1. BACKGROUND

To understand the full taxonomic and functional profile of soil microbial communities, RhizeBio uses shotgun metagenomics – a technique that sequences all DNA present in a sample. This captures a broader range of genetic information than targeted sequencing methods, which are reliant upon an existing taxonomic database and lab-validated assays that have limited scalability. With metagenomics, all microbes of interest can be identified using a single test, while also capturing functional data from the unidentified soil microbes that make up to 99% of the community. It is the gold-standard technique in environmental microbiology to study microbial diversity and interactions, as well as in clinical diagnostics to identify pathogens and genetic markers of diseases.

RhizeBio combines shotgun metagenomic sequencing with patented bioinformatics to assess the structure, health, and functional capacity of the root-associated microbial communities (the rhizosphere) which play a central and important role in crop growth, nutrient availability and uptake, resistance to diseases and pests, and ultimately crop yield.

The purpose of this study was to use soil chemistry data, rhizosphere metagenomics data, and plant tissue nutrient data together to evaluate the effect of treatment with EnSoil Algae, a live cell biostimulant (*Chlorella vulgaris*). This analysis was conducted to assess row-cropping systems across different crop growth stages and field conditions, and to evaluate nutrient uptake efficiency, soil carbon, plant stress, and overall crop health.

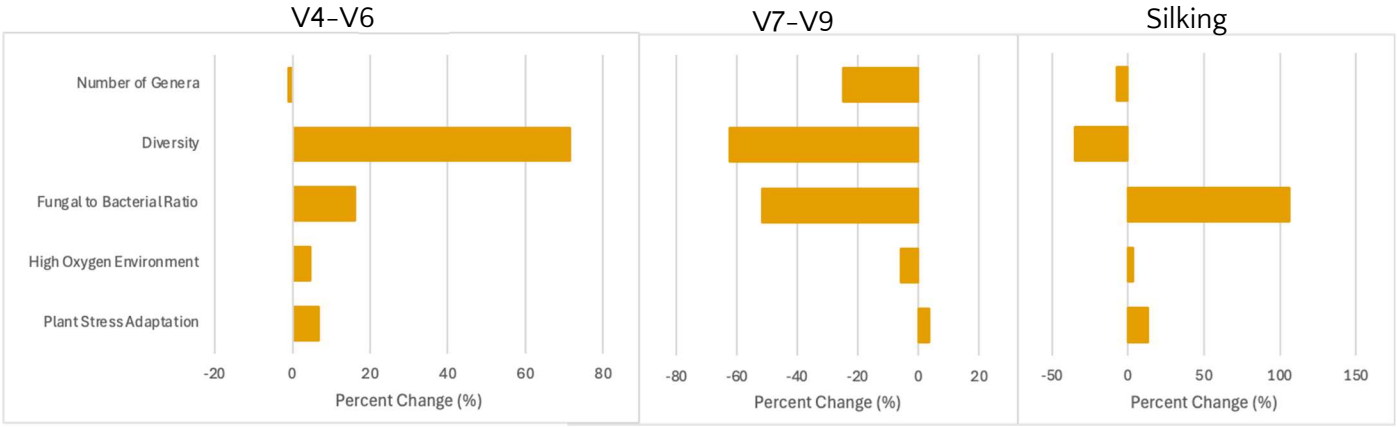
2. RESULTS

Treatment with EnSoil Algae led to varying changes in soil health and soil nutrients, microbial activity, and plant tissue nutrient levels, depending on the crop (Corn, Soy, Milo) and the growth stage of the crop. In this study, we analyzed 7 pairs of rhizosphere samples from corn (where each pair consisted of a treated and untreated sample; n=3 pairs for growth stage V4-V6, n=2 for V7-V9, and n=2 for silking.) We analyzed 2 pairs of soybean samples and one milo sample pair. Several general trends emerged across all crops and growth stages. Results for each crop are summarized below, and detailed descriptions of the different measurements can be found in Appendix A. Each of these metrics are displayed as a percent change between untreated samples and samples treated with EnSoil Algae.

