# ROOTS TO SHOOTS

## A Multi-Growth-Stage Evaluation of Biological Treatment Efficacy

## 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 constitute 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.

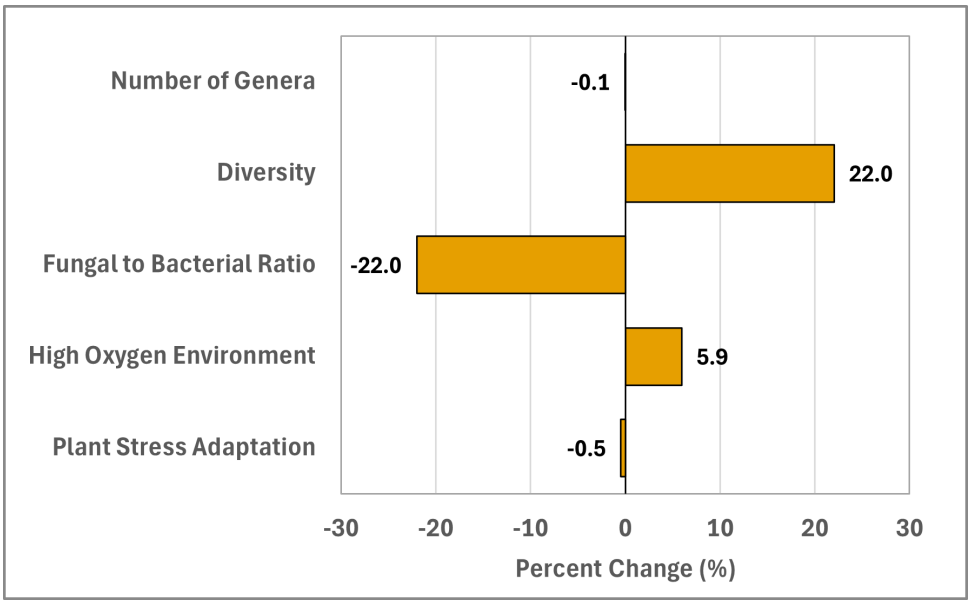
The purpose of this study was to combine data from soil chemistry, rhizosphere microbiome, and plant tissue to evaluate the effect of treatment with Fish Head Farms' *Fish Sh!t* organic soil conditioner, a biological product comprised of over 4,000 microbial species. This analysis was conducted to assess row-cropping systems (in this case, corn) 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 *Fish Sh!t* led to varying changes in soil health and soil nutrients, microbial activity, and plant tissue nutrient levels, depending on the growth stage of the crop. In this study, we analyzed four pairs of rhizosphere samples from corn plants between the V4 and V6 growth stages (where each pair consisted of a treated and untreated sample). It should be noted that one set of samples were collected for both the V7-V9 and Silking growth stages, however these samples had issues with data collection and are not reported here. Results 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 *Fish Sh!t*. A comprehensive data table can be found in Appendix B.

## 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 stressors. 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. A 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.

