THESIS

IMPACT OF METHANOTROPH ECOLOGY ON UPLAND METHANE BIOGEOCHEMISTRY IN GRASSLAND SOILS

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ABSTRACT

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Molecular assays of soil environments reveal tremendous microbial diversity, but it remains unclear how this diversity might be mechanistically linked to the ecology of the organisms and their biogeochemical function. Methane consumption in upland soils is arguably the simplest biogeochemical function, and there are emerging patterns in the diversity and biogeography of the organisms that carry out soil methane consumption. This simplicity may allow methane uptake in upland soils to be a model system for merging microbial ecology, diversity and biogeochemistry. Five key traits appear critical for methanotroph ecology: enzyme kinetics, nutrient demand, pH tolerance, ammonium sensitivity and desiccation tolerance. Unfortunately, few studies to date have examined the functional consequences these traits may have on methane consumption. Here, I present analysis of methanotroph community

composition and Michaelis-Menten kinetics of methane uptake across three North American temperate grassland sites of differing soil moisture regimes. Across this gradient, I observed distinct variation in community composition, and significant changes in enzyme kinetics. In addition, I find that differences in field estimates of methane activity parallel the patterns of Michaelis-Menten assays, which in turn correlate with differences in methanotroph community composition. These correlations suggest that methanotroph community composition alters ecosystem function.

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1. Introduction

Methane is a potent greenhouse gas, with 26 times greater radiative forcing than CO_2 (Lelieveld et al. 1993), but due to its lower atmospheric concentration it is second to CO_2 in actual radiative forcing (Forster et al. 2007). Upland (i.e., well-drained, oxic) soils are a net sink for atmospheric methane; as methane diffuses from the atmosphere into these soils, methane consuming (i.e., methanotrophic) bacteria oxidize it. At a global scale, soil uptake is the most important biological sink of atmospheric methane, offsetting emissions by about 30 Tg y^{-1} (Denman et al. 2007). Without this sink, Ojima et al. (1993) estimated that atmospheric methane through the 1990's would have increased at 1.5x its observed rate.

Spatial and temporal variability in upland soil sink strength is driven by both physical and biological mechanisms. Physically, methane uptake is affected by the soil's diffusivity, or capacity to conduct gas transport via diffusion (Castro et al. 1995, Ball et al. 1997). Gas diffusivity is, in turn, affected by both soil structure (Dörr et al. 1993) and water content (Mosier et al. 1996, Bowden et al. 1998). Biologically, the oxidative activity of methanotrophic bacteria declines at low soil moisture due to desiccation stress (von Fischer et al. 2009). In addition, studies have repeatedly observed that increased

soil nitrogen levels reduce methane uptake rates (Hütsch et al. 1994, Mosier et al. 1996), and the strength of reduction has been shown to depend on the chemical form of nitrogen (Reay and Nedwell 2004, Dunfield and Knowles 1995). This effect appears to be driven, in part, by biochemical inhibition of the methane mono-oxygenase enzyme by ammonium (Dalton 1977,Carlsen et al. 1991, Dunfield and Knowles 1995).

A number of more poorly understood biological and ecological properties of methanotroph communities could also affect methane uptake rates. The total methane oxidizing activity of the methanotroph community is influenced by both community size and the per-capita activity. Total community size depends on the balance between growth and mortality while per capita activity can be characterized by the Michaelis-Menten kinetics of their methane mono-oxygenase enzymes. Very little work has documented differences in methanotroph community biomass among sites or over time, with a few researchers showing changes in community and biomass under lab manipulated conditions (Henckel et al. 2000, Maxfield et al. 2006). More has been done to characterize the range of Michaelis-Menten kinetics among soils or across cultured strains (Gulledge et al. 2004, Knief and Dunfield 2005, Pawlowska and Stepniewski 2006), but because we cannot yet predict the Michaelis-Menten kinetics of methane uptake of a particular soil's methanotroph community, it has not been possible to build these kinetic differences into methane biogeochemistry models.

Surprisingly, there has been little work conducted thus far to evaluate how the soil methane sink will respond to future climatic change; all of the IPCC scenarios assume a constant soil methane sink (IPCC 2007) despite the fact that global change has potential to alter the rate that methane diffuses into the soil (via alteration of the soil moisture

regime of ecosystems). Moreover, as levels of atmospheric methane continue to rise, there is potential for a "methane fertilization effect" to increase the soil sink strength via coupled physical and biological mechanisms. Physically, the fertilization effect can be driven by increased diffusion of methane into soils due to a stronger atmosphere-soil concentration gradient (Ojima et al. 1993). More specifically, as methane concentrations rise at the soil surface, the diffusion of methane into to soil increases resulting in methanotrophs experiencing more methane. Biologically, a greater supply of methane to soil methanotrophs can increase their biomass and thus elevate the total levels of methanotroph activity, and potentially change methanotroph community composition.

Therefore, the challenge to predicting future methane biogeochemistry is to incorporate both physical and biological controls into next-generation models of methane uptake. Because the biological controls depend upon both instantaneous (physiological) responses and longer-term ecological and biogeographic mechanisms, there emerges a need to better integrate the growing evidence that methanotrophs show geographic distributions, that methanotrophs differ in their responses to environmental variation, and that these variations are biogeochemically important.

2. Review

2.1 Methane uptake in upland soils

Methane uptake in upland soils varies both spatially and temporally (Mosier et al. 1996, Epstein et al. 1998). To evaluate the controls on variability in methane uptake rates, Del Grosso et al. (2000) assembled methane consumption datasets from 28 field sites covering natural and managed systems, including temperate grasslands and forests and

tropical grasslands. They found that methane uptake rates were highest at intermediate water content, and that the uptake rates also responded to temperature and agricultural influence. The hump-shaped response of methane uptake to soil moisture (Fig. 1) arises from the separate effects of moisture on gas transport and methanotroph activity. Soil diffusivity declines with increasing soil moisture, as soil pores fill with water and limit the free movement of gases (Hilell 1998, Moldrup 2003). The reduction in methane uptake rates at low soil moisture (left side of hump in Fig. 1) is a persistent feature in xeric systems (e.g., Schnell & King 1996, Gulledge & Schimel 1998, von Fischer et al. 2009) where desiccation stress restricts the activity of soil microbes (Potts 1994), including methanotrophs.

Despite the recognition that both methanotroph activity and diffusivity jointly control the rate of methane consumption in upland soils, there remain a surprisingly small number of studies where *both* methane consumption and diffusivity are measured in the field. In one of the few such studies, Ball et al. (1997) measured soil diffusivity and methane uptake in Scottish forests, tilled fields and pastures. In these mesic systems (mean gravimetric water content = 24%), Ball found that differences in methane uptake rates among study sites were correlated with the diffusivities of the sites, but that changes in methane uptake within sites (i.e., temporal patterns) were generally not related to diffusivity. Instead, the variation was attributed to variation in the activity of methanotrophic bacteria. Similarly, when von Fischer et al. (2009) measured both soil diffusivity and methane uptake in xeric grasslands in Colorado, desiccation stress to methanotrophs was found to be the primary driver of temporal variability in methane uptake rates.

2.2 Methanotrophs

Methanotrophs are highly specialized bacteria, defined by their obligate use of methane as a carbon and energy source, with oxidation of this substrate catalyzed by methane monooxygenase (MMO) enzymes (Hanson & Hanson 1996, Bowman 2006). What we know of methanotroph diversity was based initially on culturing these organisms in the laboratory. Phylogenetic analysis of the gene that codes for the 16S subunit of RNA in cultured strains revealed two major lineages of methanotrophs: Type I methanotrophs that fall within the α-proteobacteria, and Type II methanotrophs that are γ-proteobacteria (Hanson & Hanson 1996, Bowman 2006). Culture dependent methods are inherently biased by the fact that some bacterial strains are easier to culture than others (e.g., Vorob'ev and Dedysh 2008), and so it remained unclear if the soil organisms responsible for methane uptake really were represented by those that had been cultured. To address this question, several groups have used stable isotope probing (SIP) to identify the active methanotrophs in soils and sediments (reviewed by McDonald et al. 2005). In SIP experiments, soils are incubated in the presence of ^{13}C-labeled methane, and then the DNA is extracted and centrifuged in a density gradient solution so that the heavier ^{13}C-labeled DNA, which was synthesized from the $^{13}CH_4$, can be physically separated and sequenced. Thus far, the vast majority of organisms identified from methane SIP experiments show close relationship with the cultured strains, supporting the notion that molecular techniques developed on cultured methanotrophs will also target uncultured strains (McDonald et al. 2005). Based on this evidence, there has been a proliferation of molecular work to identify which strains of methanotrophs are present in a given soil. These culture-independent approaches have identified several new groups

of methanotrophs that are currently classified outside of the Type I and II groups. Notably, studies from around the world have repeatedly found methanotrophs from the Upland Soil Clusters alpha and gamma (Knief et al. 2003, Horz et al. 2005, Zhou et al. 2008) as abundant methanotrophs in some upland soils. However, despite their appearance in methanotroph communities worldwide, very little is known about these novel strains and their influences on methane consumption. Having only identified them by their distinctive pMMO gene, important physiological and ecological traits are not yet known. Initial studies have suggested a variety of methanotrophs have unique traits that make them successful in their niche (Knief et al 2003, Horz et al. 2005, Zhou et al. 2008), but knowledge of how they influence methane uptake and will respond to climate change is still mostly lacking.

2.3 Methanotroph traits and competitive success

Methanotroph success and community composition is dependent on the match between methanotroph traits and the environmental niche it occupies (Graham et al. 1993, Bodelier et al. 2000, Henckel et al. 2000, Macalady et al. 2002, Wu et al. 2009). From an analysis of the literature, I have identified five traits that are likely to be of primary importance for methanotroph ecology (Fig. 2): enzyme kinetics, nutrient demand, pH tolerance, ammonium sensitivity and desiccation tolerance. I present evidence that there is variation in these traits, that they are likely to be important for both methanotroph activity (and thus their distribution and abundance) and for the biogeochemical response of methane uptake rates to environmental variation.

