BSSD 2021 Performance Metric: Summary Report

Goal: Develop new omics-based techniques to understand microbiome function in environmental samples

Introduction

The LLNL "Microbes Persist" Soil Microbiome Scientific Focus Area (SFA) seeks to determine how microbial soil ecophysiology, population dynamics, and microbe-mineral-organic matter interactions regulate the persistence of microbial residues and formation of soil carbon (C) (**Fig. 1**). Our SFA research program is now four years old; it evolved from previously-funded BSSD projects at UC Berkeley, Northern Arizona University, Ohio State University and LLNL. We use stable isotope probing (SIP) in combination with 'omics analyses to measure how dynamic water regimes shape activity of individual microbial populations *in situ* and how their ecophysiological traits affect the fate of microbial and plant C. Using measures of population dynamics and microbiome-mineral interactions, we are working to synthesize both genome-scale and ecosystem-scale models of soil organic matter (SOM) turnover, to predict how soil microbiomes shape the fate and persistence of soil C. Here, we focus on approaches (and examples) that advance our understanding of activity in complex soil microbiomes.

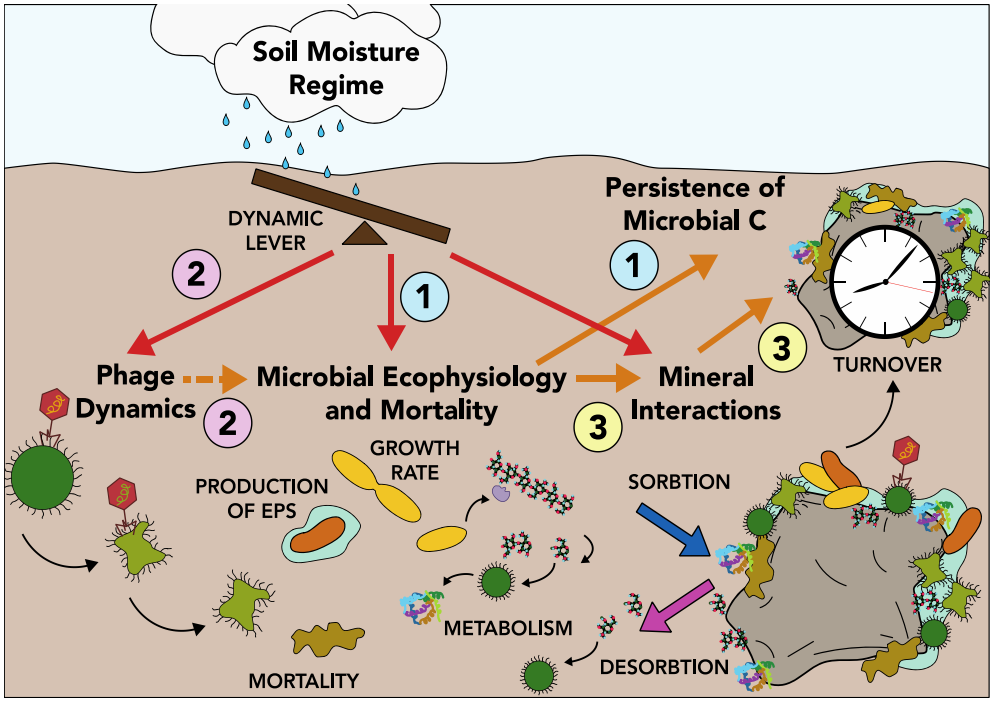

Figure 1. Conceptual figure illustrating our major objectives: 1) How soil water drives microbial ecophysiological traits linked to C persistence; 2) Microbial death: Impact of phage and other factors shaping microbial host dynamics and C cycling; 3) Role of minerals in stabilizing microbial necromass and products.

