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ABSTRACT

Dairy farms produce vast amounts of manure, which can be stored in anaerobic lagoons, facilitating the production of potent greenhouse gasses. Manure can be diverted to the field for use as fertilizer to aid in forage crop production. The viability of this practice depends on the breakdown of organic matter, making it available to the plant and soil microbial community. Microorganisms rest at the heart of this process, and assessments of how their structure and function changes throughout cropping cycles remain limited. In this thesis research, a field trial was established using three rates of manure application: high (100% of plant nitrogen stemmed from manure), low (50% plant nitrogen was provided by manure and 50% from chemical fertilizer), and zero (100% plant nitrogen came from chemical fertilizer) to grow forage crops over two years. Plots were sampled regularly, resulting in 22 sampling dates spanning agricultural milestones such as fertilizer application, plant growing season, and harvest. Here I found manure changes the soil microbial community, increasing microbial diversity, microbial biomass-carbon (MBC) and dissolved organic carbon (DOC) following application. Furthermore, manure associated microbial groups appear to be most responsive to application, increasing following application before diminishing throughout the growing season. Manure application also improves carbon use efficiency (CUE) and stable isotope probing (SIP) revealed microbial carbon incorporators temporarily increase following application before diminishing in the growing season. Assessment of functional genes in manure applied vs. zero manure soils revealed a greater abundance of genes involved in carbon metabolism and degradation in manure soils. Together, effective manure management can play a profound role in improving the structure and function of the soil microbial community, curating a more robust and efficient carbon cycle.

INTRODUCTION

Studying microbial communities in soil environments

Soil harbors a wide range of life, spanning the macroscopic to microscopic realms. Recent insights into microscopic organisms living in the soil have been largely facilitated by the widespread adoption of high-throughput sequencing (HTS). These sequencing techniques facilitate the characterization of millions of DNA sequences extracted from the soil environment. These sequencing techniques grant insight into previously inaccessible world of soil microorganisms by assessing the variation on the 16S rRNA gene and mapping it back to databases (Caporaso et al., 2011). This is particularly useful because most microbial groups remain unculturable and therefore difficult to study (Tedersoo et al., 2021). This has led to a surge of interest in the field of soil microbial ecology and the development of new bioinformatic pipelines like DADA2 and QIIME2 that facilitate the curation of massive amounts of DNA data to databases, allowing assessment of the diversity and composition of the soil microbial community (Marizzoni et al., 2020). Together these insights have shaped the way we understand soil microbial communities and their role in ecosystem functions.

Advances in analytic capabilities have reinforced and expanded our understanding that soil microbial communities vary between soil environments. This variability was notably explained by variances in soil pH between ecosystem types, suggesting local soil conditions contribute largely to their structure (Fierer and Jackson, 2006). Furthermore, microbial communities are also known to vary across time and land use, suggesting that generalizations on trends in microbial community structure across systems are particularly difficult to make (Lauber et al., 2013). Localized studies therefore represent the best way to determine how each specific environment relates to their underlying soil microbial communities (Mony et al., 2020). In an agricultural environment, practices such as tillage and fertilizer application have a profound effect on the structure of the underlying soil microbial community (Srour et al., 2020). Systems utilizing no-tillage and organic fertilizer amendment practices demonstrate improved microbial diversity (Drijber et al., 2000; Wang et al., 2017; Zhong et al., 2010). Determining how these trends persist over time and between agricultural systems presents the next step in assessing the dynamics of the soil microbial community in managed land.

Soil microbial communities in agricultural systems

In agricultural systems, we have direct control over the management of vast amounts of soil. Therefore, understanding the dynamics of the soil microbial community in these systems is exceptionally important because they drive soil conditions and ecosystem functionality. In an organic agricultural system, HTS revealed that organic farming systems have improved plant growth and decomposition and that microorganisms rest at the heart of this dynamic (Bonanomi et al., 2016). Soil microbial communities facilitate these useful ecosystem processes by breaking down and recycling soil organic matter into biomass, often with enzymes such involved in the processing of carbon containing compounds (Fernandez et al., 2016). This process feeds not only the soil microbial community, but also the surrounding plants because many of their nutrients are derived from decomposing organic matter. Therefore, understanding the soil microbial community is critical in predictions of plant health and productivity (Andreote and Pereira e Silva, 2017). For example, anaerobic stress was found to play a critical role in shaping the production of microbial volatiles that can suppress soil-borne diseases. Understanding precisely how the underlying structure of the microbial community changes, and how we can improve soil microbial diversity through management therefore has implications related to the proliferation of diseases (Van Agtmaal et al., 2015). As such, clarifying the relationship between the soil microbial communities and ecosystem function can help us curate healthier soils.

In a global analysis of long-term fertilization, it was reported that nitrogen fertilization increased the relative abundance of some of the most common microorganisms in soil, *Actinobacteria* and *Proteobacteria*. These microbial groups are often implicated in their ability to degrade complex organic compounds, which suggests management decisions can have profound impacts on the cycling of nutrients in soil systems (Dai et al., 2018). Furthermore, *Actinobacteria* and *Proteobacteria* are broadly classified as copiotrophic taxa who thrive in nutrient rich environments and with faster growth rates than other microorganisms. This relationship between higher nutrient loads and specific microbial groups was also found to extend to a greater catabolic capability, effectively linking the structure and function of soil microbial communities (Fierer et al., 2012). Overall, management decisions in agricultural environments can drive changes to the underlying soil microbial community and these changes drive soil functional processes.

Microbial role in the agricultural soil carbon cycle

The soil microbial community directly controls the way carbon enters and exits the soil environment. Extracellular enzymes released by microorganisms aid in the breakdown of organic compounds, increasing the availability of carbon to other organisms who with respire it as CO_2 or CH_4 or incorporate into their cells and then into soil organic matter (Schimel and Schaeffer, 2012). Through effective management we can maximize this effect and mitigate carbon accumulation in the atmosphere (Dungait et al., 2012). In this respect, soils can be made more sustainable by promoting the sequestration of carbon, curating a diverse microbial community capable of providing beneficial ecosystem services such as improved plant growth, while also mitigating greenhouses gas emissions that contribute to climate change. Alternatively, neglecting this relationship can lead to a positive feedback loop wherein warmer global temperatures can increase the magnitude of carbon lost from soils as greenhouse gasses, leading to warmer conditions and further release (Karhu et al., 2014).

The concept of microbial CUE describes the ratio of carbon allocated to growth relative to other microbial processes (Del Giorgio and Cole, 1998). A higher CUE suggests an increase in belowground carbon storage, which means carbon losses to the atmosphere or surrounding systems are reduced. Conversely, a lower CUE means more carbon escaped the soil environment, often as CO_2, and less carbon is maintained in the terrestrial system. Insights into this relationship have been brought about by using isotopic analysis, where radio labeled isotopes can be tracked through the soil environment. In a study that measured CUE and its relationship to microbial diversity in a model soil, it was found that microbial community composition and diversity were the strongest predictors of CUE. In this case, abiotic factors such as moisture contributed strongly to this relationship, with CUE being highest in high moisture soils (Domeignoz-Horta et al., 2020). Understanding specifically how CUE is modified across environmental conditions as well as biotic and abiotic factors could inform management practices that maximize CUE across time (Adingo et al., 2021).

Determining how the soil microbial community changes with respect to management and following their repeated patterns across growing seasons can present a clearer picture of how the agroecosystem functions at different stages of the season. When the functional capacity of the microbial community in agricultural soils was assessed throughout an entire year, patterns emerged with respect to management practices. Notably, genes related to the nitrogen cycle were more abundant following fertilization while many others were nonresponsive to agricultural milestones (Orellana et al., 2018). In a separate study, tillage events regularly increased the amount fast growing competitors in the soil microbial community, while systems utilizing no-till garnered more slow growing stress tolerant microorganisms. Furthermore, the presence of cover crops was found to shift the microbial community to ruderals that have a wide range of metabolic capacities, but only moderate growth (Schmidt et al., 2018). Together, these findings suggest certain land management decisions can drive predictable trends in the soil microbial community.

Organic soil amendments represent one of the most profound ways that management can alter the soil microbial community. Compared to mineral fertilizer, organic amendments have been demonstrated to increase bacterial diversity and stimulate the growth of microbial groups such as *Firmicutes* and *Proteobacteria* that prefer environments rich in nutrients (Francioli et al., 2016). Popular organic amendments can range from various forms of municipal waste, composts, crop residues and animal manures. Of note, compost can be derived from a wide range of sources including the other forms of organic material (Ansari et al., 2019). Compost is amongst the most favored of these organic amendments because it is known to harbor fewer pathogens, providing better food safety than other forms of organic amendment. However, composting has been shown to reduce the extent that other organic amendments such as animal manure, can modify the underlying soil microbial community. Non-composted manure had a stronger effect than composted manure on increasing the abundance of microorganisms capable of organic matter degradation and more profoundly altered overall community composition (Ouyang et al., 2022).

The dairy "poop loop" and the role of manure application in shaping soil microbial communities

Manure serves as an attractive soil amendment due to the logistical ease of its transport and application. Dairy farms are often near agricultural land, and they produce a large amount of organic waste, because dairy cows must maintain high milk production in commercially viable dairy farms (Van de Haar and St-Pierre, 2006). Estimates of manure production suggest a single dairy cow producing commercially viable yields of milk can produce more than 54 kg manure per day, compounded by hundreds to thousands of cows per dairy farm (Tauer and Mishra, 2006; Van Horn et al., 1994). This massive amount of manure is diverted around the farm, where it can end up in the coral, field, or nearby lagoons. Of these locations, lagoons represent the largest contributor to greenhouse gas emissions from the dairy farm. These lagoons, provide an ideal mixture of anaerobic conditions and vast amounts of carbon and nutrients from manure and urine that facilitate the growth of methane-producing Archaea (Owen and Silver, 2015). Thusly, minimizing the amount of manure diverted for storage in lagoons by using it as a soil amendment can reduce the harmful effects of this waste management practice (Castanheira et al., 2010).

Amendments of manure to the soil environment can change the underlying soil microbial community by serving as a food source for the microbial community (Kumar et al., 2013). Manure itself is rich in nutrients, essentially being comprised of organic matter from partially digested plant material (Zubair et al., 2020). The organic matter in manure is initially broken down with added nitrogen and carbon containing compounds used for growth. Much of the research on manure impacts on microbial communities have been conducted in laboratory scale microcosm experiments (Dong et al., 2020; Han et al., 2018; Pérez-Valera et al., 2022; Wang et al., 2018) and do not include plant growth and more realistic environmental conditions. Plants grow throughout the season and increasingly utilize soil nutrients that could otherwise be used by the soil microbial community (Kumar et al., 2013; Mohammadi et al., 2011). Assessing the microbial community across growing seasons could help us more accurately predict how soil microorganisms respond to seasonal factors such a plant growth in active copping systems.

Manure application can also modify the soil microbial community by the direct addition of microorganisms from manure to the soil. A microcosm study examining the role of poultry manure-compost in shaping the microbial communities in soils with and without a history of manure application clarified this trend. In this instance, soil without a history of application was more susceptible to invasion of manure derived taxa, suggesting that manure application has diminishing returns on its import of manure microorganism to the soil environment (Gravuer and Scow, 2021). In another microcosm study, examining the proliferation of antibiotic resistance genes (ARGs) from manure to the soil environment found that manure-borne microorganisms persist in the soil environment and engage in horizontal gene transfer with indigenous soil microorganisms. This effect was most pronounced following manure addition, and it diminished over time as indigenous soil microorganisms prevented the complete establishment of ARG containing microorganisms (Chen et al., 2017). Understanding the extent the manure microbial community invades and persists in an agricultural system over longer timescales, following and between manure application events, will add clarity to the microbial dynamics underlying these systems.

Agricultural practices utilizing high quality inputs of organic carbon such as manure (relatively low C:N) likely facilitate a greater CUE (Sinsabaugh et al., 2013). This occurs through the increased capacity to accumulate of microbial biomass and through the conversion of organic carbon to forms that are not easily accessible for respiration (Kallenbach et al., 2019).

This effect is seen across agricultural systems and environments, where manure application predictably increases soil organic carbon (Gross and Glaser, 2021). In a study examining the CUE between agricultural systems utilizing organic carbon applications vs. those using conventional mineral fertilizers, it was determined that CUE was higher in organic systems and higher amounts of soil organic carbon were correlated with increasing CUE. Additionally, organic systems had a greater abundance of soil organic matter that was derived from the soil microbial community. Organic carbon application may therefore facilitate an increased CUE through providing a robust supply of nutrients that sustains a microbial community more capable of converting those nutrients to forms of carbon that stay trapped in the soil environment (Kallenbach et al., 2015). Together, effective manure management can minimize microbial processes that convert organic carbon to potent greenhouse gases such as CH_4 and feed the soil microbial community creating a more robust belowground reservoir of carbon. Understanding the ways in which manure application changes the structure of the soil microbial community, therefore provides clarity in predicting in how management practices can be used to drive increases to CUE in agricultural systems.

Assessing the functional role of soil microbial communities in manure applied systems

Though manure application might improve CUE, the degree in which this effect occurs throughout and between conventional cropping cycles is not well known. Methodologies designed to assess CUE utilize laboratory microcosms, which indicate a potential carbon dynamic at the time soil was collected and returned to the lab (Domeignoz-Horta et al., 2020; Geyer et al., 2019). Soil microorganisms fluctuate between dormancy and feeding depending on the availability of soil nutrients. In dairy farm forage systems manure is applied at the beginning of each cropping cycle. Broadly the CUE of a given soil environment will therefore depend on the abundance of active soil microorganisms and their role in driving CUE in manure applied systems is dependent on the timing of application events (Joergensen and Wichern, 2018). In a study examining CUE at three timepoints in a grassland receiving mineral fertilizer, it was observed that sampling date was the most significant factor explaining variation in microbial growth and respiration. The authors suggest the microbial community is the likely culprit in driving these dynamics and future research should focus on microbial growth across seasons to understand the carbon dynamics of soil systems (Simon et al., 2020). Therefore, a method that can identify the microbial groups most responsible for driving changes in CUE could be paired with conventional assessment of the soil microbial community to determine how those groups change over time. Doing so could deepen our understanding of the relationship between the soil microbial community, manure amendment, and the carbon cycle.

The method of SIP allows for determination of microbial groups most responsible for processing and incorporating various forms of carbon substrates. The technique of SIP utilizes the same radio labeled isotopes permitting paired assessment of SIP and CUE in concurrent experiments. Notably, SIP is being used to determine how the microbial community contributes to the soil carbon cycle across the globe by identifying the groups most responsible for incorporating various radiolabeled carbon substrates (Barnett et al., 2021). As such, the dynamics of microbial groups identified by SIP as carbon incorporators may then be explored in microbial datasets to determine the potential changes in the carbon cycle in a variety of systems (Youngblut et al., 2018).

Ultimately, assessments of microbial taxonomic groups can be used to determine how the structure of a microbial community changes with respect to manure application, but they provide limited information on the changing functional capacity of the microbial communities. Studying a soil system's metagenome by sequencing and determining the functional genes present in the totality of extractable DNA in a soil can therefore provide us with a clearer image in how a soil's functional capacity responds to manure application (Jansson and Hofmockel, 2018). In a recent meta-analysis assessing the differences in functional diversity between organic and conventional systems it was found that functional diversity was greater in organic systems than those using mineral fertilizer (Bebber and Richards, 2022). However, this meta-analysis used studies who assessed the functional diversity of the soil microbiological community by using Biolog^{TM} Ecoplates and therefore only captured trends in functional genes placed on the chip (Checcucci et al., 2021). To date, there has been no description of how functional genes change in soil following manure application in a field receiving regular manure inputs.

