

The influence of eutrophication status on the methane oxidation in subtropical wetland soils

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Abstract

Methane (CH₄) is the second most abundant greenhouse gas and is 25 times more efficient at trapping heat than carbon dioxide. With an approximate 10 year lifespan in the atmosphere, reductions in CH₄ emissions may significantly reduce the impact of climate change. Microbially mediated CH₄ oxidation is a significant determinant of the net CH₄ fluxed from wetland soils. With the potential to reduce the soil CH₄ emissions by up to 90%, an increased understanding of this process is essential to improve strategies to reduce CH₄ emissions from wetland systems.

The kinetics of CH₄ oxidation were determined in soils along the established nutrient gradient present in WCA-2A in Florida Everglades. A laboratory manipulation study was performed using microcosms containing soils which were collected from the eutrophic (F1) and oligotrophic ridge (U3R) and slough (U3S) sites. Rates of CH₄ oxidation were measured for each soil under the added 10%

vol CH₄ concentrations along the depth profile at 0-5, 5-10, and 10-20 cm increments to determine the Michaelis-Menton kinetics. Significant differences were found in the maximum oxidation rate (V_{max}) and substrate affinity (K_m) among the sites and with soil depth. The V_{max} ($20.1 \pm 4.7 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$) at 0-5 cm in the F1 site was significantly lower than deeper depths. The V_{max} of the F1 site at 5-10 cm was significantly higher than the U3R and U3S sites. The K_m at 0-5 cm in the F1 ($684 \pm 313 \mu\text{g CH}_4 \text{ g}^{-1}$) and U3R ($1380 \pm 230 \mu\text{g CH}_4 \text{ g}^{-1}$) sites showed significantly higher affinity for substrate relative to that observed in deeper soils. The K_m values below 5 cm of the U3S site were significantly lower than both the F1 and U3R sites. Significant positive correlations with total phosphorus (TP) suggest that TP may be influencing the oxidation activity. Correlations with nitrate (NO_3^-) suggest varying influence on CH₄ oxidation activity, with higher (NO_3^-) concentrations having an inhibitory effect on CH₄ oxidation rates. Results from another study that determined the influence of ammonium (NH_4^+) on the rate of CH₄ oxidation revealed significant negative correlations between the two variables in soils from the F1 ($p = 0.0011$) and U3S ($p = 0.0078$) sites.

Introduction

Atmospheric Methane

Methane (CH₄) is a highly reactive greenhouse gas and, although much lower in concentration in the atmosphere, it is 25 times more effective at absorbing infrared radiation than carbon dioxide (CO₂). Atmospheric concentrations of CH₄ have increased significantly since the industrial revolution, rising from 830 ppb to the current concentration of 1826 ppb (Etheridge et al., 1992; US EPA, 2013; IPCC, 2007). Factors expected to contribute to continued increases in global CH₄ emissions consist of natural and anthropogenic sources. Fossil fuel industries, agricultural sources (e.g., rice cultivation, ruminant animals), waste treatments, and biomass burning are current anthropogenic sources of CH₄ which contribute approximately 400-610 Tg CH₄-C yr⁻¹, the majority of total annual global CH₄ emissions (Reddy and DeLaune, 2008). Wetlands, termites, aquatic systems, and gas hydrates are among the natural sources of CH₄. Of these natural sources, wetland soils are the largest contributor to atmospheric CH₄. Approximately 30-40% of global CH₄ emissions are released from natural wetland systems (Pester, et al., 2012).

Methane Production and Oxidation

Methane emissions are a net result of CH₄ production and oxidation in wetland soils where anaerobic conditions prevail. Methane is the final product formed during the decomposition of organic matter. Once formed, it can be

emitted into the atmosphere via three ways: 1) the aerenchyma tissue of wetland plants, 2) the formation of gas bubbles (i.e., ebullition), and 3) diffusive flux of dissolved CH₄ from soil (Fig. 1).

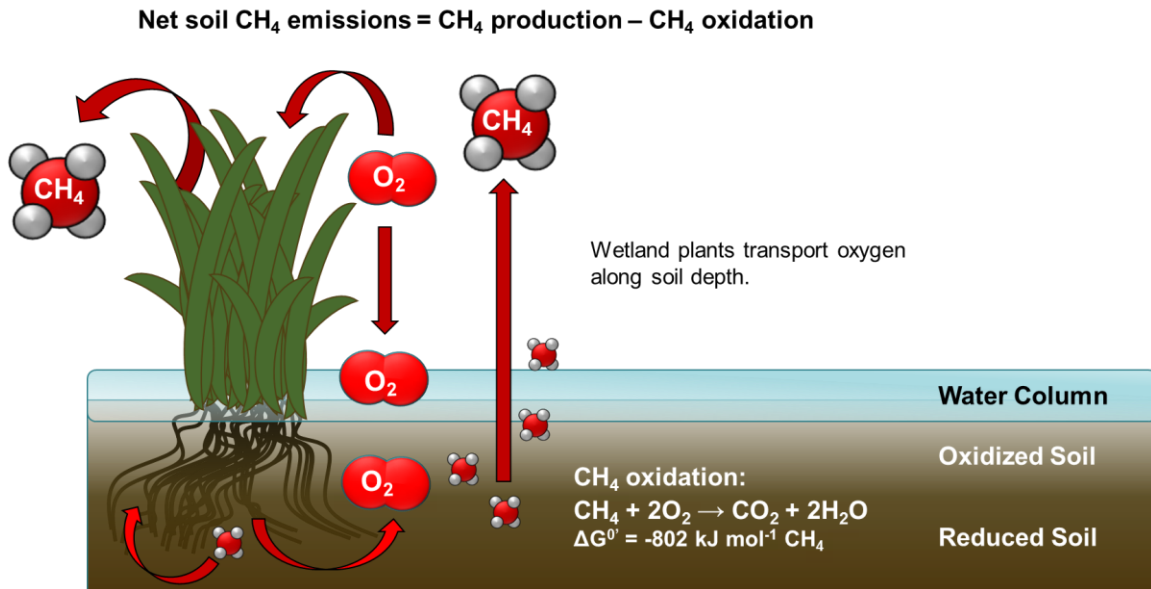
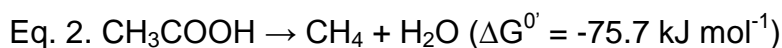
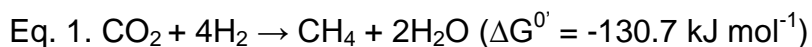


