# Microbial Memory Response: Observing History-Dependent Adaptation to Repeated Exposures of Emulsified Vegetable Oil in a Contaminated Aquifer

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## ABSTRACT

The utilization of native microbial communities to remediate and immobilize hazardous contaminants has been a common practice for decades. One technique commonly employed to enhance this process is biostimulation, where limiting nutrients are added to a contaminated system in order to stimulate favorable reducing conditions for specialized microorganisms. Many biostimulation applications have been conducted using emulsified vegetable oil (EVO), which stimulates growth of indigenous microbial communities and favorable reducing conditions. However, this practice is sometimes known to cause a lag phase before degradation can occur, lessening the overall efficiency of this practice. The studies described herein aim to reduce the lag phase of degradation by taking advantage of a history-dependent adaptation, called the microbial memory response. This is a novel concept which hypothesizes that a microbial community which has been exposed to a substrate in the past will be able to degrade it more rapidly upon a second or subsequent exposure. To do this, two experiments were designed—one laboratory scale microcosm experiment and one secondary *in situ* injection of EVO. Both experiments focus on Area 2 of the Oak Ridge Field Research Center (ORFRC), which underwent a subsurface injection of EVO in 2009. The microcosm experiment included groundwater and sediment collected from two sites: one which had been exposed to EVO before and one which had not. Both types of microcosms were amended with a small amount of EVO and monitored for changes in geochemical parameters and the microbial community. Results from this study indicated that the microbial response to EVO was similar in both types of microcosms. The *in situ* secondary injection was conducted at Area 2 in December 2017 and was monitored for 134 days for changes in geochemical parameters and microbial community. Results from this study indicated that while a distinct community of microbes responded to the EVO injection, the rate at which it was degraded was similar to the primary injection. Overall, neither of the studies showed strong conclusive evidence for the presence of a memory response but did potentially elucidate the limited duration and magnitude of the memory response.

## TABLE OF CONTENTS

Introduction ..... 1
References ..... 8

Chapter I Monitoring Emulsified Vegetable Oil Degradation in Previously Exposed and Unexposed Groundwater Microcosms ..... 13
Abstract ..... 14
Introduction ..... 15
Materials and Methods ..... 17
Results ..... 24
Discussion ..... 31
References ..... 38

Chapter II Microbial Community Response to In situ Secondary Injection of Emulsified Vegetable Oil ..... 46
Abstract ..... 47
Introduction ..... 48
Materials and Methods ..... 51
Results ..... 57
Discussion ..... 63
References ..... 69
Appendix ..... 75

Conclusion ..... 76

Vita ..... 79

## LIST OF TABLES

**Chapter I Tables:**

Table 1. Basic geochemistry measurements for downgradient wells at time of sample collection. ..... 18

Table 2. Average chemical concentration of EVO-amended microcosms. SD columns indicate standard deviations of replicate values. Nitrate and sulfate are recorded in mg/L, iron and uranium are recorded in parts per million (ppm). ..... 25

Table 3. *p*-values of geochemical factors from three-way ANOVA test. ..... 26

Table 4. Results of ADONIS test for EVO-amended and control samples. ..... 31

**Chapter II Tables:**

Table 5. Geochemical measurements for each well at each time point, by their indicated units. BD values indicate measurements that were below the detected limits of the instrument. ..... 58

Table 6. Resulting p-values from repeated measurements ANOVA. Asterisks indicate a significance factor of $p < 0.05$. ..... 60

**Chapter II Appendix Tables:**

Table 7. Time point comparison used to generate repeated measures ANOVA. Since time points in the first and second injections were not identical, similar time points were chosen and re-labeled for the purpose of generating a model for the ANOVA test. ..... 75

## LIST OF ATTACHMENTS

1. Data and Figures (MR_Figures.pdf)

## INTRODUCTION

Advancements in the study of how to utilize the natural breakdown of organic and inorganic matter by microbial species for remediation of polluted terrestrial and marine environments (1). The basic principle of bioremediation is to engineer environmental parameters to provide more favorable remediation of contaminants by the microbial community (2). Currently there are three primary bioremediation processes: monitored natural attenuation, biostimulation, and bioaugmentation. The first and least invasive is monitored natural attenuation (intrinsic bioremediation), a method which the natural microbial community is used to detoxify contaminants with natural processes. Natural attenuation can occur in sites where the native microbes are known to degrade the specific contaminant and if environment has an abundance of limiting nutrients, critical terminal electron acceptors, or other organics for co-metabolic natural attenuation (3). The second method is biostimulation, which still utilizes only naturally occurring microbes, but aims to enhance the degradation rates by adding limiting nutrients, terminal electron acceptors, or terminal electron donors (4). The third and most difficult to prove method is bioaugmentation, which requires the addition of a non-native microbe or enzyme in order to increase degradation rate (3). Several biostimulation methods have been used to quantify the effects that engineered biological degradation and immobilization events have on an ecosystem. The most common ways of monitoring the efficiency and effectiveness of bioremediation is by tracking the changes in important geochemical makers, i.e. degradation by products, and key microbial taxa who are known degraders of the present contamination (4, 5). During *in situ* groundwater remediation processes, degradation can be observed by measuring the shifting redox conditions, which indicate that more favorable electron acceptors are being utilized (4). However, any type of bioremediation process depends on the ability of the microbes to adapt to new environmental conditions, which are prone to rapid and extreme changes during remediation and meteorological events. Biostimulation is the most common method employed for bioremediation and biodegradation applications. Most of the time, the limiting nutrients added to these systems are in the forms of organic compounds such as acids or salts, but there is also a widespread use of

electron donors, such as acetate or ethanol, to stimulate growth of the indigenous microbial community capable of biodegrading or immobilizing contaminants (6). One commonly used carbon amendments is emulsified vegetable oil, or EVO, which can be degraded by certain microbes into important co-metabolites for the utilization of critical dominant terminal electron acceptors.

EVO is used in bioremediation applications via a subsurface injection, mostly into contaminated groundwater systems. It is a soybean oil-based emulsion which has been used successfully in the past to stimulate the remediation of acid mine drainage (7), chlorinated solvents (8, 9), uranium (VI) (10, 11), 2,4,6-trinitrotoluene (TNT) (12), and many others. The 60% SRS-SD Small Droplet Emulsified Vegetable Oil (Terra Systems, Inc. Claymont, DE) has been used in many bioremediation studies previously. It includes sixty-percent food grade soybean oil, four-percent food grade sodium or potassium lactate, proprietary nutrients and emulsifiers, and a small amount of vitamin $B_{12}$. The soybean oil provides large lipid molecules to be hydrolyzed into long chain fatty acids, while the small percentage of lactate is for rapid microbial consumption in order to ensure anaerobic conditions are met shortly after injection. The addition of vitamin $B_{12}$ has been described in previous studies to enhance dechlorinating ability of certain microbes (13). Once EVO is injected into an aquifer system, the oil (mainly consisting of C18 and C16 triglycerides), undergoes rapid lipid hydrolysis and subsequent glycerol fermentation using microbial lipases. Additionally, members of the *Pelosinus* spp. can ferment this glycerol into propionate and acetate (14). The resulting long-chain fatty acid byproducts: linoleic, oleic, and palmitic acids can be oxidized by members of the *Desulforegula* spp. among others (15). This process produces sulfate ($SO_4^{2-}$), hydrogen sulfide ($HS^-$), and acetate byproducts. The acetate from the long-chain fatty acid oxidation and molecular hydrogen from glycerol fermentation are then used as electron acceptors for microbial respiration by many different bacteria including—but not limited to—members of the *Geobacter, Comamonadaceae,* and *Desulfovibrio* families (16). Some of these bacteria also stimulate denitrification, metal reduction (specifically of $Fe^{3+}$, $Mn^{4+}$, and $U^{6+}$), and sulfate reduction. As these compounds are used up by the microbes, reduction/oxidation potential changes in the aquifer system and the dominant terminal electron acceptor process changes. Carbon dioxide is produced from these

many reactions, which is then oxidized into methane by methanogenic archaea *Methanobacteria* and *Methanomicrobia*. This model of subsurface EVO degradation is limited to only key taxa which have already been identified in previous EVO-studies and therefore other unclassified bacteria and archaea may also be participating in the process in unknown ways. However, the specifics of how slow release substrates can generate these persistent biodegradation conditions for long periods of time have been suggested (6, 17).

Further, the microbial stimulation that occurs as a result of EVO degradation is nonspecific and allows for the potential removal of many different types of contaminants, and changes in limiting nutrient availability. In particular, remediation of heavy metals and nitrate contaminated groundwater is a very prevalent utilization of this process. Uranium and nitrate are two of the most common groundwater pollutants in Department of Energy sites; however, treating sites with both types of contamination can be difficult. Part of the process to be observed in this study is the is the reduction of the highly soluble U(VI) to the insoluble U(IV), where it would go from being distributed in the groundwater to being precipitated out in the soil (18). The exact microbial-driven mechanisms behind the reduction events have been studied extensively. In addition to the taxa listed above, there are several well-known microbes that are U(VI) reducers specifically that are good markers for reduction events. Members of *Geobacteraceae* are common metal reducers, but so are mesophilic sulfate reducers including *Pseudomonadaceae* and others (19). In previous studies, a broad mass-spec and proteomics analysis of the microbial community showed a distinct change in abundance of proteins involved in ammonium assimilation, EVO degradation, and lipid fermentation just four days after an injection (20). An ethanol-based remediation injection conducted in 2004 used a functional gene array to observe the differences in gene abundance after the injection (21). Results from this study concluded that the functional gene abundance and richness depended heavily on distance from the injection well, and that the microbial community was indeed stimulated for U(VI) reduction optimization of geochemical and hydrological conditions. One common issue with these other electron donor substrates is that they are consumed too quickly by the microbial community at the point of injection and are unable to stimulate an entire site (22). EVO however, is

considered a “slow release” substrate, meaning it can persist in an aquifer system for months to a year after an initial injection (23). EVO, which is considered an “oil-in-water” emulsion, is more complicated and expensive to prepare than other alternative non-aqueous phase liquids (NAPLs) used in bioremediation, but it requires less oil to be injected and can be distributed at greater distances from the injection site (24). Therefore, there is plenty established information on the organisms and geochemistry involved in EVO biostimulation studies.

One such study was conducted in 2009, in order to observe the specific microbial consortia involved in EVO degradation and contamination reduction (25). The site of this study is Area 2, which is part of the Oak Ridge Field Research Center (ORFRC) at the Y-12 National Security Complex and is located directly downstream from a now-retired hazardous waste disposal site (S-3 Ponds). Area 2 was established as a part of the ORFRC due to its higher-than-average nitrate, uranium, and technetium-99 concentrations (26). The site contains a number of groundwater wells along a neutral-pH gravel pathway which ultimately impinges into Bear Creek, part of the Bear Creek Watershed system. A limited number of wells were chosen as location for an *in situ* bioremediation experiment involving a subsurface EVO amendment. During this experiment, over 3,400 liters of a 20% EVO/groundwater mixture was injected into the nitrate and radionuclide-contaminated aquifer. The groundwater was monitored for a total of 269 after the injection for changes in organic molecules, metal ions, microbial community, microbial diversity, function gene enrichment and sulfate-reducing bacteria (11, 20, 25, 27). Results from this study varied, but generally indicated that sustained U(VI) reduction was possible when using EVO as an electron donor. Microbial analysis of the groundwater post-injection suggested that only a small number of microbial taxa were directly involved in EVO degradation, and the amendment caused the enrichment of functional genes related to bio-reduction events occurring in the subsurface. This injection provided many novel insights into the particular geochemical and microbial processes associated with EVO-based biostimulation. However, two common themes among biostimulation applications are that one: in the majority of cases more than one amendment is needed to sustain reduction of contaminants, and two: the time it takes for the microbial community to become capable of reduction may either be too fast or

too slow (in the case of organic acids). So far, there has been little to no research into how these two important issues may be addressed.

The shifting of metabolic processes necessary for creating anaerobic reduction conditions can take some time, and cause what is known as a “lag phase” in biodegradation (28). The time it takes for the microbial community and environmental system to reach optimal detoxifying conditions is a concern when it comes to cost and effectiveness of certain bioremediation treatments (3). In the past, predicting metabolism kinetics was based mostly on using existing pathways to predict biodegradation (29). However, more recent methods have been developed that are able to model biodegradation processes using the lag phase as an important parameter (30, 31). Developing a means to decrease or otherwise reduce the time it takes a microbial community to get past the lag phase has a potential to make biostimulation techniques more efficient. Previous studies and industry applications have shown that oftentimes multiple amendments of substrates is required in order to achieve significant immobilization and removal of contaminants (1). Because of this, a concept has emerged to try and explain how these native microbial communities are experiencing a type of specific history-dependent adaptation known colloquially as a “microbial memory response”.

In other words, the microbial memory suggests that microbes can gain the ability to respond to and degrade nutrient amendments more rapidly if they have been exposed to that amendment before. The concept of this history-dependent adaptation in microbes has only even been explored in one previous study, written by Wolf *et al* in 2008 (32). In this study, different treatments were initially applied to individual *Bacillus subtilis* cells before undergoing the same treatment. The goal was to trigger a type of stress response that would affect the sporulation ability of the cells, as well as the abundance of a genetic degradation pathway. Results indicated that when cells were grown in low-nutrient broth and then transferred to a starvation broth, cellular growth was rapid as was the activation of specific degradative enzymes. On the contrary, cells grown in a nutrient-rich broth before being transferred to starvation media did not begin sporulation or enzyme production until many hours later. Although the implications from this study are very broad, they support the idea that the types of conditions microbes

are exposed to in the past can affect how they respond to similar conditions in the future. Other studies have come to similar conclusions, but in the more specific scope of cell growth stress-responses in pathogens (33-35). In an environmental context, the microbial memory response has only been hinted at in particular instances, such as the degradation rates of hydrocarbons after the *DeepWater Horizon* oil spill (36, 37). Because of this, it seems reasonable to believe that the same idea of history-dependent adaptation in microbes can be applied to entire communities of microbes existing in contaminated aquifers. Conceptually, the memory response would occur during a biostimulation event after several steps. First, an ecological system (in this case, an aquifer) would be exposed to a carbon amendment or other electron donor, which would eventually result in favorable degradation or reduction conditions. Next, once the amendment had been completely consumed, the microbial community and geochemical parameters would return to their post-amendment state. Finally, a secondary amendment with the same substrate as the first would be added after a period of months to years, and the microbial community would react to this amendment in the same way as the first, but measurably faster. However, this type of response has only been observed in a short term duration (38). Therefore, two separate studies were designed in order to try and observe a microbial memory response using a site which has been previously exposed to an amendment of EVO.

