BSSD 2019 Performance Metric Q3

Goal: Develop metagenomics approaches to assess the functioning of microbial communities in the environment.

Q3 Target: Report on how transcriptomics and metagenomics analyses can be used in concert to elucidate microbial community function in environmental samples.

Executive summary:

The LANL SFA in Terrestrial Microbial Carbon Cycling aims to inform climate modeling and enable carbon management in terrestrial ecosystems by discovering widespread biological processes that control carbon storage and release in temperate biome soils (primarily arid grass/shrub lands and forests). Combined use of metagenomic (MG) and metatranscriptomic (MT) techniques is essential to achieve these goals. Metagenomic inventories of the dominant taxa and/or functional genes in microbial communities are a springboard to develop hypotheses about functional changes and to down-select samples for resource-intensive metatranscriptome analyses that can provide greater mechanistic insight. Integrated use of metagenomics and metatranscriptomics for ecosystem research is still in an early phase of development. Nonetheless, the SFA has successfully developed and applied these techniques in several field studies (separately funded and led by external BER-funded collaborators) to gain insight into "active" taxa and genes involved in ecosystem response to drivers of ecosystem change.

A general finding in comparing MGs and MTs is that they differ, sometimes dramatically, in the relative abundance of specific taxa or gene types. For example, targeted MGs showed co-dominance of Ascomycota and Basidiomycota fungi in surface litter in a pine forest under long-term N fertilization, but targeted MTs suggested Basidiomycota fungi remained the most active players in cellulose decomposition [1]. Even more striking, shotgun MGs from forest soil samples have repeatedly indicated complete numerical dominance of bacteria (i.e., nearly all sequence reads are bacterial), whereas shotgun MTs show a large fungal component and have thereby provided far greater insight into fungal metabolic activities in soil [3, 5, 6]. For a Maple forest ecosystem under long-term N deposition, MTs revealed clear N fertilization effects on fungal and bacterial CAZyme activity and intriguing inter-kingdom CAZyme co-expression patterns, whereas only weak taxonomic responses occurred in parallel targeted MGs [4]. In contrast, a separate study found that inorganic nitrogen cycling pathways were not enriched in MTs as expected; instead the genes were 150-fold lower in abundance compared to MGs, illustrating unexpected challenges in use of MTs to broadly examine the integration of major biogeochemical cycles in surface soils [5]. Accurate interpretation of MT/MG data is also a challenge at this early stage of development. Higher abundance of components in MTs compared to MGs is generally considered an indicator of "active" status. Exploiting this concept, the SFA found evidence suggesting a much stronger role of dormancy in the maintenance of bacteria, but not fungi, in Maple forest litter decomposer communities [2]. However, modeling and simulation revealed the need for caution when using MT/MG comparisons for activity assessments because variation in the biology of different organisms can create limitations on data analysis that lead to erroneous conclusions if ignored [7]. Ongoing research in the SFA is addressing some fundamental challenges in use of MTs while, in parallel, integrating MT & MG to discover processes that underpin large differences in carbon cycling.

Background

Metagenome (MG) and metatranscriptome (MT) techniques can be combined in various ways to achieve greater insight into microbial community processes that underpin ecosystem behavior. MGs document the "seedbank" of organisms and gene functions in a community. Metagenomic inventories are expected to represent a mixture of active organisms, dormant organisms (e.g. spores), recently dead cells, and even some extracellular DNA from older dead cells. This motivates the use of metatranscriptomics to identify the subset of metabolically active organisms and the gene functions they express. MG and MT techniques can be applied to microbial communities in targeted or shotgun mode, sampling a specific gene or all genes, respectively. The four combinations of MG and MT modes provide different levels of functional insight with corresponding effort and expense (Table). Effective methods for soil shotgun metatranscriptomics that provide inter-kingdom (e.g. prokaryotes and eukaryotes) coverage emerged only in the past 5 years, including the method published by the SFA in 2015 [4]. The LANL SFA mostly uses targeted metagenomics with shotgun metatranscriptomics but has applied various combinations in different studies since 2012, primarily to investigate the response of soil communities to N fertilization.

