The Response of Soil Microbial Communities to Antibiotic Residues in Dairy Cattle Manure

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ABSTRACT

Antibiotic use in agricultural ecosystems has the potential to increase resistance to antibiotics in soil microbial communities since 40-95% of an antibiotic dose administered to livestock is excreted intact or as metabolites. Exposure to antibiotics is also known to alter microbial community composition, biomass, and physiology, but the potential influences of antibiotic residues on the essential ecosystem processes that microbes regulate, e.g., carbon and nitrogen cycling are not well understood. I investigated the effects of antibiotic residues associated with dairy cattle operations on soil microbial communities and the ecosystem processes they regulate. I examined the effects of antibiotic exposure on the biogeochemical functioning of soil microbial communities by measuring activity of extracellular enzymes associated with organic matter processing and nutrient mineralization in soils collected from dairy cattle operations across the United States. At each experimental station paired sites were identified by local managers that represented sites with high and low stocking rates of dairy cows who had been treated prophylactically with antibiotics to prevent mastitis. Responses varied among individual enzymes, but I found an overall significant decrease in total hydrolytic enzyme activity under high cattle stocking rates indicating a change in the functioning of the microbial community in soils exposed to antibiotic laden manure. Principle components analysis suggest that while some of the variation in enzyme activities are associated with the abundance of antibiotic resistance genes, soil organic matter (total organic, mineralizable, and particulate organic carbon) was the most significant variable accounting for differences in enzyme activities. This reflects an inherit challenge in studies of antibiotic exposure in agricultural landscapes: the difficulty of distinguishing direct effects of antibiotic residues from the organic matter and nutrient subsidy associated with manure applications. To address this concern I conducted a series of incubation experiments manipulating soils to isolate the influences of antibiotics, manure resource subsidies, and bovine microbiome inoculants into soils. Specifically, I examined soil respiration and antibiotic resistance gene counts using qPCR following treatment with cephapirin, pirilimycin and a positive and negative control. I found that pre-exposure to antibiotics and manure is important in modulating the response of microbial communities (soil respiration, and gene copy numbers of AmpC and TetO) to further antibiotic exposure. I conclude that antibiotics themselves have a direct effect on soil communities and their functioning that is additive to the effect of manure (i.e., as a resource subsidy). This effect is mediated by the history of previous exposure to antibiotics, i.e., cattle stocking density. These results suggest that antibiotic residues from dairy cattle operation may have significant effects on microbial communities and the biogeochemical cycling they regulate in agricultural ecosystems.

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GENERAL AUDIENCE ABSTRACT

Antibiotic usage has become a widespread health concern. As development of new antibiotics slows down and bacteria resistant to all but last resort antibiotics become more common the scientific and health communities have begun researching antibiotic resistance. Most antibiotics used in the United States are not used on people but are instead used on farm animals to promote growth and increase production. These antibiotics are normally given to animals before they are even sick in order to stop any potential infections. As a result of this many of the antibiotics used go through the animal unused and are therefore released in waste product like urine and manure. That same manure is often spread onto fields as a fertilizer to allow crops to grow. This means that antibiotics are being applied to fields and pastures along with manure. These antibiotics are then killing soil microbial communities (bacteria and fungus that live within soil) while simultaneously creating a store of antibiotic resistance genes. Antibiotic resistance genes are what render bacteria immune to antibiotics themselves. In addition to the immunity the soil microbes may be less efficient at nutrient cycling, the process through which nutrients in the dirt are transformed to a form useable by plants, meaning more fertilizers and manure may be needed to reap the same amount of crop from the same area of land. This research found that antibiotics, when applied with manure, are leading to changes in soil microbial communities as well as a decrease in ecosy+stem functioning. This work is significant because it indicates that antibiotic resistance has implications beyond just public health, it could be affecting food growth and have real economic consequences.

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General Introduction

Antibiotic use in livestock production has the potential to increase antibiotic resistance in microbial communities generally (Ghosh and LaPara 2007). Antibiotic resistance can also be spread directly to human pathogens from exposure to resistant organisms in the environment (Teuber, 2001), those pathogens are then resistant to antibiotics commonly used by medical doctors to combat disease causing microbes. Antibiotic resistance has come to the forefront as an important issue for human health, the World Health Organization calls it 'A complex global health challenge (WorldHealthOrganization, 2014). The implication of antibiotic resistance to humans stems from the importance of antibiotics themselves in treatment of disease, human and animal (Aminov, 2010; Hao et al., 2014; Knoblock-Hahn, Brown, & Medrow, 2016; Marston, Dixon, Knisely, Palmore, & Fauci, 2016). Antibiotic usage for both humans and livestock has been increased globally. Antibiotic use leads to the proliferation of antibiotic resistance genes (Pruden, Pei, Storteboom, & Carlson, 2006; Udikovic-Kolic, Wichmann, Broderick, & Handelsman, 2014).

Antibiotics have the potential to change the composition of soil microbial communities (Andersson & Levin, 1999; Nygaard, Lunestad, Hektoen, Berge, & Hormazabal, 1992). Changes in microbial communities may have impacts on ecosystem function as well. Since antibiotic resistance gene plasmids are costly to maintain and have measureable effects on the fitness of microbes (Bouma & Lenski, 1988; Helling, Kinney, & Adams, 1981) a constant addition of antibiotics to soil may lead to stresses on the soil microbial community as well as an increase of antibiotic resistance genes, which in turn can further stress a community. Since microorganisms facilitate many of the most essential biochemical and ecosystem processes in natural and

human systems, changes in the structure and functioning of soil microbial communities are a cause for concern, especially in in agricultural ecosystems. In cases were antibiotic additions are more sporadic the stress induced by the antibiotics is less evolutionarily forcing and microbes are forced into an evolutionary tradeoff. This means that microbes are in a tradeoff of whether or not to support additional antibiotic resistance genes. Maintaining antibiotic resistance genes takes energy and nutrients but has the potential advantage of conferring resistance to future antibiotic additions. In the absence of future antibiotic additions the maintenance of antibiotic resistance genes could be considered wasted resources since the antibiotic resistance genes would confer no benefit; the resources could be spent on growth instead.

The tradeoff soil microbe's face is the maintenance of antibiotic resistance genes. In the case of constant manure and antibiotic inputs, like areas highly stocked with cattle, antibiotic resistance genes become a necessity to survive but are easier to maintain with constant resource subsidies. In cases of sporadic additions, antibiotic resistance is beneficial when additions occur as they not only allow survival but then allow for a microbe to partake in the resource subsidy created by the combination of manure and dead microbes. In contrast, when manure isn't present those microbes are using resources to maintain the DNA, RNA and proteins associated with resistance. The microbes without antibiotic resistance will then be at a relative advantage to those with antibiotic resistance. The antibiotic resistance gene laden microbes use resources on maintenance of antibiotic resistance genes that could be used for other processes, like growth. Resources put toward growth also lead to obtaining more nutrients to support that growth. This means that nutrient cycling rates will increase in agricultural systems, a positive for agricultural managers. The situation becomes even more

complicated when one considers that manure in the absence of antibiotic residues may have an effect on antibiotic resistance.

Even in the absence of antibiotics manure could potentially increase antibiotic resistance through selection pressure. By adding nutrients into a system copiotrophic conditions are being created. Copiotrophic conditions are hypothesized to increase competition in microbial systems (Michalet et al., 2006).One form of competition used by microbes is antibiotics. This also means selection for antibiotic resistance genes will increase in order to survive antibiotics. In turn new antibiotics will be developed to allow for competition to increase. This strong selection quickly creates a system that is an example of the red queen hypothesis (Valen, 1973).Runaway antibiotic resistance is problematic for human and animal health. It is also important to the function of soil microbial communities that may be forced into a biological tradeoff that could fundamentally shift community composition. Soil microbial community composition could also be affected by the rumen microbes that survive the excretion process from the cow. Being exposed to antibiotics as well as living in a highly copiotrophic environment could make rumen bacteria particularly well suited to survival in highly trafficked sites that are receiving very high input levels of manure. Horizontal gene transfer of antibiotic resistance genes that developed in the rumen environment could also increase antibiotic resistance gene abundance in soil microbial communities. Runaway resistance through nutrient additions and horizontal gene transfer are two possible mechanisms through which manure may increase antibiotic resistance genes even in the absence of anthropogenic antibiotics (Knapp, Dolfing, Ehlert, & Graham, 2010). Runway resistance is largely the product of the heavy usage of antibiotics in animal agriculture.

Antibiotics are used for treatment of infectious mastitis in many animals, including cows (J. Barlow, 2011). Since cows infected with mastitis are unable to produce saleable milk (Seegers, Fourichon, & Beaudeau, 2003), dairy managers use broad spectrum antibiotics during the dry period in to curb potential infections. The dry period is the amount of time when a cow isn't milking and is given a chance to recover from the milking season. While the dry period has many potential benefits for the animal's health, it provides a major opportunity for mastitis to infect the teat. During milking, the teat is cleaned with an antimicrobial dips, and most cows are milked at least once or twice a day. Going from twice-daily cleanings to no cleaning at all leaves the mammary gland very susceptible to infection during the dry period. A common recommendation to help avoid mastitis during the dry period is the use of prophylactic antibiotics (Berry & Hillerton, 2007). Failure to proactively prevent mastitis, either through cleaning or antibiotics, can result in a clinical infection, known as acute mastitis.

The cost of treating a clinical infection varies by strain. Treatment for gram positive clinical mastitis costs an average $133.71 per head of cattle. Gram negative treatments cost an average of $211.03. Other sources put the average cost of infection at $95.30 per treatment, based on modeling software. The cost in the software is derived from the combination of the costs of culturing, antibiotics, lost profits from unusable milk, additional medicines (anti-inflammatories), and labor associated with administering treatments (Cha et al., 2011). From an economic standpoint, prophylactically using antibiotics makes sense to keep cows, and therefore dairies, profitable (Kiser, 1976). In the context of profitability and production antibiotics are an easy choice for farmers to gain production and efficiency with relatively little effort. Unsurprisingly antibiotic use in animal husbandry has become widespread.

An estimated 16 million kilograms of antibiotics are used in the United States, 70% of which are used sub-therapeutically in livestock production (Sarmah, Meyer, & Boxall, 2006). Sub-therapeutic treatment is intended to proactively prevent an infection rather than to treat an existing infection. If between 40% and 90% of administered antibiotics are then secreted by the animal receiving them (Gutierrez, Watanabe, Harter, Glaser, & Radke, 2010) potentially anywhere from 4.8 to 14.4 million kilograms of antibiotic is stored in manure. This manure is most often spread onto agricultural fields as a fertilizer, exposing soil communities to antibiotics (K. J. Forsberg et al., 2012; Popowska & Krawczyk-Balska, 2013).

The magnitude, regularity, and record keeping of the location of dairy cattle make dairy farms a useful model system through which to explore the effect of antibiotic resistance on soil microbial communities. Dairy farms are present across the nation which allows for across site comparisons to be drawn which allows conclusions to be drawn on a national scale rather than just locally. Additionally, antibiotics are given to dairy cattle regularly and those same cattle are allowed to graze in fields, this combination serves as a natural antibiotic amendment study that is applicable to any dairy operation that utilizes antibiotics. Information on an individual fields stocking rate is also recorded. This gives insight into the natural history of a site and allows classification of sites into low and high stocking rates. This classification allows for the comparison of the impact of pulses and presses of manure and therefore the pulses and presses of antibiotics to soil microbial communities. High stocking rate sites receive presses of manure because soils in these fields are receiving steady manure inputs from the high relative density of animals. The low stocking rate sites only receive pulses of manure since they have a relatively lower density level than the high stocking rate sites. Additionally, the lower density

means that manure is only reaching soil communities when an animal happens to pass and simultaneously secrete manure, this means that manure application to any one area is infrequent but does happen eventually. Overall, in low stocking rate sites manure will be a novel input whereas in the high stocking rate sites manure additions will be commonplace. I can take advantage of this dichotomy to examine the effects of manure, and therefore the antibiotics, that the cows are ingesting and excreting, on soil microbial communities.

Soil microbial communities are integral to agricultural management today. Our dependence on microbe's ability to cycle nutrients for plants, and therefore us, underpins our need to not only understand them but protect their functionality. Antibiotics and antibiotic resistance are being widely studied both from a human and animal health perspective. Antibiotic resistance genes are a subject of concern that even makes it to mainstream news. Researchers aren't investigating the health of the organisms receiving the brunt of antibiotic additions; soil microbial communities. I investigated the effect of manure and antibiotic additions on soil microbial communities with a focus on the potential ramifications upon soil cycling.

In chapter two I examined the response of extracellular enzymes to different stocking rates of cattle. By comparing dairies nationwide I determined that the main driver of extracellular enzyme activity was organic matter content. This indicates that in order to truly understand the effect of antibiotics and antibiotic resistance genes I need to separate out the effects of the manure itself. In chapter three using a series of incubations I tested the effects of manure, antibiotics, and manure associated bacteria.

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Effects of antibiotic residues on extracellular enzyme activity in dairy pasture soils

Introduction

Antibiotics are commonly used in livestock agriculture to proactively prevent infection and disease spread in herds and promote growth (Ungemach, Müller-Bahrdt, & Abraham, 2006). Antibiotic use in animal agriculture is a continuing human health concern because of its role in the transmission of antibiotic resistance, chiefly in pathogens (Swartz, 1989). In response to the concerns about antibiotic resistance the European Union banned the use of antibiotics to promote growth in (CEC, 2003). Antibiotics that are used on animals therapeutically can expose natural microbial communities to antibiotic residues, which then promote antibiotic resistance through selection pressure and/or horizontal gene transfer (Pruden et al., 2006). Antibiotic resistance gene spread can occur directly through contamination of animal products such as milk, beef, and poultry or via an indirect path like manure application to soils or settling ponds (Witte, 1998). While human health concerns about antibiotic exposure and resistance have received increasing attention (Ghosh & LaPara, 2007a), transmission of resistance to free-living microbial communities may alter their composition, activity, and/or the essential ecological processes they facilitate, *e.g.*, carbon storage and nutrient recycling.

Previous studies have shown that 40-90% of an antibiotic dose administered to dairy cattle is excreted either intact or as a metabolite from the livestock itself (Gutierrez et al., 2010; Kemper, 2008). Research has also shown that antibiotic use in livestock has the potential to increase the antibiotic resistance of microbial communities (Kevin J. Forsberg et al., 2012; Ghosh & LaPara, 2007a). The addition of antibiotic resistance directly alters the microbial

community structure and physiology of the microbes therein (Andersson & Levin, 1999; Liu, Wu, Ying, Luo, & Feng, 2012; Wepking et al., 2017). The shifts in microbial communities caused by antibiotic resistance could have significant consequences on essential ecosystem services such as decomposition and elemental cycling. Understanding the potential metabolic and physiological costs incurred by microbes maintaining antibiotic resistance genes may be an important component to understanding the potential effects of antibiotic use in animal agriculture.

The continuous development of antibiotics and antibiotic resistance genes places a regular selective pressure on microbes as they are forced to adapt to novel antibiotics (Hede, 2014). This regular selective pressure is a component of normal competition however, the level of selective pressure has likely increased due over the last 25 years due to greater use of antibiotics in both agriculture and human medical applications (H. Heuer, H. Schmitt, & K. Smalla, 2011). Antibiotics are naturally utilized by soil microbes for inhibition of competitors as well as for inter-cellular signaling (Martinez, 2008) and are therefore endemic to microbial communities. As anthropogenic antibiotics become more abundant in soil, antibiotic resistance genes may be under greater selective pressure. The more antibiotics that are put into a system the more antibiotic resistance genes will be selected for and therefore present in the system. This idea follows the same logic as the 'Red Queen Hypothesis' (Valen, 1973)The proliferation and selection of antibiotic resistance genes has potentially meaningful effects on microbial community structure and function; including but not limited to decomposition and elemental cycling.

The expression of antibiotic genes and the production of antibiotic resistance-associated proteins can be viewed as an opportunity cost for an individual microbe. Resources allocated to the translation of antibiotic resistance genes cannot be used for growth and reproduction. A microbial colony expressing antibiotic resistance genes has fewer resources to devote to other essential functions, including nutrient and substrate acquisition, growth, and replication. Figure 2.1 illustrates the concept of the opportunity cost of increased antibiotic resistance. Microbial resources are expended to maintain antibiotic resistance genes leaving fewer resources to invest in growth (Panel A), and in the acquisition of new substrate and nutrients through the production of extracellular enzymes. In the absence of antibiotic resistance genes more resources can be allocated to substrate and nutrient mineralization and acquisition and thus to growth of a microbial population (Panel B), however such a population may be more vulnerable to antibiotics (Panel D). In contrast the community with resistance may have greater potential growth upon the addition of antibiotics (Panel C) because the antibiotic resistance genes are already being expressed minimizing potential losses. In summary, nutrient cycling in a community with greater antibiotic resistance may be slower than in a community with little or no resistance because of a reduced investment by microbes into nutrient mobilizing enzymes. Upon the addition of antibiotics the growth potentials reverse circumstances wherein the antibiotic resistance gene laden community will have higher growth potential in comparison to the community without antibiotic resistance genes. The scenario wherein the microbial community has lower nutrient availability in the presence of antibiotic resistance genes may have negative consequences for agricultural ecosystems that rely on microbial recycling of nutrients, *e.g.*, dairy pastures.

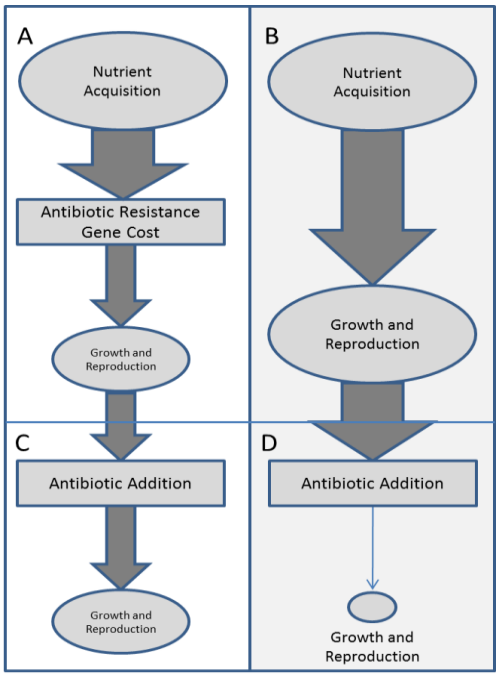

Figure 2.1. Arrow size relates to the amount of growth potential available. On panel A, a microbial system in which antibiotic resistance genes are present is represented. In panel B a microbial system without antibiotic resistance genes is represented. The size of the arrow is indicative of how much resource is being passed into growth. Panel A represents a loss of resources to antibiotic resistance genes, while panel B represents a system that expends no resources on antibiotic resistance genes. Panel C represents a continuation of Panel wherein antibiotics are added to the system. Panel D is the continuation of panel B showing the addition of antibiotics.

