Diversity of ammonia-oxidizing archaea under managed and native conditions

Dylan McCalmont

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Sally Brown
David Stahl
Steven Fransen

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Abstract

Diversity of ammonia-oxidizing archaea under managed and native conditions

Dylan Tyler McCalmont

Chair of the Supervisory Committee:
Research associate professor, Sally Brown
School of Environmental and Forest Sciences

Ammonia oxidizing archaea (AOA) contribute to a significant portion of ammonia oxidation in soil. These organisms have significant impacts on plant proliferation, as well as production of fugitive gases. AOA community distribution patterns are influenced by multiple factors, of which, biogeography has emerged as an important variable. Developing an understanding of community differences in AOA amid differing land management types may provide tools to understand differences in N use efficiency and other, broader impacts of AOA on soil and atmospheric biogeochemistry. The goal of this study was to assess whether agriculturally cropped (managed) soils displayed shifts in AOA community diversity in contrast to native (non-managed) soils located in close proximity. Soil was collected from two sites in eastern Washington with similar climate and precipitation patterns, but representing two different soil series. At both sites soil was collected from the surface horizon (0-15 cm) of the adjacent native shrub-steppe (dominated by sagebrush and bunchgrass) and from plots cultivated in switchgrass. AOA communities were evaluated by terminal restriction fragment length polymorphism (TRFLP) targeting subunit A of the *Archaeal* ammonia monooxygenase and analyzed using multivariate

statistical approaches. The AOA communities were also evaluated via sequenced clone libraries where similarity was compared using chi-squared statistical tests. At both the slightly alkalkine (Prosser) and slightly acidic (Paterson) agricultural sites, significant differences in AOA community diversity were observed based on the contribution of differing terminal restriction fragments (TRFs) to managed and native soils based on analysis of similarity (ANOSIM, R value greater than 0.6, p value less than 0.05). In contrast, native soils displayed higher similarity to one-another, despite significant spatial separation, than either agriculturally influenced site. In addition, a higher number of TRFs were observed in the non-managed areas, indicative of a more diverse AOA community. The sequences clone libraries targeting the AOA *amoA* gene clarified the TRFLP findings and indicated a much greater diversity of *Nitrososphaera* clades in the native site, but an absence of the genera *Nitrosopumilus* and *Nitrosotalea*, which were both observed in the cultivated site. At the slightly alkaline site, similar differences between native and cultivated AOA communities were also observed. At the slightly alkaline site the most abundant TRFs in the native soils were non-detectable in the cultivated areas, suggesting a complete replacement of native ecotypes. Sequenced clone libraries from the slightly alkaline site confirmed that there was greater diversity of *Nitrososphaera* clades in the native soil and an, again, an absence of *Nitrosopumilus* which was detected in great abundance in the cultivated soil and effectively replaced many of the *Nitrososphaera* clades that were observed in the adjacent native soil. These results suggest that agricultural land-management significantly alters AOA community diversity patterns and relative abundance for the soils examined. These results can inform future research to assess whether these soils are also attributed with differing rates of nitrogen usage and production of fugitive

gases, parameters that would be useful for modeling the impacts of switchgrass cultivation on nitrogen cycling soil ecosystems.

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Abstract

Ammonia oxidizing archaea (AOA) contribute to a significant portion of ammonia oxidation in soil. These organisms have significant impacts on plant proliferation, as well as production of fugitive gases. AOA community distribution patterns are influenced by multiple factors, of which, biogeography has emerged as an important variable. Developing an understanding of community differences in AOA amid differing land management types may provide tools to understand differences in N use efficiency and other, broader impacts of AOA on soil and atmospheric biogeochemistry. The goal of this study was to assess whether agriculturally cropped (managed) soils displayed shifts in AOA community diversity in contrast to native (non-managed) soils located in close proximity. Soil was collected from two sites in eastern Washington with similar climate and precipitation patterns, but representing two different soil series. At both sites soil was collected from the surface horizon (0-15 cm) of the adjacent native shrub-steppe (dominated by sagebrush and bunchgrass) and from plots cultivated in switchgrass. AOA communities were evaluated by terminal restriction fragment length polymorphism (TRFLP) targeting subunit A of the *Archaeal* ammonia monooxygenase and analyzed using multivariate statistical approaches. The AOA communities were also evaluated via sequenced clone libraries where similarity was compared using chi-squared statistical tests. At both the slightly alkalkine (Prosser) and slightly acidic (Paterson) agricultural sites, significant differences in AOA community diversity were observed based on the contribution of differing terminal restriction fragments (TRFs) to managed and native soils based on analysis of similarity (ANOSIM, R value greater than 0.6, p value less than 0.05). In contrast, native soils displayed higher similarity to one-another, despite significant spatial separation, than either agriculturally influenced site. In addition, a higher number of TRFs were observed in the non-managed areas, indicative of a more diverse AOA community. The sequences clone libraries targeting the AOA *amoA* gene clarified the TRFLP findings and indicated a much greater diversity of *Nitrososphaera* clades in the native site, but an absence of the genera *Nitrosopumilus* and *Nitrosotalea*, which were both observed in the cultivated site. At the slightly alkaline site, similar differences between native and cultivated AOA communities were also observed. At the slightly alkaline site the most abundant TRFs in the native soils were non-detectable in the cultivated areas, suggesting a complete replacement of native ecotypes. Sequenced clone libraries from the slightly alkaline site confirmed that there was greater diversity of *Nitrososphaera* clades in the native soil and an, again, an absence of *Nitrosopumilus* which was detected in great abundance in the cultivated soil and effectively replaced many of the *Nitrososphaera* clades that were observed in the adjacent native soil. These results suggest that agricultural land-management significantly alters AOA community diversity patterns and relative abundance for the soils examined. These results can inform future research to assess whether these soils are also attributed with differing rates of nitrogen usage and production of fugitive gases, parameters that would be useful for modeling the impacts of switchgrass cultivation on nitrogen cycling soil ecosystems.

Literature review

Introduction

N cycle

Archaea, *Bacteria*, some specialized fungi, and plants govern the nitrogen cycle in soils. However, the prokaryotes—*Archaea* and *Bacteria*—are the main biological actors in the nitrogen cycle as they participate in each step of the process. Since there is no one starting point in the nitrogen cycle, this summary will begin with ammonia (NH_3) or ammonium (NH_4^+), derived from nitrogen fixation or mineralization of organic matter (Figure 1).

The first two steps of the nitrogen cycle comprise the nitrification pathway, the oxidation of ammonia to nitrate (NO_3^-) via nitrite (NO_2^-). This research primarily concerns the first and rate-controlling step of nitrification, the oxidation of ammonia to nitrite and subsequently nitrate. This process can move in the reverse order, from nitrate to nitrite to ammonia, and is called assimilatory reduction. Nitrate can be oxidized to nitric oxide (NO) or nitrous oxide (N_2O), again via nitrite (Bothe et al., 2007).

The penultimate step of the nitrogen cycle is the conversion of several different species of nitrogen: ammonium, nitric oxide, nitrous oxide, and nitrite, all reacting to form dinitrogen (N_2) and other secondary products. However, the denitrification pathway is not always completed. The conversion of nitrous oxide to dinitrogen is also integral to this study. The step that completes the nitrogen cycle is the conversion of dinitrogen to ammonia or ammonium by N-fixing bacteria.

The desire to better understand the often-incomplete denitrification pathway is part of the motivation for this study. Nitrous oxide has approximately 298 times more greenhouse gas effect than carbon dioxide (CO_2), and has multiple biological origins. Thus there is significant interest within the scientific community to better understand the organisms that fuel the production of nitrous oxide, namely ammonia-oxidizing archaea and ammonia-oxidizing bacteria. N_2O is also released during ammonia oxidation by both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Francis et al., 2005). Thus, the source of nitrous oxide is also integral to this study. There is significant interest within the scientific community to better understand the organisms that fuel the production of nitrous oxide, and the relative importance of ammonia-oxidizing archaea and ammonia-oxidizing bacteria.

In the absence of oxygen, nitrate has several fates. It can be reduced to nitrite, and then to ammonia, in both assimilatory and respiratory processes, the later termed dissimilatory reduction of nitrate to ammonia (DRNA). As stated above, the nitrite also is a co-substrate with ammonia in the recently described anammox reaction, generating dinitrogen (N_2) as an end product. In denitrification, nitrate is respired through a series of intermediates of increasingly reduced nitrogen species—nitrite, nitric oxide (NO), nitrous oxide (N_2O)—to release N_2 as a final product (Bothe et al., 2007). Di-nitrogen makes up the majority of the earth's atmosphere (approximately 78.09%).

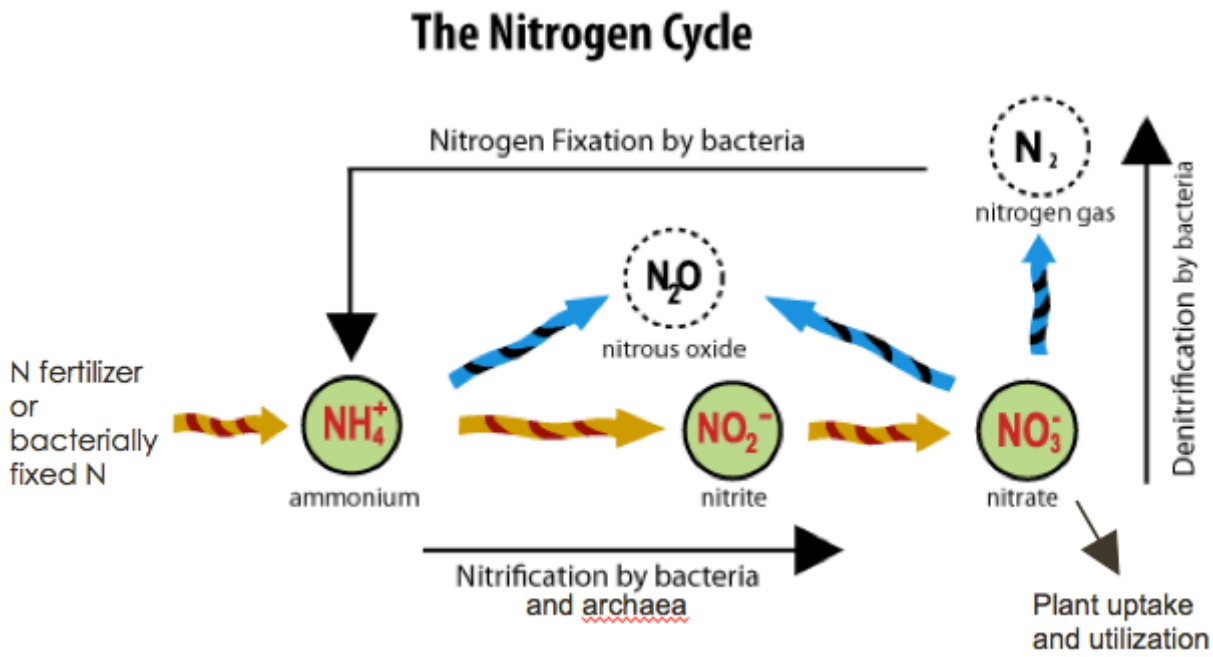

Figure 1. AOA play a key role in the nitrogen cycle oxidizing ammonium to nitrite and sometimes nitrous oxide (teachoceanscience.net).

The biochemical reactions of nitrification

Ammonia-oxidation can begin from either ammonium or ammonia. The two possible reaction paths are as follows, for ammonium:

Step 1: $2\ NH_4^+ + 3\ O_2 \rightarrow 2\ NO_2^- + 2\ H_2O + 4\ H^+$

Step 2: $2\ NO_2^- + 1\ O_2 \rightarrow 2\ NO_3^-$

For ammonia:

Step 1: $NH_3 + O_2 \rightarrow NO_2^- + 3\ H^+ + 2\ e^-$

Step 2: $NO_2^- + H_2O \rightarrow NO_3^- + 2\ H^+ + 2\ e^-$

In both cases nitrite (produced in step one) is quickly converted to nitrate (produced in step two), as ammonium degradation is usually the rate-limiting step (Brock, 2012).

Nitrous oxide formation occurs when nitrate is reduced to nitrite, nitrite is reduced to nitric oxide, and nitric oxide is reduced to nitrous oxide:

$1\ NO_3^- \rightarrow 1\ NO_2^- \rightarrow 1\ NO \rightarrow 1\ N_2O$

Agricultural nitrogen fertilization methods

Nitrogen can be added to crops in multiple forms. Organic nitrogen can be added to crops in the form of manure, biosolids, yard waste, crop residues, etc. Organic nitrogen has to undergo mineralization processes before it becomes available for biotic uses. Mineralization is the oxidation or decomposition of organic matter to form plant accessible forms of nitrogen. Mineralization takes time, temperature, and moisture for organic nitrogen to be released slowly (Brady and Neil, 2004). It is a microbially mediated reaction. The nitrogen fixation symbiosis process may also provide nitrogen to crops. An example of this is the relationship between legumes and rhizobia. The reduction of molecular nitrogen to ammonia by nitrogen fixation results in continuous, but relatively small amounts, of N

available for crops (it is important to note that nitrogen fixation can still result in N leaching). After plants uptake nitrates they use them to create amino acids and subsequently proteins which perform many cellular-level functions within plants. There are also free living nitrogen fixing diazotrophs. Free living diazotrophs include oxygenic and anoxygenic photosynthetic bacteria and various anaerobes. The most common way that nitrogen is applied to crops is in the form of synthetic nitrogen that has been fixed through a series of chemical reactions known as the Haber-Bosch process (Brady and Weil, 2004). Synthetic nitrogen can be applied to crops in a solid or a liquid form. If a crop is irrigated then nitrogen can be added to the out-going water. This process is typically referred to as fertigation or the combination of irrigation and fertilizer application. However, the more common form of synthetic nitrogen addition is in a solid pelletized form. Nitrogen is typically applied as a solid once or twice during the growing season. During irrigation or rain the nitrogen is dissolved in water and delivered to the plant roots. However, with so much available nitrogen being applied at the same time there is a potential for a significant portion of the applied nitrogen to leach out of the soil with the above and/or below ground run-off. Nitrogen leaching occurs after nitrifiers convert ammonia to nitrite and subsequently nitrate, which is highly mobile in soils. Nitrogen leaching is costly and poses significant environmental problems.

The role of microbial communities in ecosystem functioning is important because these communities and ecosystems can have global impacts. Soil microbial communities can change as a result of land-use changes (Hallin *et al*., 2009). Microbial communities can have global impact in multiple ways. Examples of this are nitrification and the production of atmospherically active gases (for example N_2O). Developing a better understanding of one of the most important nutrient cycles on earth will allow more accurate and complete evaluation of these microbial communities. However, it can be hard to isolate which variable it causing the community to change. Hallin *et al*. (2009) observed that AOA communities found in fallow and soil cultivated in annual maize were different, but that the plants were not the most determinant factor. By further controlling the test environment the mechanism of AOA community change can be more completely understood. The results of this study hint at possible AOA community differences in soil cultivated with perennial crops and annual crops.

History of AOA study

The above discussion is linked to ammonia-oxidation because it is the process that creates the principal form of plant available nitrogen (nitrate) in the soil. For nearly a century, beginning in the late 1800s, scientists believed that ammonia oxidizing bacteria (AOB) were solely responsible for oxidizing ammonia into nitrite and subsequently nitrate. Könneke et al. (2005) disproved the theory that AOB were the sole environmental ammonia-oxidizers. They were also the first group of researchers to establish a firm metabolic link between ammonia oxidizing archaea (AOA) and ammonia oxidation. The work of Könneke et al. was built on the work Carl Woese, Edward DeLong, and Jed Fuhrman. Woese established that *Archaea* represented a deep evolutionary division, comprising one of three domains of life. Bacteria and Eucarya comprise the other two domains (Woese et al., 1977; Woese et al., 1990). DeLong and Fuhrman were the first to establish that members of the *Archaea* were environmentally relevant in temperate environments (DeLong et al., 1992; Fuhrman et al., 1992).

All scientists studying ammonia-oxidizing archaea (AOA) are indebted to scientists that were able to establish a direct metabolic link from an AOA species to ammonia-oxidation. Hatzenpichler et al. built on the work of Könneke et al. and (2008) were able to confirm that *Nitrososphaera gargensis* (an AOA species) oxidize ammonia for energy. Pratscher et al. (2011) demonstrated the same ability, this time in the presence of ammonia oxidizing bacteria (AOB). Using stable isotope probing (SIP) with a focus on RNA the researchers were also able to demonstrate AOA were autotrophically fixing CO_2.

The relationship between native AOA communities—meaning the AOA communities that were present in an area prior to agricultural disruption—and the AOA communities that exist in areas with ongoing agricultural practices is not fully understood. Research has found an unexpected amount of archaeal genetic variation in native soil environments (Schleper *et al*. 2005). Further understanding this genetic diversity and how it is affected by anthropogenic activities could improve our understanding of soil nitrogen transformations, and how these transformation impact the global nitrogen cycle and climate change.

AOB diversity

AOB are less phylogenetically diverse than AOA (Figure 3). Therefore there is less niche differentiation in AOB than is seen in AOA. Prevalent AOB are *Nitrosomonas*, *Nitrosospira*, and *Nitrobacter* (not included in the figure 3 phylogenetic tree). AOA are more phylogenetically diverse and therefore have more niche differentiation is observed between the various clades; this is expected. For example, *Nitrosotalea* ("Clade D" in figure 2) is an acidophilic AOA; the other AOA clades also have niche differentiation, though most are currently less well defined than that of *Nitrosotalea*.

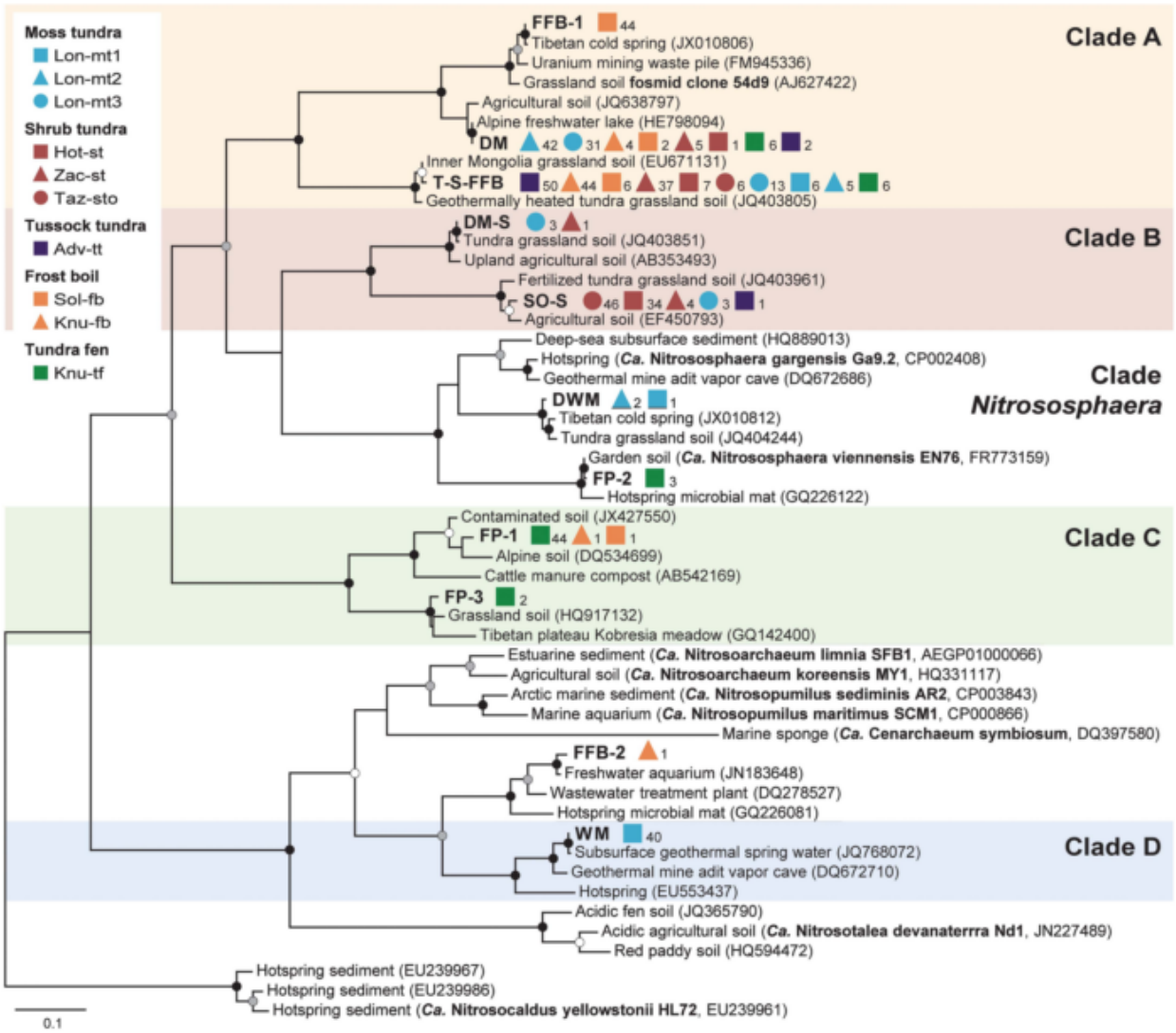

Figure 2. AOA phylogenetic tree (Alves et al., 2013).

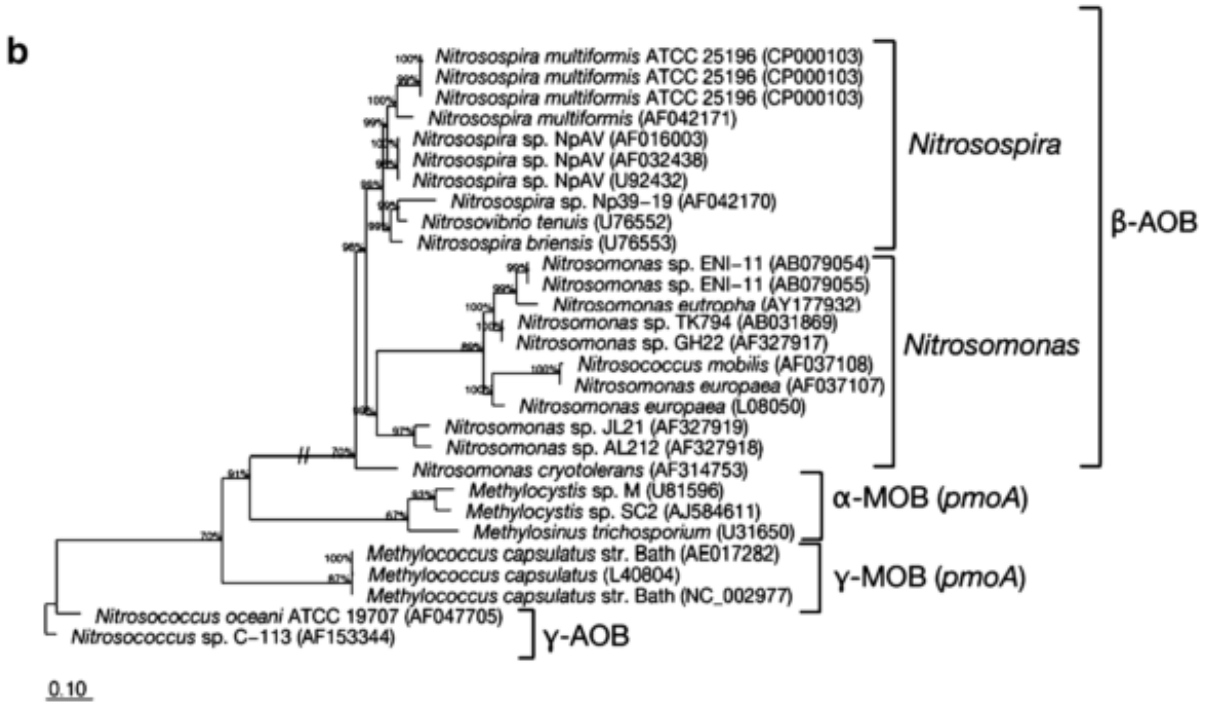

Figure 3. AOB phylogenetic tree (Junier et al., 2009).

