

Temporal Dynamics of In-Field Bioreactor Populations Reflect the Groundwater System and Respond Predictably to Perturbation

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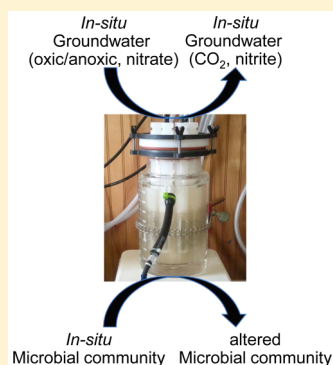
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Supporting Information

ABSTRACT: Temporal variability complicates testing the influences of environmental variability on microbial community structure and thus function. An in-field bioreactor system was developed to assess oxic versus anoxic manipulations on *in situ* groundwater communities. Each sample was sequenced (16S SSU rRNA genes, average 10,000 reads), and biogeochemical parameters are monitored by quantifying 53 metals, 12 organic acids, 14 anions, and 3 sugars. Changes in dissolved oxygen (DO), pH, and other variables were similar across bioreactors. Sequencing revealed a complex community that fluctuated in-step with the groundwater community and responded to DO. This also directly influenced the pH, and so the biotic impacts of DO and pH shifts are correlated. A null model demonstrated that bioreactor communities were driven in part not only by experimental conditions but also by stochastic variability and did not accurately capture alterations in diversity during perturbations. We identified two groups of abundant OTUs important to this system; one was abundant in high DO and pH and contained heterotrophs and oxidizers of iron, nitrite, and ammonium, whereas the other was abundant in low DO with the capability to reduce nitrate. In-field bioreactors are a powerful tool for capturing natural microbial community responses to alterations in geochemical factors beyond the bulk phase.



INTRODUCTION

One of the most difficult aspects of studying *in situ* microbial ecology is determining the fundamental ties between temporal environmental fluctuations and microbial community structure. While microorganisms are the foundation for ecosystem health, affecting local geochemistry¹ and contaminant fate,^{2–4} it is difficult to link the response of microbial species within complex communities to specific perturbations.^{5,6} Hence, it is important to develop methods to temporally interrogate natural microbial communities so that linkages between biodiversity and function may be established.

The effects of contaminants on the diversity, structure, function, and biotransformation capabilities of groundwater microbial communities have been studied at the Oak Ridge Field Research Center (ORFRC)^{4,7–18} but only provide limited data linking geochemistry to microbial community structure and ecological function. A key limitation of most ecological

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studies of groundwater systems, as at the ORFRC, is the inability to continually monitor the microbial community alongside changing environmental conditions in order to identify factors driving community responses. While bulk or large-scale hydrological and geophysical studies have been performed at this and other sites,^{19–23} smaller scale temporal studies on variations and their effects on the community are poorly documented. However, it is known that both temporal and spatial^{24–26} variability in groundwater bacterial populations can significantly impact watershed scale estimates of microbial processes, although temporal variation is thought to be greater than spatial variation.²⁶ The result is a knowledge gap regarding the local effect and response of the subsurface microbial community to rapid geochemical alterations such as the influx of oxygenated water after a rainfall event or amendments for bioremediation of organic or inorganic contaminants.

We have developed a novel in-field bioreactor system for closely approximating *in situ* conditions and monitoring the response of the groundwater microbial community to geochemical manipulation beyond the bulk phase of traditional microcosms. Bioreactor studies have been successful at the ORFRC in demonstrating the technology³ and for monitoring the effect of ethanol injections for U(VI) immobilization.⁴ However, these large, expensive systems designed for the treatment of groundwater contamination are not well suited for testing theories regarding the influence of contaminants or more common geochemical parameters on community structure and subsequent function. The bioreactors in this study are much smaller, allowing for experimental replication and can differentiate between planktonic and attached communities. Further, due to the constant influx of native groundwater, *in situ* geochemical conditions can be maintained for considerable lengths of time as opposed to closed microcosms.

The purpose of this study was to demonstrate the feasibility of manipulating the geochemical conditions within in-field bioreactors in order to capture the response of the natural microbial community. We aimed to mimic common biogeochemical shifts at the groundwater-vadose interface wherein oxygen and oxidized compounds are abundant during recharge events and decline during drought.²⁷ Thus, we examined the response of the *in situ* microaerophilic bacterial community structure to manipulation of dissolved oxygen (oxic versus anoxic) and pH in the replicated bioreactor systems. We demonstrated that a diverse bacterial community similar to that in groundwater could be established without nutritional amendment, that the temporal stochasticity of the groundwater chemistry and community were reflected by changes in the reactor community, and that the reactor community was responsive to short-term perturbations. This work shows the viability of in-field bioreactors as an approach for understanding drivers of the microbial community assembly and response to perturbation in groundwater.

MATERIALS AND METHODS

Field Site and Experimental Design. In order to accomplish the study goals, a new groundwater monitoring well, FW305, was installed in the uncontaminated area of the ORFRC in Oak Ridge, TN down to bedrock at 7.8 m (Figure S1). Groundwater from the well was pumped into the mobile laboratory and delivered to the bioreactors at a liquid phase replacement rate of 0.02/h, 50 h maximal generation time to retain a considerable fraction of the extant planktonic microbial

community. Details of the well construction, groundwater flow, and mobile laboratory are described in the supplemental text.

