Microbial Community Analysis Assignment

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Microbial community composition & diversity via amplicon sequencing (16S & 18S rRNA gene regions, ITS regions)

Method:

16S and 18S rRNA gene amplicon sequencing are considered to be the gold standards in identifying microbial community structure in prokaryotes and eukaryotes, respectively (Zhi et al. 2014). Sequences that are greater than 97% similar are assigned to the same species, 95% similar are assigned the same genus, and 80% similar are assigned the same phylum (Zhi et al. 2014). Operational taxonomic units are used to group these microbes together and allow for an easier way to compare community structure (Zhi et al. 2014).

The 16S rRNA gene is able to distinguish the mRNA initiation sites and has implications in tRNA binding and the association of the two ribosomal subunits. This gene is the most common genetic marker for examining microbial taxonomy for a number of reasons; these include its presence in all prokaryotes (often in an operon), its function not changing over time meaning that its an accurate measure of evolution, and its large size (1,500 bp) allowing for informatic analysis (Janda and Abbott 2007).

Similar to the 16S rRNA gene, the 18S rRNA gene is a useful phylogenetic marker especially for high resolution taxonomic studies. The eukaryotic cistron also contains two internal transcribed spacers (ITS) that get spliced post-transcription and are more commonly used as barcode markers for diversity studies.

To prepare samples for amplicon sequencing, the DNA must first be extracted, followed by amplification by polymerase chain reaction (PCR) where the 16S rRNA or the 18S rRNA gene is denatured, annealed, and then extended (Petti et al. 2020). Next, the PCR products are purified, and then sent through another round of PCR followed by purification.

Information Obtained:

Amplicon sequencing provides the DNA sequences of the gene of interest (either 16S or 18S rRNA) in each of the samples (Petti et al. 2020). This allows for comparison with reference sequences to determine taxonomic classification of the microorganisms; entire phylogenetic trees can even be assembled this way (Petti et al. 2020).

Example:

In a study conducted by Johnston and Behrens 16S rRNA gene amplicon sequencing was used to analyze the microbial community within activated sludge (2020). It is thought that these microorganisms remediate wastewater, and the goal of the study was to gain a better understanding of their activity patterns throughout the seasons, specifically population abundance and growth (Johnston and Behrens 2020). It is thought that the same group of microbes remains in the activated sludge throughout the year, despite the fact that seasonal abundances of individuals change (Johnston and Behrens 2020). Every ten minutes, triplicate sequencing batch reactors were sampled during reaction cycles throughout all four seasons (Johnston and Behrens 2020). The gene and transcripts of the 16SrRNA amplicons were quantified using PCR and the products were sequenced to determine community composition (Johnston and Behrens 2020). The sequences were grouped into 108 operational taxonomic units (OTUs) all having stable abundance, activity, and growth throughout all four seasons (Johnston and Behrens 2020). Significant differences ($p<0.05$) in transcript copy numbers were observed at lower temperatures (Johnston and Behrens 2020).

Advantages:

Gene amplicon sequencing is a widely used technique due to its highly targeted and sensitive method (Gupta et al. 2019). This technique does not require the sample of interest to be culturable, and the relative abundance of all microbes present in the sample can be examined (Gupta et al. 2019). Additionally, numerous sequences can be sequenced simultaneously which is a major advantage.

Disadvantages:

The major disadvantage of gene amplicon sequencing is its low phylogenetic power at the species level (Janda and Abbott 2007). It is also discriminatory towards some genera meaning that their DNA sequences will not be read as well or as much as others.

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Abstract:

Microbial community composition & diversity via amplicon sequencing (16S & 18S rRNA gene regions, ITS regions)

From Johnston and Behrens 2020…

"Activated sludge is comprised of diverse microorganisms which remediate wastewater. Previous research has characterized activated sludge using 16S rRNA gene amplicon sequencing, which can help to address questions on the relative abundance of microorganisms. In this study, we used 16S rRNA transcript sequencing in order to characterize "active" populations (via protein synthesis potential) and gain a deeper understanding of microbial activity patterns within activated sludge. Seasonal abundances of individual populations in activated sludge change over time, yet a persistent group of core microorganisms remains throughout the year which are traditionally classified on presence or absence without monitoring of their activity or growth. The goal of this study was to further our understanding of how the activated sludge microbiome changes between seasons with respect to population abundance, activity, and growth. Triplicate sequencing batch reactors were sampled at 10-min intervals throughout reaction cycles during all four seasons. We quantified the gene and transcript copy numbers of 16S rRNA amplicons using real-time PCR and sequenced the products to reveal community abundance and activity changes. We identified 108 operational taxonomic units (OTUs) with stable abundance, activity, and growth throughout the year. Nonproliferating OTUs were commonly human health related, while OTUs that showed seasonal abundance changes have previously been identified as being associated with floc formation and bulking. We observed significant differences in 16S rRNA transcript copy numbers, particularly at lower temperatures in winter and spring. The study provides an analysis of the seasonal dynamics of microbial activity variations in activated sludge based on quantifying and sequencing 16S rRNA transcripts."

Metagenomics for Microbial or Viral Communities

Method:

Advancements in ultra-high throughput sequencing technology have allowed for large numbers of DNA sequences to be read at an affordable cost; this has increased the number and overall scope of metagenomic sequencing projects (Huson et al. 2009). This method is appealing for researchers because cloning or PCR amplification is not a prerequisite for sequencing (Huson et al. 2009). Metagenomics is a culture independent genomic analysis of the microbial community within a particular niche (Sleator et al. 2008). It involves the direct isolation of DNA from samples of the environment of interest, followed by cloning of the complete genomes of each individual microbe (Sleator et al. 2008). This results in a DNA library which can be further examined for functions and sequences of interest (Sleator et al. 2008).

Viral metagenomics determines the genetic composition of viral particles from environmental or biological samples (Li et al. 2015). This technique has been frequently used for viral discovery, especially regarding human and animal viruses (Li et al. 2015). Characterization first requires non-specific nucleic acid amplification, due to the low viral nucleic acid concentrations, before DNA sequencing can occur – either plasmid cloning and Sanger sequencing or next-generation sequencing can be performed (Li et al. 2015).