Dissertation research

This dissertation research expands upon the body of knowledge surrounding soil organic amendments and how they drive changes to the soil microbial community, shaping the carbon cycle. This research is conducted in a forage cropping field trial and aims to: 1.) determine how repeated manure inputs to a forage production soil increase MBC and diversity and select for lower abundance genera, and 2.) identify microbial carbon incorporators and determines the metabolic capacity of the microbial community in manure applied soils with a higher CUE.

Chapter 1 uses a field scale experiment where forage crops typical for feed on a dairy farm were grown. This field was designed to assess how manure application changes the microbial community over a 2-year period. This field was divided into plots received high (2.24

kg/m^2, n = 4), low (1.12 kg/m^2, n=4), or zero (n=4) rates of solid dairy manure amendment at the start of each growing season. Soil was collected from this field 22 times over the span of 665 days where it was then assessed for changes in microbial community structure by 16S rRNA analysis. Sampling times were chosen to span agricultural milestones such as fertilizer application, the growing season, and harvest. In doing so, I found that higher rates of manure application spurred increases in soil DOC, MBC and changed the microbial community composition while maintaining crop yields. Furthermore, I identified manure associated taxa that increase abundance following manure application, before diminishing throughout the growing season. Finally, I described how the relationships between the microbial community and physicochemical soil properties are modified more predictably by manure inputs than soils receiving zero manure input.

Chapter 2 utilizes soils collected from the same field scale experiment to determine if manure application increases CUE relative to soils that received zero manure. To build on this, SIP was used to identify the microbial groups or "incorporators" most responsible for processing added labelled carbon. The abundance of these incorporators was then measured over the course of the field experiment where they were found to increase in manure applied soils, but not in those that received zero manure, most notably following manure application. Using a metagenomics approach, I found a larger increase in genes related to carbon metabolism in manure applied than zero manure soils, suggesting that the metabolic capacity of the soil microbial community was increased by manure application.

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Chapter 1: Repeated manure inputs to a forage production soil increase microbial biomass and diversity and select for lower abundance genera

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ABSTRACT

Adding manure to croplands restores carbon and nutrients in depleted soils, while addressing a waste disposal need. This practice depends on the presence and activity of microbial communities to break down manure inputs, which provide carbon for microbial growth and release nutrients for plant uptake. In a 2-year field study, we measured changes to soil physicochemical properties and microbial community composition in a conventional rotation of summer silage corn and winter wheat. Three ratios of manure to synthetic N fertilizer were used: high (100% of plant nitrogen stemmed from manure), low (50% plant nitrogen was provided by manure and 50% from chemical fertilizer), and zero where all plant nitrogen came from chemical fertilizer. Yields of corn and wheat were similar across treatments. Soil microbial community dissimilarity increased with higher amounts of manure, suggesting a dose dependent relationship between manure application and microbial community composition. Both microbial biomass-carbon (MBC) and dissolved organic carbon (DOC) showed the greatest response to increasing application rates, immediately following manure application, then decreasing until the next application. The Shannon index increased after a "ramp up" period following manure application, with the largest increase observed in soils with high rates of manure applied. More taxa known to be present in manure increased in high application rate soils. Microbial responses following manure application were transitory, indicating that without continual inputs of resources, changes to the microbial community following manure application are not maintained.

IMPORTANCE

Dairy farms are often adjacent to large fields where the vast amount of manure produced by dairy cows is applied. Nutrients from manure can feed crops, which can then feed cows, improving the nutrient use efficiency of the system. Soil microorganisms are critical to this process as they help break down manure into crop utilizable nutrients. We found that manure increased soil microbial diversity after each application, but the effects were temporary, while MBC increased additively with each round of application. Furthermore, microorganisms from manure itself appeared to increase most significantly following application, yet they were unable to persist without regular application. These findings contribute to more efficient management practices by providing insight into which members in the soil microbial community are most impacted as well as a timeline to those changes.

INTRODUCTION

Manure application can provide nutrients to the crops while simultaneously addressing a waste disposal problem by recycling nutrients into the production of forage crop and minimizing losses to the surrounding environment. The inputs of carbon and other nutrients from manure application greatly impact the underlying soil microbial community (Nicol et al., 2016) by increasing soil microbial diversity (Caban et al., 2018). In one such instance, a 30-year nutrient depletion trial was amended with animal slurry yearly for 20 years resulting in increased bacterial community diversity and microbial biomass compared to a mineral fertilizer control (Van der Bom et al., 2018). Similar findings were further recapitulated in a meta-analysis of 103 peer-reviewed publications where microbial biomass was found to be higher in manure applied systems relative to those receiving only chemical fertilizer. Furthermore, it was described that the longer the practice occurred, the larger the increases in microbial biomass (Ren et al., 2019).

Improvements to soil biodiversity, particularly that of microbial diversity has been demonstrated to promote functions in the soil ecosystem related to carbon degradation. Lower microbial diversity has been observed to modify decomposition towards more degradable carbon sources, thus showing a link between changes in microbial diversity to changes in the soil carbon cycle (Maron et al., 2018). Furthermore, animal manure provides readily available carbon, in addition to mineral nutrients, for soil microbes (Diacono and Montemurro, 2011). A commonly used measure of labile carbon associated with organic inputs is DOC of which a large portion is microbially available (Mentges et al., 2019). Overall, manure application may initiate a feedback loop where microorganisms can feed on the readily available organic material, increasing, and changing the composition of the microbial community, which can then facilitate the formation of soil organic matter (Cotrufo et al., 2015). Additionally, long-term applications of chemical fertilizer often decrease bacterial diversity, therefore utilizing alternative sources to meet crop nutrient demands can help maintain or increase soil microbial diversity (Sun et al., 2015).

Amendment of soil with manure can also be a source of new microorganisms, sometimes modifying the recipient microbial community (Chen et al., 2017). Recently a meta-analysis of 37 studies investigating the effects of organic vs mineral fertilizers on soil microbial diversity found that functional as well as taxonomic diversity is higher in organic systems using manure (Bebber and Richards, 2022). Added organisms can include pathogens and those carrying antimicrobial resistance genes (He et al., 2019); however, many are potentially beneficial. The abundance of copiotrophs and specific organotrophs in manure may stimulate the ability of the soil to metabolize a wide variety of carbon compounds (Toju et al., 2018; Wang et al., 2021) and potentially increase soil organic carbon (Chowdhury et al., 2021). A study examining the relationship between enzymes related to carbon compound degradation and manure application found that manure mixed into the soil induced a higher activity than soils with no manure applied showing that the capacity to degrade organic compounds likely increases following manure application (Chowdhury et al., 2021).

Other known benefits of manure amendments are on soil health properties such as moisture retention, soil aggregation, and nutrient cycling (Martínez-García et al., 2018; Reganold and Wachter, 2016; Wang et al., 2020), which, in turn, are mediated by microbial activity (Cobo et al., 2002). Models of soil carbon dynamics indicate that soil organic matter is created by inputs of carbon, metabolism by soil microbial communities, and physical protection of by-products (Grandy and Neff, 2008). Newer work clarifies these dynamics by suggesting soil organic matter can form early through microbial decomposition of organic matter, or later through physical movement into soil (Cotrufo et al., 2015). In integrated animal-forage systems, carbon enters soil as plant residue and root exudates and in the form of animal waste (Cai et al., 2019). Furthermore, addition of manure has the potential to add new microorganisms to soil or stimulate resident soil members by adding carbon and other nutrients (Mohammadi et al., 2011b). Finally, the act of applying manure itself is a form physical disruption as its incorporation often employs the use of tillage (Maguire et al., 2011). Together, manure application has the potential to impact the soil carbon cycle and soil organic matter formation through decomposition and physical integration into soil. Ultimately, the extent that soil physicochemical and microbiological characteristics are related with one another in manure applied soils informs how management can be utilized to improve the soil carbon cycle and mitigate unintended negative side effects.

The objectives of our study were to: 1) determine the relationship between manure application rate and soil DOC, MBC, microbial community composition, and crop yields; 2) identify manure-associated microbial taxa that change in abundance in soil following manure application; and 3) describe interactions between soil microbial communities and physical-chemical properties as related to manure inputs. Our field study was conducted in a conventionally managed corn-wheat forage rotation and measured 22 times over a two-year period.

MATERIAL AND METHODS

Experiment design and soil sampling

In August 2017, an experimental field on Rincon silty clay loam (pH 6.35-7.91) with an organic carbon stock of 5 kg/m^2 at 0-30cm depth was established at the University of California Davis Russell Ranch (Davis, CA) as corn-wheat rotation on a field previously used as fallow and divided into twelve experimental subplots (4.57 m by 36.57 m). Plots were established in summer for two full years consisting of four growing seasons. The crop rotation consisted of corn in summer (SC1), wheat in winter (WW1), corn in summer (SC2) and wheat again in winter (WW2). Manure was scraped from the corral of a local dairy farm located approximately 20 km from the field site and transported to the experimental location by truck. Plots received high (2.24 kg/m^2, n = 4), low (1.12 kg/m^2, n=4), or zero (n=4) rates of solid dairy manure amendment at the start of each growing season, where it was applied to the topsoil before being mixed in during bed construction. Application rates were determined based off assumed N availability to meet crop nutrient demands and crop yields were measured after each season to determine if rates were sufficient in maintaining growth and productivity. One treatment received 100% of its N from manure inputs (High), a second treatment received 50% of their N from manure and 50% N from chemical urea fertilizer (Low), and a third treatment consisted of 100% N with chemical urea fertilizer inputs (Zero) to meet crop demands.

Soil samples were collected using twist augers (7cm diameter for a 15 cm depth of sampling) on an approximate bi-monthly basis, with sampling dates designed to align with agricultural milestones such as tillage, planting, irrigation events, harvesting, and fertilizer application. In total, 22 sampling dates were recorded spanning the range of 665 days. For each sampling event, 10 soil cores per subplot were collected from bulk soil near the center of each subplot plot at randomly dispersed intervals 15m away from the edge of the field. Soil collected from each individual subplot was mixed in the field before transport back to the laboratory on ice, with a portion subsampled from each mixture and stored at -80° C to be used for DNA extraction and molecular analysis. In total, 12 representative samples with 4 corresponding to each rate of manure application (high, low, zero) were generated from each sampling event, corresponding to each of the 12 treatment subplots established at the start of the field trial. In addition, manure was collected from the manure spreader as it was applied to the field then returned to the laboratory and stored at the same conditions as soils samples.

DNA extraction, PCR amplification and sequence analysis

Total DNA was extracted from 0.25g of soils and 0.3g of manure using the DNeasy PowerLyzer PowerSoil kit (Qiagen Inc., Germantown, MD) following the manufacturer's protocol. Gel electrophoresis was used to assess quality of DNA after each extraction, to ensure minimal degradation. Yields were assessed with a Qubit 3 fluorometer (ThermoFisher, Waltham, MA) and extractions producing >15.0 ng/µL DNA were used to construct 16S rRNA gene libraries.

Libraries were prepared using a standard protocol using the 16S rRNA gene primer pair 515-F (GTGCCAGCMGCCGCGGTAA) and 806-R (GGACTACHVGGGTWTCTAAT) targeting the V4 hypervariable region (Caporaso et al., 2011). PCR was performed in duplicate using Phusion Hot Start II high-Fidelity PCR Master Mix (Thermo Scientific Inc., Waltham, MA). Reactions were conducted using a modified version of the manufacturers protocol, with 1 µL DNA template (15 ng/µL), 1 µL of each primer (10 pMol), 10 µL Master Mix, and 7 µL water to reach a final volume of 20 µL/reaction. Negative controls were used in each batch of PCR substituting 1 µL DNA template with 1 µL water and a unique reverse barcode to remove any possible contaminating DNA following sequencing analysis. All reactions were conducted using the C1000 Touch Thermocycler from Bio-Rad Laboratories, Inc. (Hercules, CA). PCR cycles included a 30s initial denaturation at 98°C, followed by 27 cycles of denaturation at 98°C for 10s, annealing at 50°C for 30s, extension at 72°C for 15s, and a 7min final extension at 72°C before being held at 4°C. Following PCR, a 3 µL aliquot of each reaction was assessed on an agarose gel to ensure specific and successful amplification. Duplicate reactions were mixed and assessed for concentration using the Qubit 3 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA). Next, 100 ng of each successful reaction was pooled and purified using the QIAGEN's QIAquick PCR Purification Kit (Qiagen, Inc., Germantown, MD) according to the manufacturer's protocol. Completed libraries were sequenced on the Illumina MiSeq PE250 system at the UC Davis DNA Technologies Core. Here, 11 million (28% PhiX) sequencing reads were generated with an overall Q30 > 90.1%. The Dada2 platform (version 1.18) was used in the R environment (www.r-progect.org) to process demultiplexed reads using the conventional pipeline recently described (Parsons et al., 2020). Dada2 uses error modeling to correct sequencing errors and group sequences into exact sequence variants (ESVs) that were used in conjunction with the Silva database (version

128) to determine phylotype counts (Callahan et al., 2016). After processing on the Dada2 pipeline, ESVs corresponding to mitochondrial and chloroplast taxonomy were removed and samples were rarefied to 12,000 reads per sample.

Soil physicochemical analysis

Fresh soils collected from the field trial were also used to evaluate the impacts of manure application on soil DOC and soil MBC. A representative bulk soil sample (8 g) was mixed with 40 mL 0.5 mol L^{-1} K_2SO_4 in polypropylene tubes and centrifuged at a force of 7,969 × g for 15 min to remove suspended solids. Supernatant solutions were retained for DOC concentrations (mg L^{-1}). The total MBC was measured by chloroform fumigation (Joergensen, 1996; Yang et al., 2016). Both DOC and MBC were measured using a Total Organic Carbon analyzer (Shimadzu TOC-VCSH analyzer, Kyoto, KYT, Japan). Reactive carbon was assessed as permanganate oxidizable carbon (POXC) was measured as outlined elsewhere (Culman et al., 2012). In brief, 2 g of soils were weighed into 50 mL polypropylene tubes prior to oxidation. The reaction was initiated by adding 18 mL of deionized water and 2 mL of 0.2 mol L^{-1} $KMnO_4$ (final reaction concentration = 0.02 mol L^{-1} MnO_4^-) to each tube containing pre-weighed soil, followed by mixing for exactly 2 min on a reciprocal shaker and then allowed samples to settle for exactly 10 min. The reaction was terminated by transferring 0.5 mL of supernatant into a fresh 50 mL tube with 49.5 mL of deionized water, which was inverted to mix thoroughly, resulting in a homogenized 1:100 dilution. To minimize variability in reaction termination time, the five analytical replicates were run in sequence. The 1:100 dilution was transferred to microcuvettes and analyzed by UV–Vis spectrophotometry utilizing the Genesys™ spectrophotometer to quantify MnO_4^- remaining in solution by absorbance at 550 nm. A separate 8 g of fresh soil was used to determine NO_3- and NH_4^+ concentrations via standard spectrophotometric methods (Doane and Horwáth, 2003; Verdouw et al., 1978). Potentially mineralizable nitrogen (PMN) was measured using an anaerobic incubation method with extraction by KCl (Griffin et al., 2017; Waring and Bremner, 1964). Electrical conductivity (EC) was measured according to Sudduth et al. (2005), specific ultraviolet absorbance (SUVA) was measured using UV absorbance to determine the aromaticity of DOC (Weishaar et al., 2003).

