Interplay between Microorganisms and Geochemistry in Geological Carbon Storage

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43 **ABSTRACT (200 words maximum)**

44 Researchers at the Center for Frontiers of Subsurface Energy Security (CFSES) have
45 conducted laboratory and modeling studies to better understand the interplay between
46 microorganisms and geochemistry for geological carbon storage (GCS). We provide evidence of
47 microorganisms adapting to high pressure CO₂ conditions and identify factors that may
48 influence survival of cells to CO₂ stress. Factors that influenced the ability of cells to survive
49 exposure to high-pressure CO₂ in our experiments include mineralogy, the permeability of cell
50 walls and/or membranes, intracellular buffering capacity, and whether cells live planktonically
51 or within biofilm. Column experiments show that, following exposure to acidic water, biomass
52 can remain intact in porous media and continue to alter hydraulic conductivity. Our research
53 also shows that geochemical changes triggered by CO₂ injection can alter energy available to
54 populations of subsurface anaerobes and that microbial feedbacks on this effect can influence
55 carbon storage. Our research documents the impact of CO₂ on microorganisms and in turn, how
56 subsurface microorganisms can influence GCS. We conclude that microbial presence and
57 activities can have important implications on carbon storage and that their presence should not
58 be overlooked in further GCS research.

1. Introduction

Geologic carbon storage (GCS) involves the capture, compression, injection, and storage of anthropogenic carbon dioxide (CO₂) in order to mitigate carbon emissions to the atmosphere. Deep (>1 km below the ground surface) sedimentary formations are one of the largest sets of likely injection targets. Pore waters in potential storage reservoirs are typically saline with ionic strengths ranging from that of seawater to levels near those of fluids saturated with halite. Injected CO₂ 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 CO₂ will alter groundwater pH and dissolved inorganic carbon (DIC) concentration, increase levels of dissolved ions, and cause both mineral dissolution and precipitation (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 may also be important, however, because microorganisms can influence 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), and alter water chemistry on a regional scale (Flynn et al., 2013; Kirk et al., 2015).

Microbial life extends deep into the subsurface, including depths of interest to GCS. The depth limit of microbial life in the subsurface is somewhat uncertain. However, active

microorganisms have been confirmed at depths greater than 3 km (Kieft et al., 2005). Their ability to adapt to a wide range of environmental conditions (Pikuta et al., 2007) together with the vast size of the habitable subsurface allow subsurface microbes to play a major role in mediating global-scale biogeochemical processes (Colwell and D'Hondt, 2013; Orcutt et al., 2013; Parkes et al., 2014).

Changes in conditions following CO₂ injection will impose stress on indigenous microorganisms, potentially triggering changes in community composition (Mu et al., 2014; Peet et al., 2015; Wilkins et al., 2014). Where CO₂ exists as a supercritical phase, it may dissolve cell membranes and cause cell death (Dillow et al., 1999; White et al., 2006). High levels of CO₂ in an aqueous solution can also be toxic to microbes because CO₂ can pass through cell membranes, acidify cytoplasm, and disrupt cellular functions (Ballestra et al., 1996).

In addition to changes in community composition driven by CO₂ stress, CO₂ injection may also shift community composition by altering redox disequilibrium. When CO₂ dissolves into water, carbonic acid is produced, which can then dissociate into protons and dissolved inorganic carbon species:

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 CO₂ abundance affects the extent to which those reactions are out of equilibrium (Harvey et al., 2013; Kirk, 2011; Mayumi et al., 2013; Ohtomo et al., 2013). Such changes can significantly affect microbial activity because the amount of energy that is available in the environment for microbial reactions affects the ability of microorganisms to compete with one another. Microorganisms that conserve energy from

more energetically favorable reactions can grow faster, and thus catalyze their reaction more rapidly, than those using less favorable reactions (Jin, 2012; LaRowe and Amend, 2015; Lovley and Goodwin, 1988; Roden and Jin, 2011).

