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Research Article

RESEARCH ARTICLE

Microbial community composition of a hydrocarbon reservoir 40 years after a CO_2 enhanced oil recovery flood

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One sentence summary: Microbial community composition was compared between samples affected by a CO_2-EOR flood and those from areas that were outside or stratigraphically above the flood region to determine if CO_2-EOR flooding impacted the microbial community, or if the reservoir was able to "reset" back to pre-flood conditions.

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ABSTRACT

Injecting CO_2 into depleted oil reservoirs to extract additional crude oil is a common enhanced oil recovery (CO_2-EOR) technique. However, little is known about how *in situ* microbial communities may be impacted by CO_2 flooding, or if any permanent microbiological changes occur after flooding has ceased. Formation water was collected from an oil field that was flooded for CO_2-EOR in the 1980s, including samples from areas affected by or outside of the flood region, to determine the impacts of CO_2-EOR on reservoir microbial communities. Archaea, specifically methanogens, were more abundant than bacteria in all samples, while identified bacteria exhibited much greater diversity than the archaea. Microbial communities in CO_2-impacted and non-impacted samples did not significantly differ (ANOSIM: Statistic R = -0.2597, significance = 0.769). However, several low abundance bacteria were found to be significantly associated with the CO_2-affected group; very few of these species are known to metabolize CO_2 or are associated with CO_2-rich habitats. Although this study had limitations, on a broad scale, either the CO_2 flood did not impact the microbial community composition of the target formation, or

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microbial communities in affected wells may have reverted back to pre-injection conditions over the ca. 40 years since the CO_2-EOR.

Keywords: carbon dioxide enhanced oil recovery; microbial ecology; carbon sequestration; bioinformatics

INTRODUCTION

As atmospheric CO_2 concentrations increase worldwide, strategies to reduce this greenhouse gas are becoming necessary to curb global climate change. One popular method for carbon utilization and/or storage is CO_2-enhanced oil recovery (CO_2-EOR). CO_2-EOR involves injecting CO_2 into depleted crude oil reservoirs in order to extract residual oil from a formation. This process typically leaves around 30% of the injected CO_2 in the target formation, thus presenting the potential to curb CO_2 emissions, while the remaining 70% is recycled back to the surface (Melzer 2012).

Increasing energy demand, depletion of oil reservoirs and rising CO_2 levels in the atmosphere are driving the initiation of more CO_2 injection projects. Changes in subsurface microbiology due to CO_2 injection can impact the long-term fate and transport of the injected CO_2, as well as impact CO_2 injectivity and possibly alter target formation and cap rock lithology (e.g. Giese *et al.* 2009). This injected CO_2 also may also be converted by *in situ* methanogens into additional natural gas (e.g. Sugai *et al.* 2012), making the injection more financially appealing and potentially converting a greenhouse gas into a usable fuel source. Therefore, it is increasingly important to understand the long-term impacts of CO_2 injection on reservoir microbial communities. Recent work has demonstrated that the innate microbial community composition may change after a CO_2 injection, albeit varying environments (Mu *et al.* 2014; Wilkins *et al.* 2014; Peet *et al.* 2015; Kirk *et al.* 2016), and cells may die as a result of CO_2 dissolving into cell membranes (e.g. White, Burns and Christensen2006). Other research has determined that microbial communities can live and thrive in high CO_2 conditions like those experienced during CO_2-EOR (Freedman, Tan and Thompson 2017; Probst *et al.* 2017). Furthermore, microbial populations have been documented to change after CO_2 injection compared to water-flooded portions of the same reservoir (Liu *et al.* 2015), but no study has analyzed microbial community composition of a reservoir decades after CO_2 injection has stopped. Even though changes in microbial communities can impact a CO_2 injection project, very few large-scale projects have monitored microbiological changes *in situ* (e.g. Michael *et al.* 2010). Furthermore, studies with a microbiological component mostly involve geochemical modeling and/or simply examine isolates from high-CO_2 natural analogue environments, such as hot springs (e.g. West *et al.* 2011; Kirk *et al.* 2016); few studies target the actual microbial communities living in geologic CO_2 sequestration or CO_2-EOR reservoirs.

The Olla Oil Field in the LaSalle Parish, Louisiana, USA has been previously studied due to its high microbial methanogenesis activity compared to surrounding oil fields. This was initially hypothesized to be due to a CO_2-EOR flood in the 1980s (McIntosh *et al.* 2010; Shelton *et al.* 2014, 2016a, 2016b). Shelton *et al.* (2014) determined that the CO_2 flood was not the cause of the increased methanogenesis observed in the Olla Field; therefore, the crude oil composition and the microbiology of the Olla Field and surrounding oil fields were also examined in an attempt to determine the drivers of increased methanogenic activity (Shelton *et al.* 2016a, 2016b). This present study analyzes how the CO_2-EOR flood may have changed microbial community structures in the Olla Oil Field. The microbial composition of the samples collected from CO_2-affected production wells (n = 2) are compared to samples from unaffected portions of the same target sand (n = 5) and unaffected younger strata in the same oil field (n = 2). This study is unique in that it allows for an assessment of *in situ* microbiology almost 40 years after a CO_2 flood, providing an opportunity to determine how long-term CO_2 injection and cessation may impact the innate microbial communities. This study is also the first of its kind in analyzing microbial communities *in situ* post-CO_2 injection and cessation.