2.3.1 Enzyme Kinetics

Probably the most important trait of methanotrophs is the rate of methane uptake. The MMO enzyme shows classic Michaelis-Menten kinetics, such that the rate of methane oxidation rises almost linearly with methane concentration at low methane concentrations, then plateaus at a maximum rate (Vmax) at high concentrations (Conrad 1993, Lontoh and Semrau 1998). The capacity of the enzyme to perform at low substrate concentrations is defined by the Michaelis-Menten constant (Km); lower Km values indicate higher affinity for the substrate at low methane concentrations. Biochemical theory indicates that a tradeoff between Km and Vmax arises because enzymes with greater affinity (lower Km) will be inherently slower at catalyzing the reaction and thus have a lower Vmax (Stryer 1988, Tcherkez et al. 2006). In principle, this tradeoff suggests that the optimal Km should vary with the level of soil methane, and there is evidence that this is the case; in wetland and landfill cover soils where methane levels are high, the Km for MMO is much higher (lower affinity enzyme) than in upland soils (Table 2 in Gulledge 2004). While there is some phenotypic plasticity in Km, the degree of plasticity is generally much smaller than the range of Km values observed in nature (Dunfield and Conrad 2000). Variation in enzyme kinetics has obvious implications for both the relative success of different methanotroph strains, and for rates of methane uptake.

It is important to note that the levels of soil methane vary not just from soil diffusivity and rates of methane uptake, but also, potentially, from methane production. While methane production is widely recognized as happening in wetland soils, there is good evidence of methane production in upland soils as well (Andersen et al. 1998, von

Fischer and Hedin 2002, Megonigal and Guenther 2008), and this has the potential to stimulate methane consumption in grassland soil (Kammann et al. 2009). Where this methane production alters soil methane levels, the optimal enzyme kinetics would also likely change, depending on how much methane production is occurring locally.

2.3.2 Nutrient demand and ammonium sensitivity

Studies of methanotroph nutrient demands have primarily investigated nitrogen and copper. SIP experiments with ^{15}N-labeled N_2 have found methanotrophs to be active N-fixers (Buckley et al. 2008), and this trait is thought to be widespread among methanotrophs (Hanson and Hanson 1996, Auman et al. 2001, Boulygina et al. 2002, Foght 2010), suggesting that methanotrophs may frequently be N-limited. In addition, high levels of nitrogen reduce methane uptake rates in upland soils (Mosier et al. 1996, Wang and Ineson 2003) because ammonium is an inhibitor of methane oxidation (O'Neill and Wilkinson 1977). However, Mohanty et al. (2006) found that Type I vs. Type II-dominated methanotroph communities responded differently to ammonium additions, such that Type I methanotrophs responded positively to N additions while their Type II counterparts were inhibited, suggesting certain methanotrophs have the ability to tolerate high nitrogen levels better than others.

In addition to N, copper is an important methanotroph nutrient. When copper levels are sufficient, methanotrophs build a particulate version of the MMO enzyme (pMMO), and when it is deficient, a soluble form (sMMO) is constructed instead (Dalton 1992, Collins et al. 1991, Leak and Dalton 1986). Although methanotrophs with the particulate version have greater growth rates due to pMMO's greater specificity and efficiency (Leak and Dalton 1986, Leak and Dalton 1986), it is thought that the lack of

correlation between copper concentration in the soil solution and methanotroph activity arises because at least some methanotrophs produce an extracellular enzyme that can liberate copper from mineral forms (Kim et al. 2004, Balasubramanian and Rosenzweig 2008).

Together, these nutrient relations suggest that nitrogen is the most important nutrient for methanotroph activity, and that the nature of response to varying N availability will depend on traits of methanotrophs that allow them to tolerate high ammonium levels and cope with N-deficient environments.

2.3.3 pH tolerance

Nearly all cultured methanotroph strains have circum-neutral pH optima (Bowman 2006). Yet, methane consumption occurs in soils ranging from pH<4 to as high as 9. In addition, there is evidence that methanotrophs growing in more acidic environments have somewhat lower pH optima than those from high pH environments (Amaral et al. 1998, Saari et al. 2004), suggesting our cultured methanotroph representatives are not representative of the true diversity. Field experiments where soils were limed to increase pH caused a reduction in methane uptake rate (Hutsch et al. 1994), further suggesting that extant communities of non-cultured methanotrophs have different optimal external pH requirements. Although little is known about pH homeostatic mechanisms unique to methanotrophs, they are likely similar to other bacteria that employ trans-membrane sodium-proton pumps, such pumps become more energetically costly as the environmental pH departs from the optimal internal pH (Booth 1985). Given this likely high energetic cost, soil pH has the strong potential to influence the competitive success among methanotrophs.

2.3.4 Desiccation tolerance

Methanotrophs living in drier soils have inherently greater supply of atmospheric methane due to the higher soil diffusivity, but low soil moisture also threatens the organisms with desiccation. Because of their simple cell membrane, all bacteria are sensitive to desiccation, and so the capacity to pass through a dry period depends on traits to endure the stress period and revive again when conditions are favorable (Potts 1994). Laboratory (Schnell and King 1996) and field data (Gulledge and Schimel 1998, 2000, von fischer et al. 2009) show that water stress strongly affects methanotroph activity, and work by Chiemchairsri et al. (2001) suggests that production of an extracellular polysaccharide sheath (EPS) is at least part of the coping strategy used by methanotrophs. EPS's are known to have high water retention (Roberson and Firestone 1992) contributing to preservation of cell structure during time of desiccation (Helm et al. 2000, Hill 1997). Like many structural or biochemical stress responses (Potts 2001), there should be a significant energetic cost associated with the production of EPS structures. In addition, various methanotrophs have the ability to go into a resting stage cyst form, possibly conferring an advantage to cells undergoing drying stress (Whittenbury et al. 1970, Malashenko et al. 1975, Hazeu et al. 1980). However, because soils differ in desiccation risk, the possession of traits to tolerate desiccation would have advantages only in some environments.

I expect that methanotrophs adapted to frequent desiccation will show unique responses to soil drying and wet up events as compared to those who experience desiccation less frequently. Figure 3 shows a theoretical depiction of two different methanotroph communities: desiccation tolerant and a desiccation intolerant community

and their response to soil drying and soil wet up events. Due to the desiccation traits discussed above, desiccation tolerant methanotroph communities should be able to sustain methane consumption longer during soil drying and revive methane consumption quicker after a wet up event than a desiccation intolerant community.

2.4.5 The methanotroph habitat & fine-scale biogeography

What is the ideal habitat for methanotrophs? Methanotrophs grow fastest on moist soil surfaces with abundant supplies of methane, oxygen and nutrients. The inherent heterogeneity of soils generates spatial patterns in the quality of these habitats, and I anticipate that soil methane concentrations will likely be one of the primary determinants of methanotroph habitat quality. Soil methane concentrations show well-recognized patterns with highest concentrations near the soil surface, and lower concentrations with depth (Koschorreck and Conrad 1993). In addition, I speculate that there can be finer scale variations in soil methane concentrations following pore size and diffusivity differences: soil macropores should have higher concentrations, while smaller micropores, which have a more tortuous connection to the atmosphere, have lower methane levels. Moreover, there can be sub-centimeter scale variation in methane concentrations associated with methanogenic microhabitats. While the loci of such microhabitats remain unknown, they may include oxygen-restricted environments like soil aggregates (Sexstone & Tiedje 1984) or regions of high carbon turnover such as the rhizosphere (Jones et al. 2009, Hinsinger et al. 2009) or the biofilms along soil macropores (Morales et al. 2010).

Soil moisture levels are likely the other primary determinant of methanotroph habitat quality. Interestingly, in mesic to xeric ecosystems where desiccation stress is a

common consequence of the environmental regime, there is a potential tradeoff between access to methane and risk of desiccation (von Fischer et al. 2009): near-surface soils and macropores, with their greater supply of atmospheric methane, are also the first to dry out. Micropores and deeper soil habitats, on the other hand, would retain moisture longer, but methane concentrations could be more rapidly depleted. Such suites of environmental conditions can select for suites of methanotroph traits, leading to specialization for different microhabitats: desiccation tolerant methanotrophs with low Km MMO in near-surface and macropore habitats, desiccation intolerant methanotrophs with very low Km MMO in deeper and micropore habitats, and desiccation intolerant methanotrophs with higher Km associated with anoxic microzones.

I hypothesize that soils with diverse spatial habitats will confer stability in biogeochemical function under a greater breadth of conditions than will more homogeneous soils.

2.3.6 Soil moisture as a master variable of methanotroph ecology

I have presented evidence that soil water content directly influences methanotroph activity (section 2.3.4 desiccation tolerance), but it should be noted that soil moisture has an influence over the other soil habitat properties that I suggest play a role in methanotroph distribution. Specifically, methane levels are affected by soil gas diffusivity, with declining supply at higher moisture. Soil pH becomes high in arid areas with carbonate precipitation, and low in wetter, productive environments where organic acids accumulate. In addition, nitrogen availability can be reduced by leaching at high soil moisture levels.

I suggest that soil moisture is a ‘master’ variable of methanotroph ecology, and may turn out to be the most important environmental property for predicting methanotroph activity and methanotroph community composition. This large influence on both methanotroph ecology and overall methane uptake is apparent in Figure 2. Future efforts to understand how different methanotroph communities respond the changes in soil water content can allow for improved methane modeling, something that is certainly needed given the IPCC predictions in soil moisture regimes shifts for many ecosystems (IPCC 2007).