New ways for comparing multiple metagenomic datasets are necessary as metagenome analysis is shifting from species and function identification of individual datasets to comparative analysis (Huson et al. 2009).

Information Obtained:

Metagenomics studies the uncultured organisms and examines the diversity, functions, cooperation, and evolution of microbes in a wide range of environments such as soil, water, and the digestive systems of humans and animals. Metagenomics can be divided into either function-driven or sequence-based analysis of uncultured microbes (Sleator et al. 2008). In function-driven metagenomics, DNA libraries are screened for a particular phenotype; some examples are salt tolerance and enzymatic activity (Sleator et al. 2008). Following this, the phylogenetic origin of the cloned DNA can be found (Sleator et al. 2008). Sequence-based metagenomics involves the screening of clones for the 16S rRNA gene for identification,

followed by the sequencing of the entire clone for other genes of interest (Sleator et al. 2008). Large-scale metagenome sequencing can also be performed to "search for phylogenetic anchors in the reconstructed genomes" (Sleator et al. 2008).

Example:

Metagenomic analysis was used in a study performed by Hendriksen et al. to examine the antimicrobial resistant bacteria found in untreated sewage (2019). Antimicrobial resistance (AMR) is an ongoing threat to the health of all organisms; however, "obtaining representative data on AMR for healthy human populations is difficult" (Hendriksen et al. 2019). For this experiment, 79 sites in 60 different countries were analyzed, and differences in abundance and diversity of AMR genes were found in Europe, North America, Oceania and Africa, Asia, and South America (Hendriksen et al. 2019). This does not account for the effect of air travel between locations. There appears to be a strong correlation between socio-economic, health, and environmental factors with AMR gene abundances around the world (Hendriksen et al. 2019). This suggests that AMR gene diversity and abundance vary, and "improving sanitation and health could potentially limit the global burden of AMR" (Hendriksen et al. 2019).

Advantages:

Metagenomics has expanded researchers' knowledge of the biodiversity of single-celled organisms by recovering the sequences of uncultured bacteria and archaea (Schulz et al. 2020). This method has also recently proven to be an accurate technique for examining the diversity of viruses which are a very unknown group of entities (Schulz et al. 2020).

Disadvantages:

There are some notable disadvantages of the metagenomics method. These include low resolution because of the lack of sequence coverage in complex environments, the incapability of classifying short metagenomic fragments, and the lack of verification of the function of the genes of interest (Sleator et al. 2008).

References and Citations

Metagenomics for Microbial or Viral Communities

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Abstract:

Metagenomics for Microbial or Viral Communities

From Hendriksen et al. 2019…

"Antimicrobial resistance (AMR) is a serious threat to global public health but obtaining representative data on AMR for healthy human populations is difficult. Here, we use metagenomic analysis of untreated sewage to characterize the bacterial resistome from 79 sites in 60 countries. We find systematic differences in abundance and diversity of AMR genes between Europe/North-America/Oceania and Africa/Asia/South-America. Antimicrobial use data and bacterial taxonomy only explains a minor part of the AMR variation that we observe. We find no evidence for cross-selection between antimicrobial classes, or for effect of air travel between sites. However, AMR gene abundance strongly correlates with socio-economic, health and environmental factors, which we use to predict AMR gene abundances in all countries in the world. Our findings suggest that global AMR gene diversity and abundance vary by region, and that improving sanitation and health could potentially limit the global burden of AMR. We propose metagenomic analysis of sewage as an ethically acceptable and economically feasible approach for continuous global surveillance and prediction of AMR."

Microbial Community Transcriptomics (whole-transcriptome shotgun sequencing; RNA-seq)

Method:

Transcriptomics is a method that examines both the coding and non-coding regions of RNA sequences and quantifies gene expression in cells, tissues, organs, and organ systems (Jiang et al. 2015). RNA sequencing analyzes the ever-changing cellular transcriptome of microorganisms using next generation sequencing methods. It is a high-throughput culture-independent method that exceeds the genetic potential of DNA-based assays (Zhi et al. 2014). It utilizes signals from both phylogenetic and metabolic markers to reconstruct the composition and activity within microbial communities simultaneously (Zhi et al. 2014). Transcriptomic analysis lays the foundation for examining the regulatory pathways and genetic interactions that influence both qualitative and quantitative phenotypes of microbes (Jiang et al. 2015). Shotgun sequencing is a method that uses nucleic acids from samples to sequence and identify the expression levels of a particular gene of interest (Thoendel et al. 2020). It involves breaking the DNA strands within a sample into small fragments (100-1000 bp in length), and then subsequently sequencing each piece.

Information Obtained:

It provides the first steps towards characterizing gene functions by providing the information required to analyze genetic interactions in cellular functions, growth and development, and various biological systems (Jiang et al. 2015). Whole transcriptomics also "produces molecular fingerprints of disease processes and prognoses" which can be used to understand host and pathogen relationships (Jiang et al. 2015).

Example:

In a study conducted by Seyler et al., metagenomic and metatranscriptomic data from low temperature, ridge-flanked environments was used to reconstruct the metabolic potential, transcript abundance, and microbial community dynamics of hydrothermal fluid circulation in the oceans (2021). The microbial life within the oceanic crustal aquifer is thought to have large impacts on biogeochemical cycles (Seyler et al. 2021). The North Pond community is motile and metabolically flexible and has the ability to use both autotrophic and organotrophic pathways;

therefore, they are important for oxidizing organic carbon within the crust (Seyler et al. 2021). These microbes also function in low oxygen conditions by using nitrate and thiosulphate as terminal electron acceptors – anaerobic processes are most commonly found at the deepest level of the aquifer (Seyler et al. 2021). This study demonstrated the heterogeneity of microbes in the subseafloor aquifer and highlights biogeochemical cycling in oceanic crust (Seyler et al. 2021).

Advantages:

The most important advantage of microbial community transcriptomics is the precision of the results, making them more reproducible and ideal for comparative studies (Zhi et al. 2014). Each determination of gene function is added to a growing database that is used by scientists all over the world for further research (Zhi et al. 2014).