METHODS

Formation water from 9 different wells that produce from the Olla Oil Field in LaSalle Parish, Louisiana, USA was collected in August of 2014 (Fig. 1). Water samples from continuously pumping wells were collected in sterile 1 L glass bottles and filtered through sterile 0.22 μm Sterivex GP filter units (Millipore®, Billerica, MA USA) using a GeoPump (Geotech Environmental Equipment, Inc. Denver, CO, USA) and sterile plastic Nalgene tubing until the filters clogged. Filters were immediately frozen on dry ice and kept frozen until analysis at the University of Colorado Next Generation Sequencing Facility. DNA was extracted at the University of Colorado at Boulder using a MO BIO Powersoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Slices of the Sterivex filters were added directly to the bead tubes of the MO BIO kit in order to extract DNA from the filter units. The 515-F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806-R (5′-GGACTACHVGGGTWTCTAAT-3′) 16S rRNA gene primer pair (Fierer *et al.* 2012) was used during amplification; these primers included Illumina adapters and error-correcting 12-bp barcodes. A GoTaq® Hot Start PCR Master Mix (Promega, Madison, WI, USA) was used for PCR. Thermal cycling (in a 25 μL reaction) consisted of initial denaturation at 94°C, annealing at 50°C for 30 s, extension at 70°C for 90 s and a final extension at 72°C for 10 min. Gel electrophoresis was used to confirm amplification, and all PCR products were quantified using the PicoGreen dsDNA assay. Samples were pooled together in equimolar concentrations, and the amplified DNA was sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA, USA), running 2 × 250 base pair (bp) chemistry.

Sequenced DNA was processed downstream using a joint QIIME (Caporaso *et al.* 2010) and UPARSE pipeline (Edgar 2013) as discussed in Shelton *et al.* (2016a). Demultiplexing was performed in QIIME, while the remainder of the downstream processing was performed using UPARSE. Quality filtering was performed with a maxee value of 0.5, sequences were dereplicated and singletons were removed from the dataset prior to determining and assigning phylotypes (all in UPARSE). Taxonomic units were mapped to operational taxonomic units (OTUs) at a minimum of 97% similarity (typically greater) using the Greengenes 13_8 (http://greengenes.secondgenome.com) database.

All subsequent steps were performed in R (R Core Team 2015), including contaminant removal, where any sequences matching mitochondria and/or chloroplast phylotypes were eliminated from the dataset, as well as any phylotypes identified at greater than 5% in the blank (i.e. control) samples (8 contaminant OTUs

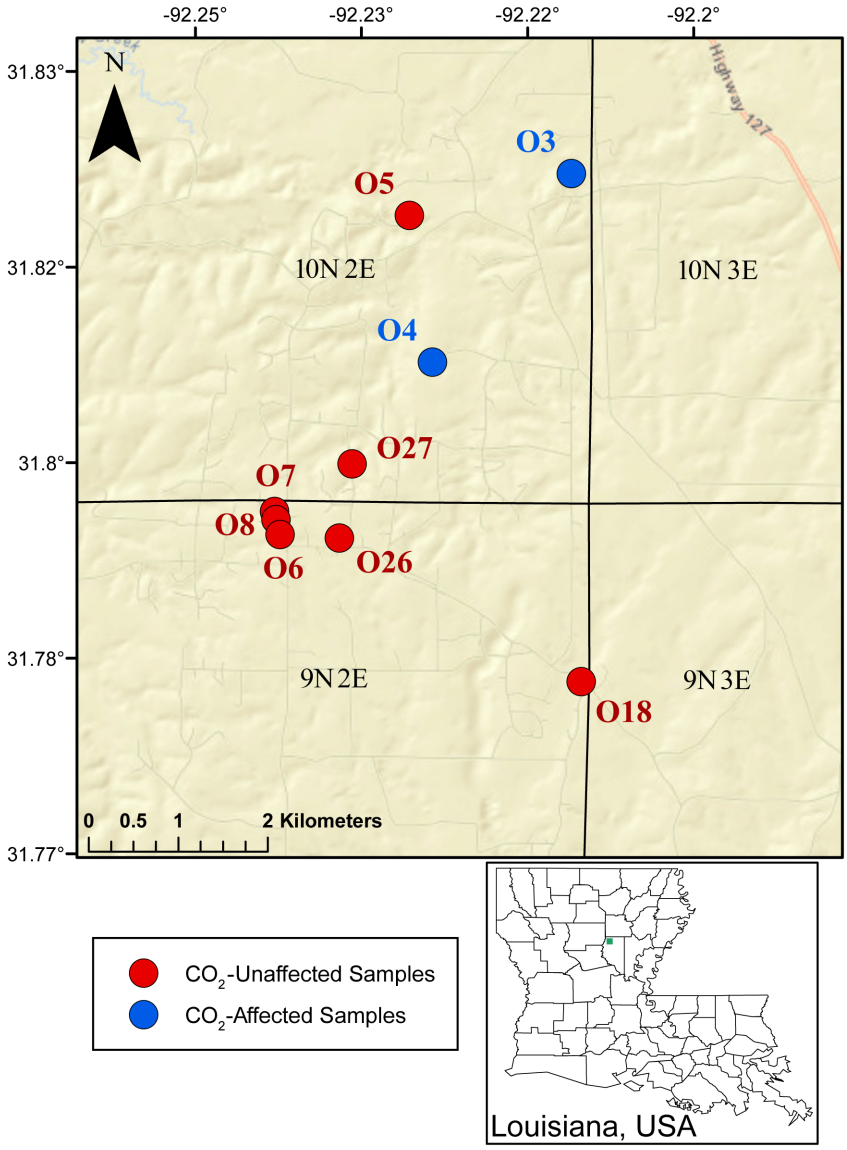


Figure 1. Map of sample locations, Louisiana, USA. The small green square on the map of Louisiana annotates the location of the inset map.

were identified and removed from the dataset). This resulted in 180 773 (n = 9) total sequences, with the minimum number of sequences per sample being 5829 (sample O4). Therefore, the entire sample set was then randomly subsampled to 5000 sequences per sample. All subsequent statistical analyses were performed in R using the vegan (Oksanen *et al.* 2014), bioDist (Ding, Gentleman and Carey 2017), RAM (Chen, Simpson and Levesque 2016) and indicspecies (De Caceres and Jansen 2016) packages. All methods requiring a distance matrix were performed using a Bray–Curtis dissimilarity matrix. Sequences reads for each sample were deposited into the National Center for Biotechnology Information Short Read Archive (SRA) under BioProject PRJNA310850 and BioSample accession numbers SAMN04457241 and SAMN04457231 - SAMN04457237.