This report highlights advances made by "Microbes Persist" SFA from 2018-2021; we summarize material found in previous quarterly reports (https://sc-programs.llnl.gov/biological-and-environmental-research-at-llnl/soil-microbiome#reports) focusing on the following topics:

1) The latest genomic-based techniques used to explore the composition of microbiomes in environmental samples (Q1 Performance Metric Report). This report focused on the approaches used by the LLNL *Microbes Persist* Soil Microbiome SFA that move beyond traditional amplicon-based assessments of soil microbial communities by pairing stable isotope probing and assessments of inactive (relic) nucleic acids with metagenomic and metatranscriptomic surveys. This allows us to differentiate between actively growing microorganisms, dead and degraded DNA, and DNA/RNA from all taxonomic groups (including viruses) that make up the soil microbiome. Stable isotope probing enabled approaches are

particularly key to our efforts, giving us an unprecedented picture of the most relevant taxa in soil ecosystems. Of equal importance are the new informatics applications we are developing, including a computational suite to automatically identify recovered genomes, detect key functional genes, link inter-trophic interactions, and predict ecological drivers on community structure. These new tools not only help us to develop a microbiome-informed predictive understanding of soil carbon persistence but also provide valuable resources to the broader scientific community.

2) Using omics-based techniques to infer activities among microbial communities in an environmental microbiome (Q2 Performance Metric Report). In this report, we described several methodologies we use to infer activity of organisms (including phages), from measuring rapid changes in gene expression (transcriptomics) and metabolic products (metabolomics and lipidomics), to the incorporation of isotopically labeled substrates into DNA. By pushing for more temporal and spatial resolution, we aim to better capture the dynamics of ever-changing subpopulations of active microorganisms as their functional traits shift with environmental drivers. We also recognize the need to consider biotic interactions—e.g. with bacterial predators, RNA and DNA viruses—which play a large but poorly recognized role as regulators of microbial growth dynamics and biogeochemistry. Combining our knowledge of the functions of active microorganisms with biogeochemical measurements at various scales will enable the implication of key microbial players in bulk-scale biogeochemical process rates. Further, studying how these microorganisms sense and respond to changing environments, and how those changes modulate their activities will help inform future predictive models.

3) Computational approaches used to analyze microbial activities in environmental microbiomes (Q3 Performance Metric Report). Our SFA has developed, tested, and applied several cutting-edge computational approaches to better understand microbial activities in soils. Our efforts to test the sensitivity, precision, and statistical power of quantitative-SIP (*qSIP* [1-3]) are designed to make sure it is used more broadly by the scientific community, and with lower costs. New metagenomics computation and curation approaches, such as MetaHipMer and FixAME will streamline assembly and interpretation of large numbers of metagenome assembled genomes. Tools in the iVirus package, that we have recently incorporated into BER's KBase, are making it more tractable to extract ecological patterns of viral sequences from complex environmental datasets. Finally, we predict that incorporating metrics of microbial function, such as the population metrics measured by qSIP, will improve biogeochemical models. Trait-based modeling is a promising way to integrate information at the genome level (e.g. minimum generation times, substrate utilization capacity, transport kinetics, biomass chemistry, and phage covariance, etc.) to predict emergent processes like total microbial biomass, community composition, turnover and respiration in a way that can dynamically scale from 'omics data to system-level fluxes.

4) The use of imaging and mass spectrometry-based capabilities to describe microbiome interactions (Q4 Performance Metric Report). This report describes *Microbes Persist* developments in imaging, mass spectrometry, and related methods that provide direct insight into the biological, chemical and physical processes that contribute to the formation and stabilization of SOM. Our project's signature techniques include NanoSIMS approaches (to understand cell-cell and OM-mineral interactions at the single cell and even viral particle scale), radiocarbon (^{14}C) analyses (to determine both the age and turnover time of soil organic matter), and a suite of soil chemical characterization techniques that we refer to collectively as '*Multi-dimensional SOM-mineral characterization*' (SEM, TEM, STXM, NEXAFS, NMR, FTICR-MS, LC-MS). Here, we focus on how imaging, NMR, beam line and mass spectrometry approaches can deepen our understanding of soil microbiomes and the soil matrix.

Below we provide summaries and highlights from our four subject reports. More details can be found in the full reports available on the *Microbes Persist* SFA website: https://sc-programs.llnl.gov/biological-and-environmental-research-at-llnl/soil-microbiome.