In this paper, we examine geomicrobiological studies conducted at the Center for Frontiers of Subsurface Energy Security (CFSES) within the context of the interplay between microbiology and GCS. In other words, we consider what our findings tell us about how GCS could affect subsurface microbes and in turn, how subsurface microbes could affect GCS. Given the potential for microorganisms to influence the geochemistry and hydrodynamics of the subsurface, understanding this interplay may be a key to ensuring secure carbon storage. Moreover, this knowledge can provide a basis for developing biological strategies to enhance GCS reservoir performance (Mitchell et al., 2010).

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 GCS and subsurface microbiology. Our research has identified and characterized an isolate from a CO₂-rich spring (Santillan et al., 2015). We used pure-culture batch reactor experiments to test the influence of mineralogy on the ability of cells to survive exposure to high-pressure CO₂ (Santillan et al., 2013). We considered how decreasing pH, a geochemical change caused by CO₂ injection, will affect the stability of bioclogging in porous media (Kirk et al., 2012). 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 CO₂ abundance (Kirk, 2011; Kirk et al., 2013). These efforts

provide insight into both sides of the two-way interactions between GCS and subsurface microorganisms.

2. Methods

The content below provides a brief summary of methods used in our investigations. For more details about these methods as well as our results, please refer back to the publications associated with each study.

2.1. Isolation

A capnophile, an microbe capable of growth in the presence of high concentrations of CO₂, was isolated and characterized as part of our effort to learn about properties of microbes in aqueous environments with high CO₂ levels (Santillan et al., 2015). The isolate was collected from Crystal Geyser spring, Utah, USA. The site is considered an analog site for GCS research and provides the opportunity to study a subsurface microbial community that has been exposed to elevated CO₂ over a long period of time (Emerson et al., 2015). CO₂ has been leaking from the subsurface near the geyser for over 400,000 years (Burnside et al., 2013).

Samples of water and microbial biomass were collected at 9.7 m depth in the spring outlet using aseptic techniques. Cultures were prepared immediately by placing filtered biomass in serum bottles that contained Luria Bertain broth amended with 15 g L⁻¹ NaCl. The bottles were then placed within a pressure vessel and pressurized to 1 MPa with ultrapure CO₂. Cultures were incubated for about 1 month and then re-cultured multiple times to cultures

containing Tryptic soy broth with 15 g L⁻¹ NaCl. After three transfers, the cultures were 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 CO₂, 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). Cell morphology was characterized using transmission electron microscopy (TEM). Lipid samples were processed according to Rodriguez-Ruiz et al. (1998) and analyzed using gas chromatography mass spectrometry (GCMS).

2.2. Pure-culture experiments

Pure-culture experiments were performed to examine factors influencing the ability of cells to survive exposure to high-pressure CO₂ (Santillan et al., 2013). Experiments were conducted with three model organisms: *Shewanella oneidensis* strain MR-1 (ATCC BA-1096), *Geobacillus stearothermophilus* (ATCC 7953), and *Methanothermobacter thermoautotrophicus* (ATCC 29096). These organisms allowed the experiments to include variation in metabolic reactions as well as cell wall structure and composition. *S. oneidensis* is a Gram negative bacterium that was grown under iron-reducing conditions, *G. stearothermophilus* is a Gram positive aerobic bacterium that is capable of sporulation, and *M. thermoautotrophicus* is a methanogenic archaeon. Species closely related to *G. stearothermophilus* and *M. thermoautotrophicus* have been detected in the deep subsurface (Kawaguchi et al., 2010;

Nazina et al., 2001). *S. oneidensis* is widespread in soils and shallow sediment and has been studied within the context of CO₂ leakage to shallow groundwater from deep storage (Wu et al., 2010).

Organisms were grown to stationary phase in batch cultures and then placed in pressure vessels (Parr instruments) and exposed to elevated CO₂ pressure at 30°C for time periods ranging from 1 to 24 hr. CO₂ pressures tested ranged from 0.3 to 6.5 MPa. At the end of the exposure period, pressure was slowly released over a period of about 2 min to limit potential impacts of pressure change on cell survival. The cultures were then removed from the pressure vessels and sonicated to disperse biofilm and attached cells. Cell survival was quantified using cultivation. Cultivable *S. oneidensis* and *G. stearothermophilus* cells were enumerated using the pour plate method. *M. thermoautotrophicus* cells were cultivated in liquid anaerobic cultures with low CO₂ content. Growth was periodically assessed in the cultures by measuring optical density at 680 nm. Iron reducing activity of *S. oneidensis* was evaluated by measuring ferrous iron concentration using the ferrozine method (Stookey, 1970). Methanogenesis by *M. thermoautotrophicus* was evaluated by measuring CH₄ partial pressure using gas chromatography.

