THESIS

NITROGEN FERTILIZER IMPACTS ON SOIL MICROBIOME AND TOMATO PLANT DEVELOPMENT

Submitted by

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ABSTRACT

NITROGEN FERTILIZER IMPACTS ON SOIL MICROBIOME AND TOMATO PLANT DEVELOPMENT

Nitrogen (N) fertilization largely supports agricultural production. Urea is a common N amendment used in agriculture and when overapplied it has negative consequences in the environment due to its highly labile and reactive form. Alternative fertilizers, such as controlled release fertilizers (CRF) have been designed to diminish the harmful effects of applied N. This thesis investigates and makes comparisons regarding N fertilizer types and their effects on microbial community composition and plant development. Both research questions were addressed by growing tomato (*Solanum lycopersicum* 'Rutgers') plants as the test crop, which serve as a good model crop for indoor greenhouse production and were grown to the vegetative stage in both studies covered in this thesis. The fertilizer types considered are urea, a quick releasing form of N fertilizer and Environmentally Smart Nitrogen (ESN), a controlled release fertilizer. The soil used in these studies was from a low N plot (5.2 mg/L NO_3) from the Agricultural Research, Development and Education Center (ARDEC) in Fort Collins, Colorado.

The first research question addressed in Chapter 2 examines how different types of N fertilizers compare under different soil conditions and fertilizer rates. Altering the soil microbiome through sterilization (via autoclave processing) allows us to understand how urea and a controlled release fertilizer compare in their impact on microbial community composition and N assimilation by a tomato crop. It was found in this study that the use of ESN promoted plant performance and enhanced soil nitrate concentration. The soil microbiome findings from this first experiment

showed that high rates of nitrogen fertilization led to higher relative abundances of nitrifying bacteria species. The second research question addressed in Chapter 3 follows a developmental study to track how N fertilizer impacts tomato plant performance, rhizosphere microbiome assembly, and plant nutrient uptake by sampling weekly for eight weeks. It was found in this study that ESN enhanced nitrogen use efficiency and plant nitrogen uptake. The soil microbiome results indicated a shift in community structure at the middle stage of the rhizosphere development. By studying the plant growth and rhizosphere microbiome response to urea and a controlled release fertilizer applied soil, we can improve our understanding on N release rates and bacteria that are responsive to these agents. This is the first research to our knowledge examining N fertilization's impact on rhizosphere development during early to vegetative growth using, especially using a weekly sampling resolution.

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Chapter 1

Introduction

Nitrogen in the Soil

Nitrogen (N) is an essential nutrient, crucial for plant development, and its bioavailability in agroecosystems is often highly limiting to production (Liang et al 2021, Pajares et al 2016). Under natural conditions, N can be found in various forms fluctuating throughout its complex cycle. The N cycle involves several biological and non-biological processes which include nitrification, denitrification, nitrogen fixation, mineralization, microbial and plant uptake of N, and ammonia volatilization (Fowler et al. 2013, Ghaly et al. 2015). Atmospheric nitrogen (N_2 gas), which represents approximately 79% of our atmosphere is the most abundant source of N (Lamb et al. 2014). Nitrogen is usually available to plants as either ammonium (NH_4^+), nitrate (NO_3^-), or amino acids (Guo et al 2021, Marchi et al 2016, Koops et al 2001).

Organic N that is present in soil organic matter, crop residues, and manure is converted to inorganic N through the process of mineralization. In this process, some bacteria can break down organic material and release NH_4. Formation of NH_4 increases as microbial activity increases. Nitrification is the conversion of NH_4 to NO_3. Denitrification is a process by which bacteria convert NO_3 to N gases, resulting in losses from the soil. These complex processes are all influenced by prevailing climatic conditions, along with the chemical properties of soil. While N does exist naturally in the environment, inorganic fertilizers greatly support worldwide agricultural production.

Nitrogen Fertilizer History

The overall use and sources of applied N fertilization have increased and changed throughout agriculture's history. Prior to the 20th century, producers largely used organic sources of fertilizers, such as animal manure (Russell et al. 1977, Larramendy 2019). The 20th century saw many advancements in the development and use of synthetic N fertilizers. The Haber-Bosch process revolutionized agriculture in 1918 (Erisman et al. 2008) by allowing for the synthesis of inorganic N sources (Humphreys et al. 2021) that were relatively cheap to manufacture and could be directly applied to agricultural fields (Prasad et al 2021). Throughout the next couple of decades, many regions across the world experienced food scarcity, which led to another historical moment in agriculture, the Green Revolution (Evenson et al. 2003, Dixon et al. 2022). Since the Green Revolution, worldwide agricultural production has seen multiple recessions and events, making fertilizer inputs costly, scarce, and research has shown chemical fertilization to have lasting negative effects on the environment (Pingali et al. 2012, Ilinova et al. 2021).

Nitrogen Economic Value and Cost

Currently, N fertilizer supplements agricultural production, as most soils are deficient and reliant on external N inputs to meet crop demands. The constant need for soil nutrient inputs can be a burden for many producers. Nitrogen inputs alone account for approximately 60% of the worldwide fertilizer utilized each year (Kang et al. 2022, Wang et al. 2018). From the year 2000 to 2015, global N fertilizer use has increased by approximately 57 % to support growing populations and associated food demand (Erisman 2008, Zhang 2021). At the same time, the cost of N fertilizer has varied dramatically. For example, the cost of anhydrous ammonia, a common type of N fertilizer, increased from about $500 per ton in 2020 to $1400 per ton in 2022, and $1026 per ton in 2023 (Schnitkey et al. 2022, DTN 2023). Urea is currently priced at $626 per ton as of May 2023 (DTN 2023). This cost fluctuation can be considered drastic, and producers may not be

able to sustain these high and unreliable costs. Researchers and economic experts predict that the use of N fertilizer will experience a 3 % growth annually (Lawrencia et al. 2021, Zhang 2021), which is correlated with the growth of worldwide population. Agricultural demands increase each year, and this rapid growth necessitates new N delivery technologies that are efficient and minimize losses to the environment.

Nitrogen Use Efficiency Strategies

Previous studies have demonstrated that excessive N fertilization above a certain threshold does not promote crop productivity but can lead to large N losses and cause a series of environmental problems (Chen et al. 2019, Ju et al. 2009). Nitrogen fertilizer, when applied with an effective nutrient management strategy, has a higher efficiency rate on the overall soil and crop health (Oliveras-Berrocales et al. 2017). NUE strategies can include consideration of fertilizer type, quantity, placement, and timing of applications based on the crop's nutritional requirements (King et al 2018, Mikkelson et al 2011). Split and single application timing rates are the most effective strategies for ensuring nutrient availability to crops (Davies et al. 2020). Split application can be performed by taking the total required rate and distributing it throughout a growing season. In contrast, single applications apply the fertilizer at point in time, typically at planting to sustain development over the course of a growing season. Nitrogen fertilizer application strategies are context-dependent and vary greatly with soil type, nutritional demands of the crop, and the developmental stages of the plant (Maynard et al. 2006). Fertilizer application can be location specific as one recommendation is not going to be applicable to every growing operation.

Studies have shown excessive fertilizer additions to soil can lead to initial nutrient accumulation followed by loss (Zhu et al. 2016, Sun et al. 2021). The time between the accumulation and loss of the fertilizer is a direct result of the nutrient management strategy (Cheng et al 2021). Nitrogen loss primarily occurs through surface runoff, leaching and volatilization

(Follett and Delgado 2002; Meisinger and Delgado 2002; Delgado 2022). Surface runoff happens when excess moisture causes fertilizer to run off the field, potentially reaching waterways through heavy rainfall or irrigation (Follett and Delgado 2002). Leaching, the movement of N past the root zone, is one of the largest pathways of N losses. Denitrification represents an important pathway of gaseous N losses, but the magnitude depends greatly soil characteristics (e.g., soil texture, soil drainage, etc.) and water balance (Peoples et al. 1995). Leaching, surface runoff, and gaseous losses could be all reduced with best management practices, including the use of controlled release fertilizers (Delgado 2002; Meisinger and Delgado 2002, Shoji et al 2001).

Enhanced Efficiency Fertilizers

Controlled release fertilizers (CRF) are a type of EEF and are made of granules with a semi-permeable coating surrounding the nutrients. These fertilizers are designed to increase nutrient availability to crops while decreasing environmental pollution through nutrient loss (Maharjan et al 2015). Similar crop yields have been found when comparing applications of general and controlled release N fertilizers (Carson et al 2014).

Controlled release fertilizers can contribute to high yields with lower N applications. For example, Shoji et al. (2001) found that potato tuber yields of about 46 Mg ha^{-1} could be produced with 107 kg N ha^{-1} of controlled release N fertilizer and 27 kg N ha^{-1} of starter urea fertilizer (total of 134 kg N ha), which produced a slightly higher total yield than that produced with the farmer's traditional fertilizer application of 269 kg N ha^{-1} of urea fertilizer .In a comparison study on N loss, researchers found that Environmentally Smart Nitrogen (ESN), a polymer coated controlled released fertilizer, reduced N loss by 52% when compared to urea, a quick release N fertilizer (Tian et al. 2015). Environmental pollution due to N loss has been proven to decrease with the use of enhanced efficiency fertilizers (EEF) (Besen et al 2021).

The coatings used in controlled release fertilizers are usually resin and polymer derivatives. CRF release mechanisms can depend on microbial activity, soil temperature, and soil moisture for the discharge of their nutrients (Lawrencia et al 2021, Vejan et al 2021). Use of enhanced efficiency fertilizers in agricultural soils can greatly mitigate the negative impact that N fertilizers have on the environment while simultaneously supporting crop productivity.

The Soil Microbiome and the Nitrogen Cycle

Soil bacteria cycle N required to sustain plant development, and many of these beneficial microorganisms are responsible for mobilizing N from sparingly soluble soil sources through processes such as biological N fixation, mineralization, and nitrification (Bakkar et al. 2012, Prasad et al. 2017). Nutrient cycling soil bacteria inhabit both the rhizosphere and bulk soil (Suresh et al 2019). Many specialized species are actively promoting nutrient acquisition and competition for resources (Kuzyakov et al 2013). Studies have shown that enough soil carbon enhances microbial growth, which in turn, can promote N acquisition (Dixon et al 2022, Prasad et al 2017). While microbes can release this macronutrient into a plant available form, excess fertilizer addition can promote a surplus of denitrifying bacteria, which leads to the loss of N via production of N_2O and N_2 gas into the atmosphere. In agroecosystems with an abundance of nitrifying and N-fixing bacterial species, deleterious effects from high N fertilization are ameliorated (Ye et al 2018).

Nitrogen conversion pathways present within soil microorganisms support plant nutrient assimilation by converting N from sparingly available to bioavailable forms (Moreau et al 2019). Common soil bacterial phyla, including Actinobacteria, Acidobacteria, and Bacteroidetes, contain members capable of nitrification and ammonification. Within the phylum Proteobacteria, the genus *Nitrobacter, Nitrosomonas*, and *Nitrosospira* are found to function as ammonia and nitrite oxidizers (Suresh and Abraham 2019, Zhu et al .2016, Koops et al. 2001). Phyla including Azotobacter, Azospirillum, and Rhizobacter are known to provide available N to crops through

their N fixing and nitrification processes which have been found to support plant development (Franche et al. 2009, Bhardwaj et al. 2014). These bacteria can commonly be found living in the rhizosphere of leguminous plants, a natural N accumulator. These beneficial microbes can function as free-living soil microorganisms who benefit crop development through N transformation from the atmosphere.

Nitrogen Fertilizer Impact on the Relationship Between Crops and Microbiome

Rather than directly interfering with plant function, many of the beneficial microbes involved in the N cycle interact within the rhizosphere of plant roots (Moreau et al 2019). Because the soil serves as the primary reservoir of microorganisms to be recruited by plants, these soil communities can also be impacted by fertilizer applications (Li et al 2023). These soil microbes have several beneficial effects like supporting plant growth and nutrient acquisition (Trivedi et al 2020).

A balanced microbial community within soil systems for healthy crop development is often associated with sustainable agricultural practices and NUE (Du et al. 2018). Nitrogen toxicity in crops has been shown to decrease vegetative growth and crop yield in tomato and wheat (Wang et al. 2011). Conventional agriculture production fields are commonly found to have lower soil microbiome because of continuous cultivation, low organic matter inputs, and excessive fertilizer additions (Zhu et al. 2016). Excessive synthetic N inputs can disrupt the soil microbiome's function and structure. For instance, when high rates of N were applied to an agricultural soil, microbial community richness and evenness decreased by 47 % (Hu et al. 2019). Previous studies have indicated that N fertilization changes soil microorganisms' community structure, diversity, and functionality (Wei et al., 2018; Chen et al., 2019, Ren et al 2020). One study found that long term N fertilization increases the relative abundance of Actinobacteria and Proteobacteria, while decreasing overall bacterial diversity (Dai et al. 2018). Furthermore, numerous studies have looked

at how long-term applications of chemical fertilizers can significantly alter the structure and diversity of soil bacterial communities (Ge et al. 2008, Mchugh and Schwartz 2015, Sun et al. 2015). Most of these studies focus on the relationship between the overall change in bacterial community abundance and the nutrient uptake of specific crops (Ortiz-Castro et al 2009, Maron et al. 2011). In contrast, there has been relatively little research looking at how the type of N fertilizer application impacts a crop's rhizosphere microbiome; and specifically, how the crop can recruit and signal for microorganisms for nutrient acquisition throughout the crop's life.