The experiments described herein focus on the “long term” response by monitoring the microbial and geochemical changes during a secondary EVO injection, approximately nine years after the first amendment. The first experiment is a laboratory-scale microcosm study, which was developed with the goal of measuring and comparing the degradation ability of groundwater and sediment which had been previously exposed to EVO, and groundwater and sediment which had never before been exposed to EVO. This experiment would also serve to potentially observe a “contained” memory response—that is to say, to observe a memory response taking place outside of the ecological system. Microcosms were constructed using both groundwater and sediment from sites that had been exposed to EVO during the 2009 injection, as well as sites that had not. The intention was to see what would happen if the two sets of microcosms were exposed to the same amount of EVO at the same

time. If a memory response did indeed occur, it would be apparent by the geochemical and microbial changes in the two sets of microcosms, with the ones from the previously exposed sites being more rapid. Analyzing 16S rRNA genes in the groundwater and sediment communities in addition to the fluctuations in important molecular degradation byproducts was used to observe the possibility of a microbial memory response. However, relating the results of this study to the memory response has possible caveats. This experiment was set up outside of the natural ecological system, where practical applications of bioremediation take place, it would be difficult to say whether or not this response was at the magnitude required for it to be impactful. Even so, the overarching goal of using a smaller scale and laboratory-controlled experiment in order to observe a type of memory effect taking place within the microbial community was an important one. The results of this experiment would help establish to what extent we might expect to observe a response *in situ*.

Thus, an experiment using a secondary *in situ* injection of EVO was designed to take place at the same site as the 2009 injection. The goals of this experiment would be comparing the microbial and geochemical changes from the primary injection and using that comparison to infer the occurrence of a microbial memory response. By analyzing changes in abundance of key EVO-degradation taxa mentioned above, along with the fluctuations of molecular degradation by-products, the results from this study should elucidate the presence of a memory response. If the microbes did exhibit a history-dependent adaptation to the secondary amendment, it would be apparent that the community would be able to degrade this injection of EVO more rapidly than the initial injection. Being able to make this observation in an *in situ* system would greatly expand understanding of the microbial memory response. The exact mechanisms behind this response would require more investigation outside of the scope of these studies. However, the data and samples received from them would provide a starting point for that investigation. A deeper understanding the microbial memory response can inform future biostimulation strategies and applications, increasing their effectiveness and utilization in contaminated sites worldwide.

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# CHAPTER I

# MONITORING EMULSIFIED VEGETABLE OIL DEGRADATION IN PREVIOUSLY EXPOSED AND UNEXPOSED GROUNDWATER MICROCOSMS

## Abstract

In this experiment, we attempted to observe a microbial “memory response”, the idea that a microbial community will degrade a substrate more rapidly if it has been exposed to it multiple times. This novel idea has the potential to increase the efficiency of many commonly-used biostimulation techniques. In order to do this, anaerobic microcosms were developed using sediment and groundwater from a low-contamination aquifer at the Oak Ridge Field Research Center which had been amended with an emulsified vegetable oil (EVO) in 2009. Four groundwater wells from the same site were used to create the microcosms—two of the wells were directly downstream from the previous injection of EVO, and the other two were upstream and unexposed to EVO. All microcosms were amended with EVO, and changes in both microbial communities and geochemical parameters were compared to see if the rate of degradation was faster in those that had already been exposed previously. A respirometer was used to measure gas production in the microcosms throughout several time points. ICP-MS analysis measured anion concentration in the water, as well as trace metals found in the water and sediment. To analyze microbial communities, both microcosm sediment and 0.2 μm [micrometer] pore-diameter groundwater filters underwent DNA extraction and subsequent 16S rRNA gene amplicon sequencing. GC analysis showed that after EVO addition, $CH_4$ and $CO_2$ was produced in both upstream and downstream samples at the same rate; similarly, IC analysis indicated nitrate and sulfate were also consumed at the same rate. Phylogenetic data indicated that the relative abundance of known sulfate-reducing taxa increased and peaked around 30 days after amendment, however, abundance was higher in downstream samples. Principle component analysis of sample OTUs show that both well locations had similar shifts in the microbial community throughout the experiment. However, the statistical ADONIS test shows a significant difference in microbial populations depending on location and time point. This data indicates that degradation occurs at the same rate in both previously exposed and un-exposed samples, and specific microbial taxa are enriched during different dominant terminal electron acceptor processes.

## Introduction

Bioremediation is the process of utilizing an environmental system's innate microbial community to reduce and immobilize harmful toxins; and these technologies and practices have seen much advancement in recent years. One common technique, biostimulation, increases the biotransformation rate of the innate microbial community by increasing limiting nutrients and promoting geochemical conditions which effect the target contaminant (1). Biostimulation has been applied to many sites in the past, typically utilizing an amendment of a carbon source or other electron donor which is added or injected into a subsurface aquifer system. A commonly used carbon source in biostimulation treatments is emulsified vegetable oil, or EVO, because the by-products formed during its degradation are known to sustain reducing conditions in anaerobic systems for months to years after being injected (2). EVO has been used multiple times in the past and present with the specific goal of reducing and immobilizing heavy metals and radionuclides (3-6). Due to the sustainable aspects of EVO degradation, it is considered a more compelling electron donor choice than other organic acids or alcohols sometimes used in biostimulation treatments. However, there are instances where biotransformation rates are too slow to be considered totally effective (7, 8). The lag phase that occurs during remediation events imposes a significant problem on the usefulness of these techniques as a whole (9). Given this, a concept based on previously observed history-dependent microbial adaptation may be applied to *in situ* biostimulation treatments in order to lessen the impact of this lag phase (10).

This concept, termed here as the "microbial memory response", is based on microbial adaptation to repeated exposures of a substrate in environmental systems. Microbes have been observed to respond to changing conditions based on what they have been exposed to in the past (10-12), however this type of memory has so far been anecdotal. The basic principle of how a memory response would occur in the environment is that an ecological system which has been previously exposed to a certain substrate would react to a secondary exposure of that substrate more rapidly. Understanding more about how this history-dependent adaptation or acclimation could be applied to *in situ* bioremediation studies could have an effect on the efficiency and implementation of certain techniques and strategies. Therefore, a small-scale

experiment was designed in order to detect a microbial memory response in native groundwater and sediment communities. By utilizing a laboratory-controlled amendment of an EVO into a series of anaerobic microcosms, this study attempted to monitor the rates of degradation in groundwater which has been both previously exposed to EVO and water which has not. The water and sediment collected from this study came from a contaminated aquifer system that had undergone an EVO in 2009.

In 2009, there was a study conducted to observe the specific microbial community changes that would occur in a reaction to a subsurface EVO injection, and potentially describe the multitude of organisms directly involved with the EVO degradation pathway (13). The injection of EVO was conducted in Area 2 of the Oak Ridge Field Research Center, part of the Y-12 National Security Complex, in late February 2009 using approximately 3,400 liters of a 20% EVO/groundwater solution. The overarching goal of that study was to collect samples after the EVO had been injected, in order to study what type of microbial and chemical changes were occurring in the system in response to the amendment (13, 14). For this study, only groundwater was collected—filters were used for DNA extraction, so therefore all microbial data was based on planktonic communities only. These wells were monitored for a total of 269 days post-injection. The main differences between sample collection in the primary study compared to the microcosm study, aside from the injection itself, are the facts that sediment was collected in addition to groundwater and only four wells were chosen total. Two of them were not involved in the previous study. Having the ability to construct microcosms that use groundwater and sediment from the previous study site could provide the means to detect a small-scale memory effect.

The overarching goal of this study is to attempt to observe a microbial memory response in a controlled laboratory experiment using environmental samples. The primary question being addressed by this particular design is whether or not previous exposure to carbon source amendments would make a microbial community more adept at degrading that carbon source. In order to do this, groundwater and sediment from wells which had been both previously exposed to EVO and previously unexposed from a contaminated aquifer, was taken and then treated them with the same EVO-amendment in microcosms. If the microbial community from previously exposed wells

did in fact have a memory response, the expectation was that those microcosms would be able to degrade the EVO more quickly than the ones who had not been previously exposed. However, because of the limited nature of the microcosms and the fact that once collected they were no longer exposed to any outside or additional influences from the aquifer system, it stands to reason that the overall patterns of changes will need to be the primary focus. Specific concentrations or microbial abundances might be greatly affected by the fact that the microcosms are isolated samples. Despite this, the general trends and rates will still be significant. Furthermore, any indication of the memory affect would support the idea of the "long-term effect" concept, as these microcosms were constructed and exposed to EVO over five years after the original injection. If a memory effect is indeed observable from a laboratory-scale experiment, it would also develop information regarding the magnitude of the response. Conversely, if the null hypothesis were to be supported (i.e. if the exposed and unexposed samples degrade EVO at the same rate) that would not necessarily indicate that there was no response, and instead might mean that the scale of the experiment was not adequate to observe one, or that the memory effect could only be observed in a short-term time frame. Either way, this experiment would provide some much-needed data on the memory response and data for comparisons to the 2009 study and the secondary EVO injection.

## Materials and Methods

### *Study site and primary injection description*

The aquifer system in Area 2 is currently contaminated with nitrate, uranium, technetium, metals, and various volatile organic compounds, sourced from the S-3 pond (now a parking lot) adjacent to the site. The S-3 pond was once a hazardous and radioactive waste dumping site, which operated from 1951 until 1983. According to past reports, the Area 2 aquifer system contain an average uranium concentration of 1 ppm, less than 100 pCi/L of Tc-99, about 40 ppm of nitrate, a higher-than-average pH (6.5), and low levels of dissolved organic carbon (less than 50 ppm) **(Figure 10)**. However, seasonal variation also affects the concentration of contaminants, as nitrate has been monitored to get above 120 ppm in the peak of summer, and then dip below 20 ppm between November and January **(Figure 10)**. Precipitation events are known to affect

contaminate levels, as extended periods of high rainfall will typically lower the nitrate concentration, and vice versa for extended periods of low rainfall **(Figure 10)**. Groundwater in this site flows through a neutral pH carbonate gravel pathway and empties into Bear Creek, which is part of the larger Bear Creek Valley watersheds. This pathway has been postulated to be the cause of the uranium and nitrate contamination found in Bear Creek, because the groundwater flow begins near the S-3 discharge site and ends by seeping into the creek bed. The wells chosen for the 2009 study followed the flow path of the groundwater in order to easily monitor changes in geochemistry and microbial community at increasing distances from the point of injection.

A map of Area 2 and sampling well layout is provided **(Figure 1).** Groundwater flow in relation to this map enter the system beginning at the top and flows southward to the bottom. The reason why the upgradient wells used in the microcosm experiment did not include the control well from the previous injection is because there was some concern that too much EVO had been added too quickly, potentially causing a backflow into the control well. Groundwater and sediment were collected from two wells—GP01 and GP03—which are located downstream from the injection points of the primary 2009 study and two wells—FW231 and GP02—which were located upstream from the injection point. The idea behind this being that the two upstream wells were located far enough away from the original sites of the injection that there was no plausible way they had been exposed to EVO. Since the groundwater conductivity is relatively high in this site (between 0.56 and 0.81 mS/cm), there is little chance that the EVO would have been able to reach either of the upstream wells. However, one of the upstream wells, FW231, while still technically located in Area 2, is still physically closer to the source of contamination (S-3 pond) than the other wells which potentially affects the results of its geochemical measurements and microbial community structure.

**Table 1. Basic geochemistry measurements for downgradient wells at time of sample collection.**

| Well | pH | DO (ppm) | Redox (pE) | Conductivity µS/cm | Temperature (ºC) |
|---|---|---|---|---|---|
| GP01 | 6.7 | 0.62 | 4.363 | 873.3 | 14.47 |
| GP03 | 6.67 | 0.32 | 4.601 | 865.9 | 16.8 |

### *Microcosm construction*

Groundwater and sediment needed to construct these microcosms came from two different groundwater well locations from the ORFRC. Sampling began in March 2015, about five years after the primary injection. Four wells were to be analyzed over four separate time points using destructive sampling. Essentially, for each of the four wells (FW231, GP02, GP01, and GP03) there were four main sampling time points. Each time point had four replicates: two bottles with EVO and two without. The replicates with no EVO would serve as controls. Therefore, there was a total of 64 microcosms constructed for this experiment. On the day of sample collection, field chemical parameters for the groundwater including temperature, pH, dissolved oxygen, hydraulic conductivity and oxidation-reduction potential were measured *in situ* using an Aqua Troll 9500 (In-Situ, Inc. Fort Collins, CO). Each microcosm was filled with 25 g of dry weight sediment and 0.8 L of groundwater. Approximately 13 L of groundwater was collected from each well using peristaltic pumps into sterile glass bottles, using the same methods reported in 2015 by Mark Smith *et al* (15). Sediment (350 g) needed was collected using the surge block method (16). Water and sediment were placed into sterile glass containers and transported back to the laboratory for microcosm construction. They were equally divided and placed into sterile 1 L glass bottles inside an anaerobic chamber. Bottles were sealed with 2-cm thick butyl rubber septum and capped with aluminum crimp seal to allow for gas headspace sampling. Additionally, 200 mL of EVO was added (in replicate) to half of the microcosms from both upgradient and downgradient locations (60% SRS® -SD, Terra Systems, Inc. Claymont, DE). The microcosms were sealed, removed from the chamber and left to incubate anaerobically at room temperature for approximately five months with intermittent destructive sampling on: the day the microcosms were created, after one week, after four weeks and then finally after 21 weeks (150 days). Samples at the start of the experiment were collected before EVO addition.

