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BSSD 2022 Performance Metrics Q1

Q1 Target: Report on the latest techniques for recovering microbial isolates from environmental samples.

Introduction

LBNL ENIGMA (ENIGMA- Ecosystems and Networks Integrated with Genes and Molecular Assemblies) SFA 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 contaminated Oak Ridge Field Research Site (ORR-FRC). ENIGMA is a consortium of 16 investigators at eleven institutions across the country led by Lawrence Berkeley National Laboratory. Established in 2009, ENIGMA researchers collaborate to create a multiscale, causal and predictive model of the reciprocal impacts of microbial communities on critical processes within the ecosystem (e.g., N-cycling). Efforts focus on studying subsurface microbiomes within the contaminated Bear Creek aquifer at the Oak Ridge Reservation, a site with complex gradients of contaminants, generated by research and production of nuclear materials, including nitrate, acidity, uranium, technetium and volatile organic carbon species, the fate of which is mediated in large part by the activity of subsurface microbial communities. The ENIGMA workflow uses sophisticated, increasingly model-driven, field experiments to discover the components of and measure the natural and anthropogenically perturbed dynamics of these geochemical and biological processes. From these we infer the chemical, physical and microbial interactions predictive of these dynamics and estimate the ecological forces, both stochastic and deterministic, that shape community function. We then deploy a unique array of culturing, genetic, physiological and imaging technologies to capture this diversity in the laboratory and map the genetic basis for observed behaviors. Laboratory consortia are used to map gene function and investigate material flow within and among cells in conditions that simulate relevant field processes.

Our ambition is to do so at sufficient resolution to causally predict the active biotic and abiotic mechanisms mediating key processes such as denitrification; dissect the dispersing and persistent microbial community components critical in space and time during these processes; and ultimately predict the future changes in contaminant fate from current observations and possibly arising from natural and anthropogenic perturbations. Outcomes are significant both in the fundamental science of community ecology and in gaining an applied understanding of biologically-mediated subsurface processes in contaminated sediments.

Critical to our goals and objectives is the cultivation and recovery of representative isolates and reduced communities from our field site (ORR- FRC).

The need for representative enrichments and isolates

Compared to animal and plant hosts, other non-human environments on Earth such as marine sediment, seawater, soil, and the terrestrial subsurface host prodigious and undescribed microbial populations, as most of them have never been cultured and characterized in the laboratory [1]. In the terrestrial subsurface, it is estimated that there are $2{,}500 \times 10^{26}$ microbial cells, of which more than 70% belong to uncultured clades and thus their physiologies and ecological impacts remain largely mysterious [1]. Despite rapid technological advances in modern molecular tools such as metagenomics, metatranscriptomics, and metaproteomics — for identification of key microbial taxa and critical metabolic processes in a given environment, a complete interpretation of omics-based data is still constrained by the unavailability of reference genomes and physiologically characterized isolates[2]. Challenges in microbial cultivation/isolation in the laboratory have impeded the ability of microbiologists to fully investigate the roles and function of microbes in terrestrial subsurface ecosystems. Further, much of the genome-based approaches used in characterizing an environmental process depend heavily on information available in databases from sequenced genomes of cultivated species. And since at most only 1-2% of microbial species have been identified and cultured in laboratories to date [3–5], the fundamental information we have access to, is incomplete and inadequate. The importance of microbial isolation and cultivation to environmental

microbiology is indisputable; it provides physiological information about microbial communities that cannot be obtained directly from genomic sequencing efforts alone and provides a context in which to corroborate the theoretical findings of gene-based technologies [6]. Ecosystems-level studies deciphering the structure and function of microbial communities can be relevant and significant only when the key microbial members are successfully cultivated, characterized, and then manipulated or perturbed to even begin to understand their ecological function. Thus, the challenge of obtaining microbial isolates from their natural milieu severely impedes our efforts to study the physiology of the majority of microorganisms that are driving critical nutrient cycles, facilitating bioremediation and catalyzing green systems for energy production and greenhouse gas capture.