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CHAPTER 2: COINCIDENT CHANGES IN METHANOTROPH COMMUNITY COMPOSITION AND ENZYME KINETICS SUGGEST THAT COMMUNITY COMPOSITION ALTERS ECOSYSTEM FUNCTION

1. Introduction

Numerous studies have now documented that soil microbial community composition varies spatially (e.g., Fierer and Jackson 2006, Oakley et al. 2010), temporally (e.g., Lipson et al. 2002) and in response to experimental manipulations (e.g., Marschner et al. 2003, Horz et al. 2005). At very coarse phylogenetic scales, ecologists have successfully used the functional differences between bacteria and fungi to explain biogeochemical patterns based on fungal:bacterial ratios (Beare et al. 1992, Six et al. 2006). But more recent studies of soil metagenomes reveal a staggering volume of diversity at finer phylogenetic scales (e.g., within bacteria; Roesch et al. 2007) that beg us to understand their functional implications. Although some studies of bacterial diversity (e.g., Balser and Firestone 2005, Strickland et al. 2009) have found that ecosystem functions do vary with community composition, they generally have not resolved the mechanisms that give rise to these patterns, and so the findings have not improved biogeochemical prediction. Thus, it remains unclear how the predictive skill of biogeochemical models might be improved by explicitly considering the

ecophysiological differences among microbial communities, and the biogeographic processes that structure these communities.

The lack of known mechanisms linking ecosystem function with bacterial community composition has led ecologists to construct ecosystem models that largely employ kinetic constants and response functions that simplify microbial influence (Schimel 2001). Allison and Martiny (2008), argue this assumption to be incorrect due to microbial communities being generally sensitive to disturbance, not rapidly resilient after a disturbance and at least some microbial taxa being functionally dissimilar. In addition, for microbial groups that specialize on a biogeochemical process (i.e. methanotrophs and methane consumption) community composition can play an even more substantial role in controlling process dynamics (Schimel 2001, Allison and Martiny 2008).

One major hurdle for evaluating the importance of microbial community composition in structuring patterns in biogeochemistry is the near overwhelming complexity of most biogeochemical processes. However, methane uptake by methanotrophic bacteria in upland (i.e., well-drained, oxic) soils has been argued to be among the simplest biogeochemical processes (von Fischer et al. 2009) and it may therefore prove to be an appropriate model system for linking microbial diversity with ecosystem function. Moreover, the basic biology, physics and chemistry of the process have been studied at discrete levels ranging from ecosystem to enzyme: the phylogenetic structure of upland methanotrophs is relatively well characterized (Murrell 2010), we have a substantial understanding of methanotroph biochemistry (Hakemian & Rosenzweig 2007), a representative methanotroph's whole genome has been sequenced

(Ward et al. 2004), and the structure of the key enzyme's active site has been determined (Balasubramanian et al. 2010).

The importance of linking methanotroph biology with methane uptake is further underscored by the fact that CH_4 is a potent greenhouse gas (Lelieveld et al. 1998) whose concentration rose persistently through the late 20th century, then stabilized (Dlugokencky et al. 2003; Rigby et al. 2008). The mechanism of stabilization remains unresolved (Fletcher et al. 2004; Bousquet et al. 2006), thus exposing weaknesses in our understanding of CH_4 biogeochemistry and highlighting our inability to predict future atmospheric CH_4 that is critical for policy planning (Meehl et al. 2007). The uptake of CH_4 by upland (i.e., oxic, well-drained) soils is a key part of the global CH_4 budget, removing an estimated 30 Tg CH_4 yr-1 (Denman et al. 2007), and thus halving the rate that CH_4 accumulated over the late 20th century (Ojima et al. 1993). Although global-scale assessments for present and future atmospheric CH_4 (Denman et al. 2007) treat the soil sink as static, numerous studies have documented the sensitivity of CH_4 uptake rates to climate and global change factors (e.g., Gulledge and Schimel 1998, Le Mer and Roger 2001; Dubbs and Whalen 2010), suggesting that interannual variation in sink strength may be significant, and that future climate change may alter the geographic distribution and magnitude of the soil CH_4 sink.

Variation in observed rates of methane uptake arise from differences in both the activity of methanotrophic bacteria, and the soil gas diffusivity. (Curry 2007, King 1997, del Grosso 2000, von Fischer et al. 2009). While soil gas diffusivity is a relatively straightforward function of soil porosity and diffusivity (Hillel 1982), methanotroph

activity is a more complex ecophysiological response of the methanotrophs to variation in their environment.

Although studies of cultured methanotrophs reveal much about the ecophysiological differences among the different methanotroph clades, soil metagenomic studies indicate that the dominant methanotrophs from many environments remain uncultured (Dumont et al. 2006, Chen et al. 2008). This metagenomic information is primarily based on sequence information from the functional gene methane monooxygenase (MMO). The MMO enzyme carries out the first oxidative step in methane consumption (Dalton 1980, Lipscomb 1994). Bacteria possessing this enzyme have been found in two phylogenetic classes (Gammaproteobacteria and Alphaproteobacteria), with groups differing in some biochemical pathways, internal cellular structure, MMO enzyme properties, ecology and phylogeny (Hanson and Hanson 1996, Bowman 2006). The membrane-bound form of MMO (pMMO) is found in nearly all studied methanotrophs (although the genus *Methylocella* is an exception (Dedysh et al. 2004)). The *pmoA* gene has been widely used as a nearly universal functional gene marker for methanotrophs. Uncultivated methanotrophs with unique *pmoA* sequences have been discovered in a number of soils (Knief et al. 2003, Horz et al. 2005, Ricke et al. 2005, Zhou et al. 2008), and many have been hypothesized to carry out atmospheric methane consumption.

A number of examples now support the idea that methanotroph activity in soils varies with community composition (Gulledge et al. 1997, Bodelier et al. 2000, Carini et al. 2008, Liebner and Wagner 2007), but unfortunately these studies have only touched the surface of methanotroph community diversity. If we are to improve our understanding

of the role of methanotrophs in methane biogeochemistry and build next-generation methane biogeochemical models (Groffman and Bohlen 1999, Schimel 2000, Green et al. 2008), there is a need to document biogeographic patterns in methanotroph composition coincidentally with ecophysiological differences among these communities. An added benefit to this work would be an improved understanding of species turnover across sites and of factors that drive methanotroph biogeography. With this need in mind, I conducted a cross-site study on three LTER grasslands in order to document methanotroph biogeographic patterns, community composition, ecophysiologies and their possible controls. The grasslands fall along a precipitation gradient, but also vary in other soil properties that may be important for methanotroph ecology.

2. Material and Methods

2.1 Sampling sites and soil characteristics.

To document biogeographic patterns, community composition, and ecophysiologies of methanotrophs in distinctly different grasslands, Konza LTER grassland (central Kansas), Shortgrass Steppe (SGS) LTER grassland (northern Colorado) and Sevilleta LTER grassland (central New Mexico) were examined in this study. The grasslands were Konza Long Term Ecological Research site (3487-hectare area of native tallgrass prairie in northeastern Kansas (39°05'N and 96°35'W), Shortgrass Steppe Long Term Ecological Research site: all research was located within the Central Plains Experimental Range (CPER) (6280-hectare area of shortgrass steppe in northeastern Colorado (40D 49’N; 104D 46’W) and Sevilleta Long Term Ecological

Research site (100,000-hectare area of Short-grass Prairie and Shrub-Steppe in central New Mexico (34°20'N and 106°43'W)). Measurements and soil samples were taken in a variety of locations across the grasslands. At Konza, measurements and soil samples were taken at both summit and toeslope topographic positions within one, four and twenty-year burn plots. At the Shortgrass Steppe, measurements and soil samples were taken at both summit and toeslope topographic positions within sandy-loam and clay-loam sites. At Sevilleta, measurements and soil samples were taken at a blue gramma (*Bouteloua gracilis*) dominated site (toeslope) and a black gramma (*Bouteloua eriopoda*) dominated site (summit).

Soil core samples (0-10cm depth, 5.5 cm diameter) were taken from each site. These cores were placed into collection bags and stored in a cooler for <6 hours. Soils were then homogenized sieved through 2mm sieves where large roots and rocks were removed. To minimize physiological effects of temperature cycling, I held these sieved samples at room temperature for <48 hours until they were subsampled for soil chemistry and enzyme kinetic assays. The remaining material was frozen to -20°C for later DNA extraction.

2.2 Soil methane uptake.

I measured soil methane fluxes in the field using Los Gatos High Precision $CH_4/CO_2/H_2O$ analyzer. Gas concentrations are measured at 1 Hz with typical precisions better than 2ppb/500ppb/500ppm, respectively. Gas fluxes reported here were corrected for water vapor dilution using the instrument algorithm that calculates the mixing ratio of CH_4 for dry air. Closed chambers were placed over the soil surface using incubation times of 3-5 minutes. Two pieces of 9m-long polyethylene tubing (6.35mm OD,

3.175mm ID) connected the chamber headspace and the analyzer, and air was re-circulated using an internal pump at 500mL/min. The instrument was powered using a 12V car battery and DC–AC power inverter. Chamber bases were opaque PVC and were inserted into the soil surface 1 hour before measurement to a depth of 8 ± 2 cm to maintain vertical gradients in gas concentrations during measurement. Chamber lids were round, 20 cm inside diameter, and vented following (Livingston and Hutchinson 1995). They sealed against the chamber base with a narrow strip of self-adhesive closed-cell weather stripping. The chamber lids were opaque PVC. To quantify the total system volume, I measured the height of each chamber above the soil surface each time I measured gas flux.

During the flux measurement phase, I excluded data from the first 90 seconds while air in the chamber headspace, tubing and analyzer equilibrated. After this equilibration period, CH_4 concentrations generally decreased linearly with time. Fluxes were calculated based on linear regression of the gas concentration change with time.

