

**SPATIOTEMPORAL DYNAMICS OF MICROBIAL COMMUNITIES
IN CONTAMINATED SUBSURFACE**

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

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May 2024

DEDICATION

To my dad, Lendell Fitzgerald. You introduced me to science when I was five years old with a microscope and a vial of pond water. I wish you could see me now. I miss you every day. Thank you for looking over me and seeing me through the most challenging times of my life thus far. I will always love you and try to make you proud.

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Hazen for his mentorship and guidance. When I started with you as an undergraduate researcher, I had no idea you would see me through to my doctorate. Thank you for supporting and challenging me throughout my academic career as well as my personal growth, Boss.

Thank you to my committee members, Dr. Karen Lloyd, Dr. Jill Mikucki, and Dr. Andrew Steen, for their support, constructive criticism, and thought-provoking questions. You all have made me a better scientist.

To Dominique Joyner, thank you for being an academic sounding board and true friend. I'm better in so many ways for knowing and working with you.

My field days were always better when I got to drag Kenneth Lowe along! "What Would Kenneth Do" should be everyone's life motto. Thank you for teaching me all that I know in field!

I could not have done this without the love and support of my husband, Cameron. You have been my rock and my partner, and I could never thank you enough. I love you.

During my time in graduate school, I gained the significant title of "Mom." I love you with all of my heart, Riley Claire, my darling daughter. Since you can grow up to be anything, grow up to be brave.

ABSTRACT

Groundwater serves as drinking water for 50% of United States residents, but a study conducted by the United States Geological Survey (USGS) found hazardous geological or manmade contaminants in approximately 20% of U.S. groundwater sources. Some contaminants were introduced due to industrial activities, such as at the Oak Ridge Reservation (ORR) in Tennessee. It is possible these contaminants could be reduced or immobilized by microbial activity, but the understanding of how spatiotemporal variability of subsurface geochemistry and the drift, dispersal, and selection of associated microbial communities is vital to understanding these potential processes. To understand the spatiotemporal relationship between microbial communities and geochemistry, a 70-day high-resolution time series survey of 27 wells was completed at the ORR. This study design was to obtain diurnal and seasonal fluctuations at three sites with mild, moderate, and high levels of nitrate and heavy metal contamination. One well at each site was selected for additional analyses by sampling for microbial communities in groundwater and sediment-associated environments simulated with sediment traps. Groundwater was filtered through 8 μm [micrometer] and 0.2 μm [micrometer] filters for 16S rRNA [ribosomal ribonucleic acid] gene sequencing and shotgun metagenomic analysis. In each of the wells used for microbial sampling, 18 unamended sediment traps were deployed throughout the sampling period for a time series soil analysis.

The results revealed distinct succession patterns in the abundant phyla and cell size of the microbes affected by the contamination levels and rainfall events. During a drought period captured over the course of the study, we established a baseline of microbial communities and geochemistry present in the groundwater at each site. We determined planktonic Actinobacteria became more abundant in all three contamination levels in the changing geochemistry after heavy rainfall. However, sediment-associated Firmicutes and Bacteroidota consistently increased after rainfall events. The largest geochemical fluctuations after rainfall were seen in the mild contamination level with sharp increases in nickel and aluminum. The drastic subsurface changes over time and space within these extreme environments show that any future bioremediation efforts at contaminated sites like the ORR require dynamic efforts that are adaptable to each site.

TABLE OF CONTENTS

Introduction to Field Site and Sampling Timelines	1
Ecosystems and Networks Integrated with Genes and Molecular Assemblies (ENIGMA)	1
Background: Oak Ridge Research Center	1
BER ORNL Research Site and the S-3 Ponds	1
Previous ENIGMA Campaigns and Findings that Led to Current Research.....	2
Pilot Time Series Overview.....	3
Results and Lessons Learned	4
27-Well Survey Overview	4
Results	5
References.....	6
 Chapter 1: Time Series Study Comparing Contaminated and Uncontaminated Groundwater Microbial Communities and Geochemistry	7
Introduction	7
Materials and Methods	8
Well Selection	8
In-Field Sampling	9
Dissolved Element Analysis	10
DNA Extraction and Sequence Analysis	10
Results.....	12
Discussion.....	13
References.....	15
 Chapter 2: Spatiotemporal Dynamics of Subsurface Geochemistry and Microbial Communities in DOE Legacy Site Groundwater	18
Introduction	18
Materials and Methods.....	19
Well Selection	19
Groundwater Collection and Geochemical Analysis.....	19
Results.....	20
Discussion.....	22
References.....	24
 Chapter 3: Time Series Analysis of Sediment-Associated and Groundwater Microbial Communities in Varying Contamination Levels	26
Introduction	26
Materials and Methods.....	27
Study Design	27
Groundwater Sampling.....	28
Bug Trap Development and Deployment	28
DNA Extraction for Bug Traps	28

DNA Extraction for Groundwater Filters.....	29
Sequence Data Processing.....	29
Dissolved Element Analysis for Groundwater and Sediment	30
Results.....	31
Discussion.....	31
References.....	35
Chapter 4: DOE Superfund Sites: History, Policy Initiatives, and Current Research to Benefit Environmental Restoration	38
Introduction	38
Comprehensive Environmental Response, Compensation, and Liability Act (1980).....	38
Hazard Ranking System	39
National Priorities List	40
Financing Cleanup Efforts – Trust Fund Creation.....	40
Nuclear Waste Policy Act.....	41
Evaluation of Successes and Shortcomings	41
Relevance and Future Challenges.....	42
Superfund Green Remediation Strategies.....	42
Nitrate Pollution and Sources.....	43
Knowledge Gaps in Current Research	44
Conclusion.....	45
References.....	46
Appendices	48
Appendix A	48
Appendix B	58
Appendix C	69
Appendix D.....	83
Vita.....	95

LIST OF FIGURES

Appendix A

Figure A.1. Hydrogeologic regions at Y-12 National Security Complex	48
Figure A.2. Contaminant pathways from the S-3 Ponds	49
Figure A.3. Major flow paths in the Bear Creek Valley.....	50
Figure A.4. Background Site A Wells for Pilot Time Series	51
Figure A.5. Area 2 Wells for Pilot Time Series	52
Figure A.6. Area 3 Wells for Pilot Time Series	53
Figure A.7. Contaminated areas at the BER ORNL Field Research Site (ORRFRC)	54
Figure A.8. Area 1 Wells for the 27-Well Survey	55
Figure A.9. Area 2 Wells for the 27-Well Survey	56
Figure A.10. Area 3 Wells for the 27-Well Survey	57

Appendix B

Figure B.1. Conceptual model of subsurface flow and mixing zones	58
Figure B.2. Background Site A Aqua TROLL 600 and rainfall data	59
Figure B.3. Area 2 Aqua TROLL 600 and rainfall data	60
Figure B.4. Area 3 Aqua TROLL 600 and rainfall data	61
Figure B.5. Taxonomic analysis of groundwater communities by area, well, and pore size.....	62
Figure B.6. NMDS of groundwater communities by well and pore size	63
Figure B.7. Taxonomic richness by week and complete communities	64
Figure B.8. Taxonomic richness separated by filter size	65
Figure B.9. Beta diversity of community structure	66
Figure B.10. NMDS plots of groundwater microbial communities by Bray-Curtis index.....	67
Figure B.11. Sulfate-reducing bacteria composition of groundwater samples	68

Appendix C

Figure C.1. Drought conditions displayed by U.S. Drought Monitor	69
Figure C.2. Rainfall totals at Y-12 sampling site.....	70
Figure C.3. Water table measurements by area.....	71
Figure C.4. In-field dissolved oxygen measurements by area	72
Figure C.5. In-field pH measurements by area	73
Figure C.6. In-field conductivity measurements by area.....	74
Figure C.7. In-field nitrate concentrations by microbial sampling well	75
Figure C.8. Metals of interest across all wells and sampling period	76
Figure C.9. TC, DOC, DIC and TN in each area	77
Figure C.10. Acridine Orange Direct Counts (AODC) for microbial sampling well	78
Figure C.11. Morphological examples from each microbial sampling well	79
Figure C.12. Taxonomic analysis of each microbial sampling well	80
Figure C.13. Relative abundance of Proteobacteria by well	81
Figure C.14. Well construction data by area in relation with water table fluctuation	82

Appendix D

Figure D.1. Bug trap design	83
Figure D.2. Bug trap deployment scheme.....	84
Figure D.3. Taxonomic analysis of bug trap sediment.....	85
Figure D.4. Taxonomic analysis of Area 1 bug trap sediment and planktonic community.....	86
Figure D.5. Taxonomic analysis of Area 2 bug trap sediment and planktonic community.....	87
Figure D.6. Taxonomic analysis of Area 3 bug trap sediment and planktonic community.....	88
Figure D.7. Cations that decreased in bug traps from starting material	89
Figure D.8. Cations that increased in bug traps from starting material	90
Figure D.9. Cations that decreased in bug traps compared to groundwater samples	91
Figure D.10. Cations that increased in bug traps compared to groundwater samples	92
Figure D.11. Relative abundance of phyla in bug traps grouped by area.....	93
Figure D.12. Map of sample area with geochemical fluctuations.....	94

Introduction to Field Site and Sampling Timelines

Ecosystems and Networks Integrated with Genes and Molecular Assemblies (ENIGMA)

For the last twelve years, Ecosystems and Networks Integrated with Genes and Molecular Assemblies (ENIGMA), a Department of Energy Scientific Focus Area, has been characterizing, monitoring, and conducting field experiments at the DOE's Biological and Environmental Research (BER) Oak Ridge National Laboratory (ORNL) field research site. The focus of ENIGMA's research has been on elucidating the mechanisms and efficacy of bioreduction and bioimmobilization of Uranium (U), one of the DOE's most common waste site contaminants. This has involved several field studies including pump tests, hydrological modeling, characterization of sediment and groundwater, and characterization of amendments of ethanol, bromide, and nitrate as well as a comprehensive understanding of the geology of the site.

Background: Oak Ridge Research Center

The BER site at ORNL is located within the Y-12 plant area of responsibility and located in Bear Creek Valley adjacent to the city of Oak Ridge. The sampling locations for this study include a 243-acre previously disturbed contaminated area and a 404-acre background area for groundwater (Watson et al. 2005). The background area provides for comparison studies in an uncontaminated area and includes ancillary structures located within a 3.2-mile (5.2-km) radius of each other on the ORR. See Appendix A, Figure A.1.

Bear Creek Valley primarily consists of southeast-dipping fractured clastic low hydraulic conductivity shales, and siltstones interbedded with porous carbonate limestone and dolostone. Carbonate geology in the area is mainly karstic producing some solution-enlarged features in the region. Interbedded limestone and shale formations of the BCV strike N 55°E and dip 45°E (Shevenell et al. 1994). The flow in Bear Creek is supplemented by small tributaries originating on the southern slope of Pine Ridge and by springs emanating mainly from the base of Chestnut Ridge (Appendix A, Figure A.1) (Watson et al. 2005). In its upper reaches, Bear Creek follows a relatively straight course along the geologic strike close to the contact between the Maynardville Limestone and Nolichucky Shale. In the BCV, cracks in the saprolite clay can extend up to 7 m into the subsurface, resulting in irregular pathways of preferential flow within the subsurface. Below is a brief description of the background and contaminated areas at the BER ORNL field research site.

BER ORNL Research Site and the S-3 Ponds

The background area A consists of over 40 wells and covers approximately 163 hectares located in West BCV, about 2 km west of the contaminated area (Appendix A, Figures A.1 and A.2). This site lies directly along the geologic strike of the contaminated area and is, therefore, underlain

by nearly identical geology, mineralogy, and structure. No known contaminants have been disposed of at this location throughout the history of DOE operations. The majority of the area is heavily wooded and surface hydrology is primarily perennial and ephemeral crosscutting streams that feed into Bear Creek. The primary underlying bedrock unit for the background is Maryville Limestone.

The contaminated area at the ORR is monitored by over 400 groundwater wells across approximately 98 hectares in the BCV, just west of the main Y-12 industrialized plant area. The Y-12 complex is home to several burial grounds and disposal sites, but the well-characterized S-3 site is of interest for this research. The S-3 ponds were four in-ground impoundments developed in 1951 as a waste disposal site for hazardous liquid waste generated at Y-12 during uranium operations (Revil et al. 2013). Each pond had a capacity of 9.5 million liters. These ponds were neutralized, drained, and capped in the 1980s and now serve as a parking lot. Major pathways of interest for the S-3 Pond contaminants include Pathway 1 and Pathway 2 (Appendix A, Figure A.3) which largely flow parallel to Bear Creek. Bear Creek flows down the middle of the area adjacent to Bear Creek Road. The flow in Bear Creek is supplemented by small tributaries originating on the southern slope of Pine Ridge, and by springs emanating mainly from the base of Chestnut Ridge. The tributaries convey shallow groundwater that has discharged to the surface and storm flow. In its upper reaches, Bear Creek follows a relatively straight course along the geologic strike close to the contact between the Maynardville Limestone and Nolichucky Shale. Dozens of single and multiport wells have been installed over the last 40 years with various installation and drilling methods at the two reactive barriers sites and within the different research areas within the contaminated FRC. These wells were installed in the area by the Y-12 Groundwater Protection Program and have been made available for research purposes.

Groundwater contaminants at the research field site (S-3 ponds and By/By plume) include radionuclides (uranium, Sr-99, and Tc-99), metals (strontium, cadmium, barium, boron, mercury, chromium), volatile organic contaminants (VOCs), and nitrate (Shevenell et al. 1994). The uranium-nitrate-pH gradient present at the field test site is of particular interest for this study because the gradient creates an extreme environment for these microbial communities. Nitrate levels in the groundwater here are the highest ever recorded – approximately 30,000 ppm – and the concentration within the ponds was recorded at over 70,000 ppm (Shevenell et al. 1994). The chemical gradient at this site provides a stable transect where microbial communities and activities can be assessed. In addition, these stable transects allow for elucidation of relationships between the environment and the genetic and functional diversity of the community. Notably, the water level inside the ponds was 4.3 m above the groundwater table, which allowed for vertical infiltration of the contaminants into the groundwater of the study.

Previous ENIGMA Campaigns and Findings that Led to Current Research

In 2014, ENIGMA launched its first campaign to begin characterizing the groundwater geochemistry and microbial communities at the BER field site. 99 wells were sampled and analyzed for key microbial populations, focusing on nitrate reduction, acidophiles, metal

resistance and cation reduction, across the site and identify chemical gradients in the subsurface that would enable future experiment (Smith et al. 2015). This sampling campaign showed the microbial communities' divergence across the sites were distinct. Therefore, communities within the same well were more closely related than those in different wells. The geochemical parameters of all wells were evaluated using geochemical correlations and found to be independent from each other. This campaign also established a direct microbial-geochemical association by comparing individual geochemical parameters with the microbial community present during sampling. This connection showed that with a larger data set over an extended period the groundwater microbial community structure could be used to predict the water geochemistry.

After this in-depth look at the association between groundwater and geochemistry, the next campaign focused on understanding the sediment microbial community's relation to groundwater communities and geochemistry. A sediment core was collected from an uncontaminated, pristine background site and analyzed by depth for microbial communities and geochemistry (Wu et al. 2023). Results showed depth-related covariation in the microbial communities and a distinct geochemical gradient in the vertical space (Moon et al. 2020).

Following this study of depth-dependent microbial selection in an uncontaminated core, ENIGMA collaborators and I developed a sediment core sampling campaign to analyze contaminated subsurface environments. Two locations surrounding the S-3 Ponds – one upgradient and one downgradient – were selected to begin understanding how microbial communities in and around a contamination plume could vary by depth in the sediment. Results showed that increased concentration of heavy metals in the downgradient core heavily constrained the microbial community (Thorgersen et al. 2023). However, the bioavailability of these metals increased due to the acidic environment and were able to be utilized by this less diverse, constrained community.

These studies showed that (1) groundwater geochemistry and microbial communities were dependent on each other and predictable at any given time, (2) microbial communities change in a depth-dependent manner, and (3) sediment microbial communities from a downgradient core were constrained due to heavy metal toxicity when compared to an upgradient core. However, the questions of how these connections changed over time and how groundwater communities compared to sediment communities still need to be answered.

Pilot Time Series Overview

Due to sampling challenges and the complexity of the heterogeneous subsurface matrix that ranges across the vadose, capillary fringe, and saturated zones, few field sites have been comprehensively described and studied despite the significant ecosystem benefits associated with shallow subsurface systems. A pilot time-series study was developed to understand how

the microbial communities and geochemical parameters changed over time in pristine and contaminated environments.

The experimental focus for this study was comparing uncontaminated (background) wells to a subset of wells at the ORR (Appendix A, Figures A.4, A.5, and A.6) that provide gradients of pH, NO₃, Mo, and/or SO₄ among other key constraining variables to elucidate potential impacts from hydrology and meteorology. We performed a time series of water level and flow, functional metagenomics, biological activity, and geochemistry of sampled groundwater.

Nine wells were selected across three sampling sites the BER: background (pristine, uncontaminated), Area 2 (low levels of nitrate and heavy metals) and Area 3 (high levels of nitrate and heavy metals).

Results and Lessons Learned

Within the 8-week sampling period, there were drastic and distinct differences in the microbial communities, metals concentrations, and geophysical parameters between the Background Site and the contaminated sites at Y-12. There were significant changes in the microbial communities and geochemistry across each two weeks. When developing the 3D subsurface models, there were many hurdles due to needing more time points, and the wells being so close together led to ineffective interpolation within the model. A longer time scale with more frequent sampling and additional wells was needed to develop an accurate and descriptive model.

Due to drastic differences between the Background Site's physicochemical parameters (large water table fluctuations after rainfall and diurnal fluctuations that were not seen at the contaminated sites) and microbial community diversity and functional groups, the decision was made to forego sampling the Background Site and focus on 3 areas at Y-12 (Areas 1, 2, and 3). This decision was made in hopes they are more representative of each other and will allow for direct comparison.

All information, analyses, and results of the Pilot Time Series can be found in Chapter 1 of this dissertation.

27-Well Survey Overview

To effectively model the subsurface environment and understand the connection between microbial communities and groundwater geochemistry at Y-12, a larger area and additional timepoints were needed.

Twenty-seven wells across three areas of varying contamination at Y-12 (Appendix A, Figures A.7, A.8, A.9, and A.10) were selected for this study. All wells were sampled for geochemical

analyses (oxygen saturation, pH, conductivity, metals, nitrogen and carbon content, anions) 4 days a week from July to December for a total of 70 sampling days. One centrally located well from each area was selected as the microbial sampling well and 5 liters of water was sequentially filtered through an 8 μm and 0.2 μm filter for microbial community analysis. Acridine orange direct counts (AODC) were also completed for each day. Bug traps filled with sterilized, local sediment were also deployed within these three wells. These bug traps allowed a time series analysis of sediment-associated microbes and geochemistry to compare to the groundwater samples as well as study the founder effect of microbial communities to determine if sediment-associated microbial communities changed over time. A total of 18 bug traps were deployed in each well 3 at a time across the sampling period and left for 4, 6, or 8 weeks. Over the course of the study, detailed meteorological data and water table fluctuations were also recorded. During the sampling period, East Tennessee experienced a drought from the September to the end of October followed by substantial rainfall.

Results

Our results showed that each area has a distinct microbial community structure and geochemical fluctuations that were heavily impacted by rainfall. The less contaminated areas (Areas 1 and 2) were found to be much more diverse and dynamic and saw changes in microbial community structure on a diurnal basis. In Area 3, the most contaminated area, we see the community dominated by one phylum, Betaproteobacteria. However, the community did undergo small changes after heavy rainfall events.

The bug traps also saw changes over time and differences between each well. The microbial communities changed over the course of the time study and showed that the founder effect was not a predominant factor when using bug traps in these environments. The starting sediment was analyzed and compared to the deployed sediment. Some metals (B, Ca, Mo, W) decreased from the starting material whereas some metals (Na, Se, K, U) increased from the starting material.

All information, analyses, and results of the 27-Well Survey can be found in Chapters 2 and 3 of this dissertation.

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Chapter 1: Time Series Study Comparing Contaminated and Uncontaminated Groundwater Microbial Communities and Geochemistry

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Introduction

The shallow subsurface has historically been considered a stable environment based on studies that used traditional cell counts and cultivation techniques (Goldscheider et al. 2006, Kallmeyer 2011). Investigations of microbial biodiversity were performed via visual cell identification and cell counts (Goldscheider et al. 2006). However, most bacteria found in the subsurface are unculturable. In the last two decades, significant technological advancements have been made in the application of next-generation sequencing (Tringe and Hugenholtz 2008), high-throughput-omics approaches (López-García and Moreira 2008), single-cell methods (Lasken and McLean 2014) and methods encompassing untargeted functional potential (Rajendhran and Gunasekaran 2011). Once sequence technologies were utilized, a much greater diversity and variability were observed in these environments than previously recognized.

Temporal and seasonal dynamics influence hydrological mixing, particularly between and within the saturated and capillary fringe. Following precipitation events, the vadose zone experiences high saturation levels as vertical infiltration proceeds downward to the water table, yet residual pore water can persist creating varying levels of water and gas saturation (Jones and Bennett 2014). The capillary fringe exists at the interface of the saturated and vadose zone and is highly dependent upon fluctuations of the local water table. The capillary fringe is dynamic over time with varying physicochemical conditions resulting from water table fluctuations (Griebler and Lueders 2009). Geochemical parameters (e.g. pH and dissolved oxygen) in the transitional boundary between the vadose and saturated zones can vary drastically which in turn impacts ecosystem function in terms of geochemical cycling, biotic/abiotic filtering and buffering processes (Dobson et al. 2007, Chakraborty et al. 2012). Aquifer recharge and a fluctuating water table can occur via seasonal patterns, and the transition zones between the variably saturated and saturated zones has been shown to be an important ecotone for microbial diversity and activity (Sirisena et al. 2013, Hazen and Saylor 2016). Our goal now is to discover the key biological mechanisms, specifically related to nitrate reduction, heavy metal and low pH resistance, that constrain certain operational taxonomic units (OTUs) and/or genetic systems to

locations at a site/zone and what mediates community diversity and activity (Figure B.1). All figures for Chapter 1 can be found in Appendix B at the end of this document.

Subsurface porous media habitats can be conceptually divided into three zones (I, II and III) with respect to groundwater flow and mixing (Flint et al. 2023). The vadose zone (Zone I, including the capillary fringe) is variably saturated depending on infiltration episodes and degree of vertical water table fluctuation. Zone II is the ‘shallow’ groundwater zone wherein groundwater flow, together with seasonal changes in water table elevation, can cause multi-directional flow (i.e. vertical and horizontal fluctuations) that can result in greater mixing (Chu et al. 2000). The ‘deeper’ groundwater zone (Zone III) lies below the depth affected by seasonal water table fluctuations. The degree of mixing in Zone III is related mainly to the groundwater flow field (Bougon et al. 2012, Hermans et al. 2023). All of the wells used in this study fall within the saturated zone. Therefore, we developed three hypotheses: (1) Short-term changes in groundwater direction and flow rate due to rainfall events have only a minor effect on microbial community composition; (2) Long-term changes on an annual or seasonal basis have a significant effect on community variability; and (3) Core community composition will be increasingly stable (selectable) but lower diversity at increasingly stressed environments.

Materials and Methods

This study was completed using continuous passive monitoring and biweekly active monitoring. Passive monitoring included down-well groundwater monitors at each site (Background A, Area 2, and Area 3) and meteorological stations at the background site and at the ORR.

Passive monitoring stations were set up at Area 2, Area 3, and Background Site A in large enough wells to accommodate multiparameter data loggers, called Aqua TROLLs developed by In-Situ, Inc., that were used to collect real-time geochemical data during sampling (In-Situ 2023). A remote logging Hobo solar-powered weather station with logging was setup near the Aqua TROLL at Area 2 and Background Site A (Appendix A, Fig. A.5) on July 30, 2018, which collected temperature, wind speed, wind direction, rainfall, relative humidity, soil moisture, barometric pressure, and light intensity.

Active water sampling was completed every two weeks starting on March 20, 2019, for 8 weeks for a total of 5 sampling points. The last sampling day was May 15, 2019. This sampling method allowed for an opportunity to capture seasonal changes in microbial communities and in-depth geochemistry in addition to diurnal fluctuations with passive monitoring.

Well Selection

Passive water monitoring wells were selected in Area 2, Area 3, and Background Site A using the following criteria:

- Information from previous hydrological flow and biogeochemistry in each area (Smith et al. 2015, Paradis et al. 2017, Paradis et al. 2019)
- Inner diameter is large enough to accommodate the Aqua TROLL 600 (pH, RDO, Conductivity, ORP, Temperature, Turbidity, Pressure, Nitrate) data loggers, and
- Central location in relation to the active sampling wells.

Each prospective well was examined using a downhole well camera (Well-Vu DCC15) to ensure no damage to the well casings or intrusion from vegetation had occurred. Each well was then developed by pumping until all Aqua TROLL 600 parameters were stable, before installing the Aqua TROLL in them. The Background Site A Aqua TROLL in well FW300 was installed on 6/19/2018 with limited interruptions, due to battery life and calibrations, until the start of sampling. Area 2 Aqua TROLL with nitrate sensor in well GW-835 was installed on July 6, 2018. Area 3 Aqua TROLL with nitrate sensor in well FW126 was installed on January 9, 2019. Three Level TROLL 400s (water level, pressure, temperature) were installed at each site in designated wells between July 20, 2018 and January 15, 2019. All TROLLs logged data points every 10 minutes.

Active water monitoring wells were selected in Area 2, Area 3, and Background Site A, using the following criteria:

- information from previous hydrological flow and biogeochemistry in each area,
- screened across the capillary fringe and the saturated zone, where available, and
- each well required one screened interval at the top of the shallow aquifer.

Each well was then pumped to determine pump sampling time and volume, hydraulic conductivity, water level change after pumping, and effect on nearby well depth (Appendix A, Fig. A.4, A.5, and A.6). There were no changes seen in surrounding wells when pumped at a rate of 100 mL per minute.

In-Field Sampling

Temperature, dissolved oxygen, pH, conductivity, oxidation-reduction (Eh) potential, and nitrate were determined at the wellhead using multi-parameter Aqua TROLL 600 sondes developed by *In-Situ, Inc.* based in Denver, Colorado (In-Situ 2023). The wells were pumped until all monitored geochemical parameters had stabilized according to the sonde's real-time measurements. 10 L were pumped through a 10 μm , 0.2 μm , and 0.1 μm filter sequential. The three filters in sequence coupled with the clogging of the 0.1 μm filter created catastrophic failure to the tubing connecting the manifolds. To alleviate the pressure building in the tubing, we developed a push-pull system for this sampling using two peristaltic pumps. One pump was stationed between before the filter manifold setup and would pull water from the well to push through the filters. The second pump was placed between the 0.2 μm and 0.1 μm filter since this was where most of the pressure was building up. This push-pull system allowed for the elimination of clogged filters and additional down-the-line DNA extractions. All filters were frozen at -80C

until they could be transported for extraction. Unfiltered sample was also collected for cation analysis.

Dissolved Element Analysis

For determination of dissolved elements, 15 mL of unfiltered groundwater was collected in acid-washed polypropylene tubes and stored overnight at RT. After settling, 10 mL of groundwater was then transferred to a conical acid-washed polypropylene tube containing 2 mL of 12% (v/v) trace-grade nitric acid (VWR, Radnor, PA) - giving a final concentration of 2% nitric acid (v/v). The samples were stored at 4°C and transferred to the University of Georgia for analysis.

The samples were spun down just prior to analysis. Forty-five (45) samples were analyzed for 52 elements using an Agilent 7900 inductively coupled plasma mass spectrometer (ICP-MS) fitted with MicroMist nebulizer, ultra-high matrix introduction (UHMI) -spray chamber, Pt cones and an octopole reaction system (ORS) collision cell (Agilent Technologies, Santa Clara, CA). The data were collected using 3-point peak pattern, 3 replicates, 100 sweeps/rep, and various integration times (all >0.3 s) using either no gas or He gas in the ORS collision cell. The external calibration standards, IV-ICPMS-71A and IV-ICPMS-71B, were used to create an 11-point curve from 0 - 500 ppb for each element and 1 ppm of the internal calibration standard, IV-ICPMS-71D, was added to each sample (Inorganic Ventures, Christiansburg, Virginia). All samples from Area 3 were analyzed as above but using a UHMI method with an approximate dilution rate of 88x. For the UHMI samples, the external calibration standards, IV-ICPMS-71A and IV-ICPMS-71B, were used to create a 16-point curve from 0 - 5,000 ppb for each element and additional 10,000 - 100,000 ppb for Al, Mg, K, Ca, Na, Mn and 50 ppm of the internal calibration standard, IV-ICPMS-71D, was added to each sample (Inorganic Ventures, Christiansburg, Virginia). External calibrations were performed about every 100 samples or each day, whichever occurred first. Data were processed using the nearest internal standard by mass and fitted to a linear curve though the calibration blank. All data is reported in ppb (i.e. ng/mL) and in μM with the detection limit (DL) listed as "< value" in ppb.

DNA Extraction and Sequence Analysis

For DNA extraction, groundwater samples were stored at -80°C before use. Each filter was cut evenly in half, and one half was used to extract DNA. Microbial genomic DNA was extracted by a freeze-grinding in SDS lysis extraction buffer and purified by using DNeasy PowerSoil DNA isolation Kit (QIAGEN; bead tubes were not used) (Weiser et al. 2019). DNA quality was checked by NanoDrop, and double-strand DNA concentration was measured by Quant-iT™ PicoGreen™ dsDNA Assay Kit.

For library preparation and sequencing, two-step PCR amplification method was used for PCR product library preparation (Wu et al. 2015). In the first step PCR, the standard primers were used to amplify the V4 region of prokaryotic 16S rRNA genes (515F [5'-

GTGCCAGCMGCCGCGGTAA-3'] and 806R [5'-GGACTACHVGGGTWT CTAAT-3']). In the second step PCR, phasing primers were designed and used to increase the base diversity in sequences of sample libraries. PCR amplification and purification were the same as reported previously (2), except amplification cycles (10 cycles in the first step and 20 cycles in the second step for 16S). Sample libraries were then sequenced by MiSeq platform (Illumina, San Diego, CA, USA) following MiSeq's commercial protocol.

Rarefied ASV tables were imported into R and used to create a phyloseq object using the Phyloseq package along with associated metadata. A phyloseq object representing all the ASVs detected on all filters at a specific sampling date and well was also created using phyloseq. Only unweighted analyses were used on the combined object. Ggplot2 was used to visualize all results.

Alpha diversity metrics were generated using the estimate_richness function in package Phyloseq and the function pd from the picante package. For the combined samples, only presence/absence metrics were reported. Beta diversity of the combined object was generated using unweighted unifracs distances from package vegan and NMDS ordination was performed using phyloseq function ordinate. ANOSIM and PERMANOVA analysis was performed using functions anosim and adonis2 in the vegan package, with permutations structure set with the area as blocks and wells as plots, with within set to series. Strata was set as Area, and wells were nested in Area. Function envfit was used to fit environmental variables to the ordination. Indicator species analysis was performed with function multipatt from package indicspecies using presence/absence-transformed ASV tables merged to remove filter size from consideration.

Geochemical and microbial associations were generated by first running MaAsLin2 on individual filters and wells to determine differentially abundant features with week 1 as a reference and k over A filter of 15 samples and an 0.0005 abundance applied to the whole dataset. Then, features were filtered to those that were differentially abundant in at least two filters on the same day in the same well (e.g., on week 3 an ASV was positively differentially abundant in the 0.2 μ m filter and negatively differentially abundant in the 10 μ m filter in well FW-101). Features (in this case ASVs) with a differential abundance coefficient above the coefficient of cell number change (natural log of the ratio of cells at the week of interest compared to week 1 in order to match the MaAsLin2 procedure) were selected for further analysis. Each feature was then assessed for a change in filter prevalence. If a feature was increased in one filter but decreased in another, we identified this as a change and coded it depending on the filter sizes.

To process the sequence data, QIIME2 (version 2021.2) was used to perform the ASV-based processing. After barcode and primer sequences were trimmed with zero maximum error, sequencing data were processed by DADA25 to identify exact amplicon sequence variants (ASV). The method 'consensus' was used to remove chimeras; samples are denoised independently; forward and reverse truncate positions are 220 and 170, respectively, after quality trim with a quality score 2; maximal expected error score is 2.0 when combining forward and reverse

sequences. The ASVs were identified taxonomically by RDP Naive Bayesian Classifier algorithm⁶ in DADA2 with a confidence threshold of 0.5, based on the Silva database⁷ (v138.1; silva_nr99_v138.1_train_set.fa.gz from <https://benjjneb.github.io/dada2/training.html>). After classification, ASVs identified as Chloroplast, Mitochondria, Eukaryota, or unclassified kingdom were removed. The prokaryotic ASV sequences were aligned using MAFFT⁸ in QIIME2, without mask.