The Olla Field was injected with CO_2 from 1983 until 1986 via eight injection wells (Shelton *et al.* 2014). Due to heterogeneous CO_2 flooding of the 2800' sand—the target formation—of the Olla Oil Field, only parts of the 2800' sand were impacted by the injected CO_2. Two samples, O3 and O4, were collected from wells that produced injected CO_2 during the CO_2-EOR flood (deemed the CO_2-affected wells; Shelton *et al.* 2014). Seven additional wells, O5, O6, O7, O8, O18, O26 and O27, were sampled that were not affected by the CO_2-EOR flood as they never produced injected CO_2 during the EOR project (Shelton *et al.* 2014).

Two of the CO_2-unaffected wells, O26 and O27, produce from strata stratigraphically above the 2800' sand, which was not impacted by the CO_2 flood. Samples from strata younger than the 2800' sand were added to the study to increase the robustness of the sample set, even though the hydrochemistry and lithology of this younger strata are slightly different than the 2800' sand. Although these two samples were located in different strata than the other CO_2-unaffected samples, they were statistically similar in regard to hydrogeochemical parameters (Shelton *et al.* 2016a; see Tables SI–2 and SI–3 (Supporting Information) for hydrologic and gas geochemical data). Therefore, this was used as justification to group the samples together. The remaining 5 CO_2-unaffected wells, O5, O6, O7, O8 and O18, produce from portions of the 2800' sand that were not impacted by injected CO_2. Detailed information about the study site, background and sampling methods can be

found in McIntosh *et al.* (2010) and Shelton et al. (2014, 2016a, 2016b).

RESULTS AND DISCUSSION

Alpha diversity and general community composition

The CO_2-affected wells were wells O3 and O4, while the CO_2-unaffected wells were wells O5, O6, O7, O8, O18, O26 and O27, with wells O26 and O27 producing from more shallow strata, as discussed in the Methods section. The total number of unrarefied sequences per sample ranged from 5829 (O4, CO_2-affected sample) to 45 217 (O6, CO_2-unaffected sample; average = 20 086, n = 9), while the total number of identified OTUs per sample (i.e. sample richness prior to rarefaction) ranged from 107 (O7) to 306 (O8), with an average value of 202 OTUs (Table SI–1, Supporting Information). The Shannon Diversity Index of the unrarefied dataset ranged from 1.41 (O27) to 4.19 (O3), while the Pielou Evenness ranged from 0.28 (O27) to 0.77 (O3). On average, the sample richness and Pielou evenness for the rarefied dataset are both higher for the CO_2-affected samples compared to the CO_2-unaffected samples (Fig. 2).

Every sample (n = 9) is dominated by a methanogenic archaea (Fig. 2). Twenty-eight of the 876 identified OTUs were archaea. The observed microbial community in eight of the nine wells sampled is dominated by *Methanothermococcus* spp., where *Methanothermococcus* spp. ranges from 14.2% (O3) to 74.1% (O27) of the total measured abundance across these eight samples. *Methanothermococcus* sp. has been identified as a hydrogenotrophic methanogen (Takai, Inoue and Horikoshi 2002) with preferential growth requirements well within the hydrogeochemical parameters observed in these eight wells. The microbial community in the water sampled from well O6 is dominated by *Methanohalophilus halophilus*, comprising 68.2% of the total measured abundance in that sample. *Methanohalophilus halophilus* typically prefers a high salinity environment, 1200 mM Cl^- (Kendall and Boone 2006); although sample O6 was concentrated in Cl^- at 1394 mM Cl^- (Table SI–2, Supporting Information), it was not significantly more saline than the other wells sampled in this study. There was no geochemical reason for well O6 to have different dominating OTUs than the other eight wells (i.e. the geochemistry of the gas and water from well O6 was not remarkably different than any other well sampled; Tables SI–2 and SI–3, Supporting Information). Most notably, it is not the most saline sample, nor did it have more chemical or isotopic indicators of methanogenesis than the other eight samples (Shelton *et al.* 2016a; Shelton *et al.* 2016b). The other archaea identified at greater than 1% abundance in at least one of the nine samples are *Methanolobus* sp., the order E2 within the class *Thermoplasmata*, *Methanomicrobiales*, *Methanosaeta* sp., *Methanocalculus* sp. and the order NRA6 of the class *Methanomicrobia*.

The bacteria identified in these nine samples are much more diverse than the archaea: 847 different bacterial OTUs were identified out of 876 total identified OTUs. Every sample contains high amounts of low abundance (<1% of total sample abundance) bacterial OTUs; no single bacterial OTU was identified at greater than 6.2% abundance in any of the nine samples. The dominating bacterial OTUs identified were *Thermovirgaceae*, *Alicyclobacillus* sp., the order BA021 of the phylum OP9 (Atribacteria), *Deferribacter* sp. and *Acinetobacter lwoffii*. Two of these dominating bacteria have been identified in crude oil reservoirs, *Thermovirgaceae* (Piceno *et al.* 2014) and *Deferribacter* sp. (Greene, Patel and Sheehy 1997). However, only one has been associated with high CO_2 environments, *Deferribacter* sp., which is typically identified in hydrothermal vents growing autotrophically off of CO_2 (Slobodkina *et al.* 2009; Takai *et al.* 2003). *Alicyclobacillus* sp. is usually associated with fruit juices (e.g. Chang and Kang 2004), BA021 has been identified in anaerobic digestors (e.g. Wang, Hou and Su 2017), while *A. lwoffii* is typically associated with animal environments (e.g. Debarry *et al.* 2007). In some cases, when considered together, these low abundance bacterial OTUs dominate the community compositions of some of the samples collected (Fig. 2).