The bioreactors were custom designed and obtained from Biosurface Technologies (Bozeman, MT) and Allen Scientific Glass (Boulder, CO) (Figure S3). Each bioreactor contained 800 mL of groundwater sample (planktonic community) with ~100 mL of atmospheric headspace as well as eight removable biofilm coupons that were filled with pulverized sediment (5 g). To begin the experiment, the triplicate bioreactors were filled with groundwater and allowed to acclimate for 24 h. As aerobic groundwater conditions are common at our site at the depth of our well (although not during the experimental time course), headspace gas was initially atmospheric and was introduced into the bioreactors via the secondary line on the drip tube with a sterile 0.22 μm filter to prevent contamination. The planktonic phase was stirred slowly (<40 rpm) using stir plates below the bioreactors in order to maintain homogeneity.

Temporal Sampling. Sampling of the groundwater and planktonic portion of the bioreactors was performed approximately every Monday, Wednesday, and Friday (M/W/F) for 11 weeks. At each sampling time, 85 mL was taken from the primary container for the groundwater, and 85 mL was taken from the outlet of each bioreactor for various analyses as detailed below. The biofilm coupons were harvested at the conclusion of the experiment. Samples for DNA analysis were immediately frozen in the mobile laboratory and stored at -80°C until the conclusion of the experiment, when all samples were processed and sequenced.

Environmental Perturbations. Triplicate in-field bioreactors were fed unfiltered groundwater for 11 weeks, with groundwater and bioreactors being sampled every 2–3 days. Once a stable DO and pH were maintained (day 48), the bioreactor headspace was altered from atmospheric air to anoxic via the addition of 80:20% $\text{N}_2:\text{CO}_2$ gas resulting in low oxygen conditions within the reactors and a decrease in pH due to higher dissolved CO_2 . Anaerobicity was maintained from days 48–56 and then returned to atmospheric for days 56–63. Once again, the bioreactor atmosphere was changed to anaerobic at the same rate for days 63–78.

Biogeochemical Analysis. The monitoring of geochemical parameters included temperature, pH, dissolved oxygen (DO), dissolved CO_2 , metals, organic acids, anions, oxidation–reduction potential (redox), and conductivity.

Groundwater geochemistry was monitored using a Multi-parameter Series Troll 9500 (In-Situ Inc.), while the bioreactors were monitored using an Orion VERSA STAR Multiparameter Benchtop Meter (Thermo Fisher Scientific). Both the troll and benchtop units had probes attached which allowed the simultaneous measurement of temperature, pH, oxidation–reduction potential, conductivity, and DO. Bioreactor geochemical measurements were taken within 30 s of sample removal from the bioreactors every day that biochemical sampling was performed. The Troll 9500 was inserted into the primary container for the duration of the experiment so that groundwater measurements did not disturb the inflowing groundwater. Groundwater geochemical data was collected on the same days as bioreactor sampling.

The organic acids and anions subsamples (5 mL) were collected and filter sterilized via syringe and 0.22 μm filter into sterile 15 mL falcon tubes and kept at 4°C for transport. All samples were placed onto the Dionex ICS 5000+ Dual Pump, Dual Column system (ThermoFisher Scientific; Waltham, MA) within 1 h. Anions and organic acids were analyzed

simultaneously using an AS11HC column with a KOH gradient of 0–60 mM, and sugars were analyzed on a CarboPac SA 10 column with an isocratic flow of 1 mM KOH, per the manufacturer's instructions. Quantification at each time point included lactate, acetate, propionate, formate, butyrate, pyruvate, succinate, fumarate, citrate, fluoride, chloride, bromide, nitrate, sulfate, and phosphate. Calibration of each parameter was accomplished using 5-point calibration curves with the prepackaged standards from Dionex. The calibration curves were performed at the beginning of each run and included check standards after every 15 samples.

For dissolved CO₂, liquid samples from the groundwater and bioreactors were placed into empty, sterile serum bottles containing N₂ gas and sealed with butyl rubber stoppers and aluminum crimp seals. Samples were kept at 4 °C until returned to the laboratory and then analyzed on a SRI 8610C gas chromatograph equipped with a methanizer and Flame Ionization Detector with a 1.82 m by 0.32 cm HayeSep D packed column (SRI Instruments, Torrance, CA) as previously described.²⁸ All reported values are total gas (headspace and dissolved) concentrations and are reported per mL water basis.

Fifty-three metals were monitored over the course of the experiment. At each sampling, 10 mL of groundwater and 10 mL of bioreactor effluent were taken and stored in acid-washed 15 mL conical tubes and stored at –20 °C until they were analyzed. A representative subset consisting of 16 groundwater and 27 bioreactor samples was thawed and prepared for analysis as previously described.²⁹

DNA Extraction and Sequencing. Community DNA was extracted from the frozen water or sediment using a freeze-grinding method.³⁰ Bacterial 16S rRNA gene sequences were amplified using standard methods³¹ in triplicate and combined, purified with an Agencourt AMPure XP kit (Beckman Coulter, Beverly, MA, USA), eluted in 50 µL of water, and aliquoted into three new PCR tubes (15 µL each).

A second PCR was performed using phasing primers including Illumina adapters, spacers, target primers, and barcodes on the reverse primers using standard methods³² in triplicate. PCR products from triplicates were combined and quantified with PicoGreen. All PCR products were pooled at equal concentration and sequenced in the same MiSeq run using standard methods. The pooled mixture was purified with a QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD, USA) and requantified with PicoGreen. Sample libraries for sequencing were prepared according to the MiSeq™ Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA).