Results

The in-field physical and geochemical parameters, including depth to water (DTW) measurements (indicating the subsurface water table level), groundwater temperature, pH, dissolved oxygen (DO), and conductivity, are plotted together with the soil moisture and precipitation data to show how precipitation events affect the water table and geochemistry. Figures B.2, B.3, and B.4 show data from each of the three areas. As the figures show, the background site increases and decreases in DTW have a strong relationship with rainfall totals with fluctuations up to 2.5 meters in 3 hours. There is also a decrease in DO, pH, and conductivity following rainfall events. In Area 2, there is very little increase or decrease over time, even with rainfall. Small decreases in DO, pH and conductivity following only very heavy rainfall events (>3mm per 8 hours). Most DTW changes were less than 0.5 meters. In Area 3, there is minimal fluctuation in DTW even with heavy rainfall events, but there is still a decrease in DO, pH, and conductivity with heavy rainfall. There was a small diurnal flux, but minimal when compared to the Background Site's ≥ 1 m per day.

Observed ASVs varied greatly over the course of the 9-week monitoring period. The bacterial communities from contaminated wells were similar in diversity and structure as compared to those in uncontaminated wells (Fig.B.5). While alpha- and/or beta-Proteobacteria generally dominated, the phylum Bacteroidetes significantly increased or even predominated in small-size bacteria after late April, mainly attributed to the genus *Hydrothalea*. The bacterial communities from contaminated wells were similar in diversity and structure to those in uncontaminated wells (Fig. B.6). Small-size bacteria from different areas became convergent after late April, implicating the influence of migration corresponding to precipitation changes in April and May.

Notably, the complete communities (i.e., all filters combined) of Area 3 had significantly lower phylogenetic and taxonomic richness when all filters were combined (Fig. B.7, $p < 0.01$). However, no significant trend over time in richness metrics was detected. Separated by filter, 0.2 μm filters showed higher diversity metrics for Area 2 and the background area. In comparison, Area 3 had consistently low diversity by all metrics used for the communities detected on the 0.2 μm filters (Fig. B.8). No patterns were detected over time. In contrast, the 0.1 μm filters showed no clear trend in diversity between areas or over time, although most metrics showed slightly lower diversity in the 0.1 μm filters than all others. The 10 μm filters showed no trends in time or area by most metrics. Richness metrics were positively correlated with several measured environmental variables, including total carbon (primarily dissolved organic carbon,

acetate, and formate), with additional weak positive correlations with Mo and Ca. Both were negatively correlated with Al, Cr, Mn, Fe, and P.

Beta diversity indicated that community structure was variable throughout the monitoring period but depended primarily on the specific sampling location. Ordination of unweighted Unifrac distance demonstrates clustering by area and well (ANOSIM by area $R=0.73$, $p=0.06$; by well, $R=0.90$, $p=0.001$). PERMANOVA analysis of beta diversity of the complete community also indicated that community variance was strongly and significantly influenced by the well sampled (and therefore Area) ($R^2=0.54$, $p=0.09$), significantly influenced by the week of sampling ($R^2=0.02$, $p=0.002$), and significantly different based on the week and well sampled ($R^2=0.12$, $p=0.016$). Notably, GW-823 was substantially separated from the other 2 wells sampled in the background area. Fitting of the measured environmental variables to the NMDS ordination of unweighted Unifrac distances showed that variables negatively correlated with diversity were associated with Area 3, while carbon, total nitrogen, and sulfate were not significantly associated with any area. Excluding GW-823, all measured environmental variables corresponded to Area 2 and Area 3 compared to the background wells (Fig. B.9).

To identify geochemical and microbial associations over the course of the monitoring period, we performed differential abundance analysis on the independent filter samples followed by comparisons of differential abundance for each feature identified as differentially abundant in at least two filters at one timepoint. We then identified taxa that switched the filter with which they were associated. We found 291 ASVs in the 8 wells with Week 1 cell count readings that changed filters. The majority were Proteobacteria (mostly Gammaproteobacteria and Alphaproteobacteria), with Patescibacteria (primarily of class Parcubacteria) the next most common ASV. We also performed this at higher taxonomic levels in order to control for stochastic processes acting at the ASV level.

Discussion

Within the 8-week sampling period, there were drastic and distinct differences in the microbial communities, metals concentrations, and geophysical parameters between the Background Site and the contaminated sites at Y-12. There were large changes in the microbial communities and geochemistry across each two-week period.

Both alpha and beta diversity corresponded more to area than to the sampling week in nearly all cases. Alpha diversity was substantially lower in Area 3 wells than in Area 2, while the background wells were highly variable. The differences in alpha diversity were much more related to the sampled area than week, as the geochemical conditions are more related to the sampling area than seasonality (Hemme et al. 2015, Paradis et al. 2022). Analyses of beta diversity demonstrated the high similarity between wells from the same area, again with the background wells as the exception – GW823 was less similar to the other background wells than

to the Area 2 and Area 3 wells, although still notably separate. Wells within each area were also distinct from each other (Fig. B.10).

We identified several ASVs that were to be found predominantly in one filter for one timepoint but a different filter pore size during the next time point. The standard protocol for sample collection involves a pre-filter through a 10 μm filter to capture larger particulates and prevent clogging of the following filters with pore sizes of 0.2 μm and 0.1 μm , filtered sequentially. However, when we identified differentially abundant ASVs and compared their prevalence on each filter, we found that several taxa changed which filter they were associated with. Syntrophobacterales was initially associated with the 10 μm pre-filter in the Background Site (Fig. B.11). A taxon shifting to a 10 μm filter likely represents a shift to a more particle-attached lifestyle, while a shift to the 0.2 μm filter represents a more planktonic lifestyle. A shift to the 0.1 μm filters may represent a change in morphology, either cell size or shape, allowing the cell to pass through the 0.2 μm filter. Conversely, the same taxa, Syntrophobacterales, was found on the 0.1 μm filter the first week and then seen almost exclusively on the 10 μm for the following 3 weeks until we see it again the last week on the 0.1 μm . This indicates that it became more particulate-associated during the drought period before associating again after rainfall. Other taxa, such as Desulfovibrionales, switched between the 0.1 and 0.2 μm filters, especially in Area 2, which could indicate a change in cell size due to changes in nutrient concentrations. While 16S rRNA presence is not a proxy for activity, these shifts frequently agree with the literature about which filters taxa are most often associated (Nimptsch et al. 2014, Byappanahalli et al. 2021). Additionally, it is important to note that this filter jump could also be related the stress response of the cell in changing pH and increasing heavy metal concentrations (Mueller et al. 2019, Groß et al. 2021).

Previous studies have shown that soil type, geological structure and contaminate type play a significant role in shaping microbial community structure (Jeong et al. 2019, Zhu et al. 2022, He et al. 2023) Due to the differences between the Background Site's physicochemical parameters and microbial community diversity, the decision was made to forego sampling the Background Site and focus on 3 areas at Y-12 (Areas 1, 2, and 3). Area 1 is located near the S-3 ponds but not in the groundwater flow path of the subsurface contaminate plume affecting Areas 2 and 3. This will allow ENIGMA to compare three geologically similar and more closely located areas for future studies.

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Chapter 2: Spatiotemporal Dynamics of Subsurface Geochemistry and Microbial Communities in DOE Legacy Site Groundwater

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Introduction

Approximately 1% of Earth's water resources are safe and available for human use (Danielopol et al. 2003, Griebler et al. 2014); groundwater accounts for roughly 95% of that available freshwater (Smith et al. 2018). The shallow subsurface, where most groundwater is found, is home to diverse microbial communities. They maintain the recycling of matter through biogeochemical processing (Goldscheider et al. 2006, Deharveng et al. 2009), but we still not do fully understand the relationship between these low-nutrient environments, which significantly impact the microbial ability for nutrient cycling (Mermillod-Blondin 2011). However, more research in the last decade has been focused on the shallow terrestrial environments to better understand these mechanisms mainly due to public influence (Stigter et al. 2011, Wilson 2017, Denham et al. 2020). In the most recent United States Geological Survey (USGS), they determined that more than 20% of the United States drinking water was contaminated with geological and manmade materials that exceeded human health hazard levels (Leslie et al. 2015, Li et al. 2021). Many of these water sources were contaminated due to industrial runoff or improper waste practices of hazardous materials (Winter 1998, Hasan et al. 2021).

For example, the Bear Creek Aquifer at the Oak Ridge Reservation, our field test site, contains complex contaminants, including high nitrate levels, mixed metals, and uranium. The contaminants were generated from proliferation of nuclear materials at the site and disposed of in improperly lined subsurface reservoirs, known as the S-3 ponds, with a 9.5 million liter capacity each (Brooks 2001, Revil et al. 2013). This study sets out to address how the geochemical properties and microbial communities present in this contaminated groundwater change over time across three different areas near the S-3 ponds of varying contamination level (mild, moderate, and severe). 27 wells were selected across this site and sampled 4 days per week from July to December of 2019 to study geophysical parameters (water table fluctuations, temperature, dissolved oxygen, conductivity, pH), heavy metals, and organic/inorganic carbon and nitrate. One well from each area was selected as the microbial sampling well. This well was additionally sampled for direct cell counts, groundwater microbial communities and archived samples for future isolations.

The following hypotheses were developed to drive this study: (1) Geochemical and geophysical parameters are driving changes within groundwater microbial communities; (2) In higher levels of nitrate and metals, microbial communities will have decreased diversity than in lower levels of nitrate and metals; and (3) During severe weather conditions, such as drought or heavy rainfall, microbial communities and geochemistry will change significantly, but eventually, return to baseline.

Materials and Methods

Well Selection

When selecting the wells to use in this survey, we were limited to pre-established wells at the site. Many of these wells are decades old and have been installed using various installation methods over the years. They were also installed at differing depths in each area for multiple reasons (i.e., project questions, bedrock interference, etc.). Therefore, well selection was based on several factors. The field equipment we used required specific well attributes, including well diameter and similar depths in each area. First, wells that were chosen in each area were required to have an inner diameter of $>2''$. This ID allows for the installation of an Aqua TROLL 500, manufactured by In-Situ, Inc. based in Denver, Colorado, which allows parameters to be directly measured without the need to pump groundwater with a low-flow pump setup. Secondly, the remaining wells (sub-2" diameter) were chosen based on spatial location in the areas of interest and the physical condition of the well and screen depth. This method allowed for a total of eight to ten wells to be chosen at each of the areas (Appendix A, Figures A.7, A.8, A.9, and A.10) of interest: Area 1, Area 2, and Area 3.

Groundwater Collection and Geochemical Analysis

The groundwater sampling occurred 4 days per week over 17 consecutive weeks (July-December) to develop a seasonal time series analysis of the effects of external weather on groundwater geochemistry, as well as groundwater microbial communities. Temperature, dissolved oxygen, pH, conductivity, oxidation-reduction (Eh) potential, and nitrate were determined at the wellhead using the Aqua TROLL 600s. The wells were pumped until all monitored geochemical parameters had stabilized according to the sonde's real-time measurements. Microbiological sampling wells with Aqua TROLL 600s were purged by pumping 3 L of water before sampling.

Acridine Orange Direct Counts provide a direct estimate of the total number of bacteria in the environment, regardless of their ability to grow on any media that might be used. Biomass from 10 mL unfiltered groundwater samples fixed with 37% vol/vol formaldehyde in the field will be filtered through a 0.2 μm pore size black polycarbonate membrane (Whatman International Ltd., Piscataway, NJ) supported by a vacuum filtration sampling manifold (Millipore Corp., Billerica, MA). Filtered cells will be stained with 25 mg/mL of Acridine orange for 2 min in the

dark. Unbound Acridine orange will be filtered through the membrane with 10 mL filter sterilized 1X PBS (Sigma Aldrich Corp., St. Louis, MI) and the rinsed membrane mounted on a slide for microscopy. Cells will be imaged with a FITC filter on an epifluorescence microscope. The number of cells stained with Acridine orange, are counted by epifluorescent microscopy (Hazen et al. 2010).

For determination of dissolved elements, 15 mL of unfiltered groundwater was collected in acid-washed polypropylene tubes and stored overnight at RT. After settling, 10 mL of groundwater was then transferred to a conical acid-washed polypropylene tube containing 2 mL of 12% (v/v) trace-grade nitric acid (VWR, Radnor, PA) - giving a final concentration of 2% nitric acid (v/v). The samples were stored at 4°C and spun down just prior to analysis. One thousand, eight hundred and eighty-eight (1,888) samples were analyzed for 52 elements using an Agilent 7900 inductively coupled plasma mass spectrometer (ICP-MS) fitted with MicroMist nebulizer, ultra-high matrix introduction (UHMI) -spray chamber, Pt cones and an octopole reaction system (ORS) collision cell (Agilent Technologies, Santa Clara, CA). The data were collected using 3-point peak pattern, 3 replicates, 100 sweeps/rep, and various integration times (all >0.3 s) using either no gas or He gas in the ORS collision cell. The external calibration standards, IV-ICPMS-71A and IV-ICPMS-71B, were used to create an 11-point curve from 0 - 500 ppb for each element and 1 ppm of the internal calibration standard, IV-ICPMS-71D, was added to each sample (Inorganic Ventures, Christiansburg, Virginia). All samples from Area 3 were analyzed as above but using a UHMI method with an approximate dilution rate of 88x. For the UHMI samples, the external calibration standards, IV-ICPMS-71A and IV-ICPMS-71B, were used to create a 16-point curve from 0 - 5,000 ppb for each element and additional 10,000 - 100,000 ppb for Al, Mg, K, Ca, Na, Mn and 50 ppm of the internal calibration standard, IV-ICPMS-71D, was added to each sample (Inorganic Ventures, Christiansburg, Virginia). External calibrations were performed about every 100 samples or each day, whichever occurred first. Data were processed using the nearest internal standard by mass and fitted to a linear curve though the calibration blank. All data is reported in ppb (i.e. ng/mL) and in μ M with the detection limit (DL) listed as "< value" in ppb.

10 mL of unfiltered groundwater were collected and filter through a 0.2 μ m syringe filter and placed into a glass tube with no headspace for dissolved organic carbon (DOC) and inorganic carbon (DIC), total nitrogen (TN), and dissolved organic nitrogen (DON). To minimize bacterial decomposition of some components within the groundwater sample, samples were stored at 4°C. These samples were measured using combustion analysis at Lawrence Berkley National Laboratory. Filtered groundwater samples (0.2 μ m filter) were collected in injection vials with no headspace. There is a small gap from October 15-18 in Areas 1 and 3 data due to sample breakage during shipment.

Results

All figures for the Chapter 2 can be found in Appendix C of this document.

During the sampling period (July 29 – December 4, 2019), Tennessee experienced a significant drought (Fig. C.1). Drought conditions are broken into 5 categories ranked from D0 to D4: abnormally dry, moderate drought, severe drought, extreme drought, and exceptional drought (NIDIS 2019). East Tennessee experienced what is defined as severe drought (D2) for 35 days and extreme drought (D3) for 12 days prior to and 16 days after the severe drought. Precipitation totals (Fig. C.2) were collected from the HOBO weather stations. As shown, the September to October drought was bookmarked by heavy rainfall, approximately 1.6 inches greater than the normal averages for Oak Ridge, Tennessee. The water table response (measured by in-well depth to water measurements) to rainfall varied by area and well (Fig. C.3). Area 1's water table increased with rainfall more quickly than Areas 2 and 3. Area 3 was much slower to respond and integrated the water table increases across rainfall events (i.e., close rainfall events did not allow time for the water table to decrease to baseline in Area 3). Area 2 water level was consistent throughout the sampling period, including the drought. This is explained by the proximity of Bear Creek to Area 2 wells. There is positive feedback from the surface water of Bear Creek into the groundwater table in Area 2.

When analyzing the water table fluctuations, it was noted that some wells' screened intervals were within the variably saturated zone of the subsurface due to the drop in water table during the drought. A visual representation of this data is shown in Figure C.15, where the green dot denotes the top of the screened interval, the black dot represents the bottom of the screened interval, and the box plot shows the depth to water (ft) below ground surface range over the sampling period. The water table range for 7 of the 10 wells sampled in Area 2 never goes above the screened interval. The depths of the wells in each area varied, but the average middle of the screened interval for each area is as follows: Area 1 is 15.89 ft (4.8 m), Area 2 is 18.99 ft (5.79 m), and Area 3 is 41.32 ft (12.59 m).

In-field geochemical measurements taken with Aqua TROLL 600s varied in response to rainfall. Dissolved oxygen (DO mg/L, Figure C.4) was the fastest to increase after a rainfall event in Area 1 when compared to Areas 2 and 3. However, EU07 in Area 1 and TMW12 in Area 2 were constantly higher in DO than any other well in the area. The most likely explanation is related to well construction, but there are no obvious defects in the casings. Area 3 mainly was anoxic over the sampling period. pH was lowest in Area 3 (Fig. C.5). Conductivity ($\mu\text{S}/\text{cm}$) was higher in Area 3 by two orders of magnitude (Fig. C.6). In-field nitrate measurements were only taken for the microbial sampling well (Fig. C.7). Nitrate in Area 3 is on average 6 times higher than in Area 2. However, Area 1 nitrate measurements are near zero. In Area 2, TMW12 was constantly fluctuating during rainfall events. After analyzing this data, we discovered there was a crack at the top of the well casing that was allowing water from higher in the water table to infiltrate into the well. The action of the water falling into the well created this disturbance in the dissolved oxygen measurement.

Eight metals of interest due to site history (Ni, U, Mo, Al, Co, Fe, Mn, and Cu) were analyzed (Fig. C.8). Nickel is two orders of magnitude higher in Area 3 than Areas 1 or 2. Iron is highly variable across all three areas and from well-to-well within each area. Molybdenum is consistent except

for in FW026 in Area 3 over the last month of sampling where there was a steady increase. Aluminum is above detection limits in Area 3 for majority of wells throughout the sampling period. Also, there is an unexplained dip in Al at the beginning of sampling in FW026 in Area 3. In Area 1, FW066 and FW065, the closest wells in Area 1 to the S-3 ponds, also show high Al levels when compared to the rest of the wells in Area 1. Additionally, manganese is extremely high in all wells in Area 3 as well as FW065 and FW066 in Area 1. Total carbon was consistent in all three areas at approximately 60 ppm. The remaining measurements (DOC, DIC, TN, DON) varied drastically in FW106 in Area 3 when compared to EU05 (Area 1) and GW-835 (Area 2). DOC was 10 times higher; DON was 40 times higher, and TN was 1000 times higher. However, DIC was actually decreased by 0.25 times more than in the other areas.

Total carbon, total nitrogen, and dissolved inorganic/organic carbon and nitrogen (DIC, DOC, DIN, DON) measurements were analyzed by area (Fig. C.9). Total carbon was consistent in all three areas at approximately 60 ppm. The remaining measurements (DOC, DIC, TN, DON) varied drastically in FW106 in Area 3 when compared to EU05 (Area 1) and GW-835 (Area 2). DOC was 10 times higher; DON was 40 times higher, and TN was 1000 times higher. However, DIC was actually decreased by 0.25 times more than in the other areas.

Observed ASVs varied greatly over the course of the sampling period in Areas 1 and 2 (Fig. C.13). *Proteobacteria* dominated a majority of sampling days for these areas. However, the microbial communities had increased diversity before and after the drought and Area 2 saw the most diversity over time. Area 3 is predominately *Proteobacteria*, but there is increased diversity after the heavy rainfalls. When comparing the relative abundance of *Proteobacteria* in relation to rainfall events (Fig. C.2 and Fig. C.14), it is evident that rainfall or geochemical changes impacted by rainfall are the dominate cause of this increase in diversity.

Discussion

The drought conditions followed by heavily rainfall events seen during this study gave a unique look at how changing weather patterns could effect these subsurface communities. During the the study, Area 2's consistent geochemical (Fig. C.4-9) and geophysical parameters (3) are most likely due to its highly-permeable lithography (H. L Boston 1992) and connectivity with nearby surface water of Bear Creek (Lewandowski et al. 2020). However, Area 2's microbial community did see changes throughout the sampling period most likely referring back to the microbial transfer of sediment microbes as the surface water infiltrated the subsurface (Fillinger et al. 2021).

Aluminum, cobalt, and nickel all increased in various wells with the influx of rainfall, which is supported by the contaminant levels taken in the S-3 ponds at the time they were capped (Brooks 2001). With these increases seen only after rain events, it is hypothesized the plume is being washed out from either an upstream flow path or a downward push from surface water (Lin et al. 2022). To accurate address this concern of continued contamination coming from the

S3 ponds, isotopic analysis of upstream groundwater as well as falling rain water would be necessary (González-Trinidad et al. 2017). Additionally, total carbon, dissolved organic carbon, dissolved inorganic carbon, and total nitrogen all increased with heavy rainfall in the Area 3 well, which is additional evidence of continued plume contamination.

In Areas 1 and 2, *Proteobacteria* is the predominant phylum. However, we do see increased diversity when compared to Area 3 (Fig. C.13). In the higher levels of nitrate and heavy metals (i.e., Area 3), we see severely decreased diversity. Interestingly, *Proteobacteria* abundance significantly decreases after rainfall events in Areas 1 and 2 ($p < 0.05$) (Fig. C.2 and C.13). However, we do not see that decrease in Area 3. This is most likely due to the depth of the wells in Area 3 compared to Areas 1 and 2. As seen in Fig. C.15, the water table is much higher than the screened interval of Area 3, so the likelihood of extreme interference from precipitation events is much lower.

Throughout the study, major microbial community changes are seen approximately one week after a heavy rainfall event ($>3\text{mm}/8\text{ hours}$). On the other hand, significant microbial community changes are seen starting at 10 days into drought conditions and continue to become less diverse as the drought continues and dissolved organic matter decreases (Qiu et al. 2021). Since the Area 3 wells are so unique from Areas 1 and 2 (pH, nitrate, DO, conductivity, heavy metal concentration, etc.), it is difficult to determine if the lower diversity is due to the lack of perturbation from the surface rainfall or if it is due to the extreme geochemistry.

With the difficulty in being able to accurately compare these wells due to the discrepancies in depth and geological characteristics, it is now evident that wells must be installed in a consistent, hydrogeologically connected manner. This is ENIGMA's next priority in order to address these hypotheses more accurately and build a descriptive flow model.

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Chapter 3: Time Series Analysis of Sediment-Associated and Groundwater Microbial Communities in Varying Contamination Levels

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Introduction

The water found in the terrestrial subsurface holds most of Earth's freshwater resources. There is approximately 100 times more fresh water in the subsurface than in surface waters and more than one third of the global population relies on groundwater for fresh drinking water (Danielopol et al. 2003, Li et al. 2021). For this reason, understanding our subsurface environment and the connection between subsurface geochemistry and microbial communities is crucial to protecting our freshwater resources. Subsurface geochemistry effects microbial community composition in the sediment and groundwater, which are vital to these environments for nutrient cycling, biotransformation and biogeochemical processing (Christensen et al. 2018, Huang et al. 2021). These communities are sensitive to perturbation and can be used as potential biosensors in contaminated environments (Smith et al. 2015). These communities have the ability to create microenvironments within an extracellular polymeric matrix (ECPM) consisting of polysaccharides, proteins, lipids and DNA (Di Martino 2018). We know that biofilm structure has distinct advantages for the microorganisms, such as nutrient availability, biotransformation of vital compounds and quorum sensing, compared to the planktonic community (Stoodley et al. 2004, Kirisits et al. 2007). The interaction between these attached communities (i.e., biofilms) and the free-floating planktonic microbes is poorly understood (Qin et al. 2021).

In studying, the connection between groundwater and sediment microbial communities, the current methodologies for obtaining samples (i.e., drilling sediment cores, extensive groundwater sampling, etc.) can be intrusive, cost-prohibitive, and permanently alter the subsurface environment (Hazen et al. 1991, Holm et al. 1992). Additionally, these methods only provide a snapshot of the geochemistry and community composition and do not consider how the subsurface geochemistry changes over time. Other laboratory methods of studying these interactions (i.e., flow-through cells, bioreactors, flow-through columns, etc.) do not allow for natural perturbation that we would see in the subsurface.

In this study, we designed an in-situ apparatus that allowed us to study the attached (or "sediment-associated") microbial community over time to begin to understand how these

communities can change as the geochemistry and community composition of the groundwater changes. We created “bug traps” using slotted conduits filled with local, heat-sterilized, homogenized sediment and deployed into preestablished groundwater wells in Oak Ridge, TN, within the Oak Ridge Field Research Center (ORFRC) at the Y-12 National Security Complex. These bug traps allowed groundwater flow through the sediment to mimic the conditions seen in the terrestrial subsurface. Microbial communities were, hypothetically, able to create those microenvironments within our bug traps while we were able to continue taking groundwater samples for microbial and geochemical assessment. The bug traps were deployed in triplicate in a time series format to allow for a varied look of the founding communities. The following hypotheses were developed to drive this study: (1) In higher levels of nitrate and metals, attached microbial communities will have decreased diversity less change over time than in the unattached microbial communities; (2) Geochemical changes related rainfall fluctuations, such as DO concentration, conductivity and pH, will increase diversity than during baseline conditions; and (3) Microbial community in simulated-sediment environments (i.e., bug traps) do change overtime and are not dictated by the pioneer community that colonizes them. All figures for Chapter 3 can be found in Appendix D of this document.

Materials and Methods

Study Design

Groundwater and sediment-associated microbial communities were studied in three preestablished wells at the ORFRC. The three wells are located in known but varying levels of contamination of heavy metals, radionuclides, nitrate, and low pH. These communities were studied through the analysis of filtered groundwater and deployed bug traps from July to December 2019. The design of the bug traps can be found in Figure D.1. The samples have been coupled with continuous geochemical monitoring in the chosen wells to see if/how geochemistry and local microbiology interact on a daily and seasonal basis as well as how planktonic microbial communities differ from sediment-associated communities. The unique time-series design of the bug trap deployment has also given the opportunity to see how microbial communities in the sediment change over time in relation to the surrounding geochemistry as well as study the pioneer community effect in bug trap experimental designs.

For each well, six sets of bug traps in triplicate were deployed for 4, 6, or 8 weeks (total of 18 bug traps per well over time) (Fig. D.2). This design serves multiple purposes. It allows:

1. The study of the founder effect of the pioneer microbial community to see if it changes over time.
2. The comparison between groundwater microbial communities over time with the sediment-associated communities deployed within the same well as the groundwater sample was derived.
3. The comparison between groundwater and sediment geochemistry over time.

Groundwater Sampling

For in-field geochemistry, temperature, dissolved oxygen, pH, conductivity, oxidation-reduction (Eh) potential, and nitrate were determined at the wellhead by specific probes with an in situ multi-parameter Aqua TROLL 600 sondes. Sondes were calibrated weekly and checked daily for accurate measurements.

Before groundwater samples were taken, each well was measured for stabilization. Microbiological sampling wells with Aqua TROLL 600s were purged by pumping 3 L of water before groundwater filtering and sampling. Unfiltered water samples for direct counts were collected in 15 mL conical tubes, fixed with 1 mL of formaldehyde (37% vol/vol), and stored at 4°C. For nucleic acid extractions and microbiological studies, 10 L of groundwater were filtered onto 10 µm and 0.2 µm filters, frozen at -80°C, and transported for extraction. For determination of dissolved elements, 15 mL of unfiltered groundwater was collected in acid-washed polypropylene tubes and stored overnight at RT. After settling, 10 mL of groundwater was then transferred to a conical acid-washed polypropylene tube containing 2 mL of 12% (v/v) trace-grade nitric acid (VWR, Radnor, PA) - giving a final concentration of 2% nitric acid (v/v). The samples were stored at 4°C for transfer.

Bug Trap Development and Deployment

For the bug traps, local, homogenized sediment was dried and sieved through a standard 78-700 soil sampling sieve set – separating the sediment into six size categories. The sediment was then autoclaved to eliminate microbial activity. The bug traps were built using a 1-inch slotted PVC well screen cut to 6-inch lengths and capped with 1 inch PVC caps (Fig. D.1). The areas of the trap contained by the end caps were filled with sediment between 500 and 2000 microns and covered with a fine PVC mesh. The slotted area of the trap was then filled and packed with sediment less than 500 microns. This layering technique was used to prioritize our limited sample of local sediment and to attempt to mimic the fine slit clay found around the deployment wells.

Once built, bug traps were deployed down well in groups of 3 at 6 time points for 4, 6, or 8 weeks over 17 weeks (Fig. D.2). 18 bug traps were deployed in each of the 3 microbial sampling wells. During collection, the bug traps were kept in an anoxic environment and then stored at -80°C until the end of the experiment when they could be processed. The sediment was then distributed to collaborators for cation and 16S rRNA analyses outlined below.

DNA Extraction for Bug Traps

Fifty-six (56) sediment samples were sent to the University of Oklahoma's Institute for Environmental Genomics. Microbial genomic DNA was extracted by a freeze-grinding method as described previously (Zhou et al. 1996) and purified by using DNeasy PowerSoil DNA isolation

Kit (QIAGEN; bead tubes were not used). A two-step PCR amplification method was used for library preparation as described previously (Wu et al. 2015). In the first step PCR, the standard primers were used to amplify the V4 region of prokaryotic 16S rRNA genes (515F [5'-GTGCCAGCMGCCGCGGTAA-3'] and 806R [5'-GGACTACHVGGGTWT CTAAT-3']), and the ITS region of fungal rRNA genes (ITS7F [5'-GTGARTCATCGARTCTTTG-3'] and ITS4R [5'-TCCTCCGCTTATTGATATGC-3']) (White et al. 1989). In the second step PCR, phasing primers were designed and used to increase the base diversity in sequences of sample libraries. PCR amplification and purification were the same as reported previously (Wu et al. 2015), except amplification cycles (the first + second step: 10 cycles + 20 cycles for 16S, and 12 cycles + 25 cycles for ITS). Sample libraries were then sequenced by MiSeq platform (Illumina, San Diego, CA, USA) as described previously (Caporaso et al. 2012).

DNA Extraction for Groundwater Filters

Four hundred and twenty (420) groundwater samples were stored at -80°C before use for DNA extraction. Each filter was cut evenly in half, and one half was used to extract DNA. Microbial genomic DNA was extracted by a freeze-grinding in SDS lysis extraction buffer and purified by using DNeasy PowerSoil DNA isolation Kit (QIAGEN; bead tubes were not used). DNA quality was checked by NanoDrop, and double-strand DNA concentration was measured by Quant-iT™ PicoGreen™ dsDNA Assay Kit.

For library preparation and sequencing, two-step PCR amplification method was used for PCR product library preparation (Wu et al. 2018). In the first step PCR, the standard primers were used to amplify the V4 region of prokaryotic 16S rRNA genes (515F [5'-GTGCCAGCMGCCGCGGTAA-3'] and 806R [5'-GGACTACHVGGGTWT CTAAT-3']). In the second step PCR, phasing primers were designed and used to increase the base diversity in sequences of sample libraries. PCR amplification and purification were the same as reported previously (2), except amplification cycles (10 cycles in the first step and 20 cycles in the second step for 16S). Sample libraries were then sequenced by MiSeq platform (Illumina, San Diego, CA, USA) following MiSeq's commercial protocol.

Sequence Data Processing

QIIME2 (version 2021.2) was used to process the sequence data for ASV-based processing. After barcode and primer sequences were trimmed with zero maximum error, sequencing data were processed by DADA2 to identify exact amplicon sequence variants (ASV). The method 'consensus' was used to remove chimeras; samples are denoised independently; forward and reverse truncate positions are 220 and 170, respectively, after quality trim with a quality score 2; the maximal expected error score is 2.0 when combining forward and reverse sequences. The ASVs were identified taxonomically by RDP Naive Bayesian Classifier algorithm in DADA2 with a confidence threshold of 0.5, based on the Silva database (v138.1; silva_nr99_v138.1_train_set.fa.gz from <https://benjjneb.github.io/dada2/training.html>). After

classification, ASVs identified as Chloroplast, Mitochondria, Eukaryota, or unclassified kingdom were removed. The prokaryotic ASV sequences were aligned using MAFFT in QIIME2, without mask.

Dissolved Element Analysis for Groundwater and Sediment

The stored samples were spun down before analysis. One thousand, eight hundred and eighty-eight (1,888) samples were analyzed for 52 elements using an Agilent 7900 inductively coupled plasma mass spectrometer (ICP-MS) fitted with MicroMist nebulizer, ultra-high matrix introduction (UHMI) -spray chamber, Pt cones and an octopole reaction system (ORS) collision cell (Agilent Technologies, Santa Clara, CA). The data were collected using 3-point peak pattern, 3 replicates, 100 sweeps/rep, and various integration times (all >0.3 s) using either no gas or He gas in the ORS collision cell. The external calibration standards, IV-ICPMS-71A and IV-ICPMS-71B, were used to create an 11-point curve from 0 - 500 ppb for each element and 1 ppm of the internal calibration standard, IV-ICPMS-71D, was added to each sample (Inorganic Ventures, Christiansburg, Virginia). All samples from Area 3 were analyzed as above but using a UHMI method with an approximate dilution rate of 88x. For the UHMI samples, the external calibration standards, IV-ICPMS-71A and IV-ICPMS-71B, were used to create a 16-point curve from 0 - 5,000 ppb for each element and additional 10,000 - 100,000 ppb for Al, Mg, K, Ca, Na, Mn and 50 ppm of the internal calibration standard, IV-ICPMS-71D, was added to each sample (Inorganic Ventures, Christiansburg, Virginia). External calibrations were performed about every 100 samples or each day, whichever occurred first. Data were processed using the nearest internal standard by mass and fitted to a linear curve through the calibration blank. All data is reported in ppb (i.e. ng/mL) and in μM with the detection limit (DL) listed as "< value" in ppb.

