Agricultural output of crops with high nutrient demand (e.g., maize) requires soils to be amended with nitrogen fertilizers for economical production. Following the green revolution and development of the ability to synthesize mineral fertilizers, agricultural output has increased significantly to feed the growth of the human population. This increase in production is paralleled by increased mineral fertilizer usage and agricultural output, with environmental side effects. Soluble mineral fertilizers are prone to leaching and losses to water ways, where they contribute to eutrophication and degraded water quality. Additionally, fossil fuel use in the production of fertilizers contributes to increases in atmospheric greenhouse gases. To cope with the demands of and increasing human population, agricultural production must become both more sustainable and more efficient to minimize impacts on the environment.

Soils under crop production in agricultural ecosystems are a source of nitrogen pollution in surface and groundwaters, the use of highly mobile synthetic fertilizer exacerbates this issue. Nitrogen fertilization with organic amendments is an alternative to synthetic fertilizers with a wide range of potential soil health benefits from the additional organic matter. Nitrogen bearing compounds from plant litter, microbial cells and animal wastes are the primary input of organic nitrogen in agroecosystems utilizing organic amendments. Plants require mineral nitrogen for growth and cycling nitrogen between organic and mineral forms is a crucial process to understand for efficient use of compost, manures and green manures. The soil nitrogen cycle from organic to mineral/inorganic N is at least partially mediated by the microbial community; however, little is known about the specific bacteriome dynamics in soils amended with organic nitrogen materials.  The rate at which mineral nitrogen is converted from organic forms is an important consideration for crop production and environmental quality, the timing between mineralization and nutrient uptake, dictates weather a nutrient is used by the crop or lost from the agroecosystem.

Organic matter in soils, particularly N-bearing compounds, must be depolymerized into smaller compounds that can be assimilated by microbes or acted upon by extracellular enzymes before assimilation. Depolymerization regulates N cycling and controls N entering the microbial pool where it can then be mineralized into plant available forms. Balancing the rate of depolymerization and mineralization of nitrogen from soil organic matter to closely match the needs of the growing plant will provide a more efficient supply of nutrients to plants while minimizing losses to the environment. This biologically mediated process has been associated with litter/amendment C: N ratios below 25:1. When C: N ratios of more than 25:1 are observed in litters and amendments, immobilization has been observed, which is the utilization of nitrogen compounds for microbial growth at the expense of plant available mineral nitrogen. Thus C: N ratios are a critical factor affecting the impact of organic fertilizers on levels of plant available nitrogen in the soil.

Improving our understanding of the biological players involved in nutrient release

could help improve agricultural production while still maximizing environmental benefits from complex amendments. A key knowledge gap for varying organic management strategies is understanding the soil nitrogen (N) pool and the role of associated microbial communities as drivers of N cycling. In this study, we study the impacts of amendments of both alfalfa and compost, provided at 3 equal rates of total nitrogen, to a soil but with differing C: N ratios chosen to result in immobilization (compost) or mineralization (alfalfa). We characterize both the chemical and microbial response to these amendments and hypothesize that specific microbial communities will respond to initial nitrogen and carbon availability and that this membership will be specific to varying amendments. We expect that these distinct early microbial responders will dominate soil microbial communities in response to organic amendments and will decrease in abundance through time. Our objective was to characterize these early responding microbial communities for various organic amendments and to identify potential microbial membership within organic amendments that may be involved in plant nutrient availability.

########### Materials and Methods ###########

Three organic nitrogen amendments plus control were chosen based on current use in agriculture and predicted effects on soil nitrogen cycling: (1) an amendment of alfalfa residue with low C: N ratio, simulating plow down of alfalfa hay as a nitrogen source before maize production; (2) stable composted horse manure with a high C: N ratio used as an amendment replicating the use of composted manure on many organic farms; (3) an amendment consisting of a mixture of alfalfa residue and compost was constructed to represent a neutral C: N ratio; and (4) a control treatment receiving no amendment included in the study as a reference.

