over the remainder of the column (i.e., the unclogged portion) remained at pre-growth levels.

Pores and biomass were imaged with a scanning laser confocal microscope before and after acidification. Culturable cell concentrations in column effluent were monitored periodically in all of the experiments. The culturable cell content of 1 cm column segments was also measured in one pH 4 and one pH 5.7 experiment after the experiments were terminated. The concentration of live and dead cells in the column effluent of two pH 4 and three pH 5.7 experiments was also quantified.

*Mixed-community experiments*

The experiments were carried out in duplicate using anoxic semi-continuous bioreactors inoculated with a mixed-microbial community from a freshwater aquifer (Kirk et al., 2013). Two sets of experiments were performed: one with low $CO_2$ partial pressure (~0.002 MPa atm) in the headspace of the reactors and one with high $CO_2$ partial pressure (~0.1 MPa). A fluid residence time of 35 days was maintained in the reactors by replacing one-fifth of the aqueous volume with fresh medium every seven days. The aqueous medium was composed of groundwater amended with small amounts of acetate (250 µM), phosphate (1 µM), and ammonium (50 µM) to stimulate microbial activity. Synthetic goethite (1 mmol) and sulfate (500 µM influent concentration) were also available in each reactor to serve as electron acceptors.

Reactors were incubated for 15 weeks. During that time, influent medium and reactor effluent were regularly sampled and analyzed using a variety of techniques. The ferrozine method was used to analyze ferrous iron concentration (Stookey, 1970). Ion chromatography was used to analyze anion concentrations. Gran alkalinity titrations were used to evaluate alkalinity. Atomic adsorption and inductively coupled plasma optical emissions spectroscopy were used to measure cation concentrations.

Well-mixed samples of reactor solids and fluid were collected at the end of the incubations for analysis of acid-volatile sulfide (AVS) abundance and microbial community analysis. AVS was measured as described by Kirk et al. (2010). Total community DNA was extracted from microbial samples using an Ultraclean® Microbial DNA Isolation Kit (MO BIO) and then sequenced using 454 pyrosequencing. Sequences were then processed using QIIME (Caporaso et al., 2010).

Rates of acetate oxidation, iron reduction, and sulfate reduction were evaluated using mass-balance calculations based on measured reactor chemistry.

### 1.3. Numerical

Bioenergetics calculations were applied to data collected during two field $CO_2$-injection experiments (Kirk, 2011) and data collected from the mixed-community experiments (Kirk et al., 2013). In both cases, the calculations assessed changes in energy available ($\Delta G_A$) for microbial metabolism. As defined previously (Bethke et al., 2011), $\Delta G_A$ is the negative of the free energy change of microbial metabolic reaction ($\Delta G_r$) and can be calculated in units of kJ·mol$^{-1}$ as follows:

$$\Delta G_A = -\Delta G_r = -[\Delta G_T^\circ + RTln \prod_i (\gamma_i \times m_i)^{v_i}]$$

where $\Delta G_T^\circ$ is the standard Gibbs free-energy change for reaction *r* at temperature *T* (°K), *R* represents the gas constant (kJ·mol$^{-1}$·K$^{-1}$), $\gamma_i$ and $m_i$ are the activity coefficient (molal$^{-1}$) and molality of the $i$th chemical species in the reaction, and $v_i$is the stoichiometric coefficient of that species, which is positive for products and negative for reactants.

Standard Gibbs free energy values at *in situ* temperature were calculated using the Geochemists Workbench® software package (Bethke, 2009) and the Lawrence Livermore National Laboratory thermodynamic database (Delany and Lundeen, 1990). Activities were calculated from chemical data with Geochemists Workbench® software using an extended form of the Debye-Hückel equation, the *B*-dot equation (Helgeson, 1969).

Calculations for the mixed-community experiments considered iron reduction and sulfate reduction, the two groups of microorganisms that account for all of the microbial activity during the experiments. Calculations for the field studies considered iron reduction, sulfate reduction, and methanogenesis. Those groups were selected because they are the three most common groups of respiring microorganisms in the subsurface (Bethke et al., 2011; Lovley and Chapelle, 1995; McMahon and Chapelle, 2008). As such, they are likely present in many potential storage reservoirs that contain active microbial populations.

Field experiment data used in our calculations was collected during the Frio Formation experiment and the Zero Emissions Research and Technology (ZERT) experiment (Kharaka et al., 2006; Kharaka et al., 2010). To account for errors associated with activity modeling and

limited data constraining electron donor concentrations, results from the bioenergetics analysis of the field data are normalized relative to conditions present prior to $CO_2$ injection, as follows:

$$\Delta G_A^{CO_2} - \Delta G_A^{initial} = \Delta G_A^n$$

where the superscript "$CO_2$" designates each value calculated during or after $CO_2$ injection began, "initial" designates the value calculated prior to injection, and "n" represents the normalized value. As such, our analysis of the field data considered how energy available changed as a result of $CO_2$ injection, not absolute values of energy available.