Antibiotics have two major modes of action, bacteriostatic and bactericidal (Pankey & Sabath, 2004). Bacteriostatic antibiotics stop growth and/or reproduction while bactericidal antibiotics kill bacteria. Cephalosporin is a class of bactericidal antibiotics, used on dairy cows, that works by inhibiting synthesis of the cell wall (Walsh, 2000). Pirilimycin is a bacteriostatic

antibiotic that prevents cellular replication by binding the 50s ribosomal subunit which blocks peptide exit from the ribosome (Tenson, Lovmar, & Ehrenberg, 2003), this effectively suspends cellular action because proteins are unable to be synthesized. An additional consideration is that each antibiotic resistance gene confers protection from specific antibiotics. This means that a microbe must not only possess and maintain an antibiotic resistance gene but have the proper gene that confers resistance to the antibiotic the microbe is being exposed to. By investing in resistance a microbe is essentially "betting" that antibiotics will be added to the system and the microbes' suite of antibiotic resistance genes will allow it to survive the addition. Microbes preferentially allocate their resources into nutrient acquisition to maximize their growth rate. Antibiotic resistance genes can increase or decrease microbial growth rates depending on the environment. For agricultural systems microbial growth rates are important because they are directly involved in nutrient acquisition and turnover, which provides much of the plant available nutrients (Schimel and Bennett 2004).

Manure is commonly collected and applied to agricultural fields (e.g., row crop and pasture systems) to subsidize soil nutrient availability ensuring maximum levels of crop or forage production, thus antibiotics administered to and subsequently excreted by livestock are routinely released to agricultural landscapes. The manure may also contain bacteria with associated antibiotic resistance genes. Additionally, manure additions represent a significant nutrient and carbon addition to the system (Sommer, 2013). Examination of the impacts of antibiotic inputs and manure to soil microbial communities must be examined together because they are applied together in modern animal agriculture systems.

Dairy cattle manure has been shown to increase soil organic matter, total and inorganic nitrogen, total and inorganic phosphorus, as well as various other micronutrients (Chang, Sommerfeldt, & Entz, 1991). Additionally, carbon and nitrogen amendments have been shown to change mineralization rates in lab experiments (R. Chen et al., 2014), indicating that microbial communities and their function are also being affected. Manure itself represents a significant resource subsidy, antibiotics notwithstanding, and nutrient effects on the soil have to be determined in order to see the effect of antibiotic resistance genes on soil microbial communities.

Microbial hydrolytic extracellular enzymes catalyze the hydrolysis of organic substrates into smaller molecules and constituent nutrients that are more soluble and often small enough to be absorbed by microbial cells (R. S. Sinsabaugh, 1994). For example Beta-glucosidase breaks up glycosydic bonds to release glucose (Jeng et al., 2011), a simple sugar that is more accessible to microbes. Extracellular enzyme activity is used to assess microbial activity levels, often with respect to the limitation of key nutrient elements (Kotroczo, Fekete, Toth, Tothmeresz, & Balazsy, 2008{Sinsabaugh, 2002 #212; Luo, Meng, & Gu, 2017). Microbial nutrient requirements and resource allocation are a trade-off. If a microbe has enough of a certain nutrient then it would not expend resources to create an enzyme to acquire more of that nutrient (Allison, Weintraub, Gartner, & Waldrop, 2011; Mooshammer, Wanek, Zechmeister-Boltenstern, & Richter, 2014). Based on the economic theory put forward by (Allison et al., 2011) the extracellular enzymes present in the soil are a reflection of the demands of the soil microbial community. The activity level of individual enzymes indicates the demand for that particular substrate; thus the demand extracellular enzyme activity for a substrate is a good indicator of

nutrient cycling (Luo et al., 2017). Each extracellular enzyme has a specific mode of action that is tied to acquiring a specific substrate. Specific enzyme activity levels will indicate demand for a substrate and therefore indicate ecosystem processing.

The degradation of cellulose is integral to carbon cycling in almost all environments. Cellulose is the most abundant organic compound in the biosphere compromising nearly half of the organic matter synthesized by photosynthesis (Eriksson, Blanchette, & Ander, 1990). The carbon in cellulose must be made available to microbes through the action of enzymes; beta-glucosidase and alpha-glucosidase both catalyze the hydrolysis of oligosaccharides into more labile monosaccharides (Dick, 2011). Specifically beta-glucosidase is important to the breakdown of cellulose into glucose by hydrolysis of glycosydic bonds; alpha-glucosidase similarly hydrolyzes bonds to release an alpha-glucose (Dick, 2011); cellobiohydrolase is another enzyme utilized in the breakdown of cellulose, it can additionally be used by microbes to breakdown other beta1-4 glucans (Ljungdahl & Eriksson, 1985). Beta-glucosidase and cellobiohydrolase are the two most commonly measured carbon-acquiring enzymes (Allison, Sinsabaugh, & Weintraub, 2007)) but there are other enzymes that can break down carbon into simpler forms. In addition to alpha-glucosidase, beta-glucosidase and, cellobiohydrolase I also measured beta-xylodase and n-acetyl-beta-glucosaminidase as carbon acquiring enzymes (Elzobair, Stromberger, & Ippolito, 2016; Robert L. Sinsabaugh et al., 2008). N-acetyl-beta-glucosaminidase serves a dual purpose. In addition to carbon acquisition it also aids in freeing nitrogen from complex molecules. This is achieved by cleaving beta 1-4 glycosydic bonds in molecules that contain N-acetylglucosamine. N-acetylglucosamine is commonly found in chitin and peptidoglycans (R. L. Sinsabaugh, Reynolds, & Long, 2000).

Nitrogen and phosphorus are often the limiting nutrient for primary productivity in most ecosystems (Elser et al., 2007). Nitrogen and phosphorus are typically required at proportionally lower amounts than carbon, but unlike the open cycle of carbon, these nutrients are internally recycled among plants, decaying organic matter, microbes, and readily available inorganic pools. Much of the turnover of nitrogen and phosphorus in soil ecosystems is facilitated by microbes depolymerizing organic compounds in soils through the release of extra-cellular enzymes that cleave inorganic nutrients, e.g., nitrate, ammonium and phosphate from functional group or low molecular weight organic compounds (Schinnel and Bennett 2004). In this study I measured N-acetyl-beta-glucosaminidase to represent microbial demand for nitrogen, and alpha-phosphatase (acid-phosphatase) to represent microbial demand for phosphorus (Robert L. Sinsabaugh et al., 2008).

The goal of this study is to examine the effects of antibiotic residues on nutrient cycling; extracellular enzyme activity characterizes the energetic (carbon) and nutrient (nitrogen and phosphorus) demands of soil microbial communities. I examined the differences between two manure application levels in cattle pastures, as determined by stocking rates in a nationwide study of soils at dairy farms. I used extracellular enzyme activity to elucidate the differences in microbial nutrient cycling between sites under high and low levels of cattle stocking densities. Sites with high levels of cattle stocking and therefore manure, will be subject to constant antibiotic inputs and selection but may also have the requisite antibiotic resistance genes built up to survive the additions. Sites that have low stocking rates and therefore rarely receive manure additions may be unaccustomed to antibiotic additions. Low stocking rate sites will not have the suite of antibiotic resistance genes necessary to easily survive antibiotic additions

however; they will also be relieved of the metabolic cost associated with maintaining antibiotic resistance genes and their subsidiary proteins. I sought to examine the differences between high and low cattle stocking rates in nutrient cycling via extracellular enzyme activity.

Methods

Nationwide Site Description

Soil samples were collected from top 10 centimeters of soil at agricultural experimental stations across the continental U.S. Institutions included Vermont (VT), Georgia (GA1), George Madison (GA2), Cornell (NY), Washington (WA), Florida (FL), New Hampshire (MH), Mississippi (MS), West Virginia (WV) and Kansas (KS). Managers at each site sent two soil samples: one soil was taken from an area that is heavily traveled by dairy cattle and is presumed to have heavy inputs of manure; hereafter 'High'. A second sample was taken from an area of low dairy cattle traffic and assumed sporadic manure inputs, these sites are labeled 'Low'. The goal of the study is to compare soil enzymatic activity between high and low sites to determine what effects dairy cow manure inputs have on soil microbes and their functioning (Wepking et al., 2017).

Enzyme Activity Procedure

The activity level of six different enzymes was estimated using fluorescence measurements. The hydrolytic enzymes assayed were (β-1-4-glucosidase (BG), β-1-4-N-acetylglucosaminidase (NAG), α-1-4 glucosidase (AG), alkaline phosphatase (AP), beta-D-cellobiohydrolase (CHB), β-xylodase (XYL). Soil slurries were created by adding 0.25 grams of soil to 125 mL of a neutral buffer solution (pH ~ 7). Slurries were created by blending buffer and soil then continually stirred until sampled into 96-well plate. Each plate included reference

standards, sample absorbance, sample quenching and, negative controls. All wells included 200 µl of neutral buffer solution and 50 µl of sample. Reference and sample quench standards were created with 50 µl of 200 µM methylumbelliferone (MUB). Negative controls received an additional 50 µl of buffer. Samples received 50 µl of soil slurry. All samples were replicated eight times within the plate. Fluorescence was measured after adding 15 µl of .5M NaOH. Measurements were done using an Infinite M200 Pro, Tecan Inc. (Saiya-Cork, Sinsabaugh, & Zak, 2002).

Microbial Biomass Procedure

Microbial biomass was determined through chloroform fumigation-extraction (Noah Fierer & Schimel, 2002). Samples were weighed into glass tubes, 20 grams per tube and two tubes per sample. The soils were then mixed with 40 mL of .5M K_2SO4. One tube from each sample set received .5 ML of ETOH free chloroform. Tubes were then capped and shaken for 3 hours at 150 rpm. All samples were then gravity filtered and samples that received chloroform were bubbled (O_2) for 1 hour to remove excess chloroform. Samples were then frozen and stored at -20 °C until they were subjected to TOC and colorimetric analysis (N. Fierer & Schimel, 2003).

Statistics

ANOVAs were performed using the AOV function in R and R-studio with alpha set at 0.05. For post hoc analysis we used a Welch's T-Tests between site and treatment combinations with alpha set at 0.05. Normality was not tested for as an even amount of samples were run on both sites allows for non-normality (Box, 1954). Principal coordinates analysis done in R and R-

studio. Methods for using principal coordinates from the environmental PCA (Fig 2.10) as loadings for the response PCA (Fig 2.11 and 2.12) are taken from (Burke, 1989) Linear models were created in R and R-studio using the LM function.

Results

A two way analysis of variance showed that enzymatic activity for each individual enzyme measured differed significantly between low and high cattle stocking rate, and among geographic sites (Table 2.1). The interaction between geographic site and stocking rate was also significant. The analysis of variance table shows very high levels of significance for all factors and interactions, table 2.1 displays this information. Microbial biomass for each site showed that high traffic sites had higher levels of carbon with the exception of the New Hampshire site (Fig 2.2). The high traffic sites on average have a lower enzymatic activity per unit of microbial biomass carbon then the low traffic sites. This indicates low traffic sites are exhibiting higher levels of enzyme activity. Beta-xylodase hydrolytic enzyme activity (Fig 2.3) was significantly different between the high and low cattle input levels ($p=<0.001$), with low input sites exhibiting higher levels of enzyme activity in all sites except for the soils taken from the New Hampshire. Alpha-glucosidase enzymatic activity levels did not exhibit a significant difference ($p=0.301$) between high and low cattle input levels (Fig 2.4). Beta-glucosidase enzymatic activity was also measured, and a significant difference between high and low cattle traffic levels was observed ($p=>0.001$) in activity levels corrected to microbial carbon. I observed that the low cattle traffic sites have greater enzyme activity levels than the high cattle traffic sites in (Fig 2.5), with the exception of the samples collected from New Hampshire. Beta-D-cellobiohydrolase activity levels were measured in the nationwide samples. The activity levels

between high and low cattle traffic levels contain a significant difference (p=0.009) in beta-D-cellobiohydrolase levels corrected for microbial carbon. In this case low cattle traffic sites saw higher enzyme activity levels, as seen in figure 2.8, with the exception of soils taken from New Hampshire sites.

I detected a significant difference (p=>0.001) between low and high cattle traffic levels in alpha phosphatase activity levels corrected with microbial carbon. Figure 2.6 indicates that every site but New Hampshire exhibited more activity in low traffic sites than in high traffic sites. N-acetyl-beta-glucosaminidase activity was measured for each sample taken. A significant difference (p=>0.001) in N-acetyl-beta-glucosaminidase activity levels, corrected with microbial carbon, was found in the activity levels found in figure 2.7. In all site comparisons, except for New Hampshire samples, there was a higher enzyme activity levels in low cattle traffic sites.

Total measured hydrolytic enzyme activity. Figure 2.9 illustrates the total measured hydrolytic enzyme activity, i.e., the sum of the six different hydrolytic enzymes measured. There is a significant difference between the high and low cattle traffic sites(p=<0.001); in every location but New Hampshire the total measured hydrolytic enzyme activity levels are higher in low cattle traffic soils relative to high cattle traffic soils. Figure 2.10 illustrates the site by site comparison of total measured hydrolytic enzyme activity. Table 2.1 includes the results of all the anovas ran for enzyme activity.

Principal coordinates analysis of the environmental variables depicts the variation among soil parameters (Fig 2.11). The soil parameters included in this principal coordinates analysis were pH, microbial carbon, microbial nitrogen, mineralizable carbon, mineralizable

nitrogen, particulate organic carbon, total carbon, total nitrogen, fungal to bacterial ratio, *TetO* gene abundance, *AmpC* gene abundance, *TetW* gene abundance, and *ErmB* gene abundance. This environmental space is a graphical representation of variation within the soil environment. In the graphical space of the analysis markers that are close together have more similar biogeochemical environments than markers that are further apart. Principal coordinate 1 (PC1) explains 95 percent of the variance and is heavily loaded with total carbon (TotC,-0.814) as well as mineralizable carbon (MinC,-0.411) and particulate organic matter carbon (POMC) (-0.403). Principal coordinate 2 (PC2) explains an additional 5 percent of the variance and is also heavily loaded by POMC (-0.709) and MinC (0.698). The low cattle density sites cluster in a quadrant of the ordination space illustrating a negative relationship with total carbon and a positive relationship to PC1.This shows that variation among sites is largely driven by amount of organic matter in the system.

The principal coordinates plot of enzyme activity across environmental space (Fig 2.12) shows that high density sites tend to cluster to the right, opposite of total measured hydrolytic enzyme activity. Low density sites are spread throughout the area indicating a less similar environment. All other enzymes also correlate negatively on PC1 away from the cluster of high density sites. PC1 is mainly loaded with total enzymes (-0.859), as well as BG (-0.204), AP (-0.45), and NAG (-0.1). PC2 is mainly driven by AP (0.669) and BG (-.0702). Additionally, PC2 is loaded with CHB (-0.137) and All (-0.182). This indicates that AP and BG are the two dominant enzymes driving the significant differences between high and low cattle stocking rates.

Figure 2.13 excludes the total measured hydrolytic enzyme activity to allow for a clearer picture of enzyme activity against environment. Similar to figure see that principally the high

sites cluster positively on PC1. PC1 is predominately loaded by AP (-0.97) with NAG (-0.185) and BG (-0.141). PC2 is loaded heavily with BG (-0.959), with CHB (-0.212), and AP (0.165). This indicates AP is driving most of the variance structure in enzymatic activity with BG accounting for a moderate amount as well. Linear modeling shows that only in the case of total hydrolytic enzymes can any meaningful predictions from the environment be seen. The model of total hydrolytic enzymes is heavily influenced by PC1 of the environmental data, indicating that environmental factors are important to understanding total hydrolytic enzyme activity.

Discussion

I used dairy cattle stocking rates across a national network of sites to study the putative effects of antibiotic inputs from manure on soil extracellular enzymatic activity. I found that there was a significant difference between high and low cattle stocking rates for of the study sites in both total measured hydrolytic enzymatic activities as well as for specific nutrient-acquiring enzymes, e.g. phosphorus as indicated by phosphatase. There was a significant difference in total measured hydrolytic enzymatic activity between soils exposed to high and low cattle densities and antibiotic resistance gene exposure in 9 of the 10 sites (Fig. 2.9). Analysis of variance indicates that high and low input sites are significantly different in total measured hydrolytic enzyme activity per gram of microbial carbon.

The hydrolytic enzyme data was corrected back to microbial carbon in order to discern differences between the high and low sites. As suggested by (Allison et al., 2007) in order to compare sites to one another enzyme activity should be normalized to microbial carbon or soil organic carbon (Sinsabaugh et al. 1994). The correction ensures that the effect being seen isn't

due to the density of microbes but to the per capita allocation to enzyme production. This effect was especially pronounced in the high versus low cattle stocking sites. High stocking sites had greater enzymatic activity and microbial carbon than the sites with low stocking. The only exception to this was the New Hampshire sites which have lower microbial carbon levels in the high traffic sites (Fig 2.2). Upon correction with microbial carbon, the data show that the low cattle traffic sites have higher levels of enzymatic activity, with the exception of the New Hampshire sites. Additionally, t-tests (Table2.2) show that significant differences exist between high and low input sites at each site for each measured enzyme. Significantly different levels of enzymatic activity indicate differences in nutrient demands between high and low cattle traffic sites (Fig 2.9). This suggests that the nutrient demands of the soil microbial communities are significantly influenced by high inputs of manure and antibiotics into the system, which appear to be altering the structure and functioning of soil microbial communities. This conclusion is corroborated by (Wepking et al., 2017) whom reported that antibiotic exposure influenced carbon use efficiency in these sites. Manure inputs represent a significant subsidy of nutrients, microbes, genetic information, as well as antibiotic residues which are affecting the soil communities they are being added to.