Choice of substrates for enrichments and isolations:

Successful recovery and cultivation of environmental microbes in the laboratory critically depends on appropriate growth media and incubation conditions that best mimic the ecological habitat of the bacteria, that includes temperature, moisture, salinity, pH, specific trace metals and nutrients among others [3]. Enrichment culturing is a common initial step in microbial isolation to select for microorganisms with specific metabolisms within the total microbial population. The choice of organic substrate is of paramount importance in enrichment media composition. Yeast extract and simple organic compounds such as glucose, acetate, lactate, pyruvate, and casamino acids are amended routinely, either as an individual carbon (C) source or as a mixture, with the understanding that most microbes utilize these C substrates [7]. However, these simple C compounds commonly lead to selective and biased growth of microorganisms with faster growth rates, generally considered the 'weeds' of the microbial world [3,5], and have rarely recovered slow growing yet metabolically active and relevant microbes from the environment [8]. Partly for this reason, despite the rapid advances in "omics" technologies, we have still only been able to cultivate less than 2% of microbes on Earth in the laboratory [4,9,10].

Rationally designed growth medium that closely mimics the natural environmental habitats of microorganisms has proven to be an effective strategy in recovering diverse and previously uncultivated organisms from various environments[11–16]. Specifically, microorganisms in the subsurface are reported to grow optimally under oligotrophic conditions, when nutrient availability is low [17]. In groundwater, dissolved organic matter (DOM) derived from the adjacent sediment contributes to the available C source for microorganisms. Our previous study shows that sediment DOM contains a myriad heterogenous organic compounds—mostly recalcitrant C such as lignin-like compounds and a small portion of relatively labile C such as carbohydrate- and protein-like compounds [18]. Other natural C sources available for microorganisms in groundwater can derive from dead, lysed microbial biomass turnover. Despite the potential of these natural C sources for cultivation of diverse microorganisms under laboratory conditions, reports on the application of sediment DOM or microbial cell lysate for cultivation/isolation of microorganisms from the terrestrial subsurface environment is lacking.

We initiated microcosm enrichments amended with different natural C sources as the first step to recover a greater diversity of bacterial species from Oak Ridge FRC groundwater under aerobic conditions and evaluated two types of complex natural organic matter (NOM): FRC sediment-extracted NOM and bacterial cell lysate. The cell lysate was prepared using a native, extremely abundant bacterial strain isolated from FRC groundwater to mimic representative cell lysis products available for groundwater microorganisms. For comparison, we also evaluated six simple organic C sources, conventionally used in cultivation media-simple C source (glucose and acetate), naturally occurring compounds (benzoate, oleic acid, and cellulose), and mixed vitamins. After enrichment cultivation, conventional direct plating was conducted to obtain axenic bacterial isolates from enrichment cultures amended with complex natural organic C source (i.e., NOM or cell lysate).

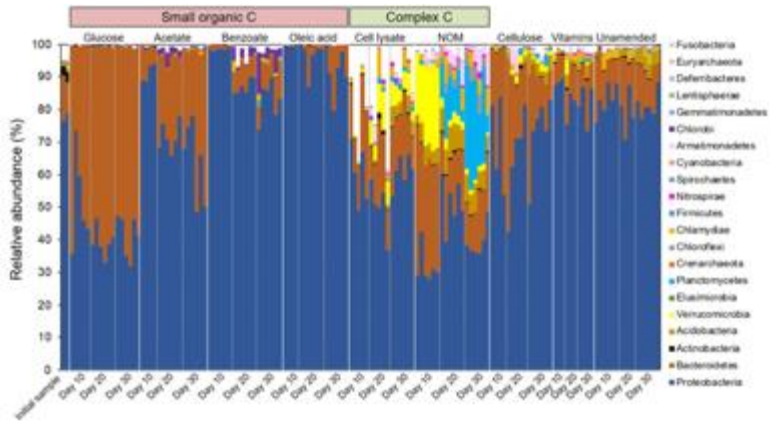

Figure 2 *Temporal community structures of the initial groundwater sample and each C-amended group reported as relative abundance of taxonomic phyla over 3 timepoints (Days 10, 20, and 30).*