For total element analysis, three aliquots of approximately 1 g each of homogenized sediment from each of the 56 bug traps was dried overnight at >120°C. The moisture content and dry mass of samples were determined. The dried sediment was microwave-digested in 10 mL of concentrated trace-grade nitric acid (VWR, Radnor, PA) as described in EPA Method 3051A (EPA, 2007) using a CEM MARS 6 (Matthews, NC). After microwave digestion, samples were diluted for ICP-MS analysis, resulting a final dilution of 1:50 (250x dilution factor) or 1:500 (2,500x dilution factor) in 2% (v/v) nitric acid. The diluted samples were analyzed for 52 elements (see supplementary data for details) using an Agilent 7900 inductively coupled plasma mass spectrometer (ICP-MS) fitted with MicroMist nebulizer, ultra-high matrix introduction (UHMI) - spray chamber, Pt cones and an octopole reaction system (ORS) collision cell (Agilent Technologies, Santa Clara, CA). The data were collected using 3-point peak pattern, 3 replicates, 100 sweeps/rep and various integration times (all >0.3 s) using either no gas or He gas in the ORS collision cell. The external calibration standards, IV-ICPMS-71A and IV-ICPMS-71B, were used to create an 11-point curve from 0 – 1,000 ppb for each element and 1 ppm of the internal calibration standard, IV-ICPMS-71D, was added to each sample (Inorganic Ventures, Christiansburg, Virginia). Undiluted metal concentrations (in ppm) are normalized against the dry weights of the sediment samples (in kg). Final concentrations are reported as mg per kg sediment. Elements present at higher concentrations (Mg, Al, K, Mn, and Fe) were determined

from the 2500X dilutions and the remaining elements were determined from the 250X dilutions. Measurements of certain elements (Fe) in some samples were slightly above the quantification range of the instrument for both dilutions measured. External calibrations were performed about every 50 samples or each day, whichever occurred first. Data were processed using the nearest internal standard by mass and fitted to a linear curve though the calibration blank. All data are reported in ppm (i.e., mg/kg soil).

Results

Taxonomic analysis of the bug trap sediment shows distinct differences from the starting material and by area of deployment (Fig. D.3). *Proteobacteria* dominates the microbial community, which is also seen in the groundwater samples from these areas. However, *Acidobacteria* and *Bacteroidota* are seen as other predominant communities in Areas 1 and 2. Area 3 does not see such an increase in these phyla but does see a significant increase in *Firmicutes*.

When attempting to study founder effect of bug traps, which are suspended in a predominant flow path of a groundwater well, it is important to determine the microbial community present in the groundwater at the time of deployment. Figures D.4, D.5, and D.6 show the groundwater taxonomic analysis at deployment time for each triplicate set of bug traps in each area, respectively. Area 1 groundwater community is more diverse than we see in the bug traps at any timepoint (Fig. D.4). *Patescibacteria* and *Verrucomicrobiota* are present in the groundwater at the time of deployment but not seen or barely present in the bug traps. Area 2 is also more diverse in the groundwater than the bug trap sediments – particularly at time points 4 and 5 (Fig. D.5). *Patescibacteria* are heavily present in the groundwater but do not permeate into the bug trap community. Interestingly, Area 3 bug traps are more diverse than the groundwater (Fig. D.6). The groundwater is predominantly (>97%) *Proteobacteria* at all time points. Still, we do see diversity increase in the bug trap samples. Specifically, we see significant increases in *Firmicutes*, *Actinobacteria*, and *Acidobacteriota* throughout all bug trap time points.

Cation data was analyzed in comparison to the starting material placed into the bug traps (Fig. D.7). There were 8 metals of interest that were analyzed more in depth: B, Ca, Mo, W, Na, Se, K, and U. B, Ca, Mo, and W decreased from the starting material and continued to decrease over time. However, Na, Se, K, and U increased from the starting material. Of particular note, uranium continued to increase in Area 3 (well FW106) the longer the bug traps were deployed.

Discussion

Through this study, there were a lot of distinct differences in the microbial communities within the bug traps compared to groundwater samples. The differences in microbial phyla between the groundwater and bug traps likely stem from a combination of environmental stability present in the porewaters created within the bug traps as opposed to the free-flowing nutrients

found in the groundwater, geochemical variations, interactions between the microbes within the bug trap, and ecological interactions (Hazen et al. 1991, Hug et al. 2015, Kinsman-Costello et al. 2017).

The most abundant phyla found in all bug traps was Proteobacteria (68.4%-99.2%), which is commonly the most prevalent in sediment and aquatic communities since it plays a strong role in metabolism and degradation of various nutrients (Reardon et al. 2004, Griebler and Lueders 2009). When comparing the bug traps across each site, Areas 1 and 2 are distinct from Area 3 (Fig. D.11). After Proteobacteria, Acidobacteriota and Bacteroidota comprise the remaining communities in Areas 1 and 2. However, the next most abundant phyla in Area 3 were Firmicutes and Actinobacteriota. The most likely explanation of this is the dissolved oxygen content between the three areas. Acidobacteriota and Bacteroidota are widely considered aerobic (Wexler 2007) (few species of Acidobacteriota have found to be facultative anaerobes) (Kielak et al. 2016), whereas Firmicutes can be facultative anaerobes (Wright et al. 2017) and Actinobacteriota have numerous anaerobic members found in sediment across the country (Lewin et al. 2016).

One of the main reasons for designing a time series bug trap experiment was to study the “founder hypothesis,” described as the ability of the founder cells, or inoculum, to drive the future microbial composition of a substrate or biofilm (Waters et al. 2013, Litvak and Bäuml 2019, Eigentler et al. 2022). As shown in Fig. D.3, the replicates do vary with time. However, there are a limited number of phyla that have increased or decreased abundance over time. For example, in Area 1 TP1, we see a decreased abundance of Acidobacteriota from the starting material in 1.1.1, but the subsequent time points see a gradual increase in the same phyla. However, in TP6, there is a decrease in Acidobacteria from replicate 1 to replicate 3 and an increase in Firmicutes. Sequential changes are also seen throughout Areas 2 and 3. Area 2 TP6 sees the same stair step increase of Acidobacteria and Bacteroidota shown in Area 1. Area 3’s trends are more focused on Actinobacteria and Firmicutes. For example, Area 3 TP6, there is a steady decrease in Firmicutes with longer incubation time, and Firmicutes are drastically decreased in the following time point.

Additionally, comparing the starting material within the bug traps to any of the replicates, we see little overlap or consistency over time. The starting material was more diverse than any of the bug traps analyzed. With Proteobacteria making up only 29% of the abundant phyla in the starting material, it is almost 40% less than any bug trap analyzed. Additionally, Actinobacteria and Firmicutes were the next most abundant with 15% and 36%, respectively, which were also higher than any of the bug traps by at least 15% each. With these distinct changes across replicates over time and space and from the starting material, we have noted that founder effect had little effect on the long-term microbial composition within this bug trap experiment.

Analyzing dissolved element measurements in the bug traps showed some interesting trends. Four metals, B, Ca, Mo, and W, decreased during incubation from the starting material, whereas four others, Na, Se, K, and U, increased. Ca decreased the most in Area 3, but U and Na saw

large increases and also cumulative increases with increased incubation time, especially during a drought period seen during sampling. This showed that metals in heavily contaminated areas have the tendency to accumulate within the sediment over time (Feijtel et al. 1988, Huisman et al. 1997, Sundby et al. 2004, Husson et al. 2019).

When looking at the same metals data from the bug traps and comparing them to the groundwater metal concentrations, additional trends become evident (Fig. D.9 and D.10). The groundwater metals concentrations were averaged by well for the duration of each bug trap's incubation period to compare the aqueous concentration to the bug trap. B, Ca, Na, and U were all found to be higher in the groundwater than in the bug traps. However, W was prevalent in all bug traps across all areas, but only found consistently in the groundwater in Area 1. Mo showed an interesting trend across all three areas. Aqueous Mo concentrations in Area 1 were double what was found bug traps; Area 2 were just slightly elevated; and Area 3 showed higher Mo in bug traps than in soil pore water. Previous studies at this site have characterized a metal-resistant *Bacillus* strain, Firmicute phylum, with a high molybdate affinity from a bore hole near the Area 3 incubation well (Ge et al. 2020). There is evidence to suggest that the Firmicute and Mo increase seen in the Area 3 bug traps could be contributed to *Bacillus* colonization.

With this data, we were able to analyze how the groundwater community present during bug trap deployment could impact the bug trap microbial community. At every TP in Area 1 and Area 3, the bug trap microbial compositions were less diverse than the groundwater composition at the time the bug trap was deployed (Fig. D.4 and D.6). However, Area 2 saw some variability over time when looking at diversity. At TP1 and TP3 bug trap deployment, the groundwater was dominated by Proteobacteria (96.9% and 90.24%, respectively). TP2, TP4, TP5, and TP6 saw increased diversity with Patescibacteria abundance increased (4.22%, 31%, 29.2%, and 13.98%, respectively).

When comparing the microbial composition of Area 3 bug traps to groundwater, we see drastically increased diversity in the bug traps. This is most likely due to the extreme environment and limited within Area 3. During the sampling period and bug trap deployment, Area 3 stayed anoxic with a pH between 2.72 and 6.93, nitrate concentrations between 150 and 8615 ppm, and conductivity as high as 36,942 uS/cm (all areas shown in Fig. D.12). The microenvironments created by biofilms within the bug traps can allow microbes to survive within a community structure that has not been seen within the planktonic environment. Numerous studies have been done that show the increased potential for bioremediation in sediments versus planktonic communities due to the longer-term effects (Cardenas et al. 2008, Xu et al. 2015, Yan et al. 2019), which could drastically benefit contaminated sites, like DOE Superfund site where this study was conducted.

The observed shift in microbial phyla between groundwater and the in-situ sediment incubations, known as bug traps, are most likely attributed to various geophysical/environmental conditions (i.e., rainfall, water table fluctuations, lithography, etc.) and subsurface geochemistry. This study set out to find a less intrusive sampling method that

would bridge the knowledge gaps surrounding the interactions between sediment and planktonic microbial communities. With additional experiments and data analysis, this study concludes that bug traps can be an effective, but not perfect, substitute for subsurface sediment sampling.

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Chapter 4: DOE Superfund Sites: History, Policy Initiatives, and Current Research to Benefit Environmental Restoration

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Introduction

The United States Department of Energy (DOE) Superfund program is a crucial initiative to address and clean up hazardous waste sites across the United States. Established in 1980 under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), this program is managed by the Environmental Protection Agency (EPA) in collaboration with the DOE (DOE, 2023). The Superfund program focuses on sites contaminated with hazardous substances that pose significant risks to human health and the environment (EPA, 2023). These substances can include toxic chemicals, heavy metals, and radioactive materials. DOE's involvement in this program is primarily related to its responsibility for managing and cleaning up sites where nuclear weapons were tested or produced, such as the Y-12 National Security Complex.

The DOE Office of Environmental Management (EM) directs the most comprehensive environmental cleanup program in the world (USDOE, 1999). They have been responsible for 107 sites in the US, out of which 92 are considered completed, leaving only 15 active cleanup sites (EPA, 2023). There are currently 18 DOE sites in the National Priorities List (NPL). There are an estimated 10,000 contaminated plumes on DOE land, but many DOE sites have not been comprehensively characterized (USEPA, 2004). There is contaminated groundwater, soil, and sediment at 72% of DOE sites.

The management and disposal of hazardous and nuclear waste have long been a contentious issue due to its potential hazards to both human health and the environment. During the first 40 years of nuclear proliferation, there were no legislative guidelines or regulations regarding the storage or disposal of radioactive waste. There was also very little research on neutralization or remediation of this waste for long-term storage. The first legislation to address the issue of hazardous waste disposal was CERCLA in 1980.

Comprehensive Environmental Response, Compensation, and Liability Act (1980)

The Comprehensive Environmental Response, Compensation and Liability Act of 1980 (CERCLA), now known commonly as Superfund, was passed on December 11, 1980 and is run by the Environmental Protection Agency (EPA) (EPA 2022). This law was enacted due to the growing concerns of environmental and health risks posed by abandoned or uncontrolled hazard waste

sites created by the chemical and petroleum industries. CERCLA was the government's response to one of the greatest environmental catastrophes in American history known as the "Love Canal Tragedy" (Gibbs 2010). Love Canal, located outside of Niagara Falls, became home to a toxic waste landfill in the 1920s harboring the waste generated by the Hooker Chemical Company from byproducts of producing industrial solvents. The landfill was eventually capped, but produced health effects, such as leukemia, various cancers, epilepsy, reproductive health issues in women, and mental breakdowns (Gensburg et al. 2009, Gensburg et al. 2009, Austin et al. 2011). The United States government viewed this disaster as a call to action.

CERCLA empowers the EPA to take emergency actions when immediate threats to public health or the environment are identified. This authority allows for swift response and containment measures at contaminated sites. Under CERCLA, the EPA created the Hazard Ranking System and the National Priorities List. CERCLA also developed a trust fund to finance the cleanup of Superfund sites.

Hazard Ranking System

The EPA developed the Hazard Ranking System (HRS) in 1981 as a critical tool for assessing and prioritizing hazardous waste sites for cleanup. This system was developed to ensure that limited resources are allocated efficiently and effectively, targeting the most urgent environmental threats. One key aspect of the HRS is its emphasis on transparency and public involvement. The EPA actively seeks input from communities affected by hazardous waste sites during the ranking process. This ensures that local concerns are considered when determining site priorities. Moreover, the HRS also evaluates long-term risks associated with a site. It considers not only current conditions but also potential future impacts such as climate change or population growth. By considering these factors, the EPA can determine which sites pose the greatest risk to public health and the environment. This approach allows for better planning and preparation in addressing environmental hazards.

The HRS evaluates sites on a scale of 0 to 100 (100 being the highest hazard risk) based on various factors, including the potential for human exposure to hazardous substances, the toxicity of those substances, and the likelihood of contamination spreading to other areas (EPA 1998). The scores are based on an initial assessment, limited investigations to assess the risk of human and environmental harm, and the possibility of additional assessments in the future. The HRS score is determined by the investigation of four pathways of contamination and risk. The pathways are based on routes of exposure: groundwater, surface water, soil and air (EPA 1992). To begin scoring, the EPA must first identify and characterize the source(s) and then their potential pathways. The next critical step in assessing a site is to identify targets for the significant pathways. All of these assessments are assigned various scores to be included in that pathway's score. Each pathway consists of 25% of the total 100 points. If a site scores greater than 28.5, it is placed on the National Priorities List.

National Priorities List

The NPL acts as a guidebook for the EPA to determine the sites that required additional investigation before remediation can occur. Firstly, it helps identify and prioritize sites for cleanup based on their potential impact on public health. By evaluating factors such as toxicity, proximity to communities and potential for migration, the EPA can allocate resources efficiently to address the most urgent cases. Secondly, the NPL promotes transparency and public involvement in environmental decision-making. The inclusion of a site on the list triggers a formal process that allows affected communities to participate in cleanup decisions to ensure local concerns are taken into account. There are currently 1,888 superfund sites listed by the EPA and 1,336 of them are on the National Priority List. Being listed on the NPL can provide access to federal funding for remediation efforts. The EPA allocates resources based on site ranking and severity of contamination. This financial support enables cleanup operations that might otherwise have been delayed or hindered due to lack of funding.

Financing Cleanup Efforts – Trust Fund Creation

One of the key provisions of CERCLA is its liability scheme. It created a trust fund, financed by taxing industries that were generating hazardous waste, to cover the expenses of cleanup efforts when the responsible party could not be identified. It holds liable any person or entity involved in the generation, transportation, disposal, or handling of hazardous substances. This provision ensures that those responsible for contaminating a site bear the financial burden of its clean up. If responsible parties cannot be identified or are unable to pay, funds from a dedicated trust called the Superfund Trust Fund are used. The Superfund process involves several steps, including site assessment, remedial investigation/feasibility study, selection of a cleanup plan, implementation of remedial action, and long-term monitoring. Through these stages, community involvement and public participation play a vital role in decision-making processes.

Since its enactment, CERCLA has been instrumental in cleaning up thousands of hazardous sites across America. However, it has also faced criticism for its lengthy and costly cleanup processes. During the first five years of CERCLA, remediation efforts were minimal but costly. Over \$700 million had been spent to clean up 16 of the 799 designated superfund sites at the time. After five years of dismal remediation efforts, a congressional investigation was launched and an act amending CERCLA was written in 1986. The Superfund Amendments and Reauthorization Act of 1986 (SARA) addressed many of the issues and pitfalls seen since CERCLA's authorization. First, SARA highlighted the necessity of developing permanent solutions to hazardous waste sites. Additionally, the trust fund was increased from \$1.6 billion to \$8.5 billion to increase the number of site clean-ups attainable at a time.

Nuclear Waste Policy Act

After CERCLA outlined and regulated environmental hazard concerns and due to growing public concerns over the safe and effective handling of radioactive waste, the United States Congress enacted the Nuclear Waste Policy Act (NWPA) in 1982 (Nuclear Waste Policy Act As Amended with Appropriations Acts Appended, 1982). The NWPA established a clear division of responsibilities between the federal government and individual states regarding the management of nuclear waste. It designated the Department of Energy (DOE) as the responsible agency for the safe and permanent disposal of high-level nuclear waste, particularly spent nuclear fuel, while giving states the authority to regulate low-level waste. This allocation of responsibilities sought to provide a centralized approach to high-level waste management, ensuring uniform safety standards and continuity in the disposal process.

The Nuclear Waste Policy Act amendment of 1987 also authorized the establishment of a permanent geologic repository for high-level nuclear waste, commonly referred to as the proposed Yucca Mountain repository. This repository, located in Nevada, was intended to provide a long-term solution for the disposal of nuclear waste by utilizing deep geological formations to isolate the waste from human contact.

Evaluation of Successes and Shortcomings

While the NWPA represented a significant step towards addressing the complexities of nuclear waste management, it has faced numerous challenges and criticisms over the years. One of its notable successes is the establishment of a comprehensive framework for waste management, which provided a sense of direction and long-term planning (A New Era, 2010). This framework aimed to ensure transparency, public involvement, and stakeholder participation in decision-making processes, enhancing public trust in the disposal methods employed. However, the NWPA's implementation faced significant setbacks, most notably regarding the proposed Yucca Mountain repository. Through the last 40 years, the efficacy of actually using Yucca Mountain has debated due to limited core lithography data currently available from Yucca Mountain cores obtained by the DOE (MacFarlane et al. 2006). Numerous simulations and models have shown the highly-permeable, heterogeneous geological structures underlying Yucca Mountain create an unfavorable and potentially dangerous opportunity for subsurface contaminate spread (Arnold et al. 2003, Swift and Bonano 2016, Schwartz 2020). It has been shown at far too many superfund sites how difficult subsurface contamination is to remediate and the extensive research that is required before even beginning remediation efforts. Additional flow models and a deeper understanding of the geology will need to be obtained before the DOE will officially make Yucca Mountain a deep geologic repository. Despite substantial investments, these findings and debates have stalled the repository's development, calling into question the effectiveness of the Act.

Additionally, the Act lacks clarity on procedures and timelines for siting and constructing repositories, leading to delays and a lack of confidence in the overall process (NASEM 2023). The NWPA has been criticized for neglecting interim storage solutions for nuclear waste. This omission has strained existing storage facilities, posed potential safety risks and impeded progress towards a comprehensive waste management strategy. The Act's focus on a single repository concept at Yucca Mountain has hindered exploration of alternative options that could meet the nation's interim storage needs.

Relevance and Future Challenges

The NWPA, while flawed, remains relevant in addressing the present and future challenges of nuclear waste management. The Act's emphasis on public involvement and transparency sets essential precedents for engaging stakeholders and communities in the decision-making processes.

As the United States continues to generate nuclear waste, the need for a comprehensive, science-based approach to waste management becomes increasingly urgent. However, the NWPA must be adapted and expanded to meet contemporary challenges. Immediate attention should be given to developing interim storage solutions, enhancing the safety and capacity of existing facilities, and advancing research and development into innovative waste treatment technologies. Furthermore, revisiting siting criteria and adopting a more flexible approach in evaluating potential repository sites could help overcome the challenges faced by the Yucca Mountain project.

The Nuclear Waste Policy Act of 1982 set a significant precedent for the management, storage, and disposal of nuclear waste in the United States. Despite its successes, the Act has faced notable shortcomings in implementation, particularly concerning the proposed Yucca Mountain repository and the lack of attention to interim storage solutions. Looking ahead, the Act's relevance hinges upon adapting and expanding its provisions to meet the evolving challenges of nuclear waste management. By prioritizing research, innovation, and public engagement, the United States can ensure the safe, efficient, and sustainable handling of radioactive waste for current and future generations.

Superfund Green Remediation Strategies

The environmental impact of remediation efforts at DOE Superfund sites has garnered public attention over the last few years. In 2009, the EPA developed the Superfund Green Remediation Strategy, which aims to reduce energy use in remediation efforts, preserve natural resources and decrease emissions created during site remediation (EPA 2009). Green remediation prioritizes energy efficiency in cleanup efforts and focuses on minimizing the environmental footprint of cleanup activities while achieving the desired outcomes. It emphasizes sustainability and promotes the use of renewable energy sources, recycling and reusing

materials, and reducing greenhouse gas emissions. By incorporating these strategies into clean efforts, Superfund sites can be restored without causing further harm to the environment.

Within these efforts, the EPA published Best Management Practices (BMP) for remediation with numerous options for greener solutions. Bioremediation, a remediation technique that uses biological processes to degrade or remove contaminants, aligns with the principles of green remediation and has been recommended as one of these BMPs. Bioremediation, which often relies on natural processes and organisms, is considered a more sustainable approach compared to other energy-intensive remediation methods. EPA recognizes that the feasibility of bioremediation depends on factors such as the type and extent of contamination, site conditions and the specific contaminants involved; feasibility assessments are crucial to determine the appropriateness of bioremediation for a given site.

Nitrate Pollution and Sources

Nitrate pollution in groundwater is a significant environmental concern in the United States, primarily due to agricultural activities, wastewater discharges, and other human-related sources and is one of the main issues in groundwater management (Menció et al. 2016). The excessive use of nitrogen-based fertilizers in agriculture is a major source of nitrate pollution and heavily impacts areas with intensive farming activities. Groundwater is particularly vulnerable to high nitrate concentrations due to its slow movement and limited natural attenuation (Nolan and Hitt 2006).

Current estimates of total reactive nitrogen on Earth show that reactive nitrogen species are more than double what they were during the pre-industrial era due to our agricultural practices, including nitrogen-based fertilizers (Galloway, 2004)(Ascott et al. 2017). However, most of these estimates do not consider the amount of nitrogen stored as nitrate in the vadose zone. The unsaturated (vadose) zone between the top of the soil and the top of the water table is where most nitrate is stored (Ascott et al. 2017). Precipitation then releases the nitrate and transports it into the water table. Once nitrate enters groundwater, it can persist for years, gradually moving through aquifers and spreading the contamination. This vadose zone nitrate is of particular concern because it would mean that changes to the agricultural industry would still not give immediate relief to the issue of nitrate contamination (Wang et al. 2013).

High nitrate levels in drinking water can pose serious health risks, especially for infants. Nitrate ingestion can lead to methemoglobinemia, commonly known as “blue baby syndrome,” a condition that reduces the blood’s ability to carry oxygen and can lead to death (Majumdar 2003). Aquatic ecosystems are also heavily impacted by nitrate pollution. Excessive nitrogen in bodies of water can lead to algal blooms and hypoxia (oxygen depletion), which causes die-off of aquatic organisms (Martín et al. 2024). Obviously, this creates a massive devastation to these affected ecosystems.

The EPA has set a Maximum Contaminant Limit (MCL) for nitrate in drinking water at 10 milligrams per liter (mg/L) (Safe Drinking Water Act, 2023). However, there are aquifers in the United States that far exceed that limit, such as the aquifer present at the Oak Ridge Reservation in Oak Ridge, Tenn. At this DOE Superfund site impacted by industrial waste and nuclear proliferation, nitrate concentrations in the groundwater have been recorded at over 35,000 mg/L but are persistently 600 to 1,000 mg/L with a pH of 3.5 (Groundwater, 2001). These nitrate concentrations are the highest ever recorded and this site has been designated for research to assess remediation potential for nitrate and mixed waste contamination in groundwater environments (Paradis et al. 2019).

Knowledge Gaps in Current Research

There are persistent challenges and knowledge gaps in the environmental remediation of DOE Superfund sites and areas impacted by contamination, such as nitrate pollution. Understanding the long-term impacts and effectiveness of remediation efforts at DOE Superfund sites is crucial. However, there is almost no researching focused on assessing the persistence of contaminants and the sustained success of implemented remediation strategies over extended periods. Studies, such as the 27-Well Survey and extended surveying efforts, are critical in efforts to understanding how these environments are maintaining remediation efforts at active sites and sites that are impacted by nitrate pollution from various sources.

Many DOE Superfund sites involved complex contamination from various sources including chemical and radiological contaminants. Major knowledge gaps exist when trying to understand the synergistic or cumulative effects of these contaminants and developing comprehensive remediation strategies. There are also many sites that have been left abandoned or retired where the extent of contamination is not fully documented or effectively communicated over the decades since the site was active. Interdisciplinary efforts and comprehensive analysis are required for sites that are impacted by multifaceted contamination sources.

Developing adaptive remediation strategies that can be adjusted based on continuous or long-term monitoring and new information is an ongoing challenge. Research is needed that focuses on creating flexible approaches that can respond to changing conditions and emerging challenges. This is especially true of active DOE research sites and areas impacted by the ever-changing agricultural pollution. As new pesticides, herbicides, feeds, and animal care needs are developed, the long-term environmental impacts will need to be addressed and researched.

Additionally, more research is needed to explore and develop innovative technologies for more efficient and cost-effective remediation and monitoring. This includes advancements in in-site treatment methods, monitoring technologies that allow for comprehensive remote monitoring and the use of sustainable and green remediation practices. Research conducted at the Y-12 National Security Complex is utilizing in-field, real-time groundwater monitoring created by In-

Situ, Inc. to analyze nitrate, dissolved oxygen, and pH. However, this monitoring system is costly and limited in what measurements are currently accessible.

Understanding the health effects of exposure to contaminants at Superfund sites is critical. Biomonitoring and studies could help assess the real-world impact of remediation efforts on human health. Real-time, in-field monitors focused on National Primary Drinking Water Regulations (NPDWR) outlined by the EPA with immediate public health implications, such as chlorine, arsenic, lead, cyanide, and mercury, would allow the public and industries to have real-time information about the safety of the available drinking water. A recent study showed that living near a superfund site negatively effects life expectancy by up to 1.22 years (Kiaghadi et al. 2021).

Conclusion

The examination of DOE's superfund sites underscores the complexity of addressing historical legacies of contamination resulting from nuclear activities. Policy initiatives, such as CERCLA and the NWPA, play pivotal roles in guiding the cleanup and management of these sites. The challenges posed by nitrate pollution further emphasizes the need for a multifaceted and adaptive approach to environmental remediation.

The policy landscape surrounding nuclear waste management is marked by ongoing efforts to strike a balance between energy needs, environmental protection, and public safety. The exploration of consolidated interim storage solutions and community-based consent approaches reflects an evolving understanding of the intricate challenges associated with long-term nuclear waste management.

As we move forward, future research efforts must address persistent knowledge gaps to enhance the efficiency of environmental remediation. Investigations into the long-term impacts of cleanup strategies at DOE Superfund sites, innovative technologies for contaminant removal and the integration of community preferences and safety are crucial. Additionally, research should consider the dynamic nature of environmental challenges, including the impact of climate change on contaminant transport and the emergence of new pollutants.

In this era of environmental consciousness, collaborative endeavors involving government agencies, scientists, local communities, and industry stakeholders are paramount. Bridging the gap between scientific advancements and practical implementation is essential to achieving sustainable and effective environmental remediation strategies. Through continued research and technological innovation, there is hope for a cleaner, safer, and more resilient environment for future generations.

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Appendices

Appendix A

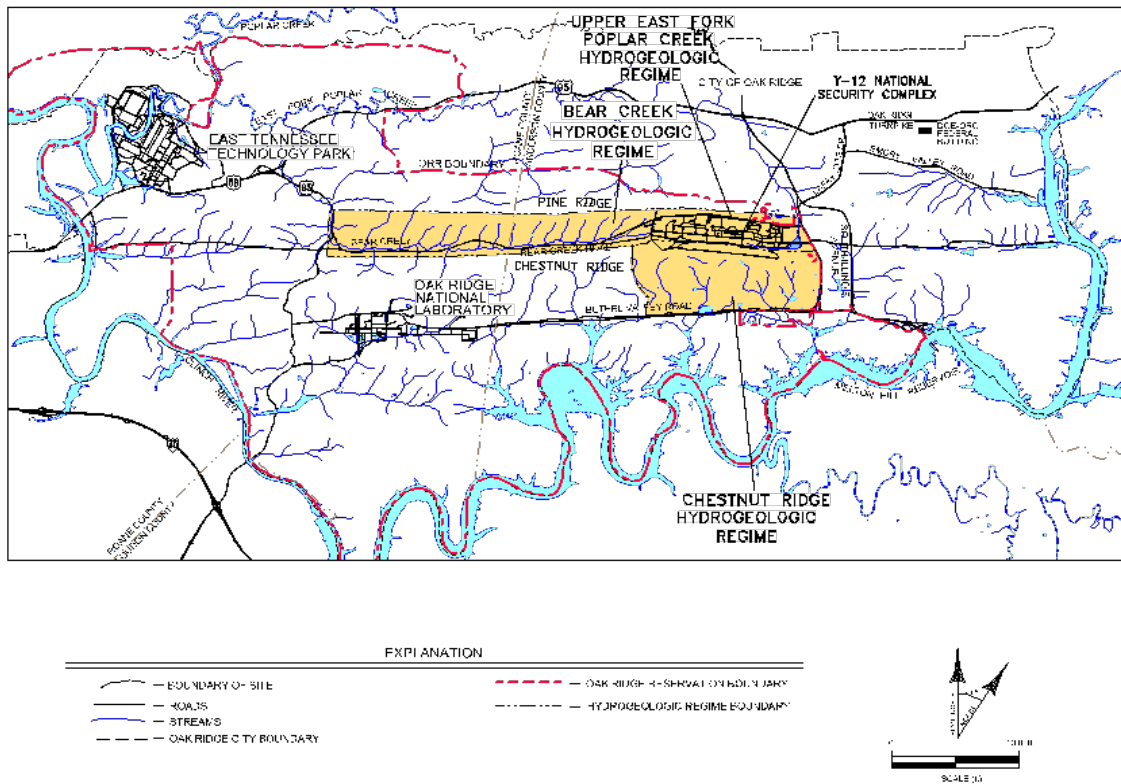


Figure A.1. Hydrogeologic regions at Y-12 National Security Complex.

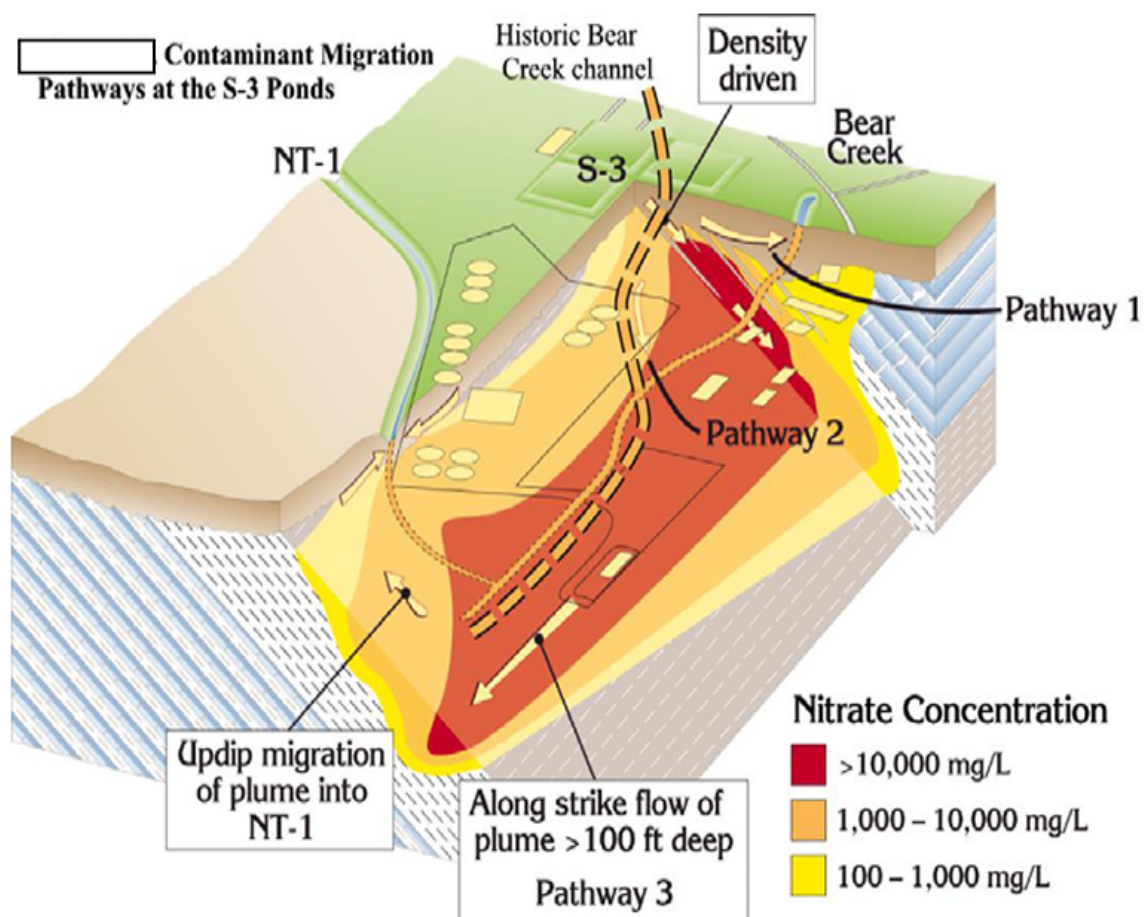


Figure A.2. Three contaminant pathways from the S-3 Ponds with their average nitrate concentration.