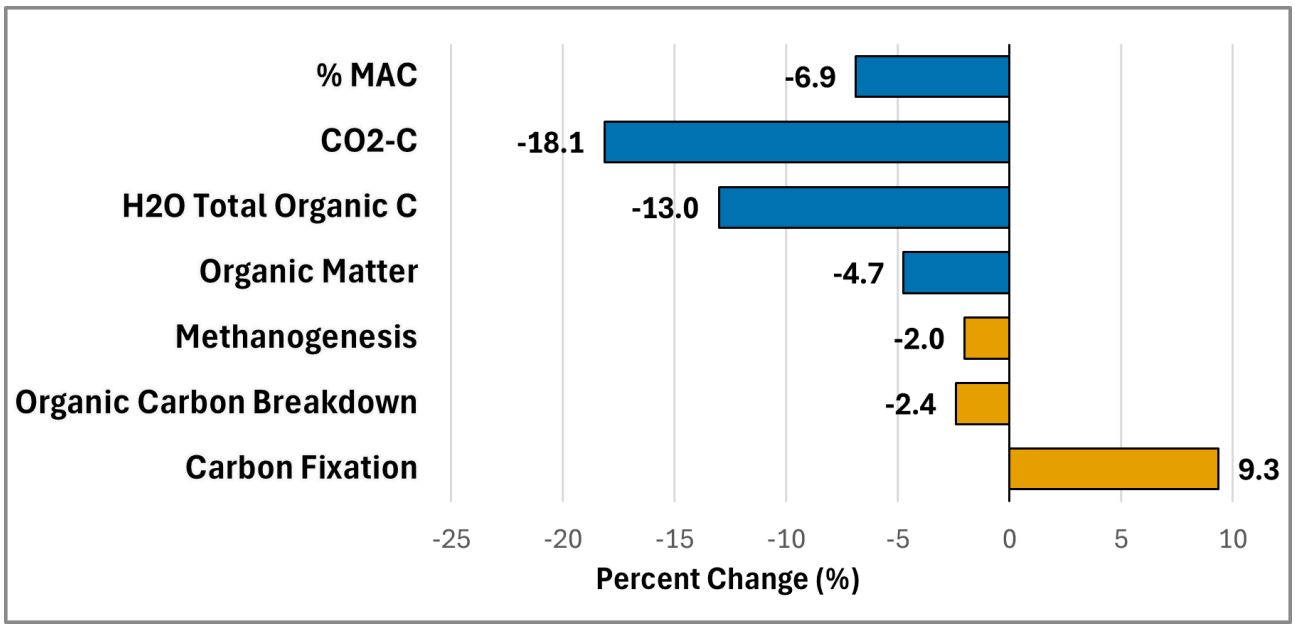



| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Community Structure** | 13.4 | 65% | | 11.2 | 64% | | -16.4% | -1.1% | |
| Number of Genera | | 1481.7 | | | 1480.0 | | | -0.1% | |
| Diversity | | 4.8% | | | 5.9% | | | 22.0% | |
| Evenness | | 3.0% | | | 3.8% | | | 23.3% | |
| Mycorrhizae Abundance | | 79% | | | 73% | | | -6.9% | |
| Plant Stress Adaptation | | 75% | | | 75% | | | -0.5% | |
| Bacteria to Fungal Ratio | | 0.026 | | | 0.018 | | | -22.0% | |
| 1:1 Soil pH | 6.4 | | | 6.2 | | | -3.4% | | |
| Soil Health Calculation | 13.4 | | | 11.2 | | | -16.2% | | |
| 1:1 Soluble Salt | 0.2 | | | 0.2 | | | -4.9% | | |
| Organic C:N | 10.6 | | | 10.8 | | | 1.5% | | |
| High Oxygen Environment | | 79% | | | 83% | | | 5.9% | |
| **Environmental Stressors** | | 33% | | | 34% | | | 18.7% | |
| Anoxic Environment | | 33% | | | 34% | | | 18.7% | |

Metagenomic analysis revealed an increase in microbial community diversity and soil oxygenation. Notably, the ratio of fungi to bacteria decreased by more than 20%, suggesting a significant proliferation of bacterial populations in response to the treatment. This shift in microbial balance likely stems from the biologically-diverse composition of *Fish Sh!t*, which appears to favor bacterial growth over fungal development in the rhizosphere ecosystem.

