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THAT CONSUME ATMOSPHERIC METHANE

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COMPARISONS OF METHANOTROPH COMMUNITIES IN SOILS THAT CONSUME ATMOSPHERIC METHANE

By

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ABSTRACT

COMPARISONS OF METHANOTROPH COMMUNITIES IN SOILS THAT CONSUME ATMOSPHERIC METHANE

By

Uri Yitzhak Levine

Methane is a potent greenhouse gas that is 21-25 times more efficient at trapping heat (infrared radiation) than carbon dioxide. Methane oxidation is mediated by methane-consuming microbes (methanotrophs), but only in upland soils does the activity of aerobic methanotrophs account for a net uptake of atmospheric methane. The conversion of native lands to row-crop agriculture diminishes the strength of the soil methane sink, typically dropping the rate of methane consumption by 70%.

To determine the relationship between the rates of methane consumption in soils and the diversity of microbes that catalyze them, we conducted molecular surveys of methanotroph communities across a range of land uses at The Kellogg Biological Station Long Term Ecological Research Site (KBS LTER) and correlated our findings to measurements of the *in situ* fluxes of methane. Rates of methane consumption and methanotroph diversity were positively correlated, as conversion of lands to row-crop agriculture led to a 7-fold reduction from maximal rates of consumption in the native deciduous forests. In fields abandoned from agriculture both methanotroph richness and the consumption of methane were estimated to require approximately 75 years to return to the present diversity and consumption rate of the native deciduous forests. The linear trajectory for recovery of both measures suggested that managing lands to conserve or restore methanotroph diversity would yield increases in the rate of consumption of atmospheric methane in KBS LTER soils.

Long-term fertilization is one aspect of row-crop agriculture that is likely to be a significant disturbance to the methanotroph community. We hypothesized that a consequence of this disturbance would be its association with decreases in methane consumption and methanotroph richness in fertilized forest sub-plots at KBS LTER, but neither rate nor methanotroph richness declined due to long-term fertilization alone at KBS LTER. A meta-analysis examined the effect of long-term fertilization in other sites, and revealed no consistent decline in methanotroph richness in fertilized soils. The methanotroph communities did display a distinct biogeography with communities clustering together based on geographic location. As a consequence, the composition of the unique soil methanotroph community probably plays a role in dictating the response of the methanotroph community to changing land use and its disturbances.

The causes behind the change in methanotroph richness and the correlated decrease in methane consumption associated with row-crop agriculture at KBS LTER remains unclear as it is not caused by fertilization alone. The quantification of the effect of other variables associated with agricultural management is necessary to determine which management strategies at KBS LTER could be utilized to enhance methanotroph richness. However, a management strategy determined at KBS LTER to be beneficial to the methanotroph community may not be applicable to other paired sites, as the unique methanotroph community and environmental characteristics from each geographic location will probably yield a dissimilar response to the management practice.

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Uncertainties in the Global Methane Budget and Biological Influences on the Concentration of Atmospheric Methane

Introduction

To better understand the soil methane sink and its capacity for atmospheric methane consumption, the studies in this thesis attempt to link variation in methane flux associated with different land uses to changes in the richness and composition of communities of methane consuming bacteria (methanotrophs). We then investigate the effect of long-term fertilization, one of the factors associated with the land use changes, to determine if it is associated with methanotroph community changes. As an introduction to these studies, this chapter reviews the role of the soil methane sink in the global methane budget, the methane consuming bacteria that are responsible for the methane sink, the microbial pathway and enzyme that facilitates methane oxidation, the effects of changing land use on the methane sink, and an overview of the following chapters.

The Concentration of Atmospheric Methane

Methane (CH_4) is a potent greenhouse gas whose atmospheric concentration, as of 2005, is 1774 ppb (1). Methane contributes approximately 15% to the atmosphere's total radiative forcing (1), and is 21-25 times more efficient at trapping heat (infrared radiation) than carbon dioxide due to a longer lifetime in the atmosphere and greater radiative efficiency (2). The present concentration of atmospheric methane represents its highest concentration in at least the past 650,000 years (1). Since pre-industrial times (ca. 1900), the concentration has increased 250% largely due to human agricultural

practices and fossil fuel use (1). Following almost a decade with little change in atmospheric methane concentrations, as of 2007, there are renewed increases measured at all worldwide monitoring stations (3).

The balance between methane emissions and the strength of the largest methane sink, photochemical oxidation by hydroxyl radicals in the troposphere, primarily determines the concentration of atmospheric methane (4). Therefore, it is thought that the current increase in methane is either due to increases in wetland emissions in Siberia, or a weakening of the hydroxyl radical sink (3). However, ambiguity in our knowledge of multiple facets of the global methane budget has resulted in our inability to definitively identify the cause of either the current increase or the decade of little change in the concentration of atmospheric methane (3). Many other methane sources apart from wetlands, the largest source of emissions, could be changing the magnitude of their flux, and the strength of other methane sinks could also be changing.

Sources of Atmospheric Methane

There are numerous environments that are methane sources (yield a net production of methane to the atmosphere). Many anaerobic environments produce methane due to the activity of methanogenic archaea, and their activity accounts for more than 70% of methane emissions. These environments include wetlands, landfills, oceans, domestic and wild ruminant animals, poorly drained soil, and termites (5). Other sources of methane include methane hydrates, wildfires, fossil fuel mining and use, and biomass burning (5). Despite the identification of so many methane sources, the existence and magnitude of emissions from additional methane sources is currently debated.

For example, plants producing methane under aerobic conditions was reported by Keppler et al. (6), and originally estimated to be contributing between ~10-40% of annual methane emissions. Critiques of their methods and data from ice cores revised their estimates downwards to 0-10% of total annual emissions (7, 8). Attempts to replicate the initial observation have been inconsistent, with some studies confirming the initial observation of methane emission from plants (9-12), while others have been unable to corroborate those findings (13-15). Some studies have reported that the observed methane is created through UV degradation of plant pectin (9, 11, 12), and if shown to be the causal mechanism it would also explain the inability of some studies to replicate the initial findings as UV light was not included in experimental conditions (13, 14). However, the level of UV light exposure that causes methane emissions due to pectin degradation far exceeded the amount of natural UV light exposure (11), and normal UV light conditions would not yield large methane emissions from plants (13). Therefore, the contribution of plants to methane emissions remains controversial.

Another possible contributor to methane emissions that is not typically accounted for in current methane budgets is methane emissions originating from geological sources. The methane is produced from microbial and thermogenic processes in Earth's crust and subsequently released into the atmosphere through faults, seepage, and other mechanisms. Current measurements have considerable error associated with them, but they are estimated to possibly contribute 6% of total annual emissions (5). Thus, two potentially significant sources of methane emissions remain controversial, and the magnitude of their flux and their contributions to levels of atmospheric methane remain unconstrained.

Methane Sinks

The number of methane sinks (environments that yield a net uptake of methane from the atmosphere) is far fewer than the number of methane sources, but there is similar uncertainty in the quantification of their impact on the concentration of atmospheric methane. Approximately 90% of the methane consumed by methane sinks is due to the photochemical oxidation by hydroxyl radicals in the troposphere. The other methane sinks, stratospheric loss and uptake by well-drained soils, split the remaining 10% of methane sink consumption (5).

The hydroxyl radicals in the troposphere are very short lived, and producing accurate measurements of the strength of the sink and its variability remains challenging despite recent methodological improvements (4). Consequently, accurate modeling of the troposphere sink and the ability to attribute changes in the concentration of atmospheric methane as an effect of changes to the troposphere sink remains difficult (3). In addition, factors causing variations to the hydroxyl radical sink are unknown. Due to the strength of the troposphere sink, modifications to it will have large implications for the concentration of atmospheric methane. Like other methane sources and sinks, future investigations will have to clarify the role of the troposphere sink in determining the concentration of atmospheric methane.

Compared to the troposphere sink, the atmospheric methane consumed by well-drained soils is small: 30 ± 15 Tg of methane, roughly 3-9% of the total amount of methane removed from the atmosphere (16). However, well-drained soils are the only biological sink of methane (5). The uptake of methane in well-drained soils is due to the direct consumption by aerobic methane-oxidizing bacteria (methanotrophs) in soils

(Reviewed by (17)). Methanotrophs are ubiquitous, and will consume a significant portion of the methane produced in most of the environments that are methane sources (i.e. (18)), but only in well-drained soils does their activity yield an environment with a net uptake of methane (see comment above). In a reaction mediated by their methane monooxygenase enzyme (MMO), methanotrophs consume methane via an initial oxidation with oxygen to produce methanol with the following stoichiometry:

$$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3\text{-}OH + H_2O + NAD^+$$

After several chemical conversions (Figure 1.1), the carbon from methane is either incorporated into biomass or respired as CO_2.

Aerobic Methanotrophs

Aerobic methanotrophs are phylogenetically distinct bacteria, and are found within the *Proteobacteria* and *Verrucomicrobia* phyla. *Verrucomicrobia* methanotrophs have only recently been described, having been isolated from extremely acidic aquatic environments (19-21). Their optimum methanotrophic activities are typically in the range of pH 2.0-2.5 (21), are capable of oxidation down to pH 0.8-1 (20), and do not contain the intracellular membranes typical of *Proteobacteria* methanotrophs (19). Although one strain has had its genome sequenced (22), our knowledge of these methanotrophs remains limited. Thus far, *Verrucomicrobia* methanotrophs have only been discovered from aquatic environments, and *Verrucomicrobia* methanotrophs have not been found in soils.

Isolates of *Proteobacteria* methanotrophs have been well studied in culture, but the importance of cultured isolates to the consumption of atmospheric methane and the soil methane sink is limited (see discussion below). *Proteobacteria* methanotrophs are

found in the gamma-*Proteobacteria* and alpha-*Proteobacteria* subdivisions. Type I methanotrophs are gamma-*Proteobacteria*, and include the genera *Methylomonas, Methylobacter, Methylococcus*, and *Methylomicrobium*. Type II methanotrophs are alpha-*Proteobacteria*, and include the genera *Methylosinus* and *Methylocystis*. In addition to their subgroup classifications, Type I and Type II *Proteobacteria* methanotrophs can be distinguished on the basis of the following traits: dominant phospholipid fatty acids, serine verses RuMP carbon assimilation pathways, cellular morphology, ability to fix nitrogen, G+C content of DNA, and the ability to form resting stages (reviewed in (23)). Notably, some authors define *Methylococcus capsulatus* (Bath) and similar strains as Type X methanotrophs due to the presence of both serine and RuMP carbon assimilation pathways, and possession of traits similar to Type II methanotrophs.

Other Methane Oxidizing Microbial Communities

Anaerobic methane oxidation has also been observed, and like aerobic methane-oxidation it can attenuate a significant portion of the methane emitted from methane sources (24). Details of metabolism are still being unraveled, but the process is attributed to methanotrophic archaea (ANME) who are most likely performing reverse methanogenesis within a microbial consortia (reviewed by (24)). ANME have typically been found in close association with sulfate-reducing bacteria (25), but other bacteria also appear to be involved in the microbial consortia (26). Denitrifying bacteria anaerobically oxidizing methane without ANME has also been reported (27), further highlighting the possibility that other electron acceptors could be involved (24). ANME have thus far only been found in environments that are net methane sources and due to

their requirement for anoxia, it is unlikely that they contribute to the consumption atmospheric methane in well-drained soils.

Ammmonia-oxidizing bacteria have also been thought to potentially contribute to the strength of the soil methane sink as MMO is evolutionarily related to ammonium monooxygenase (AMO) (28), and both enzymes are capable of oxidizing a wide range of substrates that includes the other enzyme's substrate (reviewed in (23)). However, while AMO is capable of oxidizing methane, its K_m for methane is much higher than MMO's K_m, such that about a thousand ammonia-oxidizers are required to achieve the same rate of CH_4 oxidation as a single methanotroph (29). No evidence has been found for ammonia oxidizers playing a significant role in methane oxidation under low or high methane levels in either microcosms or the environment (29-31). Therefore, while it is possible for ammonia-oxidizers oxidize to methane, their *in situ* contribution to atmospheric methane consumption is, at best, minimal. As a result, we can assume that the strength of the soil methane sink is mostly, if not entirely, due to the activity of aerobic methanotrophs.

Methane Monooxygenase

Methane monooxygenase (MMO) is the key enzyme in the oxidation of methane, and is found in both soluble and particulate forms. In the methanotrophs that produce both forms, soluble MMO (sMMO) is expressed under low copper conditions, but there is sufficient copper available in nearly all environments that it is rarely expressed in the environment. Conversely, the particulate MMO (pMMO) is found in all known methanotrophs (32) except two *Methylocella* strains (33). Due to the near ubiquity of the particulate MMO in methanotrophs, the gene encoding the A subunit of pMMO, *pmoA*, is

typically used for non-culture based assessments of the methanotroph community in the environment (17). Methanotrophs can be also be identified through their *pmoA* gene due to its phylogeny being congruous with their 16S ribosomal genes (17). 16S ribosomal genes can also be used for the identification of environmental methanotrophs, but many of the primer pairs will capture methylotrophs in addition to methanotrophs (reviewed by (17)). In addition, use of *pmoA* is advantageous for methanotroph detection instead of ribosomal genes because unlike 16S sequences, the inference of methanotrophy is not constrained by our phylogenetic knowledge of cultured methanotrophs. For instance, if a novel *pmoA* is found it can be assumed to be a novel methanotroph, but if a novel 16S gene is discovered, the assumption of methanotrophy is uncertain.

pMMO is made up of three subunits encoded by the genes, *pmoC*, *pmoA* and *pmoB*, which are found consecutively in an operon under the control of a σ^{70} promoter (34). There are typically two nearly identical copies of the *pmoCAB* operon in the genome (34, 35). pMMO is an integral membrane protein, and as a result it has proven difficult to study as purified protein preparations are unstable with low specific activities (32, 36). Therefore, our knowledge of pMMO's biochemistry remains limited and controversial. The pMMO active site remains unknown despite solved protein structures (37, 38), and there are numerous hypotheses as per the location and nature of the active site. The active site could potentially lie in any of the three subunits that make up pMMO (subunits A, B and C), and data supports the active site metal center as either, or some combination of: diiron (36), mononuclear (37), dinuclear (37, 38) or trinuclear copper metal centers (39, 40). Further hampering our understanding of pMMO is the continued

inability to correlate the reaction of substrates or products with any of the hypothesized active sites (reviewed by (41)).

Methanotrophs Responsible for Consumption of Atmospheric Methane

Regardless of its biochemistry or the role it plays in catalyzing the oxidation of methane, the *pmoA* gene remains an effective method of characterizing and identifying the methanotroph community. Surveys of *pmoA* in well-drained soils have rarely found the well-studied cultivable *Proteobacteria* methanotrophs detailed above (reviewed in (17)). These observations were not completely unexpected, as the inability of cultured methanotrophs to grow on atmospheric methane had led to the hypothesis that uncultured "high affinity" methanotrophs are responsible for the strength of the soil methane sink (42). Most of the cultured methanotrophs have a half-saturation constant (K_m) that is too high to allow growth on atmospheric methane. Only three *Methylocystis* strains (sp. DWT, sp. LR1 and sp. SC2) have been reported to approach the required atmospheric methane oxidation kinetics, and to be capable of prolonged survival at atmospheric methane concentrations (43-45). For at least *Methylocystis* sp. SC2, and in all probability the other strains, their ability to use low methane concentrations is due to a pMMO isozyme (43). *Methylocystis* sp. SC2 has two *pmoCAB* operons, each encoding a different pMMO, and Baani and Liesack (43) demonstrate that survival at low methane concentrations (10-100 ppm) is due to one isozyme, while growth at high methane concentrations (>600 ppm) is due to the other isozyme.

However, while the strains have been reported to survive for up to three months under low methane concentrations, no growth has been observed when they have been incubated under atmospheric methane concentrations (1774 ppb; 1.74 ppm) (29, 43-46).

It is possible that *Methylocystis* strains are contributing to the consumption of atmospheric methane in well-drained soils by surviving via atmospheric methane oxidation for maintenance energy in between exposures to higher concentrations of methane that allow for its growth. West and Schmidt (47) found support for such a possibility, as they were able to stimulate methanogenesis after exposing a well-drained arctic tundra soil to anaerobic conditions. Upon the soil's subsequent return to aerobic conditions atmospheric methane was consumed at a faster rate; indicating that the exposure of methanotrophs to greater methane concentrations is advantageous to the methanotrophic community and the soil methane sink. Thus, so long as the *Methylocystis* strains are occasionally exposed to greater levels of methane produced by methanogens stimulated via anaerobic conditions from anoxic soil microsites or occasional soil flooding, they will be able to persist for a prolonged time on atmospheric methane and contribute to the strength of the soil methane sink.

While these findings indicate that at least some cultured methanotrophs are contributing to the oxidation of atmospheric methane they are not likely to be the methanotrophs responsible for the majority of atmospheric methane oxidation. Instead, methanotrophs that have not yet been cultured are hypothesized to likely be responsible. Multiple studies looking at soils from throughout the world using culture independent molecular surveys have found as yet uncultured novel numerically dominant and phylogenetically distinct *pmoA* in well-drained soils (29, 46, 48-61). These *pmoA* sequences form distinct phylogenetic clusters apart from the cultured gamma- and alpha-*Proteobacteria* and *Verrucomicrobia* methanotrophs (Figure 1.2), and a selection of their names are: JR1, JR2, JR3, MR1, Cluster I, Cluster II, Cluster III, Cluster V, Upland Soil

Cluster-γ (USC-γ or WB5F-H), Upland Soil Cluster-α (USC-α or RA14), and RA21 (44, 46, 48-50, 55, 57).

As the sequences from these clusters are numerically dominant and commonly found in well-drained soils throughout the world, it is plausible to conclude that they are the methanotrophs responsible for the majority of biological atmospheric methane oxidation. However, it has not yet been demonstrated that any of these clusters can grow on atmospheric methane. Kolb et al. (29) found *in situ pmoA* expression for USC-α in a German forest soils, but could not detect Cluster I *in situ pmoA* expression. Cluster I is the only one of the clusters that has cultured representatives, but the strains have only been minimally characterized (55). None of the exact methane oxidizing capabilities (K_m, V_{max}, etc.) of any of the above clusters is known, and therefore a determination of the magnitude of their contribution to atmospheric methane oxidation awaits further supporting data.

Linking Methane Consumption to the Methanotroph Community

Although a full understanding of atmospheric methane oxidation will require the culturing of many uncultured methanotrophs, our ability to measure methanotroph diversity through the retrieval of the *pmoA* gene allows for the linking of methanotroph diversity and community structure to rates of methane consumption. An example of a model system where the link between the methanotroph community and methane consumption can be tested is in well-drained soils of the same soil type that differ in their land use.

The soil methane sink is significantly affected by land use with conversion of soils to agricultural use, based on worldwide measurements, leading to an approximately

70% reduction in net methane consumption, and an approximately 100 year recovery to the methane consumption rates of the native land use (reviewed by (62)). Land use conversion to agricultural land-use introduces many environmental changes, and studies examining total methane flux have found that fertilization, tillage, pesticide and herbicide application, changes in water filled pores space, dry bulk density, and pH can attenuate methane consumption in agricultural soils (reviewed in (63, 64)). In particular, fertilization is an acute disturbance to methanotrophs. Ammonia is a known inhibitor of MMO (63, 65, 66), and long-term application of fertilizer negatively impacts the methanotroph community (30, 54).

However, paired sites that differ in land use share many environmental characteristics like climate and soil type that better facilitates our ability to identify factors that are impacting the methanotroph community and rates of methane consumption due to conversion to agricultural soils. An example of one such paired site, and the one used to investigate the methanotroph community in this thesis is the Kellogg Biological Station Long Term Ecological Research Site (KBS LTER; http://lter.kbs.msu.edu).

KBS LTER features a range of different agricultural treatments as well as an entire successional gradient (early, mid and late successional soils). There are at least three replicates of each land use, and for many of the treatments *in situ* measures of methane consumption have been regularly made between March-December since 1992. Like other paired sites, the magnitude of the soil methane sink at KBS LTER is affected by land use. Rates of methane oxidation increase along a successional gradient: conventional row-crop agriculture soils consume the least amount of methane, mid-

successional fields have intermediate rates of methane consumption, and late successional forests have the highest rates. On a yearly average, the late successional forest soils consume 9.17 g CH_4-C ha^{-1} day^{-1}, roughly 6 times more methane than the conventional row-crop agricultural soils (1.62 g CH_4-C ha^{-1} day^{-1}) (67).

The robust gas measures, the large rate differences between the soils of the successional gradient, and replicated plots make KBS LTER an ideal choice for exploring the link between methanotroph diversity and community structure to rates of methane consumption. In addition, KBS LTER features sub-plots in the late successional forest that allow for the determination of how one factor associated with row-crop agricultural management, long-term fertilization, affects methane consumption and the methanotroph community.

Previous studies have not tried to correlate methanotroph richness to rates of methane consumption as methane consumption rates have been well examined in soils of varying land use (reviewed by (62)), but studies of the methanotroph community in comparable sites have typically only taken a limited number of rates measurements (46, 53, 56, 59, 60). The results from these studies have been inconclusive with no clear relationship observed between methanotroph richness and methane consumption (46, 53, 68). Methanotroph community changes have been observed across other successional gradients, with patterns of methanotroph diversity changing along the successional gradients of reforested cornfields (46) and reclaimed pasture lands (68). KBS LTER features more land use types in its successional gradient then either of those study sites and coupled with robust gas measurements can better resolve patterns between the

methanotroph community, rates of methane consumption, and land use then any previous study.

Summary

In order to fully understand the causes of the increase in atmospheric methane, there are many areas of the global methane budget that require greater understanding. One such area is the soil methane sink, the only biological methane sink, where the activity of aerobic methanotrophs results in the net consumption of atmospheric methane. The methanotrophs that are probably responsible for the consumption of atmospheric methane have not yet been cultured, and a range of non-cultured methanotrophs have been revealed in culture-independent molecular surveys. Land use changes, particularly the conversion of native lands to row-crop agriculture, result in a dramatic drop in the rate of methane consumption, but changes in land use have not been linked to changes in methanotroph community diversity and structure. Paired sites of differing land use and changed rates of methane consumption, like those featured at KBS LTER, represent ideal model systems for linking methanotroph diversity to rates of methane consumption.

Thesis Overview

The main goals of this thesis is to (1) determine how the methanotroph community changes along with rates of methane consumption, (2) to begin to investigate factors that may be causing the changes, and (3) to determine if our findings at KBS LTER are applicable to other sites. By doing so, insights can be gained into how lands might be managed to enhance the methanotroph community, and in turn, the capacity of soil methane sink.

Therefore, the main overall questions of this thesis were:

(a) Do soils of various land uses at KBS LTER, with different rates of methane consumption, harbor different methanotroph communities?

(b) Can the differences in the methanotroph community be directly related to changes in the rate of methane consumption?

(c) At KBS LTER is a decline in methanotroph richness associated with long-term fertilization alone?

(d) Are there typical changes to methanotroph communities in response to long-term fertilization?