Our data suggests that the majority of change exhibited between high and low cattle input levels is due to the organic matter subsidy represented by manure inputs. This is inferred from the relationship between total carbon and enzymatic activity in multivariate ordinations. Organic matter is also comprised of more than just carbon; it is rich in phosphorus, nitrogen and micronutrients. Figure 2.11 illustrates the relationship between total carbon and input levels. The total carbon vector strongly points away from the cloud of low input sites. The total

carbon arrow also represents all measured organic matter fractions including total organic C, particulate organic matter, mineralizable carbon, and microbial carbon. Similarly (Luo et al., 2017) found similar results with SOM significantly affecting the activity levels of AG,BG, and NAG; they concluded that hydrolytic enzyme measurements are a good indicator for elemental cycling furthering the confidence I have in the selected measurements. Figure 2.11 also has each of the antibiotic resistance genes weighted on vectors. In comparison to the size of the carbon associated vectors there not as much variance explained through examining the quantity of antibiotic resistance genes. This does not indicate that antibiotics are not playing a role in this system or that the scenario presented in figure 2.1 is null. The results of the principal coordinate's analysis in figure 2.11 do indicate that carbon is an important variable that is a bigger factor in differentiating sites

Organic matter appears to be the main environmental driver of differences between enzyme activities in high and low cattle input sites. The low input sites all cluster in negative space to total carbon suggesting that a lack of organic matter and nutrients is what makes low sites similar, and drive their higher enzyme activity levels. Fig 2.12 illustrates the relationship between the environment and enzymatic activity, showing that enzyme activity (mainly total enzyme activity) is negatively correlated to high cattle input sites, further indicating that enzymatic activity has a negative relationship with total carbon and this organic matter. Many of the enzymes I measured were for acquisition of carbon. Presence of end products may inhibit extracellular enzyme activity (Mandelstam & Jacoby, 1965). The large amount of SOM in high input sites may inhibit hydrolytic enzyme activity. In order to examine individual enzymes and further examine the difference of effect between antibiotics resistance genes and carbon

Figure 2.13 shows the relationship without the total enzymatic value; which then indicates that the two enzymes driving this trend are AP and BG (by length of lines).

BG allows for the degradation of cellulose by hydrolyzing glycosydic bonds resulting in the release of glucose (Jeng et al., 2011). BG activity correlates away from sites with high stocking. This agrees with our environmental data as the high sites are enriched in total carbon and therefore may have less need for carbon acquisition enzymes. Additionally, the labile carbon could repress enzyme activity of carbon acquiring enzymes, like BG (Collins et al., 2008). This means that the difference in carbon acquiring enzyme activities could be derived from a shift in microbial activity or from a buildup of carbon. The manure subsidies present in the high traffic sites could account for both carbon buildup and a shift in microbial activity. AP activity is also strongly associated with this trend. AP seems to trend away from most sites indicating that generally phosphorus is not lacking in any sites. Since manure is being added to all sites this indicates that any level of manure addition may relieve phosphorus stress and therefore lower AP activity levels. The shift in AP enzymes as well as carbon acquiring BG supports the idea that the shifts I see are from microbial shifts and not just carbon repressing enzyme activity. This also indicates that carbon is driving the relationships and not antibiotics.

Linear models for each enzyme (Table 2.3) indicate that carbon alone can explain 32% of total enzymatic activity in all sites, this relationship is developed from all sites and input levels. Other studies have shown the importance of temperature and moisture to enzyme activity (Collins et al., 2008), which may explain the site by site variation. Soil texture can also have an effect on the enzyme activity (Burns et al., 2013; Song, Han, Zhu, & Herbert, 2012). This is because it can be difficult for enzymes to reach their intended substrates in soils with high clay

content. Clay then represses enzyme activity, which results in lower readings. Since I wanted to look at the effect on enzymes as a whole and not site by site I decided to include a summation of total measured hydrolytic enzymes to be able to look at trends across all sites and not just artifacts of individual sites. Despite the site differences the strength of the relationship between total measured enzymatic activity and carbon confirms that manure is an influential subsidy of nutrients that alters soils, and may be more of an effect on soil microbial communities that then antibiotic residues that accompany it.

Previous studies (R. Chen et al., 2014) have shown that raw nutrient subsidies have effects on microbial activity levels, and our findings are in line with this conclusion. Enzymatic activity is a proxy for nutrient demand and since our high sites show a drop in activity levels this indicates manure is acting as a nutrient subsidy. Interestingly, a different study finds that in soils exposed to manure that AmpC (antibiotic resistance gene) gene copy number is an important variable in determining stress of the microbes. The authors also note that manure and soil carbon were not controlled and thus merit consideration and that manure exposure increases antibiotic resistance gene copy numbers (Wepking et al., 2017). Therefore to properly investigate the effect of dairy associated antibiotics on soil microbes the raw nutrient subsidy of manure has to be separated out, other studies corroborate the narrative that nutrient subsidies alone can increase antibiotic resistance (Udikovic-Kolic et al., 2014). With such a significant amount of variation being explained by organic matter alone regardless of the exposure to antibiotic resistance genes, it's difficult to distinguish the potential influence of antibiotic resistance genes or antibiotics themselves on soil microbial communities from the large resource subsidy represented by manure inputs.

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Distinguishing the effects of manure from antibiotic residues on soil microbial communities

Introduction

A large percentage of antibiotics dosed to animal livestock are excreted into the soil, for example from 40 to 95% of an antibiotic dose administered to livestock is excreted intact or as a metabolite into soil (Gutierrez et al., 2010). Previous work has shown that exposure to these antibiotics and antibiotic residues can directly alter microbial community composition, biomass, and physiology (Andersson & Levin, 1999; Liu et al., 2012). The addition of antibiotic laden manure to soil microbial communities increases antibiotic resistance gene counts (Ross & Topp, 2015) indicating that antibiotics have an active effect on the soil microbial community. The study (Ross & Topp, 2015) included several different treatments including raw manure, digested manure, dewatered manure, and composted manure all of which showed a significant increase in antibiotic resistance gene counts in soil microbial communities over one week. Antibiotic resistance genes are known to change soil microbial communities , for example, (Ghosh & LaPara, 2007a) reported a change in the type of bacteria present wherever chlortetracycline- resistant genes were present in sufficient amounts. This shift in microbial community could affect ecosystem processes by creating a community that isn't structured for efficient growth and nutrient acquisition. Instead the soil microbial communities also may have to devote resources to acquisition and maintenance of antibiotic resistance genes. Maintaining antibiotic resistance genes is energetically costly taking away from potential growth. Such changes to microbial communities following antibiotic exposure could have significant consequences on essential ecosystem services, e.g. decomposition and nutrient cycling

(Wepking et al., 2017), which are relevant to issues of broader environmental concern, such as carbon sequestration and soil fertility.

Antibiotics naturally occur in soils and are utilized by microbes to inhibit competitors as well as for cell to cell signaling (Martinez, 2008), which in turn necessitates some natural level of antibiotic resistance. Therefore, a level of selection is placed on antibiotic resistance genes naturally as well because they are necessary for communication and competition between microbes which both can increase production of a community. This means that antibiotic resistance genes are naturally present in soils without exposure to anthropogenic antibiotics, but the level of antibiotic resistance genes has likely increased due to greater use of antibiotics in medical and agricultural applications (Holger Heuer, Heike Schmitt, & Kornelia Smalla, 2011). As antibiotics become more common in soil, there is increased selection pressure for microbial antibiotic resistance.

Antibiotic resistance genes become more common via two potential mechanisms. First, an antibiotic resistance gene could be transferred through horizontal gene transfer. The most likely method of horizontal gene transfer is transformation, where DNA is taken up by bacteria from the environment and is added into the bacteria's genome (Chen, Christie, & Dubnau, 2005). Transformation often occurs when during changes in a bacteria's environment, including nutrient subsidies (Thomas & Nielsen, 2005). Since the antibiotics and resistance genes are being added in tandem with manure in most livestock operations this creates an ideal situation for transformation to take place. Additionally, common genes are more likely to be picked up (Lopatkin, Sysoeva, & You, 2016) through transformation. Since manure microbes are also exposed to antibiotics there is a good chance antibiotic resistance genes are common within

manure. The second possible method of horizontal gene transfer is conjugation. In conjugation two cells in direct contact can exchange plasmids (Frost & Koraimann, 2010), meaning the antibiotic resistance gene must be contained on the plasmid itself. Studies estimate that this may be the most common method of horizontal gene transfer (Lopatkin et al., 2016) because there are many potential receptors for plasmids (Klumper et al., 2015) and plasmids often carry antibiotic resistance genes (M. Barlow, 2009; Holmes et al., 2016). Finally, horizontal gene transfer can occur through transduction. Transduction is mediated by phages and requires specific receptors (H Ozeki & Ikeda, 1968), it is not considered to be an important mechanism of antibiotic resistance gene spread (Lopatkin et al., 2016) but antibiotic resistance genes can be horizontally transferred via transduction (Drulis-Kawa, Majkowska-Skrobek, Maciejewska, Delattre, & Lavigne, 2012).

The second method of antibiotic resistance gene proliferation is natural selection of existing genes within the microbial metagenome (Ghosh & Lapara, 2007b; Martinez, 2008). Selection will mandate a certain level of antibiotic resistance in order to survive in the environment as more natural antibiotics are created (Bennett, 2008). As antibiotics are transferred into the environment through agricultural antibiotic use the selection pressure on antibiotic resistance genes will increase above the natural level. In order to survive and proliferate in an environment laden with veterinary antibiotics antibiotic resistance will be a necessity. Antibiotic resistance genes will be heavily selected for and therefore proliferate throughout the environment. Between selection pressures mandating antibiotic resistance genes and horizontal gene transfer microbes will become increasingly laden with antibiotic

resistance genes. Agricultural antibiotic use is increasing the amount of antibiotic resistance genes in the soil microbial community (Holger Heuer et al., 2011)

An abundant vector for antibiotic residues in agricultural ecosystems is manure, which itself is a substrate for soil bacteria. Manure has substantial levels of carbon, nitrogen, phosphorus, as well as its own resident microbial community (Nicholson, Groves, & Chambers, 2005; Pell, 1997). Additions of organic carbon and mineral nitrogen have been shown to accelerate the activity of soil microbes in organic matter decomposition (priming) and increase the gene copy numbers of antibiotic resistance genes (R. R. Chen et al., 2014). This indicates that resource additions alone changes soil microbial activity levels and antibiotic resistance, necessitating the consideration of antibiotic exposure and nutrient subsidies separately. The proliferation of antibiotic resistance genes through horizontal gene transfer, selection, and substrate additions must distinguished to fully understand the effects of agricultural antibiotic use on microbial communities and ecosystem processes.

Given that nutrient additions change community composition, looking at the effects of antibiotic residues in manure additions without considering or controlling for other factors may result in misinterpretation of results. Manure consists of three major components that can affect antibiotic resistance gene levels; antibiotic residues, resources and, the addition of antibiotic resistance genes in living and dead rumen biota. Resource subsidies select for copiotrophic bacteria, and may also directly select for antibiotic resistance genes independent of antibiotic residues because of the increase in competition (Noah Fierer et al., 2012). Manure subsidies alone could increase antibiotic resistance gene levels through creating a more copiotrophic environment. The selection of copiotrophs may lead to a more competitive

environment because rather than expending resources to obtain nutrients those resources can now be put into competing with nearby organisms for their resources. It follows that nutrient increases can result in antibiotic resistance gene increases as shown in (Zhou, Qiao, Wang, & Zhu, 2017) in which artificial fertilizers increased the abundance of antibiotic resistance genes. This indicates that supplying nutrients in the form of manure may increase antibiotic resistance genes. Another supporting study found that antibiotic resistance genes have been increasing in abundance since the 1940s in a consistently managed soil plot (Knapp et al., 2010). A chronosequence of archived soils from a dairy farm showed that soils exposed to manure and nutrients in the Netherlands have a natural buildup of antibiotic resistance genes. This indicates that cattle stocking levels may change microbial soil communities through nutrient additions, but it may also change the microbial community in a more direct manner.

Cow rumen microbes are added to the soil microbial communities when manure is applied to fields. The rumen microbes bring their own unique taxa and genetic information, *e.g.* antibiotic genes. Most microbes in manure are dead; though some may survive and are active in a completely novel environment (Kudva, Blanch, & Hovde, 1998; Parfrey et al., 2014). This potentially means that rumen microbes could survive and even proliferate in soil. A study showed that microbes survived for up to 21 months within manure slurries in ideal environmental conditions (Guan & Holley, 2003) indicating gut flora can survive transmission to soil. Even if no rumen microbes survive and/or proliferate in the soil they may contribute genetic information through intact plasmids. The plasmids can then be utilized by soil microbes through horizontal gene transfer. Potentially one of these transferred genes could allow for a certain group to gain an advantage altering community structure.

Horizontally transferred plasmids can become active in host microbes. Hypothetically speaking this could give soil microbes that pick up these plasmid a unique gene advantage For example the ability to better process nutrients from the animal gut. This hypothetical gene would be assumedly common in the cow's rumen. If manure is added to soil the rumen associated nutrients could also end up in the soil. If a soil microbe took up the hypothetical gene that allowed it to more efficiently utilize the gut nutrients then the microbe with the gut gene would then be at an advantage compared to other soil microbes. The advantage would be more efficient nutrient acquisition. Plasmid additions and selection could change the community composition from its natural state to a permanently altered one. This altered group may have a different level of ecosystem functioning than the natural community. Antibiotic resistance genes can also be transferred on plasmids. It isn't difficult to replace the hypothetical nutrient gene with an antibiotic resistance gene in the hypothetical situation. The antibiotic resistance gene would confer protection to manure associated antibiotics allowing for survival of resistant microbes.

In order to understand the effect of antibiotic resistance on soil communities these three major components of manure; antibiotic metabolites and raw antibiotics, nutrients, and rumen microbes; that potentially affect antibiotic resistance gene abundance need to be examined separately. Each of these components are normally applied together in agricultural manure application making it difficult to separate out the exact effect of each component on antibiotic resistance genes and nutrient cycling. In order to determine the effect of antibiotics and the resistance genes that follow the three major components of manure needed to be separately addressed. Figure 3.1 illustrates this by showing the three constituents. These three

constituents of manure, along with history, are the four major mechanisms proposed to determine antibiotic resistance and need to be examined in order to determine the effect of antibiotic resistance on ecosystem functioning.

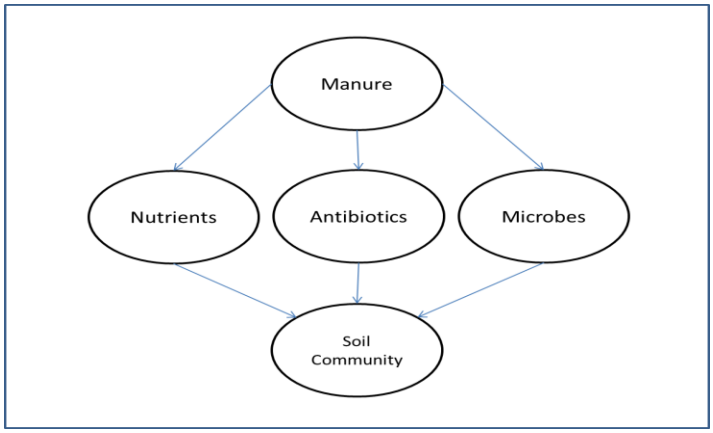

Figure 3.1. A diagram showing the different constituents of manure that could have significant effects on the size, composition and, functioning of soil microbial communities. Nutrients include organic matter, ammonia, and phosphorus. Antibiotics are the residues and metabolites excreted from the cow into the manure. Microbes include live and dead microbes as well as the genes and vectors associated with them

In this study I created soil incubations to distinguish the effects of nutrient subsidies, antibiotic residues, and rumen microbes on soil specifically wanted to look at the effects on microbial communities, antibiotic resistance genes, and microbial functioning. Soil samples were collected from the Virginia Tech Dairy, from areas of high and low cattle traffic. The 'high' sites are from areas with high cattle traffic and therefore receive increased levels of manure input. The 'low' sites are from areas with low cattle traffic that should receive sporadic manure inputs. The driving idea behind the high and low sites is to determine if pre-exposure to

antibiotics and antibiotic resistance genes affects the response of soil microbial communities to antibiotic additions.

In the first incubation high and low soils were amended with three different types of manure, two with antibiotics and one without. Some soil samples received no manure to serve as a control. This was to determine if antibiotic residues and manure have an effect of soil microbial communities. I hypothesize that the high traffic soils will have a weaker response to antibiotic additions because they already have antibiotic resistance genes to negate the effects of antibiotic additions. In contrast the low traffic soils will have a stronger response since the antibiotic will be novel to them. This means that because they have no pre-exposure they will have fewer antibiotic resistance genes and will be heavily impacted by antibiotic additions.

A second incubation of high and low soils was also setup. An extraction of each of the manure's bacteria was added to each soil type. This was to determine what effect live bacteria from manure in the absence of nutrients associated with manure. An additional extraction of sterilized microbes from each manure type was also added to each site to distinguish the effect of the dead microbes as a source of nutrients from the living microbes themselves. In this incubation I expect to see that antibiotic resistance gene copy numbers will increase in soils exposed to the live bacteria because they will introduce antibiotic resistance genes into the community along with antibiotics that will select for those genes. Antibiotic resistance gene abundance data as well as respiration data was collected to test for resistance buildup and community activity response in both incubations.

Methods

Incubation Setup Description

Incubations were set up to quantify carbon dioxide fluxes as a measure of total microbial activity and to estimate antibiotic resistance gene counts. Soil was collected from the upper 10 cm of soil at Virginia Tech's dairy facility. Two soil sample types were taken from areas of low and high cattle traffic. High sites were collected from near feeding troughs where manure input to soil is relatively high. Low sites were collected from adjacent pasture lands on the same soil type where manure inputs were relatively low. Manure was collected from cattle at Virginia Tech's dairy facility, where access to antibiotics exposure is controlled and tracked. The amount of manure used was based on normal input levels of Virginia Tech's dairy farm and came out to 0.49 grams of manure per incubation. This is equal to roughly one year of normal manure input to an average field at Virginia Tech's dairy farm, based on stocking rates as well as animal manure production. Six grams of soil was incubated for 100 days, at 20 degrees C ° with differing treatments depending on the incubation group. The first group received one of three types of manure; manure with cephapirin(C) present, manure with pirilimycin (P) present or manure that was un-amended with antibiotics (Con+). Additionally a negative control set of soils with no manure added was run (Con-). Cephapirin is a bactericidal antibiotic; its mode of action kills the bacteria, and in contrast pirilimycin is bacteriostatic, which instead arrests growth of bacteria. Cephapirin was used on the cows present at our collection site whereas pirilimycin was not. Cephapirin is used in the treatment and prevention of mastitis in dairy cattle (Ray, Knowlton, Shang, & Xia, 2014). Each of the two cattle stocking levels was tested with each manure type for a total of 8 treatment combinations.