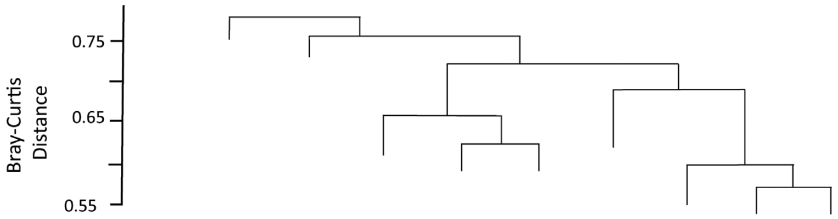
There are no obvious differences between the CO_2-affected (O3 and O4) and CO_2-unaffected (n = 7) samples when looking at the alpha diversity metrics and the general microbial community composition. Any major observed variation exists mostly in the low abundance bacterial communities. Unfortunately, concentrations of DNA were not measured and therefore, differences in total biomass could not be compared between the CO_2-affected and CO_2-unaffected samples.

A previous study by Gulliver, Gregory and Lowry (2016) compared the microbial communities of different samples that they exposed to different partial pressures of CO_2, emulating CO_2 sequestration conditions in a saline aquifer and a depleted crude oil formation. The study found no relationship between microbial community diversity and the partial pressure of CO_2 (pCO_2) in formation water associated with an oil field (similar to that of the Olla Field), while in the saline aquifer, microbial diversity decreased with increasing pCO_2 (Gulliver, Gregory and Lowry 2016). An additional study by Gulliver, Lowry and Gregory (2014) also found that increasing pCO_2 over a specific threshold initiated decreases in microbial community diversity in the target formation. The CO_2-affected samples in this study generally had higher Shannon Diversity values than the unaffected samples, opposite to what was observed in the Gulliver, Gregory and Lowry (2016) study for a saline aquifer and the oil field formation water. Conversely, the Gulliver, Gregory and Lowry (2016) study found that changes in the microbial community were site-specific (e.g. aquifer versus oil field formation water) and highly dependent on pH. The microbial community in the oil field formation water shifted from *Pseudomonas* in no- to low-pCO_2 samples to *Escherichia* in the high pCO_2 samples (Gulliver, Gregory and Lowry 2016). This study did not identify any dominating *Escherichia* OTUs in the CO_2-affected samples; however, *Pseudomonas* was present in higher abundances in the CO_2-affected versus the CO_2 unaffected samples (Fig. 2), albeit not at a statistically significant difference.

Beta diversity

In order to determine if the microbial composition of the CO_2-affected samples were distinct compared to the CO_2-unaffected samples, the data were analyzed using a variety of statistical methods. As shown in Fig. 2, no obvious grouping of the CO_2-affected samples can be observed in a dendrogram based on hierarchical clustering of Bray–Curtis distances between the sites (Fig. 2). The O3 and O4 samples were separated into two different, distinct clusters, both being more similar to CO_2-unaffected samples than to each other.

The same result occurred when performing a principal coordinates analysis (PCoA) on the dataset. The dataset was evaluated 4 different ways in order to determine if a relationship exists between the microbial communities of the CO_2-affected samples versus the CO_2-unaffected samples. Four different PCoA plots were constructed: one at the OTU level that included both the identified archaea and



	O7	O6	O8	O18	O3	O4	O5	O26	O27
Methanothermococcus	42.7	2.2	37.8	51.7	14.2	37.8	61.1	46.1	74.1
Methanohalophilus	0.0	69.0	0.4	0.8	0.5	5.8	0.1	0.1	0.0
Methanolobus	0.0	0.2	0.0	0.8	13.2	0.4	0.2	1.5	0.5
Chitinophagaceae	6.6	1.0	1.0	0.2	1.4	0.1	0.0	0.4	0.1
Alicyclobacillus	1.2	0.4	4.8	3.8	5.0	2.8	0.7	1.2	0.5
Unclassified *BA021*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	5.5
Kaistobacter	3.0	0.6	1.5	0.4	2.0	1.3	0.0	0.0	0.0
Unclassified *Thermovirgaceae*	0.0	5.1	0.1	0.0	1.0	3.5	5.0	9.9	7.6
Acinetobacter	5.2	1.4	1.2	1.4	4.0	0.2	0.3	0.2	0.2
Pseudomonas	2.2	0.9	3.5	2.8	5.9	3.8	0.5	2.1	0.4
All Other	39.1	19.2	49.7	38.1	52.8	44.3	32.1	37.7	11.1
Rarefied Species Rank Dataset									
Shannon Diversity Index	2.88	1.91	3.83	2.93	4.19	3.34	2.14	2.91	1.41
Sample Richness	109	146	306	230	232	137	214	175	168
Pielou Evenness	0.615	0.383	0.670	0.539	0.769	0.680	0.399	0.563	0.276

Figure 2. Coupled dendrogram (based on hierarchical clustering of Bray–Curtis distances between the sample locations) and a species abundance-based heat map. Dark green indicates a greater % abundance while white indicates a smaller % abundance. The Shannon Diversity Index, sample richness and Pielou Evenness after rarefaction (at the species rank) are also listed. 'All Other' indicates all of the remaining identified OTUs that were present at less than 3% abundance in at least one sample.

the identified bacteria (Fig. 3A), one at the order level that included the identified archaea and bacteria (Fig. 3B), one at the OTU level that only considered the identified bacteria (Fig. 3C) and one at the OTU level that only considered the identified archaea. The datasets were evaluated this way due to the alpha diversity statistics; as there was much more bacterial diversity than archaeal diversity, the archaea could have been masking any bacterial differences between the CO_2-affected and CO_2-unaffected samples. However, the CO_2-affected samples (O3 and O4) neither cluster together nor do they cluster distinctly from the CO_2-unaffected samples in any of the four scenarios (Fig. 3).