To answer questions (a) and (b), chapter 2 presents the findings of assessments of the methanotroph community from the successional gradient at KBS LTER. We find that the conversion of native lands to row-crop agriculture causes the loss of methanotroph richness, and a correlated decrease in the rate of methane consumption. We also find that land uses harbor different microbial communities that appear to change in parallel to differences in the plant community.

To determine the importance of methanotroph diversity to rates we find that the recovery of both methanotroph richness and methane consumption is linear, concurrent and will take approximately 75 years following abandonment from agriculture. The linear trajectory and lack of a step-wise increase in methane consumption rates following any increase in methanotroph diversity suggests that every methanotroph taxon, and not just a few taxons, are contributing to the rate of methane consumption at the KBS LTER. Further supporting the finding of complementarity within the methanotroph community is the comparison of correlations of methane consumption with methanotroph richness, carbon dioxide efflux with total bacterial richness, and of both gas fluxes with moisture

and temperature. We find that carbon dioxide efflux, a process that is the result of a highly redundant microbial community does not correlate with total bacterial richness, and that more of the carbon dioxide flux can be explained by moisture and temperature.

To answer questions (c) and (d), in chapter 3 I present the findings from assessments of the methanotroph community from KBS LTER late successional forest fertilized sub-plots, from agricultural and forest soils from the Rothamsted Research site, and from previously published studies of methanotroph communities. We find that long term fertilization alone is not causing a decrease in methanotroph richness nor in rates of methane consumption at KBS LTER. This result is consistent with our findings in Chapter 2 that linked methanotroph richness and rates of methane consumption, and led to the expectation that the response of richness and rates of methane consumption to long-term fertilization would be the same. No consistent decline in methanotroph richness is observed in other long term fertilized soils, and methanotroph communities display a distinct biogeography with communities clustering together based on geographic location. There is no pattern of typical methanotroph community changes in accordance with long-term fertilization.

Based on these findings we conclude that the each methanotroph taxon at KBS LTER provides a similar contribution to rate of methane consumption, and with every additional methanotroph in the soil there is an increase in the rates of consumption. The causes behind the change in richness remain unclear, as they are not caused by long-term fertilization alone, and other agricultural land-use associated changes are implicated as helping to cause the decrease in methanotroph richness in the Ag HT treatment at KBS LTER. The results also indicate that the findings at KBS LTER may not be directly

applicable to other soils possibly due to each site's unique methanotroph community and soil properties. Further experiments that would clarify the effects of land use on the methanotroph community are discussed in Chapter 4.

Three appendices are also provided. One details the PCR bias associated with the reaction conditions used in this study, and how the finding led to exclusion of abundance measures in the methanotroph community analyses. The second appendix presents NH_4^+ and NO_3^- nutrient data from a fertilized late successional forest sub-plot at KBS LTER before and after fertilization. The sub-plot's methanotroph community was not assessed, but the findings reinforce the expectation that the methanotrophs in the fertilized sub-plots are exposed to ammonia following fertilization, and highlight the active nitrification in KBS LTER late successional forest soils. The third appendix confirms the finding of methanotroph biogeography in long-term fertilized soils by assessing the clustering of methanotroph community composition in a variety of well-drained soils.

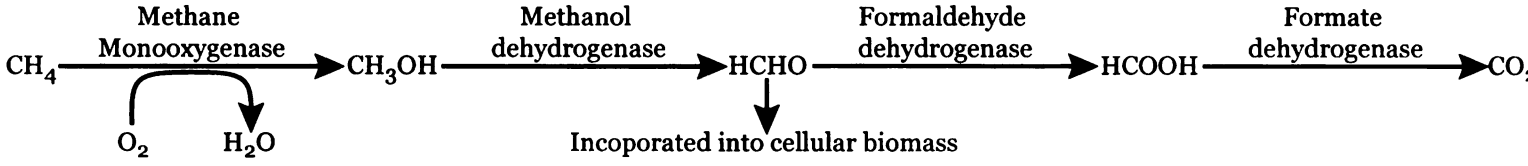

Figure 1.1. A simplified schematic of the metabolic pathway of methane oxidation. The key enzyme is the methane monooxygenase, which facilitates the oxidation of methane into methanol.

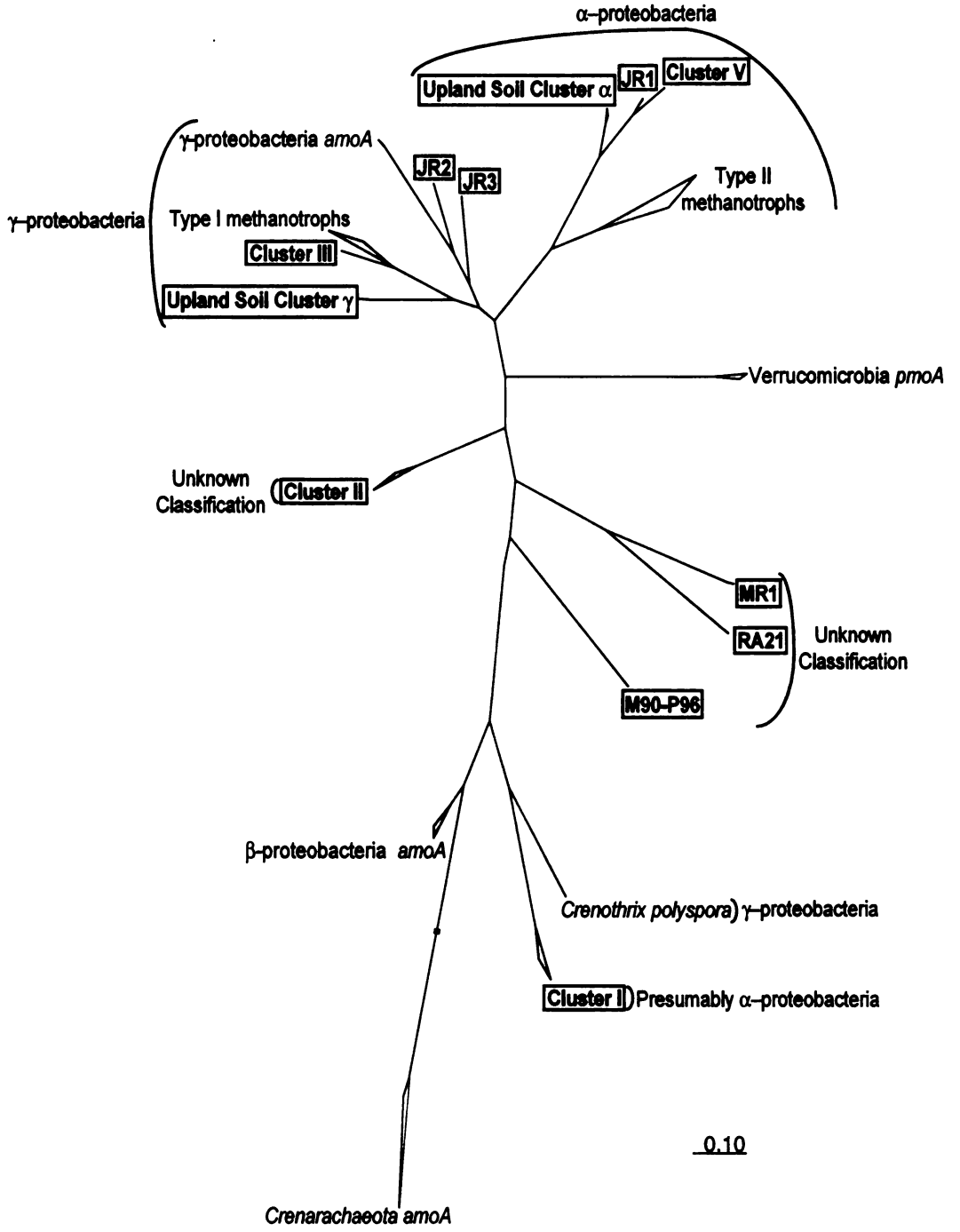

Figure 1.2. Phylogenetic tree of selected partial pmoA and amoA protein sequences from public databases. The tree is based on 164 amino acid positions using Phylip Protein Maximum Likelihood as implemented in ARB (Ludwig et al. 2004). Boxed labels are indicative of pmoA clades that have been found in soils, have not been cultured, and are thought to likely play a substantial role in the oxidation of atmospheric methane. The scale bar represents 10 PAM units.

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Chapter 2

Agriculture's Impact on Microbial Diversity and the Flux of Greenhouse Gases

Abstract

Row-crop agriculture impacts both the production of carbon dioxide and the consumption of methane by microbial communities in upland soils - Earth's largest biological sink for atmospheric methane. To determine if there are relationships between the rates of these ecosystem level processes and the diversity of microbes that catalyze them, we measured the in situ fluxes of methane and carbon dioxide, and conducted molecular surveys of methane–oxidizing bacteria (methanotrophs) and total bacterial diversity across a range of land uses. Rates of methane consumption and methanotroph diversity were positively correlated, as conversion of lands to row-crop agriculture led to a 7-fold reduction from maximal values found in native deciduous forests. In fields abandoned from agriculture the diversity of methanotrophs and the consumption of methane increased monotonically, suggesting that managing lands to conserve or restore methanotroph diversity could help mitigate increasing atmospheric concentrations of this potent greenhouse gas. In addition, methanotroph diversity was more capable at explaining methane consumption than was moisture and temperature. Conversely, total bacterial diversity did not correlate with changes in carbon dioxide emission, but did correlate to moisture and temperature. Taken together, these results are consistent with the prediction that ecosystem processes are more likely to be influenced by microbial diversity when microbial communities have limited species richness.

Introduction

Carbon dioxide (CO_2) and methane (CH_4) are responsible for approximately 80% of the positive radiative forcing of the atmosphere from long-lived greenhouse gases (approximately 62% and 18%, respectively) (1). The atmospheric concentrations of both gases now exceed any of their respective levels over the past 650,000 years, and continue to rise (2, 3). Fluxes of both gases are affected by land-use changes – especially deforestation and row-crop agriculture (4, 5). Conversion of native upland soils (e.g. forest, grassland) to agricultural management reduces their capacity for methane consumption by an average of 71%, and recovery of methane consumption is estimated to be ca. 100 years following the cessation of agriculture (reviewed in (5)). Deforestation and agricultural development increases the efflux of carbon dioxide from soil (4, 6), and is estimated to have contributed approximately 25% of the increase in radiative forcing from carbon dioxide since 1850 (1).

Microbes directly control methane consumption, and portions of soil carbon dioxide efflux (7, 8). Aerobic methane-oxidizing bacteria (methanotrophs) consume methane in well-drained soils, and the consequential net uptake of methane constitutes Earth's largest known biological sink for atmospheric methane (9, 10). Soil respiration is a large biological source of atmospheric carbon dioxide, and is mainly a reflection of microbial respiration (reviewed in (11)) plus respiration from roots. The relative contribution of root and heterotrophic microbial respiration has proven difficult to disentangle (12). Nevertheless, changes in soil respiration have been associated with variations in the microbial community (8, 13).

How row-crop agriculture changes the methanotroph and the heterotrophic microbial community and their associated rates of methane consumption and carbon dioxide efflux remains unclear. The Kellogg Biological Station Long Term Ecological Research Site (KBS LTER), with replicated plots of the same soil series (14) that just differ in land use (Table 2.1), represents an ideal system for determining how row-crop agriculture impacts microbial communities and the greenhouse gas fluxes they control. Past studies have typically investigated either gas flux or the microbial community at analogous sites, but rarely has the function mediated by the microbes and the microbial community been investigated simultaneously in agricultural soils or across a land-use gradient. We correlated *in situ* fluxes of carbon dioxide and methane to assessments of microbial diversity across a successional gradient featuring 5 different land uses at KBS LTER (Table 2.1).

Methane consumption rates have been well examined in soils of varying land use (reviewed by (5)), but studies of the methanotroph community in comparable sites have either not measured rates of methane consumption (15), taken a maximum of two rate measurements (16-19), not featured replicated sites (16, 18, 19), and have only examined a limited number of successional stages (15-19). No clear relationship has been observed between methanotroph richness and methane consumption in any study (18-20), and, to our knowledge, ours is the first study that has attempted to correlate methanotroph richness to rates of methane consumption. Methanotroph community changes have been observed across other land-use gradients, with patterns of methanotroph diversity changing along a gradient of grazing intensities (20), and along a successional gradient of reforested cornfields (18). KBS LTER features more land use types in its successional

gradient then any previous study, and coupled with robust gas measurements can better resolve patterns between the methanotroph community, rates of methane consumption, and land use.

Similarly, few studies have attempted to determine the effects of row-crop agriculture on both soil respiration and the heterotrophic microbial community. Studies have documented land-use associated changes to the microbial community due to the conversion of forest to pasture (20, 21), but rates of soil respiration were not measured. Others have linked changes in soil respiration to changes in the microbial community (8, 13, 22), but agricultural soils were not included in any of those studies.

We hypothesize that microbial diversity is more likely to be important to a specialized metabolic process like methane oxidation than it is to be a factor in a metabolically redundant process like carbon dioxide production (23-25). Specialized metabolic processes like methane consumption are typically the result of relatively taxonomically narrow microbes, and a limited number of species contribute to the ecosystem process, resulting in a lack of functional redundancy. On the other hand, soil respiration is a process that is the result of a multitude of taxonomically diverse microbes and enzymatic processes, and the number of microbes found to be contributing to carbon dioxide production is likely to be high. Therefore, we expect that methanotroph richness will correlate to rates of methane consumption, but total bacterial richness will not correlate to carbon dioxide efflux.

In addition, by measuring methanotroph diversity and methane consumption in fields that had been abandoned from agriculture for various lengths of time, we should to be able to infer if the postulated positive relationship between net methane consumption

and methanotroph diversity is due complementarity or selection. The complementarity and selection hypotheses are actively debated to explain positive relationships between the magnitude of an ecosystem process and species richness (26, 27). The complementarity hypothesis gives importance to every species in determining the magnitude of the ecosystem process, and postulates that increased ecosystem function results from the combined activity of species in complementary niches. The selection or sampling effect hypothesis offers an alternative explanation: the positive relationship between diversity and function is driven by one or a few dominant species particularly proficient at performing the process under study. Discerning between the two hypothesis is important because if the postulated positive relationship between net methane consumption and methanotroph diversity is observed, it will be useful to identifying whether all methanotroph diversity, or only select species need to be conserved or restored in order to possibly enhance the methanotroph community, and in turn, the capacity of the soil methane sink.

Materials and Methods

Site Description

This study was conducted at the Kellogg Biological Station Long Term Ecological Research Site (KBS LTER; http://lter.kbs.msu.edu) located in Hickory Corners, Michigan. Soils of five treatments were examined in this study: conventional agricultural management of historically tilled land (Ag HT), early successional plant communities on fields that had been abandoned from agriculture in 1989 (Early HT), mid-successional plant communities on either historically tilled land (Mid HT) or never

tilled grassland (Mid NT), and a never tilled late successional deciduous forest (Late DF). Additional site descriptions can be found in Table 2.1. All of the KBS LTER soils are located within 3 km of the main experimental site, are all of the Kalamazoo/Oshtemo soil series, and are well-drained Typic Hapludalfs (fine or course loamy, mixed).

Rate Measurements

In situ rates of methane consumption were measured using closed-cover flux chambers (28). Rate measurements were typically made twice monthly between March and December at 3 or 4 replicate plots between 1992 and 2007 for Ag HT, Early HT and Late DF, and between 1992 and 1997 for Mid NT and Mid HT. Mid HT rate measurements were also made in 2002. Individual rate measurements and detailed methods are available at http://lter.kbs.msu.edu. To determine rate differences based on treatments, analysis of variance (ANOVA) was performed using PROC MIXED (SAS Inc, 2002).

Soil Sampling

Methanotroph diversity was assessed in 5 soil cores (2.5 x 10 cm) collected from 3 replicates along a gradient of 5 land uses (Table 2.1). These 75 samples, collected from a total of 15 experimental fields on 13 June 2006, as well as samples used for additional molecular surveys collected on 8 December 2004, 6 June 2005, and 13 June 2007 were transported to the laboratory on ice where they were mixed thoroughly and flash frozen in a dry ice/ethanol bath, then stored at -80°C until processing.

Total bacterial diversity was assessed in 5 soil cores (5 x 10 cm) from two Ag HT and two Late DF replicates in December 2006. Samples were pooled, transported to the laboratory on ice, sieved and stored at -80°C.

DNA Extraction and *pmoA* PCR Reactions

For the assessment of total bacterial diversity, DNA was extracted according to Zhou et al. (29), followed by a cesium-chloride gradient purification (30). For the assessment of methanotroph diversity, DNA was extracted from soil samples with the Mo Bio PowerSoil™ DNA Isolation Kit, following the manufacturer's protocol, except that mechanical cell lysis was performed by bead beating for 45 seconds.

All soil samples were screened for genes coding for the A subunit of particulate methane monooxygenase (*pmoA*) via PCR amplification with the primer sets A189 (5'-GGNGACTGGGACTTCTGG-3') -A682 (5'-GAASGCNGAGAAGAASGC-3') (31), and A189-mb661 (5'-CCGGMGCAACGTCYTTACC-3') (32) in order to encompass all known *pmoA* genes (33). No amplification was observed from A189-mb661.

Amplification reactions contained either 25, 45 or 90 ng of undiluted DNA, 1.25 µl 1% BSA, 10 mM dNTPs, 0.2 µM of each primer, 2.5 µl 10x PCR buffer (200mM Tris-HCl (pH 8.4) and 500 mM KCl), 0.5 µl 50 mM $MgCl_2$ and 1.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) in a total volume of 25 µl. Reaction conditions for the 62°C-56°C clone libraries and for tRFLP (see below) were 95°C for 5 minutes, 15 cycles of a 'touchdown PCR' of 95°C for 1 minute, 62°C for 1 minute (-0.4°C each cycle to 56°C), and 72°C for 1 minute, 15 cycles using 56°C as the annealing temperature, and a final 10 minute extension at 72°C. To ensure that no methanotroph diversity was missed, additional libraries were constructed under the same conditions, but with varying annealing temperatures: 60°C to 51°C (-0.6°C each cycle to 51°C), 48°C, and 51°C (Table 2.2).

Clone Libraries

Cloning was performed with the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) using either vector pCR 4 or pCR 2.1 as per the manufacturer's protocol. Transformants were screened via PCR reactions with the primers F2 (5'-CAGTCACGACGTTGTAAAACGACGGC-3') and R4 (5'-CAGGAAACAGCTATGACCATG-3') (34). One-1.25 μl of the PCR products were purified via incubation with 0.25 μl of ExoSAP-IT (Usb, Cleveland, OH) for 30 minutes at 37°C. Sequencing was completed at the Research Technology Support Facility at Michigan State University (RTSF).

Sequences identified by BLASTX (35) as *pmoA* or the A subunit of ammonia monoxygenase (*amoA*), which A189-A682 also amplify, were imported into Arb (36). In Arb, sequences were translated and aligned using Clustal W. Nucleic acid sequences were aligned according to the protein sequence. Sequences from clone libraries were determined to be the same species if they were $\geq$ 94% identical (37) as determined by DOTUR (average neighbor grouping) (38).

Soil cores collected in December 2004 and June 2005 were used to construct 62°C-56°C clone libraries from 5 cores from the 1st replicates of Ag HT and Late DF. For June 2005 samples, libraries represent sequences pooled from separate DNA extractions, PCR reactions and cloning reactions. These libraries also include clones whose identities were inferred from identical banding patterns in restriction fragment length polymorphism analyses using the AlwI enzyme (Neb, Ipswich, MA). The same June 2005 soil samples were also used to construct libraries from 48°C, 51°C, and 60°C-51°C annealing temperatures. For these annealing temperatures, separate DNA

extractions were pooled for 4 replicate PCR amplifications, which were subsequently pooled into 1 cloning reaction.

Libraries from June 2006 samples were constructed from duplicate 60°C-51°C PCR reactions from each of 5 soil cores from the 1st and 3rd Late DF and 1st and 2nd Ag HT replicates which were pooled according to replicate. Prior to cloning these PCR products, as well as those from June 2007, were digested with the restriction enzyme PflFI (Neb, Ipswich, MA), and gel extracted with the PrepEase kit (Usb, Cleveland, OH) to reduce the incidence of cloning *amoA* and non-specific PCR products.

Additional 62°C -56°C clone libraries were constructed from a collection of the June 2006 soil cores from various treatments where large unidentified peaks were observed in the tRFLP analysis (see below). The libraries were constructed in order to match more tRFLP peaks with known sequences, and therefore were not sampled until *pmoA* rarefaction curves were asymptotic.

Sequences representing each of the *pmoA* species found in this study are deposited in GenBank under accession numbers FJ529724 - FJ529808, and GQ219582-GQ219583.

Terminal Restriction Fragment Length Polymorphism (tRFLP) Analysis

For tRFLP (39) each of the 75 soil cores from June 2006 was amplified for *pmoA* with A682 labeled with fluorophore 6-carboxyfluorescein (6-Fam), and template addition was always 45 ng. To obtain enough DNA for tRFLP at least 4 replicate PCR reactions were pooled. PCR products were purified using a MinElute column (Qiagen, Valencia, CA). In a 50 µl reaction, 300ng of purified product was digested with 1.5 U TauI (Fermentas, Glen Burnie, MD) by incubating at 55°C for 1 hr 30 min. To inactivate the

enzyme the DNA was precipitated as follows: The sample was diluted to 500 µl, followed by the addition of 50 µl 3M sodium acetate, 1 µl or 2.5 µl 10 mg/ml glycogen, and 500 µl isopropanol, and holding on ice for at least 5 minutes. The DNA was then pelleted by centrifugation at 16,000 x g for either 5 (1µl glycogen) or 10 minutes (2.5 µl glycogen). The supernatant was decantated, and the pellet washed with 500µl of 80% ethanol followed by centrifugation for 2 minutes at 16,000 x g, and removal of the supernatant. After a 30 second centrifugation additional ethanol was removed, and the DNA was air dried for 5-10 minutes before resuspension in 20 µl of water. In an 18-22 µl reaction, either 140 ng (1µl glycogen) or 160 ng (2.5µl glycogen) of DNA was digested with 2.5 U SspI (Neb, Ipswich, MA) in a 1hr 30min incubation at 37°C. After heat inactivation at 65°C for 20 minutes 6 U of BstUI (Neb, Ipswich, MA) was added, incubated at 60°C for 1hr 30min, and inactivated by adding 0.8 µl of 0.5 mM EDTA. Capillary electrophoresis of the tRFLP reactions was then performed with a 5 fu cutoff at RTSF.

To ensure that the tRFLP profiles could be compared, the distribution of the total fluorescence was compared with PROC UNIVARIATE (SAS Inc, 2002), and any outliers were excluded. Individual peaks were distinguished from the background signal and binned using TRFLP-Stats (40). In TRFLP-Stats default settings were used except for the standard deviation cutoff, which was increased to 4.5. The resulting cutoff of approximately 25 fu excluded any profile whose highest peak area was not greater than 350, and ensured that peak areas below 300 were not identified as true peaks in greater than 90% of the samples. PCR products from clones representing 14 *pmoA* OTUs, 1 *amoA*, and 21 non-specific (neither *pmoA* or *amoA*) were run as tRFLP at least once.