Disadvantages:

A disadvantage of transcriptomics is the technical challenge of optimizing sample preparation for pathogen detection while trying to minimize as much background signal and contamination as possible (Thoendel et al. 2020). Furthermore, metatranscriptomic shotgun sequences contain numerous reads making them challenging to analyze and interpret, especially if no close matches exist in the database (Thoendel et al. 2020; Zhi et al. 2014).

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Microbial Community Transcriptomics (whole-transcriptome shotgun sequencing; RNA-seq)

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Abstract:

Microbial Community Transcriptomics (whole-transcriptome shotgun sequencing; RNA-seq)

From Seyler et al. 2020…

"The oceanic crustal aquifer is one of the largest habitable volumes on Earth, and it harbors a reservoir of microbial life that influences global-scale biogeochemical cycles. Here, we use time series metagenomic and metatranscriptomic data from a low-temperature, ridge flank environment representative of the majority of global hydrothermal fluid circulation in the ocean to reconstruct microbial metabolic potential, transcript abundance, and community dynamics. We also present metagenome-assembled genomes from recently collected fluids that are furthest removed from drilling disturbances. Our results suggest that the microbial community in the North Pond aquifer plays an important role in the oxidation of organic carbon within the crust. This community is motile and metabolically flexible, with the ability to use both autotrophic and organotrophic pathways, as well as function under low oxygen conditions by using alternative electron acceptors such as nitrate and thiosulfate. Anaerobic processes are most abundant in subseafloor horizons deepest in the aquifer, furthest from connectivity with the deep ocean, and there was little overlap in the active microbial populations between sampling horizons. This work highlights the heterogeneity of microbial life in the subseafloor aquifer and provides new insights into biogeochemical cycling in ocean crust."

Microbial Community Proteomics

Method:

Proteomics is a field of research that studies the entire protein complement within a complex microbial community at a certain point in time (Wang et al. 2016). This method has the potential to determine microbial diversity, metabolic potential, ecological function, and microbe-environment interactions (Wang et al. 2016). Genome decoding technique and high-throughput sequencing has expanded microbial community proteomics within a variety of environments including marine water, sediments, activated sludge, groundwater, and acid mine biofilms (Wang et al. 2016).

Due to the complexity of environmental samples, two different methods for protein separation and identification have been created (Wang et al. 2016). The first method is gel-based. Mixed proteins are separated by either 1D or 2D polyacrylamide gel electrophoresis (Wang et al. 2016). Following this, the target protein bands are cut out of the gel and digested into peptides with an enzyme such as trypsin (Wang et al. 2016). The peptides are analyzed using mass spectrometry, database searching, and bioinformatic analysis (Wang et al. 2016). The second method is liquid-chromatography based. The whole proteome is digested into a complex peptide mixture without having first been separated in a gel (Wang et al. 2016). Next, the peptides are separated using strong cation exchange chromatography or microcapillary reverse-phase coupled with tandem mass spectrometry and the data is examined for protein identification and then bioinformatic analysis (Wang et al. 2016). The second method increases the proteome coverage meaning that high-throughput identification can be done for a large number of proteins in a shorter amount of time (Wang et al. 2016)

Information Obtained:

Proteomics provides insight to the regulation of gene expression, protein synthesis, the stability of mRNA, and the translation of mRNA to proteins in response to environmental conditions and stressors (Wang et al. 2016). This method also helps to link microbial species to their ecological function via use of the environmental proteomic database (Wang et al. 2016).

Metaproteomics considers physiological activity alongside translational regulation of microorganisms in their environments (Wang et al. 2016).

Example:

The proteomics method was used in a study conducted by Xie et al. to examine a layer in the ocean known as the deep chlorophyll maximum (DCM); this is a critical layer that is high in biomass and productivity as well as determines marine productivity, biogeochemical cycling, and carbon sequestration (2020). The microbial community structure and metabolic activities are not very well known in this layer of the ocean, thus, this study aimed to characterize proteins of three size fractions using a proteomic approach (Xie et al. 2020). Overall, 17,724 non-redundant proteins were identified with the most abundant being cyanobacteria, SAR11, nitrite-oxidizing bacteria, archaea, eukaryotic phytoplankton, and phototroph-associated viruses (Xie et al. 2020). These organisms were active in light-dependent energy transduction, carbon fixation, nitrification, sulfur metabolism, dissolved organic matter uptake, and methylated compound oxidation (Xie et al. 2020). *Nitrospinae* and *Thaumarchaea* complemented carbon fixation pathways within the DCM (Xie et al. 2020). This experiment offered a comprehensive view of microbial community and metabolic processes – underlining the new biogeochemical processes present in the DCM (Xie et al. 2020).

Advantages:

The major advantage of proteomics is the large-scale output of quantitative information with high accuracy and precision; this is particularly useful for comparative and quantitative proteomics where the main goal is to determine differences in protein expression in different biological states or environmental gradients (Wang et al. 2016). Additionally, this label-free approach has increased the flexibility of several different comparisons (Wang et al. 2016).

Disadvantages:

One of the main challenges of proteomics is sample collection and preservation, especially for low-abundance proteins (Wang et al. 2016). The information provided by the metaproteome identifies the abundant microbes, not the rare species (Wang et al. 2016).

