Ammonia-Oxidizing Bacteria and Archaea Under Continuous Maize: The Influence of Tillage, N Input and Aggregation on Abundance and Community Composition

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AMMONIA-OXIDIZING BACTERIA AND ARCHAEA UNDER CONTINUOUS MAIZE: INFLUENCE OF TILLAGE, N INPUT AND AGGREGATION ON ABUNDANCE AND COMMUNITY COMPOSITION

by

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AMMONIA-OXIDIZING BACTERIA AND ARCHAEA UNDER CONTINUOUS MAIZE: INFLUENCE OF TILLAGE, N INPUT AND AGGREGATION ON ABUNDANCE AND COMMUNITY COMPOSITION

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University of Nebraska, 2014

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Nitrification involves the oxidation of ammonium ($\text{NH}_4^+$) and is an important component of the overall N cycle. Nitrification occurs in two steps; first by oxidizing ammonium to nitrite, and then to nitrate. The first step is often the rate limiting step. Until recently ammonia-oxidizing bacteria (AOB) were thought to be the sole contributors to this process; however, the discovery of crenarchaeota, ammonia-oxidizing archaea (AOA), in marine environments has led to further study of their role in nitrification. Current literature supports the dominance of AOA over AOB in terrestrial ecosystems; however, little is known about what drives their abundance. To investigate the role of cropping system management on soil nitrifier abundance we sampled long-term continuous maize (25+ years) under two tillage treatments (tillage and no tillage) and five N fertilizer rates (0, 40, 80, 120, 160 kg ha$^{-1}$ yr$^{-1}$). Samples were collected three times during 2012; Pre-plant (5/1/12), after planting (5/14/12) and mid-season (7/6/12). Results show that AOA greatly outnumber AOB. The low AOB abundance may be attributed to niche differentiation between archaea and bacteria, as it was found that AOB were less resilient to N rate and tillage than AOA in monoculture maize. Little, if any, literature has examined soil structure as a possible niche. In previous studies, AOA have
been shown to be more resilient to environmental conditions, than AOB. AOB abundance and community structure have been shown to be influenced by tillage, N-rate, and possibly plant growth; they also exhibited spatial heterogeneity. Given the different microenvironments in aggregate size fractions and AOA resistance to environmental conditions and habitat modification a second experiment examined the role of aggregate size fractions on AOB and AOA abundance. AOB were found to respond to soil depth and differed in concentration among aggregate size fractions although N rate still did not influence their abundance. AOA on the other hand was overall unresponsive to soil depth, N rate or aggregate size fraction; however, an N rate by depth interaction affected AOA abundance. Understanding the factors affecting AOB and AOA abundance are important to determine better soil management practices.
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CHAPTER 1

Introduction

1.1 The Nitrogen Cycle

Nitrogen (N) is an important, often limited, nutrient needed for biological processes in plants. Many factors can affect available soil N, including its interaction with soil organic carbon (SOC). The N cycle (See Figure 1.1)(Gruber and Galloway, 2008) is composed of six parts: fixation, mineralization, immobilization, volatilization, denitrification and nitrification.

Nitrogen Fixation

Dinitrogen gas (N$_2$) is abundant in the earth’s atmosphere, yet is considered to be a rate-limiting nutrient. Plants and animals cannot use N in this form and must be fixed by nitrogen-fixing bacteria. A small amount of N-fixation occurs anaerobically through lightning. Agriculture chemical fertilization is dependent on the industrial production of fertilizers. Chemical fertilizers are produced industrially using the Haber-Bosch process which requires high pressure and temperature. The energy requirements for N-fixation are high and current food production would not be possible without the Haber-Bosch process. The majority of N-fixation occurs through specialized microorganisms and only prokaryotes are known to possess the ability to fix N. Due to the inhibitory effects of O$_2$ on the nitrogenase enzyme (responsible for N-fixation), N-fixation must occur anaerobically even if the organism is considered aerobic. Some free-living aerobic N-fixers include species from the commonly studied genera *Azotobacter* (chemoorganotroph), while the anaerobes include *Clostridium* and *Desulfovibrio*
(chemoorganotrophs). *Rhizobium* (chemoorganotrophs) are a common group of bacteria that form symbiotic relationships with legumes. However, plants will use available soil N first in order to reduce energy consumption. Although the organisms mentioned are considered chemoorganotrophs, N-fixing bacteria encompass a wide range of organisms, including phototrophs (Bernhard, 2010; Vitousek et al., 2002; Zehr et al., 2003).

**Mineralization (Ammonification)**

Mineralization is the conversion of organic forms of nitrogen (i.e. manure) to ammonium. This process is carried out by many types of microbes and soil fauna. Mineralization and immobilization typically go hand-in-hand and can occur at the same time in soil microsites. Mineralization occurs under certain conditions when there is a low carbon (C) to nitrogen ratio, i.e. C/N ratio (<20). This is due to the fact that as microbes consume C for energy, they require other nutrients such as N. If their N requirements are low and they are consuming rich N detritus, then they will release extra N into the soil. The N is then available for uptake by plants. This process usual occurs in warm, moist soils that are aerated well. Extreme temperature and water content can increase or decrease the activity (Bernhard, 2010; Johnson et al., 2005).

**Immobilization**

Immobilization is the opposite of mineralization. This involves the uptake of N by microorganisms making the N unavailable to crops. This typically happens when there is a high C:N ratio. In this case, microorganisms do not have enough N in the detritus they are consuming and must scavenge soil available N. Many microorganisms can be involved in this process. Immobilization is largely determined by the amount and
availability of soil N and bioavailable C. The balance between immobilization and mineralization is considered either net immobilization or mineralization (Johnson et al., 2005; Paul, 2006).

**Volatilization**

Volatilization involves the conversion of the ammonium ion to gaseous ammonia. This is not a microbially-mediated process, but does result in the loss of ammonium from the soil. Environmental conditions that influence this are soils with high pH and fertilization using organic sources such as surface applied urea or manure (Johnson et al., 2005).

**Denitrification**

Denitrification involves the anaerobic conversion of nitrate (NO$_3^-$) to gaseous forms of N (dinitrogen (N$_2$) and nitrous oxides (N$_x$O$_x$)) (Bernhard, 2010). Denitrification uses NO$_3^-$ as a terminal electron instead of oxygen (O$_2$) and is therefore linked to nitrification. Denitrification can be a beneficial or undesirable process depending on the environment. For the management of wastewater, denitrification is a desirable process because it removes NO$_3^-$ from groundwater and replenishes fixed N$_2$ to the atmosphere. Since N$_2$O can deplete the ozone, an unwanted greenhouse gas, denitrification would be considered undesirable. In agricultural soils, rates of denitification have to be controlled as N-fertilizer can be lost before reaching the crops. Prokaryotes are thought to be the only organisms capable of denitrification and, much like N-fixation, there are many species from different genera involved: e.g. *Bacillus*, *Pseudomonas*, *Thiomicripora* and *Paracoccus* (Bernhard, 2010). This process usually occurs in poorly aerated soils, anaerobic microsites in aggregates, or in microsites of high O$_2$ demand such as in the
rhizosphere or sites of decomposing residues creating transient anaerobiosis. Since denitrification returns N to the atmosphere it is considered the closing the global N cycle.

Nitrification

Nitrification is an important part of the global N cycle. Some plants are not capable of taking up the ammonium ion (NH$_4^+$) and prefer N as NO$_3^-$. Nitrification is the conversion of NH$_4^+$ to NO$_3^-$ through microbial processes. This is an aerobic process carried out by both bacteria and archaea (known as nitrifiers) (Bernhard, 2010) There are two major steps in nitrification. The first step is carried out by ammonia-oxidizers, converting ammonia (NH$_3^+$) to nitrite (NO$_2^-$). This involves the enzyme ammonia monooxygenase (AMO) which converts NH$_3^+$ to hydroxylamine (NH$_2$OH). The hydroxylamine oxygenase (HAO) enzyme is responsible for the conversion of NH$_2$OH to NO$_2^-$ which is then converted to NO$_3^-$ by nitrite oxidizing bacteria (AOB) (Hooper et al., 1997; Prosser, 1986). Ammonia oxidation was thought to be carried out exclusively by bacteria, although mesophilic archaea have been recently shown to be capable of nitrification (Treusch et al., 2005; Venter et al., 2004). Ammonia-oxidizing bacteria are mainly chemolithoautotrophs and the main species involved belong to the genera Nitrosomonas, Nitrosococcus, and Nitrosospira. The physiology of ammonia-oxidizing archaea is still unknown as very few have been grown in pure culture (Hatzenpichler, 2012). The species belong to the large group Crenarchaeota (now Thaumarchaota) (Brochier-Armanet et al., 2008; Spang et al., 2010) and are typically identified by their environmental association with marine, terrestrial and freshwater, Group I.1a, I.1b and I.3, respectively (Figure 1.2) (DeLong, 1998; Spang et al., 2010). Nitrite-oxidizing bacteria include the genera Nitrobacter, Nitrococcus and Nitrospira. Nitrification, like
mineralization, requires warm moist soils. It can be inhibited by extreme temperatures or pH.

Nitrification is an important part of the global N-cycle and involves the transformation of the more stable NH$_4^+$ to more mobile nitrate NO$_3^-$ form of N. Although nitrification does not contribute to direct inputs of N into soil, it may result in losses to the system through provision of nitrate for denitrification. Denitrification can result in the loss of N applied fertilizer to the atmosphere as a greenhouse gas (Bernhard, 2010; Johnson et al., 2005). The rate of nitrification and understanding the management practices that allow for the most efficient rate of NO$_3^-$ production in a soil system are important to N use efficiency of crops and therefore economic and environmental sustainability. Loss of N in a cultivated system means less fertilizer reaches crops and may affect yield. For these reasons, it is important to understand the drivers behind the microbial mediated transformation of N.

Soil C, including POC and dissolved organic carbon (DOC), total N, vegetation type, climate, fertilizer type, pH, soil moisture and altitude are all important factors that can affect AOB and AOA communities and abundance (He et al., 2007; Prosser and Nicol, 2012; Shen et al., 2008; Yao et al., 2011). These factors have not been studied on a large scale and it is difficult to make comparisons due to the different primer sets used, extraction methods and lack of soil structure and chemical data. It is difficult to correlate an individual soil property to either AOB or AOA abundance. It is suggested that a combination of field, molecular, and microcosm experiments be performed to gain a better understanding of their drivers. Even so, there is most likely not one single
environmental driver for these communities (i.e. soil pH, fertilizer type, input amount) (Prosser and Nicol, 2012).

1.2 Biochemistry of Ammonia Oxidation

Ammonia (NH\textsubscript{3}) is first oxidized to hydroxylamine (NH\textsubscript{2}OH) by AMO enzyme found in the membrane and then the HOA enzyme is used to convert it to NO\textsubscript{2}\textsuperscript{-} in the periplasm (Hooper et al., 1997; Hyman and Wood, 1985) The ammonia monooxygenase gene (\textit{AmoA}), responsible for the active site of AMO, was first discovered in bacteria (AOB). The AMO enzyme uses NH\textsubscript{3}, not NH\textsubscript{4}\textsuperscript{+}, and NH\textsubscript{3} can become less available in acidic soils, affecting the growth of AOB (Suzuki et al., 1974). The proposed mechanism for the oxidation of NH\textsubscript{3} is shown in Figure 1.3 (Klotz and Stein, 2008).