Sequence Data Processing. The sequencing data was quality filtered, demultiplexed, and overlapped with custom Python scripts (<https://github.com/almlab/SmileTrain>) that call USEARCH for quality filtering and overlapping paired end reads and Biopython³³ for file format input and output. After processing with USEARCH and custom Python scripts, sequences were progressively clustered to 90% with UCLUST,³⁴ aligned to the silva bacterial database with mothur, align.seqs, and processed with distribution-based clustering (DBC) as previously described³⁵ with *k_fold* 10 to remove sequencing errors. Groundwater and bioreactor/biofilm sequences were processed separately, and merged output files, reclustered with DBC *p-value* parameter of 0.0001, *distance* parameter of 0.06 (hamming distance in substitutions per site), and no abundance criteria (*k_fold* 0). OTU representatives were defined during clustering as the most abundant sequences

in the OTU. Taxonomic identification was made with RDP³⁶ using a 0.50 confidence threshold³⁷ as suggested for sequence lengths of up to 250 bp and used the nonredundant nucleotide database (excluding uncultured and model organisms) to generate a hypothesis about the functionality of OTUs in the group in Figure 3A. This was done by identifying the most similar cultured isolates or strains with whole genomes sequences with at least 99% identity across the entire length of the sequences. Representative sequences were aligned using muscle (v3.8.31³⁸) and aligned sequences used to generate a phylogenetic tree with fasttree (v2.1.7³⁹) under the *-nt -gtr -gamma* settings. Each sample was rarified to 6,458 sequences for downstream analysis.

Community Diversity Analysis. We examined the change in α -diversity (total within-sample bacterial phylodiversity) over time and the percentage of shared OTUs between each bioreactor and the most recent groundwater sample to measure the similarity (UniFrac β -diversity) between bioreactors and groundwater. We also examined an additional metric of community assembly status using 16S copy number to determine if numbers increase during establishment or disturbance periods.⁴⁰

Groundwater and bioreactor similarity was tested using the ANOSIM⁴¹ function in the vegan package (<http://CRAN.R-project.org/package=vegan>) in R (<http://www.R-project.org>) using weighted and unweighted UniFrac distance as the β -diversity metric.⁴² We used ADONIS⁴³ to progressively partition community variance in UniFrac abundance weighted phylo- β -diversity across reactors as explained by the fixed effects and random variables in the study. ADONIS analysis identified the disparity between groundwater and bioreactors and tested if perturbations had a significant effect. Because ADONIS partitions variance in the order variables are input, random variables appeared first (e.g., time), followed by the fixed effects (e.g., Sample Type (groundwater or bioreactor)) tested. The random variable, Replicate, was input after Sample Type because groundwater had only one replicate and was followed by the remaining fixed effects of the study period (Establishment [days 1–20], Oxidic [days 21–47, 57–63], and Low Oxygen [days 48–56, 64–78]), DO, pH, Nitrate, Nitrite, Acetate, and Groundwater OTU Richness (only variables explaining >1% of the variance were included in the analysis).

Clade-correlation methods⁴⁴ identified all nonoverlapping clades of phylogenetically related OTUs significantly correlated with the strongest treatment-responding biogeochemical factor, nitrite.

Test for Nonstochastic Community Assembly. To further test the bioreactors ability to replicate a growing groundwater microbial community, we adapted the neutral theory of community assembly⁴⁵ to create a null model simulating neutral community assembly. To create simulated data, the null model assumed that bioreactor OTUs for each time point were random samples of the current groundwater OTUs and the previous simulated bioreactor time-point. Groundwater community was not simulated because there were no propagule pool measurements external to the groundwater samples. The OTU richness (the number of randomly selected OTUs) of each simulated sample was set equal to the richness observed in the corresponding real-world bioreactor sample at that time point. After all OTUs were chosen for a simulated sample, OTU abundances were simulated by randomly assigning abundances from the real bioreactor sample. Thus, the simulations randomized OTU