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Microbial Community Proteomics

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Abstract:

Microbial Community Proteomics

From Xie et al. 2020…

"The deep chlorophyll maximum (DCM), a critical layer in the ocean characterized by the highest biomass and productivity, determines marine productivity, biogeochemical cycling and carbon sequestration. However, a comprehensive understanding of microbial community structure and metabolic activities in this layer is lacking in several parts of the oceans. Here, we characterized the whole spectrum of proteins covering three size fractions (0.7-200 μm, 0.2-0.7 μm, 10 kDa-0.2 μm) in the DCM of the South China Sea using a metaproteomic approach. A total of 17 724 non-redundant proteins were confidently identified. Proteins from Cyanobacteria, SAR11, nitrite-oxidizing bacteria, Archaea, eukaryotic phytoplankton and phototroph-associated viruses were abundant. These organisms were actively involved in diverse biogeochemical processes including light-dependent energy transduction, carbon fixation, nitrification, sulfur metabolism, dissolved organic matter (DOM) uptake, and C1 and methylated compounds oxidation. Furthermore, chemolithoautotrophic activity of Nitrospinae and Thaumarchaea complemented carbon fixation pathways in this habitat. Notably, photoheterotrophic activity of SAR11 and PVC (Planctomycetes, Verrucomicrobia and Chlamydiae) bacteria and mixotrophic activity of photoautotrophs suggested diverse regulation channels of light on microbe-mediated DOM recycling. This in-depth metaproteomic study provides a holistic view of microbial community and metabolic activities in the DCM, and uncovers novel biogeochemical processes, especially those previously ignored but potentially active in the smallest fraction."

Microbial Community Metabolomics

Method:

Metabolomics is a technique used to identify and quantify all of the metabolites in a microbial sample (Idle and Gonzalez 2007). It is important to avoid any metabolic changes during the pre-analysis phase of metabolomics to ensure reliable data in the end – the samples must reflect the physiological state as they did at the moment of sampling (Meyer et al. 2013). Rapid sampling is also necessary due to the rapid turnover of certain metabolites, such as ATP (Meyer et al. 2013). The most commonly used sampling methods in metabolomics are direct quenching, cold centrifugation, and fast, vacuum-dependent filtration (Meyer et al. 2013). Adenylate energy charge (EC) is a parameter used to analyze the sampling quality; it represents the energy state of the cell and is defined as (ATP + ½ ADP)/(AMP + ADP + ATP) (Meyer et al. 2013). In the metabolomics method, EC is to monitor cell stress which is shown in metabolic changes (Meyer et al. 2013). Most adaptation processes are ATP dependent meaning that, when stress is induced, ATP decreases causing a reduction in EC (Meyer et al. 2013).

Information Obtained:

These metabolites alongside their interactions within a biological system make up the metabolome. The objective of this method is to determine how the physiological conditions of a microbe are linked to external events, such as how they are able to interact with their environment. Substrates, intermediates, and products of metabolism are determined and analyzed.

Example:

Metabolomics was used in a study by Han et al. to determine the connections between microbial strains in the human gut and host phenotypes (2021). Gut microbes play an important role in maintaining the health of humans (Han et al. 2021). An integrated mass-spectrometry pipeline was created to identify the microbiota-dependent metabolites in a range of sample types (Han et al. 2021). The metabolic profiles of 178 gut microbe strains were determined based on a constructed library of 833 metabolites (Han et al. 2021). This microbiome-based

metabolomics pipeline and interactive profile, together, provide an efficient way for characterizing the interactions between microorganisms and their hosts (Han et al. 2021). Deviations in the connections between phylogeny and metabolism were used to determine a novel type of metabolism in *Bacteroides*, and subsequent biochemical pathways were identified using comparative genomics (Han et al. 2021).

Advantages:

Metabolomics provides a close link to the phenotypes of microorganisms. An advantage of this method is the use of nuclear magnetic resonance spectroscopy and mass spectrometry to enhance the resolution of the comparison of numerous different chemical substances in the microbial samples of interest (Idle and Gonzalez 2007). This method has also led to the expansion of small molecule biochemistry (Idle and Gonzalez 2007).

Disadvantages:

A major drawback of the metabolomics method is that it is sensitive to genetic and environmental stimuli (Johnson and Gonzalez 2012). Several factors have to be considered when executing this technique so that confounding factors are limited (Johnson and Gonzalez 2012). Another drawback to this method, specifically involving the direct quenching sampling method, is that intracellular metabolites may leak out when an organic solvent is added to induce cell lysis (Meyer et al. 2013).

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Microbial Community Metabolomics

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Abstract:

Microbial Community Metabolomics

From Han et al. 2021…

"Gut microorganisms modulate host phenotypes and are associated with numerous health effects in humans, ranging from host responses to cancer immunotherapy to metabolic disease and obesity. However, difficulty in accurate and high-throughput functional analysis of human gut microorganisms has hindered efforts to define mechanistic connections between individual microbial strains and host phenotypes. One key way in which the gut microbiome influences host physiology is through the production of small molecules[1,2,3], yet progress in elucidating this chemical interplay has been hindered by limited tools calibrated to detect the products of anaerobic biochemistry in the gut. Here we construct a microbiome-focused, integrated mass-spectrometry pipeline to accelerate the identification of microbiota-dependent metabolites in diverse sample types. We report the metabolic profiles of 178 gut microorganism strains using our library of 833 metabolites. Using this metabolomics resource, we establish deviations in the relationships between phylogeny and metabolism, use machine learning to discover a previously undescribed type of metabolism in *Bacteroides*, and reveal candidate biochemical pathways using comparative genomics. Microbiota-dependent metabolites can be detected in diverse biological fluids from gnotobiotic and conventionally colonized mice and traced back to the corresponding metabolomic profiles of cultured bacteria. Collectively, our microbiome-focused metabolomics pipeline and interactive metabolomics profile explorer are a powerful tool for characterizing microorganisms and interactions between microorganisms and their host."

Stable Isotope Probing for Tracking Microbial Metabolism Through Biomarker Analysis

Method:

Stable Isotope Probing (SIP) is an *in situ* method that identifies active microbes within a particular complex environment that carry out specific metabolic processes (Zhi et al. 2014). Even though samples enriched with nitrogen and oxygen are abundant in the environment, SIP experiments are characterized by incorporation of isotope-labelled substrates (Zhi et al. 2014). SIP describes an entire suite of methods all involving the exposure of a sample to a stable, isotope-enriched substrate and then labelled with a biomarker such as DNA or RNA (Dumont and Murrell 2005). A key component of the substrates is that they do radioactively decay.