Plants require different nutrients from the soil as they enter new developmental stages. The rhizosphere microbiome, in conjunction with plant development also undergoes a multitude of differences (Chaparro et al. 2014). A study looking at bacterial functional genes related to N assimilation and carbon degradation were found to be enriched at later developmental stages in maize (Xiong et al. 2021). Bioavailable nutrients that are readily available to soil microorganisms are known to compete with plants for N (Capek et al. 2018). Nitrogen excess in soils can increase the fluctuating acquisition of resources between crops and microbes.

Conclusion

The goal of this thesis is to contribute to our understanding on how various types of N fertilizer applications affect soil microbiome composition, rhizosphere microbiome development, and tomato plant development and nutrient uptake. Understanding how these amendments impact agroecosystems can further promote N management strategies to sustain soil health and crop production.

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Chapter 2

Controlled Release Nitrogen Fertilizer Promotes Tomato Development and Soil Nitrate Availability while Shifting the Soil Microbiome Composition

Introduction

Nitrogen (N) is an essential nutrient for plant growth and development (Liang et al. 2021, Hestrin et al. 2021). Agricultural crops often require considerable N inputs because of their high productivity and nutrient limitation in most agricultural soils Nitrogen applications tend to be excessive in agriculture due to the difficulty of matching available soil N and crop N demands (Gastal et al. 2002, Shoji et al. 2001). Volatilization of ammonia (NH_3) contributes to atmospheric pollution. Nitrogen leaching into the waterways can lead to toxic algae blooms and hypoxia in aquatic life. Additional consequences of improperly applied N fertilizer include decreased biodiversity, accelerated competition of N between N cycle dysfunction (García et al. 2021). Such high N levels detrimentally impacts the environment by increasing volatilization and leaching (Shoji et al. 2001). Volatilization of ammonia (NH_3) contributes to atmospheric pollution. Nitrogen leaching and runoff into waterways can lead to toxic algae blooms and hypoxia in aquatic life. Additional consequences of improperly applied N fertilizer include decreased biodiversity,

accelerated competition of N between N cycle dysfunction (García et al. 2021). Thus, long-term effects of inorganic N fertilizer on the soil are a global concern because of the negative impact these agents have on environmental pollution and agroecosystem health (Miner et al. 2020).

Nutrient management strategies can reduce these environmental impacts and improve overall soil and crop health (Machado et al. 2022, Owens et al. 2022, Oliveras-Berrocales et al. 2017). Nutrient management strategies can include reduced applications of fertilizer and proper timing of application based on the crop's nutritional requirements and the season. Split application rates are effective strategies in enhancing nutrient availability to crops (LopezBellido et al. 2005, Burton et al. 2008). Split applications can be performed by distributing the total recommended amount of fertilizer throughout a growing season. In contrast, single applications are performed by applying the fertilizer once, typically at planting (Hartz et al. 2009, Abbasi et al. 2012). Single applications of fertilizers are most common in commercial agriculture (Ladha et al. 2005). However, these types of applications are the ones most prone to generate leaching and volatilization of N into the environment. Thus, innovative technologies such as the use of controlled release N fertilizers have been found to enhance nitrogen management in agricultural systems by the slow and sustained release of N (Gao et al. 2018, Shoji et al. 2001, Garcia et al. 2018, Chen et al. 2019).

Nitrogen fertilizer alters soil microbial community structure (García et al. 2021), which affects the N cycle and plant's nutrient uptake (Dixon et al. 2022). Specific soil microbes, such as *Rhizobium leguminosarum,* can fix N_2 into ammonium (NH_4+) which is preferred by plants (Lindstrom et al. 2021). While nitrate (NO_3-) can be used by plants, it can also be metabolized by free-living soil microbes, such as *Pseudomonas putida* (He et al. 2019, Schmidt et al. 2022). It has been found that urea tends to increase the abundance of bacteria with nitrification roles in the soil, such as *Nitrosomonas* and *Nitrospira* (Wang et al. 2021). While a community of nitrifying bacteria

is needed for proper N cycling, situations involving an abundance of denitrifying and nitrifying microbial communities can cause competition for N with crops and create an imbalance in the soil microbiome (Zhang et al. 2017). Soil pH, which can be altered through N fertilization, has also been found to reduce the nitrifying bacteria group, Ammonia Oxidizing Bacteria (AOB), due to low osmotic potential and toxicity in the soil (Jiang et al. 2021, Tang et al. 2016, Omar et al. 1999). Furthermore, short-term N applications decrease a soil's microbial biomass, while long-term N applications increase or do not influence microbial biomass (Jiang et al. 2021).

Soil sterilization is a methodology used in agriculture to alter a soil's microbial community to diminish pathogen loads (Guo et al. 2018, Sennett et al. 2021). Controlled burns, chemical fumigation, and solarization have historically been used to control pathogens and disease in agricultural soils (Hays et al. 2005). Experimentally, soil can be disturbed through steam pressure or by autoclaving (Tian et al. 2015). Soil microbial biomass has been shown to significantly decrease after soil autoclaving, and microbial re arrangements tend to happen when a crop is grown in the disrupted soils (Li et al. 2019, DiLegge et al. 2022). Autoclaved soil has been shown to promote plant biomass in previous studies (Li et al. 2019). In the context of N fertilization, using autoclaved soils offers the experimental possibility to compare the effect of microbial competition for applied nitrogen and investigate how the alteration of a soil's ecosystem can impact the native microbiome. It is important to mention that the autoclave is not a complete sterilization process, it only decreases the microbial load.

To further understand the impact of N fertilizer types at a microbiological scale, we compared the impact of urea (46-0-0) with Environmentally Smart Nitrogen (ESN), a controlled release fertilizer (44-0-0), in nonsterile and sterile (autoclaved) soil conditions. We reasoned that within

the controlled release fertilizer treatments, microbial communities would be more diverse, therefore promoting plant performance and reducing N loss in the nonsterile soil condition. We reasoned that the autoclaved soil condition would reduce the microbial load allowing for a strong host selected microbiome that would promote N toxicity resilience. We also hypothesized that microbes associated with nitrification and denitrification would be in higher abundance in the fertilized treatments.

Material and Methods

Selection of Tomato Seeds and Growing Conditions

The study was performed in a greenhouse at the Horticulture Center of Colorado State University (CSU), Fort Collins, Colorado from October 22nd – December 10th, 2021. 'Rutgers' tomato, *Solanum lycopersicum* cv. *Rutgers,* was used to evaluate the N fertilization effect on soil microbial communities and plant performance. Tomato seeds were sterilized with 3% sodium hypochlorite and rinsed with distillate water. Seeds were pre-germinated on wet filter paper and stored in petri dishes for seven days. Small plants were transplanted to a potting mix to promote root establishment for 12 days. Plants were then transplanted into 15-cm diameter pots with drainage holes that contained the air dried 1.2 kg of collected agricultural field soil. The soil used in the study was a low N content (5.25 NO_3 ppm) soil from the Agricultural Research, Development, and Education Center (ARDEC) in Fort Collins, Colorado. The soil was collected from long-term study control plots with 20 years of not being fertilized with nitrogen fertilizer (USDA-ARS). The soil was a clay loam soil (fine-loamy, mixed, mesic Aridic Haplustalfs). Soil was collected, sieved with a 1 cm sieve, and dried prior to the study via air drying. The study lasted for eight weeks after transplantation in the greenhouse with a 14-hour photoperiod. Plants were

watered to field capacity (24.9 % volumetric) once per day via top watering. Pots contained drainage holes for excess moisture to be able to drain out. The range of average minimum and maximum temperatures in the greenhouse during the experiment was 20 to 33 °C. Treatments were arranged in a completely randomized design with 10 replicates per treatment. Each treatment consisted of a pot with or without a tomato plant filled with either sterilized or nonsterilized soil and amended with either urea or ESN fertilizer.

Soil Autoclave and Preparation Process

One half of the treatments were planted in non-sterile soil from the low N plot at ARDEC in Fort Collins, CO. The other half of the treatments were grown in the low N ARDEC soil that was autoclaved with the purpose of reducing microbial biomass and altering the native soil microbiome to determine how fertilizer affects its development. The high temperatures that the autoclave process creates conditions that many soil microorganisms cannot survive in, however many thermophilic and spore producing bacteria are known to survive under these conditions. Soil was steam pressurized in a Lindig soil steamer (Crops Research Laboratory USDA-ARS, Fort Collins, CO) at 76 °C for 6 h. Soil was then autoclaved using a STERIS autoclave for three 15-min liquid cycles at 121 °C. Soil was dried before administering soil into pots.

Fertilizer Selection

Two N fertilizers, Environmentally Smart Nitrogen (ESN) (44 %N) and urea (46 %N), were used for the study. Fertilizer was applied to the pots one week after transplantation in increments of 2 g urea (0.92 N), 0.5 g urea (0.23 N), 2.05 g ESN (0.88 N), 0.55 ESN (0.22). The fertilizer applied was in granular form and was lightly buried (5 cm depth) in four places

surrounding the plant. Slightly higher amounts of ESN were used to provide the same N application rate for both fertilizer treatment types.

Bulk Soil Collection

After eight weeks of plant growth in pots, we collected bulk soil for DNA extractions and nitrate analysis by collecting four soil cores around the diameter of the pot. Sampled soil was packed into individual 15 mL falcon tubes and stored at -20 ºC for DNA extractions. Soil was dried and packed into individual bags for nutrient analysis.

Plant Biomass Sampling

The shoots and roots immediately following harvest were cleaned. For this study, shoots refer to the tomato plant's total aboveground biomass. Cleaning consisted of gently rinsing the plant shoots and roots with water. After cleaning, the shoots and roots were placed in a drying oven at 80 °C, and the dry biomass of shoots and roots were weighed and totaled for analysis.

Soil Nutrient Analysis

Bulk soil was dried and sieved (2 mm sieve size) and sent to Ward Laboratories (Kearney, NE) to be analyzed with for NO_3 **-.**

DNA Extraction

Total DNA was extracted from each 0.25 g of the bulk soil sample using a DNeasy Powersoil PRO isolation kit and QIAcube (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The DNA was then quantified using a Qubit Fluorometer (Invitrogen Qubit 4 Fluorometer). Extracted DNA was stored in individual tubes at -20 ºC.

16S Amplicon Sequencing with Minion Flow Cells

Based on Qubit concentrations (ng/μL), extracted DNA was diluted 10x with HPLC water to lower DNA concentrations. Mastermix consisted of 10 μL Phusion HSII master mix, 7.2 μL H_2O, 0.4 μL forward primer, and 0.4 μL reverse primer for a total of 18 μL Mastermix per 2

μL sample. Bacterial primers used were Bact_27F-Mn (5' – TTTCTGTTGGTGCTGATATTGC AGRGTTYGATYMTGGCTCAG – 3') and Bact_1492R-Mn (5'ACTTGCCTGTCGCTCTATC TTC TACCTTGTTACGACTT – 3'). Polymerase chain reaction (PCR) settings were 98 °C for 30 sec, 98 °C for 15 sec, 50 °C for 15 sec, and 72 °C for 60 sec for 25 cycles, and 72 °C for 5 min. After the first PCR, equal volumes of DNA and beads were mixed. A 96-pronged magnetic stand was used to move beads with adhering DNA into two 30 second rinses of 70% EtOH. DNA was eluted in a 96 well plate with 40 μL PCR grade water and beads were removed using a magnetic stand. DNA was quantified using a Qubit fluorometer with high sensitivity assay solutions. The second PCR settings were 98 °C for 30 sec, 98 °C for 15 sec, 62 °C for 15 sec, and 72 °C for 60 sec for 25 cycles, and 72 °C for 5 min.

After a second PCR, DNA and barcodes were pooled in AMPure bead solution in a 96 well plate. Wells with suspended DNA and barcodes were pooled into a clean Lo-Bind tube. MinION sequencer was loaded with a flow cell (R9.4.1) and was prepared for DNA loading. To prepare the flow cell, air (~20 μL) was removed using a pipette. The flow cell was then primed with flush buffer, and pooled DNA was loaded into the sampling port. MinKNOW software was used to sequence the pooled library for 48 hours at the USDA-ARS facility in Fort Collins, CO. Raw data was downloaded and base-called and demultiplexed using Guppy v6.0.1. Sequences were filtered based on length (V34: 300-600 bp; Full: 1000-2000 bp) and a minimum q-score of 70 using Filtlong v0.2.1 (Wick 2017) and Cutadapt v3.2 (Martin 2011). Chimeras were filtered using

vsearch (Rognes et al. 2016), and taxonomy was assigned with minimap2 v2.22 (Li 2018) using the default NCBI-linked Reference Database from EMU. Error-correcting was done with Emu v3.0.0 (Curry et al. 2022) which applies an expectation minimization algorithm to adjust taxonomic assignments using up to 50 sequence alignments per sequence reads. Samples with less than 10000 reads were removed from all down-stream analyses.

Functional gene abundances classified by KEGG ontologies (Table 1) were estimated for the entire EMU reference database (Curry et al. 2021) using PICRUSt2 (Douglas et al., 2020). The first two steps of the default PICRUSt2 pipeline were performed. First, the python script (place_seqs.py) which utilizes HMMER (Eddy et al. 2011) was used to add the query sequences to the default PICRUSt2 prokaryotic 16S rRNA phylogenetic tree using EPA-NG (Barbera et al. 2019). Second the python script (hsp.py) which utilizes the castor R package (Louca & Doebeli, 2018) was used to predict 16S rRNA and functional gene copies per genome. Functional gene abundances (copies g^{-1} soil FW) for each sample and N-cycle gene of interest were calculated as follows:

$$Gene.CPS = \sum_{i=1}^{S_{obs}} \frac{n_i}{N} \times \frac{Gene.CPG_i}{16S.CPG_i} \times 16S.CPS$$

Gene.CPS = functional gene copies g^{-1} soil FW
S_{obs} = number of observed Amplicon Sequence Variants (ASV) n_i = number of sequenced reads in ASV i
N = number of sequenced reads
$Gene.CPG_i$ = functional gene copies per genome for ASV i
$16S.CPG_i$ = 16S rRNA copies per genome for ASV i
16S.CPS = 16S rRNA copies g^{-1} soil FW

Table 1. KEGG orthologues selected for PICRUSt analysis.