### *Geochemical and gas sampling*

Important geochemical indicators of EVO degradation were measured using several different methods, depending on the analyte being measured. Chemical oxygen

demand, ferrous iron ($Fe^{2+}$ [Fe2+]) and sulfide ($S^{2-}$ [S2-]) were measured using a DR3900 Laboratory VIS Spectrophotometer (Hach, Inc. Loveland, CO) and corresponding kits. Prior to all geochemical analysis, groundwater was filtered through 10 and 0.2 µm pore-diameter [microliter] membrane filters. Chemical oxygen demand (COD) was measured using high range COD Digestion Vials, 100 mL of sample was digested, 2 mL of digested sample was added to a proprietary reagent and read in the spectrophotometer. Ferrous iron was measured using 10249 FerroVer® [Reserved] Powder Pillows, 1 mL of filtered groundwater mixed with EDTA solution and proprietary reagents before being observed through the spectrophotometer. Sulfide measurements were taken using the 10254 Methylene Blue methods, 10 mL of sample mixed with proprietary sulfide reagents before being observed with spectrophotometer.

Nitrate ($NO_{3-}$ [NO3-]) and sulfate ($SO^{2-}_4$ [SO2-4]) were measured using ion chromatography with a Dionex ICS-2100 system (Thermo Fisher Scientific, Waltham, MA). The Dionex system used an AS9 column with a carbonate eluent (U.S. EPA Method 300.1) to measure anionic concentrations by chromatographic separation and conductivity by making comparisons to a standard curve. Approximately 10 mL of 0.2 µm [micrometer] filtered groundwater was injected into the AS9 column. Calibration curves for each analyte were created using standard concentrations.

Trace metals in the groundwater are indicators of changing metal-reducing conditions and provide evidence to support the efficacy of EVO to stimulate immobilization of radionuclides like uranium (17). Filtered groundwater samples were measured using inductively coupled plasma mass spectrometry using a Perkin-Elmer SCIEX Elan 6100 with a dual stage discrete dynode electron multiplier (U.S. EPA Method 200.8). High concentration samples measured the levels of sodium, magnesium, aluminum, potassium, calcium, scandium, and manganese. Low concentration samples measured levels of lithium, beryllium, aluminum, potassium, chromium, iron, manganese, nickel, cobalt, copper, zirconium, gallium, arsenic, selenium, strontium, silver, cadmium, cesium, barium, lead, bismuth, and uranium. The Elan 6100 separated cationic analytes based on measured mass-to-charge ratio and the calculated instrumental detection limit of each analyte based on isotopic abundance.

The instrument used an internal standard and measured standard spikes in order to generate a relative percent difference between sample duplicates.

Dissolved gas measurements for carbon dioxide ($CO_2$ [CO2]) and methane ($CH_4$ [CH4]) were taken using an SRI 8610C gas chromatograph (GC) using microcosm headspace samples. The sampling method used to collect headspace gasses was similar to ones described in previous studies (18). For each sample including replicates, a smaller volume of groundwater and sediment collected was put into sterile glass scintillation vials and sealed with a rubber cap. At each time point, the bottles would undergo destructive sampling and headspace collection. Samples collected were analyzed on the SRI 8610C instrument for carbon dioxide and methane concentration.

***DNA extraction and 16S rRNA gene amplicon sequencing***

Groundwater was removed by peristaltic pump in the laboratory for each destructive microcosm sampling, as pumps were attached to two separate filter apparatuses, one with a 10.0 µm [micrometer] pore-diameter nylon filter and one with a 0.2 µm [micrometer] pore-diameter polyethersulfone (PES) filter (Sterlitech, Inc. Kent, WA). The water went through both filters and into another sterile container. Sediments were collected after groundwater was poured out by aseptically transferring it into a separate container. The 0.2-micron filters and sediments were collected aseptically into 50 mL Falcon tubes and stored in a -80°C [degrees Celsius] freezer until transportation to the laboratory where DNA extraction occurred. DNA extraction was done on both groundwater filters and sediments using a modified Miller method (19, 20). Sediment and filters (cut in half) were placed into a Lysing Matrix E tube (MP Biomedicals, Solon, OH). Miller phosphate and Miller buffer were both added at a 1.5 mL volume and mixed. A 3.0 mL volume of both phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform were added to the tubes then underwent bead beating on medium/high speed for five minutes. Samples were relocated to sterile 15 mL Falcon tubes before being centrifuged at 10,000 x *g* at 4°C [degrees Celsius] for ten minutes. Supernatant was removed from the tubes and an equal volume of chloroform was added. This process was repeated again, and supernatant was added to another tube with an equal volume of S3 solution (MoBio Power Soil, Carlsbad, CA). Aqueous sample was put onto a spin column with a

multifilter vacuum apparatus filtered completely. A small 500 µL [microliter] volume of solution S4 was added to the filters and centrifuged at 10,000 x *g* for thirty seconds. Flow through from this process was removed and centrifuged once more to ensure quality filtration. Finally, 100 µL [microliters] of S5 solution was added to recover samples in—all extracted DNA was stored at -20ºC [degrees Celsius] until library amplification.

DNA samples were amplified according to the process described in Wu *et al* (2015) (21). DNA was amplified using a two-step PCR in which the first step consisted of amplifying 16S rDNA genes for ten cycles using universal 515F and 806R primers. Secondly, the product from the first step was then amplified for an additional twenty cycles using primers with spacers in order to increase base diversity, barcodes, Illumina adaptors and sequencing primers, and target 515F, 806R primers. Agarose gel electrophoresis was used to guarantee amplification efficiency. The amplified PCR products were combined in equal molality and purified with QIAquick gel extraction kits (Qiagen Sciences, Germantown, MD). A more detailed description of library preparation and PCR set up can be found in Smith *et al* (2015) (15). Amplified sequencing libraries were prepared following the MiSeq™ [trademark] Reagent Kit Preparation Guide (Illumina, San Diego, CA) and the method described in Caporaso *et al* (2012) (22). Combined sample library was diluted to 2 nM before being denatured by combining an equal volume of 10 µL [microliter] of diluted library and 0.2 N NaOH solution and incubated at room temperature for five minutes. Sequencing was performed for 251, 12, and 251 cycles for forward, index, and reverse reads, in that order, on an Illumina MiSeq using a 500-cycle v2 MiSeq reagent cartridge.

***Computational sample processing and analysis***

Quality control for amplicon sequencing data generated from the MiSeq included several different processing steps. Raw FASTQ files from the MiSeq were uploaded to the IEG Galaxy Pipeline (University of Oklahoma, Norman, OK). The pipeline first removed PhiX sequences and then put sequences into split libraries, with a set maximum limit for barcode errors at zero. Primers were trimmed using a sequencing method developed by Wu *et al.* (2015) (21). Average read length was calculated using

Flash to join paired-end reads and filter poorly overlapped and low-quality sequences. A combined .fasta file was generated and any undetermined bases were deleted from the sequences. Sequences were filtered according to length, with the minimum length being between 240 and 250 and the maximum length being between 256 and 260. Data was processed using the ribosomal RNA gene reference database SILVA (23).

Operational taxonomic units (OTUs) were generated using a combination of the algorithms UChime (24), in order to remove chimera sequences and UClust (25) for sequence clustering. The sequences were first processed with UChime, which generated a redundancy map for identical sequences. UClust was used to cluster sequences using a threshold of 97% similarity. The OTU table was generated in Galaxy. Total number of sequence reads were calculated for each sample and resampled until each was rarefied to the same number in each sample. OTU classifiers were made using the RDP Classifier (Michigan State University, East Lansing, MI) as described in Wang *et al* (2007) (26). Sequence alignment and tree generation were done in the Galaxy pipeline, using PyNAST alignment tool to align representative sequences (27). Phylogenetic tree construction was completed using FastTree tool (28). Once the OTU table, classifiers, and phylogenetic tree are all generated, relevant data files were loaded into different statistical software for analysis and figure generation.

COD, pH, nitrate, sulfate, acetate, iron, and uranium concentrations in upgradient vs. downgradient samples was done using ANOVA tests on SAS (SAS Institute, Cary, NC). $CO_2$ [CO2] and $CH_4$ [CH4] levels were analyzed for statistical difference using ANOVA tests in R. All 16S microbial data was analyzed for alpha and beta diversity changes, community dissimilarity, distance matrices and subsequently ordinated using a combination of the R packages Phyloseq (29), vegan (30), and ggplot2 (31), along with several base packages included with R software. Phyloseq objects were trimmed and filtered by removing any OTUs which did not occur more than once in more than 15% of samples. OTUs were further filtered by removing all sequences not in the “Bacteria” taxonomic domain. All distance matrices were calculated using weighted UniFrac distances, which were also used in in permutational multivariate analysis of variance ADONIS tests. Canonical analysis of principal coordinates (CAP) ordination was obtained using weighted UniFrac distances and a general formula including a

model of interacting environmental factors. These factors included: pH, COD, nitrate, sulfate, iron, and uranium levels. Using the weighted UniFrac dissimilarity measure and the given environmental “constraints”, the CAP ordination performs a linear mapping of microbial community in response to changing factors (32). A principle coordinates analysis ordination was constructed using a weighted UniFrac distance matrix, which allows for an insight into the weighted importance of certain environmental factors (33). Samples were also tested for significant differences between control samples and replicates in both filter and sediment samples. Geochemical data came from the microcosm groundwater only, so values for analytes were applied to sediment samples as well to enable CAP ordination with both filter and sediment DNA. A preliminary survey of the top microbial taxa found in these samples was conducted. Nine was chosen as an arbitrary amount of taxonomic families to include. Family was the lowest taxonomic rank chosen due to the uncertainty of accuracy in Illumina sequencing results at more specific ranks (34).

## Results

### *Chemical and geological parameters*

The average concentration and standard deviation for each of the measured geochemical parameters is provided **(Table 2).** Nitrate, sulfate, and iron concentrations did not vary between wells of both upgradient and downgradient locations. Nitrate, at time point 0, was slightly higher in the upgradient wells than downgradient, but not by a significant amount. Likewise, sulfate levels were even among all wells at this point as well, with the exception of upgradient well FW231, which started with a higher amount than all other wells. Iron (II) was below detection limits in all wells at time point zero. Uranium levels at the same time were similar in all wells with the exception of FW231—which had significant variability between replicate samples. This caused the standard deviation to be higher and average to be lower than the other wells. In the past, ICP-MS has been shown to be an accurate measurement of even very low concentrations of radionuclides (35, 36). All other wells started out with uranium concentrations of over 1 ppm, which is average for the Area 2 aquifer.

**Table 2. Average chemical concentration of EVO-amended microcosms. SD columns indicate standard deviations of replicate values. Nitrate and sulfate are recorded in mg/L, iron and uranium are recorded in parts per million (ppm).**

| | Day | $NO_3$ | | $SO_4$ | | Fe | | U | |
|---|---|---|---|---|---|---|---|---|---|
| **FW231** | 0 | 16.380 | ± 3.960 | 50.460 | ± 8.780 | BD | - | 0.514 | ±0.727 |
| | 7 | 0.820 | ± 0.470 | 52.120 | ± 10.090 | 0.116 | ±0.162 | 3.516 | ±0.103 |
| | 30 | BD | - | 0.050 | ± 0.080 | 8.021 | ±1.992 | 0.309 | ±0.071 |
| | 150 | BD | - | 0.130 | ± 0.120 | 25.772 | ±2.925 | 0.104 | ±0.007 |
| **GP02** | 0 | 12.930 | ± 1.780 | 30.420 | ± 42.830 | BD | - | 1.075 | ±0.006 |
| | 7 | 1.780 | ± 0.500 | 40.600 | ± 3.57 | 0.019 | ±0.006 | 1.220 | ±0.008 |
| | 30 | 0.040 | ± 0.050 | 10.520 | ± 5.940 | 5.175 | ±4.128 | 0.414 | ±0.087 |
| | 150 | BD | - | 0.030 | ± 0.050 | 26.808 | ±2.382 | 0.047 | ±0.002 |
| **GP01** | 0 | 12.820 | ± 3.490 | 40.790 | ± 9.600 | BD | - | 1.107 | ±0.001 |
| | 7 | 0.280 | ± 0.400 | 65.220 | ± 15.200 | BD | - | 1.256 | ±0.005 |
| | 30 | BD | - | 5.330 | ± 0.370 | 1.349 | ±0.647 | 0.149 | ±0.087 |
| | 150 | BD | - | 0.210 | ± 0.110 | 8.567 | ±0.289 | 0.041 | ±0.014 |
| **GP03** | 0 | 10.380 | ± 0.270 | 42.790 | ± 0.190 | BD | - | 1.189 | ±0.001 |
| | 7 | BD | - | 70.110 | ± 1.160 | 0.166 | ±0.098 | 1.359 | ±0.023 |
| | 30 | BD | - | 2.970 | ± 0.070 | 1.695 | ±0.135 | 0.399 | ±0.065 |
| | 150 | BD | - | 0.290 | ± 0.000 | 10.257 | ±1.424 | 0.034 | ±0.007 |

Most reduction occurred between time points 7 and 30. In all wells, nitrate levels had effectively depleted between day 0 and day 7 in both upstream and downstream wells. Upgradient well GP02 was the only sample which still had detectable nitrate at day 30, albeit in very low concentrations (0.04 mg/L). Sulfate levels increased in all wells between day 0 and day 7, with the highest increases happening in the downgradient wells. Sulfate levels then dropped in all wells between days 7 and 30, with the highest decreases (relative to previous concentrations) also happening in downgradient wells. Iron increased significantly in all wells between days 0 and 7, with the exception of downgradient well GP01, which was measured at a negative value on day 7. Between days 7 and 30, concentration of iron was increased by an order of magnitude or more by every well. Uranium levels slightly increased between days 0 and 7, but subsequently decreased by an order of magnitude between days 7 and 30. Similar to iron, the difference in concentrations between sample replicates for most wells seems to occur on day 30. This difference is not apparent in nitrate or sulfate levels.