These clone controls allowed the identification of specific tRFLP peaks as either *pmoA* OTUs, *amoA*, or non-specific PCR products, and allowed for the exclusion of *amoA* and non-specific PCR product bins. Those bins that were identified as either were excluded from the analysis. To obtain a better representation of an entire replicate, and to control for soil heterogeneity, bins from the same replicate were summed. For every replicate, tRFLPs from at least 3 soil cores were obtained and summed. In total, tRFLP profiles were obtained from 68 of the 75 soil cores.

At least two negative controls (no DNA PCR reactions) were included on each plate of tRFLP reactions, and any profile whose highest peak area was not greater than 300, the highest peak area in these controls, was excluded from the analysis. To assess the reproducibility of the method, twenty samples had technical replicates whose results were then pooled after analysis in TRFLP-Stats.

Each tRFLP bin then served as an Operational Taxonomic Unit (OTU), and the OTUs were used in a linear regression (PROC REG, SAS Inc, 2002) against the rate of methane consumption at KBS-LTER in summer (June-August), against the rate of methane consumption between March-August, and to calculate β-diversity with PAST (41) or Estimate S (http://viceroy.eeb.uconn.edu/EstimateS).

16S Tag Sequencing

16S tagged sequencing and analysis was performed on a 454 Life Science's pyrosequencer as described by Sogin et al. (42). Richness estimates were then regressed against mean carbon dioxide measures taken between 2005-2007 using simple linear regression (PROC REG, SAS Inc, 2002).

Multiple regression

To determine the ability of soil moisture and maximum air temperature to predict rates of methane consumption and carbon dioxide emission, we used multiple regression (PROC MIXED, SAS Inc, 2002) with data taken between 2005-2007 from Late DF and Ag HT. Moisture and soil temperature measurements were taken on the same day that gas flux was measured. Individual measurements are available at http://lter.kbs.msu.edu/datasets.

Results

Rate measurements

Maximal rates of methane consumption and carbon dioxide emission were observed during the summer months for all treatments (Figure 2.1). Carbon dioxide emissions peaked May through August, and significant treatment effects were found (Figure 2.2a). The conventional row-crop agricultural soil (Ag HT) had the lowest average rate of soil respiration, and the early (Early HT) and mid-successional sites (Mid NT and Mid HT) had maximal rates. On average, the early and mid-successional sites emit approximately 79% more, and Late DF emits 18% more, carbon dioxide than Ag HT per day.

Rates of methane consumption also changed according to treatment (Figure 2.2b) with the greatest consumption in Late DF followed by the mid-successional soils, Early HT, and the lowest rates in Ag HT (Figures 2.1 and 2.2b). The overall difference in rates between the highest rate in Late DF and the lowest rate in Ag HT is approximately 7-fold (9.98 g CH_4-C ha^{-1} day^{-1} and 1.29 g CH_4-C ha^{-1} day^{-1}, respectively), resulting in 7 times more methane being consumed per day in the late successional deciduous forest as

compared to the conventional row-crop agricultural soils. The difference between the Late DF and Ag HT treatments grew 2-fold with the inclusion of rates from 2000-2007 (28). The highest rates of methane consumption were observed June through September (Figure 2.1), the 7-fold difference in rates remained the same during those months, and coincided with when we obtained the majority of the soil samples used to assess the methanotroph community.

Methanotroph Diversity and Community Composition

***pmoA* Clone libraries**

Clone libraries of the *pmoA* gene, encoding the A-subunit of the particulate methane monooxygenase, the first enzyme in the pathway of methane oxidation and the defining enzyme of aerobic methanotrophs (43), were used to assess methanotroph diversity. Libraries from Late DF and Ag HT were constructed from various annealing temperatures, soil samples (Methods, Table 2.2), and until rarefaction curves were asymptotic (Figure 2.3) to ensure that all methanotroph diversity had been captured. The libraries constructed from the various annealing temperatures revealed that the 60°C-51°C annealing temperature yielded the most methanotroph species in an individual library (Table 2.2). Due to PCR bias, measures of *pmoA* abundance were excluded from comparisons of the methanotroph community (Appendix A).

Although the A-subunit from some ammonia monooxygenase genes (*amoA*) amplified with the *pmoA* primers (31), *amoA* sequences were distinguished based on diagnostic amino acids (44, 45), and their clustering in phylogenetic trees (Figure 2.4, Figure 2.5). Digestion of the PCR products with the restriction enzyme PflFI (Neb, Ipswich, MA) was found to greatly reduce the incidence of cloning *amoA*, and to have no

change in the overall methanotroph richness recovered from individual libraries (Table 2.2).

Phylogenetic analysis of *pmoA* genes revealed that methanotrophs in KBS LTER soils cluster within seven clades (Figure 2.4). Six *pmoA* clades - Cluster I, Cluster II, KBS1, JR1, MR1, and Upland Soil Cluster α - were found in Late DF, compared with just 2 clades, Cluster I and Cluster II, in Ag HT. Cluster RA21 was only recovered from Mid NT (Figures 2.4). Grouping the *pmoA* sequences from the KBS LTER clone libraries at a species level (94% average nucleotide similarity) revealed an even more dramatic difference in the methanotroph richness between the Ag HT and Late DF land-use treatments: 24 methanotroph species in Late DF, and only 7 species were present in Ag HT (Table 2.2, Figure 2.5). Of the 7 methanotroph species found in Ag HT, 2 were unique to the treatment while the other 5 species were also found in Late DF. In both Late DF and Ag HT, more methanotroph species were found in Cluster I then in any other cluster, with 12 Late DF and 6 Ag HT species. Next was Cluster II with 7 Late DF and 1 Ag HT species. The other clusters had no more than 2 methanotroph species. Cluster KBS1 was represented by 1 Late DF sequence from the libraries in this study, and additional sequences from the KBS1 clade are reported from a clone library constructed from a subplot of the 1st Late-DF replicate that was sampled on 13 June 2007 with 60°C-51°C as the annealing temperature (Chapter 3) to further confirm the presence of this unique cluster (Figure 2.5).

Terminal Restriction Fragment Length Polymorphism (tRFLP) Analysis

To survey methanotroph diversity across the entire gradient of KBS LTER land uses (Table 2.1), and in three replicates of each treatment, terminal Restriction Fragment

Length Polymorphism (tRFLP) of the *pmoA* gene was used with soil sampled during the peak period of methane consumption in 2006. The tRFLP assay distinguished 11 Operational Taxonomic Units (OTUs) that were confirmed as *pmoA* gene fragments through sequence analysis, and the requirement for comigration with terminal restriction fragments from cloned controls. Similar to the clone libraries, richness differences between treatments were observed. The Late DF sites averaged 7 OTUs compared to 2 OTUs in Ag HT, and intermediate numbers of *pmoA* OTUs were found in the successional sites (Figure 2.6).

In addition, following release from row-crop agriculture (Ag HT), the composition of the methanotroph communities in soils abandoned from agriculture (Early HT and Mid HT) became more like the native sites (Late DF and Mid NT) (Figures 2.8 and 2.9). These methanotroph communities are statistically different from one another according to a one-way analysis of similarities ($p < 0.005$, ANOSIM), with methanotrophs in the early successional soil beginning to diverge from the row-crop agricultural plots. Divergence continued in the Mid HT soils that have been abandoned from agricultural for ca. 50 yrs., such that these communities began to overlap in composition with communities of methanotrophs in native fields (Figures 2.8 and 2.9).

Correlation of methanotroph diversity and methane consumption

Simple linear regression revealed a strong positive correlation between summer (June-August) rates of methane consumption and *pmoA* OTUs ($r^2=0.62$, $p<0.001$) (Figure 2.6). If *pmoA* OTUs are regressed against rates of methane consumption from March-December a nearly identical relationship is found ($r^2=0.64$, $p<0.001$). The same strong positive correlation is also exhibited when, in addition to the *pmoA* OTUs, tRFLP OTUs

that could not be identified (Methods) are included and correlated with summer (r^2=0.43, p=0.008), and March-December methane consumption rates (r^2=0.48, p=0.004).

We tested whether the positive relationship between methane consumption and methanotroph diversity at the KBS LTER was more likely to be explained by either the complementarity or selection hypotheses by determining the trajectory of recovery of richness and consumption in successional soils (Early HT and Mid HT) after intensive row-crop agricultural management (Figure 2.7). There was a linear trajectory for the recovery of both methane consumption (r^2=0.69, $p < 0.001$) and methanotroph richness (r^2=0.99, $p = 0.020$) over time. Extrapolation of the trajectories reveals that if methane consumption and methanotroph diversity continue at the same rates since cessation of agriculture, both would return to the current level of their equilibrium community, the late successional deciduous forest, in approximately 75 years.

Total Bacterial Diversity

Estimated average total bacterial richness (Chao I) was determined from 454 16S tag sequencing from December 2006 soil samples, and found to be 11,105 in Ag HT, and 7,762 in Late DF. We assumed that every bacterium would contribute to carbon dioxide emissions, and preformed a linear regression with total bacterial richness and average March-December carbon dioxide efflux from 2005-2007. No relationship was found between the two measures (r^2=0.22, p=0.522) (Table 2.3). Despite the limited power in the linear regression due to the limited number of samples (n=4), using the same number of samples from comparable methanotroph richness measurements yields a dramatically different result (Table 2.3). Using *pmoA* clone libraries from Ag HT and Late DF June

2006 soil samples (Table 2.2), and average methane consumption measurements from the same 2005-2007 dates, we found a significant positive linear relationship between average March-December methane consumption measures and methanotroph diversity (r^2=0.96, p = 0.018).

Multiple Regression

Multiple regression with the Ag HT and Late DF gas fluxes from 2005-2007 against soil moisture and maximum air temperature from the same dates and replicates discerned the ability of these general microbial metabolic regulators to explain the observed fluxes. Moisture and temperature were found to be able to explain portions of both fluxes, but were 10 times more effective in explaining the efflux of carbon dioxide (r^2=0.37, p < 0.001) then the rates of methane consumption (r^2=0.03, p = 0.005) (Table 2.3).

Discussion

Microbial Diversity and the Flux of Greenhouse Gases

Correlating ecosystem process rates to their respective microbial diversity measures across the KBS LTER land-use gradient conformed to our hypothesis: methane consumption rose as methanotroph diversity increased (Figure 2.6, Table 2.3), and increases in carbon dioxide emission were not correlated to increases in bacterial richness (Table 2.3). The lowest flux of both greenhouse gases were found in Ag HT as both carbon dioxide and methane consumption fluxes were diminished by row-crop agriculture (Figures 2.1 and 2.2). The difference in the correlations was instead due to the effect of land use on microbial diversity: Row-crop agriculture was associated with a

dramatic decrease in methanotroph richness, but total bacterial richness was greater in Ag HT then it was in Late DF (Figure 2.6, Tables 2.2 and 2.3).

As postulated by our hypothesis, the ability of microbial diversity to explain the rate of the gas flux is likely the result of the functional redundancy of the microbial community mediating the ecosystem process. Thousands of bacterial species contribute to the efflux of carbon dioxide from soil – reducing the likelihood that diversity influences the process rate. There are comparatively few methanotroph species, which likely results in little or no functional redundancy, as methanotrophs probably occupy non-overlapping niches and every methanotroph species contributes to the rate of methane consumption.

Evidence for methanotrophs occupying separate niches at KBS LTER was gained by testing to see if the positive relationship between net methane consumption and methanotroph diversity could best be explained by either the complementarity or selection hypotheses. Ideally this determination would be able to be made with measures of species-specific methane oxidation rates and the reconstruction of defined communities, but the absence of cultured methanotrophs representative of the clades found in KBS LTER soils preclude such measures. As has been found in other soils, the methanotroph community consuming atmospheric methane at KBS-LTER is composed of uncultured methanotrophs. Our findings further indicate that these phylogenetic clusters are those that are largely responsible for the consumption of atmospheric methane (10, 19). No clones were found from the Type I or Type II methanotrophs that are well represented in culture (Figures 2.4 and 2.5). Rather, *pmoA* clones belonging to 6 clades that have been identified previously in other culture independent investigations of

upland soils (10, 18, 19, 45), and 1 new clade, identified as KBS1, were found in KBS LTER soils. Of the 7 clades that were recovered, only Cluster I methanotrophs, presumably ∝-proteobacteria, have been reported to be cultured, but they remain poorly characterized (46).

Despite the absence of cultured methanotrophs, we determined whether the complementarity or selection hypotheses could best explain the relationship between methane consumption and methanotroph richness by plotting the recovery of both measures in soils that had been abandoned from agriculture for various lengths of time. The trajectory of the recovery of both methane consumption and methanotroph richness over time is linear, with no indication of abrupt step-wise increases in methane consumption that would be expected to accompany the establishment of a particularly productive methanotroph species in accordance with the selection hypothesis (Figure 2.7). Instead, the concurrent and incremental recovery of methane oxidation and methanotroph diversity following the abandonment from row-crop agriculture is consistent with complementary roles of methanotroph species, and suggests that every methanotroph OTU is important to rates of methane consumption.

Additional support for complementarity in the methanotroph community at KBS LTER is found in the ability for general abiotic microbial metabolic regulators, moisture and temperature, to explain 10 times more of the variation in CO_2 flux then it did for methane consumption (r^2=0.37 and 0.03, respectively; Table 2.3). Without an influence of diversity on rates of soil respiration, the ability of moisture and temperature to exert controls on the efflux of carbon dioxide was more apparent. In contrast, with diversity explaining most of the rate of methane consumption, moisture and temperature could

only explain a minimal amount of the variation in the rate of methane consumption (Table 2.3).

Other studies have similarly failed to find a correlation between total microbial diversity (24, 47, 48) and soil respiration. These studies have also found that changes in the microbial community were correlated to more metabolically specialized processes like N_2O production (24) and nitrification potential (24, 48). Studies that have not directly measured total microbial diversity have also found support for the microbial community not influencing carbon dioxide production, but influencing specialized microbially mediated processes ((49), reviewed in (23, 25)). However, not all studies have found that diversity always influences specialized physiological processes. Enwall et al. (50) found rates of denitrification to be independent of microbial diversity, and Wertz et al. (47) found similar results for denitrification and nitrification. There is also evidence of microbial diversity being correlated to soil respiration (8, 13, 22). Additionally, Bell et al. (51) found bacterial respiration to be correlated with increasing bacterial diversity in laboratory microcosms, but with a maximum of 72 bacterial species, their ecosystem was likely not nearly as functionally redundant as the thousands of bacterial species contributing to carbon dioxide production in soil.

Methanotroph richness changing with rates of methane consumption has also been observed in other sites, but an overall pattern is unclear. In Mono Lake, the depth with the highest rate of methane oxidation was found to have the greatest richness of methanotrophs (52), and two pine forests had greater methanotroph richness and methane consumption rates than paired pasture soils in New Zealand (16). However, that same study found that a paired shrubland and pasture soil had the same methanotroph

richness despite different rates of methane consumption, and other studies have been inconclusive with no clear relationship observed between methanotroph richness and methane consumption (18-20). Therefore, the relationships we have observed between methane consumption and methanotroph diversity is consistent with some previous findings, and the lack of an overall pattern may at least be partially due to methodological differences. Most assessments of the methanotroph community are performed at a broader phylogenetic level, with fewer replicates, and/or compared to less robust measures of the rate of methane consumption. In addition, this is the first study to correlate methanotroph richness to rates of methane consumption.

The Impact of Agriculture on Methanotroph Diversity

At KBS LTER, there is unambiguous evidence linking the diversity of aerobic methanotrophs to the observed rates of methane consumption, and for agricultural management to diminish methanotroph species richness. The 24 methanotroph species that we recovered in Late DF (Figure 2.5, Table 2.2) is, to our knowledge, the most methanotroph richness recovered from a single soil; other studies have reported between 1-13 methanotroph species (16, 18, 19, 45). Conversion of soils to row crop agriculture dramatically reduced that richness to a small subset of the methanotroph richness found in Late DF.

In addition, the recovery of methanotroph diversity and methane consumption after cessation of agriculture is projected to take approximately 75 years (Figure 2.7). This slow recovery of methanotroph richness and methane consumption at the KBS LTER is consistent with worldwide observations that suggest a recovery period of ca. 100

years for methane consumption following the cessation of agriculture (5). The lack of a quicker recovery following the stopping of agricultural management practices, especially those like fertilization that represent a disturbance to the methanotroph community, indicate that many aspects of methanotroph niches are distressed due to row-crop agriculture. It will be important to identify these pivotal variables and their applicability to other sites if we are to manage lands to conserve or restore methanotroph diversity and enhance the capacity of soil to serve as a sink for this potent greenhouse gas.

The methanotroph community changes across the successional gradient at KBS LTER mirrors changes in the plant community, and further underscores the notion that methanotroph niches are slowly re-established and colonized following row-crop agriculture (Figures 2.8 and 2.9). There is a discernable pattern of recovery, and an apparent succession of methanotrophs. Similar methanotroph community changes across other successional gradients have also been observed, with patterns of methanotroph diversity along successional gradients of reforested cornfields (18) and reclaimed pasture lands (20). In each of these studies, as well as our study, the methanotrophs changed along with the plant community. King and Nanba (53) also found distinct methanotroph communities in volcanic deposits with different plant communities. However, while these observations suggest that plant diversity and/or community composition influences methanotroph diversity, there is not an obvious causal connection between plant and methanotroph diversity.

Conclusion

In conclusion, our results suggest that every methanotroph OTU is important to methane consumption rates in KBS LTER soils, and both methane consumption and the diversity of methanotrophs decline in response to row crop agriculture. The decline of both methane consumption and methanotroph diversity in row-crop agricultural soils, and the long time required for recovery of methanotroph diversity suggests that multiple aspects of the methanotrophs habitat are disrupted. It will be important to identify and quantify the effect of these pivotal variables if we are to manage lands to conserve or restore methanotroph diversity, and enhance the capacity of soil to serve as a sink for this potent greenhouse gas. There is no relationship between soil respiration and bacterial richness, and the contrasting result from methane consumption and methanotroph richness is consistent with the prediction that microbial diversity is more likely to be important to a specialized metabolic process whose a microbial community is likely to be of limited richness and consequently functionally redundant.

Acknowledgements

All methane rate measurements and were taken by G. Philip Robertson and members of his laboratory. Soil sampling and DNA extraction for the assessment of total bacterial diversity was performed by Dion Anotonopoulos.

Table 2.1. KBS-LTER Sites Investigated in this study.[1,2]

Treatment	Abbreviation	Description
Deciduous Forest	Late DF	Late successional deciduous forest, two of the three replicates have never been cut, one replicate cut 100 years ago; all have never been tilled
Mid-Successional Forest	Mid HT	Abandoned from agricluture sometime between 1950-1960
Mid-Successional Grassland	Mid NT	Forest until harvested ca. 1960, never been tilled, and is annually mowed
Early Successional	Early HT	Abandoned from agricluture in 1989, and is perodically burned
Conventional Agriculture	Ag HT	Wheat-corn-soybean rotation, chisel plowed, conventional fertilizer and pesticide inputs.

[1] Additional site details can be found at http://lter.kbs.msu.edu

[2] All soils are of the Kalamazoo/Oshtemo series and are well-drained Typic Hapludalfs (fine or course loamy, mixed).

Table 2.2. Summary of *pmoA* clone libraries

Soil Sample				PCR Annealing	*pmoA* Sequences	*pmoA* species at 94%
Treatment	Replicate	Month	Year	Temperature	in Libraries	Sequence Identity
Late DF	1	December	2004	62-56	51	4
Late DF	1	June	2005	62-56	57	11
Late DF	1	June	2005	51	110	9
Late DF	1	June	2005	48	96	6
Late DF	1	June	2005	60-51	89	14
Late DF	1	June	2006[1]	60-51	43	10
Late DF	3	June	2006[1]	60-51	48	8
Ag HT	1	December	2004	62-56	52	1
Ag HT	1	June	2005	62-56	63	2
Ag HT	1	June	2005	48	58	3
Ag HT	1	June	2006[1]	60-51	55	5
Ag HT	2	June	2006[1]	60-51	52	4
All Late DF Sequences					494	24
All Ag HT Sequences					280	7
All Ag HT and Late DF Sequences					774	26

[1]PCR products from these soil sample were digested with the PflFI restriction enzyme (Neb, Ipswich, MA)

Table 2.3. Correlations between the flux of greenhouse gases, species richness and environmental conditions.

		r^2	P Value
CO_2 Production[1]:			
	vs. bacterial richness[2]	0.22	0.522
	vs. temp and moisture[4]	0.37	<0.001
CH_4 Consumption[1]:			
	vs. methanotroph richness[3]	0.96	0.018
	vs. temp and moisture[4]	0.03	0.005

[1] Rate measurements were typically made bimonthly between March and November of 2005 through 2007.

[2] Linear regression (n=4) with total bacterial richness estimated with Chao I and based on 220,996 16S tag sequences determined from samples collected in December 2006. Rate measures are arithmetic averages from the same plots from which richness was determined: The second and fourth replicates of Ag HT, and the first and third replicates of Late DF.

[3] Linear regression (n=4) with methanotroph richness determined at a 94% *pmoA* sequence identity from clone libraries from samples collected in June 2006. Rate measures are arithmetic averages from the same plots from which richness was determined: The first and second replicates of Ag HT, and the first and third replicates of Late DF.

[4] Multiple regression (n=280) with maximum air temperature and soil moisture for the day rates were measured. Rate measures are from four replicates of Ag HT, and from three replicates of Late DF.

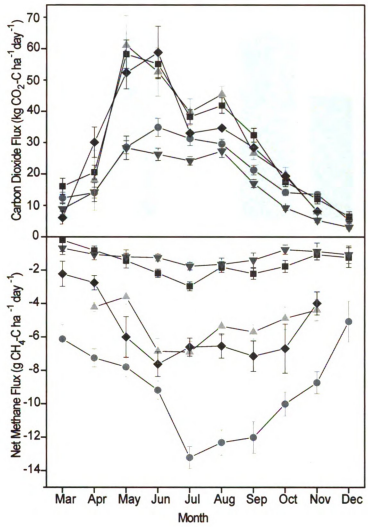

Figure 2.1. Average monthly carbon dioxide production and methane consumption based on current land management and historical land use at the KBS-LTER: Agricultural management of historically tilled land (Ag HT; ▼), early successional plant communities on fields that had been abandoned from agriculture in 1989 (Early HT; ■), mid-successional plant communities on either historically tilled land (Mid HT; ▲) or never tilled land (Mid NT; ◆), or a late successional deciduous forest (Late DF; ●).