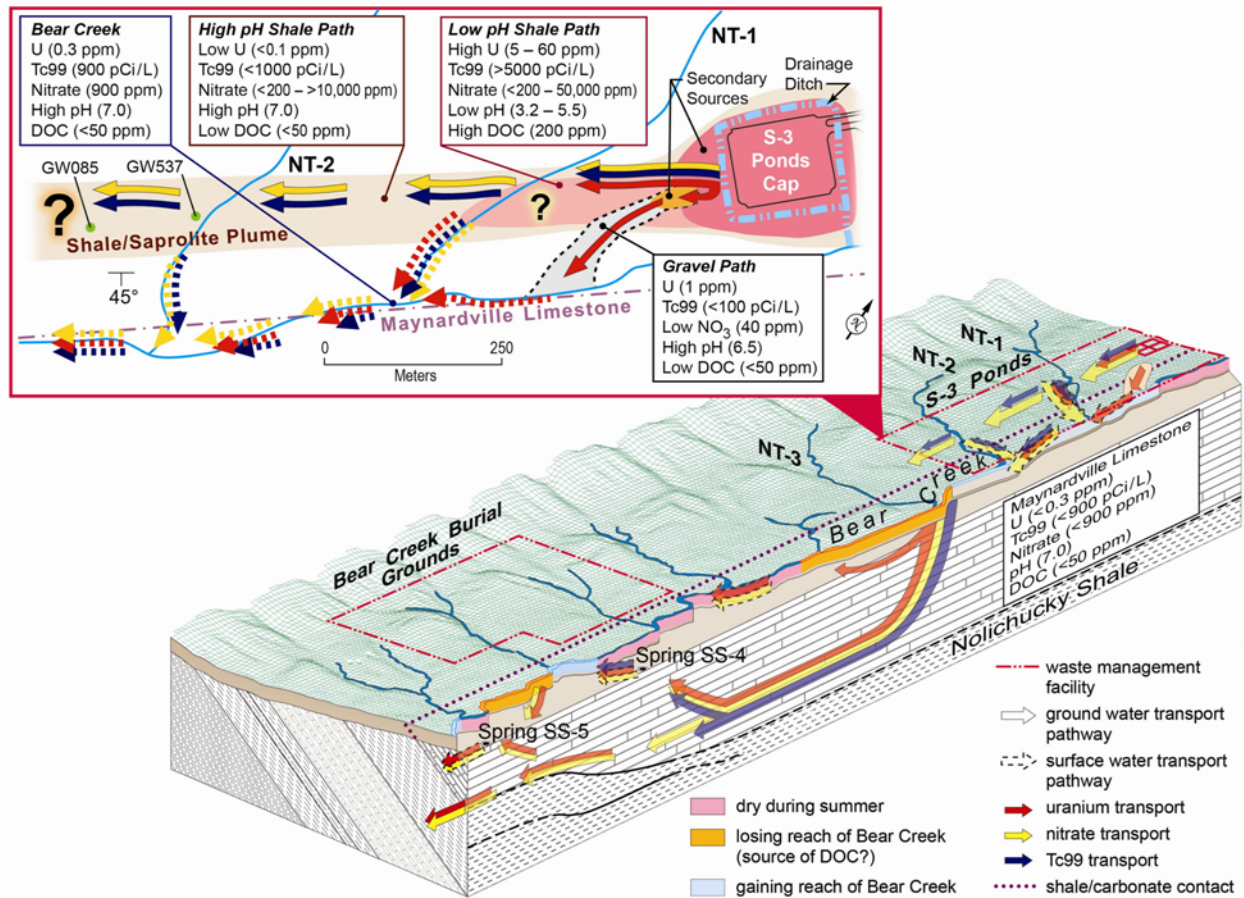


Figure A.3. Major flow paths in the Bear Creek Valley.

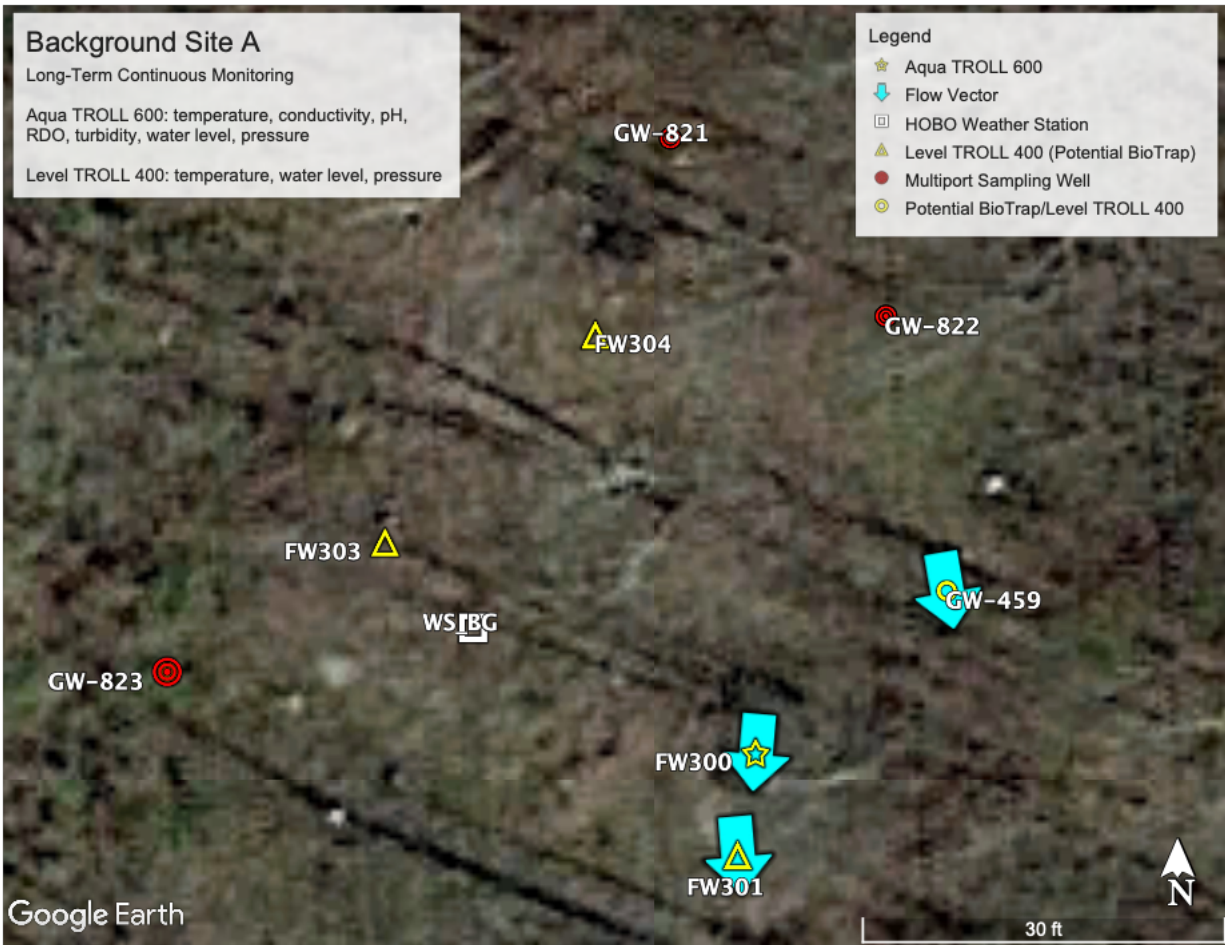


Figure A.4. Background Site A Wells showing Aqua TROLL 600 (FW300), multiport sampling wells (GW822, GW821, GW823), water level monitoring wells (FW301, FW303, FW304), water level/Bio-Trap wells, Hobo weather station. Note: FW300, FW301, and FW303 were Global Survey Wells (Smith et.al. 2015).

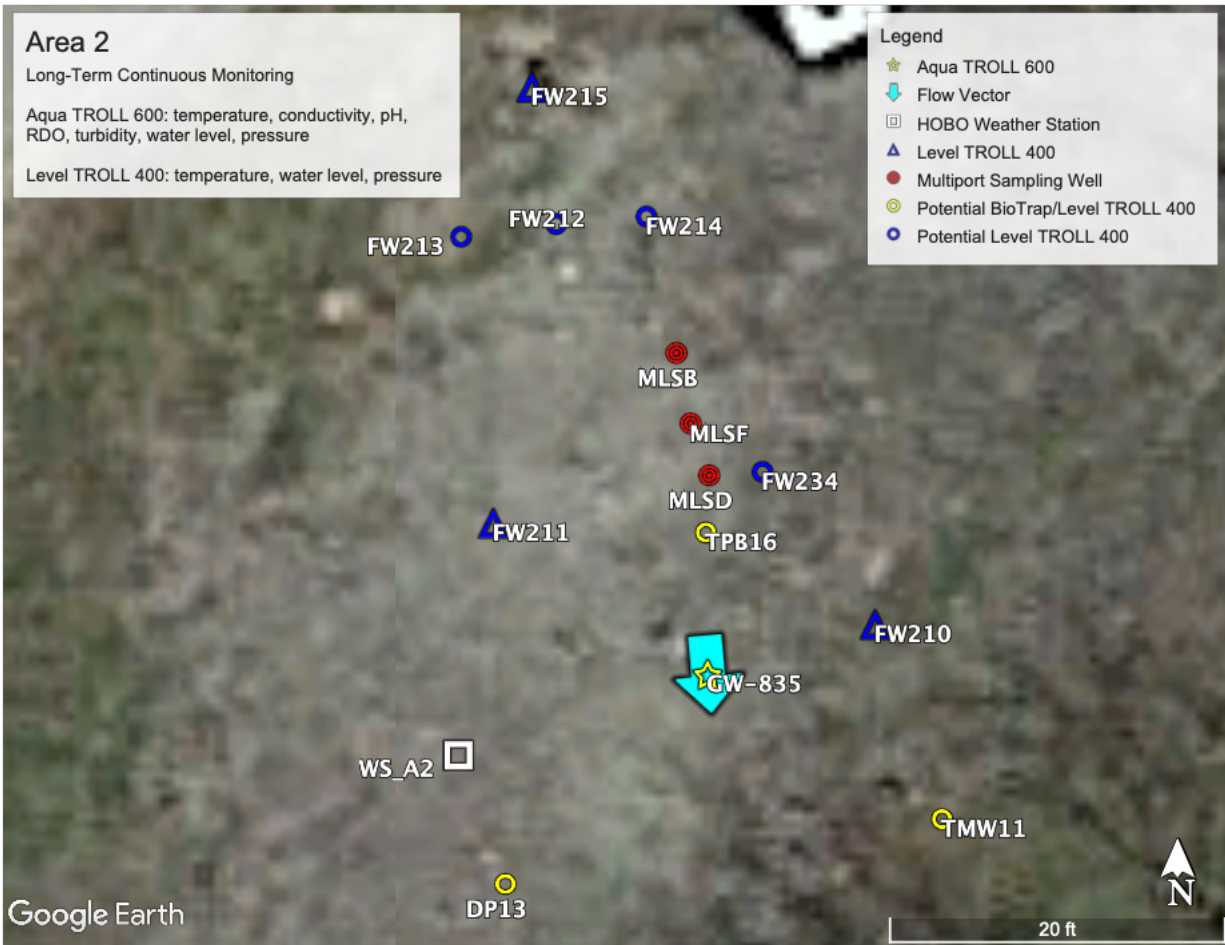


Figure A.5. Area 2 Wells showing Aqua TROLL 600 (GW835), multiport sampling wells Area 2 (MLSB, MLSB, MLSF), water level monitoring wells (FW210, FW211, FW215), water level/Bio-Trap wells, Hobo weather station. Note: FW215, TPB16 were Global survey wells (Smith et al. 2015), FW215, FW213, FW212, FW214, FW216, MLSB3 were used in the IFRC EVO injections and in two ENIGMA EVO related studies (Adams, 2018; McBride, 2018; Charles J. Paradis, 2017).

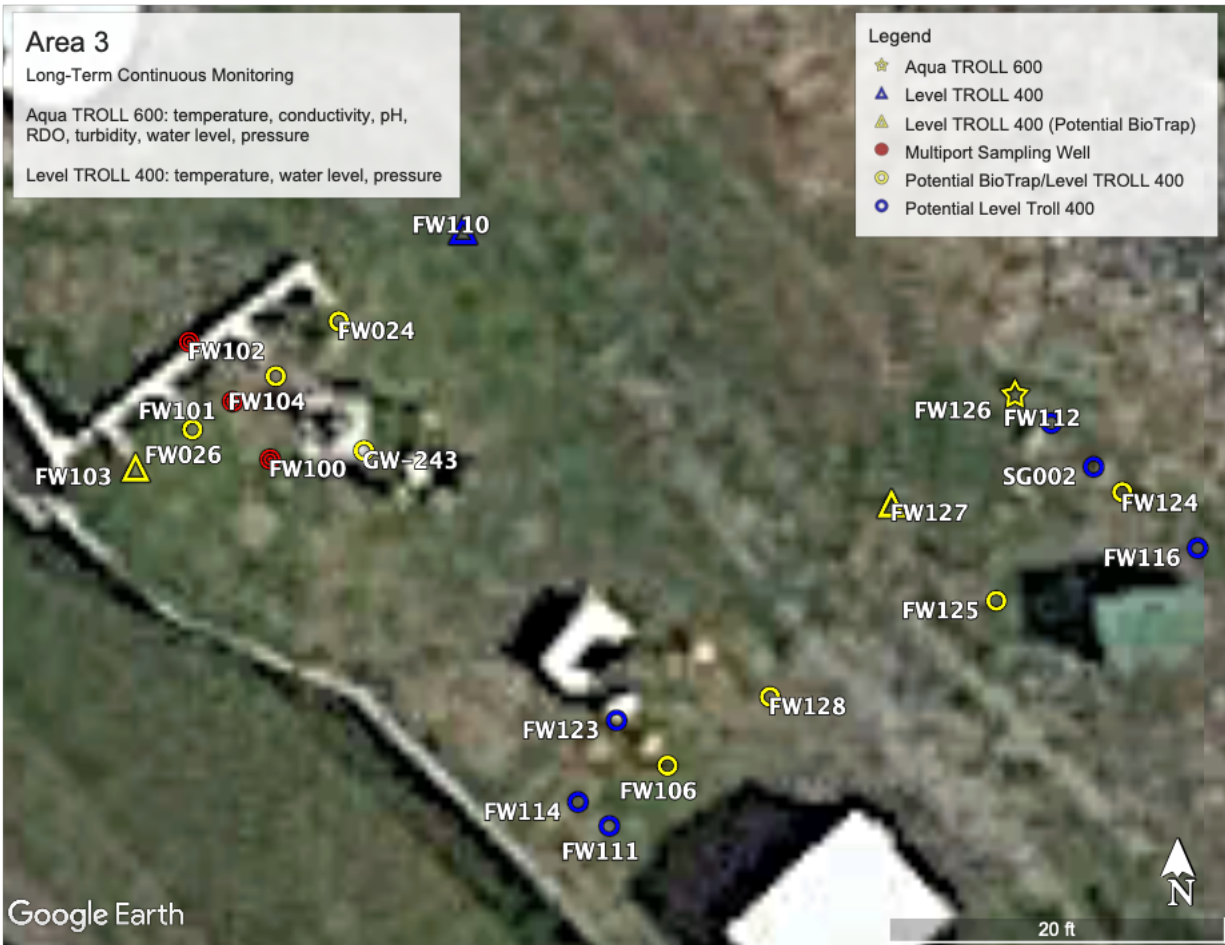


Figure A.6. Area 3 Wells showing Aqua TROLL 600 (FW126), multiport sampling wells (FW100, FW101, FW102), water level monitoring wells (FW215, FW211, FW210), water level/Bio-Trap wells. Note: FW104, FW126, FW106 were Global survey wells (Smith et al., 2015).

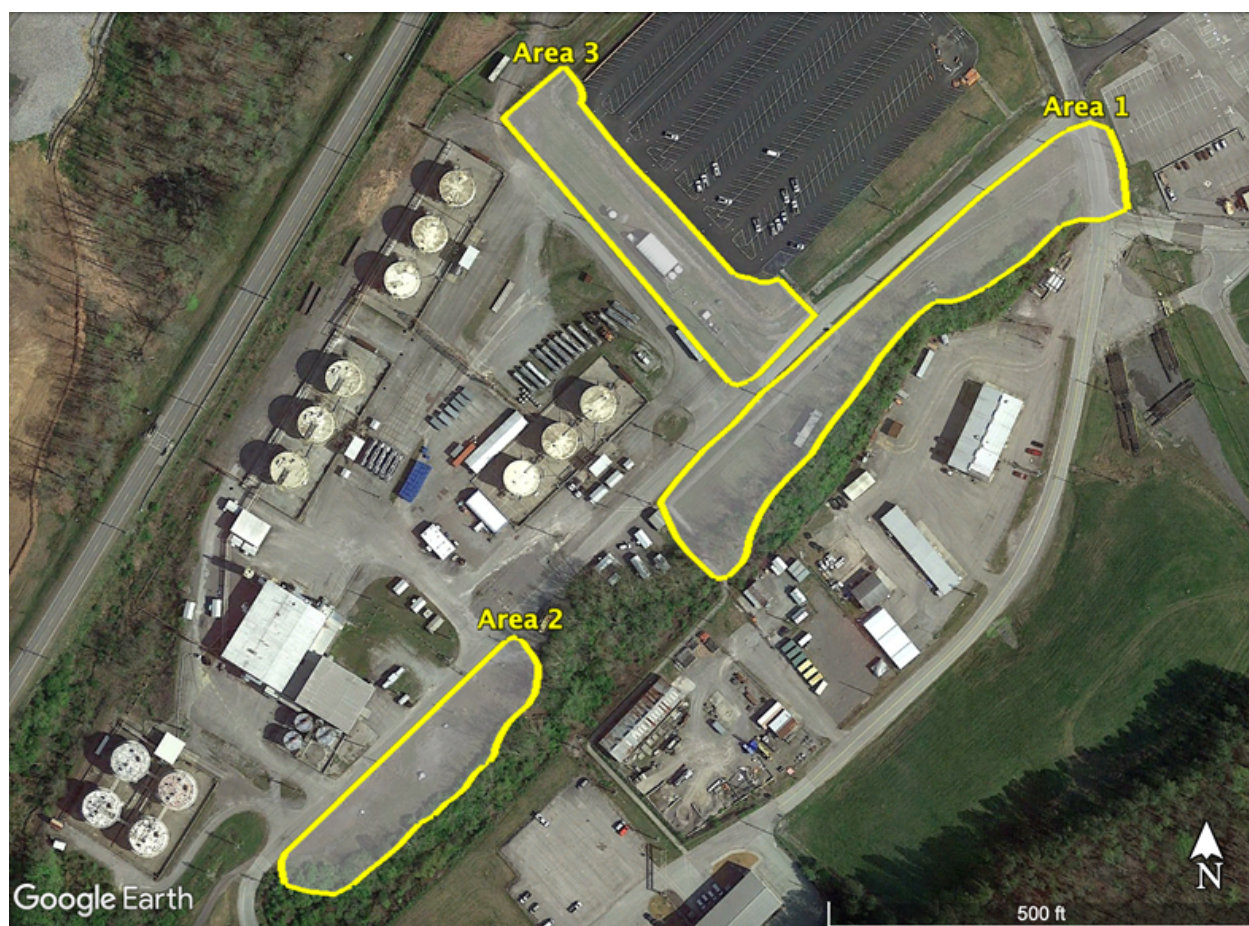


Figure A.7. Location of the contaminated areas at the BER ORNL Field Research Site (ORRFRC).

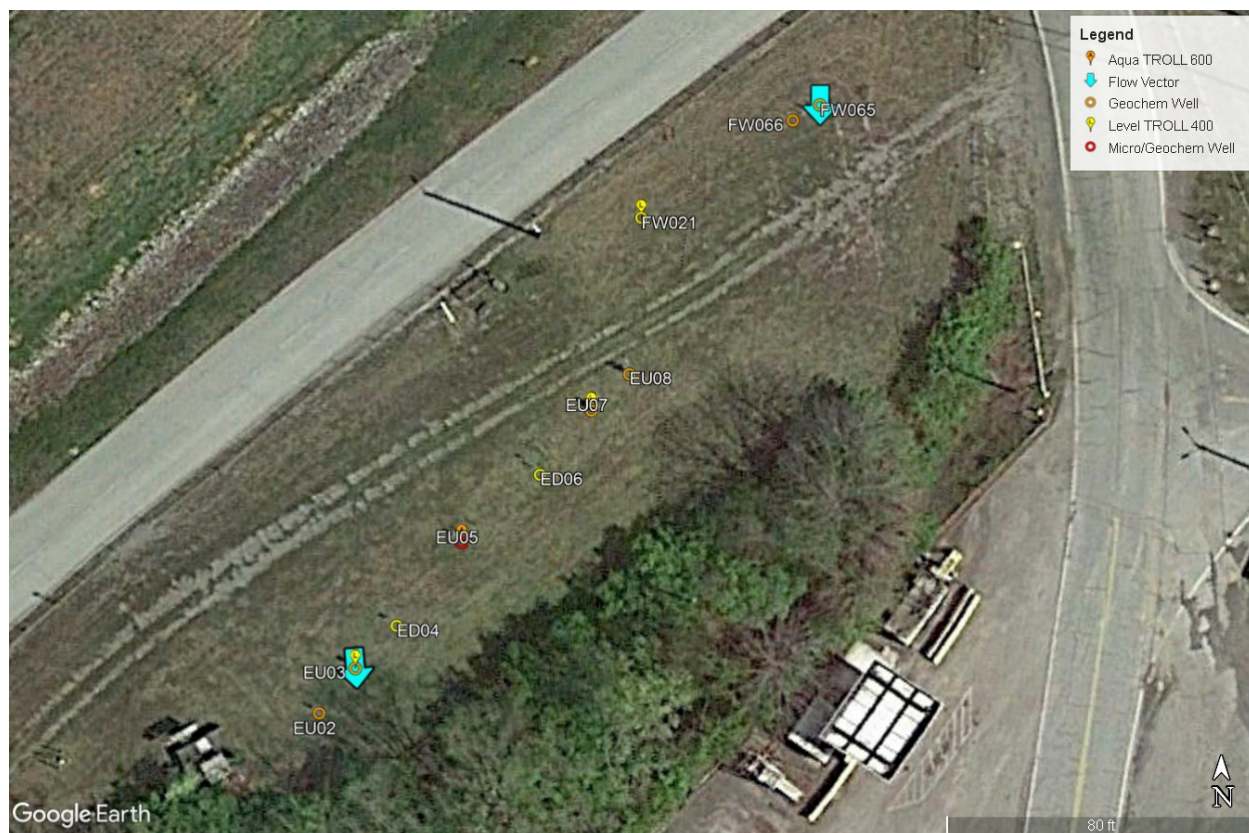


Figure A.8. Area 1 Wells with continuous monitoring and microbial analysis well denoted.

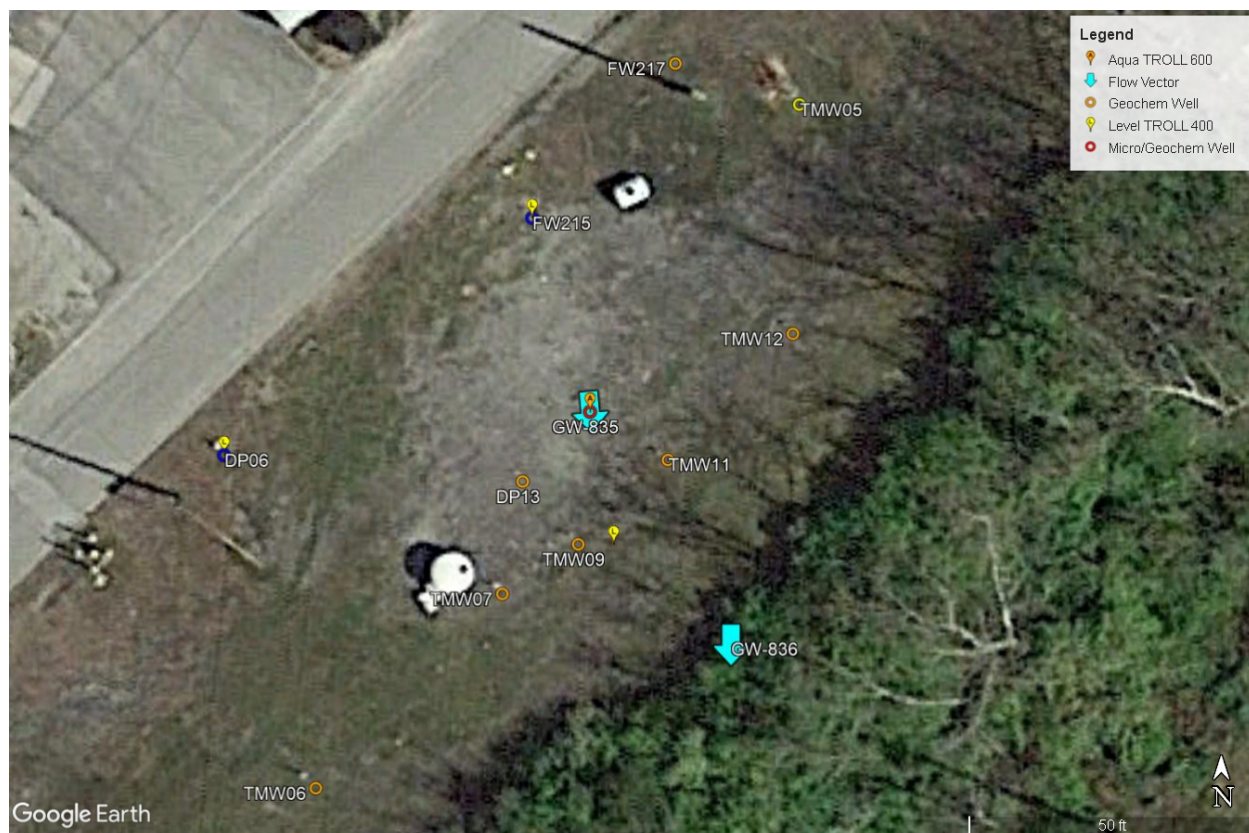


Figure A.9. Area 2 Wells with continuous monitoring and microbial analysis well denoted.

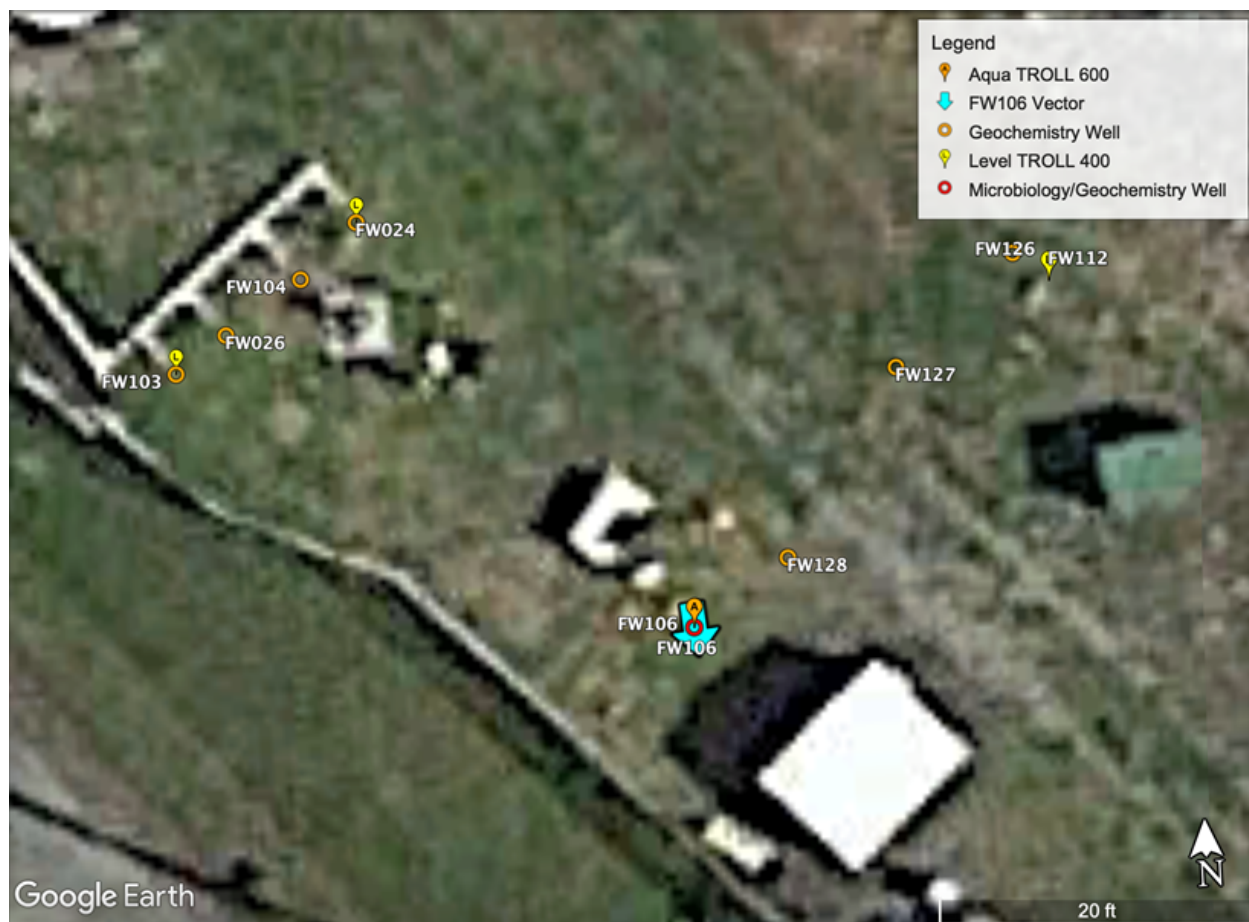


Figure A.10. Area 3 Wells with continuous monitoring and microbial analysis well denoted.