Gene	Process	Reaction	KEGG Entry
nifH	N-fixation	$N_2 -> NH_3$	K02588
pmoA-amoA	Nitrification	$NH_3 -> NH_2OH$	K10944
hao	Nitrification	$NH_2OH -> NO_2^-$	K10535
nirK	Denitrification	$NO_2^- -> NO^-$	K00368
nosZ	Denitrification	$N_2O -> N_2$	K00376

Statistical Analysis

All data were analyzed with R version 4.1.2 (R-project, 2021). A two-way ANOVA was run for plant biomass (Biomass ~ Fertilizer Rate + Fertilizer Type). There was a high, low, and control fertilizer rate. Fertilizer type includes the control, ESN and urea. A two-way ANOVA was run for the soil NO_3 concentrations (Soil NO_3 ~ Fertilizer Rate + Fertilizer Type). A Tukey's HSD (honest significant difference) test was used for comparisons of means and statistical differences were assigned at alpha less than 0.05. For the NO_3 analysis, data was log transformed to achieve residual normal distribution. To test for the effects of the treatments on microbial community composition, a permutational multivariate analysis of variance (perMANOVA) was used to test for significant differences in microbial community composition using Bray-Curtis distances on Hellinger-transformed relative abundances. Partial db-RDA ordination (i.e., constrained by fertilizer treatment with soil and plant effects removed by partialling out) was used to visualize differences in microbial community composition using Bray-Curtis distances on Hellinger-transformed relative abundances. Differences in bacterial species abundances between fertilizer treatments were tested by differential abundance analysis (DESeq2) with a false-discovery rate of 0.05 as the accepted threshold for the adjusted p-value. A three-way ANOVA was run for the total abundance (gene copies g^{-1} soil) of selected N cycling genes (Gene copies ~ Fertilizer Rate, Fertilizer Type, Soil Condition (autoclaved or nonautoclaved).

RESULTS

Total Plant Biomass

Tomato plant biomass was significantly different between fertilizer treatments but not soil treatments (autoclaved vs non-autoclaved) with no interaction ($p<0.05$). A post-hoc Tukey test showed biomass was significantly higher in both rates of ESN, but not urea in the autoclaved soil condition (Figure 1).

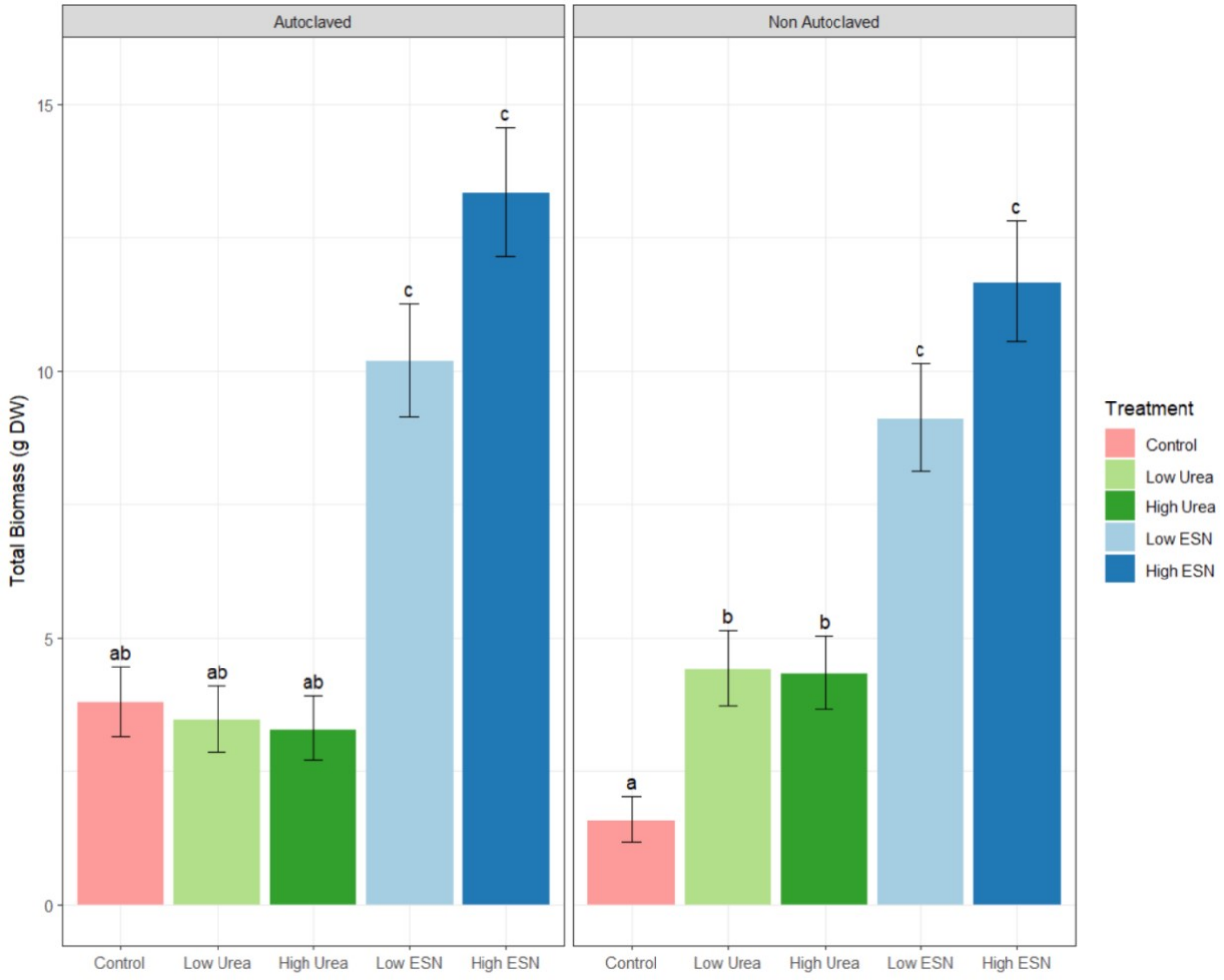

Figure 1. Biomass of tomato plants (g DW) under different nitrogen fertilizers for eight weeks. **A.** Effect of ESN and urea on tomato plants grown in autoclaved soils. **B.** Effect of ESN and urea on tomato plants grown in nonautoclaved soils. Bars with different letters are significantly different ($p<0.05$) based on a Tukey HSD post-hoc test. Each bar is the least-square mean averaged over soil treatment levels, and error bars are the pooled standard error.

Soil Nitrate Analysis

At the end of the 8-week growing period, no significant differences between soil NO_3 concentrations in nonsterile and sterile soils; however, a significant difference was observed for both the plant and fertilizer treatments (Figure 2) ($p<0.05$). ESN promoted the highest soil NO_3 availability and urea promoted higher soil NO_3 in comparison to the control.

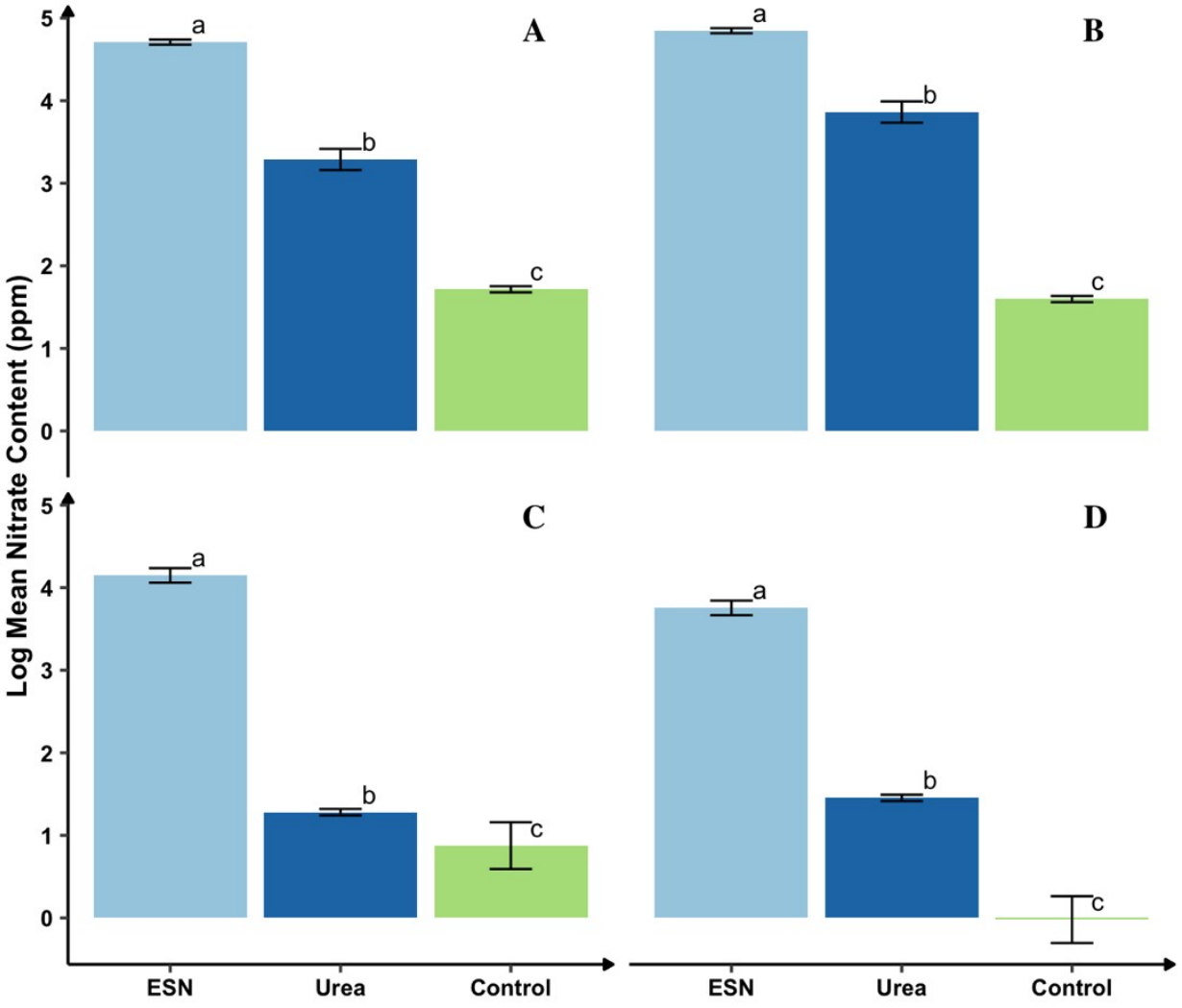

Figure 2. Soil NO_3 contents (ppm) under different fertilizer regimes eight weeks after applying fertilizer in (A) non-sterile soils with no plant, (B) non-sterile soils with tomato plants, (C) sterile soils with no plant, and (D) sterile soils with tomato plants. Bars with different letters are significantly different ($p<0.05$) based on a Tukey HSD post-hoc test. Each bar is the backtransformed least-square mean averaged over soil treatment levels, and error bars are the pooled standard error.

Soil Microbial Community Composition

Soil microbial community composition was determined by 16S rRNA amplicon sequencing and a perMANOVA showed that all three treatments had a significant effect on species-level composition with soil ($F = 270$, $p = 0.001$) having the greatest impact, followed by the fertilizer treatment ($F = 3.23$, $p = 0.001$), and finally the presence of a tomato plant ($F = 7.57$, $p = 0.003$). Compositional differences in soil microbial communities were significantly related to soil sterilization (Figure 3). Bulk soil microbiome analysis across all treatments indicates a clear divide between sterile and nonsterile microbial assembly ($p< 0.001$).

Since differences in tomato plant biomass were only significant between fertilizer treatments and not soil, differences in the microbial community between fertilizer treatments was assessed by partial db-RDA (i.e., constrained by fertilizer treatment with soil and plant effects removed by partialling out). The first two constrained axes of the db-RDA explained 6.7% of the variation in community structure with the high urea and high ESN treatments separating from the control along axis 1 (Figure 4). Pairwise perMANOVA tests, conducted separately for the nonautoclaved and autoclaved soils, confirmed that the high urea and high ESN fertilizer treatments were different than the control ($p < 0.007$); but not the low urea or low ESN ($p > 0.208$) fertilizer treatments. The species scores from the db-RDA show that *Nitrospira mulitformis, Nitrobacter winogradsky, Nitrosomonas communis,* and *Nitrospira briensis,* all known nitrifiers, were positively correlated with axis 1 and the high urea and ESN fertilizer treatments.