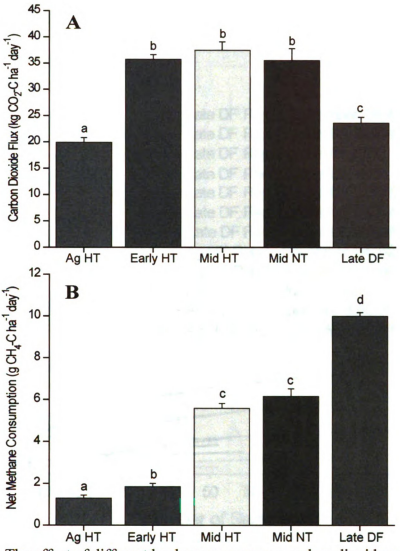

Figure 2.2. The effect of different land uses on average carbon dioxide emission (a) and net methane consumption (b) at KBS LTER. Different letters represent significant differences ($p<0.05$) between treatments. Rate measures are the same as those in Fig. 2.1. Error bars represent standard errors. Land use treatments are: agricultural management of historically tilled land (Ag HT), early successional plant communities on fields that had been abandoned from agriculture in 1989 (Early HT), mid-successional plant communities on either historically tilled land (Mid HT) or never tilled land (Mid NT), or a late successional deciduous forest (Late DF).

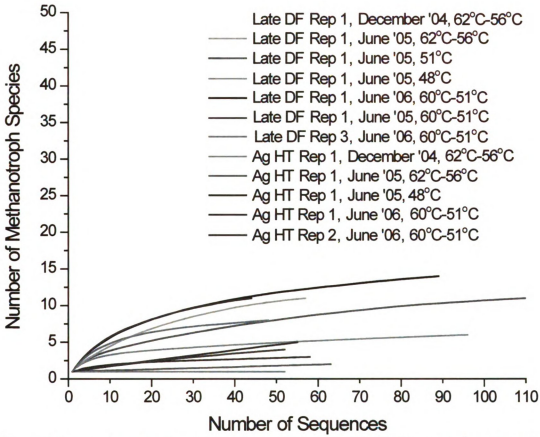

Figure 2.3. *pmoA* rarefaction curves from all of the KBS-LTER *pmoA* clone libraries constructed from the Late DF and Ag HT treatments. Libraries were constructed from DNA extracted from soil sampled in December 2004, June 2005, and June 2006 with various annealing temperatures (For additional details see Table 2.2, and Methods). All curves were constructed using data from neighbor joining matrixes from Arb (Ludwig et al. 2004), with curves calculated by DOTUR (38). Methanotroph species are defined as *pmoA* sequences having 94% average nucleotide sequence similarity. Error bars representing 95% confidence intervals were omitted for the sake of clarity.

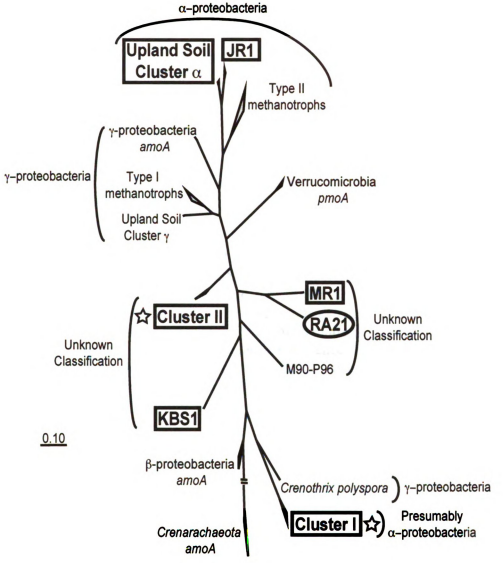

Figure 2.4. Phylogenetic tree of selected partial *pmoA* and *amoA* protein sequences from public databases and translated from PCR-based clone libraries from KBS-LTER soils. The tree is based on 164 amino acid positions using Phylip Protein Maximum Likelihood as implemented in ARB (Ludwig et al. 2004). Boxed or circled labels are indicative of *pmoA* clades recovered from KBS-LTER soils. Cluster I and Cluster II clades (boxed and starred labels) were recovered from Ag HT and Late DF, while clades KBS1, JR1, Upland Soil Cluster α, and MR1 (boxed labels) were recovered from Late DF. Clade RA21 (oval) was only recovered from Mid NT soil. The scale bar represents 10 PAM units.

Figure 2.5. Neighbor joining phylogenetic tree of the partial nucleic acid sequences of *pmoA* and *amoA* from reference sequences, and KBS-LTER clone libraries from soil sampled in December 2004, June 2005, June 2006, and June 2007. The tree is rooted with the branching from the *amoA* sequences from *Nitrosomonas eutropha* and *Nitrosococcus mobilis*. Nodes representing the 27 different methanotroph species (defined as *pmoA* sequences having 94% average nucleotide sequence similarity) identified at KBS-LTER are highlighted. The colors of the highlights reflect species' membership in the seven *pmoA* clades depicted in Figure 5. The *pmoA* sequences included in this tree are deposited in GenBank under accession numbers FJ529724 - FJ529808, and GQ219582-GQ219583.

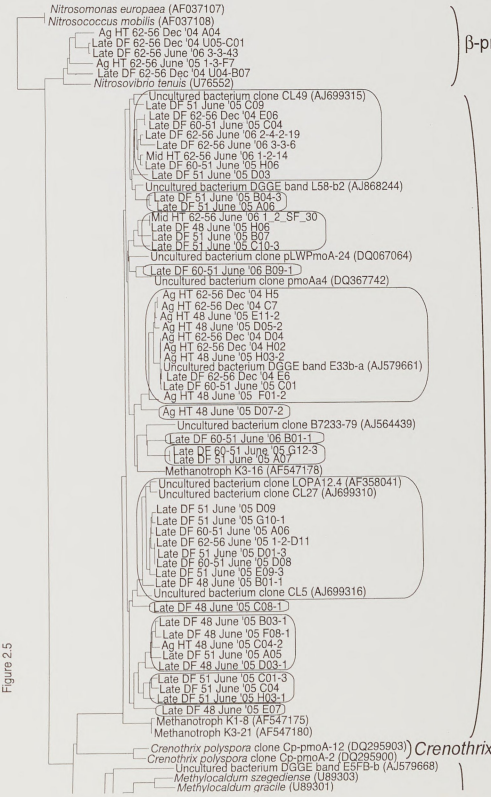
Nitrosomonas europaea (AF037107)
Nitrosococcus mobilis (AF037108)
Ag HT 62-56 Dec '04 A04
Late DF 62-56 Dec '04 U05-C01
Late DF 62-56 June '06 3-3-43
Ag HT 62-56 June '05 1-3-F7
Late DF 62-56 Dec '04 U04-B07
Nitrosovibrio tenuis (U76552)
β-proteobacteria amoA
Uncultured bacterium clone CL49 (AJ699315)
Late DF 51 June '05 C09
Late DF 62-56 Dec '04 E06
Late DF 60-51 June '05 C04
Late DF 62-56 June '06 2-4-2-19
Late DF 62-56 June '06 3-3-6
Mid HT 62-56 June '06 1-2-14
Late DF 60-51 June '05 H06
Late DF 51 June '05 D03
Uncultured bacterium DGGE band L58-b2 (AJ868244)
Late DF 51 June '05 B04-3
Late DF 51 June '05 A06
Mid HT 62-56 June '06 1_2_SF_30
Late DF 48 June '05 H06
Late DF 51 June '05 B07
Late DF 51 June '05 C10-3
Uncultured bacterium clone pLWPmoA-24 (DQ067064)
Late DF 60-51 June '06 B09-1
Uncultured bacterium clone pmoAa4 (DQ367742)
Ag HT 62-56 Dec '04 H5
Ag HT 62-56 Dec '04 C7
Ag HT 48 June '05 E11-2
Ag HT 48 June '05 D05-2
Ag HT 62-56 Dec '04 D04
Ag HT 62-56 Dec '04 H02
Ag HT 48 June '05 H03-2
Uncultured bacterium DGGE band E33b-a (AJ579661)
Late DF 62-56 Dec '04 E6
Late DF 60-51 June '05 C01
Ag HT 48 June '05 F01-2
Ag HT 48 June '05 D07-2
Uncultured bacterium clone B7233-79 (AJ564439)
Late DF 60-51 June '06 B01-1
Late DF 60-51 June '05 G12-3
Late DF 51 June '05 A07
Methanotroph K3-16 (AF547178)
Cluster I
Uncultured bacterium clone LOPA12.4 (AF358041)
Uncultured bacterium clone CL27 (AJ699310)
Late DF 51 June '05 D09
Late DF 51 June '05 G10-1
Late DF 60-51 June '05 A06
Late DF 62-56 June '05 1-2-D11
Late DF 51 June '05 D01-3
Late DF 60-51 June '05 D08
Late DF 51 June '05 E09-3
Late DF 48 June '05 B01-1
Uncultured bacterium clone CL5 (AJ699316)
Late DF 48 June '05 C08-1
Late DF 48 June '05 B03-1
Late DF 48 June '05 F08-1
Ag HT 48 June '05 C04-2
Late DF 51 June '05 A05
Late DF 48 June '05 D03-1
Late DF 51 June '05 C01-3
Late DF 51 June '05 C04
Late DF 51 June '05 H03-1
Late DF 48 June '05 E07)
Methanotroph K1-8 (AF547175)
Methanotroph K3-21 (AF547180)
Crenothrix polyspora clone Cp-pmoA-12 (DQ295903)
Crenothrix polyspora clone Cp-pmoA-2 (DQ295900)
Crenothrix polyspora
Uncultured bacterium DGGE band E5FB-b (AJ579668)
Methylocaldum szegediense (U89303)
Methylocaldum gracile (U89301)

Figure 2.5

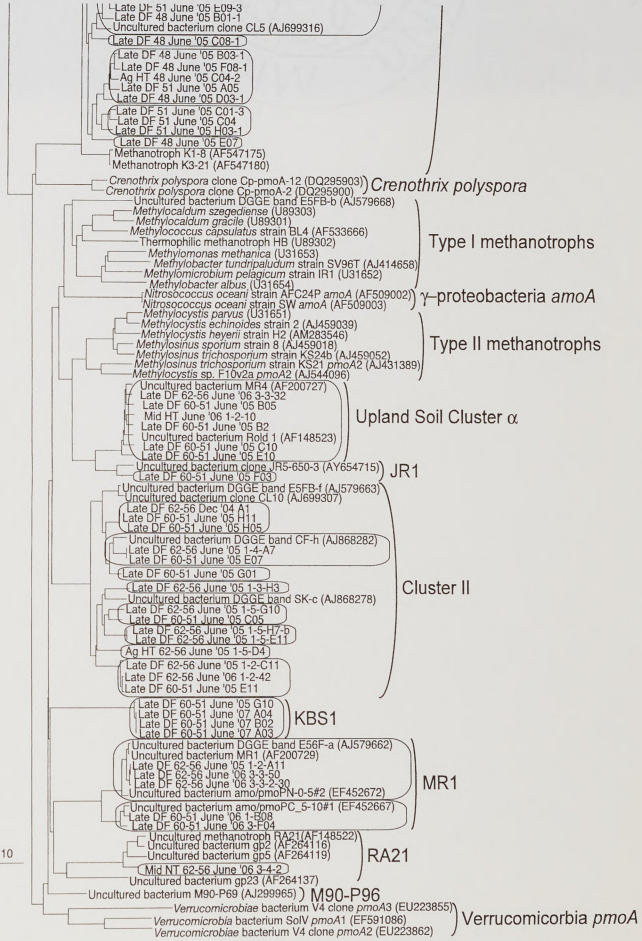

Figure 2.5 Continued

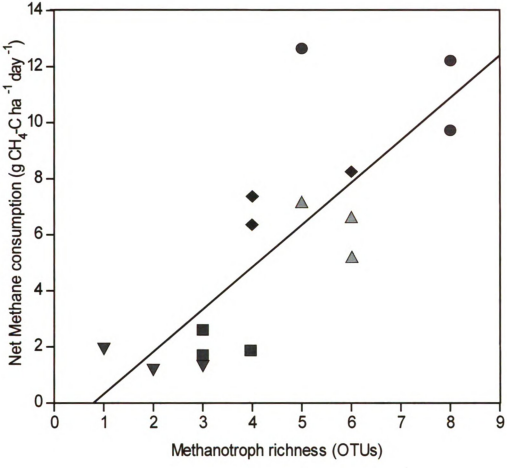

Figure 2.6. The correlation between methanotroph diversity and methane consumption at KBS LTER. Relationship between summer methane consumption (June-August) and methanotroph richness (both represent averages of 3 replicate plots) across landscapes at the KBS LTER. A simple linear regression is presented (r^2 =0.62, p <0.001) with operational taxonomic units (OTUs) defined as peaks in the tRFLP analysis that have been identified as a *pmoA* gene. Symbols are as follows: Agricultural management of historically tilled land (Ag HT; ▼), early successional plant communities on fields that had been abandoned from agriculture in 1989 (Early HT; ■), mid-successional plant communities on either historically tilled land (Mid HT; △) or never tilled land (Mid NT; ◆), or a late successional deciduous forest (Late DF; ●).

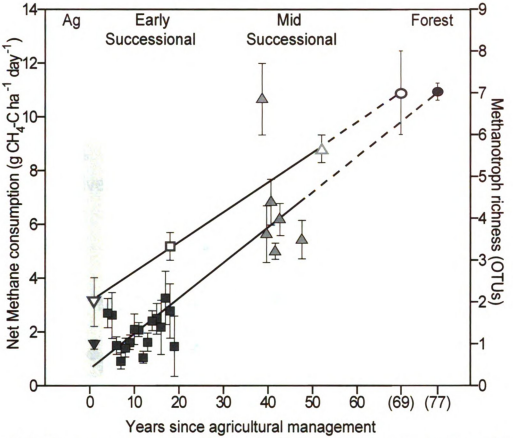

Figure 2.7. The recovery of methanotroph diversity and methane consumption at KBS LTER following row-crop agriculture. Increase in methanotroph diversity (open symbols) and methane consumption (closed symbols) as a function of time since cesssation of agriculture. Measurements of the deciduous forest (Late DF) are positioned based on projections from linear regression used to fit methanotroph diversity ($y = 0.07x + 2.05$; $r^2 = 0.99$, $p = 0.020$) or methane consumption ($y = 0.13x + 0.80$; $r^2 = 0.69$, $p < 0.001$). Error bars represent standard errors; symbols are as follows: Agricultural management of historically tilled land (Ag HT; ▼), early successional plant communities on fields that had been abandoned from agriculture in 1989 (Early HT; ■), mid-successional plant communities on either historically tilled land (Mid HT; ▲) or never tilled land (Mid NT; ◆), or a late successional deciduous forest (Late DF; ●).

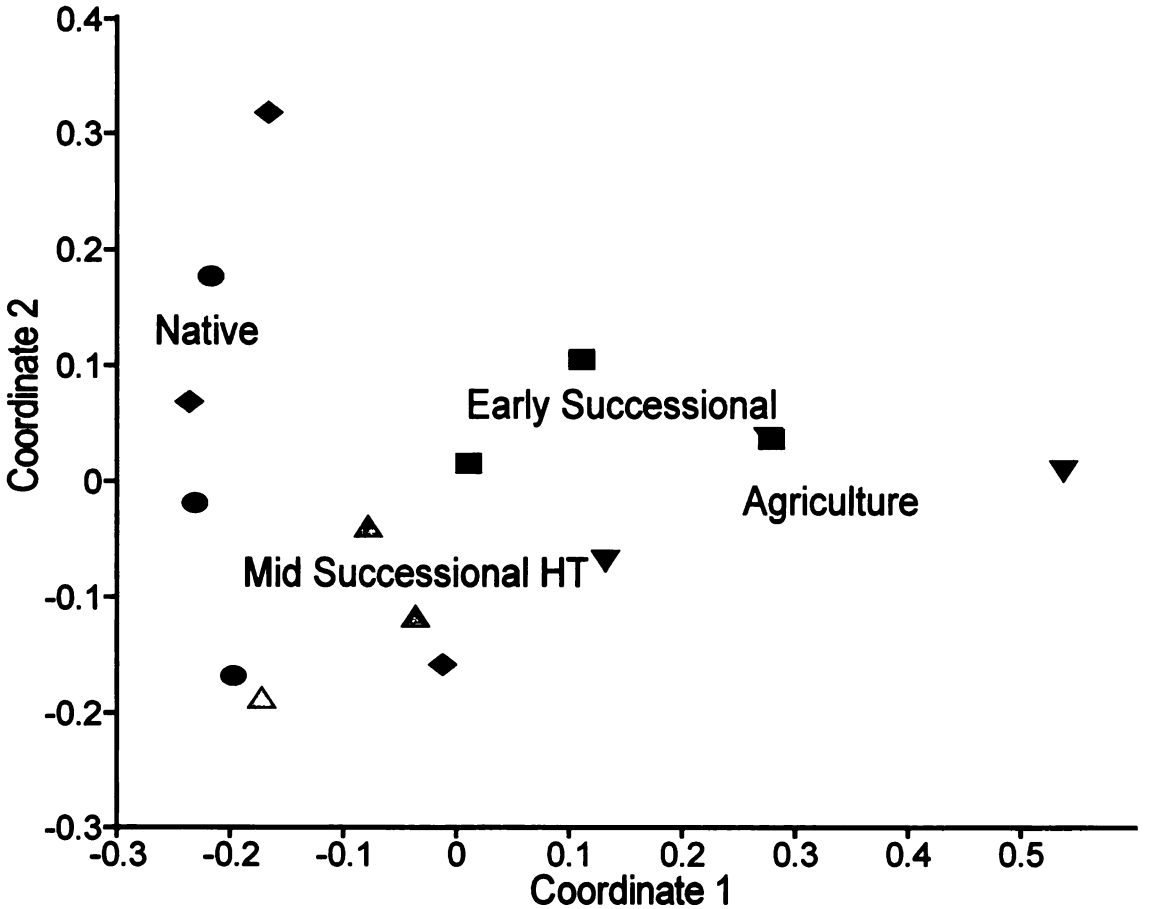

Figure 2.8. Similarity of methanotroph communities among KBS-LTER treatments. The Sørenson index was calculated for each pairwise comparison of methanotrophs using OTUs for all confirmed *pmoA*s, and then plotted using two-dimensional non-metric multidimensional scaling (Hammer et al. 2001). Symbols are as follows: Agricultural management of historically tilled land (Ag HT; ▼), early successional plant communities on fields that had been abandoned from agriculture in 1989 (Early HT; ■), mid-successional plant communities on either historically tilled land (Mid HT; ▲) or never tilled land (Mid NT; ◆), or a late successional deciduous forest (Late DF; ●).
Figure 2.9 contains the same data represented as a dendogram.

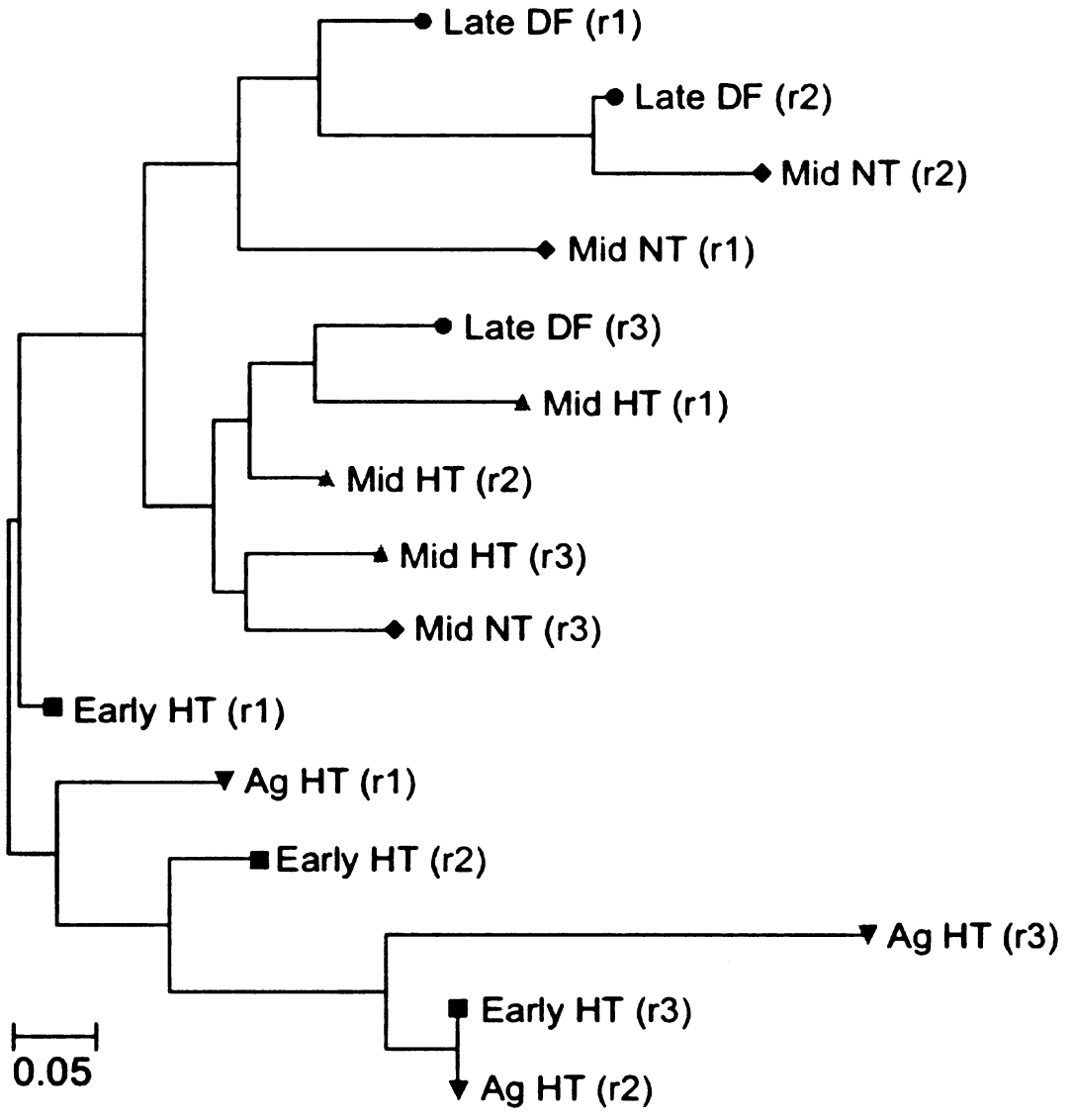

Figure 2.9. Similarity of methanotroph communities among KBS-LTER treatments. The Sørenson index was calculated for each pairwise comparison of methanotrophs using OTUs for all confirmed *pmoA*s, and then clustered using neighbor-joining with MEGA (54). Symbols are as follows: Agricultural management of historically tilled land (Ag HT; ▼), early successional plant communities on fields that had been abandoned from agriculture in 1989 (Early HT; ■), mid-successional plant communities on either historically tilled land (Mid HT; ▲) or never tilled land (Mid NT; ◆), or a late successional deciduous forest (Late DF; ●).