Figure 1. Pathway of aerobic CH₄ oxidation.

During the process of the formation of CH₄ (methanogenesis), methanogenic archaea (i.e., methanogens) utilize compounds such as CO₂, H₂, and acetate to produce CH₄ under highly reduced conditions (<-200 mV). The two pathways of hydrogenotrophic and acetoclastic methanogenesis are presented as equations 1 and 2, respectively (Reddy and DeLaune, 2008; Mitsch and Gosselink, 2007).



Type I and Type II Methanotrophs

The two main classes of aerobic methanotrophs are the Type I (*Methylobacter spp.*, *Methylomonas spp.*) and Type II (*Methylocystis spp.*) methanotrophs. Although both Type I and Type II methanotrophs have the ability to oxidize CH₄, Type II methanotrophs are more efficient at performing this function at lower O₂ concentrations and have a preference for higher CH₄ concentrations (Chowdhury and Dick, 2013; Hanson and Hanson, 1996). In flooded rice fields, populations of Type I and Type II methanotrophs have been strongly correlated with CH₄ concentration in the soil pore water, with the population of Type II increasing with increasing CH₄ availability. It was also shown that the ratio of Type I/Type II methanotroph populations decreased with depth (Macalady et al., 2002).

Methane Monooxygenase

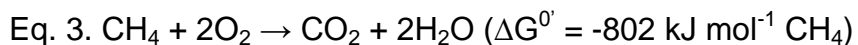
The consumption of CH₄ by methanotrophic bacteria begins with the conversion of CH₄ to methanol by the enzyme CH₄ monooxygenase (MMO). This enzyme is found in both the particulate (pMMO) and soluble (sMMO) forms and has a highly broad specificity. The ubiquitous form, pMMO, has been found in most methanotrophs, excluding the genera *Methylocella* and *Methyloferula* (Chauhan et al, 2012), while sMMO is limited to few Type II species.

Methane monooxygenase (MMO) enzymes are released by both Type I and Type II methanotrophs and convert CH₄ to methanol as the first step in the

oxidation of CH₄ (Hanson and Hanson, 1996). It is believed that methanotrophs have the ability to withstand anoxic conditions and periods of depleted CH₄ concentrations (Roslev and King, 1994) due to the broad specificity of the MMO enzymes. This broad specificity of MMO may allow for utilization of alternate substrates, such as ammonium (NH₄⁺), which may be of benefit during periods of low CH₄ availability and allow the methanotrophic population to be sustained. The broad specificity of MMO may also act to enhance competitive inhibition of CH₄ oxidation due to this same mechanism when the availability of alternate substrates exceeds CH₄ availability (Cai and Mosier, 2000).

Methane Oxidation

Methane oxidation occurs as methanotrophic bacteria consume CH₄ as it fluxes from the soil (Zheng et al., 2012). Commonly, zones of methanotrophy in soils are located at the soil surface and near the plant rhizosphere, as these are the areas of relatively higher oxygen (O₂) availability. Methanotrophs are obligate aerobes which consume CH₄ with O₂ to produce carbon dioxide (CO₂) for energy generation as well as utilize the carbon (C) from CH₄ for biomass according to the following equation:

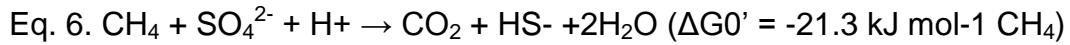
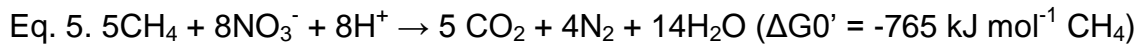
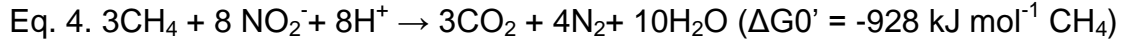


As a sink for atmospheric trace gases, soils may significantly reduce CH₄ emitted to the atmosphere by conversion of CH₄ to CO₂ via microbially mediated CH₄ oxidation as the gas passes through the aerobic zone of the soil water

interface. Due to the low solubility of CH₄, diffusion through flooded soils is often limited and allows for the oxidation of CH₄ to CO₂. This oxidation process results in a significant reduction in the net CH₄ emitted from the soil. Approximately 30 Tg CH₄-C yr⁻¹ may be fixed by this process globally (Dlugokencky et al., 2011).

Studies have reported that elevated CH₄ concentrations (> 1000 ppm) may stimulate CH₄ oxidation in soils by providing an abundance of substrate (Benstead and King, 1997; Bender and Conrad, 1992). In a study of soils from a mixed hardwood deciduous forest, Benstead and King (1997) showed that the rates of CH₄ oxidation increased as the concentration of CH₄ increased and deduced that low CH₄ availability did not reduce the potential of methanotrophs in the soil to oxidize CH₄ over time. In contrast, a study of forest soils exposed to CH₄ levels lower than sub-atmospheric concentrations (< 0.03 ppm) were shown to lead to a decreased ability of oxidation (Schnell and King, 1995). These differences were attributed to the variability in affinity of the methanotrophic populations for CH₄.

