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Bacterial and Archaeal Nitrifier Communities after Seven Years Surface Ground Cover and Nutrient Management in an Orchard Soil

Mashael Albalawi
University of Arkansas, Fayetteville

Bacterial and Archaeal Nitrifier Communities after Seven Years Surface Ground Cover and Nutrient Management in an Orchard Soil

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Mashael Albalawi
University of Tabuk
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University of Arkansas

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_____
Dr. Mary Savin
Thesis Director

_____
Dr. Kristen Gibson
Committee Member

_____
Dr. Burt Bluhm
Committee Member

Abstract

Nitrification is oxidation of ammonia to nitrite and subsequently to nitrate. The rate limiting step of nitrification, ammonia oxidation to nitrite, was thought for many years to be carried out primarily by ammonia oxidizing bacteria (AOB). However, using molecular techniques and investigating the *amoA* gene, ammonia-oxidizing archaea (AOA) were discovered. Understanding how long-term application of surface ground covers and organic fertilizers to perennial systems affect microbial communities is critical for sustainable soil management. An organically managed experimental apple orchard soil received seven years (2006 – 2013) of annual, surface applications of poultry litter or commercial fertilizer compared to no fertilizer combined with compost (C), wood chips (WC), shredded paper (P), or mow-and-blow (MB) ground cover mulches. This research investigates AOB and AOA community composition based on the *amoA* gene in the soil surface as affected by treatments from 2007, 2009, and 2013, and as related to soil properties. The AOB community exhibited low and variable diversity investigated by using PCR-denaturant gradient gel electrophoresis, and richness ranged between 0.67 and 6.0. The AOB diversity did not consistently change over time and clustering did not distinctly separate communities. The log of the AOB abundance measured using qPCR of the *amoA* gene fragment ranged from zero to 4.3 with smallest numerical abundances in two of the paper treatments and the largest in compost with poultry litter. The AOA richness ranged from zero to 6.0 across all treatments and years. The AOA community composition clustering suggested that the community shifted from 2009 to 2013. The AOA diversity increased from 2009 to 2013 in compost and wood chip treatments, but diversity decreased in paper with no or poultry litter fertilizer, and in mow-and-blow with poultry litter from 2009 to 2013. Equitability increased with time in compost, decreased in wood chip and

paper treatments, and depended on fertilizer in mow-and-blow. Compost supplied the greatest organic matter and dissolved organic N (DON) but the smallest ammonium-N concentration. Poultry litter increased both nitrate and DON. The AOA and AOB were correlated to soil properties, but correlations did not indicate strong linear relationships between communities and particular soil variables.

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Dedication

This thesis is dedicated to my great parents, Aziza and Fahad.

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1. Introduction

1.1. Background

Microbial communities play critical roles in the soil environment. They are essential players in maintaining soil functions, including toxin degradation, recycling of organic matter, promotion of plant growth through plant root symbiosis, and importantly they cycle nutrients such as carbon, nitrogen, phosphorus, sulfur, and micronutrients. Advances in molecular techniques have raised questions about our understanding of biogeochemical cycles such as the nitrogen (N) cycle. The nitrogen cycle is important for regulating availability of the plant-growth limiting nutrient in agriculture, but it can lead to serious environmental hazards if managed poorly. Nitrification, the progressive oxidation of ammonium to nitrite followed by nitrate, a negatively charged form of available N, results in a more mobile form for plant uptake but also in a more leachable form because most soils carry a net negative charge (Brady and Weil, 2016). Nitrate may be denitrified, which can lead to emission of the greenhouse gas nitrous oxide. Leaching and nitrous oxide emission both result in losses of N from the soil and can lead to serious pollution problems (Brady and Weil, 2016).

Ammonia oxidizing bacteria (AOB) were isolated in pure culture in 1890 by Sergei Winogradsky. Nitrifiers are small fraction of the microbial communities in soil (Hermansson and Lindgren, 2001) The AOB belong to two major bacterial classes: Betaproteobacteria (*Nitrosomonas* spp. and *Nitrosospira* spp.) and Gammaproteobacteria (*Nitrosococcus* spp.) (Nicolaisen and Ramsing, 2002). For almost a century, it was thought that ammonia oxidization, the rate limiting step in nitrification, was carried out only by these bacterial chemolithoautotrophs. However, heterotrophic nitrification is performed by fungi and

heterotrophic bacteria (Verstraete and Focht , 1977), although heterotophic nitrification is not linked to deriving energy from ammonium.

In the late 1990's, archaea that oxidize ammonia were discovered (DeLong, 1998). Archaea in soil were found to possess a homologous gene to that in bacterial nitrifiers, *amoA*, a gene that encodes for the subunit A of ammonia monooxygenase (Treusch et al., 2005). In the same year as Treusch et al.'s publication (2005), a marine ammonia oxidizing archaea (AOA), *Nitrosopumilus maritimus,* was isolated in pure culture (Könneke *et al*., 2005). This archaeon is able to oxidize ammonia in the absence of organic carbon and at a rate similar to the AOB. Later, *Nitrososphaera viennensis* was isolated from soil (Tourna et al., 2011). This archaeon has chemolithoautotrophic growth and also can grow when pyruvate is applied at low concentration (up to 0.1 mM) (Tourna et al., 2011). Most recently, Lehtovirta-Morley et al. (2016) isolated *Nitrosocosmicus franklandus,* an archaeon from arable soil with pH 7.5. This archaeon has a higher tolerance to nitrate and ammonia concentrations than typical AOA and is similar to soil AOB as growth was possible at 100 mM ammonium (NH_4^+).

Ammonia oxidizing archaea are present in soil, sediment, and water (Morimoto et al., 2011). What is less clear is how management impacts the contributions of AOA and AOB in soil under different management schemes. Leininger et al. (2006) showed via qPCR that archaeal *amoA* is 1.5 - 230 times more abundant than bacterial *amoA* in pristine and agricultural surface soils. In the other studies, AOB were more abundant in grassland soils than AOA (Di et al., 2009). Di et al. (2009) observed a 3.2 to 10.4-fold increase in AOB numbers after addition of urine**.** The AOA and AOB likely respond differently to environmental factors such as pH (Nicol et al., 2008), temperature (Tourna et al., 2008), and ammonia concentrations, which may suggest

distinct niches of AOA and AOB. The AOA have been more abundant in acidic environments (Nicol et al., 2008), while AOB appear to prefer neutral and alkaline soils (Shen al., 2008).

Ammonia concentration is a key factor regulating the community structure of AOA and AOB. In general, the AOB seem to thrive at high ammonia concentrations while AOA thrive in low ammonia concentrations (Martens-Habbena et al., 2009; Tourna et al., 2011; Verhamme et al., 2011). In most studies the AOB, *Nitrosomonas europaea*, do not grow at small ammonia concentrations. The optimal ammonia concentration for their growth is in medium containing 1–5 mM while the mesophilic crenarchaeon '*Candidatus Nitrosopumilus maritimus*' strain SCM, for example, has a 200-fold lower half-saturation constant K_m value, 0.133 µM ammonia, than AOB isolates (Martens-Habbena et al., 2009),

In addition, how ammonia becomes available, whether through mineralization or applied as inorganic fertilizer, may affect AOA and AOB differently. Ammonia available at small concentrations derived from mineralization of soil organic matter may stimulate AOA, while inorganic fertilization may stimulate growth of AOB (Jia and Conrad, 2009; Kelly et al., 2011). Organic amendments that release inorganic N at metered rates during decomposition may stimulate AOA (Kelly et al., 2011). Unlike synthetic nitrogen, organic amendments need microorganisms, time, temperature, and moisture to decompose and mineralize N (Brady and Weil, 2016).

Organic amendments applied to the soil surface as ground covers and organic nutrient sources are used to enhance soil fertility as they protect the soil surface, add organic matter, and contain a combination of nutrients released upon decomposition (Brady and Weil, 2016). Organic amendments can be applied in many forms and include waste products such as poultry litter, composted yard waste, shredded paper, or plants residues. Organic amendments may be

applied to the ground surface or tilled into the soil, and the no-till strategy employed in an orchard ensures that resides are maintained on the surface (Ismail et al., 1994). As a result, N is expected to be available to plants in relatively smaller amounts as compared to inorganic fertilization; however, if organic amendments are poorly managed, N losses might occur (Pimentel et al., 2005). The increased demand for organic fruit along with efforts to reduce greenhouse gasses in the environment has drawn attention to organic management (Kramer et al., 2006), and more data about the effects of management on the activity and diversity of AOA and AOB are needed to clarify niches of ammonia oxidizers in contributing to the processing and fate of N in organic orchards, especially in the Mid-Southern U.S.

1.2. Objectives and Hypotheses

Objectives

There were three objectives of this thesis research. The first objective was to determine AOA and AOB abundance in soil from an organically managed apple orchard that received seven years of annual applications of twelve combinations of three organic fertilizers (poultry litter, commercial, or no fertilizer control) and four ground cover (compost, wood chips, paper mulch, or mow-n-blow) treatments.

A second objective of this research was to determine AOA and AOB diversity in soil from an organically managed apple orchard that received seven years of annual applications of twelve combinations of organic fertilizer (poultry litter, commercial, or no fertilizer) and four ground cover (compost, wood chips, paper mulch, or mow-n-blow) treatments.

A third objective was to determine whether AOB and AOA were related to soil properties in the organic orchard soil.

Hypotheses

It was hypothesized that repeated annual application of ground covers and/or organic nutrients would change the AOA and AOB community abundances measured after seven years of annual treatment applications. The AOB outcompete AOA at high N concentrations. However, AOA may have greater affinity for N at low concentrations and thus organic amendments may favor AOA if N is slowly released. It was hypothesized that AOB would dominate over AOA for the treatments that resulted in large available ammonium concentrations.

It was hypothesized that repeated annual application of ground covers and/or organic nutrients would change the AOA and AOB community diversity. Increasing organic matter may increase microbial diversity. However, treatments supplying readily decomposable organic matter or that lead to abundant supply of ammonium for nitrification may select for particular populations and thus decrease diversity.

Finally, it was hypothesized that changes in soil properties would be related to AOA and AOB community size and diversity, which would change in response to organic matter as indicated by dissolved organic carbon (C) and N, ammonium concentration, and soil pH.

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2. Literature Review

2.1. Organic Management

The increased demand for organic fruit along with efforts to reduce greenhouse gasses in the environment has drawn attention to organic management (Kramer et al., 2006). The United States Department of Agriculture (USDA) defines organic agriculture as production by approved methods which "integrate cultural, biological, and mechanical practices that foster cycling of resources, promote ecological balance, and conserve biodiversity. Synthetic fertilizers, sewage sludge, irradiation, and genetic engineering may not be used" (USDA, 2015, P. 1). One of the goals of organic management is to build or at least maintain nutrient reserves and promote nutrient recycling to reduce the use of external inputs. Maintaining soil fertility that ensures optimal crop yields is a major challenge in organic management, which excludes the use of synthetic chemicals for pest control and fertilizers.