## 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.

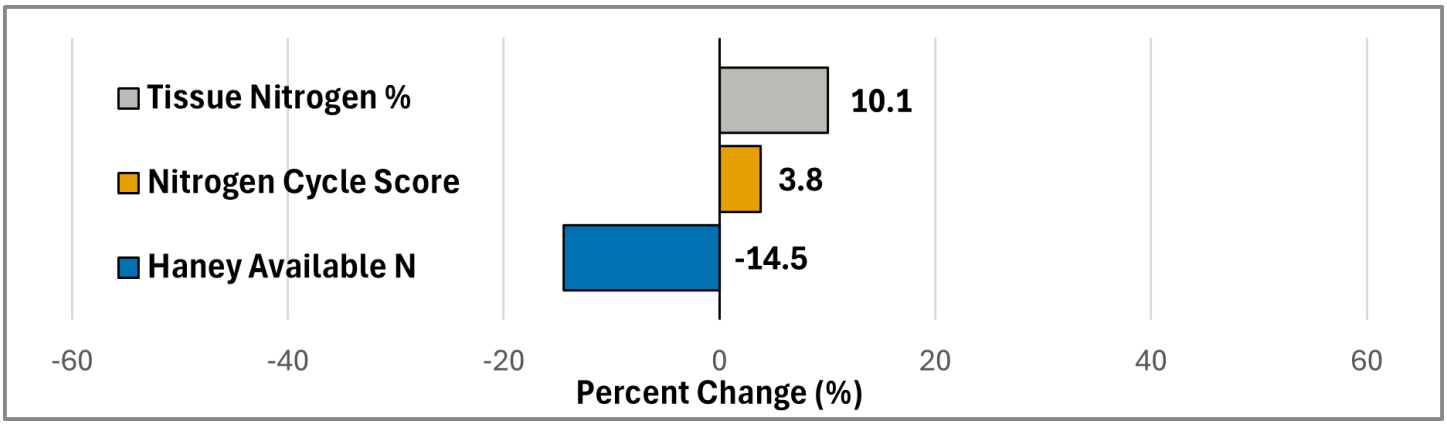



| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Carbon** | 174.7 | 67% | | 153.5 | 69% | | -13.0% | 2.6% | |
| CO2-C | 89.4 | | | 67.2 | | | -18.1% | | |
| Organic Matter | 3.6 | | | 3.4 | | | -4.7% | | |
| Carbon Fixation | | 77% | | | 84% | | | 9.3% | |
| Organic Carbon Breakdown | | 67% | | | 66% | | | -2.4% | |
| Methanogenesis | | 43% | | | 42% | | | -2.0% | |
| H2O Total Organic C | 174.7 | | | 153.5 | | | -13.0% | | |
| % MAC | 63.0 | | | 48.3 | | | -6.9% | | |

Although the microbial potential for carbon cycling in the rhizosphere showed no significant difference compared to untreated corn, there was a notable change in total organic carbon within the soil. This shift likely indicates active carbon processing, potentially driven by microbial activity stimulated by the treatment.

## 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. The biological Nitrogen Score includes denitrification, nitrification, nitrogen fixation, nodulating bacteria, and organic nitrogen breakdown.



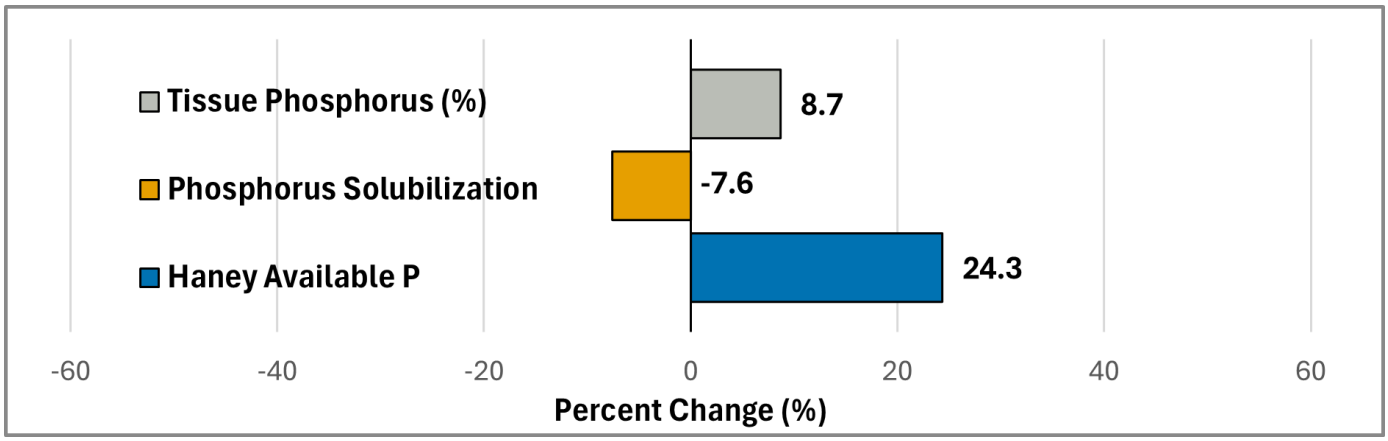

| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Nitrogen** | 109.1 | 62% | 3.71 | 92.4 | 63% | 4.12 | -14.5% | 3.8% | 10.1% |
| Nitrogen Fixation | | 77% | | | 76% | | | -2.3% | |
| Nodulating Bacteria | | 64% | | | 62% | | | -2.1% | |
| Organic Nitrogen Breakdown | | 72% | | | 78% | | | 10.8% | |
| H3A Ammonium | 8.3 | | | 11.3 | | | 25.8% | | |
| Nitrification | | 36% | | | 40% | | | 15.1% | |
| H3A Nitrate | 21.0 | | | 13.5 | | | -28.1% | | |
| Denitrification | | 40% | | | 41% | | | 35.9% | |
| H2O Total N | 42.3 | | | 33.3 | | | -19.1% | | |
| H2O Organic N | 16.3 | | | 13.8 | | | -14.0% | | |
| H3A Inorganic Nitrogen | 29.2 | | | 24.7 | | | -12.2% | | |
| Organic N Release | 16.3 | | | 13.8 | | | -14.0% | | |
| Available N | 109.1 | | | 92.4 | | | -14.5% | | |
| Total Nitrogen, % N | | | 3.7 | | | 4.1 | | | 10.1% |

