AMMONIA OXIDIZERS IN A GRAZING LAND WITH A HISTORY OF POULTRY LITTER APPLICATION

by

ABHA MUNDEPI

(Under the Direction of Mussie Y. Habteselassie)

ABSTRACT

Poultry litter (PL) is widely applied on grazing lands in Georgia, but it is not clear how it affects the function and community structure of ammonia-oxidizing bacteria (AOB) and archaea (AOA). In 2009, soil samples were collected from plots that received PL for 15 years at 8 Mg ha^{-1} annually and those plots were resampled in 2013 after 2 years of PL stoppage, with the objective of examining the long-term impacts of PL and its legacy effects on AOB and AOA. In 2009, the abundance of AOB and AOA were significantly higher in PL treated soils (6.98 and 7.06 log copies g $soil^{-1}$ for AOB and AOA, respectively) than controls plots that received equivalent amount of nitrogen (N) in the form of urea ammonium nitrate (6.39 and 6.53 log copies g $soil^{-1}$ for AOB and AOA, respectively). In 2013, AOB abundance decreased significantly in response to the discontinuation of PL application. We used dicyandiamide to separate the roles of AOB and AOA in nitrification in PL and CL (no N added) soils. At early stage of incubation, AOA dominated nitrification in both PL and unamended CL, accounting for ≥70% of nitrification potential (NP). With time, AOB contribution to NP grew in PL, accounting for ≥50% at high ammonium level. We designed a laboratory study to examine how AOB and AOA respond to elevated levels of Zinc (Zn) and Copper (Cu). Zn application resulted in greatest decrease in abundance of AOB (78%)

and AOA (85%) at the high ammonium level over 28 days. Likewise, Cu application significantly reduced AOB (92%) and AOA (63%) abundance at the high ammonium level over 28 days. The relative contribution of AOB to NP was significantly higher in both Zn (~60%) and Cu (~70%) treated soils despite the numerical dominance of AOA over AOB. Overall, AOB were the dominant players in PL soil. As such, understanding the effects of land application of PL on AOA and AOB communities and their respective roles in nitrification is essential to target the right group to achieve efficient N use.

INDEX WORDS: poultry litter, ammonia oxidizing bacteria, ammonia oxidizing archaea, amoA, nitrification, nitrification potential, alpha and beta diversity, trace metals

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DEDICATION

This dissertation is dedicated to my husband, Sadanand Singh who has been supportive of my work and has encouraged me to go through the challenges of graduate school. This work is also dedicated to my parents whose constant love has sustained me throughout my life.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

More than two-thirds of the total U.S. broiler chicken production is located in the southeastern United States (National Agricultural Statistics Service, 2012). This generates a large quantities of poultry litter (PL), that is commonly land applied as a source of nutrients, particularly nitrogen (N) and phosphorus (P), to perennial pastures and hay fields (Dou et al., 2001; Edwards et al., 2007; Soupir et al., 2006). The conversion of PL contained N into plant usable forms is achieved through microbially mediated processes of mineralization and nitrification (Bitzer and Sims, 1988; Cabrera et al., 1994). Mineralization results in the conversion of organic N to ammonium, which is subsequently oxidized via nitrification. Nitrification is of particular importance from an agriculture and environmental perspective, since it produces inorganic N forms (nitrate and nitrite) that can easily be lost through leaching and denitrification (Conrad, 1996; Knowles, 1982; Smil, 1997).

Nitrification is the oxidation of ammonia into nitrate via nitrite. The conversion of ammonia to nitrite is the rate limiting step in nitrification and is mediated by a narrowly defined groups of organisms called ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA)(Hermansson and Lindgren, 2001; Leininger et al., 2006; Treusch et al., 2005). Until 2005, AOB were thought to be the only microbes involved in ammonia oxidation (Treusch et al., 2005), but since then a number of studies have confirmed the role of AOA in nitrification in soils, with some studies reporting AOA as more abundant than AOB (Leininger et al., 2006; Nicol et al.,

2008; Zhang et al., 2012). Since numerical abundance does not necessarily translate into functional importance, further studies are required to elucidate the relative role of AOA and AOB in ammonia oxidation, especially in PL treated soils where such research is lacking.

The relative importance of AOB and AOA varies in arable soils depending on the environmental factors with one or the other being more competitive under a given set of conditions. Factors such as ammonium availability, soil pH, temperature, salinity and mixotrophy are believed to influence the relative roles of AOA and AOB in nitrification thereby defining their niche specialization (Prosser and Nicol, 2012). AOA have a higher substrate affinity for ammonia than AOB and dominate in oligotrophic soils, with ammonia derived from mineralization of organic matter (Jung et al., 2011; Martens-Habbena et al., 2009). AOB tend to dominate in soils with high ammonium availability that comes from application of inorganic N fertilizers (Di et al., 2010a). There are studies that have also examined the influence of the other factors mentioned above (Levičnik-Höfferle et al., 2012; Nicol et al., 2008) but not in PL treated soils, which present some unique conditions, including elevated levels of trace metals.

Chemical inhibitors are commonly used to separate the role of AOA and AOB in nitrification. Dicyandiamide (DCD) is such an inhibitor that specifically target the enzyme for ammonia oxidation produced by AOB (McCarty and Bremner, 1989; Offre et al., 2009). By using this technique, Dai et al (2013) reported AOB to be functionally more important than AOA in pasture soils amended with urea and urine N substrate and DCD. Using a similar approach, Giguere et al (2015) reported that AOB and AOA had higher nitrification activity in summer than winter, with AOB and AOA being the dominant players in nitrification in cropped soils and non-cropped soils, respectively. Ouyang et al (2016) also reported AOB to be the bigger contributors to potential nitrification in agricultural soils amended with ammonium sulfate and steer waste compost.

Long term PL application can result in trace metal accumulation in soils (Jackson et al., 2003; Sims and Wolf, 1994). This makes PL receiving soils different from soils that receive other forms of animal waste. Trace metals (e.g., Zn, Cu, As) are added in poultry and livestock diet as essential nutrients to help in physiological functions and metabolism, improve health and feed efficiency, increased egg production and prevent diseases (Angel and Powers, 2006; Sims and Wolf, 1994). These metals end up in soil with land application of PL. Several studies have examined the impact of metals on ammonia oxidizers (AO) (Mertens et al., 2010; Ruyters et al., 2010; Liu et al., 2010; Ollivier et al., 2012) but the soils were not treated with PL nor did they have a history of PL application, making it hard to extrapolate their findings.

There are few studies that have attempted to study AO and nitrification in PL amended soils, but they did not cover AOA, that could potentially play a dominant role in nitrification in any terrestrial environment (Dharni et al., 2010;Tomlinson et al., 2015). Our study was, therefore, designed to address this research need in PL treated soils. Our study examined the change in function and community structure of AO in pasture soil with a 15 year history of PL application. The role of AOA and AOB in nitrification were defined under normal and elevated trace metals levels to reflect the effect of long-term PL application. Proper understanding of the roles of AOA and AOB in nitrification in PL treated soils will help design a management system that targets the right group of organisms in order to control nitrification and avoid excessive N loss.

Literature Review

Poultry litter as a source of nutrients

About 8.6 billion broilers and 13 million Mg of PL are produced annually in the United States (National Agricultural Statistics Service, 2012) with the largest contribution by the southeastern states. PL is a mixture of excreta, bedding material, feathers and waste feed, applied

to land as a fertilizer for a variety of crops (Kelleher et al., 2002; Nahm, 2003). The high content of N, P, K, and Ca and other plant nutrients in PL makes it a frequently used organic amendment in US agricultural soils (Edwards and Daniel, 1992; Sims and Wolf, 1994). Application of PL increases the organic matter, oxygen diffusion rate, water holding capacity, and aggregate stability of soils with more pronounced effects in cultivated soils than pastures (Adeli et al., 2009). But, the high transportation cost of PL typically limits disposal to nearby areas. As a result, this excess PL production leads to over application in the local fields and accumulates excess nutrients that are harmful to both the crops and environment (He et al., 2009). The application of PL at adequate rates improves soil quality and increases total soil carbon and microbial biomass (Watts et al., 2010). Studies have shown that excessive application of PL resulted in accumulation of nitrate and phosphorus (Sharpley et al., 1998) and increased soil salinity at the surface (Kingery et al., 1994).

The transformation of organic nitrogen (N) within the litter into usable forms by plants and microorganisms (ammonium and nitrate) is achieved through a sequence of microbially mediated processes (Fig. 1.1). N mineralization, also called ammonification, is the first process that converts the organic N forms (mainly uric acid and urea) into ammonium or ammonia, depending on the soil pH. The ammonium is then converted to nitrate via nitrite through a process called nitrification. In the absence of enough oxygen, the nitrate produced through nitrification is converted into nitrous oxides and dinitrogen gases through denitrification. Ammonia, nitrate, and nitrous oxide gases can easily be lost from the soil system through volatilization and leaching (Cabrera et al., 1993; Gordillo and Cabrera, 1997).

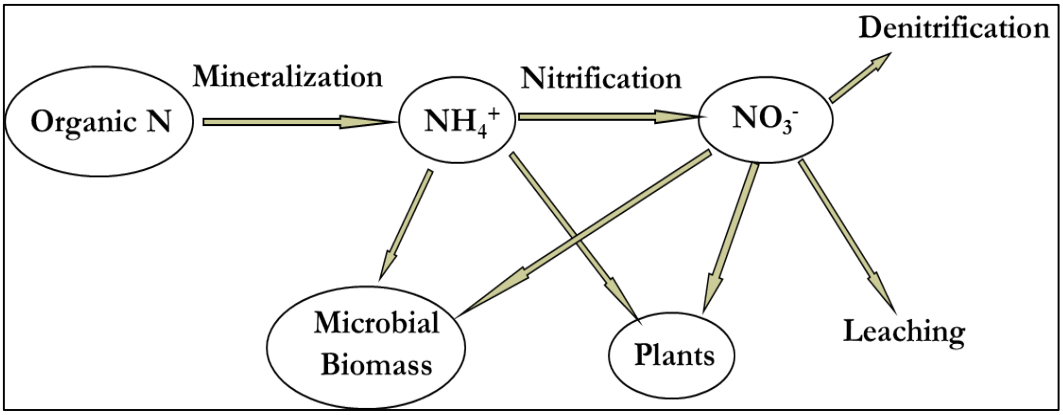

Figure 1.1. Simplified representation of N cycle in soils

Continuous application of litter or manure increases the level of primary and secondary nutrients in soils by creating a reservoir of nutrients for several years after application (Ginting et al., 2003). However, not all of the N and other nutrients are made available for plant uptake by the soil microbes during the first year of application. About 55% of N in PL becomes available to plants in first year, with 45% being available for the succeeding years (Eghball et al., 2004). Sharpley et al (1993) showed that the continual application of PL for several years resulted in 2.5 and 1.2 fold increase in NO_3^--N and total N, respectively, in treated plots compared to untreated plots. There is an increased interest in quantification of N mineralization in soils due to the environmental risks associated with the use of organic wastes in agricultural production (Sharpley et al., 1998). However, studies on mineralization of land applied PL are few compared to other wastes. Sanchez and Mylavarapu (2011) showed that higher rates of PL application during 60 days of incubation yielded greater N mineralization rates, while organic N mineralization rates of 0.4-5.8% and 25.4-39.8% in composted and non-composted PL, respectively, were observed by (Tyson and Cabrera, 1993) after 8 weeks of incubation at 25°C.

Significant ammonia losses also occur due to the high rate of mineralization of organic N when PL is applied on the soil surface in pastures or no-till fields (Cabrera et al., 1993). To decrease these losses, application of composted PL to pastures (slower rate of N mineralization) is a good management practice when the amount of litter produced exceeds the area of land available and transportation to other areas is not economically feasible (Brinson et al., 1994). Such management practices and others discussed to minimize N loss from land applied PL are often effective but their impact on soil microorganisms that mediate key processes in the transformation of the waste is not clear.

Role of ammonia oxidizing bacteria and archaea in nitrification

Autotrophic nitrification is the process of conversion of ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-) and is mediated by groups of bacteria and archaea (Fig. 1.2). The conversion of ammonia to nitrite is the rate limiting step in the nitrification in various environments and is critical to N cycling in soils (Choi and Hu, 2008; Kowalchuk and Stephen, 2001). Two major microbial groups are involved in ammonia oxidation: ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Chemolithotrophic AOB belong to β and γ-Proteobacteriaceae which includes genera *Nitrosomonas* and *Nitrosospira* and are widely used in microbial ecological studies (Kowalchuk and Stephen, 2001). AOB use ammonia, carbon dioxide and molecular oxygen as an energy source, carbon source and electron acceptor, respectively. The reactions in ammonia oxidation step of nitrification are shown below:

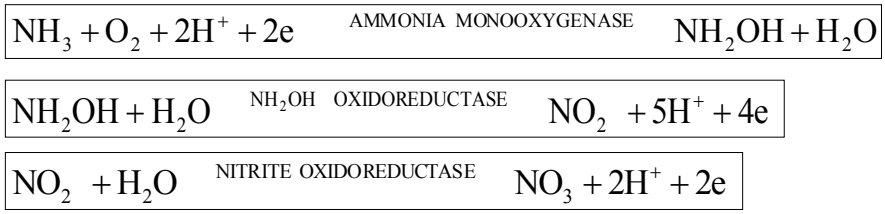

Figure 1.2: Reactions in ammonia oxidation to nitrate

For many years, AOB were considered as the only organisms involved in the nitrification process but recent microbiological studies have shown the existence of novel lineages of mesophilic Crenarchaeota capable of performing ammonia oxidation (De La Torre et al., 2008; Treusch et al., 2005). The cultivated representatives of this group can grow with ammonia as the sole energy source by conversion to nitrite and incorporation of inorganic carbon (Konneke et al., 2005). The first evidence of the role of archaea in ammonia oxidation was given by the metagenomic studies in which genes encoding the subunits for ammonia monooxygenase (AMO) enzyme were detected on the genomic fragments of archaea from the phylum Crenarchaeota (now included in phylum Thaumarchaeota) (Schleper et al., 2005; Treusch et al., 2005). Genes encoding the *amoA*, *amoB* and *amoC* subunit of the AMO enzyme were also found in the genome of *Crenarchaeum symbiosum*, (Hallam et al., 2006; Preston et al., 1996). *Nitrosopumilus maritimus* is a chemolithotrophic marine AO possessing *amoA*, *amoB* and *amoC* gene homologues similar to those of chemolithotrophic AOB (Konneke et al., 2005; Prosser and Nicol, 2008).

AOA were originally thought to be present only under certain environments, but recent studies have shown their presence in a wide variety of environments such as in hot/thermal springs (Hatzenpichler et al., 2008), marine/ocean ecosystems (Beman et al., 2008), estuaries/fresh water bodies (Santoro et al., 2008), soil/terrestrial ecosystems (Leininger et al., 2006; Tourna et al., 2008) and wastewater treatment plants (Park et al., 2006).

In autotrophic AO, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) are the two key enzymes involved in the ammonia oxidation process. The two enzymes are co-dependent, with AMO catalyzing the oxidation of ammonia to hydroxylamine with further oxidation to nitrite performed by HAO (Arciero et al., 1991). The AMO enzyme is encoded for by at least three contiguous genes (*amoC*, *amoA*, *amoB*) that are arranged in a highly conserved gene cluster in all AOB (Sayavedra-Soto et al., 2006; Stein et al., 2007). The *amoA* gene coding for the first subunit of AMO enzyme helps in the study of genetic differences in AOB populations. Polymerase chain reaction primers are used to amplify sequences of *amo* operon by targeting the region encoding the C-terminus of the *amoA* subunit, to discriminate between AOB of the β and γ-Proteobacteria and ammonia and methane oxidizers belonging to the γ subclass (Horz et al., 2000; Rotthauwe et al., 1997).

The discovery of anaerobic ammonia oxidizing (Anammox) bacteria has changed this concept of aerobic ammonia oxidation (Kuypers et al., 2003). Anammox bacteria were first discovered in the wastewater treatment facility in Netherlands and confirmed using ^{15}N labelling experiments (Mulder et al., 1995). The thermodynamic calculations of ammonia oxidation in the absence of oxygen but presence of nitrite/nitrate supports the discovery (Broda, 1977). This process is mediated by bacteria belonging to order *Brocadiales*, a part of phylum *Planctomycetes* (Jetten et. al., 2010). Kuypers et al (2003) reported the discovery of first marine anammox in the Black Sea. Since then, the activity of these bacteria has been found in many marine ecosystems and up to 67% of dinitrogen production in marine sediments has been attributed to this process (Dalsgaard et al., 2005). Electron acceptor for the anammox process is nitrite.

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.5} + 2.03H_2O$$

Figure 1.3: Equation for anaerobic ammonia oxidation (Strous et al., 1998).

These bacteria have a single internal bound compartment called as “anammoxosome” (Van Niftrik et al., 2009) that is a center of anammox catabolism and the organisms involved includes *Ca. Brocadia*, *Ca. Kuenenia*, *Ca. Scalindua*, *Ca. Anammoxoglobus* and *Ca. Jettenia* (Jetten et al., 2005; Quan et al., 2008). *Brocadia fulgida* grows well in a bioreactor environment when acetate is added to ammonium, nitrite and nitrate (Kartal et al., 2008). Zhu et al (2011) reported that anammox activity in paddy soils results in 4-37% of the soil N production. These bacteria have been successfully applied in removing ammonia at low cost from wastewater treatment systems as they do not require an organic electron donor that is needed for conventional nitrification/denitrification. Aeration expenses are reduced to half of the wastewater ammonia needs to be nitrified to nitrite with the remaining used as electron donor (Abma et al., 2007). These bacteria contribute significantly to global N cycle as being an important producer of dinitrogen in marine ecosystems.

Another important recent discovery is that of *Nitrospira* bacteria with the ability to mediate the complete oxidization of ammonia to nitrate (comammox) changing the idea that nitrification is always mediated by two separate groups of organisms (Daims et al., 2015; Pinto et al., 2015; Santoro, 2016). Complete AO were isolated from biofilms in an aquaculture treatment system (van Kessel et al., 2015) and sequence analysis of *Nitrospira nitrosa* and *N. nitrificans*, the two microbes grown in enrichment culture, showed the presence of *amo*, *hao* for ammonia oxidation and *nxr* gene for nitrite reduction. In a similar study, Pinto et al (2015) observed the presence of comammox in drinking water treatment plant in Ann Arbor, Michigan, USA using DNA sequencing of samples from biologically active filters.

Methods for studying AO

Functional activity of AO can be expressed as gross and net rates of nitrification and nitrification potential. Gross nitrification represents the total amount of nitrate produced from ammonia oxidation without accounting for nitrate losses via leaching, immobilization or denitrification and is commonly measured using ^{15}N tracer techniques. Net nitrification is the rate of conversion of ammonia to nitrate excluding the losses (Hart et al., 1994; Norton and Stark, 2011). Nitrification potential is the maximum rate of conversion of ammonia to nitrate under non-limiting substrate availability and has been used as a sensitive indicator of disturbances (Norton and Stark, 2011; Zhou et al., 2015).

Molecular diversity of soil AO and nitrifiers is frequently studied using 16S rRNA or functional genes such as *amoA, amoB, hao, ureC*. However, there is a no single primer pair which includes all AO or nitrite oxidizers (Ward and Arp, 2011). The use of these genes in the study of soil AOB helped in classifying the bacteria into β and γ -AOB (Klotz and Stein, 2011). The 16S rRNA genes are ubiquitous, structurally and functionally conserved in the organisms. These genes may contain some variable and highly conserved regions. The diversity of the organism obtained using 16S rRNA gene is far greater than the diversity associated while using functional genes (Liu and Jansson, 2010). However, multiple heterogeneous copies of 16S rRNA genes are present in the genomes of microorganisms. Microbes which differ substantially ecologically and physiologically can have the same 16S rRNA genes which occurs due to the conserved nature of RNA gene which changes very slowly over time. But since AO make only a small part of the total bacterial population, use of functional genes to study their diversity is a more suitable approach (Liu and Jansson, 2010). The use of functional genes helps in establishing the connections between the presence of a specific microbe and the corresponding function observed. The sequences for

these functional genes are very specific and target only the AO population which are capable of performing ammonia oxidation.

Molecular techniques like quantitative polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE), *amoC-amoA* intergenic spacer analysis (AISA) and high throughput sequencing (e.g. Illumina sequencing) are increasingly used to determine the abundance and composition of AOA and AOB communities. Quantitative PCR has high accuracy, rapid analysis and reproducibility and can be used to quantify the copy numbers of AOB and AOA from environmental samples. Low DNA recovery and PCR artifacts are the two major drawbacks of this method (Rastogi and Sani, 2011). The abundance of *Nitrosomonas* and *Nitrosospira* species in natural and engineered systems is evident from the 16S rRNA and *amoA* gene sequences derived from cultures and environmental clones (Park et al., 2002).

DGGE pattern analysis by Shen et al (2008) showed that the fertilization of N, P, K and organic matter results in significant shifts of the AOB community but not the AOA community. DGGE coupled with qPCR not only determines the abundance of AOA and AOB but also characterizes the microbial community diversity, composition and shifts in population under different fertilization managements (He et al., 2007).

High throughput sequencing techniques offer greater depth of sample coverage and can be easily used for in-depth studies of unknown microbial community diversity (Roh et al., 2010). Illumina sequencing is fast and less expensive compared to Sanger sequencing and helps to generate billions of reads at low cost with high speed and explore the deeper layers of the microbial communities (Rastogi and Sani, 2011). The approach used is “sequencing by synthesis” coupled with bridge amplifications on the surface of a flow cell. It is more effective at sequencing homo-polymeric stretches and generates shorter reads with high sequencing accuracy.

Relative importance of AOB and AOA in nitrification

Several studies have reported AOA to be more abundant in soils than AOB, with a potentially greater role in nitrification in soils (Leininger et al., 2006; Nicol et al., 2008; Zhang et al., 2012). In soils and oceanic waters, AOA exceed AOB by two to three orders of magnitude (He et al., 2007a; Leininger et al., 2006). However, functional redundancy is present between the two groups since ammonia oxidation can be taken over by AOA when AOB are suppressed by use of antibiotics (Schauss et al., 2009) or nitrification inhibitors (Dai et al., 2013). Culture studies enable quantitative analysis of kinetics and provide strong evidence of the link between the function and specific genes if the isolates are representatives of phylogenetic groups and environmentally relevant populations (Prosser and Nicol, 2012).

Population size and community structure of bacteria and archaeal *amoA* is related to environmental conditions such as soil pH, ammonium availability (Nicol et al., 2008), vegetation type (Chen et al., 2008), salinity or N fertilization practices (Nicol et al., 2008; Shen et al., 2008). AOA have a higher substrate affinity for ammonia than AOB due to their smaller size and greater surface to volume ratio (Jung et al., 2011; Martens-Habbena et al., 2009), and as such dominate in low ammonium oligotrophic soils. AOB, on the contrary, grow well in high ammonium environments (Di et al., 2010a). The niche separation of AOB and AOA also varies with the source of N input; AOB and AOA grow well in soils with ammonia derived from urea/ammonium fertilizer and mineralization of organic matter, respectively (Di et al., 2010a; Jia and Conrad, 2009; Levičnik-Höfferle et al., 2012). AOA prefer a wide soil pH range for growth and are abundant in acidic soils whereas, AOB are found to be abundant in soil with neutral pH (Nicol et al., 2008). AOA are found to be transcriptionally more active and abundant in soils with low pH (acidic soils), thus contributing to higher nitrification rates (Zhang et al., 2012). Increase in AOA abundance

with increase in soil pH was reported by (Jia and Conrad, 2009). However, Nicol et al (2008) reported a negative correlation between AOA abundance and soil pH.

AOA are reported to have the potential to use different organic compounds other than ammonia as a source of energy (i.e mixotrophic growth); however, autotrophic AOB only use ammonia as an energy source. AOA utilize a 3-hydroxypropionate pathway, or the Kreb's cycle, for autotrophic carbon fixation, while the Calvin cycle is used by AOB (Ward et al., 2007). AOA are also found to be abundant and active at higher temperature ranges, while AOB dominate in the mesophilic temperature range (Zhang et al., 2008). There is a need to identify factors linking diversity, abundance and activity of AOA and AOB as it still remains unknown whether population sizes, community composition or edaphic properties control the relative contribution of AOA and AOB to nitrification activity (Zeglin et al., 2011). DNA-based stable isotope probing and transcription analysis are increasingly used to demonstrate the role of AOA in ammonia oxidation and to understand the link between AOA abundance, diversity and ecosystem function (Shen et al., 2012; Tourna et al., 2008).

Nitrification inhibitors and their impact on AOA and AOB

N use efficiency in agricultural systems depends largely on nitrification. N is an essential plant nutrient and increasing available N is beneficial to sustain the world's growing population (Erisman et al., 1998). But, excess N has also produced detrimental effects to the environment such as global warming, eutrophication of aquatic systems etc. (Di and Cameron, 2002; Galloway et al., 2003). Nitrification represents a potential source of N loss by which plant available N is leached to deeper soil layers in the form of nitrate or emitted as nitrous oxide (Di et al., 2010b). Agricultural soils are identified as a source of nitrous oxide emissions which also causes destruction of the stratospheric ozone layer (Di and Cameron, 2006; Galloway et al., 2003).

Therefore, nitrification inhibitors (NI) are increasingly used to prevent these N losses from agricultural fields. They help in the accumulation of ammonium in soils by restricting the microbial activity of AOB *Nitrosomonas* and *Nitrosospira* (Zacherl and Amberger, 1990). The synthesis of Nitrapyrin [2-chloro-6 (trichloromethyl) pyridine] in 1962 revolutionized the use of NI in agriculture (Zerulla et al., 2001). Nitrapyrin, combined with fertilizers, has been shown to reduce N losses; however, in a study by Shen et al (2013), a weak inhibiting effect of Nitrapyrin was found on *Nitrosospira multiformis* and a moderate effect on the archaeon *Nitrososphaera viennensis*.

Dicyandiamide (DCD; 2-Cyanoguanidine), is another NI that is commonly used (Di and Cameron, 2002; Merino et al., 2001). DCD is a non-volatile, non-hygroscopic, relatively water soluble (23 g litre^{-1} at 13°C), chemically and physically stable NI, which is usually broadcasted onto soil surface and breaks down in soils to non-toxic products. Several studies have reported the decreased nitrate leaching and nitrous oxide emissions through the application of DCD (Dai et al., 2013; Di et al., 2010b) but it's efficiency depends on factors such as fertilizer type, temperature (Di and Cameron, 2005), application rate (Di and Cameron, 2006), soil moisture (Kumar et al., 2000) and the method of DCD application (Di et al., 2007).