S. oneidensis, the model organism most susceptible to CO₂ exposure of those tested, was selected for a second set of experiments that examined the effects of mineral solid phases on CO₂ toxicity (Santillan et al., 2013). Minerals and rock samples (Ward's Natural Science, Rochester, NY) were crushed to the size of coarse sand, cleaned of any magnetite they may have contained using 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. Test cultures were then exposed to 2.5 MPa CO₂ for up to 8 h. The impact of CO₂ exposure on cell survival was assessed by comparing the culturable cell content of test cultures to identical cultures that were not exposed to high-pressure CO₂. In both cases, the cultures were sonicated prior to culturing to disperse cells and cell survival was evaluated using pour plating. Samples of minerals and cells were imaged using scanning electron microscopy (SEM) following termination of the experiments.

For our pure-culture tests, control experiments were performed to assess the impact of sonication and pressure changes on cell survival. Results indicate that neither factor significantly impacted the culturable cell concentrations we measured. A set of control experiments was also included to examine the extent to which biofilm protected cells during exposure to high-pressure CO₂. For those controls, the cultures were sonicated prior to CO₂ exposure to disperse biofilm cells.

2.3. Bioclogging experiments

Column experiments were performed to examine how sudden acidification of water would impact the stability of biofilm in porous media (Kirk et al., 2012). The experiments were run in 10 cm long square capillary tubes with a 1 mm² cross-sectional area packed with 105–150 µm diameter glass beads. 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, a fluorescent dye, was included for pore-space imaging. pH was adjusted using HCl. Medium was pumped through the columns at $0.015 \text{ mL min}^{-1}$ (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 colony forming units (CFU; stdev 0.3) of *Pseudomonas fluorescens* tagged with a green fluorescent protein (GFP). Biofilm production by *P. fluorescens* is well characterized, including growth in flowing systems (e.g., Pereira et al., 2002; Simoes et al., 2007; Simoes et al., 2005). A strain tagged with GFP was chosen to allow biomass growth to be monitored nondestructively. 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.

The average saturated hydraulic conductivity over the entire length of each column was evaluated for each of the three phases of the experiments based on pressure measurements. Pores and biomass were imaged with a scanning laser confocal microscope during the experiments. Culturable cell concentrations in column effluent were measured periodically throughout the experiment by plating effluent samples. For two pH 4 and three pH 5.7 experiments, effluent cell abundance was also quantified using live-dead staining. This approach provides a measure of cell viability that, unlike plating, is not influence by any cultivation bias. After the experiments were terminated, the culturable cell content of 1 cm column segments was measured in one pH 4 and one pH 5.7 experiment.

2.4. Mixed-community experiments

Experiments were carried out with bioreactors containing a mixed-microbial community to examine how changes in CO₂ abundance could alter interactions between groups of microbes that naturally co-exist (Kirk et al., 2013). Unlike the pure-culture experiments, which isolate factors that influence cell survival, these experiments consider how an increase in CO₂ could affect interactions between different functional groups of microorganisms.

The experiments were carried out in duplicate using anoxic semi-continuous bioreactors. Microbes and groundwater for the experiments were obtained from a freshwater aquifer. Two sets of experiments were performed: one with low CO₂ partial pressure (~0.002 MPa) in the headspace of the reactors and one with high CO₂ partial pressure (~0.1 MPa). Hereafter, we refer to these experiments as the low-CO₂ bioreactors and high-CO₂ bioreactors, respectively. 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. Rates of acetate oxidation, iron reduction, and sulfate reduction were directly evaluated using mass-balance calculations based on measured reactor chemistry.