Management practices employed in organic production include use of tillage, ground cover mulches and incorporation of green manure crops, livestock manure and organic fertilizers, among other practices. Ground covers and organic nutrient sources are used to enhance soil fertility as they contain a combination of nutrients such as nitrogen, potassium, and phosphorus that can be slowly released upon decomposition. In addition, no-till strategy ensures that resides are maintained on the surface, which maintain the nutrient content (Ismail et al., 1994; Johnson et al., 2005).

Organic mulch amendments influence soil biological, chemical, and physical properties. For example, compost applications significantly increase soil organic C, microbial activity, and water content (Brown and Cotton, 2011). Neilsen et al. (2003) studied the effect of adding organic mulches such as compost, paper and hay in an apple orchard and reported an increase in

soil microbial activity and N turnover which led to greater N fertilizer-use efficiency. Similarly, Leon et al. (2006) studied the impact of fresh or composted paper mill residual by-products on soil total carbon and soil moisture. There was a positive correlation of total C and soil moisture content. Water holding capacity increased with increasing soil C. The authors noted that this positive change promotes healthier plants as there was more available water, and it suppressed a common snap bean root rot.

Soil management impacts N cycling. Poudel et al. (2002) compared the effect of organic management utilizing cover crops and composted animal manure to conventional systems on N pools. While the mineralization potential was 112% greater in the organic system compared to the conventional system, the rate of mineralization was 100% higher in the conventional system. The higher concentration of N did not release rapidly. This indicated a reduced risk of nitrate (NO_3^-)–N leaching and groundwater pollution. Kramer et al. (2006) compared denitrification potential in soil from organic, integrated, and conventional management systems. Greater organic matter (OM), denitrification potential, denitrification efficiency, and microbial activity were observed under organic management. More nitrate leaching occurred under conventional management while nitrous oxide (N_2O) emissions increased with organic management. Denitrification results in a loss of gaseous N. Reduction to N_2 as the end product is critical to decrease the environmental hazards from N_2O emissions.

Organic management aims to provide soil with N required for optimum plant growth without exceeding plant growth requirements so as not promote N losses. Pimentel et al. (2005) compared nitrate leaching in two organic plots compared to conventional management. Nitrate leaching was similar or higher in the organic plots than in the conventional plot. The higher N losses were caused by the amount of the organic legumes that exceeded the N demand during

winter rains. Thus, organic management is not always more efficient in N cycling and N retention.

2.2. Nitrification

Nitrification is oxidation of ammonia to nitrite and subsequently to nitrate. The conversion of ammonium (positively charged ion) into a more mobile form of available nitrogen for plants (negatively charged ion) also puts nitrogen in a form that can be leached from soil (Mobarry et al., 1996) or denitrified, emitting the greenhouse gas nitrous oxide. Both processes result in loss of N from the soil and can lead to serious pollution problems.

The second step of nitrification, conversion of nitrite (NO_2^-) to nitrate (NO_3^-) is carried out by nitrite-oxidizing bacteria (NOB). The oxidation of ammonia is considered to be the rate-limiting step in the process of nitrification. Nitrite oxidation is rapid; therefore, its concentration in soil tends to be small. There are different organisms that govern the nitrification process. Chemolithoautotrophic nitrifying bacteria, which rely on inorganic nitrogen as their energy source, carry out autotrophic nitrification. In addition, heterotrophic nitrification is performed by fungi and heterotrophic bacteria (Pedersen et al., 1999).

For a long time, ammonia oxidation was thought to be carried out only by the ammonia oxidizing bacteria (AOB). A marine archaeon, *Nitrosopumilus maritimus,* an ammonia oxidizing archaeon (AOA), was isolated in 2005 (Könneke et al., 2005). The contribution of AOA and AOB to nitrification is investigated through molecular techniques often targeting detection of *amoA*, a gene that encodes for the subunit A of ammonia monooxygenase. From use of molecular analyses, it has been discovered that ammonia oxidizing archaea are abundant in different environments, such as soil, sediment and water (Morimoto et al., 2011). What is less

clear is how management impacts the contributions of AOA and AOB to ammonia oxidation in soil.

2.3. AOB and AOA

There are conflicting results about the dominance of AOA an AOB in soil. Leininger et al. (2006) examined 12 soils from different climates under different management practices. The AOA copy number was 3,000-fold more numerous than the AOB copy number. Similarly, He et al. (2007) investigated abundance and composition of AOB and AOA in agriculturally managed ferrosols that had received organic manure, remained fallow, or received a combination of N, P, and K for 16 years in China. The AOA genes were more numerous than AOB, with the AOA : AOB ratio ranging from 1.02 to 12.36 in a soil at a depth of 0 – 20 cm; although, the community composition of AOA varied among treatments.

Although AOA may be more abundant in soil than AOB, the contribution of AOA to nitrification is not well understood. Jia and Conrad (2009) reported that AOB is functionally more predominant than AOA, although AOA is more abundant. In that study, nitrification was stimulated by ammonium and was inhibited by acetylene. The changes in nitrification activity was correlated with AOB, but not with AOA. In contrast, Bustamante et al. (2012) studied the effects of adding water and drying on semiarid soil. Water was added to reach 60% of water holding capacity (WHC) which increased the nitrate content in the rewetted soil microcosm. The community structure of AOA, revealed in terminal restriction fragment length polymorphism (TRFLP) profiles, changed slightly while the community structure of AOB did not change. The abundance of AOA and AOB were determined by the Most Probable Number coupled to

Polymerase Chain Reaction (MPN-PCR). Adding water increased the abundance of AOA, but the abundance of AOB did not change.

Soil pH is another factor that affects nitrification by impacting the production and availability of substrates (Kemmitt et al., 2006). In pure cultures, the optimal growth of autotrophic nitrification ranges from 7.0 to 8.5. Studies were conducted to investigate if AOA are responsible for nitrification in acidic soil. Nicol et al. (2008) observed a change in the AOA and AOB community structures across a soil pH gradient (4.9–7.5). There was a positive correlation between nitrate concentration and *amoA* gene abundance of AOA in very acidic soil (pH < 4.5), indicating that AOA are dominant in acidic soil.

Gubry-Rangin et al. (2017) studied the influence of pH on the temperature range of the AOA activity. The ammonification, net nitrification, and AOA abundance and diversity was studied in soil with pH ranging from 3.7 to 7.5 and incubated at 20, 30 or 40 °C. Net nitrification was higher at 20 or 30 and was not detectable at 40 °C. The community composition was different, with variations in optimal temperature between soils when incubated at 20 or 30 °C. A qPCR analysis of the *amoA* gene indicated that archaea were more abundant at 20 °C in the acidic soils (pH 3.7). However, they were more abundant at 30 °C soil in neutral soil (pH 7.5). The authors suggest that soil pH has a great impact on AOA physiology such as temperature optima.

Temperature influences nitrification rate and AOB and AOA community composition. The optimum temperature for nitrification activity ranges from 10°C to 30°C (Stark and Firestone, 1996**).** Tourna et al. (2008) studied the influence of temperature on the abundance and activity of AOA and AOB in soil microcosms. The soil was collected from the top 10 cm of an agricultural field in which pH was 7.0. The soil was incubated at different temperatures ranging

from 10-30 °C. The transcription of 16S rRNA and *amoA* genes was measured. Tourna et al. (2008) suggested that AOA was solely responsible for nitrification because there was a significant change in the abundance and activity of the AOA but not the AOB in the soil during nitrification activity. Similarly, Offre et al. (2009) found that AOA was responsible for nitrification at 30 °C in soil microcosms obtained from the top 10 cm of an agricultural field. The researchers used qPCR to assess the abundance of the *amoA* gene and DGGE to analyze the diversity of *amoA* genes. During nitrification activity, the abundance of AOB slightly decreased and the diversity did not change; however, the abundance and diversity of AOA changed. The correlation between the nitrification and AOA growth, but not AOB, led the authors to conclude that AOA dominate nitrification in these soil microcosms. In addition, adding acetylene suppressed both nitrification and AOA growth which indicated that AOA performed ammonia oxidation.

Tourna et al. (2011) isolated *Nitrososphaera viennensis*, which is able to consume greater concentrations of ammonia than *Nitrosopumilus maritimus*, the marine isolated archaeon. Even though this organism has a higher tolerance to ammonia concentration, its ability to tolerate ammonia is significantly less than the ammonia concentrations that AOB are able to tolerate. Ouyang et al. (2017) studied the effects of contrasting soil management, conventional and organic N management, on AOA and AOB responses to temperature and ammonium substrate concentration. Four treatments, control (no fertilizers), ammonium sulfate at two rates and compost, were applied on a cornfield for four years. A specific inhibitor against AOB, 1-octyne, was used to distinguish between AOA and AOB contributions to nitrification in slurry assays. The AOB comprised greater than 90% of the nitrifying community at a concentration of 1.0 mM ammonium, but AOB nitrified less than 50% of the ammonium. The AOA contributed least at

5 °C and increased to carrying out 100% of ammonia oxidation at 50 °C. Kinetic analysis showed that AOB maximum nitrification activity (V_{max}) ranged from 0.32 to 4.8 mmol N $kg^{-1}d^{-1}$ with a saturation constant (K_m) ranging from 14–160 μM ammonium; greater values occurred in parameters in treatments with ammonium sulfate. The AOA maximum nitrification activity (V_{max}) ranged from 0.24 - 4.28 mmol N $kg^{-1}d^{-1}$ with no effects of the treatments. The niche specializations of AOB and AOA need further study to elucidate.

2.4. Ammonia oxidation in perennial versus annual systems

There are important differences between perennial and annual plants. Perennial plants are long-lived plants. Their root systems are extensive and deep in the soil allowing them to have access to the nutrients and water to which annual plants do not have access. Their extensive root systems add many benefits to the soil. For example, perennial systems are effective in building organic matter, reducing erosion (Syswerda et al., 2012), and reducing nitrate leaching (Culman et al., 2013). In addition, stimulating organic matter input may decrease decomposition, thus increasing soil C (Kell, 2011). On the other hand, annual plants are short-lived plants that need to be planted every year. Unlike perennial plants, annual plants tend to have shallow root systems that do not allow access to the nutrients that lie deeper in the soil.

Mao et al. (2013) studied the impact of three types of bioenergy crops on N, AOA, and AOB. The three crops were maize, switchgrass and *Miscanthus* in seven sites that form a climatic gradient of temperature and precipitation. In this study, the variation between sites was greater than the variation between plants. In addition, the microbial communities under each crop in all sites did not assemble on a typical species assemblage, and *amoA* genes were significantly different between the crops at the different sites. Mao et al. (2013) also observed that the main difference across all sites was between the maize and the perennial grasses:

switchgrass and miscanthus, even though the site variation was greater than crop variation. The nitrification functional genes evaluated by qPCR did not vary significantly among sites. The abundance of AOA was positively correlated with total N and C. However, no correlation was observed with ammonia or nitrate.