Several researchers have used DCD to differentiate the contribution of AOB and AOA in soil nitrification (Dai et al., 2013; Di et al., 2010a). Zhang et al (2012) used DCD in a microcosm study to differentiate AOB and AOA contribution in acidic soils in China and found AOA to be functionally dominant over AOB, with strong positive correlations with nitrate concentrations. Likewise, Dai et al (2013) found that AOB played a dominant role in nitrification with a higher abundance than AOA in a grazed pasture soils amended with urea/urine-N and DCD. In a

laboratory incubation study using DCD, Di et al. (2010a) reported a significant relationship between nitrification rate and AOB abundance in soils amended with urine-N substrate.

The use of 1-Octyne (an aliphatic C-8 alkyne) has also been suggested to differentiate the relative contribution of AOB and AOA in the soil nitrification process, producing an inhibitory effect on the activity of AOB but not on the AOA (Taylor et al., 2015). Giguere et al (2015) used 1-Octyne to differentiate the contribution of AOB and AOA on nitrification activity in samples collected in summer and winter from paired cropped and non-cropped Oregon soils. AOB and AOA had higher nitrification activities in summer compared to winter, with AOB and AOA being the dominant players in nitrification in cropped soils and non-cropped soils, respectively. Ouyang et al (2016) using 1-Octyne reported that AOB were the major contributor to potential nitrification in agricultural soils amended with ammonium sulfate and steer waste compost.

These results suggests that archaeal and bacterial communities responds differently to NI due to structural and functional differences in AMO enzymes but further investigation is needed to draw such conclusions, especially in PL amended soils where metals and other chemicals are also introduced.

Impact of trace metals on AOB and AOA

Excessive application of PL has been shown to result in trace metal pollution in soils (Jackson et al., 2003; Toor et al., 2007). These trace metals are added in poultry and livestock feed as essential nutrients, to help in physiological functions and metabolism to improve health and feed efficiency, to increase egg production in poultry and to prevent diseases (Angel and Powers, 2006; Sims and Wolf, 1994). Trace metals are known to function as catalysts or co-factors in the functioning of several enzymes and cannot be substituted by other metals (NRC., 1994). These metals can have negative impact on soil microbes, plants and animals (Adriano, 2013) and their

excessive accumulation poses a threat to soil fertility by creating an imbalance in soil ecosystems (Komárek et al., 2010). Decreases in microbial species richness and diversity, with the development of metal resistant microbial population due to long-lasting toxic effects are frequently observed in trace metal contaminated areas (Yao et al., 2003). Inhibition of microbes by trace metals is caused by complexation of enzyme substrate and reaction with the enzyme-substrate complex (Nannipieri et al., 1994).

Zinc (Zn), copper (Cu), arsenic (As), nickel (Ni) and lead (Pb) are few of the trace metals frequently found in high concentrations in agricultural soils amended with PL (Ashjaei et al., 2011; Toor et al., 2007). These metals play an important role in cellular functions; but excess of these metals have been known to cause cellular death (Luo et al., 1999). About 90% of Cu added in swine diet was excreted in feces (Kornegay et al., 1976) and a decrease in soil microbial biomass with a change in the microbial diversity have been reported in soils with high levels of Cu (Chander and Brookes, 1991). Nicholson et al (1999) observed that 25-40% of annual input of Cu, Ni and Zn to agricultural lands in England comes from the application of animal manure.

Schomberg et al (2009) observed that 10 years of PL application in Cecil soil in Piedmont, GA resulted in a five-fold increase in extractable Zn in 0-15 cm soil layer with a greater increase in no-till than conventional tillage. Van der Watt et al (1994) reported that application of PL at 6 Mg ha^{-1} for 16 years resulted in an increase in concentrations of Cu, Zn and Mn to toxic levels. Kingery et al (1994) observed that long-term application of broiler litter to Ultisols in Alabama resulted in greater concentration of extractable Cu and Zn (2.5 and 10 mg kg^{-1}) compared to untreated soils (0.75 and 2.2 mg kg^{-1}) on the surface layer and accumulation of these metals up to 45 cm depth in broiler litter applied soils, respectively.

Heavy metals like Cu (Mertens et al., 2010), Zn (Ruyters et al., 2010) and Hg (Liu et al., 2010) are known to affect soil microbial populations and shape the ecological niches of AO populations. In a study with mine waste soils contaminated with As and Pb, Ollivier et al (2012) reported a decrease in AOB and AOA abundance with high levels of As (83 g kg^{-1}) and Pb (15g kg^{-1}) compared to control soils. Liu et al (2010) reported that application of Hg resulted in a decrease in potential nitrification rate with no significant differences in the abundance of AO. More studies have examined the impact of metals on AO (Mertens et al., 2010; Ruyters et al., 2010; Liu et al., 2010; Ollivier et al., 2012), but the soils were not treated with PL nor did they have a history of PL application. This suggests the need for research to determine the impact of trace metals on AO in PL amended soils.

Study Justification

Even though soil N cycling is widely studied there is still lack of research in regards to PL amended soils. Previous studies mainly looked at changes in process rates without simultaneous examinations of microorganisms that mediate these processes. Moreover, the discovery of new players in soil nitrogen cycling (e.g., AOA) justifies the need for more studies when it comes to PL treated soils. This research topic is of particular importance to Georgia, which is the largest poultry producing state in the country, with the subsequent generation of large amount of PL. Hence, proper understanding of the transformation of this waste applied to soils is required to avoid undesirable environmental consequences. The study has the added advantage of using long established plots with many years of PL application.

Objectives

Objective I: Examine changes in the activity and community structure of soil AO after 15 years of PL application and assess the response of the AO to the residual effect of PL application after 2 years of stoppage

Objective II: Determine the relative contributions of AOB and AOA to soil nitrification in pasture soils with a history of PL application and relate it to changes (if any) in the abundance and community composition of AO

Objective III: Determine differential contributions of AOB and AOA to nitrification under elevated trace metal levels at different substrate concentration in PL treated soil

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CHAPTER 2

CHANGES IN FUNCTION AND COMMUNITY STRUCTURE OF AMMONIA OXIDIZERS IN A GRAZING LAND WITH A HISTORY OF POULTRY LITTER APPLICATION

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Abstract

Poultry litter (PL) is widely applied on grazing lands in Georgia. It is not clear how repeated application of PL affects soil microorganisms and key nitrogen (N) transformations they mediate. We examined changes in function and community structure of ammonia oxidizing archaea (AOA) and bacteria (AOB) in a grazing land with a history of PL application. Soil samples were collected in 2009 (after 15 years of PL) and 2013 (after 2 years of stopping PL). The abundance and community composition of ammonia oxidizers (AO) were determined with molecular methods that targeted *amoA* gene. Nitrification potential (NP) was used for measuring the function of AO. In 2009, the abundance of AO was significantly higher in PL treated (6.98 and 7.06 log copies g $soil^{-1}$ for AOB and AOA, respectively) than controls plots (6.39 and 6.53 log copies g $soil^{-1}$ for AOB and AOA, respectively) that received an equivalent amount of N in the form of urea ammonium nitrate. This was in agreement with NP rate, which was higher in PL treated (0.78 mg kg^{-1} soil hr^{-1}) than control (0.50 mg kg^{-1} soil hr^{-1}) plots in 2009. The alpha (α) diversity of AO was higher in PL than control plots in 2009. In 2013, both the numerical dominance of AOB over AOA and NP decreased significantly in response to the discontinuation of PL. The α-diversity decreased in PL plots while it increased in control plots in 2013. Nonmetric multi-dimensional scaling analysis showed clustering by treatment and year, indicating the presence of distinct AO communities in the two types of plots. Correlation analysis suggested that AOB were functionally more important than AOA in nitrification, indicating the need to target AOB for efficient management of N in PL receiving soils. Overall, we observed that the change in nitrification was caused by both the change in abundance and community composition of AO.

Introduction

Georgia is the leading poultry producer in the U.S., resulting in the production of large amounts of poultry litter (PL) that is land applied on pastures and hay fields as a way of disposal and to take advantage of the nutrients contained in it (Moore et al., 1995; Pote et al., 2003; Sauer et al., 2000; Sims and Wolf, 1994). Surface broadcasting is the most common way of application (Cabrera et al., 1994; Sistani et al., 2009). While continuous application of PL improves soil fertility by creating a reservoir of nutrients (Eghball et al., 2004; Ginting et al., 2003), excessive application can lead to soil quality problems from heavy metals, which are commonly added to poultry feed to minimize health disorders and avoid diseases (Angel and Powers, 2006; Jackson et al., 2006; Sims and Wolf, 1994).

The use of PL as a source of N in soil is governed by the transformation of organic N into plant usable forms (Cabrera et al., 1993; Sistani et al., 2008). This transformation of organic N is achieved through a sequence of microbially mediated processes. The first process in that sequence is mineralization, which is the conversion of organic N forms (mainly uric acid and urea) into ammonia. Ammonia is then converted to nitrate via nitrite through nitrification. The conversion of ammonia to nitrite is the rate limiting step in the nitrification process in various environments and is critical to N cycling in soils (Choi and Hu, 2008; Hu et al., 2003; Kowalchuk and Stephen, 2001). Nitrite is unstable in soils and is quickly converted to nitrate, which can be lost via leaching or denitrification. As a result, the function of soil microorganisms that mediate this process is crucial for proper and efficient management of N.

Ammonia oxidizing bacteria (AOB) were assumed to be solely responsible for autotrophic nitrification (Leininger et al., 2006; Purkhold et al., 2000). But, more recent studies have shown archaea are also capable of performing ammonia oxidation (De La Torre et al., 2008; Konneke et

al., 2005). Studies have reported ammonia oxidizing archaea (AOA) to be more abundant than AOB in some soils, with a potentially greater role in nitrification (Leininger et al., 2006; Nicol et al., 2008). The relative importance of AOB and AOA varies in arable soils depending on a number of environmental factors, with one or the other being more competitive under a given set of conditions (Wessén et al., 2010). Understanding the dominant player in this process is essential for the development of practical N management practices, such as use of nitrification inhibitors (Di et al., 2009).

PL amended soils have a unique set of conditions as compared to soils with a history of other animal waste application. There is a lack of research on the role of ammonia oxidizers (AO) in soils amended with a history of PL application (Dharni et al., 2010; Tomlinson et al., 2015). Previous studies have focused mainly on changes in process rates and trace metal content in the PL amended soils without simultaneous examinations of microorganisms that mediate these processes (Toor et al., 2007). Moreover, the discovery of new players in soil N cycling (e.g., AOA) justifies the need for more studies when it comes to PL treated soils. This research topic is of particular importance to Georgia because of its leading position in poultry production in the nation. Hence, proper understanding of the transformation of nutrients contained in the waste and its impact on soil microorganisms is required to avoid undesirable environmental consequences.

In this work, we used soil samples collected in 2009 and 2013 from plots that received PL applications between 1994 and 2011. The samples in 2009 were used to assess the impact of PL on the activity and community structure of AO after 15 years of PL application. The 2013 samples were used to assess the response of the AO to the residual effect of PL application after 2 years of stoppage. In addition to measuring the activity and community structure of AO, we characterized

the samples for a range of soil parameters that might indirectly affect the microorganisms, including trace metals that are commonly introduced to soils via PL application.

Materials and Methods

Field Plots and Soil Sampling

The experimental plots are located at the Water Quality plots, College of Agricultural and Environmental Sciences Central Georgia Research and Education Center near Eatonton, Georgia (39°24'N, 83°29'W, elevation 150 m). Soil samples were taken from four 0.8 ha PL amended (ID 1, 2, 4, 5) and two control plots (ID 7 and 8) with no PL application (Figure 1). The soil series for the area is Cecil (fine, kaolinitic, thermic Typic Kanhapludults), with sandy loam soil texture (Ashjaei et al., 2011). PL was surface applied twice a year in spring and fall from 1995 to 2011 at an average yearly rate of 8.3 Mg ha^{-1} except in 2003 and 2004 when no PL was applied. On average, the PL was composed of 2.76, 0.28 and 0.13% total-N, ammonium-N and nitrate-N, respectively. Based on a laboratory based incubation study, it was estimated that 55% of the organic N mineralized in the first year of application (Watts et al., 2010). Subsequently, the 8 Mg ha^{-1} application rate provided 227 kg of available N ha^{-1} $year^{-1}$ after considering the N coming from the organic, ammonium and nitrate pools. The PL application was discontinued in 2011. The control plots did not receive any PL application but received equivalent amount N in the form of liquid nitrogen fertilizer that combined urea and ammonium nitrate (UAN). The plots were used for growing pastures which were mainly Bermuda grass (*Cynodon dactylon*) and tall fescue (*Festuca arundinacea*) (Ashjaei et al., 2011).

The plots were sampled twice, in June 2009 and August 2013. The sampling times were chosen to account for the changes in N processes and ammonia oxidizer community structure after 15 years of PL application (2009) and the residual impact of PL application after 2 years of

stoppage (2013). In 2009, soil samples were collected from a depth of 0-15 cm. In 2013, soil samples were collected from two depths, 0-5 cm and 0-15 cm. For each plot, multiple sub-samples were collected and made into a composite sample with thorough mixing. N transformation measurements were done as soon as possible or within a three week time period during which they were stored at 4°C. Portions of the samples were stored at -20°C for the molecular analysis (Carter, 1993). For the analysis of physical and chemical properties, soil samples were air dried, homogenized and sieved through 2 mm sieve.

Soil testing and N content measurements

Soil organic carbon content was determined using the potassium dichromate oxidation method (Walkley and Black, 1934). Gravimetric moisture content of the soil was measured by drying ten gram soil at 102°C for 24 hr in an oven (Gardner and Klute, 1986). The soil samples were analyzed for basic soil properties, including pH, cation exchange capacity (CEC), percent base saturation (BS) and Mehlich-1 extractable P and trace metals at the Agricultural and Environmental Services Laboratories of the University of Georgia (http://aesl.ces.uga.edu/).

For inorganic N measurements, fifteen gram soil and 75 ml of 2M KCl were mixed in 250 ml Erlenmeyer flasks, shaken for 1 hr and filtered through a pre-rinsed Whatman No.1 filter paper. The filtrates were frozen until analyzed for NO_3^--N and NH_4^+-N by the Rapid Flow Analyzer (Alpkem Corp, Clackamas, O.R). Inorganic contents of PL were similarly determined. Total N in PL was determined by using the Kjeldahl method (Sharpley et al., 1993).

Nitrification potential (NP) was measured by the shaken soil slurry method as previously described (Hart et al., 1994). Fifteen gram of sieved moist soil from each plot was placed in 250 ml Erlenmeyer flasks with 100 ml of phosphate buffer and 1mM NH_4^+-N. The flasks were continuously shaken for 24 hr at 200 rpm. Ten milliliter aliquots were withdrawn at 2, 4, 22 and

24 hr intervals. The aliquots were centrifuged at 8000 × g for 10 min and analyzed for (NO_2^-+ NO_3^-)-N as stated above. The slope of linear regression of concentrations of (NO_2^- + NO_3^-)-N versus time was used to describe the nitrification potential for the soil samples.

Quantitative PCR

Soil DNA was extracted from 0.25 gram soil using the PowerSoil® DNA extraction kit (MO-BIO laboratories, Carlsbad, C.A). Genomic DNA of the bacterial pure culture strains *Nitrosomonas europaea* 19718 and *Nitrosospira multiformis* 25196 (ATCC, Manassas, V.A) was extracted using the DNAzol kit as per the manufacturer's instructions (Molecular Research Center Inc, Cincinnati, Ohio). The pure culture strains were used for standard preparation and as reference for the AOB community composition analysis as described below.

The abundance of bacterial and archaeal *amoA* genes in the soil DNA extracts were quantified in analytical duplicates using a modified protocol described by (Leininger et al., 2006). The amoA1F/2R and ArchamoAF/AR primers were used for the quantification of AOB and AOA, respectively. Quantitative PCR standards for AOB and AOA were prepared using genomic DNA of *Nitrosomonas europaea* 19718 and soil DNA extract, respectively, as described in Habteselassie et al. (2013). Dilution series of the stock were made to prepare a standard curve covering four orders of magnitude, from 3.0×10^3 to 3.0×10^7 copies of template per assay. Quantitative PCR for the standards were performed in triplicate with StepOne Plus™ thermocycler (Applied Biosystems by Life Technologies, Foster City, C.A). The PCR conditions for both AOB and AOA were slightly modified with the number of cycles reduced to 40 and an elongation time of 3 min (Leininger et al., 2006). The PCR product specificity was performed through the melt curve analysis by measuring fluorescence as the temperature increased from 57ºC to 94ºC, 0.3ºC per reading. The reaction volume of 20 µl was used along with 2 µl of the DNA, 10 µl of SYBR Green

Master Mix (Applied Biosystems by Life Technologies, Foster City, C.A) and 0.8µl of each forward and reverse primer (Eurofins MWG Operon LLC, Louisville, K.Y). Data analysis was done using the StepOne Plus software v2.1. The log *amoA* copies g soil^{-1} were used as proxy for the AOB and AOA abundance. Each cell of AOB contains 2-3 copies of *amoA* gene whereas the copies of *amoA* gene in AOA is still unknown (Norton et al., 2002).

Molecular fingerprinting techniques for AOB and AOA

Denaturing gradient gel electrophoresis (DGGE) was used for finger printing the AOA community in the soil samples as described in (Jia and Conrad, 2009) with slight modifications in the conditions of gel electrophoresis. In brief, archaeal *amoA* gene was targeted using amoA19F* and amoA 643R primer pair. For a total volume of 25 µl, the amplification was performed in a BIO-RAD thermocycler (Bio-Rad laboratories, Hercules, C.A) using a Hot Start GoTaq Master mix (Promega Corporation, Madison, W.I). Primers were synthesized by Eurofins MWG Operon LLC, Louisville, K.Y. Polymerase chain reaction (PCR) products were checked for size and quality with agarose gel electrophoresis (1.5%) and visualized after staining with ethidium bromide. For separation of PCR products, DGGE of archaeal *amoA* gene was performed using a D-code Universal Mutation Detection System (Bio-Rad laboratories, Hercules, C.A). Acrylamide gel of 6% containing 70-30% denaturing gradient was used to represent the community composition for AOA. The gel electrophoresis was performed using 1X Tris buffer for 20 hr at following conditions:- potential of 56 V, temperature of 54°C for AOA. Before UV illumination, the gels were first stained using SYBR Green I (Lonza, Rockland, M.E) for 20 minutes and de-stained for 15 minutes in distilled water. Acrylamide gel images were visualized under a 302 UV source using the Kodak Gel Doc 100 system (Kodak Co, Rochester, N.Y).

For fingerprinting the AOB community, the intergenic region between *amoA* and *amoC* was targeted using amoC305F and amoA310R primers (Habteselassie et al., 2013) with some modifications that are described below. This method was shown to be a quick and effective way of profiling AOB community composition. The following PCR conditions were used – (a) 94°C for 4 min, (b) 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 4 min and (c) a final extension step of 72°C for 10 min. Genomic DNA from *Nitrosopira multiformis* 25196 and *Nitrosomonas europaea* 19718 were used as positive controls. The amoAC intergenic region sizes for the two strains are 446 and 277 bp, respectively. The PCR products were run on a 6% acrylamide gel with a 0% denaturing gradient. The acrylamide gel electrophoresis was performed in 1X Tris buffer with a potential of 60 V for 13 hr at 20°C using a D-code Universal Mutation Detection System (Bio-Rad laboratories, Hercules, C.A). Gel images were obtained using the Kodak Gel Doc 100 system (Kodak Co, Rochester, N.Y) under a 302 UV source after staining with SYBR Green I (Lonza, Rockland, M.E) for 20 min. A cluster analysis was performed to examine the banding patterns using the Gel Compar II software (Applied Maths, Austin, T.X). For both finger printing techniques, the Shannon index of general diversity (H) was calculated using the following formula,

$$H = -\sum(n_i/N)\log(n_i/N)$$

where n_i is the height of the band peak and N is the sum of all peak heights of the densiometric curve (Boon et al., 2002).

Community structure of AO using high throughput sequencing

Bacterial and archaeal *amoA* libraries were prepared for paired-end sequencing using Illumina TruSeq fusion primers with custom tags (amoA1F and 2R for AOB and ArchamoAF and AR for AOA) as described in Faircloth and Glenn (2012). All PCR reactions were carried out in 25 µl final volume with 12.5 µl of Hot Start GoTaq Master Mix (Promega Corporation, Madison,

W.I), 6.5 µl of nuclease free PCR water, 2 µl of i-Tru forward and reverse primers and 2 µl of DNA template in a Bio-Rad MyCycler thermocycler (Bio-Rad Laboratories Inc., Hercules, C.A). Thermal conditions for AOB were: initial denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 50 sec; 60ºC for 45 sec; 72ºC for 45 sec and a final denaturation at 72ºC for 10 min. For AOA the conditions were: initial denaturation at 95ºC for 5 min, 35 cycles of 95ºC for 35 sec; 60ºC for 35 sec; 72ºC for 40 sec and a final denaturation at 72ºC for 10 min. The PCR product specificity was checked by running it on agarose gel (1.5%) and cleaned using Wizard® SV Gel and PCR clean up system (Promega Corporation, Madison, W.I). The cleaned PCR products were then pooled in equimolar concentration and submitted to UGA's Georgia Genomics Facility for sequencing with a MiSeq 300 sequencer (Illumina Inc., San Diego, C.A). Customized fusion primers used for the Illumina sequencing were obtained from Eurofins Genomics (Eurofins MWG Operon LLC, Louisville, K.Y).

To process raw sequence data generated from paired end Illumina sequencing, we used QIIME version 1.9.1 (Caporaso et al., 2010b). We used PANDAseq (Masella et al., 2012) to assemble paired-end reads. Demultiplexed sequences were trimmed for quality and clustered into operational taxonomic units (OTU's) based on 90% (for AOB) and 60% (for AOA) sequence identity (Masella et al., 2012; Norton et al., 2002). Operational taxonomic unit (OTU) picking was performed using an 'open-reference' OTU picking protocol, where sequences are clustered against existing sequences in NCBI GenBank database (Benson et al., 2005). For AOB, the sequence length was 495 bp and for AOA sequence length was 635 bp. The reference sequences from GenBank for both AOA and AOB were clustered and aligned using the MUSCLE v 3.8.31 program (Edgar, 2004). Reads that matched a GenBank reference sequence at 97% for AOB and 90% for AOA, identity were clustered to an OTU defined by that reference sequence. Reads that

did not match a reference sequence were subsequently clustered *de novo*. The centroid sequence in each cluster was chosen to represent the cluster and aligned with the corresponding GenBank sequence set using PyNAST version 1.2.2 (Caporaso et al., 2010a). Reads that failed to align with PyNAST were excluded from subsequent analyses. Phylogenetic trees were constructed using FastTree (Price et al., 2010) and taxonomic assignments were made for each representative sequences using the uclust version 1.2.22 (Edgar, 2010) consensus taxonomy assigner.

Further analysis and plotting of sequencing data was performed using the PhyloSeq package from Bioconductor version 3.3 library (McMurdie and Holmes, 2013) in R version 3.2. We evaluated six α-diversity indices, including the abundance based coverage estimators (ACE), Chao1, number of observed OTU's, Shannon index, Simpson's index and the inverse Simpson's index (Magurran, 2013; Rosenzweig, 1995). ACE and Chao1 measure the OTU richness whereas Shannon, Simpson indices measure the OTU diversity (Gotelli et al., 2013). We also compared the different samples in terms of Beta (β) diversity obtained using non-metric multidimensional scaling (NMDS) (Fasham, 1977) with the phylogenetic-based UniFrac distances (Lozupone and Knight, 2005). The final OTU table had an average of 3000 sequences per sample for AOB and 6600 sequences per sample for AOA (also known as depth of coverage), which represent a significant depth of coverage (Meyerson et al., 2010).

Statistical Analysis

SAS software version 9.3 (SAS Institute Inc., Cary, NC, 2001) was used for performing all the ANOVA analyses. All statistical analyses were performed at 95% confidence level ($p \leq 0.05$), unless specified otherwise. Statistical analysis of multiple year data for different soil properties measured at a depth of 0-15 cm was performed using repeated measures analysis of variance (Proc Mixed) with year as repeated measure factor. For soil samples collected in 2013, statistical analysis

of multiple depth data for Shannon index of diversity (H-value) was performed using repeated measures analysis of variance (Proc Mixed) with depth as repeated measure factor. The effect of different treatments on abundance of *amoA* in AOB and AOA at various depths over multiple years was analyzed using a split-split plot design ANOVA with treatment as the main plot, year as a subplot, and depth as a split-subplot. Significantly different means were separated by Tukey's Studentized Range (HSD) test.

The influence of different properties of soil on AOB, AOA abundance and diversity indices were evaluated through a correlation analysis. Nine important parameters, namely- ammonium concentration, nitrate concentration, pH, SOC, BS, CEC, trace metals (As+Cd+Pb+Zn etc.) were evaluated to determine their impact on the abundance and the diversity of AOA and AOB. The Spearman rank correlation between different parameters was calculated using MS Excel (Microsoft Corp., Redmond, WA, 2010). A correlation value in the range of $-0.40 \leq 0 \leq 0.40$ is considered to be non-significant. To analyze the effects of year and treatment on the community structure of AOB and AOA, we performed a multivariate ANOVA (MANOVA) on normalized fraction of OTU's from different genera.

Results and Discussion

Basic soil properties

Results of soil physicochemical properties are shown in Tables 2.1 and 2.2. Only treatment had a significant effect on soil pH ($p = 0.04$), CEC ($p = 0.005$) and soil P ($p = 0.05$). Neither treatment ($p = 0.22$) nor year ($p = 0.23$) had significant effect on SOC. Percent base saturation (BS) was significantly affected by both treatment ($p = 0.02$) and year ($p = 0.04$). For the metals present in soil, Zn ($p = 0.003$), Cu ($p = 0.0006$), Pb ($p = 0.008$) and Mo ($p = 0.007$) were significantly affected only by treatment. The effect of treatment or year was not significant on As

($p_{treatment}$ = 0.21; p_{year} = 0.88), Cr ($p_{treatment}$ = 0.13; p_{year} = 0.63), Mn ($p_{treatment}$ = 0.31; p_{year} = 0.20) and Cd ($p_{treatment}$ = 0.09; p_{year} = 0.40). None of the parameters were significantly affected by treatment × year interaction.