A. <u>The latest genomic-based techniques used to explore the composition of microbiomes in environmental samples</u>*:*

The field of microbial ecology is rapidly moving beyond "who is there" to "how do they assemble" and "what are they doing" and starting to link key microbial activities with important biogeochemical functions. The *Microbes Persist* SFA develops and uses genomic-based approaches that move beyond traditional assessments of microbial communities. These approaches and results are summarized below:

1) Microbes exist in different metabolic states in soils, with differing degrees of influence on the environment. To differentiate between actively growing microorganisms, dead and degraded DNA, and stabilized extracellular DNA, we have developed an approach to combine measurements of relic (extracellular) DNA with heavy water stable isotope probing (SIP) [4, 5].

2) Community RNA-Seq allows Bacteria, Archaea, and Eukarya to be studied simultaneously without amplification; it takes advantage of the naturally high coverage of ribosomal subunit genes (e.g. 16S, 18S, 28S), and yields greater sequencing depth of these regions than metagenomic sequencing. Using a tool called EMIRGE [6], we reconstructed ribosomal sequences from a shotgun RNA sequence dataset of living and decomposing roots, and showed that decomposition is a multi-trophic process involving cross-kingdom interactions [7].

3) Random Matrix Theory-based co-occurrence networks and ecological modeling of community assembly are important ways to explore composition and interactions in environmental microbiomes. While network modules are not proof of an interaction, they can suggest shared niches and putative interactions. Using RMT network analysis, we showed rhizosphere soil bacterial networks are far more complex than those in bulk soils, indicating a higher degree of interactions and niche-sharing [8].

4) Using SIP and genome-resolved metagenomics, we characterized the community of bacteria growing near roots (rhizosphere), those that could help the plant grow during stressful conditions, and plant pathogens [9]. We also identified isotopically labelled micro-eukaryotes and viruses which likely consumed or parasitized the rhizosphere bacteria [10].

5) There are pros and cons of metagenome assembled genome (MAG)-focused analyses versus amplicon approaches for community characterization. The lack of functional information provided by amplicons (and the fact that amplicons miss some microbial diversity) has made shotgun metagenomics an appealing alternative. By calculating the functions encoded on a contig and its depth of coverage, the metabolic capabilities of samples can be compared and further organized into pathways if the contigs are binned into MAGs. However, complex shotgun metagenome assemblies are typically under-sampled, yielding near-complete genomes for more abundant organisms, but partial or no genomes for less abundant organisms. The rps6 gene is a widespread marker more reliably found in our MAGs than the 16S rRNA gene.

6) Despite major advances, metagenome assemblers still produce significant errors that need to be corrected; this is currently a highly manual process. Our SFA is developing a computational suite to automatically identify and repair metagenomic assembly errors. The proof-of-concept version of this tool 'FixAME' is now being packaged for DOE's Kbase platform and can effectively repair errors in thousands of sequences in hours.

7) Traditional microbiome surveys typically miss extremely small cells and taxa with few 16S copies. Using an approach designed for recovery of viral particles allowed us to target small and overlooked microbes, including Candidate Phyla Radiation (CPR) bacteria and DPANN archaea [11].

8) To better understand the difference between viruses from bulk metagenomes versus virus-enriched metagenomes (viromes), we compared metagenomes and viral-targeted metagenomes sequenced from a boreal peatland. The metagenomes were well-sampled but the viromes were under-sampled, suggesting that the viromes could have yielded even more viral sequences with deeper sequencing [12].

9) We have conducted multiple SIP-metagenomics studies to assess how active viruses shape microbial populations and limit microbial metabolic outputs, including in a sub-freezing Arctic soil [13], in a wet tropical forest soil, and across our SFA's three focal sites in California grasslands (**Fig. 2**). In the Arctic soil, we found a diverse array of active microbes and viruses, implying an ongoing arms race between viruses and bacterial hosts even under anoxic subfreezing conditions. We have found the fraction of active viruses can vary significantly, ranging from 25% to 75%.