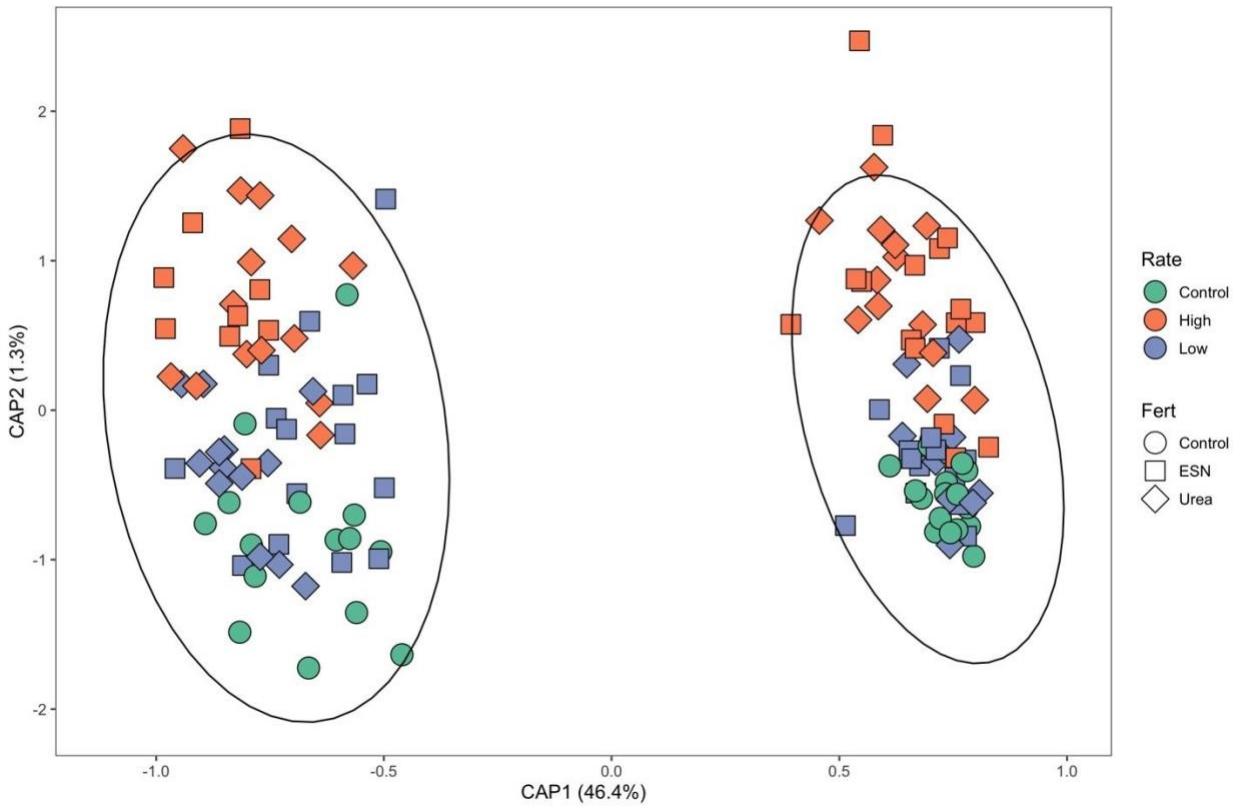

Figure 3. Constrained Principal Coordinate Analysis (PCoA) of the bulk soil microbiome sequenced data from all treatments and constrained by soil sterility and fertilizer rate using Bray-Curtis distances calculated at the species level. **Left ellipse-** Autoclaved soil condition **Right ellipse-**Nonsterile soil condition. Legend: orange – high N rate; green – no N control; purple – low N rate; circle- no N control; square- ESN; diamond- urea.

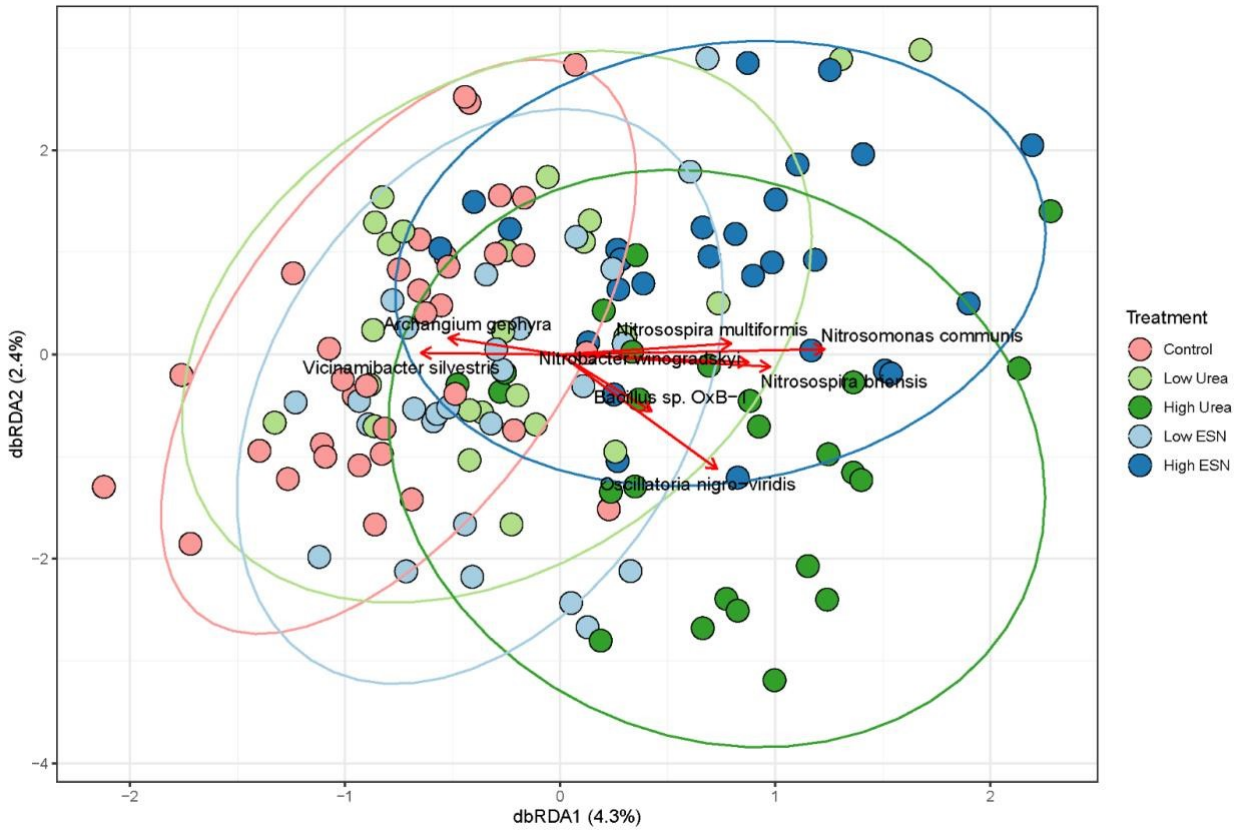

Figure 4. Partial db-RDA constructed using Bray-Curtis distances of Hellinger-transformed species relative abundances (%) from 16S rRNA amplicon sequencing on the nonautoclaved soil. The db-RDA was constrained by fertilizer treatment after partialling out both soil and plant treatments. Only species scores (red vectors) greater than 0.15 or less than -0.15 for either axis are shown for simplicity.

Differential Abundance Analysis

A differential abundance analysis was conducted to identify the species that were more abundant in the high urea or high ESN fertilizer treatments relative to the control for both nonautoclaved and autoclaved soils (Table 2). A total of 10 species were identified that were more abundant in either of the high fertilizer treatments. Like the patterns observed in the db-RDA, three taxa (*Nitrobacter winogradsky, Nitrosomonas communis,* and *Nitrospira briensis)* were enriched in both the high urea and high ESN treatments as compared to the control (Table 2). Significantly different bacterial taxa were also found when comparing the high urea and high ESN treatments

to each other (Table 3). Most of the differences were seen in the non-autoclaved soil condition. *Bacillus megaterium* is a known plant growth promoting rhizobacteria (PGPR) that promotes cytokinin production (Castro et al. 2008). *Nitrospira japonica* and *Nitrosospira multiformis* are known to function in the nitrification process (Norton et al. 2008, Fujitani et al. 2020).

The total abundance of 16S rRNA (gene copies g^{-1} soil) was determined by qPCR and used to calculate the total abundance of N cycle related genes using PICRUSt2. A three-way ANOVA for each gene showed that autoclaving significantly reduced total bacterial (16S rRNA) abundance and all five N cycle genes tested (Table 4). A significant soil interaction by fertilizer treatment was observed for *nifH*, *pmoA-amoA*, *hao*, and *nosZ* but not 16S rRNA or *nirK* (Table 4). Of the four N cycling genes with a significant interaction, only the two nitrification related genes, *pmoA-amoA* and *hao*, showed a significant increase in abundance relative to the control.

Table 2. Differential abundance for species that were enriched in either the high urea or high ESN treatments relative to the control. Analysis was conducted using DESeq2 and log2FC is the log2 fold change in abundance relative to the control. Positive values are enriched relative to the control.

	Non-autoclaved				Autoclaved			
	High Urea		High ESN		High Urea		High ESN	
Taxa	log2FC	p-value	log2FC	p-value	log2FC	p-value	log2FC	p-value
Bacillus sp. OxB-1	-	-	-	-	23.7	0.000	-	-
Geminocystis sp. NIES3709	-	-	-	-	22.0	0.000	22.0	0.000
Nitrobacter winogradskyi	8.06	0.000	8.86	0.000	4.08	0.026	4.30	0.039
Nitrosomonas communis	7.81	0.000	9.30	0.000	4.22	0.000	4.49	0.000
Nitrosospira briensis	2.44	0.001	3.08	0.000	2.65	0.002	2.48	0.030
Nitrosospira lacus	-	-	3.28	0.038	-	-	-	-
Nitrosospira multiformis	1.26	0.005	2.41	0.000	-	-	1.75	0.011
Nitrospira defluvii	-	-	8.29	0.000	-	-	-	-
Oligotropha carboxidovorans	-	-	6.76	0.002	-	-	-	-
Sphingobium mellinum	-	-	21.8	0.000	-	-	-	-

Table 3. Differential abundance for species significantly different between the high urea and high ESN treatments. Analysis was conducted using DESeq2 and log2FC is the log2 fold change in abundance relative to the control. Positive values are enriched in the high ESN treatment, negative values in the high urea treatment.

	Non-autoclaved		Autoclaved	
Taxa	log2FC	p-value	log2FC	p-value
Bacillus megaterium	1.745	0.011	-	-
[Brevibacterium] frigoritolerans	1.339	0.011	-	-
Nitrospira japonica	0.948	0.011	-	-
Adhaeribacter terreus	0.981	0.019	-	-
Patulibacter medicamentivorans	1.763	0.030	-	-
Brevitalea aridisoli	0.780	0.030	-	-
Georgfuchsia toluolica	0.772	0.030	-	-
Nitrosospira multiformis	1.150	0.039	-	-
[Polyangium] brachysporum	0.717	0.040	-	-
Chryseolinea serpens	0.944	0.049	-	-
Ohtaekwangia koreensis	1.077	0.049	-	-
Bacillus sp. OxB-1	-	-	-24.261	0.000
Roseimicrobium gellanilyticum	-	-	-5.820	0.000

Table 4. Total abundance (gene copies g^{-1} soil) of selected N cycling genes. LSmeans with different letters are significantly different ($p<0.05$) based on a Tukey HSD post-hoc test. Values are averaged over plant treatments levels.

Soil	Treatment	*Total*	*N-fixation*	*Nitrification*		*Denitrification*	
		16S rRNA	*nifH*	*pmoA-amoA*	*hao*	*nirk*	*nosZ*
Non-autoclaved	Control	1.98e09 b	1.17e08 c	2.94e07 cd	2.82e07 cd	2.13e08 bc	1.77e08 bc
	Low Urea	1.89e09 b	1.27e08 c	4.21e07 de	4.11e07 de	2.41e08 bc	1.92e08 bc
	High Urea	1.83e09 b	1.43e08 c	5.56e07 de	5.38e07 de	2.97e08 c	2.04e08 c
	Low ESN	2.01e09 b	9.95e07 c	3.15e07 d	3.02e07 d	2.01e08 abc	1.55e08 bc
	High ESN	1.83e09 b	1.18e08 c	7.22e07 e	6.64e07 e	2.94e08 c	1.83e08 bc
Autoclaved	Control	9.62e08 a	3.89e07 ab	4.01e06 a	3.94e06 a	1.59e08 ab	1.15e08 ab
	Low Urea	7.88e08 a	2.85e07 a	3.74e06 a	3.77e06 a	1.28e08 a	8.73e07 a

High Urea	8.68e08 a	3.94e07 ab	1.27e07 b	1.27e07 b	1.91e08 abc	1.30e08 abc
Low ESN	1.06e09 a	5.74e07 b	9.19e06 b	8.99e06 b	1.96e08 abc	1.53e08 bc
High ESN	8.92e08 a	3.87e07 ab	1.01e07 abc	9.54e06 abc	1.72e08 abc	1.05e08 abc

Discussion

The main goal of the study was to determine the effects ESN and urea have on plant growth, soil N concentrations, and the soil microbiome's community structure. The present study showed that ESN, when compared to urea, enhanced tomato biomass regardless of rate. The lack of growth seen with urea is indicative of toxicity and/or leaching of nitrogen. Studies have shown that controlled release fertilizers can promote crop biomass and available soil N (Carson and Ozores-Hampton 2014, Huang et al. 2016, Lawrencia et al. 2021). In the present study, the highest tomato biomass was observed in the ESN treatments.

A single dose of urea could be toxic to microbes or plants and further lost to volatilization, leaching, or denitrification. In this study, we did not measure all the possible losses of N, however we allowed the pots to naturally drain to better mimic field sites which may have allowed for leaching in the urea treatments. Interestingly, in the non-autoclaved, but not the autoclaved soil, we did see a slight but significant increase in both urea treatments to urea. At the high rate this could plant toxicity, but we did not observe typical symptoms. Regardless all these points suggest the benefits of ESN as its slow-release nature avoids many of these problems.

The sustained release of ESN throughout a plant's development when compared to urea has been suggested to provide a more optimal growing environment for the plant (Vejan et al. 2021). The more favorable growing environment could be due to several factors that all increase plant availability and uptake of N: (1) reduced N losses through leaching, volatilization and/or denitrification, (2) reduced microbial competition for N, or (3) promotion of beneficial microbes.