Plant analysis

Corn and wheat plant samples were collected at harvest from 4 randomly selected 1 m^2 locations per treatment plot. Plants were then dried at 60 °C until constant weight after harvest and then weighed to determine relative yields between treatment plots and grinded. The processed plant samples were sent to SoilTest Farm Consultants (Moses Lake, WA) for quantification of nutrient contents (Marten et al., 1989).

Statistical analysis

All statistical analysis was performed using the R environment. The Shannon index diversity was determined using the vegan package (vegan.r-forge.r-project.org), and community patterns were assessed by using the Bray-Curtis dissimilarity distance, represented by use of non-metric multidimensional scaling (NMDS). To determine the dates where changes to microbial diversity, DOC and MBC between high, low and zero were significant, unpaired t-tests were performed on high and zero, low and zero and high and low at each timepoint. Genera found to be significantly increased or decreased in abundance within the high vs. zero plots throughout the field trial were determined by Wilcoxon test. In each season (SC1, WW1, SC2, WW2) the timepoints with the most and least genera significantly changed in abundance were used to determine what percentage of those genera were also found in the source manure used throughout the field trial. The role of different rates of manure application on the relationships between soil microbial diversity, taxonomy, and their relationship to soil chemical and physical characteristics over time was assessed using Spearman rank-order correlations. Finally, ANOVA was used to assess changes in plant yield.

RESULTS

Higher rates of manure application resulted in increased differences in microbial communities

Microbial similarity patterns varied greatly between high and zero application treatments (PERMANOVA, $df = 2$, $r^2 = 0.05$, $p < 0.001$). Plots that received manure (low vs. high) had increasing amounts of dissimilarity with higher rates of manure application. Conversely, plots that received no manure had microbial community compositions that resembled one another throughout this experiment (**Figure 1-1**).

Microbial diversity, soil organic carbon, and microbial biomass increase with higher rates of manure application

Microbial diversity, as measured by the Shannon index, was greater in high than zero on days 17, 35, 64, 323, 357, and 371 (*t-test*, $p < 0.05$ all dates). These increases were observed in both corn growing seasons. In contrast, no significant differences in diversity were observed in either of the wheat growing seasons. Comparisons between manure treatments and zero in the wheat growing season resulted in less significant differences across soil physicochemical and microbiological characteristics measured in this experiment (**Supplemental Figure 1-1**). Diversity was not significantly higher at the conclusion of the field trial in high vs. zero application plots. Microbial diversity was less affected by low (50%) than high (100%), with significant increases observed on days 17 and 120, both dates closely following manure application events (*t-test*, $p <$ 0.05). Day 64 was the only occurrence where a high rate of manure application resulted in a significantly higher microbial diversity (MEAN = 5.57) than low (MEAN = 5.44) (t =3.01, df =3.55, p = 0.04) (**Figure 1-2A**).

There was a brief, but significant, increase of DOC in high (MEAN = 43.22 mg C kg^{-1}) compared to zero (MEAN = 20.28 mg C kg^{-1}) on day 17 following the first round of manure application (t = 3.63, df =3.11, p = 0.03). This was followed by a consistently significant higher concentration of DOC in high plots vs. zero throughout the two-year experiment. In contrast to trends observed in MBC, DOC was not notably higher than it was on day 1 versus day 665. DOC was similarly significantly higher in plots receiving low rates of manure application (MEAN = 45.78 mg C kg^{-1}) than plots receiving zero (MEAN = 20.28 mg C kg^{-1}) on day 17 (t =5.62, df =3.21, p = 0.01). Unlike the case of high in comparison to zero, low application plots did not possess a DOC significantly higher than zero on days 50, 87, 280, 431,451, and 469. However, at the conclusion of this trial, low application did significantly increase DOC in low (MEAN = 45.23 mg C kg^{-1}) when compared to zero (MEAN = 29.96 mg C kg^{-1}) application plots on day 665 (t =3.78, df =5.78, p = 0.01). Manure application at high did not vary significantly from low at the beginning of the field trial on days 1, 17 and 35. On day 50, high plots were significantly higher (MEAN = 40.35 mg C kg^{-1}) than low (25.75 mg C kg^{-1}) (t =5.38, df =4.97, p = 0.003). From day 50 onwards, excluding days 87, 280, and 431, DOC in high amendment plots was significantly higher than DOC in low (**Figure 1-2B**).

MBC was significantly higher in plots receiving high (MEAN = 89.03 mg C kg^{-1}) in comparison to zero (MEAN = 56.22 mg C kg^{-1}) 17 days after application (t = 4.13, df =4.29, p = 0.01). This trend continued until day 35 where subsequent timepoints in the first corn growing season no longer varied significantly. Following a second application of manure, high plots had higher MBC (MEAN = 109.61 mg C kg^{-1}) than plots receiving zero (MEAN = 62.38 mg C kg^{-1}) 120 days after initiation of the field trial ($t = 5.92$, $df = 5.91$, $p = 0.001$). This significant increase was observed throughout the remainder of the experiment, peaking on day 323 where high plots reached their zenith of MBC (MEAN = 179.43 mg C kg^{-1}) compared to those receiving zero (MEAN = 68.19 mg C kg^{-1}) ($t = 11.52$, $df = 3.79$, $p < 0.001$). These results were observed at the start of each growing cycle following manure application where the variance between high and low MBC was most pronounced. The MBC of plots receiving manure on day 1 (MEAN = 37.06 mg C kg^{-1}) was significantly higher on day 665 (MEAN = 112.89 mg C kg^{-1}), the end of the field trial ($t = -4.55$, $df = 5.99$, $p = 0.003$). The differences between low rates of manure application and zero application were less pronounced and notably, non-significant at the end of the field trial. Nevertheless, similar responses to manure application were observed on day 17 where low rates (MEAN = 89.43 mg C kg^{-1}) had a significantly higher MBC than zero plots (MEAN = 56.23 mg C kg^{-1}) ($t = 4.22$, $df = 4.31$, $p = 0.01$). The difference between low (MEAN = 113.95 mg C kg^{-1}) and zero (MEAN = 69.74 mg C kg^{-1}) plots was highest on day 343 ($t = 5.46$, $df = 3.81$, $p = 0.006$) although the difference between low and zero plots never exceeded the difference between high and zero, suggesting a dose dependent relationship. In high plots, MBC was significantly higher (MEAN = 109.61 mg C kg^{-1}) than low (MEAN = 82.25 mg C kg^{-1}) ($t = 2.92$, $df = 5.81$, $p = 0.03$). A significant difference between high and low plots remained for most of the experiment culminating at the conclusion of the field trial on day 665 where high plots maintained significantly higher MBC (MEAN = 12.885 mg C kg^{-1}) than low (MEAN = 65.225 mg C kg^{-1}) ($t = 3.92$, $df = 3.55$, $p = 0.02$) (**Figure 1-2C**).

Microbial genera that increased in abundance following manure application were genera present in manure

The most abundant genera found in manure differed from those found in soil (a breakdown of the most represented manure microorganisms can be found in **Supplemental Figure 1-2**). In manure applied plots, genera found in the source manure showed the most significant increases throughout the study. On day 1 following manure application, 27 genera significantly increased in abundance in plots receiving high rates of manure compared to zero ($p < 0.05$). This trend was maintained over the first summer corn growing season. On day 50, the date with the lowest number of genera (n = 11), 64% of those genera that showed significant increases were also found in the source manure. In the following wheat season on day 120, of the 16 genera that increased in abundance in manure, but not non-manure applied, 75% were found in the source manure. By day 280, only 2 of the original 16 genera could still be detected, of which only 1 had been detected in the source manure. Early in the second corn growing season, 31 genera increased in abundance in manure vs. non-manure plots of which 39% were also found in the source manure. Later in the season, 57% of the genera that had significantly increased in abundance in manure-applied plots were found in the source manure. At day 469 (final wheat season) and at the end of the experiment, 100% and 49%, respectively, of the genera that increased in abundance in the high plots had also been detected in the source manure.

Conversely, genera that were not detected in the manure appeared to significantly decrease in abundance in high compared to non-manure plots. During the initial corn growing season, day 1 represented had the most genera impacted by manure application, on this date, 22 genera were found to be in significantly lower abundance in plots receiving high rates of manure vs. those that received zero. On day 50, 60% of the genera decreased in abundance were found in manure, but in total only 5 genera were significantly decreased in abundance at this date, whereas 11 were found to be in increased abundance. In the first winter wheat growing season a majority (78%) of the genera found to be in decreased abundance were those not found in the source manure. On day 280, no genera were found to be in significantly lower abundance in plots receiving high rates of manure vs. those not receiving manure. Throughout the second year of the field trial, a majority of genera that decreased in abundance were not found in the source manure. On days 323, 431, 469 and 665, genera not found in manure constituted 92%, 100%, 84%, and 100% of the genera that decreased in abundance in plots receiving high rates of manure vs. zero (**Figure 1-3**). Furthermore, these trends are broadly reflected when considering the totality of this field trial. When manure application is examined alone without taking into consideration unique sampling events, 140 genera increased in abundance in high vs. zero plots with a majority (58.6%) of those genera being found to be in manure. Conversely, 51 genera had lower abundance in high vs. zero plots and a majority (84.4%) of those genera are absent from the manure microbial community (**Supplemental Figure 1-3**).

Strength of associations between soil physicochemical and biological characteristics increased in manure applied soils

Positive correlations between soil physicochemical and biological factors increased with the rate of manure application (**Figure 1-4**). In high plots, there was no significant relationship between MBC and microbial diversity. There was, however, a relationship between those factors in low ($r_s = 0.27$) and high ($r_s = 0.33$). Furthermore, there was no relationship between DOC and *Bacillus* in zero manure plots, but in low ($r_s = 0.35$) and high ($r_s = 0.42$) manure plots, the relationship was increasingly pronounced with higher rates of manure application. Not all the relationships between microbial genera and soil carbon were positive in manure applied soils. In high plots, *Sphingomonas* ($r_s = -0.16$), *Psychroglaciecola* ($r_s = -0.21$), *Gemmatimonas* ($r_s = -0.22$), *Ramlibacter* ($r_s = -0.27$), and *Mycobacterium* ($r_s = -0.39$) were all negatively associated with DOC. The negative association between genera and DOC was only reflected in *Ramlibacter* ($r_s = -0.25$) in zero plots. *Rubrobacter* ($r_s = -0.44$) and *Nitrososphaera* ($r_s = -0.43$), were negatively correlated under high application. These trends in zero plots were absent.

Plant yields did not differ between fertilizer and manure amended plots

Silage corn dry matter yield did not vary significantly between treatments in either growing season. In Season 1, it was 3.80 ± 0.25 Mg ha^{-1} in zero, 4.03 ± 0.27 Mg ha^{-1} in low, and 3.87 ± 0.20 MG ha^{-1} in high manure plots. In season 3, it was 2.77 ± 0.18 Mg ha^{-1} in zero, 2.78 ± 0.13 Mg ha^{-1}, in low, and 2.68 ± 0.13 Mg ha^{-1} in high manure plots. Similarly, wheat yields did not significantly vary between treatments with season 2 silage wheat yield being 2.68 ± 0.60 Mg ha^{-1} in zero, 2.51 ± 0.33 Mg ha^{-1} in low, and 2.35 ± 0.29 Mg ha^{-1} in high manure plots. In season 4, silage wheat yield was 2.18 ± 0.13 Mg ha^{-1} in zero, 2.15 ± 0.23 Mg ha^{-1} in low, and 1.94 ± 0.06 Mg ha^{-1} in zero, low and high plots respectively. Therefore, partially, or completely replacing mineral nitrogen fertilizer with dairy manure maintains silage corn and wheat yields for all four growing seasons. (**Table 1-1**).

DISCUSSION

Manure inputs changed microbial community composition and increased microbial diversity with high ratios of manure application resulting in greater change (**Figure 1-1)**. Providing carbon and other nutrients with manure inputs likely provided opportunities for new microbial growth, otherwise limited in low organic matter soils; similar findings were reported in a low-organic matter soil (Bastida et al., 2021). Ultimately, fluctuating diversity seen in manure applied plots drove changes in community composition because microbial diversity increased approximately two weeks after application, but not in zero manure treatment plots (**Figure 1-2A)**. In this instance manure applied plots arrived at different community compositions, while zero plots remained similar. In a study of impacts of swine manure inputs, integrase and antibiotic resistance genes increased up to 28 days following manure addition and then returned to levels observed in untreated soil (Jechalke et al., 2014). Therefore, manure application changes the soil microbial community dynamically and can contribute to a wider range of fluctuating community compositions in manure applied soil.

In our work, DOC was highly responsive to manure application where even low plots had significantly higher DOC when compared to zero and these differences were maintained throughout the experiment (**Figure 1-2B)**. Increases in DOC were detectable prior to increases in microbial diversity, suggesting that manure first provides a labile carbon pool, and later that pool may contribute to changes reflected in the microbial community. Organic matter is often decomposed by microorganisms and utilized as a carbon source for microorganisms. As such, a higher DOC pool indicates more carbon available to the soil microbial community (Nelson and Wear, 2014). Changes to DOC may therefore occur before changes to the microbial community and drive changes to soil carbon processing by heterotrophic microorganisms just as it has in marine ecosystems (Hansell, 2013).

The MBC incrementally increased over the course of the experiment, with small increases following each round of manure application (**Figure 1-2C)**. In another study examining the response of soil microbial communities to pig slurry, manure, and biochar in soils with variable soil organic matter (SOM), it was found that low SOM soil was more prone to increased MBC and activity (Yanardağ et al., 2017). This taken with our results suggests that changing community composition is more difficult than increasing the overall abundance of soil microorganisms. Furthermore, most of the taxa that did increase in abundance following manure application were also present in manure itself. We found that most manure associated microorganisms were most present following application and diminished throughout the season until the next round of application (**Figure 1-3**). It is therefore likely that few new microorganisms are added to the soil following manure application, and those that are added are dependent on the resources in manure to persist. A recent study conducted on a 9-year experiment in Moscow, Russia detailed an increase in the abundance of manure-associated genera in soil following application. In this case, annual applications of manure were used, and exogenous manure-associated microorganisms did not survive in the soil after a few months (Semenov et al., 2021). This suggests that more frequent applications of manure are necessary to curate a persistent cohort of manure-associated microorganisms and that increases to diversity are dependent on fresh applications of manure.

Our study suggests that application of manure increases the relative abundance of taxa that were rare in the original community. Of the top 20 most represented genera, only *Lysobacter* significantly increased in abundance for multiple growing seasons, with *Mycobacterium* and *Streptomyces* increasingly temporarily (**Supplemental Figure 1-1**). *Lysobacter* is one of the most abundant genera in manure (**Supplemental Figure 1-2**) and it can be resistant to tetracycline, potentially contributing to the proliferation of antibiotic resistance genes (Hayward et al., 2010). Increases to soil nitrate and PMN in manure applied plots were seen in tandem with decreased relative abundances of the most represented soil microbial genera (**Supplemental Figure 1-1**). This resulted in more negative correlations between soil nitrate, PMN and ammonium and represented microbial genera in high plots, a trend not seen in zero except for *MND1* and

Gemmatimonas. As such, it seems that microorganisms already present in manure, such as *Lysobacter*, can best utilize the nutrient pools of manure, while microorganisms already abundant in soil do not utilize the temporary increases to nutrients that follow application to the same extent. Indeed, increases to MBC were negatively correlated with the relative abundances of the most represented microbial genera (**Figure 1-4**). Therefore, increases in total MBC were happening due to relative increases in less represented microbial genera.