## 3. RESULTS AND DISCUSSION

### 1.4. Field

The springs varied greatly in their community compositions, likely reflecting geographic separation between sites and variation in conditions. The exception was Crystal Geyser and Airport Spring, the two sites located along the Little Grand Wash Fault, which had 39 phylotypes in common of the 50 operational taxonomic units (OTUs) identified at each site. Diversity, as summarized through observed taxa, the Shannon Index, and the Reciprocal Simpson's Index are summarized in Table 1. Among sandstone spring sites sampled (i.e, excluding Bravo Dome), the low-$CO_2$ springs showed more diversity than the $CO_2$ springs, with Moab Springs having much more taxa present than all the other sites. Arches showed slightly more diversity than the $CO_2$ springs sites (Crystal Geyser and Airport Spring). Of all the $CO_2$ springs sampled, Klickitat had the highest diversity. However, the diversity measured in Klickitat Springs is still considerably lower to other sampled sites in the Columbia River Basalt Group that are not exposed to $CO_2$ (Colwell et al., 1997). Moab Springs and Klickitat Springs were the only sites to test positive for Archaea with both locations exhibiting a dominance of Euryarchaeota. Of the $CO_2$-affected sites, Klickitat Springs, located in the Columbia River Basalt Group, exhibited the highest diversity in

comparison to the other sandstone locales. Previous work has suggested that the Columbia River Basalt Group is a site that can favor microbial growth (Chapelle et al., 2002; Krumholz, 2000; Stevens and McKinley, 1995). A more detailed analysis of the field data can be found in Santillan (2014).

Diversity, in general, appears to be higher at the non-$CO_2$-rich sources, in comparison to samples collected at high-$CO_2$ sites of similar lithology. However, correlations between community composition and environmental variables (including $CO_2$, water source, rock type, water chemistry) were weak at best. While $CO_2$ abundance may influence diversity at the sites, our results do not provide evidence that it is the most important factor. Instead, many other environmental variables may also contribute to varying degrees including lithology, salinity, nutrient contents, and depth. Emerson et al (2015) have also performed a survey of the Crystal Geyser microbial community and their results suggest a site that has much more diversity than our results. However, much of the differences in our findings may attribute to differences in sampling methods as well as the potential seasonality of Crystal Geyser's recharge as the source water for Crystal Geyser is a combination of deeply sourced groundwater and fresh water from the nearby San Rafael Swell (Baer and Rigby, 1978; Burnside et al., 2013; Heath et al., 2009; McPherson and Heath, 2009; Takashima et al., 2011).

Diversity studies of $CO_2$ springs reveal the presence of microorganisms in high $CO_2$ environments, including an isolate obtained from the Crystal Geyser. The isolate is considered capnotolerant, capable of survival in a broad range of $CO_2$ pressures (Santillan et al., 2015). The isolate, surprisingly, was most related (98.5%) to Lactobacillus casei, and exhibits a fermentative metabolism. It grows at $P_{CO_2}$ between 0 to 1.0 MPa and surviving up to 5 days at 2.4 MPa. CG-1 grew in neutral pH, 0.25 M NaCl, and between 25° to 45°C. It consumed glucose, lactose,

sucrose, or crude oil, likely performing lactic acid fermentation. Transmission electron microscopy reveals rod shaped bacteria at 0.1 MPa. At 1.0 MPa, cells were smaller, amorphous, and produce abundant capsular material (Figure 1).

### 1.5. Laboratory

*Pure-Culture Experiments*

For all organisms, survival was best at low $CO_2$ pressures but decreased rapidly as pressures increased. *S. oneidensis* cultures were the most sensitive to increased $CO_2$ while *G. stearothermophilus* cultures the most resilient. However, the high survival of *G. stearothermophilus* may be attributed to endospore formation as Endospores, as observed in TEM images of the *G. stearothermophilus* cultures (not shown), are attributed to the increased resilience. Both the thick cell walls of Gram-positive organisms and endospore formation have been shown to promote survival during exposure to high-pressure $CO_2$ (Damar and Balaban, 2006; Dillow et al., 1999; Furukawa et al., 2004; Nicholson et al., 2000; Spilimbergo and Bertucco, 2003; Watanabe et al., 2003; White et al., 2006; Wynne and Foster, 1948). The low survival rates of *B. subtilis* Δsp0A, an organism related to *G. stearothermophilus* but incapable of producing endospores, further corroborates our hypothesis that Gram-positive survival at high $P_{CO2}$ is linked to endospore survival rather than the resilience of the vegetative cells.

