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BSSD 2022 Performance Metric Q3

**Q3 Target**: H**ow laboratory simulations using microbial isolates informs the understanding of the microbial ecology in environmental samples.**

## Introduction

ENIGMA (Ecosystems and Networks Integrated with Genes and Molecular Assemblies) is a multi-disciplinary, multi-institutional research effort focused on addressing foundational knowledge gaps in groundwater and sediment microbiomes in the shallow subsurface at the Oak Ridge Field Research Site (FRC). Legacy activities at the FRC have identified complex gradients of contaminants including nitrate, pH, uranium, technetium and volatile organic carbon species. Nitrate levels in particular are some of the highest recorded subsurface concentrations (>10g/L) on record[1]. With these observations in mind, a central goal of ENIGMA is to causally predict the active biotic and abiotic mechanisms mediating key biochemical processes such as denitrification through characterization of the subsurface microbial communities, and environmental dynamics critical to these processes. Knowing how resource concentrations, pH, metal availability, and interactions between microorganisms in the environment differentially impact subsurface microbial activity is essential for developing a predictive understanding of the fate of geochemical species like nitrate in natural and engineered systems. To accomplish this goal ENIGMA is developing and applying sophisticated technologies and workflows for activity-based analyses in the field, genomic and phenotypic characterization of field isolates and defined synthetic communities (SynComs), as well as modeling strategies which can integrate the compendium of data being generated. Here we highlight recent progress to test hypotheses generated from field observations and gain insights into environmental phenomena relevant to the FRC using laboratory-based simulations, site derived isolates, and SynComs.

## Laboratory based simulations of environmental phenomena

Field observed correlations across biotic and abiotic factors have generated hypotheses regarding how specific environmental parameters such as pH, metal cofactors, and carbon/oxygen availability might constrain and interplay with microbial compositions and processes to drive observed phenomena at the FRC. The following sections provide examples of how these field-based hypotheses have been tested using laboratory-based simulations. These efforts provide a framework for future laboratory investigations and have facilitated the development of technologies, such a metaproteomic analysis, and will yield key resources, such as isolate and SynCom compendiums of phenotypic data (e.g., metabolomics and transcriptomics), gene regulatory networks, and metabolic models.

**Role of reduced sulfur in modulating subsurface nitrate respiration activity:** Two important modes of respiration that are commonly observed in subsurface environments are nitrate and sulfate respiration, which are facilitated by Nitrate Reducing Bacteria (NRB) and Sulfate Reducing Bacteria (SRB), respectively. While it is well understood that NRB generally outcompete SRB based on the energetics of nitrate and sulfate respiration, the inhibitory effects of hydrogen sulfide on NRB have been poorly characterized to date[2,3]. Field observations from the FRC motivated further characterization of the effects of hydrogen sulfide on NRB. SRB and NRB activity were observed to be stratified along the vertical transect of the sediment cores analyzed, which was not surprising. ***What was surprising was the association of dissimilatory nitrate reduction to ammonia (DNRA) with SRB activity***. This suggested SRB activity,

and potentially hydrogen sulfide, could impact NRB activity and promote DNRA over denitrification. This has been observed in other contexts, but the mechanism was not well understood[4].

Field observations motivated investigating the hypothesis that hydrogen sulfide could impact NRB activity and potentially influence $N_2O$ emissions. This was accomplished using the field isolate *Intrasporangium calvum C5* (*I. calvum*), a NRB with the ability to reduce nitrate to ammonia via DNRA or $N_2O$ via partial denitrification (*I. calvum* does not encode the NosZ gene (see Figure 1A)). It was discovered that hydrogen sulfide had a strong dose dependent inhibitory effect on the nitrate respiratory growth of *I. calvum* (**Figure 1**). It was also found that hydrogen sulfide promoted a shift from denitrification to DNRA, in agreement with field observations.