There is an increase in the microbial potential for nitrogen cycling which corresponds with the observed increase in the N levels of plant tissue and decreased soil N, indicating improved N availability and uptake. These findings suggest that the treatment effectively promotes nitrogen transformation and uptake, enriching nitrogen content within plant tissues.

## 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 the metrics included in this section reflect nutrient availability, so an increase in microbial potential is beneficial for crop growth. In addition to evaluating the rhizosphere microbiome through metagenomics, Haney tests and plant tissue analyses were also performed.

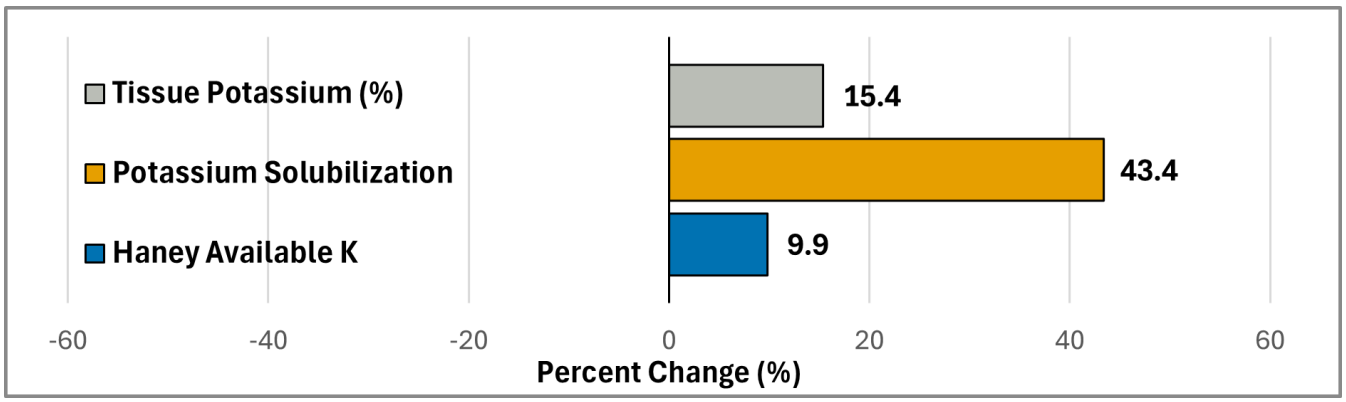
### 2.4.1. Phosphorus




| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Phosphorus** | 57.5 | 64% | 0.31 | 72.3 | 60% | 0.33 | 24.3% | -7.6% | 8.7% |
| Phosphorus Solubilization | | 64% | | | 60% | | | -7.6% | |
| H3A Total Phosphorus | 25.6 | | | 31.9 | | | 23.5% | | |
| H3A Inorganic Phosphorus | 20.5 | | | 26.2 | | | 23.1% | | |
| H3A Organic Phosphorus | 5.0 | | | 5.7 | | | 39.5% | | |
| Organic P Release | 4.5 | | | 5.2 | | | 42.5% | | |
| Organic P Reserve | 0.6 | | | 0.5 | | | -14.5% | | |
| Available P | 57.5 | | | 72.3 | | | 24.3% | | |
| Phosphorus, % P | | | 0.31 | | | 0.33 | | | 8.7% |

Plant-available phosphorus levels increased in the soil, accompanied by modest increases in plant tissue P and slight decreases in P solubilization within the rhizosphere. It should be noted that phosphorus is not at peak demand during the growth stage sampled (V4-V6), so a high change in phosphorus solubilization is not anticipated. There was a 24.3% increase in available P, most notably from H3A Organic P levels increasing to 39.5%.

