Final Scientific/Technical Report
Subcontract Award No. 7365998

Ecosystems and Networks Integrated with Genes and Molecular Assemblies

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A final report including an abstract, and description of key objectives completed during the grant period, and publications resulting from research supported by the Department of Energy.

Final Technical Report
Montana State University Sub-Contract Award No. 7365998

I. Abstract

The ENIGMA science focus area (SFA) is a multi-disciplinary, multi-institutional research effort focused on addressing foundational knowledge gaps in environmental microbial communities by studying groundwater and sediment microbiomes in the shallow subsurface at the contaminated Oak Ridge Field Research Site (FRC). We seek to discover and characterize the reciprocal interactions between the microbial communities and the geochemical and geophysical parameters of the shallow subsurface within the contamination plume. The goal of the Fields lab is to help manage and conduct laboratory experiments informed by field observations using ecological and physiological approaches to study microbial populations *in situ* as well as *ex situ* (in the laboratory). In particular, we aim to study and characterize the impact of hydrological constraints on free-living and biofilm biomass and activity with increasing spatial and temporal resolution under static and flow conditions. We also aim to help characterize novel microbial groups that are present and active under relevant field conditions, including the development of molecular techniques for *in situ* detection as well as metabolic interactions that underlie pertinent physiology and ecology.

II. Key Objectives

For the subcontract period 2017 to 2022, MSU had three main areas to conduct laboratory work: 1. *Field Process Characterization*, 2. *Interactions of Field Relevant Organisms under Porous Media-flow Conditions*, and 3. *Predictive Understanding of Microbial Interactions*. Our work is summarized under each section below.

1. Field Process Characterizations – Compare and contrast community composition, dynamics, and general microbial activity for chosen background and contaminated sites at the FRC for the bulk-aqueous and soil phases. We plan to focus on three transition zones: vadose to capillary; capillary to saturated; and into the saturated. In conjunction with ENIGMA collaborators, we will focus on the impacts of DO, pH, and conductivity on nitrate-reducing and sulfate-reducing populations driven by the turnover of NOM (natural organic matter). We will use developed techniques in my lab (radio-label incorporation, BONCAT, and stable isotope probing) to identify active, interacting populations from the field site.

The terrestrial, shallow subsurface is a complex and microbially active habitat located beneath the top-most surface soil layers, comprised of sediments, rocks, gas, porewater and groundwater. In terms of DOE research, subsurface environments contain a large diversity of microorganisms under low nutrient conditions that significantly impacts the carbon, nitrogen, phosphorus, sulfur, and mineral cycles. Typically, subsurface environments contain less labile organic matter (OM) compared to surface soils, and the degree of connectivity to surface waters (*e.g.*, rivers, streams, precipitation) can vary considerably. Despite the importance of groundwater for consumption, agriculture, and industry, the role of microbial communities in the maintenance of groundwater ecosystems is not fully understood, particularly for sites impacted by human activity. Understanding microbial community structure and function within the subsurface is critical to assessing overall quality and maintenance of groundwater; however, the factors that determine

microbial community assembly, structure, and function in groundwater systems and their impact on water quality remains poorly understood.

On an ecosystem scale, there is limited information regarding the exact relationship between microbial populations (active and transient), environmental parameters, and biogeochemical processes between groundwater and subsurface porous media that can help explain the distribution of microbial biomass and activity that ultimately impacts the fate and transport of nutrients and contaminants of interest. Our work with the ENIGMA field team has focused on aspects of chosen sites at the Oak Ridge-Field Research Center (OR-FRC) at the Y-12 Complex. The OR-FRC contains 'shallow' freshwater subsurface environments (mainly porous/granular) which typically have higher rates of groundwater flow as well as have a high degree of connectedness with the surface and are impacted by mixed wastes (*e.g.*, organic and inorganic including radionuclides). These shallow, subsurface environments are common across DOE sites that are impacted by a wide array of contaminants that have detrimental impacts on human and environmental health. For example, DOE spends ~$6B/year managing and cleaning up DOE superfund sites. Yet the roles of microbes in the subsurface at these sites are still poorly understood and underexploited.