In the second incubation group a similar setup was used, all three types of manure and a negative control with just soil were added to the two cattle traffic level soils. Instead of adding the manure directly, bacteria were extracted from manure and then added to soil incubations. Sugar centrifugation was used to separate the manure and the bacteria (Lindahl & Bakken, 1995). Centrifugation occurred at 10,000 g for 10 minutes at 4° C separating the manure from a 6 molar sugar solution. This separated the bulk manure and its associated nutrients from the lighter microbial fraction. By separating these I was able to extract just the microbes into the supernatant which left the bulk matter in the pellet. The supernatant, consisting mainly of microbial cells, was then added to the incubations, which was intended to isolate the microbial inoculation from the nutrient addition associated with manure. In an additional treatment the manure was autoclaved before extraction to sterilize microbes. Manure was sterilized using an autoclave, pressure will be set at 103.4 kPa and temperature will be set 121°C. Manure was autoclaved for 1 hour, let sit for 24 hours then once again autoclaved for 1 hour (Wolf, Dao, Scott, & Lavy, 1989). In total there were for a total of 14 treatments combinations. Five repeats were performed per sample to ensure accuracy.

CO_2 Incubation Procedure

I measured CO_2 fluxes over 28 day incubations. Six grams dry weight equivalent soil was added into 50 mL falcon tubes and was adjusted to 65% water holding capacity. Throughout the incubation the soil was rewetted to maintain the 65% level. Soils were incubated at 20°C and at 100% humidity for 28 days. Carbon mineralization rates were measured at 1, 2,3,7,14,21 and, 28 days. To measure CO_2 tubes were sealed with an airtight cap and septum. CO_2 free air was then flushed through the tube for 2 minutes and then tubes were incubated at 20°C for

approximately 24 hours. After incubation gas samples were taken from the headspace with a syringe and were read for CO_2 using a LI-7000, Li-Cor Inc, infrared gas analyzer (IRGA). CO_2 integrals were calculated and used to determine CO_2 flux per hour based on start and end times. (Noah Fierer, Allen, Schimel, & Holden, 2003)

QPCR procedure

DNA extractions were performed using MoBio Powersoil DNA extraction kits (MoBio Laboratories).A Qubit fluorometer (Thermofisher) was used to quantify DNA extractions. Successful extractions yielded between 1 and 30 ng/μL *TetO* and *AmpC* gene abundances were determined using the method outlined in (Thames, Pruden, James, Ray, & Knowlton, 2012). Each sample was run in triplicate and the gene copy numbers were estimated using a standard curve. The standard curve ranged from 10^1-10^8 copies of the gene. For *AmpC* standard curve r^2 values were between .985 and .996, efficiency values were between 98.4% and 106.4%. For *TetO* r^2 values ranged from .972 to .990, efficiency values were between 80% and 107%. Lower efficiencies and r^2 values were obtained from soils subjected to bacterial inoculations in comparison to those from manure added incubations.25 microliter reactions contained reactions contained 12.5 uL Universal SYBR Green Supermix (Bio-Rad laboratories), 1.25 uL of forward primer, 1.25 uL of reverse primer, 5 uL of PCR grade H_2O and finally 5 uL of sample. All samples were diluted down to 3 ng/μL ensuring that no samples suffered efficiency loss to over replication.

Statistics

All statistical analysis was done in R and R-Studio. Analyses of variances were performed to determine significant differences. Analysis of variance were done using the aov function, alpha was set to .05. Tukey tests were performed post-hoc to determine groupings. We used the 'agricolae' package with the HSD.test function, alpha was set to .05.

Results

Analyses of variance were performed to test for difference among treatments (Cephapirin, Pirilimycin, Positive Control, Negative Control) and cattle stocking rates (High, Low) for the manure-addition incubations, the results of which are in Table 3.1. Three separate analysis of variance were ran using three separate response variables; *TetO* gene abundance, *AmpC* gene abundance, and soil respiration as CO_2 per gram of soil. The positive control treatment was composed of soil with manure from cattle not given antibiotics. Negative control treatments consisted of soil only with no manure added.

TetO gene abundance showed no significant differences between either treatment (p= 0.14) or site stocking level (p= 0.832, Figure 3.2) in the manure incubations. Although not significant, *TetO* gene abundance in the negative control treatment (soil only) are lower than the manure amended soil treatments (cephapirin, pirilimycin, and positive control), other than the positive control treatment in the low traffic sites. This may indicate that the data are trending toward manure additions increasing *TetO* gene abundance.

AmpC gene abundances were significantly higher for the cephapirin treatment in comparison to the positive control treatment (figure 3.3) within the manure incubations. There were no significant differences between site stocking level (p= 0.255) but treatment had a

significant effect on *AmpC* gene copy numbers (p= 0.017). Additionally the interaction between treatment and site stocking level was significant (p= 0.003). To explore the significance found during analysis of variance analysis a tukey test was performed for post-hoc analysis. The tukey test found that the pirilimycin, positive control, and negative control treatments were not significantly different than one another. Additionally, the cephapirin and negative control treatments were not significantly different from each other. Although not significantly higher than the negative control, the cephapirin treatment had the highest *AmpC* gene copy number and was significantly higher than the pirilimycin and positive control treatments.

Cumulative soil respiration for the manure incubations in the high traffic sites was significantly higher in the cephapirin and positive control treatments than the pirilimycin and negative control sites as seen in figure 3.4 and figure 3.5. In contrast, soil respiration for the low sites was significantly higher in the positive control compared to other treatments. An analysis of variance of cumulative soil respiration revealed significant differences among treatments ($p<0.001$) and significant stocking rate effects, with higher levels of CO_2 evolution from high traffic sites ($p<0.001$). Additionally there was a marginally significant interaction between site stocking level and manure treatment ($p=0.056$). A tukey test was performed on the analysis of variance output to compare means within site and treatment combinations.

Soil respiration for the high traffic sites within the positive control treatment and the cephapirin treatment were not significantly different from one another. Additionally, the negative control treatment and the pirilimycin treatment were not significantly different. Pirilimycin and negative control treatments have significantly lower respiration than the cephapirin and positive control treatments. Given the significance of the interaction between

stocking rate and antibiotic treatment an additional post hoc test was performed within the sites for differences between treatments.

Analysis of the low stocking rate respiration data indicated that the positive control treatment had a higher cumulative respiration than any of the other treatments, as seen in figure 3.4 and figure3.6. The negative control treatment was significantly lower than both the cephapirin and positive control treatments but not significantly different than the pirilimycin treatment. The pirilimycin treatment was not significantly different from the cephapirin and negative control treatments, but is significantly lower than the positive control treatment. The cephapirin treatment was significantly higher than the negative control and lower then positive control treatment.

Analysis of the bacterial inoculation incubations revealed no significant differences in *TetO* gene counts (Figure 3.7) but I did find differences in the *AmpC* gene counts (Figure 3.8). An analysis of variance was run on the bacterial inoculant incubations. The factors included; treatment (cephapirin, pirilimycin, positive control, and negative control), the site history of cattle traffic levels (high and low), and type of bacterial addition (live inoculant, autoclaved inoculant). The results of the analysis of variance are in Table 3.2.In *TetO* no significant differences were found between site stocking level ($p=0.350$), manure treatment ($p=0.311$) or bacteria type ($p=0.517$). Figure 3.7 contains the graph of *TetO* copy numbers.

The negative control treatment had significantly higher AmpC gene copy numbers than the other three treatments in the inoculant addition incubations (Figure 3.8). *AmpC* gene copy numbers revealed no significant difference between bacteria types ($p=0.994$), but there were

significant differences in manure treatment (p=<0.001) and a marginally significant differences in site stocking level (p=0.079). A tukey test found that pirilimycin, cephapirin, and positive control bacteria extracted from manure are not significantly different whereas the negative control has significantly higher *AmpC* gene counts.

Discussion

I assessed the effect of dairy cattle traffic levels and manure additions on soil microbial communities by quantifying the abundance of antibiotic resistance genes and soil respiration. I measured two different antibiotic resistance genes, *TetO* and *AmpC*. I expected to see a significant increase in the *AmpC* gene counts in the cephapirin treatment above all other treatments, especially the negative control. This is because *AmpC* provides resistance to the antibiotic cephapirin which is used to treat and prevent cattle mammary gland infections (Ray et al., 2014). In contrast I did not expect to see a difference in *TetO* gene abundance since *TetO* is associated with resistance to the antibiotic tetracycline (Sougakoff, Papadopoulou, Nordmann, & Courvalin, 1987), which none of the cows in the study were exposed to. If I saw any increase in *TetO* they should be moderate in comparison to the positive control treatment and be the result of nutrient subsidies {Chen, 2014 #59}. The prediction that *TetO* will not be significantly different between treatments is supported by the results of our analysis of variance. The analysis of variance for *TetO* genes showed that no significant difference between treatments was observed. Even the cattle traffic level did not have a significant influence on *TetO* gene abundance in contrast to the findings of another study (Wepking et al., 2017), whom reported that higher levels manure were associated with greater abundance of *TetO* gene numbers, presumably because of the copiotrophic conditions. The copiotrophic conditions

were posited to have been created by the nutrient subsidy created by high stocking levels and therefore elevated levels of manure input. These contrasting results highlight the need to understand the different constituents of manure in order to elucidate mechanisms affecting nutrient cycling in antibiotic exposed soil communities, especially to determine the effect of antibiotics themselves on soil communities.

The cephapirin treatment stimulated *AmpC* gene counts above the positive control indicating that the antibiotics within the manure amendment stimulated *AmpC* gene counts. Analysis of variance between treatments of *AmpC* gene abundances showed a significant difference between treatments as well as a significant interaction among treatments and cow traffic level. A significant difference between treatments is expected based upon cattle traffic history as well as treatment applied. A post hoc tukeys test reveals interesting groupings (Figure 3.3). The negative control treatment is not significantly different from the cephapirin manure treatment. This does not follow my predictions because I would have expected the addition of cephapirin manure to stimulate *AmpC* levels above the background levels represented by the negative control. I would have expected the cephapirin treatment to have higher *AmpC* gene counts because *AmpC* genes confer resistance to the antibiotic cephapirin. These results indicate that neither antibiotic additions nor nutrient additions stimulated *AmpC* gene counts. This may indicate that a longer incubation was necessary but it certainly indicates a complex relationship between manure and antibiotics

The *AmpC gene counts* revealed that the cephapirin treatment showed significantly higher gene counts than both the positive control and pirilimycin treatments. The cephapirin treatment being higher than the pirilimycin treatment indicates that the addition of cephapirin

specifically increases *AmpC* gene counts. This indicates that antibiotics additions don't increase antibiotic resistance genes that are unrelated to the added antibiotic. The cephapirin treatment having significantly higher levels of *AmpC* genes than the Con+ treatment was also expected. Previous work has shown that copiotrophic environments have been found to select for increased antibiotic resistance gene proliferation (R. Chen et al., 2014), but having antibiotics added seems to stimulate *AmpC* gene levels more than nutrients alone. Additionally, the *AmpC* gene counts for the positive control, pirilimycin, and negative control treatments were not significantly different from each other. From the *TetO* gene counts (figure 3.2) I can only conclude that manure and antibiotic additions do not stimulate antibiotic resistance gene levels in general. This is because *TetO* genes do not confer resistance to pirilimycin or cephapirin. In order to examine the effect of antibiotics and manure on ecosystem processing I measured the respiration of the manure incubation.

The respiration data revealed a significant difference between high and low cattle traffic sites, so each site must be considered individually. In the high traffic sites the cephapirin and positive control treatments were significantly higher than the other treatments meaning cephapirin did not depress respiration but pirilimycin did. Additionally, the cephapirin and positive control treatments were not significantly different from one another. The negative control treatment had a significantly lower respiration value than the cephapirin and positive control treatments because the negative control didn't receive any nutrient subsidies in the form of manure. The pirilimycin treatments may have shown decreased respiration values because pirilimycin is a novel antibiotic to the system and suppressed microbial communities. This can be seen in the response ratio diagram (Figure 3.5), wherein the pirilimycin treatments

are lower at each time step than both the other manure treatments. Additionally, pirilimycin is a bacteriostatic antibiotic, which means rather than killing bacteria it only slows down the metabolism of the bacteria, which also would explain the drop in respiration values below the negative control treatment. This relative drop from the negative control indicates that the effect of the nutrient subsidy and the pirilimycin contained in that manure may have counter-acted one another resulting in lower total respiration in comparison to the other two manure treatments (positive control and cephapirin).Furthermore, pirilimycin was a novel antibiotic to these systems which may explain its effectiveness in suppressing respiration despite the site's history of antibiotic additions. This indicates that antibiotic additions aren't conferring general resistance only resistance to the antibiotic specifically being added which is corroborated by the results of *TetO* QPCR. However, these results are not supported by the findings of other researchers (Wepking et al., 2017) which may indicate the incubation was not long enough to see the full effects. The cephapirin and positive control treatments both exhibited an increase in respiration over the Con- treatment because they received a nutrient subsidy in the form of manure. Additionally, because of the history of pre-exposure to antibiotics in the high sites, the soil microbial community was prepared for the addition of cephapirin so it experienced no decrease in respiration levels from the addition of an antibiotic, unlike the pirilimycin incubations that were not pre-exposed and saw a decrease in respiration. To simplify, the cephapirin containing manure was essentially positive control manure for microbes in the high cattle traffic sites that were pre-exposed to antibiotics and only provided a subsidy. Figure 3.4 helps visualize this effect as the data indicates that both cephapirin and positive control manure stimulate respiration above the negative control treatment. The low traffic rate sites also saw

significant but different pairings which indicates that pre-exposure and cattle traffic levels are changing microbial community response to antibiotics

The positive control treatment was significantly higher than every other treatment in the low sites. This means that antibiotics were acting upon the low soil microbial communities and that the manure mainly served as a nutrient subsidy that increased respiration rates. Since both the other manure treatments fell below the positive control treatment this indicates that the antibiotics were depressing respiration. The cephapirin and pirilimycin treatments were not statistically different, indicating that both of the antibiotics repressed respiration. Both antibiotic treatments (cephapirin and pirilimycin) experienced suppression of respiration because the low traffic sites had not been pre-exposed to antibiotics and therefore don't have the antibiotic resistance genes to combat their effects. This is compounded by the fact that both of these antibiotics are novel and are therefore more efficacious in reducing soil microbial respiration. The negative control treatment having significantly lower respiration than the pirilimycin treatment indicates that the nutrients in the manure still boosted respiration to a degree. The significant difference between negative control and novel antibiotic treatments is in contrast to the effect I saw in the high traffic sites wherein the effect of novel antibiotics and manure roughly cancelled each other out. This may be due to the paucity of microbes in comparison to the high sites, so that the antibiotics are less effective but this is purely conjecture. The slight difference between the pirilimycin and cephapirin treatments, although statistically not significant, may be because of their different modes of action. Cephapirin is bactericidal while pirilimycin is bacteriostatic. Since the respiration values reported are cumulative (Figure 3.4), the bacteria killed in the cephapirin treatment may have become

substrates themselves which may have stimulated respiration later in the incubation, even at the first measurement point. This may explain why the pirilimycin treatment drops below the negative control treatment near the end of the incubation (Figure 3.6). A longer incubation may have seen the pirilimycin and cephapirin treatment eventually separate which suggests that pre-exposure and antibiotic mode of activity are both important factors on ecosystem processing. This incubation sheds some light on the effect of manure and antibiotics on soil microbial communities but it doesn't delve into the effect that rumen microbes may be having on soil microbial communities as suggested by figure 3.1.

In order to determine if the bacteria within the manure also have a direct effect on antibiotic resistance genes I performed a second incubation comparing inoculations of living and dead bacteria on antibiotic resistance gene copy numbers. The goal was to see if any living microbes from the rumen were taking up residence in the soil, I also hypothesized that even if the rumen microbes were non-viable their genetic information and antibiotic resistance genes may be passed into the microbial community. *TetO* gene counts showed no significant differences between site, treatments or the type (living inoculant or dead inoculant) of bacteria added. This shows that there were no significant differences in *TetO* gene copy numbers. This follows my predictions because *TetO* genes don't correlate with any of the antibiotics used in the incubations. This means that no significant amount of *TetO* genes should have been present in the manure itself which means they cannot be passed to the soil microbial community. *AmpC* gene counts also showed no significant difference between treatments with manure (cephapirin, pirilimycin, and positive control) but all three treatments with manure were significantly lower than the negative treatment that had no manure (Figure 3.8). I can infer that

the bacterial inoculation via sugar centrifugation repressed gene count numbers. This may have happened for a few reasons, I may also have not allowed enough incubation time for the gene copy numbers to respond. Another possibility is that some antibiotic residue was trapped in the microbial fraction of the extraction and was then co-applied to the sites, then without the total nutrient subsidy to promote growth I observed only decreases in the community and therefore total antibiotic resistance gene counts. This also calls into question the findings of the *TetO* QPCR data.

In summary the first incubation's data show that the history of stocking makes a difference in the microbial response to antibiotic treatments. A high stocking rate, which also means higher exposure to antibiotics, confers resistance to the effect of antibiotics used. In contrast the low stocking rate sites, without the pre-exposure conferred by a high stocking rate, are vulnerable to the effects of antibiotics. Pre-exposure leading to acclimation is not a novel concept for soil microbial communities, drought stress is another example (Williams, 2007). Comparing drought stress and antibiotic resistance buildup provides a well-established framework through which to view a more novel idea.