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Chapter 3

The Impact of Long-term Fertilization to the Methanotroph Communities in Soils

Abstract

Identifying aspects of agricultural management that are causing or contributing to the loss of methanotroph diversity is crucial if we are to manage lands to conserve or restore methanotroph diversity, and, in turn, enhance the capacity of soil to serve as a sink for this potent greenhouse gas. The effect of long-term fertilization, a separate effect from the short-term response to fertilization, is one aspect of row-crop agriculture that is likely to be a significant disturbance to the methanotroph community. We hypothesized that a consequence of long-term fertilization would be its association with decreases in methane consumption and methanotroph richness in fertilized forest sub-plots at the Kellogg Biological Station Long-term Ecological Research Site (KBS LTER). Contrary to our expectations, we found that long-term fertilization alone did not cause a decrease in methane consumption nor in methanotroph richness at KBS LTER. To determine the effect of long-term fertilization in other sites we expanded our study by sampling long-term fertilized agricultural soils from Rothamsted Research, and to include a meta-analysis of other long-term fertilized soils. Apart from the KBS LTER agricultural soil we did not find diminished methanotroph richness in long-term fertilized soils, and comparing between sites, fertilization did not select for similar methanotroph communities. Rather, methanotroph communities clustered together based on geographic location regardless of fertilization. The results suggest that each geographic location has

a unique methanotroph community, and that it is possible that some of these communities may be resistant to long-term fertilization.

Introduction

Conversion of well-drained native soils to agricultural use has been demonstrated to lead to an approximately 70% reduction in the rates of methane consumption (reviewed by (1), and to cause local extinctions of the methane consuming bacteria (methanotrophs) who facilitate methane consumption (Chapter 2). Methane is responsible for 15% of the atmosphere's total radiative forcing (2), and its atmospheric levels are currently rising (3). Therefore, identifying the aspects of agricultural management that are causing or contributing to the loss of methanotroph diversity is crucial if we are to manage lands to conserve or restore methanotroph diversity, and, in turn, enhance the capacity of soil to serve as a sink for this potent greenhouse gas.

Common row-crop agricultural practices of tillage, fertilizer, pesticides, and herbicide application can diminish methane consumption (reviewed in (4)). In addition, soil pH, water filled pores space, and dry bulk density can directly influence rates of methane consumption (reviewed by (1, 4, 5). We chose to focus upon fertilization because ammonia inhibits in situ rates of methane consumption by methanotrophs (6-8) both through direct competitive inhibition of methane monooxygenase (MMO), and indirect salt inhibition (9, 10), and has been shown to disturb the methanotroph community (11, 12). At the Kellogg Biological Station Long-term Ecological Research site (KBS LTER) Suwanwaree and Robertson (6) found that tillage had no short-term effect on the rates of methane consumption, but fertilization decreased levels of

consumption by as much as 60% in mid and late successional soils. In addition, the conventional row-crop agricultural soil at KBS LTER, which is regularly fertilized, harbors few methanotroph species (Chapter 2). We inferred that the persistent long-term application of ammonia-based fertilizers is a disturbance to the methanotroph community that is likely to have played a prominent role in causing the loss of methanotroph diversity from the agricultural treatments at KBS LTER.

There are distinct long and short-term responses to methane consumption rates to fertilization, and the long-term (chronic) effect is especially pertinent when considering the effect of fertilization as an agricultural practice on rates of methane oxidation and the methanotroph community. The short-term response of methane consumption rates after the application of ammonia-based fertilizer is a dramatic reduction in methane oxidation. Methane consumption will never fully be eliminated, and there will be a recovery to pre-fertilization levels of consumption once the ammonia has been consumed as the competitive and salt inhibition of MMO by ammonia is relieved (4, 6, 10). For instance, Suwanwaree and Robertson (6) found that approximately 7-8 weeks after the application of fertilizer methane consumption in the late successional deciduous forests (Late DF) began to recover, and had returned to pre-fertilization levels by 14 weeks. However, fertilization had no effect on no-till agricultural soil whose rate of methane consumption remained the same before and after fertilization. This lack of response to fertilization is characteristic of the long-term effect of fertilization in which the rate of methane oxidation is constantly depressed even after new applications of ammonia fertilizer (4, 6, 7).

Unlike the short-term response to fertilization, the long-term phenomenon does not have a straightforward explanation, and suggested reasons for the long-term attenuation of rates of methane consumption include: fertilization enriching the ammonia-oxidizing population, who then account for the observed methane oxidation; or fertilization reducing the methanotroph population. However, no evidence has been found for ammonia-oxidizers contributing to *in situ* atmospheric methane consumption, even after enrichment by fertilization (7, 13, 14). There is evidence that fertilization has been found to particularly impact *Methylocystaceae* (11), and reduce methanotroph abundance (11, 12). Consistent with this trend is the decrease in methanotroph richness in the long-term fertilized conventional row-crop agricultural treatment (Ag HT) at KBS LTER (Chapter 2). All of the results suggest that long-term fertilization changes the methanotroph community, and likely due to those changes, the soils consume less atmospheric methane.

Therefore, we hypothesized that long-term fertilization would be associated with decreases in methane consumption and methanotroph richness in fertilized forest sub-plots at KBS LTER. The hypothesis was tested by determining the methanotroph richness and community structure with clone libraries of the gene encoding the A subunit of the particulate methane monooxygenase (*pmoA*) from fertilized sub-plots of the Late DF treatment at KBS LTER. In addition, the effect of long-term fertilization in other upland soils was explored through additional *pmoA* clone libraries from row crop agriculture and forest soils at Rothamsted Research, and a meta-analysis that included another long-term fertilized forest (11) and agricultural soil (15) (Table 3.1).

Materials and Methods

Site Description and Fertilization history

At KBS LTER, experimental sites were established as 2x2 m plots downhill and adjacent to each of the three 1 ha replicate plots of the larger late successional deciduous forest treatment (Late DF) within the KBS LTER. The sites were established in 1995 (16), and the control treatment (Control Sub-plot) has received 0 g N m^{-2} yr^{-1}. The fertilized plot (Fertilized sub-plot) sampled in this study, 3N, initially received 3 g N m^{-2} yr^{-1}, applied in three 1 g N m^{-2} applications sometime between April-November. For 4 years (1995-1998), 3 g N m^{-2} yr^{-1} was annually applied to the plot as ammonium nitrate (NH_4NO_3). No fertilizer was applied during 1999 or 2000. Beginning in 2001, the annual application was changed to 20-30 g N m^{-2} yr^{-1}, and applied using a backpack sprayer in two or three 10 g N m^{-2} applications between April-November as either urea or NH_4NO_3. In 2007, only one 10 g N m^{-2} fertilization took place, as urea, on April 23rd in replicates 1 and 2, and on April 24th in replicate 3. Further descriptions of the KBS LTER and the Late DF sites can be found at http://lter.kbs.msu.edu/.

Rothamsted Research, located in Harpenden, Hertfordshire, United Kingdom, features the Broadbalk experiment, the longest continuous agricultural experiment in the world. Since 1843 Broadbalk has been a continuous wheat field, with different plots of the field receiving different levels of input. Broadbalk plot 8, section 1 (Broadbalk Wheat), receives 14.4 g N m^{-2} yr^{-1} as NH_4NO_3, and was chosen as the long-term

fertilized site to be studied due to its exogenous N input being roughly comparable to the conventional row-crop agricultural soils (Ag HT) at KBS LTER. Two non-fertilized soils were chosen to compare to the agricultural soil: Broadbalk Wilderness and Knott Wood. In 1882, one 0.2 ha section of the Broadbalk wheat experiment was fenced and abandoned from agricultural management. It is presently a wooded area with ash, sycamore, and hawthorn trees, and is referred to as "Broadbalk Wilderness." Knott Wood is at least 300 years old, and is a mixed deciduous woodland. The sites are located within 1.0 km of each other, and are found on silt to silty clay loams and are Chromic Luvisols classified as Batcombe series (17). Like the paired sites at KBS LTER, methane consumption has been shown to be lowest in the Broadbalk Wheat with greater consumption found in the Broadbalk Wilderness and Knott Wood soils (17, 18). Further descriptions and details of Broadbalk, Knottt Wood and Rothamsted Research can be found at http://www.rothamsted.bbsrc.ac.uk/.

Rate Measurements at KBS LTER

At the KBS LTER sub-plots *in situ* rates of methane consumption were measured using closed-cover flux chambers (19) from all three replicates of both the control and fertilized sub-plots. Chambers were constructed with 13 L containers without bottoms and inserted into the soil to establish approximately 10 L of headspace (20). The chambers had gas tight lids with rubber septa to allow for gas sampling. Four gas samples were taken at approximately 15 minute intervals (0, 15, 30, and 45 minutes). Samples were processed as detailed at http://lter.kbs.msu.edu, and methane concentrations were determined by GC-FID. Rates were calculated using a best-fit linear approach. All 4 data points were used to calculate the rate unless the last data point

indicated a plateau in consumption, in which case only the first 3 points were used to calculate the rate. A minority of samples had erroneous points excluded, which were identified based on a similar outlier also being present in the other trace gases (CO_2 and N_2O) measured from the same sample. Any rate that could not be fit with a linear line (roughly under an r^2 of 0.70) was excluded from the analysis. To determine rate differences based on treatment, a t-test was performed using the program PAST (21).

Rate measurements were made on June 20th and 27th, August 1st and 14th, and on October 16th in 2007. All rate data were taken 59-177 days after the last fertilization event. Suwanwaree and Robertson (6) found that the short-term acute effect of Late DF fertilization on rates of methane consumption began to abate between 52 and 73 days after fertilization. Therefore, the comparison of consumption rates is a test of the long-term fertilization effect, and not the short-term fertilization effect.

Soil Sampling

At KBS LTER, 3 soil cores (2.5 x 10 cm) were collected from the first 2 replicate sub-plots from the control and fertilized (30 g N m^{-2} yr^{-1}, 3N) treatments on June 13th 2007. Samples were transported to the laboratory on ice where they were mixed thoroughly and stored at -80°C until processing.

At Rothamsted Research, 2 soil cores (2.5 x 10 cm) from each site were taken on February 6th 2008. Each soil core was processed as a replicate as the Rothamsted sites are not replicated within the greater landscape (http://www.rothamsted.bbsrc.ac.uk/). All

samples were air dried for approximately 24hrs prior to sieving (2mm). Once sieved, samples were stored at -80°C until further processing.

DNA Extraction and *pmoA* PCR Reactions

DNA was extracted from soil samples with the Mo Bio PowerSoil™ DNA Isolation Kit, following the manufacturer's protocol, except that mechanical cell lysis was performed by bead beating for 45 seconds with KBS LTER soil samples, and bead beating for 30 seconds with a Fast-Prep FP12 homogenizer (Thermo Scientific, Speed 5.5) with Rothamsted Research soil samples. Three extractions were performed from each of the Rothamstead Research soil cores.

All soil samples were screened for genes coding for the A subunit of particulate methane monooxygenase (*pmoA*) via PCR amplification with the primer sets A189 (5'-GGNGACTGGGACTTCTGG- 3') -A682 (5'-GAASGCNGAGAAGAASGC-3') (Holmes et al. 1995), and A189-mb661 (5'-CCGGMGCAACGTCYTTACC-3') (22) in order to encompass all known *pmoA* genes (23). No amplification was observed from A189-mb661. Amplification reactions contained 25 ng of undiluted DNA, 1.25 µl 1% BSA, 10 mM dNTPs, 0.2 µM of each primer, 2.5 µl 10x PCR buffer (200mM Tris-HCl (pH 8.4) and 500 mM KCl), 0.5 µl 50 mM $MgCl_2$ and 1.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) in a total volume of 25 µl. Reaction conditions were 95°C for 5 minutes, 15 cycles of a 'touchdown PCR' of 95°C for 1 minute, 60°C for 1 minute (-0.6°C each cycle to 51°C), and 72°C for 1 minute, 15 cycles using 51°C as the annealing temperature, and a final 10 minute extension at 72°C.

Clone Libraries

Cloning was performed with the TOPO-TA cloning kit (Invitrogen, Carlsbad,

CA) using either vector pCR 4 or pCR 2.1 as per the manufacturer's protocol. Transformants were screened via PCR reactions with the primers F2 (5'-CAGTCACGACGTTGTAAAACGACGGC-3') and R4 (5'-CAGGAAACAGCTATGACCATG-3') (24). One-1.25 µl of the PCR products was cleaned up via incubation with 0.25 µl of ExoSAP-IT (Usb, Cleveland, OH) for 30 minutes at 37°C. Sequencing was completed at the Research Technology Support Facility at Michigan State University (RTSF).

Sequences identified by BLASTX (25) as *pmoA* or the A subunit of ammonia monoxygenase (*amoA*), which A189-A682 also amplify, were imported into Arb (26). In Arb, sequences were translated and aligned using Clustal W. Nucleic acid sequences were then aligned according to the protein sequence. Sequences from clone libraries were determined to be the same species if they were $\geq$ 94% identical (27) as determined by DOTUR (average neighbor grouping) (28). *pmoA* sequences from previously reported studies were also imported into Arb (26), and aligned as described above. Species were used to calculate β-diversity (EstimateS (http://viceroy.eeb.uconn.edu/EstimateS)) in order to facilitate the comparison between the methanotroph communities of the different treatments and studies.

For KBS LTER soils samples, clone libraries were constructed from duplicate PCR reactions from each of 3 soil cores and all 6 reactions were pooled such that there was only 1 cloning reaction per replicate. For Rothamsted Research samples, clone libraries were constructed from duplicate PCR reactions of each of the 3 DNA extractions from the each soil core and, like the KBS LTER samples, all 6 reactions were pooled such that there was only 1 cloning reaction. Prior to cloning PCR products were digested

with the PflFI (Neb, Ipswich, MA), and gel extracted with the PrepEase kit (Usb, Cleveland, OH) to reduce the incidence of cloning *amoA* and non-specific PCR products.

Results

Rate Measurements

At KBS LTER, average rates of net methane consumption, 21.5±4.6 in the control sub-plot and 18.2±2.9 in the fertilized sub-plot, did not significantly differ by treatment (t=0.58 p=0.57), and long-term fertilization has not caused a decrease in rates of methane consumption rates (Figure 3.1).

Methanotroph Diversity in KBS LTER Late DF sub-plots

All clone libraries were sampled until rarefaction curves were asymptotic (Figure 3.2a), and due to PCR bias, measures of *pmoA* abundance were excluded from comparisons of the methanotroph community (Appendix A).

At KBS LTER, an average of 9.5 methanotroph species was found in the *pmoA* libraries from the control sub-plots, while an average of 6.5 was found in the fertilized sub-plots (Figure 3.1). Despite the higher average richness in the control treatment, the difference between the treatments was not significant (t=1.18, p=0.44). Between 6-12 methanotroph species were found in the *pmoA* libraries in this study, all of which fall within the range of the 6-14 methanotroph species found in previous individual Late DF libraries constructed from June soil samples (Chapter 2), and not the range of 2-5 methanotroph species found in the individual clone libraries from Ag HT at KBS LTER (Chapter 2). In addition to the richness differences between the fertilized sub-plots and Ag HT libraries, only methanotroph Clusters I and II were previously found in Ag HT

(Chapter 2), and methanotrophs belonging to clusters I, II, and Upland Soil Cluster-α were found in the fertilized sub-plots (Figure 3.3).

Comparing the methanotroph community composition between the control and fertilized sub-plots indicated that were some differences between treatments (Figures 3.4a and 3.5). The control plots clustered together and separately from the fertilized plots (Figure 3.4a), and the phylogeny of the *pmoA* sequences recovered from the different treatments reveal different clustering between the treatments that is being driven by unshared species in Cluster I and Cluster II as well as richness differences within Cluster II (Figure 3.5). In Cluster II, 4 methanotroph species were found in the control treatments, 1 species was found in the fertilized treatments, and no species were shared between the treatments. Cluster KBS1 was also only found in the control plots, adding to the differences contributing to the clustering of the treatments. Despite the differences with the control sub-plot, the methanotroph community composition in the fertilized sub-plots was more similar to methanotroph community in the non-fertilized Late DF (Chapter 2) than they were to the methanotroph communities previously found in Ag HT methanotroph clone libraries (Chapter 2) (Figure 3.4b). Additional samples are required in order to discern, with reasonable confidence, whether the community composition changes due to long-term fertilization are statistically different.

Methanotroph Diversity at Rothamsted Research

Like the KBS LTER clone libraries, the Rothamsted clone libraries were sampled until rarefaction curves were asymptotic (Figure 3.2b), and measures of *pmoA* abundance were excluded from comparisons of the methanotroph community (Appendix A). Average methanotroph richness was highest in the agricultural soil, Broadbalk Wheat,

with an average of 8.5 species, lowest in Knott Wood with an average of 3.5, and in Broadbalk Wilderness methanotroph richness was 5 (Figure 3.6). In total, 18 methanotroph species were found in Rothamsted soils spread over 5 clusters (Figure 3.7). Cluster I and Cluster II species were found in all three treatments, clusters M90-P96 and KBS1 were only found in Broadbalk Wheat, and Upland Soil Cluster-γ was found in Knott Wood and Broadbalk Wilderness. These community composition differences drove the separate clustering of Broadbalk Wheat from Knott Wood and Broadbalk Wilderness (Figure 3.8b).

Comparison of methanotroph communities under long-term fertilization

There was an apparent common effect in the methanotroph communities under long-term fertilization (Figure 3.6). Lack or replication from all the sites precludes a robust statistical analyses with the data, but while the Ag HT treatment at KBS LTER has fewer methanotrophs then Late DF ($t=6.4$, $p=0.02$), the decrease in methanotroph richness in the fertilized sub-plots compared to the control is not significant (see above), and in all other sites methanotroph diversity increases in the long-term fertilized soils. A comparison of the methanotroph community composition between the long-term fertilized and non-fertilized paired soils reveals that the methanotroph communities cluster according to geographic location (Figure 3.8).

Discussion

The effect of long-term fertilization in Late DF sub-plots at KBS LTER

We found the lack of a difference in methanotroph richness between the control and long-term fertilized Late DF sub-plots at KBS LTER (Figure 3.1) surprising, and

contrary to our hypothesis. The richness difference between Ag HT and Late DF (Chapter 2), and Suwanwaree and Robertson's (6) finding that after applying 10 g N m^{-2} of fertilizer to Late DF there was a dramatic, although temporary, decrease in rates of methane consumption led to the expectation that a decrease in methanotroph richness would be observed in the fertilized sub-plots in Late DF at KBS LTER The fertilized sub-plots in Late DF had received 6 consecutive years of fertilization at levels that exceeded the annual amount of nitrogen applied to Ag HT. In total, 16 10 g N m^{-2} fertilizer applications were applied to the fertilized forest sub-plots prior the initiation of this study. The Ag HT plot is a corn-soybean-wheat rotation that typically receives 12 and 6 g N m^{-2} in a single application of fertilizer when corn and wheat, respectively, are grown. Each fertilizer application applied to forest sub-plots was therefore similar to the amount applied to Ag HT soil, and due to the frequency of applications the annual amount of nitrogen applied to the forest sub-plots far exceeded the amount of nitrogen received by Ag HT. Due to the many years of increased fertilizer application we expected that, like Ag HT (Chapter 2, Figure 3.6), methanotroph richness would decline in the Late DF fertilized sub-plots.

However, in contrast to our prediction, we found that there was neither attenuated rates of methane consumption nor methanotroph richness due to fertilization (Figure 3.1). The agreement between these two measures is consistent with the previous finding that at KBS LTER methanotroph richness correlates to rates of methane consumption (Chapter 2). Therefore, without a change in the number of methanotroph species in the Late DF sub-plots, we would not have expected, nor was there, a change in the rates of methane consumption. The results are also consistent to the previously observed short-term

repression of methane consumption in response to fertilization (6) as the measurements of methane consumption in this study quantified the long-term effect of fertilization, and care was taken to ensure that methane consumption rates were measured after the short-term effect of the fertilization had previously been found to subside (6). Thus, while fertilization of the late successional deciduous forest at KBS LTER is an acute temporary disturbance to rates of methane consumption (6), its long-term effect was insignificant to both rates of methane consumption and methanotroph richness.

Our results suggest that the observed decrease in methanotroph richness associated with agricultural conversion at KBS LTER (between the Ag HT and Late DF treatments) was not caused by long-term fertilization alone as the methanotroph community is resistant to the fertilization disturbance, and consequently rates of methane consumption are resilient to the fertilization disturbance. It is possible that the effects of fertilizer application are somehow buffered by the Late DF soils such that it does not represent a disturbance to the methanotroph community, but results from other fertilized Late DF sub-plots confirm that the methanotrophs would have been exposed to the ammonia in the fertilizer (Appendix B and (6)), and to have experienced the short-term inhibition associated with the fertilization (6). Regardless of the mechanism that allows for the methanotroph community to persist after long-term fertilization the results from the long-term fertilization in the Late DF sub-plots implicates other changes associated with agricultural land-use as either causing, or interacting with the short-term fertilization disturbances to cause the majority of the decrease in methanotroph richness in the Ag HT treatment at KBS LTER.

Supporting the possibility that the methanotroph community of the fertilized subplot is resistant to fertilization is the clustering of its methanotroph communities with the Late DF methanotroph communities as opposed to the Ag HT communities (Figure 3.4b), the presence of more methanotroph clusters in the fertilized sub-plots then is found in Ag HT (Figure 3.3), and methanotroph richness measures which are consistent with those found in Late DF, not Ag HT (Chapter 2). These results suggest that the variation in methanotroph community composition and methanotroph richness is within the typical range of variation observed in *pmoA* libraries from Late DF, and not the range of variation seen in Ag HT *pmoA* libraries. The trend of all sub-plot libraries to be similar to Late DF data indicates that the community has likely not changed after long-term fertilization and is possibly resistant to the fertilization disturbance.

The effect of long-term fertilization on methanotroph richness in other soils

The response of the methanotroph community to only long-term fertilization alone has only been studied in one other well-drained late successional forest soils (7, 11). Those studies took place at the Harvard Forest where pine and hardwood soils have been fertilized with 15 g N m^{-2} yr^{-1} since 1988, and the methanotroph community in the fertilized treatments has changes despite no apparent change of methanotroph richness (Figure 3.6): Gulledge et al. (7), after 10 years of fertilization, found a lowered K_m for methane oxidation in fertilized treatments – indicative of a methanotroph community that had changed to one less competitively inhibited by ammonium; and after 12 years of fertilization methanotroph abundance had declined, with *Methylocystaceae* abundance being most affected (11).

It is possible that additional years of fertilization would yield discernible methanotroph community changes, as the differences in the methanotroph community at the Harvard Forest were found well after 6 years of consecutive fertilization - when we assessed the methanotroph community at KBS LTER. However, Castro et al. (29) did find that the rate of methane consumption had declined 15-64% due to fertilization in the Harvard forest soils after 6 years of consecutive fertilization. The different effect on methane consumption rates between 6 year fertilized soils at KBS LTER and Harvard Forest suggests that differences between the sites, either physical properties or the endemic methanotroph and microbial communities are likely responsible for the contrasting results.

Further implicating differences in methanotroph communities as part of the reason for the contrasting results of long-term fertilization between KBS LTER and Harvard Forest, is the absence of a typical effect to methanotroph richness in other long-fertilized soils (Figure 3.6), and the clustering of methanotroph communities according to geographic location (Figures 3.8). Comparing between sites, fertilization did not select for similar methanotroph communities; rather, methanotroph communities clustered together based on geographic location regardless of whether they were or were not fertilized. Therefore, each geographic location appears to harbor a unique assemblage of methanotrophs, and the contrasting responses between soils of the methanotroph community to long-term fertilization and land use change is probably at least partially due to fundamentally different methanotroph communities. Further confirming the biogeography of methanotrophs is a similar comparison of methanotroph community composition in a variety of upland soils (Appendix C).