Alfalfa hay samples were collected following hay harvest and processed by passing fresh hay through a grinder and then through a 2mm mesh screened cyclone mill. Dry alfalfa was then stored in an air-tight vessel prior to use as amendment. Compost samples were collected on September 23rd 2015 from a large windrow of composted horse manure and saw dust bedding, the mixture was composted by the ISU Compost Facility, located at 52274 260th St. Ames, Iowa. Compost was dried, processed, and stored in the same manner as the alfalfa amendment. Soil originated from the USDA-ARS Organic Water Quality (OWQ) research site, situated near Boone, Iowa; on the ISU Agronomy Research Farm. Surface soil (0-15 cm) was taken from alfalfa plots that were in a four-year corn- soybean-oat/alfalfa/alfalfa rotation under organic management. The site was located on the Clarion-Nicollet-Webster soil association with fine-loamy texture soils. A total of 25 kg of soil was taken and processed through a 2 mm sieve, allowed to air-dry and stored in air-tight vessels before use.

Microcosms were constructed by mixing soil and each amendment treatment. Each microcosm consisted of 50g of air dried 2mm soil plus amendment, applied at a rate of 134.55 kg/ha of total nitrogen. The amended and control soil samples were incubated for 97 days under aerobic conditions at 300C in 3.79 l glass jars. During the incubation, samples were aerated every 24 hours by removing the lid and kept moist via addition of deionized water to the bottom of the 1-gallon jar. Soil samples were wetted to 60% water-filled pore capacity and placed into the incubator for a pre-incubation at 300 C before analysis of the first samples on day seven. Samples were subsequently analyzed on day 14, 21, 35, 45, and 97 yielding 336 incubated samples for the four treatments with n = 12 for replicates. Samples representative of Day 0 conditions were constructed by extracting DNA from un- wetted soils mixed in the same ratios as incubated samples.

The bacterial community was characterized by phylotyping using DNA sequencing. Microcosm soils were homogenized during destructive sampling and a sub sample of soil was frozen immediately using dry ice, for preservation until extraction could be performed. DNA extraction was performed using the HTP 96 well power soil kit from Qiagen using 0.25 g of soil. Following extraction, 16S rRNA genes were sequenced on an Illumina MiSeq using 16S V4 primers at Argonne National Lab in Lemont, Illinois. Mothur version 1.41.0 pipeline was used for sequence processing of the 151 bp paired end reads and operational taxonomic units (OTUs) were defined based on 97% genomic similarity. Taxonomic assignment of OTUs was completed by alignment to the most similar representative gene in the Silva 16S ribosomal database version 123.

Microbial biomass was quantified using moist microcosm soil subsampled during destruction of microcosms on sampling days. Microbial biomass carbon was calculated and measured using standard soil fumigation-extraction methods modified for a 20 g sample. Briefly, two sub-samples of 20 g of moist soil from each microcosm were weighed into 50 ml beakers and 125 ml bottles. The 125 ml bottles with 20 g of soil were extracted with 0.5 M K2SO4 and carbon was quantified. This soil fraction represents the non-fumigated portion of the microbial biomass. The 50 ml beakers with 20 g of soil were placed into a fumigation chamber and fumigated with chloroform overnight and extracted after 24 hours with 0.5 M K2SO4. This soil fraction represents the fumigated portion of microbial biomass carbon. Dissolved organic carbon in the filtrate was determined using flow injection technology using a Torch TOC Combustion analyzer (Teledyne Tekmar, Mason, Ohio) and carbon associated with microbial biomass was calculated using the correction factor (k=0.33).

Inorganic nitrogen was determined by extraction with 2.0 M potassium chloride from homogenized moist microcosm soil. Concentrations of NO3 and NH4 were quantified in the filtrate using Lachat Instruments flow injection analyzer (Lachat Instruments, Milwaukee, WI).