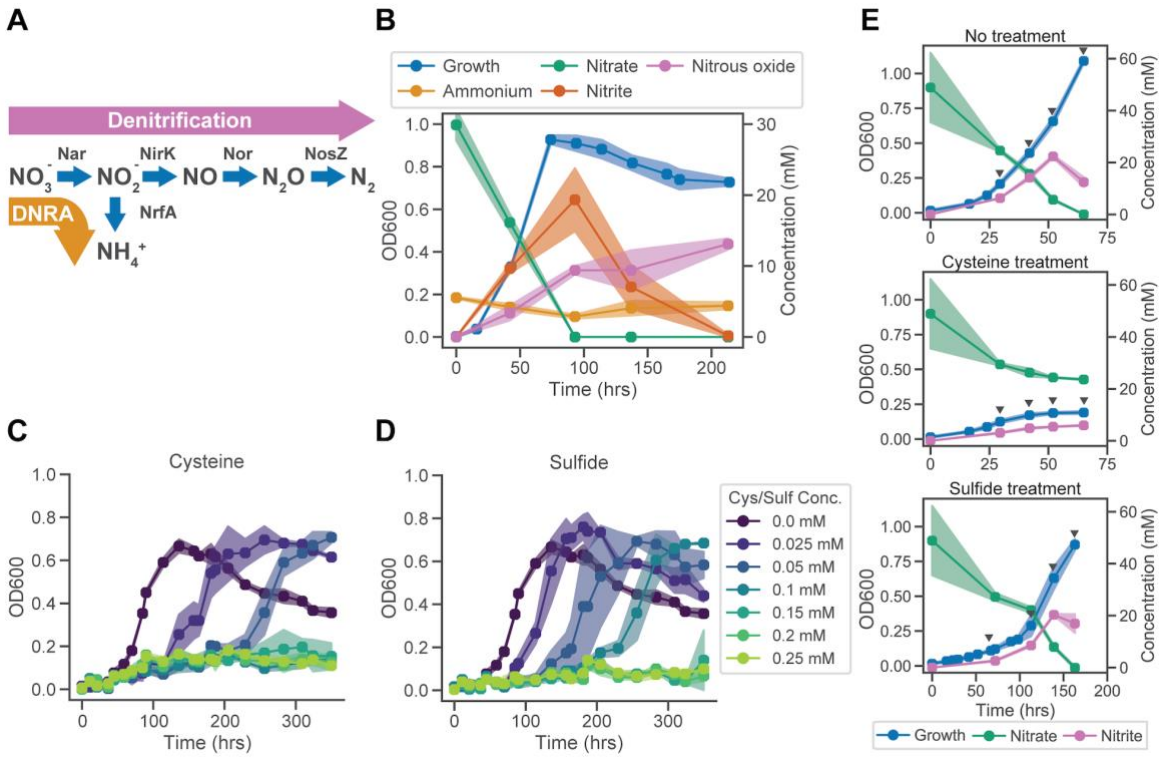


**Figure 1. Nitrate-reducing phenotype of *I. calvum* and the growth inhibitory effects of cysteine and sulfide.** (**A**). The genome sequence of I. calvum encodes two nitrate reduction pathways: partial denitrification to nitrous oxide and dissimilatory reduction to ammonia (DNRA). (**B**). Growth characteristics and dynamics of nitrate reduction by *I. calvum* with 30 mM nitrate and 20 mM lactate. Growth characteristics of *I. calvum* in the presence of increasing concentrations of cysteine (**C**) and sulfide (**D**). **(E)** Growth characteristics of cultures sampled for transcriptomic and metabolomic profiling. Profiling timelines varied across conditions based on growth characteristics. Cysteine treatment cultures contained 0.25 mM cysteine. Sulfide treatment cultures contained 0.25 mM sulfide. Triangles indicate points at which samples were collected. Samples for transcriptomics were collected as biological triplicates at all four time points, whereas samples for metabolomics were collected as five biological replicates at the first two time points for each condition. Shaded regions in all plots represent standard deviation across biological replicates ($n \geq 3$).

Systems analysis, using time-resolved transcriptomics and metabolomics, revealed that inhibition was driven in part by dysregulation of branch chain amino acid (BCAA) biosynthesis and carbon uptake (**Figure 2**). The mechanisms identified by systems analysis were further validated experimentally, supporting the role of pathway dysregulation. This work demonstrates how an observed field phenomena can be dissected and characterized using FRC isolates, laboratory-based experiments, and systems level analyses[5].