2.3 Enzyme Kinetics.

I determined kinetics of methane oxidation as a function of methane concentration using slurried soil samples. Within 48 hours of soil collection, 10 g dry weight homogenized soil was placed in 120 ml. glass vials and, 10 mL of water was added. Vials were sealed with rubber septa (GeoMicrobial Technologies) and aluminum crimp tops. Gas concentrations were manipulated by adding CH_4 to reach desired concentrations in the headspace. To quantify any headspace gas loss during incubation sampling, 40 mL of lab air was injected into triplicate “negative control” vials before incubations began. Soils from three samples from each site were incubated at five different CH_4 concentrations.

The incubation was carried out in bench top water bath shaker tables at 30°C and 140 oscillations per minute.

I sampled headspace gases every 10-12 hours for 48 hours. At sampling, I removed 10 mL of air and analyzed the samples for methane concentration on a LGR DLT-100 methane/carbon dioxide analyzer (Los Gatos Research, CA) for CH_4 concentrations. Samples were injected into a stream of N_2 gas that flowed into the instrument, and sample peaks were integrated using an SRI data acquisition system and PeakSimple software. Calibration was against commercially prepared and certified standard gases of known methane concentration. Lineweaver-Burk plots were used to calculate V_{Max} and K_M values. Data from each site's samples were averaged to calculate V_{Max} and K_M for each site.

2.4 Soil Chemistry.

Within 48 hours of soil collection, soil pH, gravimetric water content and exchangeable inorganic nitrogen were measured. The nitrogen assay, used 10 g (dry weight) of soil and 50 mL of 2.0 M KCl, stirred for 1 hour. The solution was filtered through Whatman no. 1 filter. Concentrations of nitrate and ammonium were determined colormetrically on an Alpkem.

2.5 Data Analysis

I analyzed my laboratory and field data using ANOVA, with the software program JMP 7.0 (SAS Institute, Inc. 2007), using α=0.05 for all statistical analyses. Enzyme kinetic data differed significantly from normal distribution, and were log transformed before statistical analysis.

2.6 DNA extraction and PCR amplification.

DNA was extracted from 0.4 to 0.5 g subsamples of soil using PowerSoil DNA Isolation Kits (MoBio Laboratories, CA) following the manufacturer's protocol. As recommended my MoBio, extracted DNA was stored at -20°C until future analysis was required. PCR amplification reactions were performed in 25.5 microliter (μL) reaction mixtures in a Veriti 96 well thermal cycler with a hot lid (Applied Biosystems, CA). The PCR reaction mix contained 22.5 μl of Platinum PCR SuperMix (Invitrogen Corp.), 1 μL of 10 mM A189F forward primer (GGNGACTGGGACTTCTGG), 1 μL of 10 mM mb661R reverse primer (CCGGMGCAACGTCYTTACC) and 1 μL of extracted DNA. The thermal profile consisted of an initial denaturing and enzyme activation step of 94°C for 5 min, followed by 40 cycles of 94°C for 45 sec, 52.5°C for 45 sec. and 72°C for 45 sec. A final extension was carried out at 72°C for 10 min.

Annealing temperature was experimentally optimized to increase PCR yield and limit amplification of undesired products. Three primer sets were tested (previously described in Bourne et al. 2001), with the above primer set showing optimal PCR yields with the samples used in this study (Data not shown). The size and purity of each PCR product was analyzed in a 1% agarose gel against a 100 base pair ladder. If multiple bands were present, correct sized products were gel extracted and cleaned using the Wizard SV Gel and PCR Clean-up System (Promega Corp., WI) as directed.

2.7 Cloning and sequencing of pmoA genes.

PCR products were then ligated into the pJET1.2/blunt cloning vector using the CloneJet PCR Cloning kit (Fermentas Incorporated, MD) according to the manufacturer's

instructions. One Shot TOP10 Chemically Competent E. Coli cells (Initrogen Corp.) were then transformed with the ligated pJET1.2 plasmid using the manufacturer's protocol. Clones were then screened for *pmoA* gene inserts using vector-specific primers (pJET1.2F and pJET1.2R). DNA sequencing was carried out by the Colorado State University Proteomics and Metabolmics DNA sequencing facility using an ABI 3130 Genetic Anaylyzer and by the University of Chicago Cancer Research Center DNA sequencing facility using a 3730XL 96-capillary automated DNA sequencer.

2.8 Phylogenetic analysis.

Nucleotide-based alignments were obtained using the default alignment parameters in MUSCLE ver. 3.6 (Edgar, 2004). Manual adjustments to the MUSCLE alignments were performed in MacClade ver. 4.03 (Maddison and Maddison, 2001) using the procedure outlined by Simmons (2004), following Zurawski and Clegg (1987). Five out-of-frame insertions were inferred for four different sequences: a 2-bp insertion for KNZ27_12, two 1-bp insertions for AY786014 Γ gamma-proteobateria GCS245, a 1-bp insertion for FJ868567 *Methylococcus*, and a 1-bp insertion for AM698044 clone 49. All of the insertions were autapomorphic and hence no gap characters (Simmons et al., 2007) were scored. There were no ambiguously aligned nucleotide positions excluded from the phylogenetic analyses, but 31 nucleotides in regions of individual sequences that could not be unambiguously aligned with the remaining sequences were scored as ambiguous. The nucleotide alignment consisted of 477 positions, representing 157 codons (not including the autapomorphic insertions described above). Terminals with identical nucleotide sequences were identified and merged using MacClade.

Phylogenetic analyses were performed using three different character-sampling strategies: all nucleotide characters, nucleotide characters from first and second codon positions only, and amino acid characters. The matrix of all nucleotide characters was found to exhibit significant nucleotide-frequency heterogeneity for the parsimony informative characters, as determined by the chi-square test implemented in PAUP* ver. 4.0 b10 (which ignores phylogenetic correlations; Swofford 2001). Shifts in nucleotide composition are typically concentrated at third codon positions (e.g., Prager and Wilson, 1988; Hasegawa et al., 1993) and convergent changes in nucleotide composition can exacerbate long-branch attraction (Felsenstein, 1978; Woese et al., 1991; Lockhart et al., 1992). Therefore, the phylogenetic analyses based on the amino acid characters and first and second codon positions only were expected to be more robust to this potential problem than the analyses of all nucleotide characters. No significant differences in nucleotide-frequency heterogeneity for the parsimony informative nucleotide characters from the first and second codon positions only were inferred.

Equally weighted parsimony jackknife analyses (JK; Farris et al., 1996) were conducted for each data matrix using PAUP* with the removal probability set to approximately e^{-1} (36.7879%), and "jac" resampling emulated. Two-thousand jackknife replicates were performed with 100 random addition TBR searches (each with a maximum of ten trees held) per replicate.

I used jModeltest ver. 0.1.1 (Posada, 2008) and ProtTest ver. 2.2 (Abascal et al., 2005) to select the best-fit likelihood model for each data matrix using the Akaike Information Criterion (Akaike, 1974). Following Yang (2006) and Stamatakis (2008), invariant-site models (Reeves, 1992) were not considered because models that

incorporated the gamma distribution (Yang, 1993) were considered. The models selected were GTR + Γ for all nucleotide characters, TVMef + Γ for first and second codon positions, and JTT + Γ + F for amino acid characters.

Maximum likelihood (Felsenstein, 1973) analyses of nucleotide characters from each of the molecular data matrices were performed as (fallible; Gaut and Lewis, 1995; Siddall, 1998) tests for long-branch attraction. Likelihood analyses were conducted using RAxML ver. 7.03 (Stamatakis, 2006). Given that RAxML only implements GTR Q-matrices for nucleotide characters, these matrices were applied to both nucleotide-based character-sampling strategies. Optimal likelihood trees were searched for using 1,000 independent searches starting from randomized parsimony trees with the GTRCAT and PROTCATJTTF models for nucleotide and amino acid characters, respectively. Likelihood bootstrap (BS) analyses (Felsenstein, 1985) were conducted with 2,000 replicates and ten searches per replicate using the "–f i" option, which "refine[s] the final BS tree under GAMMA and a more exhaustive algorithm" (Stamatakis, 2008: 9).

All phylogenetic analyses were repeated after excluding seven terminals with the greatest amount (16-41%) of missing / inapplicable data to test for increased resolution and branch support after elimination of these potential "wildcards" (Nixon and Wheeler, 1991). Phylogenetic trees were created using TreeGraph 2 (Müller and Müller 2004; Stöver and Müller 2008).

All phylogenetic analyses performed assume hierarchical relationships among the sampled terminals. This assumption could be violated by recombination among the sampled lineages. The pairwise homoplasy index (PHI) test of recombination (Bruen et

al., 2006) was performed in SplitsTree 4 (Huson and Bryant, 2006) to the matrix of all nucleotide characters using the default window size of 100 bp and 1,000 permutations to test for significance. No significant evidence for recombination was inferred ($p = 0.67$).

3. Results

3.1 Soil Chemistry and Field Flux Rates.

The three study sites differed in most of the physical and chemical properties that I measured (Table 1). Soil moisture followed the mean annual precipitation of each site, with Konza having the wettest soils at the time of sampling, Shortgrass Steppe was intermediate and Sevilleta the driest ($p<0.001$). Soil temperatures also differed with Sevilleta soils showing higher temperatures than either Konza or SGS soils ($p<0.001$). Soil pH levels were similar and circumneutral at the Shortgrass Steppe and Konza, but were significantly more basic at Sevilleta ($p<0.001$). Our measures of extractable inorganic nitrogen revealed differences in available ammonium and nitrate. Ammoniun levels differed significantly between all three LTER grasslands ($p<0.001$), with Konza exhibiting the highest concentrations and Sevilleta the lowest. Nitrate levels were lowest in Sevilleta, as compared with the other sites. Exchangeable copper was the only property that did not differ among sites ($p=.9$). All sites had copper levels around 25 mg/Kg soil.