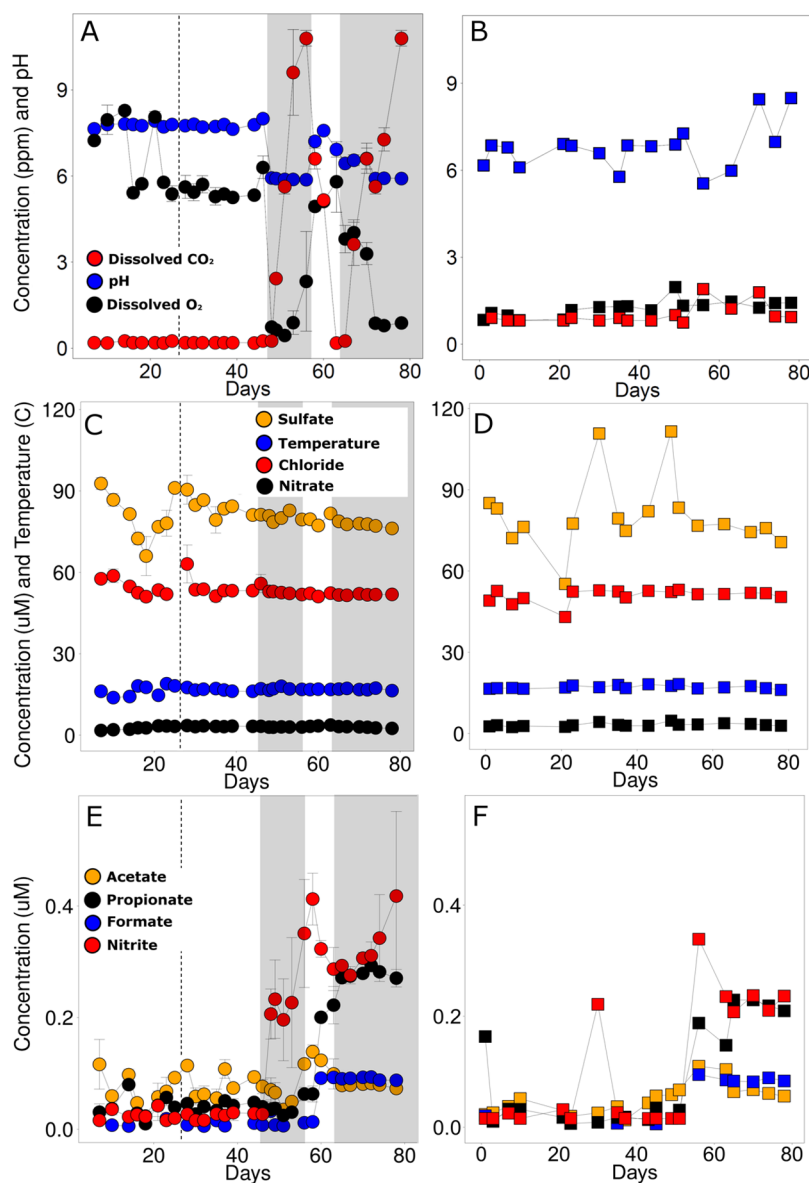


Figure 1. A comparison of the temporal geochemistry over time in the bioreactors (left; A,C,E) and the native groundwater (right; B,D,F). Dissolved O_2 (DO) and CO_2 concentrations and pH for (A) bioreactors and (B) groundwater. The three most concentrated anions and temperature for (C) bioreactors and (D) groundwater. The three most abundant organic acids as well as nitrite for (E) bioreactors and (F) groundwater. Over the first 20 days the DO levels in the reactors fluctuated, and after 25 days of stable DO, $N_2:CO_2$ gas was added. Bioreactors were returned to oxic conditions at Day 57 and perturbed to anoxic again at Day 63 for the remainder of the experiment. Gray regions highlight experimental perturbations to low oxygen conditions.

presence but kept the abundance distribution within a sample the same as the experimental findings. In order to test bioreactor divergence from the null model, we compared the abundance-weighted UniFrac β -diversity matrix from the average of 100 null models to the observed distances using a Mantel Test.⁴⁶

Co-Occurrence Clustering of OTUs over Time. OTUs were normalized to a relative abundance in each sample by dividing each OTU count by the total counts per sample. Only the 400 most abundant OTUs across all bioreactor samples (based on summed relative abundances) were used. For each OTU, relative abundances were normalized by the total relative abundance summed across all samples for that OTU, transforming each OTU into profiles with similar magnitude regardless of total relative abundances. Euclidean distance between OTUs was the dissimilarity metric for hierarchical

clustering.⁴⁷ The cluster dendrogram was divided to produce 50 candidate co-occurring groups. If a co-occurring group had at least one OTU with a mean Pearson correlation of less than 0.75 with the other OTUs in the cluster, the lowest mean correlated OTU was removed and filtering repeated until every OTUs mean correlation was ≥ 0.75 . Groups with one OTU were excluded. Because artifacts can be generated,⁴⁸ we shuffled the counts within samples to randomize the OTU matrix and repeated the analysis. No resulting clusters contained as many co-occurring organisms suggesting the co-occurrence patterns are not an artifact. Clustering co-occurring scripts are available (<https://github.com/spacocha/Distribution-based-clustering>).

Successional OTU Patterns Visualization. The 49 most abundant OTUs across all bioreactors ($>0.5\%$ of all reads across bioreactors and groundwater) were organized by timing of their peak relative abundance in reactor 1. Each OTU

relative abundance was normalized so their maximum abundance was 1 and was plotted using the heatmap function in R (R Core Team (2012); <http://www.R-project.org/>). All normalized OTU relative abundances were plotted for groundwater in the same manner to determine if the bioreactor patterns were derived from changes in the groundwater.

Data Accessibility. Data have been deposited into the public GenBank SRA under the accession number SRP072431.

RESULTS

Geochemistry. Apart from DO and pH (which were manipulated by changing the headspace) bioreactors maintained similar geochemistry to the groundwater (Figure 1). During the first 46 days, an air headspace resulted in a fully oxic DO (6 mg/L), which was distinct from the influent microaerophilic groundwater (1 mg/L). Oxic conditions were used to simulate the geochemistry at the groundwater-vadose interface while providing a maximum difference from the later experimental perturbation toward a low oxygen condition (1 mg/L). Bioreactor temperatures were maintained at groundwater temperature ($18\text{ C} \pm 2\text{ C}$; Figure 1). During the aerobic phases, dissolved CO_2 did not exceed 0.8% in the bioreactors as compared to <1.5% in the groundwater (Figure 1A, B). During the two anaerobic perturbations, all three bioreactors reacted similarly and achieved dissolved CO_2 concentrations of 10.5–11.5% in each episode (Figure 1A).

Metals (Table S1), ions, and metabolites (Figure 1C,D) were similar between the groundwater and bioreactors over the course of the experiment with the exception of nitrite. Nitrite levels repeatedly increased during low oxygen conditions and decreased during oxic conditions (Figure 1 C,D). Changes in nitrite were at levels equivalent to 1% of the nitrate concentration and, thus, may not have been strong enough to separate from the background variability in nitrate concentrations. Nevertheless, the increases in nitrite during low oxygen conditions suggest active nitrate-reduction.