Liu et al. (2017) studied the effects of fertilization and land use conversion from rice paddies to citrus orchards on the abundance and community structure of AOA and AOB on an acidic red soil of southern China. The community structure was measured by T-RFLP, cloning and sequence analysis, and the abundance was measured using qPCR. The AOA abundance was increased by land use conversion, but AOB did not change. Meanwhile, land use conversion caused distinct variations in the soil AOA and AOB communities. However, fertilizers affected AOB community in both rice paddies and citrus orchards, and affected the AOA community only in the citrus orchards. Based on phylogenetic analyses of *amo*A genes, AOB were comprised predominately of *Nitrosospira.*

2.5. N addition, AOB, and AOA

Many studies indicate that AOA dominate over AOB in abundance in soil (Leininger et al., 2006; Offre et al., 2009; Verhame et al., 2011). However, soil management can affect the abundance and diversity of AOB and AOA, and this could be because of the differences in the soil nutrient concentrations. Agricultural amendments may affect AOA and AOB differently. Ammonia-N available at small concentrations derived from mineralization of soil OM may stimulate AOA, while inorganic fertilization may stimulate AOB as the concentration of N is readily available. Organic amendments are slowly decomposing, which may stimulate AOA. There are not much data about the effects of management on the activity and diversity of AOA and AOB. Furthermore, most studies have been conducted in a short period of time, which is not

enough to understand the changes that occur to the microbial community from repeated amendments to soil (McCalmont, 2014).

Fan et al. (2011) studied the effects of different inorganic and organic fertilizer treatments in soil in China on the potential nitrification rate and the community structure of AOA and AOB. The potential nitrification rate increased with the length of organic treatment. All the treatments reduced the abundance of AOA to result in a six- to 60-fold reduction in AOA: AOB ratio. Kelly et al. (2011) studied the effects of adding biosolids or synthetic fertilizer to corn fields after three years of applications. The nitrification rate correlated with the *amoA* gene copy number. Application of biosolids at 27 Mg hectare^{-1} year^{-1} increased AOA abundance while there was no change in AOB, but synthetic fertilization did not affect AOA abundance. However, AOA were more abundant in all the treatments.

Segal et al. (2017) studied the impact of tillage and fertilizer on AOA and AOB communities and overall microbial communities. Soil samples were collected from a long-term rainfed, continuous maize field. No-till or disk tillage and N fertilizers (ammonium nitrate) at different rates were applied. Real-time qPCR was used to assess AOA and AOB abundance and fatty acid methyl esters (FAME) was used to study the microbial community change. The AOA were more abundant than AOB at abundances of 10^8 versus 10^4 g^{-1} soil, respectively. Overall, the microbial community was significantly affected by sample date, tillage, and N rate. The AOB were lower in the disk tillage plots, and a moderate correlation ($r = 0.423$) between AOB and N rate was observed. However, tillage did not affect the AOA abundance, and a moderate correlation ($r = 0.552$) was observed between AOA and water-filled pore space.

The addition of inorganic N may stimulate AOB. Di et al (2010) examined the AOA and AOB in grazed dairy grasslands soils in three locations in New Zealand. The AOA and AOB

were characterized in relation to soil depth with different N availability. The bacterial *amoA* numbers were more abundant in the soil surface of all three soils, while the AOA were greater in number in one of the three subsoils. The addition of urine substrate (inorganic N) stimulated the AOB. The AOA were only observed growing in the control plot where the urine substrates were not added. Nitrification rates, which were significantly higher in the top soil than subsoil, were correlated with AOB abundance, but not with AOA. Marusenko et al. (2015) studied the effects of long-term management in the desert topsoils of Arizona in terms of AOB and AOA community structure and nitrification rate. Real-time qPCR was used to determine the abundance of AOA and AOB. Interestingly, the addition of inorganic fertilizers increased the abundance of both AOA and AOB.

Shen et al. (2008) showed that 17-years of fertilization management in an alkaline (pH ranging from (8.3 to 8.7) sandy-loam soil affected the AOB, but not AOA. The eight fertilizers treatments included a control and a treatment that contained a different combination of N, P, K and organic manure. The AOA abundance and community structure did not change, whereas the community structure of AOB was slightly changed. The AOB were more abundant under the plot where the inorganic N was added, although the abundance of AOB did not change.

Recent evidence suggests that archaea have a more important role in fertilized soils than previously suspected. Lehtovirta-Morley et al. (2016) isolated, based on 16S rRNA gene analysis, *Nitrosocosmicus franklandus*, an archaeon from arable soil with pH 7.5. This archaeon has a higher tolerance to nitrate and ammonia than typical AOA and is similar to soil AOB as growth was possible at 100 mM NH_4^+.

2.6. Ground covers, AOB, and AOA

Organic mulching, such as wood chips and shredded paper, has many benefits including suppressing weeds, reducing soil compaction, and increasing soil organic C, microbial activity, and water content (Brown and Cotton, 2011). Mulching also influences soil properties such as nutrient concentrations and pH (Billeaud and Zajicek, 1989). Because a small fraction of the nutrient is readily available for the plants in the first year of application, organic mulch influence can last for several years (Hartl and Erhart, 2005). However, the impact of organic mulching on soil microbial communities has received little attention.

Few studies have addressed the impact of wood chips on the diversity of microbial communities. Tiquia et al. (2002) used T-RFLP of bacterial 16S rRNA genes to study the impact of using composted yard waste and ground wood pellets with or without chemical fertilizers on rhizosphere bacterial communities. Tiquia et al. (2002) reported a higher community diversity resulting from mulching.

Martir-Torres (2010) studied the abundance of AOA and AOB and the diversity of AOA in a soil from mulched (gravel, bark, and lawn mulch) and unmulched plots. Evaluated by real-time qPCR, the AOB abundance was similar in all the plots. The abundance of AOA decreased under the bark mulch. A clone library to evaluate the diversity of AOA under the treatments was created. The authors noted that although the community richness did not change under the different mulch treatments, it did affect the community composition.

2.7. Molecular methods

Many molecular methods are based on analyzing DNA or RNA. Often 16S rRNA genes or specific functional genes are amplified using polymerase chain reaction (PCR). To use PCR

methods in soil, one approach is to extract organisms from the soil matrix and then extract DNA; although, often the approach is to first extract DNA from soil directly. The PCR is then used to make, theoretically, millions of copies of DNA fragments. PCR consists of three steps that depend on temperature. First, the temperature increases and the double stranded DNA denatures into two separate DNA strands. The temperature then cools to allow the primers to anneal which marks a specific sequence to be copied. Finally, the temperature increases and new strands will be synthesized with the help of *Taq* polymerase which is derived from the thermophilic organism *Thermus aquaticus*. Primers used in PCR are short sequences that start the DNA strand synthesis (Smith & Osborn, 2009).

Denaturing gradient gel electrophoresis (DGGE) is a PCR-based method in which DNA fragments that have a negative charge are forced by electricity toward a positive electrode through a polyacrylamide gel containing a denaturing gradient created with solutions using urea and formamide (Muyzer and Smalla, 1998). The DGGE separates DNA fragments based on their sequence discrepancies which differs from agarose gel electrophoresis which separates DNA fragments based on length. Fischer and Lerman (1983) found that DNA fragments with the same length but differing in one or more base pair sequences, migrating in a denaturing gradient, are denatured at different denaturant concentrations. Separation of different DNA sequences at different migration distances in a denaturant gradient is based on the fact that a GC-base pairing that has three hydrogen bonds is more stable than an AT base pairing. As a result, sequences with higher GC content will denature at higher denaturant concentrations. Each DNA fragment stops moving at a unique point, forming a band, and each band represents a population (an assumption of the method). As a result, DGGE gives a picture of the community.

A CG clamp between 33 to 40 GC-only base pairs in length and is usually added to one of the primers' 5' ends. This helps to prevent a complete denaturing of gene fragments in DGGE. A complete denaturing of the fragments would prevent the cessation of PCR product migration during electrophoresis and lead to inconsistent results about the melting characteristics of sequences during DGGE analysis (Myers et al., 1985; Sheffield et al. 1989). Ethidium bromide, silver, or SYBR green staining are used to visualize the DGGE bands.

DGGE is a popular fingerprinting method in microbial ecology. It is suitable to investigate shifts in the microbial community across multiple samples resulting from environmental changes. The optimal duration and gradient to achieve best separation of the fragments can be experimentally determined (Muyzer and Smalla, 1998). Further analysis can be completed by cutting DNA bands out of the gels and sequencing them. However, DGGE has some limitations. First, only small fragments that have up to 500 base pairs can be analyzed (Myers et al., 1985). Secondly, only populations that comprise 1% or more of the community can be detected by DGGE. In addition, gel preparation might be difficult. Biases that arise as with any PCR based technique are another limitation of DGGE.

The method of qPCR is used to amplify and simultaneously quantify gene (or transcript) numbers within an environmental sample (Smith and Osborn, 2009). As with regular PCR, qPCR can be used to target a functional marker as specific as at the bacterial strain level (Smith and Osborn, 2009). The thermal cycle is similar to the traditional PCR in that it consists of heating and cooling stages where DNA is denatured and a targeted gene is synthesized by adding primers and replicating multiple copies of the targeted gene. Either TaqMan fluorogenic probes or SYBR Green I dye can be used to label PCR products throughout the thermal cycling process. The quantity of gene is determined at the end of each cycle which is a unique feature in qPCR,

thereby making qPCR a fast, effective, and highly sensitive approach to enumerating microbes in environmental samples. Biases of qPCR, as with any PCR based method, increase with the increased number of cycles and is a limitation of qPCR (Smith and Osborn, 2009). Furthermore, as with regular PCR, design of primers requires knowledge of sequence information, which can be derived from cultured organisms, but is a limitation for investigation of unknown and uncultured organisms.

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3. Ammonia Oxidizing Bacterial and Archaeal Communities in Organically Managed Apple Orchard Soil

3.1. Abstract

The rate limiting step of nitrification, ammonia oxidation to nitrite, was thought for many years to be carried out solely by ammonia oxidizing bacteria (AOB). Using molecular techniques the homolog of the gene that encodes for the subunit A of ammonia monooxygenase, *amoA,* was discovered in ammonia-oxidizing archaea (AOA). Soil properties are among factors that affect AOA and AOB community size, diversity, and activities. Because understanding of how long-term, organic management practices in perennial systems affect microbial communities is critical for sustainable soil management, the relationships between AOA and AOB and soil properties after seven years of annual surface applications of ground cover and organic nutrient amendments to an apple orchard soil were investigated. Poultry litter, commercial fertilizer, or a no-fertilizer control were combined with compost, wood chips, shredded paper, or mow-and-blow ground cover mulches in a 4 x 3 full factorial design for a total of twelve treatments. Soil organic matter and dissolved organic nitrogen increased while ammonium-N was lower in compost compared to the other ground cover treatments. The AOB richness, diversity, and equitability were low and variable and not consistently related to treatments. The log of the AOB abundance measured using qPCR of the *amoA* gene fragment ranged from 1.07 to 3.69 with smallest numerical abundances in two of the paper treatments and the largest in compost with poultry litter. The AOA richness, diversity, and equitability, while also low and variable, changed more consistently within ground cover treatments where diversity and equitability increased with time in compost but diversity and equitability decreased in most paper treatments. Clustering of communities analyzed by PCR-DGGE suggested that AOA changed over time.

Analyses with greater resolution in population changes within communities may provide greater insight into archaeal ammonia oxidizer responses to multiple years of surface organic amendment applications in perennial orchard soil in the Mid-Southern U.S.

