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Concentration-dependent effects of CO_2 on subsurface microbial communities under conditions of geologic carbon storage and leakage

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Abstract

Geologic carbon storage (GCS) is a crucial part of a proposed mitigation strategy to reduce the anthropogenic CO_2 emissions to the atmosphere. During this process, CO_2 is injected as super critical carbon dioxide (SC-CO_2) in confined deep subsurface storage units, such as saline aquifers and depleted oil reservoirs. The deposition of vast amounts of CO_2 in subsurface geologic formations may ultimately lead to CO_2 leakage into overlying freshwater aquifers. Introduction of CO_2 into these subsurface environments will greatly increase the CO_2 concentration and will create CO_2 concentration gradients that drive changes in the microbial communities present. While it is expected that altered microbial communities will impact the biogeochemistry of the subsurface, there is no information available on how CO_2 gradients will impact these communities.

The overarching goal of this dissertation is to understand how CO_2 exposure will impact subsurface microbial communities at temperature and pressure that are relevant to GCS and CO_2 leakage scenarios. To meet this goal, unfiltered, aqueous samples from a deep saline aquifer, a depleted oil reservoir, and a fresh water aquifer were exposed to varied concentrations of CO_2 at reservoir pressure and temperature. The microbial ecology of the samples was examined using molecular, DNA-based techniques. The results from these studies were also compared across the sites to determine any existing trends.

Results reveal that increasing CO_2 leads to decreased DNA concentrations regardless of the site, suggesting that microbial processes will be significantly hindered or absent nearest the CO_2 injection/leakage plume where CO_2 concentrations are highest. At CO_2 exposures expected downgradient from the CO_2 plume, selected microorganisms emerged as dominant in the CO_2

exposed conditions. Results suggest that the altered microbial community was site specific and highly dependent on pH. The site-dependent results suggests no ability to predict the emerging dominant species for other CO_2 exposed environments.

This body of work improves the understanding of how a subsurface microbial community may respond to conditions expected from geologic carbon storage and CO_2 leakage. This is the first step for understanding how a CO_2-altered microbial community may impact injectivity, permanence of stored CO_2, and subsurface water quality.

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Chapter 1

Introduction, Problem Identification, and Research Objectives

1.1 Introduction

Carbon capture and storage (CCS) is likely to be part of a comprehensive solution to reduce CO_2 emissions from fossil energy combustion. Atmospheric CO_2 concentrations continue to rise,[1] increasing the risk of more frequent extreme weather events.[2] The International Energy Administration (IEA) predicts that to reduce this risk by 2050, 8.2 gigatonne CO_2/yr will need to be captured for carbon storage.[3]

Geologic Carbon Storage (GCS) is the end process of CCS. After capture of CO_2 from fossil energy burning for industrial or municipal power production, CO_2 is injected into deep subsurface rock formations, termed geologic storage units, for long-term sequestration from the surface environment. Geologic storage units that have been targeted for future sequestration include both brine aquifers and oil reservoirs. Optimizing the storage capacity of the formations is an important goal of GCS scientists and engineers. In addition, the CO_2 storage deposits should be permanent, with little risk of release to the overlying environments. Overlying environments at risk includes shallow potable aquifers and the surface environment. The storage capacity and permanence of CO_2 deposits will be impacted by a variety of

geophysical, geochemical and biogeochemical processes. This dissertation focuses on the microbiology that will drive the biogeochemical processes.

1.2 Problem Identification

Geochemical parameters, such as salinity, dissolved metal concentration, and pH determines the microbial community that adapts to thrive in the subsurface. Although these microorganisms are capable of inhabiting a wide range of environmental conditions, there remain some limits to microbial survival in extreme conditions. For example, many microorganisms do not survive in environments with temperature above 110 ˚C, pH below 0.7, pH above 11, or salinity above 30% NaCl.[4] Nonetheless, a diverse microbial community is known to thrive in the deep geologic subsurface.[5, 6]

The microorganisms that thrive in the deep subsurface drive biogeochemical reactions that impact the fate of carbon and other minerals and nutrients in those environments.[7-9] Microorganisms may affect the reservoir capacity to store CO_2 and the permanence of stored CO_2 through biomineralization of CO_2,[10, 11] biofilm formation that alters flow and storage,[12-14] dissolution of carbonate minerals through acid production,[12, 15, 16] and dissolution of minerals through metal mobility.[17] These microbial processes are known to have a large impact of the geochemistry and geology in the deep subsurface and are therefore likely to affect the fate of

CO_2 following GCS. Therefore, microbial populations that adapt to CO_2 exposure are likely to play a vital role in carbon storage. In order to understand the biogeochemical reactions that will affect CO_2 storage, the microbial populations that will thrive in these systems must first be identified.

While bacteria are likely to have a large impact on reservoir capacity and CO_2 security over the lifetime of geological carbon storage, no information is available about the microbial populations that will survive and/or thrive following exposure to CO_2 at temperatures and pressures that are relevant to storage conditions. *The overarching goal of the research is to understand the microbial communities and populations that are likely to arise in the subsurface following geologic carbon storage.*

1.3 Research Objectives

This dissertation research aims to understand changes in subsurface microbial communities following exposure to $CO_{2(aq)}$ at concentrations expected during CO_2 storage. Since GCS is most likely to begin in saline aquifers and depleted oil reservoirs, the research will address the microbial community changes in these critical deep subsurface environments. In addition, this dissertation research aims to understand changes in subsurface microbial

communities following exposure to $CO_{2(aq)}$ at concentrations expected during CO_2 leakage from the deep subsurface into overlying shallow, freshwater environments.

The goal of this dissertation research will be met through achieving three main objectives. These objectives are to 1) define the microbial community changes and populations that arise following CO_2 exposure under GCS conditions on subsurface samples from carbon storage environments (saline aquifers and depleted oil reservoirs), 2) define the microbial community changes and populations that arise following a leakage scenario of CO_2 into freshwater environment, and 3) determine if changes in the microbial communities and populations that arise in these environments are similar or vary among the subsurface sites. These objectives are met through the completing the following the tasks:

Task 1: Measure changes with various $CO_{2(aq)}$ concentrations of microbial community from saline aquifer samples

Task 1 aims to understand the microbial community response to increasing $CO_{2(aq)}$ concentrations that would be expected following heterogeneous flow of injected CO_2 in a saline aquifer. Relevant microbial communities are examined in fluid samples and suspended solids from the proposed carbon storage site, the Arbuckle Aquifer. The changes in the microbial community are examined under pCO_2 exposure of 0%, 1%, 10% and 100% under site pressure

and temperature for up to 56 days. Population cell numbers are quantified by quantitative polymerase chain reaction (qPCR), and microbial population characterized by clone libraries.

Task 2: Measure changes with various $CO_{2(aq)}$ concentrations of microbial community from depleted oil reservoir samples

Task 2 aims to understand the microbial community response to increasing $CO_{2(aq)}$ concentrations that would be expected following heterogeneous flow of injected CO_2 in depleted oil reservoirs. Relevant microbial communities are examined in fluid samples and suspended solids from the proposed carbon storage site, the Mirando Oil Field. The changes in the microbial community are examined under pCO_2 exposure of 0%, 1%, 10% and 100% under site pressure and temperature for 56 days. Population cell numbers are quantified by quantitative polymerase chain reaction (qPCR), and microbial population characterized by clone libraries.

Task 3: Measure changes with various $CO_{2(aq)}$ concentrations of microbial community from fresh water aquifer samples

Task 3 aims to understand the microbial community response to increasing $CO_{2(aq)}$ concentrations that would be a result of CO_2 leakage into a freshwater aquifer. Relevant microbial communities for Task 3 are examined from fluid samples of the Plant Daniel fresh water aquifer. The Plant Daniel Aquifer has been flooded with dissolved CO_2 to represent a leakage scenario. Samples are collected both up-gradient and down-gradient of the CO_2

injection. The changes in the microbial community of the up-gradient samples are examined under pCO_2 exposure of 0%, 1%, 10%, and 100% under site pressure and temperature for up to 56 days. Population cell numbers are quantified by qPCR, and microbial population characterized by clone libraries and by pyrosequencing.

Task 4: Compare the microbial community changes and populations that disappear and arise among all of the samples examined.

Task 4 determines if microbial community changes are similar with CO_2 exposure, regardless of initial conditions, or if microbial community changes with CO_2 exposure vary amongst the initial conditions in different subsurface sites. The structure of each microbial community is directly compared between the three sites, and the extent of community differences and similarities is visualized using a UniFrac plot and a community tree. In addition, trends in cell numbers with increasing pCO_2 exposure amongst the different communities are discussed.

This work uses difficult to obtain samples of water and solids from the subsurface. The microbial community from each sample is exposed to increasing pCO_2 under the temperature and pressure of the subsurface site, in order to best simulate conditions and geochemistry expected to occur after CO_2 injection or CO_2 leakage. The high pressure was required in order to maintain supercritical conditions for CO_2 in the vessels. It is the first study to examine the initial

(~2 months) response of a subsurface microbial community to different $CO_{2(aq)}$ concentrations that may be expected following geologic carbon storage or a SC-CO_2 leak.

This dissertation begins to fill in knowledge gaps in subsurface microbial community response to CO_2 storage. The subsurface often contains up to 10^7 cell/ml,[18] and may consist of thousands of species; it is a laborious task to study all the microbial processes of this diverse system. However, I hypothesize the microbial community that adapts to CO_2 exposure will be a smaller population and less diverse. The microbial processes of relevance will be from this community of reduced diversity. This project serves to identify the adapted microbial communities from carbon storage environments at relevant formation temperature and pressures. The project may guide future studies on biogeochemical processes of the most relevant species expected to survive and thrive in a formation following CO_2 exposure. Identifying the microbial communities that are selected for by CO_2 exposure will help to identify the types of bioprocesses that may play a role in long-term carbon storage.

1.4 Structure of Dissertation

This dissertation is structured as distinct chapters aimed to define and achieve the overarching goal. Chapter 1 this introductory chapter, and Chapter 2 provides a background on geochemistry during carbon storage, biological processes in the subsurface, and biological

processes studied thus far during carbon storage. Chapter 3 describes the research results obtained for Task 1, the investigations of the microbial community of a saline aquifer after CO_2 exposure. The results from Chapter 3 are published the manuscript entitled "CO_2 concentration and pH alters subsurface microbial ecology at reservoir temperature and pressure, RSC Advances, 2014.[19] Chapter 4 describes the research results obtained for Task 2, the investigations of the microbial community of two depleted oil reservoirs after CO_2 exposure. Chapter 5 describes the research results obtained for Task 3, the investigations of the microbial community of a freshwater aquifer after CO_2 exposure during a leakage scenario. Chapter 6 describes the research obtained for Task 4, a comparison of the changes in microbial community with CO_2 exposure amongst the saline aquifer, depleted oil reservoir, and freshwater aquifer. Finally, Chapter 7 summarizes this project in context of GCS and suggests future work.

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Chapter 2

Background

Geologic carbon storage is expected to result in complex interactions between geophysical processes, geochemical processes, and biogeochemical processes. These complex interactions must be understood in order to provide a foundation for the research problem addressed in this dissertation. In order to understand these complex interactions and define the current knowledge gaps, this chapter provides a background on geologic carbon storage, geochemical processes following CO_2 exposure, and finally relevant biological processes. In addition, a review of the current research in the microbial ecology of carbon storage environments will be discussed.

2.1 Geologic Carbon Storage and Leakage Processes

Before the geochemical and biological processes of carbon storage and CO_2 leakage can be discussed in detail, it is important to understand the process of geologic carbon storage, the process of CO_2 leakage, and the properties of CO_2 in the subsurface. This section describes these subjects to provide a basic understanding of subsurface environments addressed in this dissertation.

Geologic carbon storage, the end process of carbon capture and storage (CCS) is the containment of injected $CO_{2(g)}$ in permeable but structurally isolated subsurface aquifers known as storage units. Proposed storage units are saline aquifers and oil reservoirs (Figure 2.1). After carbon capture from a point source, such as a fossil-fueled powered electricity generating plant, a compression pump drives $CO_{2(g)}$ into the storage units at pressures of 7.4 MPa and above, generally to depths over 800 m. At the pressures and temperatures typical of

these deep storage units, CO_2 will primarily be in the super critical CO_2 (SC-CO_2) phase, a high-temperature high-pressure phase with the effusive properties of a gas but density of a liquid. Gaseous and aqueous CO_2 phases will exist away from the bulk of the CO_2.

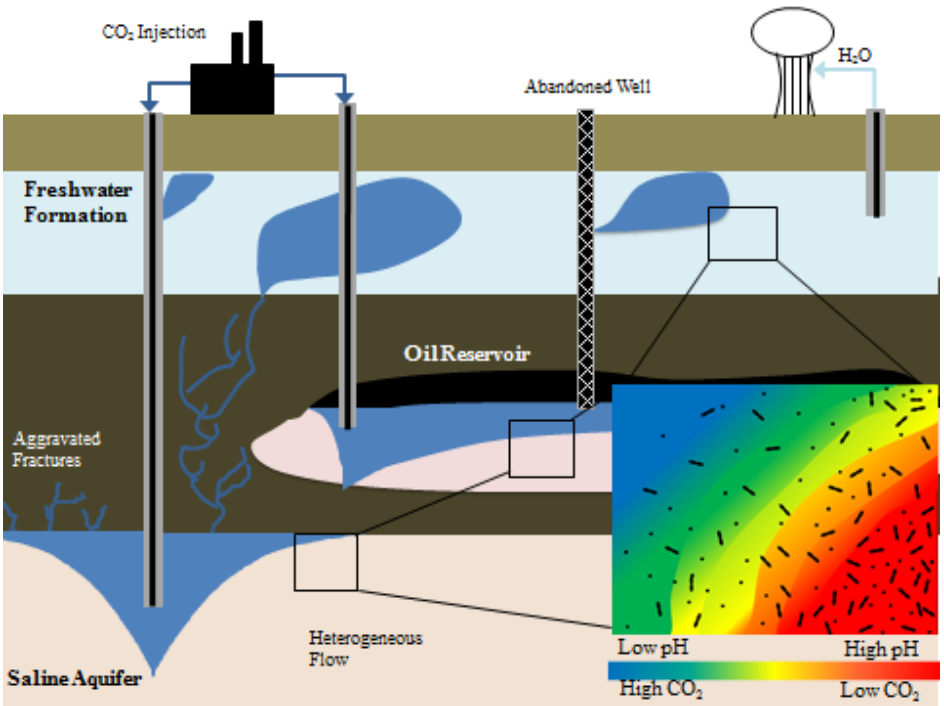

Figure 2.1. Overview of a) heterogeneous flow of injected CO_2 into potential a saline aquifer and oil reservoir as storage units, b) a potential leakage scenario into shallow formations, and c) the expected gradients of $CO_{2(aq)}$ and pH following injection and leakage.

The capacity of the storage units and permanence of geologic CO_2 storage will depend on the efficacy of four different trapping mechanisms for the injected SC-CO_2: structural trapping, residual trapping, solubility trapping and mineral trapping[1] (Figure 2.2). Immediately during injection, SC-CO_2 will displace the existing interstitial fluid within the pore space. The SC-CO_2 will be physically contained within the pore space of a storage unit and confined by an overlying and underlying low-permeability unit. This containment is termed structural trapping, and is modeled to be the dominant trapping mechanism in the

short term (less than 30 years).[2] As SC-CO_2 injection continues, pressure increases on the growing SC-CO_2 plume and the interstitial fluid within the storage unit, causing the SC-CO_2 plume and the interstitial fluid to transport. SC-CO_2 has a lower viscosity than water, and the SC-CO_2 plume will transport through the pore space of the storage unit at a higher rate than the interstitial fluid. As the SC-CO_2 plume transports, some residual SC-CO_2 may become trapped in pore throats due to capillary pressure (Fig. 2.2). This process is termed residual trapping. The residual SC-CO_2 will ultimately dissolve in the subsurface fluids, leading to solubility trapping (Fig. 2.2). Residual and solubility trapping are expected to be the dominant trapping mechanisms at longer times (between 30 years and 200 years).[2] Depending on the rock matrix and reservoir conditions, the dissolved CO_2 in solubility trapping may react with calcium, magnesium, or iron to form carbonate minerals. This process is termed mineral trapping, and converts the SC-CO_2 phase into a dense, non-mobile solid phase. CO_2 that has undergone mineralization has a relatively low risk for remobilization and release from the storage unit. The different physical and chemical properties of the carbon under each trapping mechanism will result in variable $CO_{2(aq)}$ concentrations in the storage unit. This geochemical heterogeneity is likely to result in variable water chemistry. The $CO_{2(aq)}$ effects on water chemistry is further described in section 2.3.

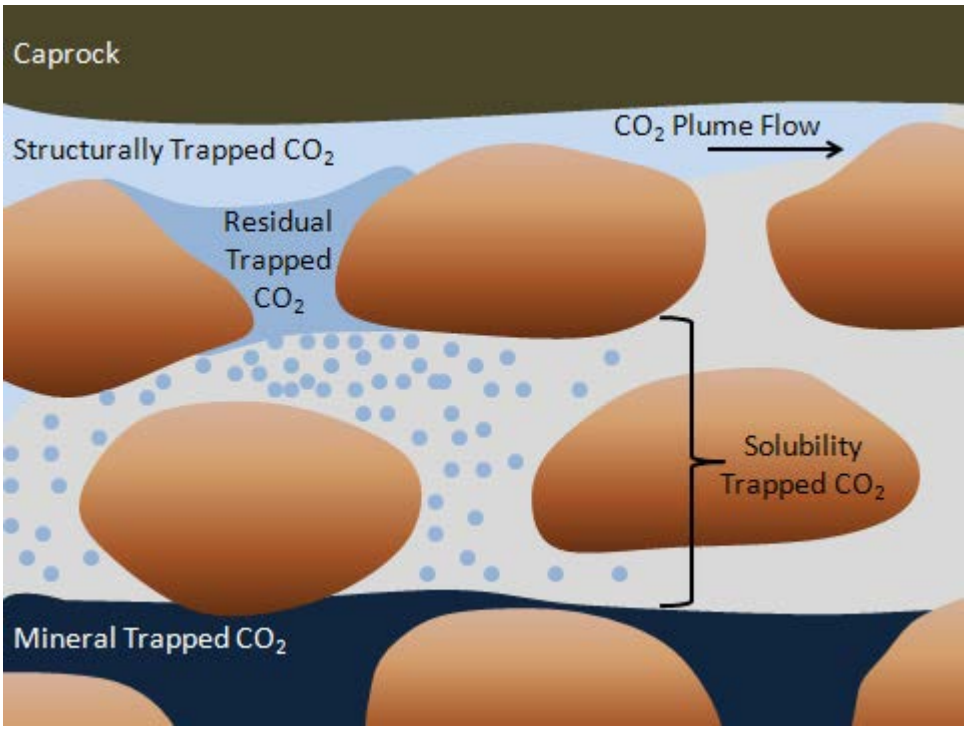

Figure 2.2. Trapping mechanisms during carbon storage

The application of geologic carbon storage raises concern for leakage of injected SC-CO_2 from storage units to overlying aquifers that may be used as sources for drinking water. Leakage may occur through two mechanisms: 1) transport through aggravated caprock fractures and, 2) transport through pre-existing wells. Aggravated fracturing is most likely to occur during geologic carbon storage in saline aquifers, as CO_2 injection is not utilized for fluid displacement (as is the case for oil reservoirs), resulting in formation pressure increase.[3] In addition, many brine formations have poorly characterized caprock;[4] caprock may contain structurally weak zones that will easily fracture with additional pressure. CO_2 leakage may also occur via transport through pre-existing wells that have decayed or faulty cement. Oil reservoirs are often accessed by numerous wells abandoned using uncertain and undocumented closure methods. For example, the state of Texas alone has over 1,500,000 oil

and gas wells, many of which were abandoned over a century ago and are without suitable records.[5]

Once leakage has occurred, $CO_{2(g)}$ will migrate up through the subsurface, reaching fresh water aquifers, where it may be retained by three trapping mechanisms: structural trapping, permeability trapping, and solubility trapping[1, 6]. $CO_{2(g)}$ may be contained under a low-permeability cap-rock (structural trapping) or under a less dense layer of soil air. Trapping mechanisms in freshwater aquifers are often beneficial, as they prevent re-emission of $CO_{2(g)}$ to the atmosphere. In freshwater aquifers, horizontal flow is favored over vertical flow due to anisotropy of the aquifer. Horizontal transport of the $CO_{2(g)}$ plume (a form of permeability trapping) will result in variable degrees of solubility trapping. Differential permeability trapping and solubility trapping will give rise to variable $CO_{2(aq)}$ concentrations within the freshwater aquifer. Similar to the storage unit, this geochemical heterogeneity will result in variable water chemistry. The $CO_{2(aq)}$ effects on water chemistry is further described in section 2.3.

2.2 Biogeochemical Parameters in the Subsurface

The extent and effectiveness of the various trapping mechanisms in a given storage unit depends on the geochemistry and microbiology of the site (further discussed in section 2.3 and 2.4 respectively). Storage units vary in geochemical characteristics (salinity, inorganic and organic content, and rock material), and geophysical conditions (reservoir pressure and temperature). These geochemical and geophysical variations lead to differences in the initial microbial community (activity, growth, ecology, diversity, and population sizes). As these initial microbial communities vary amongst geologic storage units, it is expected

that the biogeochemical processes that occur after SC-CO_2 injection may also vary. These site-dependent post-CO_2-injection biogeochemical processes will affect the trapping mechanisms and therefore permanence of CO_2 storage for a given storage unit.

Similar to containment in storage units, the containment of $CO_{2(g)}$ within freshwater aquifers will depend on the natural geochemistry and microbiology (further discussed in section 2.3 and 2.4). Freshwater aquifers vary in geochemical characteristics, microbial ecology, and geophysical conditions. The freshwater biogeochemical processes that occur after an influx of $CO_{2(g)}$ and $CO_{2(aq)}$ will vary site to site, as will the extent of trapping. These biogeochemical processes will also determine the ability of the freshwater aquifer to remain potable after $CO_{2(g)}$ leakage.

2.3 Geochemical Processes in the Subsurface

While most of the work in this dissertation focuses on understanding the microbial community changes during CO_2 exposure in subsurface units, geochemical processes are intertwined in the relationship between microbial communities and CO_2 trapping mechanisms. For this reason, it is important to understand geochemical processes that may occur in CO_2 exposed environments. A discussion of geochemical processes driven by CO_2 exposure is first considered here, followed by section 2.4 discussing relevant biological processes.

Dissolution of injected SC-CO_2 in storage units will lead to carbonate species in the interstitial fluid. While SC-CO_2 injection will lead to desiccation in portions of the storage unit,[7], much of the storage unit will experience a range of $CO_{2(aq)}$ species and concentrations due to the heterogeneous nature of CO_2 distribution. As the SC-CO_2 equilibrates with the

reservoir fluid and rock, CO_2 will be present as the dissolved species: $CO_{2(aq)}$, $H_2CO_3^*$, HCO_3^-, and CO_3^{2-} (eqn 1).

$$CO_{2(g)} + H_2O \leftrightarrow CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3^* \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad \text{(eqn1)}$$

The concentration of $CO_{2(aq)}$ and resulting pH will control the carbonate speciation of the system. Therefore, sections of the subsurface unit with a low amount of CO_2 dissolution will remain near pre-CO_2-injection pH of a neutral 7-8 and have HCO_3^- as the dominant carbonate species. Sections of the subsurface unit with high CO_2 dissolution may have a pH as low as 3.5 (Kharaka, 2009), and will have $CO_{2(aq)}$ as the dominate species. Traditionally, the dominate carbonate species of these high CO_2 concentration conditions is described as carbonic acid ($H_2CO_3^*$), which is a ratio of 860:1 of $CO_{2(aq)}$ to H_2CO_3.[8]

Carbonate speciation and pH are also affected by the mineralogy of the system. Buffering of the system in dependent on the geology, which may be silica based, such as sandstone aquifers, or carbonate based, such as dolomite aquifers. These subsurface systems will contain accessory minerals and clays with a variety of carbonates, silica, and metal oxides which will also affect buffering. Introduction to CO_2 may lead to the formation of carbonate minerals, such as calcite and aragonite ($CaCO_{3(s)}$), siderite ($FeCO_{3(s)}$), magnesite ($MgCO_{3(s)}$), and dolomite ($CaMg(CO_3)_{2(s)}$), depending on the pH and available cations in the system. On the other hand, the decreased pH and change of redox conditions may lead to the dissolution of carbonate and non-carbonate formation solids, releasing ions and carbonate in solution. This precipitation or dissolution of carbonates will lead to additional geochemical reactions, as sorption-desorption processes will adjust to the new conditions. The dissolution and precipitation reactions will affect the permeability of the subsurface unit.

The complexity of subsurface geochemical processes with introduced SC-CO_2 has been demonstrated in several previous carbon storage studies (Table 2.1). Both laboratory and *in situ* experiments have demonstrated that introduction of SC-CO_2 to subsurface geologic units result in an initial drop in pH accompanied by a spike in cation concentrations. These geochemical changes are followed by a decrease in cation concentrations to levels slightly elevated from background levels, while the pH often remained reduced. For example, injection of 1600 tonnes of SC-CO_2 in a deep sandstone saline aquifer initially leads to a drop of pH from 6.5 to 5.7 and an increase of Ca, Fe, Mn, DOC, and alkalinity, followed by a slow decrease in these components to levels slightly elevated from the pre-injection concentrations while the pH remained reduced.[9] Another study found that exposure of a sandstone core to SC-CO_2 leads to a decrease in pH from 7.9 to 4.8 and an increase in concentrations of Ba, Ca, Fe, Mg, Mn, and Sr by an order of magnitude.[10] The pH then slowly increases from 4.8 to 5.4, and some cations, such as Ba, Ca, Mg, and Mn remain at elevated concentrations. Other cations, such as Fe slowly decrease to background levels, likely due to reprecipitation of minerals that were dissolved with the CO_2-driven pH reduction. Previous studies on the altered geochemistry after SC-CO_2 introduction to an arkose material show sudden decreases of pH from 6.7 to 3.4, leading to spikes of ions such as B, Ca, Cl, K, Mg, Na, and SO_4, followed by a decrease and stabilization to 120%-150% of initial conditions.[7] Similarly, an *in situ* injection of 3.5 bar CO_2 in a freshwater aquifer lead to a pH drop from 8.6 to 5.5, and a spike in Ba, Ca, Cr, Fe, Mg, Mn, and Sr by about 1 order of magnitude, followed by the decrease to concentrations only slightly elevated from the background concentrations.[11] The increase in cation concentrations in all these cases is likely the result of the dissolution of formation rock due to the carbonic acid driven decrease in pH,

followed by various ion exchange and adsorption-desorption processes amongst accessory clays and minerals.[12] The dissolution processes lead to a slight increase in pH, and precipitation of new clay minerals and carbonates.[10]

Table 2.1. Geochemistry of CO_2 exposed subsurface studies

Rock	Study type	High pH	Low pH	Cation increase	Study
Deep sandstone aquifer	in situ	6.5	5.7	Ca, Fe, Mn	Kharaka, 2009[9]
Sandstone core	laboratory	7.9	4.8	Ba, Ca, Fe, Mg, Mn, Sr	Smyth, 2009[10]
Arkose core	laboratory	6.7	3.4	B, Ca, Cl, K, Mg, Na, SO_4	Kaszuba, 2002[7]
Freshwater sand aquifer	in situ	8.6	5.5	Ba, Ca, Cr, Fe, Mg, Mn, Sr	Trautz, 2013[11]

2.4 Biological Processes in the Subsurface

The microbial community and microbial processes will affect the system geochemistry and therefore the efficacy of trapping mechanisms. Microbial processes have affected geochemical processes and determined current conditions of the biosphere through nutrient cycling,[13, 14] metal depositions,[15] and mineral dissolution.[16] The natural geochemistry of the subsurface is impacted by the activity of diverse communities.[13, 17, 18] Anaerobic microorganisms such as iron reducers,[19-21] sulfate reducers,[19, 21-24] methanogens,[19, 21-26] and heterotrophs[22, 23] affects the natural geochemistry in subsurface systems. Bioprocesses that may occur in CO_2 future storage sites and leakage scenarios are biomineralization, biofilm formation, microbial acidolysis, metal mobility, and metal immobility.

Biomineralization is the biological catalysis of precipitation reactions that are otherwise thermodynamically unfavorable or kinetically slow geochemical reactions. Biomineralization occurs through several mechanisms. One mechanism is the biologically-induced increase of the pH in a system that would otherwise be too low to

thermodynamically allow carbonate precipitation. *Halomonas*, *Marinobacter*, *Salinivibrio*, and *Pseudomonas* bacteria have been found to increase the pH by secretion of extracellular products,[27, 28] causing precipitation of otherwise instable carbonates. Microorganisms may also induce biomineralization by utilizing cell surface area as a nucleation site; this is believed to be the mechanism behind precipitation of dolomite.[29] Some bacteria accumulate Ca^{2+} and Mg^{2+} on cell surfaces, creating a micro-niche of cation supersaturation and resulting in calcite deposition on the surface of cells.[27, 30] Many common saline aquifer microorganisms, such as *Pseudomonas*,[31] *Bacillus*,[31, 32] *Vibrio*,[31] *Halomonas*,[28, 33] and *Marinobacter*[33] can biomineralize carbonate in the presence of Mg^{2+} and Ca^{2+}. Boquet (1973) suggests that the microbial ability to biomineralize is common among most environmental bacteria.[30] Biomineralization has been receiving much attention in the field of carbon storage,[34, 35] as this process results in a denser, non-mobile carbon phase, which is the ideal trapping mechanism for CO_2 storage (mineral trapping).

Biofilm formation is another biological process that occurs in the environment, controlled by common subsurface bacteria, such as *Halomonas, Bacillus, Pseudomonas, Pseudoaltermonas,* and *Vibrio*.[36] This microbial process starts with the attachment of transported cells to a surface; after some accumulation of cells, the community begins production of extra cellular polymer substance (EPS), a hydrogel[37] substance made of polysaccharides, proteins, and nucleic acids.[38] The EPS coats the microbial community, allowing coexistence amongst different microbial species in a high biomass concentration. A biofilm conveys resilience against changing or toxic conditions that may emerge. For example, microbial communities within biofilms are resistant to UV exposure, heavy metals, pH changes, and salinity changes.[37] Within a multispecies biofilm, the high diversity of

microorganisms allows an accelerated rate of gene transfer as a mechanism of resilience.[39] In addition, the close coexistence amongst microorganisms allows the release and detection of extracellular signal molecules that may not occur in more dilute environments. This process, known as quorum sensing, is thought to induce increased acid tolerance response upon immediate detection of a decreased pH.[40] The EPS layer also serves as a diffusion barrier against toxic molecules. For example, biofilms of *Pseudomonas aeruginosa* were two fold more resilient to high Pb exposure and 600 fold more resilient to Cu exposure than planktonic cultures.[38] Upon examination of the biofilm *Pseudomonas aeruginosa*, 99% of the cells in the outer layer of EPS were dead, while only 20% of the interior biofilm cells were dead; this finding suggests the EPS sequestered much of the transport of the heavy metals from the communities in the interior of the biofilm. The EPS capability of binding to certain components would explain the pH and chemical concentration gradients measured throughout biofilms.[39] A third mechanism of biofilm resilience is the presence of slow growing or dormant cells, called "persistors", deep within the biofilm that are highly resilient to most types of environmental stress.[37] Regardless of the mechanism, biofilm forming microorganisms have already been found to withstand the stress induced by $CO_{2(aq)}$, outcompeting planktonic cultures. Biofilm strains of *Bacillus mojavensis* were found to have a 1 log reduction of biomass when exposed to 19 minutes of SC-CO_2 compared to the 3 log reduction of the planktonic counterpart.[41] Biofilm formation may reduce permeability, affecting the pore volume within the reservoir, and therefore affecting the volume of CO_2 that may be stored via structural and residual trapping mechanisms.