Averaged over time, PL amended plots had higher soil pH (6.05) than control plots (5.64) (Table 2.1). CEC and BS were 13% and 24% higher in PL treated than control plots, respectively. BS decreased by 9% between 2009 and 2013 when averaged over treatment. On average, P, Zn, Cu, Mo and Pb were 13, 4.4, 4.3, 2 and 1.3-fold higher in PL treated than control plots. The increase in soil pH values associated with PL application is expected as calcium carbonate is commonly added to poultry feed (Hue, 1992; Kingery et al., 1994; Sharpley et al., 1993). PL is also a source of organic matter, cations and other nutrients (Kingery et al., 1994; Wood et al., 1996; Daigh et al., 2009). As a result, the increase in CEC, BS and P in the PL treated plots in this study is not surprising. Metals (e.g., As, Cu and Zn) are commonly added to poultry feed to prevent diseases, improve feed efficiency and increase weight gain (Miller et al., 1991; Toor et al., 2007). The metals end up in soils during land application of PL. Although we did not see any significant build-up of As in the PL treated plots, other studies have reported otherwise. (Han et al., 2004) reported three times higher As level (8.4 mg kg^{-1}) in the long-term PL amended plots (PL applied for 25 years) as compared to plots that did not receive PL (2.68 mg kg^{-1}). Similarly, the build-up of metals in PL treated soils had been reported by Jackson et al. (2003) and Sims and Wolf (1994). Some of these metals (e.g., Pb, Cr, Zn, As) could have toxic effects on soil micro-organisms, including AO (Frostegård et al., 1993; Kandeler et al., 1996; Mertens et al., 2009; Stephen et al., 1999). The effect of these metals on AO in soils amended with PL has not been studied before. Previous studies mainly focused on impacts of PL application on physical and chemical soil properties and crop yield (Sainju et al., 2010; Sharpley et al., 1993) .

The absence of year effect in our study suggests that the metal concentrations in the soil did not decrease appreciably after two years of stopping PL application. A number of factors influence how fast the metal concentration comes down after stopping PL application, including plant uptake, leaching, soil texture and organic matter (Daigh et al., 2009). Although all conditions are not exactly comparable to our study, Daigh et al (2009) suggested that a significant decrease in metal concentrations would require more than 5 years after PL application is stopped in a Captina silt-loam soil.

Nitrification Potential and Inorganic N content

The effects of treatment ($p = 0.008$) and year ($p = 0.01$) were significant for NP, although treatment × year interaction effect was not significant ($p = 0.52$). NP was about 2-fold higher in PL treated (0.56) than control plots (0.23) when averaged over time (Table 2.3). Average NP decreased between 2009 and 2013 by three orders of magnitude from 0.64 to 0.23 mg kg^{-1} soil hr^{-1}. NP is a measure of the maximum capacity of nitrifiers to convert ammonium to nitrate (Fortuna et al., 2003) and is commonly used as an indicator of the size of the active nitrifying population in soils (Jenkins and Kemp, 1984). When used in tandem with abundance data, NP provides an estimate of the active nitrifying microbial population size. Our data indicate that PL amended plots had more active populations of AO than control plots. As reported below, the abundance data support this observation. The presence of more active AO populations in PL amended soils could partly be because of the higher pH (He et al., 2007; Nicol et al., 2008). Our NP rates are comparable to what others have reported in waste treated soils without metal accumulation. As such, the metals in PL did not seem to have affected the activity of AO negatively. In soil amended with dairy waste for 90 days, Shi et al (2004) reported NP in the range of 0.19-0.34 mg kg^{-1} soil hr^{-1}. Yao et al (2011) had also reported NP rates of 0.20-1.32 mg NO_3^--N g^{-1} hr^{-1} in the fertilized acidic soils of

the Chinese tea orchards. Soil NP in soils amended with dairy slurry at 300 kg N ha^{-1} ranged from 0.083-0.79 mg NO_3^--N kg^{-1} hr^{-1} and was significantly correlated with the AOB abundance (Fortuna et al., 2012).

The effects of treatment ($p = 0.01$), year ($p = 0.02$) and treatment-year interactions ($p = 0.01$) were significant for NH_4^+-N. On average, the concentration of NH_4^+-N was 35% higher in control plots (13.5 mg of N per kg soil) than PL treated plots (9.9 mg per kg soil). This makes sense as the oxidation of ammonium is higher in PL than control plots, as indicated by the NP rates above. Moreover, the bulk of N in the PL is in the organic form (urea and uric acid), which is relatively slowly released through microbial action unlike the N in UAN that was applied in the control plots. The lowest NH_4^+-N concentration was in PL treated plots in 2013 (5.78 mg of N per kg soil). This is most likely a reflection of an active nitrifying population in PL treated plots that resulted in oxidization of the ammonium in the absence of additional PL input. In general, ammonium level in PL ranges between 0.1 to 20.1 g N per kg of PL (Edwards and Daniel, 1992), which is significantly higher than other wastes such as dairy or sheep manure (Tiquia and Tam, 2002). However, a significant amount of the ammonium is lost in the form of ammonia through volatilization due to the high pH of PL (Cabrera et al., 1993). For NO_3^--N, only year had significant effect ($p = 0.0004$) (Table 2.3). The soil NO_3^--N content increased by 5-fold from 2009 to 2013. This is due to increased ammonia oxidation that produces nitrate and discontinuation of PL application, which supplies ammonium. Since nitrate is subject to loss in the forms of leaching and denitrification, it might not stay within the sampled depths to accurately reflect the treatment effects.

Abundance of bacterial and archaeal *amoA*

The average bacterial *amoA* (AOB) abundance in PL amended and control plots and for 2009 and 2013 are shown in Table 2.4. The 2013 data included the (0-5 cm and 0-15 cm) depths while 2009 data included only the 0-15 cm depth. We decided to sample the 0-5 cm depth in 2013 as PL was surface applied, and we felt the residual impact of PL would be better reflected at this depth. Only treatment effect was significant on AOA in 0-15 cm ($p = 0.02$) whereas treatment ($p < 0.001$), year ($p = 0.03$) and treatment × year interaction ($p = 0.009$) were significant for bacterial *amoA* (AOB) abundance. Treatment effect was significant for both AOB ($p = 0.04$) and AOA ($p = 0.04$) in the top 5 cm depth. AOB abundance was higher in PL treated (7.31 log *amoA* per g soil) than control plots (6.78 log *amoA* per g soil) in the 0-15 cm on average. Similarly, AOA was higher in PL treated (6.97 log *amoA* per g soil) than control plots (6.43 log *amoA* per g soil) on average. AOA abundance did not decrease significantly between 2009 and 2013. The lowest AOA abundance was for control plots at 0-5 cm depth in the year 2013. The AOB and AOA abundance in PL and control plots fall within the range previously reported in soils amended with animal wastes and inorganic N fertilizers (Fortuna et al., 2012; Habteselassie et al., 2013; Shen et al., 2008). Average AOB abundance in soils amended without or with dairy slurry at 300 kg N ha^{-1} ranged from 6.56 to 7.38 log copies g $soil^{-1}$ and was higher than the AOA abundance (Fortuna et al., 2012).

The results for AOB abundance in the 0-5 cm were similar to the 0-15 cm in that it was higher in PL treated than control plots. AOA abundance, however, did not show any significant difference between the PL treated and control plots in the top 0-5 cm, suggesting AOB were more responsive than AOA in the layer where the PL is directly applied. This is consistent with the data for bacterial to archaeal *amoA* abundance ratio (AOB/AOA) (Table 2.4). AOB/AOA values greater

than 1 are indicators of the numerical dominance of AOB over AOA. Although these ratios were consistently higher than 1 for all the treatments and years, the ratio is 4 to 5-fold higher in the 0-5 cm than 0-15 cm depth. AOB are more responsive to ammoniacal fertilizer than AOA, which often dominate in soils where the substrate (ammonia) comes from slowly mineralizing organic N (Jung et al., 2011; Martens-Habbena et al., 2009). The organic matter content was not significantly different between the two treatments in this study. Nor was the treatment's N rate. The difference is because PL has organic N forms that can sustain the N supply over a longer period of time unlike the control. Unlike other animal wastes, the bulk of the organic N in PL is easily hydrolysable (Nahm, 2003). This might explain the higher abundance of AOB in PL than control plots.

Fingerprinting AOA and AOB communities

For both H_{AOB} and H_{AOA}, only the effect of year was significant (H_{AOB}, $p = 0.03$ and H_{AOA} $p = 0.004$; Table 2.5). We observed significant increase in H_{AOB} (12%) and H_{AOA} (24%) values at 0-15 cm depth from 2009 to 2013. This shows that the cessation of the treatments resulted in changes in community diversity of AOB and AOA. AISA profile showed that majority of the AOB in PL and control plots were similar to *Nitrosospira spp*. AISA and DGGE profiles of AOB and AOA at 0-5 and 0-15 cm depth for 2009 and 2013 are shown in Figures 2.2a-c and 2.3a&b, respectively. In a similar study by Tomlinson et al (2015), annual application of alum-treated and untreated PL on a Captina silt loam soil for 8 and 9 years showed no significant changes in community composition of AOB using DGGE.

Community structure of AO with high throughput sequencing

We observed significant effect of treatment ($p = 0.036$) and treatment × year ($p = 0.04$) interaction on the AOB community structure. The average number of observed OTU's per sample were higher in PL amended (3000) than CL plots (1500) (Figure 2.4). The qualitative measures

Chao1 and ACE were higher in PL amended than CL plots whereas quantitative measures, Shannon and Simpson were comparable for the two soils. In PL amended plots, Chao1 and ACE were higher in the year 2009 than in 2013 while Shannon and Simpson indices were higher in 2013 than 2009. In CL plots, Chao1 and ACE were higher in 2013 than 2009, whereas Shannon and Simpson indices had overlap in 2009 and 2013. Beta diversity (β) analysis showed distinct AOB communities in 2009 and 2013 in both PL and control plots (Figure 2.5). The impact of year on β-diversity was stronger in control than PL plots, i.e., there was bigger separation (difference) in AOB communities in the control plots between 2009 and 2013 than those in PL plots. Phylogenetic analysis showed that the majority of AOB belonged to cultured and uncultured *Nitrosospira spp* in PL and control plots (data not shown). *Nitrosospira spp* are known to dominate the agricultural soils amended with inorganic fertilizers and animal wastes which could be attributed to their higher substrate affinity for NH_4^+-N (Dharni et al., 2010; Wu et al., 2011; Xia et al., 2011).

Significant effect of treatment ($p = 0.018$) and year ($p = 0.044$) were observed on the AOA community structure. The average number of observed OTU's per sample were higher in CL plots (6000) as compared to PL amended plots (5000) (Fig. 2.6). Chao1 and ACE were higher in PL plots compared to CL plots whereas Shannon and Simpson indices means were comparable for the two soils. In PL plots, α-diversity was higher in 2013 than 2009. In control plots, we observed higher α-diversity in 2009 compared to 2013. β-diversity for AOA showed distinct clusters of AOA communities both by treatment and year (Fig. 2.7). The differences in clustering of AOA communities in PL plots and CL plots was much more pronounced compared to differences in AOB communities for the two soils. A majority of AOA belonged to uncultured *Nitrosopumilus spp* and *Nitrososphaera spp* in PL and control plots. These organisms known to be abundant in

marine and terrestrial environments with low nutrient concentrations (Jung et al., 2011; Martens-Habbena et al., 2009).

PL application for 15 years resulted in higher diversity of AO than the control. This could be attributed to the introduction of unique AO from PL application (Lovanh et al., 2007). It could also be that PL selected certain AO groups with the capability to utilize the N form in PL more efficiently. For the control plots, however, UAN would be limited to selecting fast growing AO that respond to immediate high ammonium availability. Our results are similar to previous studies that reported on the importance of N forms in affecting community composition. Changes in community composition of AOB was caused as a result of ammonium fertilizer in an agricultural silage corn system (Ouyang et al., 2016). Similarly, studies such as (Chu et al., 2007; He et al., 2007b; Wu et al., 2011) have reported changes in AOB and AOA community structure due to long-term N fertilizer and organic manure applications.

We used both simple fingerprinting (DGGE and AISA) and high throughput sequencing techniques to characterize the community structure of AO. The fingerprinting techniques were able to capture changes in community composition by time (year) and not by treatment. The high throughput method captured differences in composition by both year and treatment. The fingerprinting techniques are commonly used as a faster and simpler way of capturing a 'snap-shot' of the communities to determine effects of management practices on the soil microbial communities and provide limited sequence information (Rastogi and Sani, 2011). High throughput sequencing techniques, on the other hand, offer higher depth of sample coverage and can be used for in-depth studies of unknown microbial communities (Roh et al., 2010). Overall, the choice on the use of community fingerprinting or high throughput techniques depends on the research objective and expected results (van Dorst et al., 2014).

Correlation Analysis

We looked at correlations to examine the interdependence of the observed variables in 2009 and 2013 (Table 2.5). During active PL application (2009), we observed significantly positive correlations between AOB abundance and NP, NH_4^+-N, NO_3^--N, soil pH, SOC, CEC, BS and trace metals, while AOA abundance showed smaller but still significant positive correlations with soil pH, SOC, BS, CEC and trace metals. The stronger correlation of AOB abundance with NP and inorganic N contents suggests that AOB most likely played a more important role than AOA in in regards to nitrification in 2009. This suggests that management strategies (e.g., use of nitrification inhibitors) to maximize N efficiency would be more effective if it were to target the AOB (Dai et al., 2013; Di et al., 2010b) during times of PL application. The positive correlation of AO abundance to trace metals suggest that the metals were not at the level that would inhibit the growth of the organisms. In 2013, two years after PL application stopped, significant correlations of AOB abundance were observed with only NO_3^--N content, SOC and CEC. The lack of correlation with NP indicates that discontinuation of PL application had significant impact on AOB activity. Conversely, in 2013, AOA abundance was significantly correlated with all the observed variables except CEC. This suggests that AOA's role in nitrification increased with the cessation of the treatments.

Various researchers have attempted to study the links between the AO community structure and function in response to N in the form of inorganic fertilizers or animal wastes in agricultural soils (Mahmood and Prosser, 2006; Ouyang et al., 2016; Prosser and Nicol, 2012; Webster et al., 2005). In our study, we observed a potential link between AO community structure and function and that the change in the function (NP) of AO seemed to have been caused both by the change in abundance and community composition. PL plots had higher NP, AO abundance and diversity

than control plots. Cessation of PL application for two years was followed by lower NP, AO abundance and diversity.

Conclusions

The long term application of PL changed the abundance, function and community composition of AO. PL application resulted in greater function and abundance of AO, as compared to CL plots with UAN despite the significant accumulation of some trace metals. PL also resulted in greater diversity in AO communities than UAN. After two years of stopping PL application, AOA abundance did not change significantly while AOB abundance increased significantly, indicating the selective residual impact of PL. Stopping PL application also resulted in lower AO function and diversity. Correlation analysis suggested that AOB were functionally more important than AOA in regard to nitrification and that management practices aimed at improving the efficiency of N use should focus on them in pasture lands where PL is applied.

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Tables and Figures

Table 2.1: Soil pH, cation exchange capacity (CEC), percent base saturation (BS) and soil organic carbon (SOC) contents for poultry litter (PL)-amended and control plots in 0-15 cm depth in 2009 and 2013. Values with same letter suffix are not significantly different at P = 0.05. Comparison is valid only between treatments or years. All reported values have been averaged over three replicates.

Parameters	Treatment		Year	
	PL	Control*	2009	2013
pH	6.08^{a}	5.64^{b}	6.01^{a}	5.71^{a}
Cation Exchange Capacity (meq 100 g^{-1} soil)	10.18^{a}	8.88^{b}	8.39^{a}	10.66^{a}
Base Saturation (%)	78.4^{a}	63.5^{b}	74.09^{a}	67.76^{b}
Soil Organic Carbon (g kg^{-1} soil)	1.24^{a}	1.02^{a}	0.97^{a}	1.29^{a}

*received equivalent amount of available N as in PL treatment in the form of UAN (urea ammonium nitrate)

Table 2.2: Mehlich-1 extractable phosphorous and trace metals in poultry litter-amended (PL) and control plots in the top 15 cm depth. Values with same letter superscripts are not significantly different at $P \leq 0.05$. Comparison is valid only between treatments or year. All reported values have been averaged over three replicates.

Parameters (mg kg^{-1} soil)	Treatment		Year	
	PL	Control*	2009	2013
P	115.8^{a}	8.8^{b}	59.5^{a}	65.1^{a}
Zn	10.6^{a}	2.4^{b}	5.3^{a}	7.8^{a}
Cu	6.4^{a}	1.5^{b}	4.1^{a}	4.0^{a}
As	2.4^{a}	1.5^{a}	1.9^{a}	2.0^{a}
Cr	0.23^{a}	0.14^{a}	0.19^{a}	0.18^{a}
Pb	0.74^{a}	0.56^{b}	0.69^{a}	0.61^{a}
Mn	35.0^{a}	31.3^{a}	38.8^{a}	27.5^{a}
Mo	0.25^{a}	0.13^{b}	0.23^{a}	0.15^{a}
Cd	0.05^{a}	0.03^{a}	0.04^{a}	0.03^{a}

*received equivalent amount of available N as in PL treatment in the form of UAN (Urea ammonium nitrate)

Table 2.3: Nitrification potential and inorganic N contents in poultry litter-amended (PL) and control plots in the top 15 cm depth. Values with same letter superscripts are not significantly different at P ≤0.05. Comparison is valid only between treatments or years. All reported values have been averaged over three replicates.

Parameters	Treatment		Year	
	PL	Control*	2009	2013
Nitrification Potential (mg kg soil^{-1} hr^{-1})	0.56^{a}	0.31^{b}	0.64^{a}	0.23^{b}
NH_4^+-N (mg kg soil^{-1})	9.9^{a}	13.5^{b}	13.0^{a}	10.5^{b}
NO_3^--N (mg kg soil^{-1})	20.0^{a}	17.8^{a}	6.1^{a}	31.6^{b}

*received equivalent amount of available N as in PL treatment in the form of UAN (Urea ammonium nitrate)

Table 2.4: Abundance of *amoA* and Shannon index of general diversity (H) for ammonia oxidizing bacteria (AOB) and archaea (AOA) in poultry litter-amended (PL) and control (CL) plots in the top 5 and 15 cm depths. H value is based on the *amoC-amoA* intergenic spacer analysis (AISA) for AOB and denaturing gradient gel electrophoresis (DGGE) for AOA. Values with same letter suffix are not significantly different at P ≤0.05. Comparison is valid only between treatments or years. All reported values have been averaged over three replicates.

Parameters		Treatment		Year	
	Depth	PL	Control*	2009	2013
Bacterial amoA (log copies/ g soil)	0-5 cm	8.03^{a}	7.7^{b}	NA	NA
	0-15 cm	7.31^{a}	6.78^{b}	6.69^{A}	7.4^{B}
Archaeal amoA (log copies/ g soil)	0-5 cm	7.02^{a}	6.15^{b}	NA	NA
	0-15 cm	6.97^{a}	6.43^{b}	6.79^{A}	6.61^{A}
Ratio of AOB to AOA	0-5 cm	10.23	8.31	NA	NA
	0-15 cm	2.18	2.23	0.79	6.16
H value (AISA for AOB)	0-5 cm	3.53^{a}	3.49^{a}	NA	NA
	0-15 cm	3.27^{a}	3.26^{a}	3.12^{A}	3.51^{B}
H value (DGGE for AOA)	0-5 cm	2.21^{a}	2.27^{a}	NA	NA
	0-15 cm	1.90^{a}	2.15^{b}	1.81^{A}	2.25^{B}

*received equivalent amount of available N as in PL treatment in the form of UAN (Urea ammonium nitrate). NA= not determined

Table 2.5: Spearman rank correlations (ρ) between log copy numbers of *amoA* for ammonia oxidizing bacteria (AOB) and archaea (AOA) and soil properties in 2009 and 2013.

Parameter	AOB		AOA	
	2009	2013	2009	2013
Nitrification Potential	0.63	ns†	ns	0.88
NH_4^+-N	0.75	ns	ns	-0.89
NO_3^--N	0.49	0.76	ns	0.76
pH	0.90	ns	0.66	0.91
Soil Organic Carbon	0.89	0.55	0.60	0.81
Base Saturation	0.89	ns	0.66	0.94
Cation Exchange Capacity	0.79	0.69	0.60	ns
Trace Metals*	0.84	ns	0.63	0.92

†ns=non-significant
*Trace metals include As, Cd, Pb, Zn, Cr, Mn, Mo, Fe and Ni

Figure 2.1: Long-term poultry litter (PL)-amended and control (CL) plots in Eatonton, GA. The figure shows an aerial view of the plots from where the soil samples were collected. Numbers from 1 to 6 and from 7 to 10 represent PL-amended and control plots, respectively.

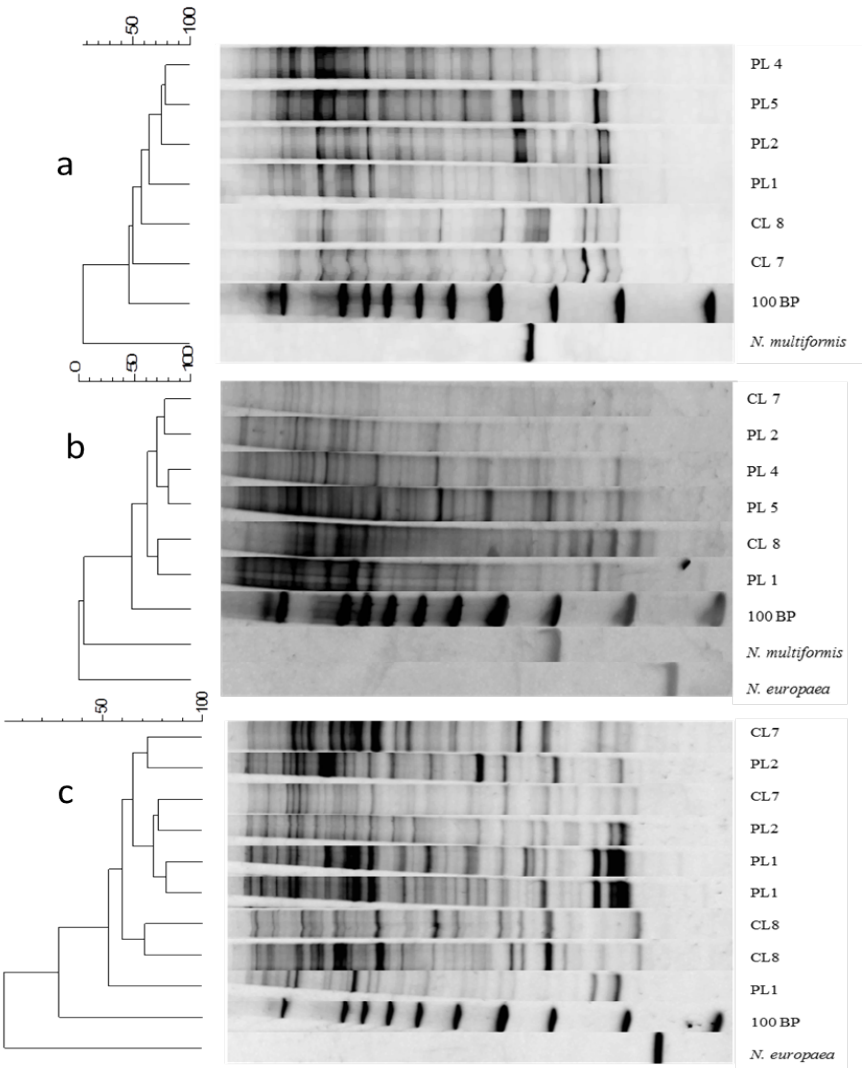

Figure 2.2: Cluster analysis of AOB *amoC-amoA* intergenic region in poultry litter (PL) amended and control (CL) plots at (a) 0-5 cm (b) 0-15 cm depths in 2013 and (c) at 0-15 cm in 2009. Genomic DNA of *Nitrosomonas europaea* 19718 and *Nitrosospira multiformis* 25196 used as positive controls with *amoC-amoA* amplicon sizes of 277 and 446 bp, respectively.

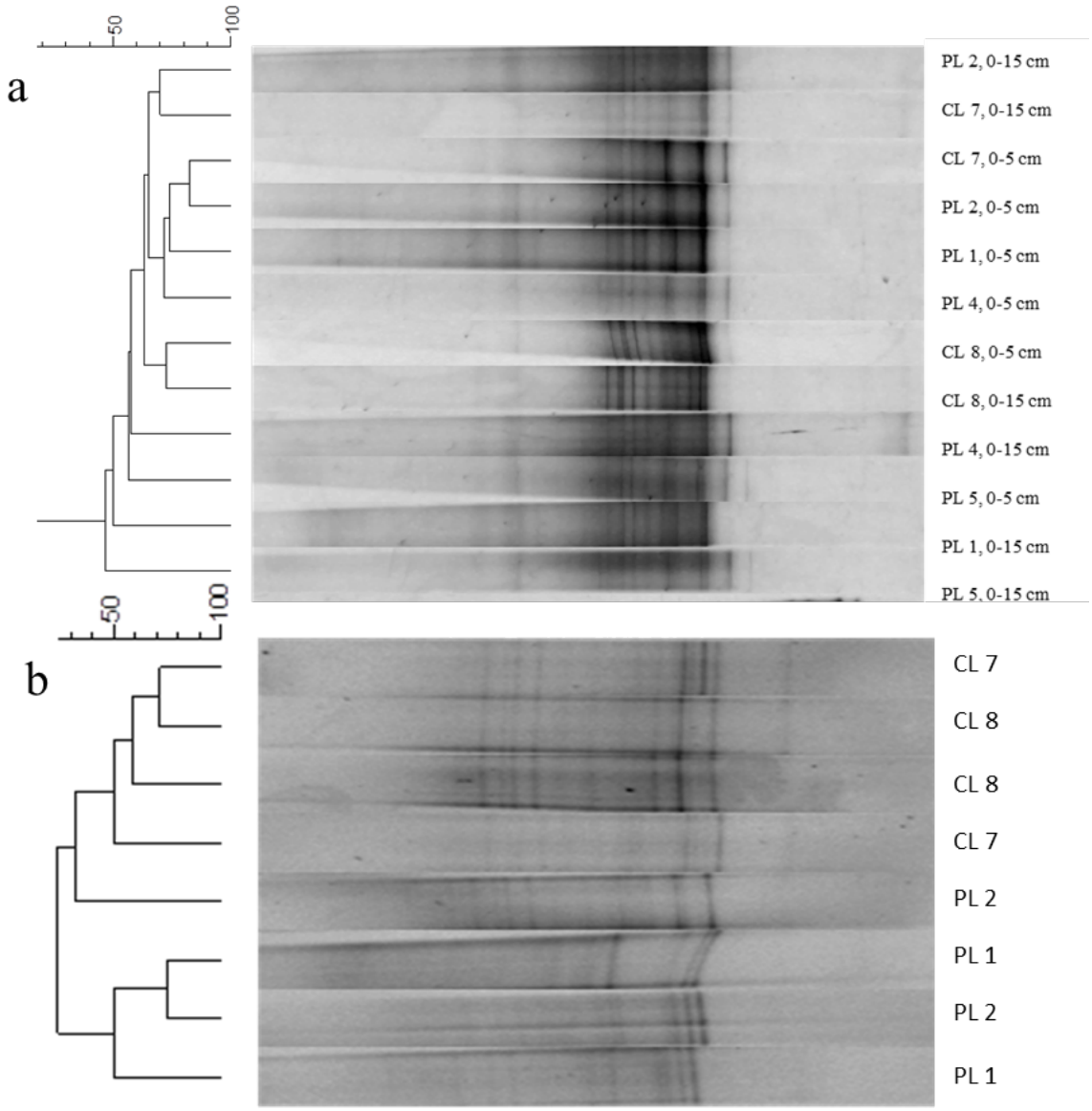

Figure 2.3: Cluster analysis of denaturing gradient gel electrophoresis (DGGE) profile of ammonia oxidizing archaea (AOA) in the poultry litter-amended (PL) and control (CL) plots for (a) 0-5 and 0-15 cm depths in 2013, and (b) 0-15 cm depth in 2009. Poultry litter-amended plots are represented by PL1 to PL5 and controls by CL7 and CL8.