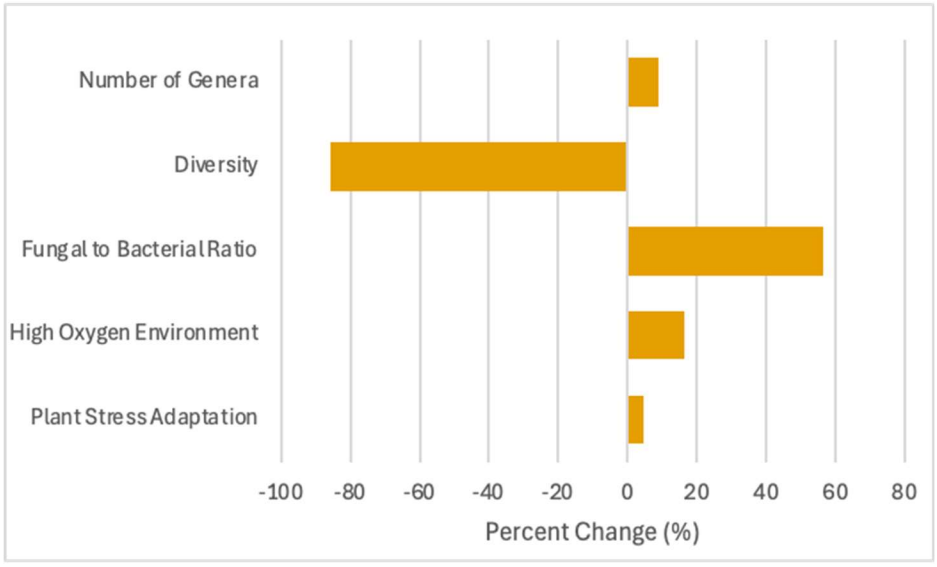
2.1. Microbial Populations and Stress Adaptation

Metrics in this category describe high-level features of the rhizosphere microbial community, as well as the capacity of a community to confer resistance to plant stress. Included here are: number of genera, diversity, fungi:bacteria ratio, abundance of bacteria that thrive in a high oxygen environment, and plant stress adaptation. Each of these contribute to the overall health of a soil system; an increase in the number of genera or diversity can indicate a system more resistant and resilient to both abiotic and biotic stressors. An decrease in the fungi to bacteria ratio is generally better for annual crops grown in tilled soils with high nitrogen inputs because bacteria are more effective at nutrient mobilization in this environment. An increase in bacteria that thrive in a high oxygen environment indicates a highly oxygenated soil, which is beneficial for rapid crop growth and nutrient availability. An increase in the plant stress adaptation metric is favorable and indicates an increase in resistance and resilience to plant stress.