The likelihood of biofilm formation in the subsurface is not well understood. Although biofilms have been able to withstand low nutrient conditions,[40] biofilm production

often occurs under high nutrient conditions, with enough carbon and nutrient supply for biomass production as well as EPS production. Therefore, the extent of this bioprocess in the subsurface without an extraneous supply of nutrients it unknown. However, there has been extensive previous research on this bioprocess as it is known to decrease rock material permeability[34, 42] and may therefore help seal leakage pathways.

Microbial acidolysis is the process of rock dissolution via biological acid production, and may occur as a result of common metabolic pathways in a broad spectrum of the known bacterial life in the shallow and deep subsurface. The production of acid reduces the system pH, resulting in dissolution of formation rock materials and accessory clays. Chemolithoautotrophic bacteria produce nitrous acid, nitric acid, and sulfuric acid as a waste product during respiration of their electron donor.[16] Likewise, fermentative bacteria produce organic acids, such as formic acid, acetic acid, lactic acid, succinic acid, and pyruvic acid as end-product metabolites.[43]

Rock dissolution via bacterial processes is well documented in natural and engineered systems. For example, modeling suggests over half of the natural weathering of the Middendorf aquifer, SC is due to microbially-produced acids.[44] Microbial acidolysis may also cause corrosion of metals. The production of sulfuric acid by sulfate reducers from oil reservoirs has long been known to be the cause of accelerated pipeline corrosion.[24] Similarly, fermentative *Clostridial* species cause biocorrosion of metals through the production of acetic acid.[45]

The production of acid as metabolite of bacterial activity has the potential to cause dissolution of rock material, increasing the permeability of subsurface units, and ultimately increasing the capacity of a formation unit for CO_2 storage through additional structural and

residual trapping mechanisms. On the other hand, if rock dissolution occurs in overlying caprocks, existing rock fractures may be exacerbated, increasing the risk of leakage. The production of acid and accompanying reduction of pH will also alter the overall geochemistry of the system. This will affect the carbonate speciation, and therefore reduce the capacity of CO_2 dissolution (solubility trapping). Reduction of pH will reduce the favorability of carbonate precipitation, and therefore decrease to capacity of CO_2 storage through mineral trapping.

Microbially-induced metal mobilization is the release of precipitated or adsorbed metals in solution through microbial metabolic processes.[46] Anaerobic microorganisms commonly utilize metals such as iron and manganese as electron acceptors, producing a more soluble redox state of these components. This mechanism of metal mobility is sometimes termed oxido-reductive biocorrosion, since these processes lead to dissolution of rock material.[16] Metal mobilization may also occur from microbial products such as siderophores, a biologically secreted ligand that solubilizes many metals making them more bioavailable. Biologic dissolution of minerals could further aggravate other metal mobility due to the reduction of adsorption surfaces.[47] Studies have found that microorganisms are capable of mobilizing metal cations and oxoanions, causing potable aquifers to become contaminated with toxic metals such as arsenic[48, 49] or copper.[50] Bacteria are known to contribute to the mobilization of arsenic, whether by reducing iron adsorptive surfaces by metabolically reducing Fe^{3+} to the more soluble form Fe^{2+}, or by directly reducing As^{5+} to the more soluble form, As^{3+}.[49] The biological release of heavy metals in solution may result in contamination of potable water if it occurs in freshwater aquifers. The deterioration of material through biological utilization of the metals results in an increased permeability, increasing the pore

volume of the storage unit and increasing CO_2 storage capacity through structural and residual trapping. Similar to acidolysis, the biologically driven release of metals in solution changes the fluid chemical equilibrium, changing the pH and the carbonate speciation, and affecting solubility and mineral trapping.

Microbial induced metal immobilization is the biologically-induced precipitation or adsorption of metals from solution.[46] One mechanism of metal immobilization is the metabolic utilization of metals resulting in a less soluble redox state and corresponding precipitation. For example, utilization of iron and manganese by bacteria such as *Geobacter* and *Shewanella* have been known to result in the precipitation of magnesite, siderite, vivianite, and other iron and manganese oxides.[15, 51] The biological reduction of sulfur also often results in iron and sulfur immobilization, as these components readily react and precipitate iron sulfide solids.[15] A variety of heterotrophic organisms are capable of chromium immobilization via the reduction Cr^{6+} to the less soluble form of Cr^{3+}.[52] Many common bacteria such as *Pseudomonas* and *Geobacter* may immobilize uranium by reducing U^{6+} to the less soluble form of U^{4+}.[53] Another method of metal immobilization is the processes of biosorption.[54] Column studies have shown that EPS has been capable of adsorbing copper,[50] and microbial cellular surfaces naturally have a binding affinity for chromium, cobalt, copper, and nickel.[54] Microorganisms are also capable of metal immobilization via intracellular uptake of such metals as chromium, copper, and lead.[54] Biological metal immobilization through mineral precipitation results in a changed permeability, decreasing the pore volume. This would decrease the capacity of CO_2 storage through structural and residual trapping. Reducing free metal ions in solution changes the

geochemistry of the system, and may change the pH and carbonate speciation. These changes affect solubility and mineral trapping.

Many of the biogeochemical reactions described above depend on the geochemical conditions of the system. For example, most carbonate precipitation resulting in biomineralization and metal immobility will not occur in pH conditions below 5 or without a high concentration of cations such as calcium, iron, and magnesium. The interaction between thermodynamically favorable bioprocesses and geochemical processes that will occur after SC-CO_2 injection are still not understood, and the likelihood of the described biological processes occurring in the subsurface after $CO_{2(aq)}$ exposure is therefore also not well understood.

The biogeochemical process are also dependent the microbial ecology of the system. For example, microorganisms in subsurface systems often affect other species within the community through such interactions as amensalism, symbiosis, and competition.[17, 18, 55] Because each non-transient species occupies a unique niche in a habitat that is coupled with the other species, a chemical effect on one species may drive metabolic and ecological changes in other species of the community. Therefore, it is important to understand the microbial ecology in storage formations and how it changes with exposure to CO_2. The biological processes that result from this altered microbial ecology will affect the geochemistry and subsequent efficacy of CO_2 trapping mechanisms.

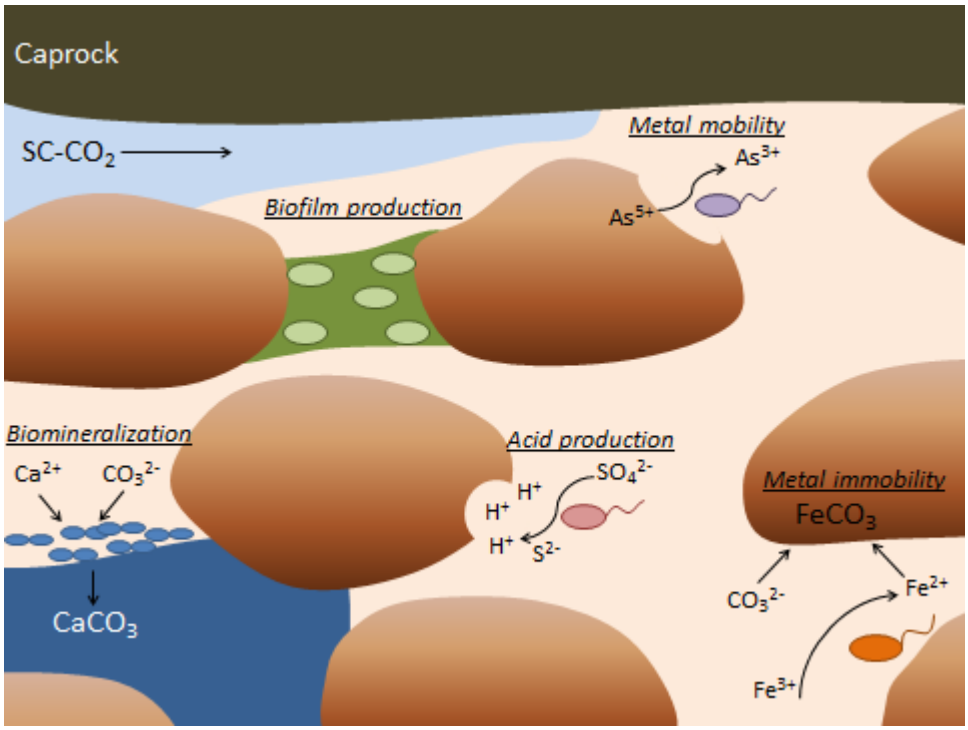

Figure 2.3. Biological processes that are known to occur in the subsurface include biomineralization, biofilm formation, acid production, metal mobility, and metal immobility.

2.5 Current Knowledge of Microbial Ecology in Carbon Storage Environments

Although many of the microbial processes described above have been examined for applications other than CO_2 storage, few studies have directly addressed the behavior of microbial populations in CO_2 storage scenarios. However, analogous environments exist, and there is an evolving understanding of the importance of microbial communities on geochemical processes that may be extended to address their importance for geological carbon storage.

The Zero Emission Research and Technology (ZERT) laboratory based at Montana State University has examined some microbial processes that may lead to pore plugging for the purpose of sealing CO_2 leakage pathways. Biomineralization of CO_2 through the ureolysis has been of most interest. Utilizing urea, calcium, and $CO_{2(aq)}$ as reactants,

microorganisms may hydrolyze urea ("ureolysis"), precipitating $CaCO_{3(s)}$. While ureolysis has no net carbon capture due to the addition of urea-based carbon, it does increase the pH, allowing more dissolution of $CO_{2(g)}$. More importantly, this bacterial process decreases matrix permeability, which may help to mitigate CO_2 leakage. The bacterium, *Sporosarcina pasteurii* was found to precipitate all of the available Ca^{2+} in the form of $CaCO_{3(s)}$, and 65% of the carbon was from the added $CO_{2(g)}$.[35] *S. pasteurii* produced a "no flow" system in a porous core after 20 hours of flow of a calcium, urea and $CO_{2(aq)}$ solution.[34, 35] In a more recent study, biofilm induced ureolysis of *S. pastereurii* reduced the permeability of core fractures by 61%-99%.[56] Researchers at ZERT have also studied biofilm formation as a means to decrease matrix permeability of fractured caprock, increasing carbon storage security. Similar to biomineralization, the EPS of biofilm formation is a potential method for sealing leakage pathways to prevent the transport of SC-CO_2 from the storage formation. Biofilms from *Pseudomonas fluorescence* and *Shewanella fridgidmarina* decreased permeability by 99% and 91% respectively.[34] The latter was found to decrease permeability after 71 hours of exposure to SC-CO_2 and 363 hours of starvation.[57] While the *Shewanella fridgidmarina* did not survive the starvation period, the remaining EPS maintained the reduced permeability.

While previous research conducted by ZERT presents a promising method of utilizing microbial processes to reduce the risk of CO_2 leakage, the studies thus far do not address issues that may arise during CO_2 storage. Thus far, ZERT studies have been on pure laboratory cultures. Although pure cultures may thrive in laboratory conditions, these species may not survive low nutrient, high temperature conditions of the subsurface. In addition, there will be nutrient competition by the better adapted populations that decreases the

likelihood of survival of introduced species.[18, 55] Microorganisms from the terrestrial surface may not be able to adapt to subsurface geophysical and geochemical conditions as well as adapt to CO_2 exposure.

For the purpose of researching geophysical, geochemical, and biological alterations that would occur during carbon storage, the Geoforum Zentrum (GFZ) in Potsdam, Germany injected 60 tonnes SC-CO_2 at a depth of 600 m in the Triassic Stuttgart saline aquifer, Ketzin, Germany. GFZ studies suggest that some subsurface species may adapt to $CO_{2(aq)}$ environments. Using fluorescence in situ hybridization (FISH), GeoForum researchers found that after SC-CO_2 injection, cell numbers originally decreased from 10^6 cell/ml to 10^5 cell/ml after CO_2 arrival. However, after 5 months following the start of CO_2 injection, the microbial population rebounded to 10^6 cell/ml.[58, 59] The growth pattern was found to be attributed to a shifting community, as methanogens began to outcompete a largely dominate sulfate reducing community. Incubations of these saline aquifer samples detected *Burkholderia fungorum* and *Propionibacterium* after 24.5 months of 5.5 MPa CO_2 exposure.[60] These studies suggest that subsurface microbial communities will change, as a few microorganisms adapt to the new conditions presented by SC-CO_2 injection.

Although the previous studies from the GeoForum Zentrum were rare experiments of subsurface microbial community changes with CO_2 exposure, these experiments lacked a thorough analysis of the microbial community. For example, the *in situ* study lacked analysis of the dominant microbial species present, and the laboratory incubations lacked a quantitative analysis of the population size. In addition, these experiments were all performed on a single site. It is still unknown whether the microbial trends of Ketzin saline aquifer will be observed in other CO_2 storage sites.

The limitations of prior studies in this field demonstrate that there is still little knowledge on the relevant microbial communities in CO_2 exposed environments. This project fills in the knowledge gaps of the microbial community response following CO_2 injection. In order to understand the dominant biogeochemical reactions that will affect CO_2 storage, the microbial communities that will thrive in these systems must first be identified. By defining the microbial population intolerant of CO_2 exposure and the microbial population adaptable to CO_2 exposure, future studies will research the relevant biological processes that arise during carbon storage.

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Chapter 3

Effects of $CO_{2(aq)}$ Concentration on a Microbial Community from Saline Aquifer Samples under Geologic Carbon Storage Conditions

3.1 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.

3.2 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-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-CO_2 into the unimpacted formation (Figure 3.1). While exposure to the SC-CO_2 phase may sterilize the subsurface[10, 11], heterogeneous distribution and dissolution of SC-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-CO_2.

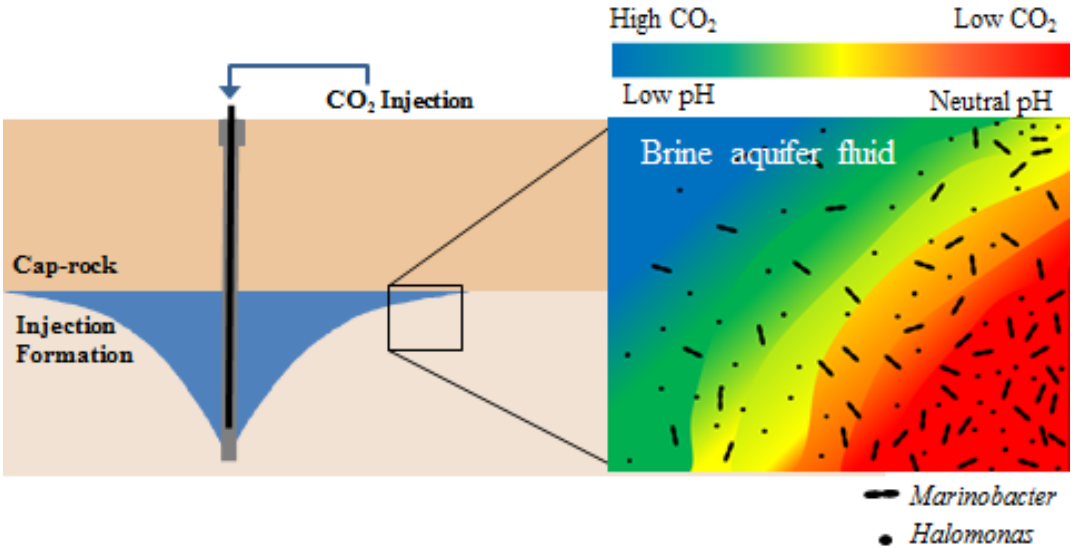

Figure 3.1. Conceptual model of heterogeneous flow of SC-CO_2 and resulting CO_2 concentration and pH gradients.

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.

3.3 Materials and Methods

3.3.1 Site Background

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[28] 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[28]. For a detailed description on the geochemistry and mineralogy of the Arbuckle Aquifer, please refer to Barker et al., 2012[29]. The total dissolved solids of this reservoir ranges from 10,000 to 250,000 ppm[29].

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[30, 31]. 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[32] . 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[32]. 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.

3.3.2 Pressurized Vessel 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[33]. 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.

3.3.3 Microbial Community Analysis

DNA was extracted using a modified method described previously in Holmes et al, 2004[34]. 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'[35] with the 519 reverse primer 5'-GTATTACCGCGGCTGCTGG-3'[35] and the 338 forward primer 5'-ACTCCTACGGGAGGCAGC-3'[36] with the 907 reverse primer 5'-CCGTCAATTCMTTTRAGTTT-3'[37]. The archaeal 16S rRNA fragments were amplified using the 344 forward primer 5'-ACGGGGCGCAGCAGGCGCGA-3'[38] with the 915 reverse primer 5'-GTGCTCCCCCGCCAATTCCT-3'[39]. 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[40].

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'[41], 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[42] to ensure sufficient coverage for the given diversity. Good's coverage values are reported in Table A.1, Supplementary Information. Sequences were trimmed using FinchTV and chimeras were detected using Bellophon[43]. Sequences with 97% similarity were grouped into OTU's on the genus level using Mothur and NCBI Blast[42, 44]. 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[45]. 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[34]. A community tree was produced on Mothur utilizing the

Yue Clayton measure, and visualized using TreeView X.

3.3.4 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 analysed for selected elements by ICP-OES (PerkinElmer Optima 7300 DV) using EPA method 6010C. Supernatant anions were also analysed via a Dionex Ion Chromatograph using EPA method 300.1. Supernatant samples were stored at -4 °C until analysis but were otherwise unpreserved.

3.3.5 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.

3.4 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 3.2). 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 3.3). 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.

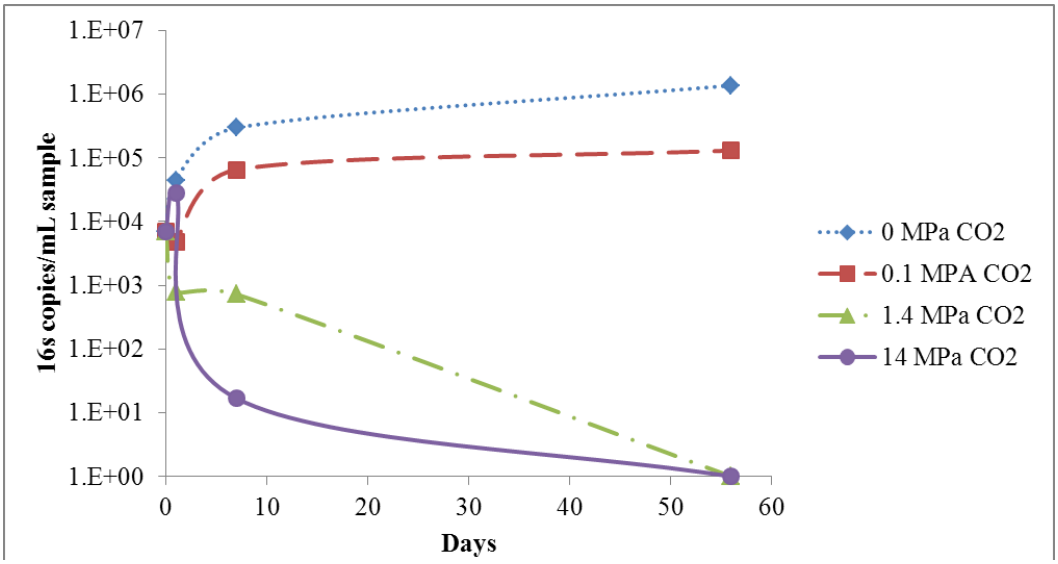

Figure 3.2. Concentration of 16S rRNA gene copies recovered from reactors exposing unfiltered saline aquifer samples to increasing pCO_2 as revealed by qPCR for 0 days, 1 day, 7 days, and 56 days of exposure. Concentrations of genes are reported as gene copies/mL of drill stem test sample.

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[46].

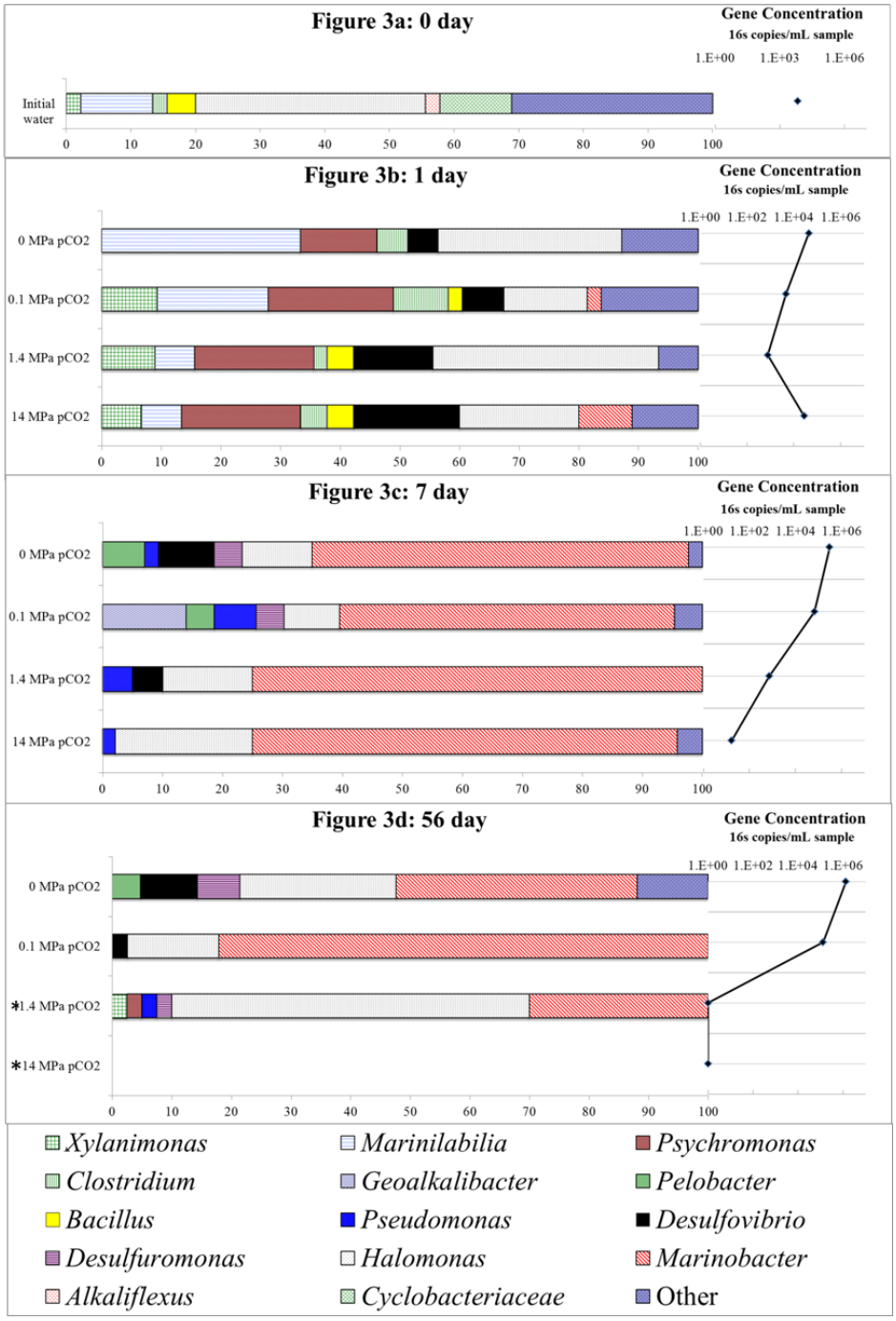

Figure 3.3. Relative proportions of phylotypes recovered from reactors exposing unfiltered saline aquifer samples to increasing pCO_2 as revealed by 16S rRNA gene clone libraries and qPCR for a) initial drill stem test sample, and following b) 1 day of exposure, c) 7 days of exposure, and d) 56 days 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

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[20].

Results reveal that increasing pCO_2 decreases microbial diversity (Figure 3.4). 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 3.4). 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.

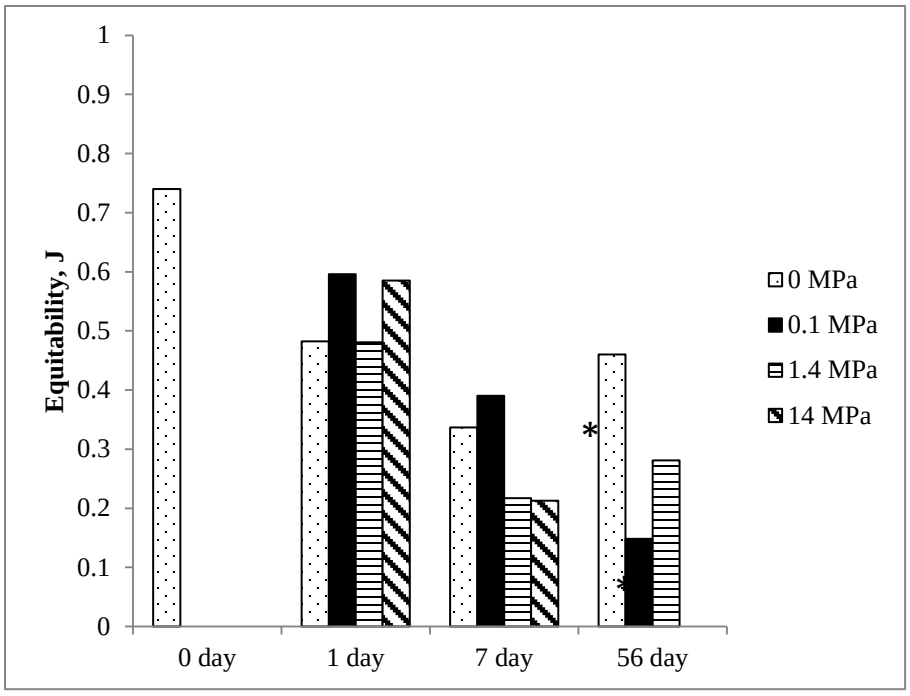

Figure 3.4. Impact of exposure time and pCO_2 on microbial diversity of unfiltered saline aquifer samples. Results show the comparison of Equitability values (J) for each pCO_2 concentration examined with time. *sample required nested PCR. A J = 0 is a pure culture, whereas J = 1 if each clone is a unique 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 3.3). 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 3.3). 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 [47, 48]

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 3.3). 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*[49], *Bacillus*[50, 51], *Marinilabilia*[52, 53] and *Psychromonas*[54] 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 3.3).

The microbial communities of each pressure vessel were compared on a community tree (Figure 3.5). 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.

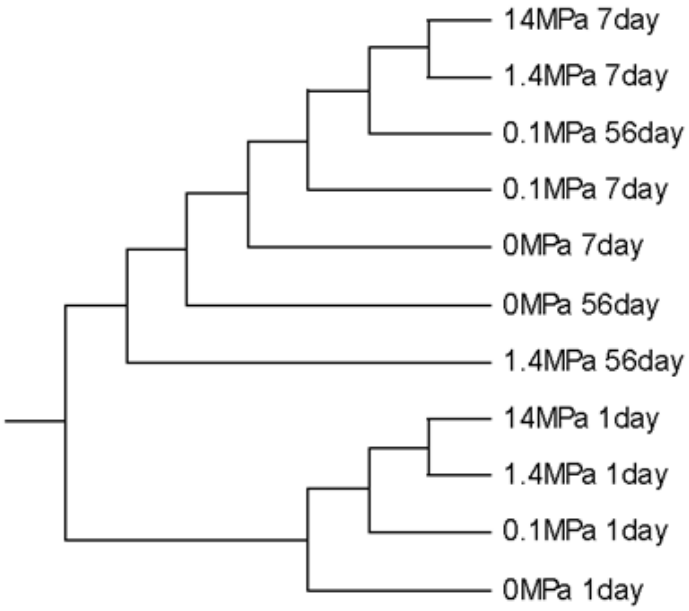

Figure 3. 5. In order to compare microbial communities amongst reactors, a community tree was constructed utilizing Mothur and TreeViewerX. The distance matrix was calculated with the Yue and Clayton measure.

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[55]. 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[47, 48, 56]. *Halomonas* were found in oil field samples[24] and marine and hypersaline environments[57-60] and are generally known for an ability to grow across a broad

range of temperature and pH conditions[61, 62]. Similarly, *Marinobacter* have been found in a variety of marine environments[63, 64] as well as oil fields[24, 65]. Strains of *Desulfovibrio* have been isolated from ground water of deep granitic formations[66], as well as marine sediments[67] and saline waters of a natural gas reserve[68]. *Desulfomonas* has been found in saline subsurface environments such as hydrothermal vents[69] 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 3.1).

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[70]. 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[71, 72] 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 3.1) and measured initial sample alkalinity (of 388 mg/L HCO_3^-) [32]. 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 3.6). 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 3.7). 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.

Table 3.1: 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 pCO_2	0.1 MPa pCO_2	1.4 MPa pCO_2	14 MPa pCO_2
Ca	mg/L	2502	2071.0	2186.0	2186.0	2104.0
Fe	mg/L	nd	nd	nd	0.5	2.5
K	mg/L	259	251.7	255.9	253.2	256.6
Mg	mg/L	702	567.8	578.4	577.1	590.7
Mn	mg/L	0.9	0.7	0.9	0.9	0.9
Na	mg/L	22690	18280.0	18440.0	17530.0	17640.0
P	mg/L	1.5	3.4	1.4	2.4	2.0
S	mg/L	631	593.9	618.4	601.0	613.1
Si	mg/L	10	9.4	10.1	10.8	11.0
Zn	mg/L	0.2	0.1	0.8	1.8	2.4
Cl	mg/L	31528	31992.3	32397.2	31639.1	37757.3
SO4	mg/L	1190	369.9	1007.4	953.7	1096.3
TOC	mg/L	2333.3	--	--	--	--
Alkalinity	mg $CaCO_3$/L	607	--	--	--	--
Modeled pH		7.8	7.50	5.50	4.38	2.88

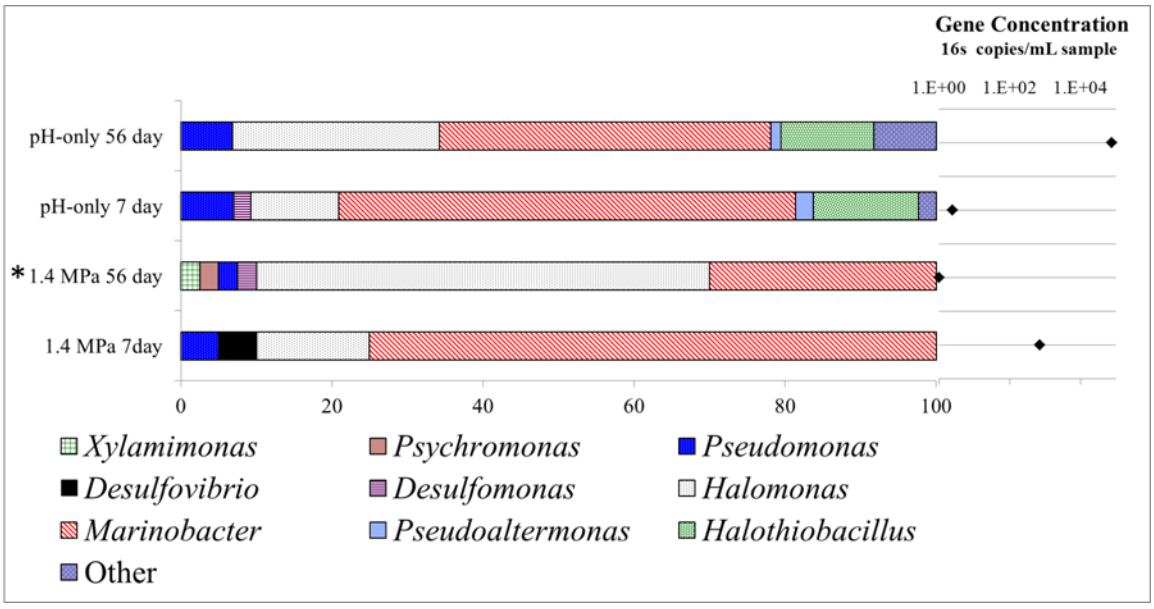

Figure 3.6. Microbial ecology characterized by 16S rRNA gene clone libraries and microbial population quantified by qPCR methods for 7 day and 56 day exposures to 1.4 MPa pCO_2 or the equivalent pH-only adjustment to 4.4. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other". * = required nested PCR.