3.2 Field Flux Rates.

Soil methane uptake rates differed across grasslands (Figure 4). On average, SGS had significantly higher methane uptake rates than either Konza or Sevilleta grasslands ($p<0.001$). Some Konza sites showed a moderate amount methane emission, which did

not occur at either SGS or Sevilleta grasslands. Ecosystem respiration was significantly higher in Konza soils than either SGS or Sevilleta soils ($p<0.001$) (Appendix Tables A1-A3).

3.3 Enzyme Kinetics.

Despite a small sample size ($n \sim 6$) for each site, I had sufficient statistical power to detect significant differences in methane oxidizing enzyme kinetics among grasslands (Figure 5 and 6). Maximum oxidative rate (V_{Max}) followed soil moisture patterns, with Konza soils exhibiting significantly higher rates than Sevilleta soils ($p=0.0303$), and SGS rates falling in between (Figure 6). Enzyme affinity (K_M) showed the opposite pattern, with Konza soils having the lowest affinity enzymes for methane and Sevilleta with the highest ($p=0.0056$) (Figure 5).

3.4 Methanotroph Community.

Our *pmoA* clone libraries consisted of 48 clones from Konza soils, 48 clones from SGS soils and 44 clones from Sevilleta soils. A simplified consensus phylogram is presented in figure 7 (for full detailed trees see support figures (S1-S13)).

All DNA based trees showed a deep and well supported branching feature between alpha and gamma proteobacteria methanotrophs. This division is well documented (Horz et al. 2005, Lin et al. 2005, Bowman 2006, Singh and Tate 2007), with major differences in biochemical pathways and GC content between groups (Hanson and Hanson 1996). All sequences found in this study fell into the gamma proteobacteria division, although other methanotroph lineages are also illustrated in Fig. 7. Moving up the gamma proteobacteria section of the tree, there is weak support for branching

between Type I, including 38 sequences amplified from Konza soils, and the rest of the gamma proteobacteria methanotrophs from the DNA based maximum likelihood analysis, while the DNA based parsimony analysis contradicts this branching. Moving further up the tree, there is a well supported division between USCγ, previously suggested to be involved in atmospheric methane consumption (Knief et al. 2003), and a clade that includes environmental clones and previously described strains and our clones. In particular, 26 sequences, mostly from Sevilleta soils, group into the USCγ clade. All DNA based trees showed a common evolutionary origin for what I am labeling the "grassland methanotrophs" clade and the *amoA* gene expressed in *Nitrosococcus oceani*, an ammonia-oxidizing bacterium that is suggested to be limited to aquatic systems (Jones and Morita 1983, Horz et al. 2005). The remaining sequences fell into this grassland methanotroph clade, that houses previously described clades and two novel clades. The first (SGS) clade, was formed with two sequences amplified from SGS soils and a pmoA sequence found in Inner Mongolian steppe soil (Zhou et al. 2008). The second (SEV) clade, is composed entirely from sequences amplified from Sevilleta soils. Both clades show close relatedness to JR2 and JR3 clades (Horz et al. 2005, Zhou et al. 2008), which grouped with 45 and 21 sequences respectively from our study.

Horz et al. (2005) contend that JR2 and JR3 clades represent methanotrophs, in part due to their conserved amino acid residues shared between *pmoA* and *amoA* sequences (Holmes et al. 1999, Tukhvatullin et al. 2001, Ricke et al. 2004) and shared residues considered diagnostic of *pmoA* (Holmes et al. 1999). I conducted a similar analysis on our newly discovered SGS and SEV clades, and find similar results (Table 2). SGS sequences shared all 16 conserved amino acid residues shared between pmoA and

amoA sequences, while SEV sequences shared all but one. SGS and SEV sequences also shared all diagnostic amino acid residues of pmoA as reported in Horz et al. (2005). Notably, a sequence that grouped with the SEV clade (SEV3 14), showed 17 unambiguously optimized synapimorphies, as mapped onto the DNA parsimony jackknife tree, with several of these being in conserved and diagnostic amino acids.

3.5 Community Composition.

The distribution of methanotroph clades found in soils from each grassland is presented in Figure 8. Konza, the wettest site, exhibited the lowest methanotroph diversity and was dominated (79%) by sequences in the *Methylococcus capsulatus* clade, with JR2-like sequences present as well. At the intermediate Shortgrass Steppe site, methanotroph communities were dominated by sequences from the JR2 clade (67%) with JR3 also abundant (25%), while SGS and USCγ sequences made up a minor portion of the overall SGS community. At the dry end, Sevilleta methanotrophs were dominated by USC_{γ} sequences (55%), while SEV and JR3 sequences also made a substantial contribution to the communities.

My observation of the Shortgrass Steppe methanotroph community in 2008 is consistent with a preliminary *pmoA* clone library that I developed in 2006. That work showed very similar methanotroph community composition to the 2008 sampling, with the communities dominated by JR2 and JR3 sequences. The only difference in the 2006 data was in the less abundant strains: three sequences (< 4%) resolved with *Methylococcus capsulatus*, and no USC_{γ} or SGS sequences were found (appendix Figure A1).

4. Discussion

4.1 Amino acid conservation.

The conservation of functional and diagnostic amino acid residues suggests the novel sequences that make up SGS and SEV clades code for methane monooxygenase enzymes as opposed to ammonia monooxygenases (Holmes et al. 1999, Tukhvatullin et al. 2001, Ricke et al. 2004, Horz et al. 2005). My phylogentic analyses suggest weak to moderate support for the grassland clades being sister to a *Nitrosococcus* clade (59 and 75 jackknife support values for parsimony and maximum likelihood analyses respectively), the only known ammonia-oxidizing bacteria among the gamma proteobacteria. Based on key amino acid differences Horz et al. (2005) argues there is strong support for JR2 and JR3 clades (clades that group with my novel SGS and SEV clades) being methane-oxidizing bacteria. My novel SGS and SEV clades share all key amino acid differences with the JR2 and JR3 sequences as shown in Horz et al. (2005), also suggesting these novel clades to be methane-oxidizing bacteria as well (Table 2).

4.2 Methanotroph biogeography.

Examination of extracted DNA (as is done in this study) is not necessarily an examination of the active pool of organisms present in a sample (Nannipieri et al. 2003**,** Molin and Givskov 1999). However, because DNA degrades relatively quickly in soil (Romanowski et al. 1992, Widmer et al. 1996, Dale et al. 2002), the composition of the soil DNA pool must result from a dynamic equilibrium where the losses from the soil DNA pool are balanced by inputs, presumably from growth of the resident methanotroph populations. I interpret my community composition results as reflecting the genetic

potential of the sites, with the relative abundances of different clades reflecting temporally integrated population sizes. The temporal stability of this genetic potential is supported by the repeatability of Shortgrass Steppe clone libraries in 2006 and 2008. Future work can more directly evaluate the identity and dynamics in the active pool of methanotrophs using $^{13}CH_4$ stable isotope probing or another molecular assay of activity.

A review of the literature shows that some methanotroph groups appear to have a restricted biogeographic distribution. For example, strains in the JR2 and JR3 clades have only been found in arid grasslands (Horz et al. 2005, Zhou et al. 2008 and present work), while upland soil cluster gamma strains are have, thus far, only been found in soils with a pH >6 (Knief et al. 2003, Zhou et al 2008 and this study).

These emerging biogeographic patterns may be a result of methanotroph strains specializing on key environmental properties, but there is much current debate, both in microbial ecology and in the broader field of ecology, about the relative importance of neutral processes (Hubbel 2001) vs. competitive interactions (Tilman 1982 and Tilman 1994) for structuring the composition of communities. Under the competition framework, methanotrophs with the optimal suite of traits for the local environment will become dominant in the local community. Using this paradigm, my results, along with other studies, suggest that the dominant methanotrophs at each grassland are abundant because they are best suited to local condition. I hypothesize that *Methylococcus capsulatus* strains are better suited to wet soils, JR2 and JR3 strains are best suited for arid grasslands and USCγ have traits that allow it to outcompete other methanotrophs in more alkaline soils. Future testing of these hypotheses would allow us to predict the methantroph community composition, based on soil habitat properties.

4.3 Enzyme kinetic differences.

My observed methane oxidation kinetics were similar to those found in similar soils. High-affinity methane oxidation (K_M 4 – 740 nM) has been consistently measured in upland soils (Knief et al. 2003, Dunfield and Knowles 1995, Saari 2004), while low-affinity oxidation ($K_{M(app)}$ 1.1– 66 mM) is typically found in soils with a larger methane supply (i.e. landfill cover soil or wetland soil) (Megraw Knowles 1987, Whalen et al. 1990, Whalen and Reeburgh 1996). Although the measured kinetics showed variability, the Sevilleta and SGS soils showed methane affinities typical of atmospheric methane oxidation (99 and 518 nM respectively). Konza soils, which were wet and even weakly emitting methane in some cases, showed average kinetics similar to bogs and other methane emitting soils (1.3×10^3 nM).

Maximum uptake rates (V_{Max}) showed a similar pattern to Km. Sevilleta and SGS soils exhibited an order of magnitude higher V_{Max} rates than has been measured in non-arid (Knief et al. 2003) and comparable values to several hardwood and pine forest soils (Gulledge and Schimel 1998, Bull et al. 2000, Gulledge et al. 2004). In contrast, Konza showed similar V_{Max} values to other high methane soils, such as bogs and landfill soils (Whalen et al. 1990, Whalen and Reeburgh 1996).