10) Studying soil viruses requires a very different informatics toolkit than is currently available. We have made advances on viral informatics tools in three ways: 1) better viral taxonomic assignment [14], 2) improving data generation capabilities with long-read sequencing [15, 16], 3) democratizing the existing 'iVirus' analytical toolkit by implementing its core components in DOE's Systems Biology KnowledgeBase (KBase) [17]. In general, we are finding that soil viruses are vastly undersampled. For example, our newly generated database is almost as large as the current database of cultivated and uncultivated viruses (IMG/VR v3) which contains only 43,586 vOTUs from 1994 genomes or metagenomes from soils.

11) Most environmental virus surveys focus on DNA viruses. By comparison, RNA viruses are understudied and unknown players in environmental samples. In a recent publication [18], we measured the diversity and ecology of RNA viruses and their hosts, and identified a large diversity of soil RNA viruses, suggesting that soils may be reservoirs for novel RNA viruses.

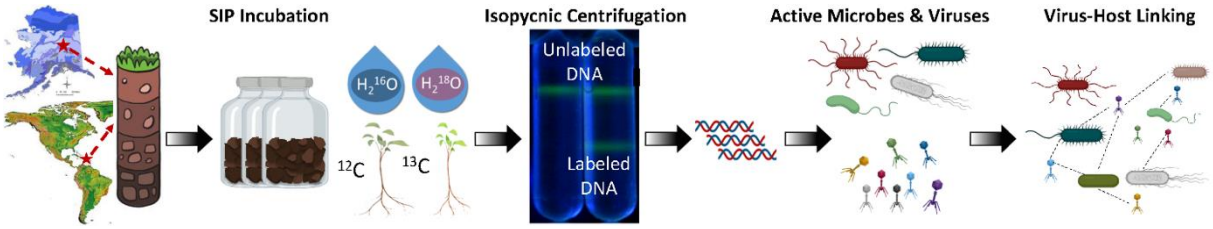

Figure 2. Generalized workflow for a SIP-metagenomics study, to identify and track active viruses, and link them to microbial hosts using CRISPR sequence matching and whole-genome similarity.

B. Using omics-based techniques to infer activities among microbial communities in an environmental microbiome:

Simultaneous identification of active microbes, their genomic capacity, and metabolic states has been a longstanding goal in microbial ecology, and methods to achieve this have accumulated in our molecular toolboxes—even for highly complex systems like soil. Here, we describe a variety of approaches we use to infer activity of organisms (including phage), from measuring rapid changes in gene expression (transcriptomics) and metabolic products (metabolomics and lipidomics), to the incorporation of isotopically labeled substrates into DNA.

1) Real-time activity and rRNA profiles in environmental samples do not always correlate due to differences in life histories, life strategies, and non-growth activities[19]. Therefore, rRNA analysis may not be a reliable metric of currently active microbial communities.

2) To assess the short-term response of soil microbes to a change in C availability, we added glucose and measured changes in soil metagenomes and metatranscriptomes over time[20]. This research shows that in combination, metagenomes and metatranscriptomes are powerful in helping understand microbial functions relevant to soil health at a foundational biochemical level.

3) Genome-resolved time-resolved transcriptomics is a powerful way to identify microbial niches in soil; we used this approach to compare microbial functions in rhizosphere, detritusphere, and combined rhizosphere-detritusphere habitats[21]. We identified distinct groups ('guilds') of microbes that express carbohydrate active enzyme (CAZy) genes in a spatially or temporally coherent manner. We also identified all the expressed genes involved in organic and inorganic N cycle processes, and

found that the presence of litter and/or living roots significantly alters the trajectory of N cycling gene expression [22]. Genome-centric analyses like these allow us to track transcription in individual populations, which may be a more relevant approach than grouping transcripts across disparate classes or phyla.