Metagenome	Metatranscriptome	Functional insight
targeted	targeted	Lowest
targeted	shotgun	↓
shotgun	targeted	↓
shotgun	shotgun	Highest

MTs identify an "active" subset of cellulolytic fungi in MGs under long-term elevated CO_2 and N fertilization

The SFA began developing soil metatranscriptomics in 2011, successfully applying targeted MT/MG comparisons to examine cellulolytic fungi contributing to carbon cycling at the Duke (North Carolina) loblolly pine plantation Free Air Carbon Dioxide Enrichment (FACE) site [1]. Because cellulose-degrading microorganisms are not phylogenetically cohesive, ribosomal gene surveys are unable to detect specific responses of cellulolytic microorganisms that may be central in altered soil carbon cycling patterns. Published PCR primers targeting the gene coding for the catalytic subunit of fungal glycosyl hydrolase family 7 cellobiohydrolase I (cbhI), a key enzyme involved in cellulose degradation, enabled monitoring a subset of cellulolytic soil fungi using DNA- and RNA-based approaches. The SFA generated DNA- and RNA-based profiles of

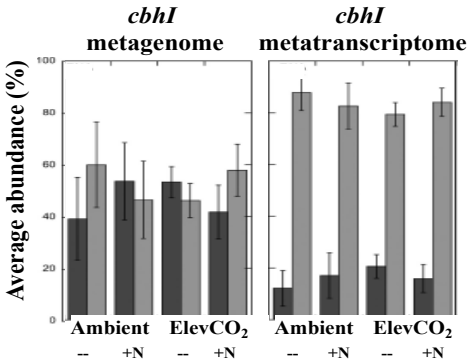

Figure 1. Average relative abundance of Ascomycota versus Basidiomycota *cbhI* sequences among 12 targeted metagenome clone libraries (dark gray bars) versus the average abundance of the types in metatranscriptome libraries (black bars) from Duke Pine Forest FACE site soils (3 replicates per treatment). From [1].

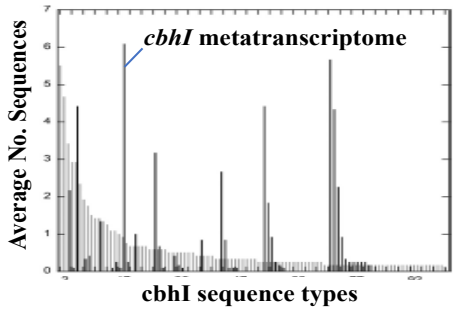

Figure 2. Average abundance of the top 100 *cbhI* sequence types among 12 targeted metagenome clone libraries (light gray bars) versus the average abundance of the types in targeted metatranscriptome libraries (black bars) from Duke Pine Forest FACE site soils. From [1].

the *cbhI* gene from soils collected from the Duke FACE site. This site contained replicate plots exposed to elevated atmospheric CO_2 or ambient CO_2 (control plots) for more than a decade. Half of each CO_2 treatment or control plot was fertilized with ammonium nitrate, which allowed the combined effects of altered CO_2 and N on soil fungal communities to be examined.

The parallel MT and MG profiles differed in important ways. The *cbhI* MG suggested similar abundance of cellulolytic Ascomycota and Basiodiomycota, whereas the *cbhI* MT suggested the Basidiomycota were the primary cellulose consumers (Figure 1; from [1]. Only a third of the *cbhI* types in the MGs were also recovered in the MTs (Figure 2) [1], consistent with the notion that metatranscriptomes can identify the active subset of a larger seedbank.

Shotgun MTs reveal fungal functional genes obscured in MGs

Bacterial cells are often more abundant that fungi in soils, which can pose a challenge to metagenomic assessments of fungal functions, but metatranscriptomes address some of this challenge. When measured by culturing, bacteria often outnumber fungi by a factor of 100. This numerical abundance can overwhelm shotgun metagenomic inventories such that only bacterial are represented. The SFA found evidence consistent with this phenomenon.

In 2012, MGs of soil samples from the Duke FACE site were almost entirely bacterial reads, whereas MTs from the same samples were primarily eukaryotic and mostly of fungal origin (Figure 3)[3, 6]. Similarly, 2017 MGs of soil samples from the Duke site used in a laboratory microcosm study were almost entirely bacterial reads (Figure 4A; from[5]). The corresponding MTs from the same samples contained a substantial fraction of fungal genes (Figure 4A; from [5]).

The greater abundance of fungal transcripts in the MT data may reflect either greater fungal versus bacterial gene expression in the samples, or greater degradation of bacterial messenger RNA transcripts during sample processing. This is one of many emerging technical issues that must be addressed for environmental metatranscriptomics. The SFA is exploring use of internal controls to address this and other technical challenges that must be solved to advance the field.