Between days 30 and 150, nitrate levels were below detectable limits in all wells at the final time point. Sulfate decreased in every well between days 30 and 150, with the exception of FW231 which increased. However, concentration of sulfate was so low in FW231 by day 30, this increase is negligible. Iron continued to increase significantly in both up-and-downgradient wells. Concentration of iron increased by an order of magnitude in all samples between days 30 and 150, with the exception of well GP01, which had a lower concentration of iron in general throughout the entire experiment. Uranium levels decreased in all wells between days 30 and 150 by an order of magnitude except in well FW231. Overall, the changes in these important geochemical markers follow the pattern of hierarchical terminal electron acceptors, mentioned in past studies (37). The potential significance of interactions between four main factors (well location, individual well, day, and both location and day) was measured with three-way analysis of variance (ANOVA) tests. The *p*-values generated from these tests are provided **(Table 3)**, with limit for significance set at $p > 0.05$. Location was the only a significant factor for iron levels, while the interaction between location and day was only significant for iron and uranium. Considering the substantial changes of concentrations at each time point, day was a significant factor for each analyte.

**Table 3. *p*-values of geochemical factors from three-way ANOVA test.**

| | Location | Well | Day | Location:Day |
|---|---|---|---|---|
| **Nitrate** | 0.0522 | 0.4468 | 2.8E-12 | 0.1425 |
| **Sulfate** | 0.215 | 0.671 | 5.82E-10 | 0.248 |
| **Iron** | 0.000945 | 0.940253 | 6.77E-09 | 1.43E-08 |
| **Uranium** | 0.298 | 0.308 | 4.11E-06 | 9.94E-06 |

Geological parameters, pH and chemical oxygen demand (COD), also varied between each well. The changes in pH of the microcosm water were relatively the same for GP02, GP01, and GP03. The pH started in between 7.7 and 8 for all samples, increased from days 7 to 30, and decreased between days 30 and 150. Downgradient well GP01 increased the most during the experiment and ended with a pH of 8 at the final time point. Upgradient well FW231 had a similar pH to GP01 from days 0 to 7, but then experienced a decrease in pH from 8.1 at day 7 to just above 7.5 at day 30. The

pH increased after and then rebounded to pre-amendment levels. COD measurements (ug/L), started the same in each sample at day 0. All wells increased in COD from days 0 to 7, with GP03 and FW231 increasing the most and GP02 and GP01 increasing a small amount. Both upgradient wells began decreasing in COD between days 30 and 150, while both downgradient increased in COD at the same time interval. Downgradient wells ended with higher COD levels at the final time point than the upgradient wells. Graphs for both pH and COD changes for EVO-amended microcosms are located in the appendix **(Figure 11)**.

Methane and carbon dioxide production in all wells over the duration of the experiment in EVO-amended and control samples is provided **(Figure 2 and Figure 3)**. Both up-and-downgradient samples produced methane after day 45, which; the concentration increased exponentially until day 117 and leveled out between then and the final time point. The control microcosms had no production of methane. Methane production in these samples follows the expected pattern of the energetics involved in methanogenic degradation (38). In upgradient well FW231, $CO_2$ [CO2] production was relatively similar in both the EVO-amended and control samples. This was not the case for the rest of the samples. In EVO-amended samples, $CO_2$ [CO2] production increased at a linear pace until the final few time points, when it leveled out. Downgradient well GP03 ended with a slightly higher $CO_2$ [CO2] than the other wells. In the control samples (other than FW231), carbon dioxide levels dropped off towards the middle of the experiment and had slight increases until the final time point.

***Microbial community response and EVO-degradation***

Alpha and beta diversity were measured for sediment and filter DNA. For alpha diversity measurements, the full and unpruned OTU data was used, meaning singletons and other “rare” taxa were left in. The alpha diversity changes in filter communities using several different alpha diversity measurements is provided **(Figure 4)**. Throughout all alpha diversity measurements, the downgradient sediment samples were more diverse than upgradient sediments. Alpha diversity measurements decreased once samples were amended with EVO. Day 30 in both up-and-downgradient wells had the lowest diversity. By the final time point, most of the wells had increased since day 30,

but none were close to the amount of diversity found before EVO was added. Chao1 showed that overall richness was greater in sediment samples compared to filter samples, and upgradient wells had less species richness than downgradient samples, especially at early time points. Shannon indices show similar results when it comes to overall diversity in sample types—sediment samples and downgradient samples had a higher number of individual species—but there are differences within the wells. Downgradient sediment samples show a significant difference between day 0 samples and all later samples, while the downgradient filter samples show that the day 0 samples are actually relatively similar to other time points. This is the opposite case with the upgradient samples. Both Simpson and Inverse Simpson indices show a similar pattern. Although microbial diversity and richness started out higher in the upgradient wells than in the sediment samples, they were still generally lower than the downgradient samples **(Figure 5)**. However, these samples followed the same pattern of a significant decrease in diversity following the EVO amendment, the lowest diversity at both day 7 and day 30. Day 150 samples had increased slightly but were still much lower than they had been before EVO was added.

A distance matrix was constructed in order to show if the phylogenic groups between samples were significantly different by focusing on sample location (upgradient or downgradient) and time point (39). The pruned OTU table was used to calculate the dissimilarity matrix that the PCoA ordination is based on. According to the PCoA, microbial clustering follows a clear pattern between each time point for both up-and-downgradient samples, with significant community overlap **(Figure 6)**. There was no overlap in downgradient samples between the initial time point and day 7, indicating that the phylogenetic shift was more significant in downgradient samples between these times. Day 30 communities have the largest spread and least amount of phylogenetic clustering. PCoA also shows that the least amount of change in the communities happens between days 30 and 150. Overall, the PCoA shows that the upgradient and downgradient samples are shifting at relatively the same rate, although small differences are present.

The CAP ordination of EVO-amended microbial community samples and the response to different environmental factors is provided **(Figure 7)**. Nitrate levels had the

most impact on the day 0 samples, since in most wells nitrate was undetectable in later time points. Sulfate and uranium were the most important factors in the day 7 samples. Sulfate and uranium both decreased significantly after day 7, meaning there was not a high enough concentration of it left to impact the later samples. Iron levels increased throughout the experiment, and the samples most impacted by iron levels were the day 150 samples. Iron continued to increase throughout the microcosm study and was highest in the final samples, and the CAP analysis showed it being most important during that time. Changes in pH and COD were most significant in the day 30 samples. By combining the results of the PCoA and CAP ordinations, the phylogenetic shifts in microbial communities and the environmental factors which impact them the most become apparent.

The top nine bacterial families found in both upgradient and down gradient wells are displayed (**Figure 8** and **Figure 9**). Abundance graphs were made by calculating total abundance of top OTUs in pruned OTU tables for both sediment and filter samples. The top nine microbial families in downgradient wells included *Bradyrhizobiaceae*, *Comamonadaceae*, *Helicobacteraceae*, *Natranaerovirga*, *Neisseriaceae*, *Oxalobacteraceae*, *Prolixibacteraceae*, *Rhodocyclacea*, and *Ruminococcaceae*. The abundances of each of these taxa vary widely between the two individual wells, GP01 and GP03. In fact, only one family, *Bradyrhizobiaceae*, is found evenly in both wells. *Prolixibacteraceae* is found in high abundance in GP01 at day 30 and 150, and the in GP03 only in day 150. Some are only really found in one well and not the other—like *Comamonadaceae* and *Natranaerovirga* in GP01 and *Helicobacteraceae* in GP03. Overall, the total abundances of the microbial families in downgradient samples are not evenly dispersed but follow patterns based on changing environmental factors. A similar amount of variation can also be seen in upgradient samples. The top nine microbial families in the upgradient samples are *Brevinemataceae*, *Comamonadaceae*, *Cytophagaceae*, *Desulfobacteraceae, Enterobacteriaceae*, *Neisseriaceae*, *Pseudomonadaceae*, *Rhodocyclaceae*, and *Thermoanaerobacteraceae* (abbreviated on the figure). In the upgradient samples, there are no microbial families that are evenly distributed in both wells. There are actually multiple families that only appear in one well or are vastly more abundance in one well over the other, including *Brevinemataceae*

and *Cytophagaceae* in GP02 and *Pseudomonadaceae*, *Neisseriaceae*, and *Desulfobacteraceae* in FW231. With the exception of *Bradyrhizobiaceae* in the downgradient wells, the highest abundance of these families is only around 6,000 reads or just above it. There is a great amount of variation between the microbial taxa appearing in the samples, and at what abundance.

A statistical analysis of the dissimilarity between microbial communities was performed. This analysis consisted of an ADONIS test, a nonparametric statistical method based on a distance matrix and mapping file and uses them to determine sample grouping form. For these samples, the distance matrix was once again calculated using weighted UniFrac. The ADONIS test determines an $R^2$ [R2] value, which indicates the percentage of variation explained by supplied mapping category, in addition to a *p*-value of significance. This test set the significance level at $p > 0.05$. The dissimilarity calculations for upgradient versus downgradient samples is listed for both EVO-amended samples and control samples, using day, location, and the interaction between location and day (**Table 4**). Unpruned and unfiltered OTU tables were used to calculate distance matrix. Results from the ADONIS test indicate there is a statistical significance in the variance of the means for each sample. Location, day, and the interaction between the two are all statistically significant factors in the EVO-amended microbial communities observed, although the $R^2$ [R2] value for location is lower than day, indicating that the sample location does not explain much of the variance in these samples. As for the control samples, location and day are also significant, but the interaction between the two is not. The ADONIS test does not necessarily mean anything related to the rate of degradation. The control samples are also significantly different by location and day, and the $R^2$ values generated are lower than with EVO-amended samples.

**Table 4. Results of ADONIS test for EVO-amended and control samples.**

EVO and Pre-treated

| Factor | $R^2$ | p-value |
|---|---|---|
| Location | 0.03758 | 0.001* |
| Day | 0.43484 | 0.001* |
| Location:Day | 0.07247 | 0.001* |

Control and Pre-treated

| Factor | $R^2$ | p-value |
|---|---|---|
| Location | 0.06185 | 0.003* |
| Day | 0.27475 | 0.001* |
| Location:Day | 0.09716 | 0.071 |

**- indicates significance of p > 0.05*

## Discussion

Measured geochemical parameters indicate that the rate of EVO-degradation is relatively the same among upgradient and downgradient wells. Rate of production and consumption of terminal electron acceptors was similar in microcosms from both well locations. This is the case for aqueous soluble compounds as well as headspace gas. Stimulation with vegetable oils has been observed to support anoxic growth such as sulfate reduction and methane production for over fourteen months *in situ* (40). However, the microcosms showed a rapid consumption of nitrate and sulfate and may be a result of the closed-system nature of the microcosms. Harkness *et al* (2013) used a similar sediment column study, and observed quick depletion of terminal electron acceptors (41). Biological denitrification occurred at a very rapid pace in all samples, indicating that nitrate is an electron acceptor being utilized by the microbial community immediately after EVO-amendment. Sulfate levels increased in all wells between day 0 and day 7, likely a result of biological processes from degradation of EVO, with the highest increases happening in microcosms from downgradient wells. Fe(III) reduction to Fe(II) occurred after nitrate is depleted in all wells except for one downgradient microcosm from well GP01 and continues until the end of the experiment. Although this reduction takes place in GP01 in the next time point, this delay may possibly be

explained by low initial concentration of Fe(III) in that sample. Subsequently, iron was the only one of the environmental factors that was found to be significantly different by well location. By the final time point, there was much less iron found in the microcosms from downgradient wells than the microcosms from upgradient wells. However, all other geochemical parameters measured began and ended with relatively similar concentration in both up-and-downgradient wells.

In the 2009 injection, most wells had depleted nitrate levels 16 days after the EVO injection. However, there was also a slight increase by day 31, before concentrations rebounded (13). In the microcosms, denitrification started by day 7 and nitrate was depleted in all microcosms by day 30. Therefore, the rate of denitrification was slightly accelerated in the microcosms compared to the 2009 injection. Also, in the original study, sulfate had a substantial decrease in concentration between days 16 and 31 days after the injection, before increasing again. The microcosm samples saw sulfate levels decreasing the most between days 7 and 30; however, because of the amount of time between these two samplings, it is difficult to tell if sulfate reduction was actually more rapid in the microcosms. After the 2009 injection, iron levels in some of the wells monitored did begin to increase after just four days. In other wells—including GP01 and GP03—iron concentration increased until 31 days after the injection before beginning to decrease again. Reduction of uranium was delayed in the microcosms when compared to the 2009 injection. Wells after the 2009 EVO injection began decreasing in uranium concentration just four days after the injection and continued to do so until day 31. Microcosm levels of uranium did not begin to decrease until after day 7. All microcosms did have less uranium concentration at the beginning of the experiment than the wells did before the initial injection of EVO. Due to limitations of laboratory microcosm experiments, it is difficult to conclude why the previously exposed and previously unexposed samples had degraded the EVO at the same rate. Past studies have observed how the use of microcosms to study natural systems greatly restricts environmental diversity (42, 43), which may affect EVO degradation rate. It is possible that the small volume of sediment and groundwater and the sealed-off nature of the microcosms themselves caused these rates to be so similar. So, when monitoring

geochemistry alone, the rates of changes in the dominant terminal electron acceptors is too similar to strongly conclude the presence of a memory response.

Carbon dioxide and methane production rate was similar in both microcosm well locations. The only significant difference was the fact that $CO_2$ [CO2] was generated in the control samples of FW231 at the same rate as the EVO-amended samples. This was not the case for any of the other wells. There is a possibility that compounds in the control microcosms could also sustain the production of $CO_2$ when other control samples could not, as well as a possible bottle effect. Overall, each of the samples is showing signs that the EVO is in fact, being degraded and that soluble uranium levels are decreasing (44). Chemically speaking, results strongly indicate there is no significant increase of degradation rate between previously exposed and unexposed samples. Gas production was not measured in the 2009 study, so there is no way to compare the two. However, based on results from the microcosms, there is no difference in carbon dioxide or methane production in any samples.