We saw no evidence that the losses of N were associated with denitrification as the abundance of denitrifying bacteria did not differ between fertilizer treatments. This finding could possibly be explained by the loss of N through leaching from the pots as the pots were top watered and allowed for draining.

The current study suggests that not only does ESN improve plant growth relative to urea, but lower rates of ESN fertilizer may also be used to obtain sufficient plant growth. First, we observed a significantly higher total plant biomass in our low ESN rate (0.5 g) compared to both the low and high urea rates (0.5 and 2 g). Second, although there was a trend for lower biomass in the increased biomass in the higher ESN compared to the low ESN rate, no statistical differences were observed. The lower N concentration in the soil suggests that the losses of nitrogen with the urea treatment due to leaching from the pot may have given an advantage to the ESN. We suggest that the lack in the growth response observed from the urea treatments compared to the ESN than treatments may have been due to toxicity that reduced the plant's ability to develop or leaching. These results suggest that ESN maintains a larger supply of N to the tomato plant and increases in total plant growth when compared to urea fertilizer under greenhouse conditions.

In this study, it was found that autoclaving the soil, which significantly changed the composition of the soil microbial community, did not have a significant impact on tomato plant biomass or soil nitrate contents. Although Zhu et al. (2016) showed that at excessive N fertilizer rates microbial competition for N can reduce plant growth, this appears to not be a factor in the current study. In a previous study, Li et al (2019), using a soil obtained from a peach orchard with replant disease, autoclaving the soil increased tomato plant biomass. However, we did not see a similar effect here presumably due to the lack of general pathogens and/or replant disease in our study soil.

Independent of fertilizer treatment, we observed that soil microbial community composition was altered only at the high fertilizer application rates. The impact of N fertilization rate on soil microbiome assembly has been demonstrated in other studies (Zeng et al. 2016, Zhu et al. 2016). Zhu et al. (2016) reported that increasing N rate impacted total microbial biomass and relative gene abundance in the denitrification and nitrification processes. In the present study, the microbial compositions showed an increase in the abundance of nitrifiers, based on both taxonomic assignments (*Nitrospira mulitformis, Nitrobacter winogradsky, Nitrosomonas communis*, and *Nitrospira briensi*) and the abundance of the *pmoA-amoA* and *hao* nitrification genes. Nitrifying bacteria convert the most reduced form of soil N, NH_4, into the most oxidized form, NO_3. Many of these species are important for soil agroecosystem function by their ability to cycle N (Bei et al. 2021, Rice et al. 2016, Yue et al. 2022). The differential abundance analysis showed differences in bacterial taxon between the fertilizer types and soil conditions. These differences include higher amounts of N cycling bacteria in the ESN treatment and higher degree of beneficial taxa in the non-autoclaved soil condition.

Conclusion

Our results demonstrate that the use of ESN, improved plant growth, and even when applied at one-quarter the rate of urea resulted in greater plant biomass. The application of ESN in the lesser rate promoted greater plant biomass while resulting in higher available soil N concentrations. A treatment specific shift in the microbial community composition was detected between ESN and urea. The decline in total abundance seen in the autoclaved soil condition suggests that the alteration of a soil's agroecosystem decreases the nutrient acquisition function in the soil. Taken together, the results of this study can inform the development of management

strategies to maximize growth under reduced N fertilization while maintaining healthy soil microbiomes.

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Chapter 3

TEMPORAL VARIATION IN RHIZOSPHERE MICROBIOME DEVELOPMENT AND TOMATO PLANT DEVELOPMENT FOLLOWING NITROGEN FERTILIZATION

Introduction

Nitrogen (N) fertilizer applications, while needed to support crop nutrient demands, are often over applied in agricultural systems to ensure that crops are not N limited. Fertilizer applications also add to agricultural pollution and runoff, water contamination, eutrophication, and more. The above challenges indicate the need for strategies to improve the effectiveness of N additions and decrease the negative effects that soil amendments have on agroecosystem health (Rocci et al. 2019, Miner et al. 2020, Kelly et al. 2022).Nitrogen inputs account for approximately 60% of the worldwide fertilizer consumed each year (Kang et al. 2022, Wang et al. 2018), however, recent shortages of N fertilizers have increased the cost of N applications and leading to considerable hardships for farmers. For instance, the cost of anhydrous ammonia has increased from about $500 per ton in 2020 to $1400 per ton in 2022 (Schnitkey et al. 2022).

Strategies to improve nitrogen use efficiency (NUE) are needed to mitigate the economic and environmental consequences of N fertilizer losses (Stevens 2019). Such strategies can include the implementation of controlled release fertilizers (CRF) as well as precision agriculture techniques, focused on applying fertilizer in the correct amount and timing to maximize

availability when the crop requires the nutrient (Li et al. 2007). The 4R's of nutrient management in agriculture are known as selecting the right rate, source, placement, and timing for fertilization applications (Fixen et al. 2020, Wang et al. 2020). When nutrient management practices are implemented according to the 4R principle, this can provide nutrients for optimal crop nutrient uptake, increase NUE, and can help mitigate the nutrient loss to the environment, which subsequently, could minimize the adverse consequences fertilization has on the environment (Ayankojo et al. 2021, Dixon et al. 2020).

The use of N fertilizers can contribute to aboveground performance throughout a crop's life cycle. Microbial communities are known to differentiate throughout a crop's development (ref?). This leads to changes in soil functioning, as applications of inorganic N amendments have been shown to increase total and relative abundance of denitrifying and nitrifying genes in the rhizosphere soil early on in plant development (Zhu et al. 2016). At the same time, long-term N application has been shown to increase the abundance of Actinobacteria species in the rhizosphere at the flowering stage of plants, and the study showed rhizosphere bacteria taxon has been shown to stabilize at around 8-10 weeks of development (Zhang et al. 2018, Ren et al. 2020).

Crops acquire N primarily in the forms of ammonium (NH_4) and nitrate (NO_3) at various stages throughout their development, respectively. Nitrogen use by plants involves several complex step including uptake, assimilation, translocation, recycling, and remobilization (Masclaux-Daubresse et al. 2010). A physiological NUE index can be expressed as the dry matter produced per N content in a plant (Good et al. 2004). Studies have shown that some plants tend to require more N at later stages of development to support flowering and grain or fruit production (Malagoli et al. 2004, Rossato et al. 2003), while other plants like tomatoes use N heavily in early growth stages (Hartz et al. 1993, Machado et al. 2008). In contrast the typical paradigm for nutrient

management in agricultural systems, plants under natural conditions rely more on evolutionary associations with rhizosphere microbes that facilitate N availability via N fixation or mineralization of organic matter into plant available forms (Caballero-Mellado et al. 2007, Pérez-Izquierdo et al. 2019, Liu et al. 2020, Kelly et al. 2022).

Plant roots can recruit distinct rhizosphere microbial communities found to be taxonomically and functionally distinct from the bulk soil (Schmidt et al. 2019). The rhizosphere microbiome associated with a plant's root system contains many N cycling bacteria (Franche et al. 2009, Richardson et al. 2009, Islam et al. 2013). Bacteria known to function as nitrifying and denitrifying species have a role in the uptake and loss of N from agroecosystems (Moreau et al. 2019, Henneron et al. 2020). The rhizosphere microbiome also contains many free-living N fixing species responsible for nutrient acquisition (Chaparro et al. 2014, Dixon et al. 2022). Plant development following N application is well understood. However, knowledge regarding how a crop's rhizosphere microbiome develops following N application is not. Studies utilizing the *Arabidopsis thaliana* and *Oryza sativa* plants have found that the plants can recruit various microbes during growth and according to the specific needs of the plant (Chaparro et al. 2014, Edwards et al. 2015, Zhang et al. 2018). This recruitment could be indicative of functional genes associated with nutrient cycling during the varied stages of a plant's growth cycle. Chaparro et al. (2014) found greater amounts of N cycling functional genes during vegetative development and N fixation genes were expressed in later stages of growth in *Arabidopsis*. Despite these findings, there is a knowledge gap regarding how the rhizosphere assembles following N fertilizer application and if there are differences regarding the type of fertilizer. Thus, to further improve NUE strategies, an understanding of how fertilizer application impacts the rhizosphere microbiome development is necessary. Here we explore how bacteria involved in N cycling change across plant developmental stage and with form of applied N fertilizer. We also studied

functional N cycling genes expressed by bacteria that are important for tomato plant growth during different developmental stages under two different fertilizer types, Environmentally Smart Nitrogen (ESN) and urea by sampling weekly over the course of eight weeks.

Material and Methods

Growth Conditions

This study was performed in a controlled greenhouse setting at the Horticulture Center of Colorado State University (CSU), Fort Collins, Colorado from May 10th to July 5th, 2022. The greenhouse range of average minimum and maximum temperatures was 20 to 33 °C, and was managed with a 14-hour photo period. We used the 'Rutgers' tomato variety (*Solanum lycopersicum* cv. *Rutgers*) to evaluate the N fertilization effect on rhizosphere microbiome and plant development by weekly sampling over the course of eight weeks. Tomato seeds were sterilized with 3% sodium hypochlorite, rinsed with distillate water, and were germinated in a potting mix. The plants were first grown in potting mix to promote root establishment for 14 days after germination. Plants were then extracted from the potting mix and rinsed to remove any excess soil, and then transplanted into 15-cm diameter pots with drainage holes containing about 1.2 kg of soil.

Soil Selection and Growing Conditions

The soil used in the study came from the Agricultural Research, Development, and Education Center (ARDEC) in Fort Collins, Colorado and contained a low N content (5.25 NO_3 mg/L) soil. The soil came from a corn production plot that has not received N fertilizer in 20 years (USDA-ARS). Upon collection, the soil was sieved and dried prior to the study. Each replicate consisted of a pot with a tomato plant and the soil from ARDEC, amended with the fertilizers, as described below. The study lasted for eight weeks after transplantation, and plants were watered to field capacity once per day.

Nitrogen Fertilizer Type and Rate Selection

Two N fertilizer types, Environmentally Smart Nitrogen (ESN) (Nutrien Ag. Solutions) (44-0-0) and urea (46-0-0), were used for the study. Fertilizer was applied to the pots one week after transplanting from the potting mix to create a total of four treatments: 1) high urea (0.46 g N per pot), 2) low urea (0.23 g N per pot), 3) ESN (0.44 g N per pot), and 4) a control (CK) with no N additions. We used a completely randomized design with five replicates per treatment and 8 sampling times, for a total of 160 replicated pots.

Rhizosphere Soil Collection for Microbiome Analysis

The rhizosphere soil was collected by destructive sampling of the pots each week for 8 weeks. Sampling was conducted by gently removing plants from each pot and carefully brushing soil from the plant roots into 15 mL Falcon tubes and stored at – 20 °C.

Plant Biomass Collection and Nutrient Concentrations

Plant shoots and roots were rinsed with water and dried at 80 °C, and the dry biomass of both shoots and roots were separately recorded. Dried shoots (stems and leaves) were ground and analyzed for total N on a CHN elemental analyzer, based on the combustion of organic matter in an oxygen atmosphere at 925°C.

Rhizosphere DNA Extraction

Total DNA was extracted from each 0.25 g of the rhizosphere soil samples using a DNeasy Powersoil PRO isolation kit and QIAcube (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was quantified using a Qubit Fluorometer (Invitrogen

Qubit 4 Fluorometer). Extracted DNA was stored in individual tubes at -20 ºC before amplicon sequencing.

16S Amplicon Sequencing and Minion Library Preparation

Using the Qubit concentrations (ng/μL) from previously extracted DNA, a dilution was made with ulta-pure water to lower the DNA concentrations. A master mix solution was made with the following reagents: 7.2 μL H_2O, 0.4 μL forward primer, and 0.4 μL reverse primer, and 10 μL Phusion HSII master mix, to bring to mix to a total of 18 μL Mastermix per 2 μL sample. The reverse and forward bacterial primers used were: Bact_27F-Mn (5' – TTTCTGTTGGTGCTGATATTGC AGRGTTYGATYMTGGCTCAG – 3') and Bact_1492R-Mn (5'ACTTGCCTGTCGCTCTATC TTC TACCTTGTTACGACTT – 3').

The settings for the polymerase chain reaction (PCR) were set to a cycle of 98 °C for 30 seconds, 98 °C for 15 seconds, 50 °C for 15 seconds, and 72 °C for 60 seconds with 25 cycles, and 72 °C for a 5-minute cycle. The PCR product, DNA product, and the AMPure beads (40 uL) were then measured in equal volumes and gently flicked to combine in a sterile tube. By utilization of a 96-pronged magnetic stand for support, the beads with the attached DNA were then washed into two ~30 second 70% EtOH rinses consecutively for cleaning. The DNA product was eluted with 40 μL nuclease free water in a 96 well plate and the beads were then completely removed by use of a magnetic stand and with a pipet to remove excess liquid. The bead cleaned DNA was quantified using a Qubit fluorometer set to have high sensitivity assay solutions. The second PCR run followed the same PCR cycle mentioned above.