4) After transcriptomics and proteomics, metabolomics is another way to interrogate the active metabolic state of a microbiome. Soil metabolites can be measured several ways; we describe results from several studies collected with LC-MS-MS and Fourier-transform ion cyclotron resonance-mass spectrometry (FTICR-MS) [23-25]. We also used LC-MS lipidomics, a related approach, to trace plant- versus microbial-derived C in soil [26]. Many of the mineral-associated lipids that we found were microbially-derived, including a large fraction of fungal lipids.

5) Stable isotope probing (SIP) is one of the few approaches that can identify the ecophysiology of active microorganisms in their native environments, making it one of the most powerful techniques in microbial ecology. Our SFA team's novel SIP methods quantify element fluxes with high taxonomic resolution [1, 27-30].

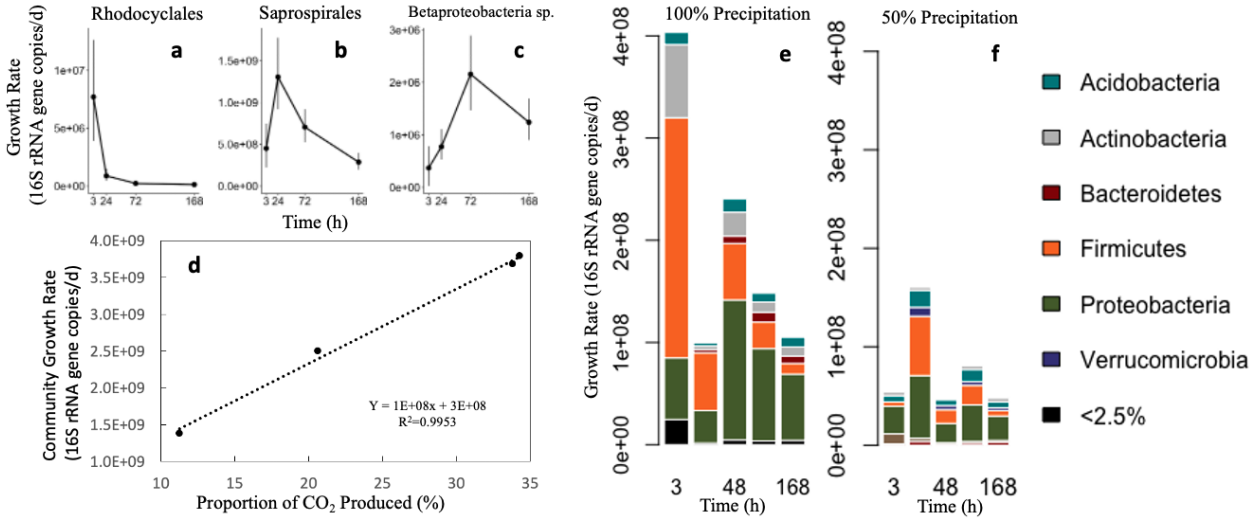

Figure 3. a-c) Examples of taxon-specific growth rate response patterns (a=primary, b=secondary, c=tertiary). Delayed responders are not shown because most of these taxa only grew at the final timepoint. d) Correlation between the portion of CO_2 produced vs. community growth rate. e,f) Phylum level growth rates for (e) 100% and (F) 50% precipitation treatments following wet-up, calculated with qSIP.

6) In seasonally dry California annual grasslands, during the autumn wet-up, a large portion of soil carbon is lost to CO_2 respiration. Using qSIP, we have characterized taxon-specific growth activities just after this critical period [4]. Bacterial growth occurred rapidly after wet-up, and approximately 40% of the measured taxa grew within the following week (**Fig. 3a-d)**, although the pattern of growth differed by genus. We also used qSIP to assess how reduced spring rainfall affects the amount of C subsequently lost after re-wetting and phage-driven bacterial mortality. Our results suggest a soil's historic rainfall patterns significantly impact microbial activities, turnover, and persistence (**Fig. 3e,f**).

7) We have also used qSIP to better understand the microbial communities actively degrading plant carbon in wet soils where soil oxygen availability oscillates [23, 31]. ^{13}C qSIP revealed aspects of microbial activity and litter C assimilation that were not apparent from our traditional 16S rRNA community analyses.