Increased understanding of AOA and/or AOB diversity shifts and why they are happening will hopefully allow people to more efficiently utilize agricultural land.

Motivation for the research

The activity of ammonia oxidizing microorganisms affects soil and atmospheric processes in several ways. Highly soluble and mobile nitrite and nitrate are lost from soils and enter groundwater and surface water systems (resulting in eutrophication and water quality degradation). Another minor factor is the depletion of positively charged ions in soil (the positively charged ions are leached from the soil system because of ionic interactions with the negatively charged nitrate). Nitrification also impacts the atmosphere due to its contribution to emissions of the potent greenhouse gases—nitrous and nitric oxides—from soils. These environmental impacts, of long recognized significance in agriculture, have elevated importance when considering increased production of bioenergy crops. Nitrogen fertilization may lead to greater emission of reactive N including water quality degradation via nitrate leaching and gas emissions via nitrous oxide and nitric oxide off-gassing and ammonia volatilization. Thus, effective nitrogen delivery to plants is essential for mitigating both economic and environmental costs of an expanded bioenergy industry. However, the relative contribution of AOB and AOA to ammonia-oxidation in different soils and under different cropping systems is not well understood. This study will elucidate further ecosystem conditions that affect AOA community diversity and thus nitrification.

Biases in community diversity analytics

Primer bias is possible any time primers are used as a tool to interrogate microbial community ecology. However, the best way to counter any possible biases is to have sufficient plot, sample, and laboratory analysis repetition. Repetition reduces the

likelihood that the same bias will be happen in every sample that is analyzed, thus increasing the veracity of the findings.

Functional significant of AOA and AOB in natural and managed soils

The following section will establish the merit and importance of examining the role of AOA in soil.

Introduction to AOA v. AOB

The following section will introduce the first research on non-thermophilic soil AOA and a few general ideas that help frame the general understanding of the role of AOA.

There are currently conflicting research results on the relative contribution of archaea and bacteria to ammonia-oxidation in soils. Before 2006 it was widely believed that ammonia-oxidizing bacteria (AOB) controlled ammonia-oxidation in both aquatic and terrestrial systems. However, Leininger et al. (2006) observed high abundance of AOA, relative to AOB, in different soils. This observation put AOB dominance into question. The evidence for a significant role of AOA in soils systems has continued to mount since 2006. While there is a lot of conflicting evidence, respected minds in the field have suggested that AOA are the dominant ammonia-oxidizers in soil (Stahl and de la Torre 2012).

Other research suggests dominance of AOB. For example, in an early experiment looking at AOA activity Nicol et al. (2004) manipulated soil from grasslands used as cattle pasture in a controlled environment to see how the AOA and AOB communities changed. After increasing and reducing the pH, applying ammonium nitrate, and applying sheep urine in different trials the team observed no change to the AOA community in any of the trials. They did however observe changes in the AOB community composition and abundance in all of the experiments, demonstrating that AOB had a response to increases in soil N. The group hypothesized multiple reasons for the apparent lack of AOA activity, including that the incubations may have been too short (28-30 days). The researchers did not differentiate between a no activity and a constant level of activity. However, both suggested that AOA were not playing a significant role in ammonia-oxidation in this soil system. Nicol et al. also lacked a true control, or un-manipulated grassland soil, in their study. This early examination of the relationship between AOA and AOB, despite some of its possible methodological shortcomings, seemed to demonstrate that AOA might play a small role in soil ammonia-oxidation.

The interaction between AOA and AOB is not well understood. Schauss et al. (2009) examined the functional redundancy of AOA and AOB. The researchers examined soils that were amended with pig manure spiked with an antibiotic (sulfadiazine or SDZ). The soil was collected from two agricultural areas in Germany, however the authors did not say what crops were being cultivated. The relative growth rates of AOA and AOB, in correlation with nitrification data, indicated that continued ammonia oxidation in the presence of antibiotic inhibited AOB could be attributed to the AOA. This overlap in nitrification capability demonstrated functional redundancy between AOA and AOB. Schauss et al. (2009) also observed that AOA were 73 times more abundant than AOB in their trials.

Establishing that AOA and AOB were functionally redundant was important because it showed that understanding the relationship and potential competition between the two taxa was important. If the two taxa were not functionally redundant it would have changed how researchers approached the entire field of study. However, the demonstration of functional redundancy necessitated the examination not only of AOA and AOB individually, but AOA and AOB together.

Zhang et al. (2010) demonstrated that AOA, like AOB, have the ability to grow autotrophically. The researchers tested whether AOA grew using organic or inorganic carbon. They found, using stable isotope probing, that AOA incorporated ^{13}C-enriched carbon dioxide. Furthermore, the team observed that nitrification was correlated with increases in AOA *amoA* gene abundance and changes in AOA *amoA* gene diversity. Additionally, no change was observed in bacterial *amoA* genes. This was direct evidence that ammonia-oxidation, in this soil, is being controlled by AOA and not AOB. Zhang et al. also observed that the majority of the AOA growing autotrophically in their test soils were from group 1.1b (the genus *Nitrososphaera*), but AOA from group 1.1a (the genus *Nitrosopumilus*) were also observed growing autotrophically in the soils.

Research has suggested that the microbial community structure influences the other microbial species that occur in a given area (Horner-Devine *et al*. 2007). Horner-Devine et al. conducted an evaluation by looking at microbial co-occurrence patterns across several different ecosystems. While the researchers did not focus on AOA and AOB, it is likely that their findings are applicable to a broad range of ecosystems. These researchers suggested several contributing factors, including: "competitive interactions, non-overlapping habitats, mutualistic or syntrophic relationships, and/or historical effect." Ammonia-oxidizing microbes and several other relationships were discussed in the article. Microbial community influence presents a challenging set of relationships to assess. However it is important to note that these relationships play a significant role in microbial ecology.

The influence of microbes on one another, especially regarding AOA, remains a relatively unstudied field. However, to study AOA biogeography it is important to begin to identify possible environmental variables influencing distribution patterns, even if they have yet to be quantified.

AOA, AOB Niche differences

Researchers have attempted to discern under what circumstances will AOA or AOB dominate in a particular soil system. Both AOA and AOB were found to play a role in nitrification in relatively acidic (3.7-6.0) upland soil in the Hunan province of China (south-central China) (He *et al*. 2007). The highest *amoA* gene copy number for both AOA and AOB was observed in the plot where both organic matter (OM) and standard fertilizer (in this case NPK) were applied. Conversely, both AOA and AOB *amoA* gene copy number was at their lowest where only N was applied. AOA were similar to or more abundant than AOB in all trials (ratios varying from 1.02-12.36). A positive correlation was observed between AOA and AOB abundance and increases in soil pH and the measurable nitrification rate. These results indicate that both AOA and AOB were playing a role in ammonia-oxidation in this soil. Additionally, the researchers observed that the AOB community did not vary between any of the plots. Interestingly, the AOA community did vary between plots that

had received different forms of long-term fertilization. These investigators also observed that the AOA community was made up of representatives from the S cluster (*Nitrososphaera*) and the M cluster (*Nitrosopumilus*). The clusters were named for the location of their initial observation, soil and marine respectively, however those are not the only locations where they are observed. *Nitrosopumilus* seemed to dominate in unbalanced/incomplete fertilizer supply plots (N, NP, NK, and PK), while *Nitrososphaera* were dominant in the fallow soil, control without fertilizer, and NPK, and NPK + OM treatments. However, the results were not statistically definitive.

The work of He et al. (2007) began to establish the complexity of the relationship between AOA and AOB. This study demonstrated the necessity of not only further examining the relationship between these two different taxonomic groups of organisms, but the relationships within each taxonomic group.

AOB generally dominate ammonia-oxidation in inorganic-N rich environments: The importance of soil N concentration and form

A trend that has begun to emerge from research on the ecology of ammonia-oxidizing microorganisms is that AOB tend to dominate in soil systems that have high concentrations of inorganic nitrogen; this nitrogen is typically applied as agricultural fertilization. AOB functionally dominated ammonia-oxidation in an agricultural soil in Germany (Jia and Conrad 2009). While AOA were more abundant in the soil, it was shown that AOB were the dominant force in oxidizing ammonia. This was shown in two ways. First, following ammonium addition AOB abundance increased, and following the subsequent addition of acetylene (a nitrification inhibitor) AOB abundance decreased. AOA did not change in abundance during either of these experiments. Second, stable isotope probing showed that CO_2 was being assimilated by AOB, not AOA.

Research by Di et al., (2009) also suggested that AOB were primarily responsible for nitrification in N-rich grassland soils. The communities were evaluated using qPCR. AOA were more abundant at four of the six test sites, but after the addition of urine as a source of ammonia the AOA abundance did not change. However, after the urine addition the abundance and activity of AOB increased 3.2-10.4 fold and 177 fold respectively. The increase in AOB abundance and activity correlated with an observed increase in nitrification. This study was conducted in six grassland soils in New Zealand. The AOA community was comprised primarily of *Nitrososphaera* and to a lesser extent *Nitrosopumilus*. The researchers observed AOA to be more abundant in the control plots than the plots treated with urine at two of the six sites. Di et al. also observed that in the urine treatment AOA RNA copy numbers, determined via reverse transcription, were $1/50^{th}$ of the AOB RNA copy numbers. This study supports the work of Jia and Conrad. It is also notable that Di et al. observed AOA to be most abundant in the low-N control plots. This observation begins to establish another trend, that AOA are the dominant ammonia-oxidizers in low-N environments.

Di et al. followed up their first study with a second study the next year (2010). They again observed that AOB were the dominant ammonia-oxidizers in N-rich grassland soils of New Zealand. The study was conducted in three intensively grazed dairy pasture grasslands in New Zealand, characterizing AOA and AOB community differences in relation to soil depth

and N availability (Di *et al*. 2010). AOB were more abundant in all three surface soils, while AOA were more abundant in one of the three sub-soils. Furthermore, AOB grew well when supplied with nitrogen. Growth was evaluated via *amoA* gene copy numbers. Conversely, AOA only grew in the control plots where N was not supplied. Nitrification rates were higher in topsoils than sub soils. Additionally, nitrification rates were correlated with AOB abundance and not AOA abundance. These results reinforced the findings of Jia and Conrad and Di et al. (2009) and observation by other researchers (Leininger et al., 2006; Hansel et al., 2008; Di et al., 2010) of AOA dominance in deeper soils.

In general these studies and others are consistent with a preference by AOA for low N conditions. Martens-Habbena et al. (2009) did some novel and elegant research that showed that AOA could grow under extreme nutrient limitation. The research was done in an aquatic setting, but is relevant to this thesis as well. The dominance of AOB in high nitrogen conditions in three different studies (Jia and Conrad, 2009; Di et al., 2009; Di et al., 2010) is strong evidence that AOB commonly dominate ammonia oxidation in high-N conditions.

Some research has indicated that most AOA cannot tolerate high concentrations of ammonia in soils. To investigate this further, Tourna et al. (2011) focused their research on a single AOA species. Tourna et al. advanced the collective understanding of AOA when they cultured *Nitrososphaera viennensis*. This organism is unique from other cultured AOA because it can tolerate higher concentrations of ammonia (approximately 10 times more) than other cultured AOA. However, it is still less tolerant of ammonia than all AOB (approximately 2.5-50 times less tolerant). Tourna et al. were able to cultivate two ecotypes of the organism, which had different ideal growth temperatures. This suggests that more ecotypes may exist. However, the discovery of a more ammonia-tolerant AOA does not offer strong evidence against previous work showing AOB dominance of ammonia-oxidation in inorganic nitrogen rich environments. However, it does indicate that collective knowledge regarding AOA growth habitats is still limited. Another study, Bates et al. (2011), which was primarily focused on AOA diversity in native soils (and will be discussed more thoroughly in that section), also observed a related trend in AOA and AOB. In several instances, when the concentration of soil-N increased, Bates et al. observed a corresponding decrease in AOA abundance and an increase in AOB abundance. As more studies are conducted and more organisms are cultivated, it will continue to become clearer what the true growth boundaries are for AOA.

AOA dominate ammonia-oxidation in low inorganic-N environments.

Several studied have noted the dominance of AOA over AOB in low N environments. Pratscher et al. (2011) determined that AOA, specifically *Nitrososphaera* and *Nitrosopumilus*, were present in the study environment (maize plots in Germany) at a numerically relevant abundance (Pratscher et al. defined "numerically relevant" as 4.96×10^7 or greater). The researchers brought soil cores back to the lab and applied either 15 μg of N per gram of dry soil or 100 μg of N per dry gram of soil weekly. The group observed that AOA only grew in the trial that had 15 μg N applied (N was added as ammonium sulfate). The AOA were not observed to grow in the trial that had 100 μg N applied per gram of dry soil on a dry weight basis.

AOA, not AOB, were observed to dominate ammonia-oxidation in two agricultural soils (Gubry-Rangin *et al*. 2010). The test soils were incubated at 20°C over 30 days both in the presence and absence of acetylene. Soils were also kept at two pH levels: 4.5 and 6. High rates of nitrification were observed in both soils in the absence of acetylene. Quantification of *amoA* genes showed significant growth of AOA, but not of AOB. A positive relationship was observed between nitrification rate and AOA growth. In the presence of acetylene (a nitrification inhibitor) transcriptional activity of AOA decreased, but the transcriptional activity of AOB did not decrease. These data suggest that AOA controlled ammonia-oxidation in these soils.

Gubry-Rangin et al. only ran trials that had 30 μg N or less applied per gram of dry soil—a low amount of available N. The authors observed a significant positive relationship between nitrate concentration and AOA abundance, but not AOB abundance. A difference in the AOA communities was also observed as a function of soil pH. The authors interpreted their results as further evidence for pH specific AOA phylotypes. Referring to AOA, Gubry-Rangin et al. also noted that, "[the] results suggest a preference for low ammonia concentration may be a factor." Gubry-Rangin observed, in support of the three previously referenced studies, that AOA were dominant in a low-N environment. This observation is important for two reasons. One, it continues to establish a clear trend that AOA play a smaller role in soil environments that have high N. Two, this evidence indicates that the native soils examined in this thesis are likely dominated by AOA.

The application of dairy slurry to six different soils was found to have no effect on AOA abundance per gram of soil after 28 days (Fortuna, 2012). Across the soils AOA were, on average, an order of magnitude less abundant than AOB. The dairy slurry had high concentrations of inorganic nitrogen in it (in the form of ammonium). These results further suggested that AOA and AOB communities have different optimum growth and activity conditions. The results in this study indicated that AOB controlled ammonia-oxidation under the conditions of the experiment. However, the group observed that the AOB accounted for approximately 70% of ammonia-oxidation activity in the soils that were studied. AOA may have played a minor role in ammonia-oxidation during the experiment. While the conditions that allowed AOA to participate in ammonia-oxidation in this experiment are not known, the slow mineralization of organic nitrogen may have played a role.

A repeated methodological problem that multiple studies are guilty of is only observing the ammonia-oxidizing microbial community change over 28 days. This is a relatively short period of time and fails to capture the possible changes that occur in the ammonia-oxidizing microbial community over a single growing season.

In a separate study, AOA were observed to dominate in abundance and nitrification rate relative to AOB where municipal biosolids were the source of N (Kelly et al. 2011). These observations were made in corn fields in Illinois. The abundance calculation was based on *amoA* copy numbers and the nitrification association was based on AOA *amoA* increase in conjunction with an increase in nitrification rate. Kelly et al. made several more observations. Synthetic fertilizer did not significantly change AOA abundance. After

application of an agronomic rate of biosolids AOA *amoA* increased in abundance, while AOB *amoA* showed no change. The authors noted that organic nitrogen has been observed to mineralize slowly and theorized that this may have advantaged AOA over AOB. Kelly et al. also noted that AOA outnumbered AOB in all of their plots. While not novel, an important observation that Kelly et al. made was that the mineralization rate of applied nitrogen may have an important effect on whether AOA or AOB can best metabolize it. The slower mineralization rate of organic nitrogen means that even if there is a high amount of total nitrogen in the soil, AOA may be able to thrive because ammonia-N will be available in smaller amounts.

There is some evidence that AOA will dominate ammonia-oxidation when large amounts of organic nitrogen have been added to a soil (Kelly et al., 2011). This is consistent with previous research showing that AOA dominate in low-N systems because organic nitrogen will mineralize slowly, continually providing a smaller amount of available nitrogen over a longer period of time. In contrast, synthetic fertilizer application adds high amounts of ammonia at one point in time.

Role of temperature

Temperature may also have an impact on the relative abundance of AOA in soils. AOA were found to be more abundant than AOB in semiarid soils in northern Arizona (Adair *et al*., 2008). Adair et al. examined five different native soils, each with a different vegetative community growing in it. Using qPCR along an elevation gradient (1556-2620m) the researchers observed that AOA were between 17-1600 times more abundant than AOB across all sites. The results suggest that AOA are the primary ammonia oxidizers in these soils. Adair et al. observed that AOB were most abundant at sites with lower air temperatures and higher amounts of precipitation, however AOA population numbers were not related or did not vary based on to air temperature or quantity of precipitation. These observations lead the authors to predict that AOA are more resistant to desiccation than AOB.

Tourna et al. (2008) created a series of soil microcosms with soil collected from the top 10cm of an agricultural field. The soil had a pH value of 7.0 and the microcosms were kept at a series of temperatures ranging from 10°C-30°C. The researchers measured the transcription of 16S rRNA and *amoA* genes. Tourna et al. observed strong evidence of AOA community, activity, and abundance change during the experiment. Conversely, they did not observe any AOB community, activity, or abundance change in the soil microcosms. These observations led Tourna et al. to hypothesize that AOA were solely responsible for the observed ammonia-oxidation. The differences between the AOA and AOB communities were accentuated at upper end of the tested temperature range. The authors observed AOA groups 1.1a (*Nitrosopumilus*) and 1.1b (*Nitrososphaera*) to be present in the soil microcosm experiments. Tourna et al. cited further need for exploration into AOA and AOB biogeography to help make the results more broadly relevant.

Another study, conducted at 30°C using soil microcosms, also showed AOA dominance (Offre et al., 2009). Over a 30-day incubation period AOA were observed to dominate ammonia-oxidation (Offre *et al*. 2009). The soil microcosms were made from soils taken from the top 10cm of an agricultural plot. During the incubation AOB gene diversity did not

change (based on 16S rRNA data) and abundance decreased slightly (based on *amoA* gene data). Conversely, the AOA community increased in abundance and underwent changes in diversity; both *Nitrosopumilus* and *Nitrososphaera* were observed to increase in relative abundance during the experiment. After acetylene was added to the microcosms the growth of AOA was suppressed demonstrating that they were oxidizing ammonia for energy. These observations led Offre *et al*. to conclude that AOA were mostly responsible for the measured ammonia-oxidation. The researchers also observed that AOA dominated ammonia-oxidation at 30°C, an observation that is supported by the findings of Adair et al. and Tourna et al.

Concluding AOA versus AOB

Huang et al. (2014) wrote that they did not think AOA needed to be considered as major contributors to ammonia-oxidation in agricultural soils. The group cited previous research (Di et al. 2009, Xia et al. 2011, and Prosser and Nicol, 2012) the region they were studying, and outside of acidic soils, as evidence that AOA did not contribute significantly to ammonia-oxidation in intensively managed soils, respectively. However, there is evidence to the contrary (Kelly et al, 2011; Zhalnina et al., 2013) and experts in the field may not agree with the assertion made by Huang et al (Stahl and de la Torre 2012). While this exclusion may have been justified in this case, the current scholarship does not indicate an ecology where AOA can be excluded from examination when attempting to understand the ammonia-oxidizing community.

There is strong evidence for AOA and AOB dominance in various environments. For example, AOB dominate ammonia-oxidation in environments that have high levels of inorganic nitrogen, while AOA generally dominate ammonia-oxidation in environments that are low in inorganic nitrogen. However, as is expected with a relatively young field of study, there are conflicting research results. Many studies have concluded that AOA dominate ammonia-oxidation in soils (Adair et al., 2008; Tourna et al., 2008; Chen et al., 2008; Offre et al., 2009; Gubry-Rangin et al., 2010; Zhang et al., 2010; Kelly et al., 2011; Nicol et al., 2008; Leininger et al., 2006; Mao et al., 2011; Hallin et al., 2009). Other studies have found AOB to dominate ammonia-oxidation (Nicol et al., 2004; Jia and Conrad, 2009; Di et al., 2009; Di et al., 2010; Bates et al. 2011). Several additional studies have observed both AOA and AOB having significant roles in ammonia-oxidation in the same environment (He et al., 2007; Fortuna et al., 2012; Tourna et al., 2011; Yao et al., 2013; Shen et al., 2008; Ying et al., 2010; Taylor et al. 2010). While multiple trends have emerged from this scholarship, there is still a lot of investigation to be conducted regarding the relationship between AOA and AOB.

As Tourna et al. noted an improved understanding of AOA biogeography is important for an improved understanding of the N-cycle. The work contained in this thesis will build on the work of Tourna and others to improve the understanding of AOA in native soils and soils that are growing various crops. The findings from Tourna et al. support the work done by Adair et al (2007) as both saw AOA dominance in high temperature soils. However, other variables were not held constant between the two studies. This section discussed some of the key differences between AOA and AOB. The next section will examine key differences between different AOA clades.

Relationship between selected environmental factors and distribution and activity patterns

Researchers have studied a range of different environmental factors and their impact on AOA distribution in soil systems. These factors include pH, depth in the soil profile, available moisture, and the role of plants.

Role of pH as a controlling environmental variable

There are many subspecies within AOA. Research indicates that particular genera may dominate under certain environmental conditions. Kemnitz et al. (2007) made multiple observations about *Archaea* and *Bacteria* in a temperate acidic forest soil. Regarding this thesis, the most interesting observation was that group 1.1c archaea (containing the genus *Nitrosotalea*) made up the main portion (85%) of AOA. Group 1.1b (*Nitrososphaera*) made up the other 15%. This study was the first to observe the AOA genus *Nitrosotalea* dominating ammonia-oxidation in low-pH soils. Several subsequent studies have further examined the importance of soil pH in AOA populations.