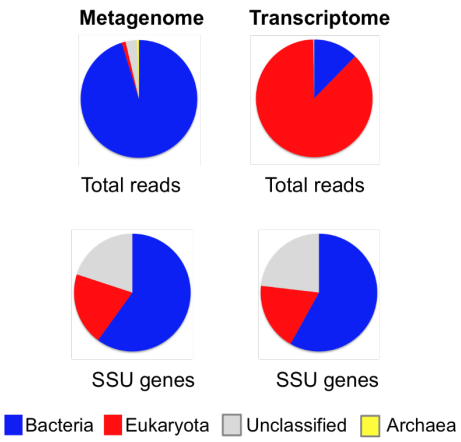

Figure 3. Composition of total sequence reads and small subunit rRNA (SSU) sequences parsed from a pine soil metagenome and corresponding metatranscriptome, illustrating the enrichment of identifiable eukaryotic sequences in the metatranscriptome. [3]

Shotgun MTs reveal N fertilization effects obscured by shotgun MGs

Using a combination of shotgun metagenomics and shotgun metatranscriptomics, the SFA gained an un-expected insight into the effect of N fertilization on expression of microbial carbon cycling genes [5]. Ecosystems are receiving increased nitrogen (N) from anthropogenic sources, including fertilizers and emissions from factories and automobiles. High levels of N change ecosystem functioning. For example, high inorganic N has repeatedly been shown to decrease the rate of microbial decomposition of plant litter, potentially reducing nutrient recycling for plant growth. Numerous studies, including our own [1, 4, 8-12], have also documented soil

microbial community changes after *long-term* experimental nitrogen (N) fertilization in natural ecosystems. However, it is unclear if the observed changes are a direct response to altered N supply, or instead a response of microbial communities to altered plant physiology or other secondary factors.

The clearest picture of direct microbial responses to N fertilization are likely to arise from measurements over a short time scale (hours to days) *immediately* after inorganic N deposition [5]. Therefore, the SFA assessed the short-term (3-day) transcriptional response of microbial communities in two soil strata from a pine forest to a high dose of N fertilization (ca. 1 mg/g of soil material) in laboratory microcosms. During the 3-day experiment, all microcosms showed substantial microbial activity, but the nitrogen fertilization treatment significantly reduced microbial respiration in the two soil strata compared to the unamended controls [5].

Consistent with published studies of fungal pure cultures, we hypothesized that N fertilization would repress the expression of fungal and bacterial genes linked to N mining from complex plant litter. However, the data did not support this hypothesis [5].

The MG data suggested little change in fungal or bacterial community composition (Figure 4B & C; from [5]), suggesting little if any changes in population abundance. In contrast, the MT data suggested substantial shifts in set of active fungal taxa (Figure 4B), and much smaller shifts in the set of active bacteria (Figure 4C) (Albright 2018). The data illustrate the potential to assess population shifts with MG data and activity shifts with MT data.

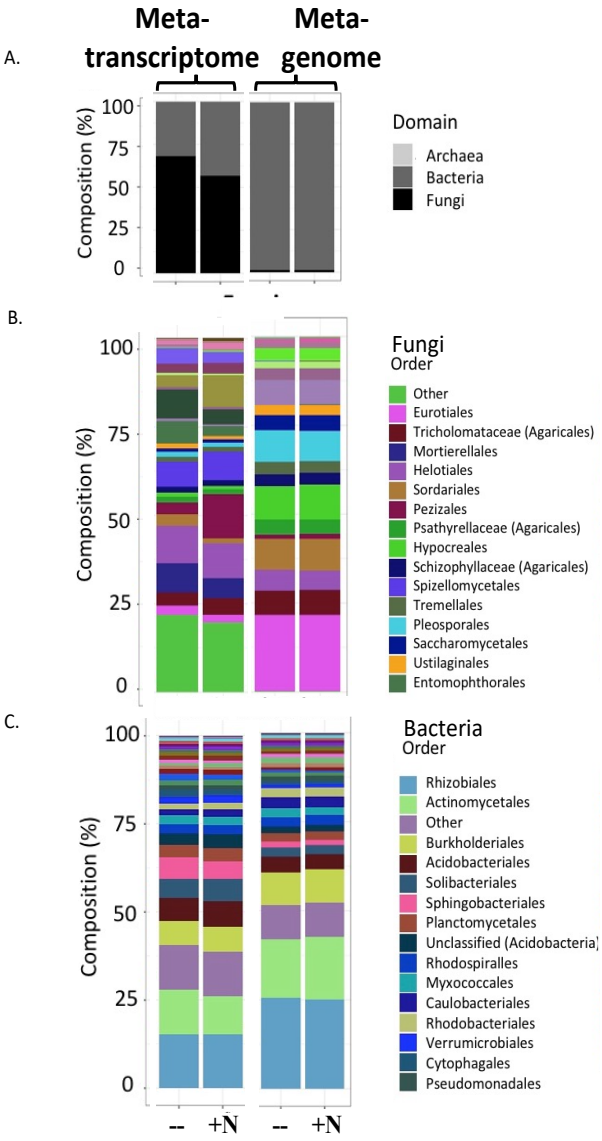

Figure 4. Response of pine forest soil litter layer microbiome to 64 hours of NH_4NO_3 exposure. Soil samples were incubated in microcosms under constant water and temperature conditions. The composition plots in panels A to C show the composite of four replicates per treatment (+/- NH_4NO_3). The NH_4NO_3 treatment significantly reduced respiration (CO_2 efflux). From [5].