Total carbon and nitrogen in microcosm soils was determined using dry combustion analysis of 2 g of air-dry, soil ground with mortar and pestle. Dry homogenized soil was combusted using Thermo Scientific FLASH Elemental Analyzer (Thermo Fisher Scientific, Waltham, MA). The pH of soils was measured potentiometric ally in a 2:1 soil-to-water slurry using a dual electrode pH meter. Soil water content was determined gravimetrically with overnight drying at 105°C.

Dissimilarities in the composition of sample bacterial communities were visualized by NMDS ordination of the Bray-Curtis distances between samples. Samples included both the microcosm inputs (i.e., the organic amendments) and the incubated microcosms (i.e., amended soils). NMDS ordinations were performed using the metaMDS() function from the Vegan package in R and were visualized with the plot\_ordination() function from the Phyloseq package. To test if the composition of OTUs was different in samples, we used the adonis() function from the vegan package in R. Adonis() uses a non-parametric multivariate analysis of variance method to test the null hypothesis that there are no differences in microbial communities.

To characterize the impact that environmental variables had on the dissimilarities of communities in each treatment we performed CAP (Canonical Analysis of Principal coordinates) analysis of the environmental variables of inorganic nitrogen, microbial biomass and C: N ratio in conjunction with the Bray-Curtis distance matrix. CAP seeks to display ordinations along with explanatory variables to reduce dimensionality and can reveal patterns in multivariate data with reference to a priori hypotheses.

All environmental variables were fit to a linear mixed effects model using the function lme() from the nlme package in R. Treatment and day were set as fixed effects in the linear model and means were estimated using the emmeans() function from the emmeans package in R. Comparisons were performed between each treatment by each day (i.e., treatment x day). ANOVA was performed on the results from the linear model. A post-hoc test for the ANOVA analysis was performed Tukeys HSD and are reported in a table found in the supplementary data.

###Results###

The response of soil microbial communities to the varying amendments were

characterized throughout the incubation. First, the characteristics (microbial community

structure and nutrient composition) were measured for all three amendment inputs. The

microbial community of each amendment was characterized through sequencing of 16S

rRNA genes, a phylogenetic marker conserved among bacteria. The resulting phylogenetic

profile was identified for each amendment, showing distinct communities for alfalfa and

compost amendments. The distribution of phyla from OTUs with abundance

greater than 2% in the alfalfa amendment is dominated by unclassified bacteria (sharing no

homology to any known phyla) and Proteobacteria, while the compost amendment is

dominated by unclassified bacteria, Actinobacteria, and Firmicutes. These amendments are

also distinct from the incubation soil used in this study, which is primarily dominated by

Acidobacteria, Actinobacteria, and Proteobacteria.

In addition to differences in the microbial communities of the inputs, we also

observed differences in the amounts of inorganic N and C: N ratio in the inputs. The alfalfa amendment was highest in inorganic nitrogen concentration at 25.53 ppm and lowest in carbon to nitrogen ratio with 20.14 parts carbon to one-part nitrogen. The compost amendment was lowest in inorganic nitrogen at 13.91 ppm and had the highest C: N ratio at

28.97. It has been previously reported that a C: N ratio below 25:1 in an input will result in

net mineralization while greater than 25:1 will result in net immobilization. Consequently,

the alfalfa amendment is expected to result in nitrogen mineralization, and a net increase in

inorganic nitrogen compared to reference soils, while the compost amendment is expected to result in nitrogen immobilization and a net decrease in inorganic nitrogen compared to

reference soils.

Inorganic nitrogen concentrations of inorganic nitrogen estimate the amount of potential plant available nitrogen (N). Generally, inorganic N is considered to be the sum of NO3 and NH4 concentrations in soils. In our experiment, the level of inorganic N in the starting soils were similar and increased over time, with Alfalfa soils had significantly higher inorganic N concentrations on days 14 to 97 than other treatments, and by day 97 had 2-fold

greater inorganic N compared to reference soils. Mixed soils initially were observed with

lower concentrations of inorganic N but increased to levels comparable to reference soils by

day 97. Compost amendments resulted in a significantly lower concentration on all days of

the incubation compared to the reference soil and had inorganic N concentrations less than 1

ppm on days 21, 35, and 45.