Bacterial community composition in cultures amended with small organic C compounds (glucose, acetate, benzoate, or oleic acid) are noticeably similar to each other at an early stage of incubation, and only diversify at later stages of growth - while bacterial community composition in cultures amended with complex natural organic C (bacterial cell lysate or sediment NOM) rapidly diversify at early stages of enrichment. We investigated the short-term response of bacterial community structure to different C sources in enrichment cultures via 16S rRNA gene surveys. Out of the quality-filtered reads, organisms from 21 phyla and 94 orders were taxonomically identified, covering 71–100% of all reads, except for two samples (57% and 60%) in the bacterial cell lysate-amended group. All phyla and abundant orders (with relative abundance >1% in any sample) are presented in Figure 1. Proteobacteria and Bacteroidetes are the two most dominant phyla in all enrichments. Complex C enriched highly diverse and quite distinct phyla compared to the initial groundwater sample and simple C (Figure 1). It is worth noting that the rarely cultivated phyla Verrucomicrobia, Planctomycetes, and Armatimonadetes, which are low-abundant taxa (< 0.5% at phylum level) in initial sample, are enriched abundantly in sediment NOM-amended cultures with clear succession patterns. Verrucomicrobia is enriched at early stages and then diminishes over time, with relative abundance decreasing from 18–27% at Day 10 to less than 2% at Day 30. Meanwhile Planctomycetes becomes one of the major phyla at later stages, with relative abundance increasing from 0.1–1% at Day 10 to 5–33% at Day 30. Armatimonadetes also increase during the incubation period, with relative abundance up to 10% at Day 30.

In microcosms amended with complex natural organic C, diverse taxonomic orders are enriched, including those scarcely enriched in other groups, e.g., *Sphingobacteriales, Gemmatales, Planctomycetales, Verrucomicrobiales, and Solibacterales.* Since complex natural organic C (bacterial cell lysate and sediment NOM) shows great potential in enriching diverse and distinct bacterial species, we then used the complex C-amended enrichments as inocula for further isolation. In this study, we obtained a total of 228 bacterial isolates representing 5 phyla, 17 orders, and 56 distinct species (Figure 2). Our isolates represent both abundant (3-5% relative abundance at OTU level) and rare (< 0.01%) species from the initial groundwater sample. Of the 56 distinct bacterial species isolated, nine belong to candidate novel species and three belong to candidate novel genera. Our cultivation strategy will benefit future development of effective and ecologically relevant cultivation/isolation strategies.

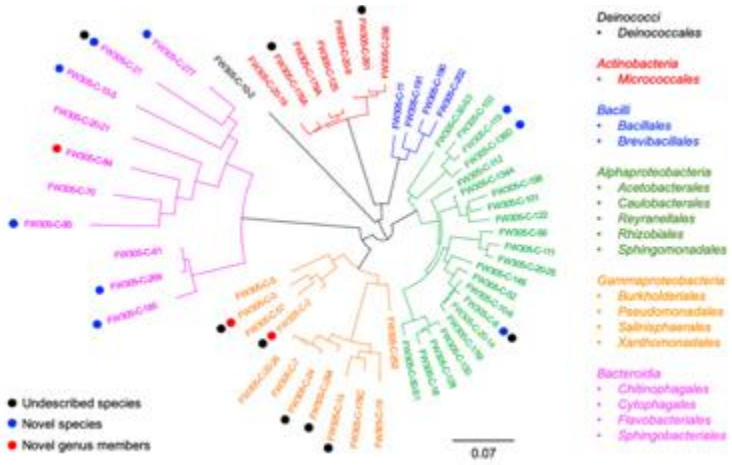

Figure 1 *Phylogenetic tree of representative isolates representing 56 distinct bacterial species. The tree is constructed from near full-length 16S rRNA gene sequences. Undescribed species (black), novel candidate species (blue), and novel candida*

Metals as a selective pressure in enrichments

Metal are important determinants in microbial community composition and functional diversity. Toxic concentrations of metals such as Cd, Pb, U, Cu, Ni, and Zn have been shown to limit microbial diversity in contaminated soils [19,20], while limitation of essential metals like Fe and Mo can change not only the microbial community structure, but affect the geochemical C and N cycles performed by the communities [21,22]. In a study comparing sandy loam soil that had either been fertilized with farmyard manure (1942 to 1967) or metal-contaminated sludge (1942 to 1961), differences in microbial community structure as well as the ratio of microbial biomass-C to organic-C were observed over 40 years later [23], stressing the impact of metals on microbial communities over longer time scales. Therefore, it is important to measure and account for both toxic metal contamination and essential metal limitation when designing enrichments for species representative of the natural community.