Following up on the observations made by Kemnitz et al., Lehtovirta et al. (2009) took a more focused look at the effect of soil pH on AOA. Within a soil pH gradient (4.5-7.5) that was maintained over 40 years in agricultural soil, Lehtovirta et al. observed changes within the AOA community over the pH gradient. While total crenarchaeota (the taxonomic kingdom in which all AOA are contained) showed no trend in abundance, group 1.1c (*Nitrosotalea*) declined in abundance as pH increased. Furthermore, the group 1.1c community diversity also changed as pH increased. Group 1.1c archaea disappeared from the community at pH above 6.0. Lehtovirta et al. also observed that group 1.1c was at its highest abundance at the lowest pH (4.5). These trends were also observed at other sites. Lehtovirta et al. also noted that at their sites crenarchaeota only made up 1.1-2.2% of the total microbial population, and of the crenarchaeotal population, *Nitrosotalea* only made up 0.1-1.8%. While *Nitrosotalea* did not compose a large portion of the microbial population, all bacteria, and most other AOA, do not grow well at such low pHs (Nicol et al., 2008) making *Nitrosotalea* the key ammonia-oxidizers in low-pH soil ecosystems.

Lehtovirta and colleagues continued to focus on *Nitrosotalea*. Two years after their initial study, Lehtovirta et al. (2011) were responsible for a significant advance in the understanding of ammonia-oxidation when they were able to cultivate the first acidophilic AOA. Ammonia-oxidation had been observed in acid soils (Kenmitz et al., 2007; Lehtovirta et al., 2009; Nicol et al., 2008). However, until this successful cultivation, no organism had been identified that was definitively responsible for the observed ammonia-oxidation at low soil pH (AOB do not grow well at acid pHs (Nicol et al., 2008)). The newly cultivated AOA, named *Nitrosotalea devanaterra*, has an optimal growth range of pH 4-5 and will not grow at pH values higher than 5.5. The species is related to the 1.1a-associated group of AOA. Work relating to AOA and pH has not been restricted to acidic soils. The three subsequent studies address AOA and non-acidic soil.

Other studies have noted the importance of pH on AOA community diversity. In a field trial that had soils ranging from pH 4.9-7.5 (~0.5 step intervals) it was observed that AOA communities adapted to their "native" pH (Nicol *et al*. 2008). The field trial was conducted

in Scotland on plots that had been maintained since 1961. The plots undergo a perpetual 8-year crop rotation. In this case adapted means the AOA communities had their highest activity at their "native" pH. In this case "native" means the pH that the AOA community had adapted to over a period of time. Other than pH, soils showed little variability between plots. The comparison was done via 16S and *amoA*, denaturing gradient gel electrophoresis (DGGE), and sequence analysis. The researchers also observed that different AOA phylotypes thrived at different pHs. Total AOA transcript abundance decreased with increasing pH (from $9.9x10^6$ to $2.1x10^6$ per gram of dry soil). AOB transcription rates did the opposite and increased with increasing pH. Furthermore, AOA transcript abundance was greater than AOB transcript abundance in the majority of the samples (AOB were 0.8-3.1% as abundant as AOA across all soils). Most AOA within the plots fell within the 1.1b group (*Nitrososphaera*), but AOA from group 1.1a (*Nitrosopumilus*) and the pSL12 group were also observed. This research helps build support for the narrative that pH has a significant effect on all AOA.

Other work has also examined pH as a possible variable impacting AOA communities, with varying results. One group of researchers found that pH was the most influential soil factor affecting AOA communities (Gubry-Rangin *et al*. 2011). The researchers found that AOA communities varied based on pH on global, regional, and local scales. The AOA communities in this experiment were quantified using the AOA *amoA* gene. Gubry-Rangin et al. observed three major AOA clades: 1.1a (*Nitrosopumilus*), 1.1a-associated, and 1.1b (*Nitrososphaera*). On a global scale the clades made up the following percentages of the AOA population 10.2%, 21.2%, 68.6%, and a negligible percent, respectively. On a regional scale—within the UK—*Nitrososphaera* were more dominant, making up 82.5% of the total population. On a regional scale group 1.1a made up a smaller percentage of the population, 0.4% (47 sites were examined with the UK, they had a wide range of pH values). 1.1a and 1.1a-associated clades were found in acid and acid-neutral soils. The 1.1b clade was found in acid-neutral and alkaline soils.

Gubry-Rangin et al. made several more observations about pH. The researchers observed the greatest AOA species richness between pH 6-8, with decreases in diversity seen at pH >8 and <5. Furthermore, pH was the only soil factor that had a significantly correlation with community formation. The group also made two novel and possibly controversial observations. One, they observed that the 1.1a-associated group dominated in acidic soils, whereas previously they had been associated with non-acidic soils. Additionally, the team observed AOA growth in soils that had high pH and high ammonia availability. Most previous work pointed to AOA growing at low pH with low ammonia availability. The group interpreted their results as strong evidence that AOA lineages are adapted to specific pH ranges, and that liming could influence AOA activity. This research further strengthens the narrative the pH is an essential factor in AOA community formation.

Published research on AOA often reports conflicting results on relative abundance and importance of soil factors. pH and location have been found to explain much of the community variation that occurs within soil. However, a recent study found that 71 environmental variables did not explain all of the observed community variability (Yao *et al*. 2013). Yao et al. also observed that the commonly cited factors of pH and location were less influential in their study than they expected. The researchers also observed that AOA

were more abundant than AOB throughout their samples, irrespective of other variables. Yao et al. conducted their study of 713 samples from soils stored in the national soil inventory of Scotland. The methods they used were TRFLP, qPCR, and sequence analysis. The 71 environmental variables explained only 40-45% of the ammonia-oxidizing microbial community variance. The authors hypothesized that the rest of the community variance could be explained by stochastic processes and/or microbial interactions (see Horner-Devine et al. 2007 for a discussion of microbial interactions), neither of which they examined in the study. Yao et al. observed that pH and soil type only explained 13-16% of the ammonia-oxidizing microbial variance.

The researchers made several other observations about their data. One observation, of particular interest to the work contained in this thesis, was that land use had a significant effect on TRFLP patterns. The authors divided the soil up into six groups: arable land, improved grassland, bogs, woodland, moorland, and semi-natural grassland. The land use types divided as followed: arable land and improved grassland grouped together, semi-natural grassland was distinct from the other land use types, and bogs, woodland, and moorland did not clearly differentiate from each other. The authors also noted that geographic location within Scotland did not have a significant effect on the ammonia-oxidizing microbial community. Furthermore, soil type was found to only explain 2.3% of the ammonia-oxidizing microbial community variation. Yao et al. also made observations about specific AOA clades. *Nitrososphaera* dominated the AOA community in arable land and improved grasslands. *Nitrosotalea* dominated in all other soil types (semi-natural grassland, woodland, moorland, and bogs). The authors noted that the data indicated that AOA were affected by climatic conditions, AOA were detectable in half of samples with a pH below 3.5, that AOA and AOB were positively correlated with nitrification potential, and that AOB dominated in managed ecosystems.

Contrary to previous evidence, Yao et al. observed that pH and location had little effect on AOA community formation. This study suggested that the study of AOA is still relatively young and a lot of conflicting scholarship will continue to emerge. The findings from this article indicate that there may not be clear variables that universally define AOA abundance or community diversity.

Influence of soil depth on abundance and diversity patterns

Depth within a soil profile may also impact AOA abundance. Other studies (Leininger et al., 2006; Di et al., 2010) have observed AOA to be relatively more abundant below the surface soil horizon (surface defined as the first 15cm of the soil). Hansel et al. examined the relationship between AOA abundance and diversity, and depth. Soil samples were taken from a relatively undisturbed area in the Melton Branch Watershed, Oak Ridge, Tennessee. Diverse microbial communities, including ammonia-oxidizing archaeal communities, have been observed on a micro-scale (Hansel et al., 2008). Within a single column of soil across different depths, highly diverse archaeal communities were observed. Several soil factors influenced the members of these communities including nutrients, water, pH, and texture. Hansel et al. used traditional soil horizon definitions and also divided up the soil horizons based on their water characteristics. The water-based horizons were: surface, vadose zone, and saturated soil. The ammonia-oxidizing community varied within each of these horizons. Carbon availability, water content, and pH were thought to drive this community

change. The AOA community observed in each horizon was mostly unique to that horizon. The researchers were unable to amplify AOA genes from the A-horizon. However, based on existing science this seems more likely the result of laboratory error, rather than a novel observation.

The AOA horizon-specific communities as described by Hansel et al. (2008) break down as follows. The saturated C horizon was comprised primarily of group 1.1b (*Nitrososphaera*). The B horizon was composed partly of 1.1a-like group (*Nitrosopumilus*). The B horizon and the unsaturated C horizon contained group 1.1c and 1.1c-associated (genus *Nitrosotalea*). This division between horizons suggested a relatively low diversity of AOA within each horizon. Additionally, the authors were surprised that group 1.1b (*Nitrososphaera*) were relatively poorly represented as *Nitrososphaera* is the genus more commonly associated with soil ecosystems. Overall, AOA had relatively low diversity within each horizon. Hansel et al. used the Francis (2005) primers to examine AOA via the *amoA* gene. The group also analyzed AOA via 16S rRNA. Other factors beyond the ones discussed by the authors could also be causing the community change by depth. For instance, the amount of water, pH, the root volume, the amount and type of soil nitrogen, and the soil texture can call change with depth.

Community structure in relationship to available water

Angel et al. (2010) studied variation in archaeal communities across a precipitation gradient (100mm/year-900mm/year). The researchers found that community variations could largely be explained by differences in precipitation and associated changes in plant community. The soils examined in this study were native soils. Angel et al. also observed that the archaeal communities were equally diverse in dry and wet regions. The communities were quantified using TRFLP of the 16S rRNA gene. However, the study did not focus on AOA specifically.

The study conducted by Angel et al. is relevant to this study because the irrigated, managed plots and native plots also form a steep precipitation gradient. Irrigation could function similarly to a wetter precipitation regime, while the native soils only receive the annual precipitation (180-250mm/year on average). Another important observation from Angel et al., that the level of soil microbial diversity did not change between dry and wet areas, is also relevant to this study because the native and managed areas can also be related to this observation.

Hu et al. (2014) observed that water addition was the most important environmental factor regulating the metabolic activity of soil ammonia oxidizers responding to environmental perturbations in dry sub-humid ecosystems. Furthermore, they observed that water was more important than fertilizer in influencing autotrophic nitrification in dryland ecosystems and that AOA and AOB responses to fertilization alone, i.e. without water, were insignificant. The researchers also noted that potential nitrification rates, AOA abundance, and AOB abundance were all higher in irrigated plots versus the non-irrigated plots. Additionally, both AOA and AOB communities underwent composition changes in the irrigated plots when compared to the non-irrigated plots. They also observed that there were no obvious effects of land use change on the AOA and AOB communities under dry conditions and that there were no significant changes in nitrification rate or AOA and AOB

metabolic activity under dry conditions. He et al. also noted that AOA became increasingly involved in ammonia-oxidation when dry soils became wetted. Given the observations they made He et al. concluded that the effects of land management and land use practices on autotrophic ammonia oxidation in dry sub-humid ecosystems are primarily regulated by the availability of water.

Role of plants

Two studies observed that the AOA community varied based on tree type. AOA communities were observed to vary in Oregon forest stands based on tree type (Boyle-Yarwood *et al*. 2008). The trees included in the study were Douglas fir and Red alder. Both grew on similar soils and under the same moisture regimes. The AOA *amoA* gene was targeted for analysis. At one of the two sites that was analyzed, the AOA *amoA* gene failed to amplify, either because it wasn't present or because of laboratory error. The community was quantified using TRFLP and qPCR techniques. Boyle-Yarwood et al. made several more observations. At the one site where both AOA and AOB were observed, they occurred in equal abundance. AOA were more abundant under Douglas fir than Red alder. As Red alder recruits N-fixing bacteria this finding appears to be consistent with previous research that indicates that AOB dominate in most high-N soil ecosystems. The soil under the Douglas fir had higher pH (the pH was 5) than the soil under the red alder (pH of 4) and lower nitrification potential. Also, Boyle-Yarwood et al. observed that nitrification potential did not correlate with either AOA or AOB *amoA* copy number.

While AOA variation under different tree types is different than variation under different agricultural crops, the variation under different tree types is still an important observation because it shows that plant type can affect the ammonia-oxidizing microbial community. It must be noted that AOA abundance varied, not the community diversity.

A later study, conducted by the same researchers, found that *Archaea*, and to a degree AOA, varied by tree and site (Yarwood *et al*. 2010). The archaeal community was examined via qPCR of the 16S rRNA. The team observed several AOA clades including: 1.1a-associated, 1.1b (*Nitrososphaera*), 1.1c(*Nitrosotalea*), and 1.1c-associated groups. 1.1a-associated AOA made of 15-17% of AOA observed, while 1.1c AOA made up 16-18% of AOA observed. These two genera did not differ significantly between site or tree species. However, 1.1b were more abundant under Red alder than Douglas fir. The group also observed that AOA were four times more abundant at one of the two studied sites. Yarwood et al. also observed that there was no correlation between archaea and any of the measured soil parameters.

The second study conducted by Yarwood et al. reinforced what they demonstrated in their first study, that AOA abundance varies by tree type. However, given that Red alder recruits N-fixing bacteria and Douglas fir does not, it is not entirely clear whether or not it was tree type, available N, both, or neither that was responsible for community shifts. The authors also observed that soil properties and location, or "ecoregion," was correlated with the AOA community composition.

Mao et al. (2011) observed gradual changes in the AOA community structure under four different bioenergy crops over their two-year establishment. The study was conducted in

Illinois and the crops examined were switchgrass, miscanthus, restored mixed tallgrass prairie, and maize. It is important to note that restored tall grass prairie may not have a microbial community that is similar to the microbial community in native tall grass prairie. Surprisingly, maize was the only fertilized crop. Although the greatest AOB growth (based on qPCR of bacterial *amoA*) was observed in this maize-soil, nitrification was correlated with the quantity of AOA, not AOB.

Mao et al. made several specific observations about the AOA community over the course of the study. First, AOA diversity declined in the second year of maize establishment. Second, the AOA communities gradually diverged under the four different crops over the two-years that the study was conducted. Mao et al. asserted that maize, the annual crop, had a larger impact on the N-cycling community than any of the perennial bioenergy feedstock crops. Mao et al. concluded that the observed microbial community variations were likely caused by the management practices or plants, not environmental conditions (i.e. precipitation or temperature) because these varied equally across all plots.

Hallin et al. (2009) conducted their study in an ongoing 50-year fertilizer trial in Ultuna, Sweden. The soil was a clay loam and the treatments were: unfertilized bare fallow, unfertilized growing corn, and fertilized (with either calcium nitrate, ammonium sulfate, solid cattle manure, or biosolids) growing corn. Corn had been planted annually since 2000. The scientists made several observations. Both AOA and AOB abundance dropped two log numbers in the ammonium sulfate plots. The AOA abundance also decreased by a factor of 100 in the plots treated with biosolids. Changes in potential ammonia-oxidation rates were correlated with AOA community size, but not AOB community size. Finally, community size appeared to be more important than community composition when developing biogeochemical process models. Said in another way, they observed that the number of AOA present, rather than which AOA genotypes were present, had the more significant impact on ammonia-oxidation. The wide range of different outcomes occurring in one plant type suggests that plants are not the only factor affecting the AOA community.

Another study, Bates et al. (2011) (which will be discussed more thoroughly in the following section), observed that across 146 native sites in N. and S. America, vegetation type was the environmental factor that most closely correlated with AOA abundance.

Native versus Cultivated Soils

The number of microorganisms that have been successfully cultured in the laboratory represents a fraction of a percent of the total microbial diversity on the planet (Giovanonni et al., 1990). However, the inability to culture most microbes does not preclude learning about them and their community structure (Urich *et al.* 2008). The focused examination of environmental samples, like the work conducted for this study, can reveal a lot about a given communities' ecology.

Leininger (2006) observed that AOA were far more abundant than AOB in "pristine" or native soils and agricultural soils (Leininger et al., 2006). AOA were more abundant than AOB (assessed via the *amoA* gene) in the surface horizon (1.5-230 times) and up to a maximum of 3000-fold more abundant at depth. These observations were made in each of

12 pristine and agricultural soils in three climatic zones. Additionally, Leininger et al. observed that AOA had a higher activity than AOB (this was determined using qPCR and pyrosequencing). Sampled soils had pH values ranging from 5.5-7.3. AOA also made up a larger percentage of the total microbial fraction when compared to AOB, 1-5% versus a maximum of 0.23% respectively. Leininger et al. also observed that different AOA ecotypes were present in the surface and the sub-surface. This study was significant as it established that AOA might play a significant role in global nitrogen cycles.

Urich et al. (2008) conducted their study in a conservation area on native soil. The soil in the area was relatively low in nutrients and had a neutral pH. The team observed that the predominant archaeal group was group 1.1b, or genus *Nitrososphaera*. However, archaea only made up 14.5% of the population (based on ribo-tags).

There is significant variability in the archaeal community diversity and abundance on the global scale (Bates *et al.*, 2011). The observed diversity of global archaeal communities necessitates close examinations of many environmental soils where relatively controlled conditions are maintained. To this end, Bates et al. undertook a study with a broader focus than previous studies. To achieve a broader focus the group examined 146 native soils across N. and S. America and Antarctica. Bates et al. observed several notable trends while conducting their study. The phylum *Crenarchaeota* was observed to be the most abundant archaeal phylum in the soils they examined (based on barcoded pyrosequencing of 16S rRNA). Bates et al. also observed that the genus *Nitrososphaera* were only present in a subset of the soils they examined, though still the majority of the soils they examined. The one soil chemical factor that consistently correlated with higher AOA abundance was a lower C:N ratio. However, the scientists also observed that multiple environmental variables correlated with one another. The correlation between higher AOA abundance and lower C:N ratio may not tell us a lot about the ecosystem conditions that affect AOA abundance.

The group also made several specific observations about the AOA. While the level of sampling was not thorough enough to detect the least abundant archaeal groups, it was still valuable for developing a better understanding of AOA biogeography. Within the two long-term ecological research (LTER) sites they examined (these sites are relatively undisturbed physically), Bates et al. observed that 90.9% of archaea detected were from 1.1b group (genus *Nitrososphaera*). Plots within both of these sites were receiving 1, 100, and 280-290 kg N/ha/yr. Within the *Nitrososphaera* lineage the scientists observed that two phylotypes made up the large majority of AOA. The dominance of only two phylotypes was a novel observation. However, within the LTER sites archaea only made up 1.7% of the microbes detected.

Within the non-LTER sites that were sampled a few trends emerged. One, vegetation type was the environmental factor most closely related to archaeal abundance. Two, archeal group 1.1c, genus *Nitrosotalea*, were only present in forest/shrubland. Archaeal abundance was also relatively low in the non-LTER sites; only 5 of the 146 soil samples had over 5% Archaea as part of the overall microbial population.

The conversion of Amazon rainforest to other uses, primarily pastures, was found to influence the soil microbial community (Rodrigues et al., 2013). It has been known for a long time that this rainforest conversion influenced the macroscopic community, but the finding regarding microscopic life is novel (the microbial community was assessed via 16S rRNA). This finding was not about Archaea specifically, or even AOA, but it is still relevant evidence regarding the community change discussed in this thesis.

AOA community variation in different cultivated crops

Shen et al. (2008) tested relative abundance of AOA and AOB in an alkaline (pH 8.3-8.7) sandy-loam soil with different fertility treatments in plots that had been undergoing a long-term fertilization experiment for 17 years. The fertility treatments included a control that had been maintained in fallow with no fertility for several years and different combinations of N, P and K. AOA were 1-2 orders of magnitude more abundant than AOB across all treatments (based on *amoA* gene counts). Although the abundance of AOB did not change with statistical significance as a function of fertilizer treatment, the diversity of the AOB community did change. In contrast, the AOA did not change in abundance or community diversity (Shen *et al*. 2008). AOB were most abundant in the trials where inorganic N was added. Additionally, AOB had positive correlations with both pH and nitrification. However, the ratio of AOA:AOB did not change significantly within the treatments during the two years that the soils were sampled. It was inconclusive whether AOA or AOB were dominating nitrification in the system. The AOA sequences observed fell within cluster S (*Nitrososphaera*) and M (*Nitrosopumilus*). The results, based on observed community changes, suggest that long-term fertilization in an alkaline soil has a greater impact on the AOB community than the AOA community.

The absence of AOA community response to the experimental conditions may suggest that AOA were not responsible for the observed ammonia-oxidation. Additionally, the AOB community change and correlation to nitrification suggests that the AOB community may have been responsible for the observed nitrification. The observation by Shen et al. that AOB were most abundant in the trial where only N was added suggests further support for the observations discussed earlier (Jia and Conrad, 2009; Di et al., 2009; Di et al., 2010; Tourna et al., 2011; Bates et al., 2011) where AOB dominated nitrification when high levels of inorganic nitrogen were present.

Ying et al. (2010) studied AOA and AOB abundance and structure in four long-term (established in 1995) plots, each with a different type of management system. The systems included restoration (forest), degradation (pasture), cropland, and pine plantation. The soil pH in the pine plantation was acidic. They observed significant affects on the AOA and AOB communities as a result of land management (Ying *et al*. 2010). The communities were evaluated via qPCR, TRFLP, and sequencing of AOA *amoA* and 16S rRNA and AOB *amoA* clone libraries. AOB correlated with potential nitrification rate, however AOA did not. Additionally, AOA were more abundant than AOB. The land utilization types affected community structure of AOA, but not AOB. More specifically, they noted that the degradation plot was dominated by the AOA 1.1c (*Nitrosotalea*) and 1.1c-associated groups. Conversely, AOA 1.1a (*Nitrosopumilus*) and 1.1b (*Nitrososphaera*) groups dominated the other land utilization types. More than half the sequences in the restoration

or forest plot were from the 1.1a-associated group. The results suggested that the 1.1a group was associated with acidic environments (Kemnitz et al. 2007, Lehtovirta et al. 2009). Ying et al. also observed that AOB abundance was correlated with nitrate concentration, this observation is similar to the several studies cited previously correlating AOB abundance to high levels of inorganic nitrogen.

The authors noted the "profound influence of frequent human disturbance" on the ammonia-oxidizing microbial community. AOA were most abundant in the two sites with the least amount of disturbance, the two forested sites.

In four different land utilization types—forest, pasture, cropped, and fallowed (for 19 years)—AOA and AOB had different contributions to nitrification in each land use type (Taylor *et al*. 2010). The soils were sampled north of Corvallis, Oregon. AOA (not AOB or fungi) dominated pasture soil nitrification potential as it recovered following exposure to acetylene. Both AOA and AOB contributed to fallow soil recovery of nitrification potential (RNP). AOB dominated RNP in cropped soil, the soil with the highest concentration of N. RNP did not reliably recover in forest soil so it could not be evaluated (the cause of this was not determined). The authors noted that AOA dominated in the soil with the most mineralizable N, while AOB dominated in the soil with the least mineralizable-N. As previous discussed evidence has shown (Kelly et al., 2011), a likely explanation for the niche partitioning of AOA and AOB is that mineralizable-N is less readily accessible than simpler forms of N. The slow-release characteristics of the mineralizable-N likely make the ecosystems with more mineralizable-N favorable to AOA rather than AOB.