Differences in the ability of *S. oneidensis* and *M. thermoautitrophicus* cells to survive exposure may also reflect differences in the ability of $CO_2$ to penetrate cells walls and/or membranes. *S. oneidensis* cells have Gram negative bacterial cell walls and *M. thermoautitrophicus* cells have archaeal cell walls, which would differ considerably in properties and may be more amenable to extreme conditions (Arakawa et al., 1999; Gambacorta et al., 1994). In contrast, bacteria contain cell membranes composed of a bilayer of a phosphate head

group ester-linked to a hydrocarbon tail (White, 2007). In addition, unlike *S. oneidensis*, *M. thermoautitrophicus* cells consume $CO_2$ in their catabolic reaction, potentially helping them limit accumulation of $CO_2$ within their cytoplasm.

As expected, metabolism in both the Fe reducer and the methanogen generally decreased with increasing $P_{CO2}$, though the methanogen showed more ability to metabolize at higher $CO_2$ pressures in comparison to the Fe reducer. This suggests that subsurface microbial populations exposed to $CO_2$ will initially be stressed, and will exhibit decreased growth and metabolism. However, this ability will vary by organism and may eventually provide a means to select for the dominant subsurface microorganisms.

Because methanogenesis is a metabolic process that occurs intracellularly whereas Fe reduction occurs on the cell membrane, these results suggest that cell viability in high $CO_2$ environments will require an internal mechanism of detoxification in order to combat the acidification that $CO_2$ will cause in the cytoplasm. The growth and survival of CG-1 over a broad range of $P_{CO2}$ further provides evidence of this. Many fermenters often utilize $CO_2$ in metabolic processes, such as amino acid synthesis or through $C_1$ metabolism (Arioli et al., 2009; Bringel et al., 2008; Song et al., 2007). CG-1's ability to survive high $P_{CO2}$ may result from utilization of a similar pathway.

The presence of a mineral increased the fraction of cells that survive $CO_2$ exposure, with the exception of kaolinite (Figure 2). However, cell viability varied widely from mineral to mineral. Cultures grown with quartz sandstone had the smallest loss of viability. SEM imaging of various minerals reveals biofilm adhesion on the grain surface with biofilm appearance varying by mineral. Review Santillan et al. (2013) for more information on these experiments.

It appears that the mere presence of a mineral will enhance *S. oneidensis* survival at relatively high $P_{CO_2}$. This survival is likely linked to biofilm formation on the mineral surfaces. In addition, survival on unreactive minerals, such as quartz sandstone, appears highest, especially when these rough surfaces provide high surface area and provide microenvironments for protection. Unlike planktonic cultures, biofilms are surrounded by extracellular polymeric substances (EPS), likely providing a reactive barrier to slow the penetration of $CO_2$ into cells. Planktonic cells are not surrounded by EPS like their biofilm counterparts, resulting in faster death. While the data show that *S. oneidensis* cells will eventually die despite the presence of biofilm, biofilm structures will continue to exist after cell death has occurred, indicating that even when cells are dead, biofilm presence will still be an important consideration during $CO_2$ sequestration.

### *Bioclogging Experiments*

Average hydraulic conductivity for the three sterile control experiments was 0.013 cm $s^{-1}$ (st.dev. 0.003). After 4 days of growth at pH 7.2, the hydraulic conductivity decreased significantly to 0.003 cm $s^{-1}$ (st. dev. 0.002) – a 0.67 log reduction (Figure 3). Most bioclogging was observed in the front centimeter of the column. The hydraulic conductivity then increased significantly with acidification in all but one pH 5.7 experiment; bulk values averaged 0.005 cm s−1 (st. dev. 0.003) and 0.004 cm s−1 (st. dev. 0.003) in pH 4 and pH 5.7 experiments, respectively. Despite the increase in hydraulic conductivity, the column reactors remained significantly clogged relative to pre-growth levels. Most biomass in the column reactors remained intact. Though the acidification caused shifts in biomass distribution to occur, potentially contributing to the increase in K. Changes in viability and culturability after

acidification may explain differences in bioclogging persistence. Cell death and stress was greater at pH 4 than pH 5.7, reflecting the pH tolerance of *P. fluorescens*.

We conclude that bioclogging can remain largely intact after acidification, bioclogging stability reflects species pH tolerance, and shifts in biomass distribution with pH likely impact clogging persistence (Kirk et al., 2012). Stimulating growth of microbes can provide an effective strategy to limit leakage from storage reservoirs, even if those cells perish. However, because of the heterogeneous distribution of favorable mineralogy to support biofilm survival within the subsurface, surviving biofilms after GCS will likely be uneven in their distribution.