The dynamic and diverse populations of bacterial organisms captured in these wells, both in the groundwater and the sediment were measured using alpha and diversity measurements. Chao1 measurement focuses heavily on the occurrence of low-abundance taxa and species diversity decreases significantly in later time points, so method may not be suitable for accurately describing richness in later time points. The abundance-based coverage estimator (ACE) measurement suggested that richness was much lower in upgradient sediment samples but was relatively even between up-and-downgradient wells in the filter samples. However, ACE puts more of a reliance on the presence of low-abundance species, suggesting that the “rare” taxa are out-competed by the larger populations at later time points in the experiment. The divide between the results of the Simpson, Shannon, and Inverse Simpson indices might stem from the fact that the upgradient wells are located physically closer to the source contamination, meaning that over time the sediment in these wells have higher concentrations of contaminants, lowering their microbial diversity (45-47). As for the control samples, alpha diversity measurements for all samples also decrease over time, but at much more gradual pace, indicating that the microbial populations die off once nutrients are consumed. Since the planktonic populations within the groundwater would

be constantly moving and flowing through the system, they would have similar diversities.

There is a distinct shift this populations immediately following the addition of EVO, and after each additional time point. The same type of distinct community shifts is not seen in the non-EVO control samples **(Figure 12)**, indicating that these community changes are a direct cause of the EVO-amendment, a result also seen in similar past studies (48, 49). However, there is no evidence that the previously exposed samples are shifting more rapidly than the previously unexposed samples. The CAP analysis shows that the changing geochemical parameters has an equal effect on both up-and-downgradient samples. A similar ordination done after the 2009 injection (Gihring *et al.* 2011. Figure 6) shows that nitrate in both the field study and microcosm was most influential during the pre-EVO time points. However, most other geochemical parameters are different. Sulfate concentrations in 2009 were effective at the same point as nitrate, but the microcosm sulfate and uranium levels were occurring at the same, but later time. CAP analysis results suggest that microbial community changes in relation to the EVO degradation are slower than in the 2009 study, and equal between upgradient and downgradient samples. The most likely explanation for the differences between the 2009 monitoring wells and the microcosms is the closed-system nature of the microcosms themselves. Because the microcosms were not exposed to any additional environmental factors or influences after being collected, the microbial community and geochemistry present at the time of collection was all that was available to change and react during the experiment.

The ability to observe phylogenetic changes among OTUs in response to the addition of EVO offered a perspective on microbial community adaptation, especially in comparison with the 2009 injection. According to the abundance plots (**Figure 8** and **Figure 9**), there are many distinct differences between the major taxonomic groups found in the upgradient wells versus the downgradient wells. The two locations only had three of the same families found in their top OTUs: *Comamondaceae*, *Neisseriaceae*, and *Rhodocyclaceae*. *Comamondaceae*, found in downgradient wells mostly in day 7 and upgradient wells mostly in days 7 and 30, contains a number of denitrifying bacteria (50). *Neisseriaceae*, which appeared most abundantly in both well locations on day 7, is

also known to have denitrifying members (51). *Rhodocyclaceae* was found in the downgradient wells at day 7 and in the upgradient wells at day 30, and contains both denitrifying bacteria (52), and sulfate-reducing bacteria in nitrate-reducing conditions (53). The downgradient samples included a high abundance from the *Bradyrhizobiaceae* family on day 150, which includes members with sophisticated iron and manganese regulatory systems (54, 55), potentially explaining how they are present in such high abundance during the highest concentration of iron. Both of the *Helicobacteracae* and *Natranaerovirga* families had the highest abundance in downgradient samples at day 30. The former, along with many epsilon-proteobacteria, are known to thrive in sulfate-reducing conditions (56), while the latter is not very well described and has been mostly found in extreme environments (57).

The upgradient wells had a relatively high abundance of the *Entereobacteriaceae* in both wells, which decreased over time. This inclusive family is known to contain denitrifying (58), and sulfate reducing (59) organisms. Other than *Comamondaceae*, the only other family with high abundance on day 7 was *Pseudomonadceae*, which includes fatty acid fermenters (60), and denitrifiers (52). By day 30, the most abundant family *Rhydocyclaceae* in well FW231 and *Cytophagaceae* in well GP02. The latter of which has been observed to increase in abundance in nitrate, sulfate, and iron reducing conditions (61). The family *Desulfobacteraceae* was highly abundant in day 30, but mostly in well FW231. This family contains many different genera of strictly anaerobic, sulfate-reducing organisms. During the final time point, the two most abundant families were *Thermoanaerobacteriaceae* in well FW231, and *Brevinemataceae* in well GP02. *Thermoanaerobacteriaceae* has been observed in the past to be an acetate-oxidizer in methanogenic conditions (62), while *Brevinemataceae* is not very well described, but has been found to contain microaerophilic organisms isolated from small rodents (63). Taxonomic presence/absence of recognized 16S rRNA gene does not necessarily indicate presence of specific metabolic functions or genetic pathways of degradation. However, these families have a diverse range of species capable of many different types of metabolism. Combining the abundance of these families with the chemical data gives a strong indication of what types of organisms are responsible for these changes.

It is possible to compare some of the community results from the 2009 study to this study. The *Comamonadaceae* and *Ruminococcaceae* families were highly abundant in the 2009 monitoring wells on day 4 and between days 17 and 140. Specific genera were found in 2009 that belong to several of the families found in this study, such as *Vogesella* and *Desulforegula*. The 2009 injection also saw significant occurrences of organisms not found in the microcosms. For one, the important sulfate-and-metal reducing group *Geobacter* was one of the most abundant species found in the 2009 monitoring wells early on, and while *Geobacter* species were found in the microcosm samples, they never appeared in high enough abundance to make up a significant portion of the population. *Pelosinus*, known for its ability to hydrolyze lipids (64) was found in certain wells in the 2009 injection with 75% relative abundance. This group only appeared in in wells FW231 and GP03 on day 30 in relatively low abundance (under 200 reads). Although there were instances of many other members of the lipase-producing *Veillonellaceae* family (65), it was never enough to be in the top families. It is impossible to conclude that the downgradient wells had more in common with the 2009 results than the upgradient wells. However, the microcosm community suggests that the exact types of species present in each sample may be different, but each sample does contain organisms capable of utilizing all terminal electron acceptors. Samples therefore had more or less the same ability to degrade the added EVO.

Without a deeper analysis of specific functional genes or metabolic pathways in the microcosms, cannot be concluded that the results of this study support the memory response hypothesis with a long-term duration. The null hypothesis of both microcosms experiencing the same rate of degradation cannot be rejected. If a memory response is present in the downgradient microcosms—either the small scale-closed off nature of the microcosms themselves, the limited amount of EVO added, the unmitigated variety of each sample, or the amount of time that has passed between exposures is making it practically undetectable. The microcosms themselves cannot truly be considered representative examples of a natural ecological system, due to the groundwater and sediment collection methods. Groundwater, although collected before the sediments, was not conducted anaerobically, which may have affected the communities. Sediments collected using the surge block method could have reintroduced oxygen into the well

locations and disturbed or destroyed anaerobic microbial populations. However, the microcosms did have an enrichment of anaerobic taxa after the addition of EVO, but it is impossible to tell how the community might have changed after groundwater and sediments were collected. Future studies attempting to monitor an environmental system for a memory response may need to be conducted in a more consistent and sequential time frame. This study has shown that the amount of time between exposures is an important factor when measuring a memory response, and six years could be the upper-limit of microbial memory. It is possible that a secondary *in situ* injection of EVO at the same field site might elucidate these results and provide a more accurate system for exploring the possibility of a microbial memory response. For this study however, the presence of the memory response cannot be supported until a more extensive evaluation of the microbial community is conducted.

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# CHAPTER II

# MICROBIAL COMMUNITY RESPONSE TO IN SITU SECONDARY INJECTION OF EMULSIFIED VEGETABLE OIL

**Abstract**

Biostimulation is an important bioremediation technique which requires the addition of limiting nutrients, electron donors, or electron acceptors into a contaminated system, in order to stimulate growth of the innate microbial community. This technique can have a detrimental lag phase making it less efficient. However, microbes exposed to this substrate more than once may experience a “microbial memory response”, meaning it will be able to degrade the substrate more rapidly upon a second exposure. This type of long term history-dependent adaptation has been anecdotal so far. This study aims to detect a microbial memory response in a nitrate and uranium contaminated aquifer and then subjecting it to a secondary injection of emulsified vegetable oil (EVO) a decade after the first. To do this, a 20% EVO/groundwater mixture was injected into an aquifer and monitored for changes in geochemistry and microbial community structure up to 134 days after the injection. HPLC was used to measure major anions in the groundwater. Following the injection, early denitrification was indicated occurring before rapid sulfate reduction and acetate production. ICP-MS was used to measure trace metal concentrations. Showed iron, uranium, and manganese reduction approximately one week after injection, with sustained reduction up to 50 days. Cell counts measured by Acridine Orange Direct Count (AODC) method indicated total cell density increased in response to EVO amendment. Additionally, microbial communities underwent 16S rRNA gene amplicon sequencing which showed a distinctly different consortium of microbes were involved in EVO degradation when compared to the first injection. However, despite the small differences between the injections, there was not enough evidence to conclude that a memory response was indeed present. Further investigation into specific pathways present in order to determine how bioactivity was different from the primary injection is needed. This study does potentially elucidate the duration of the memory response and the differences in key microbial taxa that were enriched during EVO degradation.

## Introduction

Native microbial communities in the soil and groundwater have been used to help clean up environmental systems contaminated by hazardous compounds for decades. With the right species, conditions, and energy sources, certain microorganisms can degrade or immobilize a wide array of toxic substances found in the environment. These bioremediation practices depend heavily on these factors, however and without all three of them it can be difficult to have a significant effect on contaminated areas. Because of this, a technique known as biostimulation was developed in order to provide microbial communities with the limiting nutrients needed for bioremediation or bioimmobilization (1). Biostimulation is the process of adding limiting nutrients and/or terminal electron acceptors or electron donors into an environmental system with the intention of stimulating growth of the native microbial community which are able to degrade the contaminants. Most commonly, this method utilizes some type of electron donor, terminal electron acceptor, or both and applies it via a subsurface injection into an aquifer (2). The aquifer is then able to transfer this substrate throughout, where it is degraded by the microbial community present. One substrate commonly used in biostimulation applications is emulsified vegetable oil, or EVO.

EVO is a well-known bio-stimulant, which has been used in bioremediation applications and biodegradation of hazardous materials many times in the past (3-6). EVO is used in this study as an electron donor and limiting nutrient, to a contaminated aquifer system. It consists of a 60% emulsified soybean oil mixed with 4% food grade sodium or potassium lactate, 7.5% proprietary food grade emulsifiers and preservatives, and less than 1% food grade nutrients and vitamin $B_{12}$ [B12]. The small droplet SRS-SD™ [trademark] EVO was used to maximize the effective area once in the aquifer. One reason EVO is used in bioremediation practices is because it is considered a “slow-release” substrate, which means it is more likely to spread out and subsist in an aquifer system (7). Other organic acids or alcohols used in similar studies are normally used up by the microbial community almost immediately upon injection and do not travel far enough from the injection point to stimulate sustained reduction conditions (8). Once EVO is injected into the system, it is readily degraded and creates favorable anaerobic reducing conditions (9). A mathematical model created to simulate the biological

degradation of EVO indicated that a large amount of long chain fatty acids would precipitate out after biological EVO-hydrolysis, followed by a rapid accumulation of denitrifying and sulfate-reducing bacteria (10). This would then serve to produce enough terminal electron acceptors for the microbial community to utilize, thereby promoting the bioimmobilization of U(VI), with the most accumulation occurring at the site of injection. However, one reoccurring issue with using biostimulation techniques is the microbial lag phase, which is the time it takes from the injection to the point where the aquifer reaches metal reducing conditions (11). Decreasing the lag phase could potentially enhance the microbial community's bioremediation efficiency. Because of this, the concept of utilizing history dependent adaptation in microbial communities requires a deeper understanding.

There have been laboratory-scale instances of microbes "remembering" past cell treatments (12), and evidence of past contaminant exposures enhancing microbial degradation ability (13), but currently the evidence on how this ability could be used *in situ* is anecdotal. However, based on these previous studies, it could be possible that even at an environmental scale, microbes may retain a "memory response" to exposures of certain substrates. A microbial memory response is the idea that an environmental system's native microbial community, which has been previously exposed to an electron donor substrate will be able to degrade that substrate more rapidly upon subsequent exposures. A definite presence of a "memory effect" in an environmental system has been seen in a short term duration using ethanol (14). In order to observe a memory response *in situ* a previously contaminated or exposed system would need to be subjected to a secondary exposure and monitored for changes in geochemistry, hydrological parameters, and microbial community (1). This study utilizes a field site located in Area 2 of the Oak Ridge Field Research Center (ORFRC) that contains a nitrate and uranium-contaminated aquifer system. This site was used to test the efficiency of EVO as a slow release bio-stimulant for denitrification and the immobilization of uranium (15). This was done by conducting a subsurface injection of approximately 3,400L of a 20% EVO/groundwater mixture into the aquifer system in February 2009. The 2009 injection was used as the subject for many studies which showed that addition of EVO stimulated specific members of the microbial

community (15), sustained immobilization of uranium would be more realistic with multiple amendments (16), specific enrichment of sulfate-reducing genetic pathways (17), and increased production of EVO-degradation related proteins immediately following injection (18). The amount of data generated from the first injection make this site ideal for a comparative memory response study. This site was selected to undergo a secondary *in situ* amendment with EVO to attempt to observe a memory response nearly nine years after the initial amendment.

Results of initial injection caused many changing dynamics in the aquifer. Microbial diversity decreased dramatically following the EVO amendment—suggesting that only a narrow range of organisms capable of utilizing the EVO and its by-products out competed other species very early on (15). Analysis of sulfate-reducing *dsrA* gene indicated there was a significant inverse-correlation correlation between abundance of *Desulfovibrio*-like *dsrA* genes and soluble uranium concentration—with the increases abundance of said gene correlating to the decrease in U(VI) levels in the groundwater just a few days after the amendment (17). These results also suggested that the presence of hydrogen sulfide generated from early sulfate-reducing organisms could be an important factor in sustained U(VI) reduction. A proteomics study of the groundwater just four days after the injection showed an increase in enzymes related to EVO degradation, sulfate reduction, and denitrification (18). Ultimately, the geochemical markers and microbial diversity rebounded at different times to pre-injection conditions, but the U(VI) levels were markedly decreased for nearly one year after the injection. Initial changes in geochemistry and microbial community from the original injection will be what is primarily used and compared to in the current study in order to observe a microbial memory response.