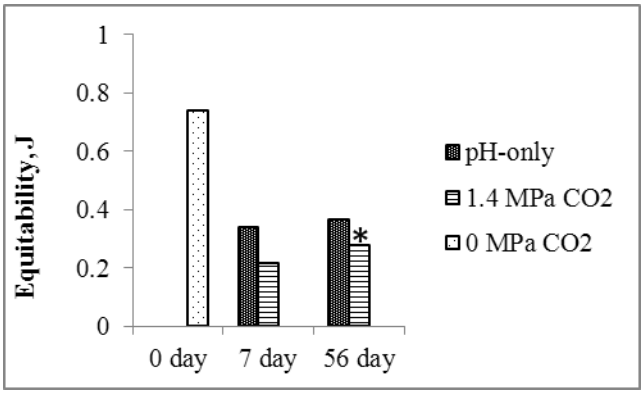

Figure 3.7. Impact of pCO_2 versus pH-only on microbial diversity of unfiltered saline aquifer samples. Results show the comparison of Equitability values (J) for each pCO_2 concentration examined with time. *sample required nested PCR. A J = 0 is a pure culture, whereas J = 1 if each clone is a unique phylotype.

It is noteworthy that high CO_2 concentrations increased the iron and zinc content in the reactor water; similar to previous studies[73] (Table 3.1). 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 A.1). These findings suggest that injected CO_2 will affect microbial communities, even when the rock matrix buffers the decrease in pH.

3.5 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[21]. 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 [74].

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[75]. 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.

3.6 Supporting Information in Appendix A

Supporting information contains Figure A1 and Table A1. References have been cited in the main text for figures and tables with an overview of material presented in the supporting information.

3.7 References

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Chapter 4

Effects of $CO_{2(aq)}$ Concentration on a Microbial Community from Depleted Oil Reservoir Samples under Geologic Carbon Storage Conditions

4.1 Abstract

Geologic carbon storage is becoming an important part of a mitigation strategy to reduce CO_2 emissions in the atmosphere. Depleted oil reservoirs are being targeted for subsurface carbon storage sites. Microbial communities within the reservoir that best adapt to post-CO_2-injection conditions may affect the injectivity of CO_2, the quantity of petroleum production, and the quality of petroleum. Assessing the potential impacts of microbial activity on the reservoir requires an understanding of how the microbial communities of depleted oil reservoirs change with CO_2 exposure. We examined the change in the microbial community in produced water samples taken from the Mirando depleted oil reservoir after 56 days of exposure to 0 MPa, 0.03 MPa, 0.34 MPa, and 3.4 MPa pCO_2 under reservoir total pressure (3.4 MPa) and temperature (40 ˚C). Additionally, the change in the microbial community in the same water after 56 days of 0.34 MPa pCO_2 exposure and $CaCO_3$ buffering was examined. The microbial community was characterized with qPCR, 16S clone libraries, and 454 pyrosequencing. The 16S gene copies concentration was found to decrease with increasing CO_2 exposure. The microbial community population appeared to be sensitive to CO_2-driven pH reduction, demonstrating an order of

magnitude higher DNA concentration in pressure vessels with a pH of 5.8 compared to vessels with a pH of 5.3. The genus *Pseudomonas* appeared to emerge as dominant in the initial sample and in reactors with 0.34 MPa pCO_2 or less and the genus *Escherichia* appeared to emerge as dominant in reactors with 3.4 MPa pCO_2. The experimental results suggest that the microbial community of a depleted oil reservoir will quickly respond to CO_2 exposure. Close to the CO_2 injection plume, where CO_2 concentrations are highest, the microbial population may greatly decrease, and microbial processes may be significantly hindered or absent. Downgradient from the CO_2 injection plume, where CO_2 concentration will be attenuated, an altered microbial community may emerge as dominant.

4.2 Introduction

Geologic carbon storage will be an important mitigation strategy to reduce anthropogenic CO_2 emissions that contribute to global climate change. Depleted oil reservoirs have high potential as early adopters for geologic carbon storage, as these reservoirs are well characterized and injection of CO_2 often results in enhanced oil recovery (EOR) which can offset the costs of carbon sequestration.[1] However, effect of CO_2 on the microbial communities associated with these reservoirs has not been characterized. It is therefore unclear if and how CO_2 injection will

alter the microbial communities, and what impact these alterations may have on the biological processes that affect petroleum quality and production.

Previous research has demonstrated that pCO_2 exposure as low as 0.1 MPa can inhibit microbial growth and a pCO_2 exposure of 1 MPa can terminate growth altogether.[2] Many microorganisms are not adaptable to the decreased pH accompanying CO_2 dissolution; for example, the common soil species *Pseudomonas stutzeri* has not been found to grow in conditions of pH less than 4.5.[3] In addition to lowering the pH, $CO_{2(aq)}$ (the predominant species of inorganic carbon at pH<6.3) may diffuse through microbial cell membranes, changing cell fluidity, inhibiting enzyme performance, and affecting intracellular pH.[4, 5] CO_2 exposure has been found to decrease cell viability of commonly cultured environmental strains, such as *Shewanella* ,[2] *Bacillus*,[6] *Pseudomonas*,[6] and *Escherichia*.[6] Gulliver et al. demonstrated that pressure vessel experiments with a mixed microbial community from a deep saline aquifer had decreased microbial population with increasing CO_2 exposure.[7] However, other studies suggest that some subsurface species may be more adaptable to CO_2 exposed environments.[8-11] For example, Morozova et. al. found that after 5 months of CO_2 injection in a saline storage aquifer, the microbial community repopulated to the initial concentration of 10^6 cell/ml.[8, 9] Incubations of these saline aquifer samples detected *Burkholderia fungorum* and *Propionibacterium* after 24.5 months of 5.5 MPa CO_2 exposure.[11] Currently, there are no studies on how a microbial

community in a depleted oil reservoir will respond to the stress induced by CO_2 exposure during geologic carbon storage.

Diverse microbial communities are known to inhabit oil reservoirs,[12, 13] which have various effects on the quantity and quality of oil recovered from the reservoir. Some microorganisms are detrimental to the quality of the oil recovered.[12, 14-17] For example, common reservoir microorganisms, such as *Desulfovibrio*, *Desulfomonas*, and *Thermococcus*, produce hydrogen sulfide.[15] Other common subsurface microorganisms, such as *Clostridium*,[18] *Geobacter*,[18] and *Thiobacillus*,[14] mobilize heavy metals via acid production and metabolic reduction.[14] Hydrogen sulfide production and metal mobilization may potentially reduce the quality of produced petroleum. Conversely, some microorganisms may be beneficial. Native microbial communities have been utilized to increase the petroleum production of depleted reservoirs via microbial enhanced oil recovery (MEOR) processes including biofilm plugging of breakthrough pathways and biosurfactant production.[19]

The stress induced by CO_2 exposure on oil reservoir microbial communities may therefore prove to be beneficial or detrimental to petroleum quality and production. The inhibition of growth of the deleterious microorganisms could prevent the occurrence of detrimental bioprocesses such as hydrogen sulfide production[20] and heavy metal mobilization, improving the petroleum quality. However, injection of CO_2 may result in a reduced biofilm or

biosurfactant producing microbial population that may prevent future success of MEOR application.

Magot et al. reviewed the various types of microorganisms found in oil reservoirs,[12] but no research has been conducted on how the microbial communities in a depleted oil reservoir respond to the stress induced by CO_2 injection. An understanding of how the microbial community responds to CO_2 exposure will improve our understanding of the types of microbial processes that may be expected to occur in a CO_2 flooded oil reservoir. The objectives of the experiment described are to 1) determine the effect of CO_2 exposure concentration on a microbial population in produced water samples collected from a depleted oil reservoir, and 2) identify the organisms that are selected for in a CO_2 exposed microbial community. The produced water samples from the Mirando depleted oil reservoir in Zapata, TX were exposed to increasing CO_2 concentrations (0%, 1%, 10%, and 100% pCO_2) under reservoir temperature and pressure in batch pressure vessels. The impact of buffering by reservoir rock material was also examined by comparing microbial community changes upon CO_2 exposure with and without calcium carbonate ($CaCO_3$) addition. After 56 days of incubation, the microbial communities in each vessel were characterized with qPCR, 16S rRNA clone libraries, and 454 pyrosequencing. The results suggest the depleted oil reservoir to be inhabited by sensitive microbial community that will quickly respond to CO_2 exposure. CO_2 exposure may lead to a decreased microbial

community population close to the injection plume, and correlating microbial-driven processes will be greatly reduced. Downgradient to the injection plume, where CO_2 concentrations are attenuated, an adapted microbial community will survive; this downgradient microbial community may affect the injectivity, petroleum quality, and petroleum quantity

4.3 Materials and Methods

4.3.1 Site Background

The Mirando oil field, Zapata County, Texas, is in the Eocene system, 490 m to 610 m depth, consisting of fine grained marshal sand. A detailed description of the geology of the oil reservoir is given in Brace, 1931.[21] Average reservoir pressure is 3.4 MPa, and average reservoir temperature is 40 ˚C. In June, 2014, this reservoir had not been injected with CO_2, and was producing about 10 barrels/day. Fluid slurry sample was withdrawn from Chargos Creek Well A (latitude 27.1506936, longitude 98.9972297) from the pump jack at the end of a routine oil draw to maximize the formation water sampled and minimize any contamination from drilling. The samples had an 8-10% oil water cut, a pH of 8.2, an alkalinity of 1154 mg $CaCO_3$/ml, and less than 0.5 g/L suspended solids. Additional chemical data of the initial sample is given in Table 4.1.

4.3.2 Pressure Vessel Exposure Experiments

Pressure vessels were manufactured by Thar Technologies Inc., and are described in Gulliver et. al.[7] Briefly, reactors were 1-L, Teflon-lined stainless steel static vessels capable of maintaining pressures up to 27.5 MPa and constant temperatures up to 90 ˚C. A volume of 200 mL of Mirando oil field fluid slurry sample was placed in a pressure vessel, and pressurized with pure CO_2 gas first to achieve the desired pCO_2, followed by pure N_2 gas to maintain a fixed total pressure closely resembling the site conditions. The reactors were pressurized with 0, 0.03, 0.34 and 3.4 MPa pCO_2 which represent 0%, 1%, 10% and 100% of the total pressure respectively. Reactors were maintained at a constant temperature of 40 ˚C which closely resembles oil reservoir temperature. Sampling was performed by opening vessels after 56 days of CO_2 exposure. A fifth vessel was pressurized with 200 mL fluid slurry sample, 0.34 MPa pCO_2, and 10 g/L $CaCO_3$ for 56 days in order to understand the impact of pH buffering by reservoir solids on the effects of CO_2 exposure on microbial communities.

After 56 days, prior to fluid sample collection, headspace samples of gas were collected in 500 ml Tedlar bags for immediate analysis by gas chromatography as described below. The fluids was then sampled and centrifuged at 4,000 g for 25 minutes to concentrate the solids for biological analysis. The top 195 ml of the supernatant was filtered for chemical analysis, and the bottom 5 ml containing the solids was used for microbial community analysis.

4.3.3 Microbial Community Analysis

The solids were resuspended in the remaining 5 ml of the centrifuged sample, and DNA was extracted from 500 µl of the remaining 5 ml utilizing the MoBio power soil kit. PCR amplification followed shortly using universal bacteria and archaea primers. The bacteria 16S rRNA gene fragments was amplified using the 8 forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' [22] with the 519 reverse primer 5'-GTATTACCGCGGCTGCTGG-3' [22] and the 338 forward primer 5'-ACTCCTACGGGAGGCAGC-3' [23] with the 907 reverse primer 5'-CCGTCAATTCMTTTRAGTTT-3'. [24] The archaeal 16S rRNA fragments was amplified using the 344 forward primer 5'-ACGGGGCGCAGCAGGCGCGA-3' [25] with the 915 reverse primer 5'-GTGCTCCCCCGCCAATTCCT-3'.[26] 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 35 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 *Methanococcus maripaudis* as a positive archaea control. No

Archaea was detected in the initial sample or the vessel samples. No further analysis of Archaea was performed.

The PCR products of the two bacterial primer sets were mixed, and cloned using the Invitrogen TOPO TA cloning kit according to the manufacturer's instructions. 96 clones per sample were sequenced at Functional Biosciences (Madison, WI).

To increase the microbial coverage captured by sequencing, 454 pyrosequencing was performed on the initial sample and samples collected from the vessel experiments. The universal primer 515 forward primer 5'-GTGCCAGAMGCCGCGGTAA-3' was used with the roche adapter A-CCATCTCATCCCTGCGTCTCTCGACTCAG and coupled with the 806 reverse primer 5'- GGACTACVSGGGTATCTAAT-3' and the roche adapter B-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG.[27] Each primer set was run in a PCR mixture with a total volume of 50 μl containing Qiagen HotStart Taq along with the DNA template. The thermocycler was run with a taq initiation step at 94 ˚C for 10 min, followed by 30 cycles of a denaturing step of 94 ˚C for 1 min, an annealing step at 55 ˚C for 1 min, and an elongation step at 72 ˚C for 1 min. After the 30 cycles, a final extension occurred at 72 ˚C for 10 min. Efficacy of the pyrosequencing reaction was tested via electrophoresis gel, using *E. coli* DNA as a positive control. The reactions were sent to the Ohio State University genomics facility for pyrosequencing.

16s rRNA clone library sequences were trimmed using FinchTV, and pyrosequencing was trimmed using Mothur.[28] Chimeras were detected using Bellophon[29] for the 16S rRNA clone library sequences and Uchime for the pyrosequencing sequences.[30] Sequences with 97% similarity were grouped into OTU's on the genus level using Mothur and NCBI Blast.[28, 31] In cases where OTUs are less than 97% similar to a phylotype of a cultured organism, the microorganisms are described as "other".

Bacteria 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.[32] The PCR mixture consisted of a total volume of 20 μl containing Applied Biosystems TaqMan Master mix along with the primers, probe, and DNA template. Diluted samples of known concentrations of *E. coli* were used as bacteria standards. The *E. coli* was quantified with pico green procedures described in Invitrogen Quanti-iT kit. The DNA 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.

In order to determine the microbial diversity represented in each clone library, sequences were grouped into phylotypes based on 16S rRNA gene similarity of > 97%. Diversity was

represented by equitability (J), which was calculated from Shannon-Weaver indices. Equitability was calculated at the species-level 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.[33]

4.3.4 Chemical Analysis (Gas and Liquid)

To determine if gas production or consumption was occurring, samples from the pressure vessels were analyzed for H_2, CO_2, CH_4, O_2, 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.

Basic water quality parameters including major cations and anions, and total organic carbon (TOC) were measured for the initial water and for water collected from the vessels after 56 days of exposure to CO_2. All sampled water was filtered through a 0.2 μm filter before any chemical analysis. Sample water was analyzed for selected elements (Ca, Fe, K, Mg, Mn, Na, P,

S, Si, Zn) by ICP-OES (PerkinElmer Optima 7300 DV) using EPA method 6010C. Supernatant anions (Cl, NO_3, NO_2, PO_4, SO_4, Br) were also analyzed using a Dionex Ion Chromatograph using EPA method 300.1. Chemical components that were non-detect for all samples are not reported. Total organic carbon was diluted by 100X, and was measured in 10 ml triplicate volumes via 1010 Total Organic Carbon Analyzer. Alkalinity was measured by titration of 30 ml volumes with sulfuric acid to a pH of 4.5.

4.3.5 Water Chemistry Model

Since the pH of the sample fluid in the vessels could not be directly measured in the pressurized reactors, it was estimated using Geochemist Workbench. The initial system was defined using the measured alkalinity and initial pH. In addition, cation concentrations above 10 mM (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 real 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 reactors with 0, 0.03, 0.34 MPa CO_2 exposure, and was calculated to be 0.83 for the 3.4 MPa CO_2 exposure.

4.4 Results and Discussion

To explore the effect of CO_2 on microbial communities in depleted oil reservoirs, the change of the microbial community with CO_2 exposure was investigated with produced water samples from the Mirando Oil Reservoir, TX. Samples were placed in batch reactors with increasing pCO_2 (0, 0.03, 0.34, and 3.4 MPa) at reservoir total pressure (3.4 MPa) and temperature (40 ˚C). Reactors were depressurized after 56 days and the microbial community was analyzed with qPCR, 16S rRNA clone libraries, and 454 pyrosequencing. The chemical analysis (ICP-OES, IC-anion, alkalinity, and TOC) of the initial water and after 56 days of exposure to different concentrations of CO_2, as well as the modeled pH for CO_2-exposed samples are given in Table 4.1. The pyrosequencing data is given in the supplementary information in Figure B1.

Table 4.1. Elements, anions, total organic carbon (TOC), alkalinity, and modeled pH in the reactors with samples from the Mirando oil reservoir after 56 days of exposure to 0 MPa, 0.03 MPa, 0.34 MPa, and 3.4 MPa pCO_2. "nd" = non-detect.

	Units	Initial	0 MPa pCO_2	0.03 MPa pCO_2	0.34 MPa pCO_2	3.4 MPa pCO_2	0.34 MPa pCO_2 w/ $CaCO_3$
Ca	mg/L	23.8	19.5	27.1	59.5	14.9	63.2
Fe	mg/L	nd	0.015	0.024	nd	nd	nd
K	mg/L	52.5	22.5	20.9	20.8	20.9	15.5
Mg	mg/L	nd	11.2	10.7	10.7	10.6	11.2
Mn	mg/L	nd	0.03	0.01	0.24	nd	nd
Na	mg/L	3888	4030	3803	3789	3816	3888
P	mg/L	nd	0.03	nd	0.03	0.02	nd
S	mg/L	nd	0.63	0.53	1.96	0.70	nd
Si	mg/L	24.54	24.46	24.18	25.02	25.00	26.50
Zn	mg/L	nd	nd	nd	nd	nd	0.068
F	mg/L	nd	1.2	1.2	1	1	nd
Cl	mg/L	6596	6563	6325	6233	6283	6766
SO4	mg/L	nd	nd	nd	5.8	nd	nd
Br	mg/L	18	17.9	17.5	17	17.1	18
TOC	mg/L	238	2381	2238	2280	1629	951
Alkalinity	mg $CaCO_3$/L	1247	1154	1233	1225	1241	1467
Modeled pH		8.42	8.42	6.44	5.40	4.34	5.80

Figure 4.1 indicates an adverse effect of CO_2 exposure on microorganisms in the produced water. Extractable DNA decreased significantly with increasing pCO_2 in the vessel. While the DNA concentration in the initial sample, the 0 MPa reactor, and the 0.03 MPa reactor remained around 10^4 gene copies/ml sample, the DNA concentration decreased to 10^2 gene copies/ml sample in the 0.34 MPa reactor and 10^1 gene copies/ml sample in the 3.4 MPa reactor. This decrease in total extractable DNA (presumed to correlate to biomass) is consistent with previous reports, demonstrating increasing CO_2 exposure resulted in a decrease in microbial population.[5, 6] Gulliver et al, 2014, demonstrated a 56 day pCO_2 exposure of 0.1 MPa inhibited growth of the deep saline aquifer microbial community by an order of magnitude, and 56 day

pCO_2 exposure of 1.4 MPa or higher decreased DNA concentrations below detection limits (<1 gene copy/ml sample).[7] These results suggest that in areas close to the front of the injected CO_2 plume, where CO_2 concentrations are greater than 0.03 MPa, microbial-driven processes of interest may not proceed at appreciable rates. However, at lower pCO_2 exposures, total extractable gene copies DNA (and hence biomass) were not affected. This suggests that microbial communities may remain active at distances farther from the CO_2 front, where CO_2 concentrations are less than 0.03 MPa.

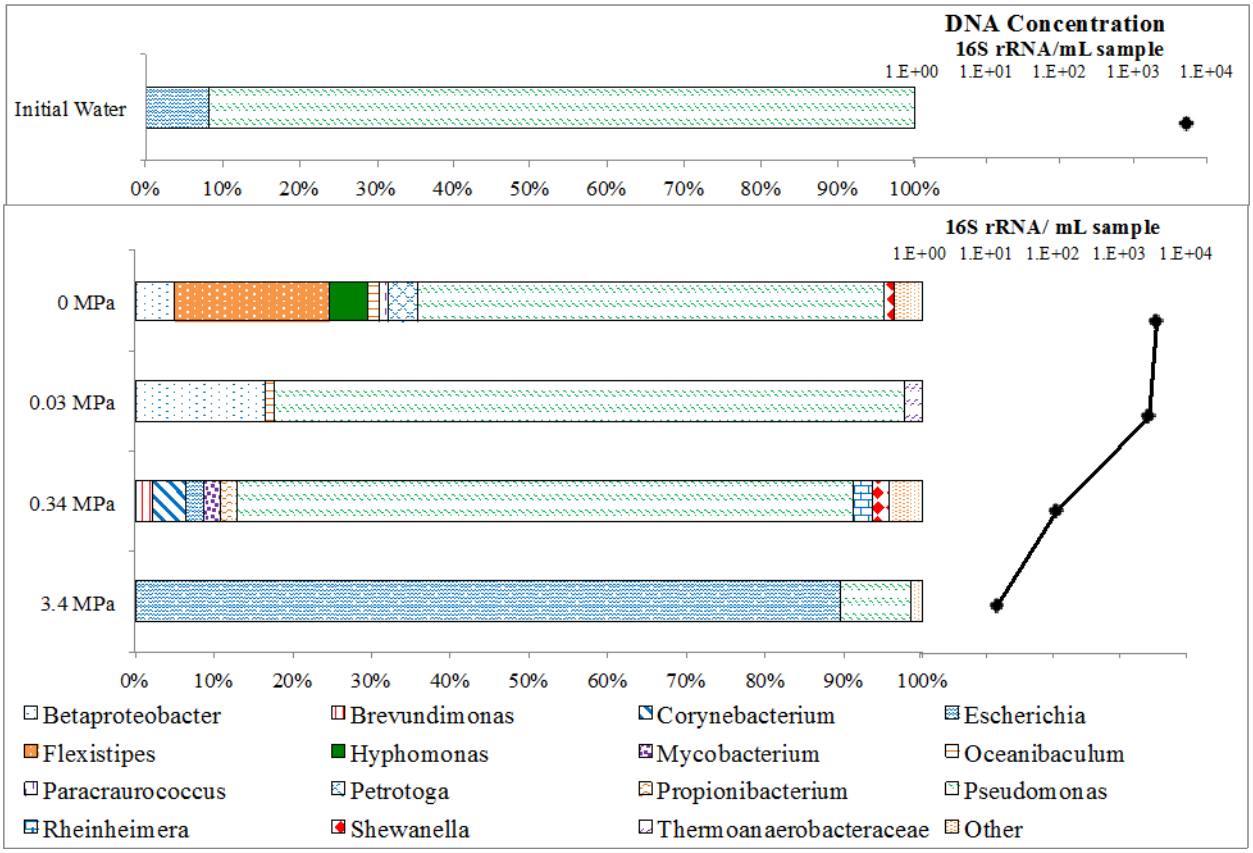

Figure 4.1. Relative proportions of phylotypes recovered from reactors exposing unfiltered produced water samples to increasing pCO_2 as revealed by 16S rRNA gene clone libraries and qPCR for a) initial produced fluid sample, and following b) 56 days of exposure to different pCO_2 values. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

The microbial community diversity did not display a clear trend with increasing pCO_2 (Figure 4.2). Equitability was 0.42, 0.25, and 0.35 for the 0 MPa, 0.03 MPa, and 0.34 MPa pCO_2 reactors respectively. For pCO_2 exposures of 0.34 MPa or less, a majority of the sequences recovered from the microbial community were most similar to the genus *Pseudomonas*. This genus comprised 59-80% of the microbial community (Figure 4.1). For the pCO_2 exposure of 3.4 MPa, a majority of the sequences recovered from the microbial community was most similar to the genus *Escherichia*, representing 90% of the microbial community.

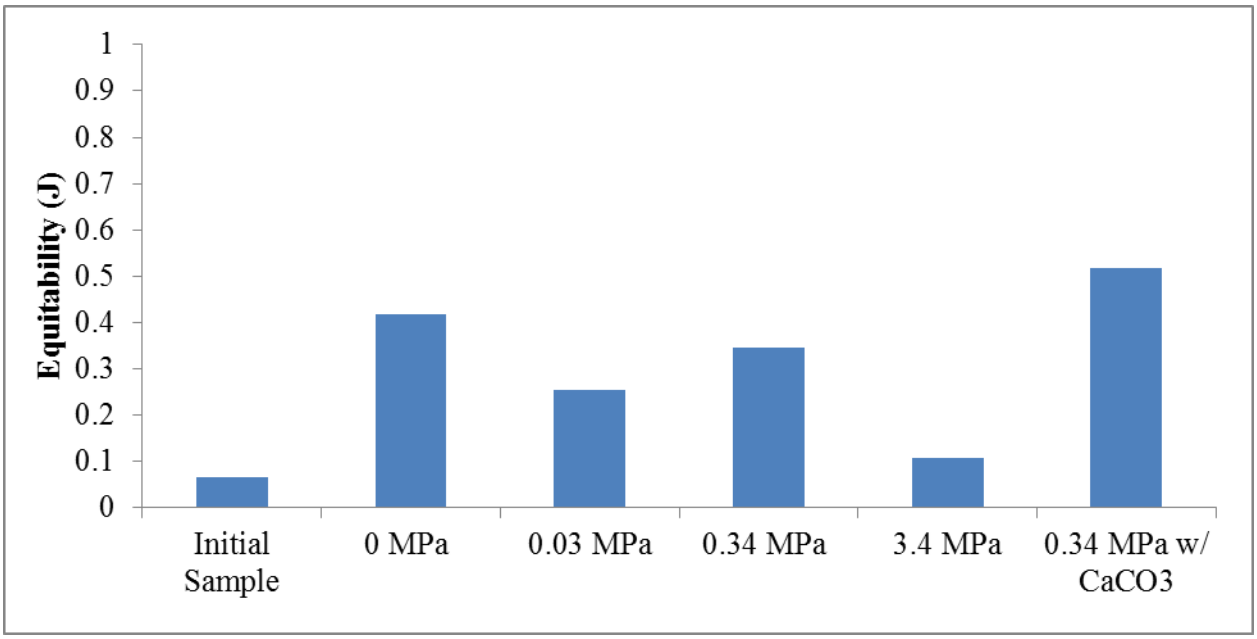

Figure 4.2. Impact of 56 days of pCO_2 exposure on microbial diversity of unfiltered produced water samples. Results show the comparison of Equitability values (J) for each pCO_2 concentration examined. A J = 0 occurs for a pure culture, whereas J = 1 occurs if each clone is a unique phylotype.

Sequenences most similar to the genus, *Pseudomona*s, is present in all of the samples, and dominant for pCO_2<0.34 MPa. A significant percentage (69%-97%) of the *Pseudomonas* genus is closely related to the species *Pseudomonas stutzeri*, a common denitrifying species that has been isolated in soils, wastewaters, sea waters, and marine environments.[3] Despite the absence of nitrate or nitrite in the produced water, the ubiquity of *Pseudomonas stutzeri* may be due to the versatility in metabolism, as this species is capable of utilizing a wide range of carbon sources as well as electron acceptors.[3] The ability of the genus, *Pseudomonas*, to utilize gasoline-derived carbons and petroleum-derived carbons has previously been documented.[34, 35] Due to the prevalence of *Pseudomonas stutzeri* in the Mirando oil reservoir, even after CO_2 exposure, further study in the adaptability and biological processes of this species is warranted.

The 3.4 MPa reactor had the lowest diversity of 0.11 equitability (Figure 4.2) accompanied by the lowest proportion of sequences closely similar to *Pseudomonas* (Figure 4.1). While *Pseudomonas* appeared to be dominate in the initial sample, and the 0 MPa, 0.03 MPa, and the 0.34 MPa reactors, it only represented 9% at the highest CO_2 exposure of 3.4 MPa. *Pseudomonas stutzeri* has previously been shown to be incapable of surviving a pH less than 4.5.[3] The pH of the water in the reactor pressured with 3.4 MPa of CO_2 was modeled to have a pH of 4.3 (Table 4.1); the *Pseudomonas stutzeri* may not have been adaptable to this decreased pH, resulting in cell decay.

At the highest pCO_2 exposure (3.4 MPa), a majority of sequences recovered were most closely similar to *Escherichia* (Figure 4.1). The species was most closely related to *Escherichia coli*, representing 94%-100% of this genus amongst all samples. While *Escherichia* is often used as an indicator of wastewater contamination, this genus is commonly introduced in natural sediments; *Escherichia* then adapts to the environmental conditions, becoming a dominant part of the native microbial community.[36] The detection of *Escherichia coli* may be due to an introduction of the species during development of the Mirando production wells, followed by species adaption and survival in the reservoir. While *Escheria* appeared to be the most resilient to CO_2 exposures of 3.4 MPa or greater, the DNA concentration was only 10^1 gene copies/ml sample in this reactor. This suggests this genus is surviving, rather than thriving in this environment. Whether this species would continue to survive with longer exposure times still needs to be determined.

Although the Mirando oil reservoir material was primarily fine grained sand, some pH buffering may occur due to the dissolution of accessory clays. To determine if pH buffering can lessen the impact of CO_2 on the microbial community, an additional reactor was pressurized with sample, 10 mg/L $CaCO_3$, and 0.34 MPa pCO_2 at reservoir temperature (40 ˚C) and total pressure (3.4 MPa). The $CaCO_3$ addition to the vessel resulted in a final pH of 5.8 compared to a pH of 5.3 for the 0.34 MPa pCO_2 unbuffered reactor.

The buffered reactor had an increase DNA concentration by an order of magnitude compared to the 10^2 gene copies/ml sample in the unbuffered reactor (Figure 4.3). This suggests the microbial community is stressed considerably at pH of 5.3 in the unbuffered 0.34 MPa reactor compared to the pH of 5.8 in the buffered 0.34 MPa reactor. However, the DNA concentration of the buffered reactor was still almost an order of magnitude less than the initial sample and the 0 MPa reactor, suggesting the buffered CO_2 exposure and pH=5.8 still stresses the microbial community. Moreover, the large effect on recoverable DNA for a 0.5 pH unit decreases suggests that the microbial communities are sensitive to changes over the pH range of 5.3 to 5.8; this incremental change in pH is expected to result from CO_2 injection.[37-39] Therefore CO_2 injection may lead to a decrease in microbial population even with buffering of accessory clays. Future studies on the effects of pH on the viability of microorganisms in this pH range are warranted.