The S-3 pond's contamination plume at ORR-FRC is a unique subsurface region of elevated concentrations of nitrate as well as multiple metals including Al, Mn, U, Fe, Co, Cu, Ni, and Cd. With the goal of identifying molecular mechanisms that enable microorganisms to survive in this harsh environment, we have developed an environmentally informed approach to enrichment and isolation. The first step of this approach is to characterize geochemical parameters (measurements taken in the field and in the lab by multiple different ENIGMA research groups such as pH, temperature, dissolved O_2, ion concentrations, and natural organic matter, 46 different elements simultaneously by ICP-MS). Using this information, enrichments are designed that mimic the observed geochemical parameters, for example a contaminated ORR metal mix was developed to use in enrichments containing the eight metals which are elevated in concentration near the S-3 ponds.

Using the environmentally informed high throughput enrichment strategy, 22 different metal tolerant microbial strains were isolated from ORR groundwater and 88 different strains from ORR sediment. Using a multi-omic approach combining RB-TNSeq and activity metabolomics, multiple metal to microbe interactions were discovered for one of these isolates (*Pantoea* sp. strain MT58) including a connection between arginine synthesis and Al^{3+} tolerance [24]. Additionally, the observation that Mo (a metal required for nitrate reduction) is less available in ORR contaminated sediment led to the isolation of *Bacillus* strain EB106-08-02-XG196, which can reduce nitrate in the presence of sub-nanomolar concentrations of Mo. This strain was found to contain a high affinity molybdate transporter [25,26].

Future enrichment plans include taking environmentally informed enrichments to the next level with "personalized enrichments" where an enrichment approach precisely matches an individual environmental sample in terms of nitrate and metal concentrations, rather than the average values based on multiple samples. Actual metal concentrations in an individual environmental sample can be more than an order of magnitude higher than the average from the 100-well survey.

Flow through porous medium as a selective pressure for enrichment

To fully understand how environmental factors impact microbial community dynamics, interactions, succession, colonization, and dispersal in the shallow subsurface environment it is essential to understand the link between microbiology and hydrology. An up-flow packed bed reactor (PBR) was designed to simulate select field conditions (*i.e.*, flow rate and particle size) observed at ORR-FRC to observe how environmental factors, including hydraulic variables such as average pore velocity, influences metabolic activity, community establishment, and cell distribution in a micropore environment. The goals were to understand how environmental variables impact distribution and metabolic activity of microbial cells in a pore microenvironment using native sediment bug trap material under hydraulic properties based upon field conditions (flow rate and particle size). 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 ORR-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 from the ORR-FRC. The inocula for the PBRs consisted of sediment

material in samplers that were incubated down-well and retrieved from three FRC wells each at distinct pH values (4, 6.3, or 7). Following 4 months of incubation, the pH 4 reactor had the largest biomass and highest activity but had the lowest diversity amongst the pH conditions. The two circumneutral reactors (pH 7 and 6.3) had lower biomass concentrations and activity but had microbial communities that were more diverse than pH 4.

The two circumneutral pH reactors were predominated by a mixture of sequences indicative of low G+C Gram-positive bacteria as well as a-, b-, and g-Proteobacteria and had more similar community structure with each other compared to the pH 4 reactor. The low pH reactor was predominated by *Burkholderia*, *Rhodanobacter*, *Burkholderia-Caballeronia-Paraburkholderia* groups (4 groups comprising over 55% of total), most likely driven by low pH and high nitrate levels [27,28]. The *Burkholderia* and *Rhodanobacter*-like sequences were not observed at significant levels in the circumneutral reactors. It is interesting to note that despite similar reactor conditions (particles, flow rate, C/N sources, dissolved oxygen), the three reactors supported very different levels, distribution, and diversity of microbial biomass similar to observations in the field (possibly driven by pH and nitrate). Future work is planned to use developed methods to ascertain the distribution of microbial biomass in-situ.