To confirm the lack of significant differences between the CO_2-affected and CO_2-unaffected samples, an ANOSIM test was also performed on the four different scenarios, testing the CO_2-affected ($n = 2$) samples against the CO_2-unaffected ($n = 7$) samples (Fig. 3). None of the ANOSIM results indicated a significant difference between the microbial community composition of the CO_2-affected versus the CO_2-unaffected samples for the data. Scenarios considered were all taxa at the OTU level (Statistic R = -0.2597, Significance = 0.769), just the bacteria at the OTU level (Statistic R = -0.2208, Significance = 0.785), just the archaea at the OTU level (Statistic R = 0.1494, 0.269), and all taxa at the Order level (Statistic R = -0.2338, Significance = 0.779).

These three pieces of evidence—the dendrogram of Bray–Curtis distances, PCoA and ANOSIM tests—indicate that there is no statistical difference between the total microbial community compositions of the CO_2-affected samples compared to the CO_2-unaffected samples. It is important to note that these data cannot confirm whether or not the microbial communities in this reservoir were ever impacted by the injected CO_2, meaning that the microbial communities of the impacted areas of the reservoir may not have changed during the CO_2 flood to begin with. However, these wells did produce injected CO_2 during the EOR project (Shelton *et al.* 2014), confirming that the well areas of the CO_2-affected wells were impacted by injected CO_2. If the microbes were indeed modified during the CO_2 flood, these results suggest that reservoirs impacted by a CO_2 flood (or perhaps a CO_2 leak) may have the ability to rebound back to their pre-flood microbial composition. Furthermore, geochemical data found in Table SI–2 (Supporting Information) also provide evidence for the rebounding of these CO_2-affected wells back to their pre-injection condition. Many hydrologic parameters of formation water change during a CO_2 injection. The pH decreases, the alkalinity increases, dissolved iron content increases and the $\delta^{18}O$-H_2O decreases (Kharaka *et al.* 2006; Zheng *et al.* 2012). These geochemical and isotopic changes are not observed in our sample set (Table SI–2, Supporting Information): the pH values for O3 and O4 are near neutral and close to those of the CO_2-unaffected samples, the alkalinity values of O3 and O4 are high, but not significantly higher than the CO_2-unaffected wells, iron was below the detection limit for most samples, and the $\delta^{18}O$-H_2O values of the CO_2-affected wells are similar to those of the CO_2-unaffected wells. This evidence supports the conclusion that the CO_2-affected portions of the reservoir may have reverted back to pre-flood conditions, given the CO_2-unaffected wells are currently representative of pre-flood conditions.