Traditionally, the shallow subsurface can be separated into three distinct zones based on moisture content in relationship to water table location termed the vadose, capillary fringe and saturated zones. The vadose zone represents the upper most boundary of the subsurface, the capillary fringe exists at the interface of the saturated and vadose zone and is highly dependent upon fluctuations of the local water table, and the saturated zone (i.e., at/below water table) consists of porous material and voids filled with water. Generally, the direction of water flow in the saturated zone can be 3-dimensional depending on hydraulic gradients and porous media properties (*e.g.*, clay lenses).

With respect to the subsurface environments dictated by water content and the impact on microbial communities, much attention has been given to the water table position and sediments transported in the saturated and capillary fringe zones. At least for the OR-FRC, nutrients and contaminants of interest tend to be highest at these boundaries, and therefore, the associated constraints and the impacts on the microbial communities are of high interest. The transitional boundary between the vadose and saturated zones can experience drastic changes in geochemical parameters (*e.g.*, pH and dissolved oxygen), particularly during rain events, and thus, the impacts on microbial activity in terms of geochemical cycling is a target for modeling in order to enable prediction for the fate and transport of nutrients and contaminants in subsurface environments.

It has become increasingly apparent that microbial populations have distinct physiologies and functions in the shallow subsurface but the potential relationships between biotic and abiotic parameters of the ecosystem are not well understood, particularly hydrogeochemical parameters across the different zones at fine enough resolution. Our work has sought to synthesize these knowledge gaps and provide field data to some of these questions.

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Impact of hydrologic boundaries on microbial planktonic and biofilm communities in shallow terrestrial subsurface environments. Subsurface environments contain a large proportion of planetary microbial biomass and harbor diverse communities responsible for mediating biogeochemical cycles important to groundwater used by human society for consumption, irrigation, agriculture and industry. Within the saturated zone, capillary fringe and vadose zones, microorganisms can reside in two distinct phases (planktonic or biofilm), and significant differences in community composition, structure and activity between free-living and attached communities are commonly accepted. However, largely due to sampling constraints and the challenges of working with solid substrata, the contribution of each phase to subsurface processes is largely unresolved. Here, we synthesize current information on the diversity and activity of shallow freshwater subsurface habitats, discuss the challenges associated with sampling planktonic and biofilm communities across spatial, temporal and geological gradients, and discuss how biofilms may be constrained within shallow terrestrial subsurface aquifers. We suggest that merging traditional activity measurements and sequencing/-omics technologies with hydrological parameters important to sediment biofilm assembly and stability will help delineate key system parameters. Ultimately, integration will enhance our understanding of shallow subsurface ecophysiology in terms of bulk-flow through porous media and distinguish the respective activities of sessile microbial communities from more transient planktonic communities to ecosystem service and maintenance.

> Smith, H.J., A.J. Zelaya, K.B. De León, R. Chakraborty, D.A. Elias, T.C. Hazen, A.P. Arkin, A.B. Cunningham and M.W. Fields. 2018. Impact of hydrologic boundaries on microbial planktonic and biofilm communities in shallow terrestrial subsurface environments. *FEMS Microbiol. Ecol*. 94:fiy191

Microbial populations can change over days time-scale between and within wells in uncontaminated groundwater. Three shallow wells (FW301, FW303, FW305) in a noncontaminated shallow aquifer in the ENIGMA-Oak Ridge Field Research Center (Oak Ridge, TN) were sampled approximately 3 times a week over a period of three months to measure changes in groundwater geochemistry and microbial diversity. It was expected that the sampled microbial diversity from two historic field wells (FW301, FW303) would be relatively stable, while diversity from a newer well (FW305) would be less stable over time. The wells displayed some degree of hydrochemical variability over time unique to each well, with FW303 being overall the most stable well and FW301 being the most dynamic based upon dissolved oxygen, conductivity, and nitrate. Community analysis via ss-rRNA paired-end sequencing and distribution-based clustering revealed higher OTU richness, diversity, and variability in groundwater communities of FW301 than the other two wells for diversity binned over all time points. Microbial community composition of a given well was on average > 50% dissimilar to any other well at a given time (days), yet functional gene diversity as measured with GeoChip remained relatively constant. These results indicated that up to half of a microbial community could change within a couple of days, likely related to hydrogeochemical changes at the local scale. In addition, despite high turnover in microbial populations, the overall metabolic functions of the entire community would not change significantly which indicated some degree of functional redundancy in terms of microbial functions. Similarities in community structure across wells were observed with respect to the