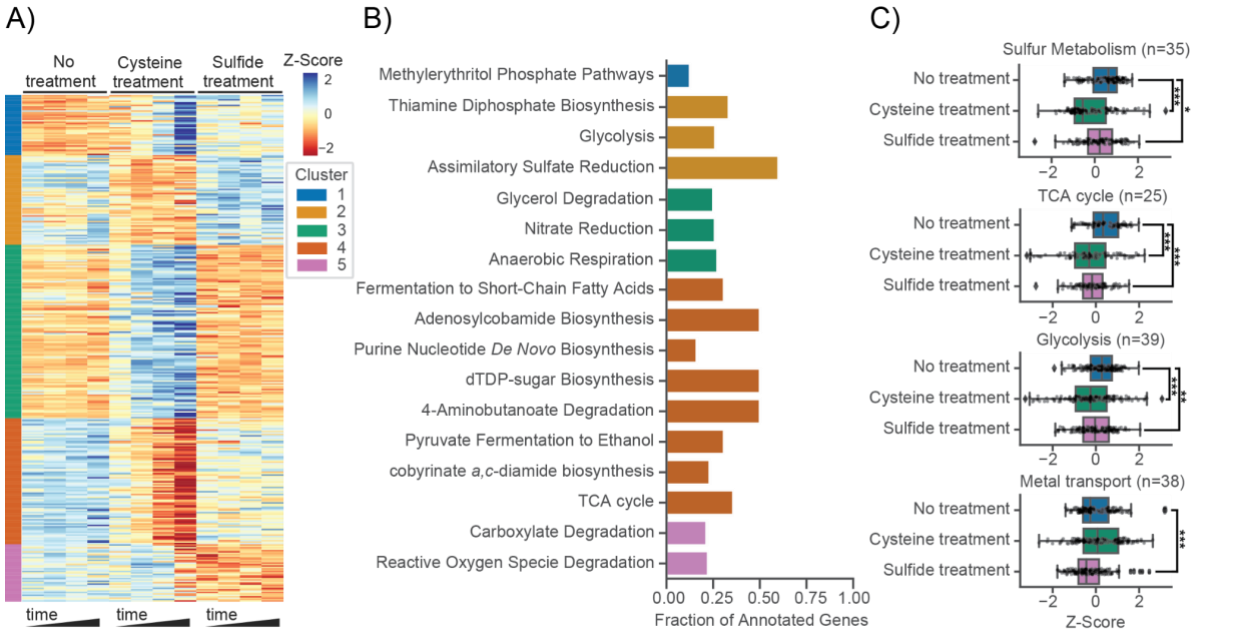


**Figure 2. *I. calvum*'s transcriptional responses to cysteine and sulfide treatment.** (**A**). Differentially expressed genes (DEGs) clustered into five groups using k-means. Expression levels displayed were normalized using the Z-score. MetaCyc pathway terms enriched in each cluster (**B**) and average Z-score normalized expression of select pathways and processes (**C**). Bars indicate comparisons for which differences were significant. *, $P < 0.05$; ***, $P < 0.001$. Boxplots display estimates of data minimum (left whisker), median (line within box), maximum (right whisker), and interquartile range (box dimensions) as well as possible outliers (points beyond whiskers). Data are overlaid as scatter points.

**Characterization of a nitrate-respiring, multi-metal-resistant bacillus species highly abundant in heavily contaminated FRC subsurface:** Predictive microbial ecology seeks to develop models that can accurately explain the impact of environmental perturbations on structure and function of microbial communities[6]. Identification of representative isolates is critical for the high-resolution physiological studies required for proper parameterization of these models[7]. Using already-generated ENIGMA datasets, we defined two major criteria for strain selection: i) high taxonomic abundance at the FRC and ii) functional relevance. Comparisons of the 16S gene sequences of current isolates to FRC sample amplicon sequence variants (ASVs) revealed an isolate with 100% identity to the most abundant ASV detected in a recent survey of the soils in the area immediately adjacent to the former S-3 ponds (Area 3) where groundwater nitrate and heavy metal concentration are highest **(Figure 3A).** This isolate is a *Bacillus cereus* species designated "CPTF". Genome analysis suggested that this strain is capable of DNRA via NarGHI

and NasDE[8,9]. Confirmatory laboratory experimentation found that nitrite and ammonium are the two major end-products of nitrate-respiration by this isolate **(Figure 3B)**. Thus, CPTF was selected as a site model organism for detailed study.