8) To investigate bacterial predators in soil, we synthesized SIP measurements from 81 experiments conducted at 14 sites--nearly 100,000 taxon-specific estimates of *in situ* bacterial growth rates[32]. We found that predatory bacteria grew more and faster than non-predatory bacteria and added C

substrates disproportionately stimulated growth rates of obligate predators compared to non-predators and facultative predators. These qSIP findings support the ecological theory that higher productivity increases food chain length and suggest that the functional significance of bacterial predators increases with energy flow.

9) One of the greatest benefits of SIP-metagenomics for viral ecology studies, is the ability to link active viruses with their active microbial hosts (using CRISPR and other approaches). In our California annual grassland rainfall gradient dataset, we used ^{18}O SIP-metagenomics to determine 1) viral diversity, 2) the 'active' viral DNA, and 3) the microbial host range of virus communities in our three grassland soil sites. Results suggest that viruses are actively preying upon key microbial taxa known to have fundamental roles in soil ecosystem function [33].

C. Computational approaches used to analyze microbial activities in environmental microbiomes:

The *Microbes Persist* SFA has developed several computational approaches to analyze and model microbial and viral activities in environmental microbiomes (Q3 Performance Metric Report). We focus on obtaining an accurate representation of the "active" component of microbial communities (e.g. qSIP), methods to ensure we have reliable genome assemblies (e.g. fixAME and virION2) and annotations (e.g. iVirus tools and multiphate2) and can predict how these community members interact with their environments (e.g. trait-based modeling).

1) To make the density fractionation strategies in metagenome-qSIP more cost-effective, we determined that by sequencing 9 fractions, one can achieve nearly the same sensitivity and statistical power as with far more effort. We also showed that fewer controls are needed. Following these guidelines can reduce the cost of metagenome-qSIP experiments by half [3]. In additional tests, we have investigated pooling strategies can optimize sequencing costs, tube corrections to adjust weighted average densities, and fractionating and sequencing only the right tails of the density curve to target abundant and more active community members. We are currently testing the next generation of metagenome assembly tools, such as Metahipmer 2, which can use orders of magnitude more sequencing data, and generate accurate assemblies in orders of magnitude less time.

2) Even highly optimized metagenome assembly software produce errors, and these errors are propagated to genomes obtained from binning. Our FixAME toolkit (and associated KBase apps) was developed to identify and correct genome assembly errors common to several more popular tools and can automatically curate and improve thousands of genomes recovered from metagenomes.

3) The Microbes Persist SFA team has begun using long-read sequencing technologies to gain new insights into viral microdiversity and novel genome arrangements. We developed virION2 [15], a hybrid workflow to combine the high-resolution of long-reads with the high-fidelity of short-reads to improve our recovered viral genomes. We also moved the iVirus suite of tools into the KBase ecosystem to allow even more researchers to identify (VirSorter), classify (vConTACT2) and match to a host (VirMatcher) in large, complex metagenomes [17].

4) Annotation of viral genomes has traditionally relied on tools designed for prokaryotes with the underlying assumptions of how prokaryotes organize their genomes. These assumptions are often not the same in viral genomes. Our SFA has contributed to new tools to resolve this concern. 1) *Phanotate,* the first viral specific structural genome annotation tool, does a better job of predicting open reading frames in viruses than other gene calling algorithms [34]. And the *multiphate2* suite of tools is designed for the rapid functional annotation comparison of viral genomes [35].

5) Finally, we are using the more accurate determinization of the "active" organisms in a community, as well as the higher confidence in their genome and predicted functions, to identify traits to feed into better trait-based modeling algorithms, and more accurately link environmental measurements to the

bacterial growth efficiencies of individual microbial community members. We are currently developing more robust methods to keep the accuracy of trait-based model predictions as the size and complexity of our experimental systems increase.