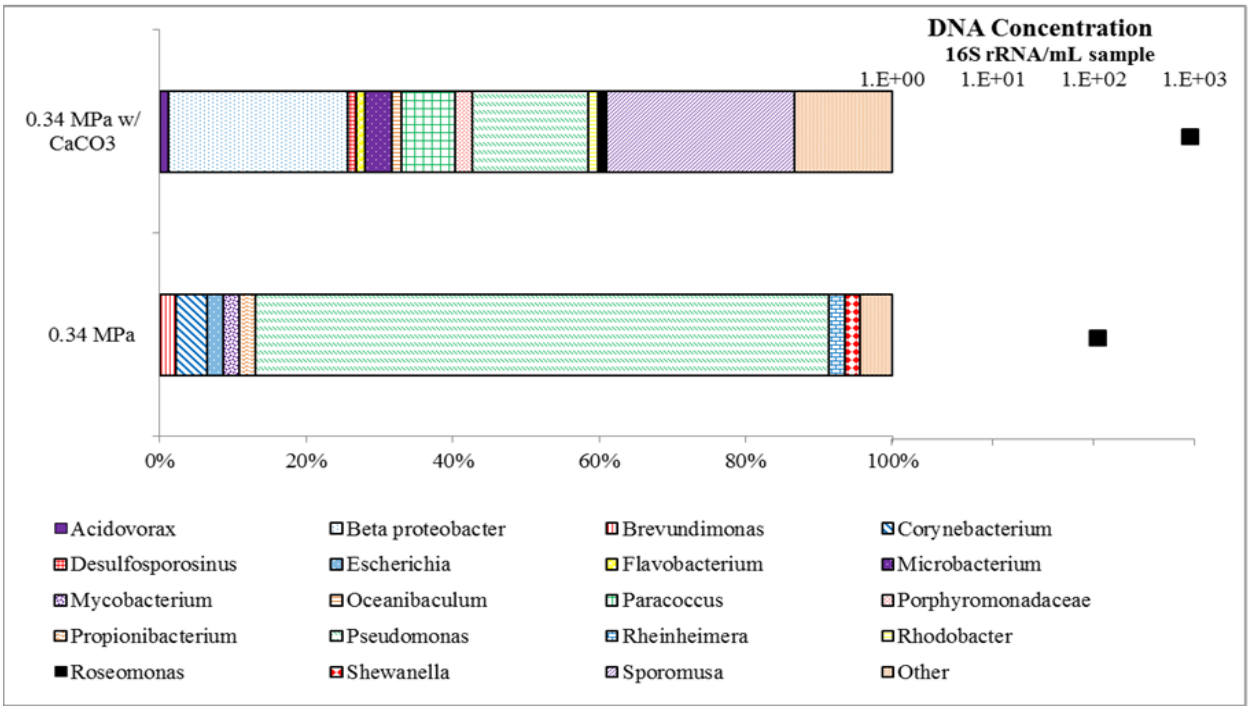

Figure 4.3. Relative proportions of phylotypes recovered from reactors exposing unfiltered produced water samples to increasing pCO_2 as revealed by 16S rRNA gene clone libraries and qPCR for 56 day exposures to 0.34 MPa pCO_2 or 0.34 MPa pCO_2 with $CaCO_3$ buffer. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

The buffered reactor also had an increased diversity of 0.52 compared to the unbuffered 0.34 MPa reactor diversity (Figure 4.2). While *Pseudomonas* appeared to remain a dominant genus, representing 16% of the detected microbial community, the increased diversity appeared to be due to an emergence of *Sporomusa*, representing 26% of the community, and Betaproteobacter, representing 24% of the community. The phylotype of *Sporomusa* is most closely related to the species *Sporomusa malonica*. This homoacetogenic species has previously been found in anoxic freshwater sediments.[40] The phylotype of Betaproteobacter is most closely

related to a nitrogen fixing strain of *Betaproteobacter* that had been isolated from wild rice (unpublished, AY235688). Gulliver et. al. also recovered sequences most similar to this species in reactors containing saline formation water and $CaCO_3$ buffer.[7] As the buffer was autoclaved, and no DNA was detected in the sterilized buffer, the addition of $CaCO_3$ may have provided a favorable condition for the emergence of this species.

Clone libraries are one technique to characterize the species present in the fluid sample. Pyrosequencing is an alternative technique to determine the array of organisms comprising the surviving microbial community. The classes of organisms detected using pyrosequencing is compared with that of the 16S rRNA clone library data in Figure B1. Overall, the classes detected by the pyrosequencing correlate with the classes detected by 16S clone libraries. Both 454 pyrosequencing and 16S clone libraries detected Gammaproteobacteria to be a dominant class, consistent with the high prevalence of sequences similar to *Pseudomonas* and *Escherichia*. The one exception to the correlation is that the class *Bacilli* was detected only in the 0.34 MPa reactor, only utilizing 454 pyrosequencing. This class was not detected in the 0.34 MPa reactor utilizing 16S clone libraries. The discrepancy between the two sequencing methods might be due to poor universal primer coverage or PCR bias. Since two primer sets were utilized during the Sanger sequencing (clone library) methods, a discrepancy due to primer coverage is unlikely. The genus *Tumebacillus* represented over 99% of the *Bacilli* found in 454 pyrosequencing. This

spore-forming genus has previously been isolated from industrial wastewater, methane hydrate-bearing sediments, and artic permafrost samples.[41-43] While the high correlation between the sanger sequencing results and pyrosequencing results suggests that the16S clone libraries did capture a majority of the dominant genera present in the microbial community, the appearance of *Tumebacillus* in the 454 pyrosequencing demonstrates a benefit to utilizing parallel methods of molecule analysis.

Ideally, the vessel experiments would have been carried out in triplicate. However, the collection of water from these sites is difficult and sample volumes are typically small. Limited volume of fluid sample and limited number of high-pressure vessels allowed only a single run for each CO_2 exposure. Nonetheless, a consistent trend was demonstrated in DNA concentration, and the dominant species were consistently detected in all samples. Although contamination of samples cannot be guaranteed, all laboratory analysis was completed with standard blanks, and sampling methods were utilized to maximize capturing the microbial community of the depleted oil reservoir.

This study begins to characterize the response of the microbial community that may be expected to occur in a depleted oil reservoir after CO_2 injection. The increasing CO_2 exposure was found the decrease DNA concentration. Results suggest *Pseudomonas* growth in the 0.03 MPa reactor, and *Pseudomonas* and *Escherichia* survival in the 0.34 MPa and 3.4 MPa reactor.

The CO_2 tolerance of *Pseudomonas* and *Escherichia* suggests these genera to be relevant for future study of microbial CO_2 tolerance and microbial processes during carbon storage. Understanding which microorganisms may survive or thrive after CO_2 exposure will ultimately provide hypotheses for which microbial processes may affect petroleum quality and petroleum production in a CO_2 flooded oil reservoir.

4.5 Supporting Information in Appendix B

Supporting information contains Figure B.1. References have been cited in the main text for figures and tables with an overview of material presented in the supporting information

4.6 References

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Chapter 5

Effect of $CO_{2(aq)}$ Concentration on a Microbial Community from Freshwater Aquifer Samples under Simulated CO_2 Leakage Scenarios

5.1 Abstract

Geological carbon sequestration is likely to be part of a comprehensive strategy to minimize the atmospheric release of greenhouse gasses, raising concerns that sequestered CO_2 may leak into overlying potable aquifers. CO_2 leakage into an aquifer is likely to impact microbial populations. A need exists to understand and identify sensitive microbial populations in freshwater aquifers that may affect the consequences of CO_2 leakage. We examined the impact of CO_2 exposure on the microbial community from a freshwater aquifer (55 m depth, 0.5 MPa, 22 °C, Escatawpa, MS) using a molecular ecology approach under controlled laboratory conditions as well as *in situ* exposure. In laboratory pressure vessels, microbial communities were exposed for 56 days to pCO_2 ranging from 0 MPa to 0.5 MPa. The *in situ* microbial community was characterized in samples taken both upgradient and downgradient from the CO_2 injection location. The number of copies of 16S genes recovered was unaffected by CO_2 exposure concentration for *in situ* exposures (max pCO_2=0.1 MPa), while the 16S genes recovered in laboratory vessels remained steady at pCO_2 exposures up to 0.05 MPa but greatly

decreased at 0.5 MPa. This suggests that microbial processes may be most impacted nearest to the CO_2 leakage and least affected where CO_2 concentrations will be attenuated. Both the *in situ* and laboratory experiments revealed the emergence of *Curvibacter* as the dominant genus within the microbial community with increased CO_2 exposure less than pCO_2=0.5 MPa. Laboratory incubations at 0.5 MPa revealed a variety of *Pseudomonas* to be the most tolerant to CO_2. Adaptability of the species *Pseudomonas* and *Curvibacter* to CO_2 exposure demonstrates a need to further research the behavior of these genera to improve understanding of the long-term consequences of CO_2 leakage.

5.2 Introduction

As geologic carbon storage (GCS) becomes part of a comprehensive solution to lessen rising atmospheric CO_2 concentration, the application of this technology raises concern for leakage in overlying freshwater aquifers.[1] During GCS, CO_2 is injected as super critical CO_2 (SC-CO_2) into a subsurface storage formation, such as a deep saline aquifer or a depleted oil reservoir. Injected SC-CO_2 in deep subsurface storage units may transport into overlying freshwater aquifers through aggravated caprock fractures or transport through pre-existing wells.[2-4] The biogeochemical processes that occur after an influx of leaking $CO_{2(g)}$ and $CO_{2(aq)}$ may impact water quality in the reservoir. A vital need therefore exists to understand the

microbial ecology that will arise after CO_2 leakage, as this community will affect the dominant biogeochemical processes in the impacted freshwater aquifer.

The introduction of CO_2 is expected to alter the chemical and physical environment in the overlying freshwater aquifer.[5] The concentration of $CO_{2(aq)}$ is expected to be highest closest to the source of incoming $CO_{2(g)}$ or dissolved $CO_{2(aq)}$, and to decrease to pre-CO_2-injection concentrations at a downgradient distance. The dissolution of $CO_{2(g)}$ results in a decreased groundwater pH and a corresponding increase in dissolved ions such as Ca, Fe, and Mg.[5-8] The extent of pH decrease and ion dissolution is a function of dissolved CO_2, and therefore also a function of downgradient distance from the source of the CO_2 plume. The groundwater that results will cover a range of $CO_{2(aq)}$ concentrations, pH, and dissolved ion concentrations.

Previous research in our lab revealed that increasing CO_2 concentration decreased the population numbers and diversity in a microbial community at a deep subsurface storage site.[9] After 56 days of exposure, pCO_2 as low as 0.1 MPa hindered microbial growth and allowed the emergence of adaptable species. At pCO_2 of 1.4 MPa or higher, the microbial population numbers were reduced to below molecular detection limits. This study indicated even the low $CO_{2(aq)}$ concentrations expected downgradient from the injection plume may alter the microbial community in a storage site. This altered microbial community is expected to be the stable

relevant community that will affect biogeochemical processes of a post-CO_2-injection storage site.

While the previous study determined the microbial community to be sensitive to CO_2 exposure under conditions of a saline formation used for CO_2 storage, it is still unknown whether the same microbial response will occur in freshwater aquifer where CO_2 is expected to arrive with attenuated concentrations. Because of the lower total pressure, $CO_{2(g)}$ and $CO_{2(aq)}$ concentrations are expected to be lower within an overlying freshwater aquifer; the lower CO_2 exposure in a leakage scenario may result in a less affected microbial community compared to the previous CO_2 storage studies with higher CO_2 exposures. On the other hand, the microbial community of a freshwater aquifer is not adapted to the harsh conditions of a deep subsurface storage aquifer, such as high salinity, high temperature, and high pressure. This initial freshwater microbial community may therefore be more sensitive to the stress induced by CO_2 exposure.

The main objectives of this study were to 1) determine if a freshwater microbial community can survive the stress of CO_2 exposure expected during CO_2 leakage, 2) if so, determine the effect of increasing CO_2 exposure concentration on the microbial community, and 3) compare results from a lab-exposed community to that from an *in situ* experiment. The microbial community in formation water from the Plant Daniel freshwater aquifer, MS was

examined in groundwater samples collected from the aquifer up-gradient and down-gradient from the CO_2 injection location (referred to here as *in situ* exposure), and in laboratory reactors exposed to different CO_2 concentrations at reservoir temperature and pressure. This is the first study to examine the response of a microbial community at conditions expected during CO_2 leakage from a carbon storage unit.

5.3 Materials and Methods

5.3.1 Site background and *in situ* study

A detailed site description was provided by Trautz et al., 2013.[5] Briefly, the Plant Daniel freshwater aquifer is an Energy Power Research Institute (EPRI) site for experimentally examining the geochemical and geophysical changes that can be expected from CO_2 leakage from an underlying geologic carbon storage site.[5, 10] The freshwater aquifer is located in Escatawpa, MS, and consists of sandy units with semi-confining clay-rich units and is about 180 ft deep and 20 ft thick. The reservoir temperature is 22 ˚C, and reservoir pressure 0.5 MPa. Additional geophysical data of the site is reported in Dafflon et al., 2013.[10]

Exposure of the microbial community to CO_2 *in situ* was performed by pressurizing extracted groundwater with 0.35 MPa CO_2 (pH=5) and then reinjecting the CO_2-water mixture into the aquifer. The groundwater was otherwise untreated. Two months after injection, three

monitoring wells downgradient of the injection, TW1, TW2, and MW3, and one background well upgradient of the injection, BG1, were sampled for formation fluid. Samples were drawn after utilizing the EPA low purge sampling protocol. Dissolved CO_2 concentration of each sample was measured at each monitoring well using a dissolved gas meter (CarboQC, Anton Paar). A detailed description of CO_2 injection and sampling locations and methods is given in Trautz et al., 2013.[5]

A volume of 1L of sample water from the four monitoring wells and from the background well was filtered for microbial analysis as described in the methods and approach section below. ICP and IC-anion data of the *in situ* samples is reported in Trautz et al., 2013.[5]

5.3.2 Pressurized Vessel Exposure Experiments

Unfiltered fluid samples from the background well, BG1 were placed in 150 mL Swagelok vessels rated for 14 MPa. The 80 mL liquid volume was pressurized to 0.5 MPa using either 100% N_2, 1% CO_2/99% N_2, 10% CO_2/90% N_2, or 100% CO_2. These CO_2 exposures represented 0%, 1%, 10%, and 100% pCO_2, representing freshwater conditions right at the CO_2 leakage plume, conditions at two downgradient distances from the plume, and conditions upgradient from the plume respectively. The vessels were stored at room temperature, which closely resembles aquifer conditions. The vessels were monitored daily for any pressure loss,

and no pressure loss occurred during the experiment. In addition, in order to understand the effect of buffering on the aquifer system, a separate reactor was pressurized for 56 days with 80 mL formation fluid, 10 g/L $CaCO_{3(s)}$ and pressurized with 10% CO_2/90% N_2 gas.

Sampling occurred after 56 days of exposure to each CO_2 concentration. Reactor vessels were sacrificed during sampling. The fluid was centrifuged at 4,000 g for 25 minutes. The top 75 ml of the supernatant was filtered for chemical analysis, and the bottom 5 mL containing microbes and any solids was used for biological analysis.

5.3.3 Microbial Community Analysis

DNA was extracted from 500 μL of the remaining 5 mL of the centrifuged utilizing the MoBio power soil kit. PCR amplification followed shortly using universal bacteria and archaea primers. Bacterial 16S rRNA genes were amplified using the 8 forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' [11] with the 519 reverse primer 5'-GTATTACCGCGGCTGCTGG-3' [11] and the 338 forward primer 5'-ACTCCTACGGGAGGCAGC-3' [12] with the 907 reverse primer 5'-CCGTCAATTCMTTTRAGTTT-3'.[13] The archaeal 16S rRNA gene fragments were amplified using the 344 forward primer 5'-ACGGGGCGCAGCAGGCGCGA-3' [14] with the 915 reverse primer 5'-GTGCTCCCCCGCCAATTCCT-3'.[15] 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. PCR conditions included a taq initiation step at 95 ˚C for 3 min, followed by 35 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 occurs at 72 ˚C for 7 min. Efficacy of the DNA extraction and PCR reaction was tested with an electrophoresis gel, using *E. coli* DNA as a positive bacteria control and *Methanococcus maripaudis* as a positive archaea control.

The PCR products of the two bacterial primer sets were mixed, and cloned using the Invitrogen TOPO TA cloning kit according to the manufacturer's instructions. The Invitrogen TOPO TA cloning kit was also utilized for any amplified DNA from the archaeal primer set. 96 clones per sample were sequenced at Functional Biosciences (Madison, WI).

The microbial community coverage was increased through use of pyrosequencing for the *in situ* experiments. The universal primer 515 forward primer 5'-GTGCCAGAMGCCGCGGTAA-3' was used with the Roche adapter A-CCATCTCATCCCTGCGTCTCTCGACTCAG and coupled with the 806 reverse primer 5'-GGACTACVSGGGTATCTAAT-3' and the Roche adapter B-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG.[16] Each primer set was run in a PCR mixture with a total volume of 50 μL containing Qiagen HotStart Taq along with the DNA template.

PCR conditions included a taq initiation step at 94 ˚C for 10 min, followed by 30 cycles of a denaturing step of 94 ˚C for 1 min, an annealing step at 55 ˚C for 1 min, and an elongation step at 72 ˚C for 1 min. After the 30 cycles, a final extension occurs at 72 ˚C for 10 min. Efficacy of the pyrosequencing reaction was tested with an electrophoresis gel, using *E. coli* DNA as a positive bacteria control. The reactions were sent to the Ohio State University genomics facility for pyrosequencing.

16s rRNA gene clone library sequences were trimmed using FinchTV, and pyrosequencing was trimmed using Mothur.[17] Chimeras were detected using Bellophon (Huber, 2004) for the 16S rRNA gene clone library sequences and Uchime for the pyrosequencing sequences.[18] Sequences with 97% similarity were grouped into OTU's on the genus level using Mothur and NCBI Blast.[17, 19] In cases where OTU's are less than 97% similar to the phylotype of a cultured organism, the microorganisms are described as "other".

Bacteria 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.[20] The PCR mixture for the bacteria primer set and probe consisted of a total volume of 20 μL containing Applied Biosystems TaqMan Master mix along

with the DNA template. Archaea 16s rRNA gene concentration will use the 806 forward primer 5'-ATTAGATACCCSBGTAGTCC-3' with the 958 reverse primer 5'-YCCGGCGTTGAMTCCAATT-3.[21] The PCR mixture for the archaea primer set consisted of a total volume of 20 μL containing Applied Biosystems SYBR Green Master mix along with the DNA template. Diluted samples of known concentrations of *E. coli* were used as bacteria standards and diluted samples of *Methanococcus maripaludis* were used as Archaea standards. The *E. coli* and *Methanococcus maripaludis* were quantified with pico green procedures described in Invitrogen Quanti-iT kit. DNA concentration were 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.

In order to determine the microbial diversity represented in each clone library, sequences were grouped into phylotypes based on 16S rRNA gene similarity of > 97%. Diversity is represented by equitability (J), which is calculated from Shannon-Weaver indices. Equitability was calculated at the species-level 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 are 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.[22]

5.3.4 Chemical Analysis of Exposure Experiments (Liquid)

Supernatant sample water was filtered through a 0.2 μm filter before any chemical analysis. Sample water was analyzed for selected elements (Ca, Fe, K, Mg, Mn, Na, P, S, Si, Zn) by ICP-OES (PerkinElmer Optima 7300 DV) using EPA method 6010C. Supernatant anions (Cl, NO_3, NO_2, PO_4, SO_4, Br) were also analyzed via a Dionex Ion Chromatograph using EPA method 300.1. Chemical components that were non-detect for all samples are not reported. Total organic carbon was measured via 1010 Total Organic Carbon Analyzer.

5.3.5 Water Chemistry Model

Dissolved CO_2 conversion from pCO_2 of the reactor vessels was estimated using a publically available solubility model developed by the Zhenhao Duan Research Group.[23]

Since pH could not be directly measured in the pressurized reactors, it 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 ideal 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.

5.4 Results and Discussion

5.4.1 Effect of CO_2 concentration on Microbial Communities in Exposure Experiments

The effect of CO_2 leakage on a freshwater microbial community was examined in reactor vessels containing samples from the Plant Daniel freshwater aquifer and exposed to a range of pCO_2 that could be expected to accompany a leakage scenario. These samples, with no prior history of exposure to elevated CO_2, were subjected to four different pCO_2 ranging from 0 MPa to 0.5 MPa at aquifer temperature (22 °C) and pressure (0.5 MPa) in batch vessel reactors. Reactors were depressurized and sacrificed after 56 days of exposure. The microbial community was analyzed using qPCR and 16S rRNA gene clone libraries. Some geochemical and water quality parameters after exposure are presented in Table 5.1, as well as the modeled pH and dissolved CO_2 for each pCO_2 condition.

Table 5.1. Elements and anions measured in the reactor water after 56 days of exposure to 0 MPa, 0.005 MPa, 0.05 MPa, and 0.5 MPa pCO_2.

	Units	Initial	0 MPa pCO_2	0.005 MPa pCO_2	0.05 MPa pCO_2	0.5 MPa pCO_2	0.05 MPa pCO_2 w/ $CaCO_3$
Ca	mg/L	2.9	2.9	2.9	2.9	3.0	350.6
Fe	mg/L	0.25	1.0	2.5	2.0	2.3	1.0
K	mg/L	8.2	7.5	7.4	7.5	7.5	9.0
Mg	mg/L	0.9	1.0	1.0	1.0	1.0	nd
Mn	mg/L	0.1	0.1	0.1	0.1	0.1	0.0
Na	mg/L	168	161.3	160.3	164.1	161.9	200.4
P	mg/L	0.1	0.1	0.1	0.1	0.1	nd
S	mg/L	nd	0.0	0.0	0.0	0.0	7.3
Si	mg/L	9.6	19.6	19.6	19.3	19.6	33.4
Zn	mg/L	nd	nd	nd	nd	nd	0.3
F	mg/L	nd	19.5	26.2	1.4	0.7	nd
Cl	mg/L	46	36.2	36.7	43.7	37.0	58.0
SO4	mg/L	17	IC	IC	IC	IC	nd
Br	mg/L	nd	IC	IC	IC	IC	nd
TOC	mg/L		12.6	22.7	21.3	47.9	
Alkalinity	mg $CaCO_3$/L	291.8	--	--	--	--	--
Modeled pH		8.2	8	6.77	5.8	4.8	6.4

While qPCR procedures were utilized for both archaea and bacteria, only bacterial 16S rRNA gene copies were recovered from qPCR. The bacterial 16S rRNA detected varied from 10^3 to 10^4 gene copies/mL, with the exception of the 0.5 MPa vessel pressurized for 56 days (Figure 5.1). This vessel had the lowest recoverable DNA concentration of only 10^1 gene copies/mL. The low DNA concentration in this vessel indicates a lethal stress of the highest CO_2 concentration. This is in agreement with previous studies, suggesting CO_2 exposure results in microbial inactivation. These previous studies demonstrated that CO_2 exposures as low as 0.1 MPa inhibit the growth of *Shewanella oneidensis*[24] and that increasing CO_2 concentrations from 0.08-0.1 MPa decreased the growth rate in pure cultures of common freshwater bacteria, *Pseudomonas putida*, *Bacillus subtilis*, *Desulfovibrio vulgaris*, and *Thauera aromatica*[25]. In

contrast to the results observed at 0.5 MPa pCO_2, the gene copy numbers were not significantly impacted at lower pCO_2 exposures (Figure 5.1), suggesting that only the community nearest to the source of the leakage may be lethally affected by the CO_2. Below 0.05MPa, the response of a mixed microbial community to CO_2 exposure may be an emergence of CO_2-resilient microorganisms.

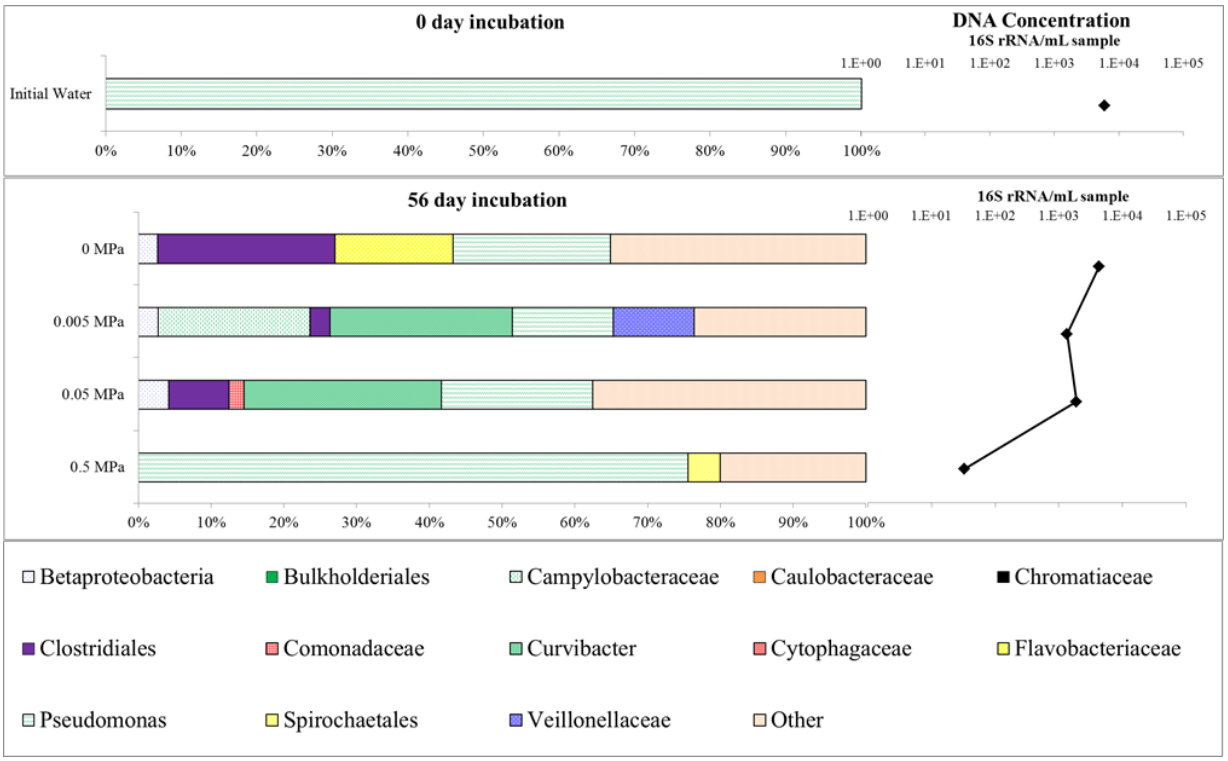

Figure 5.1. Relative proportions of phylotypes recovered from reactors exposing unfiltered freshwater aquifer samples to increasing pCO_2 as revealed by 16S rRNA gene clone libraries and qPCR for a) initial drill stem test sample, and following b) 56 days of exposure. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

Changes in the diversity of the microbial communities did not correlate with CO_2 concentration (Figure 5.2). Rather, the equitability varied from 0.31 to 0.76 with no distinct

trend with respect to CO_2 exposure concentration. Amongst the initial sample and all vessel samples, *Pseudomonas* emerges as a dominant genus in the microbial community (Figure 5.1). The most common species that represented the *Pseudomonas* genus were the ubiquitous soil species, *Pseudomonas fluorescens* (0%-30%) and arsenic-oxidizing *Pseudomonas IK-S1* (25%-35%).[26]

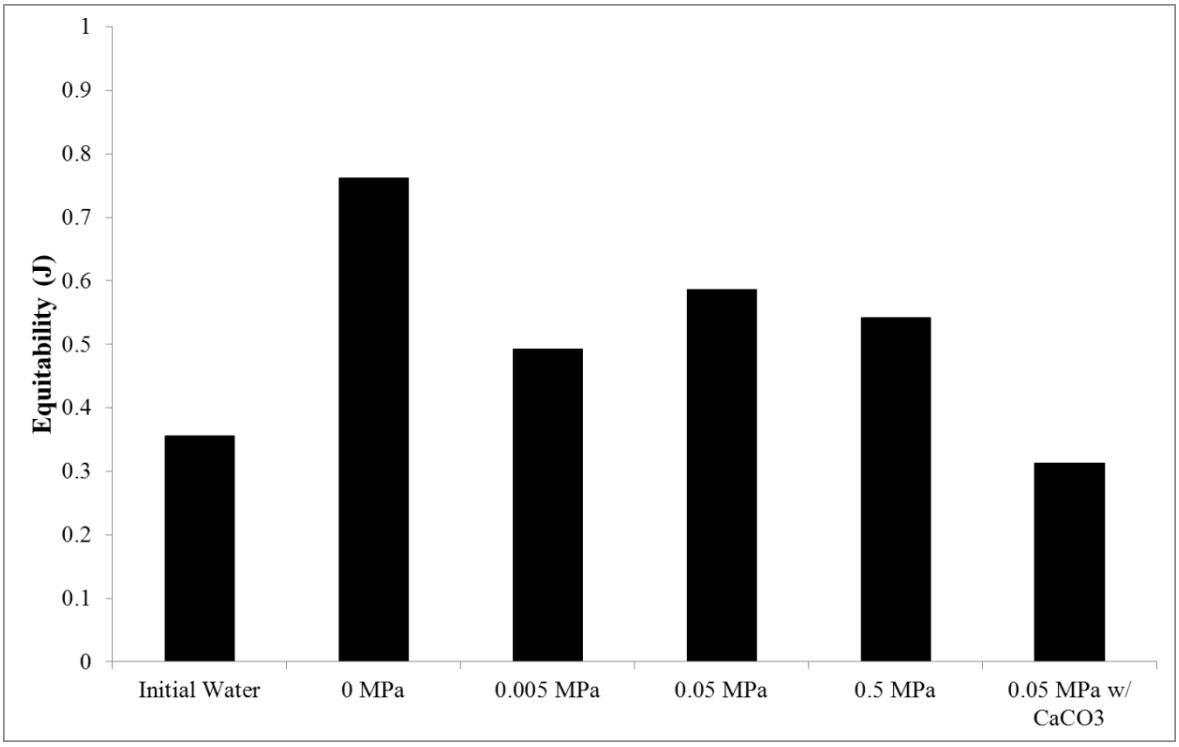

Figure 5.2. Impact of exposure time and pCO_2 on microbial diversity of unfiltered freshwater aquifer samples. Results show the comparison of Equitability values (J) for each pCO_2 concentration examined with time. A J = 0 occurs for a pure culture, whereas J = 1 occurs if each clone is a unique phylotype.

Previous studies suggest differing adaptability of *Pseudomonas* depending on the strain and experiment. A variety of *Pseudomonas* strains have previously been adaptable in different

environmental samples, such as marine sediment and water, freshwater sources, and agricultural soils.[27] However, contrary to this study, Schulz et al. found a strain of *Pseudomonas* (*Pseudomonas putida*) to be sensitive to elevated CO_2, with decreased growth after 50 hours of 0.08 MPa pCO_2 exposure.[25] Gill et. al. found 0.01 MPa pCO_2 exposure stimulated growth of *Pseudomonas fluorescens*, while 0.02 MPa pCO_2 exposure inhibited growth.[28] This study is the first to utilize a mixed microbial community from samples of the subsurface, and suggests *Pseudomonas* growth in environments with 0.05 MPa pCO_2 exposure. *Pseudomonas* is a known biofilm producer,[29-31] and has been previously researched as a microbial mitigation strategy to reduce CO_2 leakage pathways.[32] Additional research may determine the ability of *Pseudomonas* to produce a biofilm under the conditions of a CO_2-leak that may serve to immobilize heavy metals in a CO_2 impacted aquifer.