Acetate and propionate were observed in the groundwater and increased after ~60 days, suggesting recent generation from the degradation of organic material (likely humic acids and/or lignocellulose) in the groundwater (Figure 1E, F). Interestingly, in the bioreactors formate, acetate, and propionate were more abundant in the low DO periods (Figure 1E), indicating that redox conditions during aerobic phases either limited organic matter degradation or allowed for greater formate consumption. However, these fluctuations had poor explanatory power the turnover in overall microbial community composition (Table 1). It is notable that for most organic acids, the concentrations were higher in the bioreactors

than the groundwater (Figure 1E,F), suggesting similar but more active metabolism in the bioreactor communities.

Bacterial Community Structure Patterns. Bacterial 16S rRNA gene sequences showed that the oxic bioreactors maintained a planktonic microbial community with similar dominant OTUs to the groundwater but with significantly different relative abundances. The bioreactor community had a similar α -diversity (Faith's phylodiversity) to the groundwater (Figure 2A), and the bioreactors shared only 20% fewer OTUs with contemporaneous groundwater than was shared between the two temporally closest groundwater samples (Figure 2B), suggesting that the bioreactors adequately maintained a representative *in situ* microbial community for perturbation studies.

In contrast to this small difference in OTU overlap between bioreactors and groundwater (Figure 2B), the relative abundance of these OTUs changed dramatically and in a manner out of step with the groundwater with perturbation (Figures 2D, F). This translated into a significantly different average planktonic β -diversity between the groundwater and bioreactors throughout the experiment (UniFrac abundance-weighted phylo- β -diversity, ANOSIM $p = 0.02$).

Examination of the bioreactor DO, pH, (Figure 1A) and community diversity (Figure 2D, F) indicated five distinct periods. Establishment spanned days 1–20 and was based on bioreactor DO fluctuations and an increasing difference in bioreactors and groundwater relative abundances (Figure 2D, F). The first oxic period spanned days 21–47, when DO was stable and the bioreactors began to diverge from one another (Figure 2D, F). The first experimental perturbation (days 48–56) showed rapidly decreased DO/pH accompanied by a shift in β -diversity (abundance-weighted UniFrac) toward being more similar to the groundwater (Figure 2D). During the second oxic period (days 57–63), DO/pH rapidly returned to its previous level, but, similar to establishment, the community slowly shifted toward greater β -diversity from the groundwater (Figure 2D). Finally, during the second perturbation (days 64–78), there was a slow decrease in DO/pH as well as a slow increase in similarity between bioreactors and groundwater (decrease in β -diversity; Figure 2D). These β -diversity responses suggest that the bioreactor communities were diverging during the establishment and first oxic periods but that the first perturbation allowed for convergence, likely via the renewed accumulation of microaerophilic OTUs from the recent influx of groundwater. In addition, the switch from low interbioreactor β -diversity during the first perturbation to high interbioreactor β -diversity during the second low oxygen perturbation may suggest that the short second oxic period did not allow aerobic microorganisms to fully re-establish themselves.

Complementary to the β -diversity analyses, 16S copy number at each time point also indicated a significant impact of perturbations on the bioreactor communities. Changes in 16S copy number are of interest because microorganisms with greater 16S copy number are associated with a greater growth rate capacity and are more common in disturbed systems.⁴⁰ Thus, increased 16S copy numbers during the initial establishment and after perturbations indicate significant disturbances, while decreased copy numbers after periods of stable conditions suggest the bacterial community undergoes succession post disturbance.

ADONIS analysis indicated that variables including bioreactor replicate (i.e., establishment and extinction differences

Table 1. ADONIS Results for Bioreactors Alone

	Df	F Model	R ²	Pr(>F)
experimental time point	1	21.0	0.13	<0.01
replicate	2	6.6	0.08	<0.01
test period	4	7.6	0.20	<0.01
dissolved oxygen	1	1.4	0.01	0.19
pH	1	2.0	0.01	0.03
nitrate	1	3.1	0.02	<0.01
nitrite	1	1.9	0.01	0.04
acetate	1	1.1	0.01	0.33
propionate	1	1.6	0.01	0.10
groundwater OTU richness	1	9.4	0.05	<0.01