3.2. Introduction

Nitrogen (N), an essential element that is used by plants to produce amino acids and protein, thus maintaining life and productivity, is often the most limiting plant nutrient in soils. As a result, N fertilizers are widely used. Application of synthetic N has increased more than 10-fold in the last 50 years in an attempt to keep plant production in pace with the growing human population (Robertson and Vitousek, 2009). However, excessive N fertilizer applications can lead to environmental hazards from excess, resulting in losses to adjacent water bodies and from processes resulting in atmospheric pollution. Nitrification is a process in the N cycle in which nitrate is formed. Because nitrate is negatively charged, it is a mobile form of inorganic N that can be a readily available for plant uptake in soil solution. Understanding the factors controlling microbial communities mediating nitrification is important. The two-main groups of prokaryotic organisms that perform nitrification are ammonia-oxidizing bacteria (AOB) and the more recently discovered ammonia oxidizing archaea (AOA) (Könneke et al., 2005).

Archaea are generally linked to extreme environments (DeLong, 1998). However, with the advances in culture-independent techniques, archaea that survive in mesophilic conditions and oxidize ammonia were discovered in the 1990's (Fuhrman et al., 1992; DeLong, 1998). Archaea in soil were found to possess a homologous gene to that in bacterial nitrifiers, *amoA,* a gene that encodes for subunit A of ammonia monooxygenase (Treusch et al., 2005). In the same year as Treusch et al.'s publication (2005), a marine ammonia oxidizing archaeon (AOA),

Nitrosopumilus maritimus, was isolated in pure culture (Könneke et al., 2005). This archaeon is able to oxidize ammonia in the absence of organic carbon (C) and at a rate similar to the AOB. The newly confirmed phylum Crenarchaeota ubiquitously exists in a wide variety of environments (Boyle-Yarwood et al., 2008; Leininger et al., 2006; Nicol et al., 2008); although, the contribution of AOA in different environments and the conditions under which they thrive have yet to be fully elucidated.

Ammonia oxidizing archaea are present in soil, sediment, and water (Morimoto et al., 2011). Leininger et al. (2006) showed via qPCR that archaeal *amoA* is 1.5 - 230 times more abundant than bacterial *amoA* in pristine and agricultural surface soils. In the other studies, AOB were more abundant in grassland soils than AOA (Di et al., 2009). What is less clear is how management impacts the contributions of AOA and AOB in soil under different management schemes. How ammonia becomes available, whether through mineralization or applied as inorganic fertilizer, may affect AOA and AOB differently. Ammonia available at small concentrations derived from mineralization of soil organic matter may stimulate AOA, while inorganic fertilization may stimulate growth of AOB (Jia and Conrad, 2009; Kelly et al., 2011).

Organic amendments applied to the soil surface as ground covers and organic nutrient sources are used to enhance soil fertility in organically managed agroecosystems as they protect the soil surface, add organic matter, and contain a combination of nutrients released upon decomposition (Brady and Weil, 2016). Organic amendments can be applied in many forms and include waste products such as poultry litter, composted yard waste, shredded paper, or plants residues. Organic amendments may be applied to the ground surface or tilled into the soil, and the no-till strategy employed in an orchard ensures that resides are maintained on the surface (Ismail et al., 1994). Unlike synthetic nitrogen, organic amendments need microorganisms, time,

temperature, and moisture to decompose and mineralize N (Brady and Weil, 2016). Organic amendments that release inorganic N at metered rates during decomposition may stimulate AOA (Kelly et al., 2011).

Soil pH, ammonia substrate availability, and organic matter concentration are all factors that affect microbial communities in general, and ammonia oxidizers specifically. Soil pH has significant effects on microbial communities resulting from effects on the chemical form, concentration, availability of the nutrients (Kemmitt et al., 2006). The AOA and AOB exhibit shifts in their community structure in response to pH, although AOA has a wider tolerance range to pH (Nicol et al., 2008). Nicol et al. (2008) studied the abundance and diversity of AOA and AOB in a field trial in Scotland in plots with soil pH that ranged from 4.9 to 7.5. The copy number of archaeal *amoA* increased while bacterial amoA gene copy number decreased as pH decreased, indicating that AOA may be major contributors to nitrification compared to AOB in acidic soils. The community structure of both AOA and AOB were different in acidic and neutral conditions. In acidic conditions, below pH 6, acidophilic AOB may adapt to acidic conditions through biofilm formation, urease activity, or other unknown methods (Nicol et al., 2008). On the other hand, AOB appeared to be more active in neutral and alkaline soils (Shen et al., 2008; Nicol et al., 2008). Shen et al. (2008) showed that AOB abundance was correlated to pH ranging from 8.3 to 8.7 in sandy-loam soil that was under different fertilizer treatments for 17 years.

Microbial activity increases with increasing organic matter. Heterotrophic microorganisms produce a suite of enzymes that decompose organic matter and mineralize nutrients such as N. Autotrophic ammonia oxidizers benefit from released ammonia which they use as their energy source. The AOA prefer inorganic forms of N and C, although they may grow mixotrophically (Krümmel and Harms, 1982). However, AOA and AOB differ in their

tolerance to substrate concentrations (Martens-Habbena et al., 2009; Tourna et al., 2011; Verhamme et al., 2011). Different studies showed that AOA prefer organic forms of N, while AOB prefer inorganic sources of N (Di et al., 2009; Kelly et al., 2011). After addition of urine in N-rich grassland soils in New Zealand that were dominated by AOA, the abundance of AOB increased by 3.2 to 10.4-fold (Di et al., 2009). Kelly et al. (2011) observed that additions of municipal biosolids in corn fields in Illinois increased the abundance of AOA, but AOB abundance did not change. Kelly et al. (2011) explained that the slow mineralization rate may benefit the AOA. They also reported that inorganic fertilizers increased the abundance of AOB, but did not significantly affect the AOA abundance.

The AOA and AOB may be better adapted to different environmental conditions. While AOA appear to be better adapted to lower pH (3.7-7.5) (Nicol et al., 2008) and AOB may outcompete AOA for nutrients at high nutrient concentrations under fertilized conditions (Martens-Habbena et al., 2009; Tourna et al., 2011; Verhamme et al., 2011), AOA and AOB are impacted by the surrounding abiotic environment and appear to have developed niches within temperate soil. All communities are subject to the combined influences of the soil ecosystem. Conflicting results and the dearth of research in organically managed perennial orchards, especially the low organic matter soils characteristic of the Mid-Southern U.S. underlie the impetus to study the relationships between AOA and AOB and soil properties after seven years of annual surface applications of ground cover and organic nutrient amendments to an apple orchard soil managed under organic production practices. The AOA and AOB community size and diversity are expected to be impacted differently by amendments, where AOB but not AOA communities are expected to be positively related to ammonium concentration (as a measure of

available N released through mineralization), and soil pH, but perhaps negatively related to increased organic matter and dissolved organic C and N.

3.3. Materials and Methods

3.3.1. Experimental design

This study is a part of a long-term research project that was established in March 2006 located at the University of Arkansas, Main Agricultural Experiment and Extension Center, Fayetteville (Latitude: 36.1 N; Longitude: 94.1W; Altitude: 427 m). The location was prepared in 2005 as described in Choi and Rom (2011) and Ford (2015) by tilling, leveling, and applying agricultural lime to adjust the soil pH to 6.5. Composted manure was added and a cover crop was grown prior to tree planting (Choi et al., 2011; Ford, 2015; Mays et al., 2014). 'Enterprise' apple (*Malus domestica* Borkh.) trees on M.26 rootstocks were planted in 2006 on Captina (Fine-silty, siliceous, active, mesic Typic Fragiudult) and Pickwick (Fine-silty, mixed, semiactive, thermic Typic Hapludult) silt-loam soils (NRCS, 2014), with 2-m spacing within rows and 4-m between rows. The orchard has an L-shaped design consisting of two or four treatment rows in each direction with guard rows on each outer edge. The orchard was managed following the National Organic Program Standards (AMS, 2012). Each plot consisted of three trees: a treatment tree surrounded by two guard trees with ground covers and fertilizer applied to a 2 x 2 m^2 area surrounding each of the three trees in a plot.

One of four ground covers - urban green compost (GC), white shredded paper (P), wood chips (W), or a mow-and-blow green mulch as a control (M) - was applied annually to the soil surface. The compost, paper, and wood chips were applied in April each year from 2006 - 2014 at a depth of 7.5 -12 cm to each plot (Mays et al., 2014; Ford, 2015). Until 2011, compost

consisted of grass, leaves, and brush collected and composted by the City of Fayetteville, AR. After 2011, compost was obtained from PC Turnkey in Springdale, AR. Wood chips, consisting primarily of chipped yard waste of leaves and branches of hardwood species, were obtained from the City of Fayetteville, AR. The shredded paper was obtained from the University of Arkansas, Fayetteville, AR. Mow-and-blow treatments consisted of tall fescue (*Festuca arundinacea* Schreb. 'KY 31') planted between rows and other naturally occurring native, herbaceous species mowed after seed head formation each spring and periodically throughout the growing season and simultaneously blown under tree canopies with a side-discharge mower (Choi and Rom, 2011).

The nutrient sources included poultry litter or a formulated, certified organic pelletized fertilizer (Perdue AgriRecycle, Seaford, DE, or an alfalfa (*Medicago sativa*) based product by Bradfield Organics Feed Solutions, St. Louis, MO), or a no-fertilizer control. Nutrient sources were applied uniformly under the tree canopy in a 2-m width band extending the entire three-tree subplot at a rate of 50 g of N per tree per year of tree age until a maximum of 450 g N per tree was reached. Ground cover and fertilizer C and N concentrations are reported in Ford (2015) and shown here in Table 1.

3.3.2. Soil sampling and analysis

Composite soil samples were collected as described in Ford (2015) from the 0 - 10 cm depth of three blocks (three replications) after removing surface ground cover in May 2007, 2009, and 2013. Briefly, eight randomly selected cores at least 15 cm away from the tree trunk and within a 60-cm distance from the tree between trees in a row and within 45 cm distance from the tree between rows were collected using a sterilized soil probe. Soil was stored at 4 ^{o}C until sieved moist through a sterilized 2-mm sieve. A subsample was frozen at -80 ^{o}C until DNA was

extracted, and the remainder stored at 4 ºC until extracted for ammonium, nitrate, dissolved organic carbon (DOC) and nitrogen (DON), organic matter (OM), pH, and dried for soil moisture. Soil temperature around each tree at a 10 cm depth was measured at the time of sampling with a hand-held thermometer.