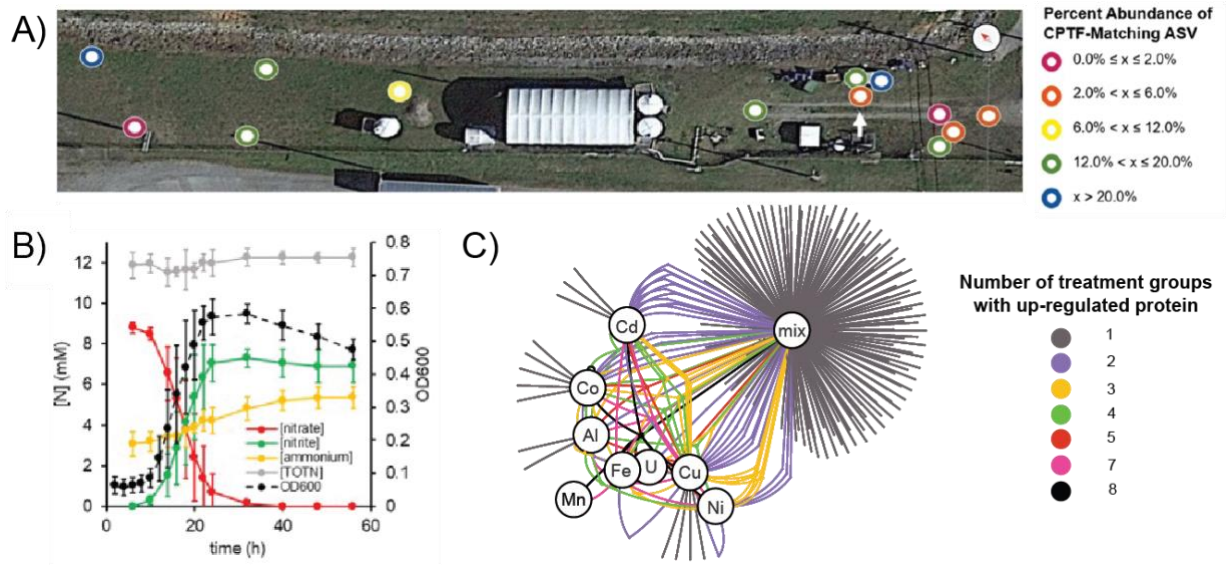


**Figure 3. Laboratory characterization and geospatial data for *B. cereus* strain CPTF** (**A**) Distribution and abundance of the CPTF-matching ASV across the Area 3 subsurface. For each dot on the plot, multiple samples at different depths were collected and analyzed by 16S amplicon sequencing. The depth with the greatest abundance of the matching ASV is shown for simplicity. The white arrow indicates the isolation source for strain CPTF. (**B**) Strain CPTF respires nitrate (red) with nitrite (green) and ammonium (yellow) as the major end-products.The total nitrogen in the system (grey line) can be fully accounted for by these three N-species. (**C**) Differential expression was determined relative to an untreated control. The metals mixture contains all of the individual metals in combination at the same concentrations they are present at in the individual treatment. Metal treatment concentrations were determined based on their average concentrations in the FRC Area 3 groundwater. Large nodes represent the treatment condition. Small nodes represent individually significantly up-regulated proteins. Edges connect proteins to treatment conditions they are significantly up-regulated under. Edge colors (indicated by legend) indicate the number of conditions a protein is up-regulated under.