At a high functional level (KEGG Level 2 cellular processes), the MG and MT profiles were quite similar between nitrogen treatments (Figure 5; from [5]), despite differences in the sets of active fungi (Figure 4B). These findings illustrate another emerging challenge in environmental

metatranscriptomics – the absence of an interpretive framework that unambiguously links metatranscriptomic profiles at various KEGG functional levels to known physiological changes. For example, the dynamic range of possible profiles at KEGG functional level 2 and the physiological states or changes represented within that dynamic range is unknown. Such knowledge would dramatically enhance data interpretation.

The most pronounced differences in functional gene expression were between soil strata rather than in response to the N addition [5]. Overall, 4% of metabolic genes changed in expression with N addition, while three times as many (12%) were significantly different across the different soil strata used in the microcosms.

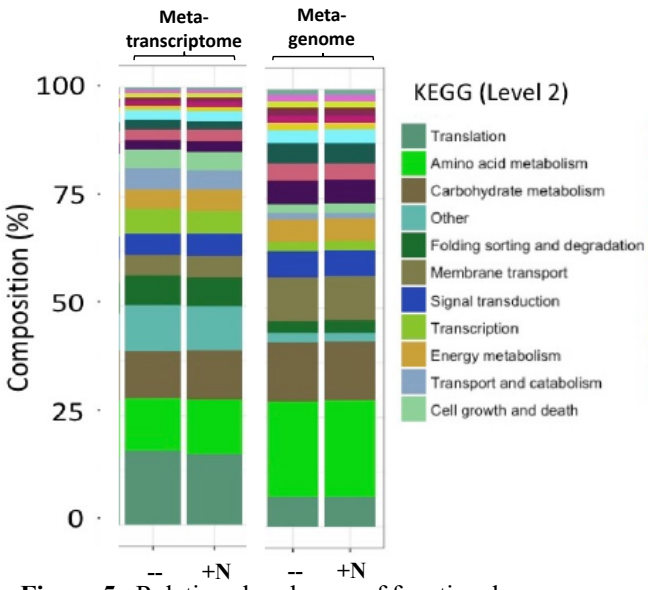

Figure 5. Relative abundances of functional groups (KEGG level 2) averaged across replicates (n = 4). From [5].

There was little evidence of repressed expression of genes involved in complex carbohydrate degradation (CAZymes) or inorganic N utilization [5]. Comparison of CAZymes in MT vs MG data suggested a subset of "active" CAZymes, but very few showed significant changes under N fertilization. The most robust response occurred with three bacterial CAZyme families: CBM52 and CE14 (bacterial enzymes) increased while CBM3 (cellulose binding, bacterial) decreased significantly with N addition in both soil strata. Inorganic N metabolic gene expression (e.g., genes *nifH*, *napA*, *nosZ*, and *amoB*) did not change significantly in response to N addition. N-cycling genes were expected to be enriched in the MT data owing to their metabolic importance under a strong pulse of inorganic N and because the gene sequences would not be diluted by the presence of non-coding sequences that occur in MG data. Contrary to expectation, N-cycling genes were 150-fold less abundant in MT data than in MG data, demonstrating the need for much greater transcriptome sequencing depth to effectively study integrated biogeochemical cycles in surface soils [5].

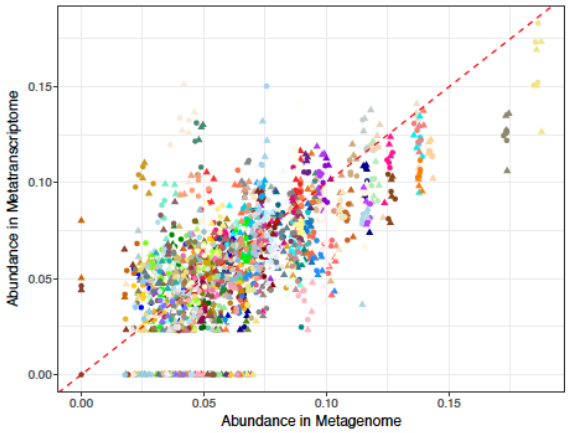

Figure 6. Correlation of CAZyme gene family abundance in paired metatranscriptomes and metagenomes. The marker colors denote different CAZymes. Dashed line indicates 1:1 correlation. Points above the line are interpreted as more highly expressed.

Collectively, the findings suggested that direct N repression of microbial functional gene expression was not the principle mechanism for reduced soil respiration immediately after N deposition [5]. Instead, changes in expression with N addition occurred primarily in general cell maintenance areas, for example, in ribosome-related transcripts. This underscores the question of

the proximal cause (community composition versus N-repression of gene expression) of differences in functional gene abundance under N fertilization in long-term field studies.