*Mixed-community experiments*

Mass-balance rate calculations demonstrate that microbial activity differed considerably between the high- and low-$CO_2$ bioreactors. Sulfate reduction was dominant in reactors with low $CO_2$ content. The reaction consumed 85% of the acetate after acetate consumption reached steady state while iron reduction accounted for only 15% on average (Figure 4). In contrast, iron reduction was dominant during that same interval in reactors with high $CO_2$ content, accounting for at least 90% of the acetate consumption while sulfate reduction consumed a negligible amount (<1%).

Pyrosequencing results are consistent with the mass-balance calculations. Sequences classified in groups that contain species related to iron reduction were abundant in samples from all biologically-active reactors but more than twice as abundant in the high-$CO_2$ reactor samples compared to the low-$CO_2$ reactor samples. Moreover, sequences classified in groups relating to sulfate reducers were abundant in the low-$CO_2$ reactor samples but nearly absent from the high-$CO_2$ reactor samples. Kirk et al. (2013) present more detailed information on these experiments.

### 1.6. Numerical

Bioenergetics analysis of the mixed-community experiments indicates that energy available for iron reduction varied directly with reaction rate (Kirk et al., 2013). After acetate oxidation stabilized (i.e., final 8 weeks), energy available for iron reduction may have been as high as 114 kJ mol-1 on average in the high-$CO_2$ reactors compared to only 60 kJ mol-1 in the low-$CO_2$ experiments. Energy available for sulfate reduction, in contrast, varied little. During the final 8 weeks of the experiment, energy available for sulfate reduction was a maximum of 65 kJ mol-1 and 62 kJ mol-1 on average in the high- and low-$CO_2$ reactors, respectively.

Bioenergetic calculations performed using data from the field $CO_2$-injection experiments demonstrate that $CO_2$ injection benefitted iron reducers much more than sulfate reducer or methanogens at both sites (Kirk, 2011). For both acetotrophic and hydrogentrophic reactions, the energy available for iron reduction increased considerably for all three iron minerals considered as electron acceptors in iron-reduction reactions (Figure 5). In contrast, energy available for sulfate reduction and methanogenesis varied relatively little.

## 4. CONCLUSIONS

The integration of our field, laboratory and numerical studies has shed light on the impact of GCS on subsurface microbial communities and in turn the impact of these communities on GCS. In the subsections that follow, we examine both sides of these two-way interactions as well as potential areas for future research.

### 4.1. Impacts of GCS on Microbiology

The results of our field and laboratory studies add to the growing body of evidence that microorganisms will be capable of adapting to perturbed conditions within GCS reservoirs.

Moreover, our findings help illustrate that numerous factors may influence microbial tolerance and response to high $CO_2$ pressures including environmental, biochemical, and structural characteristics (Dillow et al., 1999; Erkmen, 2000; Hong and Pyun, 1999; Spilimbergo and Bertucco, 2003; Watanabe et al., 2003).

GCS reservoirs are likely mostly anaerobic but much of the work examining $CO_2$-stress and physical impacts of microbial activity porous media hydrodynamics have used aerobes and aerotolerant microorganisms. Additional research that focuses on anaerobic microorganisms that may exist in GCS reservoirs is needed. Microbial communities, as well as their interactions with geochemistry, can be considerably different in anoxic environments compared to oxic environments. Our mixed-community experiments examined the impact of elevated $CO_2$ abundance under conditions more consistent with a freshwater aquifer than a GCS reservoir. Moreover, the bioreactors did not contain sediment other than goethite. Addition research is also warranted to examine how a mixed-microbial community response would vary with bedrock composition and at the higher pressure, temperature and salinity typical of the deep subsurface.

Little Grand Wash Fault, Bravo Dome, and Klickitat Springs provide opportunities to sample microbial communities that are exposed to high levels of $CO_2$. Our results show that a broad array of microorganism exists in those systems and that at least some of the prokaryotic cells present are hyper-capnophilic – microorganisms that are capable of thriving in the presence of extreme $CO_2$ concentrations. The isolation of an organism from these extreme environments also provides evidence that these organisms are metabolizing – and affecting the surrounding geochemistry. Furthermore, organisms within these environments will vary from location to location and show that each saline aquifer will host its own unique microbial community. In order to fully characterize how these communities will potentially affect a reservoir, community

analyses should be included in the reservoir characterization in addition to geochemical, lithological, and structural analyses to determine the potential geochemical effect each community may have on sequestration. We recommend the study of additional field environments, performing more detailed surveys of metabolic capabilities, and more culture attempts of capnophilic microorganisms in order to provide greater insight on two-way interactions of GCS and subsurface microbial communities. In addition, understanding the mechanisms for $CO_2$ survival in capnophilic organisms could lead to engineering of organisms that could benefit GCS.