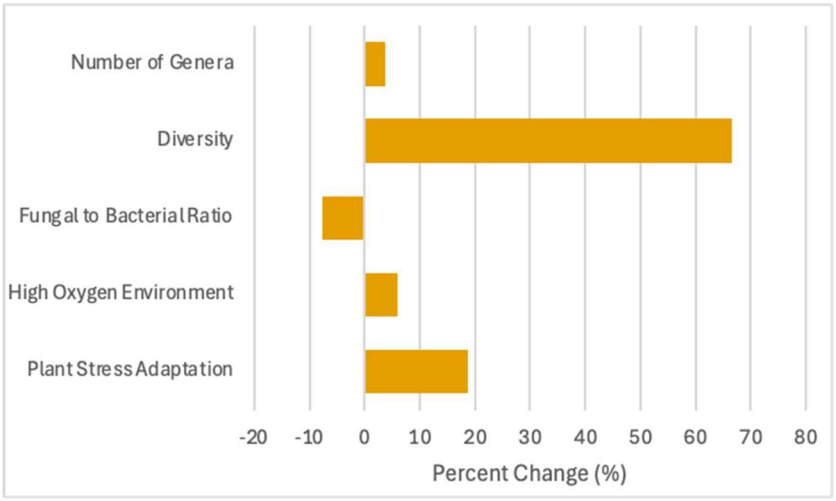
2.1.1. Corn



2.1.2. Soybean



2.1.3. Milo

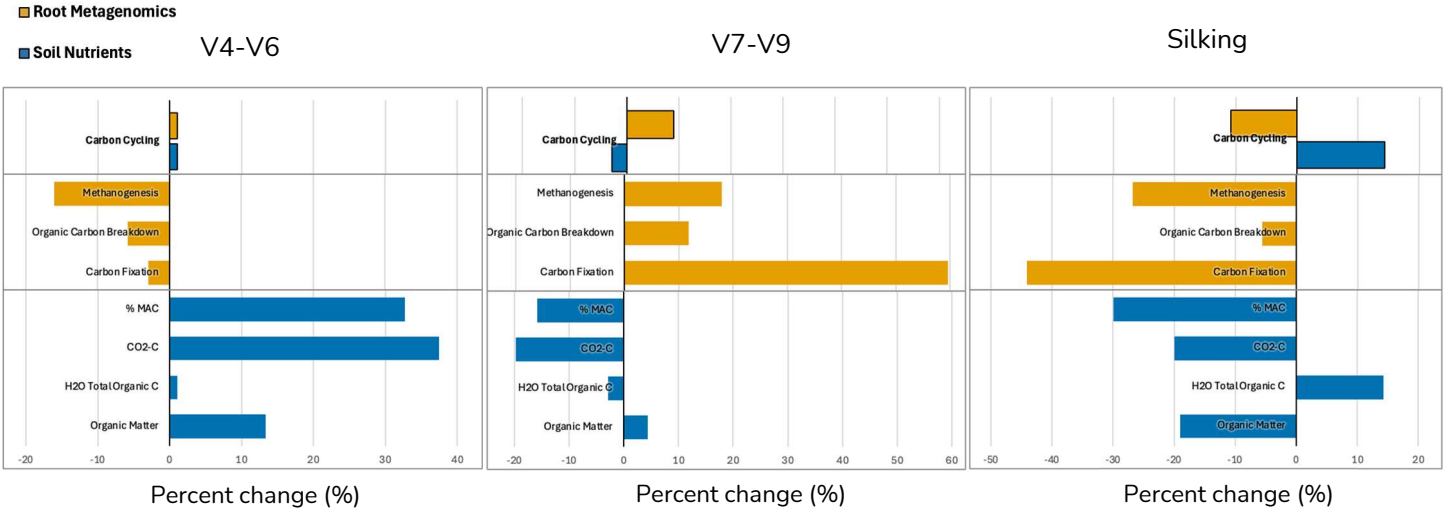


Metagenomic profiling of EnSoil Algae-treated crops revealed varied microbial dynamics across crop types and growth stages. Fluctuations number of genera, diversity, and bacteria-to-fungi ratio indicated a responsive microbial community. Despite these variations, consistent trends emerged across corn, soybean, and milo crops. The analysis showed increased abundance of microbial genes associated with high-oxygen environments, suggesting improved soil aeration or enhanced microbial activity. Additionally, there was a notable increase in genes related to plant stress adaptation across all crops. These findings suggest EnSoil Algae treatment may foster a microbial environment supporting plant resilience and improved soil conditions, regardless of crop type or growth stage.

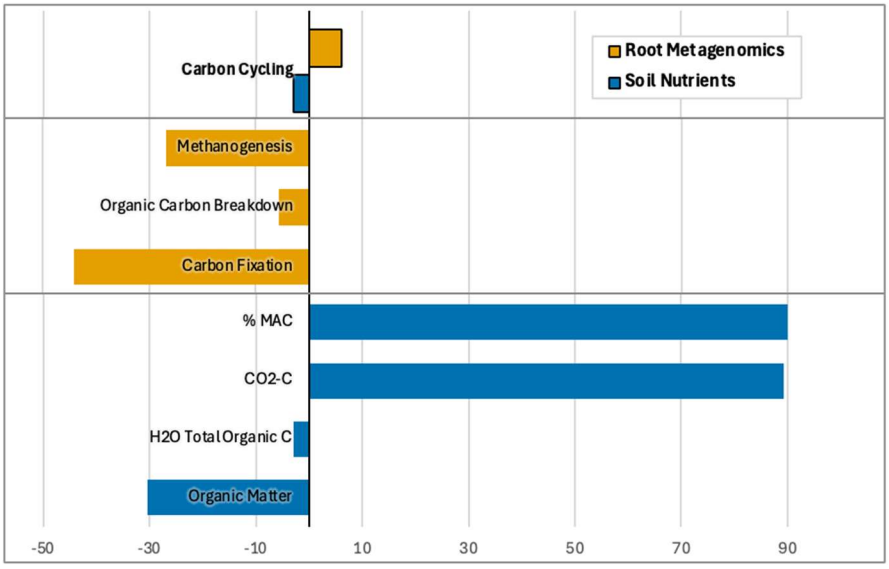
2.2. Carbon Cycling

By leveraging metagenomics to evaluate the rhizosphere microbial community, we can gain insights into the potential for carbon cycling, sequestration, and greenhouse gas emission. Combining these data with soil nutrients (Haney test) reveals insights into overall soil health. Carbon fixation and organic carbon breakdown are both microbial-driven processes that can impact the amount of carbon sequestered in a soil. Methanogenesis is the microbial release of methane, a potent greenhouse gas, so a decrease in this metric will improve the carbon footprint of an operation.