In another study using soils from Russell Ranch, a microcosm study compared microbial invasion from poultry manure-compost added to soils with a 20-year history of poultry manure compost application vs. no compost additions (Gravuer et al., 2021). The authors found that soil without a history of compost addition was susceptible to invasion of compost-derived taxa; however, the abundance of these taxa dropped substantially by a month after application. In our study, we found that continual inputs of manure over our two-year study were needed to maintain the relative abundance of manure-associated organisms, suggesting they may have limited opportunities to establish themselves in a soil environment. Together, changes to the microbial community brought about by manure likely need to be maintained by continual application.

In conclusion, we found that continual manure inputs modify soil microbial community composition and higher manure inputs were associated with greater changes. Microbial diversity showed short-term increases immediately following manure application and then dropped down to pre-input levels. In contrast, MBC incrementally increased with each manure application with the highest biomass being reached in the last season of the experiment. Most taxa that increase after manure application are those found in manure. However, other than *Lysobacter*, these taxa are not highly represented in the soil microbial community. Taken together, our work here provides a timeline to the temporary and additive changes that regular manure application can have on the microbial community. We highlight the necessity of continual applications of manure to maintain changes in the soil microbial community. Future work aimed at investigating how taxa from manure contribute to differences in soil functionality will guide precision waste management practices that can be used to create more efficient waste streams and healthier soils.

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Table 1-1. Silage corn and wheat yields for each growth season (dry weight) with zero, low and high rates of dairy manure application. The numbers to the right of each value represent the standard error of the mean. Significant differences ($p < 0.05$) based on Tukey test between all three treatments are indicated by different letters in parentheses to the right of each value.

Treatment	Season 1 silage corn yield	Season 2 silage wheat yield	Season 3 silage corn yield	Season 2 silage wheat yield
	--	Mg ha^{-1}	--------------------------------	-----------
Zero manure	3.80 ± 0.25 a	2.68 ± 0.60 a	2.77 ± 0.18 a	2.18 ± 0.13 a
Low manure	4.03 ± 0.27 a	2.51 ± 0.33 a	2.78 ± 0.13 a	2.15 ± 0.23 a
High manure	3.87 ± 0.20 a	2.35 ± 0.29 a	2.68 ± 0.13 a	1.94 ± 0.06 a

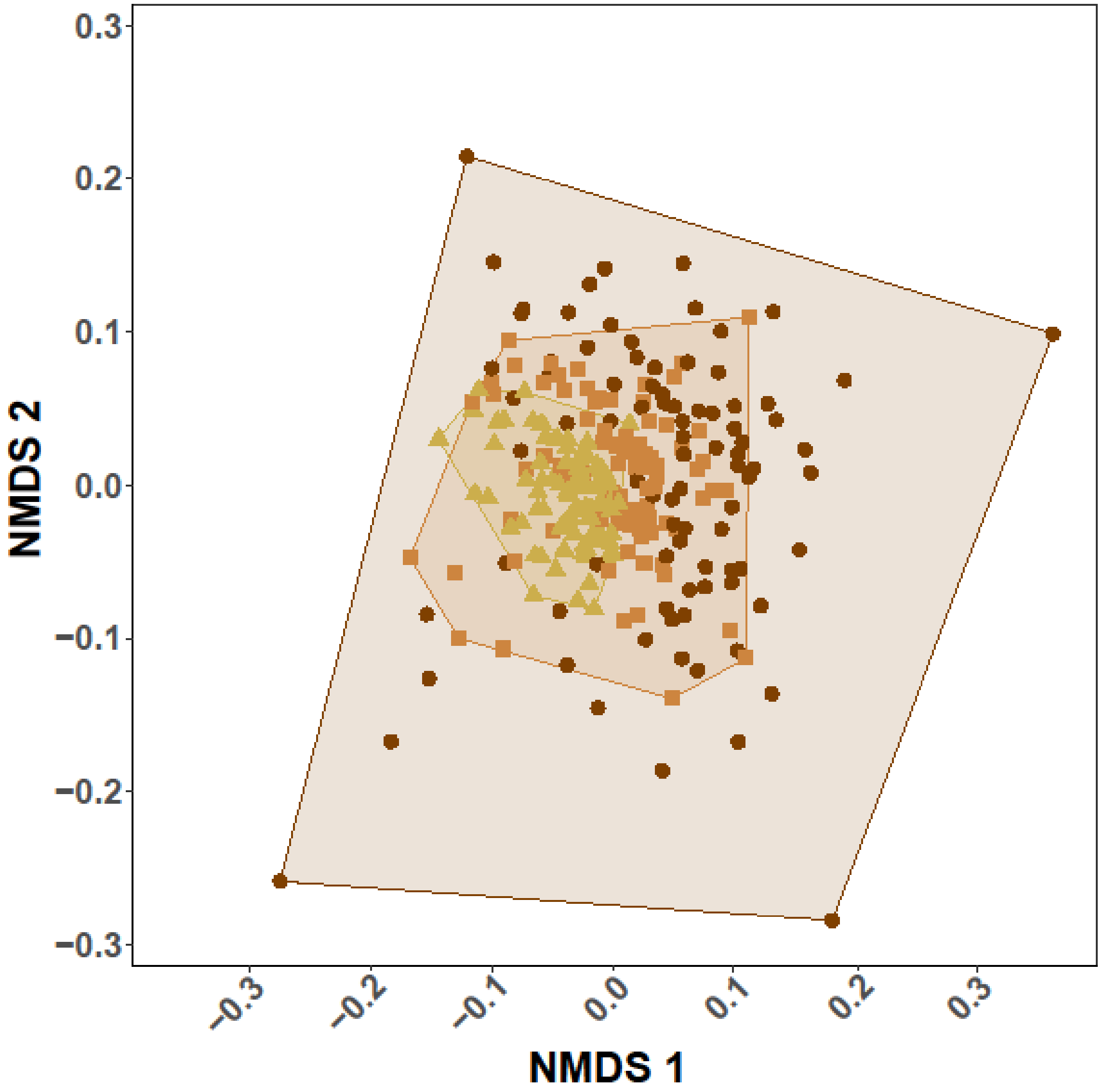


Figure 1-1. Nonmetric multidimensional scaling analysis reveals microbial similarity patterns in plots receiving high (2.24 kg manure/m^2, dark brown circles), low (1.12 kg manure/m^2, light brown squares), and a urea fertilizer control zero (0 kg manure/m^2, tan triangles).

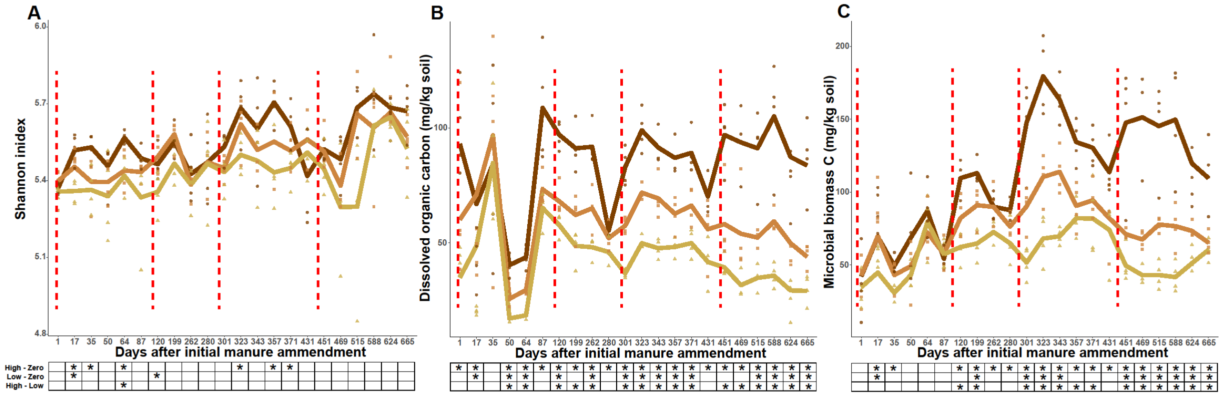
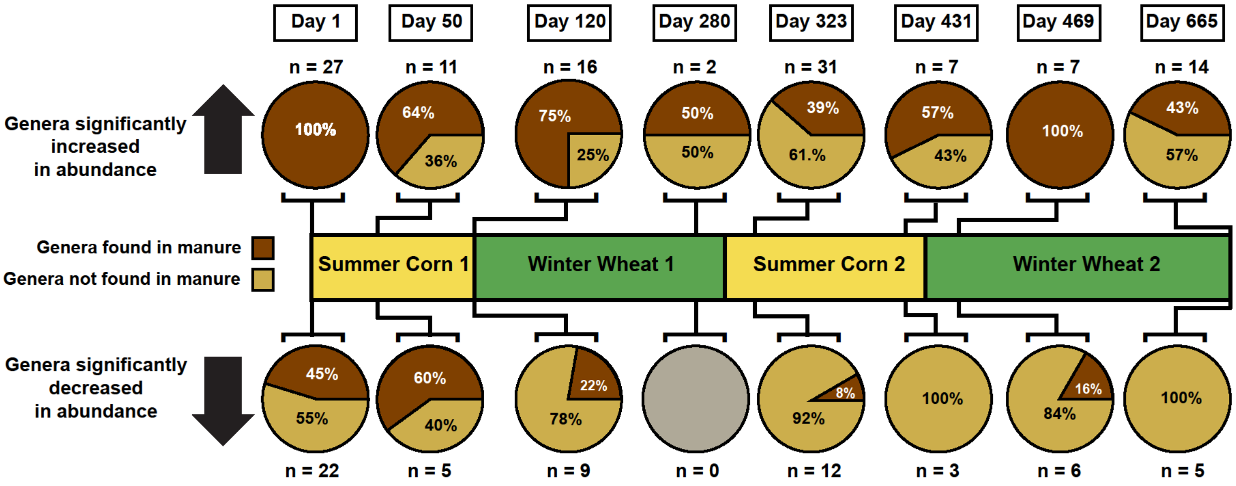


Figure 1-2. The Shannon index was used to assess microbial diversity (A), dissolved organic carbon (DOC) (B), and microbial biomass carbon (MBC) (C), over a two-year manure application regime in a simulated forage system. Manure application occurred at the start of each growing season with red dotted lines denoting each event. High, low, and zero application rates were used and represented by dark brown circles, light brown squares, and tan triangles respectively.



<u>Figure 1-3</u>. Microbial genera found to be significantly different between high (2.24 kg manure/m^2) and zero (fertilizer control) treatments. Genera that increased in abundance are on the top and those that decreased in abundance are on the bottom. Differences between the two treatments are shown in each growing season (summer corn 1, winter wheat 1, summer corn 2, winter wheat 2). Genera are either detected in manure (brown), or not (tan).