The dissolved organic C, DON, nitrate-N (NO_3^--N), and ammonium-N (NH_4^+-N), microbial biomass, OM, soil moisture, and pH were determined as described in Ford (2015). A single 0.5 M K_2SO_4 extraction from unfumigated soil samples (Jones and Willet, 2006) was used to calculate DOC, dissolved total N (DTN), nitrate-N (NO_3^--N), and ammonium-N (NH_4^+-N). Concentrations of NH_4^+-N and NO_3^--N were determined colorimetrically (Mulvaney, 1996) using a Skalar segmented-flow autoanalyzer (Skalar Inc., Norcross, GA). Dissolved organic C and DTN concentrations were measured on a Shimadzu TOC-V PC-controlled total organic carbon and attached total N analyzer (Shimadzu, Columbia, MD). Dissolved organic N was calculated as the difference from DTN subtracted by NO_3^--N and NH_4^+-N. Microbial biomass C and N was measured using the chloroform-fumigation extraction method (Vance et al., 1987). Microbial biomass was calculated from the difference in DOC and DTN concentrations between fumigated and unfumigated samples. Soil moisture was determined gravimetrically by oven-drying soil at 105 ˚C for at least 24 hr until a constant weight was reached. The soil pH was measured with electrode and pH meter using 1:2 soil-to-water (wt:vol) ratio. Organic matter content was determined using loss-on-ignition (6 hr at 550 ºC).

3.3.3. Molecular analysis of AOA and AOB

Soil DNA was extracted from approximately 500 mg soil using the NucleoSpin Soil DNA Extraction Kit (Clontech Inc., Mountain View, CA) according to the manufacturer's protocol. This method follows a bead-beating procedure which is commonly used to extract

DNA from soil. DNA was quantified using spectrophotometry (ND2002; Thermo Fisher Scientific Inc., Waltham, MA).

Polymerase chain reaction (PCR) reactions (25 µl) contained 1X PCR buffer (10 m*M* tris- HCl, 50 mM KCl, 0.01% (wt:vol) gelatin), 200 µ*M* dNTP, 0.01% BSA, 0.5 µM of each primer, 1.0 unit of Taq polymerase (Go Taq®, Promega, Madison, WI)) and 1 µl of 10 ng μL^{-1} template DNA. The bacterial *amoA* gene (AOB) corresponding to *Nitrosomonas europea* was amplified using primers Amo1F-GC [5' GGG GTT TCT ACT GGT 3'] and AmoA-2R-TG[CCCCTCTGGAAAGCCTTCTTC] (Rotthauwe et al., 1997). A GC clamp was attached at the 5' end of the forward primer for the DGGE (Myers et al., 1985; Sheffield et al. 1989). A PTC-200 DNA Engine (MJ Research, Waltham, MA) thermal cycler was used to carry out PCR reactions. Temperature conditions for the AOB were as follows: initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 45 s, with a final extension at 72 °C for 5 min. The archaeal *amoA* gene (AOA) was amplified using primers CrenamoA23f (5′-ATGGTCTGGCTWAGACG-3′) and CrenamoA616r (5′-GCCATCCATCTGTATGTCCA-3′) (Tourna et al., 2008). Temperature conditions for the thermal cycler for AOA were as follows: initial denaturation at 95 °C for 10 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 45 s, with a final extension at 72 °C for 5 min.

To confirm PCR products, 1.5% agarose gels made in 0.5X Tris/Borate/EDTA (TBE) buffer and stained with 1.8 µl of ethidium bromide were used to confirm the amplification for both AOA and AOB gene fragments. Gels were electrophoresed for 45 min at 95 V, with DNA bands visualized on a UV trans-illuminator to confirm PCR products. A Kodak EDAS 290 system and ID software package (Kodak, New Haven, CT) was used to digitally picture the gels.

To determine the concentration and the distance of migration, DNA mass standards (Bio Rad Laboratories, Hercules, CA) were used with each gel.

PCR products were separated according to sequence differences on a D-code system. Vertical gels (1.5-mm thick) containing 6% and 8% polyacrylamide (acrylamide:bisacrylamide ratio of 37.5:1) in a 1X TAE buffer pH 8.0 (30 m*M* Tris, 20 m*M* acetic acid, glacial and 1 m*M* EDTA, pH 8.0) were electrophoresed at 70 V and 60 ^{o}C for 16 hrs and 90 V 60^{o}C for 15 hrs, for bacterial and archaeal PCR products, respectively. A gradient of 45% to 65% denaturant (100% equal to 7 *M* urea and 40% deionized formamide) was created to separate AOB (Avrahami et al., 2003) and 15% to 55% denaturant separated Crenarchaeal *amoA* gene fragment sequences (Nicol et al., 2008). Gels were stained with SYBR Green I dye (1:10000 dilution, Molecular Probes, Eugene, OR) solution for 20 min. Bands were visualized using a UV trans-illuminator, and digital pictures were captured of the gels for analysis.

The abundance of AOB was measured by quantifying *amoA* genes for each group. The reaction was carried out in an iQ5 (Biorad, Hercules, CA) thermocycler. Two-fold serial dilutions of purified and quantified PCR product amplified from environmental DNA of each respective target gene fragment was used to construct standard curves. Real-time qPCR reactions (20 µl each) consisted of 10 uL of 2× SYBR Green qPCR (Invitrogen, USA), 5 pmol of each primer, 0.5 mg mL^{-1} BSA, and 1 µl of 10 ng μL^{-1} of genomic DNA as template. Thermal conditions were as follows: initial denaturation at 95 ^{o}C for 5 min, 40 cycles of 95 ^{o}C for 30 s, 60 ^{o}C for 1 min, and 72 ^{o}C for 1 min, with melt curve starting at 60 ^{o}C. The bacterial *amoA* (AOB) was amplified with primers amoA-1F (5'-GGGGTTTCTACTGGTGGT-3') and amoA-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') (Rotthauwe *et al.,* 1997). Standards and no treatment controls (NTC) were included in each master mix in triplicate and all sample reactions were

performed in duplicate for individual runs. Melt curve analysis in 0.3°C increments (10 sec each with fluorescence measurement at the end of each step) was performed at the end of each run to confirm the absence of non-specific amplification and products were electrophoresed to confirm one DNA band of the correct size.

3.3.4. Data analysis

Using the standard curve, concentrations of qPCR amplicons were calculated using equation 1 (Dhanasekaran et al., 2010).

$$\text{Number of copies} / \mu\text{L} = \frac{6.022 \times 10^{23} (\text{molecules /mole}) \times \text{DNA concentrations } (\text{g}/\mu\text{L})}{\text{Number of base pairs} \times 660\ \text{Da}} \quad (1)$$

where 6.022 x 10^{23} molecules/mole is Avogadro's number and 660 Da is the average weight of a single base pair. To calculate number of copies per g soil, each answer from equation 1 was multiplied by the total volume of extract and divided by the g soil extracted. Values were converted to log values for data analysis.

Digital pictures of the DGGE gels of both AOB and AOA were imported into Compar II (Applied Maths Inc., Austin, TX) for analysis. Soil samples that showed bands were analyzed for the presence or absence of bands and migration distances. The parameters were set to default values used by Ford (2015) except for the disk size that was set at 15%. Migration distance values of detected bands obtained by the software were manually converted into a presence-absence table, where 0 is the absence of bands, and 1 is the presence of bands. The richness (S) is indicated by the number of bands in a sample. Band intensities were normalized by dividing the individual band intensity by the greatest intensity on each gel to reduce potential differences across gels due to staining or picture quality. Using band intensity allowed for calculation of the

Shannon-Weaver index of diversity (H) and Shannon-Weaver index of equitability (E) using the equations below:

$$H = \Sigma\ pi\ ln\ pi \quad (2)$$

where pi is the proportion of the total that each band contributes to the total sample community.

$$E = H / ln\ S \quad (3)$$

and S is richness.

The intensity of DGGE bands within a lane on each gel was used to create a similarity matrix containing pairwise similarity values for 2007, 2009, and 2013 data for AOB; and 2009 and 2013 for AOA from Dice similarity coefficients (D) according to the equation

$$D = 2a / 2a + b + c \quad (4)$$

where *a* represents the number of bands in both lanes (samples) being compared (lanes A + B), *b* represents the number of bands in sample (or lane) A and *c* represents the number of bands in sample (or lane) B. From the similarity matrix, unweighted pair-group method with arithmetic means (UPGMA) was used to create a dendrogram by Ward algorithm in Gel Compar II of the *amoA* communities in order to visualize relatedness among samples (replicates, treatments and years; Applied Maths, Inc, Austin, TX).

An analysis of variance (ANOVA) using SAS (version 9.4, SAS Institute Inc., Cary, NC) was performed to determine the effects of ground cover and fertilizer treatments and year on soil variables. Least significant difference (LSD) ($\alpha < 0.05$) was used to perform mean separations. Interactions are presented only when they are significant ($P < 0.05$); otherwise, significant main effects only are presented. Because of the small range of values for richness and the number of zero values for diversity and equitability, ecological properties for AOA and AOB communities are described, but not analyzed statistically. Soil properties and AOA and AOB community

indices were analyzed by Pearson product correlation analysis regardless of treatment structure to determine if soil properties were linearly related to AOA or AOB communities.

3.4. Results

3.4.1. Soil properties

Ground covers, fertilizers and year impacted soil properties as main effects, but there were few significant interactions of ground covers and fertilizers (Table 2). There was a main significant effect of ground cover and of fertilizer on soil pH; however, no significant interaction effects were observed. Paper had the highest pH and pH in mow-and-blow was lower than in compost and wood chips, which were not different and intermediate the other two ground covers (Table 3). The pH was greater where no fertilizer was applied and poultry litter and commercial fertilizers have similar and lower pH (Table 4). Fertilizers also significantly affected nitrate–N concentration, where nitrate-N was greatest with poultry litter followed by commercial fertilizer, and was lowest in non-fertilized soil. In addition to nitrate and pH, fertilizer significantly affected DON. Poultry litter significantly increased DON compared to commercial and no fertilizer.

Ground cover significantly affected many soil properties in addition to pH, including NH_4^+-N concentration, DON, organic matter, microbial biomass C and N, and soil moisture (Table 2). Ammonium-N was significantly lower in compost than in mow-and-blow and paper, but it was not significantly different from wood chips, and microbial biomass C and N was similar and less in compost and wood chips compared to paper and mow-and-blow (Table 3). Organic matter and DON were significantly greater in compost than all other ground cover

treatments. Compost, wood chips, and paper all increased soil moisture compared to mow-and-blow.

There was a significant ground cover by fertilizer interaction in DOC (Table 2). Compost alone or combined with poultry litter resulted in a significantly higher DOC compared to any ground cover by fertilizer combination. Mow-and-blow with no fertilizer numerically had the smallest DOC concentration; however, the application of fertilizer did increase DOC in mow-and-blow ground cover compared to the absence of fertilizer (Table 5).

3.4.2. AOA and AOB communities

Using PCR-DGGE, AOB and AOA richness ranged from zero to seven taxa across all samples from all treatments and years (data not shown). Despite PCR amplification of *amoA* gene fragments of the correct size and with a clean negative control sample (suggesting no contamination), several treatments did not result in analyzable community samples in DGGE. For AOB, eight, 10, and 12 treatments did not result in any bands on DGGE in 2007, 2009, and 2013, respectively (data not shown). The absence of DGGE bands from positive PCR amplifications occurred across treatment combinations with no evident pattern. Furthermore, the treatments that did not yield DGGE patterns were not consistent each year. For AOA, none of the samples in 2007 yielded DGGE patterns that were analyzable. Although many faint bands appeared on 2007 gels, none were strong enough to be differentiated from background noise (data not shown). In 2009 and 2013, nine soils each year did not yield detectable DGGE patterns for AOA. Again, there were no discernible patterns in which samples did not show bands in DGGE and no consistency between years. Furthermore, there did not appear to be a relationship in samples between ability to detect AOB communities and AOA communities.