I hypothesize that these differences in enzyme kinetics reflect differences in soil methane supply. The Michaelis-Menten model predicts that enzyme features that improve substrate affinity (i.e., lower rates of substrate dissociation and thus lower Km) also slow the maximum catalytic rate, thus causing a lower Vmax. Such a Km-Vmax tradeoff suggests that the optimal Km rises with soil methane concentration. This prediction is consistent with studies showing a positive correlation between Km and

Vmax for methane oxidation (Table 2 in Gulledge et al. 2004). The higher Km values at Konza are consistent with endogenous methane production in those soils. Previous studies have found that some well-drained, oxic soils exhibit "occult" or hidden methane production despite net methane uptake (von Fischer and Hedin 2002). The wetter soil conditions at Konza would favor more occult methanogenesis, thus elevating soil methane concentrations. Similarly, the drier Sevilleta and Shortgrass Steppe soils are expected to have less occult methanogenesis, and so those methanotrophs feeding on atmospheric sources of methane have enzymes with greater methane affinity.

The link between enzyme kinetics and field rate of methane uptake rates is, in part, obscured by soil diffusivity which limits the degree to which methanotroph activity affects methane uptake. Quantification of the relative importance of diffusion vs. methanotroph activity requires that we simultaneously measure methane uptake and soil diffusivity (von Fischer et al. 2009), which I did not do in this study. However, if diffusivity alone drove methane uptake rates, I would expect the greatest rates of uptake in the driest Sevilleta soils. Rough estimates from a soil physics model (Millington Quirk 1961) suggest that the soil diffusivity should be four-fold higher at Sevilleta (4.5 cm^2/min) and SGS (4.0) as compared to Konza (1.1). Thus, it appears that low uptake rates at Konza are due to both low diffusivity and low methanotroph activity; low uptake at Sevilleta is primarily limited by low methanotroph activity; high rates of methane uptake at the SGS arise from the combination of high methanotroph activity and high diffusivity.

5. Conclusion.

There is considerable interest in understanding the linkage between microbial community composition and biogeochemical function. I suggest that a framework for such integration follows three steps. First, isolate a facet of the biogeochemical transformation that is under ecophysiological control by the microbial community. Second, demonstrate that ecophysiological responses differ among communities. Third, show that the community composition is predictable in time and space.

I contend that this study makes significant progress as a synthesis that improves the empirical linkage between methanotroph ecophysiology, community composition, biogeography and field rates of methane uptake. It may be easier to develop this community composition to ecosystem function linkage because methane uptake is, arguably, one of the simplest biogeochemical processes; the substrate supply is easy to quantify and the catalytic organisms are phylogenetically cohesive and relatively well known. I find that the differences in field estimates of methanotroph activity parallel the patterns from Michaelis-Menten assays, which in turn correlate with differences in methanotroph community composition. Together, these coincident changes in methanotroph community composition and enzyme kinetics suggest that community composition alters ecosystem function.

6. Future Directions.

Methane is a strong greenhouse gas, with uptake in upland soils representing the largest biological sink in the methane global cycle. Methanotrophs vary in a wide variety of traits and their rate of methane consumption. If we want to understand differences in soil methane uptake temporally, spatially and in response to climate change we have to

improve our understanding of the relationship between methanotroph traits and environment. Ultimately, improved understanding of upland methane biogeochemistry and methanotroph ecology depends on addressing these questions. Below I suggest three questions future methanotroph research should consider tackling.

5.1 Global change responses

First, how do different methanotroph communities respond to environmental variation? A methanotroph community can be thought of as a collection of traits interacting with the environment. As climate change alters ecosystems, understanding of how these different traits become more or less optimal under new environmental regimes is required to predict how methanotroph communities will change. Answering this question will require an improved understanding of the various methanotroph strains optimal growing conditions and various traits. For this reason, it will be important to expand our culturing efforts of methanotrophs responsible for methane uptake and/or perform direct soil habitat manipulations and monitor community shifts to understand how methanotrophs respond to environmental variation.

5.2 Community influence on biogeochemistry

Second, how does variation in these community shifts affect rates of methane uptake? Very few studies have looked at how methanotroph community affects methane uptake (one exception can be found with Lin et al. 2005). Climate change is expected to lengthen dry periods in many of the nations grasslands (IPCC 2007, Easterling et al. 2000), potentially giving a competitive advantage to desiccation tolerant methanotrophs. Whether this expected community shift impacts methane uptake is unknown. In a study on methanotroph community structure change during a simulated climate change

experiment, Horz et al.2005 found certain methanotroph strains changed in relative abundance in response to soil moisture manipulations. Unfortunately, how this community shift affected methane uptake was not examined. Future research should aim to couple field-based measures of methane uptake, with an improved characterization of the methanotroph community composition. Being able to link who is there with what they are doing will lead to an improved predictive power.

5.3 Biogeography

Lastly, how are biogeographic patterns of methanotrophs maintained, and can we explain these patterns by known traits? A review of the literature shows various methanotroph groups have a restricted biogeographic distribution. For example, strains in the upland soil cluster gamma group have only been found in arid grasslands (Horz et al. 2005, Zhou et al. 2008), while upland soil cluster alpha and the alphaproteobacteria cluster I are only found in acidic and neutral pH soils respectively (Knief et al. 2003, Bourne et al. 2001, Ricke et al. 2005, Kolb et al. 2005). Can we predict methanotroph distributions based on the match between their individual traits and important environmental properties?

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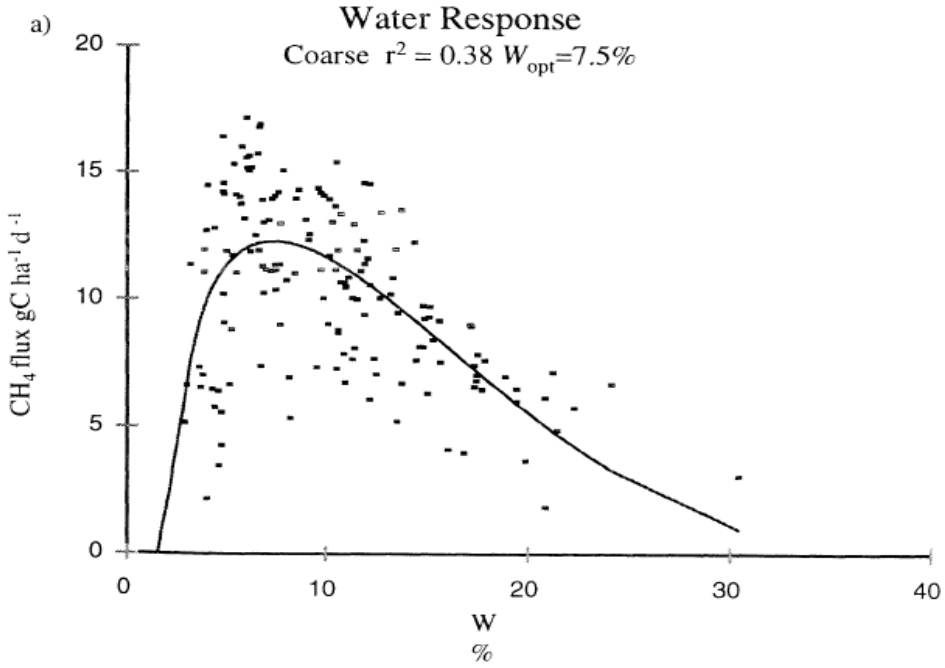

Figure 1: Methane uptake response to soil moisture

Response of methane uptake in coarse-textured soils to variation in soil water content, from Del Grosso et al. (2000).

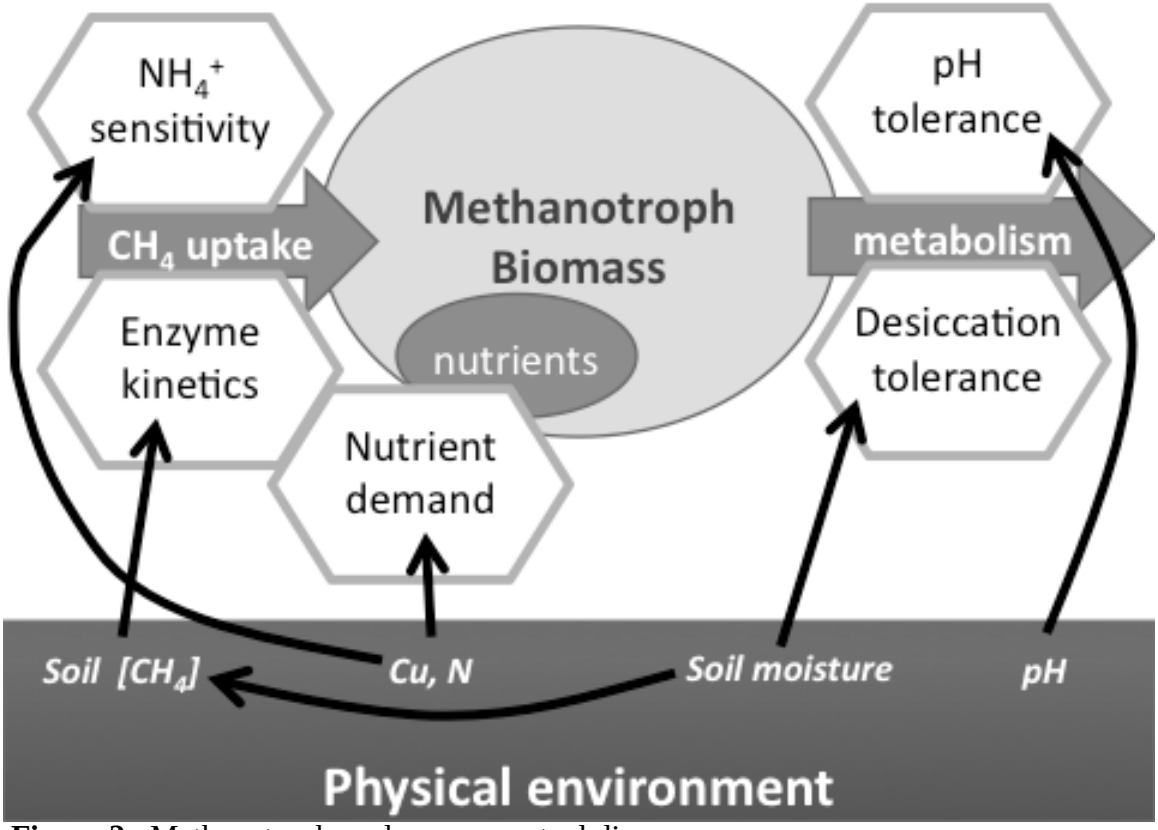

Figure 2: Methanotroph ecology conceptual diagram

Conceptual diagram for relationship between the physical environment, methantoroph traits, and biomass. Hexagons represent the methanotroph traits that mediate the response of methanotroph activity to variation in the physical environment. These traits can either affect rates of methane uptake or the rates of metabolic maintenance costs. The methanotroph biomass in a given place is the balance between methane uptake and metabolic losses.