Results from the pure-culture experiments provide insight into cellular properties that could enhance survival. $CO_2$ affects cells in multiple ways, and it appears its effect goes beyond acidification of the surrounding media. Differences in the survival of the isolates we examined may reflect the permeability of cell walls and/or membranes to $CO_2$ as well as the intracellular buffering capacity of different types of organisms. Furthermore, our findings suggest that consumption of $CO_2$ by cellular catabolism may provide a mechanism of removing $CO_2$ intercellularly, which could promote survival and activity in GCS reservoirs.

We conclude from results of our experiments that existence within microbial biofilms can promote survival of cells and that biofilms can persist following exposure to acidic formation water. These findings compliment previous research, which found that biofilms enhance cell survival and can largely remain intact when exposed to supercritical $CO_2$ (Mitchell et al., 2008; Mitchell et al., 2009). Taken together, our findings and those of other researchers highlight the role of biofilms in the survival of microorganisms and their potential for continued activity in GCS reservoirs.The interaction of microbial communities with the surrounding host rock are an additional factor impacting cell survival. Given the heterogeneous nature of mineralogy in the

subsurface, and the influence of mineralogy on cell survival in biofilms, $CO_2$ injection will cause a heterogeneous redistribution of the subsurface microbial community.

Our numerical analyses and mixed-community experiments provide a complimentary approach to these field and pure-culture laboratory experiments and provide hints as to how the surviving microbial communities will be affecting the GCS. Whereas the latter approaches help inform us about cell properties that may influence cell survival following $CO_2$ injection, our numerical analyses and mixed-culture experiments are focused on understanding how $CO_2$ injection could affect what reactions are being catalyzed by the subsurface microbial community.

Both the numerical and mixed-culture studies indicate that $CO_2$ injection has the potential to stimulate iron-reducing microorganisms where ferric iron is available. Where iron reducers compete with other groups of microorganisms for energy sources (i.e., electron donors), the activity of those competing groups may decrease in response to an increase in the rate of iron reduction with rising $CO_2$ abundance. In our mixed-community bioreactors, sulfate-reducing activity was diminished at elevated $CO_2$ abundance. Similarly, our calculations based on field injection experiment data suggest that methanogenic activity could decrease in response to increased competition from iron reducers under high-$CO_2$ conditions.

At first glance, these results seem to be in conflict with our isolate experiments. In those experiments, *S. oneidensis*, an organism capable of dissimilatory iron reduction, showed greater sensitivity to elevated $CO_2$ than *M. thermoautitrophicus*, a methanogen. However, individual isolates are not representative of an entire metabolic group of microorganisms. Cells capable of dissimilatory iron reduction, for example, have broad phylogenetic diversity and have been identified across a wide range of chemical and physical conditions, including at extreme acidic

pH and salinity (Emmerich et al., 2012; Itoh et al., 2011; Lu et al., 2010; Weber et al., 2006). The mixed-community of cells capable of dissimilatory iron reduction that may exist in a GCS reservoir, therefore, may be better able to adapt to an increase in the abundance of $CO_2$ than the individual isolate we tested. This can be further clarified by working with more model organisms and, ideally, with environmental isolate (Emerson et al., 2015; Mu et al., 2014b).

### 4.2. Impacts of Microbiology on GCS

Although prokaryotic cells are small, our findings indicate that the collective activities of microbial communities in GCS reservoir should not be overlooked. Subsurface biomass may influence the hydrodynamics of porous media in GCS reservoirs. Coupled with this effect, by catalyzing oxidation-reduction reactions, they can serve as a driver for geochemical reactions that affect the rate and mechanism of carbon storage.

The mere physical presence of microbes in the deep subsurface will alter the immediate trapping of $CO_2$ on the pore scale. Biofilms colonizing pore spaces alter permeability, affecting the movement of $CO_2$ through groundwater (Mitchell et al., 2009). Sterilization of microbes will cause a mobilization and alteration of subsurface permeability that will be difficult to quantify (Kirk et al., 2012). Biofilms colonizing the mineral surface may potentially alter wettability, as charged surfaces on EPS may change the ability for $CO_2$ droplets to adhere onto mineral surfaces. This in turn can then change how liquid and supercritical $CO_2$ droplets adhere on surfaces during residual trapping (Chaudhary et al., 2013). Moreover, the presence of biofilms can also facilitate carbonate mineralization by providing nucleation sites (Benzerara et al., 2011).

Results of the mixed-community experiments show that, where $CO_2$ injection stimulates microbial iron reduction, solubility trapping may be enhanced. Iron-reducing microbes are

strongly acid consuming. As such, their activity works to enhance $CO_2$ storage in solution by converting $CO_2$ into carbonate alkalinity. Compared to the bioreactors with low $CO_2$ abundance, the increase in carbonate alkalinity in mixed-culture bioreactors with elevated $CO_2$ abundance was six-fold greater as a result of the enhanced rate of iron reduction. Hence, iron reduction enhanced storage of carbon in solution by a wide margin.