Stable isotope probing is a method used to identify microbes in an environment that grow under the conditions of a particular substrate (Dumont and Murrell 2005). A substrate that is enriched with a stable isotope, such as ^{13}C, identifies the active microbes via selective recovery and analysis of the isotope-enriched cellular components (Dumont and Murrell 2005). DNA and rRNA provide the most information and, therefore, are commonly used as biomarkers (Dumont and Murrell 2005). ^{13}C-labelled molecules can also be purified from unlabelled nucleic acids by density-gradient centrifugation (Dumont and Murrell 2005).

Information Obtained:

An isotope array can also be used to analyze the activity of microbes in their natural environment (Dumont and Murrell 2005). An environmental sample, with the RNA previously extracted, is incubated with a ^{14}C labelled substrate, labelled with a fluorophore and examined using an oligonucleotide array that targets the 16S rRNA gene of the microbe of interest (Dumont and Murrell 2005). Researchers can then determine which microorganisms have the incorporated ^{14}C isotope in their RNA sequences by scanning the array for fluorescence (Dumont and Murrell 2005).

Example:

Stable Isotope probing is used in a study conducted by Li et al. to examine nitrogen loss in paddy soils in Southern China (2019). The Feammox pathway, which consists of anaerobic

ammonium being coupled to iron reduction, has been recently discovered as a potential cause of nitrogen loss; however, little is known about the microbes that drive this reaction (Li et al. 2019). ^{15}N isotopic tracing was used to locate Feammox in the paddy soils, and RNA-based stable isotope probing (SIP), combined with next generation sequencing, was used to examine the microbial communities involved with the pathway (Li et al. 2019). N_2 production was the most dominant pathway found in all of the collected soil samples and approximately 6.91% of the applied nitrogen fertilizers were lost through Feammox (Li et al. 2019). SIP determined that the composition of the active microbial communities within the paddy soils were dependent upon soil pH and grain size (Li et al. 2019). The presence of Geobacter, GOUTA19, Nitrososphaeraceae, and Pseudomonas during the incubation of Feammox microbes suggest that Feammox is ubiquitous, and these microbes are likely associated in natural agricultural soils (Li et al. 2019).

Advantages:

The most important advantage of SIP is that it incorporates isotope labelled elements (^{13}C, ^{18}O, or ^{15}N) into the cells of active microbes (Jiang et al. 2018). This makes stable isotope probing a powerful technique for link phylogenetic identities to the ecological functions of organisms *in situ* (Jiang et al. 2018). This is a very promising method, especially when combined with other advancements in microarrays and metagenomics (Dumont and Murrell 2005).

Disadvantages:

Because samples enriched with nitrogen and oxygen are abundant in the environment, a bias could result regarding the incubation of the sample with the stable isotope (Zhi et al. 2014). Isotope cycling within the microbial community is a potential drawback of this method.

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Abstract:

Stable Isotope Probing for Tracking Microbial Metabolism Through Biomarker Analysis

From Li et al. 2019,

"Anaerobic ammonium oxidation coupled to iron reduction (Feammox) is a recently discovered pathway contributing to nitrogen loss in various ecosystems such as paddy soils and sediments. However, little is known about the microbes driving Feammox in an agricultural ecosystem. Here, we demonstrated the occurrence of Feammox in paddy soils of Southern China using a ^{15}N isotopic tracing technique, and examined the microbial communities associated with Feammox using RNA based stable isotope probing (RNA-SIP) combined with Illumina sequencing. Feammox was detected in all collected soils with direct N_2 production as the dominant Feammox pathway. It was estimated that approximately 6.91% of the applied nitrogen fertilizers were lost through Feammox in the paddy soils. RNA-SIP results showed that the composition of enriched active microbial communities were dependent on soil properties, especially the soil pH and grain size. Geobacter were enriched in most soils across various properties. The abundance of enriched GOUTA19 were significantly higher in soils with low pH than those in soils with medium pH and high pH, and the relative abundance of active Nitrososphaeraceae and Pseudomonas only increased in soils with medium and high pH during 4-day of incubation. These results suggested Feammox is a ubiquitous and important process for N loss. Geobacter, GOUTA19, Nitrososphaeraceae and Pseudomonas were active during the incubation that favored Feammox and the growth of Feammox microbes, suggesting these microbes were potentially associated with Feammox in natural agricultural soils."

Reverse-transcriptase Quantitative PCR for Monitoring Microbial Gene Expression

Method:

Reverse transcriptase quantitative PCR (RT-qPCR) is a method used to quantify gene expression levels in samples of interest (Taylor et al. 2010). It is important that the RNA used for RT-qPCR is highly pure and integral as this is "one of the most critical points" in the experimental workflow (Taylor et al. 2010). If impurities are present in the sample, the reverse transcriptase may be inhibited leading to improper amplification of the sequence (Taylor et al. 2010). Performing the reverse transcription of RNA to complementary DNA (cDNA) early in the assay is necessary to decrease the risk of degradation via several freeze/thaws or RNase contamination (Taylor et al. 2010). The primer and target sequences must be chosen carefully to ensure efficient amplification by PCR; therefore, a RT buffer should contain a mix of random sequence primers to allow better sampling (Taylor et al. 2010). It is recommended that target sequences be unique (75-150 bp in length), contain approximately 50-60% GC content and have a melting temperature between 55°C and 65°C (Taylor et al. 2010). A validated qPCR assay has been "assessed for the optimal range of primer annealing temperatures, reaction efficiency, and specificity using a standard set of samples" (Taylor et al. 2010). This is an important step to ensure that the cDNA samples are not affected by inhibitors of Taq polymerase (Taylor et al. 2010).

Information Obtained:

During RT-qPCR, cDNA is created from an RNA sequence via reverse transcriptase and then subsequently quantified by qPCR (Zhi et al. 2014). This technique can either be used to quantify gene expression levels or to verify meta-transcriptomes (Zhi et al. 2014). qPCR will display how much mRNA is present in the sample of interest.