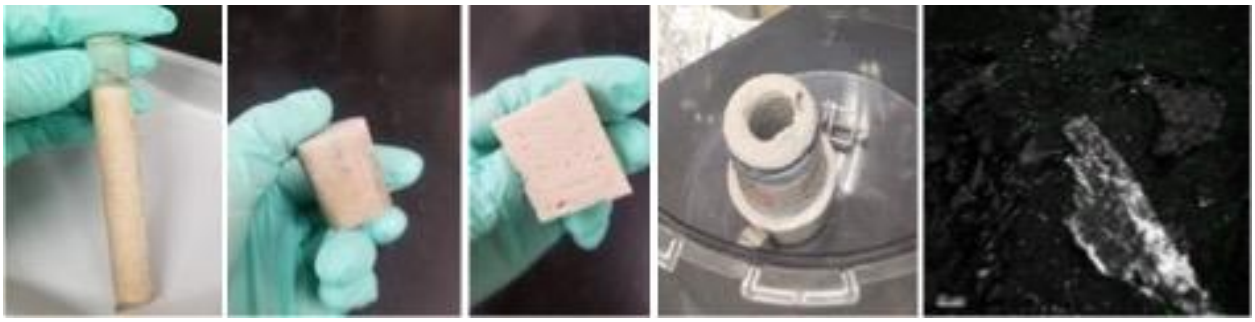

Figure 3*. Sediment cores are collected using a 5-inch plastic core (diameter = 1 inch) from the PBRs. Cores are fixed with 2% PFA for 2 hours and then receive a series of washes to remove moisture to allow the resin to fully set. Cores are saturated with LR white resin and placed in an incubator to set. Cores are then cut into sections to be imaged via Confocal. Image is an overlay of confocal and reflective images showing particles and biomass stained with SyberGold.*

A defined medium for cultivation and exometabolite profiling of soil bacteria

Exometabolomics is an approach to assess how microorganisms alter their environments through the depletion and production of metabolites. It allows the examination of how soil microbes transform the small molecule metabolites within their environment, which can be used to study resource competition and cross-feeding. While virtually any growth media can be used for exometabolite profiling, defined media are desirable in that all the metabolites can be accounted for and the media can be made reproducibly. Complex media contain components that are derived from complex organisms (e.g. yeast extract) that can vary in relative composition between batches. Using a defined medium also facilitates detection of microbial secreted products.

Towards this, we have developed a defined medium based on water soluble soil metabolites from saprolite soil collected from ORR-FRC [29]. Using both LC/MS and gas chromatography mass spectrometry (GC/MS), 96 metabolites from the soil were identified and 25 quantified, including amino acids, sugars, and carboxylic acids. This information was used to formulate a soil defined media (SDM). We used SDM to investigate the substrate preferences of *Pseudomonas corrugata* strain FW300-N2E2. Interestingly, it was found that this organism preferred lower-abundance substrates such as guanine, glycine, proline and arginine and glucose and did not utilize the more abundant substrates maltose, mannitol, trehalose and uridine (Figure 4).

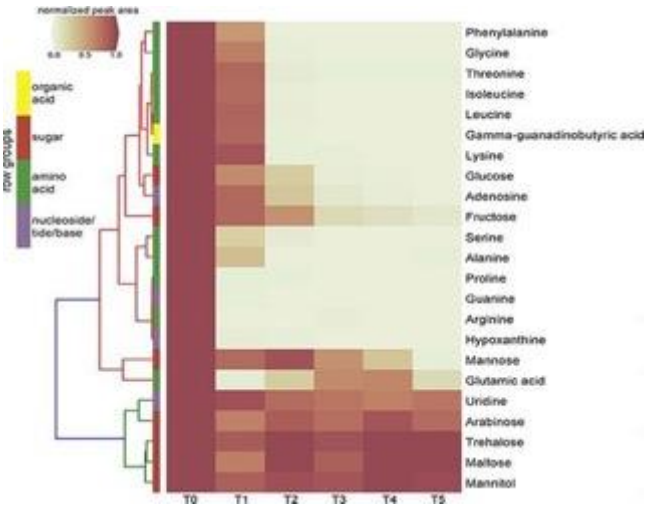

Figure 4. *Clustering heatmap of normalized peak areas for SDM metabolites across time course sampling of Pseudomonas sp. FW300-N2E2 spent media. Levels are displayed in terms of relative ratio to initial concentration at time zero (T0) with T0-5 representing 0, 3, 6, 9, 12, and 24 h time points, respectively. Metabolite row groups are colored according to the metabolite class they belong to.*