At high water levels CH₄ oxidation may be limited due to the lowered availability of oxygen to the methanotrophic bacterial populations (Schnell and King, 1995). However, recent studies have shown that in anoxic conditions, anaerobic CH₄ oxidation (AOM) may also occur in wetland systems. This process may be coupled to denitrification of nitrite (NO₂⁻), denitrification of nitrate (NO₃⁻), or be mediated by sulfate (SO₄²⁻) according to Equations 4, 5, and 6, respectively (Raghoebarsing, et al., 2006; Ettwig et al., 2010; Deutzmann and Schink, 2011).



Anaerobic CH_4 oxidation coupled to NO_2^- (n-damo; Fig. 2) is mediated by *Candidatus Methyloirabilis oxyfera* bacteria affiliated with NC10 phylum. In this process, NO_2^- serves as the terminal electron acceptor during the oxidation of NH_4^+ and CH_4 . In AOM, CH_4 is oxidized with NO_2^- to produce nitrogen gas. In NO_3^- driven AOM (Fig. 2), the process is accomplished by bacteria associated with denitrifying NC10 bacteria (Ettwig et al., 2010). A previous short term study using enrichment cultures found that the activity of anaerobic CH_4 oxidizing denitrifying bacteria was dependent on the presence of NO_2^- and that no consumption of CH_4 was shown in the presence of NO_3^- without NO_2^- (Ettwig et al., 2010). In a study by Raghoebarsing et al. (2006), similar results were obtained for short term incubations, but long term (10-20 hour) incubations showed AOM occurred and was coupled to nitrate consumption. A study of profundal sediments from Lake Constance, an oligotrophic freshwater lake in Constance, Germany, showed AOM rates, upon nitrate addition, ranged from 1.8 to 3.6 $\text{nmol day}^{-1} \text{ mL}^{-1}$ sediment and stimulated CO_2 production (Deutzmann and Schink, 2011). Another study of temperate forest soils in South Korea showed the addition of NO_3^- (0.20-1.95 $\mu\text{g N g}^{-1}$ soil) significantly stimulated rates of CH_4 consumption when CH_4 concentrations were low (1.7-2.0 ppmv CH_4). Inhibition of

CH₄ oxidation was shown, however, when CH₄ concentrations were high (300 ppmv CH₄) (Jang et al., 2011).

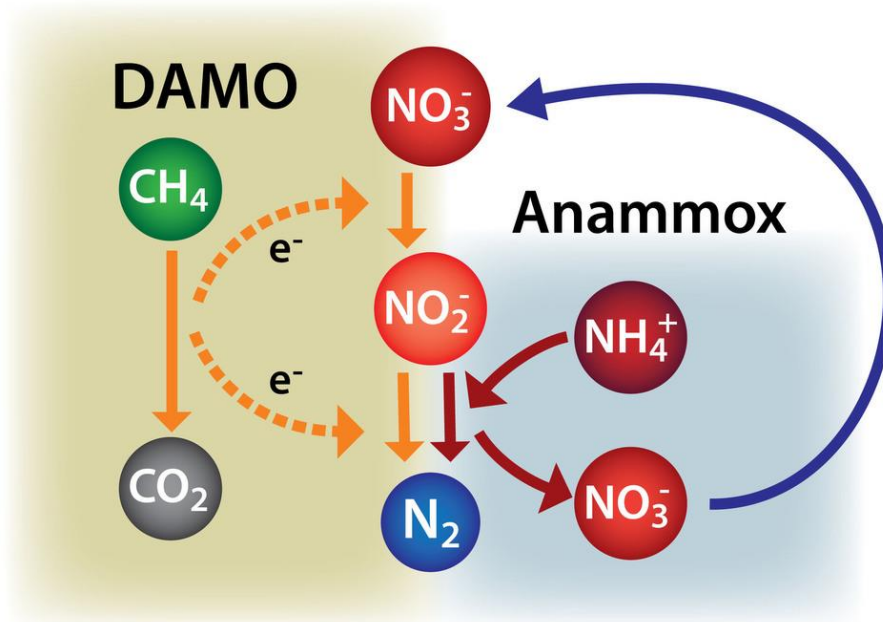


Fig. 2. Model for DAMO and anammox processes in the presence of nitrite and nitrate (Hu et al., 2015).

Sulfate (SO₄²⁻) mediated AOM has been extensively studied in marine environments. However, studies of AOM in the presence of SO₄²⁻ in freshwater systems (Fig. 3) are few. In peatlands, anaerobic CH₄ oxidation has been linked to microbial SO₄²⁻ reduction and it has been suggested that AOM is CH₄ limited (Smemo et al., 2011). Anaerobic methanotropic (ANME) archaea have the ability to oxidize CH₄ in a syntrophic relationship with sulfate-reducing bacteria (SRB). This involves the transfer of electrons from CH₄ to SO₄²⁻ and it is believed to be a form of reverse methanogenesis. In freshwater wetland soils, anaerobic CH₄ oxidation has been shown to occur under low (<1 mM) SO₄²⁻ concentrations

(Beal et al., 2011), which makes this process significant in wetlands due to the relatively low concentrations of SO_4^{2-} typically found in freshwater wetlands (Orem et al., 2011). Low SO_4^{2-} levels may be maintained in these systems as sulfur is cycled via the reduction of SO_4^{2-} to sulfide and the oxidation of sulfide to SO_4^{2-} , contributing to the oxidation of CH_4 as it fluxes through the soil and overlying water column.