With inherently different methanotroph communities at each site it would not be surprising if the response of the community to long term fertilization and/or land-use were unique for each geographic location. For instance, just using rates of methane consumption to determine the impact to the methanotroph community, distinct methanotroph communities were probably responsible for the different responses to long-term fertilization observed between birch and spruce taiga forests (30). In addition, Nyerges and Stein (31) found a broad range of sensitivities to ammonia inhibition in four methanotroph isolates, suggesting methanotroph communities of differing composition will react distinctively in response to ammonia.

In addition to the unique methanotroph community, unique physical and environmental characteristics from each geographic location are also probably playing a role in dictating the response of the methanotroph community to changing land use. For instance, if we compare the long-term fertilized forest at the Harvard Forest to the long-term fertilized Late DF sub-plots at KBS LTER, we find a number of differences that might be contributing to the observed differences in their methanotroph communities to long-term fertilization. At Harvard Forest, the forest soils are stony to sandy loams (Entic Haplorthods of the Gloucester series) (32), have low pH (3.0-4.0) (7), have a history of agricultural use (33), high nitrogen retention after fertilizer application (nearly 100%) (32, 34), and, especially in the hardwood forest, low rates of nitrification (33, 34). The absence of nitrification for at least the first 6 years of fertilization in the hardwood forest (34) and the nitrogen retention of the soils (32, 34) are signs that the methanotroph community was exposed to fertilization disturbances far longer then the methanotrophs in the fertilized sub-plots at KBS LTER. At KBS LTER fertilized sub-plots, there is robust

nitrification in the Late DF sub-plots ((6) and Appendix B), and while the methanotroph community is exposed to the fertilizer, the ammonia is consumed within approximately two months at which point rates of methane consumption begin to recover.

Therefore, the nitrification rates at KBS LTER may buffer the methanotroph community from the fertilization disturbance enough to make them resistant, and methane consumption rates resilient, to the fertilization disturbance. This possibility could be experimentally tested by inhibiting nitrification after fertilization and seeing if the methanotroph community and methane consumption rates remain unaffected by the fertilization disturbance. If the methanotroph community were found to be resistant to a fertilization disturbance and not just buffered by increased nitrification - then those would be methanotrophs that would be candidates to try to get to colonize agricultural soils. We expect fertilization resistant methanotrophs to likely be more successful in increasing the rates of methane consumption in agricultural soils as they would be able to persist in the face of one of the disturbances associated with agricultural practices. Conversely, if the methanotroph community is buffered from the fertilization disturbance by increased nitrification, then increasing nitrification in agricultural soils may provide an avenue towards increased atmospheric methane consumption in agricultural soils.

Conclusion

We found that long-term fertilization alone did not cause a decrease in methane consumption nor in methanotroph richness at KBS LTER. Comparing those results to other long-term fertilized soils found that apart from the KBS LTER agricultural soil we did not find diminished methanotroph richness in long-term fertilized soils, and comparing between geographic sites, fertilization did not select for similar methanotroph

communities. Rather, methanotroph communities clustered together based on geographic location regardless of fertilization. The results suggest that each geographic location has a unique methanotroph community, and that it is possible that some of these methanotroph communities, including the one in the KBS-LTER fertilized sub-plot, may be resistant to long-term fertilization. The results also indicate that managing land to preserve or restore methanotrophs in agricultural soils at KBS LTER and elsewhere will have to take into account many management factors, not just fertilization, and have to understand the interaction of each factor with each site's unique methanotroph and microbial communities and soil properties.

Acknowledgements

All methane rate measurements from KBS LTER and were taken by Neville Millar, who also helped with the calculations of methane flux. I'm also grateful for the help I received at Rothamsted Research from Penny Hirsch, Ian Clark, and Paul Poulton.

Table 3.1. Summary of the Sites and *pmoA* libraries used in this study.

Site	Land Use	Country	Number of pmoA sequences	Reference
Kellogg Biological Station LTER[1]	Ag HT, Rep 1[2]	United States	228	Chapter 2
Kellogg Biological Station LTER[1]	Ag HT, Rep 2	United States	52	Chapter 2
Kellogg Biological Station LTER[1]	Late DF, Rep 1[3]	United States	446	Chapter 2
Kellogg Biological Station LTER[1]	Late DF, Rep 3	United States	48	Chapter 2
Rothamsted Research[4]	Knott Wood, Rep 1	United Kingdom	32	This study
Rothamsted Research[4]	Knott Wood, Rep 2	United Kingdom	31	This study
Rothamsted Research[4]	Broadbalk Wilderness Rep 1	United Kingdom	42	This study
Rothamsted Research[4]	Broadbalk Wilderness Rep 2	United Kingdom	28	This study
Rothamsted Research[4]	Broadbalk Wheat, Rep 1	United Kingdom	45	This study
Rothamsted Research[4]	Broadbalk Wheat, Rep 2	United Kingdom	13	This study
Sakerat Experimental Station	Evergreen Forest	Thailand	5	Knief et al. 2005 (15)
Sakerat Experimenatl Station	Reforested 16 year old *Acacia* plantation	Thailand	6	Knief et al. 2005 (15)
Sakerat Experimenatl Station	Cornfield	Thailand	7	Knief et al. 2005 (15)
Kellogg Biological Station LTER[1]	Late DF, Control Sub-plot Rep 1	United States	66	This study
Kellogg Biological Station LTER[1]	Late DF, Control Sub-plot Rep 2	United States	71	This study
Kellogg Biological Station LTER[1]	Late DF, Fertilized Sub-plot Rep 1	United States	18	This study
Kellogg Biological Station LTER[1]	Late DF, Fertilized Sub-plot Rep 2	United States	29	This study
Havard Forest LTER	Pine Forest, Cntrl	United States	2	Lau et al. 2007 (11)
Harvard Forest LTER	Pine Forest, Fert	United States	6	Lau et al. 2007 (11)
Harvard Forest LTER	Hardwood, Fert	United States	6	Lau et al. 2007 (11)

[1] Replicates refer to 1 ha replicates.

[2]Represents the summing of 4 individual libraries (Chapter 2) with an average richness of 2.8 methanotroph species.

[3]Represents the summing of 6 individual libraries (Chapter 2) with an average richness of 9 methanotroph species.

[4] Replicates refer to replicate soil cores.

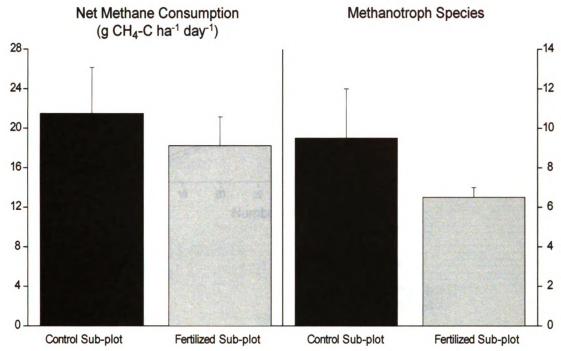

Figure 3.1. Differences between Late DF fertilized and control treatments at KBS LTER in average net methane consumption and the average number of methanotroph species (defined as *pmoA* sequences having 94% average nucleotide sequence similarity). Fertilization has no effect on either measure ($t=0.58$, $p=0.57$ for consumption; and $t=1.18$, $p=0.36$ for richness). Measures for net methane consumption are averages from 3 replicates of each treatment, while the methanotroph species are averages from 2 replicates of each treatment. Net methane consumption from the same 2 replicates as those with methanotroph species data yields the same result (20.9 ± 4.3 and 19.2 ± 3.3 average net methane consumption of the control and fertilized sub-plots, respectively, and $t=0.28$ $p=0.79$). Error bars represent standard error.

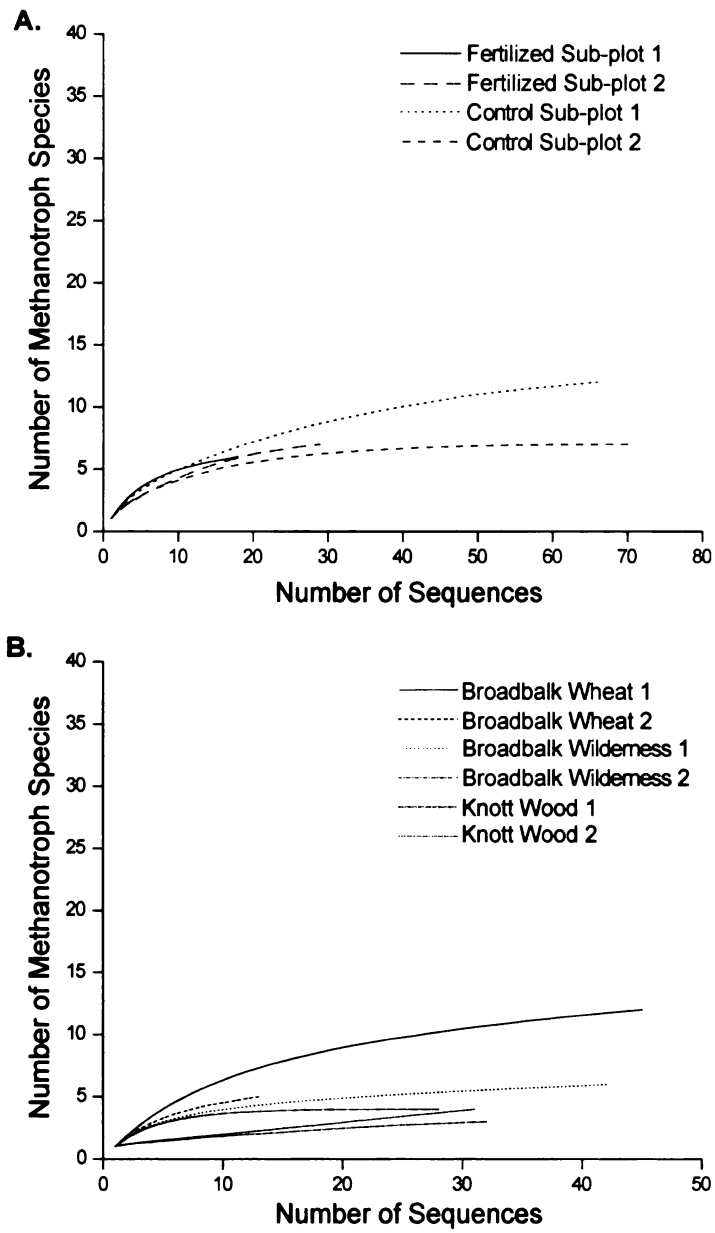

Figure 3.2. (A) Rarefaction curves from *pmoA* clone libraries constructed from the control and fertilized sub-plots of the late successional forest at KBS LTER. Libraries were constructed from DNA extracted from soil sampled in June 2007. (B) Rarefaction curves from *pmoA* clone libraries constructed from Rothamsted Reasearch soils sampled in Feburary 2008. All curves were constructed using data from neighbor joining matrixes from Arb (Ludwig et al. 2004), with curves calculated by DOTUR (Schloss and Handelsman 2005). Methanotroph species are defined as *pmoA* sequences having 94% average nucleotide sequence similarity. Error bars representing 95% confidence intervals were omitted for the sake of clarity.

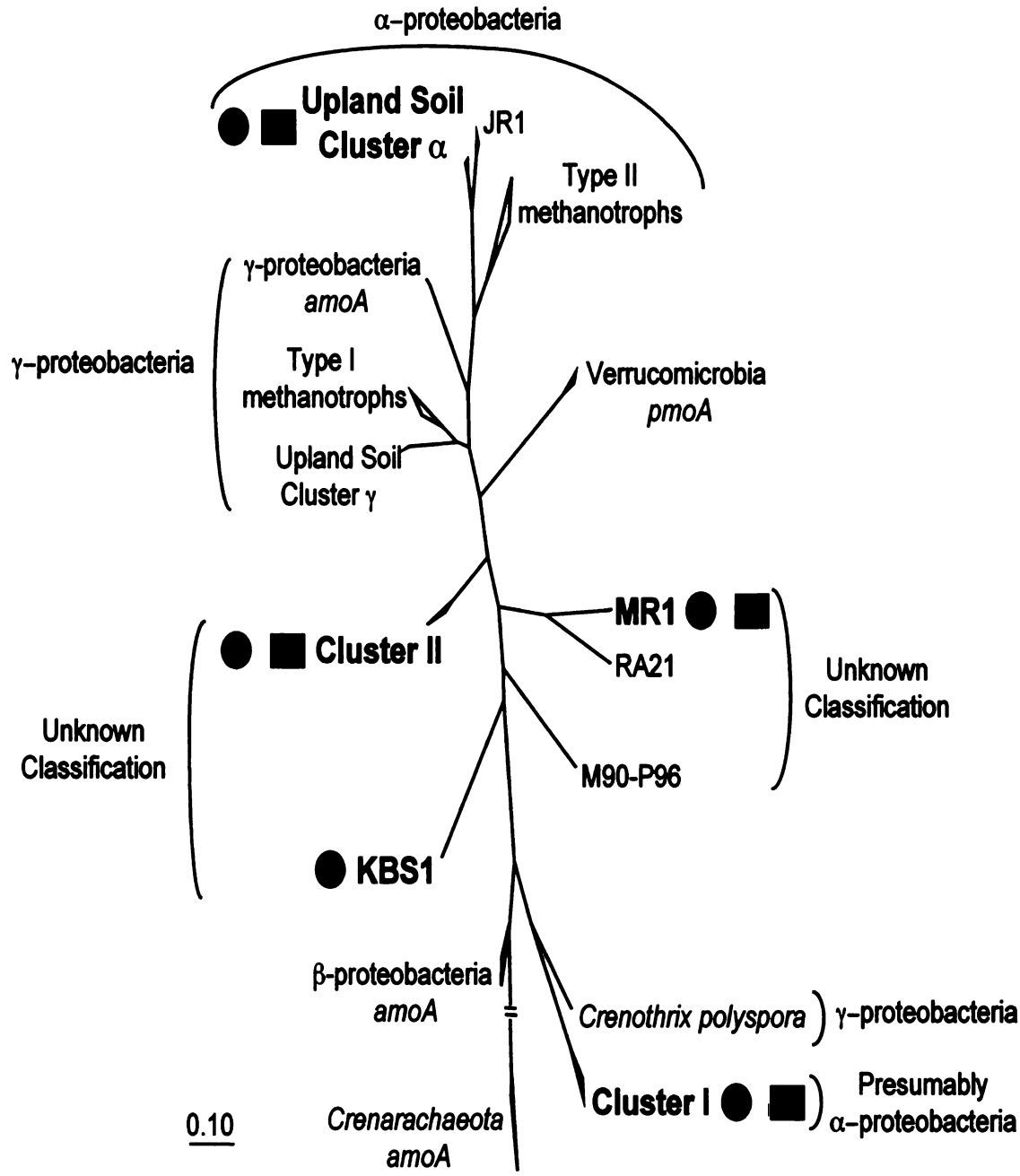

Figure 3.3. Phylogenetic tree of selected partial *pmoA* and *amoA* protein sequences from public databases and translated from PCR-based clone libraries from late successional deciduous forest sub-plots. The tree is based on 164 amino acid positions using Phylip Protein Maximum Likelihood as implemented in ARB (26). Bolded clades were recovered from the late successional forest sub-plots. The symbols adjacent to the clade reflect the clade's recovery from either the control treatment (●) or the fertilized treatment (■). The scale bar represents 10 PAM units.

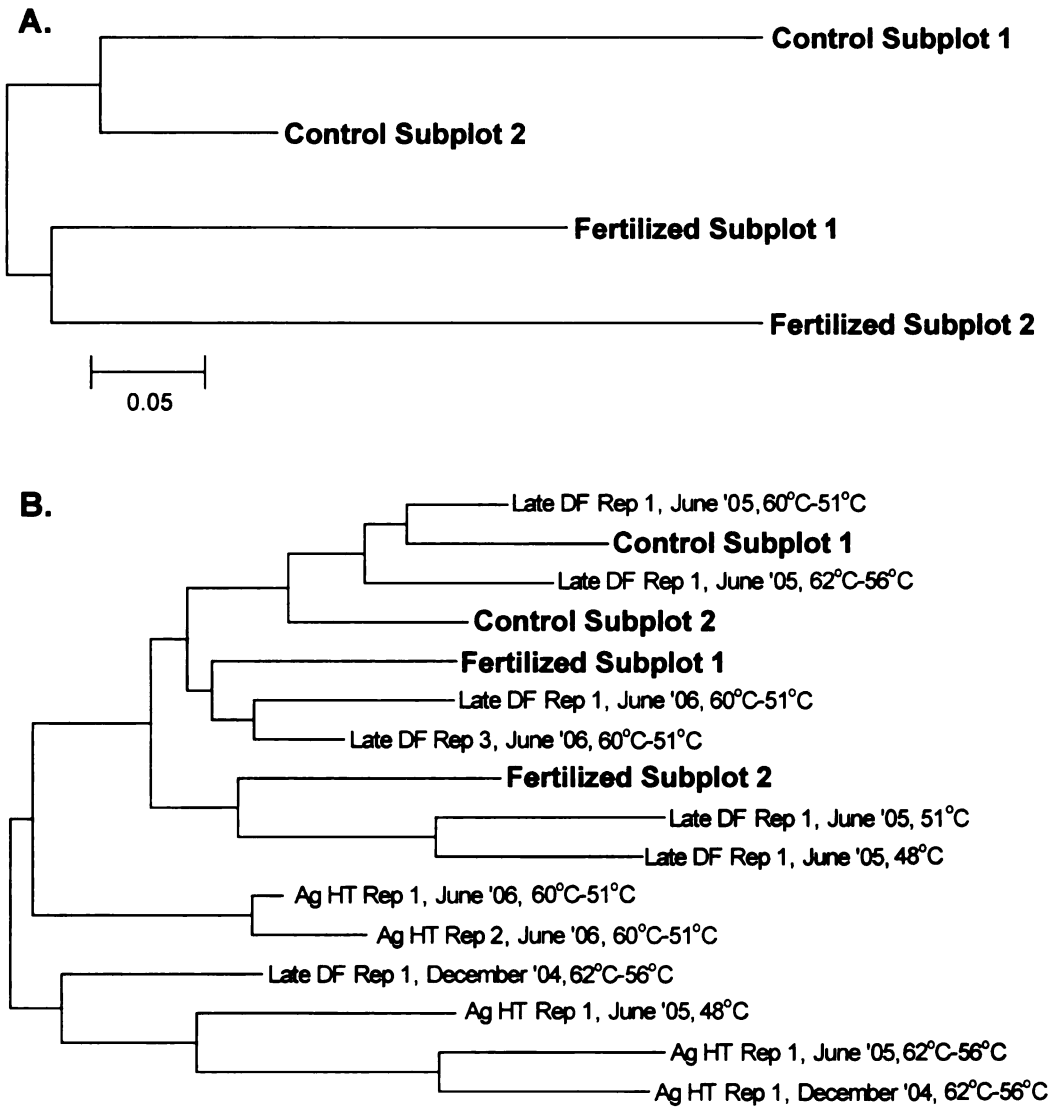

Figure 3.4. (A) Similarity of methanotroph communities among fertilized and control sub-plot replicates. (B) Similarity of methanotroph communities between fertilized and control sub-plot replicates with all other *pmoA* libraries from KBS LTER. Expect for the fertilized and control sub-plots, the *pmoA* clone libraries were first reported in Chapter 2, and details of their construction can be found there. Both dendograms are based on Sørenson index calculations for each pairwise comparison of the methanotroph communities using *pmoA* species (defined as *pmoA* sequences having 94% average nucleotide sequence similarity), and then clustered using neighbor-joining with MEGA (35). The scale bars represent at 0.05 change in the Sørenson index.

Figure 3.5. Neighbor joining phylogenetic tree of the partial nucleic acid sequences of *pmoA* and *amoA* from reference sequences, and KBS-LTER clone libraries from the control and fertilized sub-plots soil sampled in June 2007. The tree is rooted with the branching from the *amoA* sequences from *Nitrosomonas eutropha*, *Nitrosococcus mobilis*, and *Nitrosovibrio tenuis*. Nodes representing the 17 different methanotroph species (defined as *pmoA* sequences having 94% average nucleotide sequence similarity) found in late successional deciduous forest sub-plots are circled. The symbols in the circles reflect species' recovery from either the control treatment (●) or the fertilized treatment (■).