AMO consists of three subunits (A, B, and C). The first subunit is the most conserved region of the gene and is considered the active site from studies showing inactivation by acetylene (Ensign et al., 1993; Hakemian and Rosenzweig, 2007). Although AMO enzyme has not been purified (Klotz and Stein, 2008), methane monooxygenase (pMMO) has and is proposed to be structurally similar to AMO. Due to their similarity it was proposed that AMO, like pMMO, is a Copper (Cu)-containing enzyme (Hyman and Wood, 1985; McTavish et al., 1993; Shears and Wood, 1985). Copper is thought to be a cofactor for AMO because chelating agents bind to the ammonium ion and can stop activity (Hyman and Wood, 1985). It has also been shown that Cu can stimulate AMO activity (Arp et al., 2002). It is also thought that AMO and pMMO has an iron (Fe) center. However, little has been discovered for AMO composition, as it has not been purified (Ward et al., 2011).
1.3 Discovery of non-thermophilic Crenarchaeota

Archaea, discovered by Carl Woese in 1977 using 16S rRNA sequencing, has been accepted as the 3rd domain of life (Woese and Fox, 1977). Archaea were once thought to only inhabit extreme environments with little ecological significance in non-extreme environments with the exception of methanogens (DeLong, 1998). Decades after the discovery of extremophilic archaea, DeLong (1992), discovered a phyla of archaea in a coastal marine sample, belonging to the group Crenarchaeota, that was distinct from other members of the kingdom. This group was surviving in non-extreme oxic conditions while all previous members were considered extreme thermophiles. These were also found to have lower G+C contents than other hyperthermophilic phylogenetically related Crenarchaeota. This led to the hypothesis that this group is uniquely mesophilic (Fuhrman et al., 1992). Culture-independent techniques were used to categorize new mesophilic archaea because only a small percentage of archaea are culturable and the cultivivable ones belonged to only two groups: marine and terrestrial hyperthermophilic crenarchaeota (Nicol and Schleper, 2006). Molecular sequencing in soils, marine plankton, and freshwater sediments confirmed the ubiquitous existence of these mesophilic archaea belonging to a phylogenetically distinct group in the kingdom Crenarchaeota (Bintrim et al., 1997; Buckley et al., 1998; Jurgens et al., 1997; MacGregor et al., 1997; Schleper et al., 1997; Simon et al., 2000). The kingdom, once thought to exclusively house extreme thermophiles, now contain a group of mesophilic organisms found in a wide variety of environmental samples. Beside natural soils and aquatic environments, they have also been found to associate with plant roots in tomato (Lycopersicon esculentum, cultivar M82A), maize (Zea mays L.) and grassland
rhizosphere soils (Chelius and Triplett, 2001; Nicol et al., 2004, 2003; Simon et al., 2000).

Using 16S rRNA target sequences and G+C contents (51-58%), mesophilic members of Crenarchaeota were classified into environmental clusters-Marine, Terrestrial, Finnish Forest Soil Type B (FFSB) and Freshwater- divergent from thermophilic members of the Crenarchaeota group (Buckley et al., 1998); these groups are also known as Group I.1a, I.1b, I.1c and I.3, respectively (DeLong, 1998). The only members of the group I.1c were discovered in a boreal forest soil belonging to the phylogenetically branched group; this group has been shown, through 16S rRNA sequencing, to be distinct from other clusters in Group I. (Buckley et al., 1998; Jurgens and Glöckner, 2000; Jurgens et al., 1997). Members belonging to the FFSB group are thought to have diverged based on their distinctive environment of highly acidic soil (Jurgens and Glöckner, 2000). A study in a gold mine found unique, but closely related mesophilic Crenarchaeota in sediment (Takai et al., 2001). These groups may have branched from other mesophilic Crenarchaeota due to unique environmental conditions.

Molecular tools are used as a way of characterizing and understanding the possible function of these mesophilic terrestrial and marine Crenarchaeota. The first genome sequencing and fosmid formation from an environmental soil sample gave insight into the genes and function of the previously uncultivated mesophilic Crenarchaeota (Quaiser et al., 2002). Insight into the group I.1b cluster revealed existence in every soil type tested (Ochsenreiter et al., 2003). As molecular tools become more popular as a way of studying uncultivable organisms, shotgun sequencing in marine systems revealed functional genes for ammonia oxidation similar to those in AOB.
(Schleper et al., 2005; Treusch et al., 2005; Venter et al., 2004). Interestingly, the concentration of nitrate was high near the surface water where UV is typically thought to inhibit chemoautotrophs like AOB (Venter et al., 2004). To confirm the ammonia-oxidizing function gene, a microcosm study using ammonia incubations showed increased expression of amoA-like genes; suggesting that sequences found for the subunits of these genes most likely code for the three subunits- amoABC. The study found that the amo-like genes for these yet uncultivated mesophilic archaea were significantly similar to the bacterial amoA gene (Treusch et al., 2005). As mesophilic Crenarchaeota have been found in many different terrestrial systems coming from only a few lineages, interest in their diversity, distribution and ecological function has been the basis for many past and recent studies. Their abundance and ubiquity suggests global ecological significance. This could indicate the role of mesophilic Crenarchaeota in the global N cycle.

More recently, mesophilic members of the phylum Crenarchaeota were analyzed using SSU/LSU rRNA and were found to comprise their own distinct phylogenetic group. This group is not a sister group or a branch of the hyperthermophilic Crenarchaeota as previously thought (Figure 1.4) (Schleper et al., 2005), but a deeply branched group with equal stature to that of Euryarchaeota and Crenarchaeota; this mesophilic group is named Thaumarchaeota. It makes up a new phylum in the domain Archaea (Brochier-Armanet et al., 2008; Spang et al., 2010). Early literature on novel mesophilic archaea would suggest that this is a deeply branched lineage in the domain archaea, closely related to the kingdom crenarchaeota. The group is comprised mainly of mesophilic archaea but it is considered a diverse group comprised of members from
marine, freshwater and soil environments (Leininger et al., 2006; Nicol and Schleper, 2006; Wuchter et al., 2006). To further understand the roles of AOB and newly discovered AOA in nitrification, molecular based and phylogenetic studies based on varying soil properties are currently being used.

1.4 AOB and AOA Abundance in Soil

Early studies into roles of AOB and AOA in nitrification have looked at the differences behind AOB and AOA abundance and found that AOA dominate AOB in numbers (He et al., 2007; Leininger et al., 2006; Nicol et al., 2004). Multiple operon copies for amoA genes in AOB have been found but it remains to be seen if archaea also possess multiple copies (Klotz and Norton, 1998; Norton et al., 2002). Due to the abundance of marine AOA, aquatic systems have also been studied for AOA. Interestingly, a subsequent study on AO found that AOA abundance correlated with ammonium oxidation while AOB did not, even though AOB were thought to be the sole oxidizers in marine systems (Wuchter et al., 2006). This supports earlier findings by Venter and colleagues (2004) where high nitrite concentrations were measured in surface marine waters; UV light would normally inhibit AOB and the high nitrite concentration would suggest that AOA play an active role in nitrification in marine systems. Many factors have been suggested to influence AOA and AOB abundance: lower pH, higher temperature, higher altitude, higher NH₃ concentration and vegetation type; yet, correlations to nitrification rates are difficult to make. This is most likely due to inconsistencies in cropping systems, experimental site history and establishment duration, soil type and amount/type of N used in literature (Di et al., 2009; Gubry-Rangin et al., 2010; Long et al., 2012; Prosser and Nicol, 2008; Wertz et al., 2012; Xu et al., 2012).
However, separating each AO group will make it easier to understand which factors play a role in driving the abundance and community diversity.

Community shifts for AOA were observed across the different fertilizer treatments; however, this could be a response to pH. As issue arises when literature compares absolute numbers to previous papers, as PCR bias, DNA extraction efficiencies and even the amount of copies of \textit{amoA} gene in AOB are considered (Okano and Hristova, 2004). Instead, AOA and AOB abundance will be discussed in relative abundance and factors influencing the two groups will be used to characterize each one individually. It has been proposed that AOA and AOB occupy distinct niches that differ in pH, autotrophy versus heterotrophy and ammonia concentration (Prosser and Nicol, 2012). If each group is truly distinct in their role in nitrification then each group must be presented independently of the other.

\textbf{pH}

Early studies indicated pH as being a driver for AO abundance and many studies since have looked at pH as a driving factor. A long-term pH gradient-controlled field site resulted in differences in community composition of AOA \textit{amoA} gene at high and low pH (Nicol et al., 2008). This same group incubated the samples over 24 days and found that AOA \textit{amoA} transcripts separated at different pH levels (4.9-7.5), indicating that groups of AOA may be active at different pH levels. It was also observed that AOA abundance increased with soil acidity. In contrast to acidic soils, a study in alkaline soils (pH:8.34-8.65) showed low diversity of AOA and no correlation between AOA abundance and soil pH (Shen et al., 2008). Across 17 different long-term fertilization
treatments (Control, fertilizer (N) with Potassium (K), Phosphorus (P), PK, NPK, half NPK with half organic manure (OM) and OM) there were no differences in AOA abundance, indicating that fertilizer type may not be a factor in determining AOA abundance in alkaline soil. Shen and colleagues (2008) previously studied an acidic soil with long-term fertilizer treatments and AOA abundance was highest in NPK+OM (He et al., 2007), indicating that it is the differences in soil properties, not substrate type, that may influence AOA abundance. An early study in a forested system reported AOA abundance to be lower than reported in previous studies; possibly due to the lower pH in the system or differences in soil type (Boyle-Yarwood et al., 2008). On the contrary, in freshwater samples AOA actually negatively correlated with pH and ammonia availability (Herrmann et al., 2009). The many studies mentioned above have found differing responses of AOA and AOB abundance to soil pH. This indicates that their activity may vary with pH as well. A review on niche specialization for AO in soil indicated that pH may be a factor for niche specialization between AOB and AOA (Prosser and Nicol, 2012). Therefore, further review of literature into the potential activity and community shift of AOB and AOA with pH will be discussed.

In terrestrial systems, a long-term wheat-maize rotation field study with varying pH (3.47-5.81) and differences in N fertilization type (Control, NK, K, P, PK, NPK, NPK+OM and OM), resulted in high AOA numbers and a positive correlative with pH (He et al., 2007). Potential nitrification rates (PNR) were affected by pH and not differences in fertilizer substrate type, indicating that pH might be a greater factor influencing AO activity. A microcosm experiment using SIP revealed that AOA abundance and activity may be negatively affected by pH as well (Jia and Conrad, 2009).
More recent studies on soil pH and AOA abundance and community structure in pasture soils (Di et al., 2010) and an agricultural field in acidic soil (Xu et al., 2012) found that pH had no effect on AOA abundance or was positively correlated, respectively. Soil pH can affect ammonia availability and this may indicate a role of AOB and AOA thriving under differing ammonia concentrations (Xu et al., 2012). A study using a controlled pH gradient (4.9-7.5), found that AOB abundance increased as soil pH increased (Nicol et al., 2008). Even though AOA were more abundant, the gene transcripts for AOB correlated more with nitrification rates so the true contribution to nitrification would need to be further investigated. AOB bands changed intensities and even disappeared under differing pH suggesting that there are differences in pH requirements for growth of each AO. Gubry-Rangin and colleagues (2010) observed a positive correlation between nitrate concentrations and AOA abundance, but not AOB, in a pH range (4.5 and 6) microcosm study in acidic Scottish cultivate soils. After inhibition AOA abundance increased while AOB did not, indicating that they did not grow in the acidic soils after acetylene inhibition; this supports previously published literature. A study in acidic Chinese tea orchards found that in the highly acidic orchard AOB abundance was below the detection limit and was lower than AOA in all sampled soils (Yao et al., 2011). There was no correlation between pH and AOA but there was a positive correlation between PNR and AOA abundance. Another study in a Chinese subtropical forest system with highly acidic soil (pH<4.0) found that AOB was undetectable and AOA existed in large quantities (Isobe et al., 2012). This furthers the previous findings that AOB prefer higher pH than AOA. This indicates that in acidic soils AOA could be responsible for nitrification activity.
Even though much of the literature discusses that AOA and AOB react differently within a study, there are some discrepancies in literature in which they react similarly to pH. For example a study in an acidic soil with varying fertilizer treatments reported that AOA and AOB responded similarly to fertilizer treatments and that pH correlated with both groups, although, AOA abundance was considerably higher than AOB (He et al., 2007). The follow up study with the same group, but in alkaline soil, found that AOB abundance responded to different treatments and correlated with pH while AOA did not (Shen et al., 2008). Interestingly, AOB communities shifted with different treatments and AOA community, not only did not shift but, exhibited low diversity as well. This might indicate a difference in thriving alkaline versus acidic soils. As witnessed in previous studies, AOB abundance decreased with decreasing pH. Differences between studies should not rule out pH as a possible determinant of AOB and AOA response in acidic soils globally. These differences can also stem from differences in the soil types, the environment, the plant/crop grown and climate. As seen in the literature AOB and AOA do react differently to pH; however, there is no consistent trend of the response of AOB and AOA abundance, community shifts and link to PNR so further investigation and manipulation of pH in laboratory and field experiments will be needed. Another contributing factor linked with pH is ammonia availability, as pH can affect ammonia availability. The inconsistent results with AO abundance and soil pH mean that other factors may be more or equally important in determining AOA and AOB abundance and community structure.