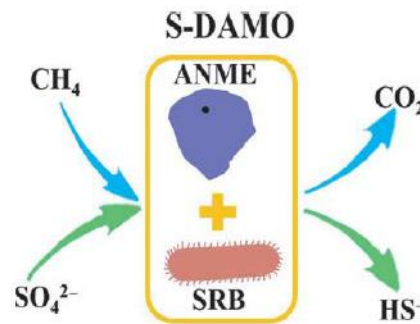


Fig. 3. Model for sulfate mediated AOM (Cui et al., 2015).

Kinetics of CH_4 Oxidation

The effect of the different regulators of methane oxidation may be shown by examining the kinetics of methane oxidation in soils. The Michaelis-Menton kinetic model (Fig. 4) is commonly used to describe the relationship between the enzymatic reaction and its substrate according to the following equation:

$$v = \frac{V_{\max} [S]}{K_m + [S]}$$

where v is the initial velocity, V_{max} the maximum velocity of a reaction that may be achieved, $[S]$ the substrate concentration, and K_m , the Michaelis constant, which defines the concentration at which half of the maximal velocity ($V_{max}/2$) is achieved (Reddy and DeLaune, 2008). The K_m is a measure of the affinity of the enzyme for the substrate of interest. High values of K_m are indicative of the low substrate affinity expressed by enzymes of broad specificity, while low K_m values indicate high affinity and a narrower substrate specificity.

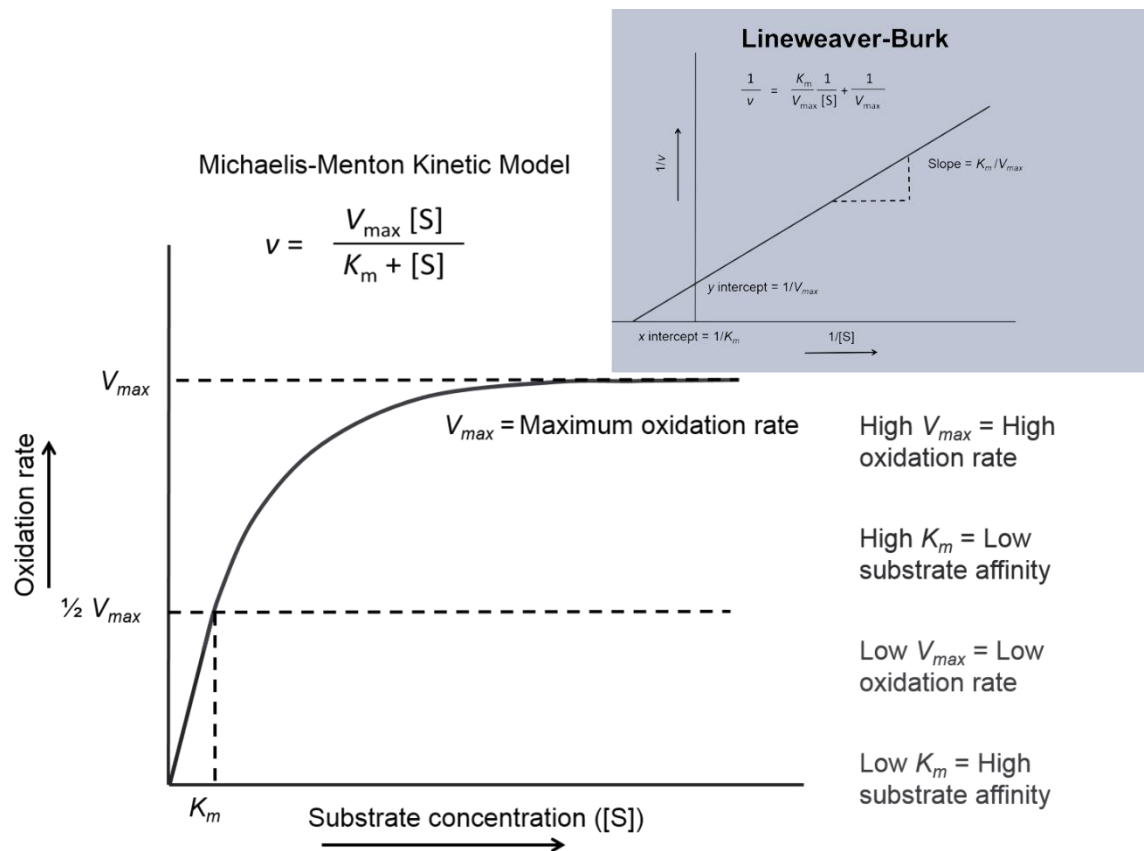


Figure 4. The Michaelis-Menton Kinetic model and Lineweaver-Burke equation.

As the concentration of the substrate increases ($[S] \gg K_m$) to the point of enzyme saturation, the velocity or rate of the reaction becomes independent of substrate concentration. For a *zero order* reaction, a constant velocity equal to the V_{max} is achieved. When $[S] \ll K_m$, the velocity is dependent on $[S]$ and the rate of the reaction is said to be *first order* (Reddy and DeLaune, 2008; Voet and Voet, 2004).

To determine the kinetic parameters of CH_4 oxidation, the Lineweaver-Burk equation may be used:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

where v is the initial velocity, K_m , the Michaelis constant, V_{max} , the maximum velocity of a reaction that may be achieved, and $[S]$, the substrate concentration, which defines the concentration at which half of the maximal velocity ($V_{max}/2$) is achieved (Reddy and DeLaune, 2008). This linear equation uses the reciprocal of the Michaelis-Menton equation. By plotting $1/v$ and $1/[S]$, the slope (K_m/V_{max}) and intercept ($1/V_{max}$) may be obtained and the values for the V_{max} and K_m may be determined (Walkiewicz et al., 2012; Voet and Voet, 2004).