The dominance of *Pseudomonas* in the 0.5 MPa vessel after 56 days suggests this genus is relatively resilient to stress caused by CO_2 exposure. However, this reactor had the lowest detectable DNA concentration of 10^1 gene copies/ml, suggesting *Pseudomonas* is merely surviving the CO_2 exposure as opposed to thriving in this system. Biological processes may therefore not contribute strongly to geochemical processes affecting water quality at this high CO_2 concentration exposure.

A wider variety of genus appear adaptable to exposure to lower CO_2 concentrations, as *Curvibacter* also emerged as dominant bacteria in the 0.05 MPa and 0.005 MPa CO_2 concentration vessels, representing 25%-27% of the community. The emergence of *Curvibacter* was accompanied by the disappearance of the spore-forming, soil bacteria, *Clostridiales*,[33] and the common chemoorganotrophic freshwater bacteria, *Flavobacteriaceae*.[34]

The enriched *Curvibacter* phylotypes were most similar to *Curvibacter delicatus*, a well water isolate that remains poorly characterized.[35] The emergence of *Curvibacter* may be due to adaptability to environmental stress, as this phylotype has previously been found to be resistant to antibiotics.[36] The lowest CO_2 concentration vessel of 0.005 MPa additionally was enriched in *Campylobacteraceae*, a common drinking water microorganism,[37] and to a lesser extent, *Veillonellaceae*, a fermentative microorganism capable of CO_2 conversion to acetate.[38] These results suggest a greater variety of genus will be adaptable at CO_2 concentrations expected further down-gradient the from CO_2 leakage source as compared to near the source of CO_2.

Previous work at the Plant Daniel site[5] revealed minimal buffering due to the dissolution of accessory clays. This buffering may result in CO_2 concentrations accompanying a pH of 5.8 compared to a pH as low as 4.8 of the unbuffered reactors (Table 5.1). To determine the effect of buffering that may have occurred in the field on the results measured here, an additional

reactor with fluid sample and 10 g/L of $CaCO_3$ was pressurized with 0.05 MPa of pCO_2 under aquifer pressure (0.5 MPa) and temperature (25 ˚C) for 56 days.

The buffered reactor demonstrated an order of magnitude higher DNA concentration as compared to the unbuffered reactor (Figure 5.3). This suggests that buffering from accessory clays may prevent the microbial population from decreasing to the extent observed in the 0.5 MPa CO_2 56 day vessel. The dominant species found in the buffered reactors is a strain of *Betaproteobacteria* (64%) after 56 days. The phylotype is most closely related to a nitrogen fixing strain of *Betaproteobacteria* that had been isolated from wild rice; this phylotype is otherwise poorly characterized (unpublished, AY235688). The emergence of this *Betaproteobacteria* in the buffered reactors and the emergence of *Betaproteobacteria* closely related to *Curvibacter* in the 0.005 MPa and 0.05 MPa 56 day vessels suggest this class may be adaptable to CO_2 exposure in near neutral pH ranges.

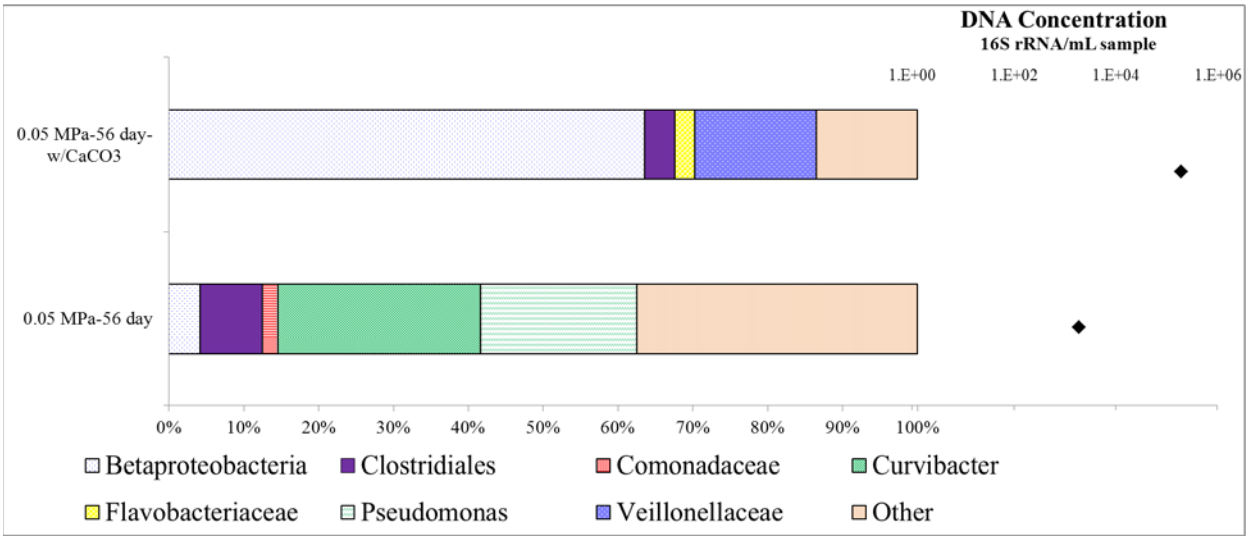

Figure 5.3. Microbial ecology characterized by 16S rRNA gene clone libraries and microbial population quantified by qPCR methods for 56 day exposures to 0.05 MPa pCO_2 or 0.05 MPa pCO_2 with $CaCO_3$ buffer. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

After 56 days of 0.05 MPa CO_2 exposure in the buffed reactor, *Methanobacterium* emerged as a dominant Archaea genus (Figure C1). While the concentration of 16S rRNA was below detection for the unbuffered vessel samples, 10^3 16S rRNA gene copies/mL sample were detected in the 0.05 MPa CO_2 vessel with buffering. *Methanobacterium* represented 100% of the detected Archaea in this reactor, with 77% most similar to the phylotype *Methanobacterium palustre*, and 33% most similar to the phylotype *Methanobacterium formicicum*. This suggests that this methanogenic Archaea was adaptable to the buffered CO_2 conditions, allowing this phylotype to grow and dominate in this environment.

The reactor vessel experiment demonstrated the mixed community's ability to adapt to the environmental stress induced by CO_2 leakage. DNA concentrations remained within 10^3-10^4 16S/ml sample at pCO_2 below 0.05 MPa. This is contrary to previous culture-based studies, which has found increasing CO_2 exposure to inhibit microbial growth.[9, 24, 25] However, similar to this study, previous *in situ* research suggests subsurface mixed communities may be adaptable to CO_2 exposure. Morozova et. al. found that after 5 months of CO_2 injection in a saline storage aquifer, the microbial community repopulated to the initial concentration of 10^6 cell/ml.[39, 40] Results from the complimentary *in situ* experiment (discussed next) further suggest the adaptability of mixed microbial communities in CO_2 exposed environments.

At pCO_2 exposures below 0.05 MPa, *Pseudomonas* and *Curvibacter* emerge as dominate in the community. Downgradient of the CO_2 leakage source, this altered microbial community may affect impacted aquifer parameters such as porosity, interstitial pH, and dissolved metals. As *Curvibacter* remains relatively uncharacterized, additional research into this microorganism may help our understanding of the effect of a CO_2 adapted microbial community on an impacted aquifer. Only at the pCO_2 of 0.5 MPa was the DNA concentration reduced to 10^1 16S/ml sample, suggesting a reduced microbial community near the CO_2 leakage source might play a limited role on the behavior of the impacted aquifer.

5.4.2 Effect of CO_2 concentration on Microbial Communities *In Situ*

In order to determine the effectiveness of measuring microbial community changes with CO_2 exposure in the vessel experiment, these results were compared to shifts in microbial communities in groundwater samples taken during an *in situ* experiment conducted at the Plant Daniel site. CO_2 saturated groundwater was injected in the Plant Daniel freshwater aquifer, and dissolved CO_2 was measured in 3 downgradient wells.[5, 10] These downgradient wells had a dissolved CO_2 concentration of 0.006 g CO_2/L, 1.153 g CO_2/L, and 1.53 g CO_2/L. The microbial community of each downgradient well and one upgradient well (0 g CO_2/L) was analyzed with qPCR, 454 pyroseqeuncing, and 16S rRNA clone libraries.

The 16S rRNA gene copies recovered by qPCR for the *in situ* experiment were between 10^3 and 10^4 gene copies/mL, and did not appear to correlate with measured dissolved CO_2 concentration (Figure 5.4). These results suggest that CO_2 exposure may not greatly affect the microbial population size at low CO_2 concentrations that would be expected from a CO_2 leak. This is in agreement with a previous *in situ* study, which found microbial communities capable of maintaining a population of 10^5-10^6 cell/ml 5 months after injection of 500 tons of CO_2 at 6.2 MPa.[39, 40] Both studies suggest an adaptable microbial ecology will develop in the subsurface after CO_2 exposure.

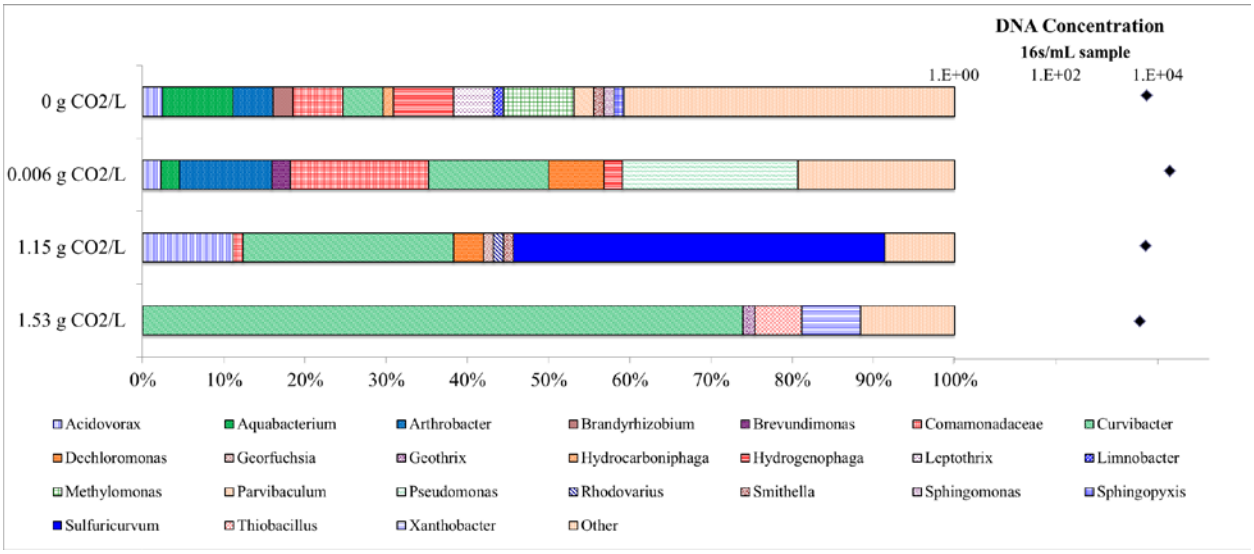

Figure 5.4. Relative proportions of phylotypes recovered from unfiltered well samples downgradient of CO_2-saturated-water injection well. Dissolved CO_2 of each well was measured by CarboQC and microbial community was revealed by 16S rRNA gene clone libraries and qPCR . Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

Although there was no apparent trend in DNA concentration with CO_2 exposure, the *in situ* experiment demonstrated a clear decrease in diversity with increasing dissolved CO_2 concentrations (Figure 5.5). Equitability decreased from 0.76 in samples with 0 MPa dissolved CO_2 to as low as 0.27 in samples with 1.5 g/L dissolved CO_2. This decrease in diversity appears to be due to the emergence of *Curvibacter* as a dominant genus (Figure 5.4). *Curvibacter* only occupied 5% of the relative abundance in the 0 g/L dissolved CO_2 fluid, which increased to 15% in 0.006 g/L dissolved CO_2 fluid, 26% in 1.15 g/L dissolved CO_2 fluid and 74% in 1.53 g/L dissolved CO_2 fluid. Similar to the reactor experiment, the enriched *Curvibacter* phylotypes were most similar to the well water isolate, *Curvibacter delicatus*. The 1.5 g/L dissolved CO_2

fluid was to a lesser degree also enriched in *Thiobacillus* (6%), a chemoautotrophic sulfur oxidizing bacteria,[41] and *Xanthobacter* (7%), a freshwater bacteria (unpublished, DQ664202). These bacteria appear to be the most tolerant of low dissolved CO_2 concentrations.

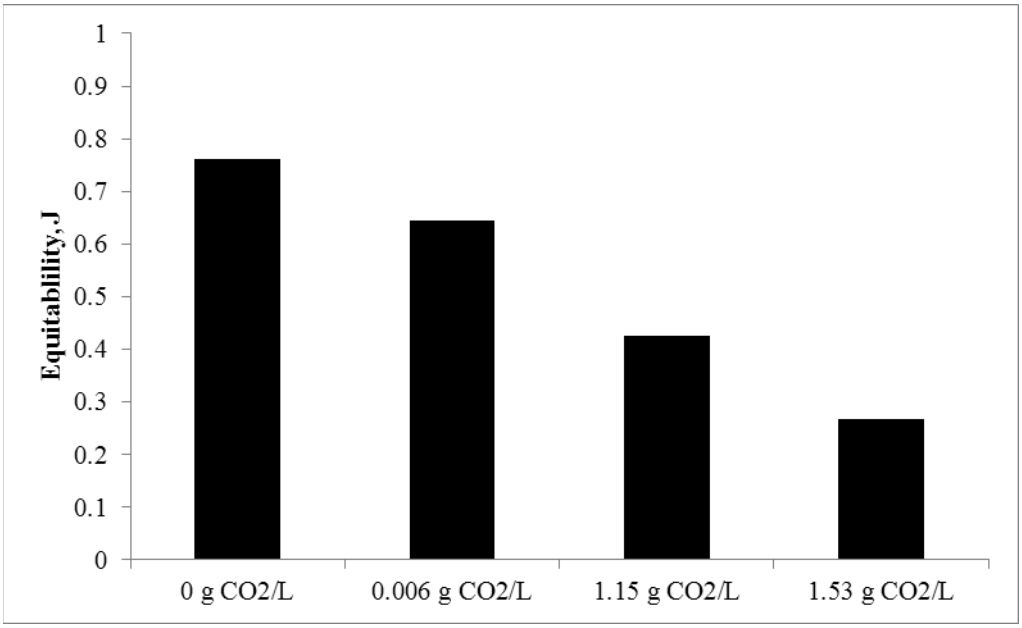

Figure 5.5. Impact of dissolved CO_2 on microbial diversity of unfiltered freshwater aquifer samples downgradient of CO_2-saturated-water injection. Results show the comparison of Equitability values (J) for each pCO_2 concentration examined with time. A J = 0 occurs for a pure culture, whereas J = 1 occurs if each clone is a unique phylotype.

The decrease of diversity was also due to the loss of abundance in ground water bacteria genus, such as the common freshwater bacteria, *Acidovorax*[42] and *Arthrobacter*,[43] *Hydrogenophaga*, a groundwater microorganism often associated with arsenic mobilization,[44, 45] *Leptothrix*, a metal oxidizing microorganism,[46, 47] and *Methylomonas*, a freshwater methanotroph.[48] These bacteria may be less resistant to low CO_2 concentrations, and will not be

involved in microbial processes that affect aquifer parameters such as porosity, interstitial pH, and metal solubility. The apparent CO_2-intolerance of metal mobilizing bacteria such as *Hydrogenophaga* and *Leptothrix* further suggests that metal solubility will be mostly dependent on geochemical processes (e.g. lowering of groundwater pH or changes in redox conditions) rather than biogeochemical processes. The reduced diversity and maintained DNA concentration demonstrates a dynamic microbial system that will adapt to various CO_2 exposures.

The pyrosequencing data displays a similar trend as the 16S rRNA clone library data (Figure 5.6). In both datasets, the overall abundant phylum was Proteobacteria representing 73%-96% of the bacteria detected by pyrosequencing and 65%-90% of the bacteria detected by 16S rRNA clone libraries. Within this phylum, Betaproteobacteria was the dominant class, representing 46%-64% of the bacteria detected by pyrosequencing and 42%-80% of the bacteria detected by 16S rRNA clone libraries. As the *Curvibacter* is in the Betaproteobacteria class, the pyrosequencing data further supports *Curvibacter* to be a dominant genus this freshwater environment. The correlation between the 16S rRNA clone libraries and 454 pyrosequencing data suggests the utilized methods accurately detected the microbial community in the environmental samples. *Curvibacter* remains relatively uncharacterized; the adaptability of this genus to CO_2 provides reason for further characterization as this knowledge may increase understanding of dominant biogeochemical processes that will occur in a CO_2 impacted aquifer.

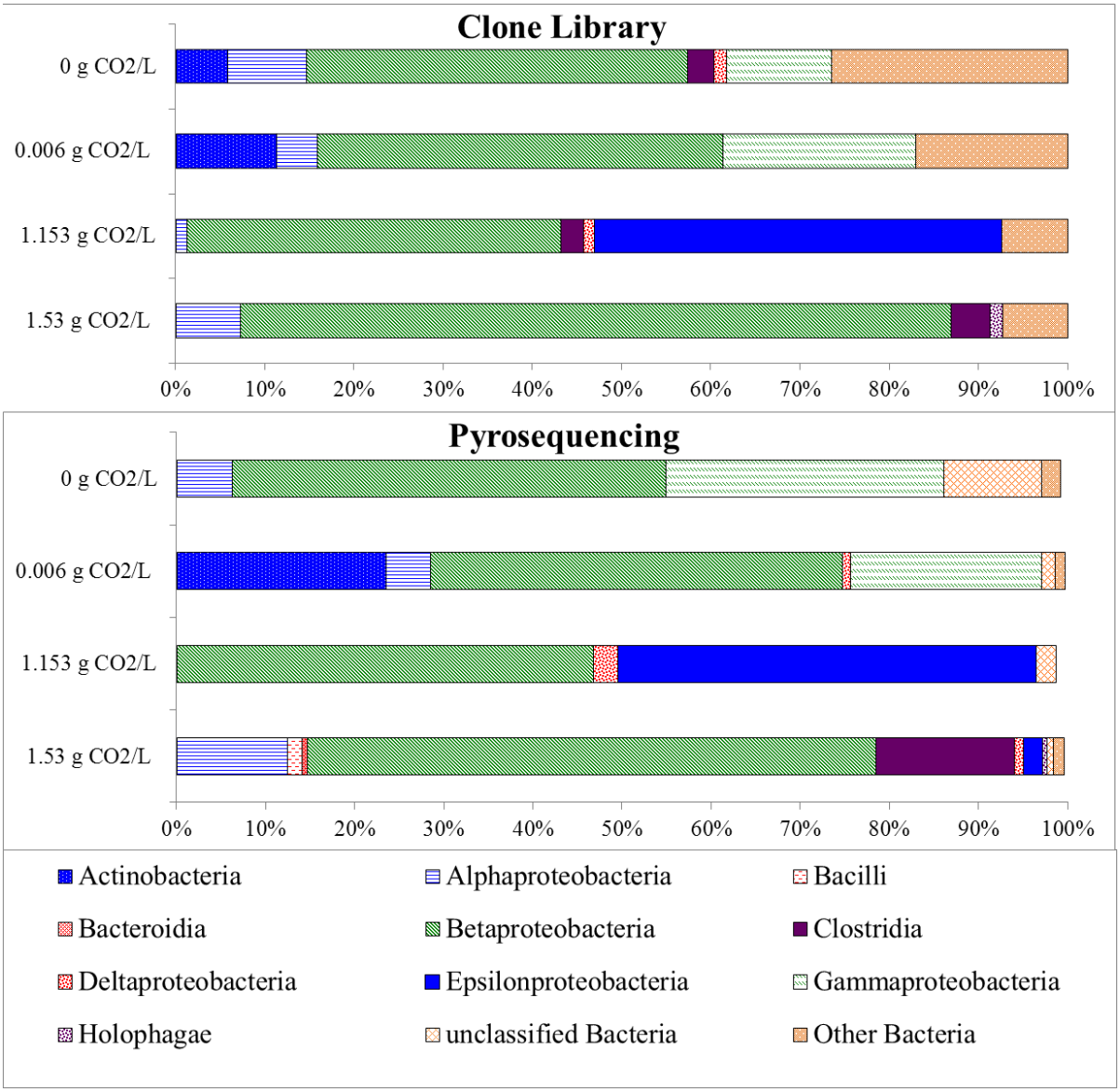

Figure 5.6. Comparison of 16S rRNA gene clone libraries and 454 pyrosequencing of relative proportions of phylotypes recovered from unfiltered well samples downgradient of CO_2-saturated-water injection well. OTUs were assigned an order based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

Although Archaea was not detected in the 16S rRNA clone library, pyrosequencing was able to detect Archaea to represent 1%-7% of the total microbial community in the samples. The

qPCR detected 10^{-1}-10^{1} gene copies/mL sample (Figure C2). The discrepancy of Archaea detection between clone library methods and pyrosequencing methods may be a demonstration of the 16S clone library limited capability to detect only the dominant microorganisms within the microbial community. The discrepancy between the two sequencing methods might also be due to poor universal primer coverage or PCR bias. However, since two primer sets were utilized during the clone library methods, a discrepancy due to primer coverage is unlikely.

The dominant classes of the Archaea present were the anaerobic methanogens, *Methanomicrobia* and *Methanobacteria* (Figure C2). *Methanomicrobia* appeared to be dominant over *Methanobacteria* at low CO_2 concentrations, representing as high as 78% of the Archaea in the 0.006 g CO_2/L fluid. However, *Methanobacteria* transitions into the dominant Archaea with increasing CO_2 concentrations, representing as high as 66% of the Archaea in the 1.53 g CO_2/L fluid. The presence of the strictly anaerobic methanogen, demonstrates an impacted aquifer will contain an anaerobic niche for these types of species to survive. However, the small percent representation of the Archaea suggests that although these microorganisms may survive CO_2 leakage conditions, methanogenesis will not likely be a dominant metabolism in the freshwater environment.

Consistent with the reactor experiment, the *in situ* experiment demonstrated an adaptable community will arise after CO_2 leakage in a freshwater aquifer. DNA concentrations remained

between 10^3-10^4 gene copies/ml sample in CO_2 exposures as high as 1.53 g CO_2/L. Increasing CO_2 concentrations accompanied a decrease in diversity as *Curvibacter* emerged as a dominant genus of the microbial community. This further suggests *Curvibacter* will be adaptable to CO_2 leakage conditions.

Although the pressure vessel experiment and *in situ* experiment utilized different initial microbial communities, some trends were similar between the results. The emergence of *Curvibacter* at mid-range CO_2 concentrations was observed in both the laboratory vessel experiment and the *in situ* experiment. In addition, both the laboratory vessel experiment and the *in situ* experiment suggested microbial population size would not be affected by CO_2 concentrations less than 1.53 g /L. The decreased gene copy detection and emergence of *Pseudomonas* in the pressure vessel experiment was not observed *in situ*; this may have been due to the differing initial communities between experiments. In addition, the high CO_2 exposure of the 0.5 MPa vessel (modeled 7.72 g/L dissolved CO_2) was not observed *in situ* (maximum 1.53 g/L dissolve CO_2). *Pseudomonas* may be most tolerant of high pCO_2 exposures not captured in the *in situ* experiment, but still expected closest to the leakage plume. Trends observed in both experiments demonstrate large changes in microbial communities, such as population decline and major shifts in dominant phylum, may be detected in laboratory experiments, while subtle changes such as shifts in non-dominant phylum, may require additional study.

There still remain many uncertainties about which biogeochemical processes may occur during CO_2 leakage in a freshwater aquifer, and if they will be significant. This study begins to fill in the knowledge gaps on the freshwater microbial response to CO_2 exposure expected during leakage. The stress of CO_2 exposure was found to result in the decay of most species, but result in the emergence of other species. The tolerance of *Pseudomonas* and *Curvibacter* to CO_2 suggests these genera to be relevant for further research in their biogeochemical processes, such as metal mobilization. Additional research in the microbial response to CO_2 exposure from alternate freshwater aquifers will further increase the understanding relevant microbial communities in CO_2 impacted aquifers. In addition, experiments of longer duration may provide more insight in the long term microbial community evolution. Understanding the microbial community of these systems will enable future studies on relevant microorganisms, tolerant to CO_2 stress and potentially lead to strategies for mitigation of deleterious impacts on water quality that may occur from CO_2 leakage. This improved understanding of the relevant microbial communities will ultimately lead to more effective prediction of long-term consequences during CO_2 leakage in a freshwater aquifer.

5.5 Supporting Information in Appendix C

Supporting information contains Figure C.1 and Figure C.2. References have been cited in the main text for figures and tables with an overview of material presented in the supporting

information.

5.6 References

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Chapter 6

Comparison of the effect of CO_2 concentration on microbial communities from a saline aquifer, a depleted oil reservoir, and a freshwater aquifer

6.1 Abstract

The injected CO_2 from geologic carbon storage is expected to impact the microbial communities of proposed storage sites including deep saline aquifers and depleted oil reservoirs. Leaking CO_2 is also expected to impact the microbial communities in overlying freshwater aquifers receiving that CO_2. The microbial community change in these subsurface sites may affect injectivity of CO_2, permanence of stored CO_2, and subsurface water quality. The effect of CO_2 exposure on microbial communities in each of these subsurface environments has been described in this thesis (Ch. 3-5). Here, the results from the different sites are compared to determine if trends emerge for the impact of CO_2 on microbial communities from all subsurface sites, or if the results demonstrate a site dependent change in microbial communities. If trends exist among sites, these results may improve the prediction of the subsurface microbial response during geologic carbon storage.

The change in DNA concentration versus CO_2 exposure and the change in DNA concentration versus modeled pH were compared among all three sites. Results demonstrate that the extractable DNA concentration decreased with increasing CO_2 exposure in all three sites

evaluated. The decrease in DNA concentration correlated well with the decrease in the pH in the reactor. The change in microbial community was statistically compared using a distance matrix and displayed as a Unifrac plot and as a community tree. Microbial communities that emerged after CO_2 exposure were highly site dependent. Comparison of results demonstrates that a decrease of microbial growth is expected to occur at other CO_2 exposed subsurface sites. However, the emerging dominant microbial species upon exposure to CO_2 appears site specific and cannot be predicted only from the shifts in microbial communities determined in the three sites studied in this thesis.

6.2 Introduction

Geologic carbon storage is a part of a proposed mitigation strategy for reducing the rate of increasing atmospheric CO_2 concentration emitted from fossil fuel combustion. During this process CO_2 is injected as supercritical CO_2 (SC-CO_2) in deep subsurface storage sites, such as a saline aquifer or depleted oil reservoir. After SC-CO_2 injection, CO_2 leakage into overlying freshwater aquifers may occur through aggravated caprock fractures or pre-existing wells.[1-3] Geologic carbon storage may therefore lead to an influx of CO_2 in any of these three subsurface sites: saline aquifers, depleted oil reservoirs, and freshwater aquifers.

An influx of CO_2 is expected to affect microbial communities within these subsurface sites. These microbial communities can in turn affect subsurface properties, such as porosity, pH, and dissolved metal ion concentration. The change in microbial communities may therefore influence the CO_2 injectivity and permanence of CO_2 in storage sites, and the potability of impacted freshwater aquifers. It is important to determine the microbial community changes that occur with CO_2 exposure in the subsurface in order to assess the potential impacts of CO_2 injection on subsurface properties.

The overall objective of this thesis is to determine the effect of CO_2 concentration on relevant subsurface microbial communities. The change in DNA concentration and microbial communities with CO_2 exposure was explored for a saline aquifer (Arbuckle, KS, Ch. 3), a depleted oil reservoir (Mirando, TX, Ch.4), and a freshwater aquifer (Plant Daniel, MS, Ch. 5). Samples from each subsurface site were exposed to 0%, 1%, 10%, and 100% pCO_2 for 56 days under site-specific temperature and pressure. The effect of buffering that would occur in subsurface sites on the microbial community response was also characterized. In these experiments, Plant Daniel water sample and Mirando water sample were exposed to 10% pCO_2 with 10 g/L $CaCO_3$ for 56 days under site pressure and temperature. Arbuckle sample was pressurized under site pressure and temperature without CO_2 but with an HCl-induced reduced pH of 4.4 (the pH expected for the 10% pCO_2 Arbuckle vessel); this vessel allowed a

comparison of the effect of a reduced pH versus the effect of CO_2 exposure on the microbial community. The microbial community for each site and each exposure condition was analyzed with qPCR and 16S rRNA gene clone library methods to monitor DNA gene copy concentration with increasing CO_2 exposure, to identify adaptable species that will emerge in CO_2 exposed conditions.

While the change in microbial community with CO_2 exposure was characterized for each of the three subsurface sites in Chapters 3 through 5, the results among these three sites have not yet been directly compared. Because each site started with a different initial microbial community and different site conditions, it is possible that the changing microbial community after CO_2 exposure will be site specific. This would indicate a need independently examine the effect of CO_2 on each subsurface microbial community. However, an influx of CO_2 may lead to an emergence of similar microbial communities, regardless of site conditions. In this case, similar results observed among Ch. 3- Ch.5 may demonstrate the ability to predict microbial community changes of future storage sites and impacted aquifers from previous research. A site comparison was conducted here to understand the general applicability and implications of the results from Chapters 3-5.

The specific objectives of this comparison were to 1) compare the effect of CO_2 concentration on DNA concentration among all three sites, 2) compare changes in microbial

community with increasing CO_2 concentration among all three sites, and 3) identify any trends that emerge across all sites. The DNA concentration was measured with qPCR as 16S rRNA gene copy/ml sample, and was compared based on both CO_2 exposure and modeled pH. The change in microbial community was compared via a distance matrix and displayed as a Unifrac plot and a community tree.

6.3 Materials and Methods

6.3.1 Site Background

A detailed site description of the potential storage site, the Arbuckle Saline Aquifer, is given in Ch. 3. Briefly, the Arbuckle saline aquifer (about 1220 to 1460 m depth) is part of the Ozark Plateau aquifer system, in Sumner County Kansas. The aquifer 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, and has a reservoir pressure of 14 MPa and a reservoir temperature of 40 ˚C. 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 after 4800 L of purging. The samples had less than 0.5 g/L suspended solids.

A detailed description of the Mirando oil field is given in Ch. 4. Briefly, the Mirando oil field, Zapata County, Texas, is part of the Eocene formation, 490 m to 610 m depth, consisting of fine grained marshal sand. Average reservoir pressure is 3.4 MPa, and average reservoir temperature is 40 ˚C. This reservoir has not yet been injected with CO_2, and currently produces about 10 barrels of oil per day. Fluid slurry sample was withdrawn from Chargos Creek Well A (latitude 27.1506936, longitude 98.9972297) from the pump jack at the end of a routine oil draw to maximize the formation water sampled and minimize any contamination from drilling. The samples had an 8-10% oil water cut, and had less than 0.5 g/L suspended solids.

A detailed site description of the Plant Daniel freshwater aquifer is given in chapter 5 and in Trautz et al.[4] Briefly, the Plant Daniel freshwater aquifer is an Energy Power Research Institute (EPRI) site used for investigating the geochemical and geophysical changes that can be expected in a freshwater aquifer from CO_2 leakage from an underlying geologic carbon storage site.[4, 5] The freshwater aquifer is located in Escatawpa, MS, and consists of sandy units with semi-confining clay-rich units and is about 55 m in depth and 6 m in thickness. The reservoir temperature is 22 ˚C, and reservoir pressure 0.5 MPa. Samples were drawn after utilizing the EPA low purge sampling protocol. Samples had less than 0.5 g/L suspended solids.