The richness and diversity were low for both AOB and AOA. The AOB richness, diversity, and equitability averaged across treatments ranged from 0.67 to 6.00, 0.00 to 1.27, and 0.00 to 0.97, respectively, regardless of treatment and year (Table 6). The AOB richness, diversity, and equitability indices were variable across treatments and did not consistently increase or decrease over time, although AOB community diversity and equitability appeared most stable across sampling time in paper and mow-and-blow without fertilizer addition. Clustering of communities by unweighted pair-group method with arithmetic means (UPGMA) also did not suggest distinct ground cover by fertilizer treatment effects. While there was some clustering of soils by ground cover treatment, these were not consistent and clustering did not indicate distinct changes with time (Figure 1).

Bacterial *amoA* abundance across treatments ranged from below detection (reported as zero) up to 4.3 (log #/g) (Table 7). The *amoA* copy numbers did not vary consistently under the different ground covers. Paper had the lowest amplicon concentration when unfertilized or applied with commercial fertilizer.

Correlation analysis showed that AOB richness was negatively related to soil moisture and DON, and diversity was positively related to organic matter, DOC, and DON (Table 8); however, correlations although significant were weak. Scatter plots of data did not indicate meaningful relationships between soil properties and AOB richness and diversity when treatment structure was not included in the analysis (data not shown).

While AOA communities appeared to be below detection in 2007 (data not shown), the average AOA richness in 2009 ranged between 0 and 5.0 and in 2013 between 1.3 and 6.0 (Table 9). The average Shannon diversity index ranged from zero to 0.97 in 2009 and 0.17 to 1.45 in 2013, while equitability ranged from zero to 0.96 in 2009 and 0.12 to 0.97 in 2013. While all

compost by fertilizer treatments increased in diversity and equitability from 2009 to 2013, most values for diversity and all for equitability decreased in the paper by fertilizer treatment combinations from 2009 to 2013. Diversity in the wood chips by fertilizer treatment combinations increased while equitability decreased from 2009 to 2013. Diversity and equitability in the mow-and-blow and no fertilizer or commercial fertilizer treatment combinations increased while mow-and-blow and poultry litter combination decreased diversity and equitability from 2009 to 2013. Clustering of communities by unweighted pair-group method with arithmetic means (UPGMA) suggested that AOA communities changed over time but did not distinctly separate by ground cover by fertilizer treatment effects (Figure 2).

Correlation analysis showed that AOA richness was positively related to organic matter, soil moisture content, and nitrate-N concentration, and diversity was positively related to organic matter, soil moisture content, DOC, and nitrate-N concentration (Table 8); however, similar to correlations found with AOB, although correlations were significant, they were weak. Furthermore, scatter plots of data did not indicate meaningful relationships between soil properties and AOA richness and diversity when treatment structure was not included in the analysis (data not shown).

3.5. Discussion

In this study soil samples were collected after 1, 3 and 7 years of annual treatment of ground cover and fertilizer combination amendments which provides insight into how AOA and AOB responded and adapted to organic management in an orchard soil. Furthermore, relationships between AOA and AOB communities and soil properties could be investigated. The results do not support our hypothesis that increased resources increase richness and diversity

of AOA and/or AOB, although AOA indicate greater changes in communities in response to soil amendments over time.

Both the AOB and AOA exhibit low diversity, and AOB abundance was low. Others have found AOB to be less diverse than AOA. For example, Shen et al. (2008) studied AOB and AOA community structure using DGGE in alkaline sandy loam soils that received different organic and inorganic treatments. The AOB abundance ranged from 1.24×10^5 to $2.79 \times 10^{6.}$ The authors detected 25 bands (richness) for AOB and Shannon diversity ranged between 1.72 and 2.45, while AOA Shannon diversity ranged between 2.91 and 3.10. However, Shen et al. (2008) noticed that AOB diversity and abundance were significantly lower in the soils receiving organic manure than the soils received inorganic fertilizers which may indicate that organic amendments may have a negative impact on AOB diversity.

The AOB have been observed to have a low diversity in cultivated fields. Meyer et al. (2014) used T-RFLP to study AOA and AOB community responses in soil from grassland ecosystems. The AOB Shannon diversity index ranged between 0.06 and 1.04 which is in the range of this study. Similarly, Chen et al. (2008) reported a low diversity in the AOB community in bulk paddy soil with or without rice plants and rhizosphere soil after five weeks of receiving different N fertilizers. The AOA diversity is commonly observed to be low in soils samples collected from a single location (Jiang et al., 2014). Similarly, Ouyang et al. (2016) studied the diversity of AOA *amoA* using 454-pyrosequencing in an agricultural silage corn system under organic and inorganic fertilization. In that study, two *Nitrososphaera* clades were dominant. The AOB was also mainly affiliated with one cluster.

The AOB and AOA communities responded differently to treatment combinations under different ground cover and organic fertilizer combinations. While the richness of AOB did not

consistently change under ground covers and organic fertilizer combinations over time, AOA showed a trend in increasing richness and diversity over time in all compost and wood chip treatments and with mow-and-blow and paper combined with commercial fertilizer. These results are consistent with other studies that found that manure application increases the AOA abundance and community structure. Ai et al. (2013) studied the effect of a long-term fertilizer experiment both in rhizosphere and bulk soil. Using DGGE, it was found that manure application increased the abundance and changed the community structure of AOA, while AOB community did not change. Similarly, Wessén et al. (2010) investigated the community structure of AOA and AOB under organic amendments such as straw and peat. The AOB diversity did not change while AOA abundance increased and AOA diversity shifted based on T-RFLP.

In contrast, some studies found AOA communities to be more stable under different fertilizer management. Shen et al. (2008) studied the effect of different fertilizers that contain different combinations of N, P and K on the AOA and AOB in alkaline soil pH (8.3-8.7) in a 17-year long experiment and found that the AOB community diversity and abundance changed. Previous studies showed that AOA and AOB populations also have distinct niches (Yao et al., 2011). Yao et al. (2011) studied the effect of fertilizers in acidic tea orchard on the diversity of AOA and AOB using T-RFLP. Specific AOA and AOB populations dominated at specific pH or nutrient contents which suggests that AOA and AOB populations adapt to different specific niches.

Organic management in a perennial system with surface applications was investigated because the release of nutrients under organic amendments was dependent in large part on microbial decomposition. Microorganisms are the primary decomposers of litter and SOM, and thus, they control the nutrient cycling in the soil. Organic amendments, unlike inorganic

fertilizers need time to decompose. The rate of organic amendment decomposition varies based on environmental factors, such as temperature, moisture and pH. Furthermore, the composition of the different organic amendments can impact decomposition. For example, a higher C–to-N ratio may result in slower decomposition and/or less N mineralization, and thus reduce nitrification (Booth et al., 2005), and indirectly alter competiveness of nitrifier populations. However, a high C: N may improve activity and tighten N cycling, resulting in less loss from the terrestrial system rather than restricting activity. For example, Yao et al. (2005) reported increasing OM and respiration and less nitrate leaching under in high C:N treatments in a silty clay loam soil of a fruit orchard in New York.

In the current study, although compost increased OM and DOC more than any other ground cover, richness and diversity of ammonia oxidizers were not greater than in the other ground covers. This could be an indicator that particular organisms were outcompeting others. Compost also had among the lowest microbial biomass concentration. On the other hand, mow-and-blow ground and paper covers, although having similar and greater C: N ratios as compared to compost, respectively, without fertilizer addition appear to be more stable in diversity and equitability but respond differently to fertilizers. Ammonium-N concentration was different across ground covers. Limitations and complexities in systems, perhaps related to substrate and release of ammonia appear to impact ammonia oxidizer communities. Although compost is expected to be recalcitrant because of prior decomposition, applications in this study which resulted in increased organic resources, dissolved organic C and N, and nitrate-N concentrations suggest rapid turnover without increase in microbial biomass. Therefore, substrates appeared to be abundant and labile in compost amended soil.

Ammonium concentration and microbial biomass are lower in compost ground cover; however, nitrate-N concentrations in compost amended soil (4.61 μg N g^{-1}) (Ford, 2015; Choi and Rom, 2011) was similar to nitrate concentrations reported in Shen et al. (2008). Nitrate in Shen et al. (2008) ranged from 4.9 to 10 mg kg^{-1} in the soil that received manure amendments. The high nitrate concentration in compost suggests that nutrient condition might select for a particular population adapted to nitrification at greater rates of N availability or higher ammonia concentrations. Further analysis is required to determine if there are populations promoted by compost amendments that produce large inorganic N concentrations.

Organic matter significantly increased with compost amendments. Organic matter when decomposed and mineralized releases nutrients such as NH_3 which ammonia oxidizers use as energy sources. Dissolved organic carbon and DON were measured as indicators of available organic matter. The greater DOC and DON concentrations in compost were expected to be coupled with larger microbial biomass from microbial growth and ammonia oxidizers as promoted from increased decomposition and mineralization of ammonia. Despite the increase in nitrate and organic matter, both microbial biomass and AOB diversity is low in compost treatment. The AOA and AOB diversity were positively correlated to organic matter, but while AOA diversity was positively correlated to DOC, AOB was negatively correlated to DOC. While these results may suggest positive and negative relationships with availability of substrate, correlations were weak and did not consider treatment structure. Furthermore, both AOA and AOB likely comprise a small portion of the total microbial community (Stahl and de la Torre, 2012).

Some researchers have observed negative relationships of AOA to soil organic matter Wessén et al. (2010) studied the AOA and AOB community structure in a 50-year research field

study that received straw or peat with or without inorganic fertilization. Soil organic carbon was negatively correlated to AOA community size. These researchers explained this relationship as a result of the competition with other heterotrophic organisms. Although AOA are autotrophic, they may also utilize mixotrophic or even heterotrophic pathways. Tourna et al. (2011) reported increasing growth of AOA after addition pyruvate, which suggests a mixotrophic pathway. Furthermore, specific populations of AOA may respond differently to different types of soil organic matter (Wessén et al., 2010). Some AOA may be stimulated by more labile organic matter. For example, the AOA abundance in a paddy rhizosphere was greater than in the non-rhizosphere soil because of the presence of root exudates (Chen et al., 2008).

Mow-and-blow and paper recycled nutrients perhaps more slowly than compost and wood chips. This can be concluded by the higher microbial biomass and ammonium concentrations in mow-and-blow and paper treatments. Although in this study, AOB were not richer or more diverse, AOB have been shown to be more abundant in soils that have high ammonium concentrations (Martens-Habbena et al., 2009; Tourna et al., 2011; Verhamme et al., 2011). The AOA also exhibit a similar trend in that richness and diversity were variable across all treatments. Studies from the research literature have found AOA to be more diverse in low ammonium concentration soils (Martens-Habbena et al., 2009; Tourna et al., 2011; Verhamme et al., 2011). The AOA diversity may allow for species to adapt to different soil conditions. While several studies have suggested that AOA are more competitive at small ammonia concentrations, Lehtovirta-Morley et al. (2016) isolated *Nitrosocosmicus franklandus*, an archaeon from arable soil with pH 7.5. This archaeon has a higher tolerance to nitrate and ammonia concentrations than typical AOA and is similar to soil AOB, as growth was possible at 100 mM NH_4. A difficulty in understanding N concentration effects on AOA and AOB arises from the fact that

ammonium concentration measurement by standard methods based on salt extractions do not account for rates of N turnover or rates of N flux to and from organisms, and therefore may not provide an adequate picture of N availability to organisms in soil.