2.2.1. Corn

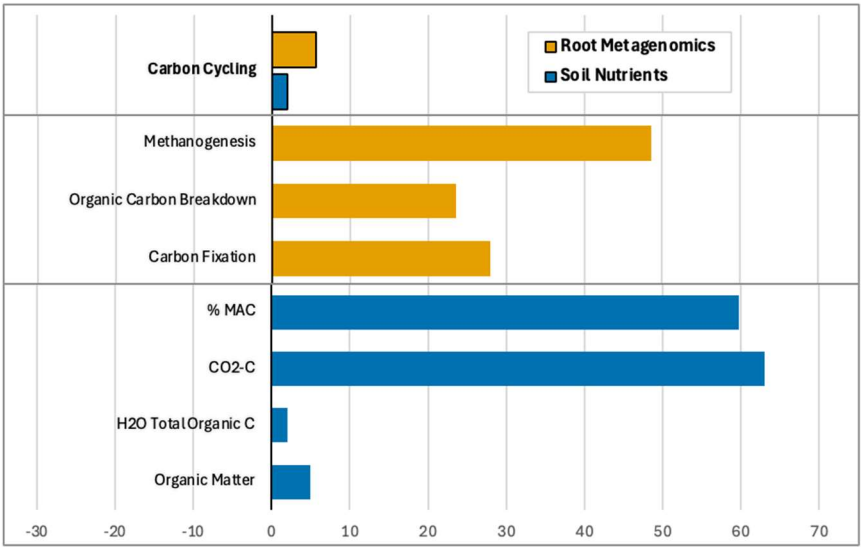


2.2.2. Soybean



Percent change (%)

2.2.3. Milo



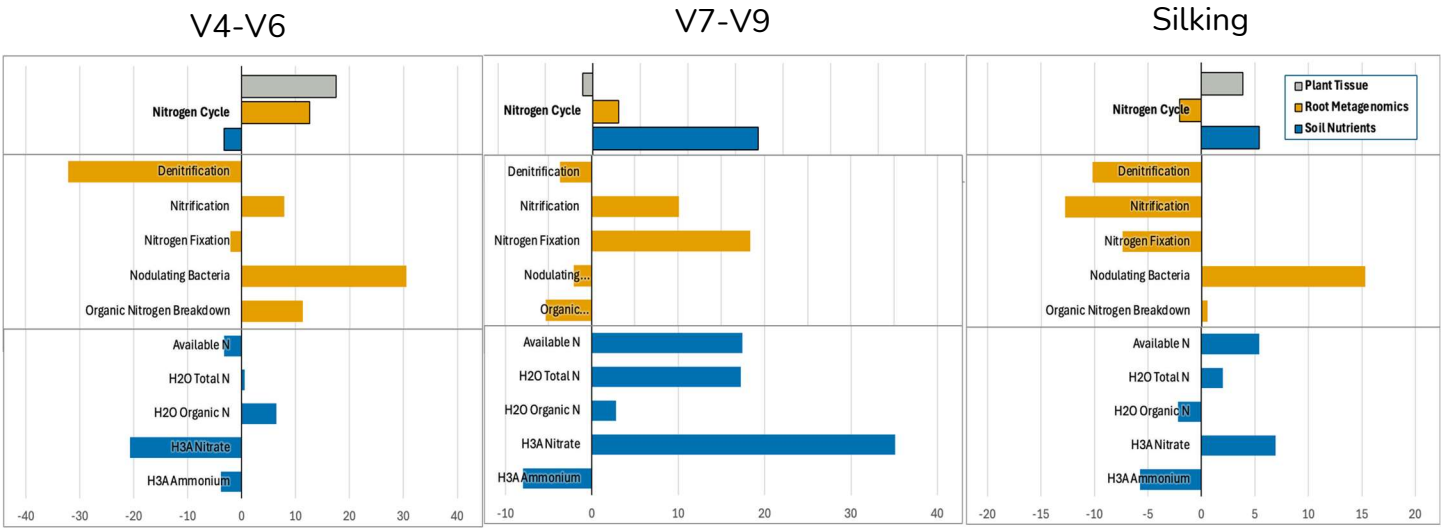
Percent change (%)

Metagenomic analysis of EnSoil Algae-treated crops reveals patterns in carbon cycling dynamics throughout the corn growth cycle. During the V7-V9 stage, there was a notable increase in the abundance of carbon cycling genes, particularly those involved in carbon fixation. These changes coincided with a slight decrease in soil organic carbon levels. However, as the plants progressed to the silking stage, the abundance of carbon cycling genes declined markedly, while total soil organic carbon content increased. These findings suggest a dynamic interplay between microbial genetic activity and soil carbon levels in EnSoil Algae-treated rhizospheres. The initial surge in carbon cycling genes during V7-V9 may reflect heightened microbial activity in processing carbon compounds, potentially leading to a temporary reduction in soil organic carbon. As the season advanced to silking, the decrease in carbon cycling gene abundance coupled with an increase in soil organic carbon implies a potential stabilization or accumulation of carbon in the soil.