The parameters measured in this study are meant to directly relate to the results of the primary injection. The field parameters measured pH, DO, temperature, conductivity, and redox potential at each well at each time point in order to check for hydrogeological effects on the aquifer as a result of the EVO amendment. The geochemical parameters monitored are all indicators of anaerobic stimulation and biodegradation. Even though neither EVO itself, nor its immediate degradation by-products (long chain fatty acids, glycerol, etc.) were directly measured, the generation of

acetate has been used in the past as an indicator for EVO degradation (19). Additionally, this study used Acridine Orange direct counts (AODC) to calculate microbial density fluctuations following EVO amendment. AODCs provide information on how cell numbers and changed as a result of EVO amendment. Finally, 16S rRNA gene data from groundwater filter samples were collected to determine the microbial community structure and diversity of the aquifer after the EVO injection. It was apparent in the 2009 study that a very limited range of microbes quickly dominated the communities after the amendment. The overarching goal of this study was to observe if the same microbial and geochemical changes detected in this first amendment occurred in the secondary amendment, and at what rate. If the microbial community does have a "memory" of the previous injection, it would respond to the current injection more quickly, and EVO would be degraded sooner than it was the first time. If a memory effect was not present, the microbial community would degrade the EVO from the secondary injection at the same rate as the first. This study would not only document the presence of a memory response for the first time *in situ* but would also give an insight as to how long the duration of memory response can last. Parameters measured in both the 2009 study and the current study will elucidate the presence of a long-term microbial community response for the first time in an environmental system.

## Materials and Methods

### *Sample site description*

Area 2 is a high permeability, pH-neutral gravel pathway that leads away from the S-3 ponds—a hazardous and radioactive waste container which is now capped by a parking lot—and impinges out into Bear Creek. This aquifer has been proposed to be the source of contamination in the Bear Creek watershed due to its connection with the S-3 ponds. The aquifer has been described as having lower concentration of contaminants (soluble uranium, nitrate, and technetium-99) than areas that are closer to the ponds, but they are still high enough to be above drinking water standards. A map of the different areas of the ORFRC and their average contamination levels is provided (Appendix Figure 1). The bedrock of Area 2 consists mostly of a saporlite-clay layer with gravel mixed at the surface. Fortunately, the permeability of this area is still very high,

and averages around $10^{-3}$ [10^-3] cm/s, which is an order of magnitude higher than the rest of the site (20). The geological and hydrological parameters of Area 2 make it a readily available site for bioremediation studies. The primary EVO amendment was conducted using three adjacent groundwater wells to actually inject the substrate/groundwater mixture. The subsequent biogeochemical changes were monitored using seven immediately downstream wells, and one upstream well as a control. The length of the entire field site from the northern most control well to the farthest south monitoring well is approximately five meters. This study uses the same injection wells and control well as the original injection but uses less monitoring wells as some had been physically damaged over time and could not be repaired. The site at Area 2 that the original injection was conducted at had not been used for any other biostimulation or remediation studies since.

***EVO injection and groundwater sampling***

The Area 2 field site consisted of three injection wells, one upstream control well, and four downstream monitoring wells. A map of the site and these wells can be seen in **Figure 13**. All wells used in this study were also used for injection, controls, and monitoring in the original injection. The secondary EVO injection was conducted on December 13, 2017 at the site. Approximately 208 liters of SRS®-SD [reserved] Small Droplet Emulsified Vegetable Oil (Terra Systems, Inc. Claymont, DE) was poured into a plastic 525-gallon horizontal leg tank. Three peristaltic pumps, connected to the injection wells, then pumped approximately 832 liters of groundwater into the same tank. Once a volume of 1,040 total liters had been reached, the EVO and groundwater were thoroughly mixed by using circulating peristaltic pumps. The solution had been mixed, the three pumps were then reconnected to the injection wells, and the entirety of the EVO/groundwater mixture was pumped back into the aquifer over a period of five to six hours, or at an approximate rate of 3 L/min. Groundwater and geochemical parameters were sampled once prior to injection; and after injection sampling was done the day after injection, then once a week for four weeks, and finally once a month for four months for a total of nine time points. Geochemical parameters including pH, temperature, dissolved oxygen, conductivity, and redox potential were measured at

each well for each time point using the Aqua TROLL 9500 (In-Situ, Inc. Camas, WA). Additionally, 10 liters of groundwater was collected for filtration and subsequent DNA sequencing at the control and monitoring wells only. For the first four time points, all water was filtered in the field using two attached pressure filter holders, through a polycarbonate (PCTE) 10 µm [micrometer] pore-diameter and nylon 0.2 µm [micrometer] filter (Sterlitech, Kent, WA) respectively. The 0.2 µm [micron] pore-diameter filters were collected aseptically and stored in 50 mL Falcon tubes on dry ice, until transported back to the laboratory for storage in a -80°C [degrees Celsius] freezer. From day 22 on, all 10 liters of groundwater were collected in sterile containers and then transported to a laboratory for filtration. All 0.2 µm [micrometer] pore-diameter] filters were stored until needed for DNA extraction. A small volume of filtered groundwater was also collected for geochemical analysis. Two 20-mL sterile scintillation vials were filled with no headspace from each well at each time point. One vial was stored at 4°C [degrees Celsius] and used for anion analysis on HPLC. The other had 1 mL of sample removed and was acidified with 100 µL [microliters] of 1M HCl for preservation and stored at 4°C [degrees Celsius] until cation analysis on ICP-MS. Unfiltered groundwater was also collected for Acridine Orange Direct Counts (AODC). For each well at each time point, a sterile 15 mL Falcon tube with a 4% formalin solution (4 mL DI water, 2 mL of formaldehyde) was filled with approximately 11 mL of groundwater in order to fix and preserve cells. AODC tubes were stored at 4°C [degrees Celsius] until prepared.

***HPLC and ICP-MS sample analysis***

Groundwater samples were analyzed for nitrate, sulfate, acetate, and other anionic compound concentrations using high-performance liquid chromatography (HPLC). This was done using a Dionex 2100 system and an AS9 column with carbonate eluent, as described elsewhere (21) (U.S. EPA Methods 300.1 and 317.0). Calibration curves were calculated using Chromeleon software (ThermoFisher Scientific, Inc., Waltham, MA) and five internal standards. Curve values were produced on HPLC using manual curves based on $R^2$ equations. Certain samples collected which still had a visible amount of EVO after being filtered were filtered through a 0.2-micron filter again

using a syringe filter. Samples which were still thought to have EVO were diluted to prevent interference with the column. Cationic compounds and trace metal concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS) on an ELAN 6100 system (PerkinElmer, Inc. Waltham, MA). Samples were analyzed for levels of sodium, magnesium, aluminum, potassium, calcium, scandium, iron, manganese, terbium, and uranium, as described previously (22). Multielemental internal standard was added to each sample in order to cover desired analytes, before being diluted with a 1% nitric acid solution and injected into the instrument with the system's autosampler. For quality control purposes, sample duplicates were run during the analysis once for every twenty samples, as well as calibration standards once every ten samples. Samples were preserved with hydrochloric acid but analyzed on the ICP-MS using nitric acid standards and dilution since the difference has not been known to cause interferences with these particular analytes in the past (23).

***Direct cell counts***

A small volume of unfiltered groundwater was collected at each well for each time point to calculate microbial cell counts. Cells were fixed by being added to a 4% formalin solution. Samples were prepared for a modified Acridine Orange Direct Count (AODC) method (13). For most samples, 1 mL of groundwater was filtered through a 0.2 µm [micrometer] pore-diameter black polycarbonate filter (Whatman International, Ltd., Piscataway, NJ) using a vacuum filtration system. However, due to excessively high cell counts or too low sample resolution, some samples were diluted to 10X concentration in DI water. Resulting filters were then stained with 25 mg/mL acridine orange and left to soak for two minutes. All filters were flushed with sterile PBS solution and before being removed from the vacuum and placed on a microscope slide to be read using a Zeiss Axioskop microscope (24). Cell count results were calculated as average cells per milliliter of water.

***DNA extraction, PCR, and 16S rRNA gene amplicon sequencing***

The 0.2 µm [micrometer] pore-diameter filters were underwent DNA extraction using a modified Miller method (25). Filters were aseptically cut into quarters and placed

into a Lysing Matrix E tube (MP Biomedicals, Solon, OH), along with a 1.5 mL volume of Miller phosphate and Miller buffer, and mixed. Next, 3.0 mL of a phenol-chloroform-isoamyl alcohol (25:24:1) solution and 3.0 mL of chloroform were added to the tubes and filters were lysed via bead beating on medium/high speed for five minutes. Samples were transferred to new tubes and centrifuged at 10,000 x *g* at 4°C [degrees Celsius] for ten minutes. The supernatants were taken from the tubes and added to an equal volume of chloroform. Centrifugation and chloroform addition was repeated and resulting supernatant was added, in equal parts, to a tube containing S3 solution (MoBio Power Soil, Carlsbad, CA). The liquid from these tubes was transferred to a multifilter vacuum spin column until all of the sample had been filtered. Then, 500 µL [microliters] of S4 solution was added to each filter and centrifuged at 10,000 x *g* for 30 seconds. Aqueous filtrate was removed and centrifuged once more, to ensure complete filtration. 100 µL [microliters] of S5 solution was added to all filtrate samples in order to recover DNA. Extracted samples were then stored in a -20°C [degrees Celsius] freezer before library amplification.

DNA amplification and library preparation was done as described in previous studies (21, 26). Briefly, DNA was PCR amplified using two steps. The first step consisted of amplifying 16S rRNA genes for 10 cycles using 515F and 806R primers. The second step takes the product from the first step and amplifies rDNA for an additional 20 cycles using primers with spacers in order to increase base diversity, barcodes, Illumina adaptor and sequence primers, and the 515F/806R target primers. Amplified samples were checked for process efficiency using gel electrophoresis. Sample PCR products were pooled together in equal molality and purified. Resulting libraries were prepared using the MiSeq™ [trademark] Reagent Kit Preparation Guide (Illumina, San Diego, CA) (27). Sample 16S rDNA was then sequenced using 251, 12, and 251 cycles for forward, index, and reverse reads on an Illumina MiSeq with a 500-cycle v2 MiSeq reagent cartridge.

### *Amplicon sample processing and statistical analysis*

Sequencing data generated from the MiSeq analysis is processed to ensure quality over several steps. First, data is combines pair-end reads and filters out poorly

overlapped and unqualified sequences using tools available through the IEG Galaxy Pipeline (University of Oklahoma, Norman OK). Sequences are then demultiplexed of raw fastq data, with barcode errors set at zero, and primer trim. The reads with average quality scores of less than 20 are completely removed by Btrim (28), before paired-end reads were combined using Flash (29). Any sequences containing unidentified bases or that had a length outside of the range (240-260 base pairs) were also removed. Both chimera sequences were removed and sample OTUs were generated using algorithm UPARSE (30), with a 97% sequence similarity threshold. OTU classifiers were generated using the reference database SILVA (31), and identified sequences taxonomically with RDP classifier based on 16S rRNA training set (32). Representative sequence data was aligned using Clustal Omega (33), and then generated OTU phylogenetic tree using FastTree (34, 35). OTUs with only one sequence read across all samples (global singletons) were removed before each sample sequences were rarefied to 44,090 reads per sample.

All statistical analyses were completed primarily with R Studio (version 3.4.4). Geochemical and cell count comparisons for statistical significance were run using ANOVA tests, included in the base software. There were several steps to processing both sets of data before they could be compared to each other because the sampling time points were not the same in each experiment. Corresponding time points had to be chosen so six similar time points between the two studies were chosen and labeled as days 1 through 6 (**Table 3**). Geochemical results from the first study were then converted from μM into mg/L for nitrate and sulfate, and μg/L for iron and uranium. Differences in the analytes concentrations were tested for statistical significance using a type III repeated measures ANOVA with a split plot repeated measures design model (36).The 16S microbial data analyses including alpha and beta diversity measurements, calculation of community dissimilarity and clustering, distance matrices, and resulting ordinations were done using a combination of R packages including Phyloseq (37), vegan (38), DESeq2, and ggplot2 (39). Beta diversity measurements were made after trimming Phyloseq objects by removing OTUs which did not occur more than once in more than 15% of samples. Weighted UniFrac distances were used in the calculation of

all distance matrices used for ordination as well as permutational multivariate analysis of variance ADONIS tests.