To explore the interplay between the different components of the FRC contaminants, cell growth and nitrogen oxide metabolism were characterized under different combinations of stressors (e.g. low pH, high nitrate, high metals) at levels determined from prior site surveys. Two key observations from these experiments have expanded our model for the impact of these perturbations on cell physiology. First, nitrite accumulation as a product of nitrate respiration can greatly enhance sensitivity to pHs below 7. Second, a metals mixture that reflects the average Area 3 groundwater concentrations of the eight major metallic contaminants (U, Al, Mn, Fe, Cd, Cu, Co, and Ni) to test the impact of multi-metal stress on nitrate respiration by this strain. While CPTF could grow in this metals mixture, the second DNRA step (nitrite reduction to ammonium) was inhibited, resulting in a greater accumulation of nitrite in the medium. Nitrite accumulation in natural systems is not only inhibitory to other microorganisms—especially at a lower pH—but could potentially support increased local chemodenitrification[10].

Following initial characterization, a high-throughput shotgun proteomics approach was used to compare the systems-level response of the cells to the metals mixture (described above) versus individual metal perturbations. While numerous studies have explored the impacts of individual metals on cell physiology[11,12], multiple metals will frequently coexist at elevated levels at contaminated industrial sites[1]. However, the literature on the impact of multi-metal combinations on cellular systems is limited and studies have primarily used binary combinations[13]. By comparing differentially expressed proteins between treatment groups, we observed 159 proteins up-regulated in the eight metals (U, Al, Mn, Fe, Cd, Cu, Co, Ni) treatment group that were not differentially expressed in any of the individual metal treatments **(Figure 3C)**. These data suggest that laboratory studies with individual metal perturbations may be informative but cannot be treated as additive when creating models that predict systems-level responses to multi-metal stress. Future work will focus on characterizing the systems uniquely up-regulated during the multi-metal perturbation event. We also will determine if these data can predict functional enrichment in metagenome samples from contaminated regions of the FRC.

**Biotic and abiotic controls of denitrification in nitrate reducing communities:** Culture-based characterization of denitrification has classically been performed using complete denitrifiers[14,15]. However, ongoing genomic and metagenomic characterization of isolates and natural communities has revealed, remarkably, that most organisms possessing genes diagnostic of the denitrification pathway lack the complete pathway (**Figure 4A**). More often the microbial communities in soil that drive denitrification are composed of a complex mixture of species that encode partial (rather than complete) denitrification pathways (**Figure 4B**). The environmental significance of pathway partitioning is mostly unexplored, but it is presumed partial denitrifiers in the subsurface complement one another via exchange of soluble nitrogen intermediates (i.e., nitrite, nitric oxide, nitrous oxide). To address the questions posed by these observations, a model SynCom composed of two FRC isolates was developed to understand how process partitioning can influence community function in denitrifying communities (**Figure 4C, D**). Initial characterization supports the notion that partial denitrifiers can complement one another. Individually neither *Rhodanobacter sp.* FW510-R12 (R12) or *Acidovorax sp.* GW101-3H11 (3H11) can perform complete denitrification but as a SynCom 3H11 and R12 complement one another to completely reduce nitrate to nitrogen gas. Additionally, we are leveraging this SynCom to dissect the influences of abiotic factors on community phenotype. We seek to understand how factors like pH, oxygen, carbon source, and metal toxicity may facilitate the production of $N_2O$.