**Temperature and soil water content**
Temperature is known to inhibit nitrification if it is too high or too low and has been shown to interact with soil water content. Soil water content affects water-filled pore space (WFPS) which can affect microbial abundance (Szukics et al., 2010) and activity (Grundmann et al., 1995). Therefore temperature may have both direct by disrupting enzymes and indirect effects through WFPS on AOB and AOA abundance and activity. The optimum WFPS for aerobic microorganisms is between 55-61% (Doran, 1980; Torbert and Wood, 1992) If water content is too low or too high this will negatively affect aerobic microbial communities through substrate and oxygen diffusion rates, respectively.

Soil water content correlations are difficult to interpret across field studies due to seasonal dynamics. A study out of Eastern Europe in a forested system found that water content, although insignificant on its own, did interact with temperature and negatively correlate with AOA abundance (Szukics et al., 2010). AOA:AOB abundance decreased significantly with increasing temperature depending on WFPS. Another study out of Australia specifically looked at the control of WFPS and AOB and AOA communities in soil and found that both AOB and AOA communities shifted with WFPS, but only AOB abundance responded to WFPS changes. (Gleeson et al., 2010). AOB increased, with maximum abundance observed at 65% WFPS. However, this same group reported lower AOA abundance than usual and site primer design as a possible issue; This could explain the lack of response from this group. An incubation study on WFPS, at 40% and 70%, in two temperate forest soils found that AOA is sensitive to water content, while AOB were affected by other factors such as N amendment (Szukics et al., 2012). A higher AOA abundance was observed at 40% WFPS. The differing responses and the manipulation of
WFPS in a laboratory study make it difficult to correlate findings with field studies as WFPS is dynamic.

Along with pH, temperature and WFPS there is a possibility that elevation affects the abundance and activity of AO. An elevation study in China found that AOA numbers outnumbered AOB by four magnitudes at higher elevations (Zhang et al., 2009); however, greater AOA abundance is also observed in many soils tested (Isobe et al., 2012; Leininger et al., 2006; Prosser and Nicol, 2012). Temperature has been found to affect microbial communities and their processes (Avrahami and Bohannan, 2007; Wallenstein and Myrold, 2006), but it still remains to be seen if this is common across climatic and altitude zones. It is difficult to gauge the importance of soil temperature on the two AO groups, as higher temperature can also interact with water availability, and can encounter a wide range over a growing season. An early study on the abundance of AOA in soil revealed that under 12 different soils and three climatic conditions, AOA dominated AOB in number (Leininger et al., 2006). It was suggested that they likely contribute to the nitrification cycle; it is unknown in what magnitude.

**Ammonia concentration**

Ammonia is the direct substrate for nitrification, and the concentration of ammonia in soils is generally low and transient, reflecting mineralization of SOC. Exceptions to this occur in managed soils, where fertilizer amount and type may lead to high concentrations of ammonia for short periods of time. A microcosm study using the application of animal urine resulted in decreased AOA amoA gene copy numbers (Di et al., 2010, 2009). The literature speculates that this could stem from AOA not using
ammonia as the sole energy source and being heterotrophic or mixtrophic (Prosser and Nicol, 2012). A study looking at ammonia uptake of marine AOA found that AOA thrived in greater number in ammonium-poor batch cultures (Martens-Habbena et al., 2009). This indicated that AOA could compete with AOB in oceanic oligotrophic environments.

A soil microcosm study on agricultural soils using stable isotope probing (SIP) found that only AOB assimilated CO$_2$ and their abundance correlated with nitrate accumulation after the addition of ammonium (Jia and Conrad, 2009). It makes sense that AOA would be adapted to low ammonia environments as they are dominant in acidic soils. In the past, nitrification is more sensitive to acidity (Boer and Kowalchuk, 2001). AOB also do not grow in acidic cultures (Gubry-Rangin et al., 2010; Nicol et al., 2008). A recent review paper covering niche differentiation of AO concluded that studies on ammonia concentration do not support niche differentiation based on NH$_3$ concentration (Prosser and Nicol, 2012). However, many field studies do not often report ammonia concentration and AOA and AOB although it has been suggested that they grow in low and high ammonia environments (Di et al., 2010), it is likely other factors such as soil heterogeneity and pH would be more important factors influencing abundance and activity. Therefore, NH$_3$ concentration in microsites could still be a factor influencing AO activity.

Vegetation and growth stage

Vegetation type and seasonal growth may also be a factor affecting AOB and AOA. Many studies include crop rotations, which can alter some of the communities
from year to year (He et al., 2007; Shen et al., 2008; Xu et al., 2012) and make it difficult to compare across the literature. Literature across different vegetation types vary in response of AOB and AOA to fertilizer treatments. An study on nitrifiers in the rhizosphere of rice paddy soils revealed that AOA was strongly affected by emerging rice seedlings and AOB was not (Chen et al., 2008). DGGE analysis on the same samples showed that AOA and AOB communities differed in plots with rice versus plots without. Root-associated AOA have also been seen in freshwater sediments (Herrmann et al., 2009). Thus, there is a possibility that these same associations could exist in other crops. Unfortunately little, if any, literature has examined cropping system as a factor and it is difficult to say if AOA and AOB form unique plant associations or if soil properties, such as aggregation differences in the rhizosphere, are influencing these differences. Although the abundance of AO groups across cultivated systems are similar in magnitude, they do vary from natural systems, which could indicate different associations with vegetation. It is also likely that the difference in management (tillage versus no tillage) could be influencing the structure and therefore the activity of AOA and AOB.

**Soil structure: aggregation**

Although soil structure per se has not been closely examined with respect to nitrifier abundance and activity, its importance could be inferred from the wide variety of soil ecosystems referenced in the proceeding sections. Two theories have emerged to explain the formation of aggregates in soils (hierarchical and non-hierarchical). Hierarchical in which smaller clay particles from smaller microaggregates with soil organic matter (SOM) and then form larger macroaggregates with fine roots and fungal hyphae (Tisdall and Oades, 1982). Non-hierarchical in which larger unstable macroaggregates are first
formed from sticky residues and clay particles and then stable microaggregates would persist and form inside (Oades, 1984). Regardless of which theory, aggregates, micro- (<250um) and macro- (>1mm) have microsites with varying levels of nutrient availability, water-filled pore space, pH gradients, and particulate organic matter (POM).

Microaggregates are known to be more stable, and have slower turnover of SOM, while macroaggregates are less stable, but would have more nutrient movement and POM availability (Six et al., 2004). Therefore management practice, cropping system and turnover of fertilizers are all important factors that could influence aggregation and therefore impact crop production and soil quality. A recent study looking at the effect of N fertilization on soil aggregation found across varying N rates and depth, aggregate size fractions significantly differed. Aggregate fractions can change microbial processes and nutrient cycling (Tisdall and Oades, 1982; Blanco-Canqui and Lal, 2004; Fonte et al., 2009) and should therefore be considered when looking at factors affecting AOA and AOB abundance and activity.

**Synthesis of factors affecting AO abundance and community structure**

Soil pH appears to be the dominant driver of AO abundance and community structure based on current literature; although, inconsistencies remain suggesting greater complexity. However, much of the literature supports AOA as dominant players in acidic and highly acidic soils. Moisture content and climate/temperature tend to go hand in hand, so it is difficult to separate the two; however, within the US, climate from east to west and water-management practices allow for interpretation of data on a global scale across many different climatic zones or moisture contents. Vegetation/cropping system is another important factor likely driving the abundance of AOB and AOA. Much of the
literature has yet to examine specific crop-AO interactions. In the current study continuous maize fields are sampled but rotational field would need to be sampled in order to suggest cropping system as a possible driver of AOB abundance. Looking into crop-specific microbial interactions may permit generalizations to be made across cropping systems, even under varying conditions. As mentioned before, there is likely not any one factor that drives AOA and AOB abundance across ecosystems, but looking into common biophysical properties of soils worldwide, will help scientists predict responses of these organisms and allow for better management practices to be in place. Currently, none of the studies mentioned have looked at the basics of soil structure for niche differentiation. Overall, AOA have been shown to be more resilient (current study) to environmental conditions and this could be due to differences in nutrient availability and moisture content in the microsites of aggregates.
PROPOSAL

With archaea being well defined in literature as a contributor to nitrification, future studies will be needed to find out if bacteria and archaea are specialized in their roles for nitrification. Currently, none of the studies mentioned have looked at the basis of soil, soil structure, to study niches. AOA have been shown to be more resilient to environmental conditions, than AOB. AOB abundance and community structure have been shown to be influenced by tillage, N-rate, and possibly plant growth; they have also exhibited spatial heterogeneity across replicate plots (current study-not yet published). This data and previous findings in literature have influenced a study into possible aggregate size fraction niches. Aggregates, micro- (<250um) and macro- (>1mm) contain microsites in which nutrient availability and water-content can vary. Microaggregates are known to be more stable, and have slower turnover of soil organic matter (SOM), while macroaggregates are less stable, but would have more nutrient movement and POM availability (Six et al., 2004). Based off of findings in previous literature there is a possibility that the differences in response to environmental factors exhibited by AOA and AOB could be due to occupy different aggregate size fractions.