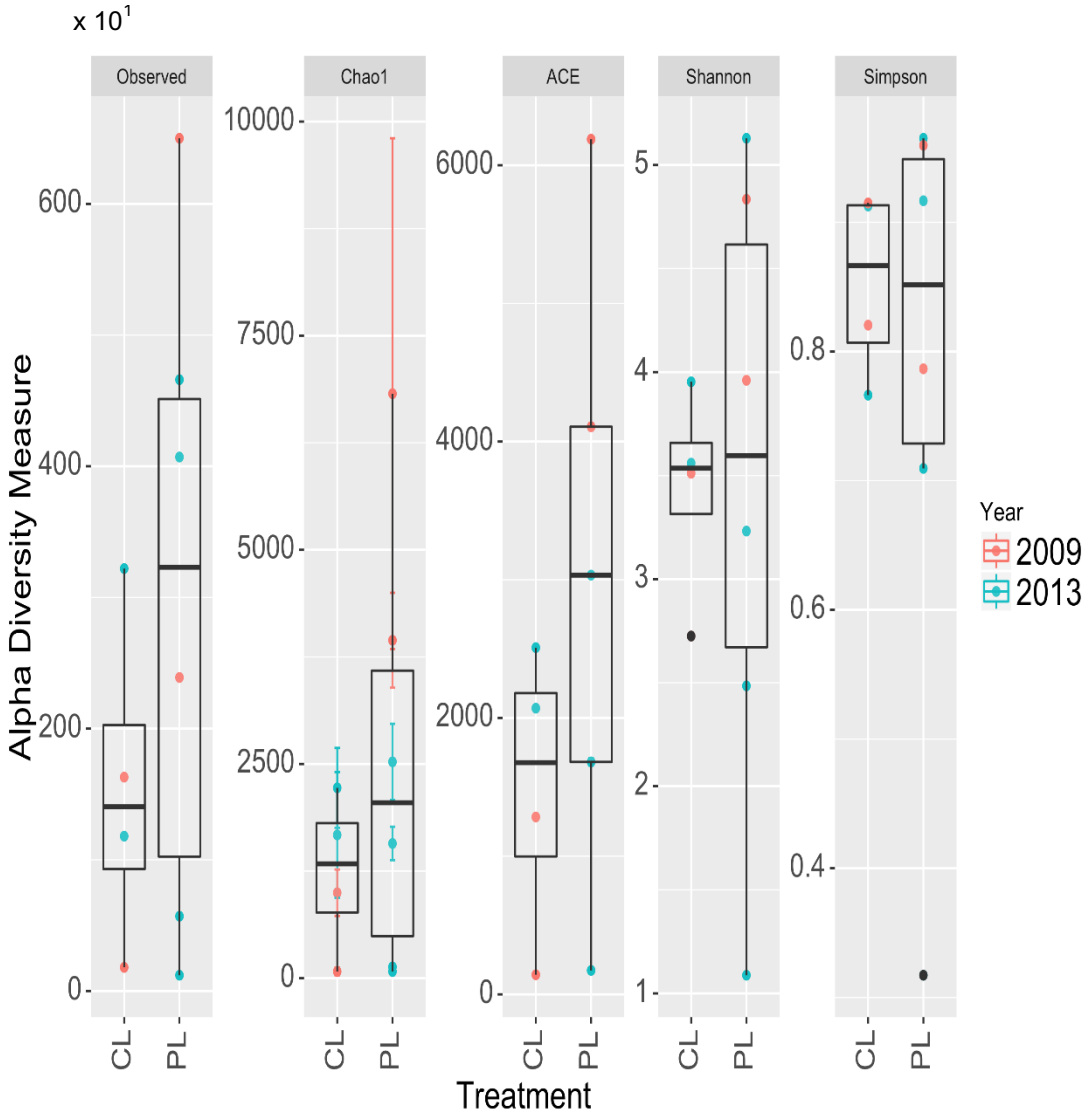

Figure 2.4: Alpha diversity indices for ammonia oxidizing bacteria (AOB) in the poultry litter-amended (PL) and control (CL) plots at 0-15 cm depth in 2009 and 2013.

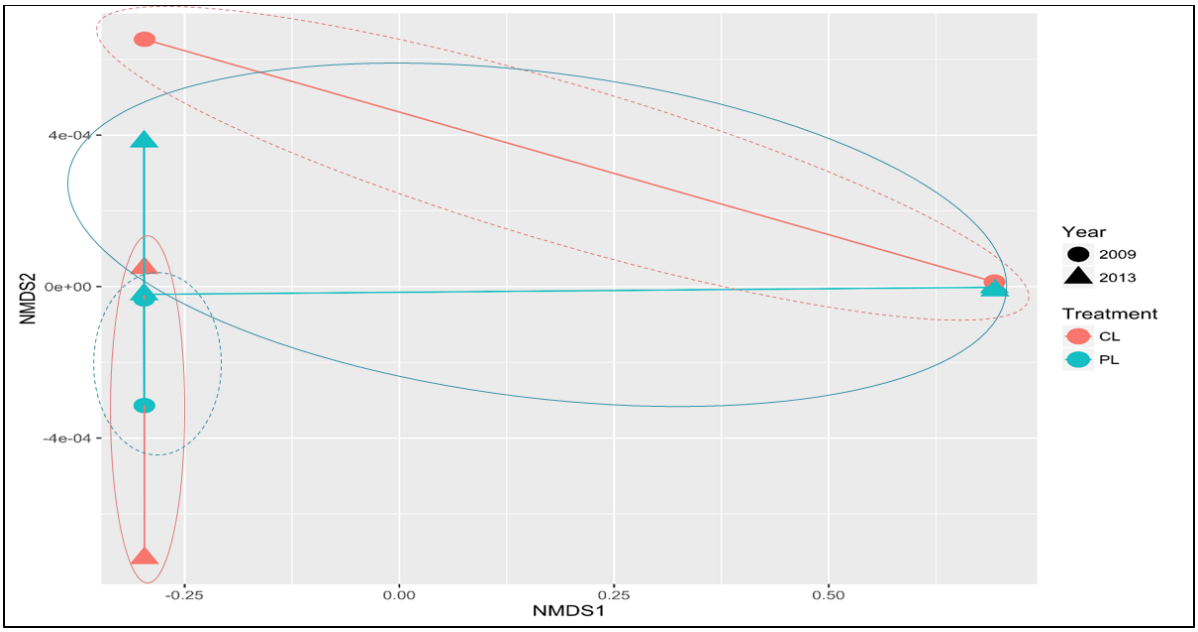

Figure 2.5: Nonmetric multidimensional scaling plots for Beta diversity in ammonia oxidizing bacteria (AOB) in the poultry litter (PL) amended and control (CL) plots at 0-15 cm depth in 2009 and 2013.

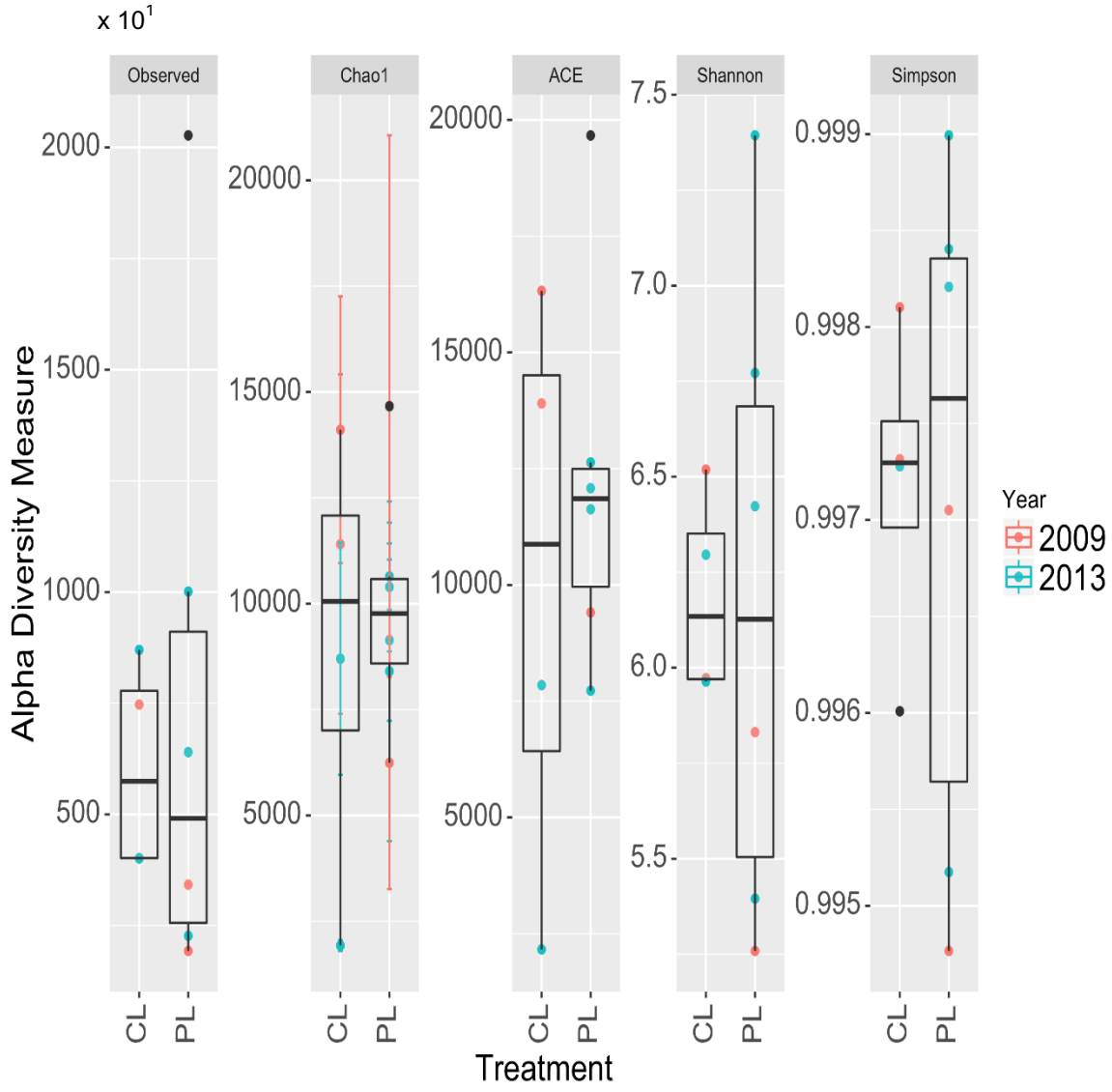

Figure 2.6: Alpha diversity indices for ammonia oxidizing archaea (AOA) in the poultry litter (PL) amended and control (CL) plots at 0-15 cm depth in 2009 and 2013.

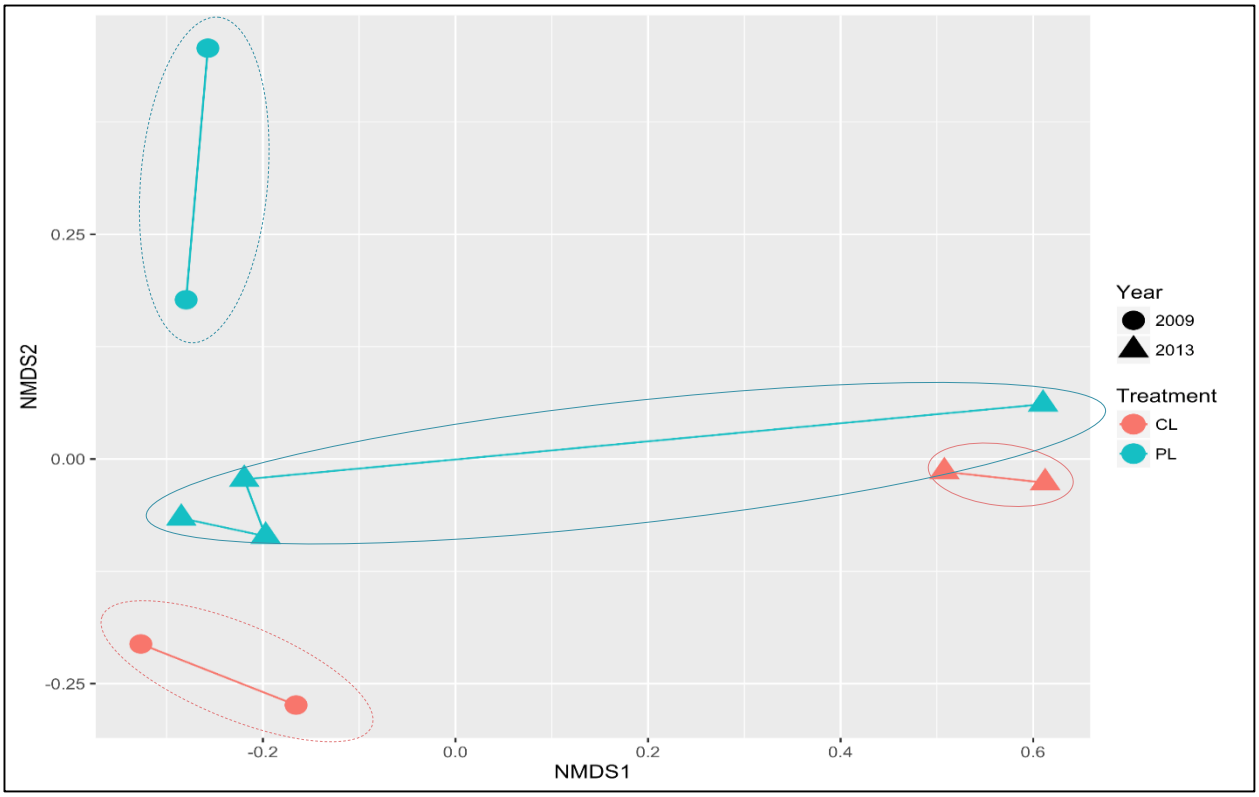

Figure 2.7: Nonmetric multidimensional scaling plots for Beta diversity in ammonia oxidizing archaea in the poultry litter (PL) amended and control (CL) plots at 0-15 cm depth in 2009 and 2013.

CHAPTER 3

ASSESSING THE DIFFERENTIAL CONTRIBUTIONS OF AMMONIA-OXIDIZING BACTERIA AND ARCHAEA TO NITRIFICATION IN A PASTURE SOIL WITH A HISTORY OF POULTRY LITTER APPLICATION

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Abstract

Ammonia oxidizing bacteria (AOB) and archaea (AOA) play an important role in nitrogen cycling in poultry litter (PL) amended soils. However, the relative contribution of AOB and AOA is not clearly understood. We conducted a microcosm study to determine the role of AOB and AOA in pasture soils with a history of PL application. Soil samples were collected at 0-15 cm from PL amended and CL plots (no N added) in Eatonton, Georgia and were amended with dicyandiamide to separate the contributions of AOB and AOA at two ammonium levels in an incubation study that lasted 63 days. The abundance and community composition of ammonia oxidizers (AO) were determined using molecular techniques that targeted the *amoA* gene. Nitrification potential (NP) and net nitrification measurements were used to characterize their function. AOA dominated nitrification in both treatment, accounting for ≥ 70% of NP early on. Over time, the AOB contribution to NP grew in PL soil accounting for ≥ 50% at high ammonium level. AOA remained to be the more dominant players in unamended CL soil. The trend was similar with net nitrification. At the end of study, AOB abundance was 76- and 63-fold higher in PL than unamended CL soil at low and high ammonium levels, respectively. AOA abundance was 42% and 7% higher in unamended CL than PL soil at low and high ammonium levels. Differences in alpha (α) and beta (β) diversities of AO between PL and unamended CL soils indicated that the difference in nitrification was caused by changes in both abundance and community composition of AO. The differing roles of AOB and AOA in these two treatments, therefore, suggest the need for different approaches that target the right group of AO to achieve efficient N use.

Introduction

Ammonia oxidizing bacteria (AOB) and archaea (AOA) play a key role in nitrogen (N) cycling by mediating the first rate-limiting step of nitrification that results in production of nitrate and nitrous oxides, two forms of N that are of agronomic and environmental concerns (Arp and Bottomley, 2006; Leininger et al., 2006; Martens-Habbena et al., 2009). It has been a common practice to use chemical inhibitors that target these organisms to slow down the process of nitrification to minimize losses of N from soils (Di et al., 2009; Kleineidam et al., 2011). Such a strategy is only effective if the relative contributions of AOB and AOA to nitrification are well understood so that inhibitors are selected to target the right group. Existing inhibitors commonly target AOB (Irigoyen et al., 2003; Zacherl and Amberger, 1990), which were originally thought to be the only group responsible for mediating the first step of nitrification.

The role of AOA was discovered recently (Könneke et al., 2005; Treusch et al., 2005) and as such they are not as well studied as AOB. Information in this area is particularly lacking in the southeastern United States where the poultry industry produces a large amount of poultry litter (PL) (a mixture of excreta, bedding material feathers and waste feed) that is commonly land applied (Edwards and Daniel, 1992; Kelleher et al., 2002). PL is rich in ammonia, in addition to easily mineralizable organic N forms, that are quickly nitrified. Therefore, proper understanding of the role of AOB and AOA in PL amended soils is needed for sound management of N and improving the N use efficiency in such soils.

There are several studies that examined the differential contributions of AOB and AOA to nitrification in soils (Dai et al., 2013; Offre et al., 2009; Zhang et al., 2012). A commonly used nitrification inhibitor is dicyandiamide (DCD), which has a bacteriostatic effect and hence works by blocking the active site of the ammonia monooxygenase enzyme in AOB (Di et al., 2010b;

Edmeades, 2004). Nitrification assays with and without DCD are used to estimate the relative contributions of AOA and AOB (Di et al., 2010a; Taylor et al., 2013). In a laboratory incubation study using DCD, Di et al (2010a) reported significant relationship between nitrification rate and AOB abundance in soils amended with urine-N substrate. Similarly, Dai et al (2013) in a field study reported AOB to be functionally more important than AOA in pasture soils amended with urea and urine-N substrate and DCD. In fallow agricultural soils with varying clay mineralogy and amended with dairy slurry at 300 kg ha^{-1}, AOB were found to be the dominant players in nitrification and AOB abundance had positive correlations with nitrification potential (Fortuna et al., 2012). Ouyang et al (2016) reported that AOB to be the major contributor to nitrification in soils amended with ammonium sulfate and steer waste compost. The results from these studies have shown that that the relative contributions of AOA and AOB to nitrification are dependent on study and site specific soil conditions that are hard to extrapolate.

The relative contribution of AOB and AOA in pasture soils amended with PL is, however, not yet explored. Studies by Dharni et al (2010) and Tomlinson et al (2015) examined the effect of PL/chicken manure addition on soil AOB only but the role of AOA in nitrification in such soils was not considered. Therefore, the main goal of this study was to evaluate the relative contributions of AOB and AOA to soil nitrification in pasture soils with a history of PL application by conducting a microcosm study. The study also examined the changes in the abundance and community composition of AO in the PL receiving soil as compared to a CL soil that received no sources of N. Measure of the differential contribution of AOB and AOA in nitrification and study of the links between abundance and community composition will help to effectively manage the losses of N in grasslands in Georgia.

Materials and Methods

Soil samples and experimental set-up

Soil samples were collected from long term PL amended plots at Central Georgia Research and Education Center near Eatonton, Georgia (Ashjaei et al., 2011) in August 2014. PL was applied in the plots at 8.3 Mg ha^{-1} from 1995 to 2011. Soil samples were also collected from a nearby plot with no history of inorganic N fertilizer or PL application and served as the control (CL) soil. Samples were taken at 0-15 cm depth with 5-10 sub-samples mixed to form a representative sample.

The incubation study was designed with two treatment (PL and CL), two ammonium levels (Low and High), two DCD levels (0 and 12 mg kg^{-1} soil) and four sampling times (0, 7, 28 and 63) as experimental variables. The PL and unamended CL soils were amended with ammonium chloride at 0 mg NH_4^+-N kg^{-1} soil (Low, served as control) and 100 mg NH_4^+-N kg^{-1} soil (High). In both low and high ammonium amended soils, DCD was applied at 0 mg kg^{-1} soil (DCD 0, served as control) and 12 mg kg^{-1} soil (DCD 12). Ammonium chloride and DCD were thoroughly mixed with 1 kg soil in a Ziploc bag and moisture was adjusted to 30% water holding capacity. DCD was applied to soils at the start and a second application of 6 mg kg^{-1} soil was done at day 45. Studies by (Chaves et al., 2006; Vallejo et al., 2006) had shown that DCD persists in soil for 30-49 days with 45% efficiency at day 49, so a second application at half the initial rate was done. Soil was then divided in three independent replicates of 50 gram each and placed in an incubator at 26ºC. Sub-samples were taken at day 7, 28, and 63 and analyzed immediately for NH_4^+-N and NO_3^--N content and NP. A small portion of the soil sample was stored at -80ºC until soil DNA extraction.

Basic soil properties, nitrification potential, net N mineralization and net nitrification

The soil samples were analyzed for basic properties such as soil pH, soil organic carbon, texture, NH_4^+-N and NO_3^--N content. Soil pH was measured in 1:5 soil: water ratio using a benchtop pH meter (Orion 3 Star, ThermoFischer Scientific, Waltham, M.A). Soil organic carbon content of PL and CL was determined using the potassium dichromate oxidation method (Walkley and Black, 1934). Gravimetric moisture content of the soil was measured by drying ten gram soil at 102°C for 24 hr in an oven (Gardner and Klute, 1986). Soil texture was determined using the particle size distribution method (Bouyoucos, 1962). Nitrification potential of the soils was measured using the shaken soil slurry method by (Hart et al., 1994). Fifteen gram of moist sieved soil (2 mm mesh) was placed in 250 milliliter Erlenmeyer flask. One hundred milliliter of combined solution of potassium monobasic phosphate (KH_2PO_4), potassium dibasic phosphate (K_2HPO_4) and ammonium sulfate were added to supply 1.5 mM NH_4^+-N and 1mM PO_4^{-3} (pH 7.2). The flasks were placed in an orbital shaker at 180-200 rpm for 24 hr. Ten milliliter aliquots were taken at 2, 4, 22 and 24 hr time interval using an auto pipette and centrifuged at 8000 × g for 8-10 minutes. The aliquots were then filtered using Whatman No. 42 filter paper and kept frozen at -20ºC. The filtrates were analyzed for (NO_2^-+NO_3^-)-N using Auto-analyzer AA1 (Seal Analytical Inc., Mequon, W.I). The slope of the linear regression of (NO_2^-+NO_3^-)-N concentrations provides the value of nitrification potential of soil samples.

The NH_4^+-N and NO_3^--N content of the samples was determined by extraction with 2M KCl. Five gram of soil and 25 milliliter of 2M KCl was added in a 250 milliliter Erlenmeyer flask and put in a shaker at 150-200 rpm for 1hr. The extracts were then filtered using Whatman No. 42 filter paper and stored at -20°C until analyzed. NH_4^+-N and NO_3^--N content of the extracts was analyzed using Auto-analyzer AA1 (Seal Analytical Inc., Mequon, W.I). The net N mineralization

and nitrification were calculated as the changes in soil inorganic-N pool over the duration of incubation (Hart et al., 1994).

Net N mineralization = $[(NH_4^+ + (NO_2^- + NO_3^-)\text{-N}]_{\text{day 63}} - [(NH_4^+ + (NO_2^- + NO_3^-)\text{-N}]_{\text{day 0}}$

Net nitrification = $(NO_2^- + NO_3^-)\text{-N})_{\text{day 63}} - ((NO_2^- + NO_3^-)\text{-N})_{\text{day 0}}$

Abundance of bacterial and archaeal *amoA*

Soil DNA was extracted from 0.25 gram fresh soil using Power Soil™ DNA extraction kit (MO BIO Laboratories, Carlsbad, C.A). The abundance of AO was quantified by targeting the *amoA* gene using amoA189F & 2R (Okano et al., 2004) and ArchamoAF & AR primer pair (Francis et al., 2005) for AOB and AOA respectively. The primer sequences were *amo*A-189F (5′-GGHGACTGGGAYTTCTGG-3′) and *amo*A-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′); ArchamoAF(5'TTATGGTCTGGCTTAGACG3');ArchamoAR(5'GCGGCCATCCATCTGTATGT3'). Quantitative polymerase chain reaction (qPCR) was performed in 20 µl reaction volume with 10 µl of 2X SYBR Select Master Mix (Applied Biosystems by Life Technologies, Foster city, C.A), 0.4 µl of primers Eurofins Genomics (Eurofins MWG Operon LLC, Lousiville, KY) at 100 nmol final concentration, 6 µl of nuclease free water and 2 µl of 1:10 diluted soil DNA in StepOne Plus™ thermocycler (Applied Biosystems by Life Technologies, Foster city, C.A).

The standards for AOB were prepared using linearized plasmid DNA of *Nitrosomonas europaea* 19718. For AOA, the conventional PCR product of the soil DNA was used as the template for the ligation reaction. The PCR product was cloned with pGEM®-T Easy Vector System (Promega, Madison, W.I) and transformed using high efficiency *Escherichia coli* competent cells. Plasmid DNA was extracted with PureYield™ Plasmid Miniprep System (Promega, Madison, W.I) and the concentration (nanograms per microliter) was measured using

Quantifluor dsDNA system (Promega) with a TBS-380 Fluorometer (Turner BioSystems, Sunnyvale, CA). Serial dilutions (1:10) of the plasmid DNA with *amoA* gene copy numbers ranging from 3×10^3- 3×10^7 were used to prepare the standard curve. For AOB, the cycling conditions were modified to 10 min at 95 ˚C, 40 cycles of 45 sec at 95 ˚C, 1 min at 60 ˚C, 4 min at 72 ˚C followed by a melt curve 15 sec at 95°C, 1 min at 60°C, and 15 sec 95°C (Okano et al., 2004). The qPCR conditions for AOA were modified to 95°C for 10 min and 40 cycles of 95°C for 35 sec, 56°C for 45 sec and 72°C for 3 min (Sher et al., 2012). Melt curve analysis was performed to determine the PCR product specificity. The qPCR reaction efficiencies for AOB and AOA varied from 83 to 92 % with r^2 from 0.93 to 0.97.