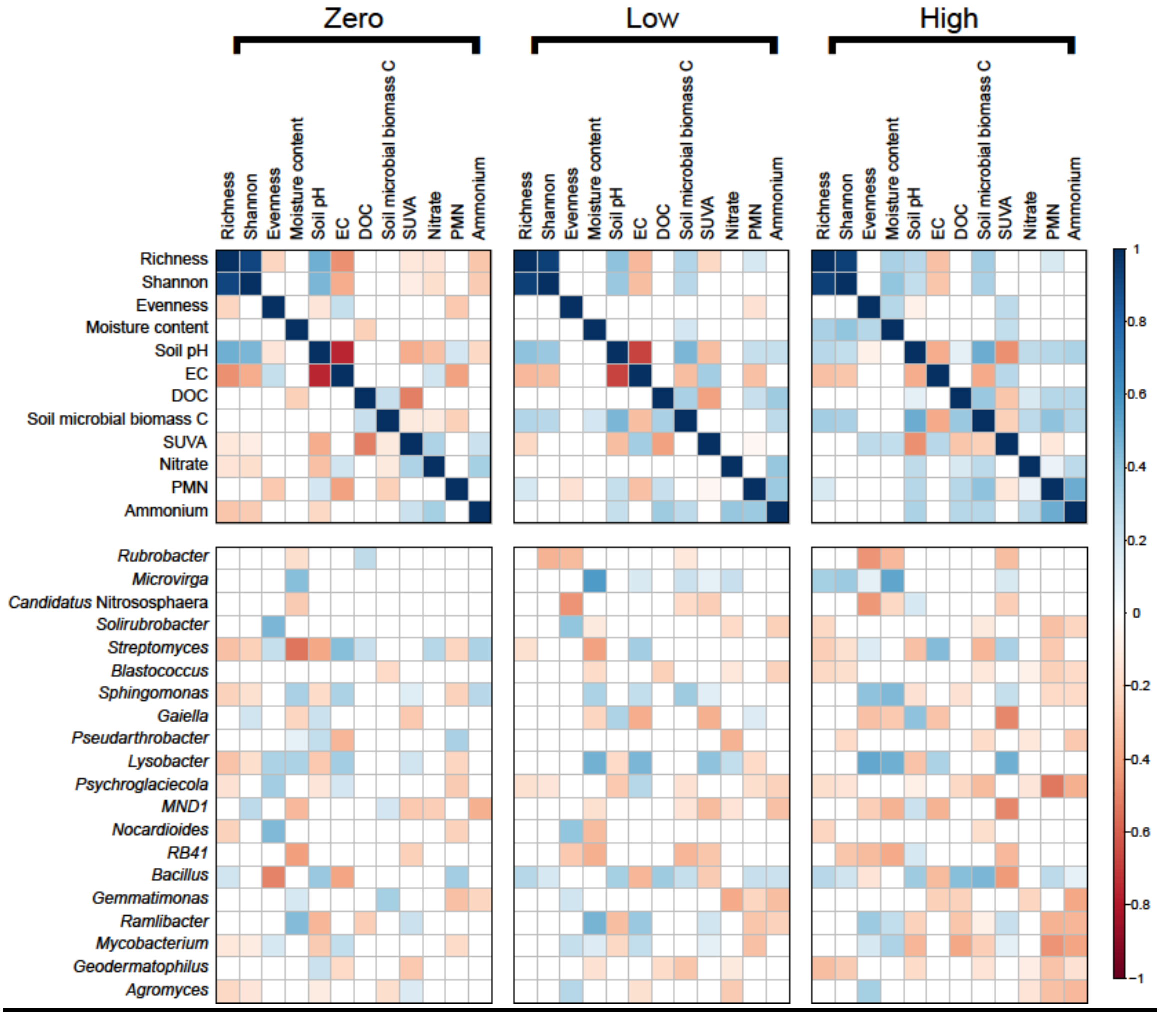
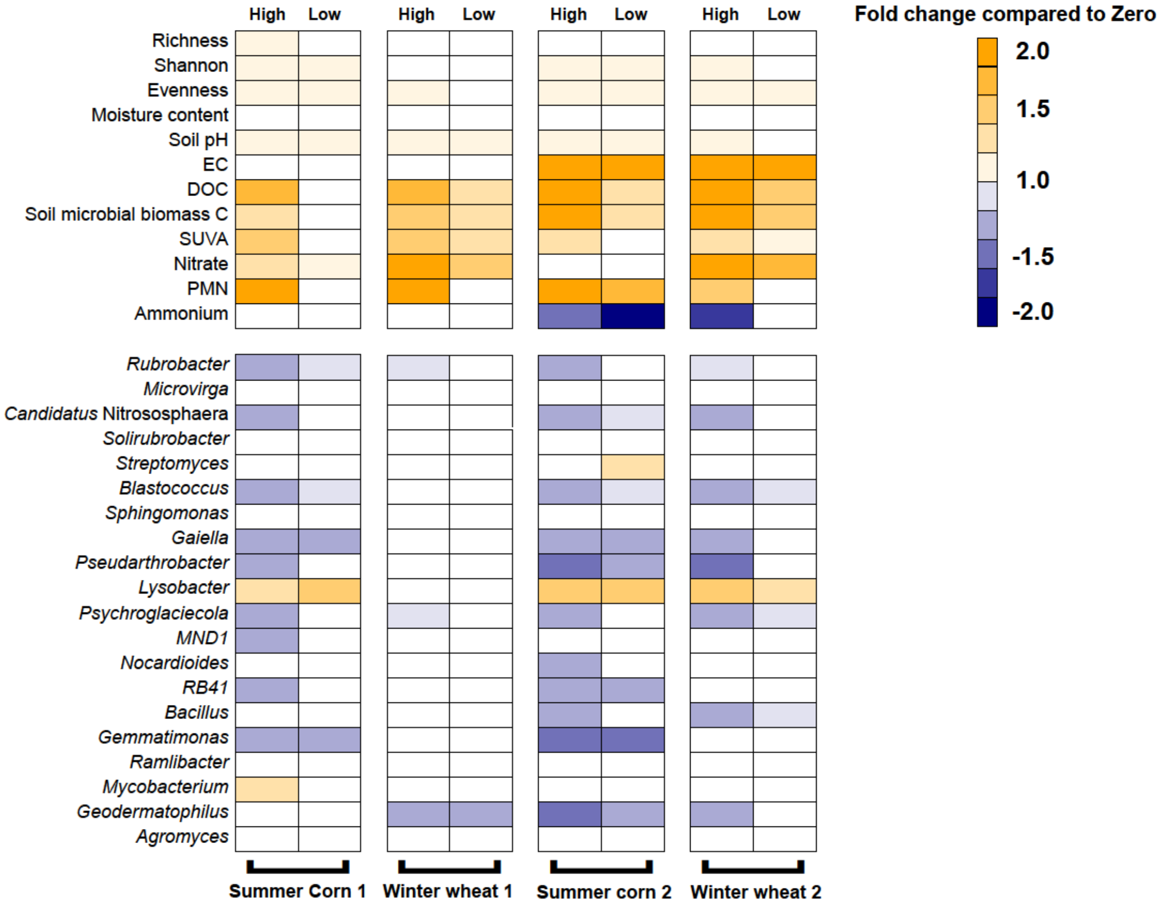
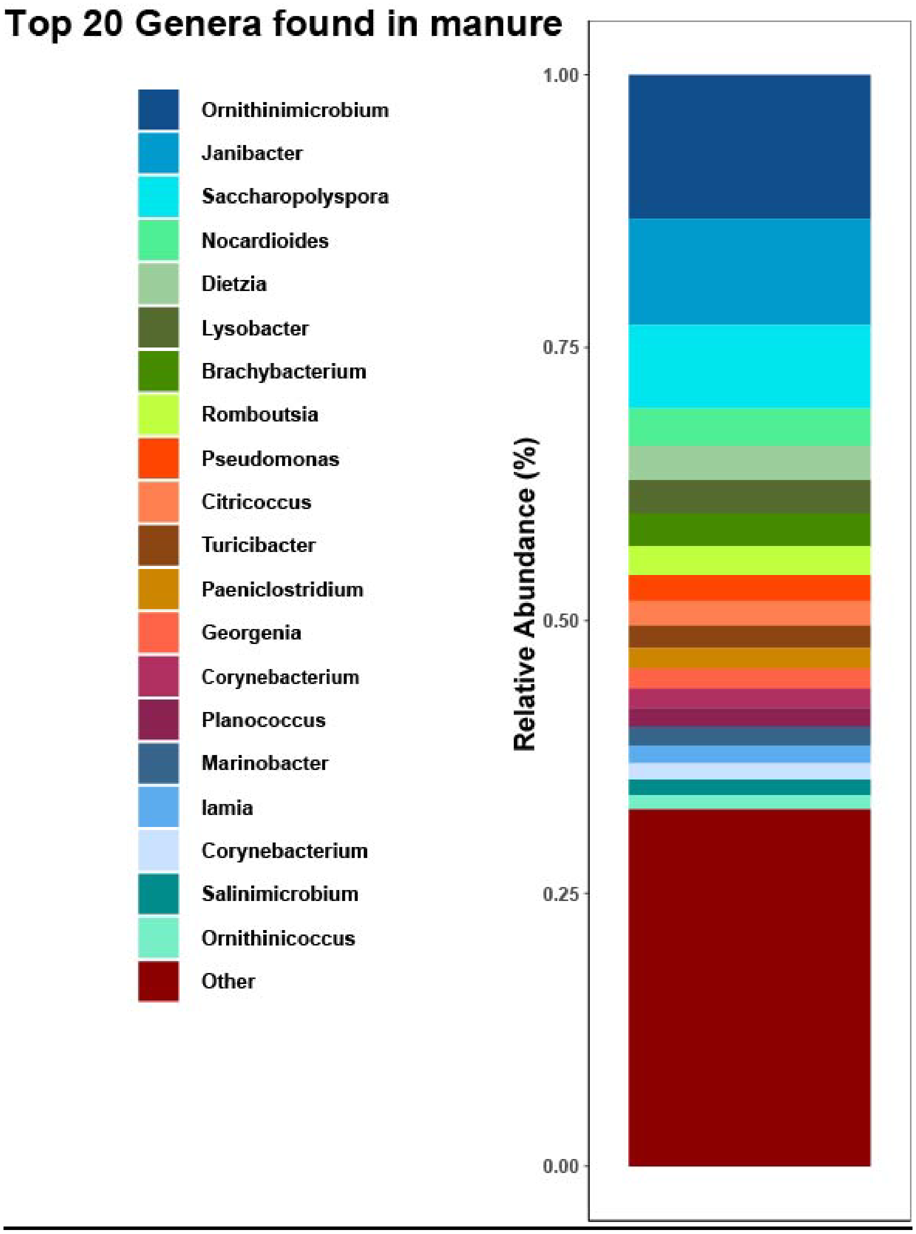


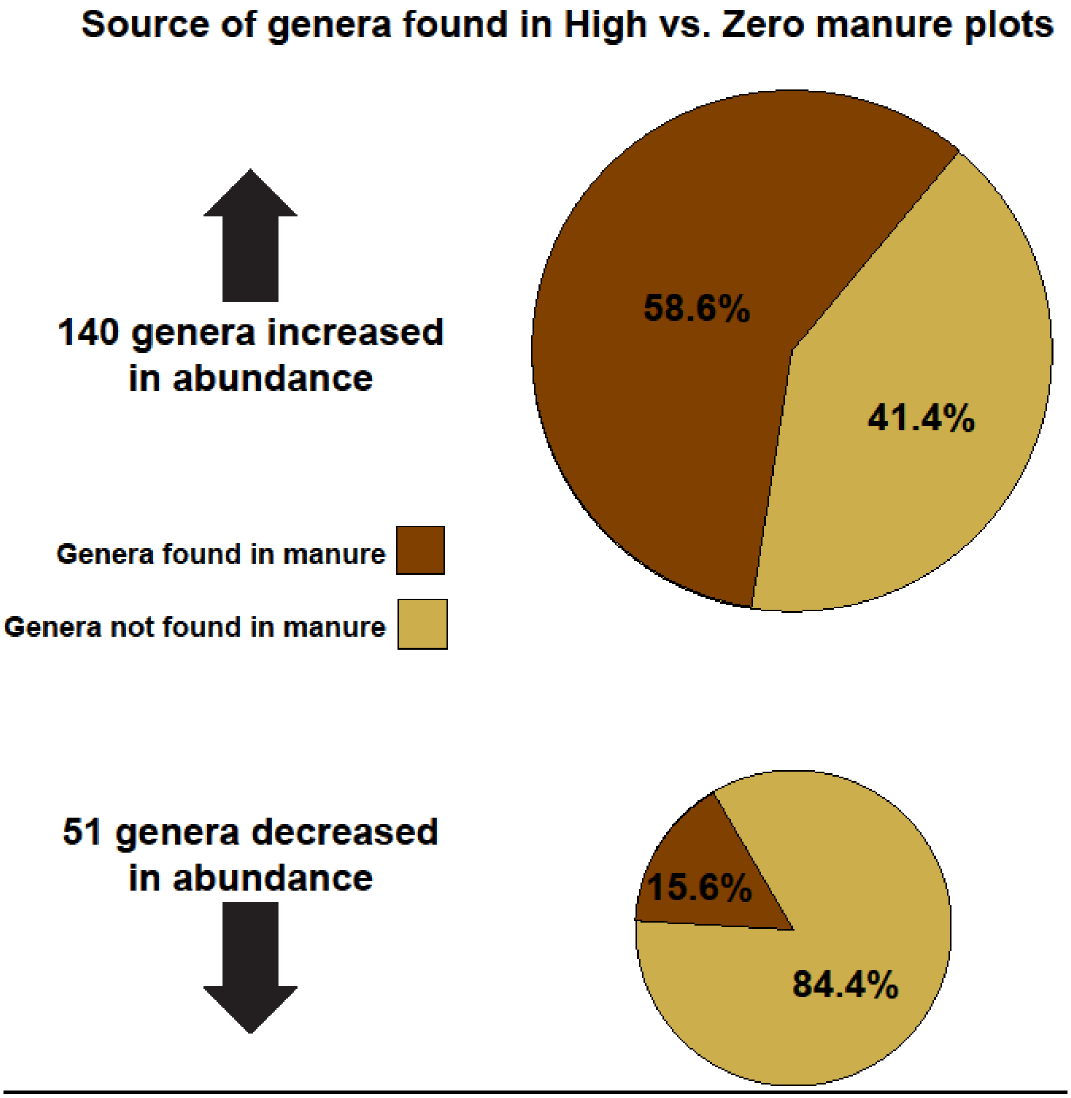
Figure 1-4. Spearman's correlations between physicochemical and biological characteristics in soils treated with urea fertilizer control zero (0 kg manure/m^2), low (1.12 kg manure/m^2) and high (2.24 kg manure/m^2). Stronger positive correlations are indicated by increased blue color intensity, negative correlations are indicated by increasing red and non-significant correlations are left blank. Richness, Shannon diversity, microbial evenness, soil moisture content, soil pH, electrical conductivity (EC), dissolved organic carbon (DOC), soil microbial biomass, specific ultraviolet absorbance (SUVA), potentially mineralizable nitrogen (PMN), and ammonium are grouped together on the top and the top 20 most abundant genera are grouped on the bottom in order of decreasing abundance.



Supplemental Figure 1-1. Unpaired t-tests were used to determine the significant relative changes between soil physicochemical and biological characteristics. Non-significant changes from low or high vs. zero are left blank and significant ($p < 0.05$) positive changes are represented by an orange gradient, with increasingly orange hues representing a greater fold change. Blue represents negative fold changes, with the darkness of the hue representing increasingly greater negative changes. The top 20 most abundant genera are included, in order of decreasing abundance with *Rubrobacter* first and *Agromyces* last.



Supplemental Figure 1-2. The top 20 genera found in manure used throughout this field trial.



Supplemental Figure 1-3. Microbial genera found to be significantly different between high (2.24 kg manure/m^2) and zero (fertilizer control) treatments when examining the field trial in its totality. Genera are either detected in manure (brown), or not (tan).

Chapter 2: Microbial carbon incorporators and metabolic capacity increase in manure applied soils with a higher carbon use efficiency

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Running title: Microbial carbon incorporation increases following manure application

ABSTRACT

Manure is a valuable agricultural resource, providing essential nutrients for plant growth and improvement of soil health. We investigated how manure amendments in an agricultural system can improve the soil carbon cycle. We established a field study on a summer corn to winter wheat rotation where manure was applied to plots adjacent to those that received synthetic N fertilizer and zero manure. Soils were collected from these plots to determine how manure application changed the microbial carbon use efficiency (CUE). We found a higher microbial CUE in soils amended than not amended with manure. Stable isotope probing (SIP) allowed us to determine the microbial groups most responsible for incorporating labile carbon in manure applied and zero manure soils. Incorporators found in manure applied soils were responsive to manure amendments for two summer seasons and increased significantly ($p < 0.05$) over the study period in manure applied soils. In contrast, incorporators present in zero manure soils were of similar abundance in both manure applied and zero manure soils. Together this suggests that manure application selects for a cohort of soil microorganisms that contribute to increased CUE. Furthermore, we sequenced the metagenomes of the soil microbial communities in manure applied and zero manure soils. We found, increased relative abundance of functional genes related to a wide variety of metabolic pathways involving the processing of carbon compounds ($p < 0.05$, $\geq$ 2.5 FC). Overall, manure applied soils possessed a greater metabolic capacity than their zero manure counterparts. Our results suggest that manure application can improve the soil microbial CUE, with possible consequences for increasing soil organic matter through the selection of a microbial community more capable of processing and incorporating carbon.

INTRODUCTION

Intensive agricultural practices deplete soil carbon and management decisions aimed at increasing soil carbon are gaining interest and acceptance as increasingly common practices (Wallenstein, 2017). Management can increase retention of soil carbon through use of cover crops or reduce carbon losses through minimizing disturbances with techniques such as no till (Kallenbach et al., 2019). Alternatively, soil carbon can be directly increased and maintained through the amendment of organic carbon. In a recent study, both straw and manure amendments were shown to increase soil organic carbon relative to a nitrogen fertilizer control in an experimental agro-ecosystem utilizing a single annual amendment (Li et al., 2021). However, in a separate meta-analysis of soil organic carbon in croplands subjected to chemical and organic fertilizer amendments, soil carbon accumulation had a high variability across climate regions and different agro-ecosystems (Han et al., 2016). Therefore, determining how the soil carbon accumulation responds to management decisions across systems is critically important in mitigating continued losses of soil carbon in agricultural systems.

The extent that organic amendments may improve soil carbon accumulation in the long term cannot be solely determined by measuring carbon inputs and losses (Kallenbach et al., 2019). The amount of soil carbon maintained in a system is largely mediated by carbon processes, which are facilitated by the soil microbial community. Adding organic substrates provides a means to promote biological interactions in soil that drive organic carbon cycling throughout or out of the system (Wood and Bradford, 2018). The soil microbial community therefore alters the amount of carbon maintained in the agricultural system and the extent organic amendments can maintain and improve the storage of soil carbon is dependent on its community structure and function (Bhattacharyya et al., 2022). As such, agricultural systems using organic amendments may alter the capability of a soil microbial community to process those amendments. This creates a potential where organic amendments such as manure curate a microbial community more capable of feeding on organic material (Cotrufo et al., 2015).