Example:

In a study conducted by Nagura-Ikeda et al. RT-qPCR was used to detect the presence of RNA of severe acute respiratory syndrome (SARS-CoV-2), more commonly known as COVID-19, and viral antigens were discovered using a rapid antigen immunochromatographic assay (2020). Saliva samples from patients with confirmed cases of COVID-19 were collected, and the RNA

was quantified using a RT-qPCR laboratory test, high-throughput system, direct RT-qPCR kits, and reverse transcription-loop mediated isothermal amplification (RT-LAMP) (Nagura-Ikeda et al. 2020). A total of 103 samples were taken – viral RNA was detected in 50.5-81.6% of the specimens and antigens were seen in 11.7% (Nagura-Ikeda et al. 2020). Significantly higher amounts of RNA were detected in samples within the first nine days of experiencing symptoms (65.6-93.4%) compared to samples taken after ten days of symptoms or from asymptomatic persons (Nagura-Ikeda et al. 2020). The RT-qPCR laboratory test, high-throughput system, direct RT-qPCR kits, and RT-LAMP has proven to be sensitive enough to the viral particles of Sars-CoV-2 and is considered a reliable test for COVID-19 (Nagura-Ikeda et al. 2020). The antigen test has a lower sensitivity; therefore, it is not recommended as a sole method for detecting COVID-19 (Nagura-Ikeda et al. 2020).

Advantages:

The major advantage of this technique it that it provides quantitative, molecular data to support phenotypic observations (Taylor et al. 2010). It provides a "fast and high-throughput detection of target DNA sequences in different matrices (Kralik and Ricchi 2017)." Amplification and visualization of new DNA amplicons occur simultaneously (Kralik and Ricchi 2017).

Disadvantages:

A disadvantage of this technique concerns the instability of mRNA molecules. According to Roberts et al., "widespread use of this technique has been limited because of its technical difficulty [to analyze total steady-state mRNA sequences] (Roberts et al. 2015)." Furthermore, qPCR cannot distinguish between viable and dead cells which is another limitation (Kralik and Ricchi 2017).

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Reverse-transcriptase Quantitative PCR for Monitoring Microbial Gene Expression

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Abstract:

Reverse-transcriptase Quantitative PCR for Monitoring Microbial Gene Expression

From Nagura-Ikeda et al. 2020,

"The clinical performances of six molecular diagnostic tests and a rapid antigen test for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were clinically evaluated for the diagnosis of coronavirus disease 2019 (COVID-19) in self-collected saliva. Saliva samples from 103 patients with laboratory-confirmed COVID-19 (15 asymptomatic and 88 symptomatic) were collected on the day of hospital admission. SARS-CoV-2 RNA in saliva was detected using a quantitative reverse transcription-PCR (RT-qPCR) laboratory-developed test (LDT), a cobas SARS-CoV-2 high-throughput system, three direct RT-qPCR kits, and reverse transcription–loop-mediated isothermal amplification (RT-LAMP). The viral antigen was detected by a rapid antigen immunochromatographic assay. Of the 103 samples, viral RNA was detected in 50.5 to 81.6% of the specimens by molecular diagnostic tests, and an antigen was detected in 11.7% of the specimens by the rapid antigen test. Viral RNA was detected at significantly higher percentages (65.6 to 93.4%) in specimens collected within 9 days of symptom onset than in specimens collected after at least 10 days of symptoms (22.2 to 66.7%) and in specimens collected from asymptomatic patients (40.0 to 66.7%). Self-collected saliva is an alternative specimen option for diagnosing COVID-19. The RT-qPCR LDT, a cobas SARS-CoV-2 high-throughput system, direct RT-qPCR kits (except for one commercial kit), and RT-LAMP showed sufficient sensitivities in clinical use to be selectively used in clinical settings and facilities. The rapid antigen test alone is not recommended for an initial COVID-19 diagnosis because of its low sensitivity."

Fluorescence *In Situ* Hybridization (FISH) Microscopy

Method:

Fluorescence *in situ* hybridization (FISH) allows for the detection of "specific DNA and RNA sequences at the individual cell level" and has many implications in cell biology, chromosome research, and cytogenetic diagnostics (Haar et al. 1994). It's high sensitivity and specificity as well as the speed at which assays can be performed allow for advances in many areas of research (Bishop 2010).

During FISH, a DNA probe is hybridized to its complementary base pair sequence within a chromosome on a prepared, fixed slide (Volpi and Bridger 2018). Probes are labelled one of two ways: directly or indirectly. Direct labelling involves annealing fluorescent nucleotides to the probe, and indirect labelling involves adding reporter molecules that can be detected by fluorescent antibodies (Volpi and Bridger 2018). These probes are visualized *in situ* by microscopy.

Information Obtained:

FISH is a powerful tool for identifying, visualizing, and quantifying specific microbes within their community (Zhi et al. 2014). This type of microscopy provides a better understanding of both the chemical and physical properties of nucleic acids and chromosomes (Volpi and Bridger 2018). Under fluorescence, the reporter molecules attached to the probe will show either the presence or absence of a particular gene aberration (Bishop 2010). Furthermore, this method has expanded to allow for simultaneous screening of whole genomes via multiplex FISH or comparative genomic hybridization (Bishop 2010).

Example:

FISH Microscopy was used in a study conducted by Bernardi et al. to analyze the tongue dorsum biofilms of patients with halitosis and those deemed healthy (control) (2019). Researchers wanted to quantify the amount of *Streptococcus spp.* and *Fusobacterium nucleatum* within the oral biofilm to examine the role that these bacteria play in halitosis (Bernardi et al. 2019). Tongue plaque samples were taken from both halitosis patients and

healthy patients and visualized and quantified using a combination of FISH and confocal laser scanning microscopy (CLSM) (Bernardi et al. 2019). Specific fluorescent probes were used to stain *Eubacteria*, *Streptococcus spp.*, and *Fusobacterium nucleatum* (Bernardi et al. 2019). By analyzing both the FISH and CLSM data, it was found that the biofilms of the halitosis patients contained significantly higher amounts of *Streptococcus spp.* and *Fusobacterium nucleatum* compared to those of the healthy patients (Bernardi et al. 2019). These two bacteria may be constituents that cause halitosis and should be considered as targets for treatment (Bernardi et al. 2019).