Verhamme et al. (2011) found that soil ammonium concentration affected the growth rates of AOA and AOB (Verhamme *et al*. 2011). Three different soil ammonium concentrations were set-up: "native" or zero N added, intermediate—20 μg NH_4^+-N added per gram of soil, and high—200 μg NH_4^+-N added per gram of soil. The soils were sampled from a long-term study that is maintained in an eight-year crop rotation cycle. There were changes in AOA community diversity in all three ammonium concentration trials, indicating growth in all three. Conversely, the AOB community only changed in the 'high' ammonium concentration trial. This evidence indicates that soil ammonium concentration contributes to define distinct ecological niches for AOA and AOB in soil. This mostly supports the evidence previously cited numerous times correlating AOB to high levels of inorganic nitrogen. Here however, AOA were also observed to grow in the high nitrogen condition; this was not observed in the previously cited studies.

Verhamme et al. conducted their studies in a sandy loam soil at pH 7.5. The team observed AOA to be more abundant than AOB in both the native and intermediate trials. The data also indicated that AOA might oxidize ammonia released through mineralization, while AOB was observed to grow better under conditions with high ammonium concentrations. Again, this observation reinforces what has already been observed several times before. Verhamme et al. noted that at the beginning of the study *Nitrososphaera* were dominant, however 28 days later (at the end of the experiment) *Nitrosopumilus* was predominant.

Shen et al. (2008), Ying et al. (2010), Taylor et al. (2010), and Verhamme et al. (2011) all focused on the affects fertilization regimes or land management styles had the AOA

community. There were two main commonalities between these four studies. All four observed, to varying degrees, the impact that available nitrogen has on AOA community diversity and/or abundance. Ying et al. (2010) and Taylor et al. (2010) both observed that land management had a significant affect on AOA community diversity. The land use types both studies looked at were forest, pasture, and cropped. The main difference between the two studies was their finding regarding cropped land. Ying et al. observed that *Nitrosopumilus* and *Nitrososphaera* were oxidizing ammonia in cropped land while Taylor et al. observed that AOB dominated ammonia oxidation in cropped land. The large number of possible crop management strategies could account for these contrary findings.

Zeglin et al. (2011) built on the work of Ying et al. and Taylor et al. by comparing the AOA communities across four types of land use (forest, pasture, cultivated, and long-term fallowed cropland) along a 10km transect. The pH range along the transect was 6.0-6.7. The researchers observed that AOA abundance did not vary significantly among the land use types, however the AOA community composition was unique in all four land use types. The group also observed that the AOA:AOB ratio ranged from 1-400:1 across the transect.

Zeglin et al. drew several conclusions regarding what factors influence AOA community composition. One observation asserted the importance of soil texture (Zeglin et al. use the phrase, "edaphic factors") in influencing the AOA community, "Given the patterns we observed, it seems that vegetation type *per se* may be less important than edaphic factors as a driver of differences in archaeal *amoA* distribution among soils from differing land-use type [sic]." The authors also determined that pH affected the AOA community regardless of other factors. Zeglin et al. also noted that there were no changes in AOA abundance or community composition because of soil-N changes within any land use type. While Zeglin et al. did observe AOA community changes by land use type, contrary to what Ying et al. and Taylor et al. observed, Zeglin et al. found that edaphic factors were statistically more significant than vegetation in predicting the effect on the AOA community.

Annual versus perennial cultivation

The effects of both annual and perennial cultivation and their effects on microbial ecology have been studied previously. Researchers concluded that the cultivation of perennial biofuel feedstock crops "had less impact on microbially mediated ecosystem services" than their annual counterparts (Watrud *et al*. 2012) and were therefore a better choice. Active bacterial biomass was observed to be lower in the sorghum (annual) when compared to switchgrass and reference grasslands. However, the researchers did not examine AOA or even archaea.

Mao et al. expanded their work on the effect of bioenergy crops on N-cycling bacteria and archaea (2013). In this study three crops were examined: maize, switchgrass (established for six years), and miscanthus (established for four years). The team chose seven sites that formed a temperature (9-14°C) and precipitation (914-1219mm) gradient (north to south) across the state of Illinois. Mao et al. observed that variation between sites was greater than variation between plant types. The group also observed that the microbial communities under each crop, across all sites, did not converge on a typical species assemblage. Fewer than 5% of the functional genes observed were significantly different between the crops at each site. However, the largest difference, at each site, was between

the maize and the two perennial grasses. Unlike the other communities that were characterized (Mao et al examined 16S, *nifH*, AOB *amoA*, AOA *amoA*, and *nosZ* genes), AOA did not statistically vary significantly based on site, even though site variation was greater than crop variation. Like the other groups of organisms measured, the AOA community did not show variation according to crop type (only 1.1% of OTUs varied).

According to the data that Mao et al. collected the climate gradient they tested was a poor predictor of AOA variation. Perhaps a steeper gradient would have a stronger effect on the AOA community. The researchers also observed that AOA abundance was positively correlated with total N and C, but showed no correlation with ammonia or nitrate. They also observed that AOA abundance was negatively correlated with soil bulk density. Regarding the AOA community composition, 15 of the 20 most dominant AOA OTUs were *Nitrososphaera* associated. The AOA community composition broke down as follows: 98.2%, 1.3%, 0.5% of OTUs were associated with *Nitrososphaera*, *Nitrosopumilus*, and *Nitrosotalea*, respectively. Mao et al. concluded that "local drivers" of microbial community composition were more influential than pervasive variables like plant type and climate. Mao et al. also questioned whether four or six years were enough to overcome the "preexisting legacies" left by prior cultivation.

Mao et al. raised several points that require further discussion. As was discussed above, there are conflicting results among the three research studies by Zeglin et al. (2011), Mao et al. and Hallin et al. (2009). The results observed by Mao et al. in 2013 conflicted with the results observed by Mao et al. in 2011. In 2011 the team observed that the AOA community diverged based on crop type over the course of two years. However, in the 2013 study, the AOA community was not observed to diverge over a longer period of time. The suggestion by Mao et al. (2013) that local factors would primarily define the AOA community would help explain some of the contradictory findings regarding AOA community formation. However, more investigation is needed before that assertion can be accepted.

It is difficult to distinguish the impact of agricultural managed sites and native sites on AOA abundance because agricultural practices can change many soil properties—pH, OM content, nitrification rates—and the microbial community (Zhalnina et al., 2013). Zhalnina et al. found that AOA, specifically *Nitrososphaera*, were more abundant in three field trials after agricultural practices began. Conversely, *Bradyrhizobium*—an N-fixing bacterium—were negatively correlated with agricultural practices. The effects of the agricultural practices were found to be reversible, meaning that the abundances of *Nitrososphaera* and *Bradyrhizobium* would reverse if the plots were not cultivated for several years.

Zhalnina et al. took their soil samples from three sites, each with an agricultural site and a non-agricultural site (one was native and two were long term fallow). The three sites were all located on long-term agricultural study land: one in the United Kingdom, one in Florida, and one in Michigan. *Nitrososphaera* was the AOA clade that was positively correlated with agriculture. Zhalnina et al. also noted that higher pH and ammonia concentrations were also positively correlated with *Nitrososphaera* (the opposite was true for *Bradyrhizobium*).

Zhalnina et al. made several additional observations. They observed that *Nitrososphaera* was the most abundant archaeal genus at all three sites and the only AOA to be significantly

correlated with agriculture at any of the three sites. They also observed that *Nitrososphaera* abundance declined slightly with time away from agricultural production —*Nitrososphaera* increased 2-7 fold depending on site, between non-agriculture and agriculture plots. The group also noted that *Nitrosopumilus* and *Nitrosocaldus* were observed in the agricultural plots at low abundance. Unexpectedly, ammonia was positively correlated with *Nitrososphaera*. Zhalnina et al. also noted that in all the plots archaea represented at most 3.7% of sequences observed (91.1-95.5 of observed sequences were bacterial). Lastly, Zhalnina et al. observed that the effects of agricultural practices were found to be reversible—meaning that the population gradually changed back over time.

Conclusions

A diverse group of non-thermophilic terrestrial and marine (meaning originally observed in a marine environment) archaea exist in a myriad of soil environments (Schleper et al. 2005). These environments include sandy soil ecosystems, pristine (similar definition to native) forest soils, agricultural fields, contaminated soils, and the rhizosphere. Succession of these archaeal populations has correlated with different environmental variables, including management practices, pollution by heavy metals, and varying rhizosphere-plant interactions. These responses to change indicate that archaea are a dynamic assemblage that changes according to environmental conditions. Archaea have been recovered from the upper layer of soil on all continents. The archaeal domain's broad distribution implies that they play an important role in global nitrogen cycles.

The work in this thesis will build on the rapidly growing field of research focusing on AOA. The goal of this is to increase collective understanding of AOA biogeography and by doing so increase the scientific communities' ability to gauge soil ecosystems' responses to humyn disturbance and global change.

Materials and methods

Experimental design and fertilizer application

Soil samples were collected from managed and native sites in Eastern WA. Both sites are used as agricultural research facilities. The first site, Paterson is located in Paterson, WA (45.939775, -119.487551). It is a research facility under the oversight of the USDA. It has been used as a research facility since approximately 1951. The second site, Prosser, is located in Prosser, WA (46.252544, -119.737838). It is a research facility under the oversight of Washington State University (WSU). It has been used as a research facility since 1919. The Paterson site receives average annual precipitation totaling 25cm while the Prosser site receives 18cm on average. In Paterson, WA the monthly average high and low air temperatures range from 6-32°C and -2-15°C, respectively. In Prosser, WA the monthly average high and low air temperature range from 6-32 and -2-15°C, respectively (NOAA). The two sites are both located in Benton County, WA and are approximately 35km apart.

Paterson samplings

Figure 4. Satellite image, soil series, vegetation overlay at Paterson. QuE = Quincy loamy sand.

Sampling at the Paterson site included samples collected from cropped switchgrass (*Panicum virgatum L.*) and native or unmanaged areas. The soil at the Paterson experiment station falls under a single classification—Quincy loamy sand (Mixed, mesic Xeric Torripsamments) (USDA NRCS WebSoilSurvey).

Switchgrass

The switchgrass sampled at Paterson was part of a field study designed to test the response of different Switchgrass cultivars to different levels and sources of fertilizer (Watrud et al., 2012). The plots (45.941050, -119.486786) for this study are organized were set up as a completely randomized design (Figure 4). The study was established in the spring of 2004. There are three, replicated 6-meter by 6-meter plots for each treatment at the Paterson site. All plots were irrigated and received 3cm of water 2-3 times per week during the growing season through an overhead irrigation system. The study design included fertilizer addition with a total of 220 kg N/ha/yr, 115.9 kg K/ha/yr, and 60.5 kg S/ha/yr (in split applications—the first application was in April and the second application was in July). Fertilizer was surface applied. Control plots with no fertilizer addition were also included in the study design. Soil from all three replicates of both the zero-N control plots and the plots that received an agronomic rate of synthetic fertilizer were analyzed for this study. All of the plots that were sampled were planted in Kanlow variety of switchgrass. The plots have not been tilled since the site was established in 2004.

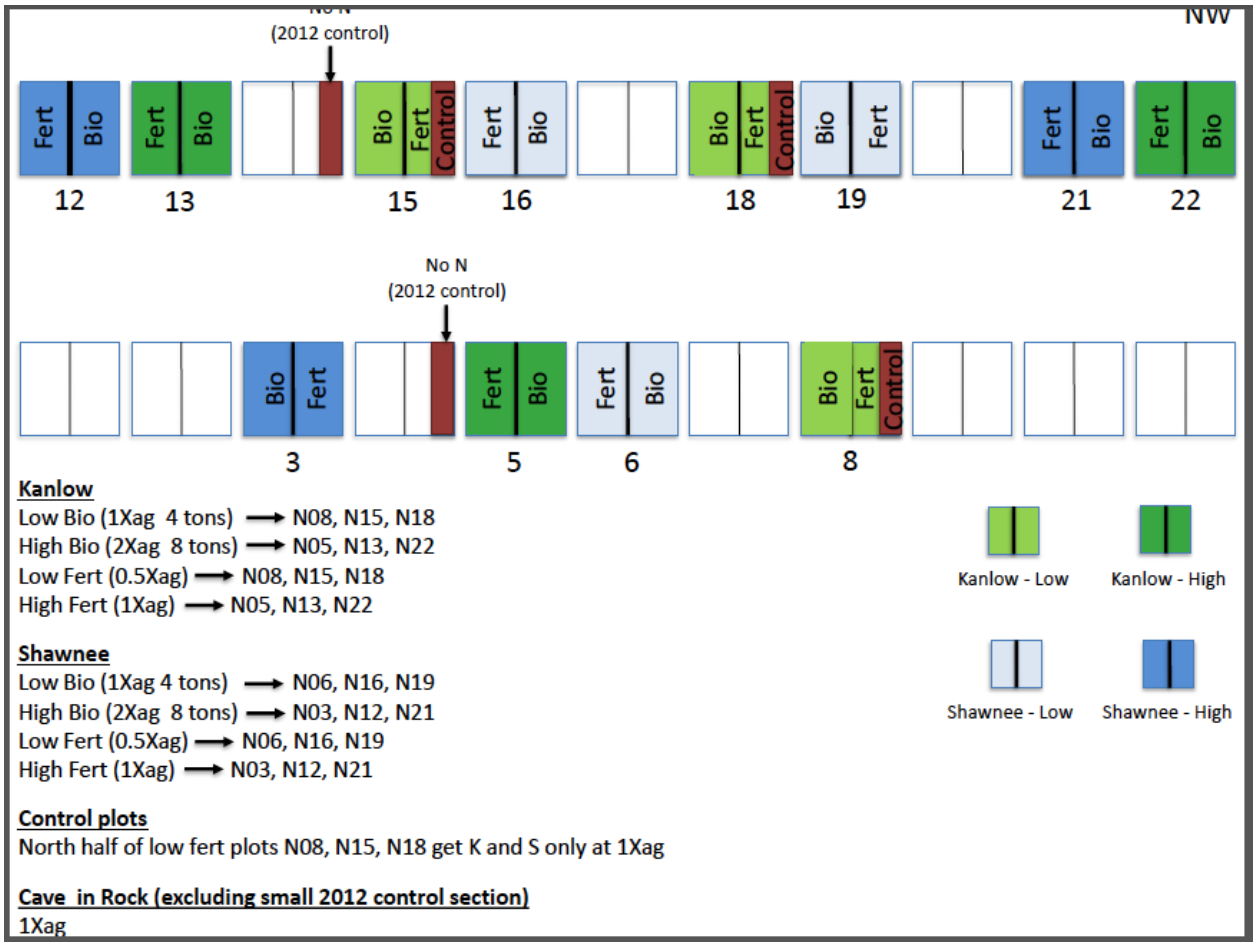

Figure 5. Switchgrass plot map at Paterson, WA.

Native

The native area (45.938938, -119.494044) at the Paterson site has been preserved in native vegetation with no history of cultivation (Figure 4). While the native area at Paterson is large, all samples for this study soils were sampled from a two-hectare area. The native plot at Paterson supports a native sagebrush steppe plant community. The native plot is approximately 2km WSW of the Paterson switchgrass plots and is in the same soil series as all of the crops grown at Paterson.

Crop	GPS coordinates	Crop in:			Fertilizer application				Irrigation
					kg.ha^{-1}				cm.ha
		2012	2011	2010	N	P	K	S	
Switchgrass	45.941050, -119.486786	Switchgrass	Switchgrass	Switchgrass	220	0	115.9	60.5	100+
Native	45.938938, -119.494044	Native	Native	Native	0	0	0	0	0

Table 1. Site history, fertilizer application, and irrigation.

Prosser samplings

Two sites were sampled at Prosser; Switchgrass and native. The Switchgrass and a portion of the native site are both on a Warden silt loam (Coarse-silty, mixed, superactive, mesic Xeric Haplocambids) (USDA NRCS WebSoilSurvey). The soil at the other native site included in the sampling is classified as a Shano silt loam (Coarse-silty, mixed, superactive, mesic Xeric Haplocambids). Both soils have very similar properties and fall under the same series classification.

Switchgrass

The switchgrass (46.256515, -119.730255) sampled at Prosser is part of a field study that was established in the spring of 2009 (Figure 6). The field study was designed to test the feasibility of interplanting alfalfa with Switchgrass to provide sufficient N for the Switchgrass crop. A no fertilizer control as well as a synthetic fertilizer treatment were included in the experimental design. A single variety of switchgrass, Blackwell, was planted. The study is organized in a randomized complete block design. There are four replicated 3x6m plots for each treatment at the Prosser site. Both fertilizer and control plots received 3cm of water 2-3 times per week from a solid set irrigation system through the growing season. The fertilizer plots received a fertilizer application of total of 220 kg N/ha/yr, 115.9 kg K/ha/yr, and 60.5 kg S/ha/yr in split applications. The first fertilizer application was in April after the grass broke dormancy and the second application was in July after the first cutting. Fertilizer was surface applied. Soil from all four replicates of the agronomically fertilized plots and the control plots were collected and analyzed for this study. The site has not been tilled since the Switchgrass was planted in 2009.

Figure 6. Satellite image, soil series, and switchgrass overlay at Prosser, WA. WdB = Warden silt loam.

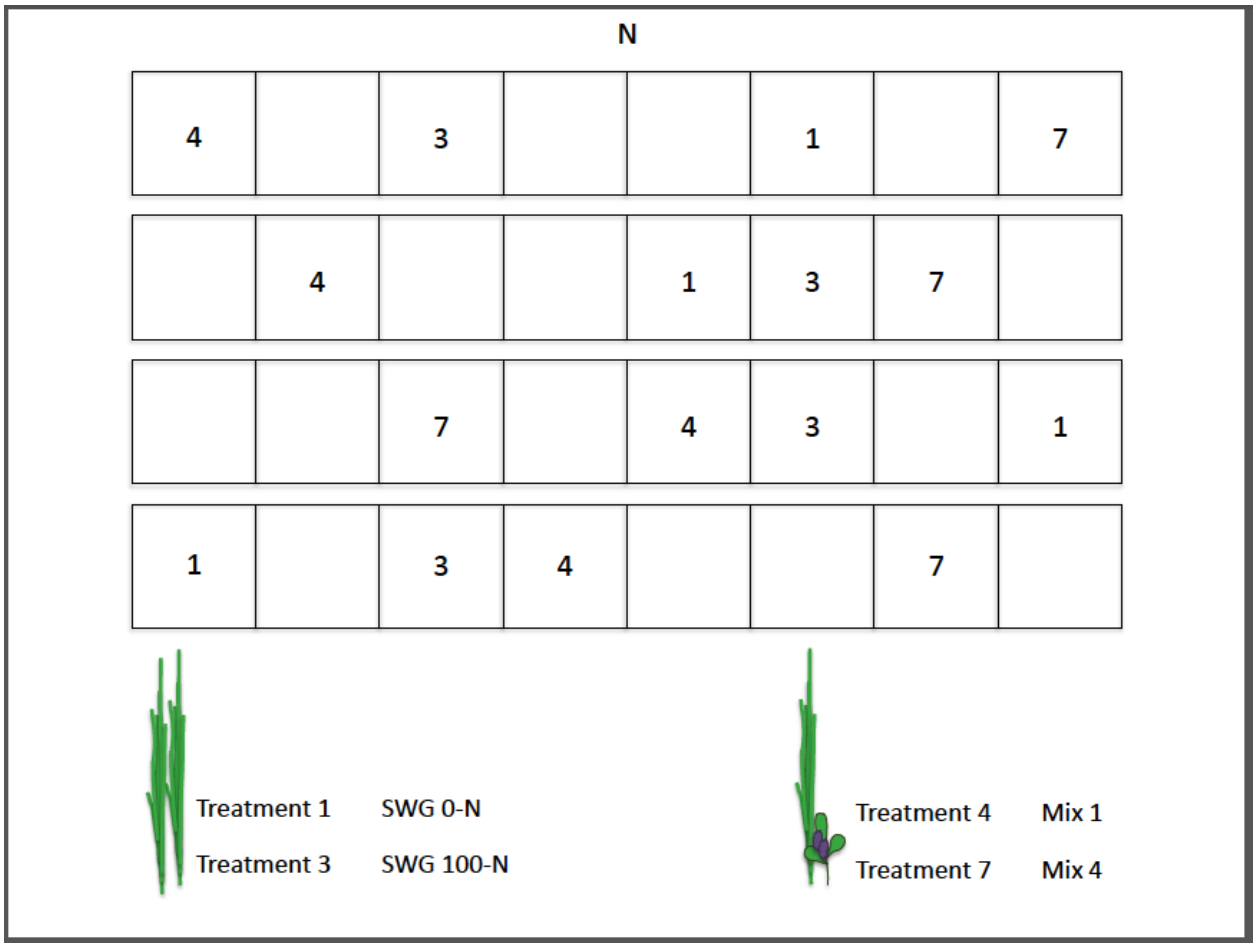

Figure 7. Prosser switchgrass plot map. Mix 1 = 70 parts switchgrass:30 parts alfalfa. Mix 4 = 50 parts switchgrass:50 parts alfalfa.

Native

Two native plots (46.293894, -119.725451 and 46.290950, -119.742660) were sampled at the Prosser site (Figure 8). Both plots have been preserved for experimental purposes and have no history of cultivation. One plot at Prosser has an area of one hectare; the other has an area of two hectares. The one-hectare native plot is approximately 13km N of the Prosser switchgrass plots. The two-hectare native plot is approximately 14km N of the Prosser switchgrass plots. Both support a native sagebrush steppe plant community. The soil series at the one-hectare plot is a Warden silt loam. The soil series at the two-hectare plot is a Shano silt loam.

Figure 8. Satellite image, soil series, and native plot location overlay at Prosser, WA. WdB = Warden silt loam. SmB = Shano silt loam.

Soil sampling

Soil samples were collected across all areas using a 1.25cm diameter aluminum soil probe. Samples were collected from all sites and plantings on May 23rd, July 17th, and August 14th 2013. The lone exception is the Prosser native sites. Samples from the Prosser native sites were only collected on the August 14th sampling date. All samples were stored on ice until they could be brought back to the lab. Once they were brought to the lab the homogenized soil cores were stored at -80°C until genetic analyses could be conducted. Before storage at -80°C a portion of the soil sample was set aside for soil-chemical analysis.

Switchgrass

Soils were collected from the agronomic-rate fertilizer and control treatments at both the Paterson and Prosser switchgrass sites. One set of triplicate soil cores was collected from random locations within each plot at each of the three sampling times during the 2013 growing season. Cores were collected from the 0-15cm depth. The triplicate cores from within a plot were homogenized into a single sample. Switchgrass breaks dormancy when temperatures begin to rise, generally in early April. The May sampling would have taken soil when the switchgrass was well into its growing cycle. The July sampling was done directly before harvesting, when the switchgrass was mature. When switchgrass is harvested it is cut to leave several centimeters of stalk. Plant growth stage for the August sampling was similar to the May sampling—when the plants had been growing from the stalks for 4-6 weeks.

Native

Three sets of triplicate soil cores were collected from random locations within each plot during each sampling time. Cores were collected from the 0-15cm depth .

Soil DNA extractions

The DNA extractions were performed using the MO BIO PowerSoil DNA Isolation Kit according to the manufacturer's instructions with one modification to the bead-beating step. Rather than vortexing the bead tubes, the tubes were shaken in a FastPres-24 instrument (MP Biobedicals) for 30s at a speed of 5.5 m/s. This was empirically determined to be a superior method for DNA extraction prior to the beginning of sample analysis because the DNA was extracted with less contamination and at a higher concentration. Duplicate extractions were performed on each homogenized soil sample, pooled, and then stored at -80°C until analyses were conducted. Duplicate extractions were compared between several samples and were found to be 70% similar on average in terms of AOA community diversity.