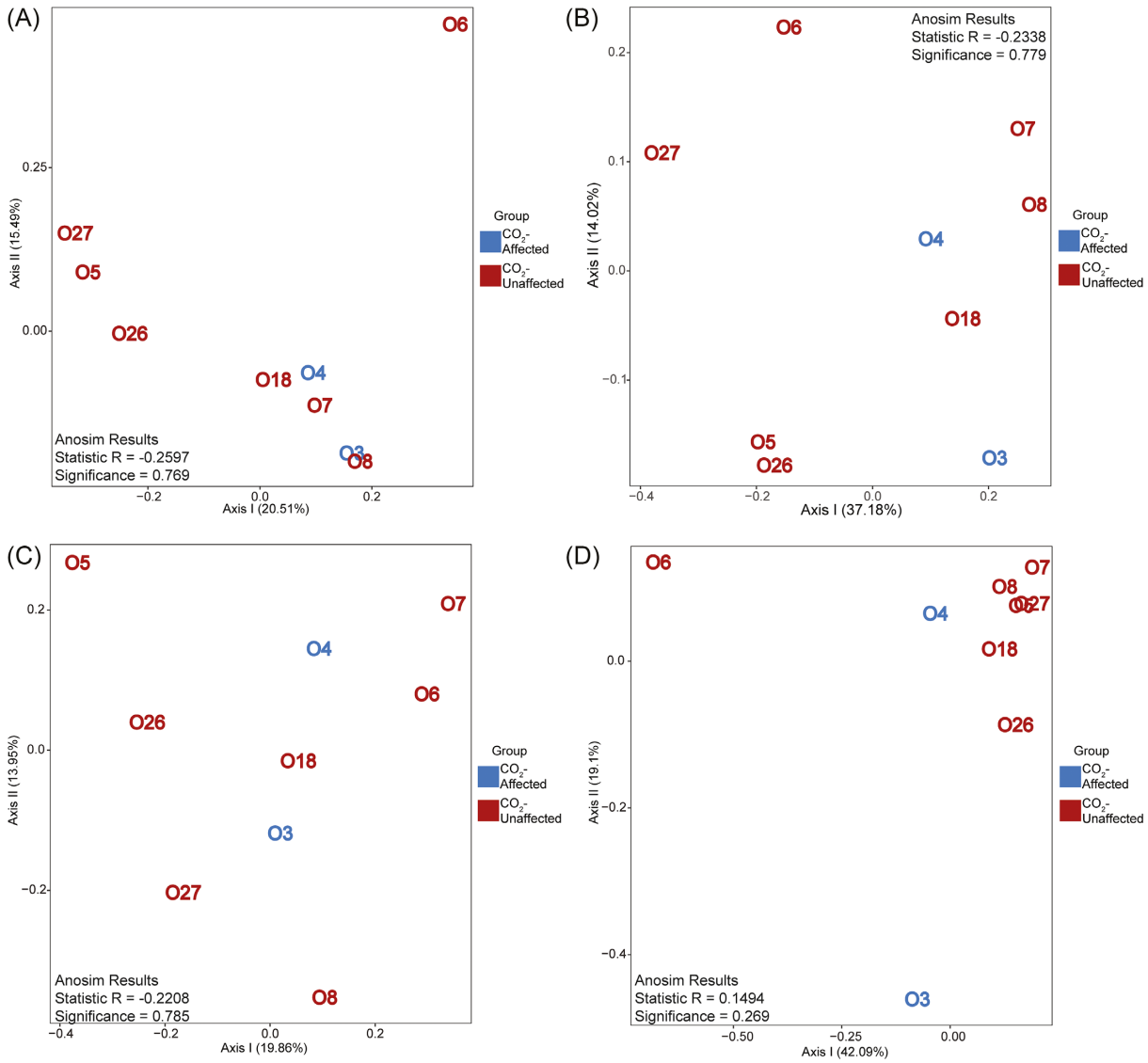


Figure 3. Principle coordinates analysis (PCoA) of sampled wells. Well names are colored based on their exposure to CO_2 (CO_2 affected = blue; unaffected = red). (**A**) PCoA of samples at the OTU level; (**B**) PCoA of samples at the Order level; (**C**) PCoA of only the identified bacteria at the OTU level; (**D**) PCoA of only the identified archaea at the OTU level.

Another issue to consider is that the small sample size may have also skewed the results, as this study only sampled two CO_2-affected wells. Therefore, it is difficult to statistically determine if the two wells were initially impacted in the same way by the CO_2 flood, or if their impact was site-specific as was observed by Gulliver, Gregory and Lowry (2016). Further, it is difficult to determine with our experiment design if the microbial communities in O3 and O4 shifted as a result of the CO_2 flood in the same manner or in a different way, meaning that the microbiology of O3 and O4 may have been modified in different ways by to the CO_2 flood.

Indicator species for CO_2-affected wells

Although the composition of the total microbial community suggested that the overall community composition of the CO_2-affected versus the CO_2-unaffected wells was not statistically different, additional tests were performed in order to see if any OTUs could serve as indicator species in the CO_2-affected samples. An indicator species (R package indicspecies::multipatt) test was performed on the group of the two CO_2 affected samples (O3 and O4) against the CO_2 unaffected samples (O5, O6, O7, O8, O18, O26 and O27). The test resulted in 14 statistically significant indicator species associated with the CO_2 affected samples, but no OTUs associated with the CO_2 unaffected samples (Table 1). These 14 organisms are *Desulfitobacter* sp., *Sporotomaculum* sp., *Corynebacterium* sp., *Syntrophomonas* sp., *Flavobacterium frigidarium*, an organism from the order *Ellin6067* and the order *Burkholderiales* and organisms from the families *Pasteurellaceae*, *Sporichthyaceae*, *Ellin6513*, *Methylophilaceae*, *Pseudomonadaceae*, *Oxalobacteraceae* and *Enterobacteriaceae* (Tables 1 and 2).

These indicator species were present in statistically higher abundance in the CO_2-affected samples versus the CO_2-unaffected samples (if at all), and could be indicative of the species that thrived in CO_2-flooded conditions. The increased abundance of these indicator species is likely not due to differences in geology or hydrochemistry, as all of these wells

Table 1. Results of the indicator species analysis (R package indicspecies::multipatt). All identified operational taxonomic units (OTUs) are associated with the CO_2-affected samples. The analysis was performed at the OTU level on the identified bacteria, the identified archaea and the whole rarefied dataset. No archaea were identified as indicator species.

Multilevel pattern analysis—CO_2 positive versus CO_2 negative				
Species	Only bacteria (OTU rank)		OTU rank	
	Stat	*P* value	Stat	*P* value
Desulfitobacter sp. (OTU 778)	0.999	0.031	0.999	0.024
Sporotomaculum sp. (OTU 3911)	0.998	0.031	0.998	0.024
Ellin6067 (OTU 142)	0.997	0.031	0.997	0.024
Pasteurellaceae (OTU 4562)	0.997	0.031	0.997	0.024
Sporichthyaceae (OTU 1093)	0.993	0.031	0.993	0.024
Ellin6513 (OTU 141)	0.988	0.031	0.988	0.024
Syntrophomonas sp. (OTU 1683)	0.985	0.031	0.985	0.024
Methylophilaceae (OTU 4351)	0.978	0.031	0.978	0.024
Pseudomonadaceae (OTU 3861)	0.975	0.031	0.975	0.024
Pseudomonadaceae (OTU 18 730)	0.965	0.031	0.965	0.024
Oxalobacteraceae (OTU 2315)	–	–	0.954	0.046
Flavobacterium frigidarium (OTU 15 553)	0.938	0.031	0.938	0.024
Enterobacteriaceae (OTU 8894)	–	–	0.936	0.046
Burkholderiales (OTU 3883)	0.922	0.031	0.922	0.024
Corynebacterium sp. (OTU 860)	–	–	0.916	0.05