Advantages:

The major advantage of FISH microscopy is that it maintains a single-cell level of analyzation while providing an intermediate degree of resolution (Volpi and Bridger 2018). This method also supports large-scale gene mapping and sequencing, and its accuracy and versatility are capitalized upon in medical research (Volpi and Bridger 2018). There are also many versions of the FISH protocol that improve the sensitivity, specificity, and resolution of the data output (Volpi and Bridger 2018). The previous factors, alongside advances in fluorescence microscopy, diversify genomic and bioinformatic resources (Volpi and Bridger 2018).

Disadvantages:

The major disadvantage of the FISH technique is its lack of ability to fluoresce targets that have a low number of DNA and RNA copies (Jensen 2014). Increases in the sensitivity of *in situ* hybridization are necessary by either amplifying target sequences before *in situ* hybridization or locating the signal after hybridization (Jensen 2014).

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Abstract:

Fluorescence In Situ Hybridization (FISH) Microscopy

From Bernardi et al. 2019,

"The present study involved a qualitative and quantitative evaluation of tongue dorsum biofilms sampled from halitosis patients and healthy volunteers. The aim of the study was to quantify the dis- tribution of Streptococcus spp. and Fusobacterium nucleatum within the oral halitosis biofilm in order to highlight the role of these bacterial members in halitosis. Tongue plaque samples from four halitosis- diagnosed patients and four healthy volunteers were analyzed and compared. The visualization and quantification of the tongue dorsum biofilm was performed combining fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM). Eubacteria, Streptococcus spp. and Fusobac- terium nucleatum were stained using specific fluorescent probes. For a comparison of the two tested biofilm groups the Wilcoxon rank-sum test was used. Morphological analysis by CLSM illustrated the distribution of the species which were tracked. Streptococcus spp. appeared to be enclosed within the samples and always associated to F. nucleatum. Furthermore, compared to the control group the biofilm within the halitosis group contained significantly higher proportions of F. nucleatum and Streptococcus spp., as revealed by the FISH and CLSM-analysis. The total microbial load and relative proportions of F. nucleatum and Streptococcus spp. can be considered as causative factors of halitosis and thus, as potential treatment targets."

NanoSIMS for Visualizing Members of Microbial Communities

Method:

One of the major goals of microbial ecology is to determine the functional roles and interactions of individual microbes within complex communities (Musat et al. 2016). NanoSIMS (secondary ion mass spectrometry) is a method that utilizes high-resolution imaging combined with stable isotope probing (nanoSIP) to trace stable isotope assimilation in microbial cells (Musat et al. 2016). This technique utilizes an ion microprobe with a primary ion beam that scans the surface of the sample of interest (Musat et al. 2016). A primary ion beam creates secondary ions from the surface of a solid sample. NanoSIMS offers high spatial resolution, high sensitivity, and high mass specificity which enables nanoSIP – a technique that typically requires the microbes of interest to phylogenetically identified (Musat et al. 2016). Consequently, *in situ* hybridization methods are preferred to help link classification to isotopic enrichment (Musat et al. 2016). The size of the analysis area ranges from 1-50 μm, and the resolution ranges from 16x16 to 2048x2048 pixels per area (Musat et al. 2016). This allows for chain-forming diatoms, filamentous cyanobacteria, microbial aggregates and even nanometer-scale microbial cells or storage granules within cells to be measured (Musat et al. 2016).

Information Obtained:

NanoSIMS, in combination with stable isotope probing, can be used to observe functions, energy flows, and biotic relationships in complex microbial communities that may have been considered uncontrollable, previously.

In situ hybridization allows researchers to determine microbial activity based on spatial relationships; this includes microbe-host interactions, microbe-microbe interactions, cell-cell nutrient and metabolite exchanges, and relationships between cells and their own organic or inorganic matrixes (Musat et al. 2016).

This method also measures phenotypic heterogeneity, which is the ability for microbes living in the same environment to display marked variability in their phenotypes (Zimmerman et al. 2015). Phenotypic characterization of a large number of single cells of the same species

within a community is experimentally difficult, thus, the relevance of heterogeneity is undetermined (Zimmerman et al. 2015).

Example:

NanoSIMS was used in a study by Wang et al. examining biological nitrogen fixation (BNF) in planted and non-planted paddy soils (2020). This process was quantified using a chamber-based $^{15}N_2$-labeling technique (Wang et al. 2020). Active diazotrophs within the soil were observed using $^{15}N_2$-DNA-stable isotope probing (SIP), and NanoSIMS was implemented to analyze the ^{15}N-enrichment of DNA in the SIP fractions (Wang et al. 2020). After twenty-eight days, this experiment demonstrated that BNF was 11.33 ± 1.90 kg N ha^{-1} in the rice-planted soil and 3.55 ± 1.18 kg N ha^{-1} in the non-planted soil (Wang et al. 2020). Most of the BNF (>95%) was found in the top 0-0.5cm of the rice-planted soil (Wang et al. 2020). Next, *nifH* genes were extracted from the surface layer and sequenced using high-throughput sequencing; results showed that the "presence of rice affected the community composition of diazotrophs" (Wang et al. 2020). NanoSIMS images portrayed the incorporation of ^{15}N in the DNA of the SIP fractions after they had undergone CsCl gradient ultracentrifugation, and analyses of the *nifH* genes demonstrated that *Nostocales* and *Stigonematales* made up most of the BNF in the planted soil (Wang et al. 2020).

Advantages:

An advantage of NanoSIMS is that it provides a tool for locating microbes in their 3D environment (Musat et al. 2016). Relating their orientation to functional features provides insight to how these microorganisms are interacting with their communities (Musat et al. 2016).

Disadvantages:

The main disadvantage of NanoSIMS is the low throughput of only five to ten images per day (Zimmerman et al. 2015). This is especially significant for bacteria in complex environments where their relative abundance is low (≤ 1%), and consequently, a sample would be represented with a single cell per image (Zimmerman et al. 2015).