Soil chemical analysis

Soil pH and ammonium and nitrate were analyzed for all collected soils. Soil pH was measured using a 1:1 (volume:volume) slurry with MilliQ water after three cycles of gentle stirring at 10 minute intervals. The soil had been oven dried prior to pH measurement. Soil pH was measured using an Accumet AP85 pH/Conductivity meter to the nearest hundredth of a unit. The meter was calibrated using pH 4 and pH 7 standards prior to analyzing the samples.

Soil ammonium and nitrate were extracted by shaking 5g (dry weight) of soil with 25 mL 2M KCl for one hour at 200 RPM, followed by filtering through a Whatman grade 41 quantitative filter paper (GE Healthcare). Two milliliters of filtrate were analyzed on a Thermo Scientific 61E ICP Spectrometer (Thermo Fisher Scientific, Waltham, MA). Duplicates were included every six samples and blanks were included every eight samples. Both duplicates and blanks were treated the same as the rest of the samples, i.e. they were shaken and filtered prior to analysis.

Total C and N was measured on all soils collected in May and July. After drying, sieving, and grinding a portion of the homogenized soil sample with a mortar and pestle 2-3 mg of soil were placed in a tin capsule. Capsules were then closed and placed in the auto-sampler of a 2400 CHN Elemental Analyzer (Perkin Elmer, Waltham, MA), and combusted at 925°C. Combustion gases were reduced and C and N detected by thermal conductivity detectors. Before running samples four blanks and five known standards were run. Every fifth sample a duplicate was run. Between every 10 samples a known standard was run.

Statistical analysis was performed on all of the soil chemical results using SPSS. The main effect of sampling date and crop were tested as well as interactions between the main effect variables. Means were separated using a Waller-Duncan test in cases where treatment was statistically significant ($p<0.05$) (SPSS, April 2014).

Terminal restriction fragment length polymorphism

Terminal Restriction Fragment Length Polymorphism (TRFLP) was conducted on the pooled DNA that was extracted from the soil samples. First the DNA was amplified with Arch-*amoA* FAM-labeled forward primers (Francis et al., 2005). The solution to amplify the

DNA contained, per reaction: 0.625 μL Arch-*amoA*F (forward primer), 0.625 μL Arch-*amoA*R (reverse primer), 12.5 μL 2X GoTaq (Promega Inc., Madison, WI), 8.75 μL PCR H_2O, 1.25 μL 1X BSA, and 1.0 μL of extracted soil sample DNA. The PCR amplification was conducted on a PTC-100 Programmable Thermal Controller. Each PCR run included both a positive and negative control. To check that the PCR worked 5 μL of each sample was run on a 1% agarose gel. Following the gel electrophoresis 8.5 μL of PCR-product were digested for one hour at 37°C with 0.5 μL HpyCH4V enzyme (Yao et al., 2013) and 1.0 μL of CutSmart buffer. Following the digestion the samples were cleaned using Qiagen QIAquick PCR purification kit according to the manufacturer's instructions with the exception that the final elution was done with 20 μL of buffer EB instead of 30 μL. This alteration was done to increase the concentration of the DNA in the solution. It was empirically determined that the alternation did not affect the quality of the DNA. Next, 2 μL of the cleaned PCR-product were mixed with 15 μL of solution composed of 288 μL HiDi and 2 μL Rox. The TRFLP was run on an ABI 3730xl DNA Analyzer. The results were analyzed using Peak Scanner v.1.0, Microsoft Excel, and Primer 6 v.6.1.13. Other enzymes were tested along with HpyCH4V prior to beginning TRFLP analysis. HpyCH4V was chosen because it provided the greatest differentiation across all of the soil samples.

Statistical analysis of ammonia-oxidizing archaeal communities via TRFLP results

Peaks representing less than 1% of total area were excluded from further analysis. TRFLP data was converted to spreadsheet form using Peak Scanner v.1.0. Sample matrices of AOA were constructed in Microsoft Excel with samples in columns and terminal restriction fragments (TRFs) in rows. Multivariate statistics were performed using Primer 6 v6.1.13. Hierarchical clustering was performed using the Bray-Curtis "cluster" function and R and p-values were obtained using the "ANOSIM" function (Primer 6 v6.1.13).

ANOSIM is an acronym for analysis of similarity. ANOSIM is a nonparametric or distribution-free for of statistical analysis. This means that ANOSIM does not make any assumptions about the distribution of the variables being assessed. ANOSIM is used to compare two matrices, in the case of this study the matrices were derived from TRFLP data.

Cloning and sequencing

Archaeal *amoA* genes for phylogenetic analyses were PCR amplified using the Arch-*amoA*F and Arch-*amoA*R primers (Francis et al., 2005). All clones were from soil collected in August. PCR was performed using approximately 20 ng of template DNA in a 25 μL reaction consisting of 1 μL of Arch-*amoA*F (forward primer), 1 μL of Arch-*amoA*R (reverse primer), 12.5 μL 2X GoTaq (Promega Inc., Madison, WI), 8.75 μL PCR H_2O, 1.25 μL 1X BSA, and 1.0 μL of extracted DNA. PCR reactions were performed on a PTC-100 Programmable Thermal Controller. Amplicons were checked using gel electrophoresis (on a 1% agarose gel). The PCR product was cleaned using the Qiagen QIAquick PCR Purification Kit. The PCR product was then cloned into the TOPO-TA vector (Invitrogen) and transformed into chemically competent TOP-10 ONEShot *E. Coli* (Invitrogen). After ligation, the clones were plated onto media containing kanamycin and x-gal in petri dishes. After a 24-hour growth period at 37°C clones containing *amoA* genes were identified using blue-white screening and transferred into 200 μL LB-broth with kanamycin and 10% glycerol in 96-well culture

plates. After another 24-hour growth period at 37°C and 300 RPM the clones were transferred again to another 96 well plate with 200 μL LB-broth, again containing kanamycin and 10% glycerol. The reference well plates (the first set) were then stored at -80°C. The second set of well plates was again grown for 24 hours at 37°C at 300 RPM before being sequenced using the Sanger method.

Nucleic acid sequence analysis

Sequences obtained by amplifying the A-subunit of the thaumarchaeal (the taxonomic phylum that contains the genera *Nitrosopumilus*, *Nitrososphaera*, and *Nitrosotalea*) *amoA* gene were analyzed in ARB (Ludwig et al., 2004) using a database of 1027 partial full length sequences (Pester et al, 2004).

DNA sequences from the AOA clones were trimmed using Geneious Pro 5.6.5 to remove vector DNA sequence. The trimmed sequences were imported into ARB and aligned using the automatic aligner. The aligned sequences were inserted into the AOA backbone tree using the parsimony tool. Based on this analysis near-neighboring sequences were identified and sequence alignments were manually corrected. Neighbor-joining trees were constructed in ARB.

Statistical analysis of ammonia-oxidizing archaeal communities via sequenced clone library results

The relative abundance of complete sequenced clone libraries was compared using chi-squared analysis of variance statistical tests. The chi-squared test is used to determine if there is a significant difference between observed counts and the expected counts. The observed counts, in this case, are the number of sequences within a clone library that associate with a given AOA clade. In all chi-squared tests expected counts are determined by: summing each row and column, calculating the 'grand total'—the sum of either the column or row sums (the sum of the column sums and the sum of the row sums will be the same), and to determining the expected value within a given cell by multiplying that cell's row total by that cell's column total and dividing by the grand total. The chi-squared tables for Paterson and Prosser can be seen below (Tables 2 and 3, respectively). The "=chitest" command in Microsoft Excel was used to calculate the p-values associated with the pairs of observed and expected tables.

	Paterson native observed	Paterson switchgrass observed	Paterson native expected	Paterson switchgrass expected
Nitrososphaera (54d9)	32	15	23.75	23.25
Nitrososphaera 8.2	3	1	2.02	1.98

Nitrososphaera (non 54d9)	36	1	18.69	18.31
Nitrososphaera 2.1	15	0	7.58	7.42
Nitrososphaera sister 2	4	0	2.02	1.98
Nitrososphaera 1.1	4	5	4.55	4.45
Nitrososphaera 4.1	2	0	1.01	0.99
Nitrosopumilus 5.1	0	7	3.54	3.46
Nitrosopumilus 1.1	0	1	0.51	0.49
Nitrosotalea 1.1	0	64	32.34	31.66

Table 2. The chi-squared test for the sequenced clone libraries at Paterson found a p-value of $2.34*10^{-24}$ indicating in strong statistical terms that the two observed AOA communities were different.

	Prosser native observed	Prosser switchgrass observed	Prosser native expected	Prosser switchgrass expected
Nitrososphaera (54d9)	12	62	37.39	36.61
Nitrososphaera 8.2	20	0	10.11	9.89
Nitrososphaera 8.1	3	0	1.52	1.48
Nitrososphaera (non 54d9)	25	1	13.14	12.86
Nitrososphaera 2.1	2	0	1.01	0.99
Nitrososphaera 3.2	32	0	16.17	15.83
Nitrososphaera 3.1	1	0	0.51	0.49
Nitrososphaera 1.1	*0*	19	9.6	9.4
Nitrososphaera 4.1	1	0	0.51	0.49
Nitrosopumilus 5.1	*0*	10	5.05	4.95
Nitrosopumilus 1.1	*0*	2	1.01	0.99

Table 3. The chi-squared test for the sequenced clone libraries at Prosser found a p-value of $2.68*10^{-26}$ indicating in strong statistical terms that the two observed AOA communities were different.

Methods challenges

The primary problem with the methods in this study was that only three samples were collected per plot per sampling time. While this worked well for the replicated switchgrass plots it did not provide enough samples to achieve statistically significant analyses for some of the tests comparing samples taken from the un-replicated native plots. If more samples were collected an additional benefit could be the reduction of standard errors. If one change could be made to this study it would be collecting more samples from the native plots or setting up a replicated plot design to facilitate the collection of more samples from the native plots.

A better understanding of the roles of AOA and AOB on a macro-scale in eastern Washington would aid farmers in the implementation of the findings from this study. Specific comparisons of their adaptations to different soil conditions, the efficiency with which they convert nitrogen to plant usable forms—nitrate, and their roles in different temperatures—increasing temperatures in the spring, consistently high temperatures in the summer, and decreasing temperatures in the fall—would help farmers more appropriate tend to their crops and prepare for the expected actions of the microbial communities in the soils they cultivate.

Organic matter was not measured in this study but would likely provide an additional and important parameter with which to evaluate AOA diversity and relative abundance. Organic matter is likely much higher in the cultivated plots than the native plots because there is a lot more plant biomass in the cultivated plots. Furthermore, the two harvests per year cause root dieback, which results in an increase in soil organic matter.

Results and Discussion

AOA communities in soils from sites of perennial switchgrass cultivation differ from those of adjacent unmanaged sites

No significant difference within the native plot at Paterson

Samples were collected from the Paterson site in May, July and August of 2013. The plant community in the Paterson native plot consists of a typical sagebrush steppe plant community (dominant plants are sagebrush (genus *Artemisia*) and bunch or tussock grasses (family Poaceae)). AOA similarity across samples was examined using TRFLP and via sequenced clone libraries.

The AOA communities found in the soil samples collected at each sampling were similar, as supported by statistical analysis. Samples collected in July and August showed the highest intra-month similarity—50% for May, 70% for July, and 80% for August. For comparison, the percent similarity for duplicate extractions run on the same soil core was 70%.

There was a possible trend showing difference with season for AOA collected from the Paterson native plot. According to statistical analysis, the May sampling time separated insignificantly from the July and August sampling times. When the May samples were compared to the July or August samples, the R statistic was 0.778, which indicates that sampling time accounted for most of the observed AOA community differences. However, the p-value for this comparison is 0.10, indicating a lack of statistical significance. The result may still be indicative of a seasonal shift in diversity at the Paterson site (Figure 9).

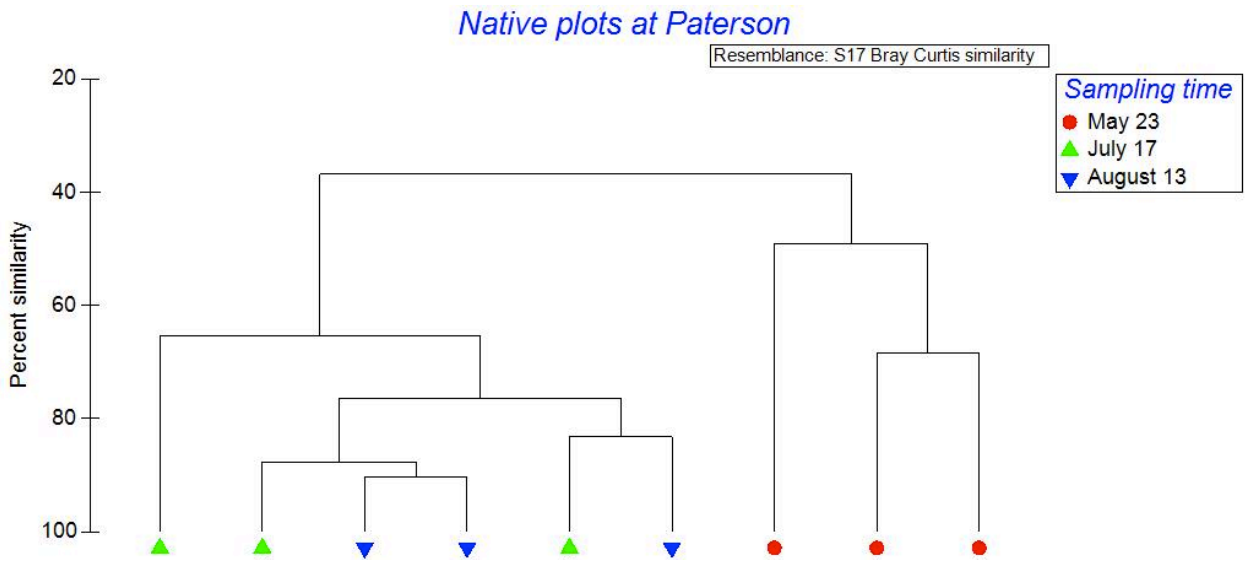

Figure 9. Percent similarity (determined by TRFLP) for AOA samples collected from the native site in Paterson, WA. Tested for AOA community difference based on the date the samples were taken.

The p-value is the chance that there will be a false negative. Said another way, the p-value is the chance that there is a correlation between data points that will be missed. Setting a p-value for a scientific test may depend on the observed variance within the samples that are collected. The greater the intrinsic variability within the samples, the greater the p-value may be acceptable to use. Furthermore, statistical significance can be viewed, and some would argue more correctly viewed, as varying degrees of uncertainty rather than as a firm boundary between significance and insignificance. Claims of significant and insignificant within this thesis are based on common practice within the field of microbiology, which generally establishes significance with a p-value of 0.05.

No Nitrosopumilus or Nitrosotalea in the Paterson native plot

Sequenced clone library data reveals additional details about the AOA community in the Paterson native soil. All cloned samples were developed from soil taken in August. Following selective PCR amplification of the archaeal *amoA*, the amplified products were cloned using an Invitrogen One Shot Top10 E. coli vector and sequenced to determine AOA clade representation and relative abundances. AOA clade representation was estimated based on the number of clones affiliated with a given clade. At Paterson 95 (of 96 possible) partial full-length *amoA* gene clones were generated from a twice DNA-extracted and pooled composite soil sample. The sample was a composite and was twice extracted in order to get better coverage of the AOA community in the soil. Available *amoA* sequences were used to assign the new sequences obtained from this study to described clades of AOA. The available AOA sequences were previously defined by Pester et al. (2004). AOA in the native soils of Paterson were associated with seven unique AOA clades (Table 4).

The most notable observation is that both *Nitrosopumilus* and *Nitrosotalea* were not observed in the native plot at Paterson (Table 4). Previous studies (Urich et al., 2008; Bates et al., 2011) have observed *Nitrososphaera* to be the dominant AOA genus in native or similar to native ecosystems. Urich et al. also made their observations in neutral pH soil, similar to the soil found in the Paterson native plot. Bates et al. (2011) did not report the pH values of the soils sampled in their study. However, the observation that both

Nitrosopumilus and *Nitrosotalea* were absent in the native soil is unique to this study. The sequenced clone library also reveals a relatively diverse population of *Nitrososphaera* in the Paterson native soil (Table 4).

AOA Clade	Paterson native
	Number of Clones
***Nitrososphaera* (54d9)**	32
***Nitrososphaera* 8.2**	3
***Nitrososphaera* 8.1**	
***Nitrososphaera* (non 54d9)**	36
***Nitrososphaera* 2.1**	15
***Nitrososphaera* 3.2**	
***Nitrososphaera* 3.1**	
***Nitrososphaera* sister 2**	4
***Nitrososphaera* 1.1**	4
***Nitrososphaera* 4.1**	2
***Nitrosopumilus* 5.1**	
***Nitrosopumilus* 1.1**	
***Nitrosotalea* 1.1**	
***Nitrosotalea* 2**	

Table 4. Number of clones observed in each AOA clade (a blank cell indicates that the clade was not detected).

Factors affecting AOA community formation

There are multiple environmental factors that could have affected the AOA community in the Paterson native soil. Adair et al. (2008) observed that temperature had an effect on the AOA population. This could be a factor as the soil temperature at Paterson in May was 17.5°C while the temperature in July (26.5°C) and August (28.6°C) was significantly higher. A second possibility for this possible seasonal difference could be soil moisture. Paterson receives on average 2 cm of rain during the month of May and only 0.5 cm of rain during the months of July and August, respectively. Additionally, in May the soil is likely wetter due to winter precipitation combined with lower evapotranspiration. In May the percent soil moisture measured in the native plot was 5.0% while in July and August the percent soil moisture was 0.5% and 0.6%, respectively (Figure 10). Other authors have also observed shifts in microbial communities as a result of differences in soil moisture (Angel et al., 2010; Hu et al., 2014).

Hu et al. (2014) observed that water addition was the most important environmental factor regulating the metabolic activity of soil ammonia oxidizers in dry sub-humid ecosystems. Furthermore, they observed that, in terms of abundance and activity, water was more important than fertilizer in influencing autotrophic nitrification in dry-land ecosystems and that AOA and AOB responses to fertilization alone were insignificant. Hu et al. also noted that AOA communities underwent composition changes in irrigated plots when compared to non-irrigated plots. They also observed that there were no obvious effects of land use

change on the AOA and AOB communities under dry conditions and that there were no significant changes in nitrification rate or AOA and AOB metabolic activity under dry conditions. He et al. also noted that AOA became increasingly abundant and active in ammonia-oxidation when dry soils became wetted. Hu et al. concluded that the effects of land management and land use practices on autotrophic ammonia oxidation in dry sub-humid ecosystems are primarily regulated by the availability of water. These results suggest that the community shift observed from May to July and August may be related to changes in soil moisture. The conditions that the Hu et al. study was conducted in are similar to the conditions this study was conducted in, possibly making comparisons between the two studies informative. The observation by Hu et al. that soil water content was the most important factor affecting the soil AOA community may be important for interpreting the results of this study.

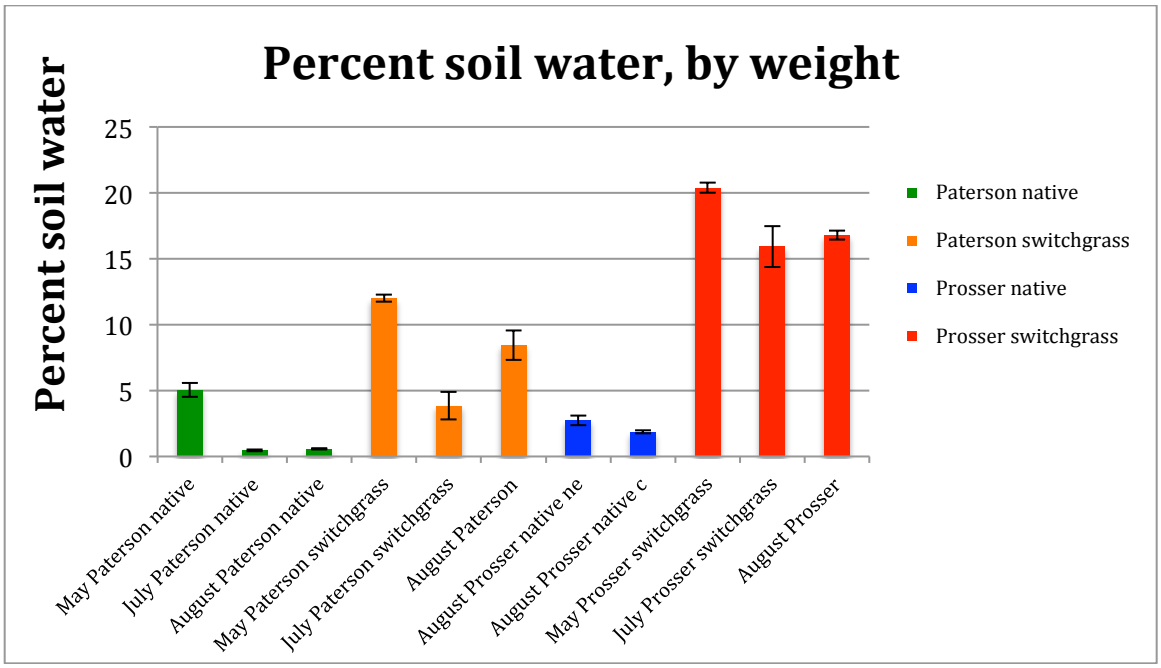

Figure 10. Percent water by weight for different plants and sites. Each plant type/site data point is the average of three percent water weight measurements.

No significant difference between the AOA communities in the native plots at Prosser

Native plots at Prosser were sampled in August 2013. Like Paterson, the plant community in the Prosser native plots consists of a typical sagebrush steppe plant community. AOA similarity across samples was examined using TRFLP and via sequenced clone libraries. Samples were collected from two sites with three triplicate samples collected from each site.

There may be an indication that the AOA populations in the native plots at Prosser differ by location within the farm (Figure 11) although the observation is not statistically significant (p-value=0.10, R statistic = 0.519). However, the AOA community in five of the six samples taken from the Prosser native plots is 85% similar to one another. That is notably similar even for samples collected within one plot. While the R-value may suggest a possible plot difference, when the data is examined more closely there is no evidence of difference between the AOA communities found in the two native plots at Prosser.