Ultimately, the soil microbial community can facilitate accumulation of carbon through increases in biomass or accelerate losses through greater respiration. This concept is defined as microbial CUE which is the ratio of carbon allocated to microbial growth relative to other microbial processes such as respiration (Geyer et al., 2019). The concept of CUE is increasingly being measured to provide insight into regulating soil storage in models of soil carbon accumulation (Cotrufo et al., 2013; Wieder et al., 2015). In soils, higher CUE increases belowground carbon storage, minimizing carbon losses to the atmosphere or surrounding systems. Agricultural systems are exceptionally relevant ecosystems in which to study microbial CUE because land management can directly impact soil carbon dynamics (Kallenbach et al., 2019). Amendments rich in nutrients are thought to increase CUE because greater amounts of carbon can be allocated to growth rather than carbon spent on extracellular enzymes for nutrient acquisition (Manzoni et al., 2017; Wu et al., 2022). In a study where conventional agricultural systems reliant on chemical fertilizer were compared to those utilizing organic amendments, it was found that CUE was higher in organic systems due to a greater abundance of microbially derived organic matter (Kallenbach et al., 2015). Though, certain organic amendments with high C:N ratios such as wheat straw have been found to decrease soil CUE despite increasing the rate that organic carbon was stored in soil aggregates (Miao et al., 2021).

To better identify microbial taxa associated with CUE, SIP has gained a renewed interest (Pepe-Ranney et al., 2016). This technique of SIP can identify microbial groups most responsible for utilizing soil carbon by tracking stable isotopes from an added labeled substrate into the DNA of the soil microbial community (Uhlík et al., 2009). Labeled isotopes such as ^{13}C-glucose can be used to simultaneously assess CUE and identify microorganisms likely playing a role in that process (Overy et al., 2021).

Metagenomic assessments can determine the distribution and diversity of functional genes within a microbial community to elucidate their relationship to terrestrial carbon storage (Dai et al., 2021). A global study found that soil microbial genetic is highest in temperate habitats and varies with respect to environmental characteristics such as precipitation and pH. This suggests that nutrient cycling varies spatially depending on the pool of microbial functions at each location (Bahram et al., 2018). In agricultural systems employing the usage of cover crops and low tillage, a positive relationship between soil carbon storage and microbial functional diversity was established. In this case, a greater amount of soil nutrients was thought to induce production of microbial enzymes which were related to an increase in genes related to the metabolism of carbon compounds (Nivelle et al., 2016). As such, management decisions likely play a profound role in shaping the functional potential of the soil microbial community. This could increase the amount of carbon that could be held by the microbial community because it would permit a wider variety of methods to process and incorporate soil carbon. To date there is no known assessment of the functional genes of soil microorganisms modified by dairy cow manure application in an active forage crop system.

Our objectives were to: 1) Determine how manure application can modify soil carbon processing improving CUE; 2) Identify and measure trends in microbial taxa that are likely to change CUE; 3) Measure impact of manure application on soil metagenome and metabolic potential. Results of this research extend our knowledge of changes in microbial communities that lead to greater efficiency in the microbial carbon cycle following organic matter inputs.

MATERIAL AND METHODS

Experiment design and soil sampling

The research experiment was carried out in Rincon silty clay loam soils (pH 6.35-7.91) with a representative organic carbon stock of 12 kg / m^2 receiving long-term manure applications at University of California Davis, Russell Ranch Agricultural Sustainability Institute (Davis, CA, GPS coordinates: 38.546470, -121.871270). This field was established as corn-wheat rotation for three years on a field previously used as fallow. Individual plots received manure (2.24 kg/m^2, n = 4) or zero manure (n=4) amendment at the start of each growing season, where it was applied to the topsoil before being mixed in during bed construction. Soil samples were collected using twist augers (7cm diameter by 15cm depth) on an approximate bi-monthly basis for the first two years, with sampling dates designed to align with agricultural milestones such as tillage, planting, irrigation events, harvesting, and fertilizer application. Twenty-two sampling dates were recorded spanning the range of 665 days. In year 3, soil was collected as previously described using twist augers from manure applied and zero manure applied plots. These soil samples were then used in a laboratory microcosm study designed to assess CUE and determine ^{13}C incorporators through SIP.

Carbon use efficiency

Soils were passed through a 5mm sieve to remove large chunks of organic matter and then wetted to bring them to field capacity (23% gravimetric moisture content). Soil was then separated into 16 microcosms, 4 of which were from manure soils utilizing a 3mg/g glucose, 4 from manure soils using 1mg/g glucose, 4 from zero manure soils using 3mg/g glucose, and 4 from zero manure soils with 1mg/g glucose. Glucose was enriched to 5% ^{13}C and each microcosm consisted of 15 g soil in a small plastic cup placed at the bottom of a 1-L mason jar. Soils were preincubated for 72 h, and then readjusted to 15g utilizing water mixed with the appropriate amount of glucose per incubation. Microcosms were then capped and incubated for 72 h, after which 20mL gas was taken with a syringe through a rubber stopper imbedded in the top of each mason jar lid. Gas was stored in 12 mL exetainer vials (Labco Ltd, High Wycombe, UK) and submitted to the UC Davis Stable Isotope Facility (SIF) for quantification of ^{13}C-CO$_2$. After completion of the microcosm experiment, soil was harvested to determine dissolved organic carbon (DOC) concentration and microbial biomass-carbon (MBC). Briefly, soil was mixed with 40 mL 0.5 mol L^{-1} K_2SO_4 in polypropylene tubes and centrifuged at a force of 7,969 × g for 15 min to remove suspended solids. Supernatant solutions were retained for DOC concentrations (mg L^{-1}). Supernatant used to determine MBC was generated by chloroform fumigation (Joergensen, 1996)(Yang et al., 2016). Following which, supernatant was submitted to SIF to determine atom % ^{13}C. Finally, CUE was calculated as follows:

$$CUE = \frac{^{13}MBC}{^{13}MBC + {^{13}R}}$$

Where ^{13}MBC represents ^{13}C in MBC and ^{13}R represents cumulative ^{13}C-CO$_2$ respired (Geyer et al., 2019).

DNA extraction, PCR amplification and sequence analysis

Total DNA was extracted from 0.25g of soils from the described field trial using the DNeasy PowerLyzer PowerSoil kit (Qiagen Inc., Germantown, MD) following the manufacturer's protocol. Gel electrophoresis was used to assess quality of DNA after each extraction and DNA yields were assessed with a Qubit 3 fluorometer (ThermoFisher, Waltham, MA). Only extractions resulting in DNA concentrations above15.0 ng/µL were used to construct 16S rRNA gene libraries.

Libraries were prepared using a standard protocol with the 16S rRNA gene primer pair 515-F (GTGCCAGCMGCCGCGGTAA) and 806-R (GGACTACHVGGGTWTCTAAT) targeting the V4 hypervariable region (Caporaso et al., 2011). PCR was performed in duplicate using the Phusion Hot Start II high-Fidelity PCR Master Mix (Thermo Scientific Inc., Waltham, MA). Reactions were conducted using the manufacturer's protocol, where 1 µL DNA template (15 ng/µL), 1 µL of each primer (10 pMol), 10 µL Master Mix, and 7 µL sterile H_2O were pooled to reach a final volume of 20 µL/reaction. Negative controls were used in each batch of PCR substituting 1 µL DNA template with 1 µL sterile H_2O and a unique reverse barcode to remove any possible contaminating DNA following sequencing analysis. All reactions were conducted using the C1000 Touch Thermocycler from Bio-Rad Laboratories, Inc. (Hercules, CA). PCR cycles included a 30s initial denaturation at 98°C, followed by 27 cycles of denaturation at 98°C for 10s, annealing at 50°C for 30s, extension at 72°C for 15s, and a 7min final extension at 72°C before being held at 4°C. Following PCR, a 3 µL aliquot of each reaction was assessed on an agarose gel to ensure specific and successful amplification. Duplicate reactions were mixed and assessed for concentration using the Qubit 3 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA). Next, 100 ng of each successful reaction was pooled and purified using the QIAGEN's QIAquick PCR Purification kit (Qiagen, Inc., Germantown, MD) according to the manufacturer's protocol. Completed libraries were sequenced on the Illumina MiSeq PE250 system (Illumina Inc., San Diego, CA) at the UC Davis DNA Technologies Core. A total of 11 million (28% PhiX) sequencing reads were generated with an overall Q30 > 90.1%. The DADA2 platform (version 1.18) was used in the R environment (www.r-progect.org) to process demultiplexed reads using the conventional pipeline recently described (Parsons et al., 2020). DADA2 uses error modeling to correct sequencing errors and group sequences into exact sequence variants (ESV) that were used in conjunction with the Silva database (version 128) to determine phylotype counts (Callahan et al., 2016). After processing on the DADA2 pipeline, ESV corresponding to mitochondrial and chloroplast taxonomy were removed and samples were rarefied to 12,000 reads per sample.

Stable isotope probing and incorporator identification

Two sets of manure and zero soils were used to construct 16 concurrent microcosms for use in a SIP experiment. Here, ^{13}C-glucose was added to the first set of microcosms at 100% enrichment at a concentration of 3mg/g soil to maximize the amount of ^{13}C integrated into microbial DNA, while all other methods of microcosm preparation were conducted as described previously. The second set of microcosms were prepared identically, but non-isotopically labeled glucose was used in place of ^{13}C-glucose. After the 72 h incubation, approximately 2.5g of soil per microcosm was used for DNA extraction, resulting in approximately 10 extractions of 0.25g soil per microcosm. Extracts were quantified and assessed for quality as described previously resulting in approximately 10 μg DNA being obtained per microcosm. The SIP protocol was conducted following a standard method outlined by Dunford et al. with some modifications (Dunford and Neufeld, 2010). Briefly, 4.6 mL CsCl (1.88g mL^{-1}), 0.9 mL gradient buffer solution (0.1 M Tris, 0.1 M KCl and 1 mM EDTA), and 5ug DNA per microcosm was pooled and then injected into 5.1 mL polpropylene quick-seal centrifuge tubes from Beckman Coulter (Beckman Coulter, Inc., Brea CA). In each round of centrifugation, ^{13}C-glucose microcosms were paired with their unlabeled glucose counterparts. Centrifugation was conducted for 44 h at 50,000 rpm at 20°C in a Beckman Coulter Optima max TL benchtop ultracentrifuge utilizing a Beckman Coulter TLA-110 Fixed-

Angle Rotor (Beckman Coulter, Inc., Brea CA) resulting in an average g-Force of 83,480 g with a maximum of 104,248 g. Afterwards, fractions from each microcosm were collected in pairs (each pair consisting of DNA extracted from ^{13}C-glucose microcosms and their unlabeled glucose counterparts) using a two-channel syringe pump system (Syringe TWO by New Era Pump Systems, Inc.) operating at 0.31 mL/min until approximately 16 fractions per sample were collected. DNA was then purified from each fraction by adding 620 uL Polyethylene glycol (PEG) and 1.5 uL Gen Elute™ (Sigma-Aldrich, St. Louis, MO) and incubating for 3 h. After incubation, tubes were centrifuged at 13,000 g for 45min, and the pellet was aspirated and resuspended in 70% EtOH then centrifuged again at 13,000 g for 30min. The pellet was then dried for 20 min in a vacuum centrifuge and resuspended in 50 uL H_2O before quantification by Qubit as previously described. All fractions that resulting in successful PCR amplification (a total of 108 libraries) were sequenced on the Illumina MiSeq PE250 system at the UC Davis DNA Technologies Core (Illumina Inc., San Diego, CA). Following this, 16S rRNA gene processing and analysis were conducted as described previously to generate ESV and taxonomy tables that were then placed into the high throughput sequencing SIP (HTSSIP) workflow to detect ^{13}C incorporators. Briefly HTSSIP is an R package that uses high throughput sequencing data generated from SIP by making paired fraction comparisons across multiple density windows to identify likely ^{13}C incorporators (https://cran.r-project.org/web/packages/HTSSIP/vignettes/HTSSIP_intro.html) (Youngblut et al., 2018).

Metagenomics and functional gene determination

Soil samples collected approximately two weeks following manure application were used to further examine differences between the metagenomes of manure applied and zero manure applied soils. Here, 1 µg DNA per sample was extracted using DNeasy PowerLyzer PowerSoil kits (Qiagen Inc., Germantown, MD) as previously described from manure (n=16) and zero (n=16) treatment plot soils. A total of 32 total soil DNA samples were submitted to the UC Davis DNA Technologies Core for sequencing using two lanes of the Illumina NovaSeq S4 PE 150 (Illumina Inc., San Diego, CA). Sequences were analyzed using part of the assembly branch of the NASA GeneLab WGS metagenomics pipeline, the reproducible workflow is available at https://github.com/cErikson/GeneLab_Shotgun_Metagenomics _Pipeline. Briefly, Cutadapt was used to trim sequencing adapters and assembly was conducted by MegaHit2 (Martin, 2011)(Li et al., 2015). Open reading frames on the resulting contigs were predicted by prodigal and predicted amino acids were functionally annotated by KoFamScan using the KEGG database. Here, KEGG BRITE provides a hierarchical classification system that organizes genes by their presence in metabolic pathways (Hyatt et al., 2010)(Aramaki et al., 2020). Functional counts were gathered by BOWTIE2 by alignment of reads to the contigs (Langmead and Salzberg, 2012). Counts were then gathered by HTSeq (Putri et al., 2022).

Statistical Analyses

All statistical analyses were performed using the R environment. Variance of CUE and atom% ^{13}C-CO$_2$ between soil treatments, and glucose amendments was assessed by ANOVA with Tukey's HSD used as a post hoc test. To determine the dates where manure and zero incorporator abundance was significantly different between treatments, unpaired t-tests were performed on manure and zero at each timepoint. Wilcoxon signed-rank test was used to assess significant differences between functional gene Shannon diversity and Simpson's evenness. The fold change of functional genes abundance between manure and zero manure soils was conducted by the R package EdgeR; all reporting at FDR ≥ 0.01 and fold change ≥ 2.5 or ≤ 2.5. Here, significance of differences between functional gene abundance in carbon related metabolic pathways in between manure and zero manure was assessed by an overdispersed Poisson model as discussed in Robinson et.al. 2010 (Robinson et al., 2010).

RESULTS

The CUE of the soil community improves after manure application

Microbial CUE values were significantly higher in manure applied soils in comparison to soils with zero manure application after addition of 1mg/g of ^{13}C-glucose (ANOVA, $p < 0.05$). In soils receiving 3mg/g of ^{13}C-glucose, CUE was higher in manure applied soils in comparison to zero manure soils, though not statistically significant. The application rate of glucose was also a significant contributor to CUE with values on average 0.15 higher in soils receiving 1mg/g of ^{13}C-glucose when compared to soils receiving 3mg/g of ^{13}C-glucose (ANOVA, $p < 0.001$) (**Figure 2-1A**). Zero manure microcosms that received 3mg/g of ^{13}C-glucose higher atom% ^{13}C-CO$_2$ than those that received manure (ANOVA, $p < 0.001$). Similarly, zero manure soils receiving 1mg/g of ^{13}C-glucose had more atom% ^{13}C-CO$_2$ than manure (ANOVA, $p < 0.001$). When using 3mg/g of ^{13}C-glucose, atom% ^{13}C-CO$_2$ was higher when compared to microcosms receiving 1mg/g of ^{13}C-glucose amendment (ANOVA, $p < 0.001$) (**Figure 2-1B**).