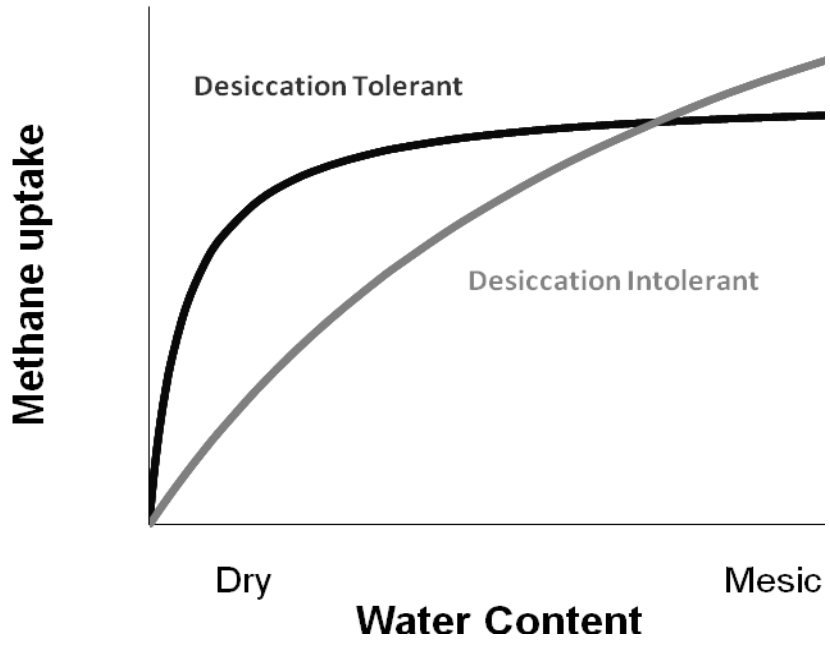

Figure 3: Theoretical methanotroph desiccation responses
Theoretical depiction of different methanotroph communities: desiccation tolerant community and a desiccation intolerant community and their response to soil moisture change.

Table 1: LTER soil characteristics
Selected soil characteristics measured from soil collected at three different LTER grasslands. All three study sites differed significantly in most of the physical and chemical properties that I measured. SGS, Shortgrass Steppe

	LTER Grassland		
	Konza	**SGS**	**Sevilleta**
Soil Moisture (Gravimetric, % water)	30.4	7.7	4.3
pH	6.6	6.4	8.2
NH_4^+ (2 M KCl, mg/L)	0.7	0.4	0.2
NO_3^- (2 M KCl, mg/L)	1.1	1.3	0.6

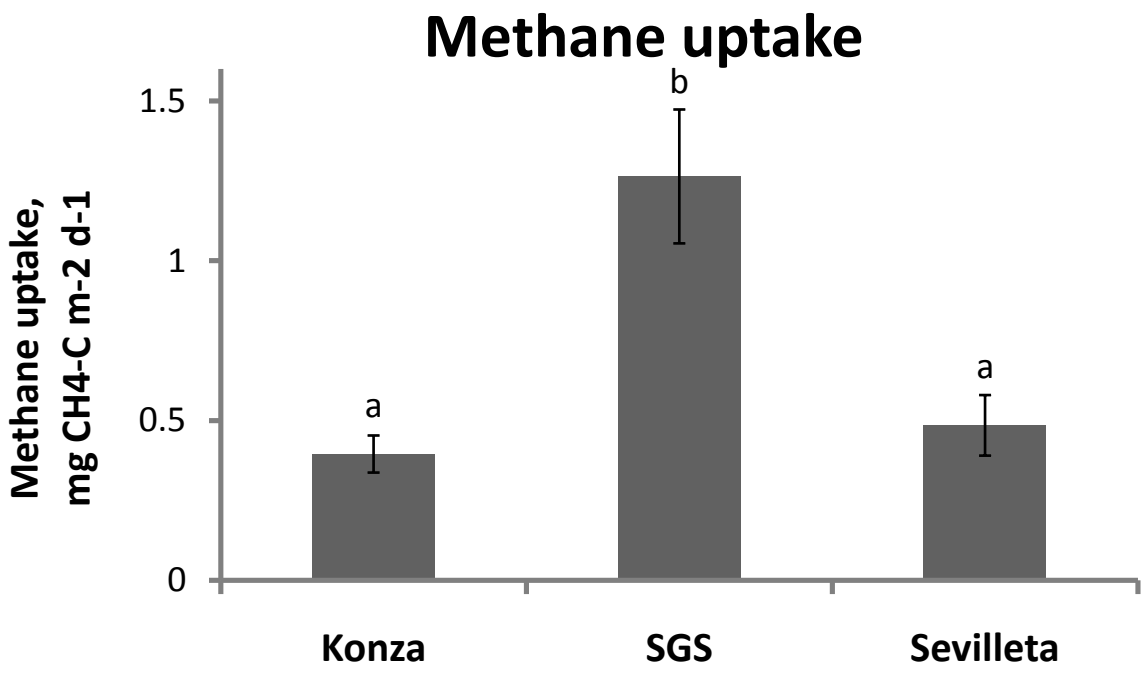

Figure 4: LTER grassland methane uptake rates
Methane uptake across three LTER grasslands (ANOVA $p<0.0001$). Bars represent 95% Confidence intervals (Konza: n = 38, SGS: n = 20, Sevilleta, n = 19). SGS, Shortgrass Steppe.

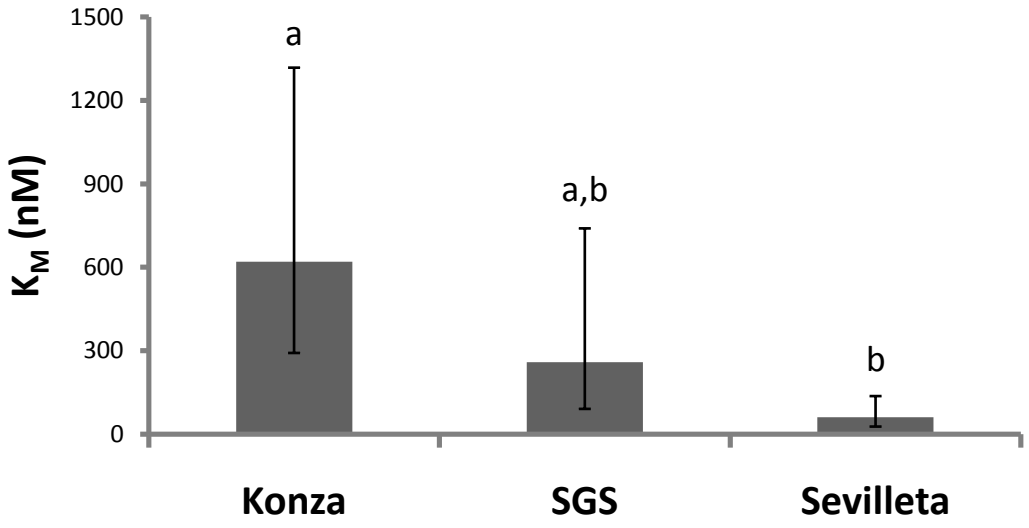

Figure 5: LTER apparent K_M
Log transformed apparent K_M concentrations from three different LTER grasslands (ANOVA p=0.0056). SGS, Shortgrass Steppe. Bars represent 95% confidence intervals (Konza: n = 8, SGS: n = 6, Sevilleta: n = 6).

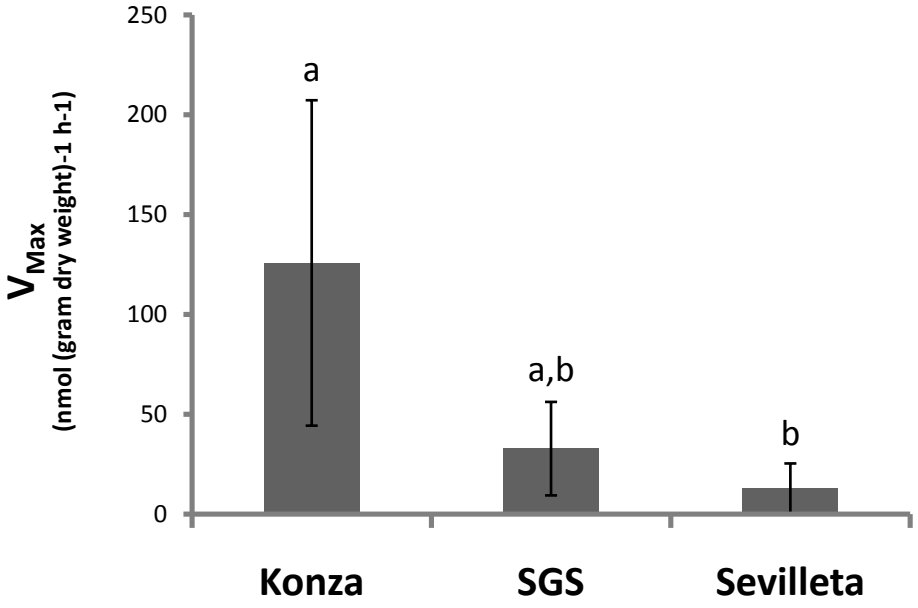

Figure 6: LTER V_{Max}
Log transformed V_{Max} rates measured from three different LTER grasslands (ANOVA p=0.0303). SGS, Shortgrass Steppe. Bars represent 95% Confidence intervals (Konza: n = 8, SGS: n = 6, Sevilleta: n =6).