MT/MG comparisons enable exploration of "dormancy" as an adaptive trait among microbial groups and its role in assembly of litter decomposer communities

The SFA combined MT and MG data to investigate the potential role of dormancy in structuring bacteria and fungi in Maple forest litter decomposer communities over an environmental gradient [2]. Dormancy, a strategy of entering a reversible state of reduced metabolic activity during unfavorable environmental conditions, is thought to foster diversity within microbial communities. Consequently, it has important implications for community function. In addition to forming "seed banks" of microbial taxa, dormancy has been hypothesized in literature to provide a mechanism for coexistence of ecologically similar species by allowing competing organisms to partition resources across time, rather than in space. The role of dormancy in assembly of communities responding to changes in environmental conditions is unclear. Quantifying changes in the phylogenetic structure of the active and dormant communities in response to changing environmental conditions provides a means to simultaneously examine the distribution of dormancy among microbial groups to better understand how dormancy will interact with environmental conditions to structure communities.

To measure the active and dormant communities of bacteria and fungi colonizing decomposing litter in maple forests, ribosomal RNA genes and transcripts from litter samples were compared across a natural environmental gradient [2]. Using an approach established by others, any OTU with a rRNA/rDNA>1 was scored as active, and any OTU with a rRNA/rDNA<1 was scored as inactive (dormant + dead).

Within bacterial and fungal communities, the active and dormant communities were phylogenetically distinct, but patterns of phylogenetic clustering varied [2]. For bacteria, active communities were significantly more clustered than dormant communities, while the reverse was found for fungi. The proportion of operational taxonomic units (OTUs) classified as active and the degree of phylogenetic clustering of the active bacterial communities declined with increasing pH and decreasing C/N. No significant correlations were found for the fungal community. The opposing pattern of phylogenetic clustering in dormant and active

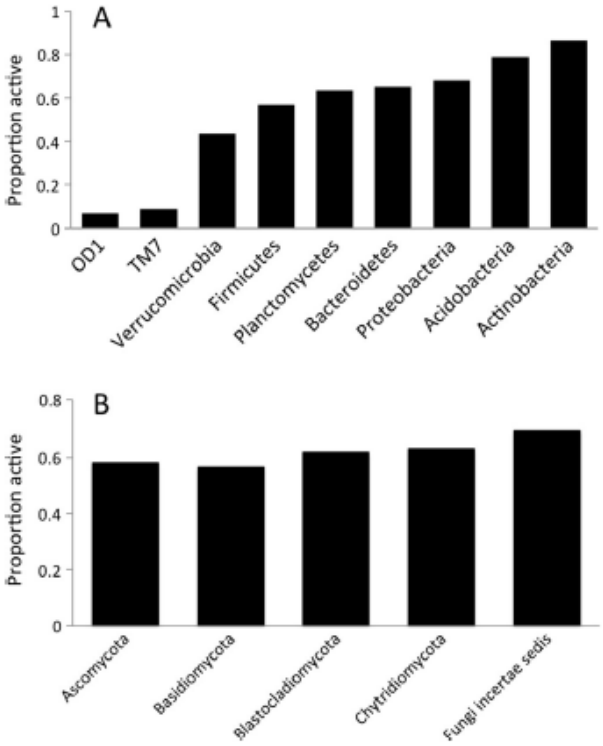

Figure 7. Putative active versus dormant taxa in Maple forest leaf litter decomposer communities based on ribosomal RNA gene ratios of RNA:DNA. The graphs show the proportion of OTUs classified as active within individual phyla for bacteria (a) and fungi (b). The activity assessments were partially validated by correlation with the abundance of Resuscitation Promoting Factor C (*rpfC*) transcripts retrieved from metatranscritome data. From [2]

communities and the differential response of active communities to environmental gradients suggest that dormancy differentially structures bacterial and fungal communities [2].

Calibration of MT/MG comparisons to classify taxa or genes as "active"

To improve interpretation of RNA/DNA ratios for identification of "active" taxa and genes, the SFA used modeling and simulation with a ground-truth dataset to assess potential errors [7]. The general use of RNA/DNA ratios is bewitching because it provides an extra layer of highly valuable information from high-throughput DNA sequencing data. It offers a means to determine not only the seedbank of taxa present in communities but also the subset of taxa that are metabolically active. Although some activity assessments can be (at least partially) orthogonally supported as in [2], there are many circumstances in which validation is not possible.