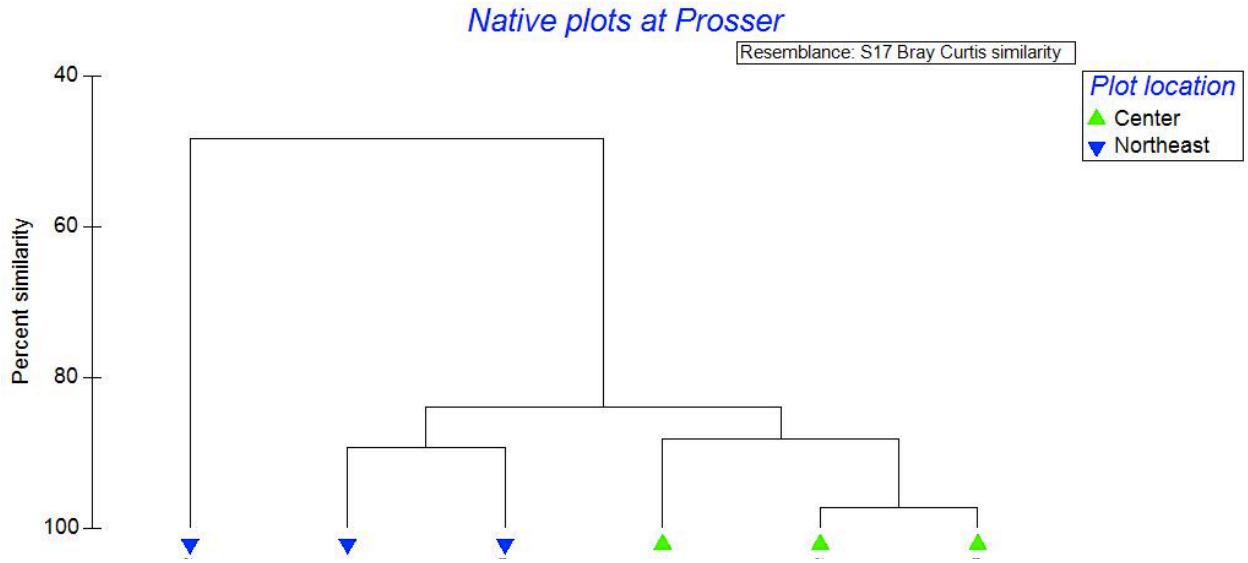

Figure 11. Percent AOA population similarity (determined by TRFLP) for samples collected at different locations in the native sites at Prosser, WA.

No Nitrosopumilus or Nitrosotalea in the Prosser native plot

The sequenced clone library data provides additional details regarding the AOA community in the native soil and Prosser. All cloned samples were developed from soil taken in August. Following selective PCR amplification of the archaeal *amoA*, the amplified products were cloned using an Invitrogen One Shot Top10 E. coli vector and sequenced to determine AOA clade representation and relative abundances. AOA clade representation was estimated based on the number of clones affiliated with a given clade. At Prosser 96 (of 96 possible) partial full length *amoA* gene clones were generated from one composite soil sample. Again, the same available *amoA* sequences were used to assign the new sequences obtained from this study to described clades of AOA. The available *amoA* sequences were defined by Pester et al. (2004). AOA in the native soils of Prosser were associated with eight unique AOA clades (Table 5).

The most notable observation is that both *Nitrosopumilus* and *Nitrosotalea* were not observed in any of the native plots. This is especially notable as neither *Nitrosopumilus* nor *Nitrosotalea* were observed in the Paterson native plot either. As was mentioned before, previous studies (Urich et al., 2008; Bates et al., 2011) have observed *Nitrososphaera* to be the dominant AOA genus in native or similar to native ecosystems. However, the observation that only *Nitrososphaera* was present in a native soil is unique to this study. This finding is particularly notable because it was observed at two different sites. The sequenced clone library data for the Prosser native site also reveals a relatively diverse population of *Nitrososphaera* in the Paterson native soil (Table 5).

AOA Clade	Prosser native
	Number of clones
***Nitrososphaera* (54d9)**	12

***Nitrososphaera* 8.2**	20
***Nitrososphaera* 8.1**	3
***Nitrososphaera* (non 54d9)**	25
***Nitrososphaera* 2.1**	2
***Nitrososphaera* 3.2**	32
***Nitrososphaera* 3.1**	1
***Nitrososphaera* sister 2**	
***Nitrososphaera* 1.1**	
***Nitrososphaera* 4.1**	1
***Nitrosopumilus* 5.1**	
***Nitrosopumilus* 1.1**	
***Nitrosotalea* 1.1**	
***Nitrosotalea* 2**	

Table 5. Number of clones observed in each AOA clade (a blank cell indicates that the clade was not detected).

Factors affecting AOA community formation

While the pHs between the two Prosser native plots were significantly different, the mean pH values at the two sites, respectively, were 6.9 and 7.2. It is unlikely that such a small difference in soil pH would have had a significant affect of the AOA community in either of these soils.

Samples from the Prosser native plots were only collected in August, therefore it was not possible to test for variation across a season, as was done for the AOA community in the Paterson native plot. As samples were collected from both sites during the summer, the similarity of the samples at Paterson and Prosser native sites from this time (July and August) can be compared. This comparison and additional analysis is contained in the following section.

No significant difference between the native plots at Paterson and Prosser

Samples from the two native sites were compared using TRFLP and sequenced clone library data. There was little differentiation between the native plot at Paterson and the native plots at Prosser (Figure 12). Excluding the May samples, as they were from a different season, and the one outlying sample from Prosser the overall percent similarity was approximately 65%. In an "ANOSIM" test for difference conducted in Primer 6 v.6.1.13, to test for the significance of 'site' (again not including the samples taken in May) the 'site' difference between the native plots was significant (p-value of 0.048), but did not account for the majority of the observed variation (R statistic of 0.43). The R statistic in this test suggests that the differing environmental variables between the two sites are not affecting AOA community diversity. Instead the relatively minor AOA community differences between the native plots may be caused by stochastic variation.

One of the principle challenges in this research was examining and parsing-out deterministic controls and stochastic controls of the population structure. Deterministic controls of AOA population structure include variables that can be controlled, especially

those that can be controlled by people. Whereas stochastic controls of population structure are variables that cannot be controlled or in many cases even measured—for instance the multitude of micro-environments or ecosystems that exist on a microscopic scale within any given soil ped.

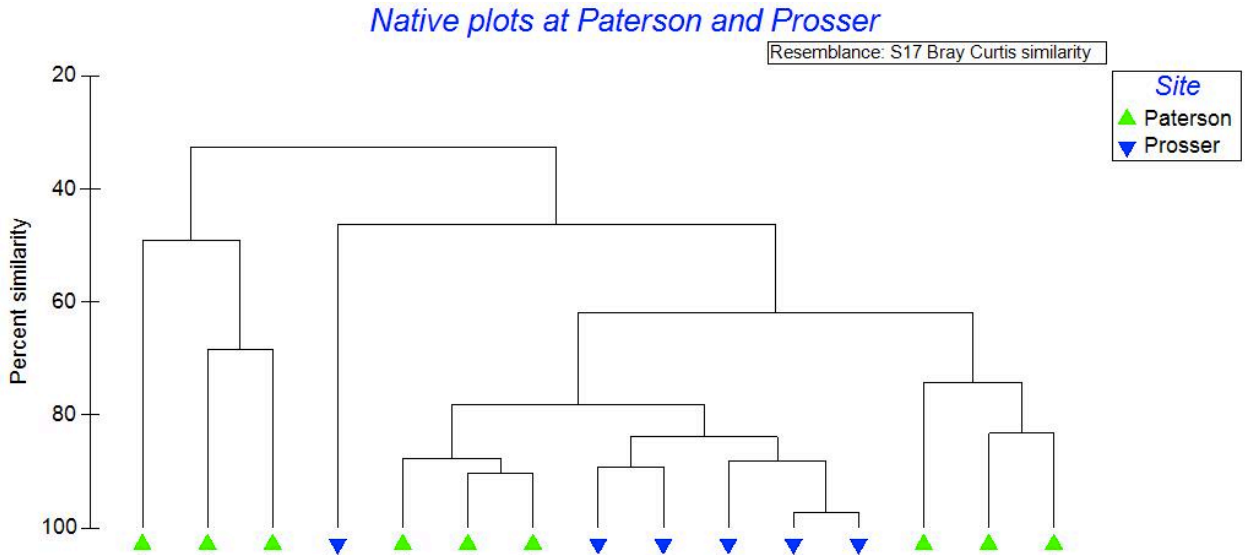

Figure 12. Percent similarity (determined by TRFLP) for AOA samples from all sampling times collected from native sites in Prosser and Paterson, WA.

At Paterson 95 (of 96 possible) and at Prosser 96 (of 96) partial full-length *amoA* gene clones were generated from one composite soil sample, respectivly. Available *amoA* sequences were used to assign the new sequences to described clades of AOA, as previously defined by Pester et al. (2004). AOA in the native soils of Prosser and Paterson soils were associated with eight unique and seven unique AOA clades, respectively (Table 6). Five of the clades were observed in both of the native sites.

AOA Clade	Paterson native	Prosser native
	Number of Clones	
***Nitrososphaera* (54d9)**	32	12
***Nitrososphaera* 8.2**	3	20
***Nitrososphaera* 8.1**		3
***Nitrososphaera* (non 54d9)**	36	25

***Nitrososphaera* 2.1**	15	2
***Nitrososphaera* 3.2**		32
***Nitrososphaera* 3.1**		1
***Nitrososphaera* sister 2**	4	
***Nitrososphaera* 1.1**	4	
***Nitrososphaera* 4.1**	2	1
***Nitrosopumilus* 5.1**		
***Nitrosopumilus* 1.1**		
***Nitrosotalea* 1.1**		
***Nitrosotalea* 2**		

Table 6. Number of clones observed in each AOA clade (a blank cell indicates that the clade was not detected).

Neither Nitrosopumilus or Nitrosotalea were observed in any native plot

The most notable observation regarding the native plots, and for the study as a whole, was that both *Nitrosopumilus* and *Nitrosotalea* were not observed in any of the native plots. While previous researchers have observed *Nitrososphaera* to dominate in native or native-like soils the absence of both *Nitrosopumilus* and *Nitrosotalea* from native soils is unique to this study. While this finding cannot be interpreted to mean that all native soils only contain AOA from the genus *Nitrososphaera*, it is an important finding for the two sites studied here, and possibly for other sites that share many of the physical, chemical, and environmental factors that are present in these two sites in eastern Washington.

The diversity of the *Nitrososphaera* communities varies between the native sites at Paterson and Prosser. The main difference between the native plots at Paterson and Prosser was the relatively high relative abundance (33%) of *Nitrososphaera* 3.2 at Prosser and the absence of this clade at Paterson (Table 6). Additionally, the clades *Nitrososphaera* 8.1 and 3.1, both representing less than 5% of the total AOA population, were present at Prosser and absent at Paterson. Similarly, the *Nitrososphaera* sister 2 subcluster and the clade *Nitrososphaera* 1.1, both representing less than 5% of the total AOA population, were present at Paterson and absent at Prosser. Overall the differences between the two native sites are minor and they share one major similarity—the absence of both *Nitrosopumilus* and *Nitrosotalea*.

Environmental and management actors affecting AOA community structure in the native plots

As stated, climate and precipitation were similar at both sites. Soil temperatures were similar at both sites during each sampling time. Percent soil water was also similar at both sites during each sampling time. However, the finer soil texture at Prosser (NRCS USDA soil survey) allowed the soil there to hold 1-2% more water than at Paterson.

Soil NH_4^+ and NO_3^- were similar at both Paterson and Prosser (Table 7). While there were measurable differences in soil ammonium and nitrate between the three soils they were not statistically significant and therefore cannot offer any explanation for the minor AOA community diversity differences that were observed between the native plots.

Plant Type	N	pH	NH_4^+	NO_3^-
			$mg.kg^{-1}$	$mg.kg^{-1}$
Paterson native	9	7.6 ± 0.08 a	1.8 ± 0.55	6.6 ± 1.7
Prosser native ne	3	6.9 ± 0.18 c	3.4 ± 0.65	11.1 ± 1.0
Prosser native c	3	7.2 ± 0.09 b	5.4 ± 2.51	12.7 ± 6.6

Table 7. pH, NH_4^+, and NO_3^- concentrations in soils collected at the Paterson and Prosser native sites. Means ± standard error is shown.

Soil pH was significantly different between the three native sites (p-value <0.05) (Table 7). It has been documented that pH has a strong effect on the AOA community (Gubry-Rangin et al., 2011; Nicol et al., 2008). However, while the pH values between the three native plots are significantly different, the changes are relatively minor, with pH values ranging from 7.6 ± 0.08 at the Paterson site to 6.9 ± 0.18 at one of the Prosser sites. Previous studies only observed significant changes in the AOA community at near neutral pHs when the pH variation shifted pHs out of a given zone, for instance from neutral-alkaline to slightly acidic (Gubry-Rangin et al., 2011). While smaller pH changes, pH value shifts of 0.5, have been observed to affect the AOA community at acidic pH values (Lehtovirta et al., 2009; Lehtovirta et al., 2011), this has not been observed in neutral and slightly alkaline soils. All three of the native soils are neutral or slightly alkaline.

Previous research has indicated that soil texture may have a significant affect on AOA community structure (Zegin et al., 2011). According to the USDA soil survey the Paterson site soil is classified as a loamy-sand while the Prosser site soil is classified as a silt-loam (NRCS USDA soil survey). This possible difference between the two sites may offer an explanation of the relatively minor AOA community differences observed between the two. However, soil texture was not tested as part of this study.

There is variation within and between the AOA communities in the switchgrass plots at Paterson and Prosser

Samples were also collected from adjacent plots planted in Switchgrass at both sites. The switchgrass at Paterson was planted in 2004 while the switchgrass at Prosser was planted in 2009. Management since planting has been similar at both sites. Grasses are fertilized in early spring and after the first harvest with surface application of synthetic N, K, and S. Irrigation based on crop demand is supplied for the entire growing season. The grasses are cut twice during the season with the first harvest typically in early to mid July and the second harvest in September or October. The grass is cut about 15cm above the soil surface to minimize damage to the stand. The samples that were analyzed for this study were collected May 23, July 17, and August 13, 2013 from each site.

No significant change in the AOA community in the switchgrass plots at Paterson over time

Sampling time showed no significant impact on AOA community diversity when the AOA communities present during each sampling event were compared to one another (R statistics <0.141 and p-values >0.258). Samples collected during July and August did not separate based on time of collection with 10 of the 12 samples showing high similarity. Samples from the May collection tended to segregate from the summer sampling, but this was not statistically significant (Figure 13).

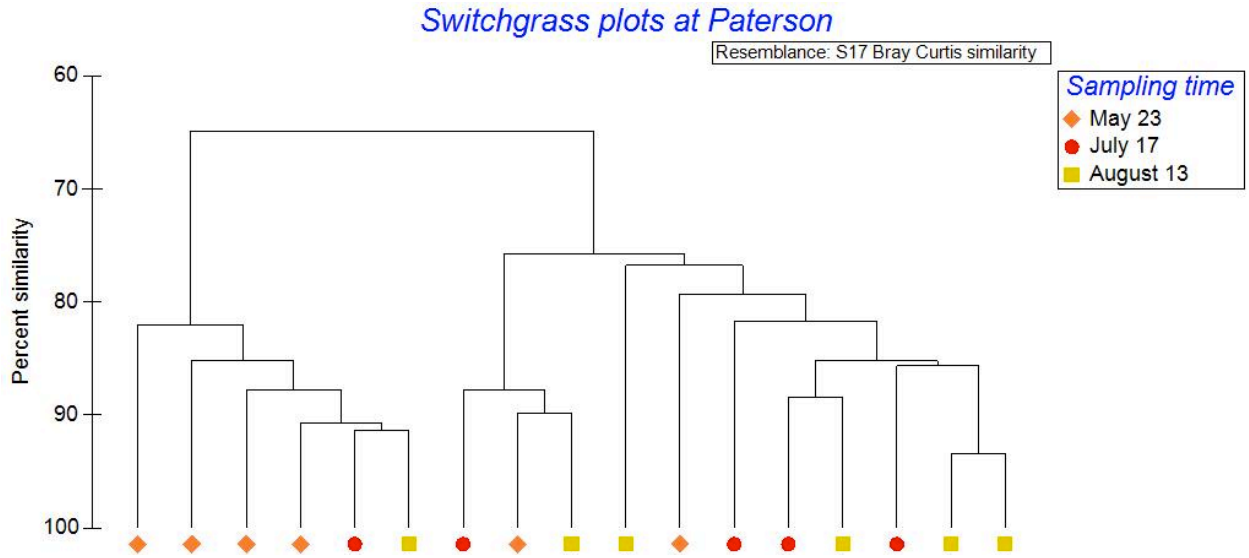

Figure 13. Percent similarity (determined by TRFLP) for AOA samples collected from switchgrass plots in Paterson, WA.

Sequenced clone library indicates that Nitrosotalea 1.1 is the most relatively abundant AOA clade in the switchgrass plots at Paterson

The AOA community in the switchgrass at Paterson has representatives from three AOA genera—*Nitrosopumumilus*, *Nitrososphaera*, and *Nitrosotalea*. *Nitrosotalea* 1.1 is the only observed clade from the *Nitrosotalea* genus, however it composes 65% of the total AOA community at Paterson. The 54d9 lineage dominates observed *Nitrososphaera* at Paterson comprising 68% of the *Nitrososphaera* population (but only 16% of the total AOA community). The *Nitrosopumilus* 5.1 clade dominates observed *Nitrosopumilus* composing 88% of the *Nitrosopumilus* population (but only 7% of the total AOA community) (Table 8).

	Paterson Switchgrass
***Nitrososphaera* (54d9)**	15
***Nitrososphaera* 8.2**	1
***Nitrososphaera* 8.1**	
***Nitrososphaera* (non 54d9)**	1
***Nitrososphaera* 2.1**	
***Nitrososphaera* 3.2**	
***Nitrososphaera* 3.1**	
***Nitrososphaera* sister 2**	
***Nitrososphaera* 1.1**	5
***Nitrososphaera* 4.1**	
***Nitrosopumilus* 5.1**	7
***Nitrosopumilus* 1.1**	1

***Nitrosotalea* 1.1**	64
***Nitrosotalea* 2**	

Table 8. The abundance of various AOA clades as observed from the creation of clone libraries.

In this study the AOA community at Paterson was not significantly different between fertilized and unfertilized switchgrass plots

The Switchgrass plots at Paterson included three no fertilizer control plots and three fertilized plots. TRFLP electropherograms for both sets of switchgrass plots showed no significant difference between plots that received agronomic rates of synthetic fertilizer and the plots that received no fertilization (Figure 14). There were significant fluctuations in soil-N in the fertilized plots, likely because the fertilizer in these plots was hand-scattered resulting in an uneven application of fertilizer. All of the control plots had consistently low values of soil-N.

The variations in soil-N did not correlate with the observed AOA community variations. Kelly et al. (2011) made a similar observation. They observed that there was no difference in AOA abundance between corn plots that received an agronomic rate of N and corn plots that received no N. While AOA abundance (measured via qPCR—like the observations made by Kelly et al. (2011)) is much different than AOA community diversity (measured via TRFLP) the absence of a response by the AOA community is an important similarity between the study conducted by Kelly et al. (2011) and this study.

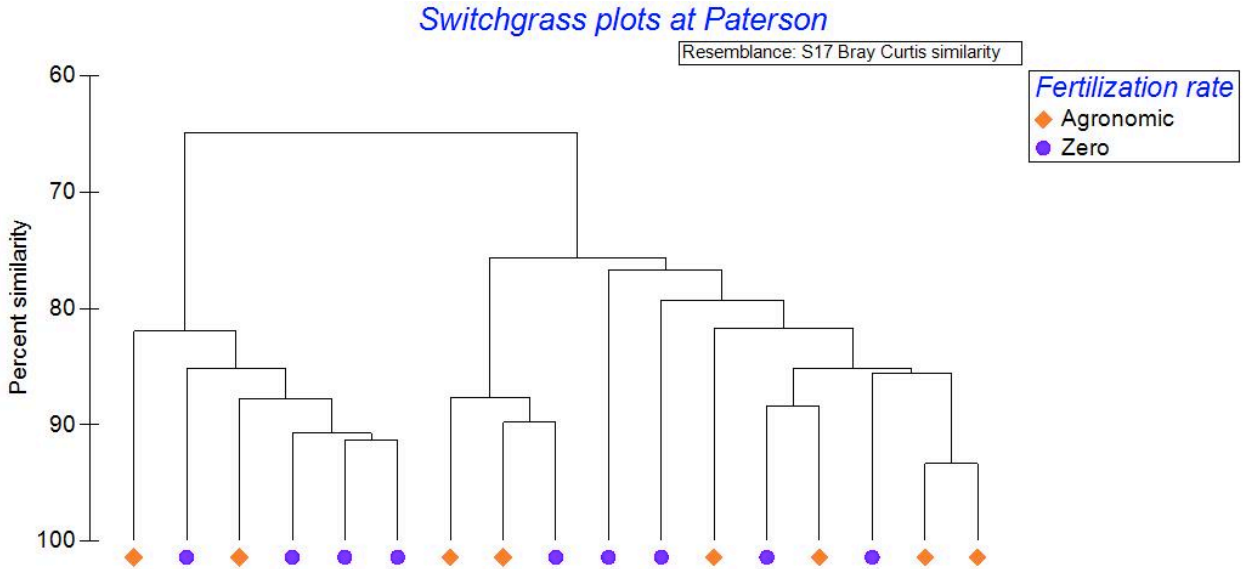

Figure 14. Percent similarity (determined by TRFLP) for AOA samples collected from fertilizer and control switchgrass plots in Paterson, WA. Test for difference: R statistic = -0.089, p-value = 0.905.

Significant change in the AOA community in the switchgrass plots at Prosser over time

In the Prosser switchgrass plots seven of the eight samples collected in August grouped together (Figure 15). This suggests that there is a significant difference between the AOA community found in the soil during the August sampling time and the AOA community found in the soil during the May and July sampling times. When compared to either of the other two sampling times for difference the R statistics were greater than 0.738 and the p-values were 0.001. The May and July sampling times did not differ from one another (R

statistic = 0.057, p-value=0.186). There are no correlated soil or environmental factors to explain this observed change in the AOA community.

The switchgrass was harvested after the July sampling at both the Prosser and Paterson switchgrass plots. This major chance in the ecosystem seems like a possible explanation for the observed change in the AOA community at Prosser from May and July to August. However, there was no change in the AOA community between July and August in the Paterson switchgrass plots. The lack of a replicated response at both of the sets of switchgrass plots suggests that the switchgrass harvest was not the factor that caused the AOA community to change between the July and August sampling times at Prosser.

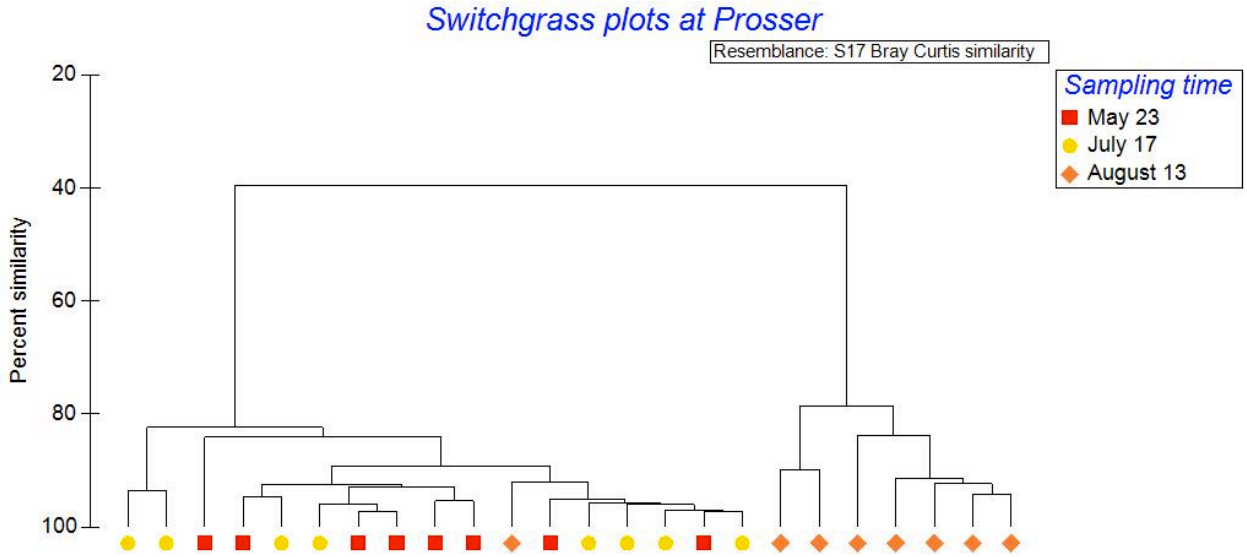

Figure 15. Percent similarity (determined by TRFLP) for AOA samples collected from switchgrass plots in Prosser, WA.