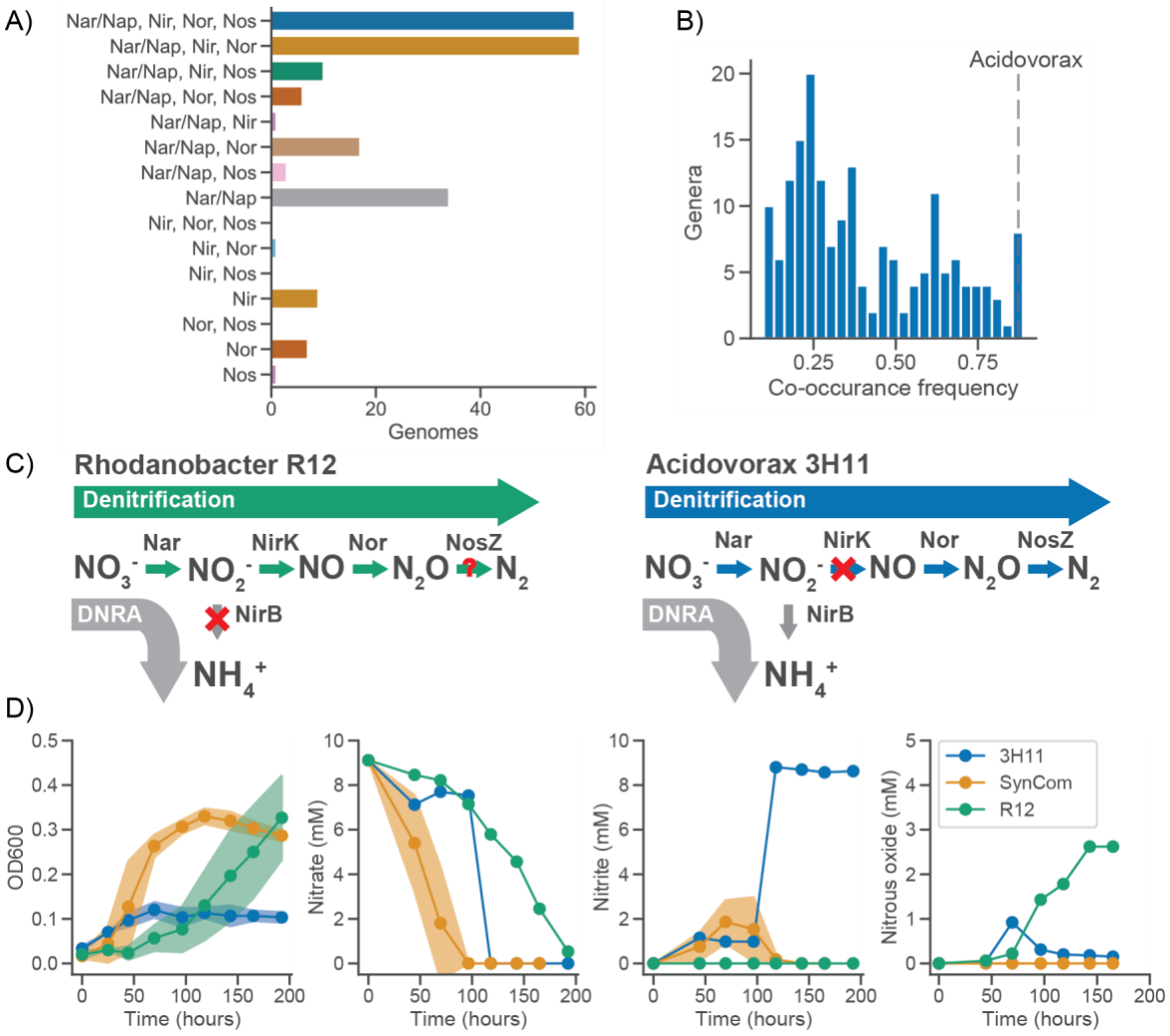


**Figure 4. Field observations and isolate characterization suggest pathway partitioning is a common feature of nitrate reducing communities at the FRC.** (**A**). Denitrification pathway composition of isolates from the FRC. Each bar represents a different combination of pathway enzymes and the number of isolate genomes with this composition. (**B**). Distribution of ground water co-occurrence for the genus Rhodanobacter with the co-occurrence fraction of Acidovorax noted. (**C**). Denitrification pathway composition for *Rhodanobacter sp.* FW510-R12 (R12) and *Acidovorax sp.* GW101-3H11 (3H11)**.** (**D**). Phenotypic characterization of 3H11, R12, and the 3H11-R12 SynCom. Shaded regions represent standard deviation across biological replicates

**Characterizing SynComs from the field requires new technologies**

Working with non-model organisms outside of their native ecosystems presents unique challenges. Unlike well characterized organisms such as *Escherichia coli*, many of the isolates in the ENIGMA strain collection lack tractable genetic systems or even complete genomes sequences which prevents use of standard molecular biology techniques with which to assay physiology (e.g., fluorescence reporters, single gene knockouts etc.). Thus, to overcome these challenges new technologies must be developed to be able to investigate cellular physiology and to better simulate environmental growth conditions which are tractable across diverse populations of bacteria. Below we highlight some of the technologies that have

been developed to address the challenges presented by working with non-model organisms, SynComs, and simulating environmental conditions.