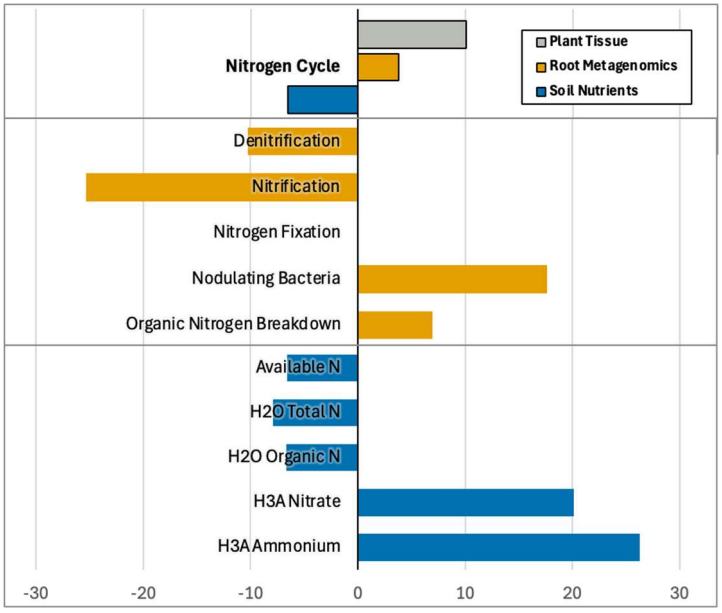
2.3. Nitrogen Cycling

Combining rhizosphere metagenomics with the Haney test and plant tissue analyses reveals intricacies of the soil nitrogen pool and cycling potentials. In general, a decrease in denitrification is beneficial, as this process decreases the amount of plant-available nitrogen and also releases a potent greenhouse gas. Increases in nitrification, nitrogen fixation, nodulating bacteria, and organic nitrogen breakdown are beneficial as these increase the amount of plant-available nitrogen.