Although SDM was successfully used for exometabolomic profiling, only half (15/30) of the screened ORR-FRC isolates grew in it. This led to the construction of a new defined medium (NLDM) that supports the growth of a wide range of diverse soil bacteria and allows in depth exometabolomic profiling. Additional compounds included were selected on the basis of their presence in R2A media, their presence in soil, and their usage across a diverse set of bacteria based on existing exometabolomic data [30]. The relative abundance of the selected metabolites was based on the knowledge of elemental stoichiometries for bacterial growth and other culture media [31].

We selected 110 phylogenetically diverse soil isolates from the ORR-FRC and compared the growth of the isolates in both NLDM and R2A. We found that only 2 out of the 110 isolates did not display significant growth on NLDM, whereas 6 out of the 110 isolates did not grow on R2A medium. (Figure 5). In fact, 63 of the 110 isolates tested reached a significantly higher maximum OD_{600} on NLDM compared to R2A. A subset of 30 isolates were characterized using exometabolite profiling on NLDM. Not only were all 64 metabolites from the NLDM medium utilized by at least 1 isolate after 24 h of growth, it revealed a high degree of phylogenetic niche conservatism for substrate use.

These efforts demonstrate the viability and utility of both the SDM and NLDM media. We anticipate that the approach of preparing environmentally relevant defined media will be applicable to diverse environments to enable more ecologically relevant isolation and examination of microbial substrate utilization. We expect that the NLDM medium will enable the examination of microbial substrate utilization for a broad range of isolates both directly and through dilution and amendments.

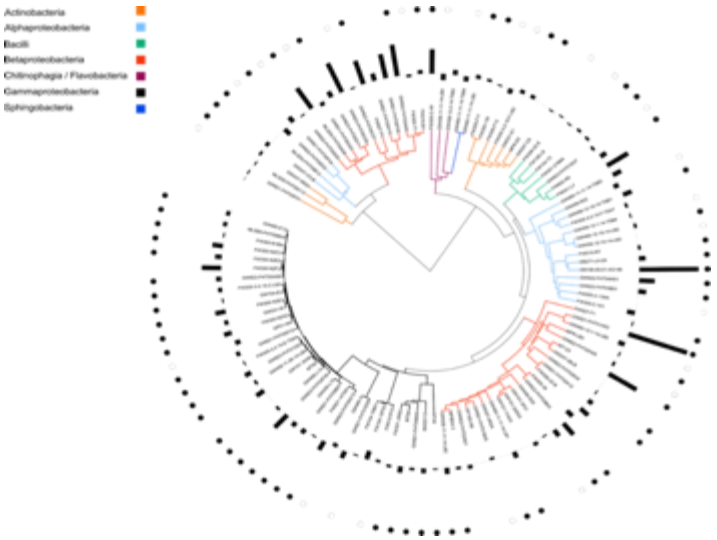

Figure 5 *Phylogenetic tree of all isolates with corresponding ratio of growth in NLDM and R2A. Label colors indicate the phylogenetic origin of each isolate by class. Bars on the outer circle indicate the average (n=2 or n=3) log2 ratio of the growth (maximum OD600) of each isolate grown in NLDM and R2A. Ratios >0 indicate that growth on NLDM was higher than in R2A and ratios <0 indicate that growth in R2A was higher than in NLDM. Closed circles indicate that growth was significantly (P<0.05) higher in NLDM compared to R2A; open circles indicate that growth was significantly (P<0.05) higher in R2A compared to NLDM (pairwise t-test).*