Changes in water availability can lead to stresses that are influential on microbial communities function (Kempf & Bremer, 1998; Yancey, Clark, Hand, Bowlus, & Somero, 1982), this is similar to antibiotic additions in manure (especially in low traffics sites).Water in drought stressed systems can come in infrequent additions that relieve water stress quickly. A common example of changes in water variability is in agricultural systems where droughts are often mitigated through irrigation. Irrigation happens relatively quickly taking a system from dry to saturated. Research has shown that this rewetting results in an immediate change in nutrient cycling

(Appel, 1998; Sørensen, 1974), the mechanisms through which are being investigated. It has been hypothesized that soil communities that are variable in their water availability are better able to handle water changes (Evans & Wallenstein, 2012; Franzluebbers, Haney, Honeycutt, Schomberg, & Hons, 2000; Williams, 2007). A study (N. Fierer & Schimel, 2003) found that a grassland soil responded less strongly to a rewetting event in comparison to a forest soil. Underlying these terrain types are associations with water variability, the grassland soil exhibited high levels of variability while the forest soil was less variable. This means that the soil communities accustomed to moisture variability were less affected by the rewetting event, effectively indicating pre-exposure matters. Pre-exposure to drought conditions minimized microbial stress levels indicating a community more resilient to disturbance. The literature shows that there is a precedent for pre-exposure to increase a soil microbial communities' resilience to change.

Moisture variability prepares soil microbial communities to withstand future moisture variability. This hypothesis is similar to my hypothesis that high traffic soils will be better able to handle manure additions because they are pre-exposed the antibiotics associated with the manure. In short antibiotic and manure exposure prepares soils microbial communities to survive antibiotic additions. The manure incubation lends some support to the importance of pre-exposure. The manure incubation shows that antibiotics that enter the environments are having an effect on soil microbial respiration in significant ways. Gene count numbers showed that *AmpC* gene copy numbers responded to cephapirin additions. This conclusion is supported by similar studies that also found that the addition of cephapirin laden manure leads to an increase in *AmpC* gene counts. The inoculation incubations were largely ineffective due to the

effect of the sugar centrifugation, thus the results are difficult to interpret. This means the difference between rumen bacteria and nutrient effect is still valuable question to answer but my study indicates that the rumen microbes are insignificant compared the nutrient subsidies and antibiotic additions.

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Tables

*Table 2.1 Analysis of variance f values for enzyme activity corrected to microbial carbon levels. This table shows the effect of Stocking Rate (High and Low), Geographic Site (NH, VT, GA, GA2, KS, NY, WA, FL, MS, WV) and the interaction between the two. Asterisks denote significance levels. * <.05, **<.01, ***<.001*

	AP	AG	BG	XYL	CHB	NAG	All
Stocking Rate	1483.5***	144.9***	1236.7***	496.8***	147.8***	658.6***	2957.5***
Geographic Site	520.5***	906.2***	863.5***	273.6***	256.5***	255.6***	753.5***
Interaction	460.6***	1443.9***	484.8***	192.9***	105.3***	304.8***	1213.4***

*Table 2.2. Table with t values from Welch two sample t-tests between the high and low sites for each given site. Individual enzymes are shown on the far left column; the all indicates the total measured hydrolytic enzyme activity. . Asterisks denote significance levels. * <.05, **<.01, ***<.001*

	NH	KS	NY	GA2	GA1	VT	WV	MS	FL	WA
AP	22.06***	--28.95***	-61.844***	-9.909***	-56.647***	-43.428***	-19.232***	-19.555***	-0.0478	-12.553***
NAG	12.813***	-6.5931***	-4.1981**	1.6469	-64.483***	-37.646***	-17.834***	6.4292***	2.539*	-11.436***
BG	27.739***	-20.918***	-55.936***	-2.1977*	-13.284***	-24.746***	-28.939***	-18.468***	15.326***	-11.632***
AG	35.988***	-15.132	-34.797***	-56.482***	29.977***	-31.573***	-10.104***	-11.419***	-4.197**	17.818***
XYL	33.229***	-15.286***	-20.577***	10.656***	12.776***	-17.751***	-10.115***	-14.374***	-5.154***	0.741
CHB	7.566***	-11.592***	-18.391***	5.1352***	-12.503***	-8.425***	-17.226***	-12.893***	-2.662*	10.941***
All	52.129***	-26.758***	-35.171***	-9.982***	-59.166***	-45.704***	-38.643***	-30.815***	9.083***	-6.282***

Table 2.3. Linear models of enzyme activity. Using enzyme activity as a response variable and scores from Figure 2.4 (environmental PCA) as an explanatory variable. The first column indicates enzyme name. The second column shows the t-value of the PC1 within the model. The third column shows the t-value of PC2 within the model. The third column indicates the p-value of the entire model using PC1, PC2, PC3, and PC4 to explain the enzyme activity. The fifth column indicates the r-squared value of the model. Finally, the sixth column indicates enzyme activity.

	PC1(T Value)	**PC2(T Value)**	**P value of model**	**R squared value of model**	**Activity**
Total Hydrolytic Enzymes	0.007	0.271	.04*	0.324	Nutrient Acquisition
Alpha-Glucosidase	0.323	0.249	0.257	0.039	Sugar Degradation
Beta-Glucosidase	0.022*	0.367	0.09	0.2312	Sugar Degradation
N- Acetyl-Beta-Glucosaminidase	0.024*	0.344	0.056	0.289	Chitin Degradation
Beta-D-Cellobiohydrolase	0.079	0.210	0.26	0.08	Cellulose Degradation
Beta-Xylodase	0.031*	0.158	0.055	0.295	Hemicellulose Degradation
Alkaline-Phosphatase	0.001	0.120	0.288	0.07	Phosphorus Degradation

*Table 3.1. A table of f-values from the manure incubation data. This table shows the effect of Stocking Rate (High and Low), treatment (Cephapirin, Pirilimycin, Positive Control, Negative Control) and the interaction between the two. Asterisks denote significance levels, and a dot denotes marginal significance. ***<0.001, **<0.01,*<0.05, <0.1*

	TetO Gene Copy Number	*AmpC* Gene Copy Number	Incubation Respiration
Stocking Rate	.046	1.307	786.470***
Treatment	1.935	3.498*	23.489***
Interaction	1.913	4.835**	2.787·

Table 3.2. A table of f-values from the bacterial inoculation incubation data's analysis of variance. Effect of Stocking Rate (high and low), treatment (cephapirin, pirilimycin, Con+, Con-), type of bacteria added (bacteria, autoclaved bacteria, Con-) and the interactions between them. Asterisks denote significance levels, and a dot denotes marginal significance

****<0.001, **<0.01, *<0.05, <0.1*

	TetO Gene Copy Number	*AmpC* Gene Copy Number
Treat	0.668	10.264***
Type	1.194	3.107 .
Site	0.888	0.000
Treat*Type	1.204	0.528
Treat*Site	0.533	1.942
Type*Site	0.954	0.411
Treat*Type*Site	1.010	0.754

Figures

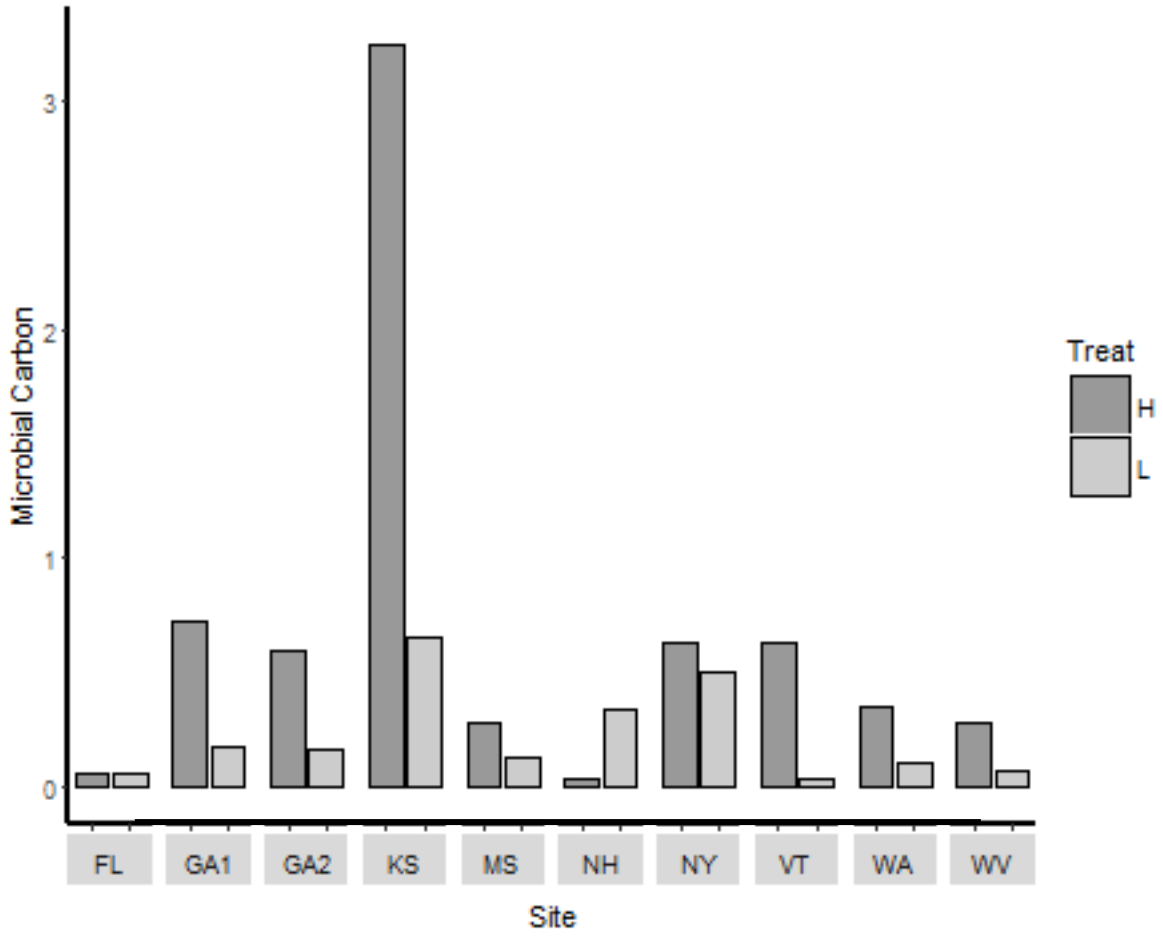

Figure 2.2 Bar graph of the microbial carbon in for each site at each stocking rate. The x axis represents site which is then subdivide by stocking rate. The y axis represents microbial carbon extracted from each sample. Error bars represent standard error.

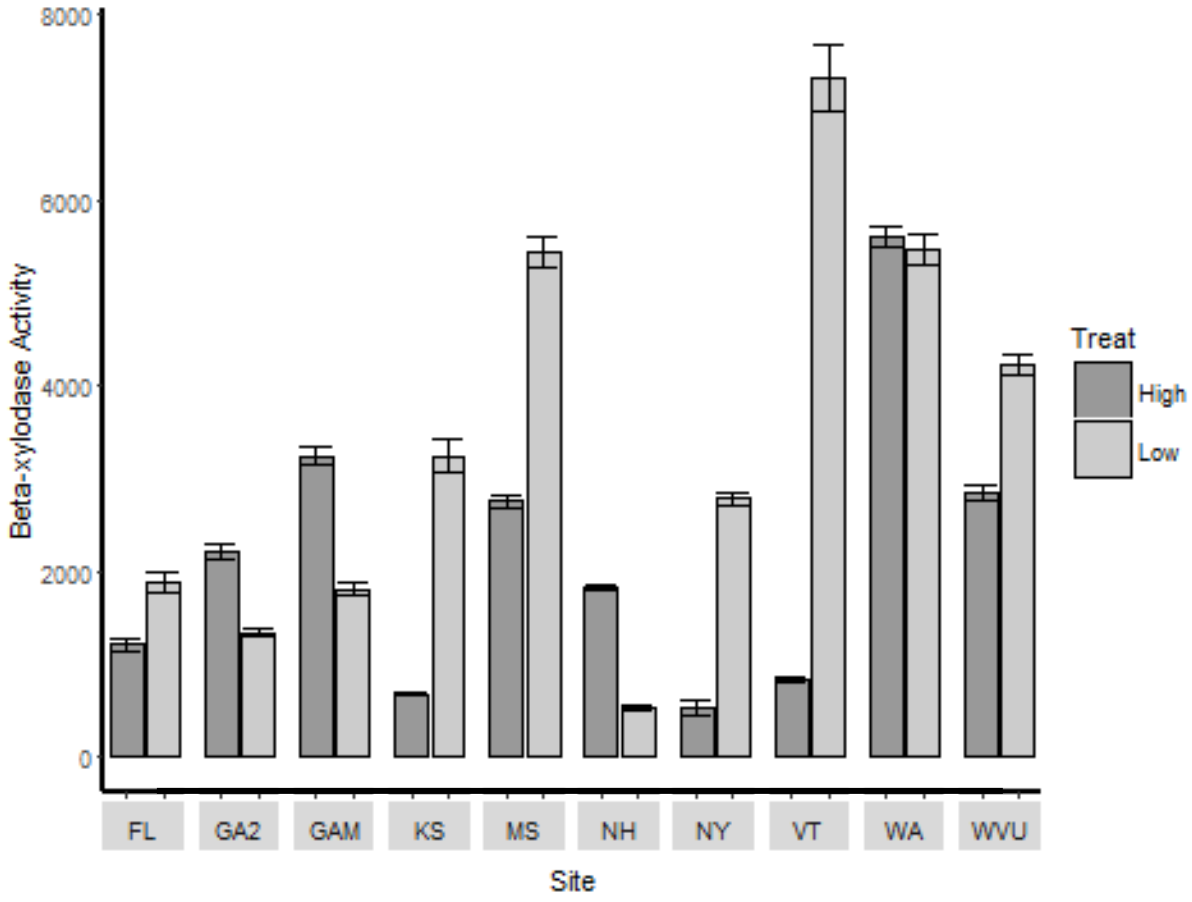

Figure 2.3 Bar graph of beta-xylodase hydrolytic enzyme activity for both the high and low input levels. All values are normalized to microbial biomass carbon to show activity per mole of carbon. A significant difference between the two treatment levels exists, p=<0.001.Error bars represent standard error. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol.uGC/MCC/hr.

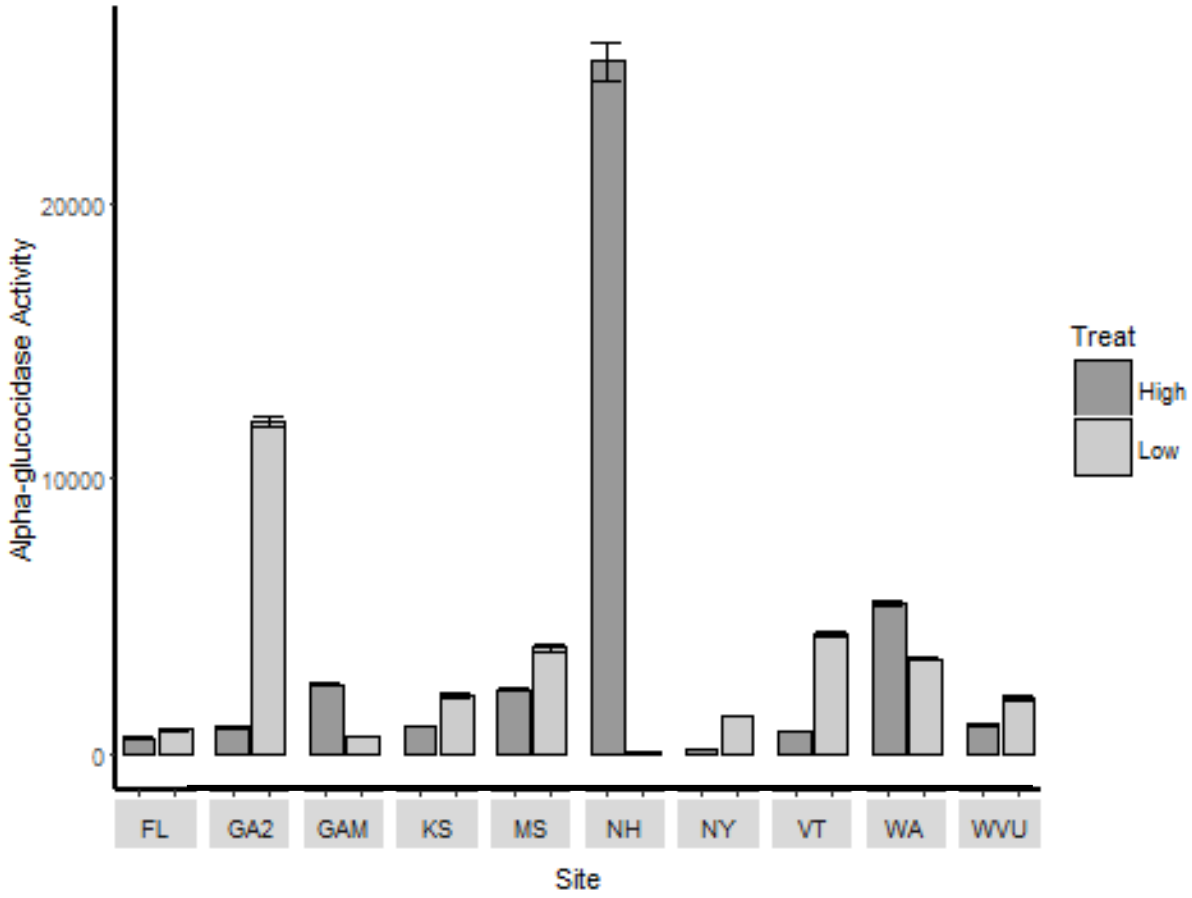

Figure 2.4 Bar graph of alpha-glucosidase hydrolytic enzyme activity for both the high and low input levels. All values are normalized to microbial biomass carbon to show activity per mole of carbon. A significant difference between the two treatment levels exists, p=<0.001. Error bars represent standard error. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