Sequenced clone library indicates that representatives of the Nitrososphaera 54d9 lineage are the most relatively abundant AOA clade in the switchgrass plots at Prosser

The AOA community in the switchgrass at Prosser has representatives from two AOA genera—*Nitrosopumilus* and *Nitrosotalea* (Table 9). AOA from clades within the genus *Nitrososphaera* compose 87.5% of the AOA community in the switchgrass soil at Prosser. AOA from clades within the genus *Nitrosopumilus* compose the remainder of the AOA community. The 54d9 lineage dominates observed *Nitrososphaera* composing 76% of the DNA reads. The 54d9 lineage is by far the most abundant AOA clade in the switchgrass soil at Prosser. *Nitrosopumilus* 5.1 is the principal clade from within the genus *Nitrosopumilus* accounting for 83% of *Nitrosopumilus* DNA reads.

	Prosser Switchgrass
***Nitrososphaera* (54d9)**	62
***Nitrososphaera* 8.2**	
***Nitrososphaera* 8.1**	
***Nitrososphaera* (non 54d9)**	1
***Nitrososphaera* 2.1**	
***Nitrososphaera* 3.2**	

***Nitrososphaera* 3.1**	
***Nitrososphaera* sister 2**	
***Nitrososphaera* 1.1**	19
***Nitrososphaera* 4.1**	
***Nitrosopumilus* 5.1**	10
***Nitrosopumilus* 1.1**	2
***Nitrosotalea* 1.1**	
***Nitrosotalea* 2**	

Table 9. The abundance of various AOA clades as observed from the creation of clone libraries.

In this study the AOA communities in the Prosser switchgrass soil were not significantly different between fertilized and unfertilized plots

The Switchgrass plots at Prosser included four no fertilizer control plots and four fertilized plots. TRFLP electropherograms for both sets of switchgrass plots showed no significant difference between AOA communities in plots that received agronomic rates of synthetic fertilizer and the plots that received no fertilization (Figure 16). There were significant fluctuations in soil-N in the fertilized plots, this was likely because the fertilizer that was applied to these plots was hand-scattered resulting in an uneven distribution of fertilizer. All of the control plots had consistently low values of soil-N.

The variations in soil-N did not correlate with the observed AOA community variations. Kelly et al. (2011) made a similar observation. They observed that there was no difference in AOA abundance between corn plots that received an agronomic rate of N and corn plots that received no N. As it was discussed in the previous section—looking at AOA community variation within the switchgrass plots at Paterson—The absence of a response by the AOA community in this study and the study conducted by Kelly et al. (2011) is an important similarity.

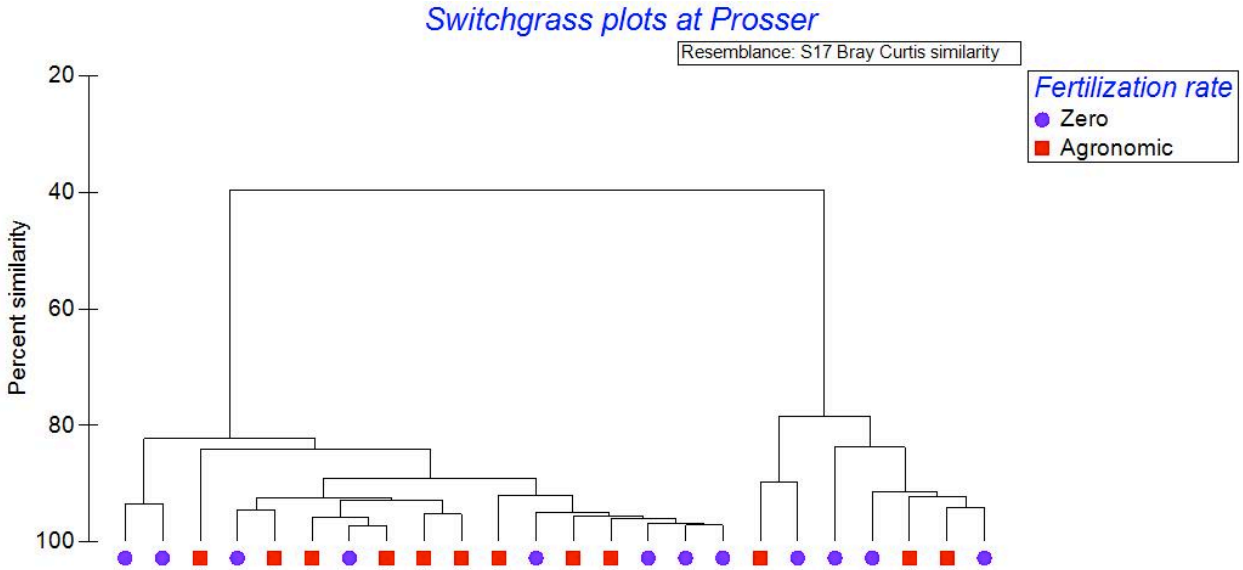

Figure 16. Percent similarity (determined by TRFLP) for AOA samples collected from fertilizer and control switchgrass plots in Prosser, WA. Test for difference: R statistic = 0.003, p-value = 0.357.

Significant difference between the AOA communities in the switchgrass plots at Paterson and Prosser

Across all sampling times the AOA communities in the switchgrass plots at Paterson and Prosser were distinctly different from each other (Figure 17). The AOA communities from the two sites are approximately 70% dissimilar. In an "ANOSIM" test for difference, conducted in Primer 6 v.6.1.13, the results suggest that 'site' accounts for most of the observed variation (R statistic of 0.871) (p-value of 0.001). The sequenced clone library data below provides a more detailed provides a more detailed explanation for the differences between the two AOA communities.

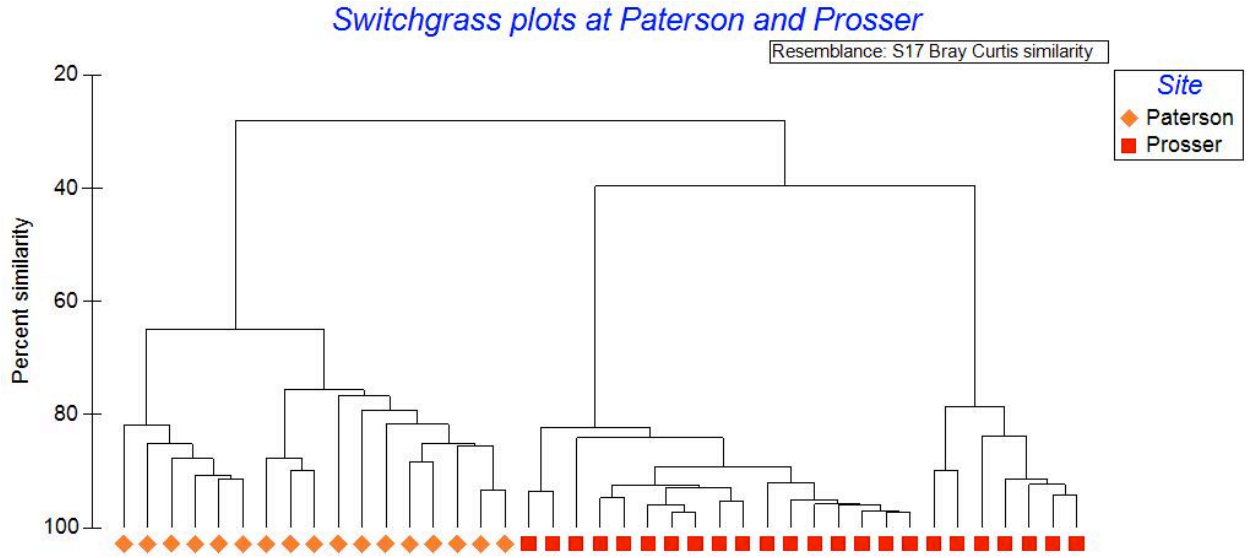

Figure 17. Percent similarity (determined by TRFLP) for AOA samples collected from switchgrass plots in Paterson and Prosser, WA.

According to sequenced clone library data the major difference between the AOA population in the switchgrass plots at Paterson and Prosser is the presence and absence of *Nitrosotalea*, respectively (Table 10). At Paterson *Nitrosotalea* 1.1 accounts for 68% of the total AOA population, while at Prosser it was not detected. The bulk of the AOA population in the Prosser switchgrass plots is made up of representatives of the *Nitrososphaera* (54d9) lineage, they comprise 65% of the AOA community. Early in the study of AOA, *Nitrososphaera* was shown to be a major AOA soil population so it is not surprising to see it constitute a large portion of the community (Stahl et al, 2012). The switchgrass soil at Paterson also contained representatives from both the *Nitrosopumilus* and *Nitrosotalea* genera. While the switchgrass plots also contained representatives from the genus *Nitrosopumilus*. An important commonality to note is that both *Nitrosopumilus* 5.1 and 1.1 are present in both sets of switchgrass plots. This commonality is important because both of these clades are absent in the native plots at Paterson and Prosser.

	Paterson Switchgrass	Prosser Switchgrass
***Nitrososphaera* (54d9)**	15	62
***Nitrososphaera* 8.2**	1	
***Nitrososphaera* 8.1**		
***Nitrososphaera* (non 54d9)**	1	1
***Nitrososphaera* 2.1**		
***Nitrososphaera* 3.2**		
***Nitrososphaera* 3.1**		
***Nitrososphaera* sister 2**		
***Nitrososphaera* 1.1**	5	19
***Nitrososphaera* 4.1**		
***Nitrosopumilus* 5.1**	7	10
***Nitrosopumilus* 1.1**	1	2
***Nitrosotalea* 1.1**	64	
***Nitrosotalea* 2**		

Table 10. The abundance of various AOA clades as observed from the creation of clone libraries.

pH may be the cause of AOA community differences between switchgrass plots at Paterson and Prosser

Soil chemical data offers a possible explanation for the observed difference between AOA communities in the Paterson and Prosser switchgrass plots. Soil pH was significantly different between the two sets of switchgrass plots. The mean pH value of the switchgrass plots at Paterson was 5.15 ± 0.1 while the plots at Prosser had a mean soil pH value of 8.00 ± 0.1 (Table 11). Previous research has shown that *Nitrosotalea* has a strong preference for low pH soils (Kemnitz et al., 2007; Lehtovirta et al., 2009; Lehtovirta et al., 2011). The pH difference may be responsible for the observed AOA community variation between the switchgrass plots at Paterson and Prosser. However, soil pH is not the overarching environmental factor controlling AOA community formation in the two sites studied. As it will be detailed in the following section other AOA communities presented in this study are different despite being found in soils with similar pHs.

As fertilizer application was similar between all of fertilized switchgrass plots, it is not surprising that soil ammonium and nitrate did not vary significantly between them (Table 11). Alternatively, the soil ammonium and nitrate levels may not have varied significantly between the two sets of switchgrass plots because the fertilizer was hand-broadcast which led to high variability of soil N measures within plots and between replicates. High

variability, in this case, means that the soil nitrogen levels were not statistically significant between any of the switchgrass plots in this study. Despite the high variability in soil ammonia and nitrate the lack of AOA community difference between the fertilized switchgrass plots and the unfertilized control switchgrass plots suggests that soil nitrogen concentration did not have a an effect on the soil AOA communities in the switchgrass plots at Paterson and Prosser.

Previous work has suggested that AOA communities diverge from one another within two years when in soil cultivated with different crops (Mao et al., 2011). The switchgrass at Paterson was planted in 2004 while the switchgrass at Prosser was planted in 2009. This suggests that management-legacy may not have influenced on the AOA communities under the two sets of switchgrass plots.

Soil texture is another factor that varies between the Paterson and Prosser sites. The NRCS USDA soil survey indicates that the soil texture differs between the Paterson (loamy sand) and Prosser (silt loam) switchgrass plots (NRCS USDA soil survey). Zeglin et al. (2011) observed this to be a significant factor affecting the AOA community in their study. However, soil texture was not measured in this study.

	pH	Ammonium (NH_4^+)	Nitrate (NO_3^-)
		mg kg^{-1}	
Paterson			
Fertilized	5.15 ± 0.1 a	10.6 ± 6.3	1.3 ± 0.3
Control		1.4 ± 0.2	0.5 ± 0.2
Prosser			
Fertilized	8.0 ± 0.1 b	9.0 ± 4.0	8.1 ± 2.8
Control		0.9 ± 0.3	0.3 ± 0.1

Table 11. Soil ammonium and nitrate levels at the Paterson and Prosser switchgrass plots.

AOA communities in soils from sites of perennial switchgrass cultivation differ from those of adjacent native sites with a sagebrush steppe ecosystem

The AOA populations in the native and switchgrass plots at both Prosser and Paterson were compared using TRFLP and sequenced clone library data.

AOA communities vary between soils from sites of perennial switchgrass cultivation and the adjacent unmanaged native ecosystem at Paterson

A comparison of the native samples at Paterson showed that the AOA community is approximately 65% similar across all samples (excluding the May sampling) (Figure 18). The AOA community in the Switchgrass plots is also approximately 65% similar across all samples (Figure 18). In contrast, the AOA communities in the native and switchgrass plots at Paterson are approximately 30% similar to each other (Figure 18). When tested for difference between the AOA communities found in the native and switchgrass plots at Paterson, respectively, the results were significant and suggested that the management difference has a large effect on AOA community formation (R statistic = 0.938, p-value = 0.001) at this site. Possible management factors that could have impacted the communities include soil moisture, plant community differences, and management differences.

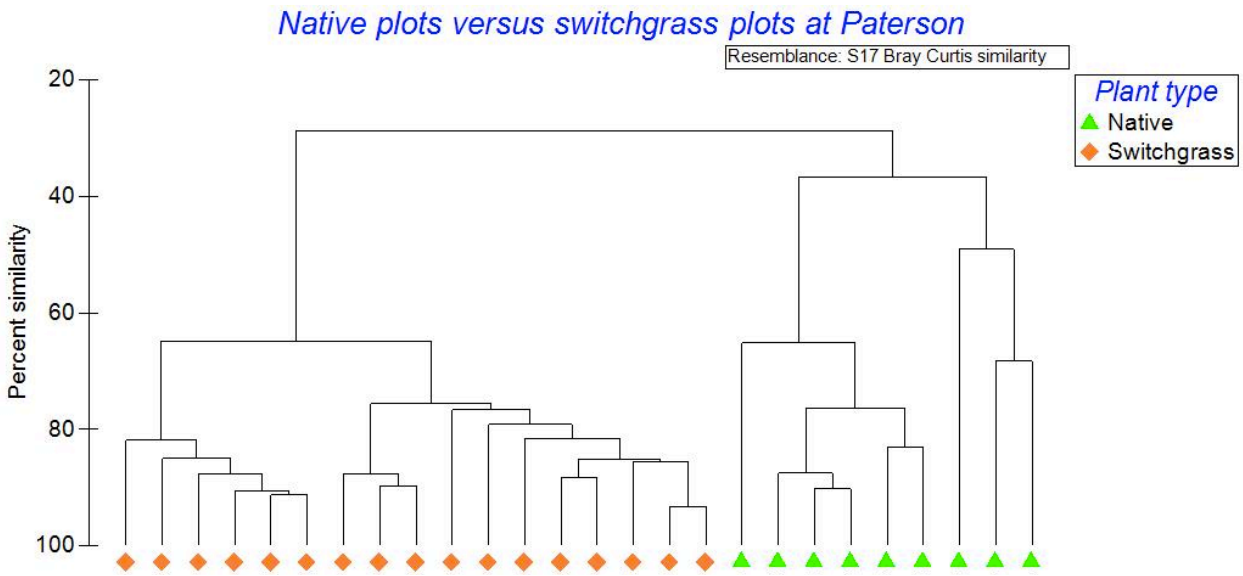

Figure 18. Percent similarity (determined by TRFLP) for AOA samples collected from native and switchgrass plots in Paterson, WA.

When the AOA communities were examined more closely using sequenced clone libraries there were notable differences between the AOA community in the Paterson native plot and the AOA community in the Paterson switchgrass plots. *Nitrososphaera* clades 2.1, 4.1, and subcluster sister 2 were present in the Paterson native plots, but were not detected in the Paterson switchgrass plots (Table 12). Additionally, the *Nitrososphaera* clade that was most abundant in the native plot is different than the *Nitrososphaera* clade that is most abundant in the switchgrass plots. Again, it is noteworthy that there were no members of *Nitrosopumilus* or *Nitrosotalea* observed in the Paterson native plots. *Nitrosopumilus* was present in the switchgrass plots composing 8% of the population. *Nitrosotalea* were highly abundant in the switchgrass plots representing 67% of the population (Table 12).

	Paterson Native	Paterson Switchgrass
***Nitrososphaera* (54d9)**	32	15
***Nitrososphaera* 8.2**	3	1
***Nitrososphaera* 8.1**		
***Nitrososphaera* (non 54d9)**	36	1
***Nitrososphaera* 2.1**	15	
***Nitrososphaera* 3.2**		
***Nitrososphaera* 3.1**		
***Nitrososphaera* sister 2**	4	
***Nitrososphaera* 1.1**	4	5
***Nitrososphaera* 4.1**	2	
***Nitrosopumilus* 5.1**		7
***Nitrosopumilus* 1.1**		1
***Nitrosotalea* 1.1**		64
***Nitrosotalea* 2**		

Table 12. The abundance of various AOA clades as observed from the creation of clone libraries.

The statistical difference between the Paterson native AOA community and the Paterson switchgrass AOA community was compared via a chi-squared test. The chi-squared test was used to test if there was a significant difference between the two microbial communities. The chi-squared test returned a p-value of $2.41*10^{-24}$, suggesting in the strongest statistical terms that the two AOA communities characterized in the above sequenced clone libraries were different. The very small p-value returned by the chi-squared test is important because it adds strong corroborating evidence to the statistical tests run on larger portions of the soil AOA communities.

AOA communities vary between soils from sites of perennial switchgrass cultivation and the adjacent unmanaged native ecosystem at Prosser

A comparison of the native samples at Prosser shows that the AOA communities are about 85% similar across all samples (excluding the single outlying sample) (Figure 19). The native plots at Prosser were only sampled during the August sampling trip; the high percent similarity may be partially related to the single sampling time. In contrast, the AOA community in the switchgrass plots is about 40% similar across the three sampling times (Figure 19). Additionally, the native and switchgrass plots at Prosser are approximately 5% similar (Figure 19). When tested for difference by crop the results are significant and suggest that the crop or management difference has a large effect on AOA community formation (R statistic = 1.0, p-value = 0.001). Factors that were potentially responsible for this observed difference include soil moisture, plant community differences, and management differences.

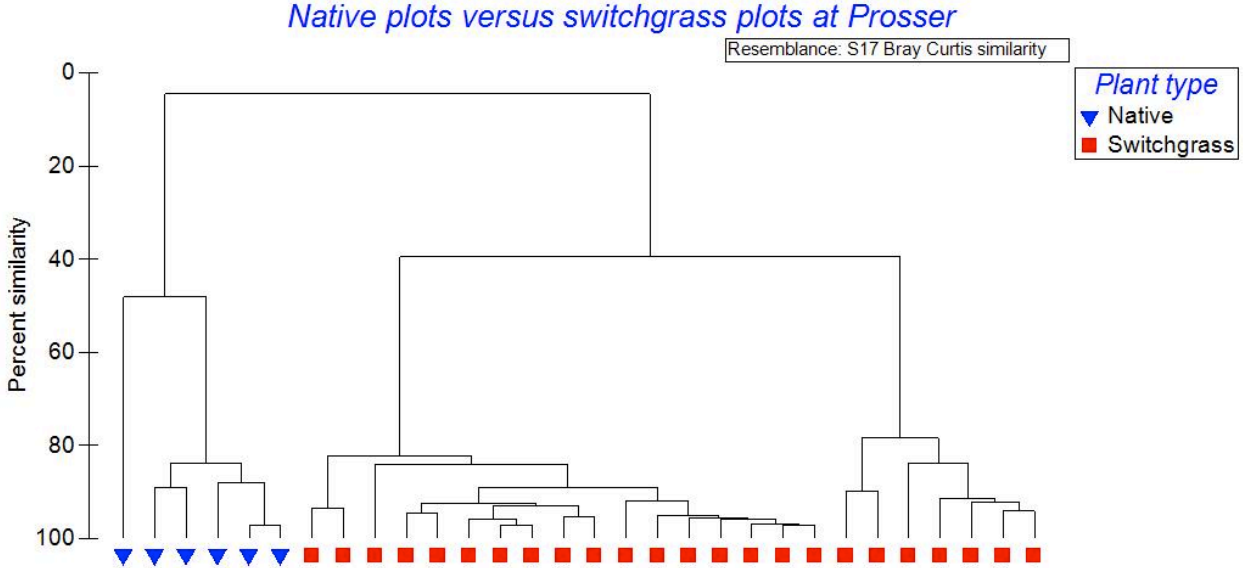

Figure 19. Percent similarity (determined by TRFLP) for AOA samples collected from native and switchgrass plots in Prosser, WA.

When the AOA communities were examined more closely using sequenced clone libraries there are some notable differences between the AOA community in the Prosser native plots and the AOA community in the Prosser switchgrass plots. *Nitrososphaera* clades 3.1 and 3.2 are present in the Prosser native plots (the clade *Nitrososphaera* 3.1 is especially dominant—accounting for 33% of the AOA community), but neither of these clades was observed in the Prosser switchgrass plots. Additionally, *Nitrososphaera* clades 8.1, 8.2, 2.1, and 4.1 were also observed in the Prosser native soil, but not detected in the Prosser switchgrass soil. Furthermore, *Nitrososphaera* subcluster 1.1 is not observed in the Prosser

native plots. However, it represents approximately 20% of the population in the Prosser switchgrass plots. This observation is of interest because it is the only time in this study that a *Nitrososphaera* clade appeared in switchgrass soil after not appearing in the associated native soil. Notably, no members of *Nitrosopumilus* or *Nitrosotalea* were observed in the Prosser native plots. Conversely, *Nitrosopumilus* were present in the Prosser switchgrass plots (Table 13).

	Prosser native	Prosser switchgrass
***Nitrososphaera* (54d9)**	12	62
***Nitrososphaera* 8.2**	20	
***Nitrososphaera* 8.1**	3	
***Nitrososphaera* (non 54d9)**	25	1
***Nitrososphaera* 2.1**	2	
***Nitrososphaera* 3.2**	32	
***Nitrososphaera* 3.1**	1	
***Nitrososphaera* sister 2**		
***Nitrososphaera* 1.1**		19
***Nitrososphaera* 4.1**	1	
***Nitrosopumilus* 5.1**		10
***Nitrosopumilus* 1.1**		2
***Nitrosotalea* 1.1**		
***Nitrosotalea* 2**		

Table 13. The abundance of various AOA clades as observed from the creation of clone libraries.