Well-mixed samples of reactor solids and fluid were collected at the end of the incubations for analysis of reactor solid phases and microbial community composition. 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). During processing, the software used AmpliconNoise to remove sequencing errors (Quince et al., 2011).

2.5. Numerical analysis

Bioenergetics calculations were used to consider how increasing CO₂ abundance affects redox disequilibrium and, in turn, microbial activity. Calculations were performed using data collected during two field CO₂-injection experiments (Kirk, 2011) and with data collected from the mixed-community experiments (Kirk et al., 2013). In both cases, the calculations assessed changes in energy available (ΔG_A) for microbial metabolism. As defined previously (Bethke et al., 2011), ΔG_A is the negative of the free energy change of microbial metabolic reaction (ΔG_r) and can be calculated in units of kJ·mol⁻¹ as follows:

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

where ΔG_T° is the standard Gibbs free-energy change for reaction r at temperature T (°K), R represents the gas constant (kJ·mol⁻¹·K⁻¹), γ_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 uncertainty regarding electron donor concentrations, results from the bioenergetics analysis of the field data are normalized relative to conditions present prior to CO₂ injection, as follows:

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

where the superscript “CO₂” designates each value calculated during or after CO₂ 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 CO₂ injection, not absolute values of energy available.

3. Results and discussion

The integration of our studies yields insight into the interplay between subsurface microbes and GCS beyond that possible within each individual study. In the subsections that follow, we examine the results of our studies within the context of these two-way interactions.

3.1 Impacts of GCS on microbiology

3.1.1. Factors influencing cell survival

Results of our isolation and pure-culture experiments indicate that cells that have properties that limit CO₂ accumulation in their cytoplasm are better able to survive exposure to high pressure CO₂. These properties include the make-up of their cell wall and membranes, the nature of their metabolic reactions, and whether they exist within biofilm.

We found that isolate CG-1 exhibits a fermentative metabolism and was most related (98.5%) to *Lactobacillus casei* (Santillan et al., 2015). It grows at CO₂ partial pressures between 0 and 1.0 MPa and is able to survive for at least 5 days at 2.5 MPa CO₂ and for at least 1 day at 5 MPa CO₂. CG-1 morphology and fatty acid composition both vary with CO₂ partial pressure. Images collected from cultures with 0.1 MPa CO₂ show rod-shaped cells. In images collected from cultures with 1 MPa CO₂, however, cells are generally smaller and encased in capsular material (Figure 1). With increasing CO₂ partial pressure, monounsaturated fatty acids decreased in relative abundance while saturated fatty acids increased. Production of capsular

material and the changes in lipid composition at high CO₂ levels are consistent with a decrease in the flexibility and perhaps permeability of the cells.

Strains tested in our pure-culture experiments varied in their ability to survive exposure to high-pressure CO₂ (Santillan et al., 2013). For all organisms, survival was best at low CO₂ pressures but decreased as pressures increased. *S. oneidensis* cells were the most sensitive to increased CO₂ while *G. stearothermophilus* cells were the most resilient.

G. stearothermophilus cells may have been better able to survive than the other strains because they possess Gram positive cell walls as well as the capacity to form endospores. Cell wall and membrane composition influence the extent to which CO₂ can penetrate cells (Bertoloni et al., 2006; Zhang et al., 2006). Gram positive cell walls are more rigid and less permeable than Gram negative cell walls. Sporulation can provide a mechanism by which cells can reduce themselves into a more durable form until CO₂ stress is removed (Furukawa et al., 2004; Watanabe et al., 2003).

Differences in survival between *M. thermoautotrophicus* and *S. oneidensis* cells may also reflect differences in the ability of CO₂ to penetrate the cells. Archaea, such as *M. thermoautotrophicus*, possess cell membranes that differ considerably from those of Bacteria. Because of those differences, they are thought to generally be better able to withstand extreme conditions (Arakawa et al., 1999; Gambacorta et al., 1994). In addition, differences in metabolism between the strains may have also contributed to variation in cell survival. Unlike *S. oneidensis*, *M. thermoautotrophicus* cells consume CO₂ in their catabolic reaction, potentially helping them limit accumulation of CO₂ within their cytoplasm. The isolation process of CG-1 suggests it may similarly benefit from intracellular CO₂ consumption. Many fermenters utilize

CO₂ in metabolic processes, such as amino acid synthesis or through C₁ metabolism (Arioli et al., 2009; Bringel et al., 2008; Song et al., 2007).