D. The use of imaging and mass spectrometry-based capabilities to describe microbiome interactions:

Imaging, mass spectrometry, and related methods enable directed interrogation of plant-microbe-mineral interactions, viral and microbial particles from soil, and the signatures plant and microbial necromass contribute as part of soil organic matter (SOM). The state-of-the-art studies our SFA has conducted combine these techniques to extract complimentary information from the same sample or experiment. Key information includes OM source and fate, spatial arrangement, and chemical transformations. These approaches can deepen our understanding of soil microbiomes and their engagement with the soil matrix.

1) ***NanoSIP***, developed at LLNL, is the most widely used NanoSIMS-enabled approach for studying microbiome interactions. It provides direct imaging of the fate of isotopically labeled substrates (**Fig. 4**) and can be used to study a broad range of microbial processes [36-41], including nitrogen transport by AMF fungi [42] and carbon transport from roots [26] and microbial necromass [43] to mineral surfaces. At the leading edge of sensitivity, we are working to characterize viral activity in soils with nanoSIP [44, 45].

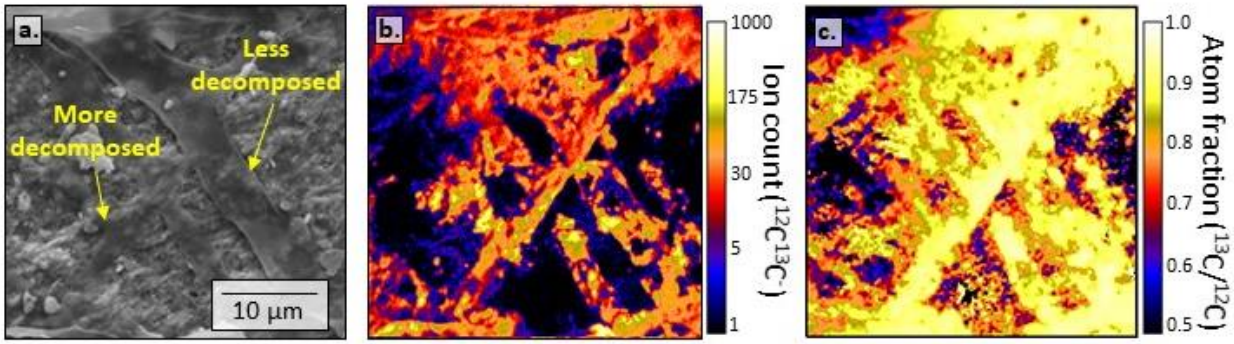

Figure 4. (a) A scanning electron microscope (SEM) image of fungal on a goethite particle and correlated NanoSIMS (b) $^{12}C^{13}C^-$ ion and (c) ^{13}C enrichment image after ~100 nm of sputtering, showing that the hyphae are the primary source of OM on the mineral surface.

2) The '***Chip-SIP'*** method our team developed uses NanoSIMS imaging to determine the isotopic enrichment of rRNA hybridized to a phylogenetic microarray [46, 47] and can be used to link identity and function in complex environmental communities. Relative to density-gradient SIP [48], ChipSIP has several benefits, including relatively low isotope enrichment, multiple isotopic tracers in the same sample, and no amplification step. This technique is works in many environments, including water, soil and insect gut [7, 46].

3) The '***BulkSIP***' method uses NanoSIMS analysis to quantify isotope enrichment in tiny amounts (~100 ng) of total ('bulk') DNA or RNA extracted from a microbial community. BulkSIP can provide important clues about average rates of substrate preference or consumption and be used as a pre-screening step for density gradient SIP. BulkSIP can provide data for multiple elements (^{13}C, ^{15}N, ^{18}O).

4) The '***STXM-SIMS'*** approach combines NanoSIMS with Scanning Transmission X-ray Microscopy (STXM) coupled with Near Edge X-ray Absorption Fine Structure spectroscopy (NEXAFS), a beamline approach that collects molecular information at a similar scale (~100 nm). STXM-SIMS has the unique capacity to yield quantitative, *in situ* information on the molecular class and elemental quantity of isotope-enriched mineral-associated organic matter [49].