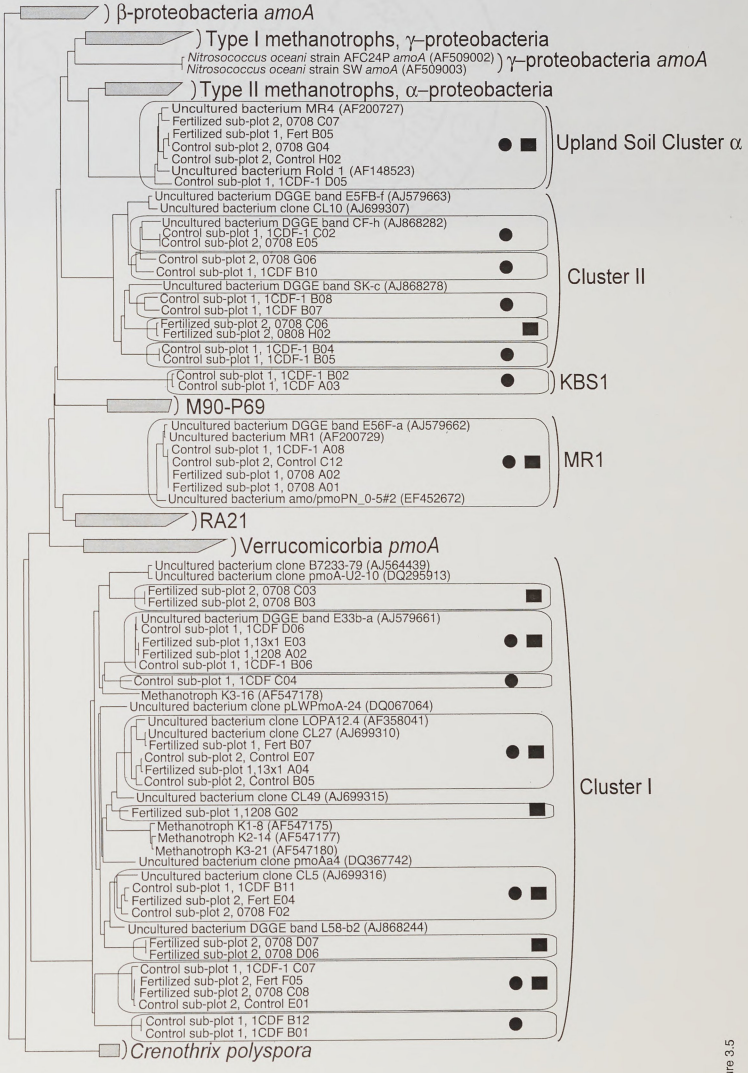
β-proteobacteria amoA
Type I methanotrophs, γ–proteobacteria
Nitrosococcus oceani strain AFC24P amoA (AF509002)
Nitrosococcus oceani strain SW amoA (AF509003)
γ–proteobacteria amoA
Type II methanotrophs, α–proteobacteria
Uncultured bacterium MR4 (AF200727)
Fertilized sub-plot 2, 0708 C07
Fertilized sub-plot 1, Fert B05
Control sub-plot 2, 0708 G04
Control sub-plot 2, Control H02
Uncultured bacterium Rold 1 (AF148523)
Control sub-plot 1, 1CDF-1 D05
Upland Soil Cluster α
Uncultured bacterium DGGE band E5FB-f (AJ579663)
Uncultured bacterium clone CL10 (AJ699307)
Uncultured bacterium DGGE band CF-h (AJ868282)
Control sub-plot 1, 1CDF-1 C02
Control sub-plot 2, 0708 E05
Control sub-plot 2, 0708 G06
Control sub-plot 1, 1CDF B10
Uncultured bacterium DGGE band SK-c (AJ868278)
Control sub-plot 1, 1CDF-1 B08
Control sub-plot 1, 1CDF B07
Fertilized sub-plot 2, 0708 C06
Fertilized sub-plot 2, 0808 H02
Control sub-plot 1, 1CDF-1 B04
Control sub-plot 1, 1CDF-1 B05
Cluster II
Control sub-plot 1, 1CDF-1 B02
Control sub-plot 1, 1CDF A03
KBS1
M90-P69
Uncultured bacterium DGGE band E56F-a (AJ579662)
Uncultured bacterium MR1 (AF200729)
Control sub-plot 1, 1CDF-1 A08
Control sub-plot 2, Control C12
Fertilized sub-plot 1, 0708 A02
Fertilized sub-plot 1, 0708 A01
Uncultured bacterium amo/pmoPN_0-5#2 (EF452672)
MR1
RA21
Verrucomicrobia pmoA
Uncultured bacterium clone B7233-79 (AJ564439)
Uncultured bacterium clone pmoA-U2-10 (DQ295913)
Fertilized sub-plot 2, 0708 C03
Fertilized sub-plot 2, 0708 B03
Uncultured bacterium DGGE band E33b-a (AJ579661)
Control sub-plot 1, 1CDF D06
Fertilized sub-plot 1,13x1 E03
Fertilized sub-plot 1,1208 A02
Control sub-plot 1, 1CDF-1 B06
Control sub-plot 1, 1CDF C04
Methanotroph K3-16 (AF547178)
Uncultured bacterium clone pLWPmoA-24 (DQ067064)
Uncultured bacterium clone LOPA12.4 (AF358041)
Uncultured bacterium clone CL27 (AJ699310)
Fertilized sub-plot 1, Fert B07
Control sub-plot 2, Control E07
Fertilized sub-plot 1,13x1 A04
Control sub-plot 2, Control B05
Uncultured bacterium clone CL49 (AJ699315)
Fertilized sub-plot 1,1208 G02
Methanotroph K1-8 (AF547175)
Methanotroph K2-14 (AF547177)
Methanotroph K3-21 (AF547180)
Uncultured bacterium clone pmoAa4 (DQ367742)
Uncultured bacterium clone CL5 (AJ699316)
Control sub-plot 1, 1CDF B11
Fertilized sub-plot 2, Fert E04
Control sub-plot 2, 0708 F02
Uncultured bacterium DGGE band L58-b2 (AJ868244)
Fertilized sub-plot 2, 0708 D07
Fertilized sub-plot 2, 0708 D06
Control sub-plot 1, 1CDF-1 C07
Fertilized sub-plot 2, Fert F05
Fertilized sub-plot 2, 0708 C08
Control sub-plot 2, Control E01
Control sub-plot 1, 1CDF B12
Control sub-plot 1, 1CDF B01
Cluster I
Crenothrix polyspora
0.10

Figure 3.5

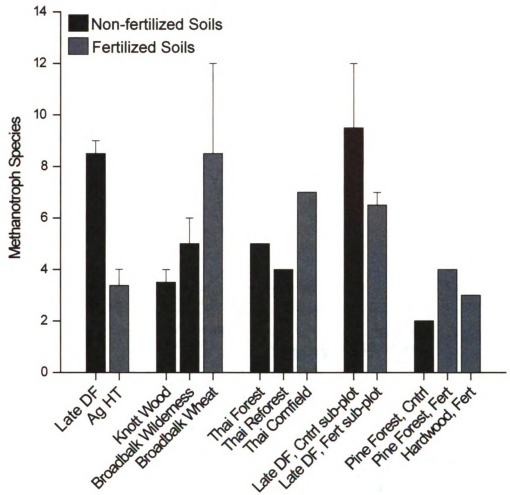

Figure 3.6. Methanotroph richness in paired sites featuring long-term fertilized (black shading) and non-fertilized soils (grey shading). The three sites to the left compare fertilized sites in the context of agricultural management, while the two sites on the right are fertilized forests whose only land management is fertilization. Error bars represent standard errors as the averages are reported for sites that featured replicated sites. Additional site details are provided in Table 3.1.

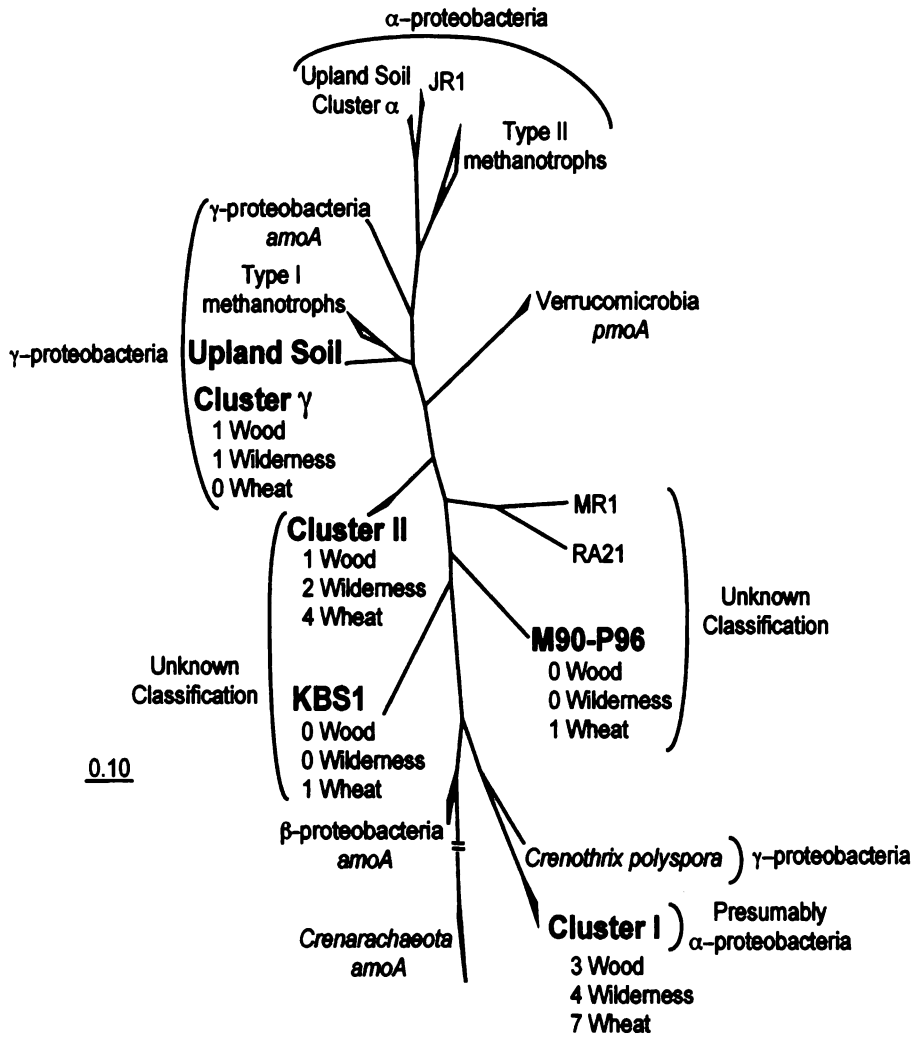

Figure 3.7. Phylogenetic tree of selected partial *pmoA* and *amoA* protein sequences from public databases and translated from PCR-based clone libraries from late successional deciduous forest sub-plots. The tree is based on 164 amino acid positions using Phylip Protein Maximum Likelihood as implemented in ARB (26). Bolded clades were recovered from Rothamsted Research soils. Beneath each bolded clade is a listing of the number of species within that clade found in which Rothamsted Research treatment: Knott Wood (Wood), Broadbalk Wilderness (Wilderness) and Broadbalk Wheat (Wheat). The scale bar represents 10 PAM units.

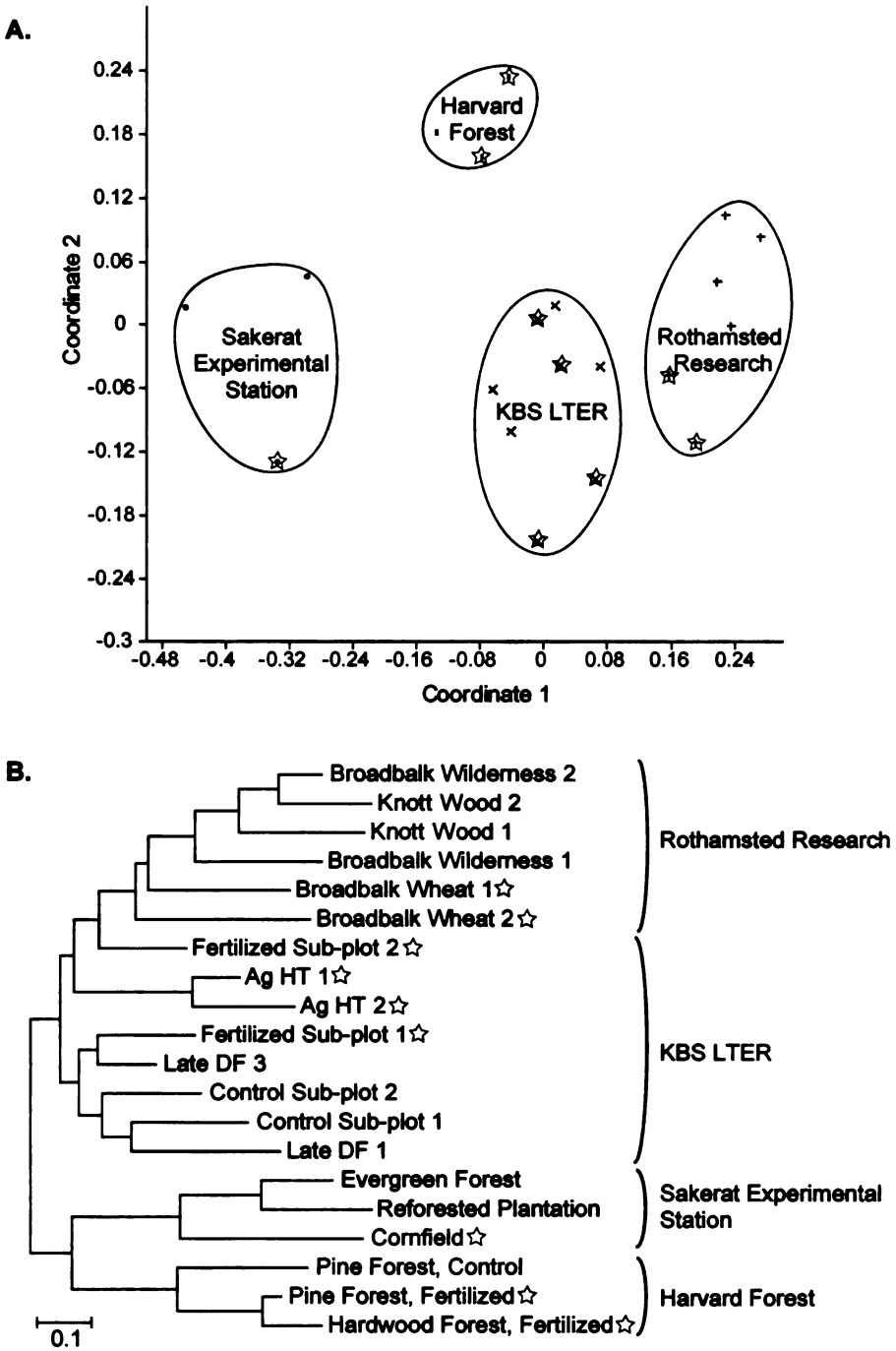

Figure 3.8. Similarity of methanotroph communities in paired sites featuring long-term fertilized and non-fertilized soils. (A) The Sørenson index was calculated for each pairwise comparison of methanotroph species using two-dimensional non-metric multidimensional scaling (21). (B) The same data as in (A), but clustered using neighbor-joining with MEGA (35) and displayed as a dendogram. Labels with a ☆ are sites that have been fertilized long-term. Additional site information is provided in Table 3.1.

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Chapter 4

Conclusions and Future directions

In this thesis there are multiple lines of evidence suggesting that multiple aspects of the methanotrophs' habitat are disrupted by row-crop agriculture at KBS LTER: (a) The decline of both methane consumption and methanotroph diversity in row-crop agricultural soils; (b) the 75 years required after the cessation of agricultural land management for methane consumption rates and methanotroph diversity at KBS LTER to achieve the current rate of methane consumption and diversity of the native soils (Chapters 2 and 3). An array of row-crop agriculture related factors, in addition to, and perhaps in interaction with long-fertilization that was investigated in Chapter 3, may all be disturbing the methanotroph community and are discussed below. Together, the net effect of row-crop agriculture at KBS LTER is the apparent destruction of methanotroph niches and subsequent loss of methanotroph richness and rates of methane consumption.

Each of the row-crop agriculture related factors could be explored in the future, as our ability to potentially manage lands to conserve or restore methanotroph diversity, and enhance the capacity of the methane soil sink, will rely on understanding the effect of these potentially pivotal variables on the methanotroph community. Only 1 of the *pmoA* phylogenetic clusters found at KBS LTER has not been reported in other upland soils (Chapter 2), and we recovered that cluster in the Broadbalk wheat soil at Rothamsted Research (Chapter 3). Therefore, while each sites' methanotroph community composition is unique (Chapter 3, Appendix C), at least some of the methanotroph species are going to be closely related to the methanotrophs that we find at KBS LTER. The discovery of the effects of land management practices to specific methanotroph

species would likely be applicable to other sites where the same or closely related species are present.

Previous studies have found that the common row-crop agricultural practices of tillage, fertilizer, pesticides, and herbicide application can negatively impact methane consumption (reviewed in (1)). These practices are all featured in KBS LTER's conventional row-crop agricultural treatment, and therefore may at least be partially causing the 7-fold land-use related decrease in methane consumption and methanotroph diversity observed in Ag HT (Chapter 2). In addition, land use related changes to soil properties like pH, water filled pores space, and dry bulk density may be contributing to the decline of methane consumption and the methanotroph community in Ag HT. Each has been shown to directly influence rates of methane consumption (reviewed by (1-3)), and changes in the methanotroph community have been found to be associated with different pHs (4, 5), changes in the successional stage of the plant community (Chapter 2 and (5, 6)), temperatures, and precipitation levels (7). None of these factors were investigated in the present study as we chose to only investigate long-term fertilization.

The short-term effect of tillage on methane consumption at KBS LTER was negligible (8), and led to the assumption that its long-term effect would be minor. However, reduced tillage has been shown increase methane consumption (reviewed by (1), and the disturbance to soil structure as a result of tillage is potentially a contributor to the 75-100 years it takes to for methane consumption rates and methanotroph richness to recover from agricultural land use (Chapter 2 and (3)), and with long-term fertilization's minor effects, tillage's long-term effect on methanotroph communities merits further investigation.

For instance, tillage may be changing the soil structure such that dry bulk density and water filled pore spaces change and cause less methane to be available to the methanotroph community. Increases in both dry bulk density and water filled pore spaces limit gas diffusion, and have been correlated to decreases in rates of methane consumption (2, 3). Dry bulk density at KBS LTER has only occasionally been measured previously at KBS LTER (http://lter.kbs.msu.edu), and therefore we cannot determine if either dry bulk density or water filled pore spaces (the determination of water filled pore spaces depends on the measure of dry bulk density) are affecting methane consumption.

Pesticide and herbicide application, other agricultural practices at KBS-LTER, have also been shown to negatively impact the rate of methane consumption at other sites (1, 9-11), and can alter the methano/methylotroph community (9). The magnitude of the chemical impact on methane consumption and the methanotroph community can greatly vary depending upon chemical and soil type (1, 9, 10, 12). For instance, long term application of atrazine and metolachlor, two herbicides among those used at KBS-LTER, has been found to not cause a difference in the rate of methane consumption, and to only cause minor changes in the composition of the methano/methylotroph community (10). Also, a study contrasting the effect herbicides and fertilization found that the methano/methylotroph community clustered according to the type of fertilization, and that methane consumption rates did not significantly decline due to herbicide treatment (12). The conventional agricultural soil at KBS LTER has been treated with a variety of chemicals (http://lter.kbs.msu.edu), so a definitive determination of herbicide or pesticide effects on the KBS LTER methanotroph community will be difficult. In

addition, the organic agricultural soil at KBS LTER despite having no herbicide or pesticide application since 1989 still has a low rates of methane oxidation (13); indicating that the influence of herbicide and pesticide application on rate and the methanotroph community is likely to minimal. However, herbicide or pesticide application may nevertheless be influencing the loss of methanotroph richness at KBS LTER.

Decreasing pH has also been correlated to reduced methane consumption (reviewed by (3)), but the reverse trend has been observed at KBS LTER. Neither rates of methane consumption nor methanotroph richness decline with pH at KBS LTER. The Late DF soils have the lowest pH (approximately 5.3, http://lter.kbs.msu.edu), but have the most methanotroph richness and the greatest rates of methane consumption, while the Ag HT soils have the highest pH (approximately 6.2) with the least methanotroph richness and the lowest rates of methane consumption. Notably, while declining pH is not affecting methanotroph richness at KBS LTER, it may be limiting methanotroph richness in the Rothamsted Research soils. There, Knott Wood has the least methanotroph richness, and the lowest pH (4.0) while Broadbalk wheat has the highest pH (6.7) as well as the most methanotroph richness (14, 15).

Future directions into determining the cause behind the decrease in methanotroph diversity and methane consumption associated with row-crop agriculture at KBS LTER might best be focused upon tillage, changes in soil structure, changes associated with the plant community (discussed in Chapter 2), and the possible interaction of these factors with fertilization and pesticide and herbicide application. Each of these directions would shed light on the niche for KBS LTER methanotrophs, and lead to insights that could

predict and explain the response of methanotrophs and, most importantly, rate of methane consumption to management changes at KBS LTER.

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Appendix A

Assessment of PCR bias

Introduction

To determine if relative abundance, in addition to richness, could be used in determining and comparing the methanotroph community structure, the bias from the polymerase chain reaction (PCR) that amplified *pmoA* in the tRFLP analysis and some of the clone libraries was assessed. PCR bias is the over-amplification of specific templates which results in the post-amplification concentrations of those templates being much greater then their pre-amplification concentrations (1, 2). If there is PCR bias then the abundance measures from PCR based community analyses are misleading, erroneous conclusions could be made regarding the dominant and rare organisms in a given community, and measures (i.e. diversity indices) that rely on relative abundance will be skewed.

The methanotroph community analyses in this thesis (Chapters 2-3) utilize the A189-A682 primer pair (3) to amplify *pmoA*. Any PCR bias associated with the primer pair has not been quantified, nor has the consistency of the output from the primers and the specific PCR conditions been addressed previously. Thus, PCR bias was assessed with tRFLP profiles whose initial templates were defined artificial communities of *pmoA* and *amoA* (The A subunit of the ammonia monooxygenase (*amoA*) is also amplified by A189-A682) that varied the concentration of initial template. Ideally, if there is PCR bias it will be consistent throughout the mixtures so that despite the bias the relative abundances measures could be included in the community analyses of the *pmoA*

community. While not suitable for quantitative comparisons, the measures would be the same regardless of concentration changes within the mix, justifying their usage.

Methods

The template for amplification reactions was 4 different mixes of purified plasmids, and each of the purified plasmids. The plasmids contained *pmoA* PCR products from soils at the Kellogg Biological Station Long Term Ecological Research site TOPO TA cloned into vector pCR 4 or pCR 2.1 (Invitrogen, Carlsbad, CA) (Chapter 2). The plasmids represented 6 *pmoA* species and 1 species of the A subunit of the ammonia monooxygenase (*amoA*), which A189-A682 also amplifies. The plasmids were mixed such that the concentrations of all the species were held approximately constant except for one *pmoA* species, Cluster I A (Table 1). Each mix was run as a tRFLP either 2 or 3 times with each tRFLP beginning with new PCR amplifications.

The PCR reactions contained a total of 30 pg of template, 1.25 μl 1% BSA, 10 mM dNTPs, 0.2 μM of each primer (A682 was labeled with fluorophore 6-carboxyfluorescein (6-Fam)), 2.5 μl 10x PCR buffer (200mM Tris-HCl (pH 8.4) and 500 mM KCl), 0.5 μl 50 mM $MgCl_2$ and 1.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) in a total volume of 25 μl. Reaction conditions were 95°C for 5 minutes, 15 cycles of a 'touchdown PCR' of 95°C for 1 minute, 62°C for 1 minute (-0.4°C each cycle to 56°C), and 72°C for 1 minute, 15 cycles using 56°C as the annealing temperature, and a final 10 minute extension at 72°C.

For each tRFLP, 4 replicate PCR reactions were pooled. PCR products were purified using a MinElute column (Qiagen, Valencia, CA). In a 50 μl reaction, 300ng of

purified product was digested with 1.5 U Tau I (Fermentas, Glen Burnie, MD) by incubating at 55°C for 1 hr 30 min. To inactivate the enzyme the DNA was precipitated as follows: The sample was diluted to 500 µl, followed by the addition of 50 µl 3M sodium acetate, 1 µl or 2.5 µl 10 mg/ml glycogen, and 500 µl isopropanol, and holding on ice for at least 5 minutes. The DNA was then pelleted by centrifugation at 16,000 x g for either 5 (1µl glycogen) or 10 minutes (2.5 µl glycogen). The supernatant was decantated, and the pellet washed with 500µl of 80% ethanol followed by centrifugation for 2 minutes at 16,000 x g, and removal of the supernatant. After a 30 second centrifugation additional ethanol was removed, and the DNA was air dried for 5-10 minutes before resuspension in 20 µl of water. In an 18-22 µl reaction 140 ng of DNA was digested with 2.5 U SspI (Neb, Ipswich, MA) in a 1hr 30min incubation at 37°C. After heat inactivation at 65°C for 20 minutes 6 U of BstUI (Neb, Ipswich, MA) was added, incubated at 60°C for 1hr 30min, and inactivated by adding 0.8 µl of 0.5 mM EDTA. Capillary electrophoresis of the tRFLP reactions was then performed with a 5 fu cutoff at RTSF.