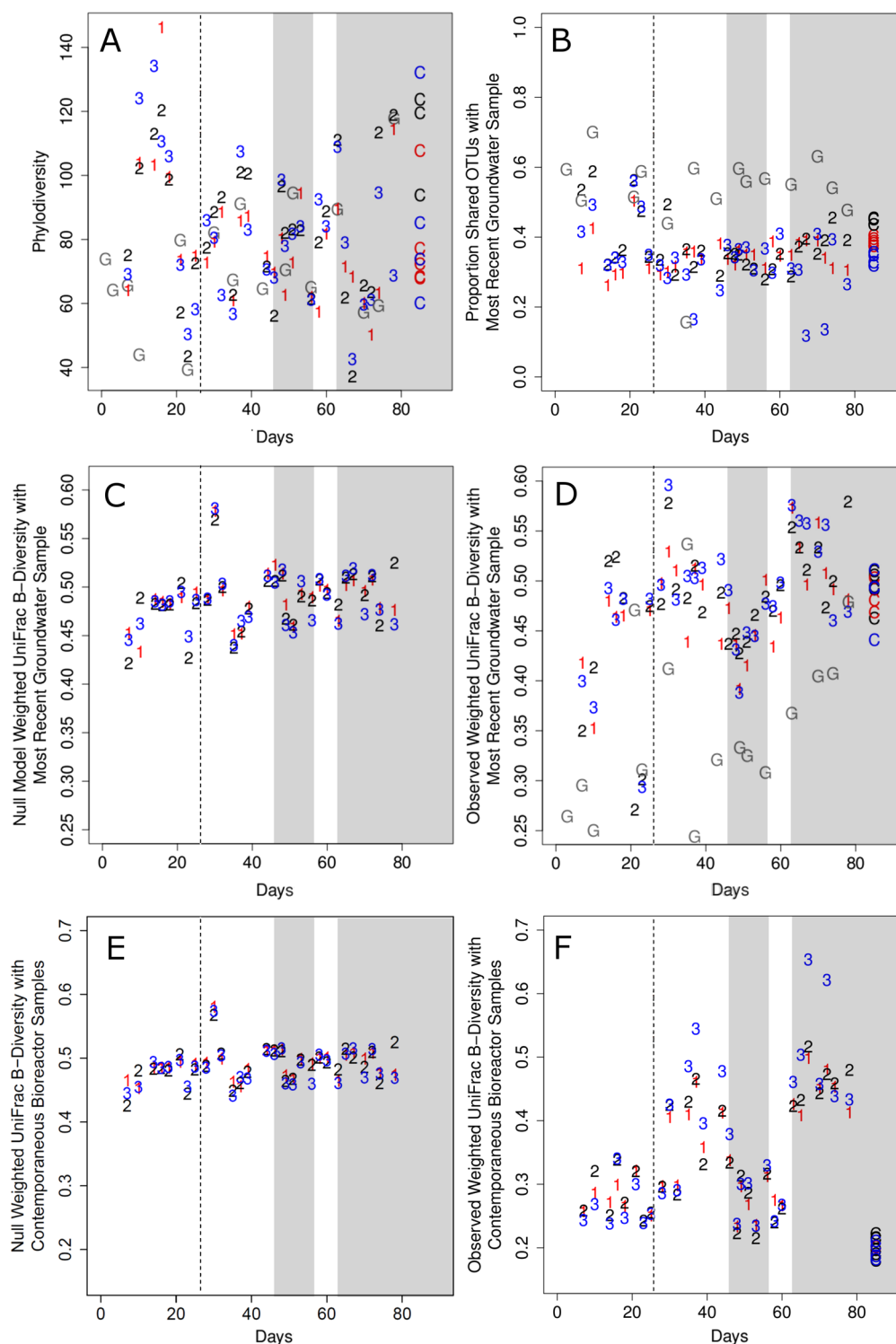


Figure 2. Plots of microbial community diversity over the 78 day time course. Numbers indicate bioreactor replicates, G indicates groundwater, and C indicates the sediment biofilm coupons (installed on day 1 and sampled only on the final bioreactor sampling date). The dashed line separates the initial establishment period from the more stable first oxic period. Gray regions highlight experimental perturbations to low oxygen conditions. (A) Within sample α -diversity at each sampling time is compared between bioreactors (1–3) and groundwater (G). (B) Shared OTU richness comparing each sample to the most recent (i.e., previous) groundwater sample to determine if the bioreactor retains groundwater OTUs over time and at what abundance level as compared to the groundwater with different DO levels. (C) One hundred stochastic assembly simulations summarized as the average of the modeled abundance-weighted UniFrac β -diversity distance between the bioreactors and the most recently observed (i.e., previous) groundwater sample and should be compared to (D) the observed weighted UniFrac β -diversity comparing each time-point to the previous groundwater time point. (E) One hundred stochastic assembly simulations summarized as the average of the modeled weighted UniFrac β -diversity distance between the bioreactors at each time point and should be compared to (F) the observed average of the weighted UniFrac β -diversity distance between each of the bioreactors at each time point.