5) Recent advances in label-free, live imaging are providing new insights into microbial community development. A state-of-the-art example is the LLNL '***Rhizo-scope'***, a custom multimodal, multiphoton imaging platform for imaging microbial community development live in soil and rhizosphere. It uses infrared light to generate multiple modes of contrast that enable soil microbes to be imaged a mineral matrix.

6) Our SFA uses fourier-transform ion cyclotron resonance mass spectrometry (**FTICR-MS**) to provide information on microbial communication and contributions to SOM pools by fingerprinting the soil exometabolome. We have used FTICR-MS to enable researchers to extract metabolite profiles and infer microbiome activities and interactions by comparing patterns from different soil treatments or C inputs [23, 26].

7) **Lipidomics** with liquid chromatography (LC) input to high resolution mass spectrometry (MS) can be used to determine the origins of organic matter. Lipids produced by different organisms, e.g. plants and microbes, have distinct signatures. In our SFA study of how root-derived C becomes associated with surfaces of different mineral types, we found more evidence of microbially-produced lipids than plant lipids [26].

8) LC-MS **metabolomics** is increasingly used to characterize soil exometabolomes [24, 25, 50]. Its strengths include quantification and identification of metabolites using authentic standards. By analyzing the isotopologues of individual metabolites, C from ^{13}C labeled inputs (such as leaf detritus) can be traced into the soil metabolome.

9) Nuclear magnetic resonance (**NMR)** is also increasingly used for characterization of soil organic matter [26]. Major chemical classes of mineral-associated organic matter can be identified by ^{13}C-NMR, allowing cross comparison of samples. Low C concentrations can be overcome with sample pooling or long ^{13}C-NMR analyses.

E. Conclusions and Future Steps

Despite many technological advances, there are still many limitations in current genomic-based approaches that must be overcome to gain a wholistic and mechanistic understanding of the soil microbiome and the fate of C in soil. One such limitation is the inability to accurately quantify key features of microbial communities in soils –such as microbial biomass, cell size, or population specific abundance. Future work and approaches need to move towards absolute quantification, since absolute quantification of microbial identity, chemistry, and activities is crucial for advancing our understanding of the global microbiome and its relationship to ecosystem function and stability. Developing and adopting uniform quantitative methods will transform our ability to understand microbial systems by enabling robust measurements of pools and fluxes of biomolecules. Consistent numerical methods are fundamental for accurate integration of microbial identity with the molecules they produce and transform, and to facilitate accurate scaling of microbial processes to understand synergistic ecosystem responses.

Despite recent progress with 'omics-based techniques and computational approaches, continued technical and methodological advancements will require additional innovation in the future. For example, even while technical hurdles involving 16S rRNA amplicon sequencing have been overcome, the field is now relying less on amplicons and more on whole genome/metagenome sequencing, generating massively more data and requiring much larger computational infrastructure. This creates potential data sharing problems, since workflows are becoming more complex and the computational workflows different groups use must be properly documented. Open computational platforms (e.g. KBase) are one solution, as are entirely new workflows meant to scale with the increase in data production (e.g. metahipmer2). Fortunately, as these annotation difficulties are resolved, other "omics" experiments with proteomics and metabolomics will also see increased benefits.

In future work, our SFA aims to better link imaging methods with bulk microbial and SOM characterization methods through experiments designed to isolate specific processes. Soil is a challenging medium for bulk characterization methods, due to the large number of compounds involved and the potential for important compounds to be rapidly consumed or sorbed by soil components. We see cross-correlation of SOM characterization methods and isotope tracing as key to moving the field forward. By linking the complimentary information gathered via the exquisite mass precision of FTICR-MS, to the ability of NMR (and STXM) to provide general classification of chemical compounds, to inferences gained through SIP-enabled metagenome resolved genomics, we envision a more comprehensive understanding of SOM. Moving forward, it will also be important to expand access to databases, instruments, and standards to broaden the base of users and practitioners and promote innovation and integration. Given the complexity of soil, it is only through the integration of imaging and mass spectrometry methods with the full breadth of 'omics approaches that we will reach a predictive understanding of how soil microbiomes shape the formation and persistence of soil organic matter.

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