The difference between the Prosser native AOA community and the Paterson switchgrass AOA community was compared via a chi-squared test. The chi-squared test was used in the same manner as it was when comparing the sequenced clone libraries from the Paterson site. The chi-squared test returned a p-value of $2.56*10^{-26}$; suggesting in the strongest statistical terms that the two AOA communities characterized in the Prosser sequenced clone libraries are different. The very small p-value returned by the chi-squared test is important because it adds strong corroborating evidence to the statistical tests run on larger portions of the soil AOA communities.

Significant difference between the soil AOA communities observed in two native plots and the AOA communities observed in two sets of switchgrass plots

AOA community structure across the two native sites was also compared to the AOA community structure across the two switchgrass sites. As discussed previously, climate was similar across all sites. Additionally, soils within each site fell under the same or highly similar soil series. The primary differences across the native and switchgrass sites were related to management practices, plant type, and irrigation. The cultivated plots were irrigated during the growing season; irrigation was similar at both sites. Likely as a result of long-term N-fertilization and irrigation (Brady and Weil, 2004), the pH of the soils in the Paterson Switchgrass plots was acidic. While there was no statistical difference between soil NH_4^+ and NO_3^- values between the fertilized switchgrass, control switchgrass and native sites, it is important to note that values for the fertilizer treatments were highly variable (likely because the fertilizer was hand scattered). This high nitrogen variability may have masked differences in soil AOA communities related to different levels of N. It is also possible that the AOA community did not respond to variability in soil N.

There is significant differentiation between the AOA community structure at the two native sites and the AOA community structure at the two switchgrass sites. The similarity between the AOA community in the native plots at Paterson and Prosser is most notable when compared to the AOA community in the switchgrass plots at Paterson and Prosser. The switchgrass plots at Paterson and Prosser are only 30% similar based on TRFLP data (Figure 20). In comparison, all but one sample from the native plots collected in July and August are greater than 65% similar based on TRFLP data (Figure 20).

The TRFLP electropherograms and the subsequent Bray-Curtis similarity matrix indicate that the native AOA communities at Paterson and Prosser and the switchgrass AOA communities at Paterson and Prosser are greater than 80% dissimilar (Figure 20). Overall, there is a significant difference between the AOA community structure observed in the native soil and the AOA community structure observed in the switchgrass soil (R statistic = 0.937; p-values = 0.001).

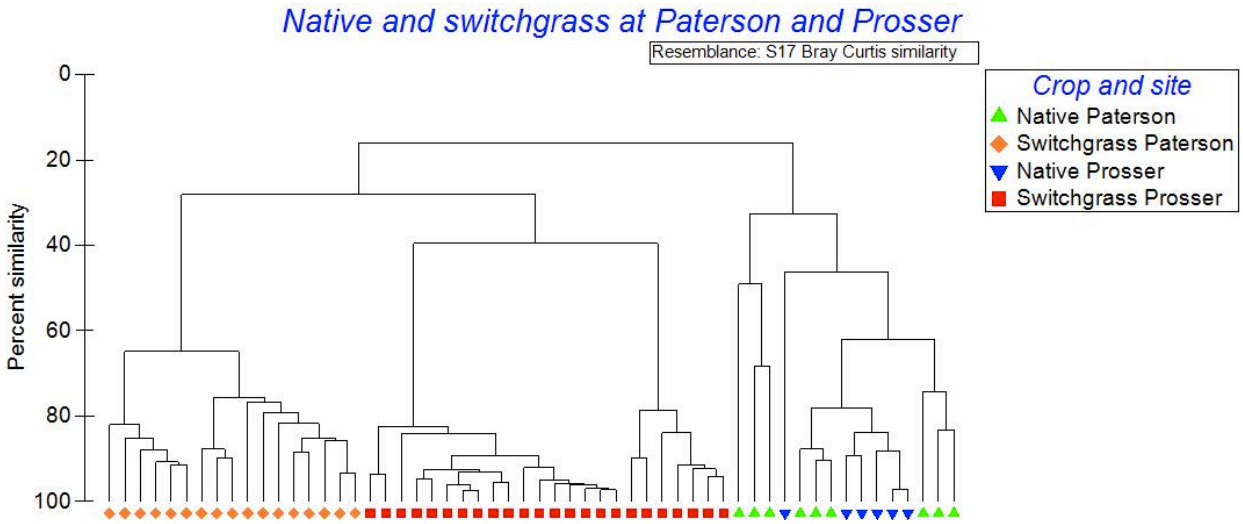

Figure 20. Percent similarity (determined by TRFLP) for AOA samples collected from native and switchgrass plots in Paterson and Prosser, WA.

Sequenced clone libraries of the AOA communities in the native and switchgrass plots at both Paterson and Prosser show in detail how the AOA community structure varies between the native and switchgrass soils. The significant difference between the native and switchgrass AOA communities is primarily caused by the absence and presence of *Nitrosopumilus* and *Nitrosotalea*, respectively (Figure 21). Specifically, the only AOA genus found in the native soil at Paterson and Prosser is *Nitrososphaera*. However, members of the genera *Nitrososphaera* and *Nitrosopumilus* are found in the switchgrass soil at Prosser, while members of the genera *Nitrososphaera*, *Nitrosopumilus*, and *Nitrosotalea* are found in the switchgrass soil at Paterson.

The presence of members of the genus *Nitrosopumilus* in the switchgrass soils and the absence of members of this genus in the native soils is especially noteworthy. This was the most significant AOA community structural change that was consistent between all of the native plots and all of the switchgrass plots that were tested.

Other studies have also observed community shift and replacements similar to the ones observed in this study. Bates et al. (2011) observed that in more than half of 143 soils examined, the majority of them native, only *Nitrososphaera*, not *Nitrosopumilus* or *Nitrosotalea*, were present. Other studies have also observed shifts in population following transitions from native to managed systems (Rodrigues et al., 2013). Rodrigues et al. (2013) observed that after a section of Amazon rainforest in Brazil in the state of Rondônia was cut down and replaced with pasture for cattle, the microbial community in the soil shifted dramatically. While a succession event has not been documented or examined in this study, significantly different microbial communities have been observed in native soil and the adjacent soil that was converted to agriculture.

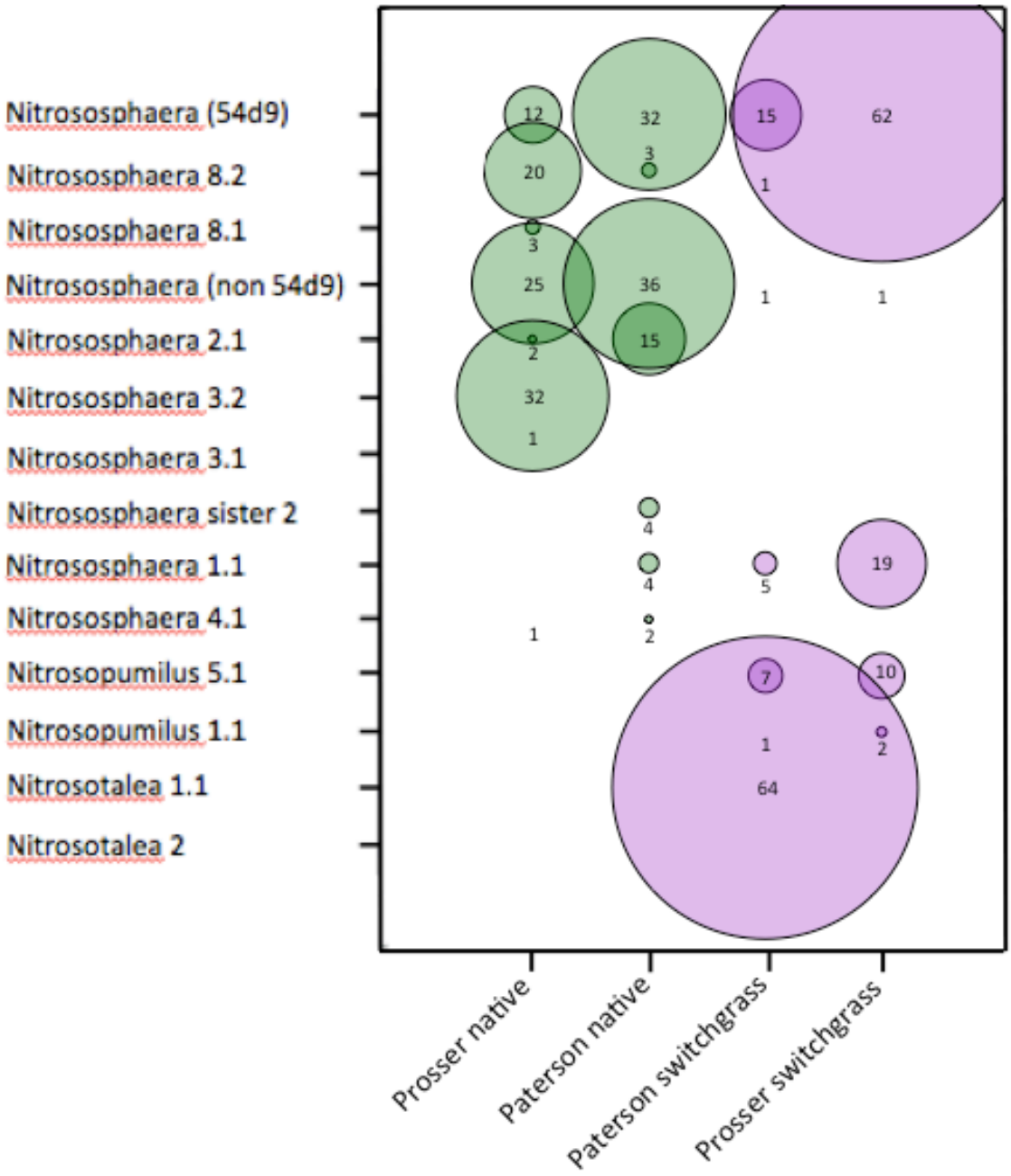

Figure 21. Relative abundance (determined via clone libraries) for AOA in samples collected from native and switchgrass sites in Paterson and Prosser, WA. AOA clades are listed on the y-axis and sampling locations are listed on the x-axis. Larger circles represent higher abundances of a given clade at a given location while no circle means that the clade was not observed.

Factors affecting AOA community formation

There are multiple factors that differ between the native and the switchgrass plots. These include irrigation, management practices, and plant type. Starting with irrigation, the native plots at both sites only receive water from precipitation. Total annual precipitation is approximately 25cm annually at Paterson and 18cm annually at Prosser (NOAA). In contrast, the switchgrass plots receive approximately 100 hectare-centimeters additional water as irrigation. Hu et al. (2014) recently observed that soil water availability was the controlling variable for AOA community formation in the ecosystem they examined.

Irrigation correlates strongly with the AOA community differences between the native and switchgrass plots. As the conditions in the Hu et al. (2014) study were similar to the conditions in this study, the observation by Hu et al. that soil water content was the most important factor affecting the soil AOA community may be important for interpreting the results of this study.

Other researchers have observed that fertilizer can affect the AOA community (He et al., 2007). However, as was pointed out during the comparison of AOA community in unfertilized-control and fertilized switchgrass, the AOA communities were similar to each other. This suggests that fertilizer alone is not a likely explanation for the AOA community difference between the native and switchgrass plots at either site.

The possible effects of management on the AOA community in the switchgrass plots are harder to parse out. The switchgrass plots have not been tilled since they were planted in 2004 (Paterson) and 2009 (Prosser), as switchgrass is a perennial crop. Previous work has shown that there are significant differences in AOA communities between annual and perennial crops potentially related to tillage (Mao et al., 2011). Lasting effects on the soil from tillage may still be affecting the microbial community, in this case specifically the AOA community, in the switchgrass soils. This cannot be confirmed or denied. Additionally, the switchgrass plots are mown twice annually—once during summer and once in the fall. When a perennial grass is cut some of its root structure dies with it. Therefore the mowing may have an effect on the AOA community. However, the lack of difference between the July and August samples at Paterson suggest that mowing is not an important factor affecting the AOA community. There is a significant difference between the July AOA community and the August AOA community in the Prosser switchgrass plots.

It is well documented that pH has a strong effect on the AOA community (Lehtovirta et al., 2009; Gubry-Rangin et al., 2011). At Paterson the mean pH in the switchgrass plots is 5.15 (Table 5) and the mean pH in the native plot is 7.6 (Table 3). The large difference in pH values between the plots may explain the presence of *Nitrosotalea*, an acidophilic genus of AOA, in the switchgrass plots. The presence of *Nitrosotalea* is one of the main differences between the native and switchgrass plots at Paterson.

At Prosser pH was not likely a factor in the strong difference observed between the switchgrass and native AOA communities. The mean pH in the switchgrass plots is 8.0 (Table 5) and the mean pH in the native plots is 7.2 and 6.9, respectively (Table 3). The similar pH values in the switchgrass and native soil strongly suggest that pH is unlikely to be responsible for the AOA community differences at the Prosser site.

Total carbon to nitrogen ratio was only measured for the Paterson site. At the Paterson site the C:N ratio did not differ significantly between the native plot and the switchgrass plots. The C:N ratio in the native plot was 18.6 ± 2.9 while the C:N ratio in the switchgrass plots was 17.8 ± 1.3. This data suggests that the total C:N ratio at the Paterson site did not have an affect on AOA community formation.

Plant type is a variable that could be affecting the AOA community. Two previous studies, Mao et al. (2011) and Bates et al. (2011) found that plant type was an environmental

variable that had a strong affect on AOA community formation. The study by Mao et al. is especially relevant because switchgrass was one of the plants they included in their study (they also examined miscanthus, maize, and restored tall grass prairie). As the native and switchgrass plots have two different plant communities growing in them, this is another possible variable causing the two AOA communities to be different. The difference in AOA communities between the two switchgrass plots indicates that other factors are affecting the AOA community besides plant type and that plant type is not the universal variable governing AOA community formation. However, this does not preclude the different plant types between the native and switchgrass plots from being a factor in the AOA community difference between the native and the switchgrass plots. The factors that seem most correlated with the AOA community differences between the native and the switchgrass sites are irrigation, management differences, and plant type.

Other researchers have observed different successional patterns for AOA communities. Similar to observations from this study, Yao et al. (2013) observed that land use significantly altered the AOA community (based on TRFLP electropherograms). However, unlike this study Yao et al. observed that *Nitrososphaera* dominated the AOA community in improved grasslands, and most notably, arable lands, while *Nitrosotalea* dominated the AOA community in semi-natural grasslands, woodlands, moorlands, and bogs. This observation by Yao et al. differs with observations made in this study and other studies (Urich et al., 2008; Ying et al., 2010; Bates et al., 2011) that link *Nitrososphaera* with less disturbed ecosystems and *Nitrosotalea* (and *Nitoropumilus*) with more disturbed ecosystems.

Zhalnina et al. (2013) also made an observation that is the opposite of the findings of this study. They observed that *Nitrososphaera* increased slightly in relative abundance in response to agriculture. This observation by Zhalnina et al. is also in conflict with other studies (Urich et al., 2008; Ying et al., 2010; Bates et al., 2011). In Zhalnina et al. not only were *Nitrososphaera* correlated with agriculture, but their relative abundance dropped in plots where agricultural practices ceased. *Nitrososphaera* was the only AOA genus that Zhalnina et al. observed to increase in relative abundance in response to agriculture. This is opposite of what was observed in this study regarding the relative abundance of *Nitrososphaera*.

Zhalnina et al. observed the relative abundance of *Nitrososophaera* to decrease with time away from agriculture. The apparent reversibility of the agricultural effect on *Nitrososphaera* in intriguing and could provide an interesting future experiment if some of the agricultural land tested in this experiment could be converted back to a native ecosystem and monitored for microbial community changes. Monitoring the microbial succession from native soil to cultivated soil and back could also be valuable for further understanding the functioning of AOA communities.

Taxonomic identification of dominant terminal restriction fragments was not possible

In this study specific terminal restriction fragments (TRFs), from TRFLP electropherograms, were unsuccessfully linked with specific AOA clades. This information would have been valuable, as it could have provided exponentially more data on which AOA clades were present in each soil sample than was provided from the limited number of

clone libraries that were built and sequenced. However, there was significant degeneracy within the TRFs. Meaning that multiple TRFs would associate with one AOA clade or one TRF would associate with multiple AOA clades (Figure 22). This degeneracy made it impossible to link specific TRFs with specific AOA clades.

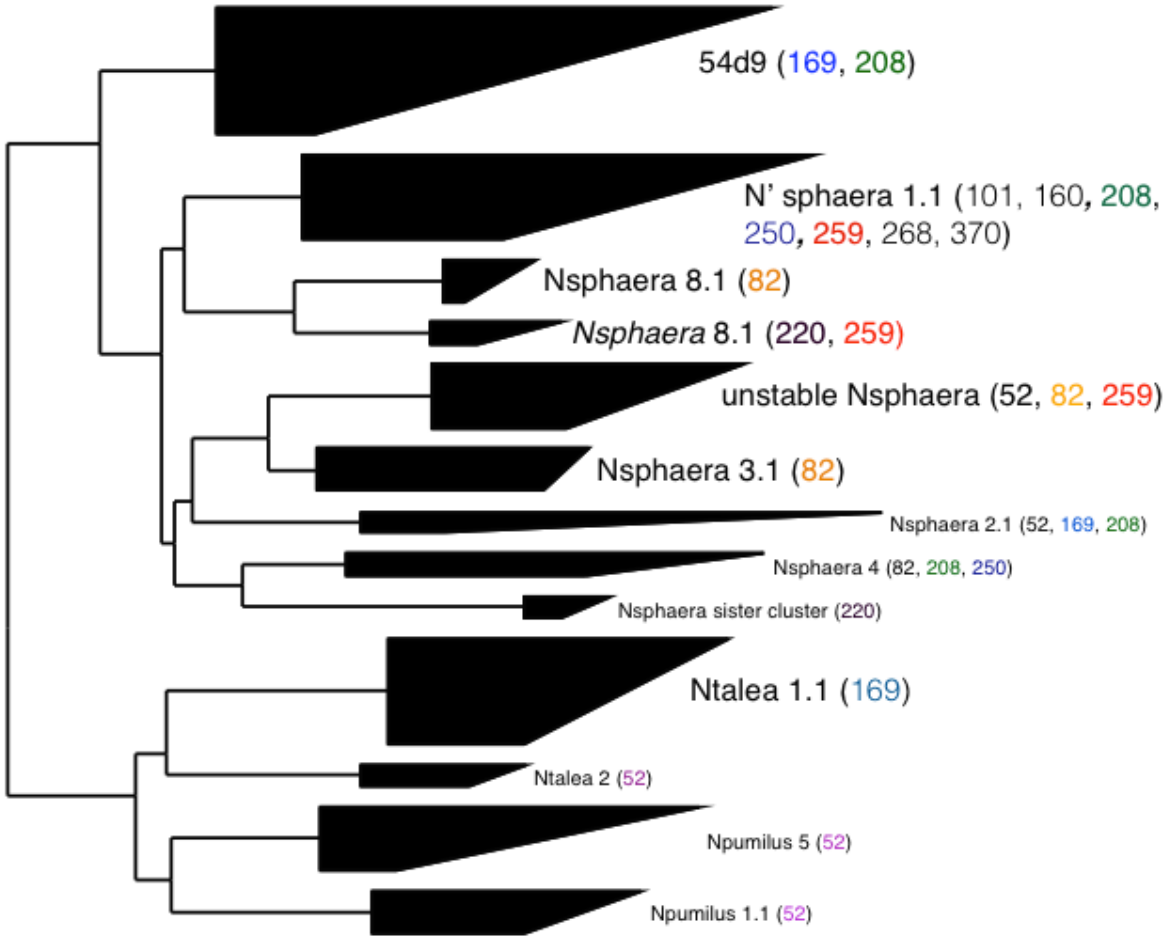

Figure 22. Multiple examples of TRF degeneracy across all samples are present in this figure. TRF 169 indicates the AOA clades *Nitrosotalea* 1.1, *Nitrososphaera* 2.1, and representatives of the lineage *Nitrososphaera* 54d9. TRF 52 indicates the AOA clades *Nitrososphaera* 2.1, *Nitrosotalea* 2, *Nitrosopumilus* 5, and *Nitrosopumilus* 1.1. TRFs 82, 208, 259, and 220 are also degenerate in this way. The figure also shows several AOA clades that are indicated by multiple TRFs, again making it impossible to distinguish which TRF belongs to which AOA clade.

TRF degeneracy was also evident within subsets of the soil samples. Within the soil samples taken from the native plot at Paterson TRF 53 was found to indicate representatives of the *Nitrososphaera* 54d9 lineage and *Nitrososphaera* 2, TRF 170 was found to indicate representatives of the *Nitrososphaera* 54d9 lineage and *Nitrososphaera* 2.1, TRF 209 was found to indicate representatives of the *Nitrososphaera* 54d9 lineage and *Nitrososphaera* 2.1, and TRF 221 was found to indicate representatives of the *Nitrososphaera* 54d9 lineage and unclassified *Nitrososphaera*. Within the soil samples taken from the native plots at Prosser TRF 81 was found to indicate *Nitrososphaera* 8.2 and

unclassified *Nitrososphaera* 2, TRF 83 was found to indicate *Nitrososphaera* 8.2 and unclassified *Nitrososphaera*, and TRF 251 was found to indicate *Nitrososphaera* 4.1 and unclassified *Nitrososphaera* 2. The degeneracy between TRFs and AOA clades both across all samples and within subsets of samples makes it impossible to determine which TRFs are associated with which AOA clades within this study.

Conclusion

The results from this study encourage follow-up studies to be conducted. While there is a strong suggestion of different AOA community structure in the native and switchgrass soil, the changing condition or conditions that are responsible for the AOA community change remain largely unclear. A follow-up study where subsets of the native plots are converted to switchgrass systems and closely monitored and intensely sampled during the transition could help explain when and possibly why the AOA communities change. Further exploration of the appearance of representatives of the clade *Nitrososphaera* 1.1 in the Prosser switchgrass plots despite the absence of representatives of this clade in the Prosser native plots also presents and appealing future study. The results from this study support previous research that has suggested that *Nitrososphaera* dominated the AOA community in native soil conditions. However, tracking *Nitrososphaera* 1.1 could reveal new information about AOA, the genus *Nitrososphaera*, and what factors led to the emergence and relatively high relative abundance of *Nitrososphaera* 1.1 in the switchgrass soil at Prosser.

The most important observation from this study is the absence of the genera *Nitrosopumilus* and *Nitrosotalea* from the native soil in the two sites investigated in eastern Washington, and the appearance of these two genera in the switchgrass soil at these two sites. While previous studies have observed *Nitrososphaera* to dominate the AOA community in native soil, the observation that the only AOA genus present in the native soil was *Nitrososphaera* is unique to this study. Further investigation of this phenomenon and additional environmental monitoring may produce results that could help the scientific community better assess which AOA genera are responsible for different ecosystem processes and thereby create conditions that encourage the beneficial processes and suppress the harmful processes in agricultural ecosystems in eastern Washington.

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