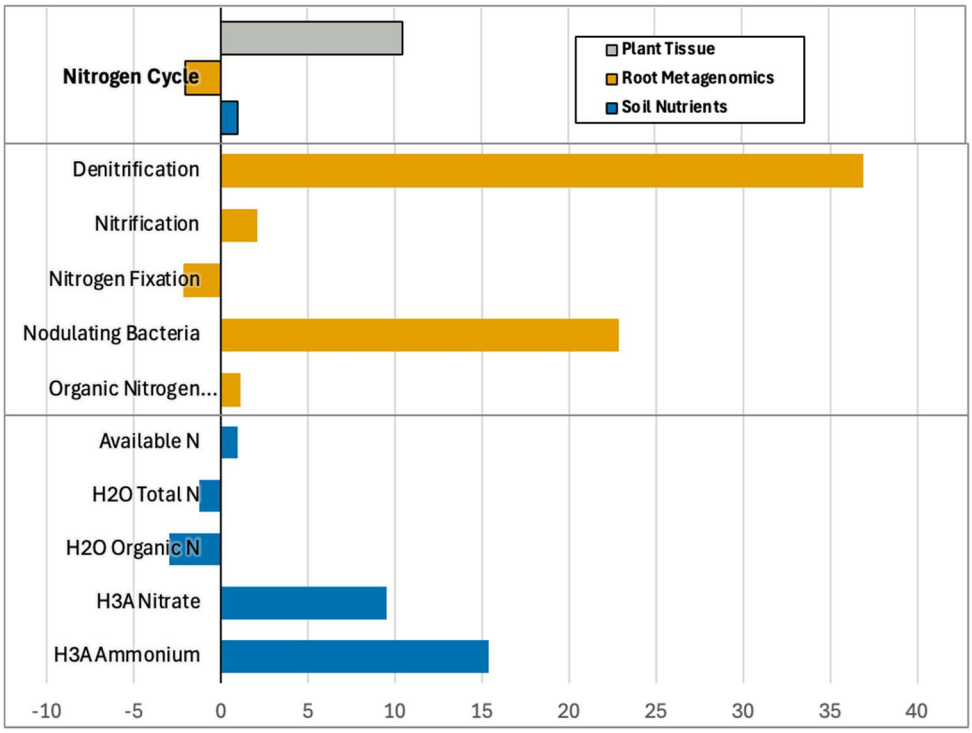
2.3.1. Corn



2.3.2. Soybean



2.3.3. Milo

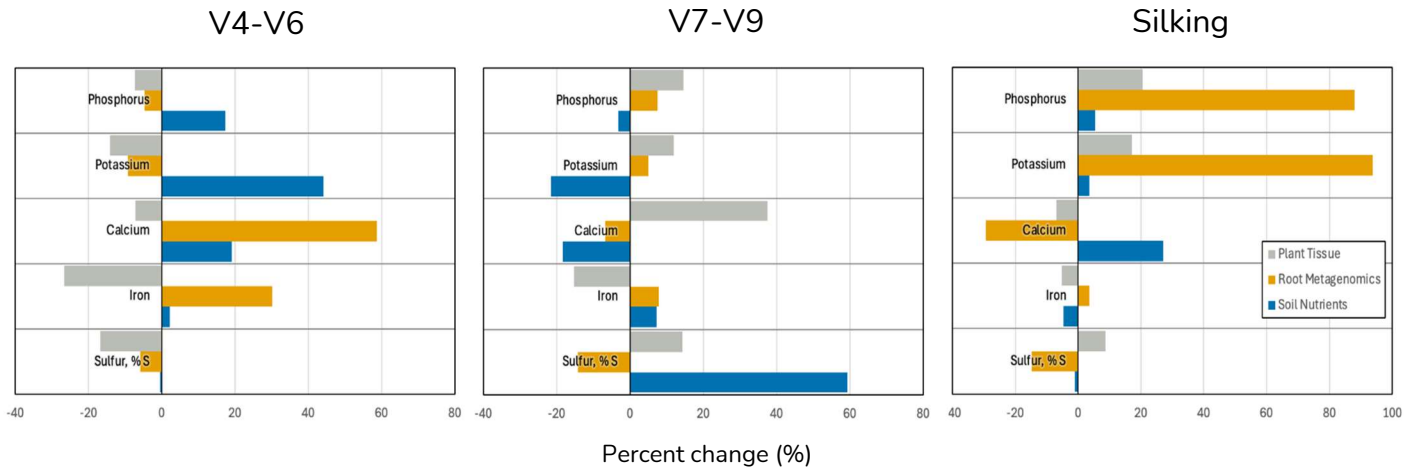


Metagenomic analysis of EnSoil Algae-treated corn rhizospheres revealed dynamic nitrogen cycling changes across growth stages. During V4-V6, increased nitrogen cycling gene abundance coincided with higher plant tissue nitrogen levels, suggesting enhanced nitrogen availability and uptake. At V7-V9, plant-available soil nitrogen and nitrogen cycling genes further increased, while plant tissue nitrogen remained stable, indicating potential soil nitrogen accumulation. The silking stage underwent a slight decrease in nitrogen cycling gene abundance, with modest increases in both tissue and soil nitrogen levels. This pattern suggests EnSoil Algae treatment enhances nitrogen cycling potential in the rhizosphere from V4-V6, leading to a significant increase in plant-available soil nitrogen during V7-V9. While this enhanced nitrogen availability could potentially improve plant growth and yield, further research is needed to confirm long-term benefits and optimize application strategies.

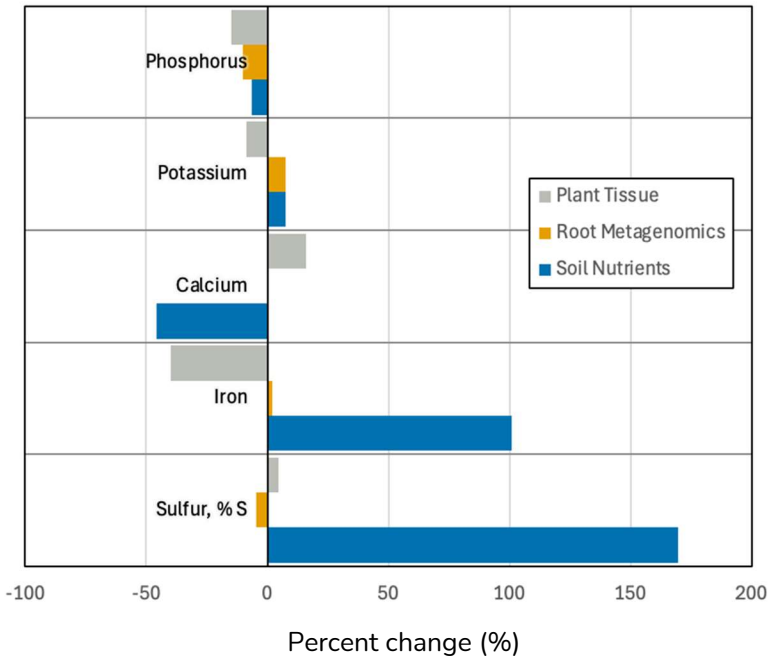
2.4. Other Nutrient Cycling

Similar to the carbon and nitrogen cycling metrics reported in previous sections, the following measurements reveal the impact of the rhizosphere microbiome on nutrient cycling and plant growth by evaluating the relevant genes through metagenomics. All of the metrics included in this section reflect nutrient availability, so an increase is beneficial for crop growth. In addition to evaluating the rhizosphere microbiome through metagenomics, Haney tests and plant tissue analyses were also performed.