Results from our experiments that included minerals, indicate that the mere presence of a mineral can enhance the ability of *S. oneidensis* cells to survive exposure to high pressure CO₂ (Santillan et al., 2013). With the exception of kaolinite, cell survival was higher in cultures containing minerals than those without (Figure 2). We hypothesize that these results reflect the shelter provided by biofilm. Unlike planktonic cells, biofilm cells are surrounded by extracellular polymeric substances (EPS), which limits their exposure to environmental stresses such as high-pressure CO₂ (Mitchell et al., 2008; Mitchell et al., 2009). Surface area available for biofilm formation was greater in cultures that contained minerals than those that did not. SEM images (not shown) confirm that biofilm formation did occur on mineral surfaces during the experiment.

3.1.2. Persistence of attached biomass

Results from our column experiments show that biofilm can remain largely intact following sudden acidification of water, even if considerable cell death occurs (Kirk et al., 2012). After 4 days of growth at pH 7.2, a 0.67 log reduction in the overall hydraulic conductivity of the columns occurred, on average (Figure 3). Acidification caused hydraulic conductivity to increase significantly in all but one pH 5.7 experiment as well as extensive cell death and stress, particularly in pH 4 experiments. However, the columns remained significantly clogged relative to pre-growth conditions. Following acidification, log reductions in hydraulic conductivity averaged 0.43 and 0.65 in pH 4 and pH 5.7 experiments, respectively.

3.1.3. Shifts in microbial reactions

Our mixed-community experiments and numerical analyses show that increasing CO₂ concentration favors microbial reactions that consume acid. As a result, microbial communities that emerge following injection of CO₂ may differ from indigenous communities not only because they are better at tolerating CO₂ stress but also because the balance between different microbial reactions has shifted.

Microbial activity differed considerably between the high- and low-CO₂ bioreactors in our mixed-community experiments (Kirk et al., 2013). Mass-balance calculations demonstrate that sulfate reduction was dominant in reactors with low CO₂ 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 CO₂ content, accounting for at least 90% of the acetate consumption while sulfate reduction consumed a negligible amount (<1%).

Results of our microbial community analyses agree with our mass-balance calculations (Kirk et al., 2013). 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-CO₂ reactor samples compared to the low-CO₂ reactor samples. Moreover, sequences classified in groups relating to sulfate reducers were abundant in the low-CO₂ reactor samples but nearly absent from the high-CO₂ reactor samples.

Bioenergetics calculations show that the rate of microbial iron reduction may have varied in response to differences in thermodynamic controls (Kirk et al., 2013). Iron reduction

was much more energetically favorable in reactors that hosted more rapid iron reduction, the high-CO₂ reactors, than those with slower iron reduction rates, the low-CO₂ reactors. After acetate consumption stabilized, energy available for microbial iron reduction was 114 kJ mol⁻¹ and 60 kJ mol⁻¹, on average in the high- and low-CO₂ bioreactors, respectively.

In contrast, thermodynamic controls on microbial sulfate reduction could not be responsible for variation in the rate of that reaction. Energy available for sulfate reduction varied little, averaging a maximum of 65 kJ mol⁻¹ and 62 kJ mol⁻¹ in the high- and low-CO₂ reactors, respectively. Instead, we hypothesize that the rate of sulfate reduction varied in response to competition for electron donor from iron reduction (Kirk et al., 2013). Where energy available for microbial iron reduction was high, the reaction occurred rapidly and little electron donor remained for sulfate reduction. However, where energy available for iron reduction was low, the reaction slowed, allowing sulfate reduction to consume excess electron donor.

Bioenergetic calculations performed using data from the field CO₂-injection experiments provide results that parallel those from the mixed-community experiments. CO₂ injection benefitted iron reduction much more than sulfate reduction or methanogenesis at both field 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.