have very similar lithology and water chemistry parameters (Shelton *et al.* 2014, 2016b). Of these 14 indicator species, to our knowledge, only four have been previously observed in or associated with CO_2-rich environments: *Desulfitobacter* sp., *Pseudomonadaceae*, Burkholderiales and *Enterobacteriaceae* (Morozova *et al.* 2011; Frerichs *et al.* 2014; Mu *et al.* 2014; Octavia and Lan 2014; Gulliver, Gregory and Lowry 2016; Ham *et al.* 2017). The other 10 organisms, *Sporotomaculum* sp., *Corynebacterium* sp., *Syntrophomonas* sp., *F. frigidarium*, Ellin6067, *Pasteurellaceae*, *Sporichthyaceae*, Ellin6513, *Methylophilaceae* and *Oxalobacteraceae,* have been identified in a variety of environments, including methanogenic sludge; sewage; in animals, human materials and plants; waterlogged soils and soils in general; marine sediment; surface waters; and aquifers (Tamura, Hayakawa and Hatano 1999; Humphry *et al.* 2001; Qiu *et al.* 2003; Baldani *et al.* 2014; Doronina, Kaparullina and Trotsenko 2014; Kim *et al.* 2014; Schink and Muñoz 2014; Stackebrandt 2014; Tauch and Sandbote 2014).

A previous study by Gulliver, Gregory and Lowry (2016) examined how various pCO_2 concentrations impacted the microbial communities of formation waters from a saline aquifer and an oil field. That study found that *Pseudomonas* dominated their low-pCO_2 samples, while *Escherichia* dominated the high-pCO_2 sample. As *Escherichia* was not present in their 0 MPa pCO_2 samples, this suggests that *Escherichia* thrives in CO_2-rich environments. If the *Enterobacteriaceae* identified as an indicator species in this study are of the *Escherichia* genus, then this could imply that, when the CO_2 flood occurred, *Escherichia* may have dominated, or at least became more prevalent, in the CO_2 affected areas of the aquifer. Given that current pCO_2 and CO_2 concentrations for the Olla Field were, at the time of sampling, much lower than typical injection conditions (Shelton *et al.* 2014), it appears that the presence of *Pseudomonadaceae* as an indicator species for the CO_2-affected wells is in agreement with the study by Gulliver, Gregory and Lowry (2016); however, neither *Pseudomonadaceae* nor *Enterobacteriaceae* dominate (i.e. are >10% abundance) the samples. This may be because sufficient time has elapsed since the CO_2 flood, allowing these CO_2-affected areas of the reservoir to revert back to close to 'pre-injection' conditions, or to microbial compositions similar to the unaffected portions of the reservoir.

Morozova *et al.* (2011) found that total bacterial cell counts in a CO_2-flooded saline aquifer initially decreased by 50% during the CO_2 flood, but rebounded up to 75% of the original pre-flood cell counts after a period of 5 months of CO_2 flooding. Sulfate-reducing bacteria (SRB), specifically *Desulfohalobium utahense*, increased in concentration 5 months after the CO_2 flood (Morozova *et al.* 2011). The SRB identified as an indicator species for CO_2-affected wells in this study were *Desulfitobacter* sp., which are within the same phylum as *D. utahense* but not more closely related. Furthermore, cell counts of archaea initially increased after the CO_2 flood, but, after 5 months of CO_2 flooding, no archaea were identified in the CO_2-affected formation waters. In contrast to the findings presented by Morozova *et al.* (2011), the CO_2-affected wells in the present study are both dominated by archaea. However, the CO_2 flood in the Olla Field ceased ca. 1 year after it was initiated, and high CO_2 concentrations were not maintained in the Olla Field over the past 40 years (Shelton *et al.* 2014).

A study by Mu *et al.* (2014) monitored microbiological changes before and after a 4 day CO_2 injection into a saline aquifer. They observed a dramatic increase in the relative abundances of *Comamonas* and *Sphingobium* ca. 30 days after the end of the 4 day CO_2 injection. Burkholderiales, which contains the genus *Comamonas*, is significantly associated with the CO_2-affected samples in this study. If the Burkholderiales OTU identified is of the genus *Comamonas*, this would be in agreement with the study by Mu *et al.* (2014) and this significant association may indeed be due to the CO_2 flood. The Mu *et al.* (2014) post-CO_2 injection samples also clustered together on a PCoA plot, unlike the samples for this study. This may be due to drastic changes to pH caused by active CO_2 flooding, which others have speculated may be the main cause for microbiological changes to the target formation during a CO_2 flood (Xu *et al.* 2010). The pH for the water samples collected for this study were close to 7 (Shelton *et al.* 2014), and had obviously rebounded back to near-neutral

Table 2. Indicator species correlated to the CO_2-affected samples, including information about the physiology, habitat and any evidence for living in a CO_2-rich environment. The references for the data provided in the columns for each row are also provided.