presence of 20 shared bacterial groups in all samples in all wells, although at varying levels over the tested time period. Similarity percentage (SIMPER) analysis revealed that variability in FW301 was largely attributed to low abundance, highly-transient populations, while variability in the most hydrochemically stable well (FW303) was due to fluctuations in more highly abundant and frequently present taxa. Additionally, the youngest well FW305 showed a dramatic shift in community composition towards the end of the sampling period that was not observed in the other wells, suggesting possible succession events over time. Likewise, these results suggested local scale effects between wells likely related to a combination of heterogeneous flow and geochemistry. Despite these differences, time-series analysis of all three wells using vector auto-regressive models and Granger causality showed unique relationships between richness and geochemistry over time in each well, highlighting local-scale effects that can be tested in the laboratory and the field. These results indicate temporally dynamic microbial communities over short time scales, with day-to-day population shifts in local community structure influenced by available source community diversity and local groundwater hydrochemistry.

Zelaya, A.J., A.E. Parker, K.L. Bailey, P. Zhang, J. Van Nostrand, N. Daliang, D.A. Elias, J. Zhou, T.C. Hazen, A.P. Arkin, and M.W. Fields. 2019. High spatiotemporal variability of bacterial diversity over short time scales with unique hydrochemical associations within a shallow aquifer. *Water Res*. 164:114917

Microbial activity is higher in sediments than associated groundwater for shallow pristine and contaminated aquifers. In order to differentiate active and non-active populations between groundwater and sediments, we used developed BONCAT techniques combined with traditional radio-isotopes to estimate active cell numbers in two sediment cores and corresponding groundwater. Within the different sediment environments, microorganisms typically reside in two distinct phases (planktonic or biofilm), and significant differences in community composition, structure and activity between free-living and attached communities are commonly accepted. However, largely due to sampling constraints and the challenges of working with solid substrata, the respective contributions of groundwater (planktonic) and sediment-associated (biofilm) cells to subsurface processes is largely unresolved. In order to directly compare the distribution of microbial biomass and activity in a shallow, subsurface environment, total cell numbers, translationally-active cell numbers (Bioorthogonal non-canonical amino acid tagging- BONCAT), and microbial activity (^{3}H-Leucine incorporation) were investigated for a low biomass pristine and contaminated groundwater and corresponding soil cores. Soil samples were analyzed as homogenized 22.8 cm sections, and groundwater was filtered (0.2 and 0.1 mm). In addition, recent work has shown the existence of ultra-small bacteria (100-300 nm) in groundwater but no work has confirmed *in situ* activity, and the ultra-small bacteria could display very different mass transport and activity distributions in porous media flow. In order to directly assess microbial activity levels between groundwater and sediments, ^{3}H-leucine uptake was compared between sediment samples from four depths that crossed the water table and compared to corresponding groundwater. The groundwater activity was assessed with a 0.2 filter fraction. The results demonstrated that cell numbers for the 0.2 mm fraction were approximately an order of magnitude higher for the pristine groundwater compared to the contaminated groundwater (10^6 v. 10^5). Cell numbers for the small fraction (0.1 mm fraction) were also at least an order of

magnitude higher for the pristine well compared to the contaminated groundwater (10^4 v. 10^2). When contaminated groundwater was compared to the pristine, there was a drastic reduction in the BONCAT activity and the contaminated groundwater was between 100-700-fold less. Additionally, the rate of leucine incorporation (^{3}H-leucine) on a per cell basis in pristine groundwater was up to 1,000 times greater than the contaminated groundwater, respectively. For pristine groundwater activity, the small cell fraction (0.1 mm) made up almost 20% of total BONCAT activity (per ml), and the small cell fraction had roughly 3-fold greater activity on a per cell basis. Overall, like total cell numbers, activity was lower (both per volume and per cell) in contaminated groundwater compared to pristine groundwater. In pristine soil, activity (^{3}H-leucine) displayed steep gradients of microbial activity in association with transition zones of water table height (*i.e.*, vadose, capillary fringe, saturated). A similar trend was also observed for the contaminated soil; however, the contaminated soil displayed an overall gradient of decreasing activity with depth. The highest activity for pristine soil was 9,253 ng C/g/d located in the transition depth between the capillary fringe and the saturated zone. Conversely, the highest activity for the contaminated soil was 9,175 ng C/g/d located in the vadose zone, perhaps the zone that is least impacted by contaminant flux. The pristine groundwater had higher activity rates than pristine sediment (per cell), but the contaminated groundwater had slower activity rates than the contaminated sediment (per cell). However, for both pristine and contaminated samples on a per volume basis, sediments had the vast majority of microbial activity compared to groundwater (80-95%). This is one of the first quantitative comparisons between corresponding groundwater and subsurface sediments, and the results suggest that field sampling schemes should consist of both groundwater and sediments in order to capture the distribution of microbial activities (higher activity but fewer cells in the groundwater versus slower activity but more cells in the sediments – on a per volume basis).