The dominating methanotrophic populations within a site are dependent on their adaptation to CH_4 concentrations in the soil, with high affinity (low K_m) microbes oxidizing CH_4 at lower concentrations and low affinity (high K_m) microbes oxidizing at higher CH_4 concentrations (Dunfield et al., 1999; Kightley et al., 1995, Bender and Conrad, 1992). Benstead and King (1997) showed that the

rates of CH₄ oxidation increased as the concentration of CH₄ increased and deduced that low CH₄ availability did not reduce the potential of methanotrophs in the soil to oxidize CH₄ over time in soils from a mixed hardwood deciduous forest. In contrast, methanotrophs in forest soils exposed to sub-atmospheric CH₄ concentrations (< 0.03 ppm) experienced a decrease in the ability to oxidize CH₄ (Schnell and King, 1995). This decrease in methanotrophic oxidative ability was attributed to the variability in high affinity vs. low affinity methanotrophic populations found in these sites. Under high concentrations of CH₄, methanotrophic populations which exhibit high CH₄ oxidizing activity and low affinity for CH₄ are often dominant (Kightley et al., 1995). Based on these previous kinetic studies, K_m may be used as an indicator of the availability of CH₄ and the methanotrophic community structure within soils.

Table 1. Kinetic parameters for CH₄ oxidation (modified from Canfield et al., (2001)).

Soil Type	V_{max} (nmol g ⁻¹ h ⁻¹)	K_m (nM)	Ref.
Temperate soil	7.1	27.7 (1.5)	1
Cultivated cambisol (20% added CH ₄)	270 (11)	1740 (34)	2
Meadow cambisol (20% added CH ₄)	410 (18)	4560 (33)	2
Forest luvisol (20% added CH ₄)	450 (8)	27900 (24)	2
Landfill cover soils	227 (18)	2600 (41)	3
Peat	110	57000	4
Preincubated humisol	15320	50000	5
Mollic gleysol	550	30660	6
Haplic podzol	443	19760	6
Eutric cambisol	138	5980	6
FL Everglades	0.3-0.7	-	7
Deciduous forest soils	1	10	8

1. Czepiel et al., (1995); 2. Bender and Conrad, (1992); 3. Whalen et al., (1990); 4. Watson et al., (1997); 5. Megraw and Knowles, (1987); 6. Walkiewicz et al., (2012); 7. King et al., (1990); 8. Benstead and King, (1997)

Nutrients as a Regulator of CH₄ Oxidation

Several studies have investigated the impact of nutrients on CH₄ oxidation potential in soils (King et al., 1990; Gerard and Chanton, 1993; Schnell and King, 1995; Macalady et al., 2002; Chauhan et al., 2012). Phosphorous (P) is often limited in wetland systems. When P is applied to a system, notable increases in ecosystem productivity may occur can affect microbial activity and biomass (Reddy et al., 1999). In a study by Conrad and Klose (2005), P additions to rice soil microcosms were shown to be stimulatory to CH₄ oxidation, however this was believed to be due to indirect mechanisms. In contrast, Zheng et al. (2013) found P fertilization to be slightly inhibitory to CH₄ oxidation regardless of a significant increase in abundance of the methanotrophic population.

Nitrogen is a major regulating element of ecosystem productivity and often considered a limiting nutrient in wetland systems. Essential for plant growth, agricultural and urban applications of N based fertilizers have greatly increased the availability of N in the environment. As a result, increases in the concentration of N have been reported in wetland systems which receive runoff from environments where N fertilization occurs. This higher abundance of N, along with availability of other limiting nutrients, such as P, increases the overall productivity of a system, resulting in increased rates of plant and microbial growth, competition, as well as increased rates of organic matter decomposition and CH₄ production. In soils under N-limitation, it is believed the addition of N-fertilizers may act to stimulate CH₄ oxidation activity by providing a necessary nutrient to the microbial population (Alam and Jia, 2012) and by reducing

competition for N between the plant and the microbial populations in areas of high root density (Bodelier et al., 2000).

Ammonia (NH_3) and NH_4^+ are inorganic forms of N commonly used as fertilizers. It has been shown that the presence of NH_4^+ may significantly affect the rate of CH_4 oxidation. Methanotrophs use CH_4 as their sole energy source while ammonia oxidizers and nitrifying microbes utilize NH_4^+ and NH_3 as their sole energy source. However, the structural similarity of CH_4 and NH_4^+ allows for cooxidation of CH_4 and NH_4^+ to occur under aerobic conditions via the CH_4 mono-oxygenase (MMO) and NH_3 monooxygenase (AMO) enzymes produced by methanotrophs and ammonia oxidizing microbes, respectively. Methanotrophs and nitrifying microbes inhabit the same niche within the soil profile allowing for the possibility of cooxidation of the respective preferred substrates.

It has been suggested that NH_4^+ may competitively inhibit CH_4 oxidation due to its ability to be taken up by the MMO enzyme (Bedard and Knowles, 1989). In a study performed by Reay and Nedwell (2004), oak forest soils were exposed to both high and low concentrations of CH_4 ($50 \text{ mL CH}_4 \text{ L}^{-1}$ and $10 \mu\text{L CH}_4 \text{ L}^{-1}$, respectively) in the presence of NH_4^+ over a range of 1 to 10,000 μM . No significant effect of NH_4^+ addition on CH_4 oxidation was detected in the soils treated to the lower CH_4 concentration. However, in the soils exposed to the higher concentration of CH_4 , oxidation was significantly inhibited when concentrations of $\text{NH}_4^+ > 5,000 \mu\text{M}$, as compared to unamended controls. In this same study, NO_3^- was also shown to strongly inhibit CH_4 oxidation in oak soil at varying CH_4 concentrations (Reay and Nedwell, 2004).