Indicator species	Habitat	Known CO_2 relationship	Optimal salinity	Metabolism	Oxygen requirements	References
Desulfitobacter sp.	Heating system pipes	Only with general sulfate-reducing bacteria	*D. alkalitolerans*: 0–5% (w/v) NaCl [0–0.5%]	Fermentative; sulfite-reducing	Anaerobic	(Nielsen, Kjeldsen and Ingvorsen 2006; Morozova *et al.* 2011)
Sporotomaculum sp.	Anoxic environments	No	*S. hydroxybenzoicum*: 0–0.2% (w/v) NaCl	Fermentative; possibly benzoate-degrading	Anaerobic	(Brauman *et al.* 1998)
Ellin6067	Soils	No	Not given	Heterotrophic; possibly ammonia-oxidizing	Aerobic	(Ye *et al.* 2016)
Pasteurellaceae	Generally animal-borne	No	Presumably salty (e.g. body fluid)	Heterotrophic; varies by Genus	Facultatively anaerobic	(Naushad *et al.* 2015)
Sporichthyaceae	Soils	No	Not given for *S. brevicatena*	Heterotrophic; nitrate-reducing	Aerobic	(Tamura, Hayakawa and Hatano 1999)
Ellin6513	Soils	No	Not given	Heterotrophic; acidophilic	Aerobic	(Beulig *et al.* 2014, Wegner and Liesack 2017)
Syntrophomonas sp.	Anoxic habitats	No	Not given	Syntrophic; fatty-acid oxidizing	Anaerobic	(McInerney *et al.* 1981)
Methylophilaceae	Surface waters, mud, activated sludge and plants	No	*Methylotenera mobilis*: No growth above 0.1% (w/v) NaCl	Obligate or restricted facultative methlyotrophs; methlyamine or methanol utilizing	Aerobic	(Kalyuzhnaya *et al.* 2006; Doronina, Kaparullina and Trotsenko 2014)
Pseudomonadaceae	Widespread	Yes if '*Pseudomonas*'	Varied	Heterotrophic; varies by species	Aerobic or facultatively anaerobic	(Frerichs *et al.* 2014; Gulliver, Gregory and Lowry 2016)
Oxalobacteraceae	Plants, soils and waters	No	*Herbaspirillum psychrotolerans*: 0–0.5% (w/v) NaCl	Heterotrophic; can be pathogenic	Mostly aerobic or facultative aerobic; Oxalobacter is strictly anaerobic	(Bajerski *et al.* 2013; Baldani *et al.* 2014)
Flavobacterium frigidarium	Antarctic marine sediments	No	Growth in up to 10% (w/v) NaCl	Psychrophilic, xylanolytic and laminarinolytic	Aerobic	(Humphry *et al.* 2001)
Enterobacteriaceae	Widespread, typically in guts of animals	Yes if '*Escherichia*'	Varies based on genus	Nitrate-reducing, glucose fermentation; may be pathogenic	Facultatively anaerobic	(Octavia and Lan 2014; Gulliver, Gregory and Lowry 2016)
Burkholderiales	Widespread	Yes if '*Comamonas*'	Varied	Heterotrophic; possibly aromatics-degrading	Varies by genus	(Offre *et al.* 2008; Mu *et al.* 2014; Tong *et al.* 2015; Probst *et al.* 2017)
Corynebacterium sp.	Widespread	No	Varies based on genus	Chemooorganotrophic; fermentative and oxidative, may be pathogenic	Aerobic or facultative anaerobic	(Tauch and Sandbote 2014)

conditions since the active CO_2 flood in the 1980s, which would have lowered the pH in the formation.

Ham *et al.* (2017) compared two naturally CO_2-rich sites and one low-pCO_2 control site as an analog for long-term microbiological changes to reservoirs impacted by CO_2 flooding. They found sequences close to *Comamonadaceae* in one CO_2-rich site and taxa related to *Anaerolineaceae*, *Nitrospirae* and methanogens in the other CO_2-rich site. This study identified Burkholderiales as significantly associated with the CO_2-rich sites, similar to the Ham *et al.* (2017) finding that *Comamonadaceae* was associated with one group of CO_2-rich sites. However, these sites were a mix of surficial springs and shallow groundwater wells, which provide geochemical conditions that are not comparable to the sites in this study.

Taken together, our study provides some evidence that the CO_2 flood in the Olla Field impacted the microbial community

structure in CO_2-affected regions of the formation. Although the majority of the microbial populations identified in both the CO_2-affected and CO_2-unaffected samples were similar (e.g. most samples were dominated by *Methanothermococcus* spp.), several low abundance taxa were significantly more abundant in the CO_2-affected samples compared to the CO_2-unaffected samples. Some of these OTUs have been linked to CO_2-rich environments; however, their low overall % abundance in the CO_2-affected wells may suggest that any long-term microbial changes to a formation due to a CO_2 flood or leak would be minor or even insignificant. It is important to note that only groundwater was sampled in this study and our findings cannot account for the response of attached microbial populations associated with biofilms or the rock matrix to the CO_2 flood. Under ideal conditions, sampling techniques that can evaluate biofilms or rock-attached microbial populations such as cores or diffusive samplers (e.g. Barnhart *et al.* 2013) would be utilized. Unfortunately, due to core material being unavailable, these methods could not be applied.

CONCLUSIONS

In summary, the present study provides a field-scale representation of how the microbial community structure of a formation may recover from a CO_2 flood or a CO_2 leak and how native microbial communities may change years after the cessation of a CO_2 flood. To the authors' knowledge, this is the only study performed at this time scale (ca. 40 years after flood). No large-scale variation was present when comparing the major microbial communities identified in the CO_2-affected and CO_2-unaffected samples. However, certain lower abundance OTUs were identified in the CO_2-affected samples that were significantly less abundant or absent in the CO_2-unaffected samples. These OTUs were found to be similar to taxa that have been shown to thrive in CO_2-rich environments. Due to their low % abundance here, this may provide evidence for the microbiology of a formation to return to pre-injection conditions after a CO_2 flood ceases or if a CO_2 leak would be stopped. At a minimum, the results of this study show that the microbial community of the CO_2-affected wells is not significantly different than the community identified in the CO_2-affected samples after 40 years post-CO_2 injection. This has wide implications for both CO_2-EOR operations and CO_2 leaks into shallow aquifers due to CO_2 sequestration. The results of this study suggest the potential for any possible microbial effects from or responses to changing the concentration of CO_2 in an aquifer or hydrocarbon-bearing formation to rebound back to pre-injection conditions if the CO_2 concentration was returned to baseline conditions.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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