Few long-term studies have been performed to ascertain the controls on nitrifier abundance in soils and assumptions about soil pH being the main environmental driver on these communities is overemphasized in the current literature. To further explore controls on nitrifier community abundance and diversity in agricultural soils, we chose a long-term field site in Concord, NE that has been continually cropped to maize since 1986. We examined AOB and AOA abundance at three time points in the 2012 growing season under five N fertilization rates (0, 40, 80, 120, and 160 kg ha\textsuperscript{-1} yr\textsuperscript{-1}) and two tillage
practices, disk and no-till. We hypothesized that (1) N fertilization rate, through effects on soil pH and aggregation, influences the abundance and community dynamics of both AOA and AOB communities; (2) tillage, through impacts on microbial habitat, alters the abundance and community dynamics of both AOA and AOB communities; (3) AOA and AOB abundance is not static but responds to crop growth and climatic shifts over the growing season; and (4) niche specialization among nitrifier communities within soil aggregate size fractions may explain the effects of soil management on the abundance of AOA and AOB in soil.
Figure 1.1. General diagram of global N cycle divided processes by land and marine ecosystems (Gruber and Galloway, 2008).
Figure 1.2. Phylogenetic tree of archaea demonstrating changes over the last two decades (Spang et al., 2010).
Figure 1.3. Model of experimentally verified (solid lines) and proposed (dotted lines) of energy flow oxidation of ammonia involved in ammonia oxidizing bacteria (AOB) (Klotz and Stein, 2008).
Figure 1.4. A phylogenetic tree of 16S rRNA sequences from archaea phyla with representation of uncultivated mesophilic Crenarchaeota (Schleper et al., 2005).
CHAPTER 2

Materials and Methods

2.1 Field Site and Sampling

This study was conducted at the Haskell Agricultural Laboratory near Concord, NE (latitude 42° 23’ N and longitude 96° 59’ W). This site has a mean seasonal temperature of 17.5 °C and an average annual rainfall of 670 mm. The site is part of the University of Nebraska-Lincoln, established in 1985 with a split-split plot design. Plots were broadcast fertilized with ammonium nitrate before tillage and rainfed irrigated.

There were four randomized complete block replicates with main plots consisting of two management systems, no tillage and disk tillage. Two crop rotations (continuous maize [Zea mays L.] monoculture and maize-soybean [Glycine max (L) Merr.] rotation) constituted the first split and N rate (0, 40, 80, 120, and 160 kg ha⁻¹ yr⁻¹) the second split. For this study, only monoculture maize plots were sampled.

For the first study samples were taken from continuous maize plots in 2012 during three critical periods of the growing season; pre-planting (SD1) (5/01/12), directly after planting (SD2) (5/14/12) and mid-season (SD3) (7/06/12). Samples taken on 7/11/12 showed maize plants at V12 growth stage. Fields were broadcast fertilized and tilled (5/02/2012), directly after the first sampling date. Maize crops were planted five days before the 2nd collection date (5/09/2012). Six soil cores (5 cm diameter) were taken per plot under two tillage treatments (tillage vs no tillage), five N rates (0, 40, 80, 120, 160 kg N ha⁻¹ yr⁻¹) and four plot replications over three sampling dates (SD1, SD2, SD3), to a depth of 15 cm and combined to form one composite sample, for a total of 120 soil
samples. Samples were then transported back to the lab and refrigerated at 4 °C until processed. Samples were sieved (4 mm) and separated into air-dried samples, for pH and EC analysis, and frozen samples at -20 °C, for FAME and DNA analysis. Soil pH and EC were analyzed on 10 g of air-dried soil using a 1:1 soil-water slurry. Readings were performed on SympHony pH/EC meter (Table 2.1a/b).

For the second study soil samples were collected from continuous maize in spring 2013 (before planting). Samples were taken as a subsample of a previous study (Blanco et al. 2014). Two depths (0-7.5 and 7.5-15 cm) from tractor cores were sliced and analyzed for C and N concentration, pH, water-stable aggregates and AOB/AOA abundance.

Samples were air-dried for 72 hrs. A portion of air-dried soil was ground through a 2 mm sieve. The samples were then roller milled on a SampleTek model 200 vial rotator. A 100 mg roller-milled subsample was analyzed for C and N concentration (Table 2.2) using a Thermo Scientific Flash 2000 Elemental Analyzer. Soil pH was determined using 10 g of air-dried soil using a 1:2 soil-water slurry. Soil pH was measured using into a Thermo Electron Corp. Orion 525A+ pH/mV/ORP/BOD meter. Water-stable aggregates distribution across the soil profile were determined by 0-7.5cm (Figure 2.1a) and 7.5-15cm (Figure 2.1b) depth across N rate previously (Blanco-Canqui et al., 2014).

2.2 AOB and AOA Abundance

Water-Stable Aggregates

Aggregates were initially dry sieved through an 8 mm sieve, using a pestle to crush larger pieces. Water-stable aggregates were separated from approximately 100 g air
dried soil using a modified wet sieving method (Blanco-Canqui et al., 2014). Once all samples passed through the initial screen, sieves were ordered from 250 µm, 1 mm and 4.75 mm in water tanks and filled with water, just under the top sieve so that the aggregates could saturate with water. After 10 mins of saturation, the samples were oscillated vertically for another 10 mins at 40 cycles/min with a stroke length of 3 cm. Samples that had not passed through 4.75 mm screen were discarded. Each range of aggregate size (<250 µm, 250 µm-1 mm and 1 mm-4.75 mm) were scraped into sterilized 50 mL Teflon tubes and immediately freeze-dried for one week. Once samples were freeze-dried, they were roller-milled for 24 hrs and then stored at room temperature for DNA analysis.

**DNA Extractions**

For the first study (2012), soil samples that were previously frozen for molecular analysis were freeze-dried overnight for long-term storage and moisture reduction. DNA was extracted from approximately 500 mg of freeze-dried soil using MoBio Ultra Clean® soil DNA isolation kits (San Diego, CA) according to the manufacturer’s ‘Alternative Protocol’ for maximum yields with a slight modification as follows: bead tubes were held in the MoBio Vortex Adapter tube holder (MoBio Catalog No. 13000-V1) and vortexed according to the protocol. Tubes were then incubated at 70 °C for 10 mins in a water bath after solution S1 was added to more efficiently break up the cells and then followed by the Alternative Protocol for maximum yields. Samples were stored at -20 °C until needed.
For the second study (2013) DNA was extracted from approximately 250 mg of freeze-dried soil using MoBio PowerSoil® DNA isolation kits (San Diego, CA) according the manufacturer’s guidelines. Samples were eluted in 100 µl of PCR grade H₂O. Samples were stored at -20 °C until needed.

Real-Time PCR Assay

The amoA gene of AOB and AOA were amplified using real-time polymerase chain reaction (qPCR) assays to target specific amoA gene sequences in AOB and AOA. DNA samples were diluted 1:10 to eliminate possible inhibition during PCR. AOB 16SrRNA genes were amplified using a 491-bp fragment specific forward primer amoA-1F (5’-GGGGTTTTCTACTGGTGTTG-3’) and reverse primer amoA-2R (5’-CCCCTCKGSAAGCCTTCTTC-3’) (Rotthauwe et al., 1997). Primers for amoA genes in AOA were amplified using targeting a 635 bp fragment forward primer amoA19F (5’-ATGGTTGTGTGGCTWAGACG-3’) (Leininger et al., 2006) and reverse primer amoA643R (5’-TCCCACTTWGACGCGGCCATCCA-3’) (Treusch et al., 2005). Each reaction was run in triplicate and Sybr Green I was used as a fluorescent dye to quantify each sample. Reactions were carried out in 20 µl reactions containing 5 µl of 1-10 ng of DNA template, 0.2 µl of each forward and reverse primer, 4.6 µl of H₂O and 10 µl of QuantiTect SYBR Green PCR Master Mix (Qiagen, Venlo, Limburg). All experiments were carried out on an Eppendorf Mastercycler realplex 4 (Eppendorf, Hamsburg, Germany). Cycling conditions for AOB and AOA as follows: Initial denaturation at 95 °C for 15 mins followed by 45 cycles of 95 °C for 1 min, annealing at 54 °C for 1 min, elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 mins. Differences in annealing temperature and length of elongation for AOA were due to optimization of the
assay. There was no non-specific annealing observed. Product specificity was confirmed using melt curve analysis. Single peaks were found for all samples used in this study. Product specificity was also confirmed using Gel Electrophoresis, by a single band at ~500 bp and ~650 bp for AOB and AOA, respectively.

Standard curves were developed using cells obtained from Dr. Kimberly Cook’s lab (USDA-ARS, Bowling Green, KY). Cells frozen in glycerol were grown up in LB broth at 37 °C for 48 hrs. Five ml of grown cells were purified using Wizard Plus Minipreps DNA purification system (Promega, Madison, WI) with slight modifications as follows: 2 microcentrifuge tubes of 2 ml were combined in step 2. Purified extracts were eluted in 50 ul of PCR grade H₂O. Concentrations were quantified using NanoDrop. 10-fold serial dilutions were made in Tris-HCL buffer ranging from 10⁻⁸-10⁻² to create a standard curve.

Each plate was run with a standard curve previously described. AOB and AOA amoA primers achieved high efficiencies between 88 % and 115 % for AOB amoA and 85 % and 99 % for AOA amoA with R² values of 0.981-0.999 and 0.990-0.999, respectively.

2.3 Microbial Community Analysis

FAMES

Fatty Acid Methyl Ester (FAME) profiles were performed on soil samples taken over three sampling dates (as described above) to track changes in total microbial biomass as well as shifts in microbial community structure (Drijber et al., 2000). Specific FAME biomarkers for arbuscular mycorrhizal fungi (C16:1cis11); saprophytic fungi
(C18:2cis9,12) and several bacterial markers were measured in response to N-rate, tillage and sampling date. The method, previously described by Grigera et al. (2007), calls for 10 g of soil but can be scaled down if soil is limited. Briefly, soil is first hydrolyzed and saponified using 0.2 M MeOH-KOH, then FAMEs are partitioned in hexane and quantified by gas chromatography using methyl nonadecanoate as an internal standard. For FAME identification, peaks are analyzed by retention time, compared with known standards then verified by GCMS.

**DGGE**

Denaturant gradient gel electrophoresis (DGGE) community analysis for bacterial and archaeal amoA genes was performed on a subset of samples (0N, 80N, 160N) from the first sampling date totaling 24 samples each for AOB and AOA. The optimal denaturant gradient ranged from 55 % to 60 % for AOB and 15 % to 55 % for AOA. Bacterial amoA genes were amplified using primers AmoA1F/2R with a GC clamp (Rotthauwe et al., 1997) and Archaeal amoA genes were amplified using primers AmoA23F/616R with a GC clamp (Sahan and Muyzer, 2008). Polymerase chain reactions were run in triplicate 50 µl reactions using 25 µl Sigma redTAQ jumpstart, 0.5 µl of each primer, 22 µl of PCR grade water, 1 µl of 12.5 µg/µl BSA as a catalyst (Juliette et al., 1995; Leininger et al., 2006), and 1 µl of DNA template. Triplicate PCR reactions were combined and concentrated to enhance bands using Wizard Kit Mini Prep (Promega, Madison, WI). Gels were run at 50 V for 16 hrs at 55 °C for AOB and AOA. The gels were stained for 10 mins with(Tian et al., 2013) 1:10,000 SYBR Green I (Qiagen, Venlo, Limburg) and scanned using a transilluminator with a 535 SYBR Green specific filter.
2.4 Statistical Analysis

For the first experiment (2012) data was analyzed using PROC MIXED module in SAS (SAS Institute, Inc., NC, USA) with a split-plot design with repeated measured for Haskell 2012. Covariance structure was set using compound symmetry as previous models were tested and this was the best fit. For the second experiment (2013) data was analyzed using PROC GLM module in SAS with a split-pot design. All two- and three-way interactions were tested and insignificant terms were dropped out. Pearson’s correlation was run with environmental factors separated by treatments on AOB and AOA abundance. The probability level (p-value) in which the null hypothesis is rejected is p<0.05. Least-square means (LSMeans) test were used whenever ANOVA was significant to determine differences among treatment and sampling dates.