6.3.2 Pressurized Vessel Exposure Experiments

Arbuckle saline aquifer and Mirando oil field formation samples were exposed to CO_2 in 1-L, Teflon-lined stainless steel static pressure vessels; details of these vessels can be found in Kutchko et al., 2007.[6] 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 for the Arbuckle vessels, and 3.4 MPa for the Mirando vessels. The experimental pCO_2 of the Arbuckle vessels was 0, 0.1, 1.4 and 14 MPa and of the Mirando vessels was 0, 0.03, 0.34, and 3.4 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 56 days of exposure to CO_2.

Identical vessel reactors were studied using Arbuckle sample, 1M HCl, and no addition of CO_2, in order to understand the effect of a reduced pH on the microbial community. The pH examined in this control vessel was 4.4, the value that resulted from exposure to 1.4 MPa CO_2. The pH-only vessel was pressurized with 99.5% N_2/0.5% H_2 gas to 14 MPa and was maintained at 40 ˚C; sampling was performed by sacrificing vessel after 56 days of exposure to CO_2. The pH of the sacrificing vessel was measured to be 5.1 in the depressurized vessel at the end of the experiment.

An additional vessel was pressurized with 200 mL Mirando sample, 0.34 MPa pCO_2, and 10 g/L $CaCO_3$, in order to understand the impact of pH buffering by reservoir solids,. The vessel was maintained at a constant 40 ˚C temperature and 3.4 MPa total pressure, and sampling was performed by sacrificing vessel after 56 days.

Unfiltered fluid samples from the Plant Daniel freshwater aquifer were placed in 150 mL Swagelok vessels rated for 14 MPa. The 80 mL of Plant Daniel sample from well BG-1 was pressurized to 0.5 MPa using either 100% N_2, 1% CO_2/99% N_2, 10% CO_2/90% N_2, or 100% CO_2. In addition, in order to understand the effect of buffering on the aquifer system, a separate vessel was pressurized for 56 days with 80 mL formation fluid, 10 g/L $CaCO_3$ and pressurized with 10% CO_2/90% N_2 gas. The vessels were stored at room temperature, which closely resembles aquifer temperature. Sampling occurred after 56 days.

After sampling, the fluid was centrifuged at 4,000 g for 25 minutes. The top 75 ml of the supernatant was filtered for chemical analysis, and the bottom 5 ml of the fluid containing solids was used for the microbial community analysis described below.

6.3.3 Microbial Community Analysis

DNA was extracted from 500 µl of the remaining 5 ml of the centrifuged utilizing a modified method described previously in Holmes et al, 2004[7] for the Arbuckle samples,[8] and the

MoBio power soil kit for the Mirando and Plant Daniel samples. 16S rRNA gene clone library methods are described in Gulliver et al., 2014,[8] and Chapter 3-5. Briefly, bacterial 16S rRNA genes were amplified using the 8 forward primer with the 519 reverse primer and the 338 forward primer with the 907 reverse. A PCR reaction in the thermocycler was run for 30 cycles, and efficacy of the DNA extraction and PCR reaction was tested electrophoresis gel, using *E. coli* DNA as a Bacteria positive control and *methanococcus* as an Archaea positive control. No Archaea DNA was detected, and all further biological analysis was only performed utilizing methods for bacteria DNA.

For samples that did not amplify with this method, nested PCR was additionally used, first using the 8 forward primer with the 1114 reverse primer, followed by the amplification using the 338 forward primer with the 907 reverse primer. 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 necessary for the Abruckle 1.4 MPa vessel and the Arbuckle 14 MPa vessel, with amplification only occurring in the 1.4 MPa vessel.

The PCR products of the two bacterial primer sets were mixed, and cloned using the Invitrogen TOPO TA cloning kit according to the manufacturer's instructions. 96 clones per

Mirando and EPRI sample and 48 clones per Arbuckle sample were sequenced at Functional Biosciences (Madison, WI).

16S rRNA gene clone library sequences were trimmed using FinchTV, and pyrosequencing was trimmed using Mothur.[9] Chimeras were detected using Bellophon.[10] The sample OTU's were clustered in R utilizing the Canberra equation.[11] Sequences were clustered utilizing the weighted Unifrac equation in Mothur with an outlier of *Methanococcus*. A community tree was constructed in Mothur with the Yue and Clayton theta diversity measure, and displayed utilizing TreeViewX. Clustered sequences were then plotted on a weighted UniFrac plot.[12]

Bacteria 16S rRNA gene concentration were determined by quantitative real-time polymerase chain reaction (qPCR) described in Gulliver et al, 2014, and Ch. 3-5.[8] Briefly, qPCR procedures used the 1369 forward primer with the 1492 reverse primer and the TAMRA 6 FAM 1389 forward probe. Diluted samples of known concentrations of *E. coli* were used as standards and DNA concentrations were amplified utilizing qPCR thermocycler methods with 40 cycles.

6.3.4 Chemical Analysis (Gas and Liquid)

To determine if gas production or consumption was occurring, Arbuckle and Mirando samples from the pressure vessels were analyzed for H_2, CO_2, CH_4, O_2, 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 before any chemical analysis. Sample water was analyzed for selected elements (Ca, Fe, K, Mg, Mn, Na, P, S, Si, Zn) by ICP-OES (PerkinElmer Optima 7300 DV) using EPA method 6010C. Supernatant anions (Cl, NO_3, NO_2, PO_4, SO_4, Br) were also analyzed via a Dionex Ion Chromatograph using EPA method 300.1. Chemical components that were non-detect for all samples are not reported. Total organic carbon was measured in 10 mL triplicate volumes via 1010 Total Organic Carbon Analyzer. Alkalinity was measured by titration of 30 mL volumes with sulfuric acid to a pH of 4.5.

6.3.5 Water Chemistry Model

Since pH could not be directly measured in the pressurized reactors, it was estimated using Geochemist Workbench. The initial system was defined using the measured alkalinity and 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 real gas law using the pCO_2, the remaining 800 mL vessel volume, and site temperature. The gas compressibility factor was assumed to be 1 for all reactors except the 100% pCO_2 Arbuckle vessel and the 100% pCO_2 Mirando vessel. These vessels were calculated to have a compressibility factor of 0.27 and 0.83 respectively.

In order to compare the geochemistry of each sample, a distance matrix was calculated with R utilizing the Canberra equation.[11] Parameters used to calculate the distance matrix were site temperature, total pressure, alkalinity, pH, and concentrations of selected geochemistry (Ca, Fe, K, Mg, Mn, Na, P, Cl, SO_4, Br). An additional distance matrix was created for the OTU shared file also utilizing the Canberra equation. The geochemistry distance matrix and the OTU distance matrix were then compared with the Mantel equation to calculate a correlation coefficient. The Mantel equation was also used to compare the Unifrac distance matrix with the geochemistry distance matrix, but this correlation coefficient was not found to be significant (>0.1).

6.4 Results and Discussion

In order to identify trends in microbial community changes that occurred as a result of CO_2 exposure, results from vessel experiments containing different subsurface site water samples

were compared. The detected 16S gene copies/ml sample decreased with increasing pCO_2 across all three sites (Figure 6.1). This suggests that CO_2 exposure in all subsurface sites will result in greater loss in cell numbers closer to the injection/leakage point where CO_2 concentration would be highest. The Plant Daniel and Mirando samples exhibited higher 16S rRNA gene copy concentration in vessels with $CaCO_3$ buffering (i.e. higher pH) as compared to vessels without buffering at the same pCO_2 exposure. This suggests the decrease in DNA concentration was primarily due to the CO_2-driven pH decrease in these two waters.

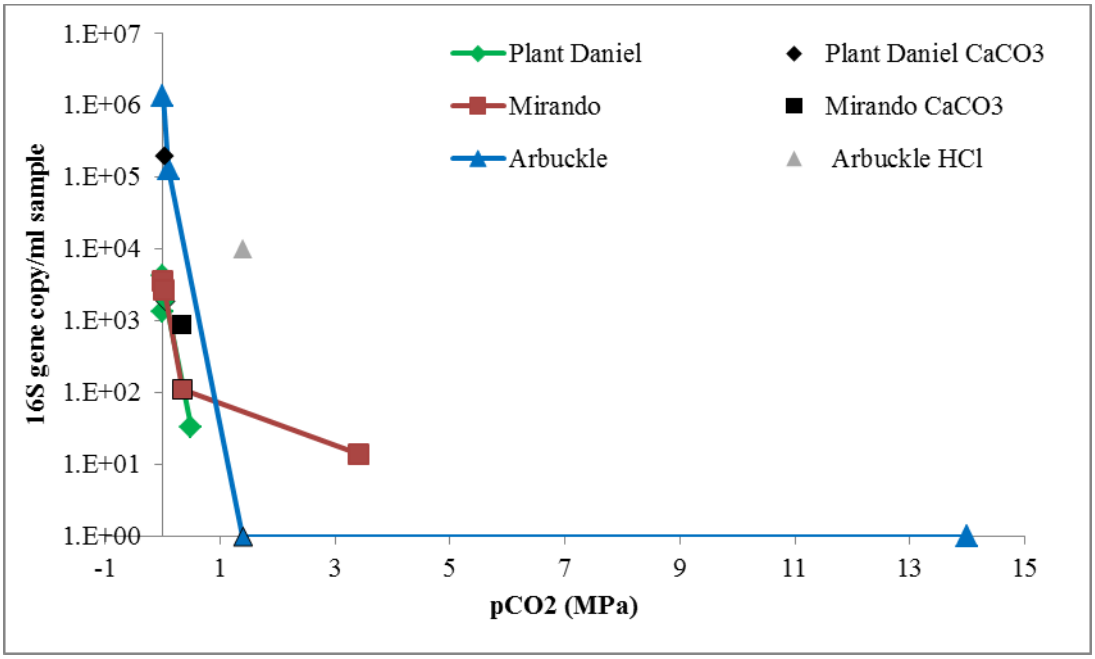

Figure 6.1. DNA concentration of the Plant Daniel freshwater aquifer, the Mirando depleted oil reservoir, and the Arbuckle saline aquifer versus 0-14 MPa pCO_2. Black data represent samples in presence of 0.05 MPa and 0.34 MPa pCO_2 and 10 g/L $CaCO_3$. The grey datum represents a sample examined to understand the effect of pH by adjusting the pH to 4.4 (initial) and 5.1 (final) with HCl in the absence of CO_2 exposure.

The decrease of 16S gene copies/ml sample detected with decreasing pH across all three sites is shown in Figure 6.2. All samples, including the $CaCO_3$ buffered samples show decreasing 16S gene copies/ml sample as pH is decreased. The effect appears greatest below about pH=5.5 to 6.5 for all sites. However, each site responds differently to this changing pH. Thus, there appears to be a site dependence of pH effect on DNA concentration. It is noteworthy that the Plant Daniel vessel with buffer increases 16S rRNA gene copy concentration by 2 orders of magnitude compared to unbuffered reactors. In this case, it appears the $CaCO_3$ buffer promoted growth within the Plant Daniel microbial community. This result might have been due to an emergence of a microbial community that benefitted from the addition of $CaCO_3$ (discussed in more detail in Ch. 5). Regardless, the results across all three sites suggest a buffered aquifer or reservoir will reduce the largely pH-driven effects of CO_2 exposure on the microbial community.

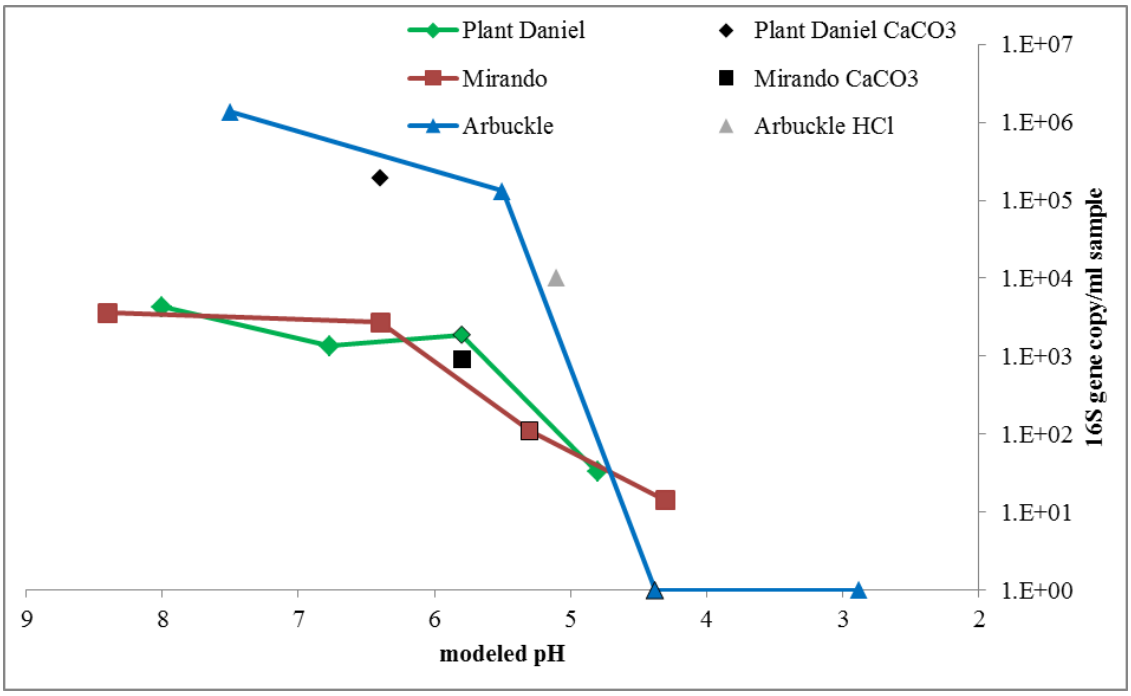

Figure 6.2. DNA concentration of the Plant Daniel freshwater aquifer, the Mirando depleted oil reservoir, and the Arbuckle saline aquifer measured by qPCR for modeled pH. Black data represent samples in presence of 0.05 MPa and 0.34 MPa pCO_2 and 10 g/L $CaCO_3$. The grey datum represents a sample examined to understand the effect of pH by adjusting the pH to 4.4 (initial) and 5.1 (final) with HCl in the absence of CO_2 exposure.

The result that microbial growth is highly dependent on the system pH contrasts previous findings that CO_2 exposure is more lethal to microorganisms than a pH reduction alone. 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] Wu et al. suggests that aqueous CO_2 is the toxic condition affecting microorganism while our results suggest that the reduced pH is primarily responsible for the observed decrease in 16S copies/mL. The pure *Shewanella oneidensis* culture utilized by Wu et. al. may have been sensitive to CO_2

exposure, but more tolerant of pH reduction. Here, the overall mixed community (without a detectable population of *Shewanella*) was found to be sensitive to pH below pH=5.5. This suggests that a CO_2 exposure in a buffered system may allow microbial community survival despite a CO_2 driven pH reduction. However, unlimited carbonate minerals may still result in a buffered pH as low as pH= 5, depending on the subsurface system. In these subsurface systems, CO_2 exposure may still lead to a reduced microbial community.

In order to visualize the magnitude of phylogenic differences between each sample, a distance matrix was made, and a UniFrac plot was created with a principle component analysis, using PC1 and PC2 as the x and y axis respectively. The Unifrac plot (Figure 6.3) clearly demonstrates that the change in microbial community due to CO_2 exposure is affected by site specific conditions. Generally, sequences clustered closest to other sequences from the same site as opposed to clustering with pH or with pCO_2 exposure. This suggests the change of microbial community with increasing CO_2 exposure will be different among various subsurface sites, and no clear trends emerged regarding a dominant microbial community. Each site had different conditions such as pressure, temperature, and dissolved ion concentrations (Table D.1-D.3, Appendix D), and the results are indicative of the strong influence of site conditions on microbial communities.

In addition, there appears to be large difference in initial microbial communities between each site (Figure 6.3). Differing initial microbial communities are expected in future geologic carbon storage sites and impacted aquifers. For example, Lavalleur et. al. characterized the initial microbial community with water samples from Columbia River Basalt, a future geologic carbon storage unit.[14] In this study, the dominant genera that was detected included *Hydrogenophaga*, *Geoalkalibacter*, *Thiovirga*, *Methylotenera*, *Methylomonas*, and *Methermicoccus*. The dominant genera detected by Lavalleur et. al. where not similar to the dominant genera detected in any of the initial microbial communities from the Arbuckle aquifer samples, the Mirando oil field samples, or the EPRI freshwater samples. The varying initial microbial communities likely contributed to an emergence of unique microbial ecologies at each site.

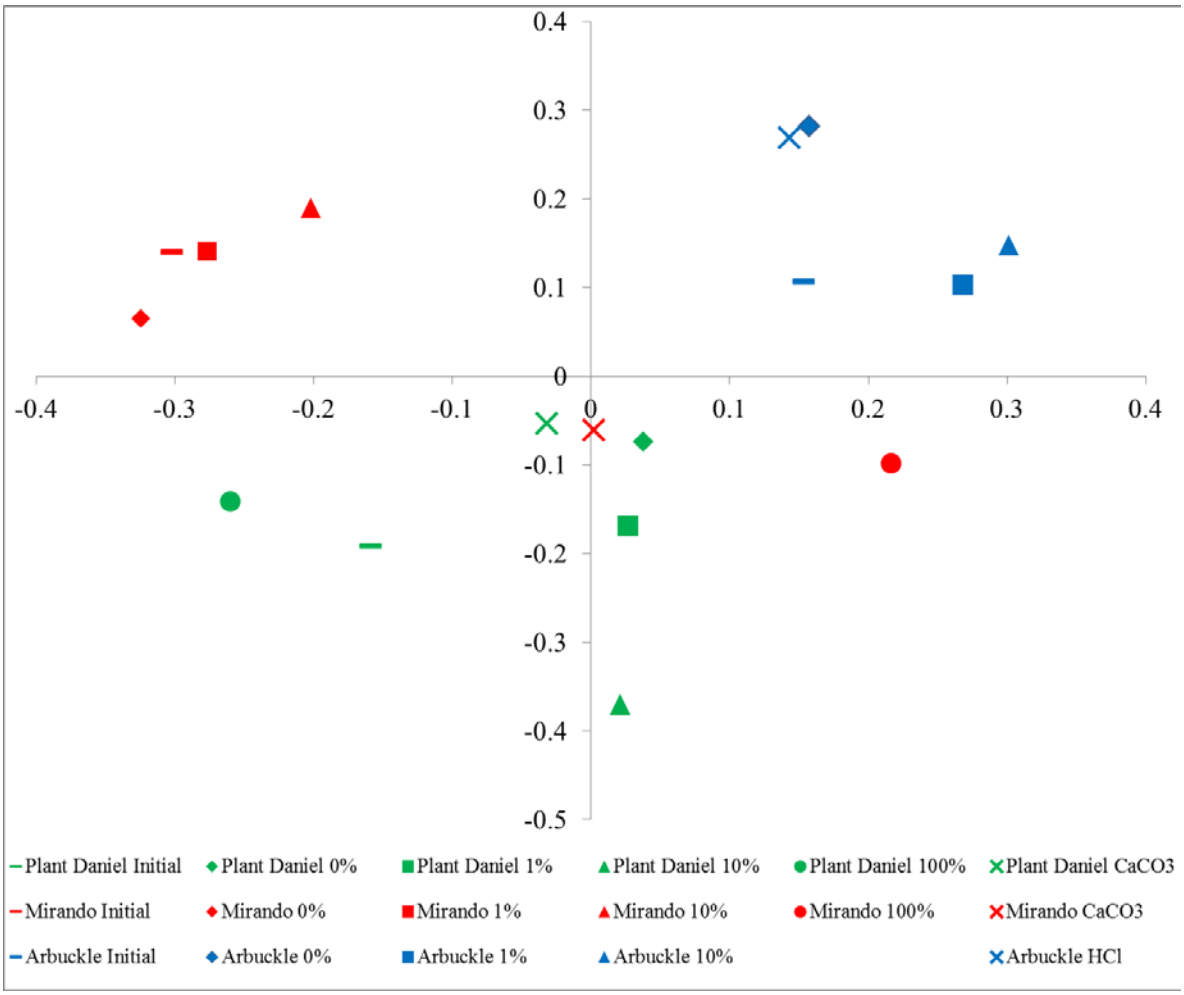

Figure 6.3. Unifrac plot of sequences recovered from vessel experiments with Plant Daniel freshwater aquifer sample, Mirando oil reservoir sample, and Arbuckle saline aquifer sample.

Previous research by Wandrey et. al. conducted a similar pressure vessel experiment, in which the microbial community from a deep saline aquifer sediment samples was exposed to 5.5 MPa pCO_2 for 24.5 months at a reservoir temperature of 40 ˚C.[15, 16] In this experiment, the genera *Rhizobium* and *Bulkholderia* emerged as dominant. These genera were not found to emerge in the deep saline aquifer studied here (the Arbuckle aquifer). In addition, although the

Wandrey et. al exposure experiment conditions were similar to the Mirando oil field exposure experiments (P = 3.4 MPa, T = 40 ˚C), none of the emerging genera in the Mirando oil field samples appeared to be similar to *Rhizobium* or *Bulkholderia*.

In another study, Frerich et. al. exposed the microbial community from natural gas field fluid samples to 10 MPa CO_2 for 30 days at a temperature of 50 ˚C. In this study, spore forming *Clostridiales* emerged as CO_2-tolerant.[17] The Frerich et. al. exposure experiment conditions were similar to the Arbuckle aquifer exposure experiments (P = 14 MPa, T = 40 ˚C). However, *Clostridiales* did not appear to emerge in the CO_2 exposed Arbuckle aquifer samples. Nor did this genus appear to emerge in the CO_2 exposed Mirando oil field samples.

The genera detected by Wandrey et. al. and Frerich et. al. were not found to dominate any of the samples in this study, despite some similarities in site conditions.[16, 17] In addition, the genera that emerged in each study appeared to be a unique to the formation and conditions used in that study. This further suggests the emerging microbial community in CO_2 exposed environments will be site dependent. The site dependent results suggest that the results determined in this thesis for the three selected sites may not provide general predictions of the dominant microbial species expected to emerge at CO_2 exposed subsurface sites.

It is interesting to note that the two $CaCO_3$ buffered vessels, one with Plant Daniel sample and one with Mirando sample, had sequences that clustered closely together. As

described in Chapter 4 and Chapter 5, the microbial community was dominated by 24%-64% *Betaproteobacteria* in both of these buffered vessel samples. This suggests that buffered subsurface sites could have similar microbial communities emerge after CO_2 exposure. However, the addition of $CaCO_3$ in the buffered vessels may have allowed a preferential emergence of microorganisms that would not occur in subsurface aquifers buffered by other accessory minerals. Additional research is warranted to determine if a predictable microbial community will arise in buffered subsurface conditions with CO_2 exposure.

While most of the sequences clustered by site, the Mirando 100% pCO_2 vessel was an outlier from the remaining Mirando sequences. Reasons for this behavior are not clear. However, the Mirando 100% pCO_2 sample contained a very low amount of biomass (10^1 gene copies/ml sample), and the community in these conditions were merely surviving rather than thriving. The ability of this surviving but unique microbial community to affect injectivity and CO_2 storage permanence warrants further study.

The community tree similarly displays the DNA sequences clustered closely by site (Figure 6.4). Due to more similar pressure and temperature conditions, it was expected that the Mirando sequences would cluster closer with the Arbuckle sequences when compared to the Plant Daniel sequences. Both the Mirando reservoir and Arbuckle aquifer are saline (11-68 ‰ salinity) and have a reservoir temperature of 40 ˚C. Plant Daniel is a freshwater reservoir (0.07

‰ salinity) with a reservoir temperature of 22 ˚C (Table D1, Appendix D). Contrary to this expectation, the sequences from the Plant Daniel aquifer and the Mirando oil reservoir cluster more closely to each other than to the Arbuckle sequences (Figure 4). The Arbuckle aquifer is the deepest of the subsurface reservoirs, at 1220 m-1460 m depth and 14 MPa pressure compared to the Mirando (490 m -610 m and 3.4 MPa) and the Plant Daniel (55 m and 0.5 MPa). The difference in pressure and depth between the Arbuckle aquifer and the other sites likely contributed to the difference in the initial and evolved Arbuckle microbial community. The results demonstrate the difficulty in predicting which site conditions will most impact the emerging microbial communities with CO_2 exposure.

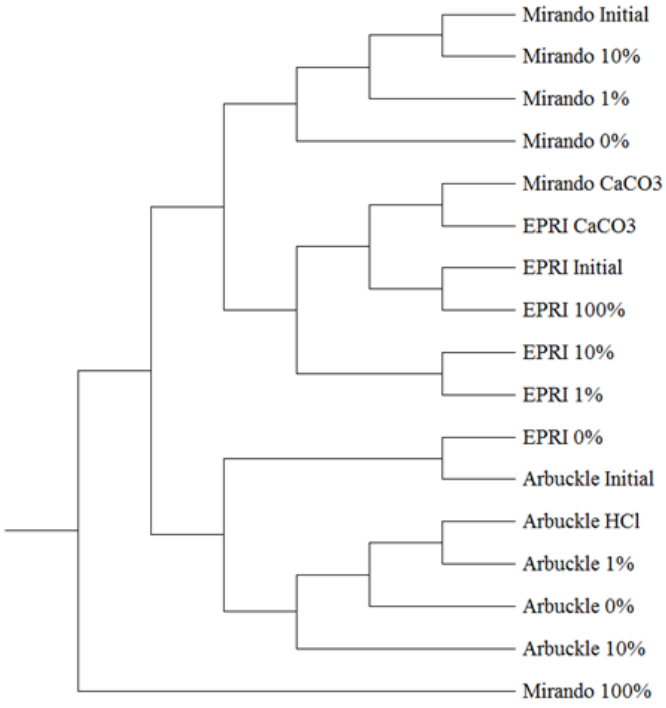

Figure 6.4. Community tree calculated by Yue and Clayton theta diversity index of sequences recovered from vessel experiments with Plant Daniel freshwater aquifer sample, Mirando oil reservoir sample, and Arbuckle saline aquifer sample.

The site dependence on the geochemistry is illustrated in Figure 6.5. Similar to the site-clustering illustrated for DNA sequences in the Unifrac plot (Figure 6.3) and microbial community tree (Figure 6.4), the geochemistry of each sample most closely clusters to samples from the same site. Although both the DNA sequences and the geochemistry appears to cluster closely by site, comparison of the distance matrix calculated by OTU and distance matrix calculated for geochemistry results in a correlation coefficient of 0.55. This suggests that the correlation between the changes in the microbial community and changes in the geochemistry is only slightly positive. The low correlation coefficient despite obvious site clustering for both distance matrices suggest a difficulty in comparing DNA sequencing models to geochemistry models. However, the site dependence of the emerging microbial communities may be closer correlated to the initial microbial community, rather than geochemical parameters.

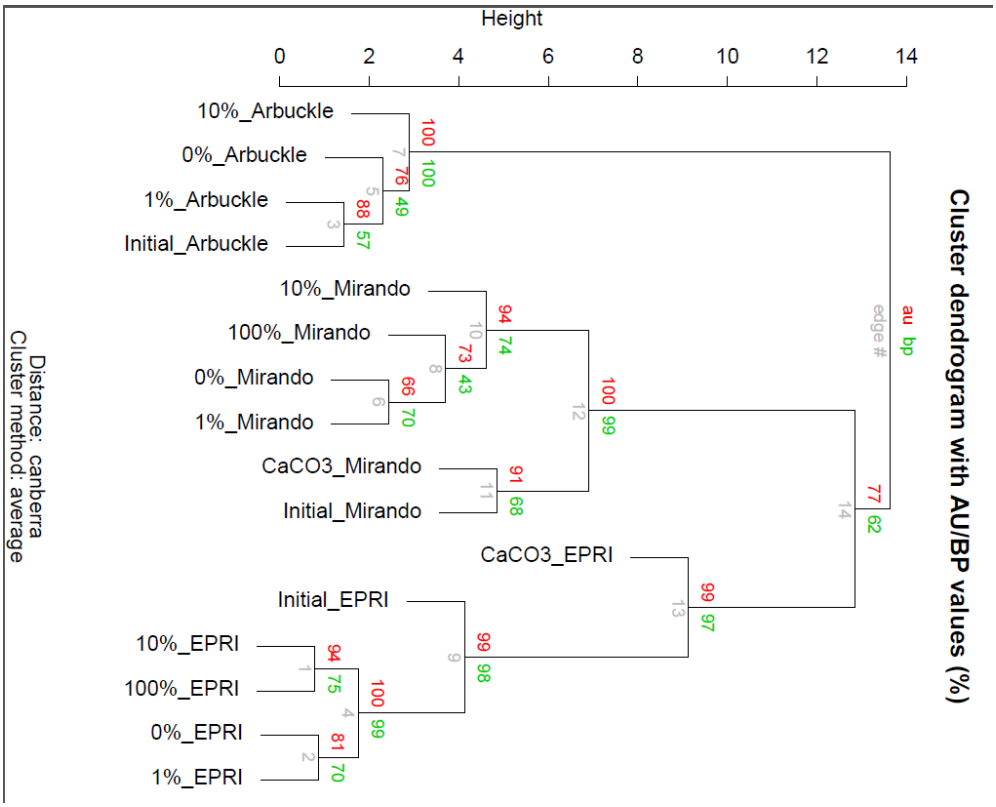

Figure 6.5. Dendogram of distance matrix calculated for the geochemistry of each sample utilizing the canberra equation.

This analysis provides some initial understanding of the effect of CO_2 injection or CO_2 leakage on microbial communities in selected environments. Microbial communities from a saline aquifer, a depleted oil reservoir, and a freshwater aquifer were exposed to increasing CO_2 concentrations and characterized with qPCR and 16S rRNA gene clone libraries. 16S rRNA gene copy concentrations decreased with increasing CO_2 exposure across all three sites. The trend in decreasing DNA concentration was found to be highly dependent on pH, with reduced pH resulting in a lower number of extractable 16S gene copies. Sequences from the dominant microbial communities at various CO_2 exposures appeared to cluster by site, suggesting the

adapted microbial community that emerges during carbon storage or CO_2 leakage will be site/condition dependent. Overall, the results imply that exposure to CO_2 will result in pH-dependent decreased microbial growth, but that no trend in the emerging microbial species can be expected.

Due to the dependence of this study on difficult to obtain subsurface samples, only three sites were characterized and compared with CO_2 exposure. Additionally, the effect of CO_2 on microbial communities under geologic carbon storage conditions has only been published for two alternate sites (Wandrey et. al. and Frerich et. al.)[16, 17] not studied in this thesis. Samples from additional subsurface sites, or published results on similar experiments would have allowed a more thorough comparison study and further illuminated trends to be expected after CO_2 injection or leakage. Further research on the effect of CO_2 on subsurface communities is warranted.

6.5 Supporting Information in Appendix D

Supporting information contains Table D.1, Table D.2, and Table D.3. References have been cited in the main text for figures and tables with an overview of material presented in the supporting information.

6.6 References

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Chapter 7

Conclusions, Research Implications, and Future Work

The goal of this dissertation was to characterize the change of microbial communities with CO_2 exposures that can be expected during carbon storage in a deep saline aquifer and a depleted oil reservoir, and resulting from CO_2 leakage into an overlying freshwater aquifer. It was hypothesized that CO_2 exposure in the subsurface would reduce both the microbial population numbers and diversity of subsurface microbial communities. The results from this dissertation suggests that high CO_2 concentrations expected near the CO_2 injection plume or leakage plume will lead to a reduced microbial population, and that a unique site-specific microbial community is expected to arise at locations away from the CO_2 source where the CO_2 concentration is diminished.