In both sets of calculations, the energy advantage gained by iron reduction with increased CO₂ levels primarily reflects changes in pH. Reduction of ferric iron in oxides and

oxyhydroxides consumes a large number of protons. As such, the energy yield of iron reduction increases sharply as pH decreases. Sulfate reduction and methanogenesis, however, consume relatively few protons. As such, the energy yield of those reactions does not vary strongly with pH.

Our numerical and mixed-culture studies indicate that CO₂ injection has the potential to stimulate microbial iron reduction where ferric iron is available. 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 CO₂ than *M. thermoautotrophicus*, 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 iron-reducing microorganisms that may exist in a GCS reservoir, therefore, may be better able to adapt to an increase in the abundance of CO₂ than the individual isolate we tested.

3.2. Impacts of microbiology on GCS

3.2.1. Impacts of microbiology on flow

Similar to our findings, previous studies have shown that biofilm can remain largely intact in porous media during exposure to supercritical CO₂ (Mitchell et al., 2008; Mitchell et al., 2009). Combined with our efforts, the results of these studies provide compelling evidence that

hydraulic conductivity will change little in response to biofilm redistribution following injection of CO₂ into GCS reservoirs where biofilms are present. If microbial biomass influences hydraulic conductivity before CO₂ is injection into a GCS, our results and those of previous studies suggest it will influence hydraulic conductivity afterward as well.

These findings imply that, in biologically activity GCS reservoirs, microbial biofilms can influence the flow of CO₂ and water away from injection wells. Consistent with this implication, previous studies found that microbial activity significantly decreased the injectivity of a CO₂-injection well at the Ketzin site (Morozova et al., 2010; Zettlitzer et al., 2010). In addition, biofilm on a mineral surface may alter the wettability of those minerals, which is a major control on residual trapping of CO₂ (Chaudhary et al., 2013).

3.2.2. Impacts of microbiology on solution and mineral trapping

Results of the mixed-community experiments show that, where CO₂ injection stimulates microbial iron reduction, solubility trapping may be enhanced. Because microbial reduction of ferric iron in iron oxides and oxyhydroxides consumes a large number of protons, the reaction works to convert CO₂ into carbonate alkalinity, thereby enhancing storage of inorganic carbon in solution (Kirk et al., 2013). Reflecting this relationship, the increase in carbonate alkalinity caused by microbial activity in high-CO₂ bioreactors was six-fold greater than that in the low-CO₂ bioreactors (Figure 6). Mitchell et al. (2010) describe a similar effect during bacterial hydrolysis of urea batch reactor experiments containing elevated CO₂ content. The results of these studies suggest that we may need to consider the response of the microbial community to CO₂ injection in order to accurately predict rates of solution trapping in GCS reservoirs.

In addition to solution trapping, microbial activity also has the potential to impact mineral trapping. Alkalinity generation by acid-consuming microorganisms works to increase the saturation state of carbonate minerals such as calcite (CaCO_3) and siderite (FeCO_3) (Kirk et al., 2013; Mitchell et al., 2010). Moreover, cells and biofilms can also facilitate carbonate mineralization by providing nucleation sites (Benzerara et al., 2011; Mitchell and Ferris, 2006). Hence, rates of mineral trapping may also be influenced by the response of the microbial community to CO_2 injection.

3.5. Future research

Our efforts and those of many other researchers have to date been weighted toward understanding one side of the interplay between microbiology and GCS: the impact of GCS on microbial activity. This area of research is important. We can understand how microbes will affect GCS without knowing what physical and functional characteristics GCS reservoirs will select for. However, we suggest that more attention needs to be paid to the impact of microbiology on GCS.

Many questions about this component of GCS geomicrobiology remain unresolved. Little is known about the nature of microbial impacts on GCS and their relative significance. For example, how will alkalinity production by acid-consuming microorganisms compare to that generated by abiological reactions between CO_2 and minerals? We also do not have a clear basis for identifying which GCS reservoirs are more likely to host significant microbial impacts. Should our attention focus on organic-rich reservoirs (e.g., depleted oil reservoirs and coalbeds) or will microbial reaction rates be significant relative to the time scale of GCS in all reservoirs?