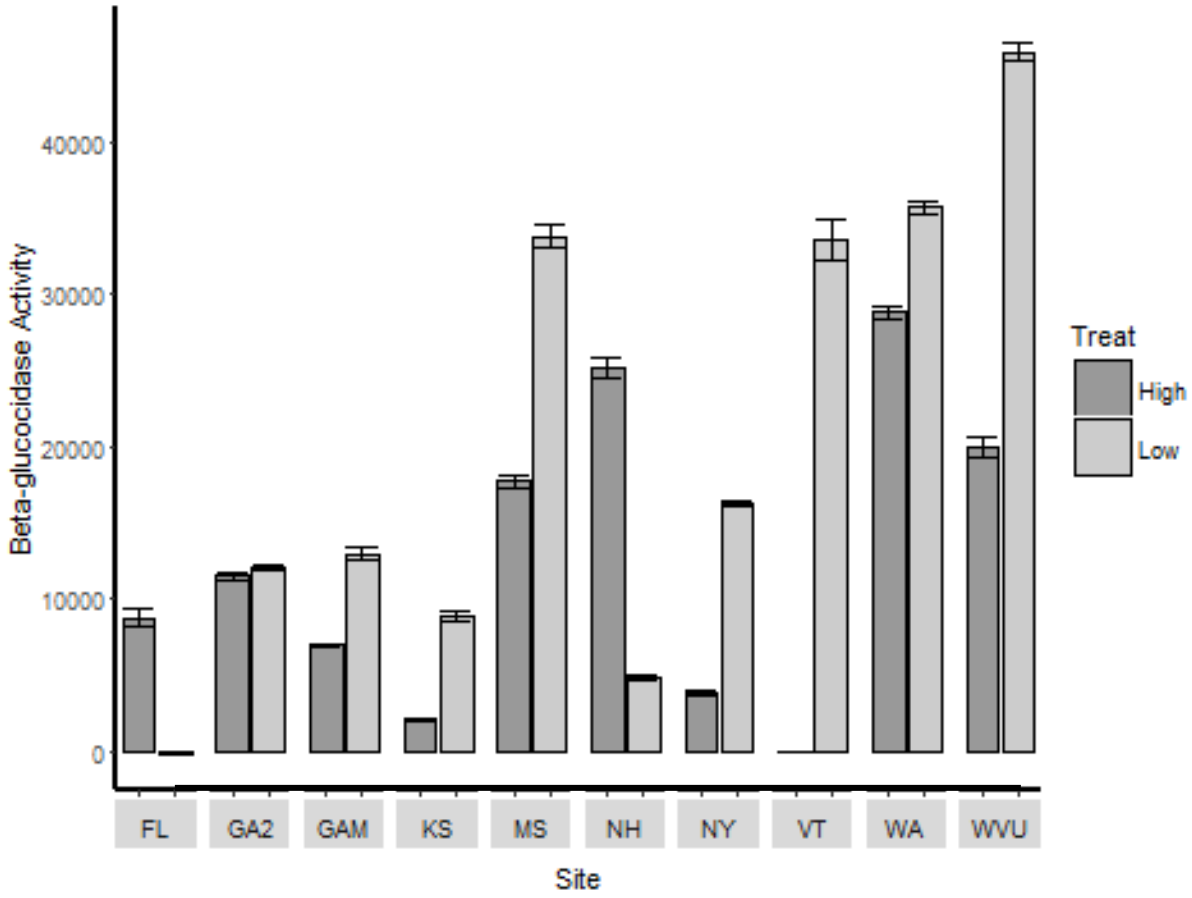

Figure 2.5 Bar graph of beta-glucosidase hydrolytic enzyme activity for both the high and low input levels. All values are normalized to microbial biomass carbon to show activity per mole of carbon. A significant difference between the two treatment levels exists, p=<0.001 Error bars represent standard error. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

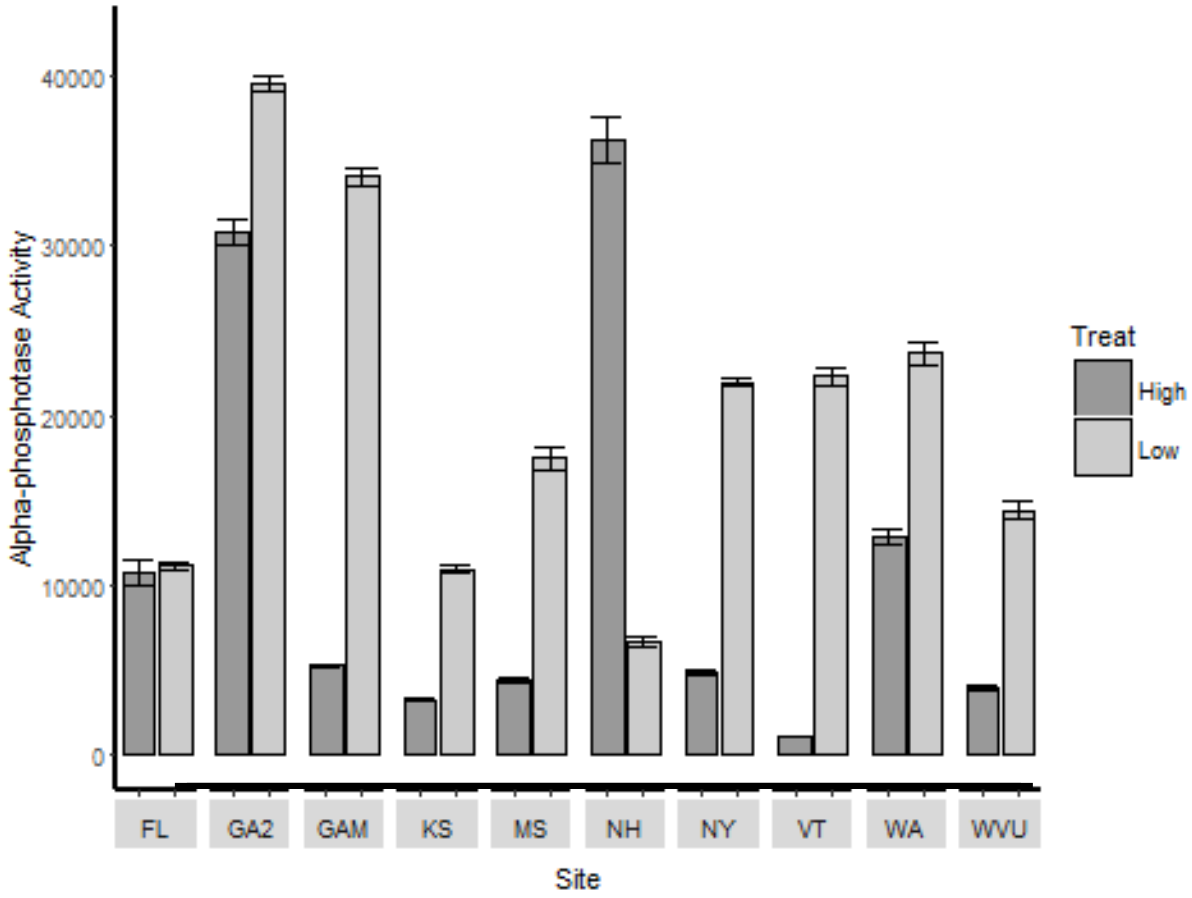

Figure 2.6 Bar graph of alpha-phosphatase hydrolytic enzyme activity for both the high and low input levels. All values are normalized to microbial biomass carbon to show activity per mole of carbon. A significant difference between the two treatment levels exists, p=<0.001. Error bars represent standard error. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

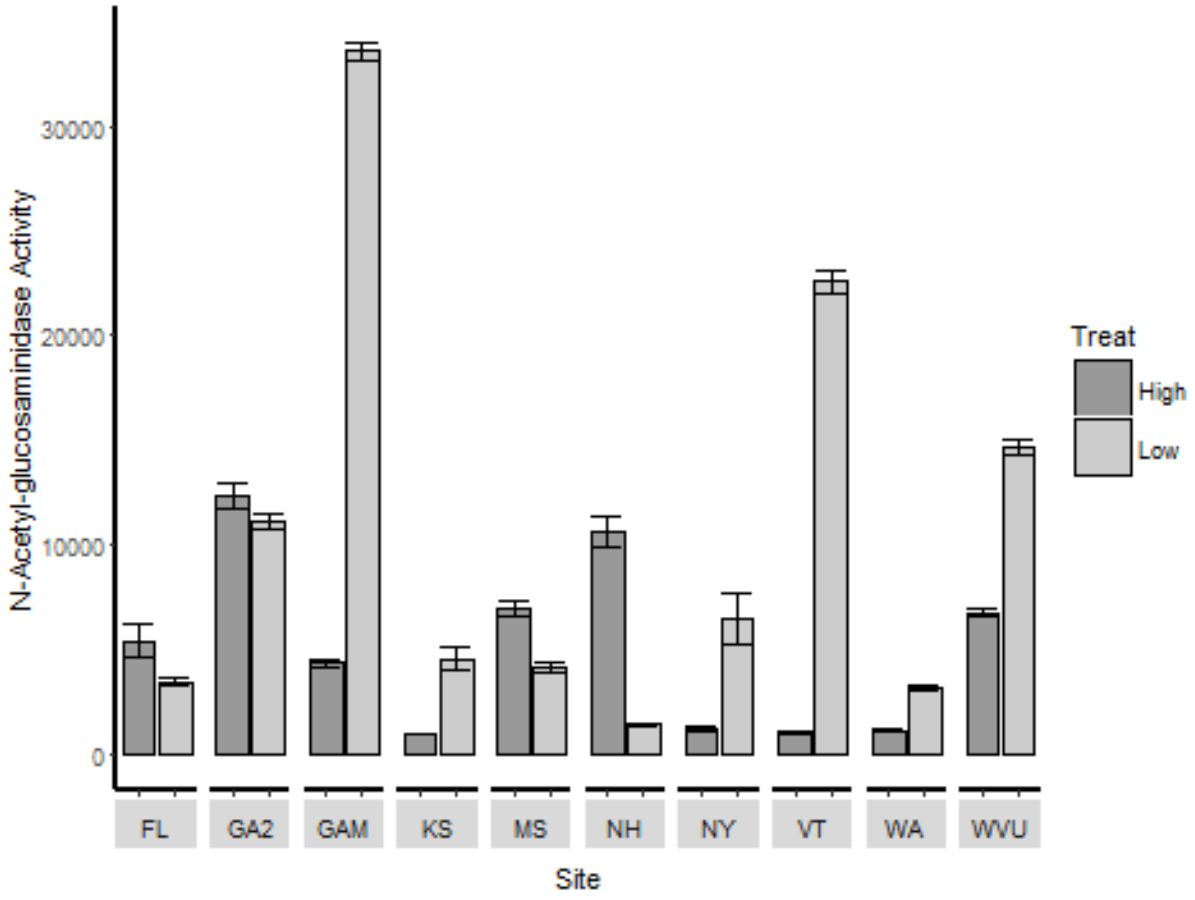

Figure 2.7 Bar graph of N-acetyl-beta-glucosaminidase hydrolytic enzyme activity for both the high and low input levels. All values are normalized to microbial biomass carbon to show activity per mole of carbon. A significant difference between the two treatment levels exists, p=<0.001 Error bars represent standard error. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

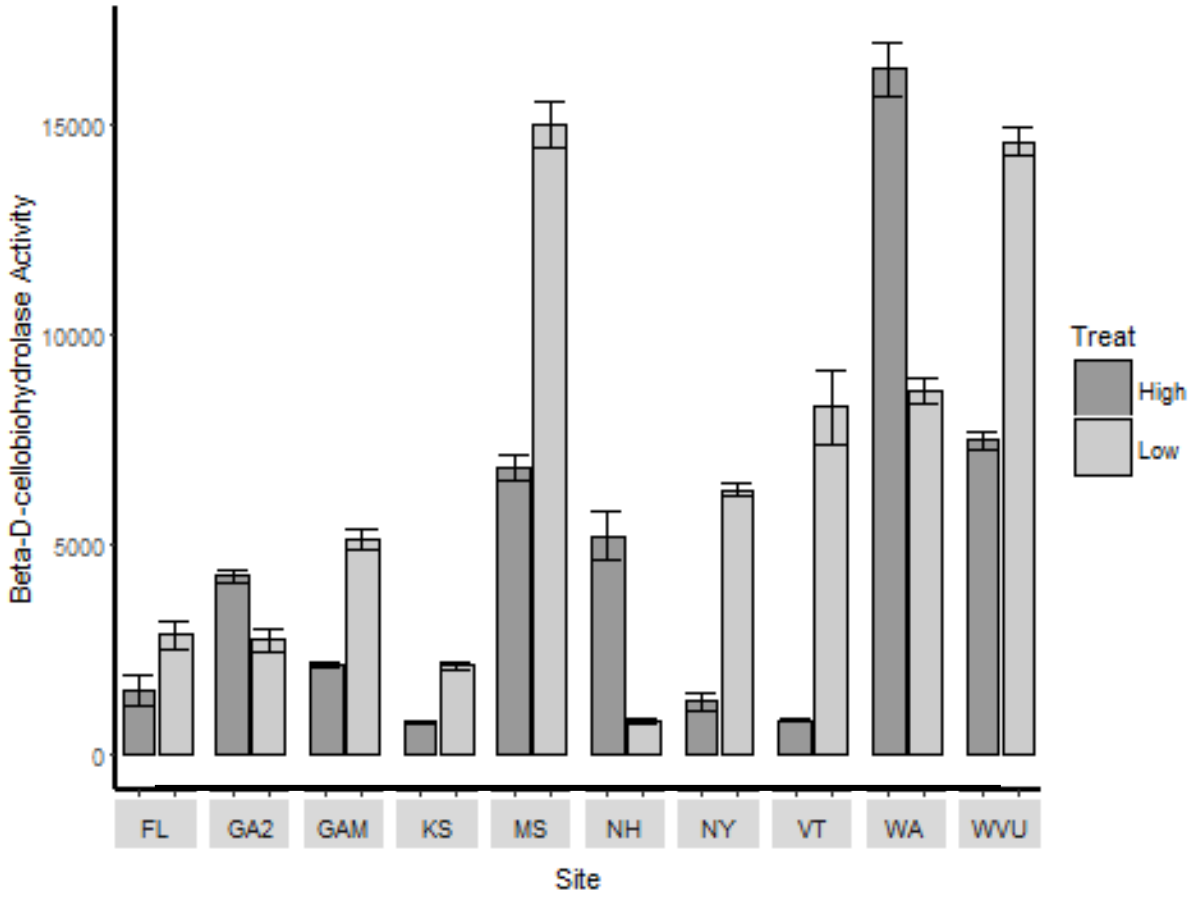

Figure 2.8 Bar graph of beta-D-cellobiohydrolase hydrolytic enzyme activity for both the high and low input levels. All values are normalized to microbial biomass carbon to show activity per mole of carbon. A significant difference between the two treatment levels exists, p=<0.001. Error bars represent standard error. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

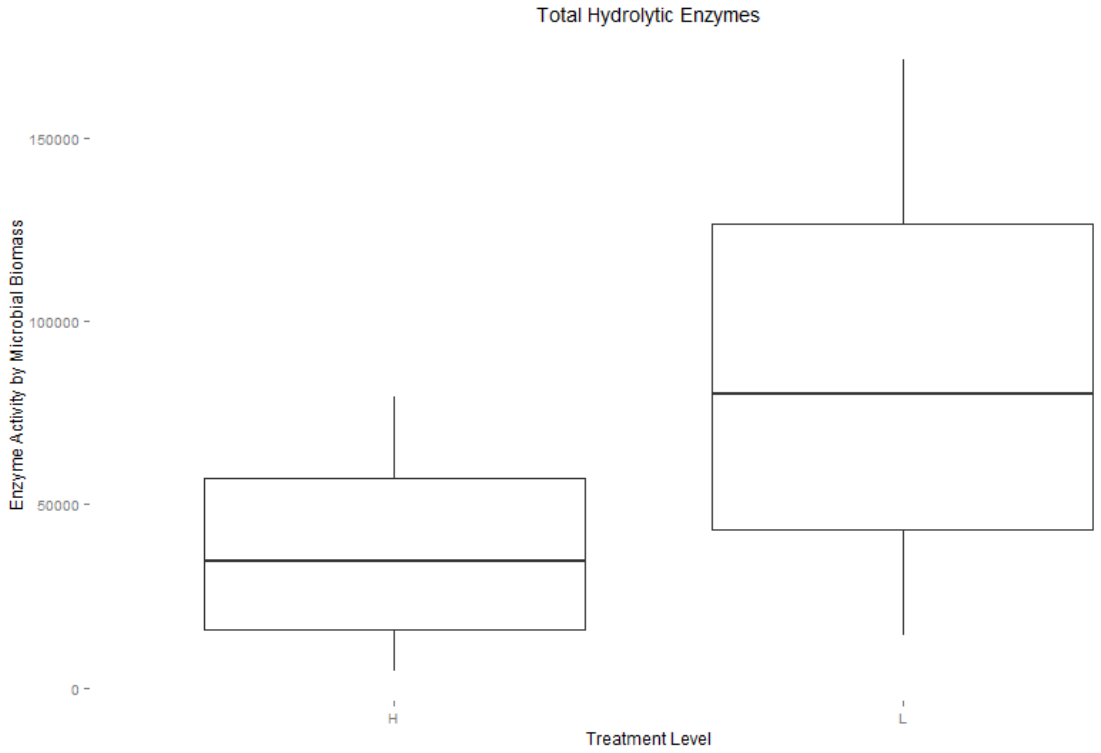

Figure 2.9. Boxplot of total measured hydrolytic enzyme activity for both the high and low input levels. Total hydrolytic enzyme activity is obtained by summing activity levels for Alkaline Phosphatase, Alpha Glucosidase, Beta-D-Cellobiohydrolase, N-Acetyl-Beta-Glucosidase, Beta-Glucosidase, and Beta-Xylodase. All values are back-corrected to microbial biomass carbon to show activity per mole of carbon. A significant difference between the two treatment levels exists, p=0.019. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