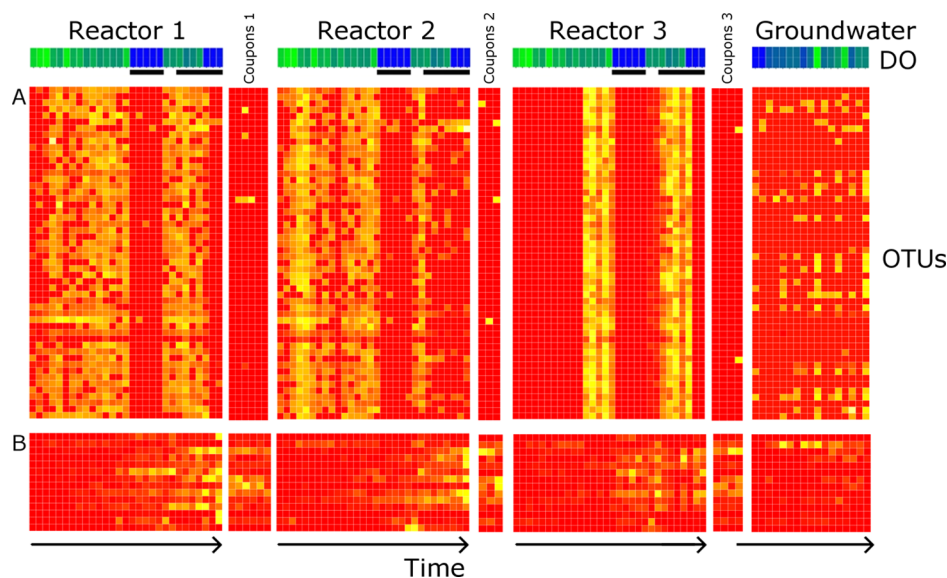


Figure 3. Temporal heatmaps of the bioreactors and groundwater showing OTU relative abundance over the course of the experiment. Low OTU abundance is red, and high abundance is yellow. Temporal DO concentrations for each bioreactor and the groundwater are blue for anaerobic conditions and dark to light green for increasing DO levels. The black bars below the DO colors indicate the anaerobic perturbations with no oxygen and lower pH. (A) The most highly co-occurring group of organisms across and within each of the bioreactors. Each OTU's relative abundance is mapped for each time point and through each phase of the experiment. (B) The most highly co-occurring group of organisms across bioreactors that correlated with increased nitrite concentrations over time.

between reactors), test phase, and incoming groundwater OTU richness were the strongest measured drivers of bacterial community composition. The ADONIS model for planktonic bioreactors explained 52% of the variance in abundance weighted UniFrac β -diversity (Table 1; only variables explaining >1% of the variance individually were included). The most explanatory manipulated variable was the test phase, explaining 20% of the total variance. DO and pH were cocorrelated with test phase, explaining 7% already explained by test phase but also an additional 2% of the remaining variance. The one organic acid and two anions contributing to the model, acetate, nitrate, and nitrite, explained an additional 4% of the variance. The final variable tested was Groundwater OTU Richness, which explained an additional 6%. The ADONIS results indicate that the unmanipulated geochemical factors measured (Figure 1, Table S1) had low explanatory power for predicating bioreactor community β -diversity.

Null modeling of the planktonic community assembly with randomized abundances indicated that bioreactor diversity was strongly stochastic over time within stable geochemical conditions (Figure 2C–F; Mantel Test $p < 0.001$, $r^2 = 0.48$) indicating that replicate bioreactors captured the groundwater variability in community composition. Further, as the ADONIS results only show strong effects from experimental manipulations, none of the ions and organics measured were likely drivers for the highly stochastic bacterial community turnover observed.

Although most OTUs were presumptive facultative anaerobes (given the oxygen state of the source groundwater and their persistence throughout the experimental manipulations), a large OTU group appeared to co-occur in all bioreactors predominantly during both oxic periods. Randomization tests showed this group of OTUs was not an artifact of compositional data since it was not reproduced when counts were randomized within libraries.⁴⁸ These OTUs decreased in abundance during anaerobic perturbations (Figure 3A) were

diverse and accounted for up to 80% of the reads in some samples. An organism similar to the sulfur-oxidizing *Thiobacillus thioeparus* was the most abundant and was as high as 25% of the total community. Ammonium- (*Nitrosomonas europaea*), nitrite- (*Nitrospira marina*), and iron- (*Acidovorax ebreus*) oxidizing organisms were also in high abundance. Others included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Deinococcus-Thermus*, *Firmicutes*, *Nitrospira* and α -, β -, γ -, δ -, and ϵ -*Proteobacteria*. All of these identifications also held using a 0.8 RDP confidence. *Thiobacillus thioeparus* was identified using BLAST, and the other organism identifications are phylum level with >0.8 threshold values.

Conversely, clade-correlation analysis identified a group of 14 clades significantly correlated with bioreactor nitrite concentration and peaked during low oxygen, accounting for as much as 45% of the reads in some samples (Figure 3B). This second group included *Acidovorax*, *Afipia*, *Aminobacter*, *Brevundimonas*, *Massilia*, *Opiritatus*, *Sphingobium*, and *Turneriella*, all of which can reduce nitrate or are associated with low oxygen systems. The repeated alteration in these two groups with changes in DO indicates that the bioreactor bacterial community responded in a repeatable manner to perturbation.

Bioreactor biofilm coupons were very similar to one another (Figure S4). The mean abundance weighted UniFrac β -diversity distance comparing coupons in separate reactors was 0.20 but were dissimilar to the groundwater (UniFrac distance of 0.44) or bioreactors (UniFrac distance of 0.41). The biofilms contained only 47% of the OTUs in the final planktonic time point and 27% of the last groundwater sample. However, all biofilm OTUs were observed in previous planktonic samples but in different relative abundances. The high similarity between biofilms but different from the planktonic bioreactor or groundwater suggests that the sediment-adhered community is under a stronger, more normalizing selective pressure. As sediment-adhered microorganisms are likely a large proportion of the subsurface microbial community, the divergent diversity

within the biofilm coupons suggests future experiments should track temporal shifts in the sediment-adhered as well as planktonic community.

DISCUSSION

We successfully introduced a microbial community in replicate oxic, in-field bioreactors that was distinct in composition from the low oxygen ORFRC groundwater, without nutrient amendment. Evidence for an active bioreactor community was found in the gradual accumulation of diversity despite smaller changes in the incoming groundwater, as well as in the marked response of bioreactor β -diversity and 16S OTU copy numbers during experimental manipulations. That the bioreactor communities became more like the groundwater during the perturbations and that the internal chemistry was similar to groundwater supports in-field bioreactors as a viable technology for testing community structure/function relationships and using such data to construct models for groundwater conditions and predicting microbial responses. Moreover, there were two consortia of strongly co-occurring bacteria one of which appeared during the oxic periods and the other during the low oxygen periods in all bioreactors despite their rarity in the groundwater. However, there was high temporal variability in both bioreactor and groundwater community composition which was unexplainable by the measured background biogeochemical fluctuations.