Soil pH is expected to influence the competitiveness of AOA and AOB populations. The AOA have been more abundant in acidic environments (Nicol et al., 2008), while AOB appear to prefer neutral and alkaline soils (Shen et al., 2008). In this study, AOA equitability increased over time in compost and wood chips, but decreased in paper from 2009 to 2013. Paper amendment resulted in the highest pH value. Previous studies showed that pH is a selective factor that shaped nitrifier communities (Lehtovirta et al., 2009; Gubry-Rangin et al., 2011). Lehtovirta et al. (2009) studied the AOA community diversity in a field that was maintained for 40 years in soils that have pH gradient (4.5-7.5) and found that different populations were observed at different pH. For example, *Nitrosotalea* group 1.1c decreased with increasing pH. Similarly, Group 1.1c archaea were not detected at pH values above 6.0. Similarly, in other research AOB community changed with different pH. In this study pH did not correlate to the AOA or AOB community structure, and the mean pH ranged from 6.5 to 7.1., which is a narrow range. As a result, the slight differences in the pH values under the different ground covers, while significant, do not appear to explain results in AOA and AOB communities.

The effect of soil moisture on AOA and AOB have been previously studied. He et al. (2007) observed that AOA and AOB are more active in wet soils than dry soils, and that AOA has a different structure in irrigated plots compared to non-irrigated plots. Moisture is directly linked to nitrification through affecting decomposing and mineralization rate. In addition, moisture can affect oxygen availability to AOB which is important for the function of *amoA* enzyme and as terminal electron acceptor. These studies suggest moisture may influence the

community structure of both AOA and AOB. Soil moisture was greater in compost, wood chip and paper ground covers compared to mow-and-blow, and was negatively correlated to AOB richness but positively correlated to AOA richness and diversity.

DGGE can provide insight into nitrifier communities with relatively low cost. However, one of the drawback of this method is the high detection limit (Myers et al., 1985). In our study, AOA richness and diversity were not obtained from positive PCR amplifications for 2007, potentially because populations were not relatively abundant enough for detection by DGGE (data not shown). Next sequence generation, such as with Illumina sequencing, could be used to avoid this limitation. Given that the same methods were utilized with all samples in this study, it is expected that the limitations and biases inherent in the PCR based protocols were similar for all of our samples, allowing the interpretation of treatment effects on AOA and AOB.

3.6. Summary and Conclusion

This study aims to investigate the effect of the four-ground cover (compost, wood chips, paper, and mow-and-blow) mulches combined with one of the three fertilizer (poultry litter, organic commercial fertilizer, and no-fertilizer control) treatment combinations applied annually for seven years in a full factorial design to low organic matter soil in an organically managed apple orchard on bacterial (AOB) and archaeal ammonia oxidizer (AOA) communities. How soil properties were affected and the relationship of the soil properties to AOB and AOA communities were also investigated. The results do not appear to support the hypothesis that increased resources increase richness and diversity of AOA and/or AOB, although AOA indicate greater changes in response to organic amendments over time. While all treatments impacted soil

over time, compost, in particular, supplied the greatest organic matter (DOC and OM) and DON, but had the smallest ammonium-N concentration.

The AOB richness, diversity, and equitability were low and variable and did not consistently change with treatments over time. Clustering of communities measured by PCR-DGGE did not clearly differentiate communities by treatments. Correlations of soil properties and AOB when significant were not clearly indicative of linear relationships. The AOA community richness and diversity indicated changes within ground cover and fertilizer treatments over time with richness and diversity increasing in most compost and wood chip treatments and diversity and equitability decreasing in most paper ground cover treatments over time. Clustering of AOA communities measured by PCR-DGGE indicated that communities changed over time but did not clearly differentiate communities by treatments. A further look at the diversity using next sequence generation would be beneficial to investigate the within-community changes that methods used in this study were not able to detect. Given that nitrifiers are a small component of the total microbial community, techniques with greater resolution into communities are needed to better identify and detect changes within the communities in this soil.

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Table 1. Nutrient contents of ground cover and fertilizer treatments applied in an organically managed apple orchard, Fayetteville, AR (n = 6).

Treatment	C[a]	N	C:N
	------ (%)------		
Compost	20.5	1.6	13.5
Wood Chips	29.7	0.7	39.2
Paper	36.8	0.2	205.9
Mow-and-Blow	40.0	2.2	15.8
Commercial Fertilizer	31.3	4.4	7.8
Poultry Litter	29.5	1.7	19.4

Data for each ground cover and fertilizer are averaged across 2006-2011.
[a]C is carbon; N is nitrogen.

Table 2. Analysis of variance (ANOVA) summary of the effects of ground cover and fertilizer treatments over time and their interactions on soil properties (0-10 cm depth) of an organically managed apple orchard silt loam in Fayetteville, AR, that was sampled in May 2007, 2009, and 2013.

Soil Property	GC[b]	Fert	Year	GC*Fert
DOC[a]	<0.0001*	0.0052*	0.0005*	0.0009*
DON	0.0005*	0.0021*	0.0061*	0.1177
NO_3^--N	0.4921	<0.0001*	<0.0001*	0.8671
NH_4^+-N	0.0294*	0.1974	0.0215*	0.2125
Microbial Biomass C	0.0018*	0.1908	0.3053	0.1471
Microbial Biomass N	0.0172*	0.1839	0.0004*	0.2705
pH	<0.0001*	0.0002*	0.0010*	0.0680
Organic matter	0.0007*	0.4077	<0.0001*	0.1764
Soil moisture	0.0011*	0.1989	0.0024*	0.3651

[a]DOC is dissolved organic carbon, DON is dissolved organic nitrogen, NO_3^--N is nitrate-N, NH_4^+-N is ammonium-N.

[b]GC is ground cover and Fert is fertilizer source.

*$P < 0.05$

Table 3. The main effects of ground cover treatments on soil properties (0-10 cm depth) of an organically managed apple orchard silt loam in Fayetteville, AR, that was sampled in May 2007, 2009, and 2013 (n = 27).

Ground Cover	pH	NH_4^+-N[a] (µg N g^{-1})	DON (µg N g^{-1})	Organic matter (%)	Bio C (µg C g^{-1})	Bio N (µg N g^{-1})	Soil moisture
Compost	7.14b*	1.01c	22.29a	4.3a	57.95b	11.02b	0.25a
Wood chips	7.01b	2.54bc	7.74b	2.7b	62.64b	9.98b	0.22a
Paper	7.44a	3.57ab	12.62b	2.5b	81.74a	15.15a	0.22a
Mow-and-blow	6.57c	3.84ab	13.23b	2.7b	97.45a	17.59a	0.18b

[a]NH_4^+-N is ammonium-N, DON is dissolved organic nitrogen, Bio C is microbial biomass C, Bio N is microbial biomass N.

*Means followed by different letters within a column are significantly different ($P < 0.05$).

Table 4. The main effects of fertilizer treatments on soil properties (0-10 cm depth) of an organically managed apple orchard silt loam in Fayetteville, AR, that was sampled in May 2007, 2009, and 2013 (n = 36).

Ground Cover	pH	NO_3^--N[a] (µg N g^{-1})	DON (µg N g^{-1})
None	7.14a[*]	13.5c	11.31b
Poultry litter	6.96b	47.5a	18.77a
Commercial	7.02b	30.1b	11.84b

[a]NO_3^--N is nitrate-N, DON is dissolved organic nitrogen.

[*]Means followed by different letters within a column are significantly different ($P < 0.05$).

Table 5. The interaction of ground cover and fertilizer treatment combinations on dissolved organic carbon (DOC) in soil (0-10 cm depth) of an organically managed apple orchard silt loam in Fayetteville, AR, that was sampled in May 2007, 2009, and 2013 (n = 9).

Ground Cover	Fertilizer	DOC ($\mu g\ C\ g^{-1}$)
Compost	None	269.4Aa*
	Poultry litter	251.8Aa
	Commercial	165.3Bb
Wood chips	None	74.9Ac
	Poultry litter	113.8Ab
	Commercial	119.0Ab
Paper	None	90.1Ac
	Poultry litter	128.7Ab
	Commercial	103.2Ab
Mow-and-Blow	None	34.9Bc
	Poultry litter	110.2Ab
	Commercial	70.1ABc

*Fertilizers within a GC followed by the same capital letter are not different. Ground covers followed by the same lower case letter are not different ($P < 0.05$).

Table 6. Mean ecological diversity index values calculated from denaturant gradient gel electrophoresis (DGGE) profiles of *amoA* gene fragments PCR amplified from ammonia oxidizing bacteria (AOB) from soil (0-10 cm depth) of an organically managed apple orchard silt loam in Fayetteville, AR, that was sampled in May 2007, 2009, and 2013 (n = 2-3).

Ground cover	Fertilizer	Richness (S)[a]			Diversity (H)			Equitability (E)		
		2007	2009	2013	2007	2009	2013	2007	2009	2013
Compost	None	1.67	1.00	3.33	0.35	0.00	0.70	0.22	0.00	0.42
	Poultry litter	2.00	2.33	1.00	0.78	0.53	0.20	0.71	0.43	0.29
	Commercial	2.67	0.67	3.33	0.78	0.00	0.73	0.87	0.00	0.63
Wood chips	None	2.67	1.00	0.67	0.60	0.27	0.20	0.43	0.24	0.20
	Poultry litter	3.00	3.33	1.67	1.03	0.97	0.37	0.97	0.80	0.53
	Commercial	4.00	2.67	2.33	0.90	0.73	0.34	0.65	0.53	0.21
Paper	None	2.67	2.67	1.00	0.53	0.73	0.63	0.55	0.73	0.58
	Poultry litter	0.67	4.33	2.00	0.60	1.23	0.43	0.87	0.85	0.24
	Commercial	4.00	1.00	4.33	0.77	0.17	0.80	0.55	0.24	0.43
Mow-and-blow	None	2.67	2.33	4.00	0.60	0.67	0.80	0.48	0.54	0.54
	Poultry litter	6.00	2.33	2.67	1.27	0.37	0.86	0.70	0.29	0.62
	Commercial	2.33	3.00	1.00	0.50	0.90	0.43	0.37	0.61	0.39

[a]S is richness; H is Shannon-Weaver index; E is Shannon-Weaver equitability.

Table 7. Ammonia oxidizing bacteria (AOB, log copy #/ g soil) enumerated by qPCR of *amoA* gene fragment DNA extracted from an organically managed apple orchard silt loam soil in Fayetteville, AR, that was sampled in May 2013 (n = 2, 3).