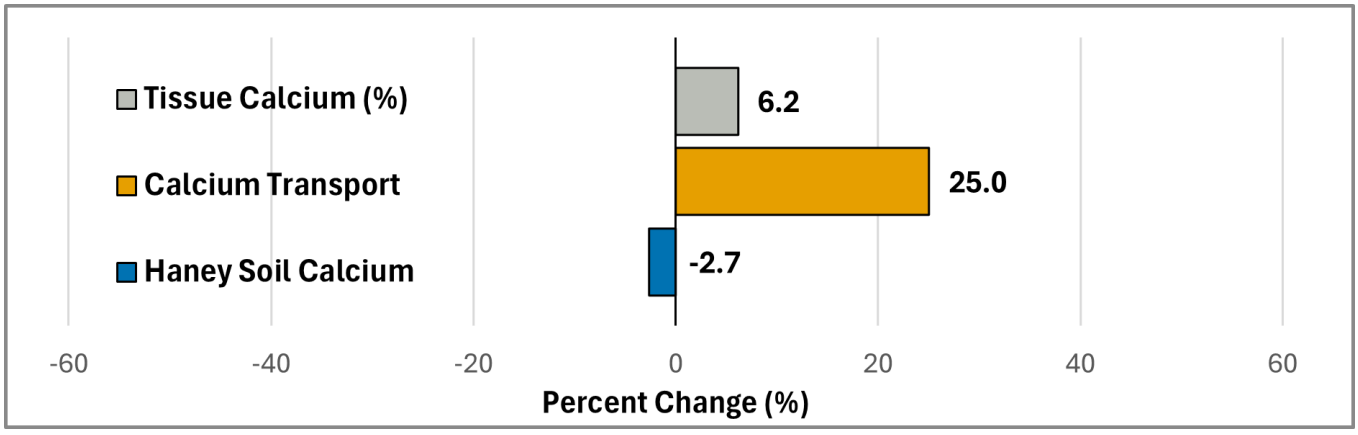
### 2.4.2. Potassium




| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Potassium** | 81.0 | 38% | 2.84 | 85.5 | 44% | 3.34 | 9.8% | 43.4% | 15.4% |
| Potassium Solubilization | | 38% | | | 44% | | | 43.4% | |
| H3A ICAP Potassium | 67.5 | | | 71.3 | | | 9.8% | | |
| Available K | 81.0 | | | 85.5 | | | 9.9% | | |
| Potassium, % K | | | 2.84 | | | 3.34 | | | 15.4% |

Potassium cycling showed significant increases in solubilization potential, which aligned with substantial increases in K levels within plant tissues.

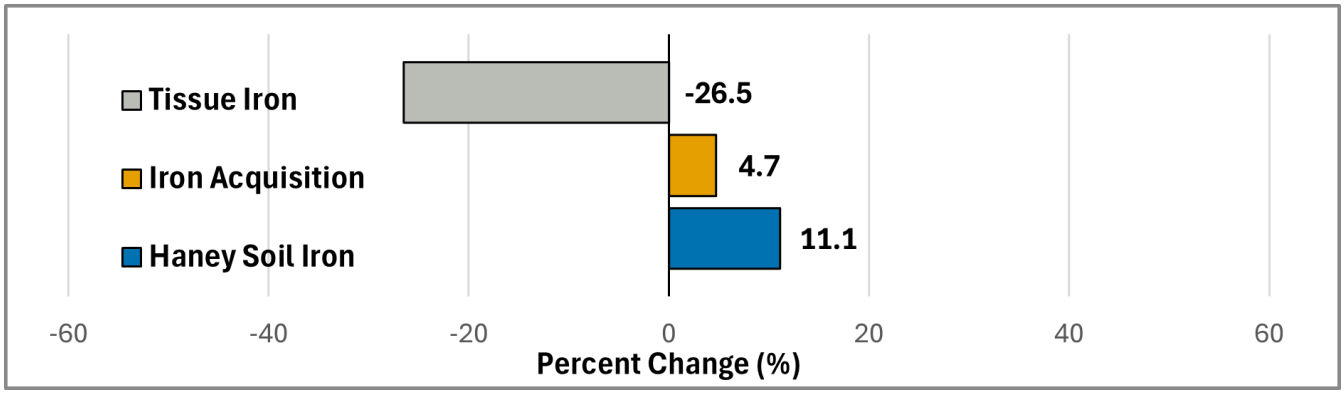
### 2.4.3. Calcium




| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Calcium** | 405.3 | 27% | 0.49 | 374.8 | 34% | 0.51 | -2.7% | 25.0% | 6.2% |
| Calcium Transport | | 27% | | | 34% | | | 25.0% | |
| H3A ICAP Calcium | 405.3 | | | 374.8 | | | -2.7% | | |
| Calcium, % Ca | | | 0.49 | | | 0.51 | | | 6.2% |

Calcium transport potential increased significantly, corresponding to higher Ca levels in plant tissues and minor reductions in soil Ca.