AOB and AOA community composition with high throughput sequencing

Bacterial and Archaeal *amoA* libraries were prepared for paired-end sequencing using Illumina TruSeq fusion primers with custom tags (amoA1F and 2R for AOB and ArchamoAF and AR for AOA) as described in (Faircloth and Glenn, 2012) for PL and CL soil samples collected at day 7 and 63. All PCR reactions were carried out in 25 µl final volume with 12.5 µl of Hot Start GoTaq Master Mix (Promega Corporation, Madison, W.I), 6.5 µl of nuclease free PCR water, 2 µl of i-Tru forward and reverse primers and 2 µl of DNA template in a Bio-Rad MyCycler thermocycler (Bio-Rad Laboratories Inc., Hercules, C.A). Thermal conditions for AOB were: initial denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 50 sec; 60ºC for 45 sec; 72ºC for 45 sec and a final denaturation at 72ºC for 10 min. For AOA the conditions were: initial denaturation at 95ºC for 5 min, 35 cycles of 95ºC for 35 sec; 60ºC for 35 sec; 72ºC for 40 sec and a final denaturation at 72ºC for 10 min. The PCR product specificity was checked via agarose gel (1.5%) and cleaned using Wizard® SV Gel and PCR clean up system (Promega Corporation, Madison, W.I). The cleaned PCR products were then pooled in equimolar concentration and submitted to

UGA's Georgia Genomics Facility for sequencing with a MiSeq 300 sequencer (Illumina Inc., San Diego, C.A). Customized fusion primers used for the Illumina sequencing were obtained from Eurofins Genomics (Eurofins MWG Operon LLC, Lousiville, KY).

To process raw sequence data generated from paired end Illumina sequencing, we used QIIME version 1.9.1 (Caporaso et al., 2010b). We used PANDAseq (Masella et al., 2012) to assemble paired-end reads. Demultiplexed sequences were trimmed for quality and clustered into operational taxonomic units (OTU's) based on 90% (for AOB) and 60% (for AOA) sequence identity (Masella et al., 2012; Norton et al., 2002). Operational taxonomic unit (OTU) picking was performed using an 'open-reference' OTU picking protocol, where sequences are clustered against existing sequences in NCBI GenBank database (Benson et al., 2005). For AOB, the sequence length was 495 bp and for AOA sequence length was 635 bp. The reference sequences from GenBank for both AOA and AOB were clustered and aligned using the MUSCLE v 3.8.31 program (Edgar, 2004). Reads that matched a GenBank reference sequence at 97% for AOB and 90% for AOA, identity were clustered to an OTU defined by that reference sequence. Reads that did not match a reference sequence were subsequently clustered *de novo*. The centroid sequence in each cluster was chosen to represent the cluster and aligned with the corresponding GenBank sequence set using PyNAST version 1.2.2 (Caporaso et al., 2010a). Reads that failed to align with PyNAST were excluded from subsequent analyses. Phylogenetic trees were constructed using FastTree (Price et al., 2010) and taxonomic assignments were made for each representative sequences using the uclust version 1.2.22 (Edgar, 2010) consensus taxonomy assigner.

Further analysis and plotting of sequencing data was performed using the PhyloSeq package from Bioconductor version 3.3 (McMurdie and Holmes, 2013) library in R version 3.2. We evaluated six α-diversity indices, including the abundance based coverage estimators (ACE),

Chao1, number of observed OTU's, Shannon index, Simpson's index and the inverse Simpson's index (Magurran, 2013; Rosenzweig, 1995). ACE and Chao1 measure the OTU richness whereas Shannon and Simpson indices measure the OTU diversity (Gotelli et al., 2013). We also compared the different samples in terms of Beta (β) diversity obtained using non-metric multidimensional scaling (NMDS) (Fasham, 1977) with the phylogenetic-based UniFrac distances. The final OTU table had 1000 sequences per sample for AOB and 6500 sequences per sample for AOA (also known as depth of coverage), which represents a significant depth of coverage (Meyerson et al., 2010).

Statistical Analysis

We performed repeated measures analysis of variance (ANOVA) to determine the effects of treatment on AOB and AOA abundance, net N mineralization, net nitrification and nitrification potential at different ammonia concentrations over time using the *afex* package in R programming language version 3.2 (Maxwell and Delaney, 2004). The bacterial and archaeal *amoA* gene copies were log transformed before the statistical analysis to meet the normality assumption for ANOVA. In the split-plot design of experimental setup, day/time variable was used as within-variable and all other independent experimental variables (treatment and ammonium level) as between-variables. Separate ANOVA were performed for each of the observables (eg. AOB abundance, AOA abundance, nitrification potential, net mineralization and net nitrification). Relevant plots were made using the *ggplot2* package in R (Wickham, 2009). A correlation analysis among different experimental observables were also performed using the *Hmisc* package of R (Press et al., 1988). All analyses were done at a significance value of $p \leq 0.05$. To analyze the effects of year and treatment on the community structure of AOB and AOA, we performed a multivariate ANOVA (MANOVA) on normalized fraction of OTU's from different genera.

Results and Discussion

The basic soil properties measured at the start of the experiment such as soil pH, soil organic carbon and NO_3^--N content were higher in PL amended compared to unamended CL soil. The NH_4^+-N content on the contrary, was higher in CL than PL soil. Soil texture for PL amended soil was sandy loam and sandy clay loam for the CL soil.

Nitrification potential, net nitrification and net N mineralization

Treatment, time, treatment × ammonium and treatment × time interaction all had a significant effect on the soil nitrification potential (NP) (Table 3.2). At day 7, PL soil had 146% and 141% higher NP than the unamended CL soil at low and high ammonium level, respectively (Table 3.3). In PL soil, NP was significantly higher (~11%) at high than low ammonium level. The relative contribution of AOA to NP in PL soil was ~70% at the two ammonium levels. At the end of the study (at day 63), PL soil showed 2-fold higher NP than the unamended CL soil at both ammonium levels. The PL soil at the high ammonium level had ~11% higher NP than at the low ammonium level. No significant effect of ammonium level on NP was observed in the unamended CL soil. At day 63, AOA contribution to NP was about ~50% in PL soil and ~70% contribution in unamended CL soil at the two ammonium levels (Table 3.4). During the incubation, we observed a consistent and significant increase in NP in the PL soil, with a corresponding increase in the contribution of AOB in nitrification.

During the early phase of the study, AOA were the dominant players accounting for more than 70% of the NP in both soil types. AOA are known to have high substrate affinity for ammonia due to smaller size and greater surface to volume ratio than AOB (Martens-Habbena et al., 2009), which makes them more competitive under substrate-limiting conditions where ammonium is released via mineralization. Since AO are slow growers, the impact of ammonium addition was

not evident on day 7. Over time, the contribution of AOA to nitrification decreased, especially in the PL soil. This suggests that AOB were able to respond to the increased availability of ammonium with increased activity and growth. This pattern was seen in both soils but it was more pronounced in PL soil where ammonium came from both fertilizer and organic N that is capable of sustaining the activity. Wessén et al (2010) had reported similar findings in which AOA were functionally dominant over AOB under ammonium-limiting field conditions, with AOB responding to ammonium addition over time.

We observed significant effects of treatment, and treatment × ammonium interaction on the net N nitrification (Table 3.2). We observed 7-fold higher net nitrification in PL soil at either ammonium levels as compared to unamended CL soil (Table 3.4). No significant effect of ammonium level was observed on net nitrification in unamended CL soil. In PL soil, high ammonium level had 30% significantly higher net nitrification than low ammonium level. The AOA contribution to net nitrification in PL soil was 67% and 45% at low and high ammonium levels, respectively, whereas the unamended CL soil exhibited a higher AOA contribution (~72%) to net nitrification. This is consistent with the NP results.

Significant effects of treatment, ammonium and their interactions were observed on the net N mineralized (Table 3.2). The net N mineralization in PL soil was 4-fold and 5-fold higher than the unamended CL soil at low and high ammonium levels, respectively (Table 3.4). In PL, significantly higher (2-fold) net N mineralization was measured at the high than low ammonium level. In unamended CL soil, 1.7-fold higher net N mineralization was measured in the high than low ammonium level.

Net N mineralization was measured to assess the potential of the soil to supply ammonium to AO via N mineralization thereby affecting nitrification. The fact that it was higher in PL than

unamended CL soil was not surprising as PL had higher soil organic matter content to start with (Table 3.1). The addition of ammonium might have also promoted the growth of heterotrophs that catalyzed N mineralization (Watts et al., 2010). This could be the reason for increased N mineralization in both soils with addition of ammonium. Our finds are similar to, Watts et al (2010) in which soil that received long-term application of PL (> 10 years) had higher rates of N mineralization compared to treatment receiving inorganic fertilizer.

Abundance of bacterial and archaeal *amoA*

We observed significant effects of treatment, time, treatment × ammonium and treatment × time interaction on the bacterial *amoA* (AOB) abundance (Table 3.2). The log *amoA* copies g soil^{-1} were used as proxy for the AOB and AOA abundance. Each cell of AOB contains 2-3 copies of *amoA* gene whereas the copies of *amoA* gene in AOA is still unknown (Norton et al., 2002). At day 7, the AOB abundance was significantly higher (~14 fold) in PL than unamended CL soil at both low and high ammonium levels (Table 3.5). However, we did not observe any significant effect of ammonium level on AOB abundance in either PL or unamended CL soil. At the end of the study, AOB abundance in PL was significantly higher than unamended CL soil by 76 fold at low ammonium level and by 63-fold at high ammonium level. In unamended CL soil, the AOB abundance decreased by 48% and 50% at low and high ammonium level with DCD application. With the progress of incubation, in PL soils, abundance of AOB significantly increased consistently, by 1.2-fold from day 7 to day 28 and 3 fold from day 28 to day 63. In contrast, the abundance of AOB in CL soils showed <1 fold differences with the progress of the incubation.

Significant effects of the treatment × time, treatment × ammonium, and treatment × ammonium × time interaction were observed on the archaeal *amoA* (AOA) abundance (Table 3.2). At day 7, the AOA abundance were 17% and 41% higher in PL than the unamended CL at low

and high ammonium level, respectively (Table 3.6). In both PL and unamended CL soil, addition of ammonium did not result in significant change in the AOA abundance. At the end of the study, AOA abundance in PL was 42% and 7% lower at low (log 6.79 copies g $soil^{-1}$) and high (log 7.03 copies g $soil^{-1}$) than in unamended CL soil. With the progress of incubation, in PL soils, abundance of AOA significantly decreased consistently, by 25% from day 7 to day 28 and by 21% from day 28 to day 63. The abundance of AOA in unamended CL soils however, remained unchanged with time. The higher contribution of AOA in NP was in the unamended CL soil that was consistent with higher AOA abundance measured in the same.

AOB and AOA abundance was in the range reported by other researchers in animal waste amended soils (Fortuna et al., 2012; Habteselassie et al., 2013; Shen et al., 2008). The higher abundance of AOB could be contributed to the high amount of N present in PL which has 70-80% of N in organic form (urea and uric acid) that can be easily converted to ammonia and nitrate by the activity of AO and nitrifiers (Kelleher et al., 2002; Nahm, 2003). Long-term application of PL (>15 years) in these pasture soils increased the soil organic matter which supported the activity of microbes involved in mineralization and nitrification (Watts et al., 2010). In line with this study, several studies have reported AOB to be abundant in soils with high ammonium content as a result of inorganic N applications (Di et al., 2009; Jia and Conrad, 2009). AOA on the contrary, are found to be abundant in soils with ammonia derived from mineralization of organic matter (Di et al., 2010a; Limei et al., 2010; Offre et al., 2009). Therefore, greater AOA abundance in the unamended CL soil could be attributed to higher substrate affinity of AOA for ammonia and the ability to grow well under low ammonium conditions. Moreover, it has been indicated that AOA are able to use organic compounds in addition to ammonia as a source of energy, which might give them a

competitive advantage over AOB under N oligotrophic environments (Hatzenpichler et al., 2008; Webster et al., 2005).

Correlation analysis

Pearson's correlation coefficients were measured to study the relationship between several observed parameters in the PL and unamended CL soils. In case of PL, we observed significant correlation (p-value ≤ 0.05) between different variables (Table 3.7). Results showed a direct correlation between ammonia level and net N mineralization, net nitrification and NP and AOB abundance. The correlations between AOB abundance and NP, net nitrification and net N mineralization were stronger than the correlations of same variables with AOA. This indicates that overall the study time, AOB played a bigger role in nitrification in PL soil than AOA. In the case of unamended CL soil, the opposite was observed. The correlations between AOA abundance and nitrification parameters were stronger than the correlations of the parameters with AOB abundance. This implies that AOA were more important players in nitrification in unamended CL.

Community Composition of AOB and AOA

We observed significant effect of treatment only (p-value 0.032 and 0.022) at day 7 and 63, respectively on the AOB community composition. At day 7, the average number of observed OTU's per sample were higher in unamended CL soils (1000) compared to PL amended soils (500) (Figure 3.1). The qualitative measures Chao1 and ACE had higher means in unamended CL soil compared to PL amended soil. The quantitative Shannon and Simpson indices had comparable mean and spread of distribution in both unamended CL soil and PL amended soil. At day 63, the average number of observed OTU's per sample were higher in PL amended (1000) compared to unamended CL soil (Figure 3.1). Chao1 and ACE measures had a higher mean and greater spread

in PL plots compared to CL plots. Shannon and Simpson diversity indices were comparable in both PL amended and unamended CL plots.

Beta diversity analysis (using NMDS plots) showed distinct clusters (differences) in AOB communities in PL and unamended CL soils for day 7 and 63 (Figure 3.2). The clusters of AOB communities were more distinct for PL amended and unamended CL soil at day 63 compared to day 7. Phylogenetic analysis for AOB at day 7 and day 63 showed that majority of the dominant OTU's in both PL and CL soil belonged to *Nitrosospira spp* (Figure 3.3a and b).

Significant effect of treatment (p-value 0.015 and 0.006) and ammonium levels (p-value 0.0009 and 0.035) were observed on the AOA community composition at day 7 and 63, respectively. At day 7, the average number of observed OTU's per sample were higher in PL amended (5900) compared to unamended CL plots (1800) (Figure 3.4). The α-diversity index Chao1 and ACE was higher in unamended CL soil compared to PL soil with higher values observed at the high ammonium level. Shannon and simpson indices had greater spread but comparable means in unamended CL plots compared to PL amended plots. At day 63, the average number of observed OTU's per sample were higher in PL amended plots (6500) compared to unamended CL plots (1000) (Figure 3.4). The alpha diversity measures (Chao1, ACE, Shannon and Simpson) were higher in PL amended plots compared to unamended CL plots. In both PL and CL soil, the α-diversity was higher at the low ammonium level. This suggests that addition of ammonium was selecting narrow but fast-growing AO, reducing the diversity with time.

We observed distinct clusters of AOA communities in PL and unamended CL soil at day 7 and 63 for the two ammonium levels (Figure 3.5). At day 7, in both PL and unamended CL soil, the AOA communities formed distinct clusters at low and high ammonium level. At the end of incubation, at day 63, we observed that the AOA communities in PL soil at low and high

ammonium level were clustered together. This shows a decrease in diversity in AOA community structure in PL soil with time. In unamended CL soil, however, AOA population had differences in community diversity as shown by separate distinct clusters at low and high ammonium level. Phylogenetic analysis for AOA at day 7 and day 63 showed that majority of the dominant OTU's in both PL and CL soil belonged to *Nitrosopumilus spp* (Figure 3.6a and b).

Our results suggests the presence of a potential link between the function (NP, net nitrification) and community composition (diversity) of AO and that the change in function could be related to changes in both abundance and community composition. The changes in community composition of both AOB and AOA over time could be attributed to the selection of specific AO with the ability to utilize N in PL and ammonium more efficiently. PL in itself has a very large and diverse microbial population which is added to soil upon application (Rothrock et al., 2008). This leads to greater variations in microbial communities in PL compared to unamended CL soil which lacked any form of N input. The AOB community has been found to be more responsive to ammonium fertilizer addition than AOA in soils with a variety of animal wastes and inorganic fertilizers being applied (Ai et al., 2013; Chu et al., 2007; Ouyang et al., 2016). Wu et al (2011) reported that 22 years of urea-N and rice straw application in soils under paddy cultivation resulted in significant changes in AOB community structure than AOA in fertilized treatments compared to controls with no fertilizer applications. He et al (2007) observed pronounced differences in the community composition of AOA in soils with long-term (16 years) of N, P, K, fertilizer and organic manures addition. *Nitrosospira spp* are abundant in agricultural soils amended with inorganic fertilizers and animal wastes as they have higher substrate affinity for NH_4^+-N (Dharni et al., 2010; Wu et al., 2011; Xia et al., 2011). The dominant OTU's of AOA belonged to

Nitrosopumilus spp, known to be abundant in marine and terrestrial environments with extremely low nutrient concentrations (Jung et al., 2011; Martens-Habbena et al., 2009).

Conclusions

The contributions of AOB and AOA to NP differed in time. Early on, AOA dominated NP in both treatment, accounting for ≥ 70% of it. With time, AOB contribution to NP grew in PL soil accounting for ≥ 50% at high ammonium level. AOA remained to be the more dominant players in unamended CL soil. Similarly, AOA accounted for the larger share of net nitrification in unamended CL at both ammonium levels and PL soil at low ammonium level. However, AOB contribution to net nitrification was more than 50% in PL at high ammonium level. Correlation analysis confirmed the dominant roles of AOB in PL and AOA in unamended CL soil. These differences are mainly attributed to difference in forms of N between the soils and the ability of AO to use them. The presence of distinct communities of AO in PL and unamended CL soil as shown by α- and β-diversity indices suggested that the difference in function (nitrification) could be explained by the difference in abundance and community composition. Efficient N management in these two types of soils, therefore, would require different approaches that target the right group of organism that mainly control nitrification.

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Tables and Figures

Table 3.1: Soil pH, soil organic carbon content, percent sand, percent silt, percent clay and inorganic N contents in poultry litter (PL) amended and unamended CL soil at 0-15 cm depth. All reported values have been averaged over three replicates.

Parameter	PL Soil	CL Soil
Soil pH (water)	6.02	5.74
Soil Organic Carbon ($g\ kg^{-1}$ soil)	1.29	0.47
Sand (%)	60	58
Silt (%)	28	10
Clay (%)	12	32
NH_4^+-N (mg kg $soil^{-1}$)	2.95	5.07
NO_3^--N (mg kg $soil^{-1}$)	10.58	0.65

Table 3.2: Results of repeated measures of ANOVA for nitrification potential (mg kg soil^{-1}hr^{-1}), net nitrification (mg kg soil^{-1}), net N mineralization (mg kg soil^{-1}) and bacterial and archaeal *amoA* abundance (log copies g soil^{-1}) in PL and unamended CL soil after 63 day of incubation.

Factors	Nitrification Potential	Net Nitrification	Net N Mineralization	Bacterial *amoA*	Archaeal *amoA*
Treatment	<0.001	<0.001	<0.001	<0.001	ns
Ammonium Level	ns	ns	0.003	ns	ns
Time	<0.001	---	---	0.0035	<0.001
Treatment × Ammonium	0.016	0.035	0.021	0.020	0.021
Treatment × Time	0.0124	---	---	0.034	0.023
Ammonium × Time	ns	---	---	ns	ns

*Significant at p-value ≤0.05, ns "non-significant" --- (data not available)

Table 3.3: LS Means for nitrification potential (mg kg soil^{-1} hr^{-1}) in PL and unamended CL soil at day 7, 28, and 63 of incubation. Letters in the superscript indicate statistical significance of the measured variable at a given soil type. All reported values have been averaged over three replicates.

Soil Type	Ammonium Level	Day 7		Day 28		Day 63	
		Total NP (AOB + AOA)	NP (AOA)	Total NP (AOB + AOA)	NP (AOA)	Total NP (AOB + AOA)	NP (AOA)
PL	Low	1.92^{a}	1.34^{a} (70%)	2.44^{a}	1.42^{a} (58%)	3.03^{a}	1.64^{a} (54%)
	High	2.12^{b}	1.51^{b} (71%)	2.71^{b}	1.44^{a} (53%)	3.37^{b}	1.45^{b} (43%)
CL	Low	0.78^{A}	0.61^{A} (77%)	0.95^{A}	0.67^{A} (71%)	1.37^{A}	0.95^{A} (69%)
	High	0.88^{A}	0.65^{A} (74%)	1.12^{A}	0.79^{A} (71%)	1.49^{A}	0.97^{A} (65%)

*Percentage (%) in parenthesis indicates the AOA contribution in nitrification potential

Table 3.4: LS Means for net nitrification (mg kg soil^{-1}) and net N mineralization (mg kg soil^{-1}) in PL and unamended CL soil after 63 days of incubation. Letters in the superscript indicate statistical significance of the measured variable at a given soil type. All reported values have been averaged over three replicates.

Soil type	Ammonium Level	Net Nitrification (mg kg soil^{-1}) (AOB + AOA)	Net Nitrification (mg kg soil^{-1}) (AOA)	Net N Mineralization (mg kg soil^{-1})
PL	Low	157.76^{a}	105.70^{a} (67%)	153.61^{a}
	High	201.87^{b}	90.84^{b} (45%)	306.40^{b}
CL	Low	24.18^{A}	17.89^{A} (74%)	38.77^{A}
	High	29.62^{A}	21.33^{A} (72%)	64.12^{B}

*Percentage (%) in the parenthesis indicates the AOA contribution in net nitrificati

Table 3.5: LS means for bacterial *amoA* abundance (log copies g soil^{-1}) in PL and unamended CL soil at 7, 28, and 63 day of incubation. Letters in the superscript indicate statistical significance of the measured variable at a given soil type and a DCD level. All reported values have been averaged over three replicates.

Soil Type	Ammonium Level	Day 7		Day 28		Day 63	
		DCD 0	DCD 12	DCD 0	DCD 12	DCD 0	DCD 12
PL	Low	7.27^{a}	7.18^{a}	7.42^{a}	6.80^{a}	7.87^{a}	7.48^{a}
	High	7.26^{a}	7.20^{a}	7.34^{b}	6.82^{a}	7.80^{b}	7.41^{a}
CL	Low	6.10^{A}	5.95^{A}	6.09^{A}	5.49^{A}	5.99^{A}	5.71^{A}
	High	6.07^{A}	5.94^{A}	6.04^{A}	5.44^{A}	6.00^{A}	5.70^{A}

Table 3.6: LS means for archaeal *amoA* abundance (log copies g soil^{-1}) in PL and unamended CL soil at 7, 28, and 63 day of incubation. Letters in the superscript indicate statistical significance of the measured variable at a given soil type and a DCD level. All reported values have been averaged over three replicates.

Soil Type	Ammonium Level	Day 7		Day 28		Day 63	
		DCD 0	DCD 12	DCD 0	DCD 12	DCD 0	DCD 12
PL	Low	7.13^{a}	7.03^{a}	6.93^{a}	6.87^{a}	6.79^{a}	6.74^{a}
	High	7.18^{a}	7.09^{a}	7.12^{b}	7.06^{b}	7.05^{b}	7.02^{b}
CL	Low	7.06^{A}	7.02^{A}	7.08^{A}	7.02^{A}	7.03^{A}	6.99^{A}
	High	7.03^{A}	6.98^{A}	7.04^{A}	7.01^{A}	7.08^{A}	7.01^{A}

Table 3.7: Pearson correlation coefficient (r) between various parameters measured in PL soil.

	Ammonia Level	Archaeal *amoA* Abundance	Bacterial *amoA* Abundance	Nitrification Potential	Net Mineralization
Archaeal *amoA* Abundance	0.54				
Bacterial *amoA* Abundance	0.58*	-0.12			
Nitrification Potential	0.60*	0.63*	0.73*		
Net N Mineralization	0.69*	0.51	0.90*	0.74*	
Net Nitrification	0.62*	0.69*	0.95*	0.90*	0.94*

'*'Significant at p-value ≤0.05

Table 3.8: Pearson correlation coefficient (r) between various parameters measured in unamended CL soil.

	Ammonia Level	Archaeal *amoA* Abundance	Bacterial *amoA* Abundance	Nitrification Potential	Net N Mineralization
Archaeal *amoA* Abundance	0.41				
Bacterial *amoA* Abundance	0.52	-0.39			
Nitrification Potential	0.55	0.64*	0.60		
Net N Mineralization	0.79*	0.70*	0.56	0.46	
Net Nitrification	0.41	0.71*	0.77*	0.56	0.79*

'*'Significant at p-value ≤0.05

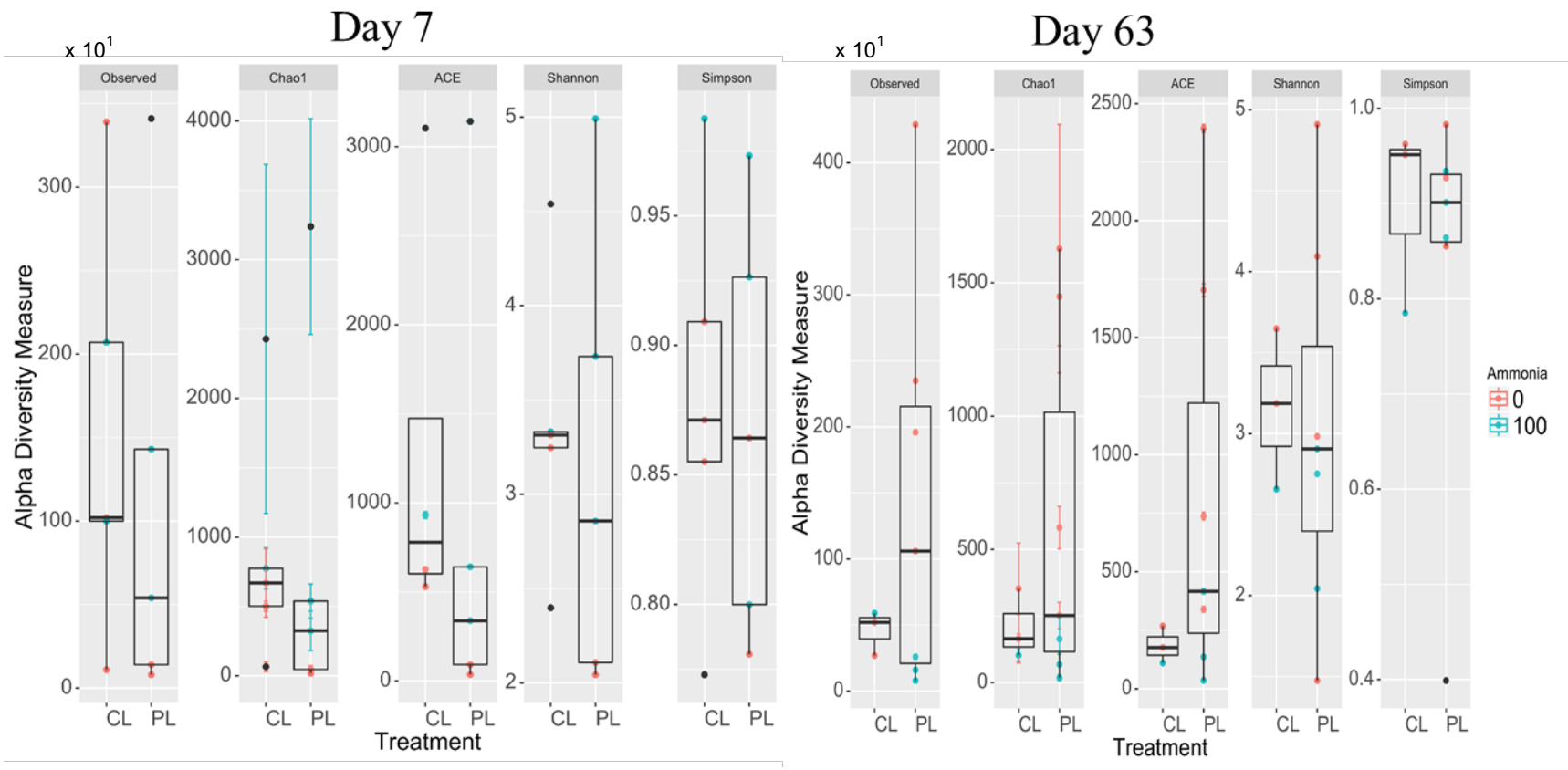

Figure 3.1: Alpha diversity for ammonia oxidizing bacteria (AOB) on day 7 and 63 in PL and unamended CL soil at low and high ammonium level.