A study by Shrestha et al (2010), observed the effects of urea and NH_4SO_4 on rice microcosms and showed Type I methanotrophs may be stimulated by the NH_4^+ based fertilizer applications. Shrestha et al. (2010) speculated that microbial community composition may be shaped by differences in N availability throughout the growing season. Higher CH_4 emissions were reported in control soils and soils treated with urea, while NH_4^+ treated soils emitted significantly lower quantities of CH_4 (Shrestha et al., 2010). In a long term study, differences in the rates of CH_4 oxidation and the methanotrophic populations were found due to the type of fertilizer applied (urea and NH_4SO_4). The NH_4SO_4 treated soils had lower CH_4 emission and oxidation rates. The authors suspected this difference to be due to an inhibition of CH_4 production by high sulfate concentrations found within these soils. No effect of N was determined in the control soils and those treated with urea (Shrestha et al., 2010).

In a study by Bodelier et al. (2000), the application of urea or $(\text{NH}_4)\text{HPO}_4$ (200 or 400 kg N ha^{-1}) stimulated CH_4 oxidation in rice paddy soils taken from plant root zones. In unplanted, unfertilized soils, Type II methanotrophs dominated CH_4 oxidation. Both Type I and Type II methanotrophs were active in the rice plant root zones of soils which had long term exposure to urea or $(\text{NH}_4)\text{HPO}_4$. The addition of NH_4^+ , along with the presence of rice plants, were shown to be the stimulating factors which activated the Type I methanotrophic population within these soils (Bodelier et al., 2000). Bodelier et al. (2000) suggested the stimulatory effect of NH_4^+ addition may be due to the high CH_4 concentration of the rice paddy soils and that elevated CH_4 availability has the capability to

diminish inhibitory effects of NH_4^+ . Toxic effects due to product of NH_4^+ oxidation may be removed by plant uptake, which may also reduce the inhibitory consequences of NH_4^+ addition to the soils. As the toxic byproducts of NH_4^+ oxidation are not readily taken up by plants in upland soils, the formation of these toxic products, coupled with low CH_4 availability, may allow for NH_4^+ inhibition of CH_4 oxidation in upland soils (King et al., 1994).

There is no clear evidence that NH_3^+ oxidizers contribute significantly to CH_4 oxidation, although their capability to oxidize CH_4 has been shown (Bedard and Knowles, 1989). Ammonium oxidizers can incorporate C from CH_4 during the oxidation process, however few studies have been done. It has been suggested that NH_4^+ oxidizers have a limited contribution to CH_4 oxidation, as it is believed the population size is too small to significantly affect CH_4 oxidation due to observed rates of CH_4 oxidation by methanotrophs being much greater than that of NH_4^+ oxidizers (Carini et al., 2003). Nitrifiers also have low affinity to CH_4 and information regarding the rate at which nitrifying archaea can oxidize CH_4 is not known (Stein et al., 2012). In a study by Bodelier and Frenzel (1999), nitrifiers made no significant contribution to CH_4 oxidation in rice field soils, although methanotrophs were shown to have significantly contributed to the nitrification process.

Study Site: Florida Everglades

Water Conservation Area 2A (WCA-2A) is a well-studied region of the Florida Everglades. Input of discharge water from the Everglades Agricultural Area (EAA) has created a gradual nutrient gradient within this 54,700 ha subtropical freshwater marsh, which provides an ideal study site. Over the last decade, nutrient inputs from this discharge water have created significant differences in regards to nutrient concentrations in this historically oligotrophic site, particularly in the phosphorous (P) concentration. The effect of this increased nutrient input can be seen in the altered vegetation along the gradient. The severely impacted eutrophic area (F1; Fig. 5) with a relatively higher nutrient status has been established in the region within closer proximity to the input source. Due to this eutrophication, a shift in the plant community composition from the once predominate native sawgrass (*Cladium jamaicense* Crantz) and periphyton vegetation to the dominance of cattail (*Typha domingensis* Pers.; Fig. 5) has occurred (Childers et al., 2003). The interior and non-impacted oligotrophic regions of WCA-2A have maintained the native vegetative communities and consist of both ridge and slough landscapes (Fig. 5). In the ridge portion, sawgrass is the dominant vegetation. In the slough areas, thick periphyton mats may be present, along with *Nymphaea odorata* and *Eleocharis cellulose*. The heterogeneity of WCA-2A allows for a study site in which comparative determinations may be made regarding the impact nutrient status and vegetation have on CH₄ oxidation in subtropical wetland soils.