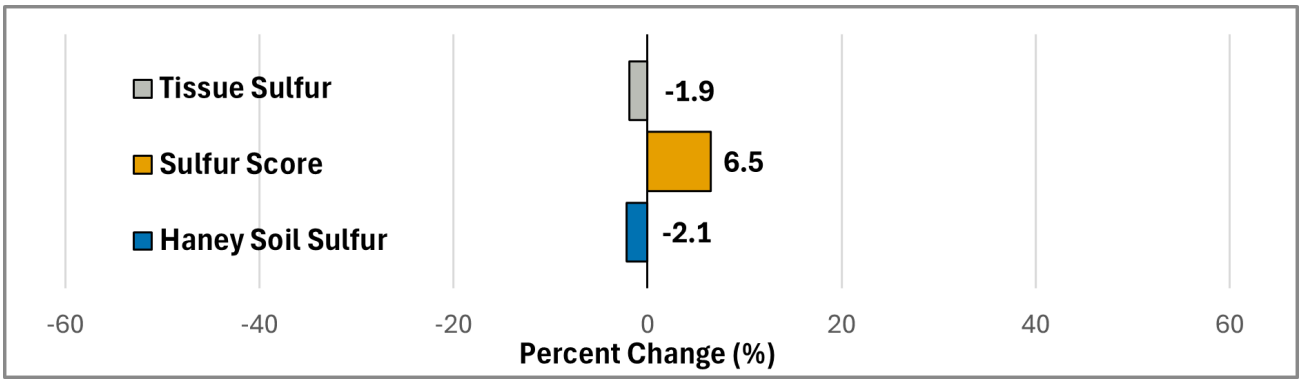
### 2.4.4. Iron




| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Iron** | 57.4 | 70% | 1051.67 | 63.7 | 72% | 609.86 | 11.1% | 4.7% | -26.5% |
| Iron Acquisition | | 70% | | | 72% | | | 4.7% | |
| H3A ICAP Iron | 57.4 | | | 63.7 | | | 11.1% | | |
| Iron, ppm Fe | | | 1051.67 | | | 609.86 | | | -26.5% |

Iron (Fe) dynamics revealed a decrease in plant tissue Fe levels despite modest increases in Fe-cycling gene abundance within the rhizosphere and higher Fe concentrations in the soil.

### 2.4.5. Sulfur




| Sample Type | Untreated | | | Treated | | | % Change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Haney | Rhize | Tissue | Haney | Rhize | Tissue | Haney | Rhize | Tissue |
| **Sulfur** | 6.8 | 43% | 0.22 | 6.8 | 43% | 0.21 | -2.1% | 6.5% | -1.9% |
| Sulfur Oxidation | | 50% | | | 50% | | | 2.1% | |
| Sulfur Reduction | | 36% | | | 37% | | | 11.9% | |
| H3A ICAP Sulfur | 6.8 | | | 6.8 | | | -2.1% | | |
| Sulfur, % S | | | 0.22 | | | 0.21 | | | -1.9% |

Sulfur cycling showed minimal changes, indicating relatively stable dynamics for this nutrient.

# 3. SUMMARY

The application of the organic soil amendment *Fish Sh!t* to corn crops resulted in significant microbial and nutrient cycling changes within the rhizosphere:

- **Microbial Dynamics**: The treatment slightly increased microbial diversity and oxygen levels in the soil while reducing the fungal-to-bacterial ratio by over 20%, indicating a notable proliferation of bacterial populations likely driven by the composition of *Fish Sh!t*.
- **Carbon Cycling**: Although carbon cycling potential remained unchanged compared to untreated crops, total organic carbon levels in the soil decreased, suggesting active carbon processing stimulated by microbial activity.
- **Nitrogen Cycling**: Enhanced nitrogen cycling gene abundance corresponded with higher nitrogen levels in plant tissues, reflecting improved nitrogen availability and uptake. This was accompanied by a reduction in plant-available nitrogen in the soil, indicating active nitrogen cycling and transformation.
- **Nutrient Cycling**:
    - **Phosphorus**: Soil P availability increased, with modest gains in plant tissue P and slight decreases in P solubilization.
    - **Potassium**: K solubilization potential and plant tissue K levels both rose significantly.
    - **Calcium**: Ca transport potential increased, leading to higher Ca levels in plant tissues and minor reductions in soil Ca.
    - **Iron**: Plant tissue iron levels decreased despite modest increases in Fe-cycling genes and soil Fe concentrations.
    - **Sulfur**: Minimal changes were observed in S cycling.