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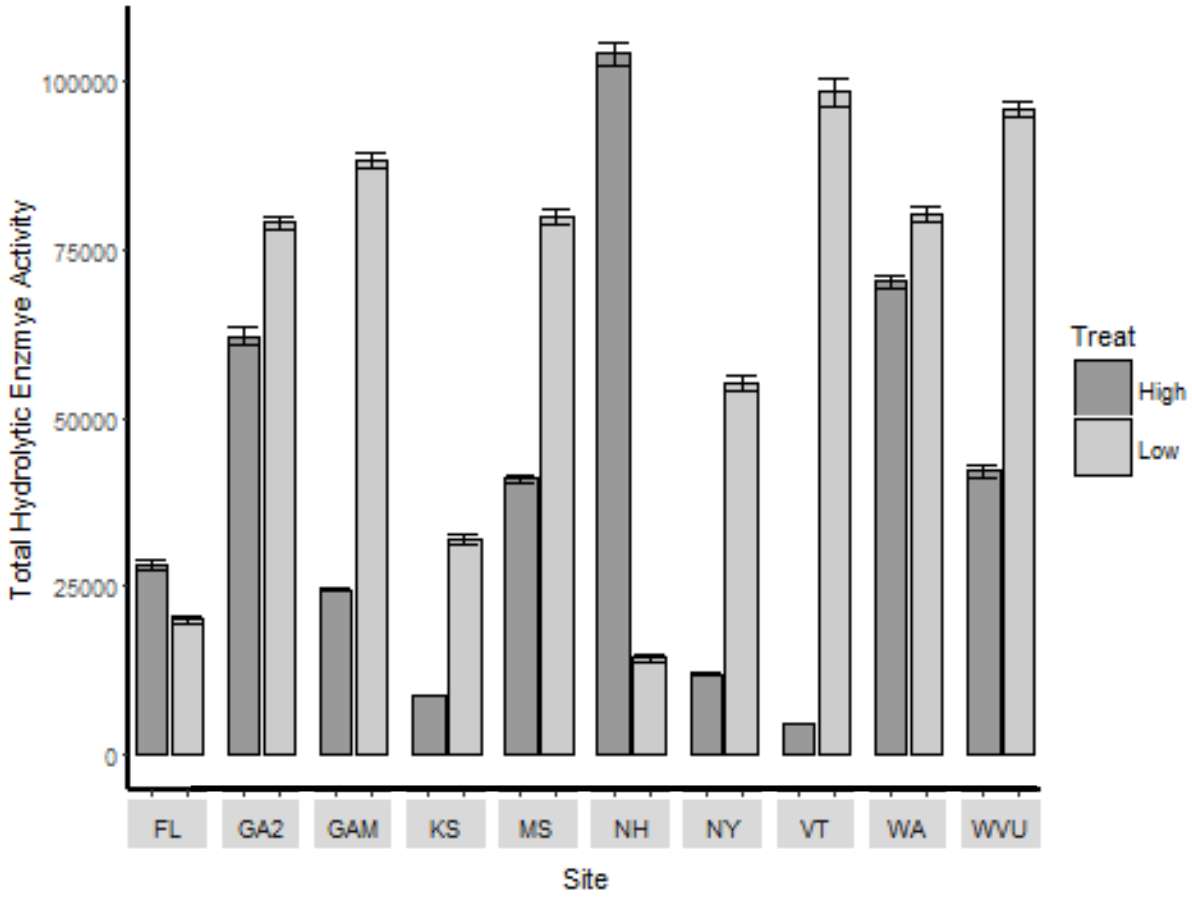

Figure 2.10. Total hydrolytic enzyme activity for both the high and low input levels. Total hydrolytic enzyme activity is obtained by summing activity levels for Alkaline Phosphatase, Alpha Glucosidase, Beta-D-Cellobiohydrolase, N-Acetyl-Beta-Glucosidase, Beta-Glucosidase, and Beta-Xylodase. Site codes are described in nationwide site description. Error bars represent standard error. Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

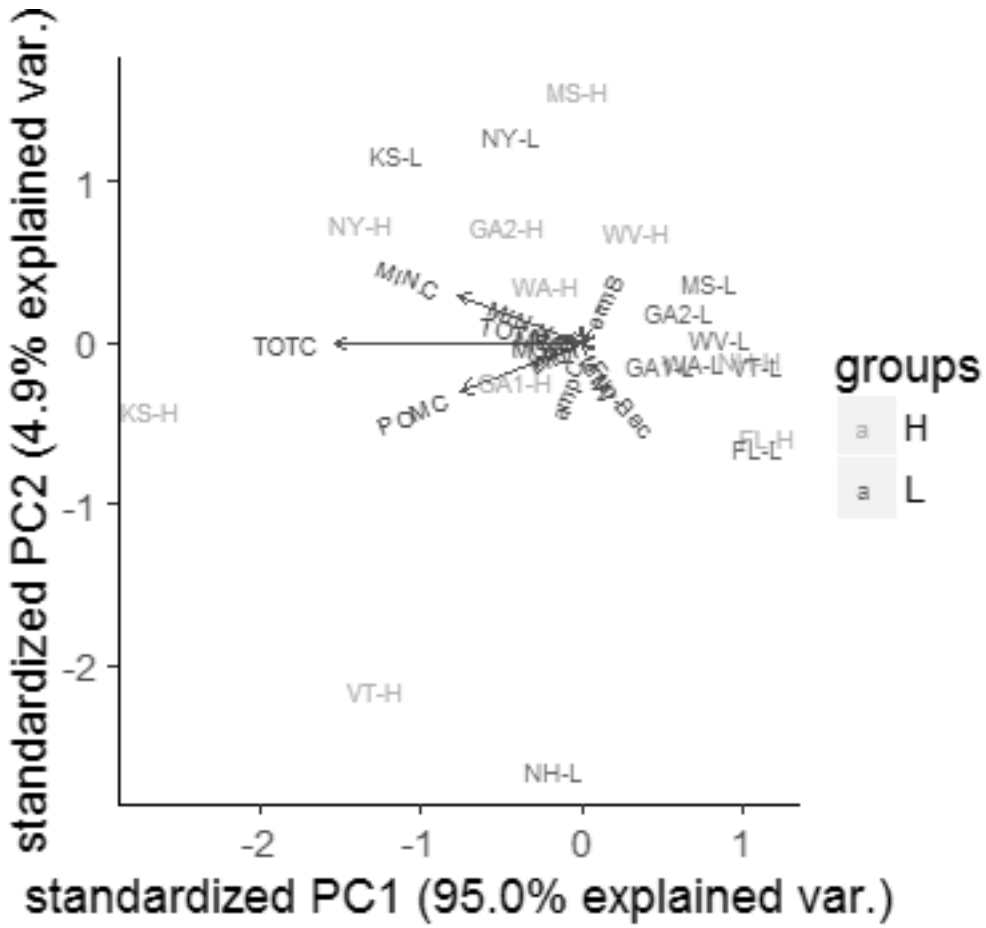

Figure 2.11. Principal coordinates ordination. High sites are shown in pink as well as represented by a "–H" indicator. Low sites are show in in blue as well as a "–L" indicator. The code indicates the site location. Variables shown in the ordination include pH, microbial carbon, microbial nitrogen, mineralizable carbon, mineralizable nitrogen, particulate organic matter carbon, total carbon, and total nitrogen, fungal to bacterial ratio, TetO gene quantity, TetW gene quantity, AmpC gene quantity, and ErmB gene quantity.

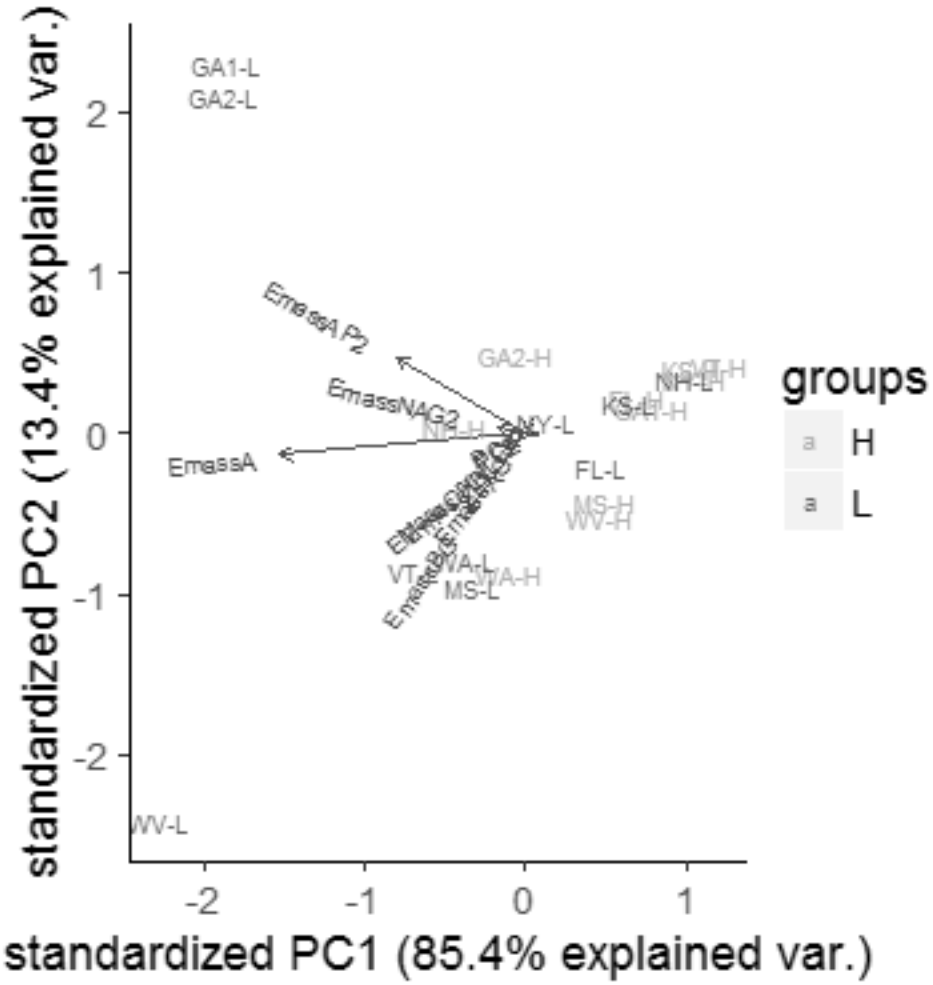

Figure 2.12. Principal coordinates analysis created using scores from Figure 2.4's 1st and 2nd principal components with individual enzyme activity as well as overall enzyme activity. High sites are represented in pink as well as by the "–H" notation. Low sites are represented in blue as well as by the "–L" notation. Code indicates individual sites. Included in the ordination are Pc1 scores and Pc2 scores from Figure 2.4, total hydrolytic enzyme (EmassA), alkaline phosphatase (EmassAP2), alpha glucosidase (EmassAG), beta-D-cellobiohydrolase (EmassCHB), N-acetyl-beta-glucosidase (EmassNag2), beta-glucosidase (EmassBG), and beta-xylodase (EMassXYL). Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

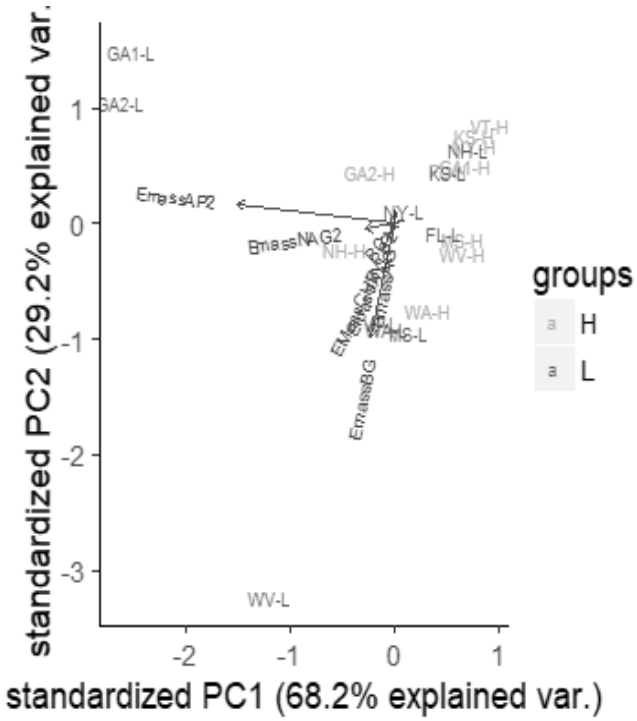

Figure 2.13. Principal coordinates analysis created using scores from Figure 2.4's 1st and 2nd principal components with individual enzyme activity. High sites are represented in pink as well as by the "–H" notation. Low sites are represented in blue as well as by the "–L" notation. Code indicates individual sites. Included in the ordination are PC1 scores and PC2 scores from Figure 2.4, alkaline phosphatase (EmassAP2), alpha glucosidase (EmassAG), beta-D-cellobiohydrolase (EmassCHB), N-acetyl-beta-glucosidase (EmassNag2), beta-glucosidase (EmassBG), and Beta-xylodase (EMassXYL). Enzyme activity is measured in nmol/uGC/hr then corrected to microbial carbon (MCC) of the sample resulting in nmol/uGC/MCC/hr.

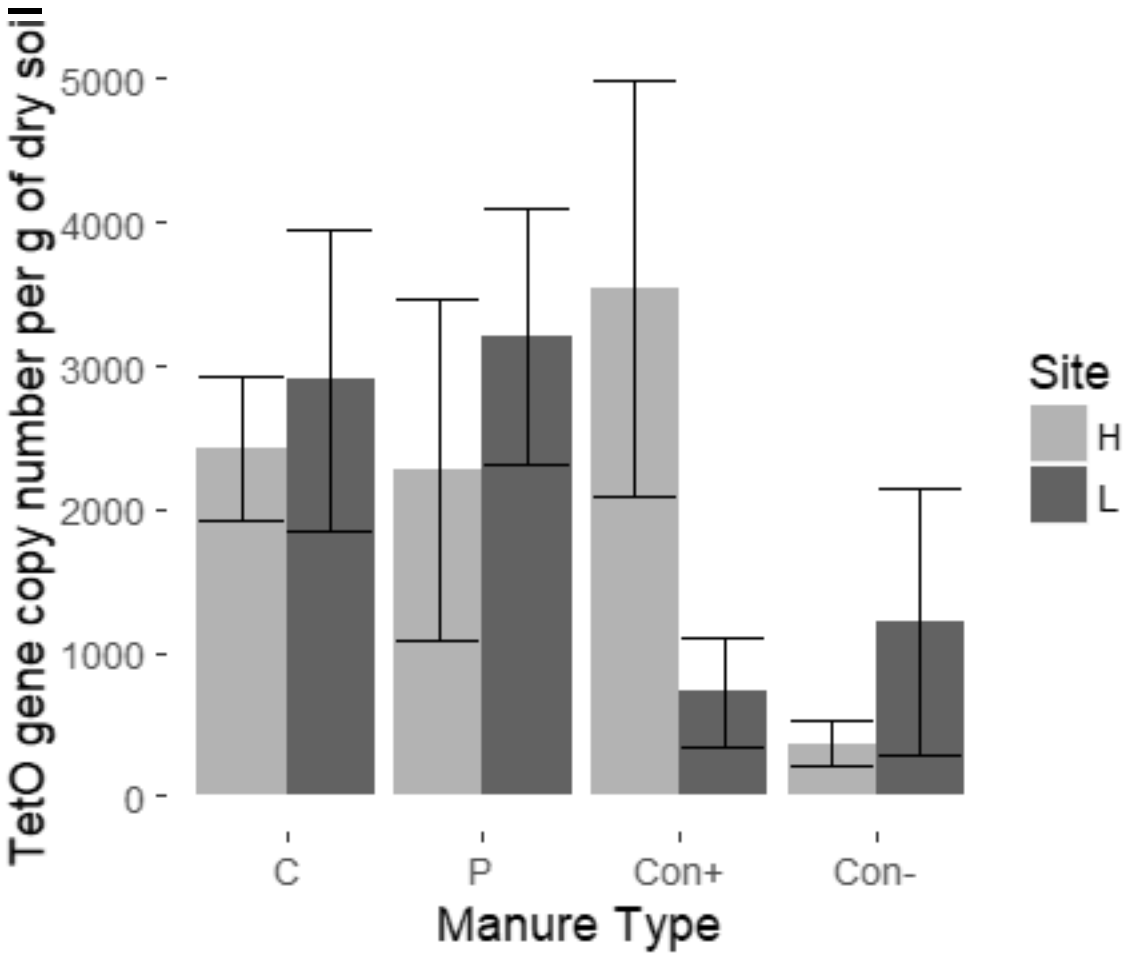

Figure 3.2.TetO gene copy number per gram of dry soil. The x axis represents the manure treatment amended. C means cephapirin, P means Pirilimycin, Con+ means positive control (no antibiotic added) and, Con- means negative control(soil that has not been amended with manure). The site legend indicates cattle traffic levels between high and low sites. No significant differences exist. Error bars represent standard error.

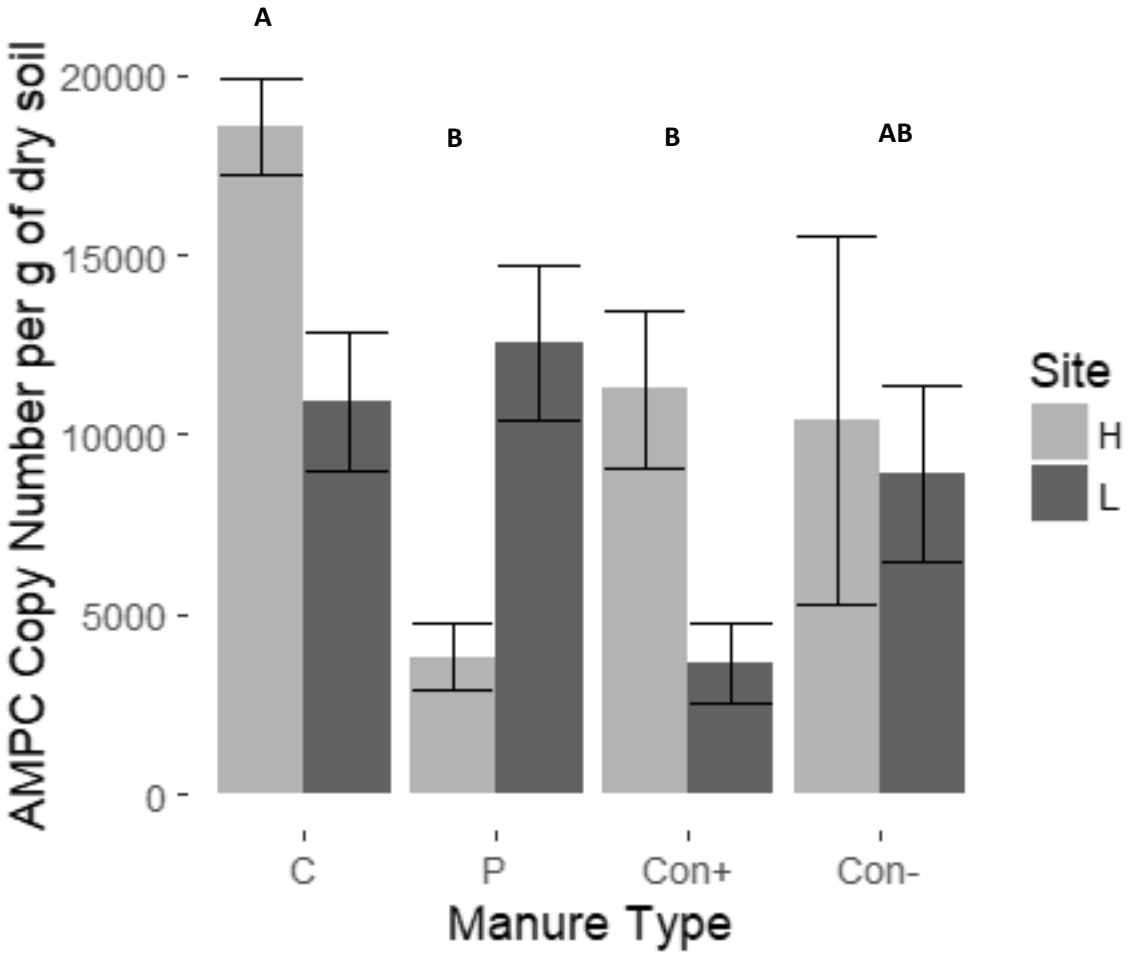

Figure 3.3.AmpC gene copy numbers per gram of dry soil. The x axis represents the manure treatment amended. C means cephapirin, P means Pirilimycin, Con+ means positive control (manure with no antibiotic added) and, Con- means negative control (soil that has not been amended with manure). The site legend indicates cattle traffic levels between high and low sites. Groupings were done using a tukey's test and in this case the groupings are based only on treatment not on cattle traffic level. Error bars represent standard error.