In addition to solution trapping, microbial iron reduction may also impact mineral trapping of $CO_2$. Much research on mineral trapping has focused on formation of calcium carbonate ($CaCO_3$) in GCS reservoirs. Our results show, however, that storage of carbon as the mineral siderite ($FeCO_3$) may also be important to consider. By simultaneously increasing levels of carbonate alkalinity and ferrous iron in formation water, microbial iron reduction supplies the necessary ingredients to form siderite.

Taken together, these observations show that shifts in microbial activity have the potential to be important feedback mechanisms on carbon storage on both short and long time scales. If $CO_2$ injection triggers increases the competitive advantage of iron reducers or other groups of microbes that are strongly acid consuming, more alkalinity and possibly carbonate mineralization will result. Numerical simulations that do not account for microbial activity, therefore, may poorly predict the rate and form of carbon trapping in GCS reservoirs.

Lastly, additional research is warranted to examine microbiological mechanisms that could create an energy return on subsurface $CO_2$ injection. For example, recent research has found evidence that $CO_2$ injection can stimulate biological conversion of crude oil into natural gas (Mayumi et al., 2013). $CO_2$ injection into depleted or heavy oil reservoirs, therefore, may

provide a strategy to enhance energy recovery from those systems and alleviate some of the economic burden of GCS.

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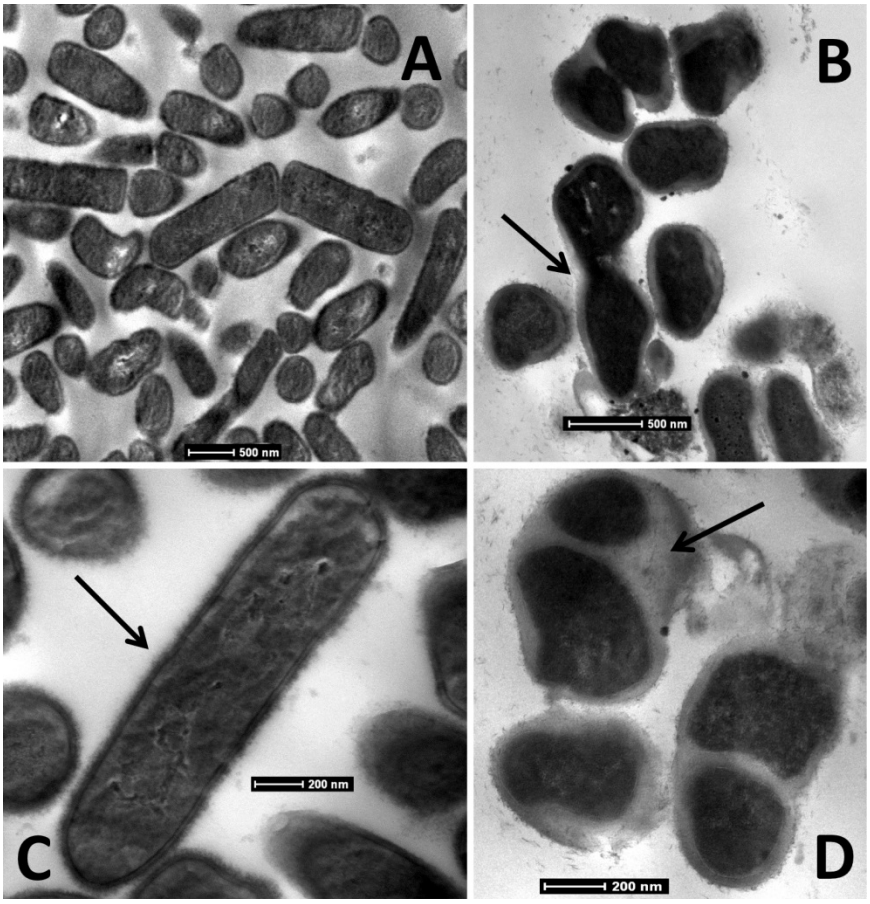
Table 1: Summary of Field Testing Site Information.