## Results

### *Geochemistry*

Geochemical changes can be seen in their exact concentrations by well and time point (**Table 5**). Nitrate levels in the wells vary but do show signs of early denitrification events. Levels in the control well (FW215) are fairly variable. The well closest to the injection point, FW216, experiences a sharp decrease in nitrate levels in the first week after injection, as does MLSB3, which is adjacent to it. FW216 nitrate levels recover after 15 days, but MLSB3 does not return to its pre-injection concentration by the final time point. The two farther wells, GP01 and GP03 experienced a marked decrease in nitrate levels by the eighth day after the injection. The day after injection, EVO was found in such a heavy volume in GP01 it was unable to be analyzed on the HPLC. Nitrate levels did not recover in GP01 until between 50 and 78 days post injection, and between 78 and 106 days post injection in GP03. Sulfate levels stayed relatively stable at all time points in the control well, as expected. FW216 experienced a decrease in sulfate levels 8 days after the injection but recovered by day 15. In the adjacent well, MSLB3, sulfate levels decreased between 8 and 22 days after injection, before beginning to recover. Sulfate depletion started after day 15 in GP01 and GP03, and levels did not start to recover until after day 50. Acetate was present in the control well at three time points in low concentration, with the highest level being at in the final day. FW216 increased in acetate 8 days after the injection, but levels were virtually undetectable in other days until another spike on the last day. MLSB3 had a significant increase in acetate between days 8 and 22, with another increase at the final day. This was similar to wells GP01 and GP03, except both of these wells still had a significant amount of acetate by day 50, and another slight increase can be found in GP03 at day 106. Iron (II) levels in the control well seem to slowly decrease throughout the experiment, while uranium levels tended to stay the same. Iron in FW216 varied concentrations fluctuated between several time points. Uranium levels stayed mostly consistent in FW216 throughout the experiment. MLSB3 iron increased between 1 and

**Table 5. Geochemical measurements for each well at each time point, by their indicated units. BD values indicate measurements that were below the detected limits of the instrument.**

| Well | Day | Nitrate (mg/L) | Sulfate (mg/L) | Acetate (μM) | Fe (μg/L) | U (μg/L) |
|---|---|---|---|---|---|---|
| **FW215** | -6 | 2.26 | 65.66 | 1.78 | 581.508 | 862.877 |
| | 1 | 8.74 | 64.49 | BD | 567.056 | 1098.664 |
| | 8 | 12.50 | 68.53 | BD | 293.135 | 1174.664 |
| | 15 | 1.49 | 60.29 | 21.08 | 203.479 | 1379.634 |
| | 22 | 19.81 | 60.13 | BD | 99.622 | 1200.032 |
| | 50 | 18.62 | 58.24 | BD | 20.234 | 1222.432 |
| | 78 | 15.0859 | 67.9834 | BD | BD | 1117.901 |
| | 106 | 13.4386 | 61.9627 | BD | BD | 1068.381 |
| | 134 | 20.6448 | 63.9647 | 63.7513 | 5.368 | 855.005 |
| **FW216** | -6 | 21.70 | 61.66 | 1.43 | 1297.843 | 979.478 |
| | 1 | 9.50 | 63.18 | BD | 13.103 | 1245.155 |
| | 8 | 2.49 | 45.15 | 94.70 | 2404.251 | 1118.615 |
| | 15 | 17.74 | 67.56 | BD | 69.362 | 1263.053 |
| | 22 | 6.94 | 56.74 | 5.79 | 316.297 | 1066.555 |
| | 50 | 13.40 | 52.95 | BD | 71.234 | 1156.562 |
| | 78 | 12.057 | 64.7197 | BD | BD | 1111.905 |
| | 106 | 19.35 | 57.4379 | BD | 278.436 | 830.286 |
| | 134 | 27.4708 | 62.0951 | 70.4136 | BD | 1133.071 |
| **MLSB3** | -6 | 25.13 | 57.32 | 1.59 | 142.478 | 1111.529 |
| | 1 | 1.87 | 65.57 | BD | 32.493 | 1273.278 |
| | 8 | BD | 33.41 | 610.66 | 4579.997 | 1076.655 |
| | 15 | 0.23 | 20.56 | 494.53 | 1760.141 | 641.159 |
| | 22 | BD | 13.36 | 274.10 | 337.422 | 508.585 |
| | 50 | 0.19 | 22.49 | 2.44 | BD | 483.509 |
| | 78 | 8.9255 | 58.649 | BD | 10.184 | 1493.579 |
| | 106 | 3.8669 | 54.0828 | BD | 44.34 | 1364.782 |
| | 134 | 6.8527 | 59.2133 | 10.0046 | 2197.104 | 675.136 |
| **GP01** | -6 | 3.76 | 53.67 | 1.55 | 9.299 | 907.072 |
| | 1 | NA | NA | NA | BD | 1474.825 |
| | 8 | BD | 52.78 | 189.67 | 146.407 | 1041.662 |
| | 15 | 0.25 | 32.74 | 314.32 | 1084.41 | 817.247 |

**Table 5 continued**

| Well | Day | Nitrate (mg/L) | Sulfate (mg/L) | Acetate (µM) | Iron (µg/L) | Uranium (µg/L) |
|---|---|---|---|---|---|---|
| | 22 | 2.96 | 19.75 | 446.82 | 403.405 | 590.684 |
| | 50 | 2.26 | 19.46 | 91.72 | 15.429 | 228.818 |
| | 78 | 16.7925 | 69.0147 | BD | BD | 1324.958 |
| | 106 | 1.7634 | 51.848 | BD | BD | 828.182 |
| | 134 | 27.4569 | 60.4031 | 105.8124 | 814.108 | 685.838 |
| **GP03** | -6 | 2.70 | 66.76 | 0.40 | 73.43 | 900.436 |
| | 1 | 19.52 | 52.22 | BD | BD | 993.735 |
| | 8 | 0.88 | 50.33 | 64.61 | 2222.183 | 989.876 |
| | 15 | 1.59 | 16.29 | 524.70 | 1570.658 | 770.454 |
| | 22 | BD | 1.95 | 773.13 | 71.616 | 618.103 |
| | 50 | 0.32 | 3.98 | 170.39 | 0.996 | 302.329 |
| | 78 | 0.4547 | 69.2189 | BD | 4.136 | 1944.831 |
| | 106 | 20.2502 | 56.1272 | 14.3145 | BD | 1349.322 |
| | 134 | 10.3373 | 59.4923 | 256.7487 | 14003.95 | 268.699 |

8 days after the injection, before slowly decreasing and then increasing again during the last three time points. before slowly decreasing and then increasing again between days 78 and 134. Soluble uranium levels in this well did decrease one day after the injection, but increased again at day 8, then decreased from day 15 to 106, before increasing again at the final time point. In the lower wells, iron levels increased in GP01 between 8 and 22 days after the injection, before decreasing between days 50 and 106, and then spiking again during the last time point. Soluble uranium concentrations decreased until approximately 50 days after the injection, and then decreased again from day 78 to day 134. GP03 increased in iron levels only between days 1 and 8, and then again at day 134. Its uranium levels changes were similar to GP01.

Geochemical values were statistically compared to the results from the 2009 injection (15). Three factors were included in the model, injection type (primary or secondary), day (time points 1-6), and well. ANOVA tables analyzed the significance between injection types, day, and the interaction between injection type and day. The p-values for each of these models are provided **(Table 6)** with a significance factor p <

0.05. Results from ANOVA tests indicate significance varies between each analyte. Nitrate levels were not found to have any significant differences when compared to the original 2009 injection. Sulfate concentration was significant only when compared to each day ($p = 0.00006$). Acetate showed a significant difference by day and by the interaction of the injection type and day; this shows that there were significant differences between levels at each time point in the primary and secondary injections. Similarly, iron was only found to be significantly different in the interaction between injection type and day ($p = 0.011$). Uranium concentrations were significant for the day and interaction factors as well. Injection type alone (primary versus secondary) was not a significant factor in any of the geochemical measurements.

**Table 6. Resulting p-values from repeated measurements ANOVA. Asterisks indicate a significance factor of $p < 0.05$.**

| | Nitrate | Sulfate | Acetate | Iron | Uranium |
|---|---|---|---|---|---|
| **Injection** | 0.577 | 0.55 | 0.0791 | 0.156 | 0.76135 |
| **Day** | 0.087 | 0.00006* | 0.0000017* | 0.093 | 0.0000095* |
| **Injection : Day** | 0.616 | 0.12 | 0.0032* | 0.011* | 0.00031* |

***Direct cell counts and microbial density***

Total cell counts were calculated as cells/mL, and changes in cell density over time for each well are displayed **(Figure 14**). At the pre-injection sampling, cell counts were low and homogenous throughout each well. Cell counts increased after the injection to day 15 in all wells including the upstream control well. Despite the increase at day 15, cell counts had not increased in any significant capacity in any of the wells, cell density in the wells decreased between days 15 and 22. Approximately 50 days after the injection, all wells had increased cell counts. The control well was the lowest at this point (630,000 cells/mL), an order of magnitude lower than the highest well, GP01 (5.3 million cells/mL). The closer wells to the injection point, FW216 and MLSB3 were higher in the first month after sampling, but were lower overall than the farther two wells,

GP01 and GP03. At day 78, both the control well and FW216 cell counts increased, while the adjacent MLSB3 and two lower wells diminished. Cell counts in the control, FW216, and MLSB increased between day 78 and 106, while the lower wells stayed relatively the same. At the final time point GP03 had returned to pre-injection counts (160,000 cells/mL). The rest of the wells had decreased since the previous month, they were still higher than the pre-injection levels. A bar graph shows the average cell counts for monitoring wells (control well was not included) over each sampling day **(Figure 15)**. The first month of sampling shows a slight increase in cell counts. Although by day 8 there were significantly more cells on average than in the pre-injection samples. However, 50 days after the injection saw the largest spike in cell counts, despite also having the largest amount of variation between wells. The last three time points are significantly lower than the counts on day 50, but the variance in each well is also high.

***Microbial community structure and phylogenetic analysis***

Observed alpha diversity and Chao1, ACE, Shannon, and Simpson indices for each well at each time point are provided **(Figure 16)**. Diversity was highest in the downstream wells and FW216 before EVO injection and lowest in upper well MLSB3. Immediately following the injection, diversity decreased in all monitoring wells except MLSB3, which increased slightly. Eight days after the injection, diversity decreased to between 800 and 1900 unique sequences for all monitoring wells. By the end of the first month, FW216, GP01, and GP03 were all still below 2000 unique sequences. Diversity in monitoring wells kept increasing, but at the final time point only MLSB3 had unique sequences comparable to pre-injection amounts (4900 and approximately 4000 respectively). Control well diversity stayed between 4800 and 5500 unique sequences for the duration of the experiment, with the exception of day 50 which had a spike of almost 7000. Both Chao1 and ACE, which to skew with high numbers of “rare” taxa (40), showed a significant difference in the diversity of each monitoring well at each time point. Shannon’s *H* calculated for each well and time point showed similar results. After EVO injection, all monitoring wells’ calculated *H* values dropped below 4 by day 15. However, by the final time point, only GP01 and GP03 had *H* values significantly below their pre-injection values. According to the Simpson index, richness and evenness in

samples did decrease after EVO injection, but most samples stayed above 0.9 for the duration of the experiment. Only GP01 and GP03 were below 0.9 for more than one time point.

A beta diversity-based distance matrix was calculated and shows how microbial communities are clustered based on individual well and time point **(Figure 17)**. The matrix for this ordination used weighted UniFrac distances, where the x-axis explains 38.7% of variation in the model and the y-axis explains 17.6% of variation in the model. The principle coordinates analysis (PCoA) ordination of microbial communities indicates that during pre-injection and one day after injection samples were similar to each other and cluster together in most monitoring wells. Well FW216 however was significantly different one day after injection when compared to the pre-injection sample. The communities at days 8, 15, and 22 are the most different compared to earlier and later samples. Communities between days 50 and 134 are similar to each other and to pre-injection clustering, with the exception of GP01 and GP03 on day 50, which were still more similar to day 22 samples. At the final time point, all of the monitoring wells are significantly different from the pre-injection communities. The control samples do change significantly 8 days after the injection, but communities are clustered for the remainder of the experiment. A similar ordination was constructed using a canonical analysis of principle coordinates (CAP) plot and includes microbial clustering in conjunction with geochemical factor changes **(Figure 18)**. This model also used a weighted UniFrac distance, where the x-axis explains 26% of variation in the model and the y-axis explains 7.4%. Similar to the PCoA, the CAP shows the pre-injection and day one samples clustered with days 78, 106, and 134 communities. The only sample to significantly change immediately after the injection was FW216. After the first month of sampling, most monitoring wells are clustered near the pre-injection and day one communities with the exception of GP01 and GP03 who, at day 50, are more closely clustered to the day 22 communities than the day 78 communities. Nitrate levels are shown to be strongly associated with the early time points between days 1 and 8. Acetate, manganese, and iron are all strongly associated with communities between days 8 and 22. Sulfate and uranium are associated with the later time points, between days 78 and 134. Both the PCoA and CAP plots show that after an initial change in the

microbial community after EVO injection, the communities became more similar to their pre-injection structure by the final time point, despite a lag in the downgradient wells.

Specific bacterial groups underwent changes within the community as well. The $\log_2$ fold changes of statistically significant taxa throughout the experiment is provided **(Figure 19)**. The 49 total bacterial families from 27 classes had a significant (adjusted p-value <0.001) increase or decrease in abundance by well and time point, the extend of which is indicated by distance from the zero level. The x-axis indicates microbial families while point color indicates class. The taxa to have the greatest increase during the experiment were *Parcubacteria*, and unclassified members of Betaproteobacteria had the greatest decrease, followed by *Gallionellaceae* which is a known iron oxidizer and denitrifier (41). The vast majority of increasing taxa came from Proteobacteria, specifically Delta, Gamma, and Epsilon. Of the top ten families with the highest increase during the experiment, three were unclassified, two were from Proteobacteria (*Desulfovibrionaceae* and *Syntrophaceae*), two are thought to be microbial symbionts—*Parcubacteria* (42) and SR1(43)—one is a member of the *Firmicutes* family (*Syntrophomondaceae)*, and one is a member of the *Clostridia* family (*Veillonellaceae*). Members of *Acidobactera* Gp2, *Gaiellaceae*, and *Chitinophagaceae* were the only organisms from a Class which only decreased during the experiment—all other significant taxa either only increased or did both depending on the organism. There were only nine families which had increasing and decreasing members during the experiment, six of which are unclassified. The remaining three are from the *Rhodocyclaceae*, *Bdellovibrionaceae*, and *Rhodospirillaceae* families. Results from the log 2-fold calculation showed that the most significantly changing bacterial families are increasing as a direct response to EVO injection.