> Smith, H.J., I. Miller, D. Joyner, K. Walker, T.C. Hazen, and M.W. Fields. Microbial activity is higher in sediments than associated groundwater for shallow pristine and contaminated aquifers (manuscript in preparation) (abstract submitted to Battelle Bioremediation Symposium, May 2023).

Interactions of field relevant organisms under porous media-flow conditions – We will design and validate reactor systems to simulate tortuous flow through a controlled particle bed, and then characterize microbial ecology and physiology that impacts larger scale processes. Currently, we plan to utilize flat plate reactors and pack column reactors with different sized particles (beads) to control flow, porosity, and permeability in order to understand the effects of flow and mass transport on microbial activity and population distribution. Reactors will be run with field material (groundwater and/or soil) as well as chosen field isolates.

Deterministic processes contribute to sediment biofilm assembly from dynamic groundwater communities. Subsurface environments are a heterogeneous matrix where the distribution of microorganisms across solid:aqueous interfaces is poorly understood. Surrogate sediments (SS) were incubated within a shallow porous aquifer in Oak Ridge, TN, and the community structure of local sediment-associated communities were compared with groundwater (GW) communities.

Community analysis of local SS and surrounding GW diversity via paired-end sequencing of SSU rRNA gene sequences and distribution-based clustering revealed higher richness, diversity, and variability in GW communities compared to SS communities. Both GW and SS communities were largely dominated by Proteobacteria; however, the relative proportions of specific taxa changed across GW:SS boundaries. Intra-well sediment biofilms displayed reproducible community structure across replicates and were distinct from GW. Community structure of inter-well sediment biofilms was distinct, with samples from each borehole harboring distinct OTUs. Null model simulations indicated that deterministic selective pressures were the dominant factor structuring SS communities in tested wells. In contrast, GW communities over time were structured by a combination of deterministic and stochastic factors. The results presented here indicate that differences in community structure within the subsurface are influenced by the available source community in combination with deterministic and stochastic assembly processes that contribute differently across groundwater and sediment fractions.

> Anna J. Zelaya, Daliang Ning, Albert E. Parker, Kathryn L. Bailey, Ping Zhang, Joy Van Nostrand, Dwayne A. Elias, Jizhong Zhou, Adam P. Arkin, and Matthew W. Fields. Deterministic processes contribute to sediment biofilm assembly from dynamic groundwater communities. (manuscript in preparation)

Development of packed-bed reactor and determination of microbial biomass distribution. Microorganisms have the potential to change the geochemical properties of the shallow terrestrial subsurface, and previous studies have uncovered significant roles that microorganisms can play in groundwater biogeochemical processes. While much attention given to the shallow terrestrial subsurface has been focused on the effects of contamination and how microorganisms function in these systems, knowledge of the distribution of microbial biomass and activity related to hydraulic properties is less understood. In this study, an up-flow packed bed reactor (PBR) was designed to emulate select field conditions (*i.e.*, flow rate, nutrient conditions, and particle size) at the Oak Ridge National Laboratory-Field Research Center (ORNL-FRC) and observe microbial biomass and activity distribution in a micropore environment. The PBR contained a porous medium of silica oxide particles (74-300 μm), and the size range was based upon particle size assessment of sediment material from the ORNL-FRC. The water phase of the system was a basal groundwater medium that contained low levels of sugars, amino acids, and nucleosides/nucleotides as the C and N sources that were based upon metabolomic characterization of sediment extracts. The inocula for the PBRs consisted of sediment material in samplers that were incubated down-well and retrieved from three FRC wells each at a distinct pH (4, 6.3, or 7). Following 4 months of incubation in the PBRs, biomass, cell concentrations, cell distribution, and microbial community analysis for each reactor were evaluated. The pH 4 reactor had the highest biomass and activity but had the lowest diversity amongst the pH conditions. The two circumneutral reactors (pH 7 and pH 6.3) both had lower biomass concentrations and activity but had microbial communities that were more diverse than pH 4. Methods were also developed to enable the embedding and sectioning of an intact core from the PBRs, and this allowed visualization of cell localization within the porous medium. The reactors showed different trends in how microbial biomass was distributed through the porous medium as well as distances to other