FAMEs were analyzed using stepwise discriminant analysis with the PROC STEPDISC function in SAS. Canonical discriminant analysis was then performed on the most influential FAMEs from stepwise analysis using PROC CANDISC in SAS. This was used to characterize changes in microbial community structure against fertilizer gradient and tillage. Significance levels were set at p<0.05. Stepwise and canonical discriminant analyses procedure were outlined in Drijber et al. (2000).
Figure 2.1. (a) Distribution of water-stable aggregate (g 100 g$^{-1}$) across N rate (0, 80 and 160 kg N ha$^{-1}$ yr$^{-1}$) sampled in depth 0-7.5 cm. (b) Distribution of water-stable aggregate (g 100 g$^{-1}$) across N rate (0, 80 and 160 kg N ha$^{-1}$ yr$^{-1}$) sampled in depth 7.5-15 cm. Adapted from Blanco-Canqui et al. (2014).
Table 2.1 (a) Mean values of soil chemical and physical properties; pH, EC and water-filled pore space (WFPS) in no-tilled plots. (b) Mean values of soil chemical and physical properties; pH, EC and WFPS in disk-tilled plots.

(a) | Sampling Date | N Rate (kg N ha\(^{-1}\) yr\(^{-1}\)) | pH  | EC    | WFPS |
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(b) | Sampling Date | N Rate (kg N ha\(^{-1}\) yr\(^{-1}\)) | pH  | EC    | WFPS |
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Table 2.2. Mean values of Nitrogen (N), Carbon (C) and pH in soil sampled by depth for Haskell 2013 study.

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<th>C</th>
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CHAPTER 3

Results

3.1 AOB and AOA Abundance

AOB Abundance

Mixed model procedure for repeated measures resulted in the main effects, sampling date (p<0.0001) and tillage (p=0.0011) being significant factors effecting AOB abundance but not N rate (Table 3.1A). The mean AOB abundance from sampling date 5/1/12 (SD1) to sampling date 5/14/12 (SD2) exhibited a decrease from $3.03 \times 10^4$ to $6.7 \times 10^3$. The abundance of AOB then increased to $2.09 \times 10^4$ by sampling date SD3 (7/6/12) (Figure 3.1). Interestingly, soil water filled pore space (WFPS) was lowest during SD3 ranging from 37.4% to 53.5%. AOB abundance was lower in disk tillage ($7.73 \times 10^3$) than no till ($3.39 \times 10^4$) plots averaged over all three dates (Figure 3.2).

Although, no clear trend with N rate emerges averaged across tillage and sampling date, abundance is lowest in 0N ($7.53 \times 10^3$) and highest in 160N ($3.18 \times 10^4$) (Figure 3.3). When separated by tillage, despite an insignificant N rate by tillage interaction, there appears to be an upward trend in AOB abundance with N rate under no tillage (Figure 3.4). There was a significant sampling date by tillage interaction (p=0.0453). Initially, after planting (SD2), there was a steep decrease in AOB abundance and then recovery by SD3 (Figure 3.5). Under no till AOB abundance differed between SD1 and SD2/3 but not between SD2 and SD3. Under disk till SD2 differed from SD1/3; however, there was no difference between SD1 and SD3 (Table 3.1B). Maize was at growth stage V12 during the week that SD3 sampling occurred.
AOA Abundance

Neither N rate nor tillage affected AOA abundance. However, AOA abundance decreased across sampling dates (p<0.0001) (Figure 3.1), which may be related to decreased water content over the growing season, as they were highly correlated (r=0.552; p<0.01) across tillage, N rate and sampling date. Mean AOA abundance of $3.79 \times 10^8$ at SD1 dropped to $1.81 \times 10^8$ at SD2 and finally to $5.32 \times 10^7$ by SD3. Given AOA abundance remained fairly consistent across N rates (Figure 3.3) and there were no interactions among N rate, tillage and sampling date, these terms were dropped out from the model (Table 3.1A).

Correlations with Soil Properties

Correlations of AOB and AOA abundance with soil properties (pH, EC and water filled pore space) were made across N rate, tillage and sampling time (Table 3.2). AOB was only correlated with pH, (r=0.275; p=0.0025) while AOA correlated only with WFPS (r=0.552; p<0.0001). Since tillage was found to be an important factor affecting AOB abundance, correlations were recalculated by tillage. This resulted in AOB being correlated with pH only under disk tillage (r=0.422; p=0.0008), but AOA abundance remained positively correlated with WFPS (Table 3.3). AOB and AOA abundances were correlated across tillage (r=0.275; p=0.0025); however, when separated by tillage, they were only correlated under no tillage (r=0.404; p=0.0014) and not disk tillage.

3. 2 Soil Microbial Community Composition and Shifts
Soil Microbial Community Structure by Fatty Acid Profiling

The concentration of bacterial fatty acid methyl esters (FAMES) did not differ significantly among treatments (Table 3.4). Bacterial biomass did, however, differ among sampling dates. Arbuscular mycorrhizal (AM) fungal biomass decreased with increasing N rate and was lower in disk tillage than no till. Saprophytic fungal biomass was also affected, albeit opposite to AM biomass, by tillage treatment being higher in the disk tillage treatment compared to no till. Microbial community composition clearly differed by tillage on the Y axis, while N rate separated out on the X-axis of the canonical discriminant plot (Figure 3.6A and 3.6B).

Denaturing Gradient Gel Electrophoresis of AOB and AOA with Tillage and N rate

Denaturing Gradient Gel Electrophoresis (DGGE) patterns of AOA were more consistent across treatments compared to AOB (Figure 3.7). For AOA, there was some clustering of disk tillage and no tilled samples (Figure 3.8) but little clustering by N rate. AOB patterns were very scattered with little clustering of treatments including across different plot locations with the same treatments (Figure 3.9). This raises the question as to whether the AOB banding pattern is due to spatial heterogeneity in the field, or a low detection limit for DGGE.

3.3 AOB and AOA Abundance in Aggregate Size Fractions

AOB Abundance

Soil provided by Blanco-Canqui et al. (2014) from the Haskell field site under no-till continuous maize and a N fertilizer gradient, was separated into three aggregate size
classes: less than (LT) 250 μm, 250-1000 μm, and greater than (GT) 1000 μm to
determine the concentration of AOB and AOA within each aggregate size class. Using
the concentrations of AOB and AOA within each aggregate class and the aggregate size
distribution reported by Blanco-Canqui et al. (2014) we calculated abundances of AOB
and AOA based on differences in aggregate distribution. AOB abundance in the soil was
affected by aggregate size class (Figure 3.10A) and depth (Figure 3.11A) but not N rate
even for concentration within aggregate (Table 3.5A & B). AOB concentration within
aggregate classes differs with a higher concentration in LT250μm aggregates than 250-
1000μm and GT1000μm aggregate size fractions (Figure 3.10B). Depth was also a
factor, with lower AOB concentrations at the 2nd depth within the aggregate classes
(Figure 3.11B). There was also an aggregate class by depth interaction (Figures 3.12A &
B) with AOB abundance based on aggregate distribution by aggregate class (Table 3.6A)
and concentration within an aggregate class (Table 3.6B). Abundance and concentration
was significantly lower at the 2nd depth for the 250-1000 μm and >1000 μm aggregate
classes only. N rate was not a factor affecting AOB concentrations within aggregates
(Figure 3.13) even when separated by depth (Figure 3.14). Insignificant interactions were
dropped out of our model (Table 3.7).

AOA Abundance

Averaged across N rate and soil depth, AOA abundance (Figure 3.10A) or
concentration (Figure 3.10B) did not differ by aggregate class. When averaged across
aggregate classes (Figure 3.11A & B), there was no difference with soil depth or N rate
(Table 3.7) however, there was an N rate by depth interaction (Figure 3.15A and Table
3.5A & B). In the surface depth (0-7.5 cm), AOA abundance (Figure 3.15A and Table
or concentration (Figure 3.15B and Table 3.5B) declined with increasing N rate, while the opposite trend occurred in the deeper depth, although not significantly.

Because the aggregate by N rate interaction approached significance (p=0.0853), we plotted AOA concentration by N rate within each aggregate class separately (Figure 3.16). There was a significant interaction between depth and N rate for the LT250 and 250-1000 µm aggregate class, but not the GT1000 µm aggregate class.

Correlations of AOB and AOA Abundance within Aggregate Class with Soil Properties

A positive correlation with Carbon (C) and Nitrogen (N) exists for both AOB (Table 3.8A) and AOA (Table 3.8B) based on aggregate size distribution. For soil pH, AOB and AOA both have a negative correlation when calculated by aggregate distribution differences and by concentration within aggregates. Correlations were also separated by depth, given its significance. For AOB abundances based on aggregate distribution, the negative correlation with pH is lost in depth 2 (Table 3.9A), as are all correlations with C and N when separated out by depth. For AOA across aggregate distribution, the negative correlation with pH is lost when separating by depth (Table 3.9B); however, there is still a positive correlation with C and N in the first depth, but is then lost in the second depth.
Figure 3.1. AOA and AOB abundance by sampling date (Sampling Date 1 (SD1): 5/1/12; Sampling Date 2 (SD2): 5/14/12; Sampling Date 3 (SD3): 7/6/12). Abundance is measured in log scale (copies g$^{-1}$ soil).
**Figure 3.2.** AOB and AOA abundance by tillage (No till and Disk tillage). Abundance is measured in log scale (copies g\(^{-1}\) soil). (NS=not significant)
Figure 3.3. AOB and AOA abundance by N fertilizer rate (0, 40, 80, 120 and 160 kg N ha$^{-1}$ yr$^{-1}$). Abundance is measured in log scale (copies g$^{-1}$ soil). (NS=not significant)
Figure 3.4. Interaction N fertilizer rate (0, 40, 80, 120 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) with tillage (No Tillage and Disk Tillage) on AOB abundance. Abundance is measured in log scale (copies g\(^{-1}\) soil). (NS=not significant interaction)
Figure 3.5. Interaction between sampling date (Sampling Date 1 (SD1): 5/1/12; Sampling Date 2 (SD2): 5/14/12; Sampling Date 3 (SD3): 7/6/12) and tillage (No Tillage and Disk Tillage) on AOB abundance. Abundance is measured in log scale (copies g$^{-1}$ soil). Different letters represent significant difference (P<0.05) between sampling date within tillage treatment.
Figure 3.6. (a) Canonical discriminant analysis of management by tillage and N rate. Disc0=Disk tillage by 0N, Disc40=Disk tillage by 40N, Disc80=Disk tillage by 80N, Disk120=Disk tillage by 120N, Disc160=Disk tillage by 160N, NT0=No tillage by 0N, NT40=No tillage by 40N, NT80=No tillage by 80N, NT120=No tillage by 120N, NT160=No tillage by 160N. (b) Correlation of FAMEs with first two discriminant functions for tillage by N rate.
Figure 3.7. (a) Denaturing Gradient Gel Electrophoresis (DGGE) pattern of AOB tillage by N rate. (b) DGGE pattern of AOA tillage by N rate. NT0=No tillage by 0N, NT80=No tillage by 80N, NT160=No tillage by 160N, DK0=Disk tillage by 0N, DK80=Disk tillage by 80N, DK160=Disk tillage by 160N.
Figure 3.8. AOB cluster analysis comparing DGGE bands by tillage and N rate. Range of similarity=0-100%. No-till 0=No tillage by 0N, No-till 80=No tillage by 80N, No-till 160=No tillage by 160N, Till 0=Disk tillage by 0N, Till 80=Disk tillage by 80N, Till 160=Disk tillage by 160N.
Figure 3.9. AOA cluster analysis comparing DGGE bands by tillage and N rate. Range of similarity=45-100%. No-till 0=No tillage by 0N, No-till 80=No tillage by 80N, No-till 160=No tillage by 160N, Till 0=Disk tillage by 0N, Till 80=Disk tillage by 80N, Till 160=Disk tillage by 160N.
Figure 3.10. (a) AOB and AOA abundance in whole soil based on aggregate distribution. (b) AOB and AOA concentration within aggregate size class. Measurements in log scale (copies g\(^{-1}\) soil). (NS=not significant). LT250=<250µm, 250-1000=250µm-1mm, GT1000=1mm-4.75mm.
**Figure 3.11.** (a) AOB and AOA abundance in whole soil by soil depth based on aggregate distribution. (b) AOB and AOA concentration averaged across aggregate size class by soil depth. Measurements in log scale (copies g\(^{-1}\) soil). (NS=not significant). Depth1=0-7.5cm, Depth2=7.5-15cm.
Figure 3.12. (a) Interaction of aggregate size class by depth on AOB abundance in whole soil based on aggregate size distribution (P=0.0004). (b) Interaction of aggregate size class by depth on AOB concentration within aggregate size class (P=0.0001). Measurements in log scale (copies g\(^{-1}\) soil). Depth1=0-7.5cm, Depth2=7.5-15cm.
Figure 3.13. Effect of N fertilizer rate (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) on AOB and AOA concentration averaged across aggregate size class. Measurements in log scale (copies g\(^{-1}\) soil). (NS=not significant).
**Figure 3.14.** Interaction of N fertilizer rate (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) on AOB concentration averaged across aggregate size class by soil depth (Depth1=0-7.5 cm, Depth2=7.5-15 cm). Measurements in log scale (copies g\(^{-1}\) soil). (NS=not significant).
Figure 3.15. (a) Interaction of N fertilizer rate (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) and soil depth (Depth1=0-7.5cm, Depth2=7.5-15cm) on AOA abundance in whole soil based on aggregate size distribution. (b) Interaction of N fertilizer rate (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) on AOA concentration averaged across aggregate size class. Measurements in log scale (copies g\(^{-1}\) soil). Different letters represent significant difference (P<0.05) between sampling date within tillage treatment. (NS=not significant).
Figure 3.16. (a) Effect of N fertilizer treatment (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) on AOA concentration within <250µm aggregates (P=0.0034). (b) Effect of N fertilizer treatment (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) on AOA concentration within 250µm-1mm aggregates (P=0.0044). (c) Effect of N fertilizer treatment (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)) by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) on AOA concentration within 1mm-4.75mm aggregates (NS=Not Significant).
Table 3.1A. ANOVA table of mean values of AOB and AOA abundance by tillage (No tillage and Disk tillage) and sampling date (SD1=5/1/12; SD2=5/14/12; SD3=7/6/12).