It should be noted that the samples included in this analysis are from the V4-V6 growth stage, which is prior to peak nutrient demand. Future data collection focusing on later growth stages may better reveal product impact on nutrient cycling and uptake during peak demand. These findings highlight *Fish Sh!t*'s ability to enhance microbial activity and nutrient cycling, improving nutrient availability and uptake while driving dynamic changes within the rhizosphere ecosystem. Finally, it should be noted that the data herein is a representation of tested 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.


# APPENDIX B

| Soil Nutrients | Percent Change (%) |
|---|---|
| 1:1 Soil pH | -3.4% |
| Soil Health Calculation | -16.2% |
| Organic Matter | -4.7% |
| 1:1 Soluble Salt | -4.9% |
| Organic C:N | 1.5% |
| CO2-C | -18.1% |
| H2O Total Organic C | -13.0% |
| % MAC | -6.9% |
| H3A Ammonium | 25.8% |
| H3A Nitrate | -28.1% |
| H2O Total N | -19.1% |
| H2O Organic N | -14.0% |
| H3A Inorganic Nitrogen | -12.2% |
| Organic N Release | -14.0% |
| Available N | -14.5% |
| H3A Total Phosphorus | 23.5% |
| H3A Inorganic Phosphorus | 23.1% |
| H3A Organic Phosphorus | 39.5% |
| Organic P Release | 42.5% |
| Organic P Reserve | -14.5% |
| Available P | 24.3% |
| H3A ICAP Potassium | 9.8% |
| Available K | 9.9% |
| H3A ICAP Sulfur | -2.1% |
| H3A ICAP Calcium | -2.7% |
| H3A ICAP Iron | 11.1% |
| H3A ICAP Zinc | 23.1% |
| H3A ICAP Manganese | -2.2% |
| H3A ICAP Magnesium | -18.7% |
| H3A ICAP Sodium | 0.3% |
| H3A ICAP Copper | -3.9% |
| H3A ICAP Aluminum | -1.3% |

| Root Metagenomic Pathways | Percent Change (%) |
|---|---|
| Number of Genera | -0.1% |
| Diversity | 22.0% |
| Evenness | 23.3% |
| Mycorrhizae Abundance | -6.9% |
| Plant Stress Adaptation | -0.5% |
| Bacteria to Fungal Ratio | -22.0% |
| Ectomycorrhizal to Arbuscular Ratio | 58.7% |
| High Oxygen Environment | 5.9% |
| Environmental Stressors | 18.7% |
| Anoxic Environment | 18.7% |
| Carbon Fixation | 9.3% |
| Organic Carbon Breakdown | -2.4% |
| Methanogenesis | -2.0% |
| Nitrogen Fixation | -2.3% |
| Nodulating Bacteria | -2.1% |
| Organic Nitrogen Breakdown | 10.8% |
| Nitrification | 15.1% |
| Denitrification | 35.9% |
| Phosphorus Solubilization | -7.6% |
| Potassium Solubilization | 43.4% |
| Sulfur Oxidation | 2.1% |
| Sulfur Reduction | 11.9% |
| Calcium Transport | 25.0% |
| Iron Acquisition | 4.7% |

| Plant Tissue Nutrients | Percent Change (%) |
|---|---|
| Total Nitrogen, % N | 10.1% |
| Phosphorus, % P | 8.7% |
| Potassium, % K | 15.4% |
| Sulfur, % S | -1.9% |
| Calcium, % Ca | 6.2% |
| Iron, ppm Fe | -26.5% |
| Zinc, ppm Zn | 7.3% |
| Manganese, ppm Mn | -18.7% |
| Magnesium, % Mg | 6.3% |
| Sodium, % Na | 79.5% |
| Copper, ppm Cu | -0.1% |
| Aluminum, ppm Al | -10.6% |
| Molybdenum, ppm Mo | -24.0% |
| Boron, ppm B | 29.6% |