After following the second PCR run, the eluted DNA and barcodes were pooled into an AMPure bead solution into a 96 well plate. The suspended DNA product and barcodes were pooled into a sterile tube. The MinION sequencer was loaded with a flow cell (Nanopore Technologies)

(R9.4.1) and prepared for the DNA loading by manufacturer's instructions. Air was removed from the flow cell with the pipet set to 20 uL and then the pooled DNA library was loaded into the sampling port. The pooled library was sequenced for 48 hours at the USDA-ARS facility in Fort Collins, CO using Nanopore's MinKNOW software. The raw data was downloaded, base-called, and then demultiplexed by use of the program Guppy v6.0.1. All the sequences were then filtered based on length (V34: 300-600 bp; Full: 1,000-2,000 bp) and with a minimum 70 q-score 70 using the Filtlong v0.2.1 (Wick 2017) and Cutadapt v3.2 (Martin 2011). The chimeras were then filtered out by use of vsearch (Rognes et al. 2016). Taxonomy was assigned with minimap2 v2.22 (Li 2018) and the use of the NCBI-linked Reference Database (NCBI). Error-correcting was done with Emu v3.0.0 (Curry et al. 2022) to adjust the taxonomic assignments with 50 sequence alignments per sequence reads. Data was rarified. Any sample with less than 10,000 reads were removed for the rhizosphere microbiome analyses.

Using PICRUSt2 (Douglas et al. 2020), function gene abundances were classified by KEGG ontologies and were estimated for our entire EMU reference database (Curry et al. 2021). We followed the defaulted first two steps of the PICRUSt2 pipeline. First, a python script (place_seqs.py) which uses HMMER (Eddy et al. 2011) was used to add the query sequences to the default PICRUSt2 prokaryotic 16S rRNA phylogenetic tree using EPA-NG (Barbera et al. 2019). The second step was to predict the 16S rRNA and functional gene copies per genome with the castor R package by use of the python script (hsp.py) (Louca & Doebeli, 2018).

Statistical Analysis

All data were analyzed with R version 4.1.2 (R-project, 2021). A two-way ANOVA was run for plant biomass and N concentration (Biomass ~ Treatment + Week). The treatments are the fertilizer type and rate combined. A Tukey's HSD (honest significant difference) test was used for

comparisons of means and statistical differences were assigned at alpha less than 0.05. A permutational multivariate analysis of variance (perMANOVA) was used to test for significant differences in microbial community composition using Bray-Curtis distances on relative abundances. Principle coordinate ordination analysis (constrained by fertilizer treatment and week) was used to visualize differences in microbial community composition using Bray-Curtis distances. Total abundance analyses to view differences based on gene expression in bacterial species between fertilizer treatments were tested by use of KEGG and the R package phyloseq. NUE was calculated using the formula: [(N content in fertilizer treatment – N content in control)/N fertilizer applied) *100]

Results

Tomato Plant Biomass

Weekly development showed total plant biomass to increase over the course of 8 weeks, with significant differences between fertilizer treatments beginning at week 2 for root biomass and week 4 for shoot biomass ($p<0.05$; Fig. 5, Table 3 and Table 4). During the second week, root biomass in high urea treatment was significantly lower than the CK. No significant differences were detected in either shoot or root DW in the third week. No differences were found amongst the biomass in the fifth week. At the sixth week, ESN and urea (0.5 g) had larger shoot DW than the CK and urea (1 g) treatments ($p<0.05$), and the ESN treatment root DW was larger than the CK and urea (1g) treatments. The seventh week development showed the ESN treatment to have higher shoot and root DW biomass accumulation compared to the other treatments ($p<0.05$). At the final stage of the study, week 8, both ESN and low urea had higher shoot and total biomass than the control, while root biomass was higher for ESN than the control ($p<0.05$).

Table 3. Dry Tomato Shoot Biomass (DWg) with letters based on a Tukey HSD post-hoc test (alpha = 0.05). Each weekly row denotes differences amongst the N fertilizer treatments over the course of eight weeks.

Week	Control	ESN	Urea (0.5)	Urea (1)
1	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}
2	0.16 ± 0.03^{a}	0.19 ± 0.03^{a}	0.15 ± 0.01^{a}	0.12 ± 0.02^{a}
3	0.37 ± 0.06^{a}	0.45 ± 0.07^{a}	0.56 ± 0.13^{a}	0.34 ± 0.10^{a}
4	1.32 ± 0.24^{ab}	2.20 ± 0.20^{a}	1.09 ± 0.24^{b}	0.90 ± 0.22^{b}
5	2.22 ± 0.37^{a}	3.93 ± 0.73^{a}	2.79 ± 0.74^{a}	4.23 ± 0.23^{a}
6	2.54 ± 0.56^{b}	5.34 ± 0.35^{a}	5.04 ± 0.78^{a}	2.59 ± 0.64^{b}
7	2.56 ± 0.51^{c}	8.57 ± 0.45^{a}	5.89 ± 0.61^{b}	3.26 ± 0.78^{c}
8	3.32 ± 0.35^{c}	10.94 ± 0.86^{a}	8.22 ± 0.94^{ab}	6.93 ± 0.77^{b}

Table 4. Dry Root Biomass (DWg) with letters based on a Tukey HSD post-hoc test (alpha = 0.05). Each weekly row denotes differences amongst the N fertilizer treatments.

Week	Control	ESN	Urea (0.5)	Urea (1)
1	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}
2	0.02 ± 0.00^{a}	0.02 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{b}
3	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.05 ± 0.02^{a}
4	0.24 ± 0.03^{ab}	0.26 ± 0.05^{a}	0.16 ± 0.03^{ab}	0.13 ± 0.02^{b}
5	0.36 ± 0.08^{a}	0.49 ± 0.06^{a}	0.33 ± 0.07^{a}	0.35 ± 0.04^{a}
6	0.51 ± 0.13^{b}	1.41 ± 0.19^{a}	0.94 ± 0.12^{ab}	0.39 ± 0.12^{b}
7	0.40 ± 0.06^{b}	1.56 ± 0.15^{a}	0.71 ± 0.12^{b}	0.45 ± 0.05^{b}
8	0.67 ± 0.08^{b}	2.20 ± 0.25^{a}	1.49 ± 0.31^{ab}	1.21 ± 0.2^{ab}

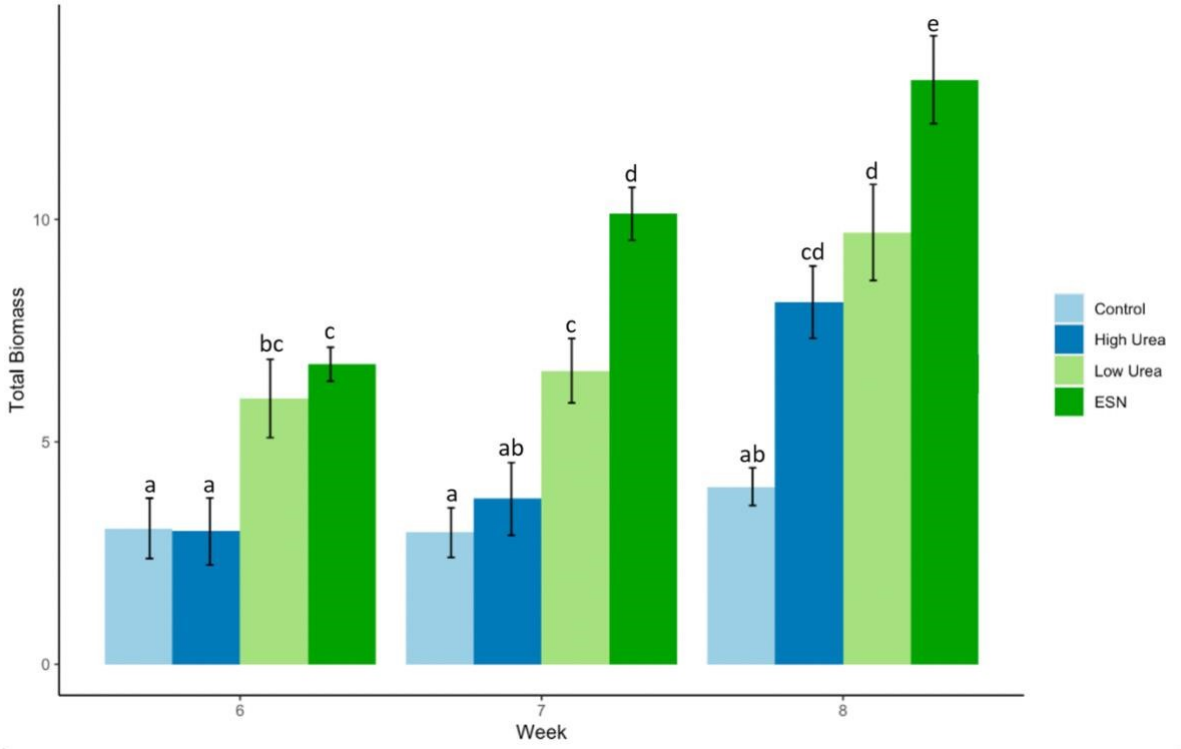

Figure 5. Mean total plant biomass under four N fertilization during the last three weeks of a greenhouse experiment with tomato plants. Letters denote significant differences based on a Tukey post-hoc between the treatments within each week. Colors represent treatment: Light blue- Control (CK), Dark blue-high urea (1 g), Light green- low urea (0.5 g), Dark green- ESN(1 g).

Tomato Plant Tissue Nitrogen Content and Nitrogen Use Efficiency

Over the course of 8 weeks, N concentration in the aboveground tomato plant tissue was found to be less in the fertilized treatments compared to the no fertilizer control, while no significant differences were observed between the fertilized treatments.

Plant N concentration was found to be higher in the urea treatments compared to the control from the second week of development until the final week of the study ($p<0.05$; Table 4).

Similarly, the ESN treatment was found to be higher than the control's plant N content excluding weeks four, six, and seven ($p<0.05$). By week five, all three N fertilizer treatments had significantly higher N content in the tomato plant tissue than the control ($p<0.05$). At week 5, the urea (1g) treatment had greater N content than the ESN (1g) treatment and urea (0.5g) treatment ($p<0.05$). The N uptake advantage of the urea (1g) treatment vanished after week five, since there were there were no significant differences in N uptake between the three N fertilizer treatments during weeks six and seven (Figure 6). Although by week eight all N fertilizer treatments continued to have a higher N uptake than the control ($p<0.05$), such that the ESN treatment had greater N concentration in the tomato plant tissue than the two urea treatments, which had similar total N content ($p<0.05$; Figure 6).

The NUE at the end of the experiment (week 8) was for 55% ESN, 46% for low urea, and 32% for the high urea treatment (Table 6). The efficiency trend increased over the course of eight weeks for ESN, but for the urea treatments values peaked around weeks 5 and 6 and then declined.

Table 5. Plant Tissue N analysis with different letters being significantly different ($p<0.05$) based on a Tukey HSD post-hoc test. Each weekly row denotes differences amongst the N fertilizer treatments.

Week	Control	ESN	Urea (0.5)	Urea (1)
2	3.67 ± 0.42^{b}	4.42 ± 0.23^{a}	4.72 ± 0.21^{a}	5.62 ± 0.24^{a}
3	2.07 ± 0.86^{b}	4.82 ± 0.18^{a}	4.91 ± 0.13^{a}	5.04 ± 0.10^{a}
4	2.47 ± 0.35^{b}	4.49 ± 0.17^{ab}	4.80 ± 0.76^{a}	5.13 ± 0.22^{a}
5	1.53 ± 0.18^{b}	4.30 ± 0.20^{a}	4.79 ± 0.38^{a}	5.15 ± 0.11^{a}
6	1.56 ± 0.21^{b}	4.01 ± 0.32^{ab}	3.77 ± 0.42^{a}	4.48 ± 0.33^{a}
7	1.14 ± 0.05^{b}	2.69 ± 0.11^{ab}	2.96 ± 0.54^{a}	3.59 ± 0.77^{a}

8	1.13 ± 0.05[b]	2.61 ± 0.16[a]	1.82 ± 0.31[a]	2.71 ± 0.24[b]

Table 6. Nitrogen Use Efficiency across the fertilized treatments.

Week	ESN	Urea (0.5)	Urea (1)
2	.65 %	.67%	.17%
3	3%	2%	9%
4	15%	11%	4%
5	29%	41%	40%
6	40%	67%	18%
7	46%	59%	25%
8	55%	46%	32%

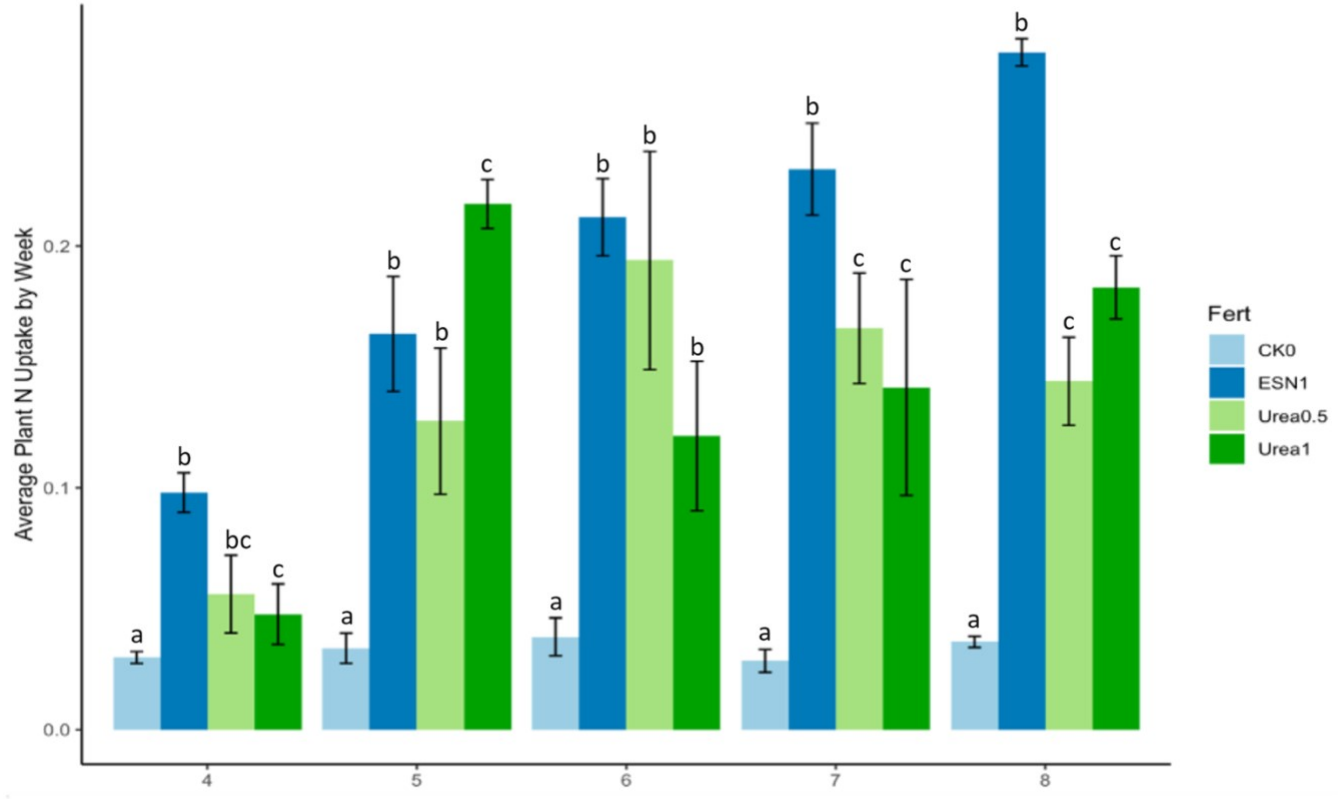

Figure 6. Bar graph demonstrating the tomato plant N uptake from weeks four to eight. Letters denote significant differences based on a Tukey post-hoc between the treatments within each week. Colors represent treatment: Light blue- Control (CK), Dark blue-ESN (1 g), Light green- urea (0.5 g), Dark green- urea (1 g).