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Abstract:

NanoSIMS for Visualizing Members of Microbial Communities

From Wang et al. 2020,

"The biological nitrogen fixation in planted and nonplanted paddy soils was quantified using a chamber-based $^{15}N_2$-labeling technique, and the active diazotrophs of soil were assessed by $^{15}N_2$-DNA-stable isotope probing (SIP). In addition, the nanometer scale secondary ion mass spectrometry (NanoSIMS) was applied to analysis the ^{15}N-enrichment of soil DNA in SIP fractions. $^{15}N_2$-labeling experiment showed that BNF was 11.33 ± 1.90 kg N ha^{-1} in the rice-planted soil and 3.55 ± 1.18 kg N ha^{-1} in the nonplanted soil after 28-day labeling. The biologically fixed ^{15}N was mainly (> 95%) recovered in the surface layer (0–0.5 cm) in the rice-planted soil. High throughput sequencing of *nifH* genes extracted from surface soil showed that the presence of rice affected the community composition of diazotrophs. The relative abundance of *Nostocales* and *Stigonematales* was significantly higher in rice-planted soil than in nonplanted soil ($P < 0.05$). After CsCl gradient ultracentrifugation, NanoSIMS images clearly showed that ^{15}N was incorporated into soil DNA in the $^{15}N_2$-labeling SIP gradient fractions. Analyses of *nifH* genes in ^{15}N-enriched SIP gradient fractions suggested that *Nostocales* and *Stigonematales* were the major contributors to BNF in the rice-soil system. Taken together, these results have highlighted the contributions of cyanobacteria to the BNF in paddy fields and improved our understanding of the close relationship between rice plants and cyanobacteria."

BioOrthogonal Non-Canonical Amino Acid Tagging (BONCAT)

Method:

BioOrthogonal Non-Canonical Amino Acid Tagging (BONCAT) is a technique used for tracking protein synthesis at the cellular level within microbial organisms and their communities (Steward et al. 2020). The non-canonical amino acids are used to track translational activity in cells and do not significantly change cell physiology – this is a key aspect of the method (Steward et al. 2020). Alterations in the metabolic states of cells could make the analysis of BONCAT experiments challenging (Steward et al. 2020).

Bioorthogonal functional groups, such as azides and ketones, have been utilized as tag proteins, glycans, and lipids in the biosynthetic mechanisms of cells (Dieterich et al. 2006). The ligation of these tag molecules with reactive probes allows for their detection – the azide group is especially notable for labelling proteins and glycans located on the cell surface (Dieterich et al. 2006). Tagged proteins can be separated from their proteome by affinity purification and then further identified by tandem mass spectrometry (Dieterich et al. 2006). The BONCAT method does not require an elution step as it allows for the direct proteolysis of affinity-purified proteins on a matrix (Dieterich et al. 2006).

Information Obtained:

The BONCAT method increases the likelihood of identifying new proteins by utilizing the selective labelling and enrichment of the subproteome of the synthesized proteins (Dieterich et al. 2006). This type of protein labelling is similar to metabolic labelling with radioactive amino acids and immediately validates candidate proteins via azidohomoalanine or d_{10}-Leu-based identification constraint modifications, which are two common non-canonical amino acids; this bypasses the need for secondary validation steps (Dieterich et al. 2006). Specific constituents of protein complexes can also be identified by tandem affinity purification of the tagged molecules and analyzed using mass spectrometry (Dieterich et al. 2006). Combining this BONCAT technique with subcellular fractionation or microdissection of tissue could allow researchers to obtain a greater picture of the temporal and spatial aspects of cellular proteomes (Dieterich et al. 2006).

Example:

The BONCAT method was used in a study conducted by Couradeau et al. to measure translationally active cells at two soil depths in Oak Ridge, Tennessee (2019). The goal of the project was to link soil microbial diversity to soil processes. To do this, technology that characterizes active microbes from extracellular DNA and from dormant cells is necessary (Couradeau et al. 2019). It was found that 25-70% of extractable cells were active (Couradeau et al. 2019). Researchers used fluorescence-activated cell sorting to distinguish the BONCAT-positive cells in order to sequence their 16S rRNA genes (Couradeau et al. 2019). They found that the composition of the active cells was distinct from the total number of cells extracted; some microbes were found at both depths suggesting that the conditions favour the survival of like species (Couradeau et al. 2019). The study concluded that the BONCAT method, alongside fluorescence-activated cell sorting, is successful in tagging the active cells in soil microbiomes *in situ* which allows for further analysis in comparing soil processes and these microbes (Couradeau et al. 2019).

Advantages:

An important advantage of the BONCAT method is analysis at the single-cell level. Other advantages, particularly involving the enrichment of the subproteome of newly synthesized proteins, include analyzing primary protein synthesis responses to internal and external stimuli and decreasing the complexity of the sample which, in turn, could allow for proteins with low levels of expression to be identified (Dieterich et al. 2006).

Disadvantages:

One of the disadvantages of the BONCAT method is that protein synthesis is the only function that can be quantified (Du and Behrens 2021). Translationally inactive cells impact ecosystem processes through proteome catalytic activities, nutrient uptake, metabolite excretion, and other cellular interactions that affect microbiomes which supports another limitation of this method (Du and Behrens 2021).

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Abstract:

BioOrthogonal Non-Canonical Amino Acid Tagging (BONCAT)

From Couradeau et al. 2019,

"The ability to link soil microbial diversity to soil processes requires technologies that differentiate active microbes from extracellular DNA and dormant cells. Here, we use BONCAT (bioorthogonal non-canonical amino acid tagging) to measure translationally active cells in soils. We compare the active population of two soil depths from Oak Ridge (Tennessee, USA) and find that a maximum of 25–70% of the extractable cells are active. Analysis of 16S rRNA sequences from BONCAT-positive cells recovered by fluorescence-activated cell sorting (FACS) reveals that the phylogenetic composition of the active fraction is distinct from the total population of extractable cells. Some members of the community are found to be active at both depths independently of their abundance rank, suggesting that the incubation conditions favor the activity of similar organisms. We conclude that BONCAT-FACS is effective for interrogating the active fraction of soil microbiomes in situ and provides a new approach for uncovering the links between soil processes and specific microbial groups."