Appendix B

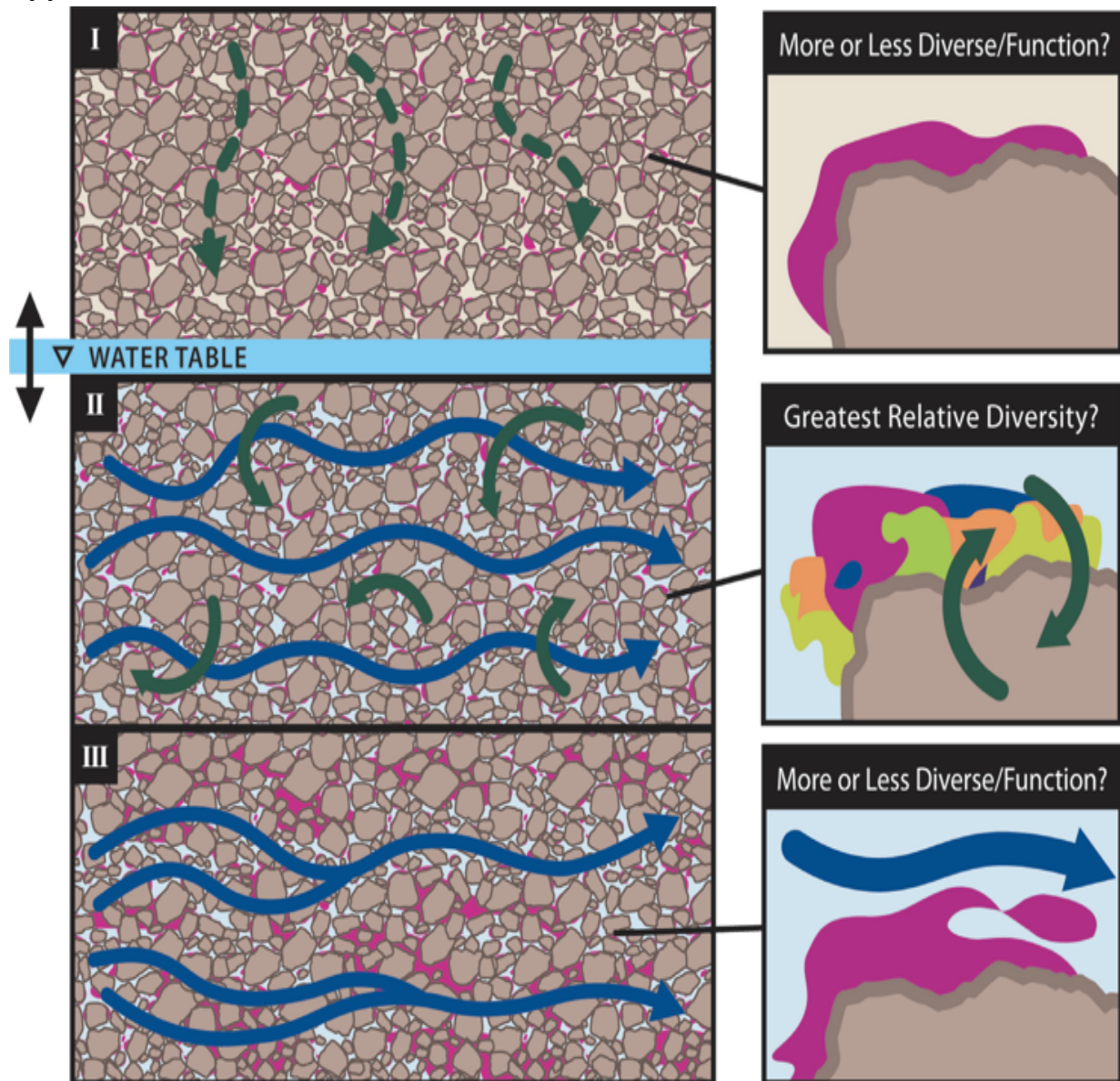


Figure B.1. Conceptual model of subsurface flow and mixing zones and potential effects on community dynamics.

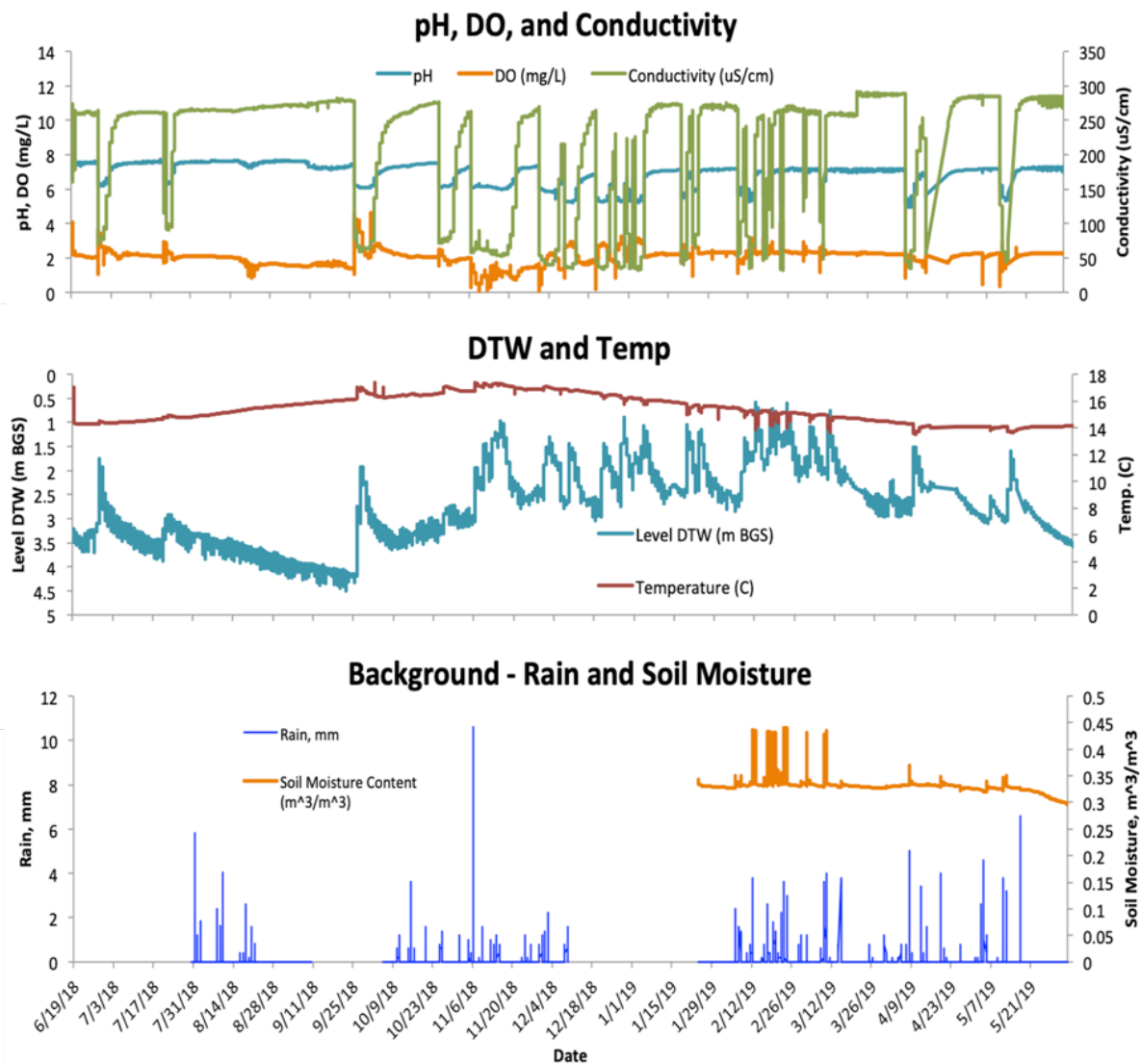


Figure B.2. Background Site A Aqua TROLL 600 data before, during and after active sampling. pH, dissolved oxygen, conductivity, depth to water (below ground surface), and temperature, were recorded. Rainfall and soil moisture data were collected from a HOBO weather station. High fluctuations in pH, DO, conductivity, and DTW during rainfall events.

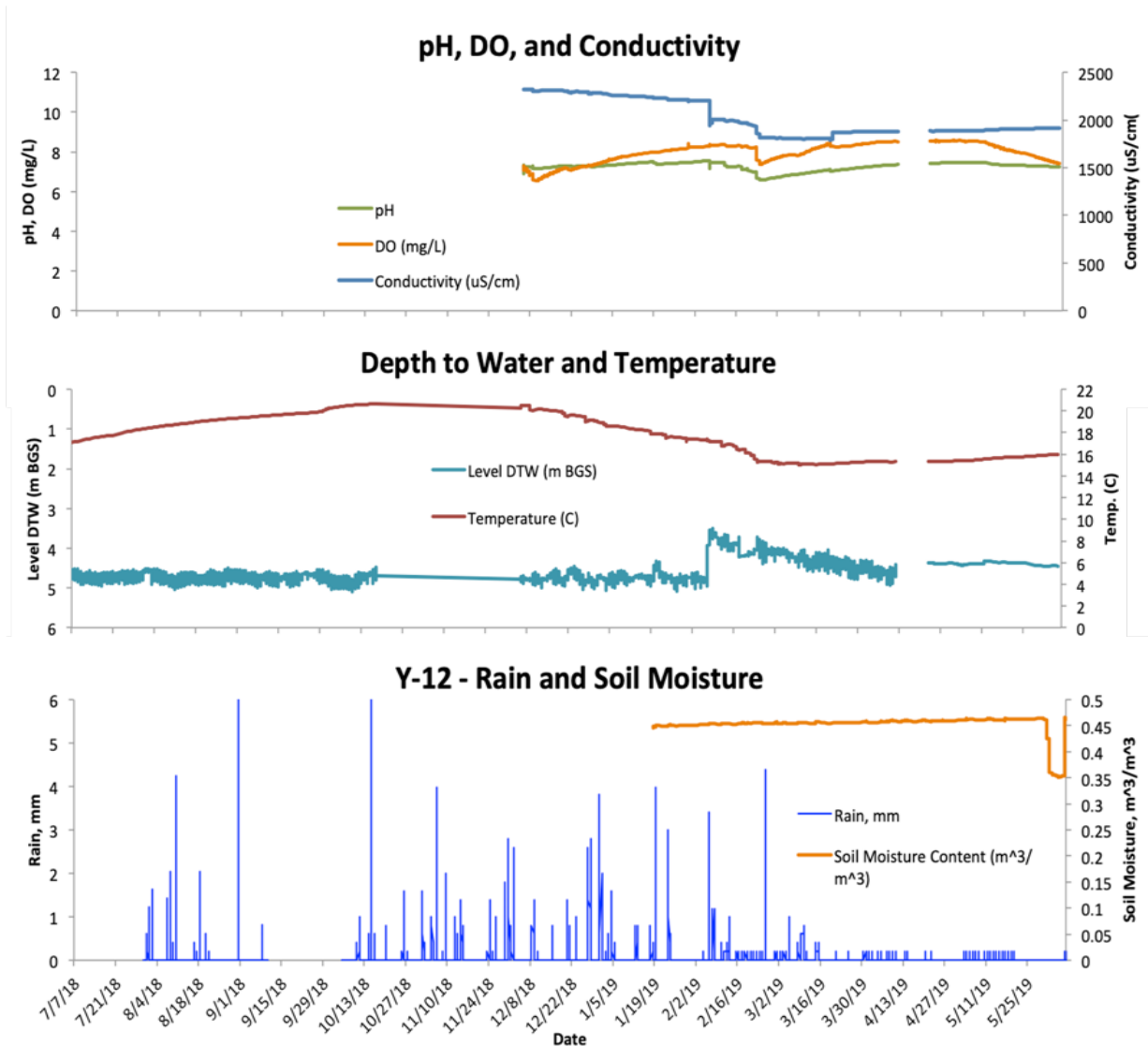


Figure B.3. Area 2 Aqua TROLL 600 data before, during and after active sampling. pH, dissolved oxygen, conductivity, depth to water (below ground surface), and temperature, were recorded. Rainfall and soil moisture data collected were collected from a HOBO weather station. Very small changes in pH, DO, and conductivity during rainfall events. Almost no change in DTW with moderate rainfall, but small change when heavy, continual rainfall occurred.

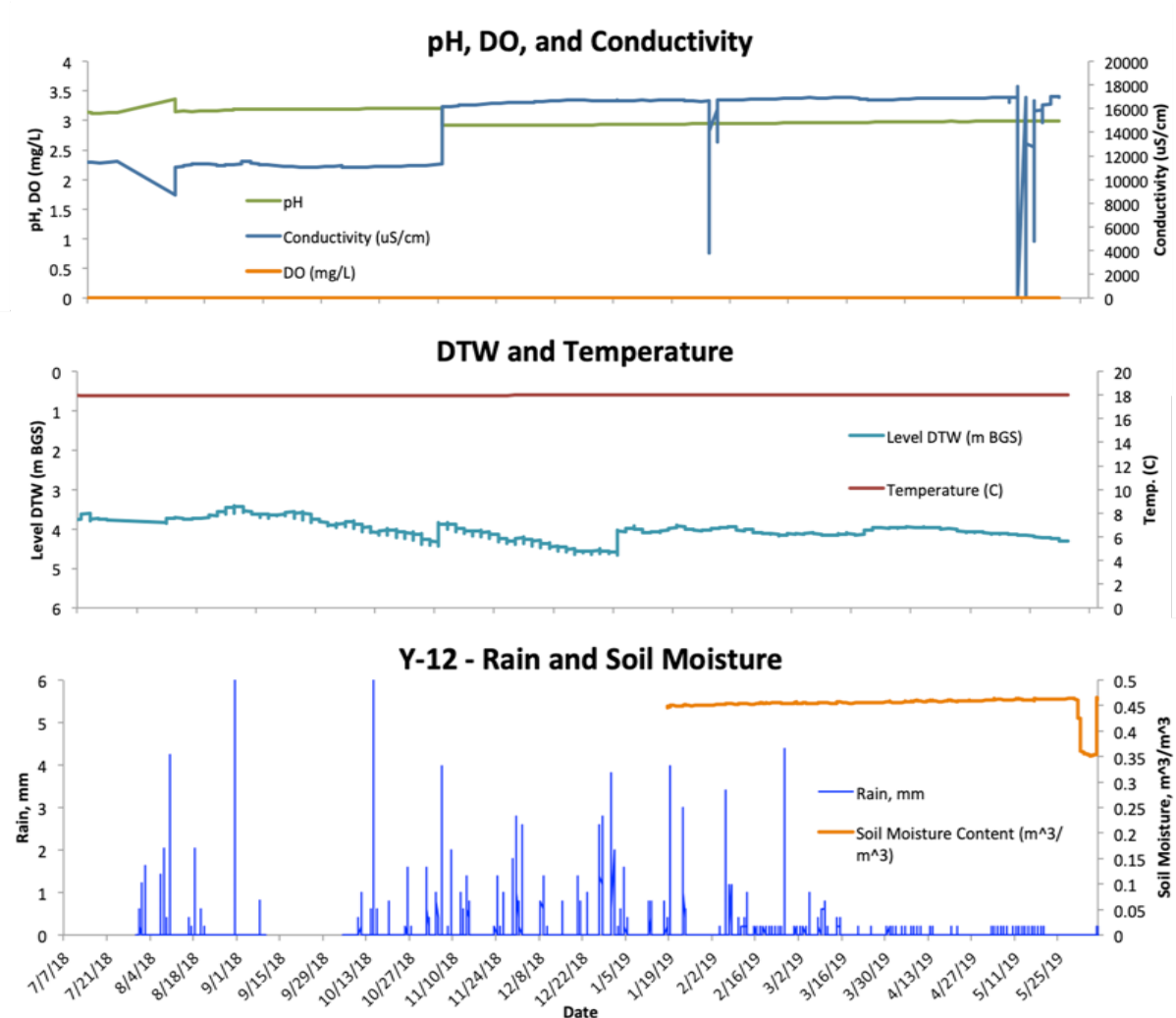


Figure B.4. Area 3 Aqua TROLL 600 data before, during and after active sampling. pH, dissolved oxygen, conductivity, depth to water (below ground surface), and temperature, were recorded. Rainfall and soil moisture data were collected from a HOBO weather station. Very little change in any parameters due to rainfall. There are misreads in the conductivity data due to equipment malfunction.

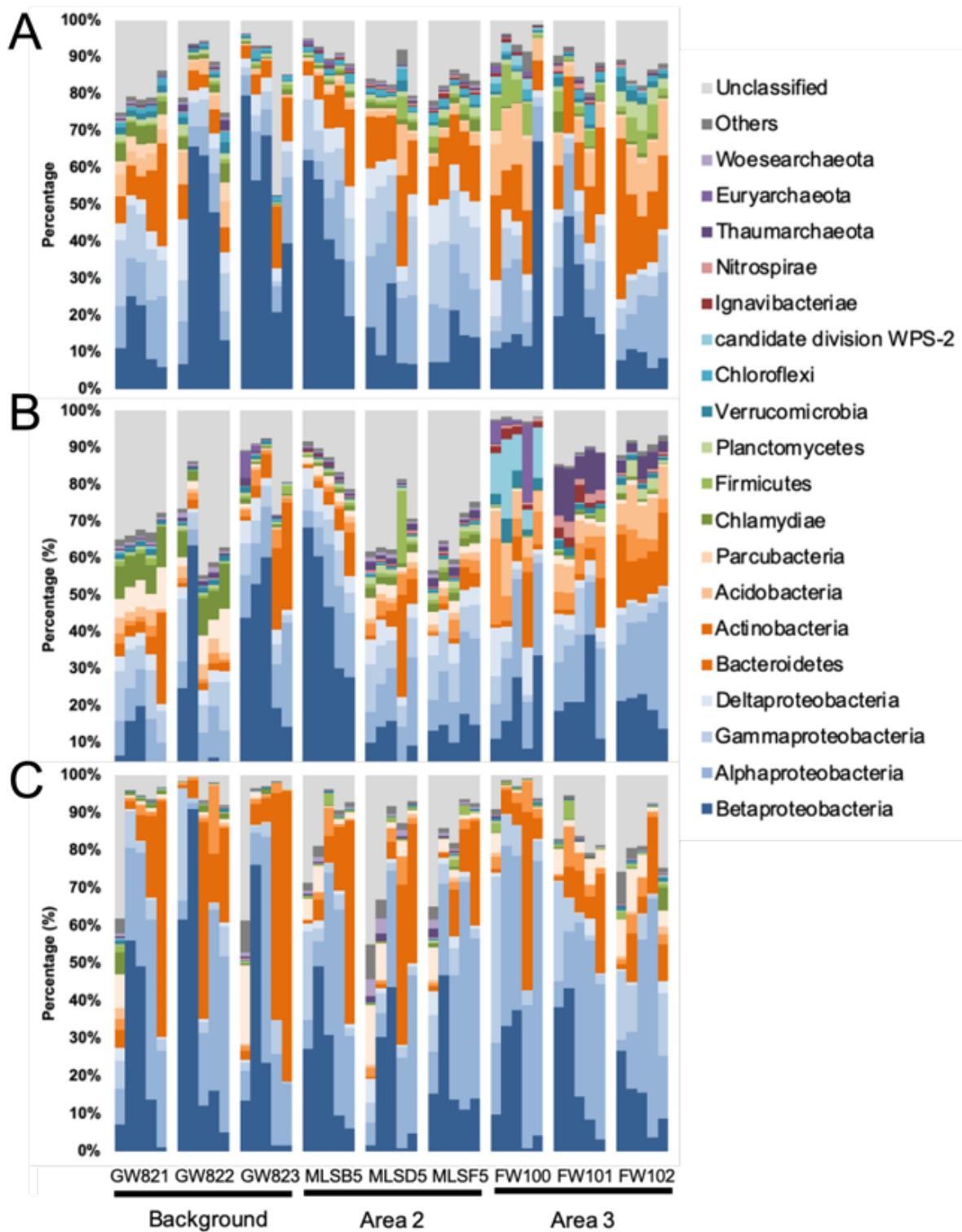


Figure B.5. All samples were grouped by filter pore size (A. 10 µm, B. 0.2 µm, C. 0.01 µm), well and area. Relative abundance of phyla found at each time point.

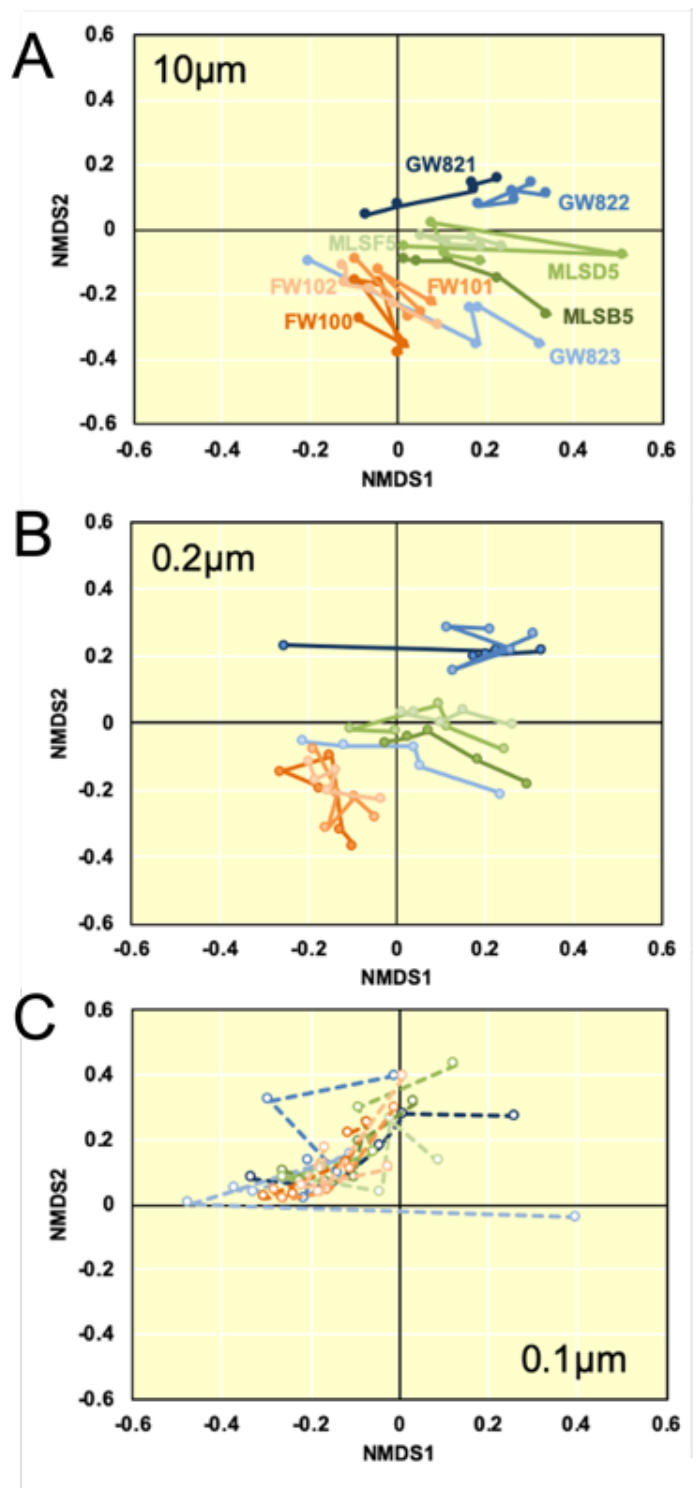


Figure B.6. Non-metric multidimensional scaling (NMDS) of taxonomic analysis ground by filter pore size (A. 10 μm , B. 0.2 μm , C. 0.01 μm) and color-coded by well.

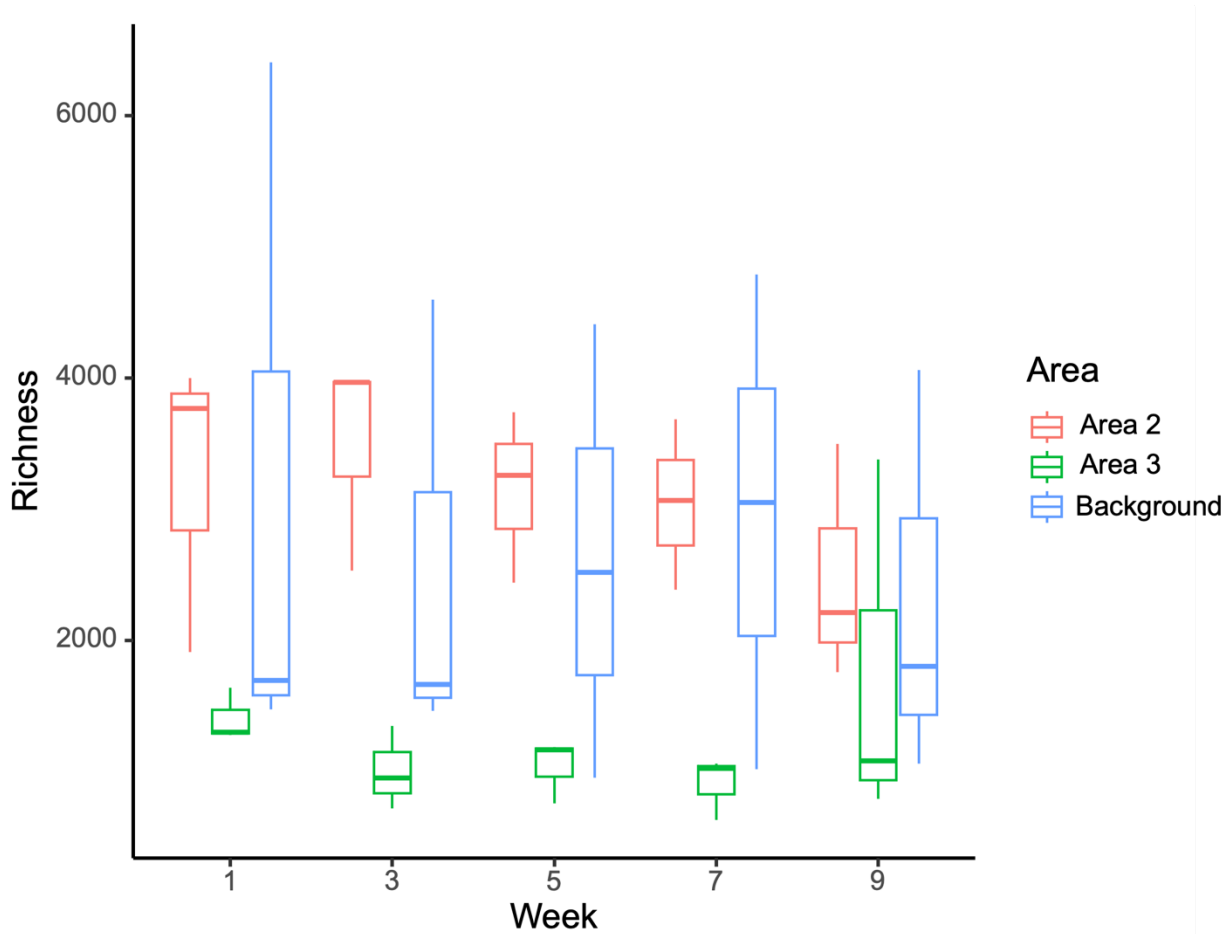


Figure B.7. Taxonomic richness by week and complete communities (i.e., all filters for each area combined).

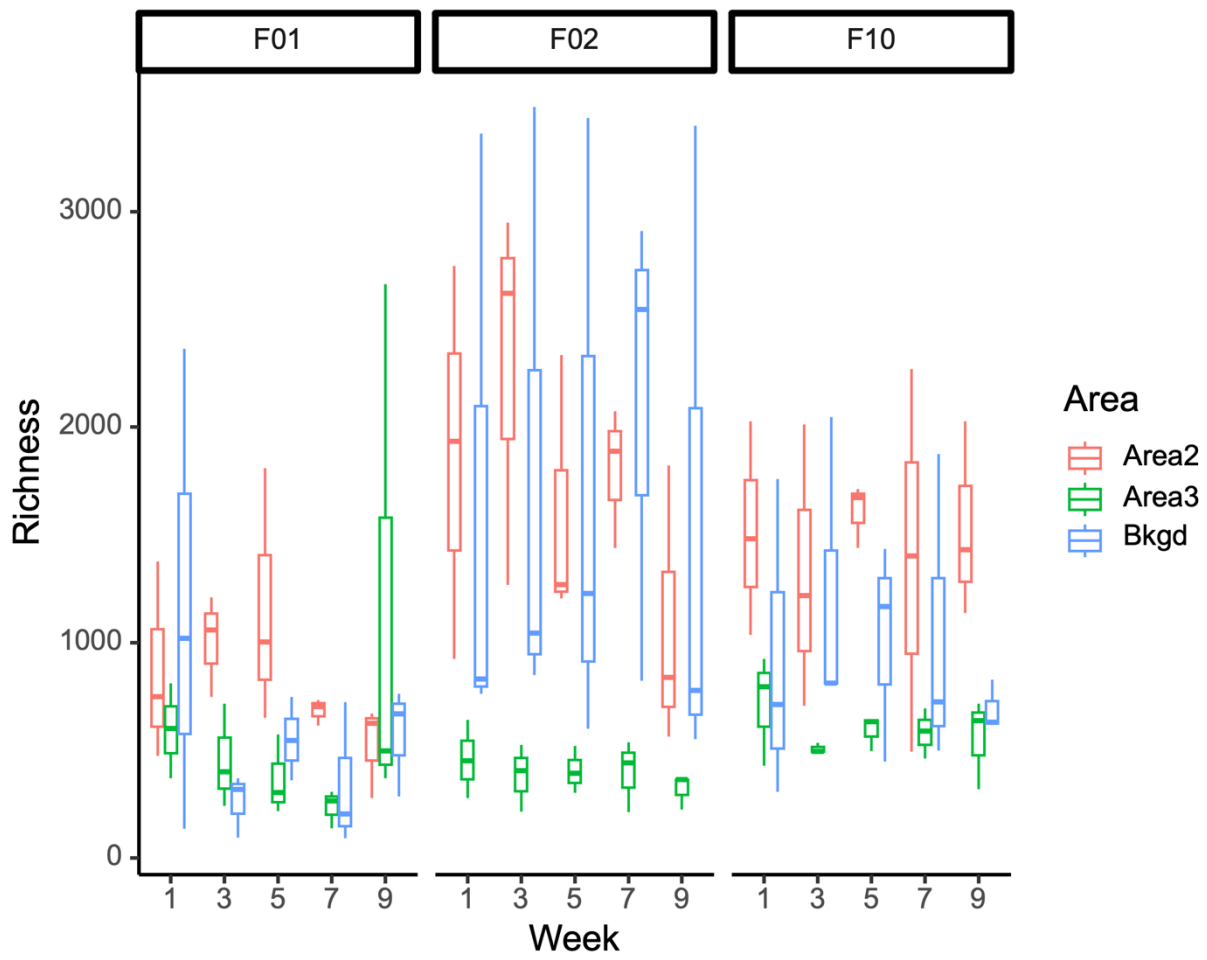


Figure B.8. Taxonomic richness separated by filter size.

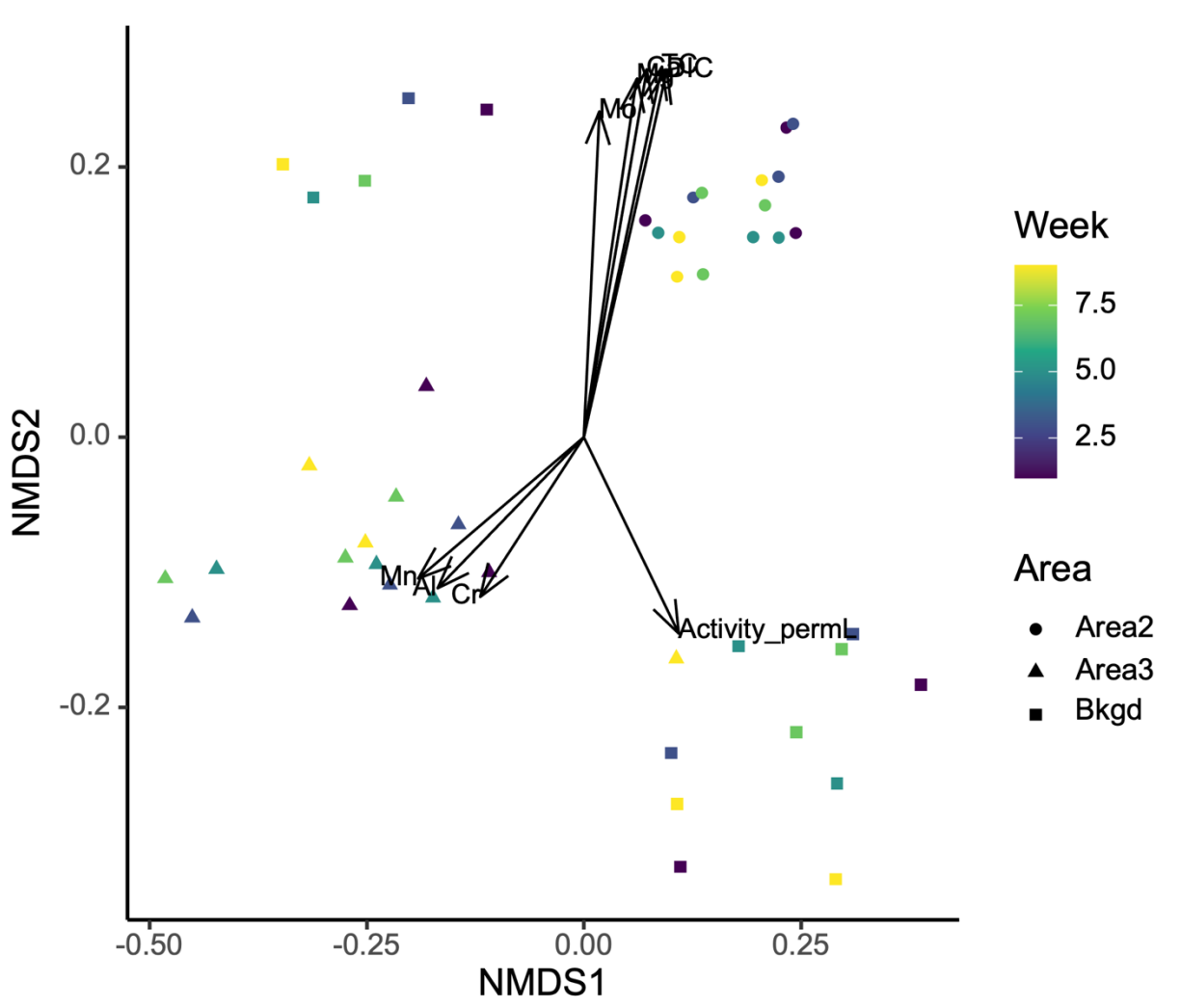


Figure B.9. Beta diversity, excluding GW-823, shows community structure was variable over time but was highly dependent on sampling area.