cells and/or cell aggregates. The measured distances were also compared to substrate concentrations over distances predicted by a model based upon diffusion coefficients for compound classes (*i.e.*, sugars, amino acids, nucleotides/nucleosides). Overall, the data and predictions demonstrated that under *ex situ* conditions meant to emulate porous media flow (*e.g.*, porosity, flow, particle size) at the ORNL-FRC, cells that are part of a diverse microbial community can be on average 15 to 50 µm apart with an average of 2 to 16 cells/particle. Based on diffusivity of potential substrates and measured distance ranges between cells, sugar levels could be approximately 5 to 20 µM whereas amino acids and nucleotides/nucleosides would be sub-micromolar between nearest cell/aggregate neighbors.

K. Massey, H.J. Smith, H. Dreesbach, S. Altenburg, Katie Walker, L.J. McKay, Y. Fan, Y. Fu, J.D. Van Nostrand, J. Zhou, T.C. Hazen, K. Davis, A.B. Cunningham, and M.W. Fields. Simulation of shallow subsurface flow conditions in packed-bed reactors to enrich surface-associated populations and estimate cellular distribution. (Manuscript in preparation).

Characterization of circularized Ultramicrobacteria (UMB) MAGs. Current circularized MAGs (Lui and Nielsen) were investigated for biochemical capacity, and the characterized bins included: Bin_14-Parcubacteria; Bin_57-Microgenomates; Bin_78-Microgenomates; Bin_110-Microgenomates; and Bin_120-Parcubacteria. The maximum likelihood phylogenomic analysis based on concatenated alignments of 16 ribosomal proteins from circularized UMB genomes demonstrated recovery of 3 members of the Microgenomates (2 Gottesmanbacteria and 1 Woesebacteria) and 2 members of the Parcubacteria (1 Wolfebacteria and 1 from the unclassified RIF21 clade). While genome lengths remained relatively short (0.6 – 1.0Mb), estimated completeness based on single copy gene detection ranged between 76-86%. Metabolic features were reconstructed from circularized UMB genomes and revealed incomplete pathways including glycolysis and TCA. Various UMB members had the potential for using a variety of sugars as starting points for glycolysis including fucose, rhamnose, mannose, glucose and fructose, interestingly these are common sugars in Gram-negative LPS molecules. All UMB populations had a complete PPP which some members may use to bypass missing steps in glycolysis. Four of the five members encoded complete ATP synthase components. UMB genomes were also scanned for extracellular features related to motility/adhesion/biofilm formation and compared to electron micrographs of cell structure in collaboration with Peter Walian. Putative sortases, filament apparati, S-layer proteins, and components of the Type IV Pilus system were distributed across the circularized UMB genomes with sortases only occurring in the Microgenomates and one S-layer protein only associated with the Parcubacteria (RIF21 clade). The presence of sortases (part of the pilus assembly machinery) in the Microgenomates could be representative of the observed “Morphotype 1” (P. Walian) with a very long, thin pilus structure. In contrast, the deduced S-layer protein detected in the RIF21 group is reminiscent of the observed “Morphotype 2”(P. Walian) which has a thick layer surrounding the ultra-small cell.

McKay, L., P. Walian, L. Lui, T. Nielson, H.J. Smith, A.P. Arkin, and M.W. Fields. Potential metabolic capacity and extracellular structures of uncultivated ultramicrobacteria in groundwater. (manuscript in preparation).