The SFA addressed this issue by focusing on a specific test case—activity assessments based on ribosomal RNA gene data [7]. As cellular activity increases, the ratio of ribosomal RNA (rRNA) to ribosomal RNA genes (DNA) is expected to increase because ribosome abundance increases much more than the genome copy number in active cells. Consequently, organisms with a higher abundance of rRNA than DNA in community surveys are proposed to be active. A critical detail of the rRNA/DNA method used in microbial community analyses is that the true ratios of rRNA to DNA occurring within cells are not measured; instead, the relative abundance of a taxon in a metatranscriptome is compared to its relative abundance in a metagenome. Although a number of studies have used rRNA/DNA ratios to characterize active microbial populations in environmental samples, there has been little effort to investigate factors that may affect data interpretation.

Simulations were performed to investigate the effects of community structure, intracellular ribosomal RNA amplification, and sequence sampling depth on the accuracy of rRNA/DNA ratios in classifying bacterial populations as "active" or "dormant" [7]. Community structure was a non-significant factor. In contrast, the extent of rRNA amplification that occurs as cells transition from dormant to growing had a significant effect ($P < 0.0001$) on classification accuracy, with misclassification errors ranging from 16 to 28%, depending on the rRNA amplification model. The error rate increased to 47% when communities included a mixture of rRNA amplification models, but most of the inflated error was false negatives (i.e., active populations misclassified as dormant). Sampling depth also affected error rates ($P < 0.001$). Inadequate sampling depth produced various artifacts that are characteristic of rRNA/DNA ratios generated from real communities. These data show important constraints on the use of rRNA/DNA ratios to infer activity status. Whereas classification of populations as active based on rRNA/DNA ratios appears generally valid, classification of populations as dormant is potentially far less accurate [7].

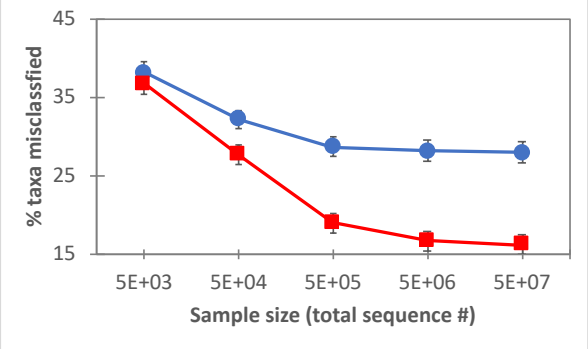

Figure 8. Effect of sample size on misclassification of activity status (dormant vs active) in simulations with two models of how the ribosome content of cells changes with activity state. From [7].

Shotgun MTs reveal long-term N fertilization effects undetected by targeted MGs

To assess the impact of long-term (16 years) of N deposition on Maple forest floor decomposer communities (Michigan), the composition and activity of bacteria and fungi in leaf litter samples from replicate plots at two geographically distant sites were examined [4]. Our hypotheses were (a) that the metatranscriptomes would illustrate community metabolic shifts that were not necessarily detectable in targeted metagenomic (ribosomal RNA gene) taxonomic surveys, and (b) the complex community in the forest floor material would show responses to N deposition that were manifest in both the bacterial and fungal CAZyme activity profiles.

Targeted MGs (ribosomal RNA (rRNA) surveys) of bacterial and fungal biomass and taxonomic composition showed no significant differences in either biomass, OTU richness, or bacterial taxonomic composition between the two sites or in response to N [4]. However, site and N amendment were significant variables for fungal community composition (Figure 9; from [4]), explaining 17 and 14% of the variability, respectively.

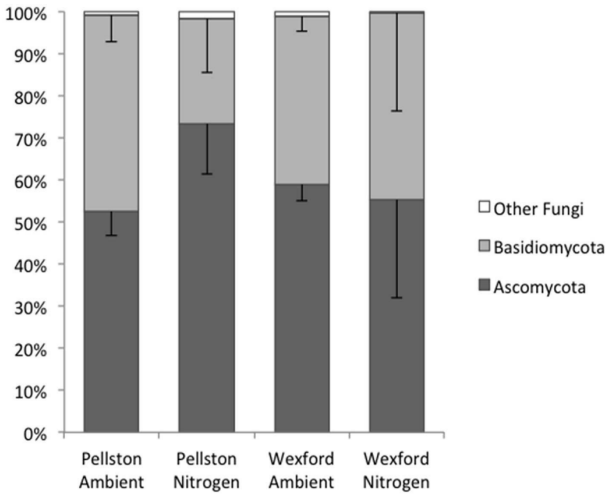

Figure 9. Phylum-level fungal composition from ribosomal RNA gene amplicon sequencing. Data are presented as plot averages (n = 3). From [4]

Functional activity of these communities was explored further using metatranscriptomes that simultaneously captured bacterial as well as eukaryotic sequences [4]. Site and N amendment responses were compared using about 74,000 Carbohydrate Active Enzyme (CAZyme) transcript sequences in each metatranscriptome. The relative abundance of expressed bacterial and fungal CAZymes changed significantly with N amendment in one of the forests, and N-response trends were also identified in the second forest. Although the two ambient forests were similar in community biomass, taxonomic structure and active CAZyme profile, the shifts in active CAZyme profiles in response to N-amendment differed between the sites. One site responded with an over-expression of bacterial CAZymes, and the other site responded with an over-expression of both fungal and different bacterial CAZymes. Both sites showed reduced representation of fungal lignocellulose degrading enzymes in N-amendment plots.