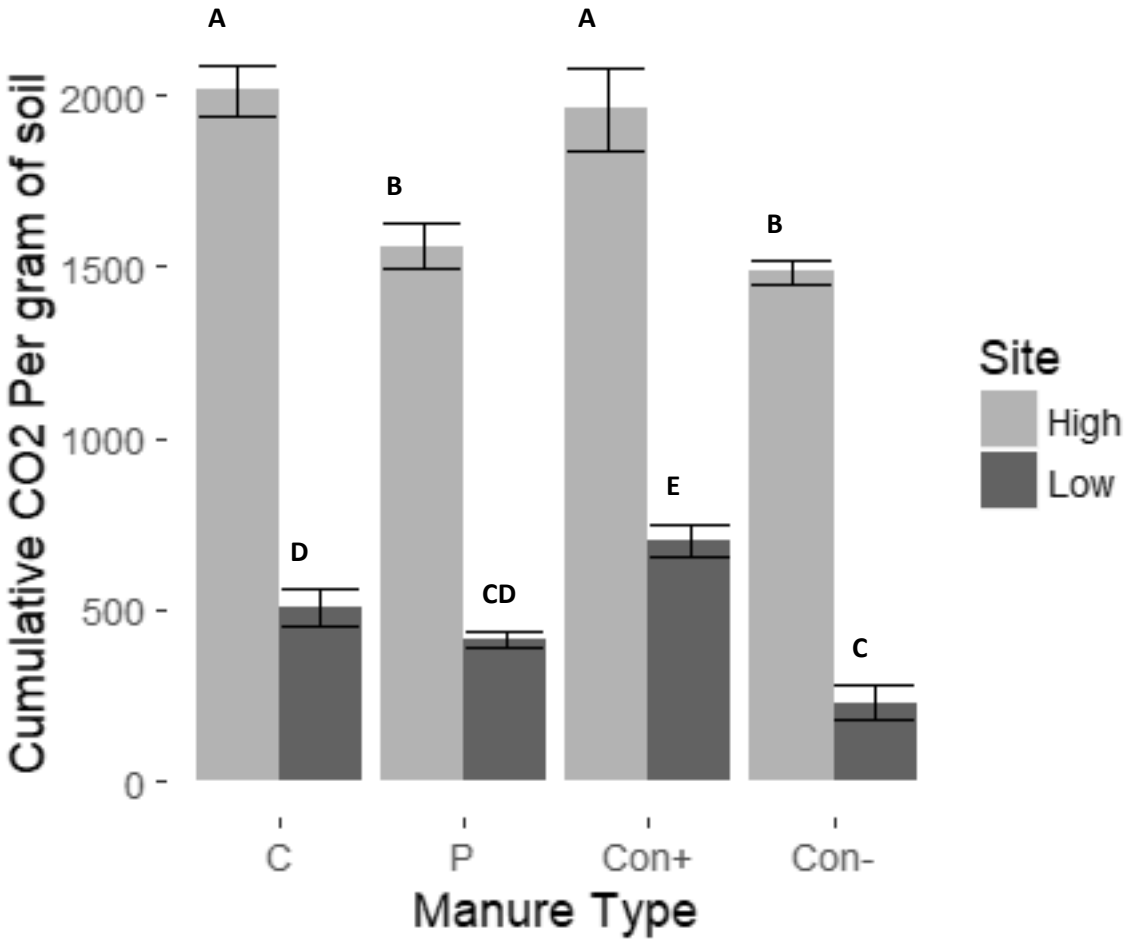

Figure 3.4. Respiration data in ug of carbon respired as CO_2 per gram of dry soil. These values are from the entire length of the incubation with each data point (Days 1, 2,3,7,14,21, and 28) summed for a total value. The x axis represents the manure treatment amended. C means cephapirin, P means Pirilimycin, Con+ means positive control (no antibiotic added) and, Con- means negative control(soil that has not been amended with manure). The site legend indicates cattle traffic levels between high and low sites. Groupings were done using a tukeys test, letter indicate that two sites are significantly similar. Error bars represent standard error.

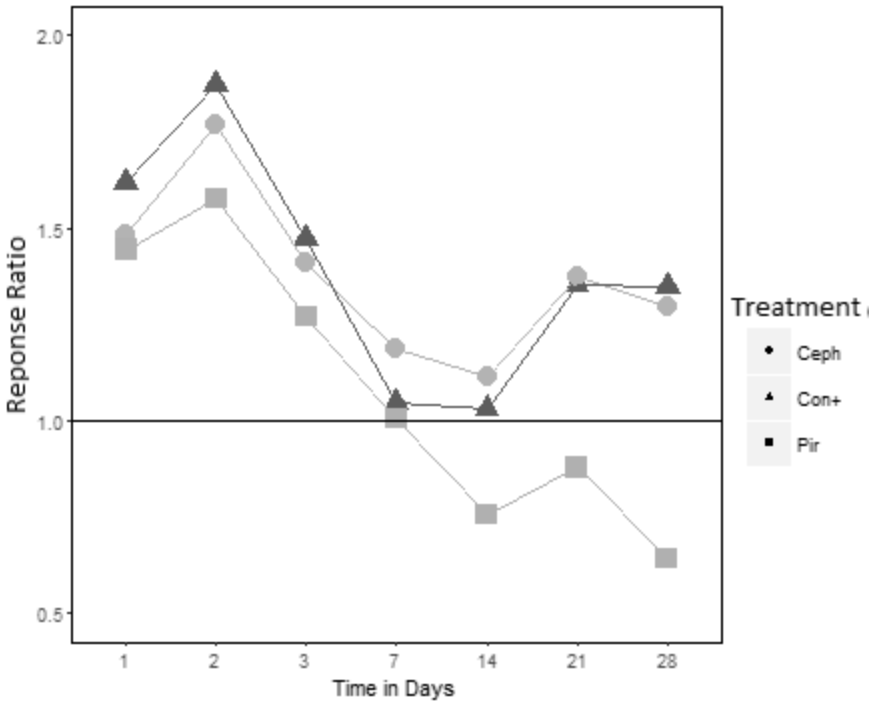

Figure 3.5. Graph of respiration response ratios over the length of the incubation for high cattle traffic soils. The y-axis is comprised of response ratios. Ratios obtained by dividing the respiration value of the treatment by the respiration value of the negative control (soil with no amended manure) at the stated time in the incubation. The X-axis is the amount of time into the incubation the CO_2 measurement was taken. The respiration values are measured in ug of carbon respired as CO_2 per gram of dry soil. The horizontal line at response ratio 1 is to help visualize the values that dip below 1 which would indicate suppression of respiration below the negative control treatment (Soil without any manure added).The C treatment stands for cephapirin manure, the Con+ treatment stands for manure without any antibiotics, and the P stands for pirilimycin manure.

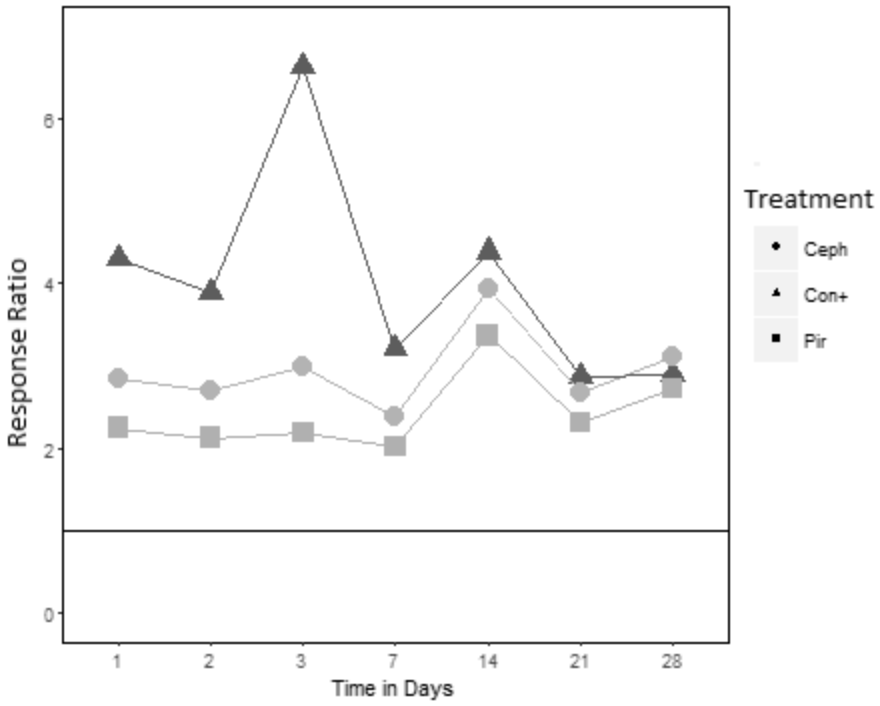

Figure 3.6. Graph of respiration response ratios over the length of the incubation for low cattle traffic soils. The y-axis is comprised of response ratios. Ratios obtained by dividing the respiration value of the treatment by the respiration value of the negative control (soil with no amended manure) at the stated time in the incubation. The X-axis is the amount of time into the incubation the CO_2 measurement was taken. The respiration values are measured in ug of carbon respired as CO_2 per gram of dry soil. The horizontal line at response ratio 1 is to help visualize the values that dip below 1 which would indicate suppression of respiration below the negative control treatment (Soil without any manure added).The C treatment stands for cephapirin manure, the Con+ treatment stands for manure without any antibiotics, and the P stands for pirilimycin manure.

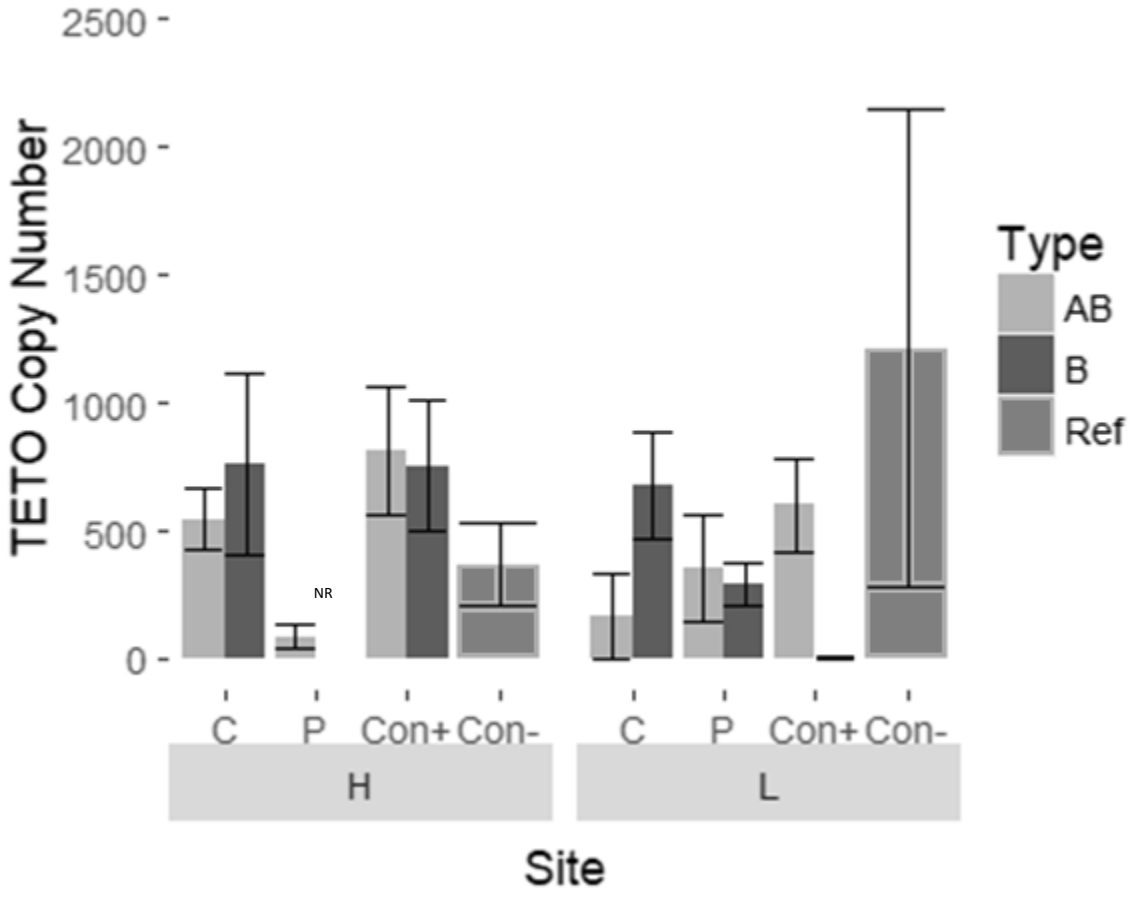

Figure 3.7.TetO gene copy numbers per gram of dry soil from the bacterial inoculation incubations. The site bars at the bottom shows cattle traffic levels. The higher level of the x-axis represents the treatment that the inoculant was extracted from. The types of manure are; C which means cephapirin, P which means Pirilimycin Con+ means positive control (no antibiotic added) and, Con- means negative control(soil that has not been amended with any inoculants). The legend indicates the type of inoculant added to the incubation. B means a live bacterial inoculant was added. AB means a dead bacterial inoculant was added.NR indicates no response. Error bars represent standard error.

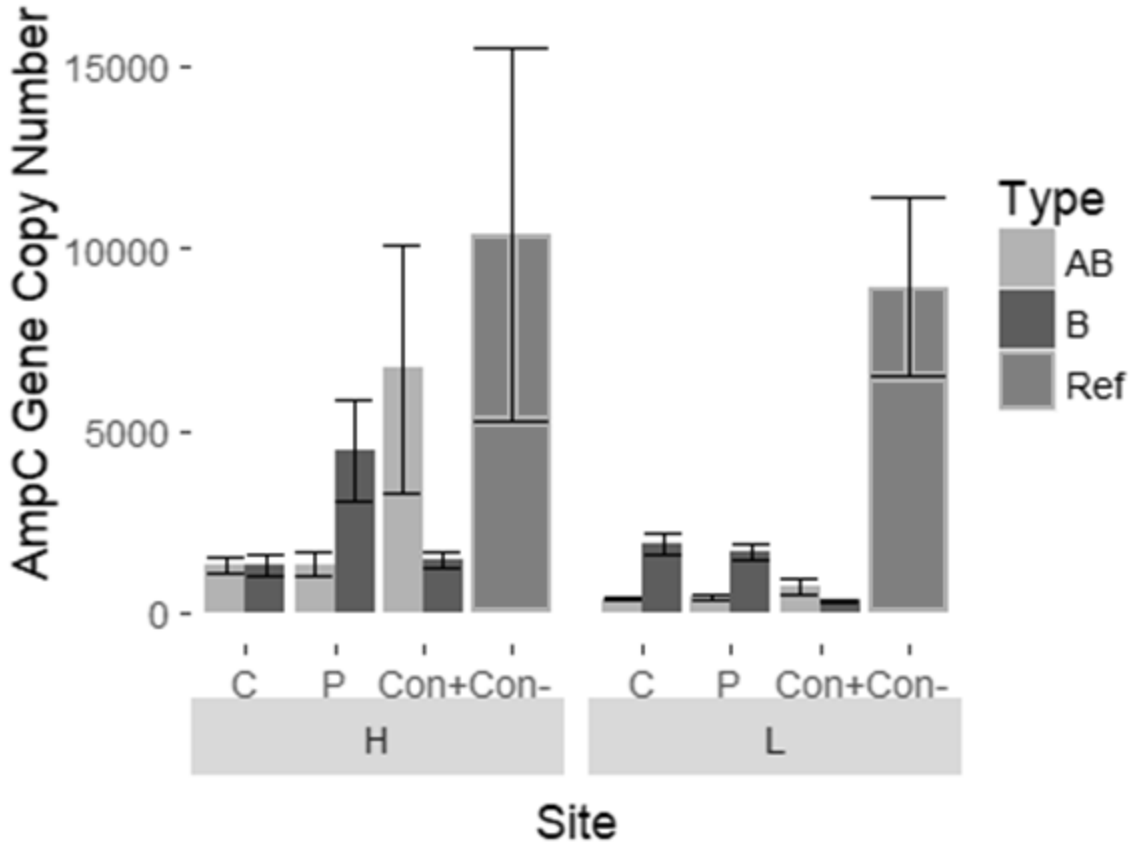

Figure 3.8.AmpC gene copy numbers per gram of dry soil from the bacterial inoculation incubations. The site bars at the bottom of the x-axis represent cattle traffic level. The higher level of the x-axis represents the treatment that the inoculant was extracted from. The types of manure are; C which means cephapirin, P which means Pirilimycin, Con+ means positive control (no antibiotic added) and, Con- means negative control(soil that has not been amended with any inoculants) The legend indicates the type of inoculant added to the incubation. B means a live bacterial inoculant was added. AB means an autoclaved bacterial inoculant was added. Error bars represent standard error.