Manure application adds unique ^{13}C incorporators

Using HTSSIP to identify ^{13}C incorporators in manure and zero manure soils revealed 102 exact sequence variants (ESV) that correspond to bacteria from the following phyla: *Actinobacteriota, Bacteroidota, Firmicutes, Gemmatimonadota, Myxococcota, Planctomycetota, and Proteobacteria*. In total, there were 61 ESV that belonged to microorganisms that incorporated ^{13}C in manure microcosms (incorporators) and 59 ESV in zero manure soils. Of the 61 ESV found in manure soils, 43 were determined to be unique to manure soils and were therefore not found in zero manure applied soils. Unique incorporators from manure applied soils belonged to *Actinobacteriota, Bacteroidota, Firmicutes, Gemmatimonadota, Myxococcota, Planctomycetota,* and *Proteobacteria*. Soils that received zero manure possessed 41 ESV that were unique to zero manure soils and not found in manure applied soils. These unique incorporators from zero manure soils belonged to *Actinobacteriota, Bacteroidota, Firmicutes,* and *Proteobacteria*. In manure applied soils, ESV from *Actinobacteriota, Proteobacteria,* and *Firmicutes* constituted most of the incorporators totaling 23, 13, and 21 of the incorporators respectively. The remaining 4 incorporators in manure soils were identified to be from the phyla *Bacteroidota, Gemmatimonadota, Myxococcota,* and *Planctomycetota.* Similarly, zero manure soils were also dominated by ESV corresponding to bacteria from the *Actinobacteriota, Proteobacteria,* and *Firmicutes* phyla, each with 24, 7 and 26 incorporators respectively with only 2 incorporators corresponding to *Bacteroidota*. Of all identified incorporators, ESV corresponding to 7 *Actinobacteriota*, 6 *Firmicutes*, and 5 *Proteobacteria* were not unique and therefore found in both manure and zero soils (**Figure 2-2**).

^{13}C incorporators found in manure applied soils increase after application

The abundance of all sequence variants determined to be ^{13}C incorporators in manure applied soils were tracked over the course of a two-year period. Throughout the two-year period incorporators detected in manure applied soils were significantly higher in manure soils than in zero soils on days 1, 35, 64, 262, 323, 343, and 357 of the field trial ((*t-test*, $p < 0.05$ all dates) (**Figure 2-3A**). Incorporators in manure applied soils exhibited fluctuating changes significantly more incorporators than in manure than zero manure applied soils in the summer growing seasons (days 1-87 and 301-431). Notably, incorporator abundance in manure applied soils increased at the start of each summer season before reaching a peak and declining. With first application event, incorporators in manure applied soils peaked on day 87 at the end of the first summer season before declining throughout the winter season. This trend was repeated on a shorter timescale following third manure application event, initiating the start of the second summer season. In this instance, incorporator abundance peaked on day 323 and then declined until the winter season where it increased briefly following manure application. In contrast, incorporators detected in soils that received zero manure, were similar between zero manure and manure applied soils only on days 1, 469 and 624 (**Figure 2-3B**). Incorporators detected in zero manure soils were less responsive to seasonality, with differences occurring only once in the first summer season on day 1 of the experiment and in the second winter season (days 451-665) on days 469 and 624. Overall, incorporators identified in zero manure soils appeared to fluctuate less frequently while being similarly present in both manure applied and zero manure soils.

Manure applied soils possess a greater metabolic capacity

Metagenomic sequencing of approximately 3.65 Tbp provided an average sequencing depth of 110 million reads per sample across 32 samples (n = 16 manure, n = 16 zero manure).

After sequence clean up and processing this resulted in a total of 6104 functional genes being found in our dataset. We found a significantly higher diversity of functional genes in manure applied soils compared to soils that received zero manure (*Wilcoxon test,* $p < 0.001$). These increases to diversity were in part caused by a significantly higher Simpson evenness in manure applied soils compared to those that received zero manure (*Wilcoxon test*, $p <0.001$). Together, soils receiving manure application possessed microbial communities that are significantly more diverse and even than zero manure soils (**Figure 2-4**). Of the 6104 genes detected in the soil metagenomes of both manure applied and zero manure applied soils, 147 were significantly higher in manure applied than zero soils (FC > 2.5, $p < 0.05$). Conversely, only 13 genes were found to be significantly lower in manure compared to zero manure applied soils (FC > -2.5, $p < 0.05$) (**Figure 2-5A**). We assessed the relative gene abundance in Brite pathways where genes can be categorized into metabolic categories (Aramaki et al., 2020). Specifically, we measured the relative abundance of genes in pathways related to metabolic and degradative processing of carbon compounds where we found 15 of the 31 pathways possessed genes with a significantly greater relative abundance in manure applied compared to zero manure soils (FC > 2.5, $p < 0.05$) (**Figure 2-5B**). These pathways correspond to ubiquinone and other terpenoid−quinone biosynthesis, porphyrin and chlorophyll metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, arginine and proline metabolism, arginine biosynthesis, Cysteine and methionine metabolism, glycine, serine and threonine metabolism, alanine, aspartate and glutamate metabolism, sulfur metabolism, nitrogen metabolism, methane metabolism, glyoxylate and dicarboxylate metabolism, amino sugar and nucleotide sugar metabolism, and pentose and glucuronate interconversions. No genes associated with metabolic and degradative processing of carbon compounds pathways showed significantly higher fold changes (FC > 2.5) in zero manure soils compared to those that received manure. Of the genes with a significantly higher fold change in manure applied soils, 5 were classified and able to receive corresponding annotations from the KEGG database. These genes corresponded to aspartate aminotransferase (FC = 4.90, $p < 0.05$), anaerobic sulfoxide subunit C (FC = 2.92, $p < 0.05$), anaerobic sulfoxide subunit B (FC = 3.64, $p < 0.05$), thiosulfate dehydrogenase (FC = 4.82, $p < 0.05$), and chitin synthase (FC = 10.39, $p < 0.05$). Aspartate aminotransferase was found in the phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, arginine and proline metabolism, arginine biosynthesis, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, nitrogen metabolism, and methane metabolism Brite pathways. The anaerobic sulfoxide subunit C, anaerobic sulfoxide subunit B, and thiosulfate dehydrogenase were found in the sulfur metabolism Brite pathway, while the chitin synthase was observed in the amino sugar and nucleotide sugar metabolism Brite pathway.

DISCUSSION

Soils that received more manure had a higher CUE, more carbon incorporators, and a more robust metabolism. Manure application led to improvements in the efficiency of the microbial carbon cycle. A higher CUE was measured in manure applied than zero manure soils, depending on the amount of glucose introduced. Though, CUE was not significantly different between the treatments, a recent meta-analysis of studies examining soil CUE utilizing glucose reports a decline in CUE with increasing amounts of glucose added (Islam et al., 2022). The glucose application rates used in our study captured this effect where 3 mg / g soil glucose resulted in a lower CUE than 1 mg / g soil. (**Figure 2-1A**). Despite this, there was significantly more atom%

$^{13}C\text{-}CO_2$ respired from zero manure soils under both glucose application rates. In systems with less nutrients, such as those in our zero manure compared to manure applied soils, microbes will typically spend more energy to acquire nutrients leaving less consumed carbon available for biomass (Craine et al., 2007; Schimel and Weintraub, 2003). This suggests that the microbial communities in zero manure soil had less available nutrients to build biomass so more was respired (**Figure 2-1B**). The extent to which a soil microbial community can absorb sudden influxes of carbon, increasing CUE, depends on the underlying metabolism of those compounds, which can change as manure application selects for more microorganisms more capable of performing that metabolic processing.

We used SIP to identify microorganisms that incorporated ^{13}C from the ^{13}C-glucose into their DNA in the two treatments. Though numbers of incorporators were similar , the community composition differed with members of the phyla *Gemmatimonadota, Myxococcota,* and *Planctomycetota* detected in manure applied, but not zero manure soils (**Figure 2-2**). Members of the phylum *Gemmatimonadota* are widely distributed and occupy a diverse range of soil habitats, being regarded as generalists with a versatile metabolism (Mujakić et al., 2022). Furthermore, most cultured *Myxococcota* are predatory bacteria that can utilize live microbial cells as well as plant and animal waste as food sources (Reichenbach, 1999). This suggests that ^{13}C from our ^{13}C-glucose may have been passed from one microorganism to *Myxococcota* through predation, though it's capacity to feed on plant waste also suggests it may have directly incorporated the ^{13}C-glucose from the surrounding environment. *Planctomycetota* are often found in agricultural soils utilizing manure (Buckley et al., 2006; Peng et al., 2021; Sharaf et al., 2021). Additionally, some members of *Planctomycetota* are observed to increase alongside degradation and decomposition of extracellular DNA in soil (Morrissey et al., 2015). As such, the incorporation of ^{13}C-glucose into

Planctomycetota may have been through the breakdown of extracellular DNA that had already been incorporated by the broader microbial community. Members of *Firmicutes* were active incorporators in both manure applied and zero manure applied soils, there were notably 7 unique *Firmicutes* incorporators found in manure soils, while there was only 1 unique *Firmicutes* incorporator found in zero manure soils. *Firmicutes* represent a well-studied phyla whose members often possess chitinolytic capability and therefore likely contribute to the breakdown of complex carbon compounds found in manure (Belova et al., 2018; Gooday, 1990). In a study utilizing SIP to track the fate of ^{13}C-chitin and identify chitin degraders, *Firmicutes* was found be one of the primary chitin degraders in soil (Wieczorek et al., 2019). The identification of a wide range of *Firmicutes* as a ^{13}C incorporators in our manure applied suggests they are also an active sink. Taken together, manure application selects a soil microbial community with a greater capacity to degrade complex carbon compounds, freeing up more labile compounds for the microbial community to access. Utilizing more complex forms of ^{13}C containing compounds, such as amino acids or plant material in SIP, may improve this understanding by linking a soil's capacity to degrade these compounds with simultaneous identification of microbial groups potentially facilitating that process (Pepe-Ranney et al., 2016).

Taxa involved in ^{13}C incorporation were more abundant in manure applied soils and primarily in the summer shortly following manure application (**Figure 2-3A**). A meta-analysis of manure's role in changing soil carbon revealed that manure application often represents an increase in soil organic carbon (Gross and Glaser, 2021). This suggests the microbial communities in our field trial had a robust supply of carbon to access from manure and therefore the abundance of microorganisms possessing the capacity to actively integrate carbon should increase following application. Notably, the extent that incorporator abundance increases in each season appears to vary throughout the experiment likely in response to immediate nutrient availability, which can be high or low depending on seasonal timing in agroecosystems (Kallenbach et al., 2019). Together, manure application likely drives a seasonal increase in manure ^{13}C incorporators that persist throughout the summer seasons, increasing the amount of carbon the microbial community can hold.

In contrast, ^{13}C incorporators found in zero manure soils rarely varied between treatments with only 4 instances of significant difference observed (**Figure 2-3B**). This indicates that zero ^{13}C incorporators are not specific to soil treatment, and manure applied soils possess both the carbon processing potential of soils that do not receive manure treatment as well as an additional set of microorganisms specifically curated by manure application. This indicates that manure application can improve soil CUE by establishing a microbial community that can hold carbon as it normally would in addition to a set of microorganisms that can add to that process. These results could clarify findings that suggest manure application improves soil microbial diversity such as in a recent meta-analysis of effects of organic vs. mineral fertilizers on soil microbial diversity, which found that functional as well as taxonomic diversity is higher in organic systems using manure (Bebber and Richards, 2022). In such a case, diversity would be improved through the simultaneous maintenance of the usual soil community, plus the addition of those from manure itself, or curated through its nutrient addition.

We utilized shotgun metagenomics to identify the genomic changes associated with manure application in soils (**Figure 2-4A**). With this method, we could identify functional genes found in both manure and soil, without being limited to a curated set of genes commonly selected by Biolog^{TM} Ecoplates, which target genes more commonly found in the fecal environment (Checcucci et al., 2021). Furthermore, the Simpson’s evenness of genes was higher in manure applied than zero manure soils (**Figure 2-4B**). This indicates that manure addition increased genes that were initially less abundant. In a study that assessed how the functional diversity of the microbial community in an oat field soil increased after supplementing mineral fertilizer with organic fertilizer, functional gene diversity was investigated. It was reported that organic supplements increased the evenness of microbial functional gene diversity, which led to greater utilization of carbon sources such as amino acids, amines, carbohydrates, carboxylic acids, and polymers (Zhang et al., 2021). Therefore, the organic nutrients added to the soil by manure application may increase microbial functional evenness through the selection of microbial groups with the metabolic capacity to break down and utilize those nutrients.

Adding manure to soil led to higher functional gene abundance within the microbial community compared to chemical fertilizer alone (**Figure 2-5A**). Combined with our observation that manure applied soils possessed a greater relative abundance (> 2.5 fold higher) of functional genes in most categorized metabolic pathways, this suggested that manure application selects for a greater metabolic functional potential (**Figure 2-5B).** In a study of pig manure impacts on soil communities, the authors presented similar findings by detecting more genes related to nucleotide metabolism and energy metabolism as well as absorption and biosynthesis of sulfate, suggesting a more robust metabolism in plots receiving manure compared to those receiving chemical fertilizer (Li et al., 2020). The increase of metabolic functional genes following manure application could be due to the diverse organic nutrients in manure feeding a certain group of microorganisms that can preferentially feed on or access those nutrients (Mendonça et al., 2011; Wallenstein et al., 2010). Therefore, manure application appears to select for a microbial community with a broader metabolic potential that is more capable of degrading and utilizing a greater variety of organic compounds.

Enzymes involved in biosynthesis increased in relative abundance in the manure amended soils. Aspartate aminotransferase, a key enzyme in the citric acid cycle which catalyzes formation of oxaloacetate (Belitsky, 2001) was successfully annotated and showed significantly greater relative abundance, in manure applied than zero manure soils (**Figure 2-5A**) (Belitsky, 2001). Other annotated enzymes were ones involved in sulfur metabolism and included the anaerobic sulfoxide subunit B, anaerobic sulfoxide subunit C, and thiosulfate (**Figure 2-5A**). This improvement to sulfur metabolism can promote the conversion of extracellular sulfate to biomass improving the capacity of the microbial community to grow, facilitating a larger biomass and higher CUE (Tabatabai et al., 1988; Zhang et al., 2016). Chitin synthase showed a substantially higher relative abundance in manure applied soils (**Figure 2-5A**). This enzyme is detected across all three domains of life, though most commonly it is associated with fungi (Gonçalves et al., 2016). The presence of growing fungi was found to be one of the largest factors influencing CUE in a study using structural equation modeling to determine the drivers of CUE in a model soil. Fungi were postulated to provide additional sources of organic nitrogen to bacteria and thus a general fungal function was important in promoting efficient community growth (Domeignoz-Horta et al., 2020). Taken together, functional genes more abundant in manure applied soils may promote a higher CUE.