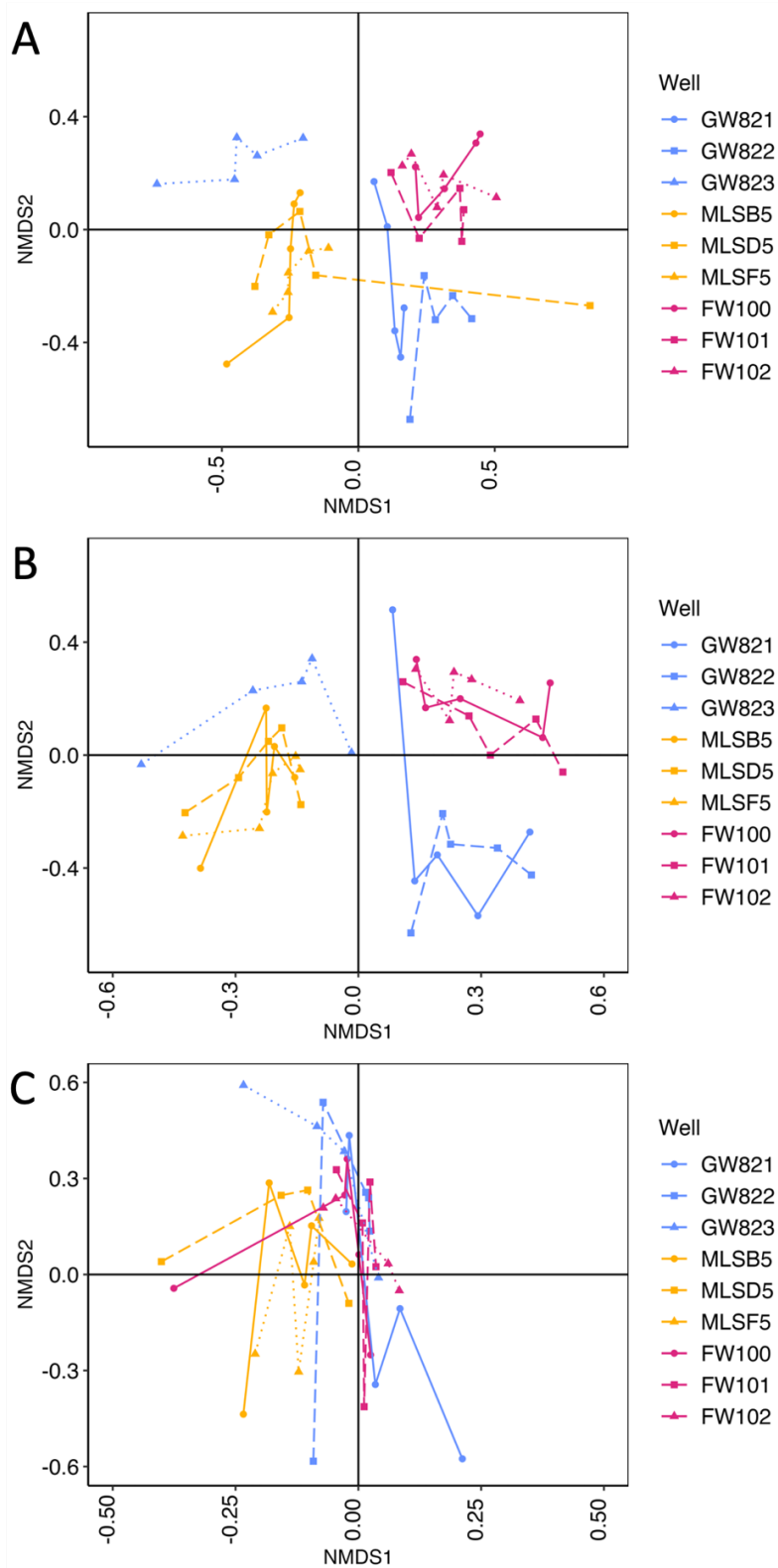


Figure B.10. Non-metric multidimensional scaling plots of groundwater microbial communities for different filter size based on weighted Bray-Curtis index. (A) 10 μm , (B) 0.2 μm , (C) 0.1 μm .

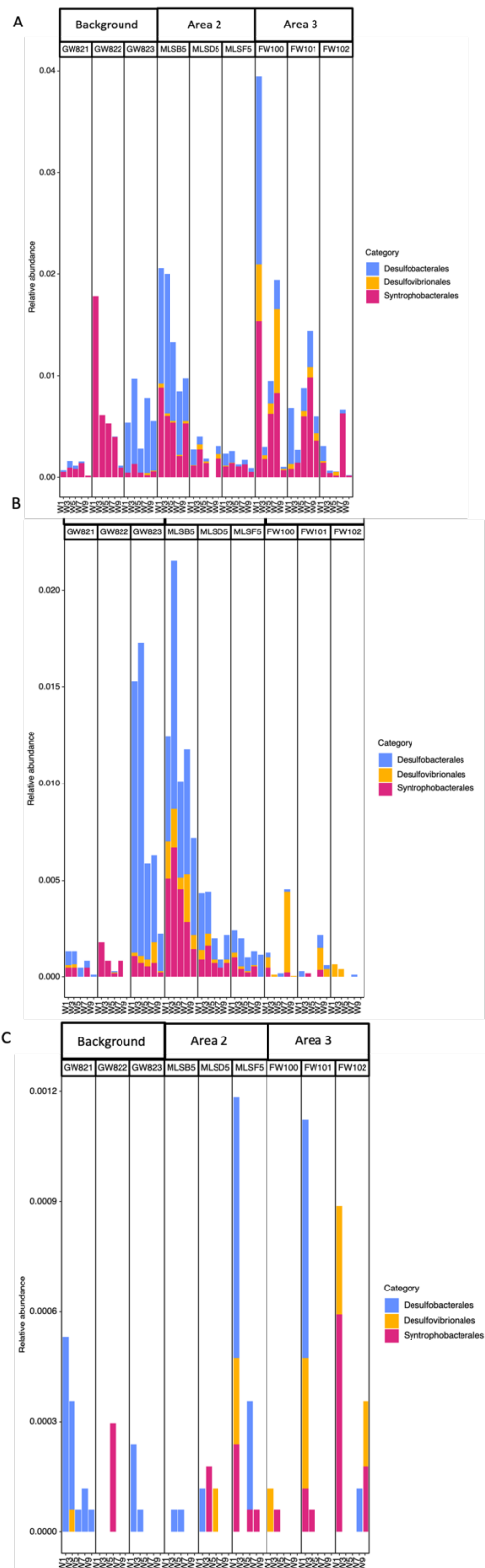


Figure B.11. Sulfate-reducing bacteria composition of groundwater samples. (A) 10 μm , (B) 0.2 μm , (C) 0.1 μm .

Appendix C

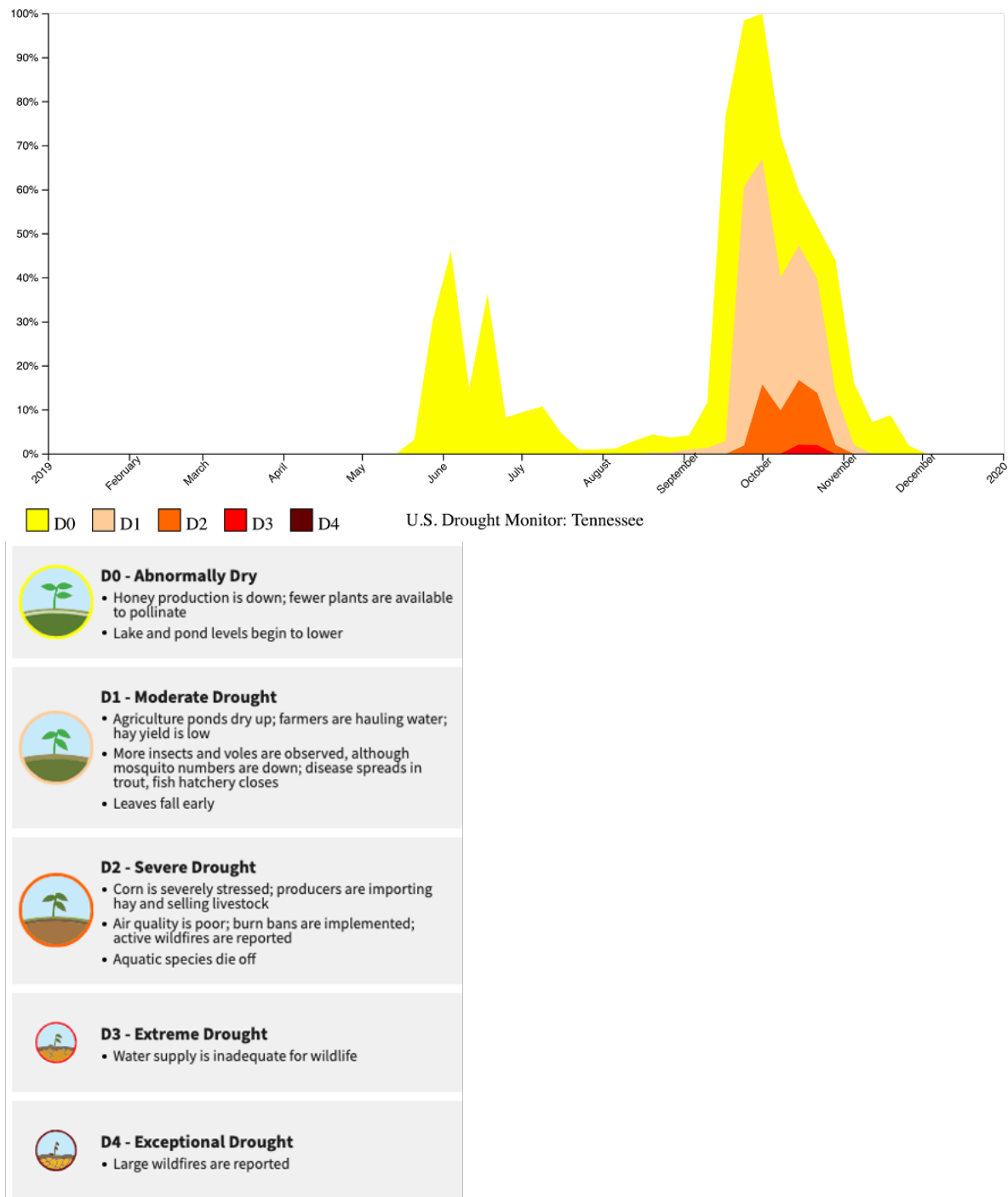


Figure C.1. Drought conditions displayed by U.S. Drought Monitor.

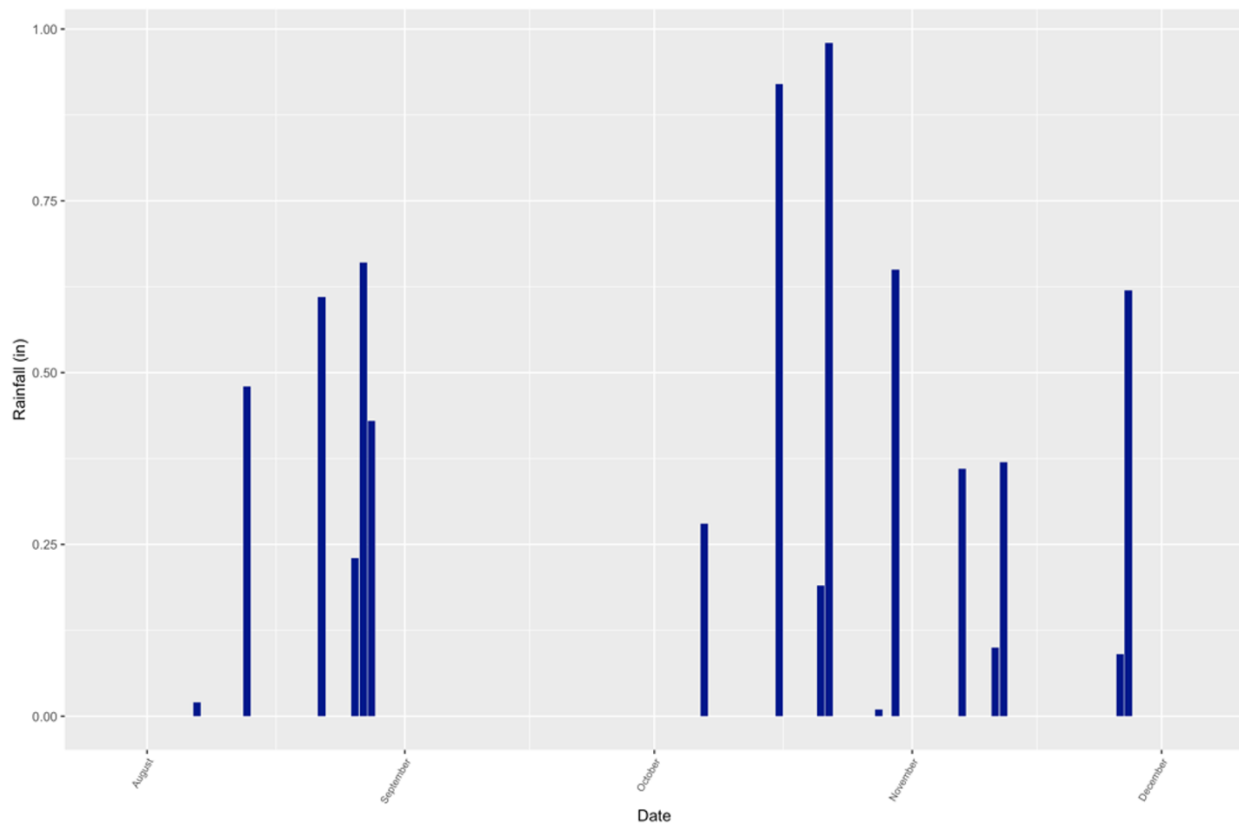


Figure C.2. Rainfall totals at Y-12 sampling site.

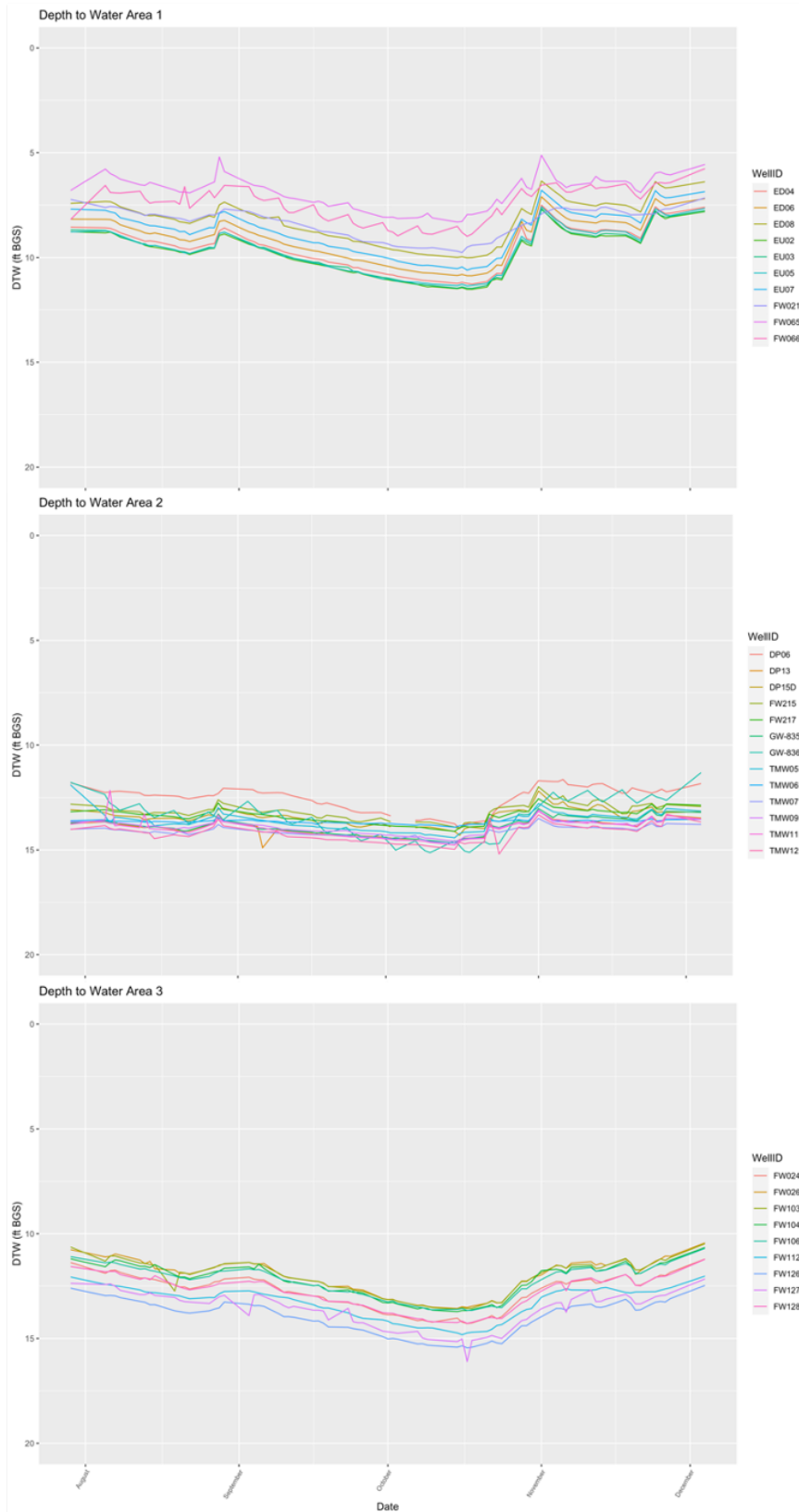


Figure C.3. Water table measurements by area.

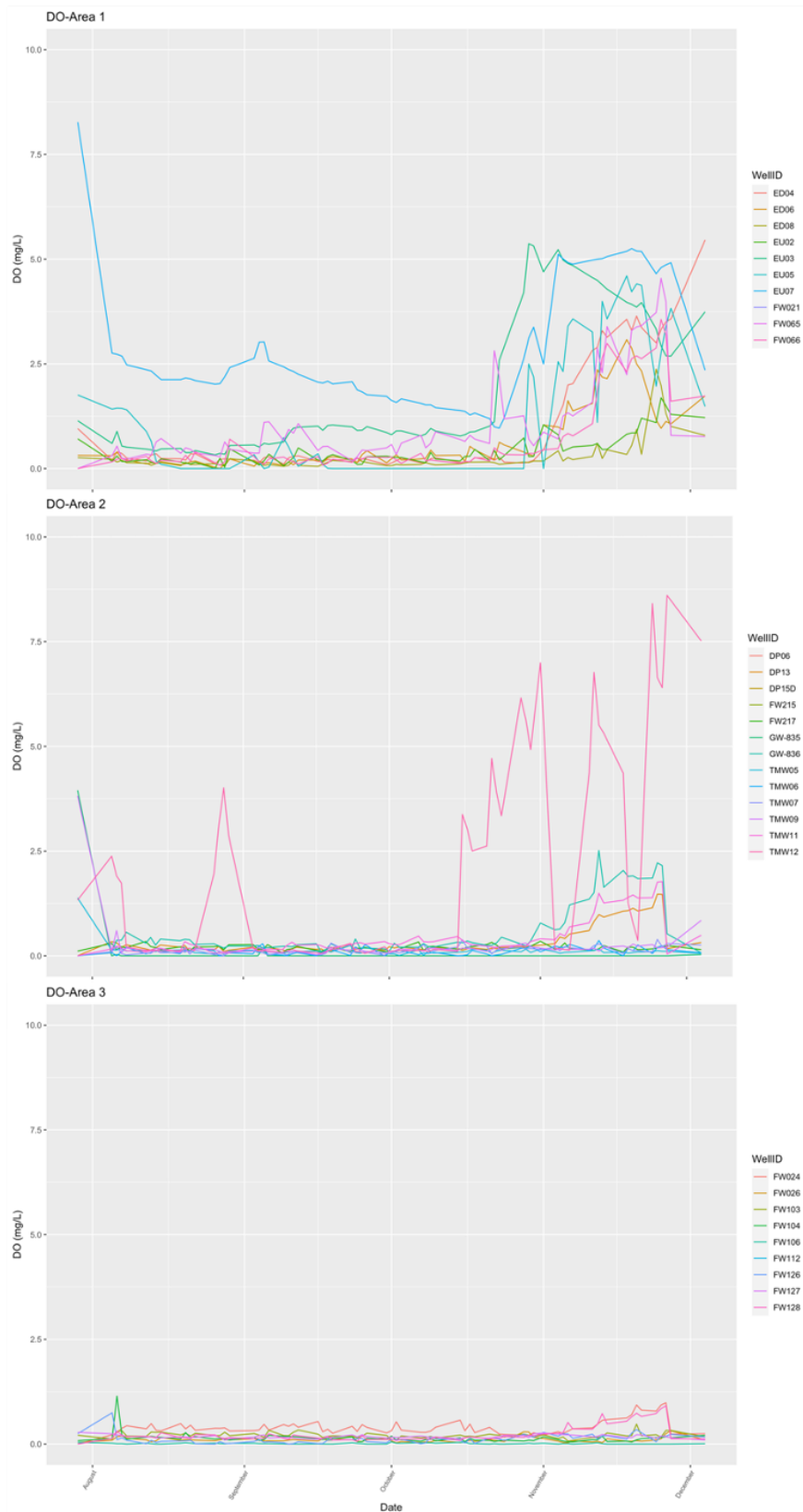


Figure C.4. In-field dissolved oxygen measurements by area.

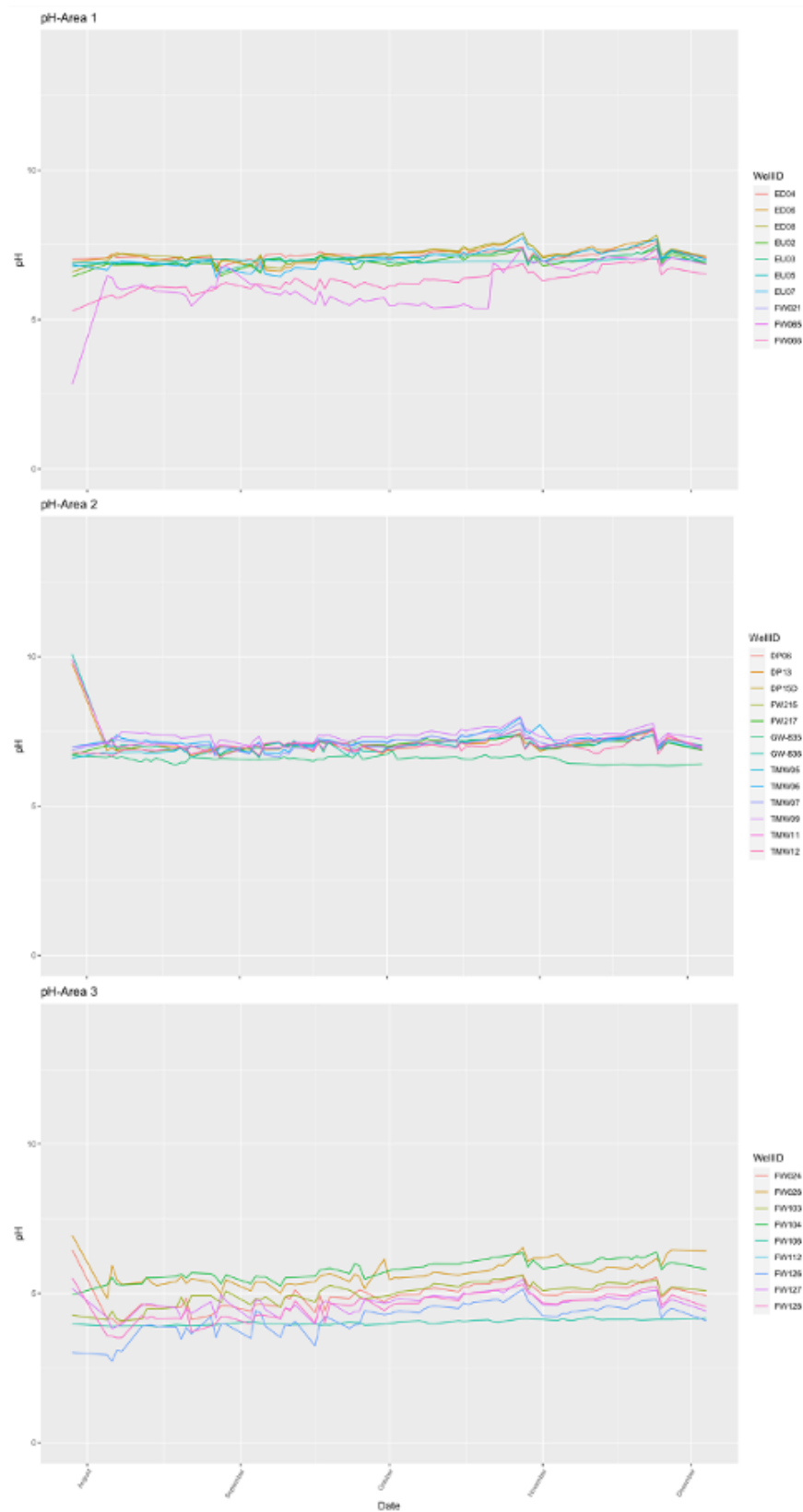


Figure C.5. In-field pH measurements by area.

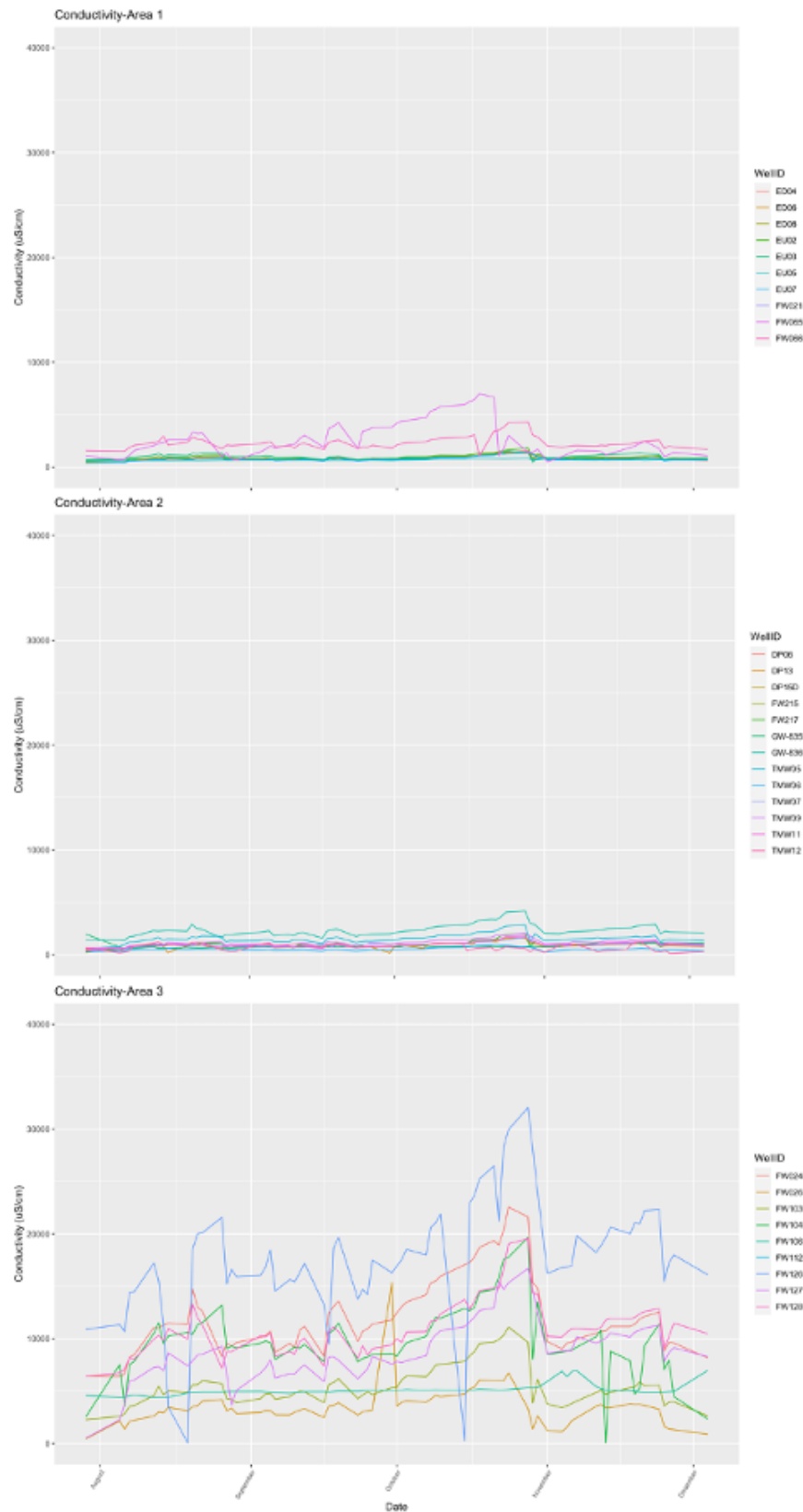


Figure C.6. In-field conductivity measurements by area.

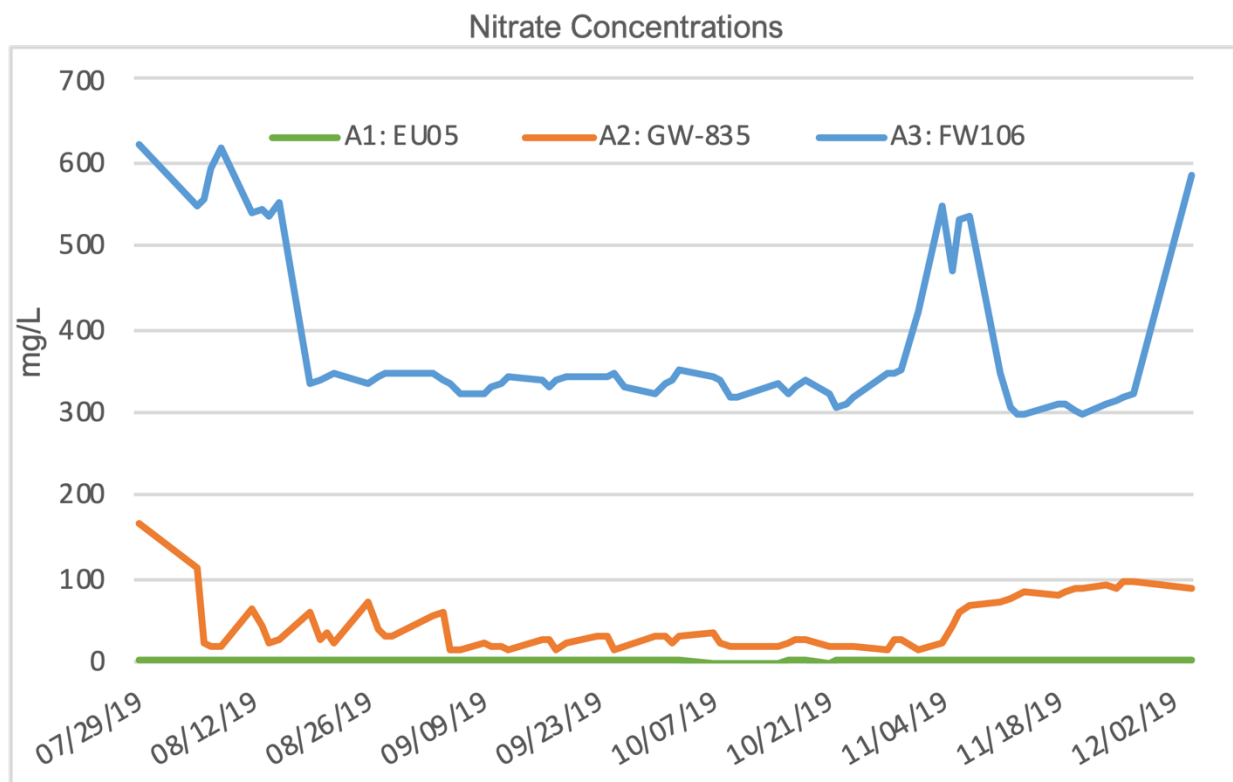


Figure C.7. In-field nitrate concentrations by microbial sampling well.