As an exploratory tool in single organism transcriptome studies, the co-expression of genes can be used to identify potential suites of co-regulated or interacting gene suites [4]. Although the interpretation of co-expression patterns in metatranscriptome samples is difficult due to the large number of genes recovered, the transcript correlation technique can still be used to identify suites of correlated, potentially interacting transcripts or organisms. Abundance correlations of the 100 most abundant CAZyme families are shown as a heat map (Figure 10; from [4]). Hierarchical clustering of these co-expression patterns showed a strong grouping of eukaryote CAZyme families (Figure 10, label A), representing many CAZyme families from all CAZyme classes (GH, GT, PL, CE, AA), most of which contain fungal lignocellulolytic activities. This cluster of genes is also negatively correlated with nearly all of the abundant bacterial CAZyme transcripts. Another striking cluster includes 15 bacterial CAZyme families (Figure 10, label B) that are highly correlated with one another. These families are associated with cleavage of beta-

and alpha-galactosidic bonds, glycogen binding, and beta- and alpha-glucosyltransferase activity (GH2, GH3, GH13, GH15). This same cluster is also negatively correlated with both the eukaryotic CAZyme cluster (A), and a cluster of five eukaryotic and six bacterial CAZy families associated with xylan and chitin degradation (GH2, GH5, GH10, GH16; Figure 10, label C), which represent multiple xylan- and chitin-degrading enzymes [4].