**Metaproteomic approaches to address challenges in characterizing ENIGMA isolates:** LC-MS/MS based metaproteomic analysis has been developed to assess the structure of microbial communities based on species proteinaceous biomass contribution. Not only does this technology provide accurate biomass composition of a given microbial community, it can provide insights into community expressed proteomes and metabolic activities. To maximize the potential of this technology we have channeled it to a high-throughput analysis pipeline with automated sample preparation, fast data acquisition, and streamlined data processing. Applying this technology we provided accurate and highly reproducible assessment of the structure change of 3H11-R12 SynCom in response to pH shifts. New sample preparation methods are developing to accommodate the analysis of much more complex and diverse microbial communities. As of today, we have successfully applied the technology to assess a community structure of over 10 ENIGMA isolates using this new method.

**Modeling approaches for examining microbial interactions using exometabolomics:** We have recently published a defined medium (NLDM) that supports the growth of diverse ENIGMA isolates and also enables detailed examination of substrate use and metabolite secretion to examine metabolic interactions in ENIGMA SynComs[16]. An important component of these studies is the prediction of metabolites that mediate interspecific interactions based on isolate exometabolomic characterization. Recently, we have conducted a time-series analysis of exometabolite profiles of individual isolates vs SynComs in NLDM and applied a published model by Behrends et al. (2009) for this purpose and found promising results[17]. Specifically, we fitted the depletion kinetics of individual NLDM metabolites by each isolate over the growth period using a sigmoid function to estimate parameters that describe their substrate use properties, including T50 (the time when half of the available substrate was depleted). By doing this, we identified NLDM metabolites that were depleted by isolates at different rates, which could be used to predict substrate competition in SynComs. Moreover, we also identified metabolites that were depleted by certain isolates, but released by the other isolates, providing evidence for cross-feeding of these metabolites in SynComs. We are now in the process of using the proteomic methods described above in combination with stable isotope probing to test these predictions.

**Reactor technologies for determining spatial-temporal community assembly (PBRs) and modeling physiological state changes (FBRs):** We have developed fluidized bed reactors (FBRs) (**Figure 5A**) that can simulate anaerobic conditions in the subsurface and allow the microbes to attach to sediment in the reactor or live as free-floating organisms (planktonic) like those observed in the groundwater. This specific type of reactor allows researchers to assemble a consortium of field isolates (i.e., SynComs) to investigate how microbes self-assemble between attached and planktonic phases and the resulting differences in their eco-physiology. For example, we are using field isolates to measure growth dynamics and the metabolic output of a mutualistic co-culture composed of the bacterium that respires on sulfate and a methane producing archaea. In anaerobic conditions, the two organisms generate methane, an important greenhouse gas. We use a systems approach to validate the partitioning of attached and planktonic communities within a recirculating FBR because they provide an all-in-one platform to simulate community partitioning dynamics while differentiating genomic diversity and functional variability between planktonic (i.e. a proxy for groundwater) or attached communities (i.e. a proxy for sediment). By assessing growth of both

planktonic and attached communities during a single simulation, one can partition the influence and interplay of each subpopulation at once, while monitoring growth dynamics coupled to metabolite profiling (**Figure 5B-E**). This assessment provides a phenotypic overview of the partitioning of between attached and planktonic communities while providing an overall view of community function within the reactor. We can couple systems-level approaches to the environmental parameters to differentiate the molecular function of each isolate in the community from distinctive growth modes (i.e., attached or planktonic) within the same environment (**Figure 5F**).