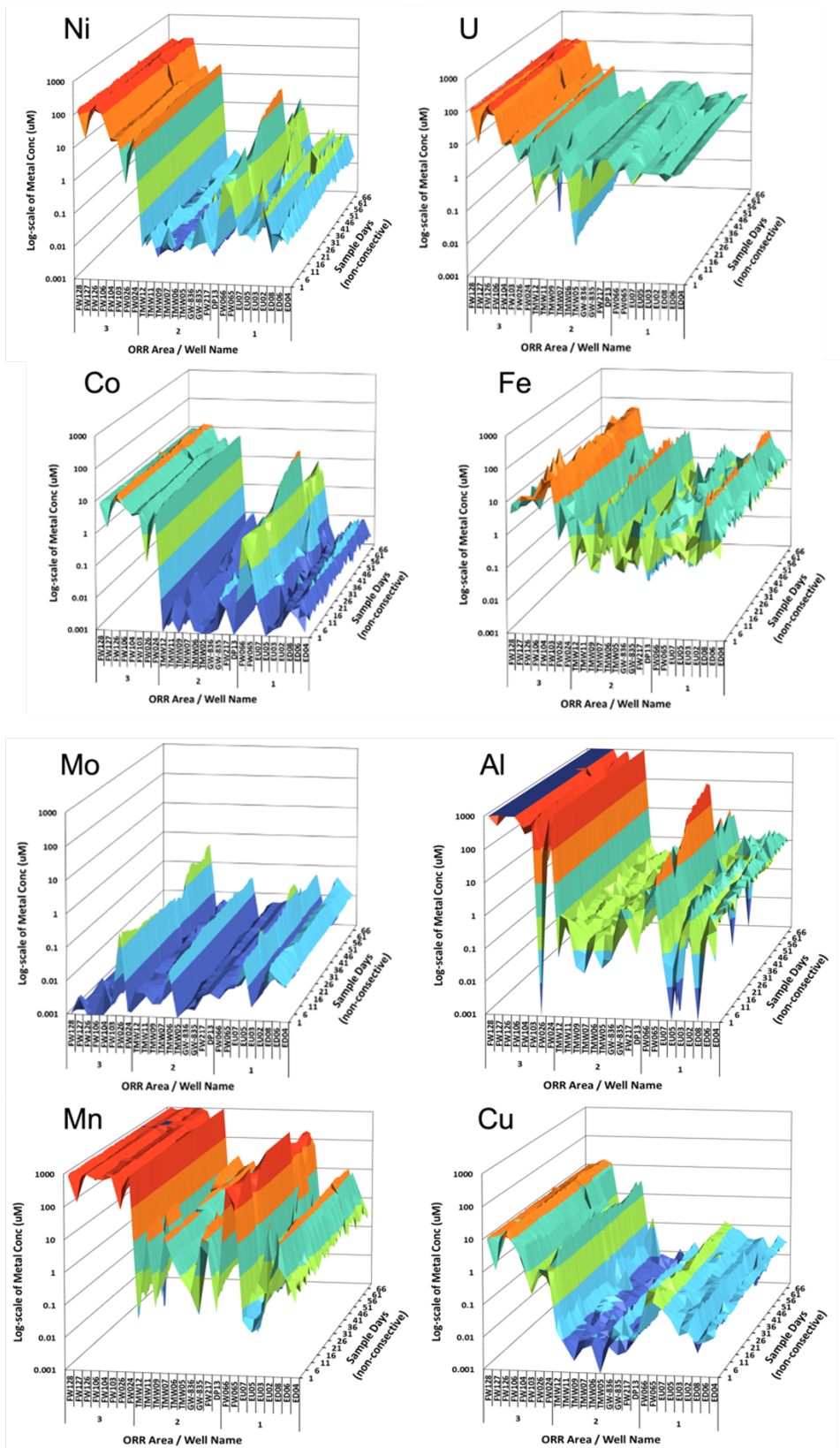


Figure C.8. Metals of interest across all wells and sampling period.

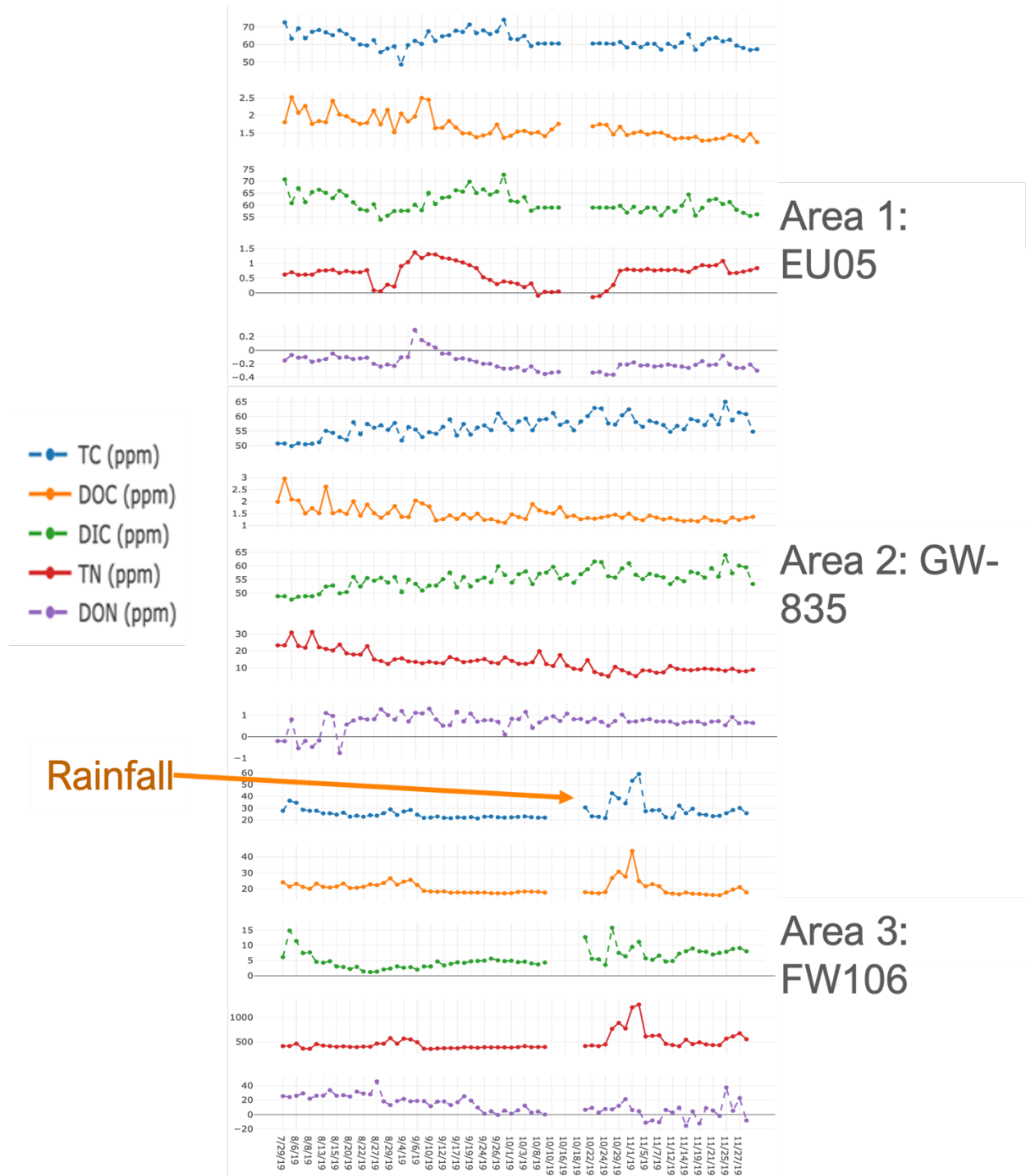


Figure C.9. TC, DOC, DIC and TN in each area.

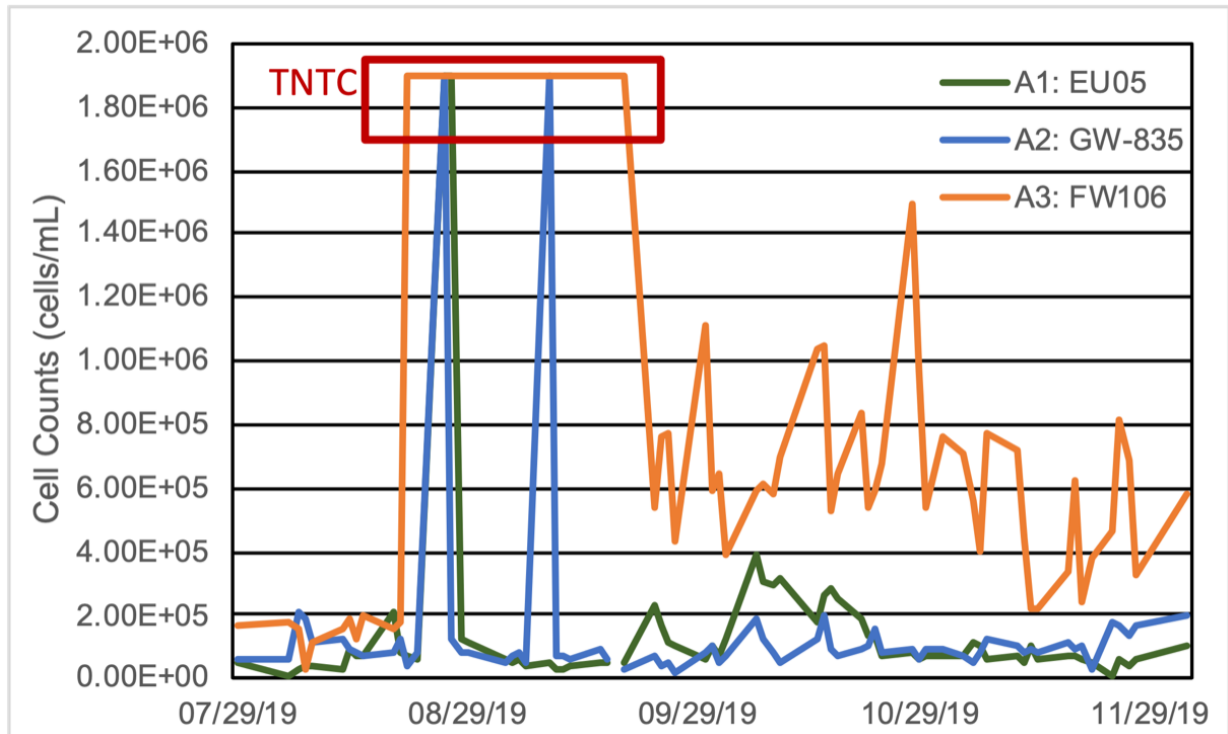


Figure C.10. Acridine Orange Direct Counts (AODC) for microbial sampling wells. Too Numerous to Count (TNTC) are denoted.

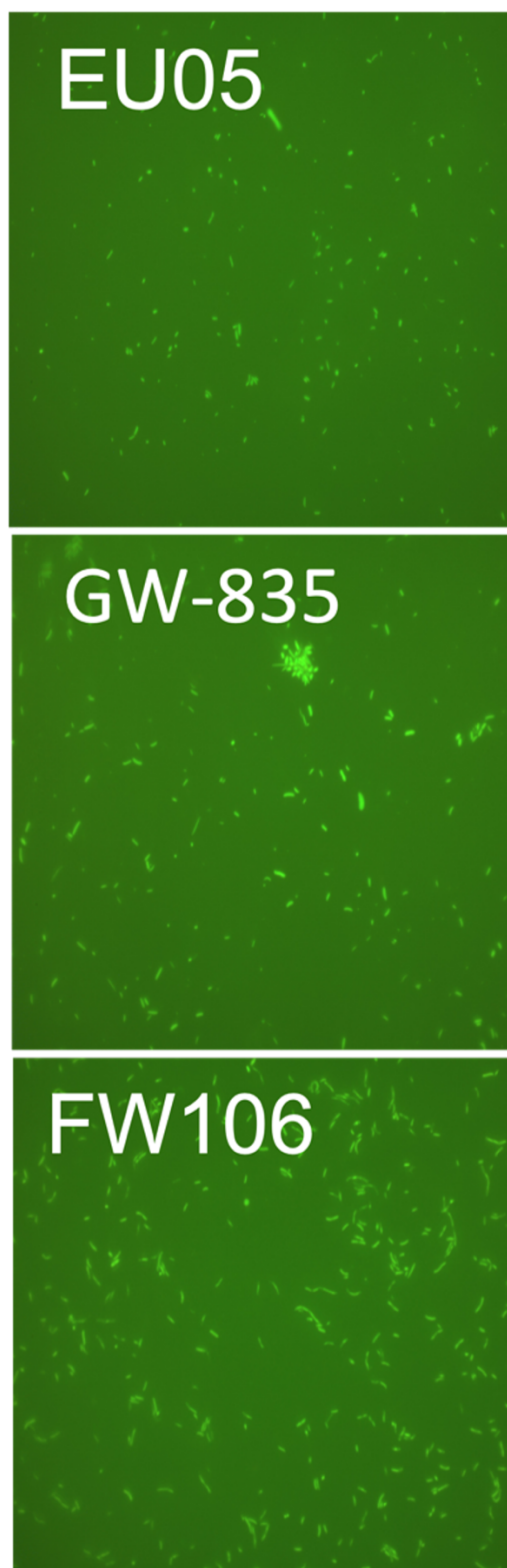


Figure C.11. Morphological examples from each microbial sampling well.

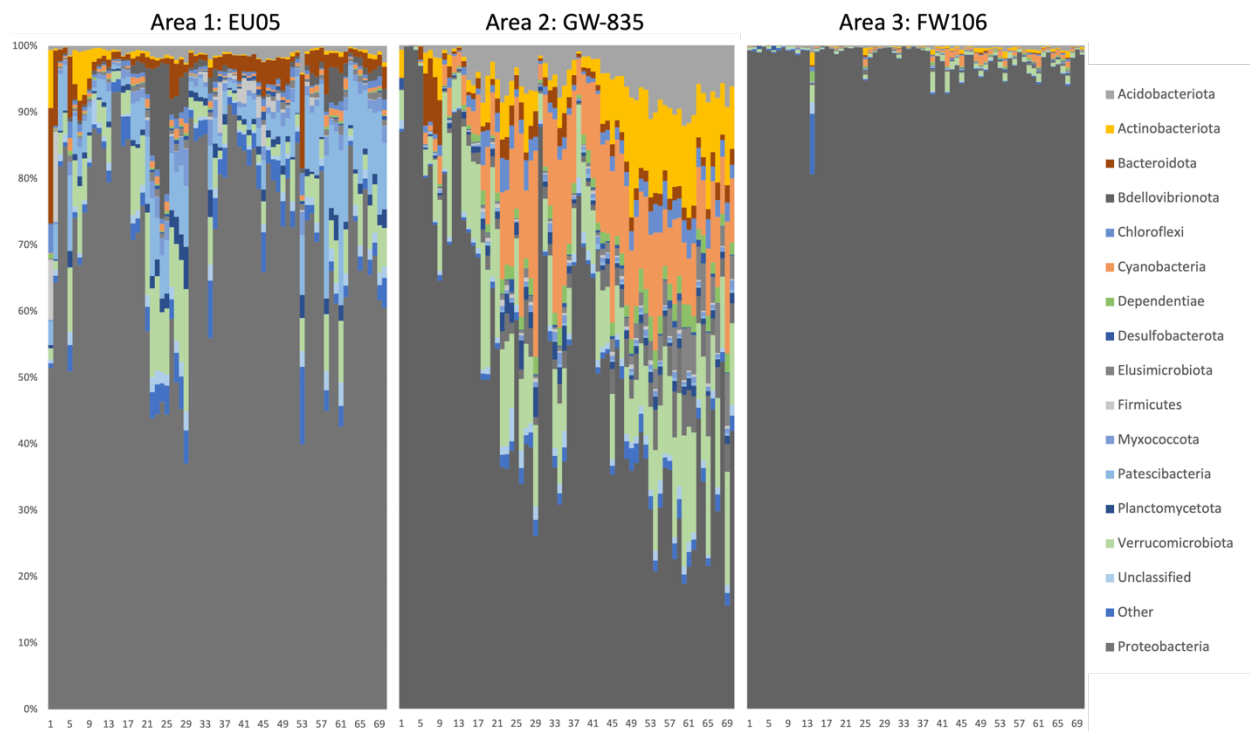


Figure C.12. Taxonomic analysis of each microbial sampling well.

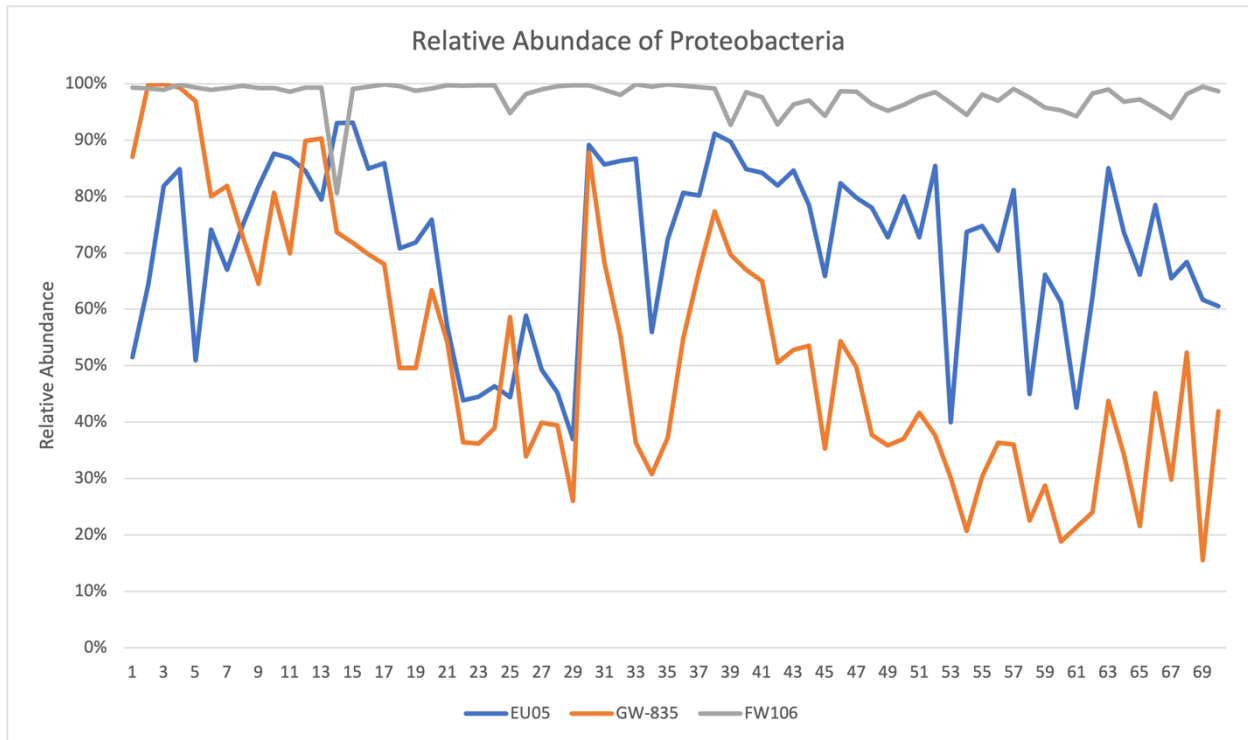


Figure C.13. Relative abundance of Proteobacteria by well.

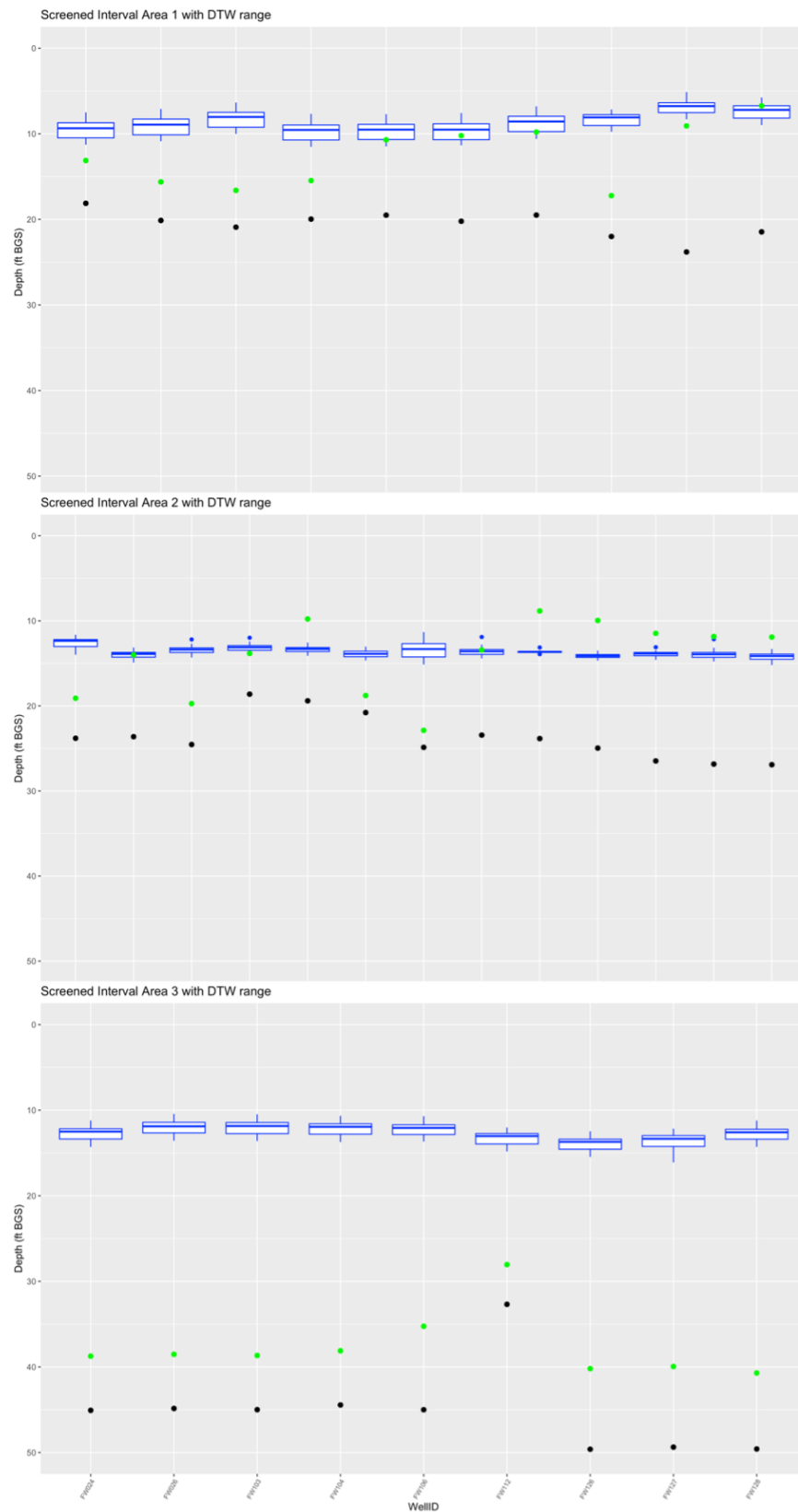


Figure C.14. Well construction data by area in relation with water table fluctuation.

Appendix D

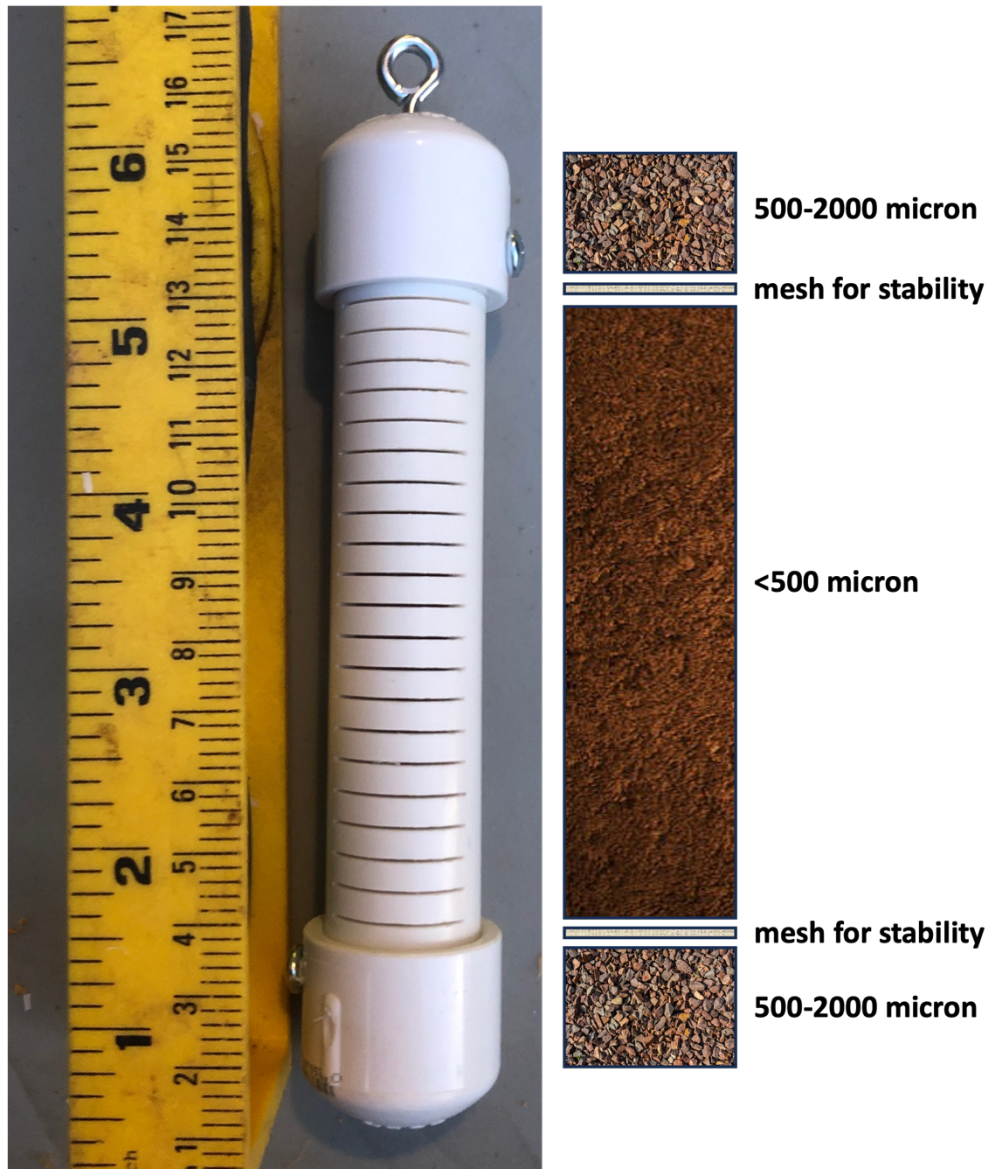


Figure D.1. Bug trap design. Utilizes local, homogenized sediment and allows the flow of groundwater to pass through the trap mimicking the subsurface terrestrial environment.

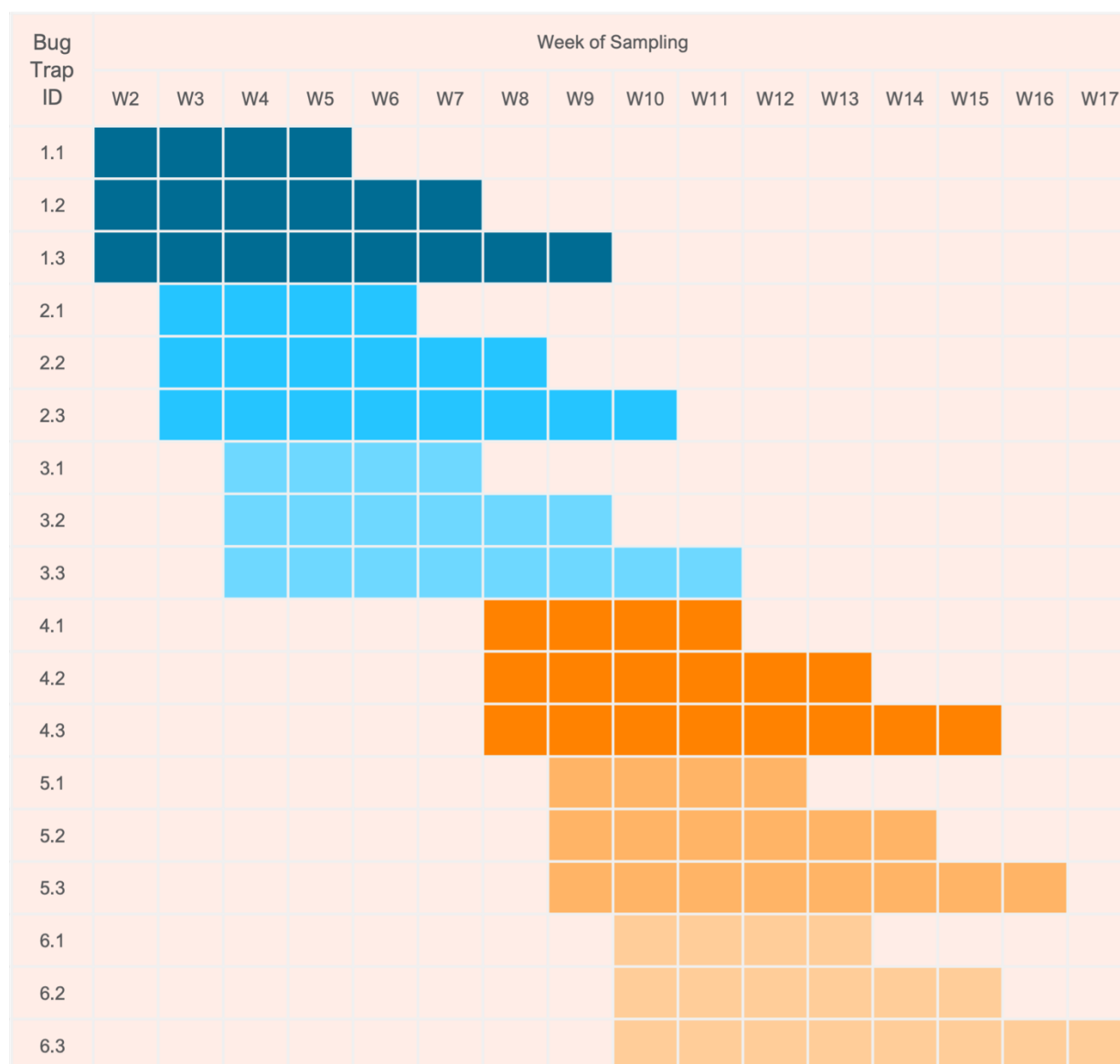


Figure D.2. Bug trap deployment scheme. Each bug trap was deployed 4, 6, or 8 weeks on a time series basis.

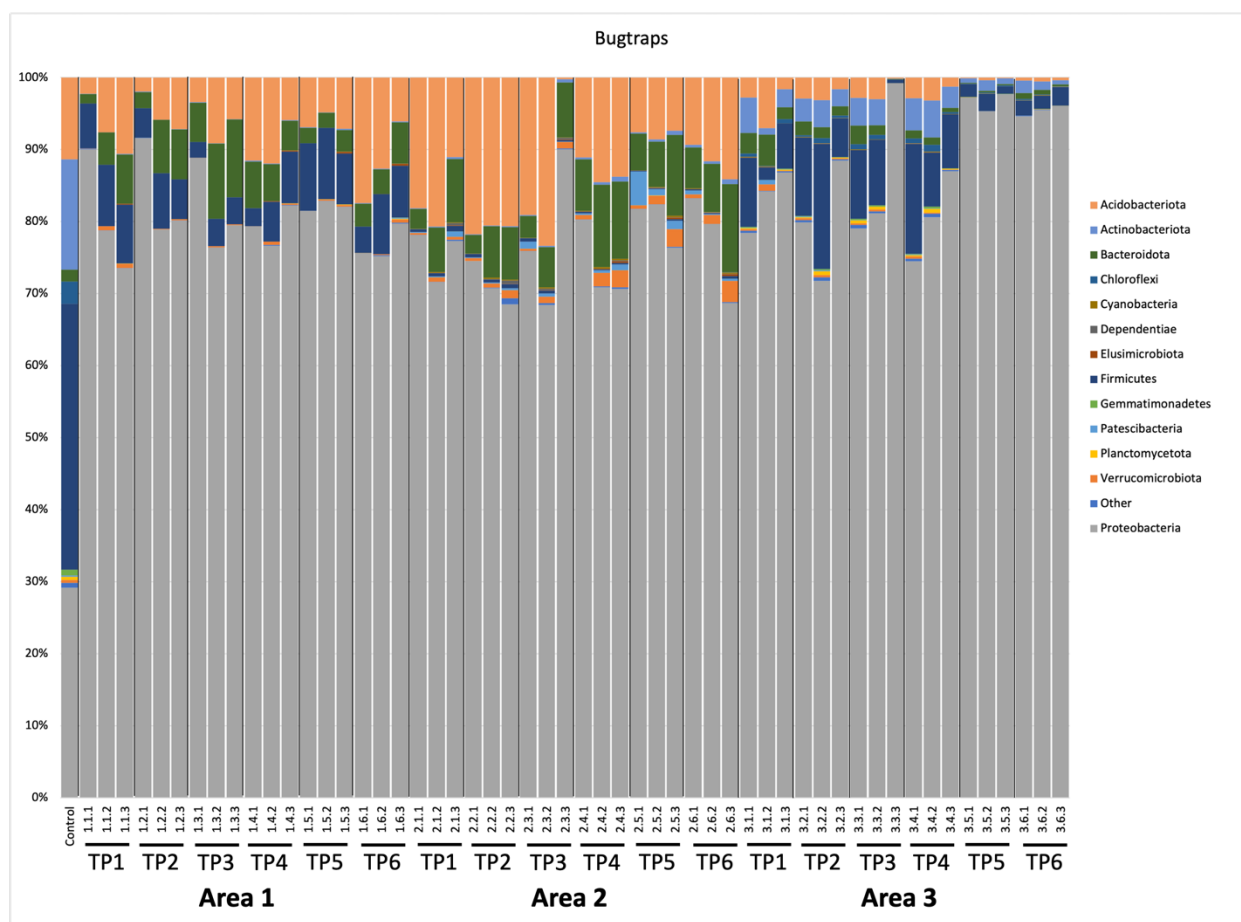


Figure D.3. Taxonomic analysis of bug trap sediment. Bug traps are grouped by area and by timepoint.