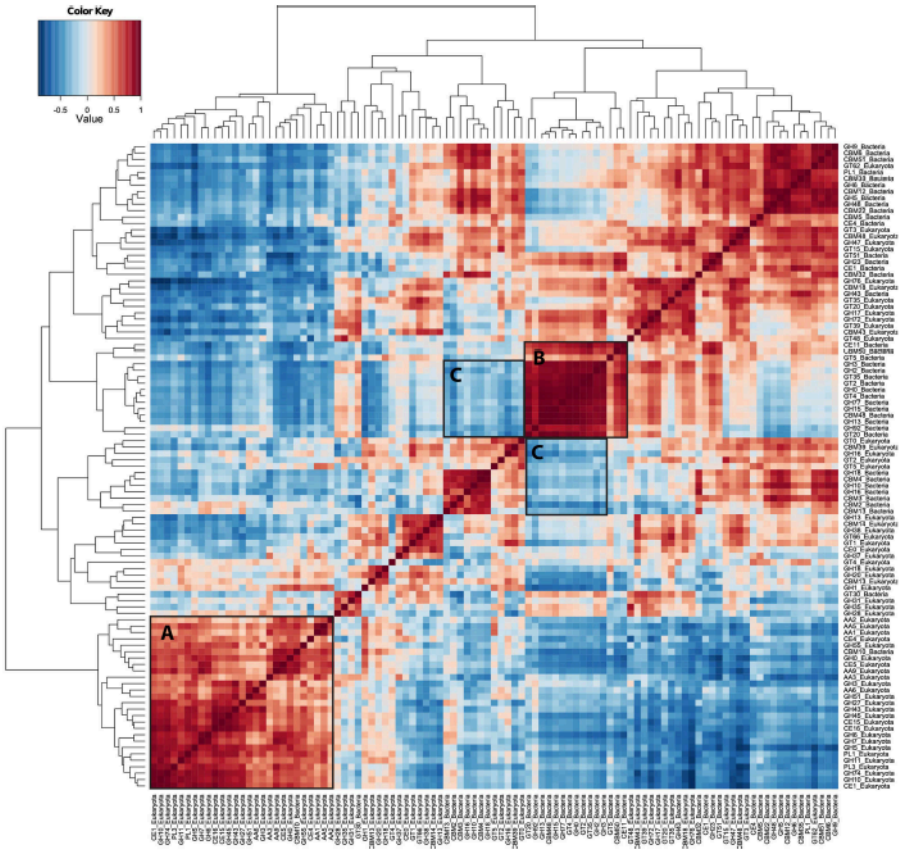

Figure 10. CAZyme transcript abundance correlation heatmap. The co-expression of many CAZymes in the metatranscriptomes illustrates that this approach is able to detect complex patterns of co-expression among carbohydrate-active genes. Pairwise comparisons of the 100 most abundant CAZyme families shows that similar patterns of abundance among the forest floor samples are illustrated as shades of red, while those with differing abundance patterns are shaded blue. Stronger correlations, either positive (red) or negative (blue), are illustrated as darker shades, while pairwise Comparisons that show no co-variation are colored white. Hierarchical Clustering on X- and Y-axes was generated using complete linkage method. CAZyme abundance correlations were calculated using the Pearson method on Wisconsin double standardized abundance data. Box A indicates a group of eukaryote CAZyme families (with one bacterial family) that are co-expressed. Box B shows a group of bacterial CAZyme families that are also co-expressed, while the C Boxes include bacterial and fungal CAZyme families that are anti-correlated with those in Boxes A and B. From [4].

Targeted MG facilitates down-selection of communities for shotgun MT investigation of functional processes

The current research direction for the SFA routinely exploits the integrated use of targeted MG and shotgun MTs. The research direction emphasizes discovery of microbial processes that create substantial variation in carbon flow *under the same environmental*

conditions. This requires screening large numbers (e.g. 100) of communities in order to sufficient cohorts that represent distinct functional states. The functional states of interest are delineated by the abundance of dissolved organic carbon from plant litter decomposition. Contrasting functional states are described as "low" and "high" DOC, respectively, and functional means for the two states differ substantially (e.g., ~2-fold).

For each functional state cohort, targeted metagenomic sequencing of ribosomal genes (i.e., taxonomic profiling) is combined with functional measurements for baseline characterization. The MG data provide a stepping stone to infer functional phenomena when there are sufficient phenotypic metadata available for distinctive taxonomic groups within each cohort. The MG data are also useful in down-selecting communities within each cohort for shotgun MT profiling.

With this approach, the SFA has screened 1600 microcosm decomposer communities (derived from over 400 natural soil samples), down-selected 500 for targeted MG sequencing, and further down-selected 78 for MT profiling. This effort has included decomposition experiments with litter from 4 different types of plants in order to discover common bacterial processes that can create different functional states regardless of plant litter type. An example of the MG data was presented in the BSSD 2019 Performance Metric Q1 report. MT data are adding depth to inferences from the MG data by demonstrating distinctive functional profiles for cohorts representing contrasting functional states. In broad terms, MT data show significant changes in the number and expression levels of CAZymes associated with the high and low DOC functional states. Results from these studies are forthcoming in papers in different stages of peer review and preparation for journal submission.

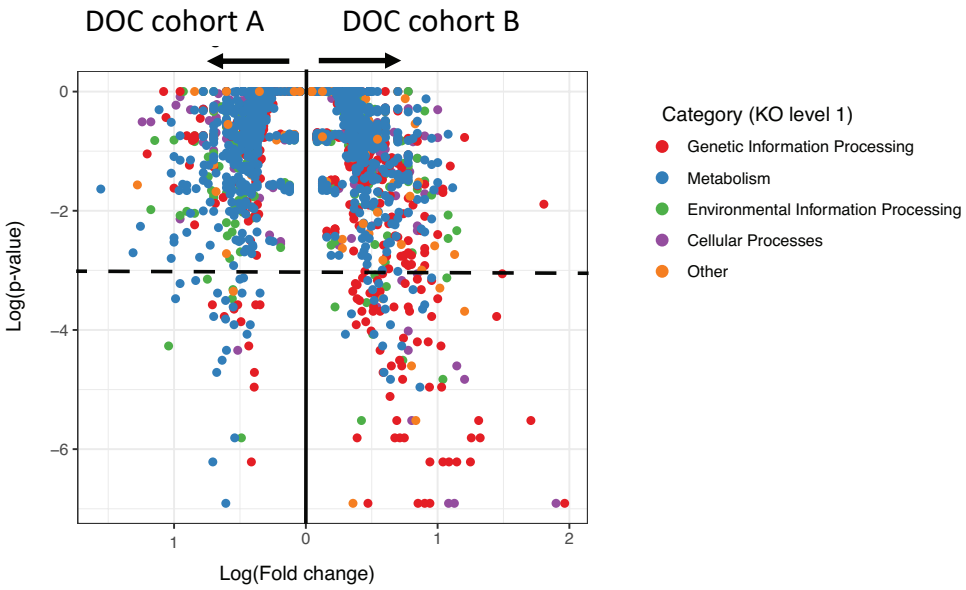

Figure 11. Volcano plot showing differential gene expression of decomposer communities (n=9 per cohort) that represent distinct patterns of carbon flow (measured as Dissolved Organic Carbon abundance) from 44 days of pine leaf litter decomposition in laboratory microcosms. The dashed line is the p-value threshold, corrected for multiple testing. Points below the line represent gene families with significant differences in expression.

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