Fluorescent microsphere beads as abiotic controls for dispersion. We attempted to repeat breakthrough curve experiments with fluorescent microsphere beads (0.1 µm and 1 µm) that would act as a model for small solutes and cells flowing through a porous media. However, the FACS instrument could not accurately count the fluorescent microspheres at low concentrations and the spheres appeared to interact (stick) with each other as well as the sand bed in the PBRs. Sand cores from the packed bed reactors were embedded with epoxy to image the microspheres beads within the porous media, but the plastic of the microspheres beads was degraded by the epoxy, thus, the microspheres beads could not be imaged.

Development of Deuterium Isotope Probing (DIP). Over the last year we have developed the in-house capacity at MSU to quantify the rate of isotopic uptake of individual microbial cells. Much of our efforts have been focused on deuterium isotope probing (DIP) to determine activity of single cells. DIP is based on the incorporation of D_2O into fatty acids. Specifically, the reductive steps of fatty acids synthesis lead to an intact retention of the three deuterium labels, providing a rapid and strong signal for activity. Incorporation of D_2O into microbial biomass changes the cellular Raman spectrum of lipids and results in a pronounced C-D peak, and our preliminary data has shown the microbial incorporation of D_2O for individual cells. DIP measurements have high sensitivity to detect differences in activity on a per cell basis and have high technical reproducibility (standard deviations <1%). In addition to D_2O incorporation we have successfully detected incorporation with Raman for ^{15}N and ^{13}C labeled compounds.

Predictive understanding of microbial interactions – We will continue to characterize fundamental bacterial – archaeal interactions in biofilm with the model system of SRB and a methanogen and transfer techniques to field isolates. We will build from transcriptomic and proteomic work to delineate the mix of cooperative and competitive interactions that impact syntrophic metabolisms.

Potential amino acid sharing in an interdomain biofilm optimizes electrogenic flow for CO_2 consumption. Symbiosis is widespread throughout the biosphere with well-studied examples in and across all three domains of life, and the syntrophy between sulfate-reducing bacteria (SRB) and methanogenic archaea is of interest because both guilds play crucial roles in many different anaerobic environments that link the carbon and sulfur cycles. *Methanococcus maripaludis* S2 and *Desulfovibrio vulgaris* Hildenborough can form a syntrophic mutualism when grown in the absence of sulfate. In order to better understand the interactions between *M. maripaludis* and *D. vulgaris*, RNA-Seq was used to create a transcriptomic profile of the co-culture biofilm as compared to the planktonic mono- and co- culture states to demonstrate unique expression and behavior in the biofilm state. Transcriptomic analysis indicated that most differential expression for co-culture biofilm compared to planktonic monocultures occurred in *M. maripaludis* with key

steps in methanogenesis down-expressed for *M. maripaludis* and electron transfer related genes were down-expressed for *D. vulgaris* Hildenborough. Many of the up-expressed genes for both populations included hypothetical proteins but also included cell surface modifications, communication via small metabolites, N-cycling, and metal homeostasis. Insight into the interactions of this anaerobic syntrophy was also explored using stoichiometric modeling. Predictions for potential amino acid exchange in the two populations identify alanine, cysteine, glycine, and serine as integral to decreasing the hydrogen or carbon dioxide requirement in *M. maripaludis*, and these amino acids are estimated to have low metabolic cost for *D. vulgaris* to produce. These results highlight unique gene expression and predicted metabolite sharing for the interdomain biofilm and indicate that the biofilm growth mode is both phenotypically and physiologically unique most likely due to specialization of lactate-oxidation in *D. vulgaris* and CO_2-reduction in *M. maripaludis* coupled to amino acid sharing that minimizes reduced electron flow to anabolic reactions in *M. maripaludis*. In order to better understand the metabolic coordination between a bacterium and archaeon in biofilm, we used deuterium and BONCAT labeling to compare levels of protein synthesis and turnover.

> Laura B. Camilleri, Kristopher A. Hunt, Aurélien Mazurie, Jennifer Kuehl, Alex Michaud, James Connolly, Egan Lohman, Zack Miller, Adam M. Deutschbauer, and Matthew W. Fields. Potential amino acid sharing in an interdomain biofilm optimizes electrogenic flow for CO_2 consumption. (Manuscript in preparation).