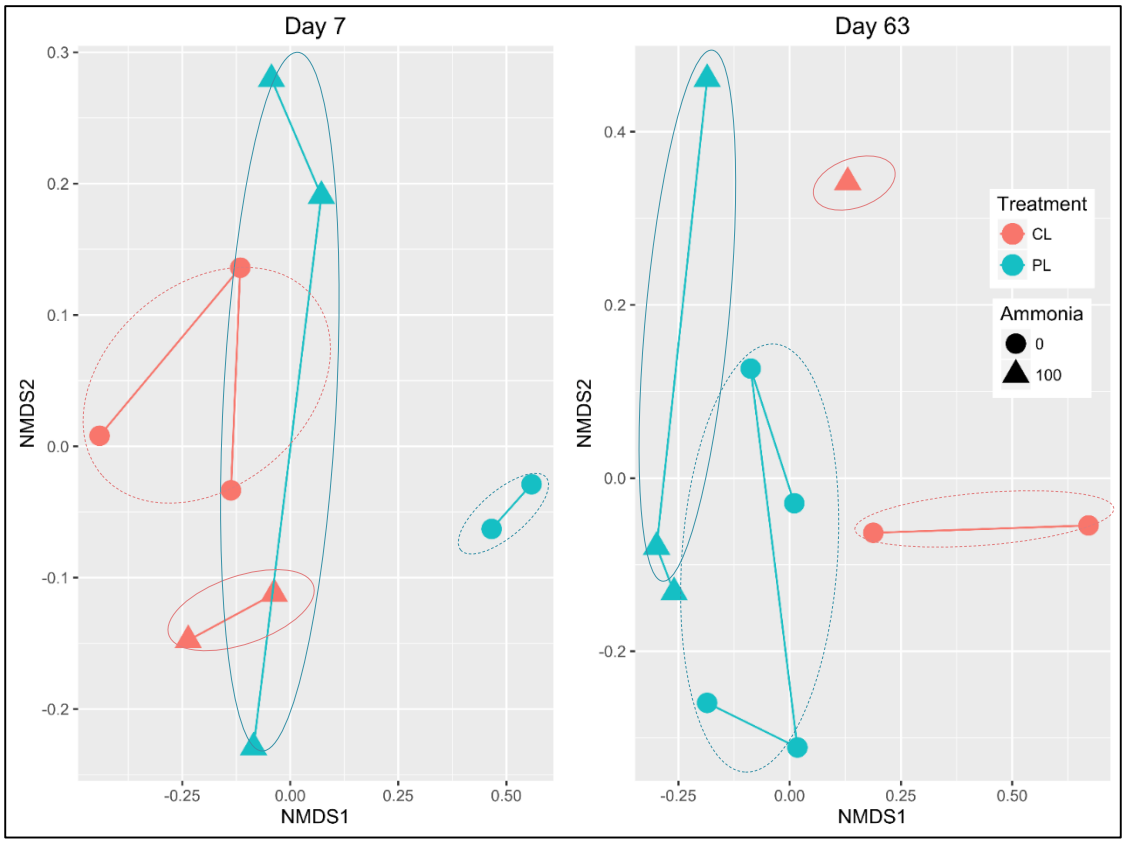

Figure 3.2: Nonmetric multidimensional scaling plot using Bray-Curtis distance matrix for ammonia oxidizing bacteria (AOB) in PL amended and unamended CL soil at day 7 and day 63.

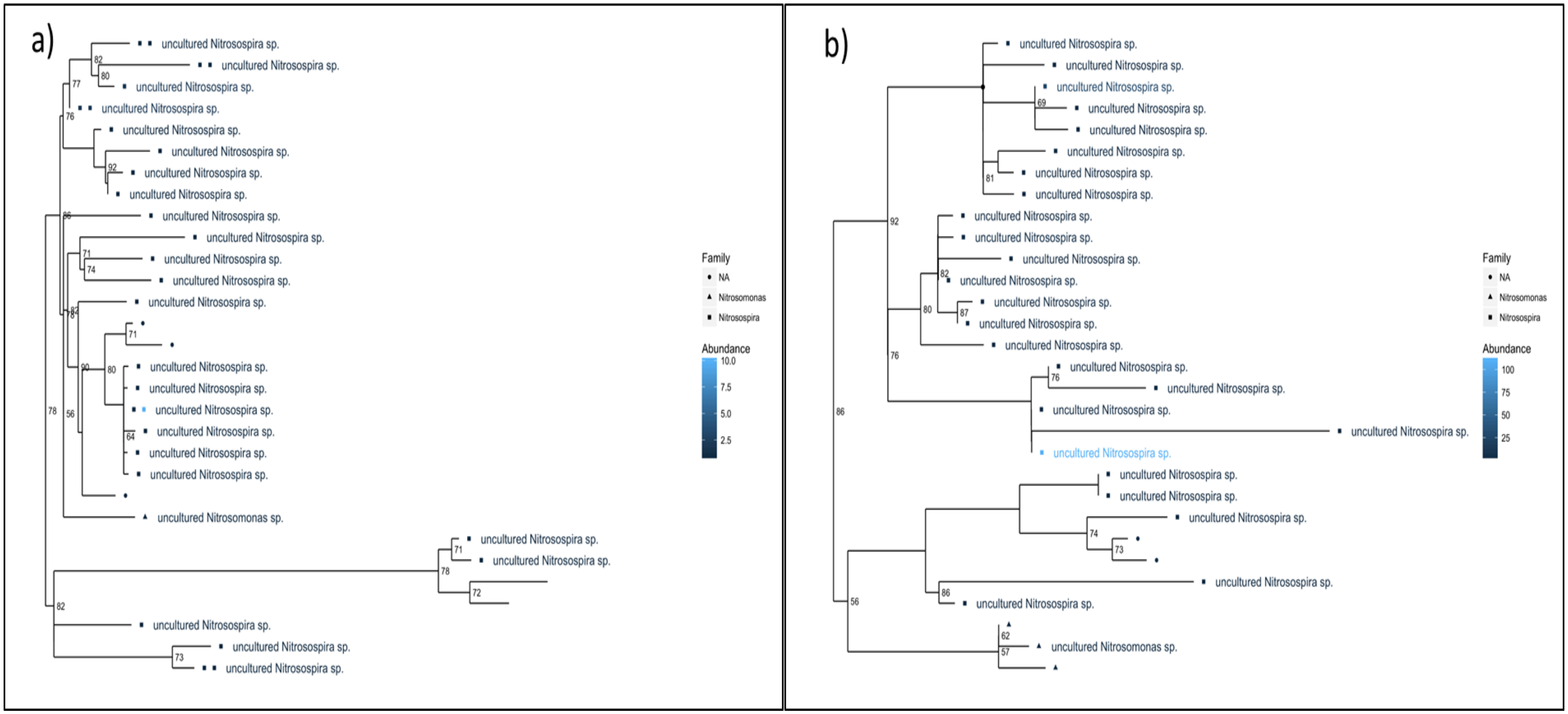

Figure 3.3: Sample phylogenetic tree for ammonia oxidizing bacteria (AOB) in a) PL soil and b) unamended CL at day 63 at high ammonia level.

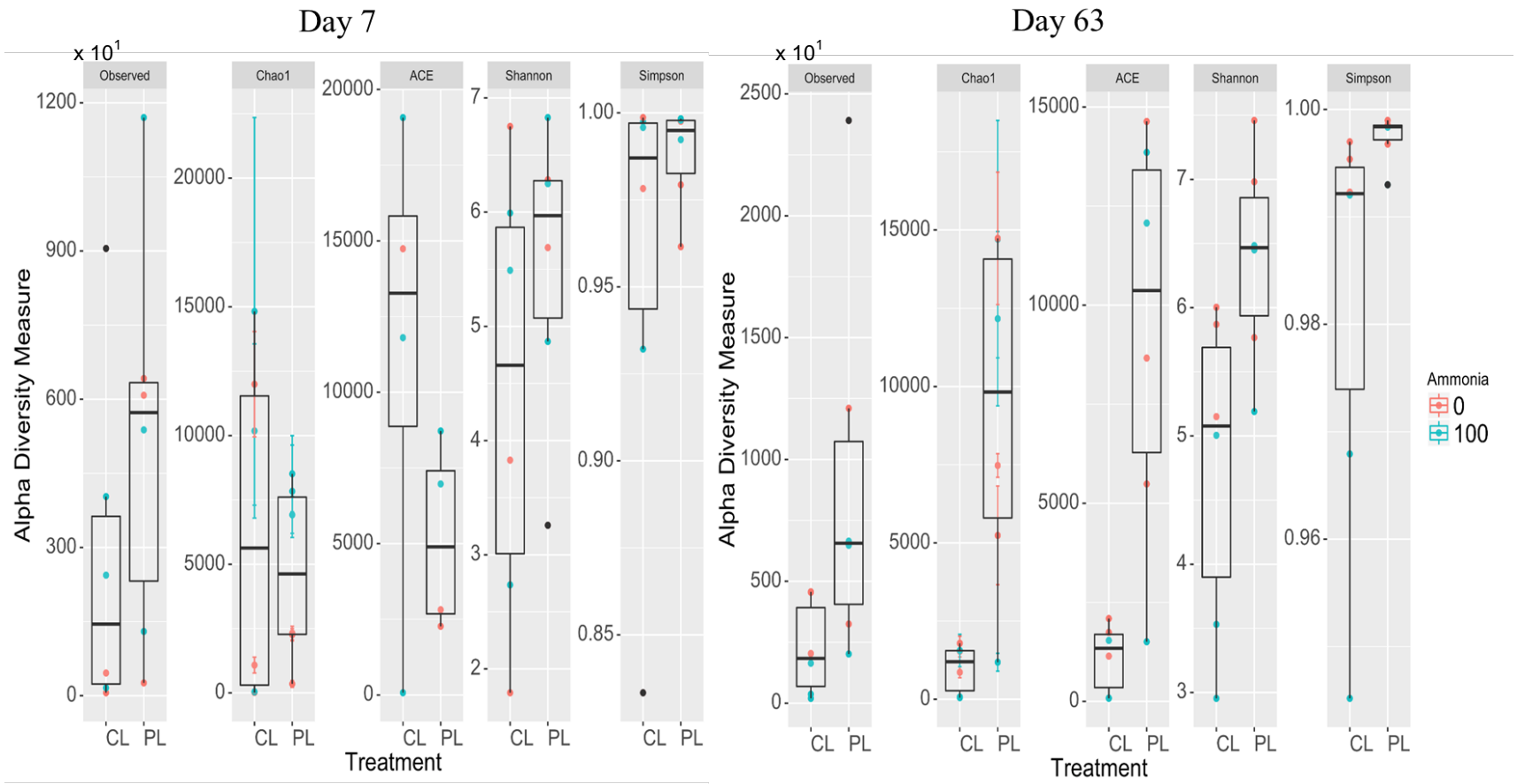

Figure 3.4: Alpha diversity indices for ammonia oxidizing archaea (AOA) on day 7 and 63 in PL and unamended CL soil at low and high ammonia level.

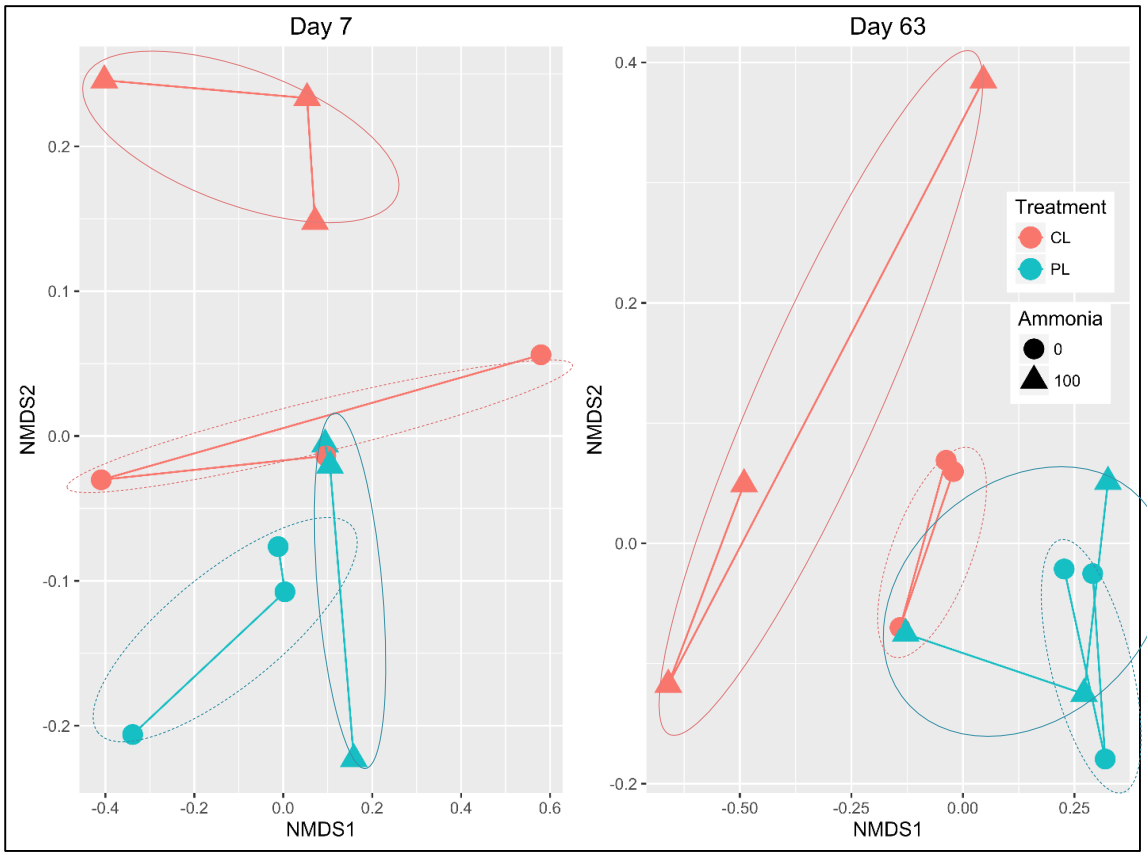

Figure 3.5: Nonmetric multidimensional scaling plot using Bray-Curtis distance matrix for ammonia oxidizing archaea (AOA) in PL and CL soil at day 7 and day 63.

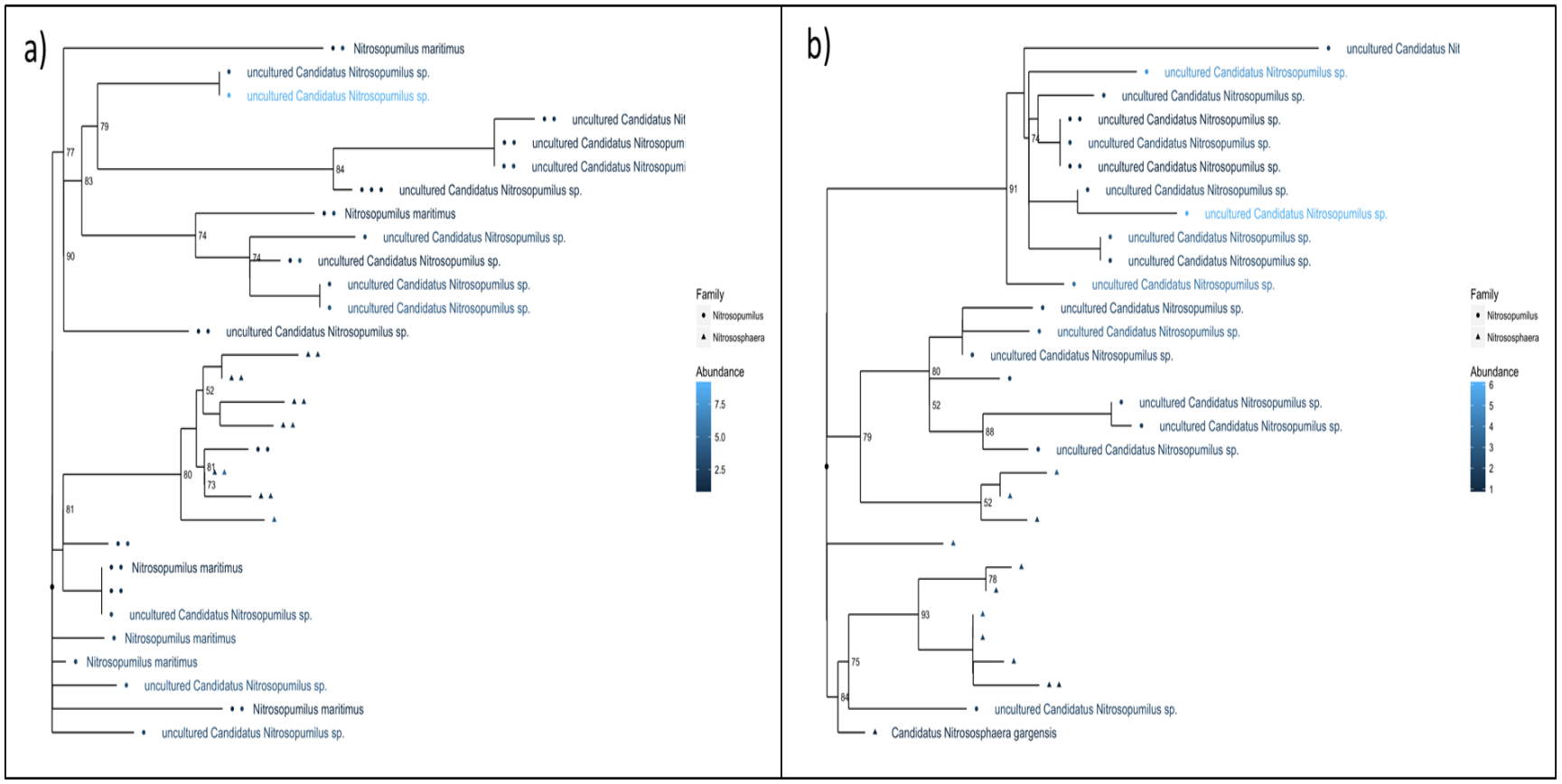

Figure 3.6: Sample phylogenetic tree for ammonia oxidizing archaea (AOA) in a) PL soil and b) unamended CL soil at day 63 in high ammonia level.

CHAPTER 4

RESPONSE OF SOIL AMMONIA OXIDIZERS TO ELEVATED ZINC AND COPPER LEVELS IN POULTRY LITTER AMENDED SOILS

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Abstract

It is not clear how trace metals introduced to soil via poultry liter (PL) affect the ammonia oxidizing bacteria (AOB) and archaea (AOA), which play a crucial role in N cycling. We conducted a laboratory incubation study to determine the response of AOB and AOA to elevated levels of zinc (Zn) and copper (Cu). Nitrification potential (NP) and net nitrification were measured to characterize changes in their function in which 1-Octyne was used in the NP assay to separate the contributions of AOA and AOB. Quantitative polymerase chain reaction was used to measure AOB and AOA abundance by targeting their respective *amoA* genes. The abundance of total bacteria and total fungi was also measured to evaluate the effect on broader groups of microorganisms. Increasing Zn levels resulted in large reductions in AOB (78%) and AOA (85%) at the high ammonium level (100 mg kg soil^{-1}) over 28 days. Likewise, increasing Cu significantly reduced AOB (92%) and AOA (63%) abundance at the high ammonium level over 28 days. Similar reductions in abundance of total bacteria and total fungi were seen under these conditions, indicating the broader effects of the metals. Increasing Zn from 21 ppm to 250 ppm and Cu from 20 ppm to 120 ppm decreased net nitrification by 46% and 27% at high ammonium level, respectively. The relative contribution of AOB to NP was significantly higher in both Zn (~60%) and Cu (~70%) treated soils despite the numerical dominance of AOA over AOB. Overall, results indicate that elevated levels of Zn and Cu depressed AOB and AOA abundance and function and that their effect was dependent on availability of ammonium. It also indicates that AOB are functionally more important than AOA under elevated Zn and Cu concentrations.

Introduction

Poultry litter (PL), which is composed of excreta, bedding material, feathers and waste feed, is frequently applied to land as a way of disposal and as source of fertilizer (Edwards and Daniel, 1992; Kelleher et al., 2002). Land application of PL has many benefits that result in improvements in soil biological, chemical and physical properties (Adeli et al., 2009; Kingery et al., 1994). However, it can also have undesirable consequences, one of which is the accumulation of metals such as copper (Cu), zinc (Zn) and arsenic (As) in soils. Metals are added to poultry feed to prevent diseases but a large percentage of the added metals is excreted in feces, within the PL (Sims and Wolf, 1994; Kornegay et al., 1976; Sharpley et al., 1998; Nicholson et al., 1999). This is highly pertinent to the southeastern region of the United States, which is the biggest poultry producer in the nation, resulting in the production of large amount of PL that is applied on pasturelands (National Agricultural Statistics Service, 2012). The fate of PL contained nutrients in soil is dependent on transformations that are mediated by microorganisms whose response to these metals is not fully understood in PL receiving soils (Kowalchuk and Stephen, 2001).

The transformation of nitrogen (N) in PL is of particular interest as it can easily be lost via leaching or volatilization (Brinson Jr et al., 1994; Cabrera et al., 1993). Organic N in PL is mainly in the forms of urea and uric acid that are converted to ammonia via mineralization (Bitzer and Sims, 1988; Havlin et al., 1999). A wide group of microorganisms, with bacteria and fungi dominating the process, mediates nitrogen mineralization (Acosta-Martinez and Harmel, 2006). The ammonia is further converted to nitrate through nitrification, which is mediated by relatively narrower groups of organisms called ammonia-oxidizing bacteria (AOB) and archaea (AOA)(Kowalchuk and Stephen, 2001). The role of ammonia oxidizers (AO) is particularly critical as it leads to production of nitrous oxide and nitrate, two nitrogen forms that can easily

escape from the systems with undesirable environmental consequences. The sound management of PL requires proper understanding of its impact on the function and abundance of these microorganisms. There is limited study on the impact of PL on soil AO despite the fact that they are widely studied in soils (Jia and Conrad, 2009; Leininger et al., 2006). Previous studies have indicated the functional and niche separation between AOA and AOB but it is not clear if it is true in PL treated soils. The differential response of AOA and AOB might suggest the need for targeted management practices to minimize N loss.

Previous studies mainly focused on the impact of PL on soil chemical properties. Repeated applications of PL led to increases in soil nitrate, total N, organic matter, extractable nutrients and metals (Zn, Cu, Mn, As) (Sharpley et al., 1993). There are limited studies that examined impact of PL application on microbial abundance and function. A study by Acosta-Martinez and Harmel (2006) reported that application of PL at rates greater than 6.7 Mg ha^{-1} for 4 years resulted in an increase in microbial biomass C and N, total bacterial and fungal populations and enzymatic activities but did not specifically looked at its impact on AO. Tomlinson et al (2015) observed changes in microbial enzyme activities and AO community richness after 8 and 9 years of untreated and alum treated PL and ammonium nitrate applications respectively but did not examine AOA whose role in nitrification is a recent discovery (Könneke et al., 2005; Treusch et al., 2005). Although there are studies that examined the impact of metals on AO (Mertens et al., 2010; Ruyters et al., 2010; Liu et al., 2010; Ollivier et al., 2012), the soils were not treated with PL nor did they have a history of PL application.

Therefore, the main goal of this study was to examine how AO respond to elevated metal levels in soil that has a history of continuous PL application. The objectives of the study were two-fold: a) to determine how AO respond in abundance and function to elevated metals levels at

different substrate (ammonium) concentrations in PL treated soil and b) to determine if AOB and AOA contributions towards nitrification differ under elevated metal levels at different substrate concentration in PL treated soil. The abundances of total bacteria and fungi were also quantified to see if changes are also reflected in the general soil microbial communities. Net N mineralization was also calculated to serve as a broad measure of bacterial and fungal activities.

Material and Methods

Soil Sampling

Soil samples were collected from 0.8 ha PL applied experimental plots (Ashjaei et al., 2011), which are located in the College of Agricultural and Environmental Sciences Central Georgia Research and Education Center near Eatonton, Georgia (39°24' N, 83°29'W, elevation 150 m) in November 2015. Samples were collected at 0-15 cm depth, with 6-8 subsamples combined to form a composite sample. These plots received PL twice a year at 8.3 Mg ha^{-1} from 1995 to 2011. Soil basic properties and initial metal concentrations were determined at Agricultural and Environmental Services Laboratories of the University of Georgia (http://aesl.ces.uga.edu/) and are shown in Table 4.1.

Experimental Set-up

The experimental set-up was designed to have three ammonium and three metal levels, resulting in nine combinations. We were interested in examining the impact of the metals on AO at different concentrations of ammonium, which is the substrate for AO. To achieve this, soil samples were amended with ammonium chloride at 0 (Low and served as control), 50 (Medium) and 100 mg NH_4^+-N kg $soil^{-1}$ (High). Trace metals Zn and Cu were applied to soils to achieve final concentrations of 21 mg kg^{-1} (initial concentration and served as control), 100 mg kg^{-1} and 250 mg kg^{-1} and 9 mg kg^{-1} (initial concentration and served as control), 20 mg kg^{-1}, and 120 mg kg^{-1},

respectively using salt solutions of zinc chloride and copper chloride. The metals and ammonium chloride solutions were thoroughly mixed with 1 kg soil in a Ziploc bag and water was added to achieve 30% water holding capacity. The soil was divided in three independent replicates for each treatment, in small plastic sample cups containing 50 gram soil. Sample cups were randomly placed in an incubator at 26°C. The soil moisture loss was monitored by weighing the soil samples regularly and replenishing the weight lost by adding water. Sub-samples were taken at 7, 14, and 28 days of incubation. A portion of the soil sample was immediately placed at -20ºC and rest was used for nitrification potential assays as described below and extraction with 2M KCl to determine the soil inorganic N content for the sampling time. Soil moisture content was determined by drying 10 gram soil in an oven at 105°C for 24 hr and measuring the loss in weight.