Rhizosphere Microbiome Community

The total sequenced rhizosphere microbiome was analyzed with the Bray–Curtis distances between samples to assess similarity of the soil microbial communities at weekly intervals (Figure 7). For simplicity, weeks 1-4 are referred to as the early stage and weeks 5-8 are referred to the late stage based on significant findings found in the plant biomass section. Principal component 1 (CAP1) explained 18.4% of the variation in the data, while principal component 2 (CAP2) explained 1% of the variation in the data within the model (Figure 7). Soil microbial communities

were clear separated between the early and late stages of plant development, while no clear separation was observed amongst the fertilizer treatments.

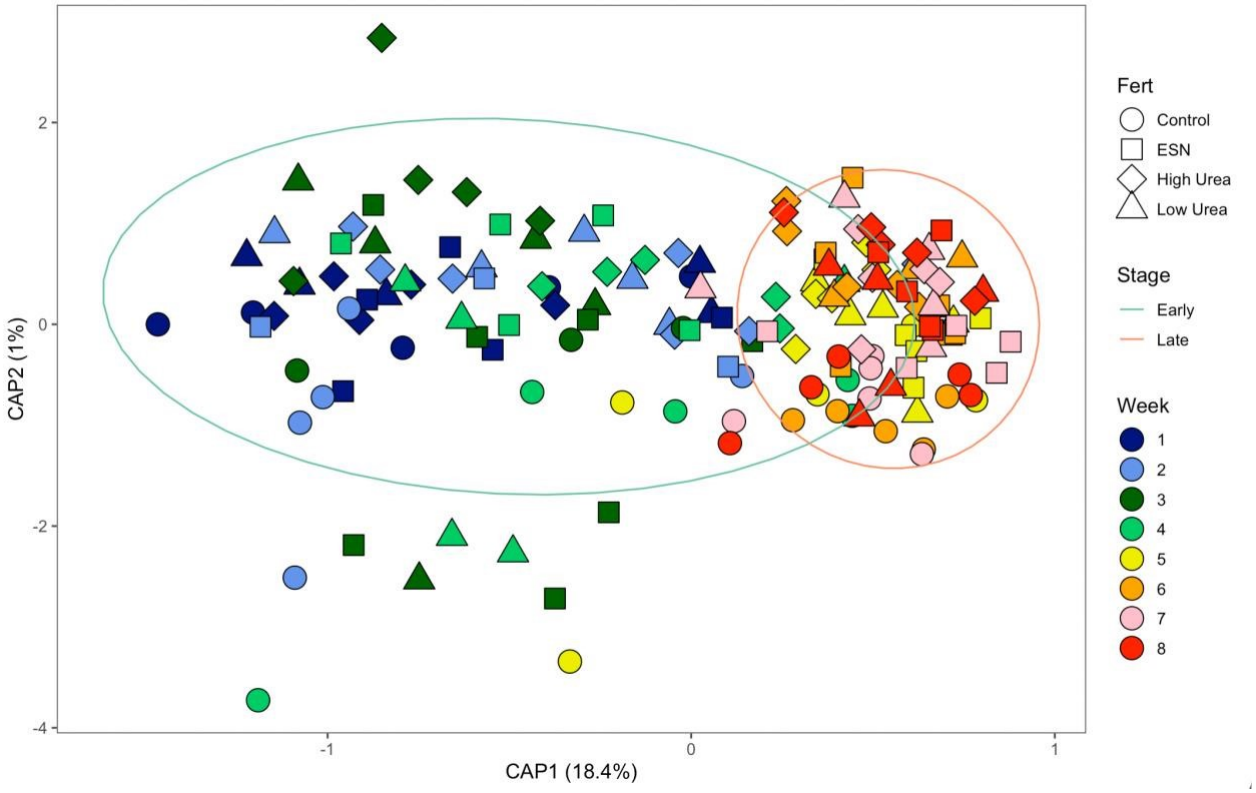

Figure 7. Principal Coordinate Analysis (PCoA) analysis of the rhizosphere microbial community throughout weekly plant development. The visualization demonstrates the pairwise community dissimilarity (Bray–Curtis index) of the rhizosphere microbial community at each week (Week 1- dark blue, Week 2- light blue, Week 3-dark green, Week 4-light green, Week 5-yellow, Week 6- orange, Week 7-pink, and Week 8-red). The developmental stage early (Weeks 1-4) and late (Weeks 5-8) have 95% confidence ellipses around each developmental stage.

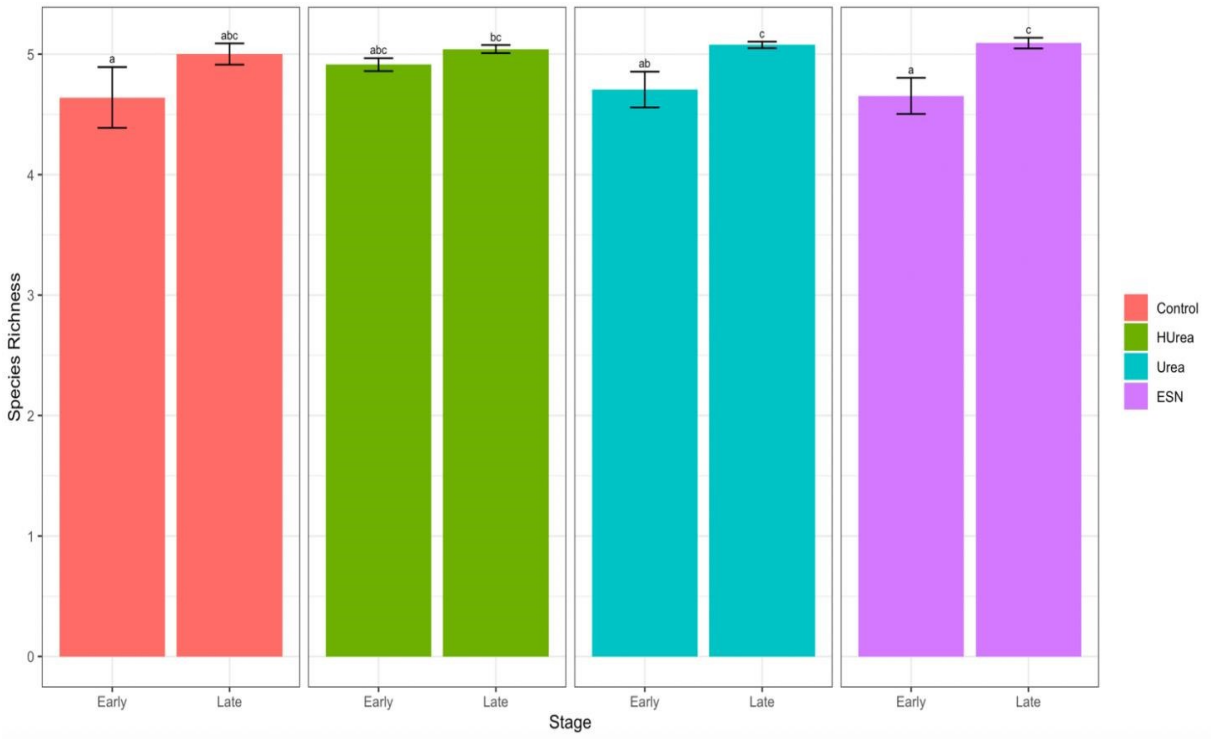

Figure 8. Species richness differences between early and late stage of microbiome development determined with Shannon Diversity index. Pink-control, Green- Urea (1g), Blue- Urea (0.5g), and Purple- ESN (1g).

Rhizosphere Microbiome Relative Gene Abundance

The relative abundance analyses of 16S rRNA (gene copies g^{-1} soil) was determined by qPCR and was then used to calculate the relative abundance of N cycling genes using PICRUSt2 (Figures 3-5). From the results of the ANOVA, the week of development was a significant factor in relative abundance differences ($p<0.05$). While the N treatments did not differ from each other in the relative abundance of various N cycling genes, they did vary compared to the control ($p<0.05$). The N fixation gene abundance were found to decrease in their enrichment until the last stage of the study. The nitrogen fixation bacteria abundance differed from the first week compared to weeks two-five ($p<0.05$) (Figure 9). Bacteria capable of nitrification were seen to differ from the first week of development to the sixth week according to the ANOVA ($p<0.05$) (Figure 10).

Similarly, denitrifying bacteria total abundance was seen to fluctuate significantly across the eight weeks of development ($p<0.05$) (Figure 11).

The nitrifying and denitrifying genes followed a trend in peaking at different weeks within their treatments. At week 4, the urea (1g) showed their highest abundance. At week 5, the urea (0.5g) showed their highest abundance. And lastly, week 6 the ESN (1g) showed their highest abundance.

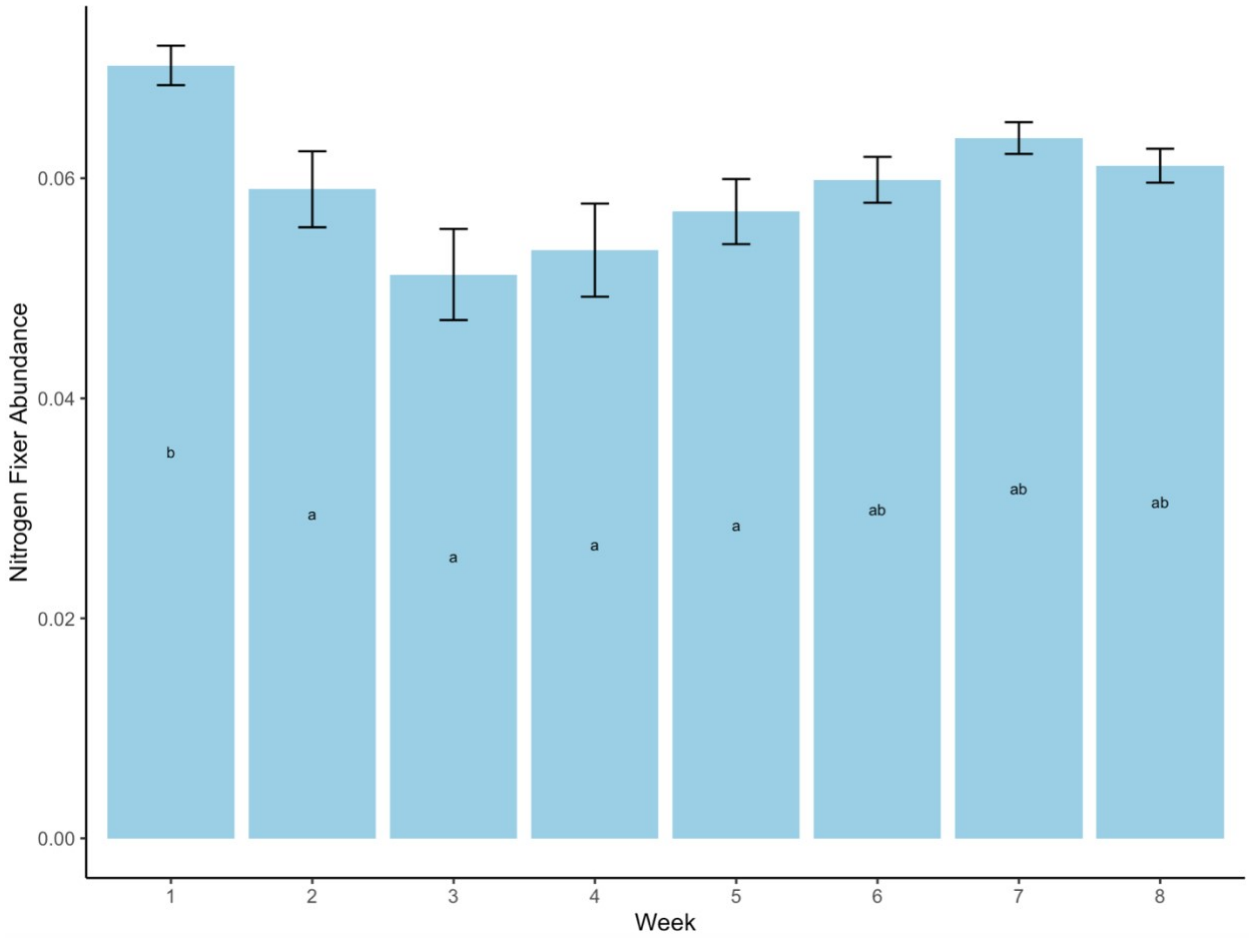

Figure 9. Nitrogen fixing bacterial community total relative abundance analysis**.** Data are the mean values of each treatment per week.