Individual peaks were distinguished from the background signal and binned using TRFLP-Stats (4). In TRFLP-Stats default settings were used except for the standard deviation cutoff, which was increased to 4.5. The resulting cutoff was approximately 25 fu. The tRFLP profiles of the individual plasmids ensured that the specific bin belonging to each species was correctly identified. Any other peaks were excluded from the analysis, and the relative abundance of the remaining peaks were normalized such that the absolute abundance of every treatment was 100. The amount of relative abundance

excluded from any one tRFLP profile before normalization was between 0-11% with all tRFLPs except one having <6% excluded.

Results

The *pmoA* species Cluster I B was consistently over-amplified in every mix, while species Cluster I A was over-amplified except for when its initial abundance was less then 10% of the initial artificial community (Table 1). The over-amplification of Cluster I A and Cluster I B species was dramatic, and in some mixes was approximately double their expected output (Figure 1, Mixes 2-4). In addition, when Cluster I A's concentration in the artificial community was increased, its relative abundance in the output also increased. However, the increase was not proportional, and increasing initial concentrations of Cluster I A led to increased over-amplification. For instance, between Mix 2 and Mix 4, the concentration of Cluster I A in the output was expected to increase 2.3 fold, but its actual output increased 2.9 fold.

Cluster II A was the only species whose actual output was consistently close to its expected output, although when Cluster I A was expected to be less then 10% of the output (Mix 1), it too was over-amplified (Table 1). *pmoA* species Cluster II B was also overamplified in Mix 1 but otherwise, it, along with *pmoA* speices Cluster I C, Cluster II D, and *amoA* species A, was under-amplified (Figure 1, Table 1). Cluster I C had the lowest amount of starting template in all the mixes, and its under-amplification resulted in the species not being recovered in two mixes (Mix 1 and 4), and *amoA* species A was also not recovered in Mix 4.

Discussion

The cause of the observed PCR bias is unclear. It is possible that it is at least partially caused by the different binding energies from the degenerate positions within the primers (1). The GC content differences from the degenerate positions can result in a 2°C difference in annealing temperature, and subsequently a greater proportion of GC-rich template can bind to the primers and cause over-amplification as compared to primers with A or T at those positions (1). Due to the ambiguous positions there are 16 template sequences that a reverse primer could bind, and 4 sequences that a forward primer could bind. Only 2 of the species in this experiment (species Cluster II B and Cluster II D) actually have identical primer binding sites. As a result, the likelihood of the primer binding sites having different binding energies is high, and is possibly contributing to the PCR bias.

However, if the degenerate primers were the primary cause of the bias we would expect to see (1) all GC-rich primer binding sites over-amplified, and (2) consistency in the pattern of PCR bias as the same species should be always be over-or under-amplified. We do not see either. With the exception of the consistent over-amplification of species Cluster I B, the results for all species were inconsistent; with some species being under-amplified in some mixes and over-amplified in others (i.e. Cluster II B). In addition, Cluster I B only has A or Ts in degenerate positions, and not G or Cs. We therefore conclude that the primer pair A189-A682 is not the primary cause of the PCR bias, and its biases are likely no worse than most other primer pairs.

Regardless of the underlying cause, the inconsistent pattern of over- and under-amplification argues for the exclusion of the relative abundance data. Supporting that

conclusion is that as the concentration of the Cluster I A species increased with each mix, its over-amplification became worse. This is disconcerting because it indicates that communities that are seemingly dominated by an over-amplified species are more likely to have erroneous measures of relative abundance. The over-amplified Cluster I A dominates the tRFLP profiles from Ag HT (Chapter 2), and therefore the relative abundance measures from the Ag HT methanotroph communities are those that are most likely to be erroneous due to the PCR bias.

For instance, the first replicate of Ag HT had Cluster I A at 98% relative abundance, and Cluster II A and MR1 were both at 1% relative abundance. Looking at that result, or if put into a diversity index (i.e Simpson or Shannon), the presence of Cluster II A and MR1 would be discounted due to Cluster I A's dominance of the community. However, the PCR bias indicates that the 98% relative abundance is likely over-amplified at least 2-fold, so the true relative abundance of Cluster 1A is probably closer to 50%. If Cluster II A and MR1 are being under-amplified, as our results indicate for Cluster II A (see Mixes 3 and 4; MR1 was not included in this experiment), then their true relative abundances are much higher then 1%. Therefore, the fairest and most representative way to present the data is to just consider whether a species is present or absent.

Despite species Cluster I C not being recovered in two mixes, its lack of recovery was not associated with either high or low species Cluster I A input; indicating that the loss of its presence may have been independent of the amount of bias in a reaction. Mix 1, with the least over-amplification, did not recover cluster I C, and neither did Mix 4, which had the greatest over-amplification of species Cluster I A. Communities with

highly over-amplified species are expected to have an increased chance of not being able to recover methanotroph OTUs (as shown by the *amoA* A species being recovered in all mixes but Mix 4), but the inability to recover Cluster I C in the least biased mix suggests that the recovery of methanotroph OTU or species that are in low abundance is variable, and just as possible in communities with limited PCR bias and those with considerable PCR bias. Considering that 5 tRFLP profiles were summed to represent one replicate (Chapter 2), the expectation of recovering a low-abundance species even in treatments with the highest over-amplification of species Cluster I A is reasonable.

Therefore, due to the PCR bias and the presence of nearly all methanotrophs in each mix, all methanotroph community analyses are presented only with presence/absence data (richness, Sørenson index), and exclude the relative measures of methanotroph abundance. The over-amplification of *pmoA* species Cluster I A and Cluster I B, and the considerable deviation of all species from their expected output led to the conclusion that abundance measures should not be included in the tRFLP or clone library community analyses of the *pmoA* community. If future investigators can identify more reproducible amplification conditions, then the use of the relative abundance measures could be justified and include in the analyses of the methanotroph community.

Table A.1. Composition, expected output and actual output of defined artifical communites of *pmoA* and *amoA* species used to assess the PCR bias of the primer pair A189-A682.

	***pmoA* or *amoA* Species**	**Amount of Template DNA in the reaction (pg)**	**Relative abundance of the Expected Output (%)**	**Average Relative abundance of the Actual Output (%)**	**Standard Error**
Mix 1	*pmoA* Cluster I A	2.5	8	9	1
	pmoA Cluster I B	4.9	16	30	1
	pmoA Cluster I C	2.9	10	0	0
	pmoA Cluster II A	4.9	16	24	1
	pmoA Cluster II B	4.9	16	22	1
	pmoA Cluster II D	4.9	16	10	2
	amoA A	4.9	16	4	2
Mix 2	*pmoA* Cluster I A	4.6	15	24	3
	pmoA Cluster I B	4.6	15	42	7
	pmoA Cluster I C	2.7	9	2	2
	pmoA Cluster II A	4.6	15	17	4
	pmoA Cluster II B	4.6	15	7	1
	pmoA Cluster II D	4.6	15	3	3
	amoA A	4.6	15	2	2
Mix 3	*pmoA* Cluster I A	6.3	21	44	10
	pmoA Cluster I B	4.2	14	31	1
	pmoA Cluster I C	2.5	8	2	2
	pmoA Cluster II A	4.2	14	10	2
	pmoA Cluster II B	4.2	14	4	0
	pmoA Cluster II D	4.2	14	4	4
	amoA A	4.2	14	3	3
Mix 4	*pmoA* Cluster I A	10.5	35	70	4
	pmoA Cluster I B	3.5	12	19	6
	pmoA Cluster I C	2.1	7	0	0
	pmoA Cluster II A	3.5	12	6	0
	pmoA Cluster II B	3.5	12	4	1
	pmoA Cluster II D	3.5	12	1	1
	amoA A	3.5	12	0	0

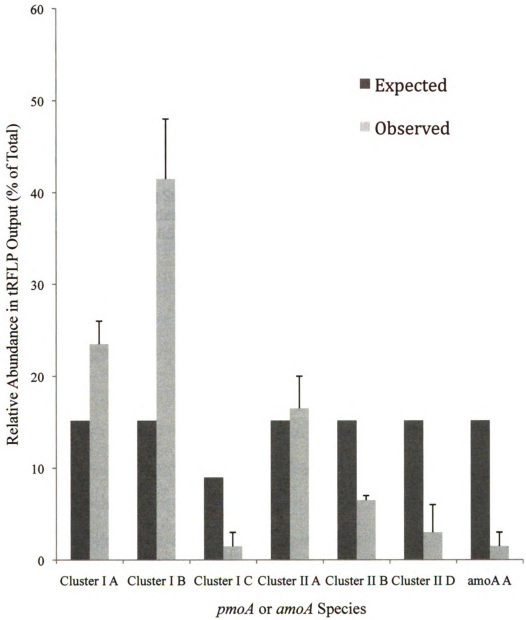

Figure A.1. Expected and actual relative abundance of the artificial *pmoA* and *amoA* community Mix 2.

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Appendix B

Ammonia and Nitrate Before and After Fertilization in a KBS LTER Fertilized Sub-plot

Introduction

In addition to sampling one of the KBS LTER Late DF fertilized sub-plots, we also established a 10x Fertilized sub-plot. The 10x sub-plot was designed to allow investigation into the short-term effect of fertilization on the methanotroph community, but its community was not investigated after we found that long-term fertilization had no effect on methanotroph richness (Chapter 3). However, the results from the nutrient analyses of the inorganic N from the 10x sub-plots from before and after fertilization is presented here to illustrate that the nitrogen in the applied urea fertilizer is converted to ammonia that can be recovered from the soil. Even though we have no direct evidence that the ammonia from the fertilization inhibits the activity from the10x sub-plot methanotrophs, we can confirm that the ammonia concentration after urea fertilization in the soil is high enough to be predicted to inhibit the rate of methane consumption. In addition, the data is also provided to illustrate the turnover of ammonia to nitrate that is indicative of considerable nitrification in the 10x sub-plot soils.

Methods

A 2x2 m sub-plot was established in each Late DF replicate adjacent to the other fertilized sub-plots. The 10x sub-plot received one application of 33 g N m^{-2} yr^{-1} as urea, which was applied using a backpack sprayer on June 5th 2007. Three soil cores (2.5 x 10 cm) were collected from all 3 replicates on the following dates: June 1st 2007, June

13th 2007, June 27th 2007, August 14th 2007, and October 17th 2007, representing 4 days before fertilization, and 8, 22, 47 and 111 days after fertilization.

Approximately 10g of each soil core was extracted with 100ml of 1 M KCl, and prepared for analysis on a Flow Injector Analyzer as per the protocol available at http://lter.kbs.msu.edu/ (Soil Inorganic N). The three measures from each plot were then averaged to be a composite measure for that replicate, and the average of three biological replicates is represented in the Figure B.1.

Results and Discussion

In the 10x sub-plot, 8 days after fertilization, a big spike in ammonia concentrations was observed as the enzyme urease quickly converted urea to ammonia (1, 2). The concentration of ammonia at that date was 8 NH_4-N ppm (or 8 μg NH_4-N g soil^{-1}). That concentration, based on modeling by Hütsch (3) is predicted to inhibit methane consumption. By 22 days after fertilization, nitrate concentrations have begun to rise as the ammonia is nitrified, and by the third measurement, 47 days after fertilization, nitrate has peaked, and concentrations of ammonia have fallen. This conversion of most of the ammonia to nitrate in 47 days after fertilization is evidence of robust nitrification, and probably lessens the effect of the fertilization disturbance upon the methanotroph community as exposure to ammonia may be relatively limited. Therefore, we conclude that the urea fertilizer was converted rapidly to ammonia, the concentrations of ammonia were high enough to have inhibited methane consumption, and ammonia was quickly nitrified to nitrate.

Acknowledgments

Neville Millar was an equal partner in the soil extractions, and was responsible for running the samples through the Flow Injector Analyzer.

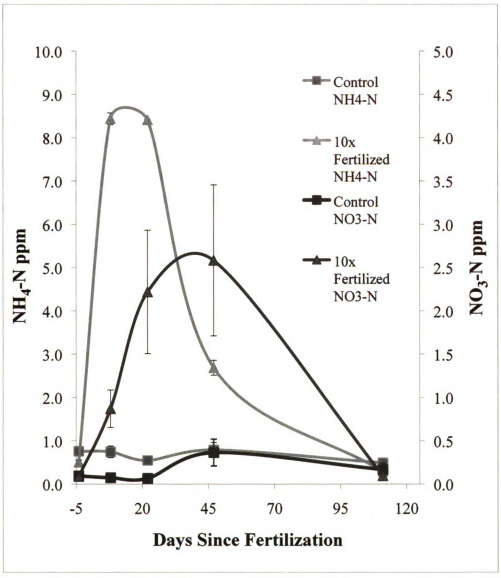

Figure B.1. Ammonia and nitrate concentrations in fertilized and control Late DF subplots at KBS LTER. Error bars represent the standard error.

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Appendix C

Biogeography of Methanotrophs in Well Drained Soils

Introduction

To confirm and extend the biogeography observed in the long-term fertilized sites in Chapter 3 (Figure 3.8), and to determine the biogeography of methanotrophs in well drained soils, a meta-analysis was performed using 27 clone libraries of the A subunit of the particulate methane monooxygenase (*pmoA*) (Table C.1). *pmoA* is found in all known methanotrophs (1) except two *Methylocella* strains (2). Due to the near ubiquity of *pmoA*, and the inference of function from the presence of the gene, it was chosen over comparing libraries that assess the methanotroph community via 16S ribosomal genes.

Methods

Using only *pmoA* sequences allowed for all of the sequences to be imported into Arb (3) where they were translated and aligned using Clustal W. Nucleic acid sequences were then aligned according to the protein sequence. Sequences from clone libraries were determined to be the same species if they were $\geq$ 94% identical (4) as determined by DOTUR (average neighbor grouping) (5). In order to facilitate the comparison between the methanotroph communities of the different soils species were used to calculate β-diversity with the Sørenson index (PAST, (6) and EstimateS (http://viceroy.eeb.uconn.edu/EstimateS)). The clustering between the communities was visualized using a neighbor-joining dendogram.

Only *pmoA* libraries from well drained soils that consume atmospheric methane were included in the analysis. Libraries from environments that are net sources of

atmospheric methane like landfill cover soils, mine soil, rice paddies, wetlands, etc, were excluded from the analysis. If a study from a well drained soil included a library from an experimental treatment of the same land use type (e.g. increased atmospheric CO_2; (7)) that library was excluded, and only the *pmoA* libraries from the control treatments were included. Only *pmoA* libraries that had at least 10 sequences were included in order to ensure that under-sampled methanotroph communities would not confound the analysis. Doing so excluded many *pmoA* libraries that were based on results from denaturing gradient gel electrophoresis (i.e. (8, 9)), and the Harvard forest and Sakerat Experimental Stations clone libraries included in the biogeography analysis of long-term fertilized sites (Chapter 3).

Included in the final analysis were 27 *pmoA* libraries from well drained soils from Germany, the United Kingdom, the United States, and New Zealand (Table C.1). The libraries were from 9 studies (Chapter 2, Chapter 3, (7, 10-14)) , and included a total of 1,560 *pmoA* sequences from a variety of forest, pasture, shrub land and agriculture soils.

Results and Discussion

The meta-analysis of methanotroph community compositions in well drained soils (Figure C.1) revealed a distinct biogeography, and confirmed the patterns seen in Chapter 3 with long-term fertilized sites. Methanotroph communities generally clustered according to geographic location. The two exceptions to that pattern was 1 library from a German forest clustering with the Rothamsted Research libraries, and the two Hawaii soils not sharing any species, and therefore clustering separately. The Hawaii forests are

on soils that vary in age by approximately 50,000 years so it is not surprising that those libraries did not conform to the pattern of biogeography evident elsewhere (Table C.1).

Table C.1. Summary of the Sites and *pmoA* libraries used in this study.

Site	Land Use	Country	Number of pmoA sequences	Reference
Kellogg Biological Station LTER[1]	Ag HT, Rep 1[2]	United States	228	Chapter 2
Kellogg Biological Station LTER[1]	Ag HT, Rep 2	United States	52	Chapter 2
Kellogg Biological Station LTER[1]	Late DF, Rep 1[3]	United States	446	Chapter 2
Kellogg Biological Station LTER[1]	Late DF, Rep 3	United States	48	Chapter 2
Kellogg Biological Station LTER[1]	Late DF, Control Sub-plot Rep 1	United States	66	Chapter 3
Kellogg Biological Station LTER[1]	Late DF, Control Sub-plot Rep 2	United States	71	Chapter 3
Rothamsted Research[4]	Knott Wood, Rep 1	United Kingdom	32	Chapter 3
Rothamsted Research[4]	Knott Wood, Rep 2	United Kingdom	31	Chapter 3
Rothamsted Research[4]	Broadbalk Wilderness Rep 1	United Kingdom	42	Chapter 3
Rothamsted Research[4]	Broadbalk Wilderness Rep 2	United Kingdom	28	Chapter 3
Rothamsted Research[4]	Broadbalk Wheat, Rep 1	United Kingdom	45	Chapter 3
Rothamsted Research[4]	Broadbalk Wheat, Rep 2	United Kingdom	13	Chapter 3
Gottingen Forest	Forest	Germany	10	Ricke et al 2005 (12)
Puruki	Pine	New Zealand	33	Singh et al. 2007 (11)
Puruki	Pasture	New Zealand	36	Singh et al. 2007 (11)
Westview	Pine	New Zealand	46	Singh et al. 2007 (11)
Westview	Pasture	New Zealand	45	Singh et al. 2007 (11)
Waiouru	Shrubland	New Zealand	33	Singh et al. 2007 (11)
Waiouru	Pasture	New Zealand	39	Singh et al. 2007 (11)
Craigieburn Range	Old-growth Beech Forest	New Zealand	41	Singh and Tate 2007 (10)
Gisburn	Alder Stand	United Kingdom	10	Reay et al. 2001 (13)
Gisburn	Oak Stand	United Kingdom	59	Reay et al. 2001 (13)
Gisburn	Norway Spruce Stand	United Kingdom	21	Reay et al. 2001 (13)
Gisburn	Scots Pine Stand	United Kingdom	23	Reay et al. 2001 (13)
Jasper Ridge	Grassland[5]	United States	32	Horz et al. 2005 (7)
Kilauea volcano, Chain of Craters Road	Mixed *Metrosideros* Forest[6]	United States	19	King and Nanba 2008 (14)
Mauna Kea lava flow	Mixed *Acacia* Forest[7]	United States	11	King and Nanba 2008 (14)

[1] Replicates refer to 1 ha biological replicates.

[2]Represents the summing of 4 individual libraries (Chapter 2) with an average richness of 2.8 methanotroph species.

[3]Represents the summing of 6 individual libraries (Chapter 2) with an average richness of 9 methanotroph species.

[4] Replicates refer to replicate soil cores.

[5] Ambient levels of CO_2, temperature, precipitation, and nitrogen.

[6] The forest is located on a >50,000 year old lava flow.

[7] The forest is approximately 300 years old.

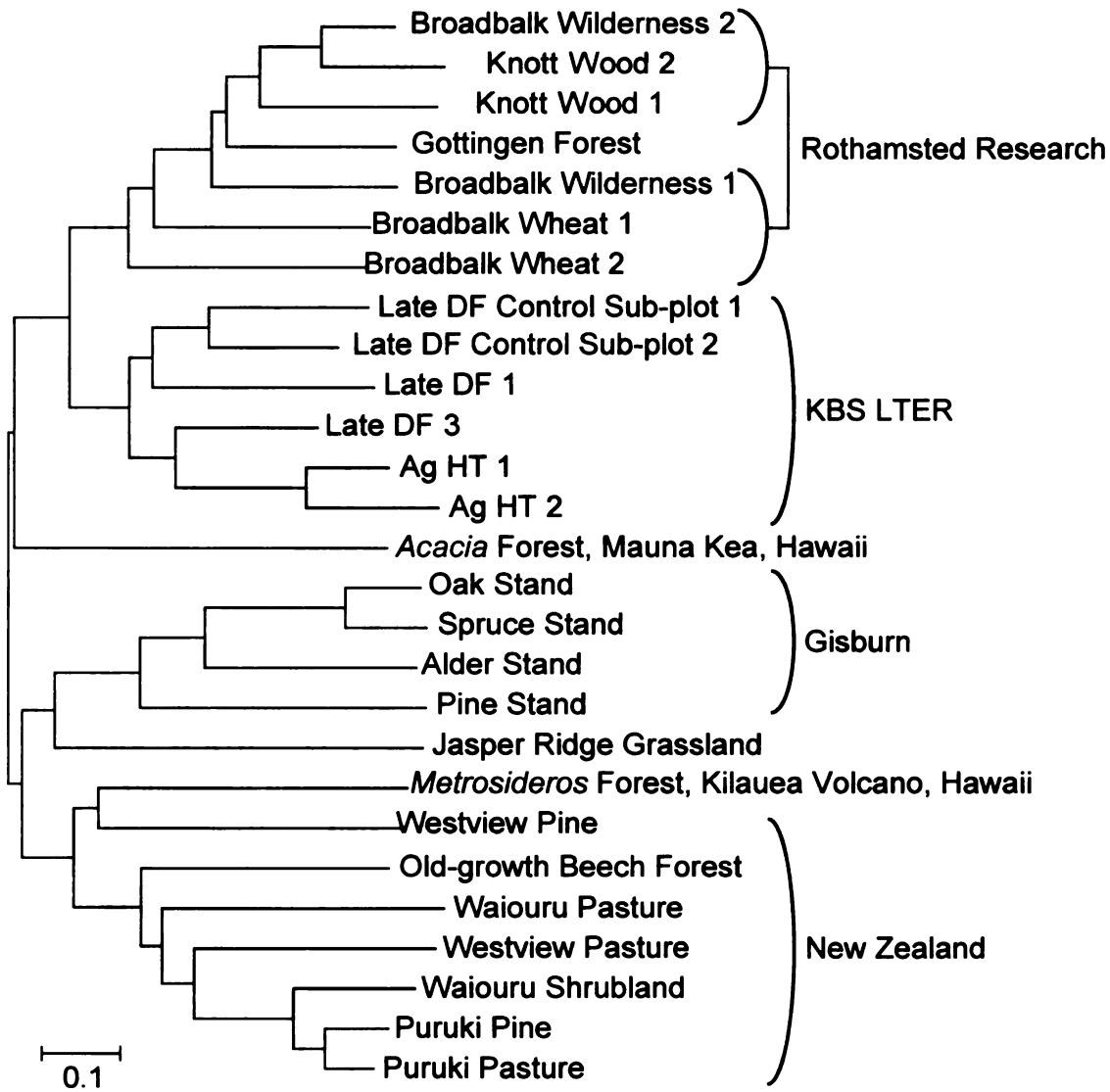

Figure C.1. Similarity of methanotroph communities in soils from around the globe. The dendogram is based on Sørenson index calculations for each pairwise comparison of the methanotroph communities using *pmoA* species (defined as *pmoA* sequences having 94% average nucleotide sequence similarity), and then clustered using neighbor-joining with MEGA (48). The scale bars represent at 0.1 change in the Sørenson index. Additional site information is provided in Table C.1.

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