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lower case letter = difference across sampling date within tillage
upper case letter = difference across tillage

Table 3.1B. ANOVA table of main effects and interactions on AOB and AOA abundance. (NS=Not Significant).

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Table 3.2. Correlation analysis of pH, EC, water-filled pore space (WFPS) on AOB and AOA abundance in Haskell 2012 (HAL2012) experiment.

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<td>0.032</td>
<td>0.052</td>
</tr>
<tr>
<td>WFPS</td>
<td>-0.006</td>
<td>0.552**</td>
</tr>
<tr>
<td>AOB</td>
<td></td>
<td>0.275**</td>
</tr>
<tr>
<td>AOA</td>
<td>0.275**</td>
<td></td>
</tr>
</tbody>
</table>

Note: **=p<0.01  *=p<0.05
Table 3.3. Correlation analysis of pH, EC, water-filled pore space (WFPS) on AOB and AOA abundance in Haskell 2012 (HAL2012) experiment separated by tillage treatment (No tillage and Disk tillage).

<table>
<thead>
<tr>
<th></th>
<th>No Tillage</th>
<th>Disk Tillage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AOB</td>
<td>AOA</td>
</tr>
<tr>
<td>pH</td>
<td>-0.128</td>
<td>-0.133</td>
</tr>
<tr>
<td>EC</td>
<td>0.181</td>
<td>0.074</td>
</tr>
<tr>
<td>WFPS</td>
<td>0.133</td>
<td>0.597**</td>
</tr>
<tr>
<td>AOB</td>
<td>0.404**</td>
<td></td>
</tr>
<tr>
<td>AOA</td>
<td>0.404**</td>
<td>0.176</td>
</tr>
</tbody>
</table>

Note: **=p<0.01 *=p<0.05
**Table 3.4** Mean and ANOVA table of known FAME biomarkers and total biomass across tillage, N rate and sampling date

<table>
<thead>
<tr>
<th>Means</th>
<th>Sum Bacteria</th>
<th>AMF</th>
<th>Saprophytic Fungi</th>
<th>Actinomycete</th>
<th>Cylcopropyl</th>
<th>Eukaryote</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-Till</td>
<td>26.977</td>
<td>4.312</td>
<td>5.185</td>
<td>4.339</td>
<td>10.101</td>
<td>1.658</td>
<td>125.32</td>
</tr>
<tr>
<td>N Rate kg/ha</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>25.53</td>
<td>5.617</td>
<td>4.947</td>
<td>4.159</td>
<td>8.818</td>
<td>1.926</td>
<td>126.9</td>
</tr>
<tr>
<td>40</td>
<td>26.976</td>
<td>4.936</td>
<td>4.862</td>
<td>4.506</td>
<td>9.71</td>
<td>1.69</td>
<td>131.27</td>
</tr>
<tr>
<td>80</td>
<td>26.422</td>
<td>3.427</td>
<td>4.664</td>
<td>4.35</td>
<td>10.052</td>
<td>1.603</td>
<td>124.08</td>
</tr>
<tr>
<td>120</td>
<td>26.623</td>
<td>3.008</td>
<td>4.763</td>
<td>4.251</td>
<td>10.842</td>
<td>1.392</td>
<td>123.3</td>
</tr>
<tr>
<td>160</td>
<td>27.123</td>
<td>2.874</td>
<td>4.721</td>
<td>4.172</td>
<td>11.353</td>
<td>1.273</td>
<td>125.93</td>
</tr>
<tr>
<td>Date</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-May</td>
<td>28.481</td>
<td>4.142</td>
<td>4.517</td>
<td>5.137</td>
<td>11.104</td>
<td>1.63</td>
<td>136.37</td>
</tr>
<tr>
<td>14-May</td>
<td>25.933</td>
<td>3.73</td>
<td>4.894</td>
<td>4.714</td>
<td>10.035</td>
<td>1.686</td>
<td>125.89</td>
</tr>
<tr>
<td>6-Jul</td>
<td>25.216</td>
<td>3.618</td>
<td>5.467</td>
<td>4.461</td>
<td>9.497</td>
<td>1.462</td>
<td>124.4</td>
</tr>
<tr>
<td>11-Oct</td>
<td>26.51</td>
<td>4.399</td>
<td>4.287</td>
<td>2.839</td>
<td>9.984</td>
<td>1.53</td>
<td>118.53</td>
</tr>
</tbody>
</table>

| Tillage     | NS     | 0.0013 | 0.0005 | NS      | NS | 0.108     | NS     |
| N Rate      | NS     | <0.0001 | NS     | NS      | <0.0001 | 0.002     | NS     |
| Date        | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.0473    | <0.0001 |
| Tillage x N rate | NS     | NS     | NS     | NS      | NS | NS        | NS     |
| Tillage x Date | NS     | 0.0312 | NS     | NS      | 0.0812 | NS        | NS     |
| N Rate x Date | NS     | NS     | NS     | NS      | 0.0743 | NS        | NS     |
| Tillage x N rate x Date | NS     | NS     | NS     | NS      | NS | NS        | NS     |
Table 3.5A. ANOVA table of mean values of AOB and AOA abundance based on aggregate distribution by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) and N fertilizer treatment (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)).

<table>
<thead>
<tr>
<th>N Rate (kg N ha(^{-1}) yr(^{-1}))</th>
<th>AOB Distribution</th>
<th>AOA Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Depth 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.04aA</td>
<td>7.13aA</td>
</tr>
<tr>
<td>80</td>
<td>4.43aA</td>
<td>6.52abA</td>
</tr>
<tr>
<td>160</td>
<td>4.88aA</td>
<td>6.19bA</td>
</tr>
<tr>
<td><strong>Depth 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.44aB</td>
<td>6.39aA</td>
</tr>
<tr>
<td>80</td>
<td>3.56aB</td>
<td>6.50aA</td>
</tr>
<tr>
<td>160</td>
<td>3.76aB</td>
<td>6.85aA</td>
</tr>
</tbody>
</table>

lower case letter = difference across N rate within depth  
upper case letter = difference across depth

Table 3.5B. ANOVA table of mean values of AOB and AOA concentration within aggregate class size by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) and N fertilizer treatment (0, 80 and 160 kg N ha\(^{-1}\) yr\(^{-1}\)).

<table>
<thead>
<tr>
<th>N Rate (kg N ha(^{-1}) yr(^{-1}))</th>
<th>AOB Concentration</th>
<th>AOA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Depth 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.61aA</td>
<td>7.71aA</td>
</tr>
<tr>
<td>80</td>
<td>5.13aA</td>
<td>7.22abA</td>
</tr>
<tr>
<td>160</td>
<td>5.62aA</td>
<td>6.93bA</td>
</tr>
<tr>
<td><strong>Depth 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.04aB</td>
<td>6.99aA</td>
</tr>
<tr>
<td>80</td>
<td>4.13aB</td>
<td>7.08aA</td>
</tr>
<tr>
<td>160</td>
<td>4.36aB</td>
<td>7.44aA</td>
</tr>
</tbody>
</table>

lower case letter = difference across N rate within depth  
upper case letter = difference across depth
Table 3.6. ANOVA table of main effects and interactions on AOB and AOA abundance based on aggregate distribution and concentration within aggregate class. (NS=Not Significant).

<table>
<thead>
<tr>
<th></th>
<th>AOB Distribution</th>
<th>AOB Concentration</th>
<th>AOA Distribution</th>
<th>AOA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregate Size</td>
<td>p=0.0016</td>
<td>p=0.0002</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>N rate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Depth</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Aggregate Size x Depth</td>
<td>p=0.0004</td>
<td>p=0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Aggregate Size x N rate</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>N rate x Depth</td>
<td>NS</td>
<td>NS</td>
<td>p=0.0005</td>
<td>p=0.0021</td>
</tr>
<tr>
<td>Aggregate Size x N rate x Depth</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 3.7A. ANOVA table of mean values of AOB and AOA abundance in soil based on aggregate distribution by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) and aggregate class (LT250=<250µm, 250-1000=250µm-1mm, GT1000=1mm-4.75mm).

<table>
<thead>
<tr>
<th>Aggregate Size Class (µm)</th>
<th>AOB Distribution</th>
<th>AOA Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Depth 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT250</td>
<td>4.69aA</td>
<td>6.66aA</td>
</tr>
<tr>
<td>250-1000</td>
<td>4.765aA</td>
<td>6.56aA</td>
</tr>
<tr>
<td>GT1000</td>
<td>4.88aA</td>
<td>6.62aA</td>
</tr>
<tr>
<td><strong>Depth 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT250</td>
<td>4.45aB</td>
<td>6.67aA</td>
</tr>
<tr>
<td>250-1000</td>
<td>2.83bB</td>
<td>6.38aA</td>
</tr>
<tr>
<td>GT1000</td>
<td>3.48bB</td>
<td>6.68aA</td>
</tr>
</tbody>
</table>

lower case letter=difference across N rate within depth
upper case letter=difference across depth
Table 3.7B. ANOVA table of mean values of AOB and AOA concentration within aggregate class size by depth (Depth1=0-7.5cm, Depth2=7.5-15cm) and aggregate class (LT250=<250µm, 250-1000=250µm-1mm, GT1000=1mm-4.75mm).