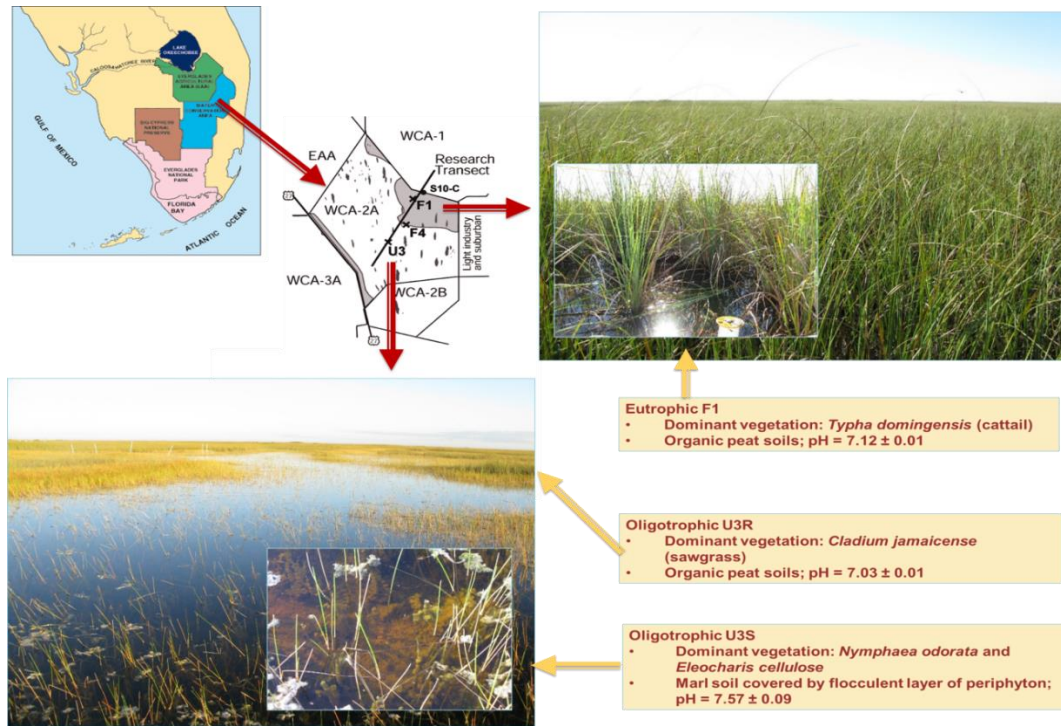


Figure 5. WCA-2A: a 54,700 ha subtropical freshwater wetland with an established P gradient which spans the marsh (FL and WCA-2A maps modified from images retrieved from USGS, SOFIA website).

As shown by Whiting and Chanton (1993), the net ecosystem production is a dominant variable in determining CH_4 emissions from wetland systems. Reported net CH_4 flux from inundated regions of the Florida Everglades varies from 4.2 to 81.9 $\text{mg CH}_4 \text{ m}^{-2} \text{ d}^{-1}$, depending on the site within the system, with vegetative community types serving as an indicator of flux (Bartlett et al., 1989). In temperate swamps and in rice paddies, aerenchyma tissue of plants is the primary means of CH_4 release and accounts for approximately 90% of CH_4 emitted (Le mer and Roger, 2001). Various aquatic plants species have the ability to exchange gases within their roots and tissues (Whiting and Chanton,

1993), as well as provide O₂ to methanotrophic microbial populations in the rhizosphere. These methanotrophic bacteria are then able to oxidize CH₄ as it fluxes from the soil. This process is a significant determining factor on the net CH₄ fluxed from a wetland system. King et al. (1990) studied the rates of CH₄ oxidation in sediment free plant roots of various plant species commonly found in the Everglades. Roots from *Cladium jamaicense* and *Sagittaria lancifolia* grown in peat showed rates of $708 \pm 382 \text{ nmol g}_{\text{dws}}^{-1} \text{ h}^{-1}$ and $526 \pm 38 \text{ nmol g}_{\text{dws}}^{-1} \text{ h}^{-1}$, respectively, while no detectable CH₄ oxidation occurred in these same species grown in marl soils (King et al., 1990). In a study of CH₄ oxidation in the rhizosphere of emergent aquatic macrophytes, Gerard and Chanton (1993) found that no methanotrophic activity occurred in the stems of *Typha domingensis*, *Cladium jamaicense*, *Sagittaria lancifolia*, and *Nymphea odorata*. Methanotrophic activity was also not observed in the rhizomes of *Nymphea odorata*. The rates of CH₄ uptake in the rhizosphere of these plants was highly variable and thought to be due to the specificity of the locations the methanotrophic bacterial populations are found in the root zone, as well as the quantity of roots present (Gerard and Chanton, 1993). In the FL Everglades, the rate of CH₄ consumption occurring in the root zones of *Typha domingensis* was shown to be in the range of 0.79-7.19 g m⁻² d⁻¹, while CH₄ oxidation occurring in the root zones of *Cladium jamaicense* was shown to be 1.66 g m⁻² d⁻¹ (Gerard and Chanton, 1993).

The methanotrophic population also differs along the nutrient gradient of WCA-2A in the Florida Everglades. The eutrophic site has been shown to harbor both Type I and Type II methanotrophs, while, in the more pristine site, only the

Type I population has been identified (Chauhan et al., 2011). Methane production and consumption in the eutrophic site was shown to be higher than in the oligotrophic site (Wright and Reddy, 2001; Chauhan et al., 2011). The abundance of methanotrophs has been shown to be higher in the eutrophic site, but it still remains to be established if the abundance of methanotrophs is correlated with oxidation rates.

Study Objectives

In order to develop better strategies to mitigate climate change, an enhanced understanding of the global CH₄ budget is critical. Methane is highly reactive, yet has a relatively short life span (approximately 10 years) in the atmosphere. Therefore, reductions in CH₄ emissions may be able to significantly reduce the impact of climate change (Dlugokencky et al., 2011). As the global temperature increases, hydrological and temperature induced changes are expected to cause increases in CH₄ emissions from wetland soils, triggering a need for improved modeling of CH₄ emissions (Kirschke et al., 2013). It has been shown that the temporal and spatial CH₄ contributions from wetland soils are quite variable, thereby creating some uncertainty in the relative contributions of CH₄ from these systems (Kirschke et al., 2013). Additional studies of the factors which determine the quantity of CH₄ fluxed from wetland soils under various conditions will aid in determining the impact of anthropogenic activities which may alter biogeochemical cycles, improve the ability to model and estimate CH₄ emissions,

and enhance strategies to mitigate climate change. Due to the temporal and spatial variability in CH₄ contributions from natural sources, uncertainty in the relative contributions of CH₄ from wetlands has led to inaccuracies when estimating the global CH₄ budget (Kirschke et al., 2013). Increasing the accuracy in the estimation of natural CH₄ emissions from wetland soils under various conditions will allow us to assess how anthropogenic activities affect greenhouse gas emissions and what changes to expect with changing climate accordingly.