Answering these questions will constrain the extent to which numerical models need to include microbial activity to accurately simulate the long-term fate of CO₂ in the subsurface.

Future laboratory research needs to simulate conditions consistent with GCS reservoirs. GCS reservoirs will commonly be anoxic, with heterogeneous mineralogy and microbiology and elevated pressure, temperature, and salinity. Many recent laboratory studies were performed under relevant conditions (e.g., Dupraz et al., 2013; Mayumi et al., 2013; Ohtomo et al., 2013; Peet et al., 2015; Wilkins et al., 2014). However, most of what we know about the impact of high pressure CO₂ on microbiology stems from food industry research into CO₂ as a sterilizing agent (e.g., Amanatidou et al., 1999; Spilimbergo et al., 2002; Watanabe et al., 2003; Zhang et al., 2006). Follow-up experiments are warranted to test some of the research questions in those studies under conditions consistent with GCS reservoirs.

Lastly, we suggest that additional research should examine microbiological mechanisms that could create an energy return on subsurface CO₂ injection. For example, recent research has found evidence that CO₂ injection can stimulate biological conversion of crude oil into natural gas (Mayumi et al., 2013). CO₂ 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.

4. Conclusions

Geomicrobiology studies performed by CFSES examine impacts of GCS on subsurface microbiology. Pure-culture and isolation studies identify factors that may influence survival, including environmental, biochemical, and structural characteristics. Our column experiments

show that biofilm can remain largely intact following sudden acidification of water, even if significant cell death and stress occurs. Mixed-community experiments and thermodynamic calculations show that the balance between microbial reactions can shift in response to changes in fluid chemistry caused by increasing CO₂ levels. Collectively, these efforts add to the growing body of evidence that microbial life will persist in GCS reservoirs, likely defined by communities that differ from those present prior to injection. Our work suggests that communities will change in response to differences in the ability of cells to tolerate elevated CO₂ levels as well as shifts in the balance of microbial reactions.

These studies also shed light on potential impacts of subsurface microbial communities on GCS. Subsurface biomass may influence the hydrodynamics of porous media in GCS reservoirs, affecting flow away from injection wells and capillary trapping of CO₂. Coupled with this effect, by catalyzing oxidation-reduction reactions, microorganisms can affect the rate and form of solubility and mineral trapping. The potential importance of microbial activity in GCS reservoirs, therefore, should not be overlooked.

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TOC figure. This paper integrates geomicrobiology research performed by the Center for
Frontiers in Subsurface Energy Security to better understand the interplay between geological
carbon storage (GCS) and subsurface microorganisms.

Figure 1. Bright-field TEM images of CG-1 at 0.1 MPa CO₂ (A,C) and 1.0 MPa CO₂ (B,D). Arrows
in: (B) show invaginations in CO₂ exposed cells that may suggest cell division; (C) show the
intact cell wall for organisms at low CO₂ exposure; (D) show the capsular material present for
CO₂ exposed cells. Modified after Santillan et al. (2015).

Figure 2. Variation with culture mineralogy in the abundance of culturable *Shewanella* *oneidensis* MR1 cells following incubation in the presence and absence (control) of high-pressure CO₂. Chart modified after Santillan et al., 2013.

Figure 3. Typical variation in hydraulic conductivity of column reactors during bioclogging experiments.

Figure 4. 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.

Figure 5. Change in energy available for iron reduction, sulfate reduction, and methanogenesis as a result of CO₂ injection during field CO₂-injection experiments. Values show the average difference between energy available prior to CO₂ 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 (Fe₂O₃), and magnetite (Fe₃O₄). All reactions were written on the basis of eight electron transfers with acetate or hydrogen serving as electron donors.

Figure 6. Average alkalinity content of effluent from the mixed-culture bioreactor experiments during the final 8 weeks of the incubations. Results are shown for biologically-active (i.e., live) bioreactors as well as corresponding sterile control reactors. Error bars show standard deviation.