<table>
<thead>
<tr>
<th>Aggregate Size Class (µm)</th>
<th>AOB Concentration</th>
<th>AOA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT250</td>
<td>5.44aA</td>
<td>7.41aA</td>
</tr>
<tr>
<td>250-1000</td>
<td>5.47aA</td>
<td>7.27aA</td>
</tr>
<tr>
<td>GT1000</td>
<td>5.45aA</td>
<td>7.18aA</td>
</tr>
<tr>
<td>Depth 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT250</td>
<td>5.18aB</td>
<td>7.41aA</td>
</tr>
<tr>
<td>250-1000</td>
<td>3.35bB</td>
<td>6.90aA</td>
</tr>
<tr>
<td>GT1000</td>
<td>4.00bB</td>
<td>7.20aA</td>
</tr>
</tbody>
</table>

lower case letter=difference across N rate within depth  
upper case letter=difference across depth
**Table 3.8A.** Correlation analysis of C, N and pH on AOB abundance across based on aggregate size distribution.

<table>
<thead>
<tr>
<th></th>
<th>AOB Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.477**</td>
</tr>
<tr>
<td>N</td>
<td>0.434**</td>
</tr>
<tr>
<td>pH</td>
<td>-0.460**</td>
</tr>
</tbody>
</table>

Note: **=p<0.01  *=p<0.05

**Table 3.8B.** Correlation analysis of C, N and pH on AOA abundance across based on aggregate size distribution.

<table>
<thead>
<tr>
<th></th>
<th>AOA Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.324**</td>
</tr>
<tr>
<td>N</td>
<td>0.336**</td>
</tr>
<tr>
<td>pH</td>
<td>-0.307**</td>
</tr>
</tbody>
</table>

Note: **=p<0.01  *=p<0.05
Table 3.9A. Correlation analysis of C, N and pH on AOB abundance based on aggregate size distribution separated by depth (Depth1=0-7.5cm; Depth2=7.5-15cm).

<table>
<thead>
<tr>
<th></th>
<th>AOB Depth 1</th>
<th>AOB Depth 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.203</td>
<td>0.089</td>
</tr>
<tr>
<td>N</td>
<td>0.354</td>
<td>0.097</td>
</tr>
<tr>
<td>pH</td>
<td>-0.595**</td>
<td>-0.163</td>
</tr>
</tbody>
</table>

Note: **=p<0.01  *=p<0.05

Table 3.9B. Correlation analysis of C, N and pH on AOA abundance across based on aggregate size distribution separated by depth (Depth1=0-7.5cm; Depth2=7.5-15cm).

<table>
<thead>
<tr>
<th></th>
<th>AOA Depth 1</th>
<th>AOA Depth 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.517**</td>
<td>0.246</td>
</tr>
<tr>
<td>N</td>
<td>0.493**</td>
<td>0.258</td>
</tr>
<tr>
<td>pH</td>
<td>-0.313</td>
<td>-0.324</td>
</tr>
</tbody>
</table>

Note: **=p<0.01  *=p<0.05
CHAPTER 4

Discussion

4.1 AOA abundance several fold greater than AOB abundance

Since the discovery of ammonia-oxidizing archaea (AOA) many studies have given insight into the drivers of their abundance. Characterizing roles of AOA and ammonia-oxidizing bacteria (AOB) is important in understanding their individual contributions to nitrification in soils. Much of the literature in soil systems have found that AOA greatly outnumber AOB in magnitude (Hatzenpichler, 2012; Isobe et al., 2012; Leininger et al., 2006). Although high in abundance, insight into the function of AOA is limited. In our soil AOA is much higher than AOB in magnitude which raises questions about their relevance to nitrification.

The limited, yet every growing, knowledge of AOA physiology has shown that AOA are autotrophs; however, they have also been shown to take in organic C and N sources as well (Spang et al., 2012). This is indicative of a role for AOA being heterotrophic or mixtrophic. We saw from our aggregate data that AOA abundance was correlated with C and N in the first depth (0-7.5cm). The no till plots are characterized with surface residues which would coincide with heterotrophic behavior of AOA. We also saw a decline with increasing N rate in the first depth. Therefore, it is possible that AOA are surviving off more organic sources. Again, the field is a long-term (25+ years) continuously managed site in which microbial communities have adapted to conditions.

There is also the possibility of AOA and AOB having different requirements for ammonia (NH$_3$) concentration in the soil (Prosser and Nicol, 2012). A study that applied
cow urine showed a negative response on AOA abundance (Di et al., 2010, 2009). However, in control soil without any N application AOA abundance increased. These studies suggest that AOA and AOB may differ in their requirements for N concentration. Another study using the cultured *Nitrosopumilus maritimus* (Könneke et al., 2005) demonstrated adaptation of AOA to oligotrophic marine environments. Likely the high abundance in our soil reflects long-term (25+ years) adaptation to soil and cropping system management giving insight into the drivers of AO abundance under continuous maize. It is likely that AOA and AOB occupy different niches where AOA are slow-turnover, persistent organisms while AOB may have a higher turnover and lower abundance but are more reactive to our treatments. If, in fact, AOA are not strict autotrophs, which some data suggests then their high abundance may not be reflective of a high contribution to nitrification and may instead be reflective of their slow growth on organic sources.

### 4.2 Temporal dynamics differ between AOA and AOB

Contrary to our hypothesis, AOA did not respond to changing pH, N-rate or tillage, but did respond to sampling date. However, the observed decrease in AOA over the growing season could be due to the decrease in water-filled pore space (WFPS).

One study found that AOA and AOB respond differently to WFPS, but it remains to be seen if this is a covariant of temperature or some other factor in the soil (Gleeson et al., 2010; Long et al., 2012; Szukics et al., 2012, 2010). Gleeson and colleagues (2010) found that AOB correlated with WFPS and AOA did not; this differs from findings in our study. A temperature by water content incubation study found that AOA abundance, but
not AOB, decreased from 55% to 30% WFPS at 5 °C. In our field study, WFPS ranged from 76%-65% at the first sampling date in early spring, which then dropped down to a range of 54%-37% by the third sampling date at the growth stage V12. It is difficult to make comparisons across studies that directly measure the impact of WFPS on ammonia oxidizer (AO) abundance in laboratory incubations to field studies where WFPS is dynamic and sampled more infrequently. However, 2012 was a particularly dry year leading to a significant and persistent (to harvest) drop in WFPS by the third sampling date allowing for trends between WFPS and AO abundance to be tested.

Although previous studies have shown the ability of AOA and AOB to respond to WFPS (Gleeson et al., 2010; Szukics et al., 2012); these are incubation studies and likely not representative of complex interactions in soil. Instead these studies with manipulated WFPS could be indicative of WFPS being an overall factor affecting substrate availability to AO. In order to better predict how AOA and AOB respond to changing soil water content in our field it would be best to measure abundance from our fourth sampling date (10/14/12) when crops were already harvested and the soil was very dry (WFPS: 43%-28%). Future work on these samples could provide more insight into the relationships between WFPS, AOA abundance, and AOB abundance.

In contrast to AOA, AOB responded to pH changes, sampling date and tillage but not the N rate gradient. Acidic soil is common in both cropping systems, where large amounts of fertilizer is applied yearly, and forested systems. It is also common in tropical regions with high rainfall. It is less common in the western corn belt where pH has been measured between 6.0-6.5 (Liebig and Varvel, 2002). This is considered slightly acidic soil, whereas the average soil pH at Haskell Agricultural Laboratory (HAL) in fertilized
versus unfertilized plots is 5.6 and 6.2, respectively with the lowest pH being reported at 4.6, putting the soil for this site into the acidic soil category. This is likely due to the long-term fertilization practices (25+years) and monocultured maize with high N requirements, as fertilization is known to cause acidification in soils (Bouman, 1992; Liebig and Doran, 1999). Within the HAL field site, AOB numbers were shown to strongly correlate with soil pH. This is interesting as nitrification rates in acidic soils have not been correlated with AOB abundance, but with AOA (He et al., 2007; Isobe et al., 2012; Yao et al., 2011). AOA seem to be ubiquitous in all soils, but react to soil pH differently than AOB (He et al., 2007; Prosser and Nicol, 2012; Shen et al., 2008). Soil pH has been suggested as a possible niche differentiation for these two organisms (Prosser and Nicol, 2012).

Based on previous literature it is surprising that AOA do not differ in abundance with soil pH. It is possible that since our site has been established for so long an equilibrium has been reached reflecting the overall acidity of our plots leading to higher abundance of AOA compared to AOB. Another explanation is that AOA response to WFPS overrides any influence of pH. There is likely not any one factor that drives AOA and AOB abundance across ecosystems, but examining common biophysical properties of soils worldwide should help scientists better predict responses and relevance of these organisms.

4.3 AOB, but not AOA abundance, is highly responsive to soil tillage

Another factor influencing AOB but not AOA is tillage. No tillage systems are characterized by increased soil organic matter (SOM), microbial biomass and plant
residues in surface soil (Calegari et al., 2008; Dalal et al., 1991; Franchini et al., 2007; Silva et al., 2013, 2010). This is apparent in our findings, as AOB abundance is markedly greater in no till than in disk tilled plots. Although a recent metagenomic study performed using shotgun sequencing on environmental samples found that there was an increase in Thaumarchaeota representatives in no tilled systems (Souza et al., 2013), we did not see a significant difference in the abundance of AOA between the two systems tested. This could possibly be due to other factors affecting soil properties such as monoculture maize which can counteract the benefit of increased residue from no till (Souza et al., 2013; Wortman, 2012). Crop rotations will need to be studied in future studies to better understand the relationships between tillage and AOA abundance.

The significance of the decrease of AOB to disk tillage is that it further confirms that AOB are more reactive in our soils to physiochemical conditions than AOA. Our findings for AOB are confirmed by other studies as well. A long-term study on manure amendments showed that AOB respond to different long-term but not short term fertilizer amendments (Wang et al., 2011). It could be that the length of establishment of our study was sufficient to differentiate AOB abundance by tillage but not AOA, suggesting AOA abundance may better reflect overall soil type and the differences in OM that exist in a no-till field versus a disk-tilled field are not great enough to override other factors dictating abundance (Bauer and Black, 1981). Tillage can affect physical properties, such as bulk density. Since AOA are correlated with WFPS, it seemed likely that AOA would respond to differences in tillage treatment. However, the average WFPS for no-tilled and disk-tilled plots was 61% and 60%, which is not enough of a difference to affect AOA abundance unlike the difference measured across sampling dates. A study comparing
conventional tillage to no tillage found that mineralizable N increased under no tillage (Doran, 1980; Paul, 2006). Thus, AOB may be responding to higher concentrations of mineralized N arising from partially degraded residues. It is clear from our study that AOA and AOB respond differently to the soil environment. When WFPS was lowest (SD3), AOB almost fully recovered for both no tilled and disk tilled plots while AOA declined over the growing season regardless of tillage. In the aggregate study we see that depth negatively affected AOB abundance, whereas AOA abundance remained consistent across depth. This supports different drivers of AOB and AOA abundance, growth and survival in soil.