Ground cover	Fertilizer	Geometric mean AOB (log copy #/g)	AOB range[a] (log copy # /g)
Compost	None	2.21	0 - 4.13
	Poultry litter	3.69	3.43 - 3.95
	Commercial	2.60	0 - 4.33
Wood chips	None	3.24	2.88 - 3.55
	Poultry litter	2.99	2.10 - 3.46
	Commercial	2.32	0 - 3.49
Paper	None	1.25	0 - 3.75
	Poultry litter	2.61	0 - 4.05
	Commercial	1.07	0 - 3.20
Mow-and-Blow	None	3.30	2.78 – 4.07
	Poultry litter	2.12	0 - 2.54
	Commercial	3.45	2.66 - 3.86

[a]Values below detection are reported as 0.

Table 8. Correlation between ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA) diversity indices and soil properties from soil (0-10 cm depth) of an organically managed apple orchard silt loam soil in Fayetteville, AR, that was sampled in May 2007, 2009, and 2013 (n = 108 for AOB and 72 for AOA).

Soil property	AOB Richness		AOB Diversity		AOA Richness		AOA Diversity	
	r	P value	r	P value	r	P value	r	P value
pH	-0.101	0.368	-0.006	0.529	0.060	0.546	-0.066	0.529
Organic matter	-0.180	0.108	0.269	0.019*	0.230	0.019*	0.248	0.016*
Soil moisture	-0.273	0.014*	0.214	0.064	0.397	0.001*	0.422	0.000*
DOC[a]	-0.130	0.084	0.337	0.002*	0.148	0.145	0.266	0.018*
DON	-0.254	0.028*	0.336	0.003*	0.048	0.629	0.031	0.764
NO_3^--N	-0.047	0.657	0.186	0.095	0.386	0.001*	0.351	0.001*
NH_4^+-N	-0.142	0.205	0.121	0.282	0.040	0.684	0.087	0.407
Microbial biomass C	0.072	0.052	0.192	0.096	0.083	0.403	-0.029	0.779
Microbial biomass N	0.095	0.522	0.060	0.609	0.001	0.995	0.126	0.226

[a]DOC is dissolved organic carbon, DON is dissolved organic nitrogen, NO_3^--N is nitrate-N, NH_4^+-N is ammonium-N.
* Significant difference at $P < 0.05$.

Table 9. Mean ecological diversity index values calculated from denaturant gradient gel electrophoresis (DGGE) profiles of *amoA* gene fragments PCR amplified from ammonia oxidizing archaea (AOA) from soil (0-10 cm depth) of an organically managed apple orchard silt loam soil in Fayetteville, AR, that was sampled in May 2007, 2009, and 2013 (n = 3).

Ground cover	Fertilizer	Richness (S)[a]			Diversity (H)			Equitability (E)		
		2007[b]	2009	2013	2007	2009	2013	2007	2009	2013
Compost	None	-	0.0	4.5	0	0.00	1.45	-	0.00	0.97
	Poultry litter	-	0.0	5.5	0	0.00	0.77	-	0.00	0.47
	Commercial	-	5.0	2.5	0	0.53	0.80	-	0.33	0.91
Wood chips	None	-	3.0	5.3	0	0.77	0.90	-	0.73	0.53
	Poultry litter	-	1.7	2.7	0	0.33	0.47	-	0.36	0.34
	Commercial	-	3.0	3.3	0	0.80	0.90	-	0.80	0.56
Paper	None	-	4.7	1.3	0	0.97	0.17	-	0.63	0.12
	Poultry litter	-	1.7	3.3	0	0.90	0.33	-	0.96	0.21
	Commercial	-	2.3	4.0	0	0.53	0.73	-	0.42	0.41
Mow-and-blow	None	-	3.0	1.7	0	0.63	0.82	-	0.58	0.94
	Poultry litter	-	3.5	2.0	0	0.93	0.40	-	0.75	0.22
	Commercial	-	2.0	6.0	0	0.50	1.13	-	0.50	0.65

[a]S is richness; H is Shannon-Weaver diversity index; E is Shannon-Weaver equitability.
[b]There were no analyzable data from 2007.

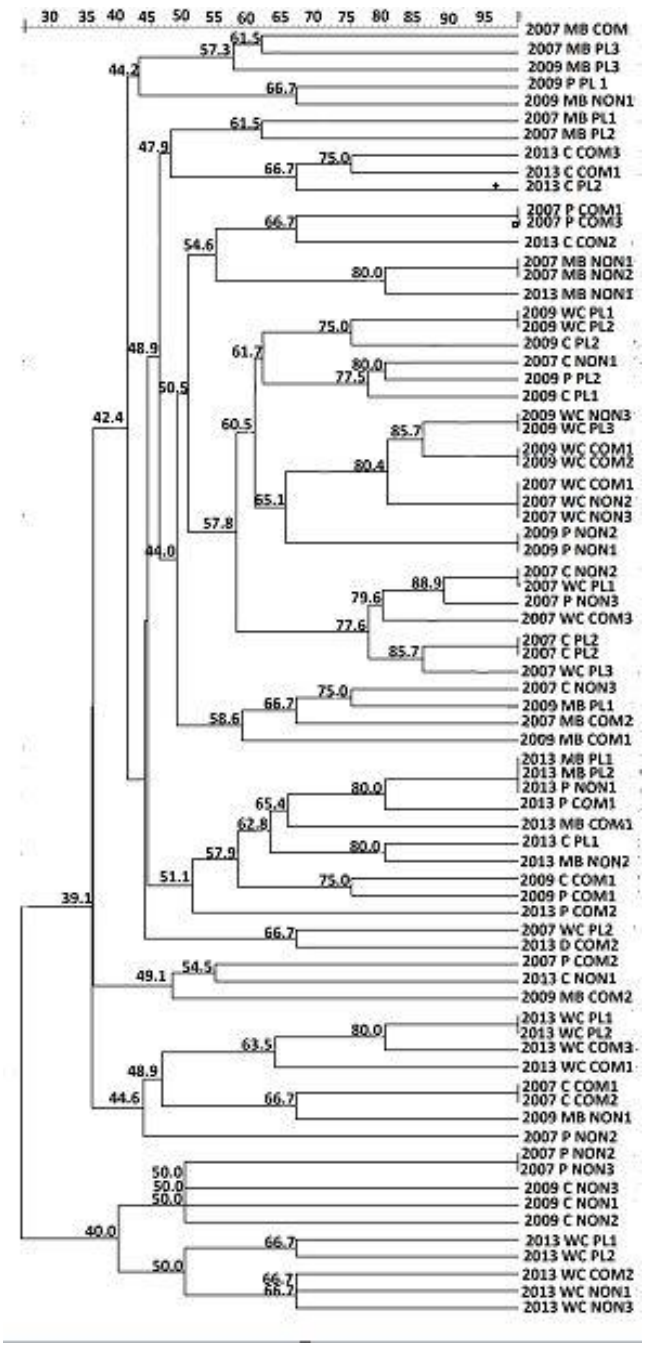

Figure. 1. Dendrogram showing bacterial *amoA* communities created from a similarity matrix calculated using Dice similarity coefficients of DGGE bands and tree topology formed using unweighted pair-group method with arithmetic means (UPGMA). Taxa names are indicated by year – ground cover – fertilizer – block. Sampling years were 2007, 2009 and 2013. Ground covers include compost (C), wood chips (WC), shredded paper (P), and mow-and-blow (MB). Fertilizers include poultry litter (PL), commercial fertilizer (Com), and a no-fertilizer control (Non).

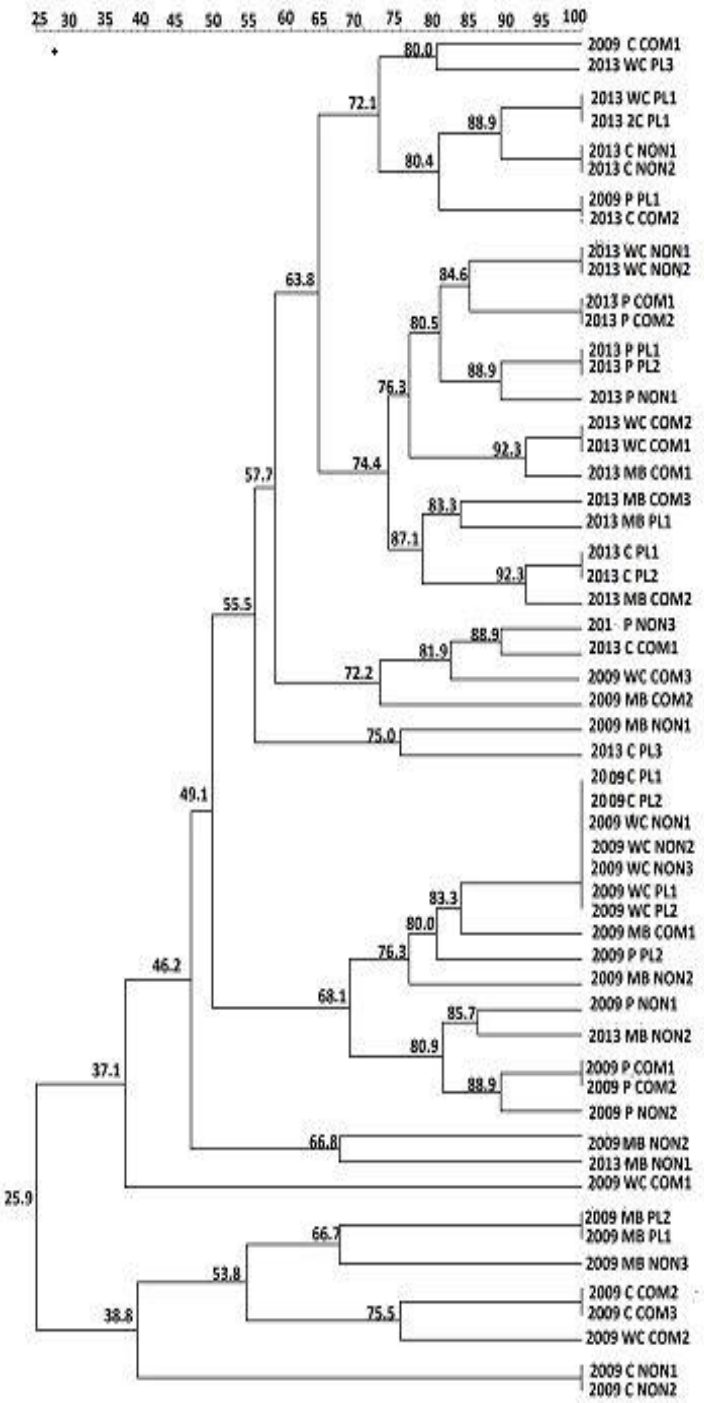

Figure 2. Dendrogram showing archaeal *amoA* communities created from a similarity matrix calculated using Dice similarity coefficients of DGGE bands and tree topology formed using unweighted pair-group method with arithmetic means (UPGMA). Taxa names are indicated by year – ground cover – fertilizer – block. Sampling years were 2009 and 2013. Ground covers include compost (C), wood chips (WC), shredded paper (P), and mow-and-blow (MB). Fertilizers include poultry litter (PL), commercial fertilizer (Com), and a no-fertilizer control (Non).