Microbial biomass provides an indication of the size of the active pool of soil organic

matter and increases in microbial biomass indicate microbial utilization of nutrients for

metabolic growth and is correlated with nutrient availability. All amended microcosms

yielded significantly higher MBC levels than reference control soils . Alfalfa had the most significant positive difference, with a two-three fold higher MBC than reference and achieving the most MBC within the experiment 780 mg C/kg dry wt. soil on day 14. Reference soils nearly doubled in microbial biomass on day 21 before declining to baseline concentrations on day 97. Mix amended soils had consistently decreasing MBC levels throughout the experiment.

To discern the impact of treatment and time on soil bacterial community composition,

we compared the Bray-Curtis dissimilarity indices between all bacterial communities

associated with each microcosm. Non-metric multidimensional scaling (NMDS) ordinations

of the resulting distances are shown for each treatment and day of soil sampling. In the ordinations, points (representing individual samples) that are close together

share similar species composition, while points further apart have different species

compositions. These results reveal that the microbial community in reference soils are

dissimilar from amended soils, with compost-amended soils being the most similar in

composition to reference soils. The alfalfa and mixed amendments had community compositions that were the most comparable throughout the incubation. The vertical spread

of samples along axis 1 suggests that amendment type explains the dissimilarity between

treatments, while the clustering of samples by day on the horizontal axis shows that time in

incubation also explains variation. Generally, day 7 communities were observed to be the

most dissimilar to day 97 communities and are most similar to day 14 and day 21

communities. As the incubation progressed, the dissimilarity between sampling times

became more pronounced regardless of treatment. Further, the first three sampling points in

time showed the most similar communities, with increasingly different communities after day

21.

To better understand the influence of the various environmental variables that were

measured on the microbial community, canonical analysis of principle coordinates was17

performed on the Bray-Curtis distance matrix, and environmental variable scores were fitted

to this ordination. We observed that measurements of C: N ratios and inorganic N concentrations were correlated to community variation along an axis, CAP1 (up to 20%

variance explained in mixed alfalfa microcosms). In addition, alfalfa-amended soils had

MBC also correlated with this axis. The second CAP axis (CAP2) had weak associations18

with pH and MBC in all treatments except alfalfa. However, CAP2 was much lower in

percentage explained, between 3.1% to 5.1% for all amendments.

To understand the microbial communities that are early responders to amendment, we

performed hierarchical clustering of the binary Bray-Curtis distance for each treatment.

These results were used to guide the definition of “early responders” to amendments. For

each amendment, bacterial communities from day 0 samples clustered together, consistent

that these samples represent similar initial conditions. These Day 0 communities will

henceforth be referred to as the baseline response group. For all amendments, communities

from sample days 7, 14, 21 cluster as early responders and 35, 49, 97 cluster as the late

responders.

For early and late response groups, the microbial communities that were unique for

each amendment was characterized. We identified specific microbial communities that

were observed to be significantly different in amendment treatments compared to reference

control soils. Significantly different communities were defined as those with a log 2-fold

relative abundance increases between amendment versus no amendment control. This19

resulted in the identification of 25 and 21 OTUs specific to alfalfa and 43 and 71 specific to

compost in the early and late response groups, respectively. The presence of these

amendment-specific early responding OTUs was next compared across all treatments

(Supplementary data).

We observed the phylogenetic distribution of OTU to be different across response

group and treatment for both alfalfa and compost amended microcosms. The 25 OTUs

unique to alfalfa in the early response group were dominated by OTUs from the

Proteobacteria and Firmicutes. The late response group in alfalfa was dominated by OTUs

from Proteobacteria and unclassified bacteria, Firmicutes were not observed in the late

response group. The compost response groups were also dominated by Proteobacteria in

both early and late, while Bacteroidetes made up a greater portion of the early responders

than late in the compost. Unclassified bacteria were a large percentage of both response

groups in the compost treated soil.