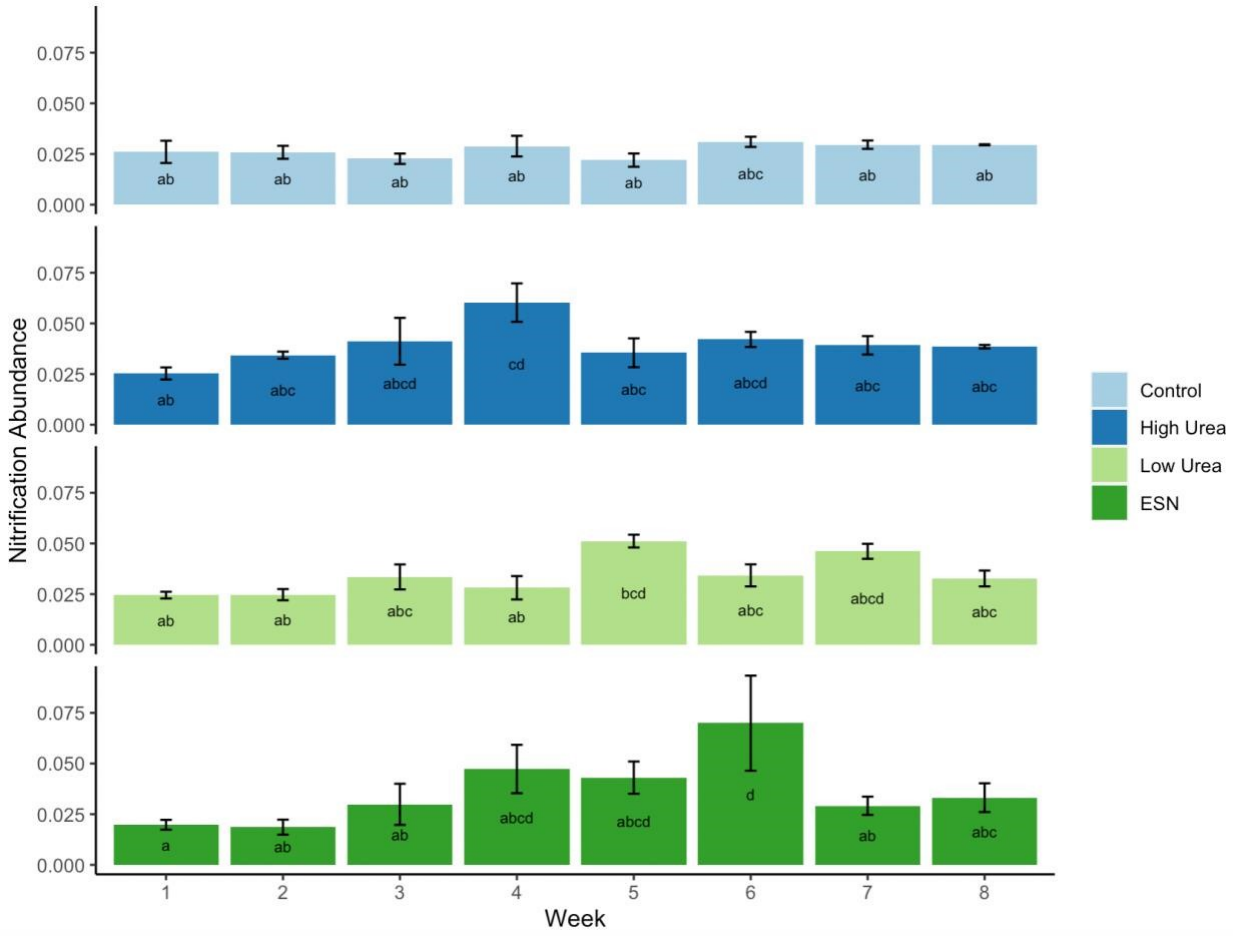

Figure 10. Nitrifying bacterial community total relative abundance analysis. Data are the mean values of each treatment per week.

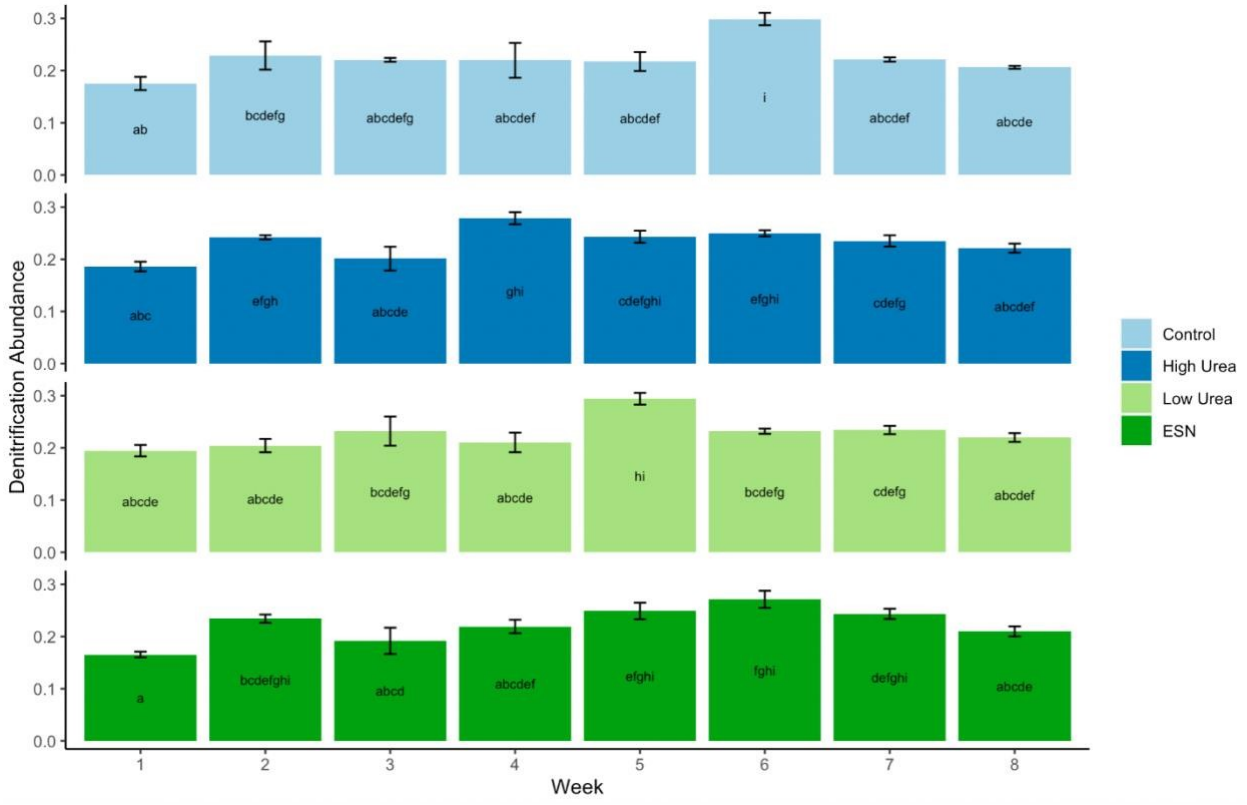

Figure 11. Denitrifying bacterial community total relative abundance analysis. Data are the mean values of each treatment per week.

Discussion

The goal for this study was to determine the effects ESN and urea on plant development and rhizosphere microbiome assembly to understand implications of different nutrient management strategies in a controlled environment.

We observed that at equal rates of N fertilization, plants accumulated greater biomass when fertilized with ESN compared to urea. We suspect that a continuous release of N with ESN supported the plant's consistent increase in biomass accumulation and led to higher overall biomass at the end of 8 weeks. In line with our findings, past studies have shown that controlled

release fertilizers can promote a steady supply of available soil N, thus leading to higher crop biomass and yields (Shoji et al. 2001, Carson and Ozores-Hampton 2014, Huang et al. 2016, Lawrencia et al. 2021). The current study suggests that applications of controlled release fertilizers can optimize biomass compared to urea when applied in similar rates. In our study, significant differences between fertilizer types began to occur at the fourth week of development. We observed a significantly higher total plant biomass in the ESN rate (1 g) compared to urea (1g) at the final stage of the study. This study helps further the notion that applying a controlled release fertilizer instead of a soluble urea fertilizer could benefit a plant's vegetative development.

Since there was a positive correlation with the higher N uptake of ESN and higher yields from week 4 to 8 (r^2= 0.99; $p<0.01$) and all N fertilizer treatments increased the tomato plant total N uptake and tomato shoot yields by week eight ($p<0.05$), we suggest that the reason that there were no differences in N uptake and yield between the 0.5 gram urea treatment and 1 gram urea treatment is that there was toxicity due to the high availability and uptake of urea-N with the 1 gram treatment shown by week five ($p<0.05$). The higher NUE of the ESN of 55% by week eight, which was significantly higher and almost double that of the NUE of high urea treatment (31%), also supports that there may have been a potential negative effect due to the higher N application rate to the tomato plants ($p<0.05$). This negative response was also seen by Samuel and Delgado (2004) and Delgado et al. (2023) in the overapplication of N on potato, the plants were negatively affected, and their tuber yields were found to be reduced.

The rhizosphere zone is an area that is inhabited by a diverse community of microorganisms. Many of these bacteria can benefit crops by aiding in nutrient acquisition from the soil (Ling et al. 2022). A profound relationship between the rhizosphere microbiome and plant functionality can be attributed to strong niche selection from the plant (Pérez-Jaramillo et al. 2016,

Schmidt et al. 2016). Hence, gaining more understanding of how fertilizer applications alter the formation of rhizosphere communities regarding crop development, could contribute to the optimization of agricultural practices, specifically nutrient management strategies (Canto et al. 2020). There has been considerable research investigating how long-term fertilization impacts a soil's microbiome, however, understanding the seedling to vegetative stage following fertilizer application is not well understood (Ai et al. 2015, Ding et al. 2019, Soman et al. 2020). Recent investigations have studied how a plant's developmental stages play a role in shaping rhizosphere microbiome structure, but further research looking into these stages could benefit NUE strategies by understanding at which stages in a crop's life cycle fertilization should be applied (Chaparro et al. 2014, Hou et al. 2013, Peiffer et al. 2013). While plants have specific nutrient requirements and are easily affected by the application of N or the lack thereof, microorganisms have other means to meet their N needs (e.g., through N fixation, mineralization) and so their response to N fertilization is less predictable.

Biological N fixation is predominantly accomplished by free-living or symbiotic bacteria groups. *Rhizobia* is well understood to establish a mutualistic association with legume species, capable of fixing N from the atmosphere to be used by both the bacteria and the host plant (Herridge et al. 2008). A field rice cropping study, a sequenced analysis of bacterial communities with applied N fertilizer showed that the relative abundance of OTUs in the genera *Bradyrhizobium*, *Methylosinus* and *Burkholderia* were higher in the rhizosphere microbiome that received a lower rate of N fertilizer than a standard ratel of N fertilizer (Ikeda et al. 2010). Our study indicated no difference in total abundance of N fixing bacteria species in the control compared to our fertilized treatments. The soil used in this study comes from a low N corn producing agricultural field and this could contribute to our understanding of the lack in differences between N fixing genes found in our analysis. This soil that we used in our study could

have had a well-established microbiome by which N fixing bacteria did not respond to with our fertilizer amendments or the tomato plant growth. We did find that N fixing bacteria species were significantly higher in the earliest stage and later stages of development. This could be indicative of microbial signaling for the need of N acquisition.

Regarding the relative abundance for genes functioning in nitrification and denitrification processes, we detected significantly higher amounts of these species in our fertilizer treatment compared to the control. Weekly, the relative abundance for nitrifying and denitrifying genes follows a trend at which point they peak within the fertilized treatments. This trend begins at week 4 with the urea (1g) showing their highest abundance of nitrifying and denitrifying genes, due to the rapid cycling of available applied N. Following at week 5, the urea (0.5g) showed their highest abundance of the nitrifying and denitrifying genes. And lastly, week 6 the ESN (1g) showed their highest abundance of nitrifying and denitrifying genes, the delay in this peak we observed could be due to a threshold by which nutrient cycling bacteria respond to.

Conclusion

Our results demonstrate that the use of ESN improved plant growth and NUE compared to urea. A shift in the rhizosphere microbial community composition was detected around the middle stage of our experiment, indicating a host selected shift in the microbiome. While the N cycling bacteria associated with fertilized treatments did not differ from each other, it was found that the urea treatments caused peaks in abundance sooner than the ESN treatment. Taken together, the results of this study can inform that the use of ESN will provide sufficient N for plants to develop and promote higher NUE the use of ESN instead of urea can benefit plant performance and promote higher NUE. Furthermore, this study adds to the knowledge that the rhizosphere microbiome differentiates as a plant develops and could be indicative that fertilizer use is adding

to the community change as well. Further research on how nitrogen applications alter rhizosphere microbiome assembly and development could add to this.

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