7.1 Conclusions

The first objective of this dissertation was to define the microbial community changes and populations that arise following CO_2 exposure under GCS conditions on representative subsurface storage unit samples. This objective was met via pressure vessel experiments discussed in Chapter 3 and Chapter 4. The second objective of this dissertation was to define the microbial community changes and populations that arise following CO_2 exposure under CO_2 leakage conditions on potential overlying freshwater aquifers. This objective was met via pressure vessel experiments discussed in Chapter 5. The third objective of this dissertation was to determine if changes in the microbial communities and populations that arise in these environments are similar or vary among the subsurface sites. This objective was met via microbial community modeling comparisons and is discussed in Chapter 6.

In Chapter 3, the effect of CO_2 exposure on the microbial community of samples from the Arbuckle saline aquifer was characterized. Increased CO_2 concentrations and exposure time decreased both the microbial population and the diversity. The findings suggest that close to the CO_2 injection plume, biological processes may be significantly hindered or absent. However, downgradient from the injection plume, where cells will be exposed to low CO_2 concentrations, microorganisms may still thrive and microbial processes that can potentially impact storage properties will likely be retained. Sequences recovered in environments with CO_2 exposures less than 0.34 MPa pCO_2 were most closely similar to the halotolerant genus *Halomonas* and *Marinobacter*. These genera may play a role in microbial processes such as biomineralization, biofilm production, or acid production, which may affect the CO_2 storage capacity and CO_2 storage security of the Arbuckle aquifer downgradient from the CO_2 injection plume.

In Chapter 4, the effect of CO_2 exposure on the microbial community of samples from the Mirando depleted oil reservoir was characterized. Increased CO_2 exposure resulted in a decreased microbial population. This again suggests that microbial processes would be significantly hindered or absent close to the injection plume, but microbial processes would be retained at a downgradient distance. In environments with pCO_2 exposures less than 0.05 MPa, greater fractions of sequences were recovered that were most similar to known *Pseudomonas* and *Escherichia* genera. While it is uncertain what role organisms from these taxa may play, they are well known to inhabit a variety of subsurface environments and may participate in important microbial processes such as biofilm production, acid production, and metal mobility, which may affect the petroleum quality and petroleum production in the Mirando oil reservoir downgradient from the CO_2 injection plume.

In Chapter 5, the effect of CO_2 exposure on the microbial community of samples from the Plant Daniel freshwater aquifer was characterized, simulating a CO_2 leakage scenario. The microbial population remained unaffected at CO_2 exposures below 0.05 MPa but decreased at CO_2 exposures higher than 0.5 MPa. This suggests that microbial processes will be retained throughout most of the freshwater aquifer, but will be hindered at closest to the leakage plume where concentrations are expected to be greater than 0.5 MPa. In environments with pCO_2 exposures less than 0.05 MPa, sequences recovered were closely similar to *Curvibacter* and *Pseudomonas*. These genera have been shown to affect metal mobility in the subsurface, which may affect the water quality of the impacted freshwater aquifer downgradient from the CO_2 leakage plume.

In Chapter 6, the effects of CO_2 on the microbial communities of samples from the Arbuckle saline aquifer, the Mirando depleted oil reservoir, and the Plant Daniel freshwater aquifer were compared. Across all three sites, increasing CO_2 exposures led to a decreased detection of 16S rRNA gene copies, suggesting a reduction in microbial population. The microbial population appeared to be most affected by CO_2-driven pH reduction, with a decreased pH resulting in a reduced microbial population. The important effect of pH on microbial populations was confirmed in experiments using $CaCO_3$ to buffer pH while still being exposed to CO_2. Sequences at various CO_2 exposures appeared to cluster by site, suggesting that the adapted microbial community that emerges during carbon storage or CO_2 leakage will be site dependent. Overall, the sum of the body of work indicates that exposures to CO_2 will result in decreased microbial growth, but no trend in the emerging microorganisms can be predicted as they are site specific.

This is one of few sets of experiments that characterize the change in microbial communities with increasing CO_2 concentrations using difficult to obtain subsurface samples. One major contribution of this dissertation is a catalogue of microorganisms that appear to thrive in these poorly understood ecosystems. A second major contribution of this dissertation is a direct comparison of the changes in the CO_2 exposed microbial communities from different but important subsurface samples representing three types of sites that may be impacted by CO_2 injection. Through this comparison, results suggest for the first time that the effect of CO_2 concentration on subsurface microbial communities is highly dependent on the resulting pH of the system. The comparison of the results also suggests the microbial communities that may emerge in CO_2 exposed environments will be site specific rather than predictable based solely on the geochemical conditions of the site.

7.2 Research Implications

Current research in the field of microbial communities in carbon storage environments investigate the ecological change at high CO_2 exposures, addressing the deleterious biogeochemical changes that occur closest to the CO_2 injection or leakage plumes. Similar to this current research, this dissertation also focused on the changes in the microbial community at high CO_2 exposures that may occur closest to the CO_2 injection or leakage plume. However, this dissertation also studies the microbial changes at low CO_2 exposures that may impact biogeochemical processes at a downgradient distance where the CO_2 concentration is attenuated.

The work in this dissertation suggests that high CO_2 concentrations near this injection or leakage plume will result in a decreased microbial population. The biological processes that

occur at these CO_2 exposures will therefore be significantly hindered or absent, and factors affecting reservoir properties or water quality closest to the CO_2 plume will be driven by the change in geochemistry or geophysics. However, microbial communities were found to survive and sometimes thrive at CO_2 concentrations expected at a downgradient distance from the CO_2 leakage or injection plume. At this down gradient distance, the change in microbial communities may impact the types and rates of biogeochemical processes that affect long-term carbon storage capacity, carbon storage security, and subsurface water quality.

Microbial processes such as biofilm formation and biomineralization are currently being researched as a mitigation strategy for reducing the porosity of leakage pathways in carbon storage environments.[1-4] These studies currently utilize cultured experiments, and demonstrate that biofilm production and biomineralization may reduce reservoir porosity by up to 99%.[1-4] However, results from this dissertation demonstrates CO_2 exposure will lead to a reduction of the microbial population regardless of the subsurface site and resulting pH, suggesting that microbial processes near the CO_2 injection plume or CO_2 leakage plume will be significantly hindered or absent. While the cultured environment demonstrates that microbial processes may be a viable solution to reducing leakage pathways, this study suggests that the microbial community is not likely to survive the CO_2 exposure near the plume, or where a leakage plume is likely to occur. An alternate mitigation method of leakage prevention will be needed at distances closest to the CO_2 plume.

While most of this dissertation focuses on the microorganism that emerge as dominant in the community after CO_2 exposure, the disappearance of microorganisms with CO_2 exposure also may affect biogeochemical properties of the subsurface. For example, the sulfate reducing genera, *Desulfuromonas* and *Desulfovibrio* appeared to represent a 7-10% of the non-CO_2-

exposed Arbuckle saline aquifer samples, but disappeared with just 1% pCO_2 exposure. This suggests that the geochemical cycling of sulfur in the saline aquifer may be affected since these S-cycling microorganisms will not survive CO_2 injection. Similarly, the fermentative genera, *Flexistipes*, appeared to represent 20% of the non-CO_2-exposed Mirando oil reservoir samples, and *Clostridiales* and *Flavobacteriaceae* appeared to represent 24% and 16% of the non-CO_2 exposed Plant Daniel freshwater samples. These genera also appeared to disappear with 1% pCO_2 exposure. These microorganisms may affect carbon cycling and metal chemistry within the subsurface units. The disappearance of these microorganisms after CO_2 injection/leakage will greatly inhibit or stop those existing microbial processes which could affect formation porosity and water quality.

The lethal effect of CO_2 on microbial communities demonstrated in this dissertation has beneficial implications for the oil and gas industry. The oil and gas industry has long researched mitigation strategies to prevent biologically induced corrosion and biological hydrogen sulfide production.[5, 6] The current mitigation strategy for these deleterious biological reactions is the utilization of chemical biocides or induced microbial competition, both of which have had limited success. The work in this dissertation suggests that microbial growth in oil reservoirs will be hindered with 1% pCO_2 exposure, and absent with 10% pCO_2 exposure. These results suggest CO_2 exposure, such as SC-CO_2 injection for Enhanced Oil Recovery, may be an effective alternative mitigation strategy for deleterious microbial processes in the oil and gas industry.

This work utilized difficult to obtain subsurface samples, allowing a rare opportunity to characterize the microbial communities of currently poorly understood environmental microbial niches. The field of geomicrobiology currently researches these microbial niches which are

often are inhabited by unique microorganisms tolerant to harsh conditions such as low nutrient concentrations, high temperature, high pressure, and high salinity. Microbial communities that have evolved to adapt to these conditions may demonstrate the ability to become tolerant of other poorly understood niches, such as deep marine subsurface sediments. The microbial communities characterized in this dissertation add to a catalogue of microbial communities characterized for subsurface environments, and may therefore improve understanding of adaptable, stress-tolerant microorganisms in the field of geomicrobiology.

7.3 Future Work

The work in this dissertation demonstrates the emerging microbial communities with CO_2 exposure to be unique to each site. This suggests that other types of carbon storage environments and impacted aquifers may not lead to the emergence of predictable microbial communities based on the research in this thesis. The three sites studied in this dissertation may not yet be a sufficient number of sites to identify trends that may be applied to other types of carbon storage sites and impacted aquifer. Additional research may support the site dependent results of this dissertation, implying the emerging microbial community of every new CO_2 exposed environment will need to be analyzed as unique. However, the inclusion of additional sites from these same types of reservoirs may demonstrate a trend in the emerging microbial communities of CO_2 exposed environments, which may then be applied to other carbon storage and CO_2 leakage sites. It remains to be seen if all saline sites, or all depleted oil reservoirs may behave more similarly to each other, than across sites types as studied here.

Once the emerging microbial community in CO_2 exposed environments have been characterized, future work should focus on understanding the metabolic pathways and secondary

biproducts of these microorganisms. Laboratory experiments should utilize detailed geochemical analysis to measure the effect of CO_2 tolerant microbial communities on water chemistry properties, such as the rate of metal ion dissolution or rate of metal ion precipitation, in order to understand the biogeochemical processes of relevant biota for geologic carbon storage or CO_2 leakage. This will increase understanding of how an altered microbial community may affect the biogeochemical processes post CO_2 injection or post CO_2 leakage.

The work presented in this dissertation relies largely on laboratory based experimental results. While best efforts were made to simulate subsurface conditions, some conditions, such as the effect of rock matrix on pH, were not replicated. As subsurface carbon storage becomes a more common practice, opportunities to collect *in situ* water and rock samples of CO_2 exposed microbial communities may arise. Future increased availability of *in situ* water and sediment samples will also allow investigation of the effect of long-term CO_2 exposure on microbial communities, without the need to account for nutrient depletion or microbial product buildup that may occur in a reactor. Characterization of these *in situ* samples will further increase understanding of relevant microbial communities that will emerge post-CO_2 injection. This future work would compare changes in geochemistry, mineralogy, and microbiology of CO_2 exposed subsurface sites. This would allow a detailed analysis of changes in biogeochemical properties that occur in carbon storage sites and impacted aquifers.

Although results from this dissertation suggests each emerging microbial community will be site dependent, it is unknown whether the adaptable microorganisms among the sites shared a trend in functional genes. For example, the increasing CO_2 may have allowed the emergence of microorganisms that varied taxonomically, but were similar metabolically. A metagenomic analysis of the DNA isolated from each site would demonstrate if any trends in functional genes

emerged with increasing CO_2 exposure. Metatranscriptomic analysis could also be utilized to detect the active functional genes; this would increase understanding microbial evolution with CO_2 exposure. Metagenomic and metatranscriptomic analysis of the emerging microbial communities in CO_2 exposed environments would also allow an investigation of potential metabolic products and secondary byproducts that may be produced during carbon storage or CO_2 leakage. These analyses will increase the understanding in microbial processes that may occur in carbon storage sites and CO_2 leakage sites, which may then increase understanding of how the emerging microbial communities will impact biogeochemical processes after CO_2 injection or CO_2 leakage.

This dissertation suggests CO_2 exposure will result in a decrease in microbial population near the CO_2 injection plume. This result has practical significance to the oil and gas industry, as CO_2 exposure may reduce microbial communities causing biologically induced corrosion and H_2S production. Investigation of the effect of increasing CO_2 exposure on microorganisms previously responsible for these deleterious biological processes, such as *Desulfovibrio*, *Desulfomonas*, and *Clostridia*[5, 6] would provide further evidence that CO_2 exposure may be an effective mitigation method for reducing biological corrosion and biological souring.

7.4 References

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Appendix A

Supporting Information for Chapter 3: Effects of $CO_{2(aq)}$ Concentration on Microbial Community from Saline Aquifer Samples under Geologic Carbon Storage Conditions

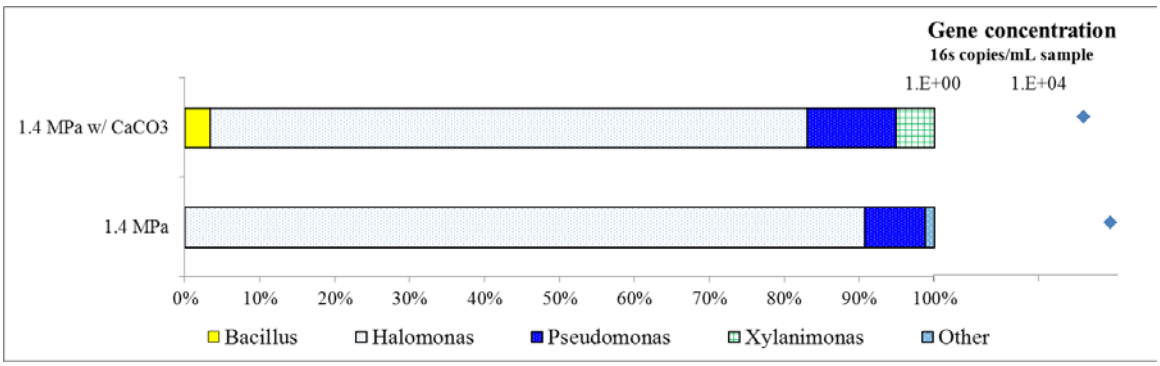

Figure A.1. In order to better understand the effect of buffering on the CO_2-exposed microbial ecology, two additional reactors were pressurized for 7 days with 1.4 MPa CO_2; one reactor only contained initial sample and one reactor contained initial sample with 1 g/L $CaCO_3$. The non-buffered reactor was modelled to have a pH of 4.4, and the buffered reactor was modelled to have a pH of 5.0. However, the initial sample used for these experiments had a long hold time (over 1.5 years) in the 4 ˚C refrigerator. For this reason, this stored initial water must be discussed as a different sample, as the initial microbial ecology had changed during the hold time. The effect of CO_2 exposure was similar in the absence and presence of calcite to buffer against pH shifts. The absence of *Marinobacter* in these two experiments is assumed to be due to the storage time of the initial sample. The diversity appeared decreased compared to the initial sample in both reactors. Communities from both reactors were overwhelmingly *Halomonas*. Gene concentration was similar between reactors.

Table A.1. Good's coverage of 1 day, 7 day, and 56 day incubations for 0 MPa, 0.1 MPa, 1.4 MPa, and 14 MPa pCO_2 exposures. Good's coverage was calculated in Mothur (Schloss, 2009). Values indicate sufficient coverage given the very low diversity in the samples.

	1 day	7 days	56 days
0 MPa CO_2	0.68	0.88	0.83
0.1 MPa CO_2	0.58	0.86	0.87
1.4 MPa CO_2	0.67	0.81	0.85
14 MPa CO_2	0.73	0.90	N/A

Appendix B

Supporting Information for Chapter 4: Effects of $CO_{2(aq)}$ Concentration on Microbial Community from Depleted Oil Reservoir Samples under Geologic Carbon Storage Conditions

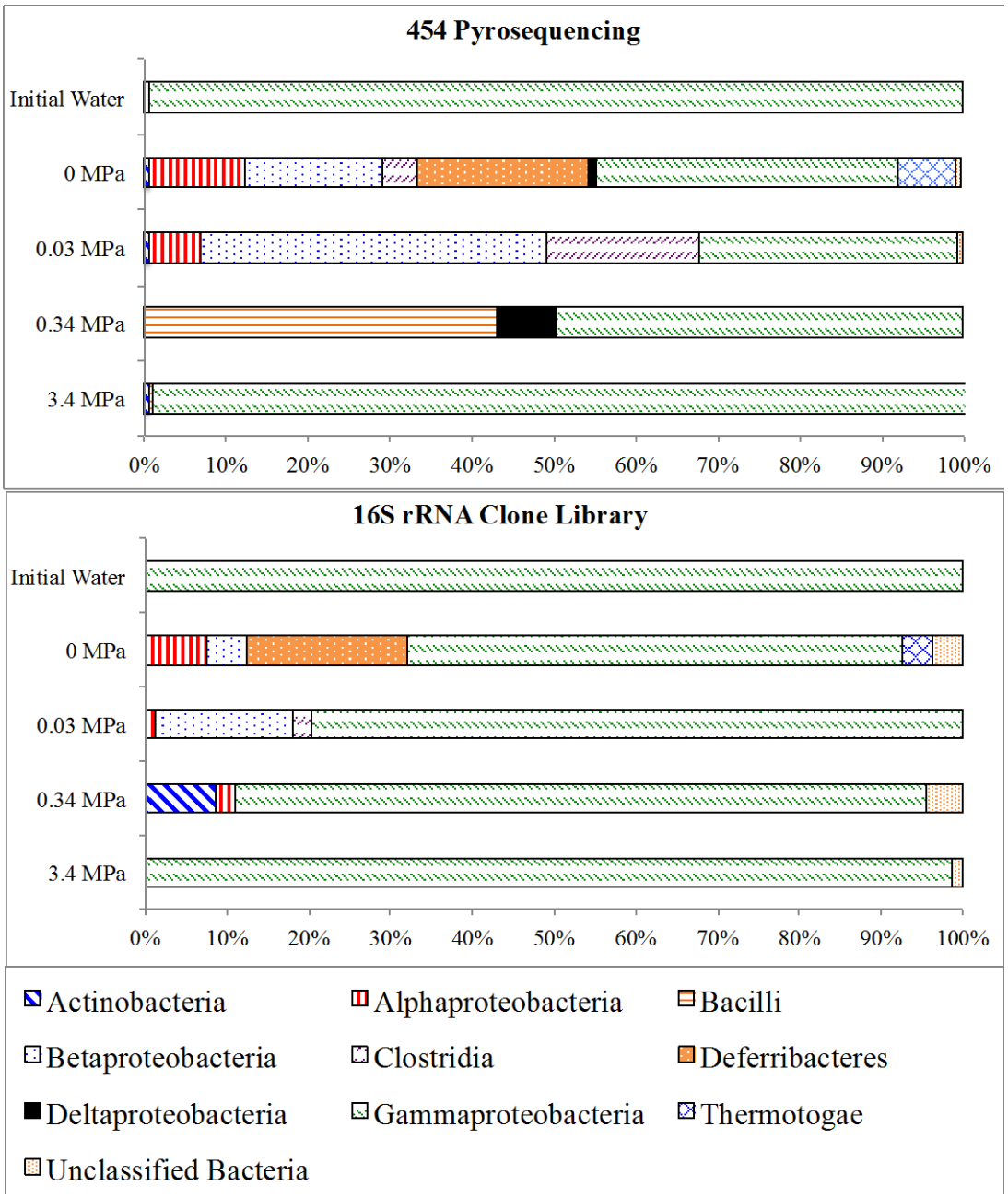

Figure B.1. Comparison of 16S rRNA gene clone libraries and 454 pyrosequencing of relative proportions of phylotypes recovered from reactors exposing unfiltered produced water samples to increasing pCO_2 for 56 days. OTUs were assigned an order based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "unclassified bacteria"

Appendix C

Supporting Information for Chapter 5: Effects of $CO_{2(aq)}$ Concentration on a Microbial Community from Freshwater Aquifer Samples under Simulated CO_2 Leakage Scenarios

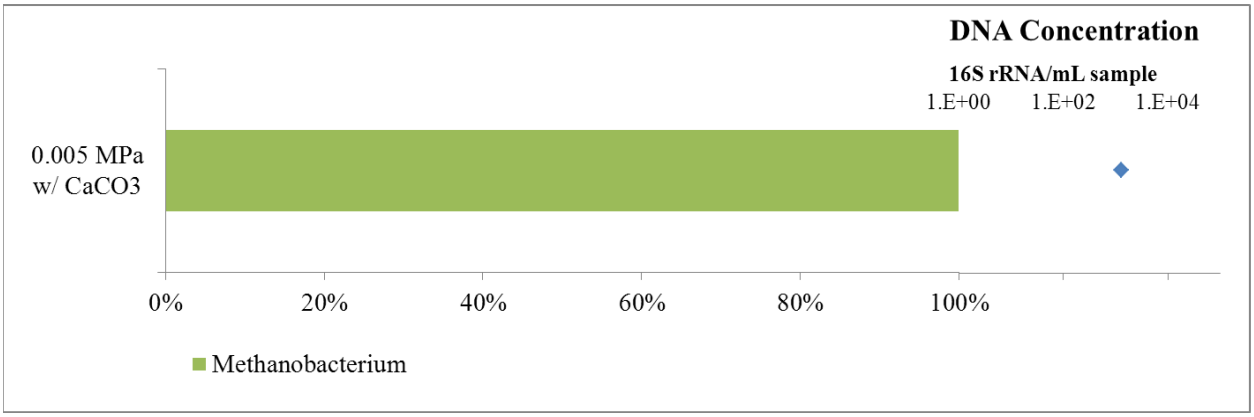

Figure C.1. Microbial ecology of Archaea characterized by 16S rRNA gene clone libraries and microbial population quantified by qPCR methods . Archaea was only detectable for 0.05 MPa pCO_2 with $CaCO_3$ buffer after 56 days of incubation. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

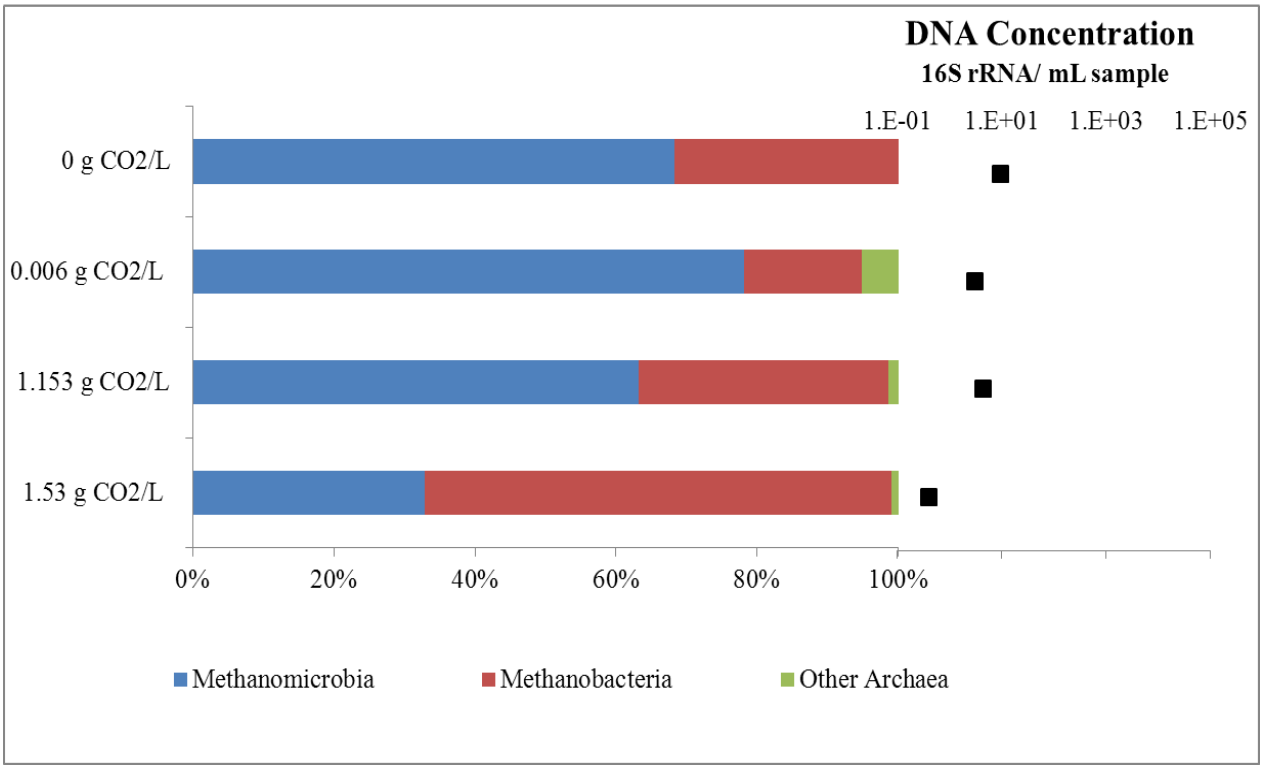

Figure C.2. Relative proportions of Archaea recovered from unfiltered well samples downgradient of CO_2-saturated-water injection well. Dissolved CO_2 of each well was measured by CarboQC and microbial community was revealed by 454 pyrosequencing and qPCR.

Appendix D

Supporting Information for Chapter 6: Comparison of the effect of CO_2 concentration on microbial communities from a saline aquifer, a depleted oil reservoir, and a freshwater aquifer

Table D.1. ICP-OES, IC-Anion, and TOC data with modeled pH for Plant Daniel freshwater aquifer vessel experiments. IC = inhibited by carbonate, nd = non-detect, -- = not measured

		Plant Daniel					
	Units	Initial	0 MPa pCO_2	0.005 MPa pCO_2	0.05 MPa pCO_2	0.5 MPa pCO_2	0.05 MPa pCO_2 w/ $CaCO_3$
Ca	mg/L	2.9	2.9	2.9	2.9	3.0	350.6
Fe	mg/L	0.25	1.0	2.5	2.0	2.3	1.0
K	mg/L	8.2	7.5	7.4	7.5	7.5	9.0
Mg	mg/L	0.9	1.0	1.0	1.0	1.0	nd
Mn	mg/L	0.1	0.1	0.1	0.1	0.1	0.0
Na	mg/L	168	161.3	160.3	164.1	161.9	200.4
P	mg/L	0.1	0.1	0.1	0.1	0.1	nd
S	mg/L	nd	0.0	0.0	0.0	0.0	7.3
Si	mg/L	9.6	19.6	19.6	19.3	19.6	33.4
Zn	mg/L	nd	nd	nd	nd	nd	0.3
F	mg/L	nd	19.5	26.2	1.4	0.7	nd
Cl	mg/L	46	36.2	36.7	43.7	37.0	58.0
SO4	mg/L	17	IC	IC	IC	IC	nd
Br	mg/L	nd	IC	IC	IC	IC	nd
TOC	mg/L		12.6	22.7	21.3	47.9	
Alkalinity	mg $CaCO_3$/L	291.8	--	--	--	--	--
Modeled pH		8.2	8	6.77	5.8	4.8	6.4

Table D2. ICP-OES, IC-Anion, and TOC data with modeled pH for Mirando depleted oil reservoir vessel experiments. IC = inhibited by carbonate, nd = non-detect, -- = not measured

		Mirando					
	Units	Initial	0 MPa pCO_2	0.03 MPa pCO_2	0.34 MPa pCO_2	3.4 MPa pCO_2	0.34 MPa pCO_2 w/ $CaCO_3$
Ca	mg/L	23.8	19.52	27.11	59.46	14.85	63.2
Fe	mg/L	nd	0.015	0.024	nd	nd	nd
K	mg/L	52.5	22.5	20.86	20.83	20.91	15.47
Mg	mg/L	nd	11.16	10.72	10.71	10.57	11.23
Mn	mg/L	nd	0.028	0.011	0.24	nd	nd
Na	mg/L	3888.0	4030	3803	3789	3816	3888
P	mg/L	nd	0.028	nd	0.034	0.023	nd
S	mg/L	nd	0.633	0.533	1.962	0.702	nd
Si	mg/L	24.5	24.46	24.18	25.02	25	26.5
Zn	mg/L	nd	nd	nd	nd	nd	0.068
F	mg/L	nd	1.2	1.2	1	1	nd
Cl	mg/L	6596.0	6563	6325	6233	6283	6766
SO4	mg/L	nd	nd	nd	5.8	nd	nd
Br	mg/L	18	17.9	17.5	17	17.1	18
TOC	mg/L	238.1	2380.9	2237.5	2280.3	1629	951.4
Alkalinity	mg $CaCO_3$/L	1247	1154	1233	1225	1241	1467
Modeled pH		8.42	8.42	6.44	5.4	4.34	5.8

Table D3. ICP-OES, IC-Anion, and TOC data with modeled pH for Arbuckle saline aquifer vessel experiments. IC = inhibited by carbonate, nd = non-detect, -- = not measured. * = concentration detected may be affected by high salinity.

		Arbuckle				
	Units	Initial	0 Mpa pCO_2	0.1 MPa pCO_2	1.4 MPa pCO_2	14 MPa pCO_2
Ca	mg/L	2502	2071.0	2186.0	2186.0	2104.0
Fe	mg/L	nd	nd	nd	0.5	2.5
K	mg/L	259	251.7	255.9	253.2	256.6
Mg	mg/L	702	567.8	578.4	577.1	590.7
Mn	mg/L	0.9	0.7	0.9	0.9	0.9
Na	mg/L	22690	18280.0	18440.0	17530.0	17640.0
P	mg/L	1.5	3.4	1.4	2.4	2.0
*S	mg/L	631	593.9	618.4	601.0	613.1
Si	mg/L	10	9.4	10.1	10.8	11.0
Zn	mg/L	0.200	0.1	0.8	1.8	2.4
F	mg/L	nd	nd	nd	nd	nd
Cl	mg/L	31528	31992.3	32397.2	31639.1	37757.3
SO4	mg/L	1190	369.9	1007.4	953.7	1096.3
Br	mg/L	nd	nd	nd	nd	nd
TOC	mg/L	2333.3	--	--	--	--
Alkalinity	mg $CaCO_3$/L	607	--	--	--	--
Modeled pH		7.8	7.50	5.50	4.38	2.88

Appendix E

Additional Microbial Community Characterization

Abstract

As geologic carbon storage becomes an important part of a mitigation strategy to reduce CO_2 emissions, a need arises to characterize the microbial communities that will affect CO_2 injectivity, CO_2 permanence, and water quality of carbon storage units and CO_2 impacted aquifers. This thesis project begins to characterize microbial community changes with CO_2 exposure that may occur during CO_2 injection/leakage, and discusses the results of pressure vessel experiments with samples from a representative geologic carbon storage site (Chapter 3), a representative CO_2-enhanced oil recovery (EOR) site (Chapter 4), and an overlying freshwater leakage scenario (Chapter 5). However, samples from several other sites were also utilized in this project, but the obtained results lack sufficient genetic information (such as quantitative DNA or phylogenic data) for publication or comparison among the other samples. One deep saline formation, Cranfield (MS), and two EOR sites, Emma (TX) and East Seminole (TX) contained too low of DNA concentrations to merit further microbial community analysis. Samples from another EOR site, Mississippi (KS) appeared to have significant PCR inhibition that prevented obtaining publishable sequence data. Lastly, 1 day and 7 day vessel samples from experiments with the Plant Daniel freshwater aquifer (MS) appeared to require more incubation time to allow the microbial community to equilibrate to experimental conditions. In order to guide future research into this broad area of study, this appendix chapter describes the sites, experiments, methods, and results from this completed work that was not discussed in the context of the other sites. In general, DNA was not

recovered after first attempt from the discussed "failed" sites samples, and several months of additional work was completed before use of these samples was abandoned altogether. Future work should focus on experimentation with samples containing easily recovered DNA, as experimentation with difficult samples is time consuming and produces unusable results.