Variability in key biogeochemical conditions (i.e., DO and pH) may be important determinants of groundwater bacterial diversity. Planktonic microbial community assembly can be deterministic under steady state conditions,⁴⁹ but groundwater is not a steady state system.^{24–26} In contrast, high spatial variability in groundwater communities has been attributed to geochemistry,^{18,50,51} but our results suggest that, as in many ecosystems, it is perturbations outside the normal range of *in situ* variability that result in strong community shifts. Indeed, drought and water table retreat destabilize microbial communities,⁵² whereas recharge events may have a homogenizing influence.^{26,53} Thus, frequent geochemical disturbances may play a role in maintaining the high groundwater microbial diversity,⁵⁴ which is important for predicting the fate of microbially metabolized compounds.⁵⁵

Although groundwater has high natural variability in microbial community composition, the in-field bioreactor approach recovered the signal of specific temporal events out of the background noise and was able to identify two large, diverse groups of organisms that responded similarly, but under contrasting conditions, during the DO and pH perturbations. Moreover, while the diversity analyses suggested that perturbations should make the samples look more like the groundwater, these clades are rare in groundwater yet become abundant in the bioreactors during these biogeochemical events. The “oxic group” decreased in abundance during periods of anaerobicity and included OTUs indicative of oxidizing reduced S-, N-, and Fe- species while others are heterotrophic (e.g., *Acidobacteria* division 4 or *Arenimonas taoyuanensis*). Hence this collective group may share resources for survival by oxidizing compounds reduced during anoxic conditions in an environment that fluctuates near the boundaries of oxic and anoxic. The second “nitrite-producing group” increased in abundance during anaerobic periods. Since all OTUs in this group are related to cultured representatives that can reduce nitrate or have been associated with low oxygen systems, this genetic potential in combination with increased

nitrite during anaerobic periods indicates active nitrate-reduction in the bioreactors. The successful identification of this nitrifying group at our site is important because it serves as a reference site for the study of a heavily nitrate and uranium contaminated groundwater system less than 5 km away.^{4,7–18}

Because organisms within both groups explain a significant component of community level variation and have a reproducible pattern across bioreactors and manipulated conditions, these organisms likely mediate key metabolic processes responding to variability in oxygen conditions within this groundwater system. The reoccurrence of groups restricted to oxidizing and reducing conditions is also in agreement with the idea that shallow groundwater systems have temporal variability in their chemistry and therefore harbor organisms that are able to remain dormant through unfavorable periods.^{19,26,52,53} Thus, depending on the range of geochemical conditions, individual groundwater wells should support diagnostic consortia (i.e., microorganisms that commonly occur at a specific location because of their adaptations to the local geochemical conditions¹⁸). However, it is poorly understood why common planktonic groundwater organism abundances vary extensively over space and time,^{18,24–26,49,50} while adhered or biofilm communities are not as variable.^{53,56} The ability to maintain groundwater communities in easily manipulable bioreactors will facilitate further investigations of the predictability of groundwater microbial functional responses, including planktonic and adhered microbes, in order to better understand the impacts of anthropogenic disturbances such as the release of hazardous substances.

In-field groundwater bioreactors have a number of advantages over benchtop bioreactors or direct groundwater amendments, but there are also limitations. While previous strategies required simulated groundwater in a bioreactor,^{3,57,58} or direct amendment injection into groundwater,^{4,59} our in-field approach allowed for easy system manipulation at a small scale while retaining the relevant influx of groundwater without the need for nutrient amendment. One drawback is the need for a dedicated well with continuously pumping into the bioreactors. Additionally, depending upon the experiment, equipment to regulate gas, temperature, or a chemical would need to be deployed. However, our results demonstrate that in-field bioreactors have an advantage in their small scale and ease of manipulation while still maintaining a microbial community that is complex, fluctuating in-step with groundwater, and responsive to perturbation. Together, these results show that in-field bioreactors are a viable approach for easily maintaining, manipulating, and testing the impacts of various geochemical or physicochemical regimes on the structure and function of groundwater microbial communities.

The study of microbial community dynamics over time is a rapidly growing area of research.^{60,61} Field condition relevant mesocosms help test theories about microbial community assembly and response to changing environmental conditions. Future work can build upon the present results and more deeply interrogate the rates of metabolite turnover and individual microbial species abundances using more accurate methods of assaying microbial species abundances such as RT-qPCR. It will be important for industrial applications of groundwater research, e.g. bioremediation or bioaugmentation, to be able to accurately predict how target microbial species abundances and associated functions respond to planned perturbations. The data derived from future in-field bioreactor studies will be a useful asset for not only studying natural

microbial communities beyond the bulk phase but also for determining the critical factors that tie community structure to function. Identifying these relationships as well as accurately capturing individual and bulk phase responses to perturbations will also contribute to the development of more accurate hydrobiogeochemical models which may aid in assessing environmental treatments and in evaluating risk management.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04751.

Supplemental methods text; Table S1, OTU identifications correlating to DO/pH alterations and nitrite, Figure S1, detailed schematic diagram of well FW305 installation and materials used; Figure S2, cartoon depicting the bioreactor experimental setup; Figure S3, pictures and description of custom designed in-field bioreactors; Figure S4, OTU abundances of biofilm coupons (PDF)

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Notes

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