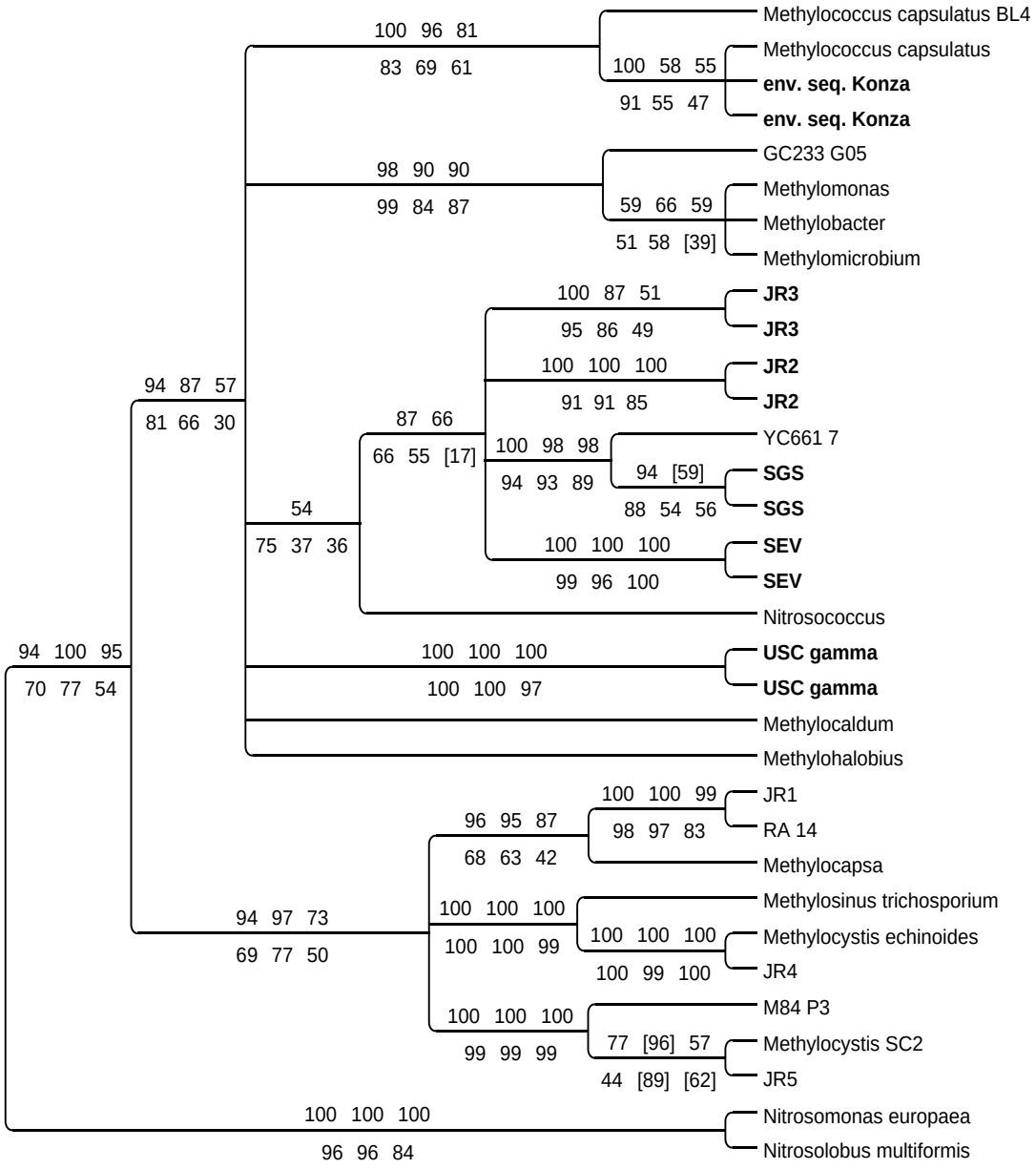

Figure 7: *pmoA* Maximum Liklihood phylogenetic tree
Consensus maximum likelihood tree showing phylogenetic relationships between *pmoA* genes sequenced in three LTER grasslands and *pmoA and amoA* genes available in public-domain databases. Values above branches represent bootstrap support values from maximum likelihood analysis, while values below branches represent jackknife support values from parsimony analysis. Left most values represent support values using all DNA nucleotides, middle values represent support values using the first two nucleotides of a codon, and right most values represent support values using amino acid translations. *amoA* sequences from public-domain databases were used as an outgroup (Ammonia-oxidizing bacteria, A.O.B.). * represents a branch is not supported by parsimony analysis.

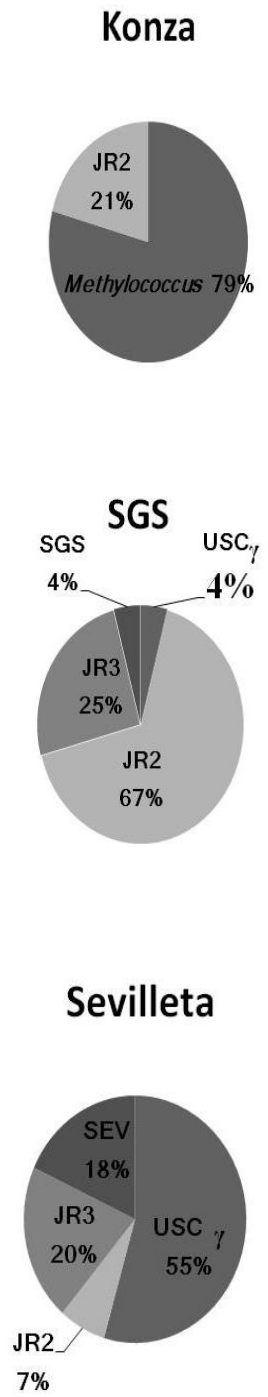

Figure 8: LTER methanotroph community composition
Methanotroph community composition (*pmoA*) found at three LTER grasslands (Konza: n = 48, SGS: n = 44, Sevilleta: n = 48). SGS, Shortgrass Steppe.

Table 2: Conserved and diagnostic amino acid summary (*pmoA* and *amoA*)

Presence of conserved and diagnostic amino acid residues in *pmoA* and *amoA* across taxa[1]. Colomns with black backgrounds represent *amoA/pmoA* conserved sites, residues in light grey (position 89) may be diagnostic of SEV *pmoA*, dark grey backgrounds represent *amoA/pmoA* diagnostic sites, outlined residues show *amoA* diagnostic sites for ammonia oxidizers in gammaproteobacteria (*Nitrosococcus*) and sites where there was considerable variability in residue identity no amino acid is shown.

amino acid position	58	62	65	70	71	76	80	86	89	96	100	101	102	109	110	111	112	113	114	115	121	140	157	158	164	165	168	172	175	182	185	190	196	197	200	204
clade																																				
Nittosomonas eropea (AmoA)	Q	V	P	T	Y	M	W	P	A	L	E	W	L	Y	W	W	S	H	Y	P	P	N	Y	P	F	G	H	V	G	D	G	R	Y	V	I	S
Nitrosospira multiformis (AmoA)	Q	V	P	T	Y	M	W	P	A	L	E	W	L	Y	W	W	S	H	Y	P	P	N	Y	P	F	G	H	V	G	D	G	R	Y	V	I	S
Nitrosococcus oceani (AmoA)	R	T	P	A	Y	q	W	P	A		E	W	A	V	g	F	T	Y	F	P	P	S	Y	P			H		G	D	G	R	Y	I		T
TYPE I	R	T	P	T	F	Q	W	P	A		E	W		W	G	W	T	Y	F	P	P	S	Y	P	I	A	H	E	G	D	G	R	Y	I	V	T
SGS (SGS3 1)	R	T	P	T	F	Q	W	P	A	L	E	W	V	W	G	W	T	Y	F	P	P	S	Y	P	L	A	H	E	G	D	G	R	Y	V	V	T
SGS (SGS2 12)	R	T	P	T	F	Q	W	P	A	L	E	W	V	W	G	W	T	Y	F	P	P	S	Y	P	L	A	H	E	G	D	G	R	Y	V	V	T
SGS (YC661 7)			P	T	F	Q	W	P	A	L	E	W	V	W	G	W	T	Y	F	P	P	S	Y	P	L	A	H	E	G	D	G	R	Y	I	V	T
SEV (SEV1 10)			P	T	F	Q	W	P	G	L	E	W	V	W	G	W	T	Y	F	P	P	S	Y	P	L	A	H	D	G	D	G	R	Y	I	V	T
SEV (SEV14 12)	R	T	P	T	F	Q	W	P	G	L	E	W	V	W	G	W	T	Y	F	P	P	S	Y	P	L	A	H	D	G	D	G	R	Y	I	V	T
JR2	R	T	P	T	F	Q	W	P	A	L	E	W	V	W	G	W		Y	F	P	P	S	Y	P	L	A	H	D	G	D	G	R	Y	I	V	T
JR3	R	T	P	T	F	Q	W	P	A	L	E	W	V	W	G	W	T	Y	F	P	P		Y	P	L	A	H	D	G	D	G	R	Y	I	V	T

[1] Modified from Table 2 found in Horz et al. 2005