Quantitative Polymerase Chain Reaction (qPCR)

Soil genomic DNA was extracted from 0.25 gram fresh soil using MO-BIO PowerSoil® DNA extraction kit (MO-BIO laboratories, Carlsbad, C.A) using manufacturer's instructions. The bacterial *amoA*, archaeal *amoA*, 16S rDNA and 18S rDNA copy numbers for total bacteria and fungi were measured using qPCR assays. The primer pairs and thermal cycling conditions used in q-PCR assays are listed in the Table 4.2. Standards for qPCR for bacterial *amoA*, 16S rDNA for total bacteria and 18S rDNA for total fungi were prepared using genomic DNA of *Nitrosomonas europaea* strain 19718 (ATCC, Manassas, V.A), *Escherichia coli* and *Saccharomyces cerevisiae*, respectively. Archaeal qPCR standards for *amoA* were prepared using PCR product of soil DNA as a template for ligation reaction. Serial dilutions of the plasmid DNA generated a standard curve with copy numbers ranging from 3×10^3 - 3×10^7. Reaction was carried out in 20 µl total volume, with 10 µl of Power^{UP} SYBR Green Master Mix (Applied Biosystems by Life Technologies, Foster City, C.A), 0.8 µl of the primers, 6.4 µl of nuclease free PCR water and 2 µl of soil sample DNA

using StepOne Plus™ thermocycler (Applied Biosystems by Life Technologies, Foster City, C.A). The specificity of qPCR product was verified by melt curve analysis and running the product on the agarose gel (1.5%). All the qPCR reactions were run in duplicate with standards in triplicate. The average amplification efficiency ranged from 79 to 93% with r^2 from 0.94 to 0.98. The log *amoA* copies g $soil^{-1}$ were used as proxy for the AOB, AOA, total bacteria and total fungi abundance. Each cell of AOB contains 2-3 copies of *amoA* gene whereas the number of copies of *amoA* gene in AOA is still unknown (Norton et al., 2002). The methods are previously described in details in Chapters 2 and 3.

Soil Inorganic N and Nitrification Potential

NH_4^+-N and NO_3^--N contents of the soil samples were measured using 10 gram soil and 50 ml 2M KCl in 250 milliliter Erlenmeyer flasks, shaken for 1 hr and the extracts filtered with Whatman No. 45 filter paper and kept at 20°C until further analysis. Soil extracts were analyzed for (NO_2^-+NO_3^-)-N and NH_4^+-N using Auto analyzer AA1 (Seal Analytical Inc., Mequon, W.I).

The net N mineralization and nitrification were calculated as the changes in soil inorganic-N pool over the duration of incubation time (Hart et al., 1994).

Net N mineralization = $[(NH_4^+ + (NO_2^-+NO_3^-)\text{-N}]_{\text{day 28}} - [(NH_4^+ + (NO_2^-+ NO_3^-)\text{-N}]_{\text{day 0}}$

Net nitrification = $(NO_2^-+NO_3^-)\text{-N})_{\text{day 28}} - ((NO_2^-+ NO_3^-)\text{-N})_{\text{day 0}}$

The differential contributions of AOB and AOA to nitrification were determined on day 7 and day 28, by using the assay previously described (Giguere et al., 2015; Taylor et al., 2013). This assay employs aliphatic alkyne 1-Octyne to inhibit AOB activity to quantify the contribution of AOA. Briefly, 9 gram soil was measured in duplicate and placed in 125 milliliter amber colored Wheaton glass bottles with 60 milliliter of 30 mM TES buffer (pH 7.2) supplemented with 1mM NH_4^+-N. Stock of 1-Octyne was prepared by adding 40 µl of liquid 1-Octyne and 100 milliliter of

air in 125 milliliter amber Wheaton bottle having several glass beads. The bottle was shaken vigorously for 30-45 seconds. One batch of soil was injected with 2.7 milliliter of 1-Octyne from stock to give a final concentration of 4μM 1-Octyne (aqueous concentration) using a gas-tight syringe and the other batch served as the control. The flasks were shaken at 120 rpm for 24 hr and 10 milliliter of extracts were taken at 2, 4, and 24 hr interval. The extracts were centrifuged at 8000 × g for 8-10 minutes and filtered using Whatman No. 42 filter paper and stored at -20°C until analyzed. Total (NO_2^-+NO_3^-)-N content of the soil extracts were measured using Auto analyzer AA1 (Seal Analytical Inc., Mequon, W.I) with nitrification potential as the linear regression of total (NO_2^-+NO_3^-)-N over time. The nitrification values in 1-Octyne-treated samples represented the nitrification potential of AOA. The bacterial nitrification was calculated as the difference between the nitrification values in 1-Octyne-untreated and 1-Octyne-treated samples.

Statistical Analysis

Repeated measures analysis of variance (ANOVA) was performed to determine the effects of different metal concentrations (Zn and Cu) on microbial abundance and function at different ammonium concentrations over time using the R programming language version 3.2. The bacterial and archaeal *amoA* gene copies, total bacterial 16S rDNA and total fungal 18S rDNA copies were $\log_{10}$ transformed before the statistical analysis to meet the normality assumption for ANOVA. Repeated measure ANOVA was performed using the *afex* package in R (Maxwell and Delaney, 2004). In this implementation, in a split-plot design of experimental setup, day/time variable was used as within-variable and all other independent experimental variables (Zn and Cu, ammonium) as between-variables. Separate ANOVA were performed for each of the observables (AOA, AOB, total bacteria, total fungi, nitrification potential, net N mineralization and net nitrification) for each sampling time too. All plots were made using the *ggplot2* package in R (Wickham, 2009).

Correlation analysis among different experimental observables were also performed using the *Hmisc* package of R (Press et al., 1988). All analyses were done at a significance value of $p \leq 0.05$.

Results

Abundance of Bacterial and Archaeal *amoA*

Zn, ammonium, time and their interactions significantly affected the bacterial *amoA* (AOB) abundance (Table 4.3). At the early stage of the study (day 7), AOB abundance ranged between 7.27 and 6.93 log copies g soil^{-1}, with no significant impact from Zn (Table 4.5). At day 28, however, Zn had an inhibitory effect, resulting in 28% to 60% decrease in AOB abundance that ranged between 6.96 and 6.31 log copies g soil^{-1} (Table 4.6). The decrease was highest (60%) at the high ammonium level between 21 mg kg^{-1} and 250 mg kg^{-1} Zn rates. At low and medium ammonium levels, however, the difference was between the 250 mg kg^{-1} and the lower Zn rates, which were not significantly different from each other.

Significant effects of Zn, ammonium, time and their interactions were observed on the archaeal *amoA* (AOA) abundance (Table 4.3). At day 7, AOA abundance was between 7.72 and 7.99 logs, with no significant impact from Zn (Table 4.5). At day 28, Zn resulted in significant decrease in AOA abundance between 21 mg kg^{-1} and the higher rates, which were significantly different from each other at all ammonium levels (Fig 4.6). Similar to AOB, the decrease in AOA abundance was the largest (71%) at the high ammonium level between 21 and 250 mg kg^{-1} Zn rates.

We also looked at the relative abundance of AOB and AOA in the different treatments in terms of their ratios (Table 4.5 and 4.6). At day 7, AOB to AOA ratio (AOB: AOA) varied from 0.16 (low ammonium level with 250 mg kg^{-1} Zn) to 0.24 (high ammonium with 250 mg kg^{-1} Zn). Overall, all values of AOB: AOA were significantly less than 1.0, indicating higher abundance of

AOA compared to AOB. At day 28, AOB: AOA ratio varied from 0.13 (low ammonium level with 250 mg kg^{-1} Zn) to 0.30 (high ammonium level with 250 mg kg^{-1} Zn). Over time, the abundance of AOB decreased by 41-78%, while AOA abundance decreased by 48-85%. Overall, the inhibitory effects of Zn was more pronounced on AOA than AOB abundance. The greatest effects of time was observed at the high ammonium level, where AOB abundance reduced by 78% while AOA abundance decreased by 85% from day 7 to day 28.

The effects of Cu, ammonium and time and their interaction terms on AOB abundance were significant (Table 4.4). At day 14, Cu had significant effect on the AOB abundance at all ammonium levels. An increase in Cu from 9 mg kg^{-1} to 20 mg kg^{-1} resulted in an increase in AOB abundance by ~90% while an increase in Cu from 20 mg kg^{-1} to 120 mg kg^{-1} resulted in a decrease by ~35% at all of the ammonium levels (Table 4.7). Similarly, at day 28, increasing Cu from 9 mg kg^{-1} to 20 mg kg^{-1} led to a 31% to 48% increase in AOB abundance at medium and low ammonium levels, respectively (Table 4.8). At the high ammonium level, however, increasing Cu from 9 mg kg^{-1} to 20 mg kg^{-1} led to a 66% reduction in the AOB abundance. Increasing Cu from 20 mg kg^{-1} to 120 mg kg^{-1} did not lead to any significant change in the AOB abundance at low ammonium level. At medium and high ammonium levels, however, increasing Cu from 20 mg kg^{-1} to 120 mg kg^{-1} led to 50% and 40% reductions in the AOB abundance, respectively. Overall, our results indicate an inhibiting effect of Cu on AOB abundance only at higher Cu and ammonium combination.

The effects of Cu, ammonium, time and their interaction effects were significant on the AOA abundance (Table 4.4). At day 14, increasing Cu from 9 mg kg^{-1} to 20 mg kg^{-1} did not have any significant impact on AOA (Table 4.7). However, increasing Cu from 20 mg kg^{-1} to 120 ppm led to a ~34% reduction in the AOA abundance at the three ammonium levels. At day 28,

significant effect of Cu was seen only at the high ammonium level (Table 4.8). AOA abundance decreased by 54% by increase in Cu 9 mg kg^{-1} to 20 mg kg^{-1} and 17% from 20 mg kg^{-1} to 120 mg kg^{-1}.

Similar to the Zn study, AOA abundance was found to be higher than AOB in all the treatments (Table 4.7 and 4.8). At day 14, AOB: AOA varied from 0.09 (low ammonium level with 9 mg kg^{-1} Cu) to 0.56 (high ammonium with 120 mg kg^{-1} Cu) (Table 4.7). A day 28, AOB: AOA varied from 0.02 (low ammonium level with 9 mg kg^{-1} Cu) to 0.29 (medium ammonium level with 120 mg kg^{-1} Cu) (Table 4.8). Over time (day 14 to 28), abundance of AOB decreased by 49-92%, while those of AOA reduced by 7-63% only. Overall, contrary to Zn, the inhibitory effect of Cu metal was more pronounced on AOB abundance than AOA abundance. The greatest effects of time were observed at the high ammonium level, where AOB abundance decreased by 92% while AOA abundance by 63%.

Total Bacterial and Fungal abundance

ANOVA of total bacterial abundance showed significant effects of Zn, ammonium, time, along with their interaction terms, except for ammonium × Zn interaction (Table 4.3). At day 7, Zn had no significant impact on the total bacteria abundance except at medium ammonium level in which an increase from 100 mg kg^{-1} to 250 mg kg^{-1} resulted in 26% decrease in total bacterial abundance (Table 4.5). At day 28, Zn caused a consistent decrease in total bacterial abundance with increasing concentration by 19% to 35% at the low ammonium level. At the medium ammonium level, the total bacterial abundance significantly increased by 26% from 21 mg kg^{-1} to 100 mg kg^{-1} and significantly decreased by 21% from 100 mg kg^{-1} to 250 mg kg^{-1}. At the high ammonium level, the total bacterial abundance decreased significantly by 52% from 21 mg kg^{-1} to 250 mg kg^{-1}.

ANOVA of total fungi population also showed significant effects of Zn, ammonium, time, along-with their interaction terms, except for the ammonia × Zn interaction term (Table 4.3). At day 7, increasing Zn from 21 mg kg^{-1} to 100 mg kg^{-1} resulted in 24% decrease in total fungal abundance at the high ammonium level (Table 4.5). No significant change in total fungal abundance was observed at low and medium ammonium levels. At day 28, increasing Zn from 21 mg kg^{-1} to 250 mg kg^{-1} resulted in a significant decrease in total fungal abundance (26% at low and 66% at high ammonium levels, respectively) (Table 4.8). From day 7 to day 28, the total fungal abundance decreased by 28-82%, the reduction being highest (82%) for high ammonium level samples, and lowest (28%) for the low ammonium level samples.

Significant effect of Cu, ammonium, time and their interactions (Table 4.4) were observed on the total bacterial abundance. At day 14, Cu application had no significant impact on the total bacterial abundance at all ammonium levels (Table 4.7). However, at day 28, Cu had an inhibitory effect on total bacterial abundance, resulting in 44% decrease at low and high ammonium level and 26% decrease at medium ammonium level with an increase in Cu from 9 mg kg^{-1} to 120 mg kg^{-1} (Table 4.8).

Total fungal abundance was significantly affected by Cu, ammonium, along with their interaction terms, except for time × Cu level interaction term (Table 4.4). At day 14, we observed 32% and 72% decrease in total fungal abundance at low and high ammonium levels with Cu increasing from 9 mg kg^{-1} to 120 mg kg^{-1} (Table 4.7). At day 28, increasing of Cu from 9 mg kg^{-1} to 120 mg kg^{-1} reduced the abundance of total fungi by 21%, 44% and 64% in low, medium and high ammonium level (Table 4.8). The total fungal abundance increased (7-25%) from day 14 to 28.

Net Nitrogen Mineralization and Net Nitrification

ANOVA showed significant effects of both ammonium and Zn on net N mineralization and net nitrification but their interaction was significant only for net nitrification (Table 4.3). Addition of ammonium resulted in an average 76% and 127% increase (at medium and high ammonium level, respectively) in net N mineralization compared to low ammonium level. Zn had an inhibitory effect leading to average 15% decrease in the net N mineralization with increase in Zn from 21 mg kg^{-1} to 250 mg kg^{-1} (Table 4.9). Application of Zn had no impact on the net N nitrification at low ammonium levels. For the medium and high ammonium level, however, Zn inhibited nitrification, resulting in 38% to 46% decrease in the net N nitrification with increase in Zn from 21 mg kg^{-1} to 250 mg kg^{-1} (Table 4.9).

ANOVA showed significant effects of both ammonium and Cu and their interactions on both net nitrification and net N mineralization (Table 4.4). Cu had no significant impact on the net N mineralization at low and medium ammonium level (Table 4.10). For the high ammonium level, however, Cu application had an inhibitory effect, resulting in 20% or 23 mg kg $soil^{-1}$ decrease in the net N mineralization from Cu 9 mg kg^{-1} to 120 mg kg^{-1}. Similarly, application of Cu had no impact on the net N nitrification at low and medium ammonium levels as well (Table 4.10). For the high ammonium level, however, Cu application resulted in 27% or 43 mg kg $soil^{-1}$ decrease in the net N nitrification from Cu 9 mg kg^{-1} to 120 mg kg^{-1}.

Nitrification Potential

ANOVA (Table 4.11) showed significant impact of ammonium, Zn, time, and Zn × time, ammonium × time interactions on the soil nitrification potential (NP). At day 7, increasing Zn from 21 mg kg^{-1} to 250 mg kg^{-1} resulted in average 37% decline in NP (Figure 4.1). Likewise, at day 28, increasing Zn concentration from 21 mg kg^{-1} to 250 mg kg^{-1} resulted in average 48% decline

in the NP. The soil NP decreased from day 7 to day 28 at all ammonium levels in Zn experiment. The decrease in NP from day 7 to day 28 was 28% with 21 mg kg^{-1} Zn at each of the three ammonium levels and 13%, 24% and 18% at low, medium and high ammonium level with 250 mg kg^{-1} of Zn, respectively.

AOA contribution to NP ranged from 38% to 56% at day 7. The contribution of AOA was higher at 21 mg kg^{-1} than 250 mg kg^{-1} of Zn. The high ammonium level had the largest contribution (56%) from AOA, while low and medium ammonium level had similar AOA contributions to NP at ~40%. As NP decreased with time, the AOA contribution declined to ~30% on day 28. AOB had a higher contribution to NP than AOA, varying from 44% to 62%, at day 7 and it increased to 66% to 70% at day 28.

ANOVA (Table 4.11) showed significant impact of time, and interactions of Cu × ammonium, Cu × time on NP. At day 7, Cu application had no significant effect on NP at three ammonium levels (Figure 4.2). In contrast, at day 28, application of 120 mg kg^{-1} of Cu led to 22%, 20% and 19% decline in the NP at low, medium and high ammonium level compared to Cu 9 mg kg^{-1}. Similar to Zn experiment, NP decreased from day 7 to day 28. The decrease in NP was 7% at 9 mg kg^{-1} of Cu and 26% at 120 mg kg^{-1} of Cu at the three ammonium levels.

AOA contribution to NP ranged from 15% to 26% at day 7. The contribution of AOA was always higher at 9 mg kg^{-1} than at the 120 mg kg^{-1} of Cu. The high ammonium level had highest contribution (26%) from AOA, while low and medium ammonium level had 19% and 23% contributions from AOA, respectively. In contrast to the Zn experiment, AOA contribution increased to 33-46% at day 28. At day 28 as well, the highest AOA contribution to NP was at the high ammonium level. The contribution of AOB varied from 74% to 86% at day 7 and it decreased

to 54% to 68% at day 28. Higher contribution by AOB in NP indicates a greater role in nitrification under the different metal and ammonium scenarios considered in this study.

Correlations among different response variables

We also looked at correlations among several observed variables in terms of Pearson's coefficients to study the interdependence of observed responses in our experiments. In the case of Zn, we find significant correlations (p-value ≤ 0.05) between AOB-total bacteria abundances, AOB abundance-NP, AOB abundance-net N mineralization, AOB abundance-net nitrification, AOA-total fungi abundance, AOA abundance-NP and net N mineralization-net N nitrification (see Table 4.12). Zn levels showed negative correlations with AOB, AOA, total fungi and NP due to inhibitory effects of Zn application. Ammonium level had significant correlation with only net N mineralization and net nitrification.

In the case of Cu, we find significant correlations (p-value ≤ 0.05) between AOA-total bacteria abundance, AOA abundance-NP, NP-total bacteria abundance, total bacteria abundance-net N mineralization and net N mineralization-net nitrification (see Table 4.13). Cu level shows negative correlations with AOA, total bacteria and NP. In contrast to the Zn experiment, AOB abundance has no significant correlation to Cu levels. We also observed significant correlation between NP and AOA and total bacteria abundance. Similar to Zn experiment, ammonium level shows significant correlation with only net N mineralization and net nitrification.

Discussion

We used the higher ends of the metal concentrations (20 mg kg^{-1} & 120 mg kg^{-1} for Cu; 100 mg kg^{-1} & 250 mg kg^{-1} for Zn) to evaluate the cumulative impacts of metals over long-term on microorganisms that mediate the key components of the N cycle. The background Cu and Zn concentration in PL soil in our experiment was 9 mg kg^{-1} and 21 mg kg^{-1}, respectively. These

concentrations are very low compared to the limits for Cu and Zn in soils amended with municipal biosolids set by USEPA 503 regulation (750 mg kg^{-1} and 1400 mg kg^{-1} respectively), (Ashjaei et al., 2011; Miller et al., 2000; US EPA., 1993). Hence, it would take several years of repeated PL application to reach the high Cu and Zn concentrations considered in our experiment. Our results indicated that elevated levels of Zn and Cu had a significant inhibitory effect on the growth of AOB, AOA, total bacteria and total fungi and their functions. The inhibitory effects of the metals were dependent on ammonium level, which is the substrate for AO. At low ammonium level, the effects of the metals were less apparent as microbial growth and activity is limited by the substrate availability (Jia and Conrad, 2009; Okano et al., 2004; Verhamme et al., 2011). With increasing ammonium concentration, however, the effects of the metals were more apparent. In addition to ammonium, the effects of metals were dependent on time. The absence of their effects at earlier days could be due to the slow growth of AO and the potential drawback of using DNA (which includes both dead and living cells) in studying abundance of soil microbes. The measure of the transcriptional activity of the *amoA* gene would provide a better estimate of the short term changes (Liu et al., 2010).

The response of AOB and AOA to the metals was different. Zn had a stronger effect on AOA than AOB. The reverse was true for Cu. This could be attributed to cellular and physiological differences (such as cell size, membrane lipids, pathway of metabolism etc.) between the two group of organisms (Prosser and Nicol, 2012; Schauss et al., 2009). This difference in sensitivity can be exploited to use the organisms as indicators for the effect of metals in soils in a way that is specific to a given metal. Although the metal types and concentrations are not exactly comparable, results similar to our study on differential response of AO to metals had been reported in soils without PL application (Liu et al., 2010; Mertens et al., 2009; Mertens et al., 2010). Subrahmanyam

et al (2014) reported AOA (7.53 log copies g $soil^{-1}$) to be more abundant than AOB (5.98 log copies g $soil^{-1}$) in combined treatment As-70 mg kg^{-1} and Cu-500 mg kg^{-1} (high Cu concentration compared to our experiment) in acidic alfisols and a significant decrease in AOB and AOA abundance with metal additions.

A study by Li et al (2009) reported 10 to 89-fold decrease in AOA abundance at the Cu dose of 1600 mg kg^{-1} to 2400 mg kg^{-1} as compared to 107 to 232-fold decrease in AOB abundance. Similarly, (Frey et al., 2008; Stephen et al., 1999) found AOB to be more responsive than AOA in heavy metal contaminated soils without any difference in the abundance of AOB.

Similar to AOB and AOA, the growth of total bacteria and total fungi was negatively affected by elevated levels of Zn and Cu but to a lesser extent. This indicates that the effects of the metals were not limited to AO. The effects of both metals were more severe on the growth of total bacterial than total fungal. This is consistent with studies that have found fungi to be more tolerant than bacteria to environmental disturbances and heavy metal toxicity (Hiroki, 1992; Khan and Scullion, 2002; Müller et al., 2001). Extracellular and intracellular sequestration of metals are the important mechanisms that increase the tolerance of fungi to the presence of heavy metals (Anahid et al., 2011). Extracellular sequestration includes chelation and cell wall binding which prevents the entry of metal ions while intracellular sequestration is the binding of metals to proteins present in the cytosol.

The effects of the metal were also apparent on microbial function. The metals caused a decrease in net nitrification, which is a measure of activity of AO. The same was true with net N mineralization, which was used as a broad measure of the activity of the bacterial and fungal communities. This agrees with the *amoA* abundance data and is not surprising as studies have shown before that Zn and Cu causes inhibition of the ammonia monooxygenase enzyme in AO

and restricts ammonia oxidation (Radniecki and Ely, 2008). Oxidative stress is one of the important mechanism known to cause microbial inhibition by heavy metals (Stohs and Bagchi, 1995). Metals are also known to interfere with the nutrient assimilation by impairing the transporters present in the cellular membrane (Hong et al., 2012; Nies and Silver, 1995). The chemical reactivity of metal ions species and their solubility, transport, absorption/adsorption and complexation reactions greatly affect the metal toxicity on soil microbes (Stohs and Bagchi, 1995). There is a wide variation in the results reported in literature on the response of total bacteria and fungi and soil AO to the heavy metals present in soils. This is partly due to difficulty in measuring the critical metals concentrations for microbes (Giller et al., 1998) or exclusion of the effect of environmental factors. Soil organic matter, cation exchange capacity and pH are the important edaphic factors known to affect the adsorption of metals (Lighthart et al., 1983; Smolders et al., 2004).

To our knowledge, no earlier studies have attempted to determine the relative contributions of AOB and AOA in nitrification in metal contaminated soils. We used NP assay to that end. NP is a measure of the maximum capacity of nitrifiers to convert ammonium to nitrate (Fortuna et al., 2003) and is commonly used as an indicator of the size of the active nitrifying population in soils (Jenkins and Kemp, 1984). The results showed AOB to be functionally dominant than AOA under both metal treatments despite the fact that AOA were numerically dominant over AOB under all conditions. This is in agreement with previous studies that reported dominant role of AOB over AOA despite their abundance being lower than AOA abundance (Dai et al., 2013; Di et al., 2009; Di et al., 2010a; Jia and Conrad, 2009). Overall, both metals resulted in reduced NP rates. This is similar to the effect on net mineralization and is in agreement with previous studies (Li et al., 2009).

The significant correlations between the growth and function variables indicate the strong link between the two. This is an indication that microbial function is considerably dictated by microbial abundance. We suggest the need of further research to determine the changes in microbial community composition as several studies have shown AO to quickly adapt to metal stresses (Mertens et al., 2009; Mertens et al., 2010). Ruyters et al (2013) in a study conducted on grassland soils spiked with 1300 mg kg^{-1} to 2400 mg kg^{-1} of Zn showed that AOB play an important role in the recovery of nitrification and Zn tolerance.

Conclusions

Elevated levels of Zn and Cu caused decreases in abundance and function of AOA and AOB in soil with a history of PL application. The negative effects of the metals were apparent at high ammonium concentration over time. Despite the numerical dominance of AOA over AOB, AOB accounted for the bigger share of nitrification than AOA under both metals. Similarly, elevated levels of Zn and Cu negatively affected the abundance of total bacteria and fungi, indicating that the effect was not limited to AO. Some of the concentrations considered in the study were high (100 mg kg^{-1} and 200 mg kg^{-1} for Zn and 120 mg kg^{-1} for Cu) and would most likely be achieved after many year of PL application and yet they did not completely inhibit nitrification. The limited reduction in nitrification could actually be beneficial by slowing down nitrate formation and hence leaching in grasslands where PL is applied. This, however, has to be viewed in light of other environmental consequences of the metals in soil.

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Tables and Figures

Table 4.1. Basic soil properties and initial metal concentrations in long term poultry litter (PL) amended soil. All reported values have been averaged over two replicates.

Parameters	Values	Parameters	Values
NH_4^+-N (mg kg soil^{-1})	2.20	Iron†	30
NO_3^--N (mg kg soil^{-1})	21.5	Potassium†	240
Lime Buffering Capacity (ppm $CaCO_3$/ pH)	597	Magnesium†	406
Soil pH	6.0	Manganese†	20
Percent Base Saturation	84	Sodium†	18
Cation Exchange Capacity (meq 100g^{-1})	17	Phosphorus†	157
Soil Organic Carbon (g kg soil^{-1})	1.29	Zinc†	21
Calcium†	2087	Lead†	1.0
Copper †	9	Nickel†	1.0

†Mehlich 1 Extractable nutrients (mg kg^{-1})

Table 4.2: Primer sequences and thermal cycling conditions used for quantitative-polymerase chain reaction.