Activity partitioning in an archaeal-bacterial biofilm. In monocultures, only *D. vulgaris* Hildenborough readily forms biofilm; however, the co-culture biofilm is evenly interspersed with *M. maripaludis*, and is thicker and filled with topographical features such as ridges, spires, and valleys. To better understand the respective levels of activity during biofilm interactions between *M. maripaludis* and *D. vulgaris* Hildenborough, deuterium-labeled proteomics and BONCAT microscopy were used to delineate activity states of the two biofilm populations. Deuterium-labeled proteins were observed in both populations, and the *D. vulgaris* labeled proteins were enriched for carbon oxidation and electron transfer while the *M. maripaludis* proteins were strongly enriched in carbon dioxide processing and methane generation. Interestingly, BONCAT labeling was observed for both organisms grown as monocultures; however, under co-culture biofilm conditions, only *D. vulgaris* was detected to be BONCAT active, yet methane was being actively produced. The data suggest that during co-culture growth, *D. vulgaris* and *M. maripaludis* have altered levels of respective cellular activity that results in streamlined specialization with respect to lactate-oxidation and methane-generation. The results suggest that an interdomain mutualistic biofilm partitions activity to optimize carbon processing and energy conservation and could have implications for bacterial-archaeal interactions that have evolved at the lower limits of thermodynamic energy conservation.

> Laura B. Camilleri, B.P. Bowen, C.J. Petzold, T.R. Northen, and M.W. Fields. Activity partitioning in an archaeal-bacterial biofilm. (manuscript in preparation)

Develeopment of a BONCAT-metagenome technique for environmental samples. We used a novel "activity-based metagenomics" technique to enrich and identify genomes of the translationally

active microbial community enriched *in situ* in a field site in Montana (site was used for ease of sampling during COVID). High-quality metagenome assembled genomes (MAGs) were recovered for a presumptive acetoclastic methanogen, *Methanothrix paradoxum*, and a presumptive novel member of the Chlorobi with the potential to generate acetate via the phosphotransacetylase-acetate kinase (Pta-Ack) pathway. Potential members of the *Geobacter* and Bacteroidetes also encoded potential acetate-producing/-consuming enzymes. All four genomic populations ('*Methanothrix paradoxum* PRB', 'Chlorobi PRB', '*Geobacter* PRB', and 'Bacteroidetes PRB') encoded several enzymes for the degradation of aromatic hydrocarbons, including ethylbenzene, phenylphosphate, phenylethanol, toluene, xylene, and phenol. Metabolic reconstructions, gene analyses, and environmental parameters indicated these populations may experience significant redox fluctuations that promote facultative energy metabolisms in the subsurface coal seam. Chlorobi PRB encoded oxidases with varying binding affinities for oxygen, as well as nitrate reductases and the ability to ferment. Remarkably, acetoclastic *Methanothrix paradoxum* PRB encoded a dioxygenase putatively involved in cleaving the ring of the acetate-containing aromatic hydrocarbon, phenylacetate. Together, these observations outline an interconnected series of metabolic reactions for the microbially mediated production of methane from subbituminous coal by translationally active, key CBM populations. The described research outlines a cell-level, activity-based approach to genome-resolved metagenomics that identifies pertinent biochemical capacities and was used to inform complex organic matter degradation to methane in natural settings. The use of activity-based metagenomics for the recovery of important MAGs promotes strategies in next generation physiology to understand ecologically relevant microbial populations.

McKay, L.J., H.J. Smith, E. Barnhart, H. Schweitzer, R.R. Malmstrom, D. Goudeau, M.W. Fields. 2021. Activity-based, genome-resolved metagenomics uncovers key populations and pathways involved in subsurface conversions of coal to methane. *ISME J.* 16:915-926

Cupriavidus 4G111 cultivation in PBRs. We have grown field isolate *Cupriavidus* 4G11 (isolated from a groundwater well at ORNL) in the PBRs. Our initial reactor run was to test different inoculation strategies to determine which methods would support the greatest biomass accumulation. Due to the location of sampling ports it is possible to inoculate reactors with different biomass concentrations. We analyzed planktonic samples for protein, dissolved oxygen, pH, dissolved organic carbon, nitrate usage, cell counts, and metabolite analysis over the course of 10 days. Additionally, cell counts are in progress for both planktonic and sediment samples, and mutant analysis (TnSeq) is planned. PBRs were inoculated with cultures at 0.1 OD, 0.05 OD, or 0.01 OD throughout the entire reactor volume. The final biomass (protein/g sediment) for the three different inoculation strategies were similar: 36.4 ± 5; 35.5 ± 10; and 35 ± 8, respectively with some variation as expected from top, middle, and bottom samples over the depth profile of the PBR.