4.4 AOB exhibit spatial heterogeneity while AOA do not

Sub-samples taken from the first experiment (2012) were run on Denaturing Gradient Gel Electrophoresis (DGGE) and revealed spatial heterogeneity between replicate treatments within AOB. Since samples were not run with laboratory replicates it is difficult to say if this heterogeneity is actually due to a detection limit bias by DGGE or representative of a highly diverse community at the field site. We plan to address this with multiple replications of the same sample. Also, lower dilutions could be performed to prevent issues with detection limits of specific phylotypes. Previous studies found banding homogeneity between AOB and AOA samples and some differences between treatments (Chen et al., 2008; Shen et al., 2008). Although DGGE has been used in many earlier studies describing AOB and AOA abundance and community structure (Chen et al., 2008; Leininger et al., 2006; Nicol et al., 2008; Shen et al., 2008) there are issues that arise from how gels are analyzed and which primer set is used. For some literature, the presence and absence of bands are counted, however, different primers and changing
gradient can alter this. Some studies do not analyze the banding pattern and instead use it to clone and excise bands to gain phylogenetic insight, but the same issues still persist, in that, primer pair bias exists and absolute phylogenetic samples cannot be generalize and are instead, site specific.

DGGE works by using a denaturant gradient to disrupt the GC bonds in DNA fragments. Since GC pairing is more stable than AT pairing, they will migrate down the gradient and denature at different concentrations. If there are not enough targets of a particular organism in a DNA extract then banding pattern across replicates will be difficult to reproduce. The high-detection limit has been noted as a drawback to DGGE analysis (Marschner, 2007). Due to this it would be easy to suspect that there could possibly be differences for AOB in our data due to low target numbers in the DNA extract. Due to the ambiguity from these results it is important to review the advantages and disadvantages to this method.

The low-cost of DGGE and accessibility to equipment compared to the most current sequencing methods (Illumina, 454 pyrosequencing) should not be overlooked as an advantage. This is the main reason for use of DGGE for community composition in this study. Although DGGE bands for target sequences can be excised from the gel and cloned and sequenced, primer and extraction bias could show preference so should only be used within a study. Depending on the primer set DGGE can take into account target organisms versus whole community 16S. For this study the former is used, so drowning out low concentration organisms is not an issue. Another advantage of this method is that community shifts due to experimental treatments can be easily seen as can temporal shifts. Banding patterns can be both visually analyzed and digitized using PCA software.
Although, this software should be used with caution as much of the settings are objective and banging patterns can be manipulated. Cluster analysis can be performed as well and this is seen as a better tool for determining the effects of our treatment on overall ammonia-monooxygenase (amoA) AOB and AOA community composition. However, even though cluster analysis for AOA in our study showed similarity by tillage and N Rate, there is also a possibility that the software recognized similarities by gel and not experimental treatment.

There are many advantages and disadvantages to using this analysis; therefore, it must be used with caution and is more of a supplementary method than a determination of the true diversity within soil. DGGE analysis is performed using target sequences for the amoA gene in AOB and AOA. DGGE is used to gain insight into the functional AOB and AOA communities and their response to experimental treatments. Due to the difficulty in getting a consistent banding pattern much of the new literature uses next generation sequencing to determine community diversity and phylogeny.

Other community analysis using fatty acid methyl esters (FAMEs) was used to track overall community shifts due to tillage and N rate. FAME analysis revealed that there was good separation of communities due to N rate and tillage that was not observed in our AOB analysis. We also cannot look into specific functional groups, so we see that AOB are reacting slightly different than the overall microbial biomass. Archaeal communities could not be tracked using this method as they are ether linked and do not possess fatty acid tails. This makes it difficult to compare bacterial and archaeal communities in our soil.
4.5 **AOB concentration is greater in microaggregates compared to macroaggregates**

A novel hypothesis is that AOA and AOB preferentially occupy different aggregate size fractions. This hypothesis came from a combination of observations, particularly that AOB seem to be more susceptible to treatments and environmental factors in our HAL 2012 study than AOA, yet AOA did respond to WFPS.

AOB abundance is dynamic and reacts to several factors including tillage. Recent results from this field site showed long-term N gradient fertilization practices influenced aggregate fraction size distribution in whole soil (Blanco-Canqui et al., 2014). We hypothesized that larger macroaggregates which are easily broken by tillage would be preferentially populated by AOB while AOA favor stable microaggregates with slower turnover and therefore are more resilient to change. The significant decline in AOA abundance with reduced WFPS; however, may suggest the opposite where declining water content impacts larger pore sizes rather than micropores within microaggregates.

However, our hypothesis of AOB concentration being highest in larger aggregates was not supported by our data. AOB concentration was highest in the microaggregates (<250µm) and AOA was not affected by aggregate size. Again, AOA seem to be more resilient to experimental and environmental factors than AOB. Our field site would be characterized by more macroaggregates especially since we are sampling in no-tilled plots. To better test our hypothesis it would be important to sample on multiple dates, especially over a growing season when roots would affect aggregate stability. It would also be important to take WFPS measurements and correlate this with overall abundance of AO in the soil. Since the field was wet due to a rain event prior to sampling in spring
2013 it is possible that AOA, which correlated with WFPS in 2012, were not reactive in the smaller microaggregates. Although, WFPS was not measured in 2013, so this would be difficult to determine. If WFPS is an important factor affecting AOA abundance then we would see the concentration of AOA fall in microaggregates differently from macroaggregates under a decrease of soil water content; this would be an important factor to test in the future. A recent study found the opposite of what is found in much of the literature in cultivated fields, that AOA community profiles were more dynamic with land-use change and AOB exhibited low diversity (Meyer et al., 2014). It would therefore be important to measure community composition of AOB and AOA within aggregate size class, as there may be a shift by aggregate size.

Our study also investigated soil depth and found that AOB were much more concentrated in depth 1 (0-7.5cm) versus depth 2 (7.5-15cm) whereas AOA concentration did not change. A study in a natural grassland, found that AOB dominated grassland top soils while AOA were found mainly in sub soils (Di et al., 2010). This could be suggestive of AOB and AOA preferring different soil fertility as the topsoils were highly fertile versus the subsoil. For the hierarchical theory of aggregate formation, stable microaggregates would form unstable macroaggregates which would be broken down over time leaving stable microaggregates, especially in tilled soils where compaction would occur. We previously saw AOB abundance decrease in tilled soils which would support the hierarchical theory. The macroaggregates, in which we hypothesized AOB would occupy, have been shown to have higher C:N rations and increased C with aggregate size (Elliott, 1986; Six et al., 2004). This could be one explanation for the AOB being much less resilient in the previous study. The observed difference by depth could
be due to the breakdown of macroaggregates and less residue and fertilizer incorporation in the second depth.

A simple correlation analysis, without WFPS measurements, showed that AOA concentration positively correlated with C and N content, but only in the first depth (i.e. shallow soils). This correlation decreased and was not significant in the second depth (i.e. deeper soils). When separated by depth, pH and AOA abundance do not correlate; however, when combined over depth, N rate and aggregate size fractions, AOA abundance negatively correlates with pH as well as the positive correlation of C and N. When AOB correlation analysis is run with C, N and pH over all the experimental factors, AOB strongly positively correlates with C and N and negatively with pH. However, the correlation with C and N drops out when separated by depth and only a negative correlation with pH is seen in depth 1. This suggests that aggregate size fractions may be driving the C and N correlation observed for AOB and AOA. It becomes apparent from our data that there is likely not one factor affecting AOB and AOA abundance and function in soil. It is also possible that AOB prefer readily available ammonium from inorganic fertilizers while AOA survive off of low concentration of mineralize N from decomposing OM and possibly C via mixotrophy. Overall, AOB and AOA have been shown to respond very differently from each other in this long-term maize monoculture. It has yet to be seen if AOA survives mainly off of mineralized C and N or if their main function is autotrophic nitrification. We can see, however, that AOA abundance is more resilient to our treatments than AOB. Likely, as more insight into AOA physiology is discovered, understanding their role, and correlations among their function and drivers of their abundance will become more clear.
CHAPTER 5

Future Work

The first experiment was a large field sampling study that took place over four sampling dates (data for the first three were shown). Although abundance data for AOB and AOA had interesting findings, it was difficult to compare to published literature as many studies use different primer sets, method collection and nucleic acid extraction. After an extensive literature review it seems that much of the findings are contradictory and this is likely due to the complexity of soil and the inability to generalize across field sites. Long-term established fields as well as incubation studies on functional gene responses will help increase our understanding of nitrification in soil and the microbes responsible. Our sampling year (2012) was unique in that Nebraska suffered from an uncharacteristically dry season with poor crop yields. It would be beneficial to resample these fields in the future under a “normal” year. Other future work to complement our field study should look into soil depth as this may be an important factor affecting AOB and AOA abundance based on the aggregate experiment. Crop rotation, with soybean for example, would also be important to compare with monoculture maize since inclusion of a N-fixing crop would likely alter nitrification processes and may provide insight into drivers of nitrification during alternative crops. Future work to build on the current study could use stable isotope probing (SIP) to gain more insight into function. A study (Jia and Conrad, 2009) employing SIP found that AOB, and not AOA, was functional in the soil. Other studies have also used SIP as a means of deducing function and found opposite results, with AOA taking up inorganic C and not AOB (Zhang et al., 2010). However, the source of ammonia, mineralized SOM, differed in the latter. This is not a new technique
in microbial ecology but is gaining popularity in the nitrifier literature (Dumont and Murrell, 2005; Radajewski et al., 2000) as it gives direct insight into the function and keys players involved in nitrification and how they metabolize substrates. By use with other downstream protocols, information about microbial abundance, function and phylogenetic status can be gathered. Although there is much room for improvement with this technique, SIP gives a better picture of function than potential nitrification rates which are so poorly correlated with abundance.

DGGE and FAMES were used to give insight into microbial community shifts resulting from long-term fertilizer and tillage treatments in our field experiment. Although, FAME analysis showed clear shifts in microbial community structure with experimental factors, it cannot be used to elucidate specific bacterial groups. Also, it would be helpful in the future to develop protocols for archaeal community analysis in order to better compare their response to treatments. DGGE as used herein was specific to nitrifier communities; however, it was a difficult protocol to develop and perfect. Although there were differences in response between AOB and AOA communities to treatment, we are unsure if this is due to low target numbers for AOB. This would have to be addressed in the future to better understand the shifts observed. There is also the possibility of using next-generation sequencing techniques. The advantage of this method involves phylogenetic trees development and also specific species composition (diversity) can be made from each sample unlike DGGE in which PCR bias can determine which bands can be excised and sequenced and FAME techniques which represent general community composition. Shifts in species representatives can also be made across treatments. This would be an interesting technique to apply to our second
study as this would give us insight into the specific AO species occupying aggregate size fractions.

Our second study, on aggregates, answered novel questions that have not been addressed in previous studies. Future work should take into account the water content of samples and aggregate porosity; these could affect the substrate availability to AOA and AOB. We also did not investigate crop rotation or tillage in this experiment. It would be interesting to investigate the effects of tillage as this was a major factor affecting AOB abundance in the previous experiment. Since different crops vary in their N input requirements, this would be important to investigate. In our middle macroaggregates we saw differences in distribution, it would be important to separate the aggregate classes into more fractions to determine if there are measurable differences between AOA and AOB concentration. As found previously (Blanco-Canqui et al., 2014) aggregate distribution shifts based on depth and N rate, it would be useful to measure AO abundance based on distribution using the whole soil profile. Lastly, it is important to remember that molecular techniques and insight into AOA physiology are ever growing and as such the key questions surrounding AO ecology, function and physiology are ever developing as well.
REFERENCES