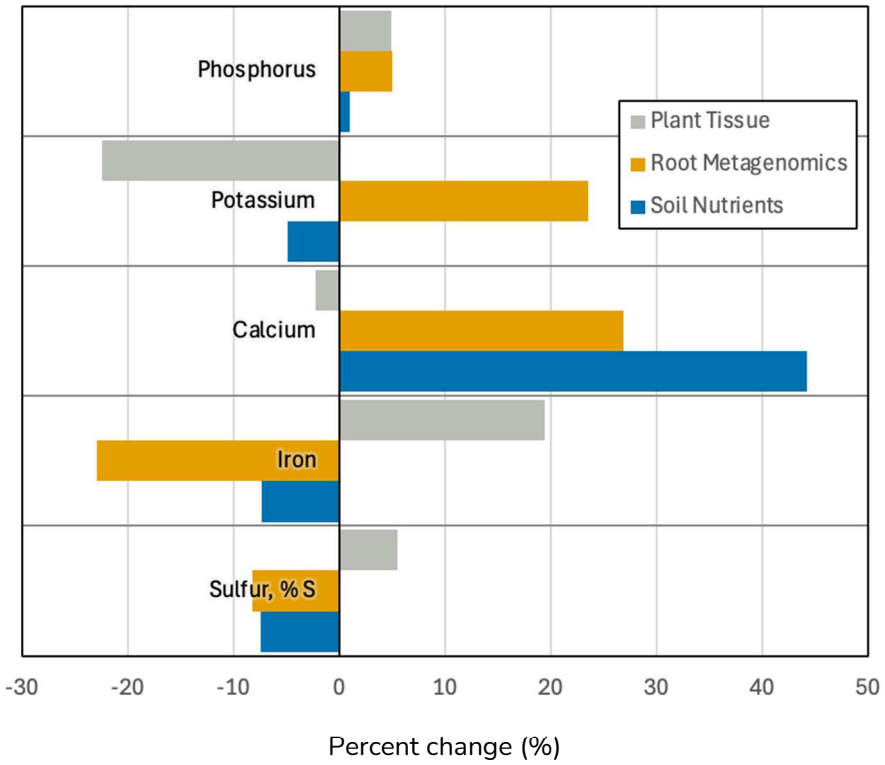
2.4.1. Corn



2.4.2. Soybean



2.4.3. Milo



Metagenomic analysis of EnSoil Algae-treated corn rhizospheres showed significant changes in phosphorus and potassium cycling. At V7-V9, increased P and K cycling gene abundance coincided with higher nutrient levels in plant tissues. During silking, further increases in rhizosphere gene abundance associated with P and K cycling were observed alongside additional nutrient uptake. This pattern suggests EnSoil Algae stimulates microbial processes that enhance phosphorus and potassium cycling and availability in the rhizosphere, potentially improving plant nutrition throughout critical growth stages. These findings indicate a positive impact of EnSoil Algae on nutrient dynamics and plant uptake in corn cultivation.

3. SUMMARY

While the application of EnSoil Algae had variable impacts on different metrics across the three different crops tested, there were several measurements that showed consistent improvement across all three crops. Nitrogen cycling metrics (nodulating bacteria, denitrification, and organic nitrogen breakdown) were improved in corn, soybean, and milo crops. Potassium solubilization and plant stress adaptation improved across the board, as well as soil oxygenation (measured as High Oxygen Environment). Additionally, data combined from the Haney test and plant tissue analyses showed an increase in nutrient movement into the crops treated with EnSoil Algae. There was an increase in carbon cycling potential mid-season in corn treated with EnSoil Algae, suggesting a more active microbial community response to crop demands during active growth. Furthermore, at the silking stage there was an increase in total organic carbon at the end of the season, revealing improved carbon sequestration and overall soil health. Finally, it should be noted that the data herein is a compilation of all farms, and each soil can respond differently.

APPENDIX A

1. Microbial Populations and Plant Stress Adaptation

   It should be noted that the addition of a microbial amendment to soils would be expected to change all the Microbial Population metrics, and not necessarily in a way that would be interpreted as an immediate improvement in soil health. For example, while a higher diversity measurement is generally seen as more beneficial for soil health, introducing a new microbial community could cause the diversity metric to decrease because the population is made less even by the addition of a microbial product.

   a. **Number of Genera Identified**: A count of all unique genera identified as being present in the soil, filtering out viruses and any human DNA (genus Homo) present. Soil microbiome richness and biodiversity supports multiple soil functions such as nutrient cycling and pathogen resistance.
   b. **Diversity**: This value is based on the Shannon diversity index, a common statistical method to evaluate the diversity of ecological populations, taking into account the number of taxa present and their relative abundance. Studies demonstrate that a biodiverse soil, containing high numbers of species, ensures functional redundancy and supports multiple functions simultaneously. It is reported here as a percentile, which is where the sample falls on a distribution curve of other soil samples.
   c. **Fungi to Bacteria Ratio**: This value is an indicator of the ratio of total fungal abundance (i.e., biomass) to bacterial abundance. F:B Ratio is an indicator of soil disturbance intensity/frequency, as soil disturbance favors the growth of bacteria at the expense of fungi. *Note: this result is represented in this report as a ratio of reads classified as fungal versus bacterial, not total numbers of fungal/bacterial species identified at a species level. This will also differ from F:B ratio determinations from other testing (i.e., microscopy), as the biomass of the organisms is not taken into account.*
   d. **High Oxygen Environment**: The abundance of genes associated with bacterial growth in a high oxygen environment.
   e. **Plant Stress Adaptation**: This metric measures the abundance of a number of methods by which bacteria promote plant growth, including the microbial production of osmoprotectants and phytohormones.