Growth of R12 and 3H11. Following SOPs developed to increase standard growth for R12 and 3H11, we grew R12, 3H11, and the co-culture with changing O_2 levels as well as with NLDM (exo-metabolite medium). At 0 ppm O_2 (anoxic), we tested growth with acetate+yeast extract,

acetate+exometabolites, and NLDM. At pH 7.0 and NLDM, we tested growth at 0, 2, and 5 ppm O_2 with 1 mM and 10 mM nitrate. At 0 ppm O_2, acetate and YE, the highest growth of both R12 and Syncom was observed. 3H11 is slightly higher than other C sources but remains low. Average growth improves under increased presence of oxygen with NLDM as C source; however, biomass yields do not reach the same levels as observed with acetate/YE. On average, oxygen is depleted around hour 100 for both Syncom and 3H11, but delayed in R12 until nearly hour 200. pH increases in all growth conditions over the course of the experiments from the buffered pH 7.0 media, to nearly 8.25 by the end of the time course. Nitrate (1mM) is depleted in 3H11 & Syncom around Hour 50, but closer to Hour 200 for R12. Conversely, nitrite is depleted in R12 & Syncom around Hour 50, but closer to Hour 200 for 3H11. 10mM Nitrate data pending IC repair. Preliminary N_20 data indicates an increasing presence in both R12 & Syncom in all oxygen levels tested, but nearly nothing in 3H11. This collection method was not ideal and will be modified for more accurate data in the next round of experiments. Analysis of data continues focusing on exo-metabolomic data under increasing oxygen levels.

III. Publications

2022

1. Peng, M.; D. Wang, L.M. Lui, T. Nielsen, R. Tian, M.L. Kempher, X. Tao, C. Pan, R. Chakraborty, A.M. Deutschbauer, M.W.W Adams, M.W. Fields, T.C. Hazen, A.P. Arkin, A. Zhou, J-Z. Zhou (2022) Genomic features and pervasive negative selection in *Rhodanobacter* strains isolated from nitrate and heavy metal contaminated aquifer. Microbiology Spectrum. [DOI]:10.1128/spectrum.02591-21 {PMID}:35107332

Legacy and Synergistic External Research

2. Schweitzer, H. D., Smith, H. J., Barnhart, E. P., McKay, L. J., Gerlach, R., Cunningham, A. B., ... & Fields, M. W. (2022). Subsurface hydrocarbon degradation strategies in low-and high-sulfate coal seam communities identified with activity-based metagenomics. npj Biofilms and Microbiomes, 8(1), 7.

2021

3. Lui, Lauren M.; E. L. Majumder, H.J. Smith, H.K. Carlson, N.S. Baliga, F. von Netzer, M.W. Fields, D.A. Stahl, J-Z. Zhou, T.C. Hazen, P.D. Adams, A.P. Arkin (2021) Mechanism across scales a holistic modeling framework integrating laboratory and field studies for microbial ecology. Frontiers in Microbiology. [DOI]:10.3389/fmicb.2021.642422 OSTI:1773742

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Legacy and Synergistic External Research

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IV. Technology Developed and Implemented

The development and use of BONCAT-enabled identification of active microbial populations in situ via both 16S- and metagenomic-targeted approaches.

V. Training (*current members)

PhD students

Anna Zeleya	Lauren Franco	Greg Krantz	Laura Camilleri

*James Marquis

MS student

KaeLee Massey

Postdoctoral

Dr. Heidi Smith	Dr. Katie Davis	Dr. Luke McKay

Research Associate

*Sara Altenburg	Dr. Kristen Brileya

Final Technical Report
Montana State University Sub-Contract Award No. 7365998

Research Professor

*Dr. Heidi Smith

Undergraduate students

Hannah Dresbach	Joby Rosenleaf	Uve Strautmanis
Caitlen Osen	Katie Stabio	*Katerina Bruhl