In conclusion, manure application to soil increased CUE and selected for a group of carbon incorporators that were not as prominent in zero manure soils. These incorporators were responsive to manure application, increasing temporarily following manure addition to soil. As such, manure applied soils also possessed a greater metabolic potential with an increase of functional genes related to carbon metabolism. A study of manure inputs in rice paddy soils found that despite the likely anaerobic conditions, manure application was followed by an increase in copiotrophic taxa and genes responsible for nutrient cycling, such as nitrate and sulfur reduction and metabolism of organic compounds. The authors suggest that manure application could enhance soil biogeochemical processes through the selection of copiotrophic members of the soil microbial community that could more rapidly utilize the diverse organic matter in manure than oligotrophs or plants. Alternatively, using chemical fertilizers alone presents a situation where nutrients are readily available and therefore do not select for of microorganisms with diverse degradative or metabolic functions (Wang et al., 2021). These results and ours suggest that nutrients in manure likely feed a group of microorganisms that preferentially process and incorporate incoming carbon, leading to an increase in metabolic functional genes and therefore metabolic potential. We expand on these findings by also providing evidence that microbial groups responsive to manure application also improve CUE. To our knowledge, this represents the first case of linking CUE to SIP by using identical microcosms for simultaneous CUE and SIP assessments and then tracking those microorganisms in the field. Here, ^{13}C was traced through respiration as well as integration into microbial DNA, following the fate of ^{13}C-glucose both above and below ground. Together, our results demonstrate manure application contributes to a more efficient soil carbon cycle. Manure management decisions that result in field application of manure could contribute to a more sustainable agroecosystem in dairy farming systems.

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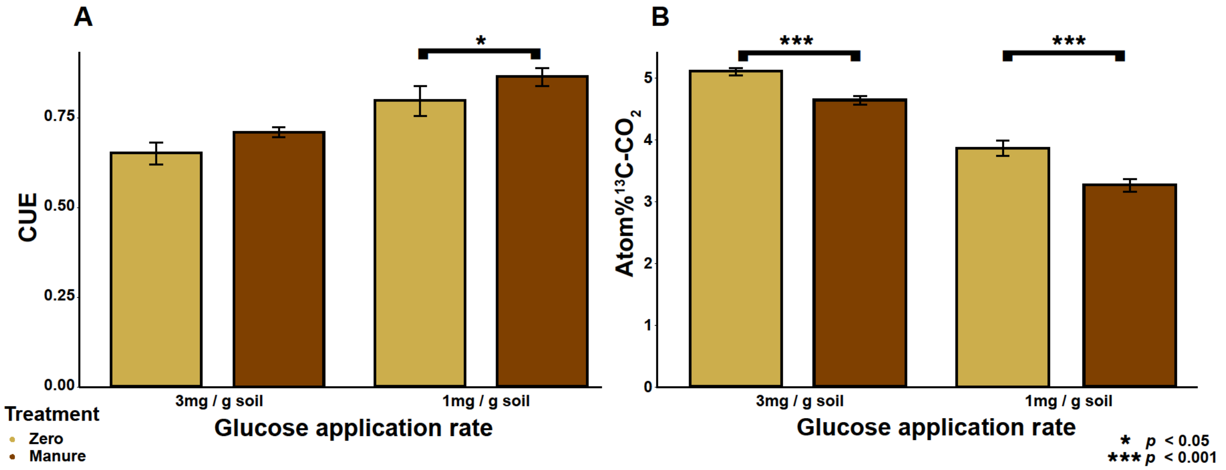


Figure 2-1. Carbon use efficiency and (A) and atom% ^{13}C-CO$_2$ (B) between soils that received zero manure (tan) and manure applied soils (brown) under two applications of glucose (3 and 1mg/g soil). Error bars represent standard deviations, where * represents $p < 0.05$ and *** represents $p < 0.001$.

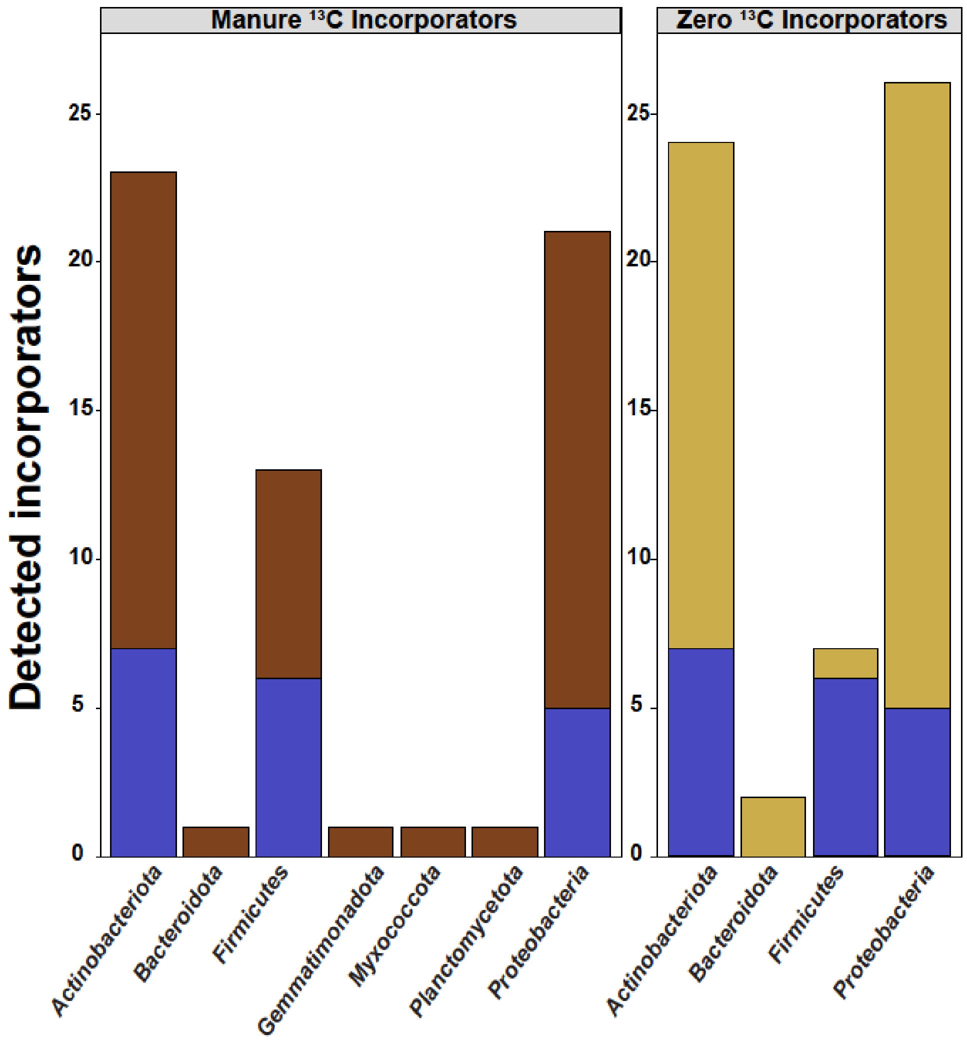


Figure 2-2. The 102 ^{13}C incorporators detected in manure and zero soils. In manure soils, ^{13}C incorporators were from the *Actinobacteriota, Bacteroidota, Firmicutes, Gemmatimonadota, Myxococcota, Planctomycetota* and *Proteobacteria* phyla. In zero manure soils, ^{13}C incorporators from *Actinobacteriota, Bacteroidota, Firmicutes, Proteobacteria* phyla were found. Incorporators only found in manure soils are brown, zero manure incorporators are tan and incorporators found across both soil types are blue.

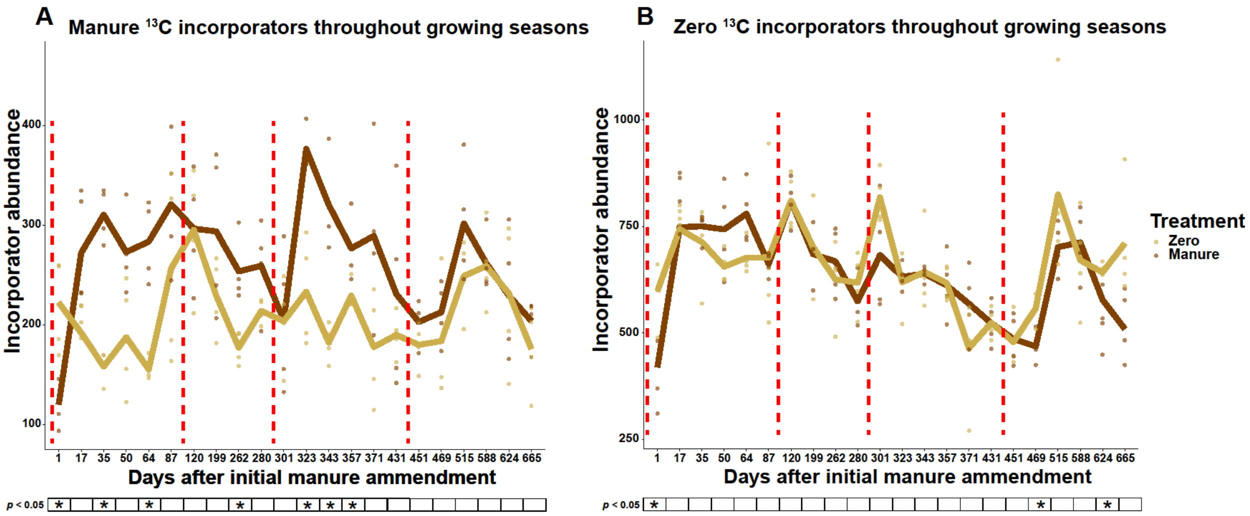


Figure 2-3. The abundance of sequence variants corresponding to ^{13}C incorporators found in manure soils (A) and zero manure soils (B) over the two year field experiment where manure (2.24 kg manure/m^2, dark brown) and zero (0 kg manure/m^2, tan) rates of manure were applied. Red dotted lines represent each manure application event. Significant differences (*p* < 0.05) between treatments are denoted for each timepoint beneath the graph.

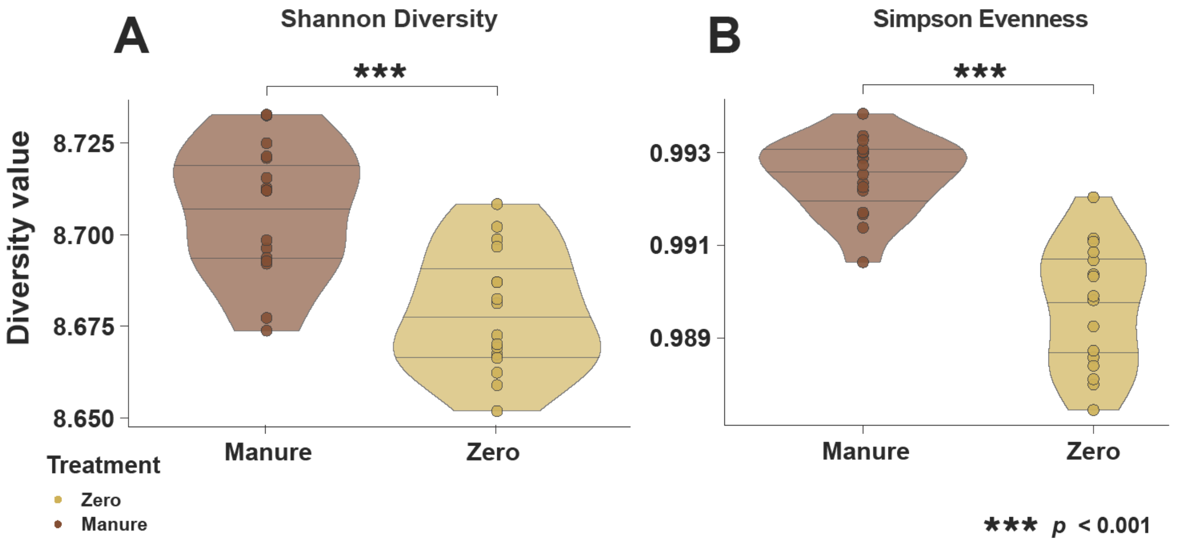
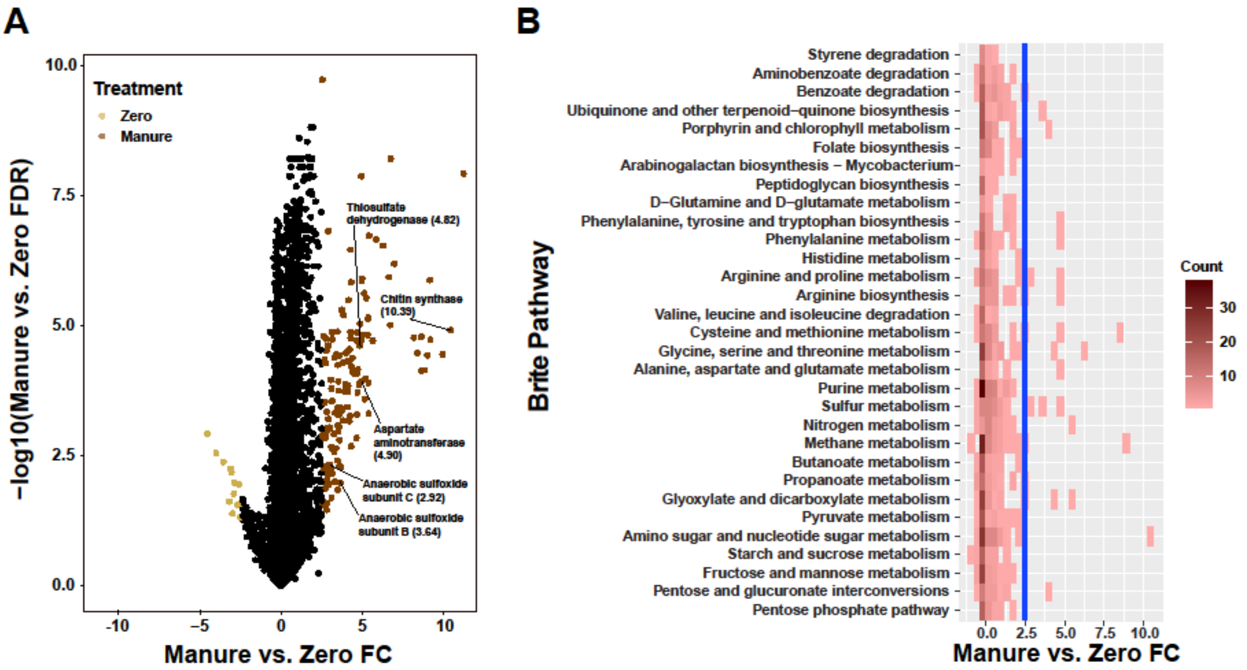


Figure 2-4. Shannon diversity (A) and Simpson's evenness (B) of genes detected in the metagenomes of manure (brown) vs zero (tan) soils. Significant differences of $p < 0.001$ are represented by ***.



<u>Figure 2-5.</u> A volcano plot of the statistical significance between (-log10(FDR) vs. magnitude of change (fold change, FC) of genes between soils that received manure vs. those that received zero manure (A). Genes found to be significantly higher ($p < 0.05$, ≥ 2.5 FC) in manure soils (brown) and genes significantly lower ($p < 0.05$, ≤ -2.5 FC) in zero soils (tan) are shown. Aspartate aminotransferase, anaerobic sulfoxide subunit C, anaerobic sulfoxide subunit B, thiosulfate dehydrogenase and chitin synthase are labeled in the figure along with their fold change. Genes found to be significantly different ($p < 0.05$) categorized by Brite pathway classifications for pathways related to carbon compound metabolism and degradation (B). The magnitude of fold change is expressed on the X-axis while darkness of red indicates the number of unique genes and their fold change in each pathway, darker reds representing greater counts. A blue line denotes the threshold where FC ≥ 2.5.