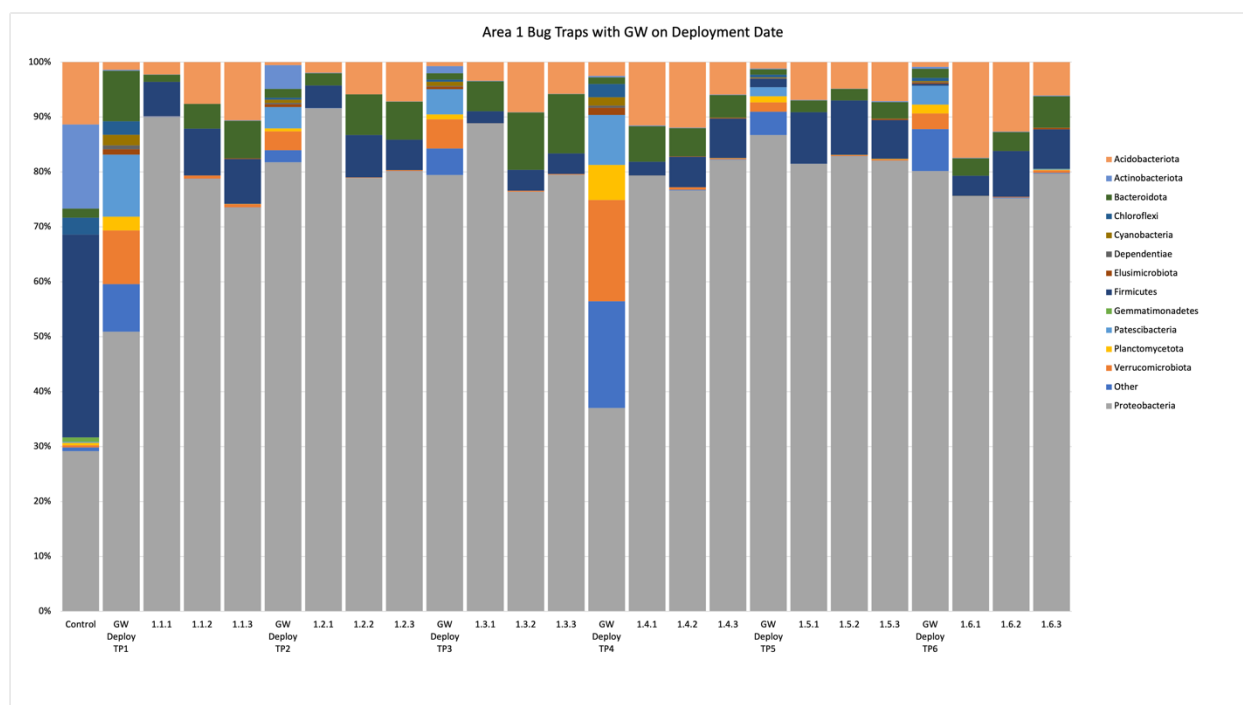


Figure D.4. Taxonomic analysis of Area 1 bug trap sediment and planktonic community. Showing taxonomic analysis of the groundwater community on the date the triplicate bug traps were deployed into the well. GW = groundwater.

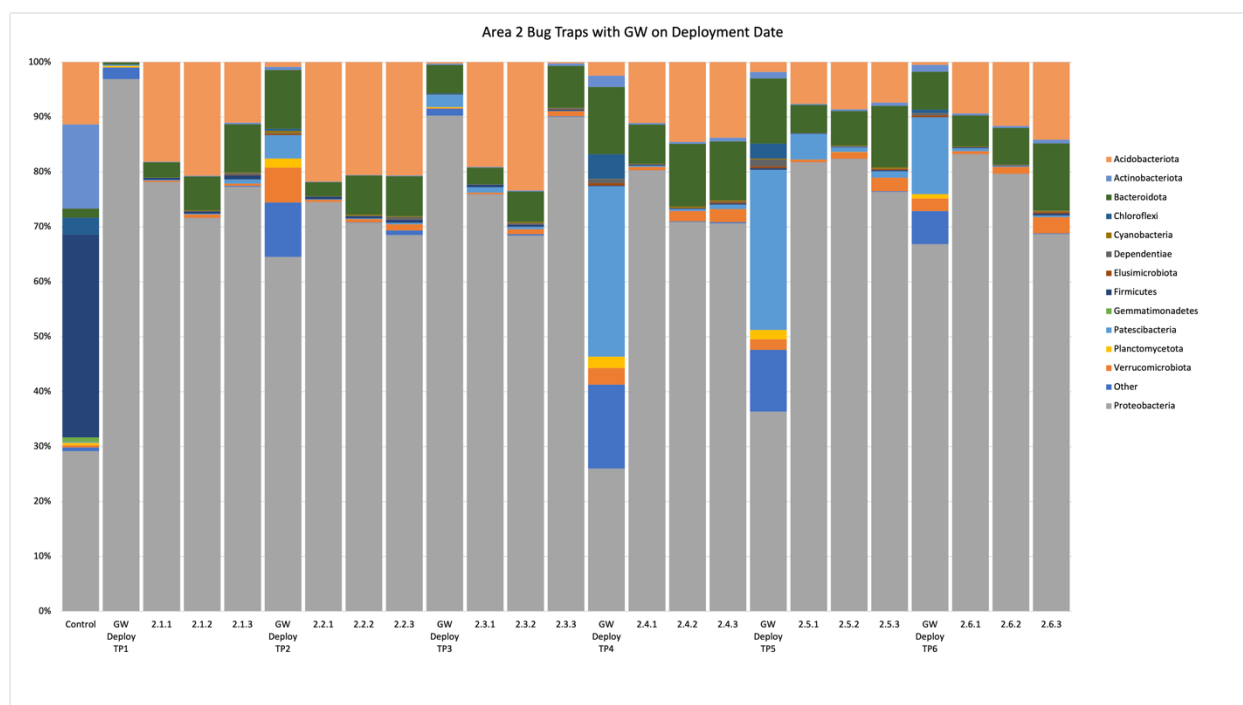


Figure D.5. Taxonomic analysis of Area 2 bug trap sediment and planktonic community. Showing taxonomic analysis of the groundwater community on the date the triplicate bug traps were deployed into the well. GW = groundwater.

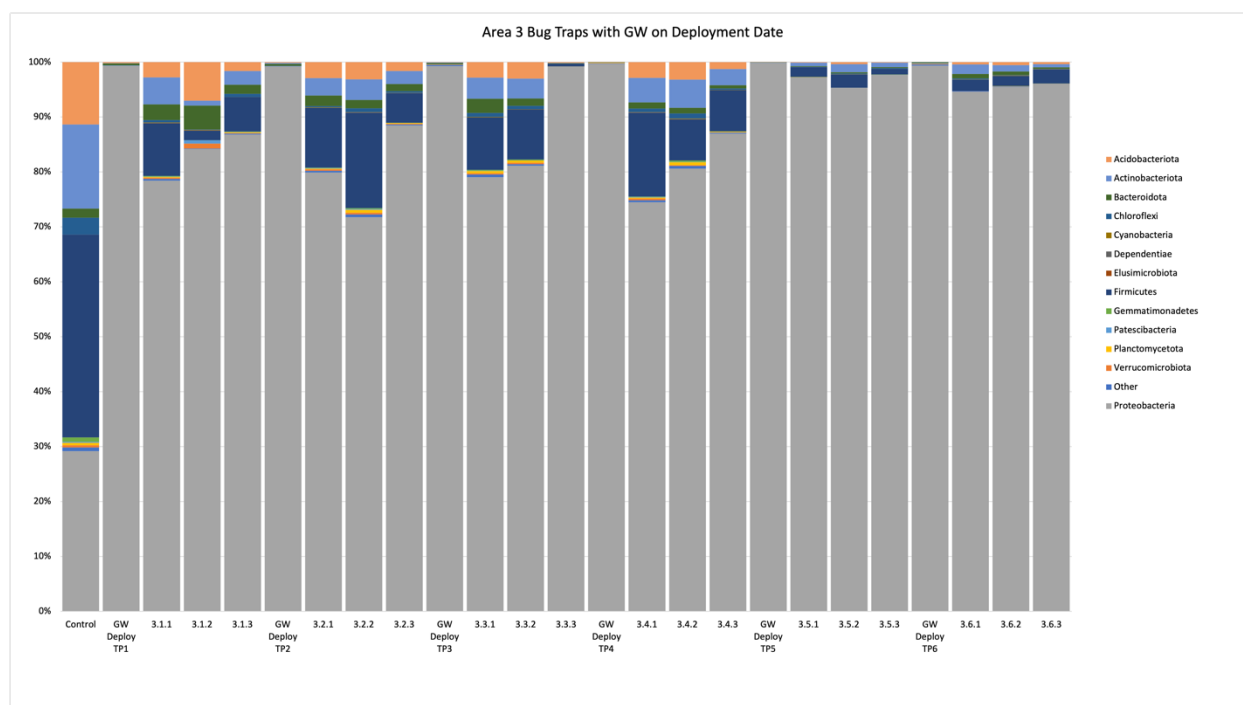


Figure D.6. Taxonomic analysis of Area 3 bug trap sediment and planktonic community. Showing taxonomic analysis of the groundwater community on the date the triplicate bug traps were deployed into the well. GW = groundwater.

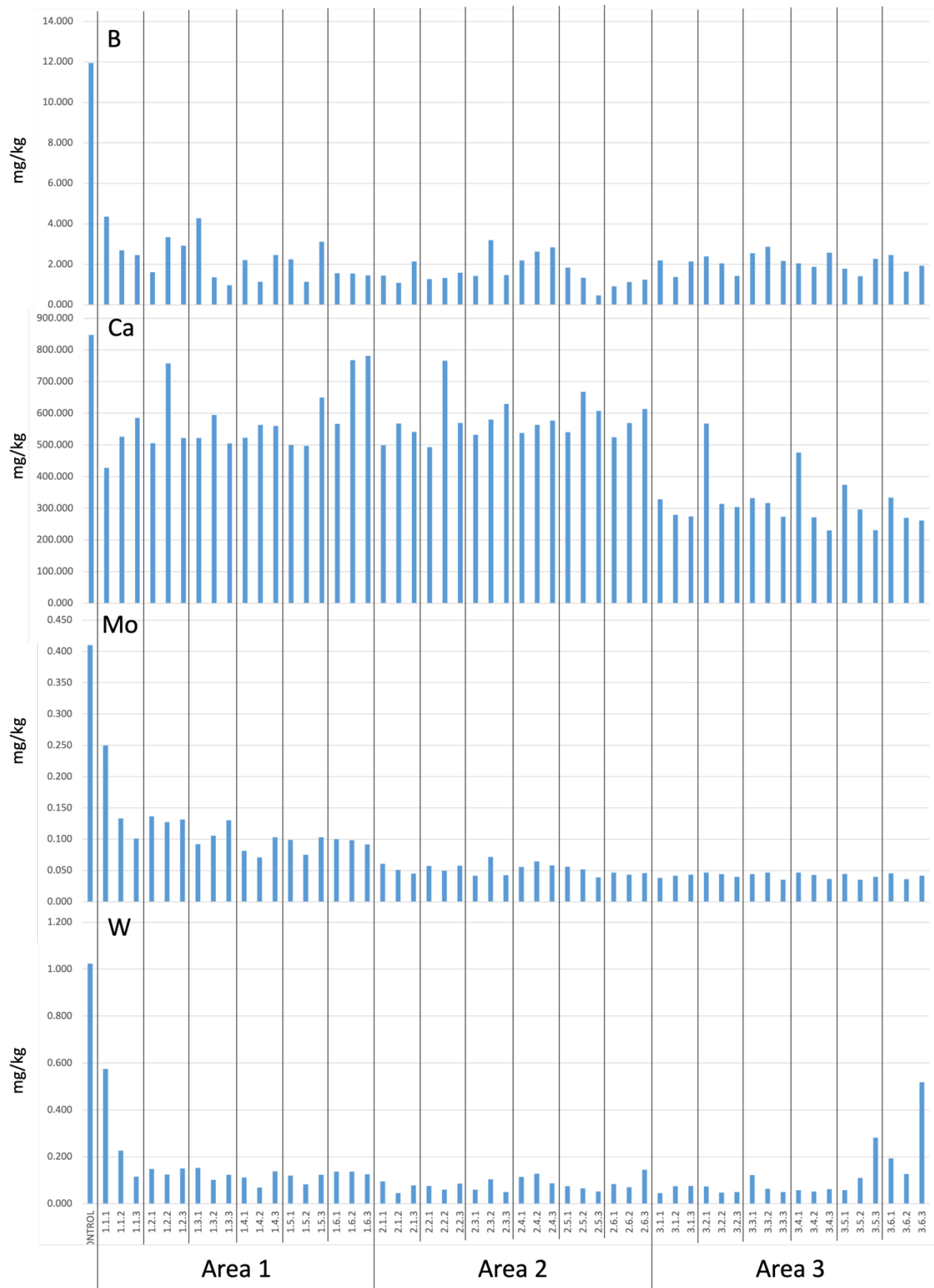


Figure D.7. Cations that decreased from starting material (control) in bug traps.

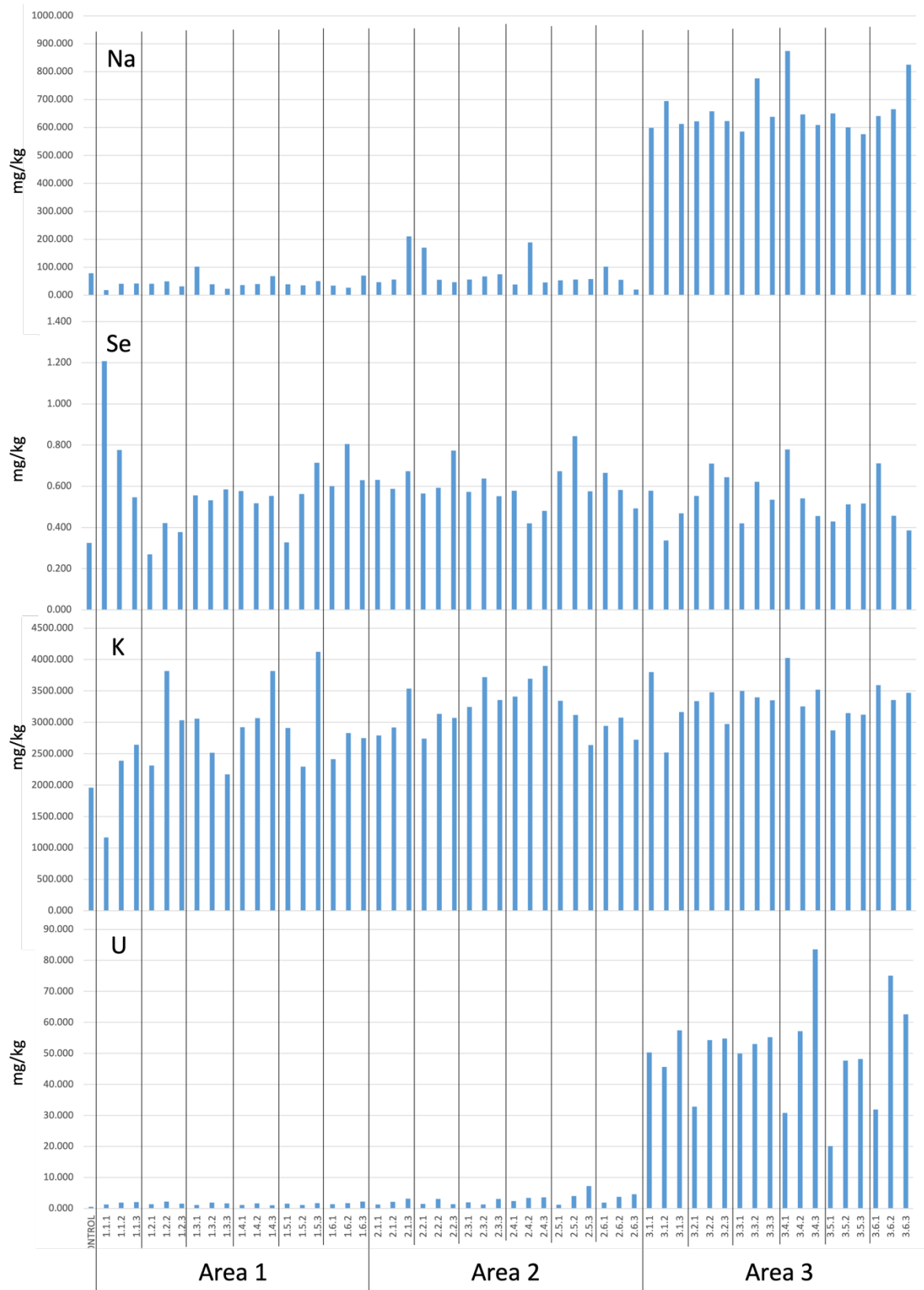


Figure D.8. Cations that increased in bug traps from starting material.

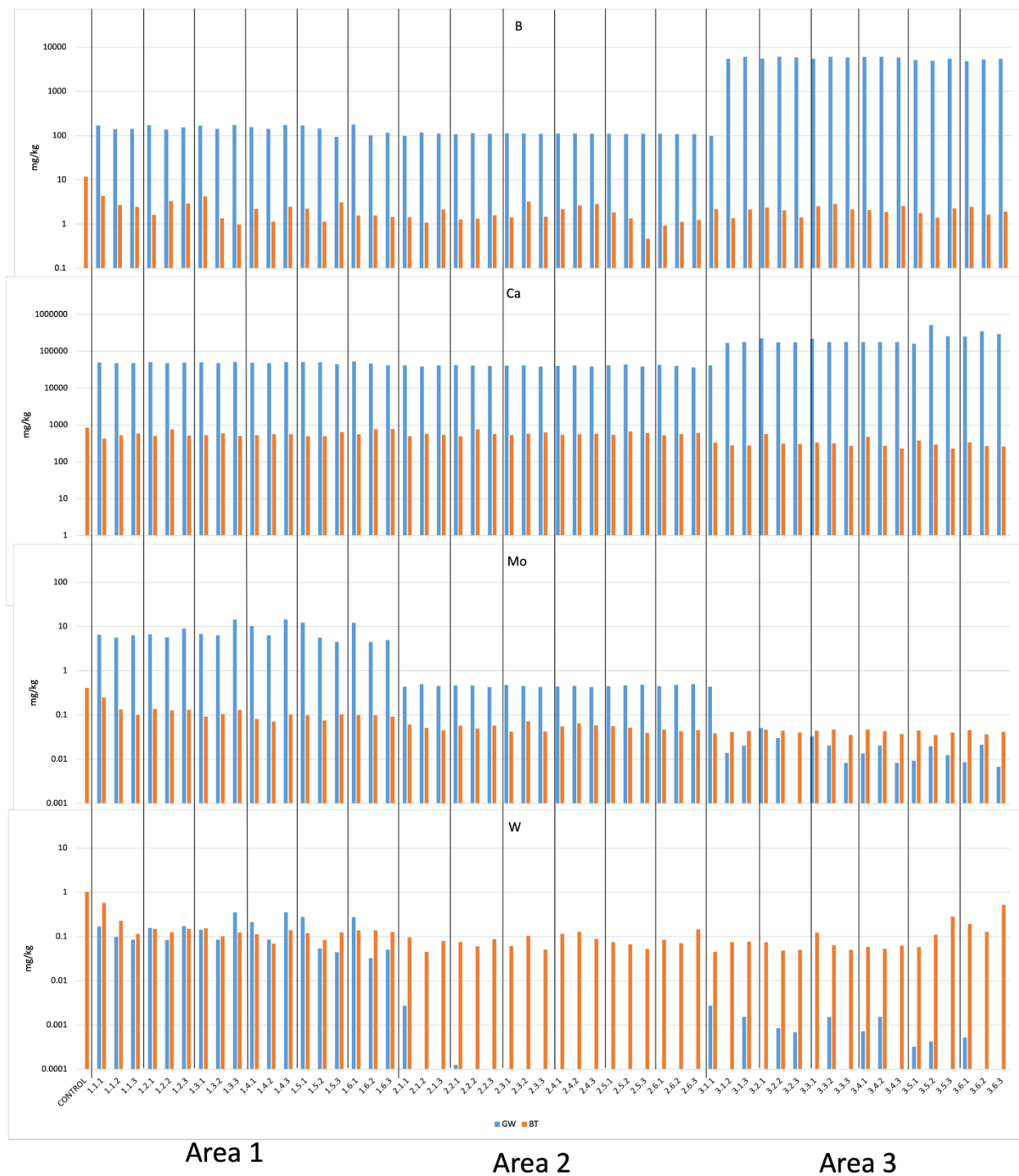


Figure D.9. Cations that decreased in bug traps compared to groundwater samples. Groundwater cation measurements were averaged over the unique incubation for each bug trap. For example, 1.1.1

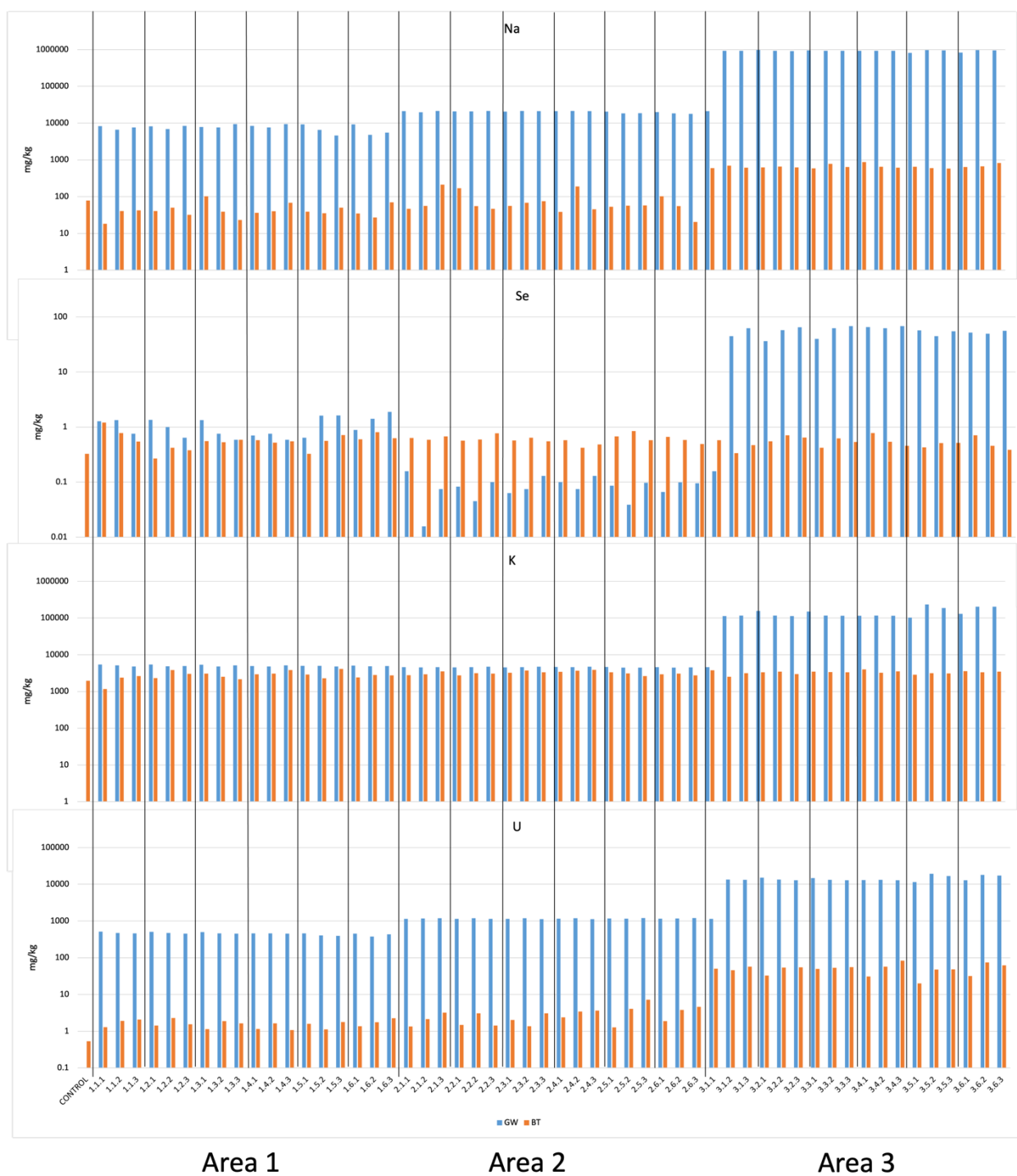


Figure D.10. Cations that increased in bug traps compared to groundwater samples.

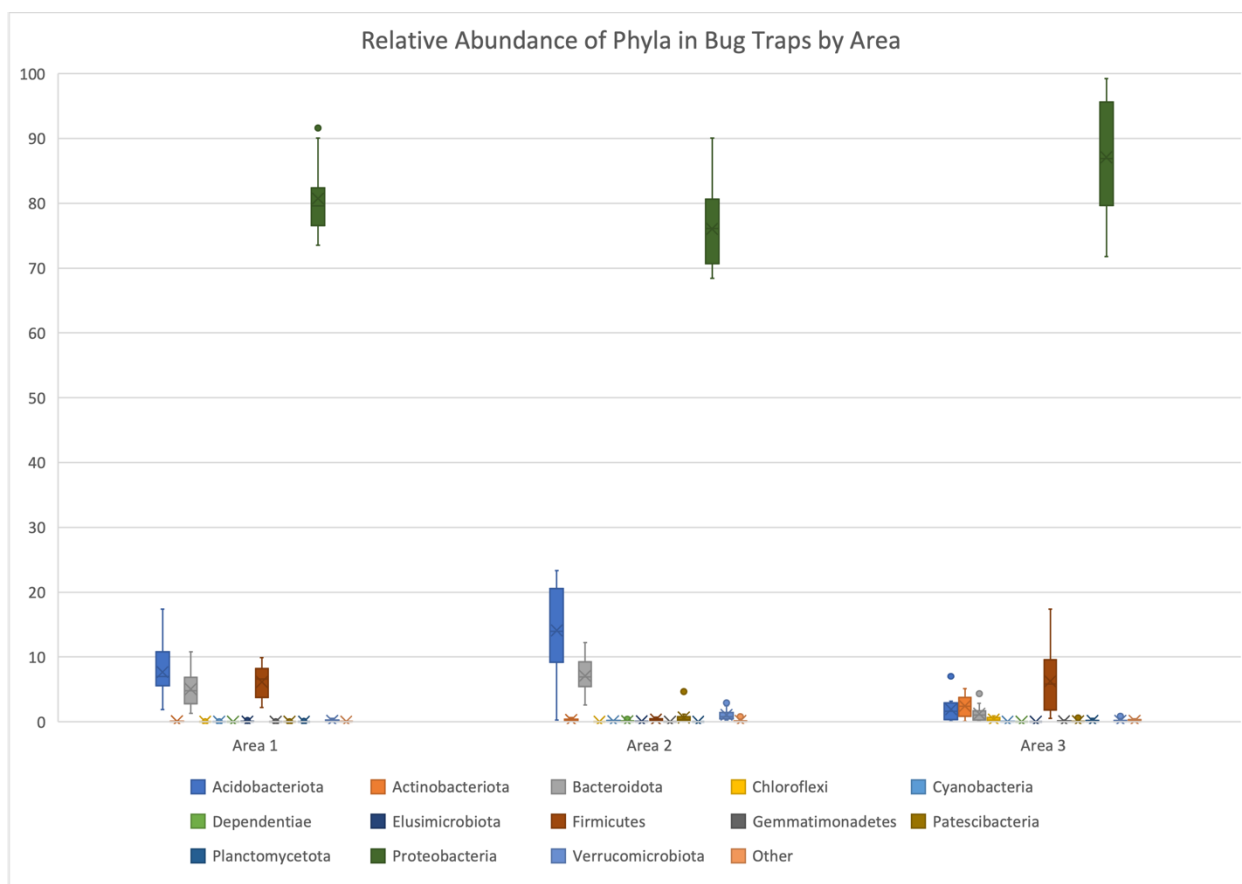


Figure D.11. Relative abundance of phyla in bug traps grouped by area.

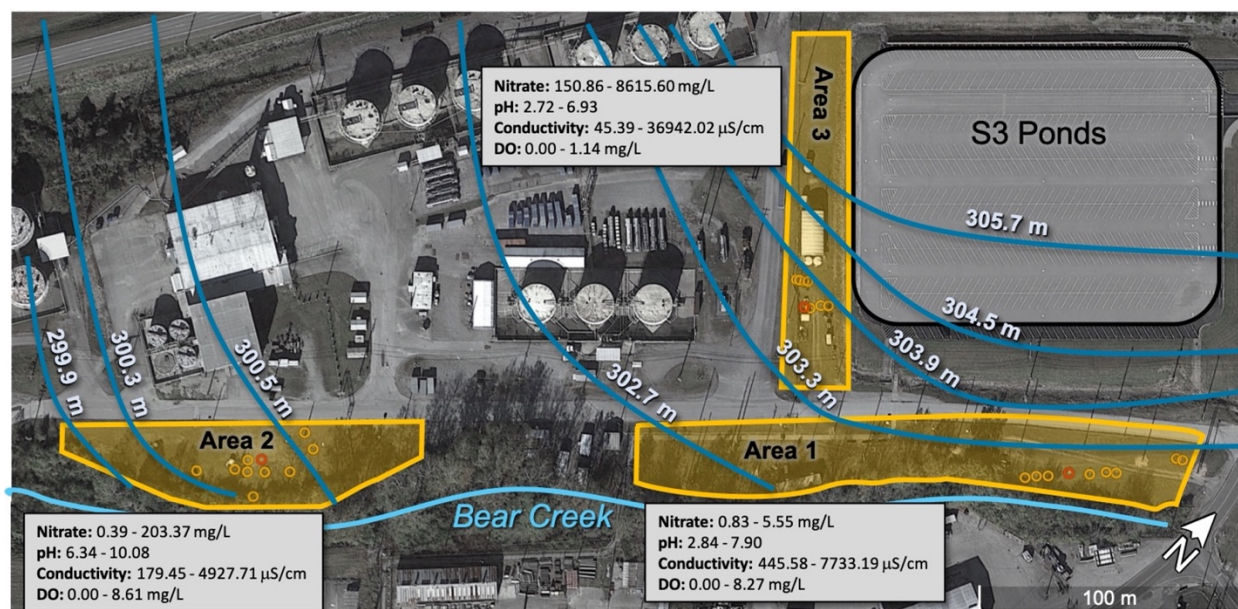


Figure D.12. Map of sample area with geochemical fluctuations.

Vita

Born and raised in Murfreesboro, Tenn., Katie traveled east to receive her bachelor's degree in microbiology from the University of Tennessee, Knoxville (UT). As an undergraduate, she participated in research with an emphasis on bioremediation, enhanced oil recovery and microbial ecology within the marine environment. She loved working in this field and realized pursuing a doctorate was the best way to continue her passion for research in bioremediation. Before beginning her graduate degree, she worked at Oak Ridge National Laboratory (ORNL) for one year as an intern. During this time, she developed a greater understanding of the research opportunities in the energy industry and the knowledge gap in the role microbial communities within this research. The Bredesen Center for Interdisciplinary Research and Graduate Education, which unites UT and ORNL for advanced research, offers a unique approach to this topic with a focus on the energy industry. She accepted a graduate position in Dr. Hazen's lab, her undergraduate mentor. Her current research is focused on applied microbial ecology with an emphasis on understanding the function and role of microbial communities in the natural attenuation and bioremediation of metals and radionuclides in contaminated groundwater and sediment. Many of her questions revolve on developing a greater understanding of how fluctuations in geochemistry and microbial communities are interconnected within these contaminated environments.

She began her research project in the Summer of 2019 with master's student Emma Dixon. They spent hours every day for 5 months collecting samples in all types of weather. From heat advisories to ice storms, they finally completed the 27-Well Survey in December 2019. Due to the COVID-19 pandemic, many samples that needed be analyzed during 2020 were delayed in shipping or processing. All samples were able to be preserved until it was safe to return to the laboratory. While waiting to get back into the lab to process samples and for data to be returned from collaborators, Katie worked with other graduate students in her laboratory to study the presence of COVID-19 in UT's campus wastewater and help analyze these data.

She is looking forward to a future career in the bioremediation or natural attenuation industry and is appreciative to all her collaborators and support system through her graduate degree.