| Site | ~ Location | $CO_2$/Non-$CO_2$ | Rock Type | Dissolved Solids | Dominant Ions | Observed Taxa | Shannon Index | Reciprocal Simpson's Index |
|---|---|---|---|---|---|---|---|---|
| Crystal Geyser | Green River, UT | $CO_2$- Rich | Sandstone | Moderate | Na, Cl | 50 | 1.5 | 1.5 |
| Airport Spring | Green River, UT | $CO_2$- Rich | Sandstone | Low | Ca, $HCO_3$, $SO_4$ | 50 | 1.4 | 1.4 |
| Moab Springs | Moab, UT | Non-$CO_2$ | Sandstone | Low | Ca, Mg, $HCO_3$ | 673 | 8.7 | 216 |
| Arches | Arches National Park | Non-$CO_2$ | Sandstone | Low | Na, Ca, Cl, $SO_4$, | 66 | 3.6 | 6.3 |
| Bravo Dome | Northeastern New Mexico | $CO_2$- Rich | Sandstone | High | Na, Cl | 80 | 3.1 | 3.2 |
| Klickitat Springs | Southern Washington | $CO_2$- Rich | Basalt | Low | Ca, Mg, $HCO_3$ | 526 | 6.6 | 14.5 |

Table 2: Summary of Organisms Tested

| Organism | From | Morphology | Examined For |
|---|---|---|---|
| *Shewanella oneidensis,* MR-1 | ATCC BA-1096 | Gram negative | Fe reduction, Gram negative survival |
| *Geobacillus stearothermophilus* | ATCC 7953 | Gram positive | Gram positive survival, spore survival |
| *Bacillus subtilis* | Bacillus Genetic Stock Center | Gram positive | Gram positive survival without spores |
| *Methanothermobacter thermoautitrophicus* | ATCC 29096 | Archaea | Methanogenesis, Archaeal survival |
| *Lactobacillus casei*, CG-1 | Isolated from Crystal Geyser | Gram positive | Capnotolerance |

Figure 1 - Bright-field TEM images of CG-1 at 0.1 MPa $CO_2$ (A,C) and 1.0 MPa $CO_2$ (B,D). Arrows in: (B) show invaginations in $CO_2$ exposed cells that may suggest cell division; (C) show the intact cell wall for organisms at low $CO_2$ exposure; (D) show the capsular material present for $CO_2$ exposed cells. Modified after Santillan et al. (2015).