Introduction

Geologic carbon storage is a part of a mitigation strategy to reduce the emitted anthropogenic CO_2. During this process, supercritical carbon dioxide (SC-CO_2) is injected in subsurface storage units, such a deep saline formation or a depleted oil reservoir. This process of geologic carbon storage increases the risk of CO_2 leakage into an overlying freshwater aquifer. Once SC-CO_2 is injected in the subsurface storage units, or once the CO_2 has leaked into an overlying freshwater aquifer, various biogeochemical processes occur that affects CO_2 injectivity, CO_2 permanence, and water quality. However, the microbial communities that affect these biogeochemical processes are still not well understood. This thesis project begins to characterize microbial community changes with CO_2 exposure of future geologic carbon storage sites and of a leakage scenario.

A diverse microbial community is known to exist in subsurface environments that are similar to targeted geologic storage sites.[1, 2] However, it has been previously difficult to obtain presentable and publishable data from subsurface environments, due to low biomass and inhibition of DNA analysis methods. While this project has successfully characterized a representative deep saline storage formation (Chapter 3), a representative

CO_2-enhanced oil recovery site (Chapter 4), and an overlying freshwater leakage scenario (Chapter 5), characterization was attempted on several additional sites. Like the successful site characterizations discussed in Chapter 3-5, these additional sites underwent pressurized vessel experiments for 56 days (or greater) and were characterized utilizing 16S rRNA sequencing and qPCR. The produced data from these sites lacked sufficient consistent information (such as quantifiable DNA or statistically reliable phylogenic data) for publication or for comparison with other results.

The objective of this chapter is to describe microbial community experiments that were completed, but failed to produce consistent or publishable data. This will provide insight on how and why these sites did not result in sufficient DNA or consistent phylogenic results for future research in this broad area of study. A site description, experimental description, and obtained data are given for each site. The sites attempted and described in this chapter are: 1) the deep saline formation for carbon storage in Cranfield, MS, 2) three potential EOR sites, the Mississippian oil reservoir (KS), the East Seminole oil reservoir (TX), andthe Emma oil reservoir, and finally,3) 1 day and 7 day pressure vessel data from Plant Daniel freshwater aquifer. The objectives of each study were to 1) Characterize the microbial ecology in fluid samples obtained from the subsurface formation prior to SC-CO_2 injection or CO_2 leakage, and 2) characterize the changes in microbial community due to CO_2 concentration exposures that can be expected from carbon storage conditions or CO_2 leakage scenarios.

Methods and Approach

Cranfield saline aquifer

Cranfield Saline Aquifer Site Background

The Cranfield storage site is a saline aquifer in the Tuscaloosa Formation, 3300 m depth, consisting of coarse-grained fluvial deposits. The Bureau of Economic Geology (BEG) characterized geochemical and geophysical properties that can be expected after CO_2 injection in this deep saline aquifer. Reservoir pressure is 26 MPa, and reservoir temperature is 120-130 ˚C. Fluid samples from the Cranfield aquifer were obtained from the UTube sampling device in December of 2009. In addition, sandstone core was retrieved from the drill auger, and preserved in foil and wax to retain native fluid. Core sediment was then sampled from the inner 1 inch of this core, to minimize contamination from handling.

Pressure Vessel Experiment

Pressure vessels were manufactured by Thar Technologies Inc., and are described in Chapter 3.[3] All reactors contained 200 mL UTube fluid sample and 2 g of core sediment sample. Two reactors were pressurized with 26 MPa of 0.5% H_2/99.5% N_2 gas for 42 days; one of these reactors was maintained at a constant temperature of 55 ˚C and the other reactor at a constant temperature of 80 ˚C. Two additional reactors were pressurized with 13 MPa of CO_2 gas and 13 MPa of 0.5% H_2/99.5% N_2 gas for 42 days; one of these reactors was also maintained at a constant temperature of 55 ˚C and the other reactor at a constant temperature of 80 ˚C. Finally, one reactor was pressurized with 26 MPa of 0.5% H_2/99.5% N_2 gas and another reactor was pressurized with 13 MPa of CO_2

gas and 13 MPa of 0.5% H_2/99.5% N_2 gas for 168 days; both of these reactors were also maintained at a constant temperature of 55 ˚C. Sampling was performed by sacrificing vessels after 42 or 168 days.

Sampled fluid was centrifuged at 4,000 g for 25 minutes. The top 199 ml of the supernatant was filtered and saved for potential chemical analysis, and the bottom 1 ml containing the solids was used for biological analysis.

Biological Analysis

DNA was extracted using a modified method described previously in Holmes et al, 2004 and briefly described in Chapter 3, Methods and Approach. DNA was also extracted using MoBio PowerSoil procedures described in Chapter 4, Methods and Approach. The qPCR, PCR, and DNA sequencing methods of the initial samples and the pressure vessel samples are described in Chapter 3, Methods and Approach. PCR procedures were also conducted on diluted pressure vessel samples (a dilution series) by 5X, 20X, and 200X to determine if PCR inhibition was occurring.

Mississippi Depleted Oil Reservoir

Site Background

The Wellington oil field, Sumner County, Kansas, contains 47 production wells and 15 injection wells. The Mississippi oil reservoir is about 1115 m -1215 m depth, and is part of the upper Mississippian series. The aquifer consists of mostly dolomite containing lenses of shale[4] and is isolated from freshwater aquifers by the 15 m thick Chattanooga shale. Reservoir pressure is 13 MPa, and reservoir temperature is 40 ˚C.

Well 1-32 (latitude 37.3154639, longitude 97.4423481) was drilled in January of 2011. Drill stem tests enabled the collection of unfiltered reservoir water from the Mississippi formations. Drill stem test water was drawn from the well at the end of the main flow period to maximize the formation water sampled and minimize any contamination from drilling. 5 L of sample were collected in sterile 1 L Teflon bottles and immediately shipped overnight on ice prior to initiating experiments.

Pressure Vessel Experiment

Pressure vessels were manufactured by Thar Technologies Inc., and are described in Chapter 3.[3] All reactors contained 200 mL of drill stem test fluid; no solid core material was available for this study, but there was about 100 g suspended solids/L drill stem test fluid. The experimental pCO_2 of the reactors was 0, 0.1, 1.3 and 13 MPa which represented 0%, 1%, 10% and 100% of the total pressure respectively. All reactors were maintained at a constant temperature of 40 ˚C and total pressure of 13 MPa. Sampling was performed by sacrificing vessels after 56 days of exposure to CO_2.

Sampled fluid was centrifuged at 4,000 g for 25 minutes. The top 199 ml of the supernatant was filtered and saved for potential chemical analysis, and the bottom 1 ml containing the solids was used for biological analysis.

Biological Analysis

DNA was extracted using a modified method described previously in Holmes et al, 2004 and briefly described in Chapter 3, Methods and Approach. The qPCR, PCR,

and DNA sequencing methods of the initial samples and the pressure vessel samples are described in Chapter 3, Methods and Approach.

Analysis by qPCR did not detect any DNA concentrations. A dilution series of the extracted DNA demonstrated PCR inhibition. DNA was amplified by diluting the extracted DNA by 200X and performing a nested PCR, discussed in Chapter 3, Methods and Approach.

East Seminole Depleted Oil Reservoir

Site Background

The East Seminole Development in Gaines County, TX was originally purchase by Mobil in 1986 for secondary oil recovery. The 1550 acres reservoir consists of San Andres dolomite and is approximately 1615 m-1710 m depth. Reservoir temperature is 43 ˚C, and reservoir pressure before water flooding was 17 MPa, and 21 MPa after water flooding. Water injection currently occurs at 1690 m depth. At the time of sample collection, production fluid had approximately 12% oil water cut.

CO_2 injection began in the fall of 2013. Pre-CO_2-injection fluid samples from the production well, E50, were taken in June, 2013. Samples were collected in sterile 1 L Teflon bottles and immediately shipped overnight on ice prior to initiating experiments. Additionally, 1.5 L of fluid from productions wells E50, L33W and E71 were filtered on site on a sterile 0.22 μm filter, and shipped overnight on dry ice prior to DNA extraction.

Pressure Vessel Experiment

Pressure vessels were manufactured by Thar Technologies Inc., and are described in Chapter 3 (Gulliver, 2014). All reactors contained 200 mL of drill stem test fluid; no solid core material was available for this study. The experimental pCO_2 of the reactors was 0, 0.2, 2 and 20 MPa which represented 0%, 1%, 10% and 100% of the total pressure respectively. All reactors were maintained at a constant temperature of 43 ˚C and a constant total pressure of 20 MPa. Sampling was performed by sacrificing vessels after 56 days of exposure to CO_2.

Samples for the initial produced fluid and the pressure vessel fluid were centrifuged at 4,000 g for 25 minutes. The top 199 ml of the supernatant was filtered and saved for potential chemical analysis, and the bottom 1 ml containing the solids was used for biological analysis.

Biological Analysis

DNA was extracted using the MoBio PowerSoil procedures briefly described in Chapter 4, Methods and Approach. The qPCR and PCR methods of the initial samples, the pressure vessel samples, and the on-site filtered samples are described in Chapter 3, Methods and Approach. PCR procedures were also conducted on diluted pressure vessel samples (a dilution series) by 5X, 20X, and 200X to determine if PCR inhibition was occurring.

Emma Oil Field

Site Background

The Emma oil field in is 43 miles south of the East Seminole oil field. This reservoir consists of San Andres dolomite and is approximately 4,200 ft in depth. Unlike the East Seminole Development, the Emma oil field has not been waterflooded, although waterflooding attempts were made in 1960 and again in 1990. At the time of sampling, production fluid had approximately 4.7% oil water cut. Reservoir temperature was 37 ˚C, and reservoir pressure was 20 MPa.

CO_2 injection began in the winter of 2014. Pre-CO_2-injection fluid samples from the production well, 1060, were taken in June, 2013. Samples were collected in sterile 1 L Teflon bottles and immediately shipped overnight on ice prior to initiating experiments. Additionally, 1.5 L of fluid from productions wells 1060, 1013 and 1113 were filtered on site on a sterile 0.22 μm filter, and shipped overnight on dry ice prior to DNA extraction.

Pressure Vessel Experiment

Pressure vessels were manufactured by Swagelok, and are described in Chapter 5. All reactors contained 80 mL of production fluid; no solid core material was available for this study. The experimental pCO_2 of the reactors was 0, 0.2, 2 and 20 MPa which represented 0%, 1%, 10% and 100% of the total pressure respectively. All reactors were maintained at a constant temperature of 37 ˚C and a total pressure of 20 MPa. Sampling was performed by sacrificing vessels after 56 days of exposure to CO_2.

Initial sample fluid and pressure vessel sampled fluid was filtered on a sterile 0.2 μm filter. The filtrate was saved for potential chemical analysis, and the solids collected on the filter were used for biological analysis.

Biological Analysis

DNA from the initial sample (well 1060) was extracted following 3 procedures. Initial sample was first extracted using a modified method described previously in Holmes et al, 2004[5] and briefly described in Chapter 3, Methods and Approach. Initial sample was also extracted using the MoBio PowerSoil procedures briefly described in Chapter 4, Methods and Approach. Last, initial sample was extracted using the MoBio PowerWater procedures. The qPCR procedure described in Ch. 3, Methods and Approach, was utilized to determine to most effective extraction procedure. PowerWater procedures were determined to result in the highest DNA concentration, and this method was used for DNA extraction of the pressure vessel samples and the onsite filtered samples.

Additionally, in order to verify DNA extraction procedures were capable of DNA extraction from Emma fluid samples, PowerWater procedures were utilized to extract 80 ml of initial fluid sample from production well 1060, spiked with *e. coli* as a positive control and 80 ml of DNA-free water, also spiked with *e. coli*. Comparison of qPCR results from the spiked Emma fluid to the spiked DNA-free water was used to determine the approximate DNA extraction inhibition of the Emma fluid samples. The qPCR procedure recovered about 10^7 16S rRNA gene copies/mL sample in both of these spiked samples, suggesting there was little to no PCR inhibition.

The qPCR and PCR methods of the initial samples, the pressure vessel samples, and onsite filtered samples are described in Chapter 3, Methods and Approach.

Plant Daniel Freshwater Aquifer

Site Background

A detailed site description of the Plant Daniel freshwater aquifer is given in Chapter 5, Methods and Approach. Briefly, the Plant Daniel freshwater aquifer is located in Escatawpa, MS and is an Energy Power Research Institute (EPRI) site. The freshwater aquifer consisted of sandy units with semi-confining clay-rich units and is about 180 ft in depth and 20 ft in thickness. The reservoir temperature is 22 ˚C, and reservoir pressure 0.5 MPa.

Pressure Vessel Experiment

A detailed description of the pressure vessel experiment is described in Chapter 5, Methods and Approach. Chapter 5 discusses experimental microbial community data obtained from the initial EPRI sample, and data after 56 days of pressurize incubation at 0.5 MPa using either 100% N_2, 1% CO_2/99% N_2, 10% CO_2/90% N_2, or 100% CO_2. However, samples were also obtained by sacrificing vessels after 1 day and 7 days. Results from the 1 day and 7 day incubations are described in this chapter.

Biological Analysis

DNA was extracted using the MoBio PowerSoil procedures briefly described in Chapter 5, Methods and Approach. The qPCR, PCR, and DNA sequencing methods of

the 1 day and 7 day pressure vessel samples is described in Chapter 5, Methods and Approach.

Results and Discussion

Cranfield saline formation

To understand the effect of CO_2 injection on microbial communities in deep saline formations storage sites, the microbial community changes in samples from the Cranfield saline formation with CO_2 exposure was investigated. Although qPCR methods did not detect DNA in core sediment sample or in fluid sample, nested PCR of DNA extracted from the core sediment (before the pressure vessel experiments) allowed microbial community characterization via 16S rRNA clone library (Figure 1).

Fluid and sediment core samples were then placed in batch reactors with and without 50% pCO_2 (0 MPa and 13 MPa) at reservoir total pressure (26 MPa) and two temperatures (55 ˚C and 80 ˚C). Although reservoir temperature was 120 ˚C-130 ˚C, the 55 ˚C and 80 ˚C were utilized in pressure vessel experiments to represent peak mesophilic conditions and thermophilic conditions. Reactors were depressurized after 42 and 168 days, DNA was extracted via two procedures, and the microbial community was analyzed with qPCR and PCR procedures. However, due to low biomass, qPCR and PCR procedures did not recover any DNA.

The genus *Pseudomonas* appeared to represent a majority of the microbial community detected in the core material (Figure 1). However, the lack of DNA in samples analyzed from any of the vessels prevented comparisons and further work on this site. The low biomass may be due to the high temperatures of the Cranfield aquifer, at

120 ˚C-130 ˚C. With a few exceptions,[6, 7] most of the known extremophiles that have adapted to survive high temperature conditions have a maximum temperature threshold of 110 ˚C.[8] It is unlikely that the lack of DNA was a result of PCR inhibition from the sample given that the dilution series did not provide improved amplification of DNA, and the other saline aquifer site (Arbuckle, KS) studied showed no PCR inhibition. Similarly, it is unlikely that the lack of DNA was a result of ineffective DNA extraction, given that two separate extraction procedures were utilized, and both failed to recover DNA. However, the lack of a positive control (DNA spiked sample) cannot rule out PCR inhibition or ineffective DNA extraction.

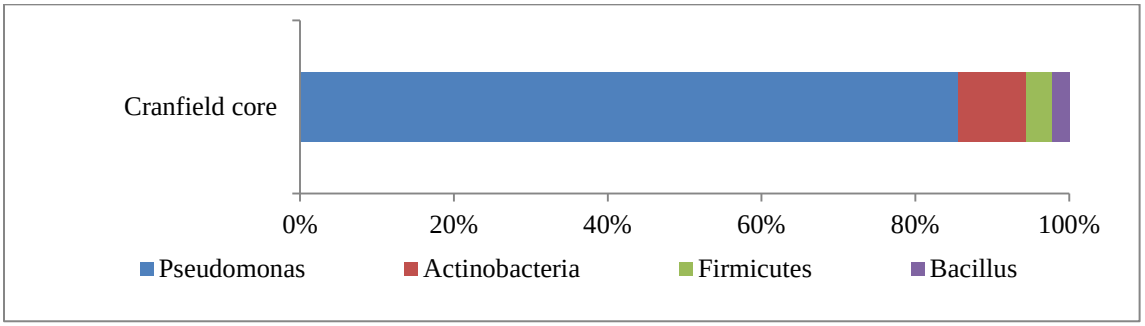

Figure E.1. Relative proportions of phylotypes recovered from core sediment material from the Cranfield saline aquifer as revealed by 16S rRNA gene clone libraries. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other".

Mississippi Depleted Oil Reservoir

To understand the effect of CO_2 on microbial communities in depleted oil reservoirs, the microbial community changes in samples from the Mississippi oil reservoir with CO_2 exposure was investigated. Samples were placed in batch reactors with increasing pCO_2 (0, 0.1, 1.3, and 13 MPa) at reservoir total pressure (13 MPa) and temperature (40 ˚C). Reactors were depressurized after 56 days and the microbial community was analyzed with qPCR and 16S rRNA clone libraries. Dilution of the extracted DNA from the exposure experiments suggested the occurrence of PCR

inhibition, and extracted DNA was diluted by 200X during the PCR procedure to optimize DNA amplification. Due to PCR inhibition, qPCR methods did not detect any DNA. Nested PCR of diluted DNA template allowed DNA analysis via 16S rRNA clone libraries (Figure 2).

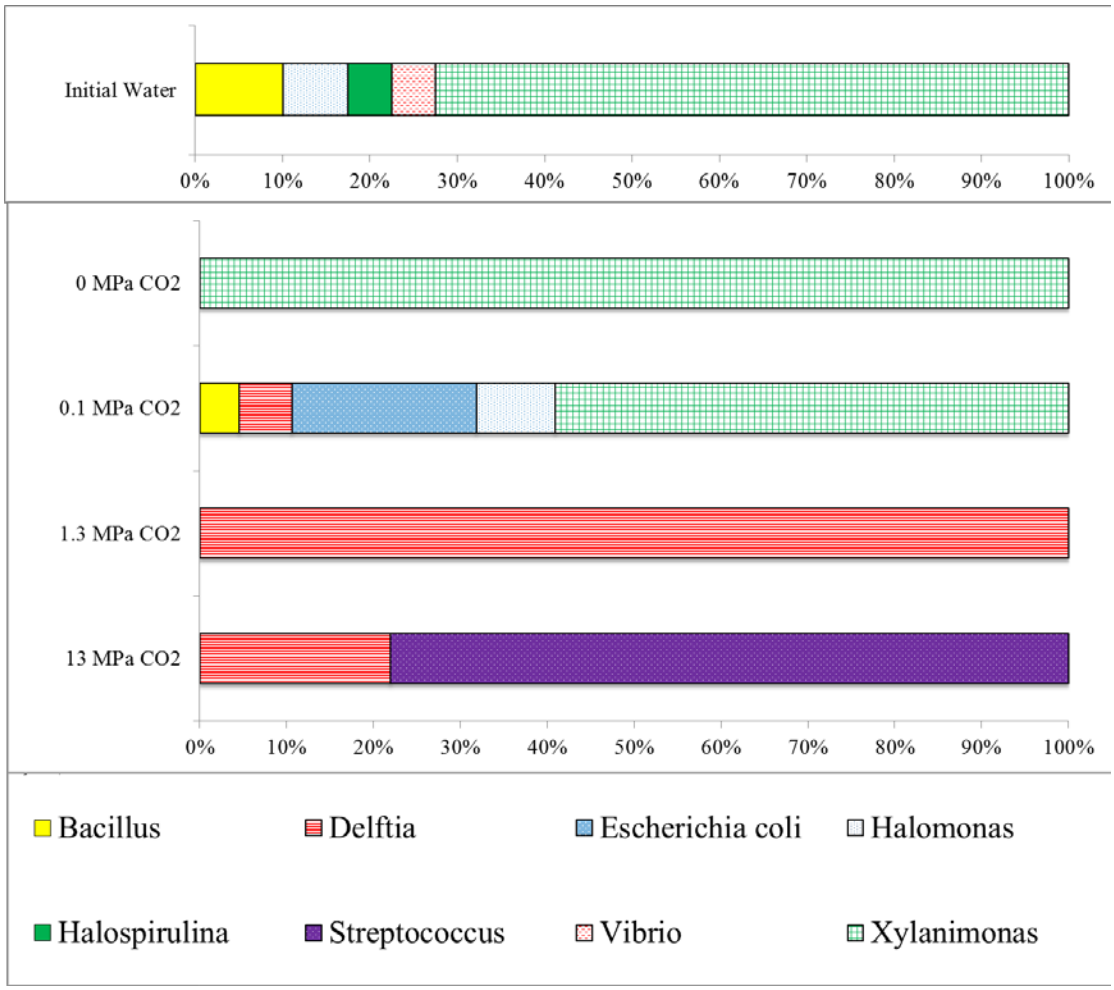

Figure E.2. Relative proportions of phylotypes recovered from reactors exposing unfiltered Mississippi oil reservoir samples to increasing pCO_2 as revealed by 16S rRNA gene clone libraries for initial drill stem test sample, and following 56 days of exposure. Clones were assigned a genera based on >97% similarity to cultured organisms. Clones with less than 97% similarity were characterized as "other". Nested PCR was utilized for all samples.

Exposure experiments with Mississippi sample followed by nested PCR procedures did result in sufficient DNA to complete 16S rRNA clone library procedures.

The phylogenetic data obtained from the 16S rRNA clone libraries is presented in Figure 2.

The 16S rRNA gene copies recovered were most similar to the genera, *Bacillus*, *Halomonas*, and *Xylanimonas*, which also appeared to be present in the underlying Arbuckle saline formation (Chapter 3). The genus, *Escherichia*, which appeared to be present in the Mississippi vessel samples, was also a dominant genus in another EOR site sample, the Mirando oil reservoir samples (Chapter 4). Although the emerging microorganisms in the Mississippi pressure vessel samples appeared to be consistent with these other site samples, this data was not publishable, due to the lack of qPCR data, and due to the potential increase of PCR-bias with nested PCR procedures. Samples from the Mississippi oil reservoir were therefore determined to have a combination of too low DNA concentration and too much PCR inhibition to merit further microbial community analysis.

East Seminole Depleted Oil Reservoir

To understand the effect of CO_2 on microbial communities in depleted oil reservoirs, the microbial community changes in samples from the East Seminole oil reservoir with CO_2 exposure was investigated. Samples were placed in batch reactors with increasing pCO_2 (0, 0.2, 2, and 20 MPa) at reservoir total pressure (20 MPa) and temperature (43 ˚C). Reactors were depressurized after 56 days and the microbial community was analyzed with qPCR and PCR. Additionally, the microbial community from filters of 1.5 L fluid sample from three producing wells was analyzed with qPCR

and PCR methods. No DNA was detected utilizing qPCR procedures and PCR procedures for the reactor samples and the produced water filters.

The low DNA concentrations from the East Seminole oil reservoir samples were surprising, as this site had a history of hydrogen sulfide production, which is a known microbial process.[9] The lack of DNA may have been due to a PCR inhibition; however, failure to recover DNA after a dilution series suggests PCR inhibition as unlikely. The produced samples contained less than 0.5 g/L solids, and the lack of DNA may be due to a filtering process that occurred before the fluid sample was received at the wellhead. For example, installation of the production well may have involved a down-hole screen that filtered sediment and biosolids before fluid collection at the top of the wellhead. It is still unknown whether the low DNA concentration in East Seminole samples was due to a filtering process before fluid collection at the wellhead, or due to a low microbial population in the reservoir. Regardless, the lack of amplified DNA from all East Seminole samples suggested the DNA concentrations were to be too low to merit further microbial community analysis.

Emma Oil Field

To understand the effect of CO_2 on native microbial communities in depleted oil reservoirs, the change of the microbial community with CO_2 exposure was investigated with samples from the Emma Oil Field, TX. Samples were placed in batch reactors with increasing pCO_2 (0, 0.2, 2, and 20 MPa) at reservoir total pressure (20 MPa) and temperature (37 ˚C). Reactors were depressurized after 56 days and the microbial community was analyzed with qPCR and PCR procedures. Additionally, the microbial

community from filters of 1.5 L fluid sample from three producing wells was analyzed with qPCR and PCR.

Despite numerous extraction procedures attempted, no DNA was detected utilizing qPCR procedures and PCR procedures for the reactor samples and the produced water filters. The lack of amplified DNA suggested the DNA concentrations to be too low to merit further microbial community analysis. PCR and qPCR analysis of a DNA-spiked produced water sample as a (positive control) from well 1060 suggested that little to no inhibition of DNA extraction procedures and PCR procedures was occurring.

Similar to sample from East Seminole, it is unknown whether the low DNA concentration in Emma samples was due to a filtering process before fluid collection at the wellhead, or due to a low microbial population in the reservoir. Regardless, the DNA concentration of the Emma samples was too low for further microbial community analysis.

Plant Daniel Freshwater Aquifer

In order to understand the effect of CO_2 on microbial communities in a leakage scenario, the microbial community changes of samples from Plant Daniel freshwater aquifer with CO_2 exposure was investigated. Samples were placed in batch reactors with increasing pCO_2 (0, 0.005, 0.05, and 0.5 MPa) at reservoir total pressure (0.5 MPa) and temperature (22 ˚C). Reactors were depressurized after 1 day, 7 days, and 56 days and the microbial community was analyzed with qPCR and 16S rRNA clone libraries.

Results from the microbial community analysis after 56 days of exposure are discussed in detail in Chapter 5. However, the microbial community was also

characterized after 1 day and 7 days of CO_2 exposure (Figure 3). A dominant portion of the microbial community appeared to be represented by *Pseudomonas* after 1 day and 7 days for all pCO_2 exposures. However, after 56 days of CO_2 exposure, the microbial community appeared to be distinct for each pCO_2 exposure. This suggested that the microbial community had not yet adjusted to the pressure vessel conditions and to each CO_2 exposure after 1 day and 7 days. For this reason, results were only discussed for the 56 day pressure vessels, as this appeared to represent a more equilibrated microbial community.

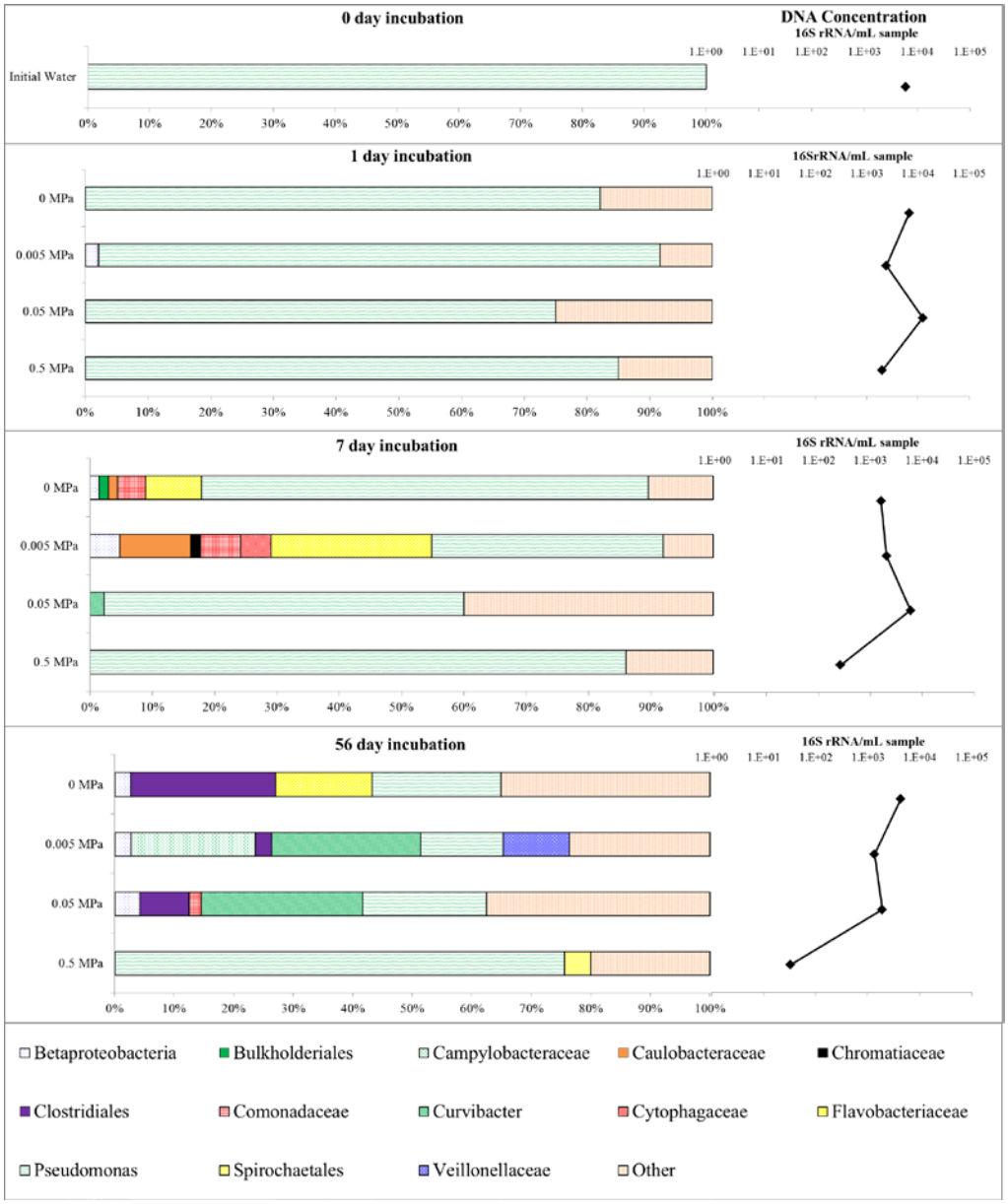

Figure E.3. Relative proportions of phylotypes recovered from reactors exposing unfiltered Plant Daniel freshwater aquifer samples to increasing pCO_2 as revealed by 16S rRNA gene clone libraries and qPCR for a) initial drill stem test sample, and following b) 1 day of exposure, c) 7 days of exposure, 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 fluid sample.

Conclusion

This work discussed in detail in this dissertation (Chapters 1-7) represents only a portion of the field work, pressure vessel experiments, and microbial community analysis that was completed. Samples from the Cranfield saline aquifer, the East Seminole

depleted oil reservoir, and the Emma oil field appeared to have DNA concentrations that were too low to obtain publishable results. Samples from the Mississippi depleted oil reservoir appeared to have low DNA concentrations and high PCR inhibition that also produced in results that were not publishable. Lastly, the results from exposure experiments with samples from Plant Daniel freshwater aquifer appeared to remain unchanged with increasing CO_2 exposure for 1 day and 7 days, suggesting the microbial community had not had sufficient time to adjust to the pressure vessel conditions and CO_2 exposures. This appendix chapter demonstrates that every successful experiment is preceded by several failed experiments, and the published record of results often represents a minor fraction of the actual academic effort.

This appendix chapter also demonstrates the inefficiency of continuing research efforts after failed first attempts to recover DNA. DNA was not recovered from initial sample of Cranfield, Mississippi, East Seminole, and Emma before further analysis. Nonetheless, work continued for several months in attempt to modify and optimize procedures to recover DNA. Despite this additional work, results were not publishable or comparable to the other sites. This suggests attempt to characterize microbial communities should be focused on samples with promising DNA recovery from the start of the experiment; experiments conducted on difficult samples may be time consuming and may produce unusable results.

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