2. Carbon Cycling

   a. **Carbon (C) Fixation**: Bacterial conversion of CO_2 to organic carbon can increase soil organic carbon and contribute to more active and diverse soil bacterial communities. This metric measures the abundances of genes involved in bacterial carbon fixation.
   b. **Organic Carbon Breakdown**: This metric measures the abundance of genes that allow microorganisms to break down complex organic molecules and liberate carbon.
   c. **Methanogenesis**: A measure of the abundance of microorganisms which are able to produce methane (CH_4). Production of methane typically occurs during the decomposition of organic matter and is strictly anaerobic. Methanogenesis represents a loss of soil carbon to the atmosphere, reducing the amount available to plants as well as acting as a potent greenhouse gas. Methanogenesis also acts as a secondary indicator of oxygen availability.

3. Nitrogen Cycling

   a. **Denitrification**: This rating measures the genetic potential for soil microbes to transform nitrate (NO_3^-) to nitrogenous gas (N_2/N_2O), resulting in loss of nitrogen from the soil as well as production of a potent greenhouse gas. This process occurs during reduced oxygen availability, often caused by wet or poorly drained soil, compaction, high temperatures and excessive decomposable organic matter. Denitrification can be controlled through improved soil drainage, use of cover crops and residues, and controlling irrigation to provide less and more frequent applications of water. The use of nitrification inhibitors has also been shown to lower denitrification.
   b. **Nitrification**: Measures the genetic potential for soil microbes to transform ammonium (NH_4^+) into nitrate (NO_3^-). Nitrate is the form of nitrogen that is most susceptible to nitrogen leaching and gaseous nitrous oxide production (denitrification). Decreasing nitrification through management is desirable to decrease nitrogen loss and increase fertilizer efficiency. Strategies to control nitrification include timing of fertilization to coincide with rapid plant uptake, use of slow-release fertilizers, and biological nitrification inhibitors.
   c. **Nitrogen (N) Fixation**: This rating measures the genetic potential for soil microbes to transform atmospheric nitrogen (N_2) into plant available ammonia (NH_3). The use of cover crops and biological fertilizers containing N-fixing microbes are strategies to improve N-fixation.
   d. **Nodulating Bacteria**: The most important source of atmospheric nitrogen fixation for plants are bacteria which can form symbiotic relationships with the roots of legumes and fix nitrogen directly within the plants' roots. This is a measure of the abundance of genes which are required for colonization of plant roots by rhizobacteria.

e. **Organic Nitrogen Breakdown**: This metric measures the abundance of genes that allow microorganisms to break down complex organic molecules and liberate nitrogen into a form usable by plants.

4. Other Nutrient Cycling
   a. **Phosphorus (P) Mobilization**: Soil microbes are effective at releasing plant-available P from the soil through solubilization of insoluble inorganic P and mineralization of insoluble organic P. Solubilization occurs through the microbial release of organic acids which are generated from central metabolism while P mineralization occurs via the activity of various microbial enzymes. This metric measures the relative abundance of microbial genes involved with P mineralization. Crop rotation and application of lime and compost have been shown to improve P mobilization.
   b. **Potassium (K) Solubilization**: Potassium solubilizing bacteria (KSB) use a variety of mechanisms to solubilize potassium in soils, primarily organic acids and siderophores. This soil analysis measures the relative abundance of microbial genes involved in solubilizing potassium.Sulfur (S) Oxidation: Some microorganisms have the capability to oxidize forms of S into sulfates, increasing plant availability, and are known as Sulfur Oxidizing Bacteria (SOB). Sulfur oxidation only occurs in the presence of adequate oxygen. This soil analysis measures the abundance of Sulfur Oxidizing Bacteria.
   c. **Sulfur Reduction**: Certain microbes are capable of metabolizing sulfates, reducing the amount of plant-available sulfur in soil. This soil analysis measures the abundance of microbes which perform sulfur reduction in soils. This process also occurs almost exclusively in anaerobic soils, and so represents a secondary indicator of oxygen availability.
   d. **Calcium (Ca) Transport**: This measures the abundance of genes which code for enzymes capable of transporting Calcium across cell membranes.
   e. **Iron (Fe) Acquisition**: This metric measures the abundance of the methods by which bacteria promote iron availability, such as through siderophore or organic acid production.