Name	Primers	Primer Sequences	Thermal Cycling Conditions	References
Bacterial *amoA*	amoA-1F amoA-2R	5'-GGG GTT TCT ACT GGTGGT-3' 5'-CCC CTC GGG AAA GCC TTC TTC-3'	95 ºC for 10 min, 40 cycles × (95 ºC, 60 sec; 57 ºC, 60 sec; 72 ºC, 3 min)	(Leininger et al., 2006; Rotthauwe et al., 1997)
Archaeal *amoA*	Archamo-AF Archamo-AR	5'-TTATGGTCTGGCTTAGACG-3' 5'-GCGGCCATCCATCTGTATGT-3'	95 ºC for 10 min, 40 cycles × (95 ºC, 60 sec; 56 ºC, 60 sec; 72 ºC, 3 min)	(Francis et al., 2005; Sher et al., 2012)
Total Bacteria (16S rDNA)	EUB 338 EUB 518	5'-ACTCCTACGGGAGGCAGCAG-3' 5'-ATTACCGCGGCTGCTGG-3'	95 ºC for 15 min, 40 cycles × (95 ºC, 60 sec; 53 ºC,60 sec; 72 ºC, 60 sec)	(Fierer et al., 2005)
Total Fungi (18S rDNA)	nu-SSU 0817 nu-SSU 1196	5'-TTAGCATGGAATAATRRAATAGGA-3' 5'-TCTGGACCTGGTGAGTTTCC-3'	94ºC for 10 min, 40 cycles × (94°C,60 sec; 56 ºC,60 sec; 72 ºC, 2min)	(Borneman and Hartin, 2000)

Table 4.3: Results of repeated measures of ANOVA for the effect of zinc levels at different ammonium levels on bacterial *amoA* copies (AOB), archaeal *amoA* copies (AOA), total bacterial and total fungal rDNA copy numbers, AOB: AOA, net N mineralization and net nitrification after 28 days of incubation.

Factor	Bacterial *amoA* Copies	Archaeal *amoA* Copies	AOB:AOA	Total Bacteria	Total Fungi	Net N Mineralization	Net Nitrification
Ammonium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Zinc	<0.001	<0.001	0.0017	0.0002	<0.001	<0.001	<0.001
Time	<0.001	<0.001	<0.001	<0.001	<0.001	---	---
Ammonium × Zinc	0.0315	0.0012	0.0012	ns	ns	ns	<0.001
Ammonium × Time	0.0023	<0.001	<0.001	0.0551	<0.001	---	---
Zinc × Time	<0.001	<0.001	0.0354	0.0304	0.00185	---	---

*Significant at p-value ≤ 0.05; ns - non-significant, --- (data not available)

Table 4.4: Results of repeated measures of ANOVA for the effect of copper application at different ammonium levels on bacterial *amoA* copies (AOB), archaeal *amoA* copies (AOA), total bacterial and total fungal rDNA copy numbers, Net N mineralization and Net nitrification after 28 days of incubation.

Factor	Bacterial *amoA* Copies	Archaeal *amoA* Copies	AOB:AOA	Total Bacteria	Total Fungi	Net N Mineralization	Net Nitrification
Ammonium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Copper	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	<0.001
Time	<0.001	<0.001	<0.001	<0.001	0.005	---	---
Ammonium × Copper	<0.001	0.002	0.009	ns	<0.001	0.041	<0.001
Ammonium × Time	<0.001	<0.001	<0.001	<0.001	<0.001	---	---
Copper × Time	<0.001	<0.001	0.006	<0.001	ns	---	---

*Significant at (p-value ≤0.05); ns - non-significant, --- (data not available)

Table 4.5: LS Means for bacterial *amoA* (AOB), archaeal *amoA* (AOA), total bacterial and total fungal rDNA copy numbers at day 7 in the zinc study; comparison is valid within one ammonium concentration. The overall averages over zinc or ammonium levels were calculated only for the variables that showed no interaction terms between the factors. All reported values have been averaged over three replicates.

Factors		*amoA* Abundance (log copies g soil^{-1})			16S/18S rDNA gene Abundance (log copies g soil^{-1})	
Ammonium	Zinc Level (mg kg^{-1})	Bacterial *amoA*	Archaeal *amoA*	AOB:AOA	Total Bacteria	Total Fungi
Low	21	6.99^{a}	7.64^{a}	0.22^{a}	9.96^{a}	7.90^{a}
	100	7.06^{a}	7.73^{a}	0.21^{a}	9.90^{a}	7.82^{a}
	250	6.93^{a}	7.72^{a}	0.16^{a}	10.00^{a}	7.79^{a}
Medium	21	7.07^{A}	7.88^{A}	0.16^{A}	10.10^{A}	8.20^{A}
	100	7.14^{A}	7.85^{A}	0.18^{A}	10.05^{A}	8.08^{A}
	250	7.10^{A}	7.78^{A}	0.23^{A}	9.92^{B}	8.16^{A}
High	21	7.20^{x}	7.99^{x}	0.16^{x}	10.31^{x}	8.29^{x}
	100	7.27^{x}	7.90^{x}	0.23^{x}	10.25^{x}	8.17^{y}
	250	7.20^{x}	7.89^{x}	0.24^{x}	10.27^{x}	8.25^{x}
Averages						
Ammonium	Low				9.95^{a}	7.84^{a}
	Medium				10.02^{a}	8.15^{ab}
	High				10.28^{b}	8.24^{b}
Zinc	21				10.12^{A}	8.13^{A}
	100				10.07^{A}	8.02^{A}
	250				10.06^{A}	8.07^{A}

Table 4.6: LS Means for bacterial *amoA* (AOB), archaeal *amoA* (AOA), total bacteria and total fungi copy numbers at day 28 in the zinc study; comparison is valid within each ammonia level. The overall averages over zinc or ammonium levels were calculated only for the variables that showed no interaction terms between the factors. All reported values have been averaged over three replicates.

Factors		*amoA* Abundance (log copies g soil^{-1})			16S/18S rDNA Abundance (log copies g soil^{-1})	
Ammonium	Zinc Level (mg kg^{-1})	Bacterial *amoA*	Archaeal *amoA*	AOB:AOA	Total Bacteria	Total Fungi
Low	21	6.60^{a}	7.36^{a}	0.18^{a}	9.65^{a}	7.75^{a}
	100	6.52^{a}	7.23^{b}	0.20^{a}	9.55^{b}	7.68^{ab}
	250	6.31^{b}	7.18^{b}	0.13^{a}	9.46^{c}	7.62^{b}
Medium	21	6.84^{A}	7.43^{A}	0.26^{A}	9.69^{A}	7.64^{A}
	100	6.82^{A}	7.08^{B}	0.58^{B}	9.79^{B}	7.51^{B}
	250	6.57^{B}	7.05^{B}	0.31^{A}	9.69^{A}	7.49^{B}
High	21	6.95^{x}	7.62^{x}	0.22^{x}	10.06^{x}	7.96^{x}
	100	6.69^{y}	7.34^{y}	0.23^{x}	10.07^{x}	7.74^{y}
	250	6.55^{z}	7.08^{z}	0.30^{x}	9.75^{y}	7.49^{z}
Averages						
Ammonium	Low				9.55^{a}	7.68^{a}
	Medium				9.72^{b}	7.55^{a}
	High				9.96^{c}	7.73^{a}
Zinc	21				9.80^{A}	7.78^{A}
	100				9.80^{A}	7.64^{AB}
	250				9.63^{B}	7.53^{B}

Table 4.7: LS Means for bacterial *amoA* (AOB), archaeal *amoA* (AOA), total bacterial and total fungal rDNA copy numbers at day 14 in the copper study; comparisons are valid within each ammonium level. The overall averages over copper or ammonium levels were calculated only for the variables that showed no interaction terms between the factors. All reported values have been averaged over three replicates.

Factors		*amoA* Abundance (log copies g soil^{-1})			16S/18S rDNA Abundance (log copies g soil^{-1})	
Ammonium	Copper Level (mg kg^{-1})	Bacterial *amoA*	Archaeal *amoA*	AOB:AOA	Total Bacteria	Total Fungi
Low	9	6.41^{a}	7.45^{a}	0.09^{a}	9.62^{a}	7.87^{a}
	20	6.69^{b}	7.52^{a}	0.12^{a}	9.54^{a}	7.72^{b}
	120	6.50^{a}	7.34^{b}	0.18^{b}	9.52^{a}	7.70^{b}
Medium	9	6.91^{A}	7.70^{A}	0.15^{A}	9.87^{A}	7.85^{A}
	20	7.19^{B}	7.77^{A}	0.21^{A}	9.79^{A}	7.83^{A}
	120	7.00^{A}	7.59^{B}	0.32^{B}	9.77^{A}	7.81^{A}
High	9	7.41^{x}	7.95^{x}	0.27^{x}	10.12^{x}	8.03^{x}
	20	7.69^{y}	8.02^{x}	0.37^{y}	10.04^{x}	7.62^{y}
	120	7.50^{x}	7.84^{y}	0.56^{z}	10.02^{x}	7.47^{z}
Averages						
Ammonium	Low				9.56^{a}	
	Medium				9.81^{b}	
	High				10.06^{b}	
Copper	9				9.87^{A}	
	20				9.79^{A}	
	120				9.77^{A}	

Table 4.8: LS Means for bacterial *amoA* (AOB) copies, archaeal *amoA* (AOA) copies, AOB: AOA ratio, total bacterial and total fungal rDNA copy numbers at day 28 in the copper study; comparisons are valid within each ammonium level. The overall averages over copper or ammonium levels were calculated only for the variables that showed no interaction terms between the factors. All reported values have been averaged over three replicates.

Factors		*amoA* Abundance (log copies g soil^{-1})			16S/18S rDNA gene Abundance (log copies g soil^{-1})	
Ammonium	Copper Level (mg kg^{-1})	Bacterial *amoA*	Archaeal *amoA*	AOB:AOA	Total Bacteria	Total Fungi
Low	9	6.06^{a}	7.42^{a}	0.02^{a}	9.64^{a}	7.90^{a}
	20	6.23^{b}	7.38^{a}	0.24^{b}	9.66^{a}	7.85^{a}
	120	6.20^{b}	7.30^{a}	0.19^{b}	9.39^{b}	7.80^{b}
Medium	9	6.55^{A}	7.45^{A}	0.12^{A}	9.71^{A}	7.90^{A}
	20	6.67^{B}	7.49^{A}	0.14^{A}	9.70^{A}	7.76^{B}
	120	6.37^{C}	7.37^{A}	0.29^{B}	9.58^{B}	7.65^{B}
High	9	7.08^{x}	7.92^{x}	0.16^{x}	9.84^{x}	7.71^{x}
	20	6.61^{y}	7.58^{y}	0.12^{x}	9.86^{x}	7.54^{y}
	120	6.39^{z}	7.50^{y}	0.06^{y}	9.59^{y}	7.27^{z}
Averages						
Ammonium	Low				9.56^{a}	
	Medium				9.66^{ab}	
	High				9.76^{b}	
Copper	9				9.73^{A}	
	20				9.74^{A}	
	120				9.52^{B}	

Table 4.9: LS Means for net N mineralization and net N nitrification after 28 days of incubation in the zinc study; comparisons are valid within each ammonium level. The overall averages over zinc or ammonium levels were calculated only for the variables that showed no interaction terms between the factors. All reported values have been averaged over three replicates.

Ammonium	Zinc Level (mg kg^{-1})	Net N Mineralization (mg kg $soil^{-1}$)	Net N Nitrification (mg kg $soil^{-1}$)
Low	21	55.49^{a}	43.22^{a}
	100	52.49^{a}	47.82^{a}
	250	50.70^{a}	48.45^{a}
Medium	21	103.37^{A}	136.29^{A}
	100	94.37^{AB}	110.29^{AB}
	250	82.28^{B}	98.61^{B}
High	21	129.96^{x}	158.65^{x}
	100	117.96^{y}	124.65^{y}
	250	111.51^{z}	84.94^{z}
Averages			
Low		52.89^{a}	
Medium		93.34^{b}	
High		119.81^{c}	
21		96.27^{A}	
100		88.27^{AB}	
250		81.50^{B}	

Table 4.10: LS Means for net N mineralization and net N nitrification after 28 days of incubation in the copper study; comparisons are valid within each ammonium level. All reported values have been averaged over three replicates.

Ammonium	Copper Level ($mg\ kg^{-1}$)	Net N Mineralization ($mg\ kg\ soil^{-1}$)	Net Nitrification ($mg\ kg\ soil^{-1}$)
Low	9	50.96^{a}	48.65^{a}
	20	47.96^{a}	45.65^{a}
	120	47.27^{a}	46.79^{a}
Medium	9	120.37^{A}	146.13^{A}
	20	111.37^{A}	145.46^{A}
	120	108.46^{A}	140.13^{A}
High	9	112.46^{x}	161.60^{x}
	20	90.46^{y}	134.85^{y}
	120	89.66^{y}	118.47^{z}

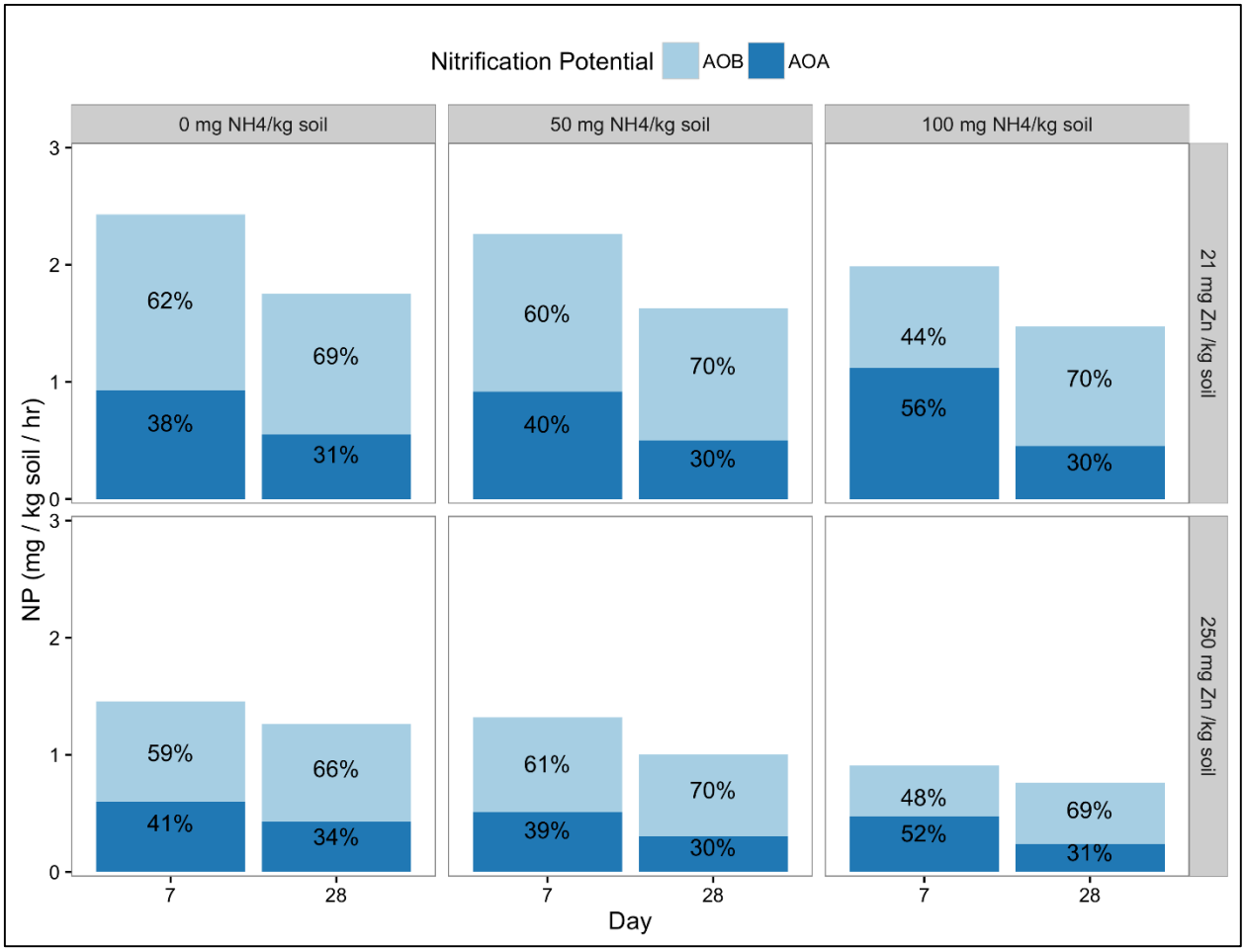

Figure 4.1: Soil nitrification potential (NP) (mg kg soil^{-1} hr^{-1}) in Zn21 mg kg^{-1} and Zn250 mg kg^{-1} treatments at 7 and 28 days of incubation with 1-Octyne application. Light blue portion denotes the contribution of AOB and dark blue as AOA contribution to soil NP.

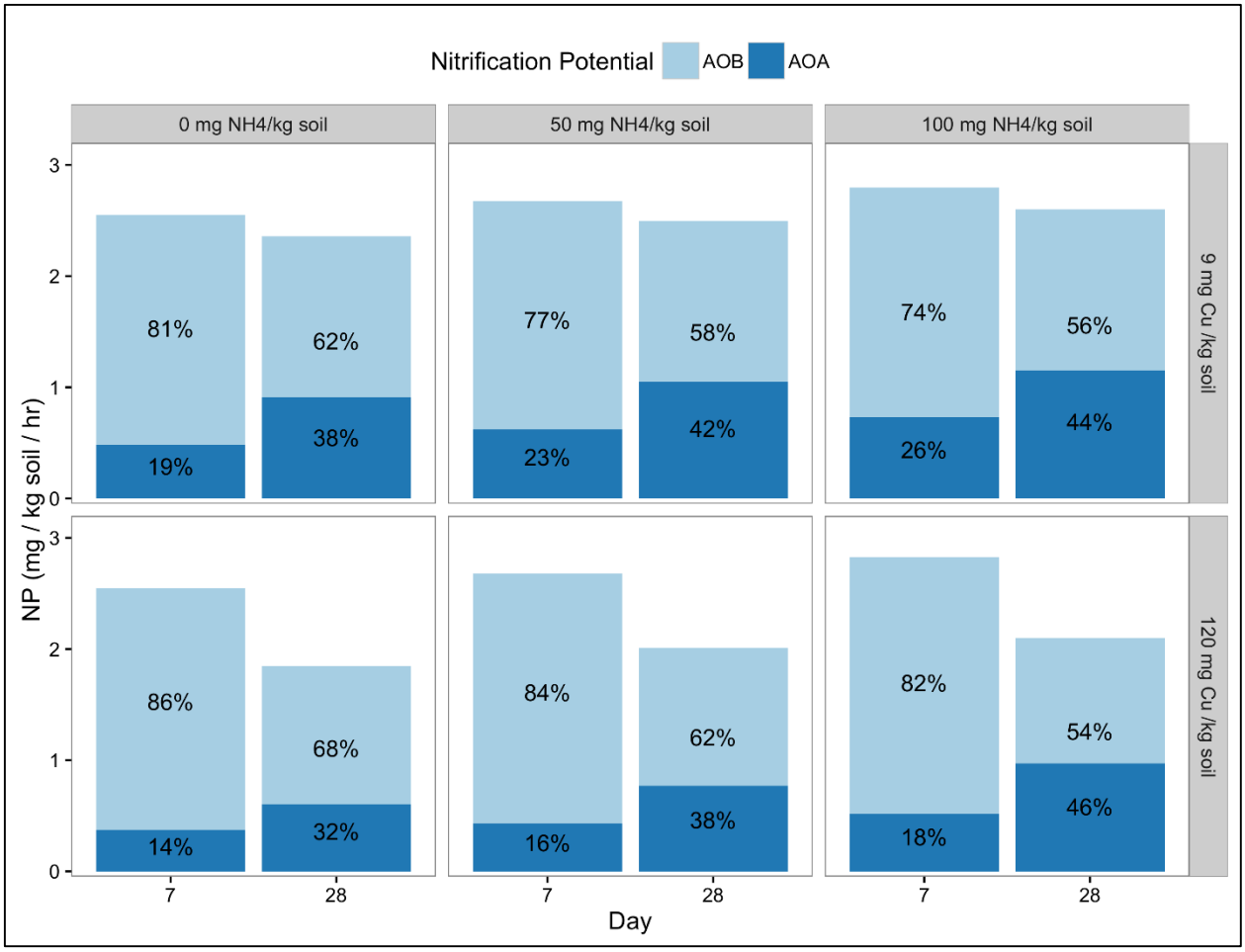

Figure 4.2: Soil nitrification potential (NP) (mg kg soil^{-1} hr^{-1}) in Cu9 mg kg^{-1} and Cu120 mg kg^{-1} treatments at 7 and 28 days of incubation with 1-Octyne application. Light blue portion denotes the contribution of AOB and dark blue as AOA contribution to soil NP.

Table 4.11: Repeated measures of ANOVA for nitrification potential with 1-Octyne to separate the contributions of ammonia oxidizing bacteria (AOB) and archaea (AOA) in zinc and copper amended soils.

Factors	p-value	Factors	p-value
Ammonium	0.007	Ammonium	ns
Zinc	<0.001	Copper	ns
Time	0.002	Time	0.007
Ammonium × Zinc	ns	Ammonium × Copper	0.010
Ammonium × Time	0.014	Ammonium × Time	ns
Zinc × Time	0.030	Copper × Time	0.027

*Significant at (p-value ≤0.05), 'ns' (non-significant)

Table 4.12: Pearson correlation coefficients (*r*) between different response variables in zinc treated soil after 28 days of incubation.

	Ammonium	Zinc level	Bacterial *amoA* Copies	Archaeal *amoA* Copies	Total Fungi	Total Bacteria	Nitrification Potential	Net N Mineralization
Bacterial *amoA* Copies	0.58	-0.72*						
Archaeal *amoA* Copies	0.14	-0.80*	0.58					
Total Fungi	0.38	-0.70*	0.43	0.93*				
Total Bacteria	0.35	-0.37	0.72*	0.49	0.49			
Nitrification Potential	0.25	-0.72*	0.65*	0.67*	0.60	0.40		
Net N Mineralization	0.96*	-0.21	0.75*	0.12	0.14	0.51	0.41	
Net Nitrification	0.79*	-0.36	0.83*	0.36	0.11	0.63	0.06	0.91*

'*'Significant at p-value ≤ 0.05

Table 4.13: Pearson correlation coefficients (*r*) between different response variables in copper treated soil after 28 days of incubation.

	Ammonium	Copper Level	Bacterial *amoA* Copies	Archaeal *amoA* Copies	Total Fungi	Total Bacteria	Nitrification Potential	Net N Mineralization
Bacterial *amoA* Copies	0.34	-0.29						
Archaeal *amoA* Copies	0.06	-0.58*	0.06					
Total Fungi	0.18	-0.53	0.05	0.33				
Total Bacteria	0.32	-0.74*	0.21	0.67*	0.37			
Nitrification Potential	0.05	-0.87*	0.36	0.63*	0.48	0.64*		
Net N Mineralization	0.70*	-0.41	0.37	0.41	0.53	0.62*	0.22	
Net Nitrification	0.82*	-0.34	0.35	0.25	0.50	0.55	0.12	0.98*

'*'Significant at p-value ≤ 0.05

SUMMARY AND CONCLUSION

This work was conducted to determine the role of soil ammonia oxidizers (AO) in nitrification in pasture soil with a history of poultry litter (PL) application. PL has a high content of N, P, K, Ca, Mg and other plant nutrients and its continuous application in soils improves soil fertility. Previous studies have focused mainly on changes in transformation rates and trace metal content in the PL amended soils and not on the role of AO which mediate these processes. This research topic is of particular importance to Georgia because of its leading position in poultry production in the nation.

Our results showed that significant changes in abundance, function and community composition of soil AO were produced with long term PL application. Greater abundance, function and diversity was observed in PL applied plots compared to control plots with urea ammonium nitrate despite the significant accumulation of some trace metals. AOA abundance did not change significantly while AOB abundance increased significantly, after 2 years of discontinuation of PL application. Function and diversity of AO decreased after stopping PL application. AOB were functionally more important than AOA in regard to nitrification as suggested by correlation analysis.

To further determine the relative contribution of AOB and AOA in nitrification, we carried out an incubation experiment using dicyandiamide. Significant differences in AOB and AOA contribution in nitrification potential (NP) were observed with time. AOA played a dominant role in NP in both PL and unamended control (CL) soil accounting for $\geq$ 70% at the start of the

incubation. Over time, the AOB contribution to NP increased to ≥ 50% in the PL soil at the high ammonium level. However, AOA remained to be the more dominant players in unamended CL soil. In net nitrification, the contribution of AOA was greater in unamended CL soil at both the ammonium levels and in PL soil at the low ammonium level. In PL soil, AOB contributed to ≥ 50% in net nitrification at the high ammonium level. This dominance of AOB in PL and AOA in unamended CL soil was further confirmed with a correlation analysis. The ability of AO to efficiently utilize the varying form of nitrogen (N) present in PL and unamended CL soil is the major factor to affect the differential contribution of AOB and AOA in nitrification. Distinct communities of AO were present in PL and unamended CL soil as shown by the α- and β-diversity indices. The differences in abundance and community composition of AO in PL and unamended CL soil resulted in variations in function (nitrification) by AOB and AOA.

Long-term PL application is shown to cause trace metals accumulation in soils. Result of the incubation study showed that Zinc (Zn) and Copper (Cu) addition at the elevated levels resulted in decrease in abundance and function of AO in soils with a history of PL application. The negative effects of metals were more apparent with time and at the high ammonium level. AOB played a greater role in nitrification in Zn and Cu amended soil regardless of higher AOA abundance. The inhibitory effects of Zn and Cu was also observed on the general microbial community as reflected by the decreased abundance of total bacteria and fungi with elevated levels of the two metals. Some of the concentrations considered in the study were high (100 and 250 ppm for Zn and 120 ppm for Cu) and did not completely inhibit nitrification. These concentrations are way below the limits set by USEPA 503 regulation for Zn and Cu in soils amended with municipal biosolids. Hence, these levels would most likely be achieved after many years of PL application. The negative effect on nitrification by trace metals can help to reduce the losses of nitrate through

leaching from PL applied grasslands keeping in mind the environmental consequences of the metals in soil.

This research work emphasizes that proper understanding of the transformation of nutrients in PL and its impact on AO is required to avoid undesirable environmental consequences such as nitrate leaching and denitrification. To improve N use efficiency in PL amended soil, we should adopt management practices aimed at the right group of organism that mainly contribute in nitrification.