## Discussion

The degradation of EVO can be tracked in the aquifer by monitoring changes in geochemistry and microbial community according to the hierarchy of terminal electron acceptors. Geochemistry in the monitoring wells immediately after the injection indicated a strong response by the microbial community and rapid degradation. Nitrate levels were either below detection limits, or greatly reduced in all monitoring wells 8

days after injection. The well closest to the injection site, FW216, showed sustained denitrification until day 106, and the next closest, MLSB3, did not return to pre-injection levels before the end of the final time point. Although the downgradient wells had low nitrate concentrations during the pre-injection sampling, both saw sustained denitrification until day 134 in GP01 and day 106 in GP03, but both ended with higher concentrations of nitrate than they began with. The control well had fluctuating nitrate levels as well, due to the fact that nitrate levels will change seasonally, as well as from precipitation events (44). Although the nitrate levels in the control well are not stable, the rate at which they are changing indicate that this well was not experiencing denitrification as a result of the EVO injection. Sulfate reduction can be seen in most monitoring wells between days 8 and 78. FW216 however, only saw a slight decrease in sulfate at day 8. This may be explained by the fact that after the EVO injection, groundwater flow may have been impeded around that well due to low hydraulic conductivity (45). Acetate is generated in all monitoring wells by day 8 and remains in the aquifer until day 50 in all wells except for FW216, which only contains measurable acetate on days 8 and 22. The acetate levels in FW216 are more similar to the control well than the other monitoring wells, which might also be explained by changing hydraulic conductivity in the wells. Iron ($Fe^{2+}$) [Fe2+] levels increase the most in all monitoring wells between days 1 and 8, however these levels began to decrease in all wells after this point. Soluble uranium (VI) levels increased immediately after the EVO injection, suggesting that introducing an aerated mixture of EVO and groundwater into the wells may have reoxidized some of the insoluble uranium as well. However, soluble uranium concentrations began to decrease in all monitoring wells after day 8 as well, however sustained decrease of soluble phase U(VI) only lasted until day 50 in MLSB3, and day 106 in GP01 and GP03. FW216 had no significant decrease of soluble phase uranium and resembled the control well. Despite the lack of response in FW216, the other monitoring wells' geochemistry is very indicative of a rapid response to the EVO injection. However, these results also suggest that the reducing conditions in the aquifer were not sustained for very long afterwards, which is contradictory to other biostimulation studies using EVO (16), including the original 2009 injection. This could perhaps be due to the fact that a much smaller volume of EVO was injected the second

time. If more EVO was available in the aquifer during the primary injection, soluble uranium concentrations could have been decreased for a longer period of time.

Microbial cell density changes indicate a slow initial increase after the EVO injection. At the first 15 days after injection, average cell density was significantly higher than it was in pre-injection samples. There was a decrease in cell density at day 22 for all wells, suggesting that the degradation process of EVO created anoxic conditions which may have caused the destruction of some of the population. During the last four months of sampling, microbial cell density increased significantly from pre-injection levels, but fluctuated from each month. GP01 and GP03 decreased after day 50 and stayed low for the rest of the experiment, while the upper wells continued to increase until day 106. There is also evidence to suggest that methane is being produced during the last months of sampling, due to the appearance of methanogenic archaea. This could indicate that between days 50 and 134 the aquifer's microbial community had begun rebounding back to pre-injection structure even though cell density is still higher in day 134 than it was before EVO was added. A potential issue with these measurements is that the planktonic cell counts may not be representative of the aquifer's total microbial community (46, 47). However, the results from the AODCs do indicate a shift in microbial density in response to the EVO addition, and demonstrate how individual wells are changing. Since microbial cell density was not measured in the original study, results cannot be compared to the current one, but monitoring the cell density changes in the aquifer do provide useful insight into the community dynamics in response to the injection.

In order to establish if there was a microbial memory response during this experiment, aspects of EVO degradation rate and the microbial organisms involved were compared. According to the ANOVA test (**Table 6**) which compared geochemistry from the two injections, sulfate, acetate, and uranium were statistically significant by day, and sulfate, iron, and uranium were significant by day and injection time. These results suggest that only a few of the geochemical markers affected by EVO degradation were different between the two injections. Shannon diversity from the original injection showed that wells were more diverse at the pre-injection time point. Diversity in the primary injection did not increase significantly after EVO injection until

approximately 80 days, which was comparable to the secondary injection. As for the bacterial groups themselves, the 2009 study noted specifically the top 15 most abundant OTUs detected in the duration of the study. Those 15 OTUs included two types of *Pelosinus* and one *Veillonellaceae*, two types of *Defulforegula*, OD1 which is now recognized as *Parcubacteria* (48), three types of *Geobacter*, one *Comamonadaceae*, one *Ruminococcaceae*, one *Vogesella,* one *Bacteroidetes* and one *Brevundimonas*. All off these OTUs either contain or are an organism which is capable of utilizing one of the dominant terminal electron acceptors in the EVO degradation process (15). The top 15 most abundant genera of organisms found in the secondary injection had similarities and differences to the ones in the first injection. Of the top 15 most abundant genera in the secondary injection, seven of them were from the unclassified groups: bacteria, *Ruminococcaceae*, *Rhodocyclaceae*, Gammaproteobacteria, Betaproteobacteria, Proteobacteria, and Deltaproteobacteria respectively. The eight known genera include *Geobacter, Carsonella* which is a bacterial symbiont (49), *Parcubacteria*, *Sulfurimonas*, *Decholoromonas*, *Desulforegula*, *Sideroxydans*, and *Undibacterium* respectively. Therefore, there were only three groups that were the same between the original injection and the secondary injection. Although the top 15 genera found in the secondary injection are also capable of utilizing one or more of the dominant terminal elector acceptors in the EVO degradation process. This comparison indicates that while both injections may have had relatively equal degradation ability but enriched different members of the bacterial community. This is very indicative of widespread functional redundancy throughout the study site. Redundancy in the microbial communities may also be a significant factor when considering how to observe a memory response in the future. Because there are so many types of species present, this redundancy can potentially cause the distinct changes in communities observed during the secondary injection.

A “site-specific” EVO degradation pathway was presented after the first injection, using the geochemical and microbial data gained during the study (15). This pathway suggested that a limited number of microbial taxa were primarily responsible for all steps of EVO degradation. Given the differences in geochemistry and microbial community structure in the secondary injection, we propose a different “site-specific”

EVO degradation pathway **(Figure 20)**. This model demonstrates not only which microbes present are likely responsible for, or being affected by, different steps of degradation, it also shows how long after injection that process lasted. The four main steps include lipid hydrolysis and glycerol fermentation, long chain fatty acid oxidation, denitrification and metal reduction, and methanogenesis. Microbes in the model were chosen based on significant (p-value ≤ 0.05) enrichment compared to pre-injection communities. They appeared at critical points during the degradation process, suggesting that they did in fact play a role in degradation. According to the geochemical and microbial changes, microbially-induced lipid hydrolysis and glycerol fermentation occurred immediately after EVO injection, and continued for the next two weeks. Long chain fatty acid oxidation and denitrification occurred at the same time. Metal reduction was occurring eight days after the injection and continued until approximately day 50. Methanogenesis (detected by the appearance of methanogenic archaea) began by day 50 and continued until the end of sampling at day 134. Methanogenic archaea appeared in the primary injection in most monitoring wells by day 80, while the first appearance of methanogenic archaea in the current study is day 50 in MLSB3. There were no time points between day 31 and 80 in the primary injection, so it is difficult to tell if this appearance was earlier. Further, other steps of the EVO degradation pathway seem to occur in the two studies at the same time points. Long chain fatty acid oxidation and denitrification was detectable in the primary injection four days after the amendment (18), which is comparable to its occurrence in the secondary injection as well. Sulfate reduction and metal reduction was detectable in the aquifer between 4 and 80 days after the primary injection but were only detectable from 8 to 50 days in the secondary injection. This may suggest that the EVO degradation did not sustain these conditions because its by-products were being metabolized too quickly, but it is difficult to tell without knowing which active metabolic pathways are present. With the geochemical and microbial community data generated after the second injection, there is not enough evidence to conclude that a memory response is present.

However, this does not definitively indicate that the microbes in this aquifer did not retain a history-dependent adaptation from the primary injection at some point. It is possible that the memory response is not apparent in this system due to the fact that so

much time has passed between exposures. If the memory response is limited by duration it would be reasonable that it would not be detectable almost a decade after a primary exposure. It is possible that certain electron acceptors are affected by past exposures, instead of the substrate as a whole, as suggested by the ANOVA results of significant geochemical parameters. Without a more in-depth analysis of the active pathways, genes, and proteins or enzymes present, it is unclear how specific bioactivity is being affected by the secondary injection. Further understanding of the metabolic and genetic aspects of the microbial communities is required before any substantial claim about the memory response is made. Studies were conducted on groundwater collected from the primary study on the abundance of specific genes and pathways (16, 17), similar studies will need to be conducted with the groundwater and amplicon libraries generated with the secondary injection as well.

The results from this injection have however, suggested the need for planned sequential sampling when attempting to observe a memory response *in situ*. Future studies of environmental microbial memory will need to be conducted with a series of multiple planned exposures and amendments to the same site, such as a time series. This would greatly increase the ability to monitor if a memory response is present and how long it lasts after the previous exposure. As one of the first *in situ* “long-term” memory response experiments, this injection demonstrates that field sites can be variable over time, but the overall reaction of the community to a carbon amendment is the same nearly a decade after a primary exposure.

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## Appendix

**Table 7. Time point comparison used to generate repeated measures ANOVA. Since time points in the first and second injections were not identical, similar time points were chosen and re-labeled for the purpose of generating a model for the ANOVA test.**

| Time Point | 2009 Injection | 2017 Injection |
|---|---|---|
| 1 | -18 | -6 |
| 2 | 4 | 1 |
| 3 | 16 | 15 |
| 4 | 31 | 22 |
| 5 | 80 | 78 |
| 6 | 135 | 134 |

## CONCLUSION

Emulsified vegetable oil used in this study, both in the microcosms experiment and field test, was degraded by the native microbial communities of the aquifer. Monitoring the microcosms for changes in terminal electron acceptors showed that there were early denitrification events, followed by a rapid increase in the soluble Fe(II) sulfate reduction, and a decrease of soluble U(VI) all within one week to thirty days after the EVO amendment. Gas production measured in microcosm headspace exhibited an increase in methane production forty days after the amendment. The secondary injection of EVO monitored groundwater changes also showed strong evidence of early remediation effects. Denitrification was occurring one day after the EVO was added in the upper wells, and by the eighth day in the lower wells. Soluble Fe(II) levels increased eight days after the injection in all wells. Sulfate was reduced in upper wells between one and eight days after injection, and in lower wells between eight and fifteen days. Acetate was being produced and soluble U(VI) levels began decreasing in the wells eight days after injection. Although there was evidence that the injection of aerated EVO and groundwater may have caused insoluble uranium to reoxidize immediate after the injection. These results are comparative to the process of dominant terminal electron acceptor changes seen in other groundwater biostimulation experiments. Geochemistry in the primary 2009 injection was similar to that of the microcosm and field studies. Most wells experienced denitrification four days after the addition of EVO, soluble Fe(II) increased and sulfate reduction occurred between four and sixteen days, and soluble U(VI) decreased after sixteen days. Acetate was also generated in all wells between 16 and 31 days after the injection. Results indicate that EVO was utilized successfully to stimulate anaerobic reducing conditions both in microcosms and in the aquifer system and caused sustained immobilization of uranium in the groundwater. Although the rate at which most of these parameters are was not found to be statistically significant in the secondary injection versus the first. Observations of the microbial communities yielded similar results.

Microbial response to EVO amendment in microcosms and the field study also indicate that biodegradation occurred. Enrichment of specific groups of taxa known to utilize the shifting dominant terminal electron acceptors was observed in both the

microcosms and secondary injection. Microcosms exhibited distinctly different microbial communities among individual wells as well as between well locations (upgradient vs. downgradient). Likewise, the types of microbes enriched by the secondary amendment had some similarities to the first, but as a whole were distinguishable. Both of the microbial communities from these studies included members known groups capable of processes which contributed to the degradation of EVO. These results indicate that the innate microbial community inhabiting this aquifer system experiences significant changes over time, but the organisms present retain the ability to biodegrade EVO, otherwise known functional redundancy. However, the rate at which these organisms in the secondary injection appeared was comparable to that of the primary injection, although they were from different phylogenetic groups—indicating that the functional redundancy present in this system had a significant effect on which microbial taxa were enriched during the microcosms and secondary injection.

Taking all of the results from these two studies into account, there is not enough strong evidence currently to conclude that microbes in the aquifer are experiencing a history-dependent response to the injection of EVO. The microbial organisms that appeared, the rate at which they appeared, and the geochemical changes that accompanied them is similar to the primary injection in both the microcosm and secondary injection studies. The aim of the first experiment was to attempt and observe a microbial memory response in laboratory scale microcosms using sediment and groundwater which was thought to contain a microbial community which had been exposed to EVO before. The second experiment endeavored to observe and measure a long term microbial memory response *in situ*, which had never been attempted before. Similar to the microcosms, a distinct microbial community responded to the secondary injection when compared to the first injection and the microcosms. Although the secondary response was rapid, it was not significantly different from the response of the primary.

There is still much to be gained from the data gathered from these studies. Despite the results from both the microcosms and field study not supporting a long term microbial memory response, this does not conclusively indicate that history dependent adaptation cannot be applied to *in situ* remediation events. Groundwater collected from

the secondary injection still needs to be analyzed further for specific bioactivity, gene enrichment, and metabolic pathways in order to have a full understanding of the microbial community reaction. Considering a short-term response was able to be detected at the same site using ethanol instead of EVO, there is evidence that longer-term memory responses can be observed *in situ* as well. Additionally, one of the goals was to gain more of an understanding about the magnitude and duration of the memory response, which was accomplished by these studies. Future studies should focus on finding the upper limit to the duration of a long-term memory response. Sequential sampling and evenly-spaced exposures in the future might elucidate even more about the presence of a memory response and its duration in an environmental system. Geochemical results from the secondary injection also suggest that only a few of the stimulated electron acceptors were significantly different when compared to the first. This could mean that the microbial community may respond to specific by-products rather than the entire carbon substrate itself. Overall, given the fact that a microbial memory response was not recognized in either the microcosms or the secondary injection, the results from these studies have still indicated that there are distinct differences between subsequent exposures to the same substrate. These results in combination with future studies of the memory response have to potential to shed light on the complex processes of microbial remediation of continuously contaminated aquifers.

## VITA

Katie McBride was born in Knoxville, TN on May 17th, 1993. She graduated from Maryville High School in Maryville, Tennessee. She received her Bachelor's of Science degree in Biological Sciences with a concentration in Microbiology from the University of Tennessee, Knoxville in May of 2016. In August 2016, she entered a graduate program through the Department of Microbiology at the University of Tennessee, Knoxville as a part of Dr. Terry Hazen's laboratory. She completed the requirements of a Masters of Science degree in August 2018.