High throughput isolation

High Throughput isolation and cultivation relies on significant scaling of the process to collect and catalogue large numbers of bacteria from a given bacterial source in order to capture as many of the different microbes present as possible. Researchers are using high throughput (HTP)

approaches to obtain isolates of interest in their area of research from a variety of environments. HTP culturing often relies on miniaturized culture volumes in 48, 96, or 384 well microtiter plates, microfluidic droplets or micro-encapsulation to increase the overall number of samples processed. Dilution to extinction is often used in combination with selective media conditions to target the microbes that match their research interest. The number of microbial cells per individual culture wells, droplet or microtiter plate well, is constrained when using dilution to extinction to ensure a large proportion of the incubations do not contain growing microbes, mostly to eliminate fast growing bacteria from dominating the final cultures. This technique has been used recently to isolate rarely-cultured *Acidobacterium spp*. from termite guts [32]. Researchers have developed very detailed HTP isolation pipeline protocols for plant root bacteria extending up to the bacterial-identification bioinformatic steps[33]. Researchers have used the extinction technique in combination with MALDI-TOF measurements to eliminate isolate redundancy, reducing effort in downstream labor-intensive steps and increasing the number of taxonomically diverse bacteria from samples collected during the course of a spring phytoplankton bloom in the North Sea[34]. The extinction technique has also been paired with specialized cell sorting machines to select for bacteria that are particularly apt at breaking down cellulosic biomass[35]. Other groups have developed microfluidic machinery to print diluted bacterial cells in tiny droplets on agar plates, and were able to capture a larger portion of bacterial populations from deep-sea sediment than with traditional plating methods[36]. HTP systems have also been developed that are able to promote the growth and isolation of unicellular cyanobacteria, with specialized incubators and liquid handling robot integration [37].

Currently, the ENIGMA SFA is building on in-house and existing technology to further optimize methods to both increase the HTP recovery of environmentally relevant bacterial strains and develop growth conditions needed for their physiological characterization. Pilot efforts have included using contaminated groundwater at the ORR-FRC as inoculum, and several hundred isolates across diverse phylogenetic clades were obtained, offering new insight into the microbes abundant at this highly contaminated site.

Boutique isolation techniques for specific microbes and metabolisms:

Metagenomic depth profiling at the ORR-FRC showed that the Clade I vs Clade II nitrous oxide reducers are differentially distributed with depth, with Clade II variants enriched closer to the surface, thus being implicated in N_2O consumption. A key objective for ENIGMA is to isolate field relevant strains coding for one or both of the NosZ variants for studies of growth kinetics, characterization of isotopic fractionation, and possible incorporation in synthetic communities. Towards this goal, multiple sets of liquid enrichments with each containing a different electron donor (Acetate, Benzoate, Vanillate, Phosphite, or Methanol) for N_2O reduction were initiated in sealed Balch tubes. Tubes were incubated horizontally and statically at 20°C with N_2O at a final concentration of 30% v/v. Liquid enrichments usually enrich for the fastest growing populations. In parallel, dilution spread platings on agar were also done and plates stored in anoxic steel pressure vessels (Figure 6) with 10%v/v final centration of N_2O at the same temperature. Dilution spread plating allows for the differentiation of slow (small colonies) and fast growers (large colonies) as well as colonies with different physical phenotypes. Combination of both approaches has helped to increase the diversity of N_2O reducing microbes isolated. Good success in obtaining growth in liquid, and colonies on plates were observed.

Figure 6 *Pressure vessels for incubation of plates anaerobically*

Summary

The collective capability for recovering relevant microorganisms from the terrestrial subsurface, especially those mediating critical biogeochemical cycles, is limited to date. This bottleneck continues to hinder a thorough investigation of microbial ecology and understanding of physiology and true metabolic potential of key organisms residing in terrestrial subsurface ecosystems catalyzing key geochemical processes. The

ENIGMA SFA has a long-standing interest in the isolation and physiological characterization of non-model, environmentally relevant bacteria. ENIGMA is pioneering the development of tools and strategies and organized workflows to address this challenge. We have initiated this process of using our field data, chemistry and metagenomics to identify microbes of interest, and are pursuing both targeted and untargeted, low and high-throughput strategies to recover those isolates representative of the genetic and functional potential observed in the field as discussed in this report. We have ~2500 diverse aerobic and anaerobic strains from groundwater and sediment to date, this collection is continuously expanding, and we call this strain collection and its taxonomic and functional mapping the ENIGMA Environmental Atlas. Through this Atlas, ENIGMA is poised to provide a foundational dataset of genotype-phenotype relationships, that will greatly enhance our ability to interpret metagenomic data and infer the functions of thousands of uncharacterized genes - key contribution to our understanding of subsurface microbial physiology.

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