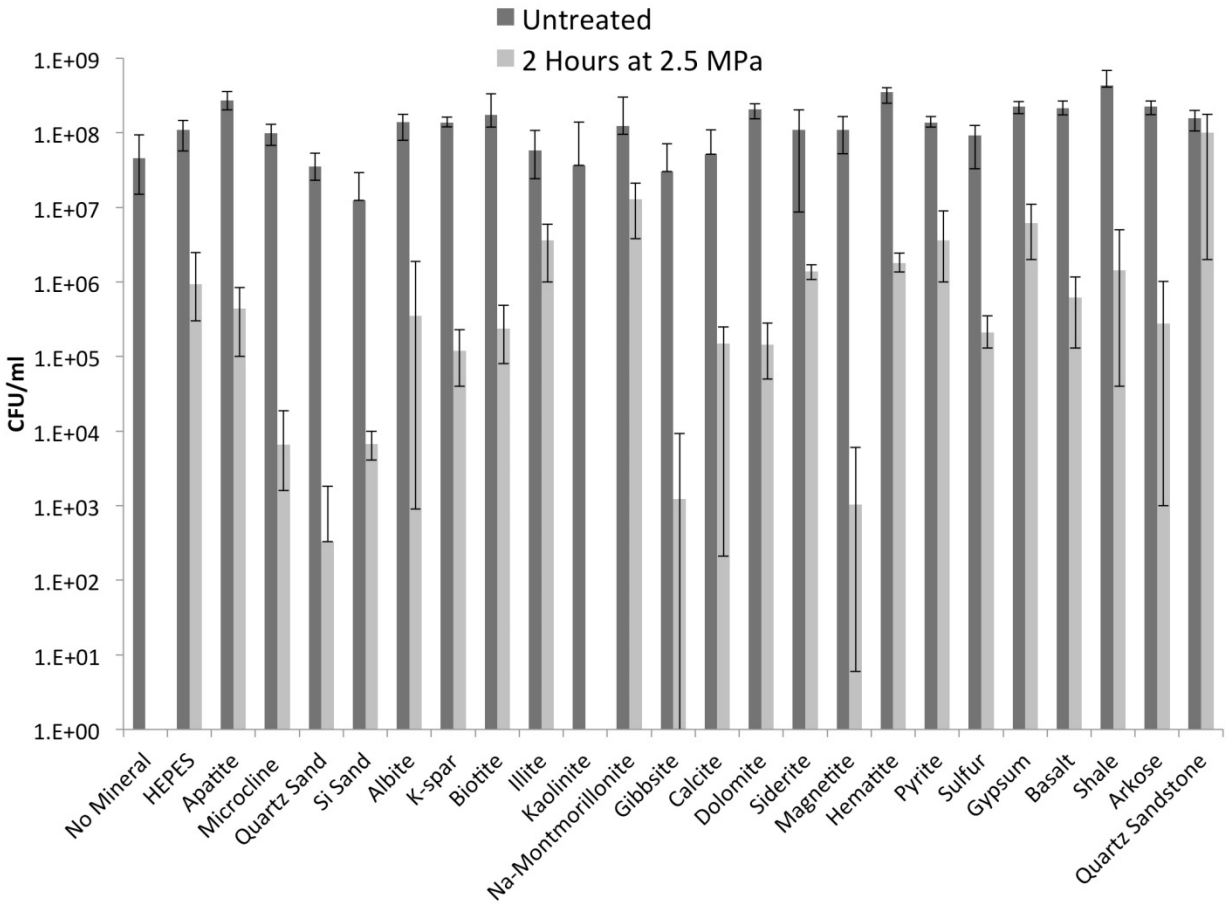



Figure 2: *Shewanella oneidensis* $CO_2$ survival in the presence of various minerals (modified after Santillan et al., 2013).

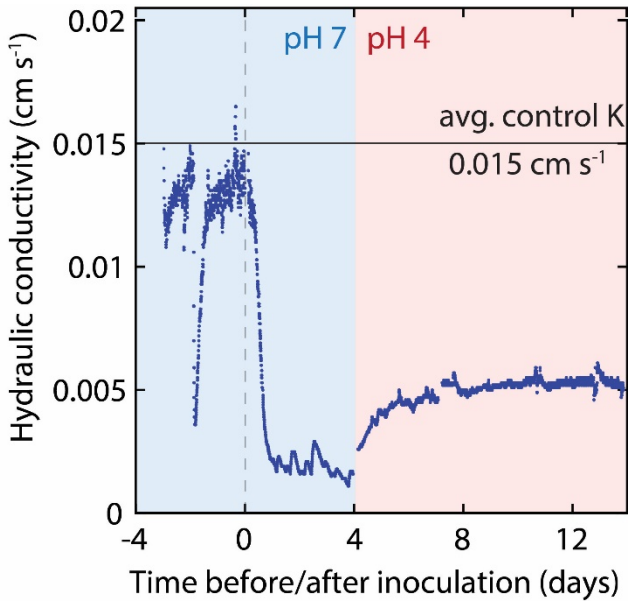



Figure 3: Typical variation in hydraulic conductivity during bioclogging experiments.

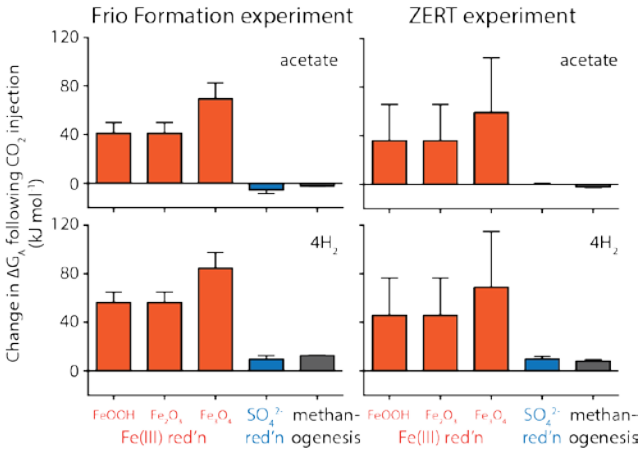



Figure 4. Change in energy available for iron reduction, sulfate reduction, and methanogenesis as a result of $CO_2$ injection during field $CO_2$-injection experiments. Values show the average difference between energy available prior to $CO_2$ injection and during. Three values were averaged for the Frio Formation experiment and eight for the ZERT experiment. Error bars show standard deviation. Calculations for iron reduction considered three sources of ferric iron (Fe(III)): goethite (FeOOH), hematite ($Fe_2O_3$), and magnetite ($Fe_2O_3$). All reactions were written on the basis of eight electron transfers with acetate or hydrogen serving as electron donors.

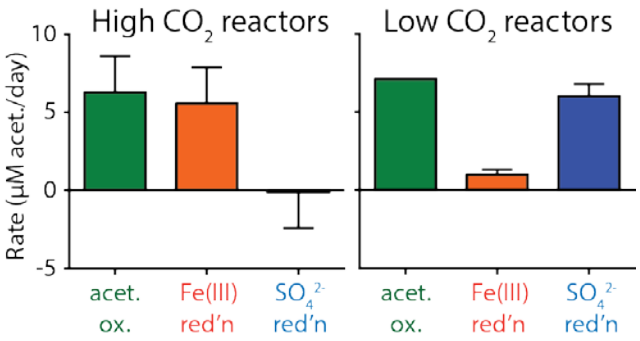



Figure 5. Average overall rate of acetate oxidation and the rate of acetate oxidation by iron reducers and sulfate reducers in the mixed-culture bioreactor experiments during the final 8 weeks of the incubations. Error bars show standard deviation.