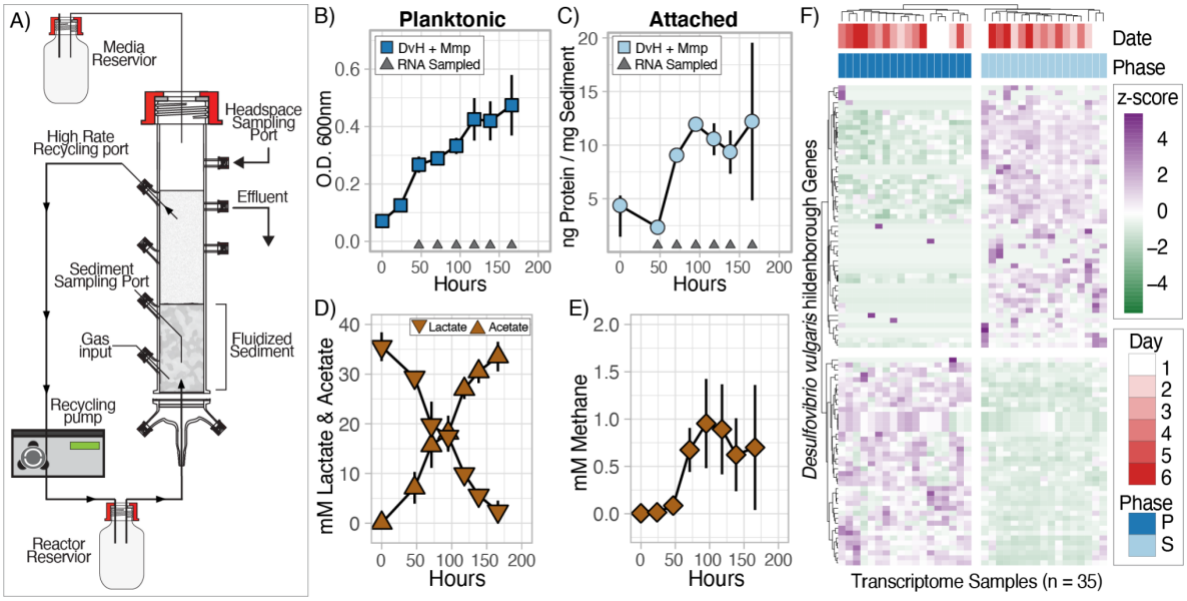


**Figure 5. Partitioning of attached and planktonic microbial communities in fluidized bed reactors can be distinguished by gene expression**. (**A**) A schematic of the custom FBRs developed to simulate biphasic growth of microbial communities by recirculating an upward fluid velocity through a column of sediment. Time-course measurements of growth dynamics between planktonic (**B**) and attached (**C**) phases of the microbial community. Metabolite profiles indicate growth by the consumption of lactate and the corresponding production of acetate (**D**) while the methanogen produces methane from hydrogen and carbon dioxide (**E**). Gene expression profiling was conducted over 6 days of reactor growth and RNA samples (n = 35, grey triangles **B** & **C**) were collected from the liquid (P: Planktonic) or from the attached (S: Sediment) phase. Differential gene expression analysis revealed 95 genes from that were significantly regulated (Fold Change[P vs. S] >= 2 & adjusted p-value =< 0.001) based on phase of growth (i.e., P or S). Heatmaps indicate the z-score of transcriptome counts for the significantly differentially expressed genes (rows) across the time-course of collected samples (columns).

## Summary and future outlook

Untangling complex interactions between biotic and abiotic factors in the field site is both challenging and resource intensive. Thus, to complement the field-based activities that are key to ENIGMA's central mission, laboratory-based studies are being leveraged to isolate variables of interest (e.g., role of heavy

metals and pH), and perform in-depth characterization of specific phenomena. A key feature of these laboratory-based simulations is leveraging field-based data and the ENIGMA isolate collection to selectively assemble SynComs to specifically test mechanistic hypotheses related to community function in well controlled environmental simulations. Here we highlighted how laboratory-based simulations have been leveraged to investigate hypotheses relevant to environmental phenomena at the FRC. These efforts have provided insights into the myriad roles microorganisms play in facilitating field relevant processes. These efforts also provide a framework for future studies and have facilitated the development of technologies with which to assay the physiology of non-model organisms and SynComs, and better simulate environmentally relevant growth conditions. Moving forward several key resources will be developed to aid future investigations. These include generation of SynCom transcriptome compendiums, gene regulatory networks, and metabolic models. Once generated, these resources can be leveraged to aid causally prediction of the active biotic and abiotic mechanisms mediating key biochemical processes such as denitrification at the FRC.

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