Several studies have focused on factors and regulators governing the production and consumption of CH₄. However, there is still paucity of information on methanotrophy as it relates to different wetland systems. In this study we focused primarily on subtropical wetland systems. Through the study of the kinetics of CH₄ oxidation, information may be gained regarding the influence of regulators, such as nutrient status, carbon quality, CH₄ availability, and microbial community structure, may have on the potential maximal rates of CH₄ oxidation (V_{max}) and the affinity (K_m) of the active methanotrophic community for CH₄ (Benstead and King, 1997; Schnell and King, 1995). Therefore, one objective of this study is to determine the kinetics of CH₄ oxidation among soils of contrasting nutrient status, vegetation, and soil depth and to determine how eutrophication status impacts the oxidation of CH₄.

As the net CH₄ emissions are a difference of CH₄ production and consumption, it is critical to determine the impact NH₄⁺ fertilization has on CH₄ oxidation potential. Conflicting results among several studies show NH₄⁺ may inhibit, stimulate, or have no effect on the rate of CH₄ oxidation. Therefore, a second

objective of this study was to determine if varying concentrations of NH_4^+ impact the rate of CH_4 oxidation potential in subtropical wetland soils.

Materials and Methods

Soil sampling

Soil cores were collected in October 2013 from the eutrophic F1 (26.3535 N, -80.35613 W) and the oligotrophic U3 ridge (U3R; 26.28516 N, -80.41005 W) and U3 slough (U3S; 26.28959 N, -80.40946 W) regions along the nutrient gradient of WCA-2A. This study site was previously described in this report. Five soil cores (0-40 cm) from each of three locations within each site were collected to yield 15 cores per site. Each core was sectioned in the field at 0-5cm, 5-10 cm, 10-20 cm, 20-30 cm, and 30-40 cm increments. The surface of the soil core was declared after discarding floc material until a visible soil structure was determined (Fig. 6). The sectioned soils were then transported in sterile, sealed plastic bags to the Wetland Biogeochemistry Laboratory at the University of Florida, Gainesville, FL. Once in the laboratory, all samples were weighed. All rocks and macro-organic material were removed from each sample and five randomly selected cores were composited so that each site had three composite replicate samples for each increment of depth. Samples were stored at 4°C prior to experimental analysis, which began within one week of sample collection.



Figure 6. Soil cores collected from the F1, U3R, and U3S sites showing soil composition, root mass, and redox zones along the depth profile.

Soil Characteristics

Biogeochemical and physio-chemical characteristics were determined for each composite soil sample. For bulk density (BD) calculations, the volume of the removed macro material was calculated by water displacement and this value was factored into the final values obtained. Soil moisture content (MC) was determined by drying an aliquot of soil from each replicate at 70°C for 72 hours. Soil pH was determined by using a 1:2 soil to water suspension. Dried, ground, and sieved (60 mm) subsamples were measured for total C (TC) and N (TN) using a Delta Plus XL Isotope Ratio Mass Spectrometer (IRMS) (Thermo Finnigan, San Jose, CA) interfaced via a Conflo-III device (Thermo Scientific,

Waltham, MA) to a ECS4010 elemental analyzer (Costech Instruments, Valencia, CA) at the Stable Isotope Mass Spectrometry Laboratory, University of Florida, Gainesville, FL.

The chloroform (CHCl_3) extraction method was used to determine microbial biomass carbon (MBC) and nitrogen (MBN) in each soil sample (Sparling et al., 1990; Brookes et al. 1985). Briefly, duplicate soil samples were incubated with and without CHCl_3 for 24 hours. Fumigated and non-fumigated samples were extracted with 0.5 M potassium sulfate (K_2SO_4) by shaking at low speed for 1 hour, centrifuging at 6000 rpm for 15 minutes, and filtering the samples via vacuum filtration using Whatman #41 filter paper. The filtrate was acidified with 1 drop of concentrated sulfuric acid. Aliquots of the acidified filtrate were then analyzed for total organic carbon (TOC) and total organic nitrogen (TON) according to US EPA Methods 415.1 (US EPA, 1993) using an automated TOC-L Total Organic Carbon Analyzer coupled with a TNM-L Total Nitrogen Measuring Unit and ASI-L Auto sampler (Shimadzu Scientific Instruments Inc., Columbia, MD). Extractable ammonium ($\text{NH}_4\text{-N}$) was determined from the filtrate using an AQ2 Automated Discrete Analyzer (SEAL Analytical, West Sussex, England) according to US EPA Method 103-A Rev 10 (US EPA, 2005).

Extractable nitrate ($\text{NO}_3\text{-N}$) was determined by extracting each soil sample with 2 M potassium chloride (KCl) and analyzing the extracts via a rapid flow analyzer (RFA-300; Alpkem Corporation, Clackamas, Oregon) according US EPA method 353.2 (USEPA, 1983). Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were determined by water extraction method using

sterile double deionized (DDI) water at a 1:10 soil to water ratio. Samples were shaken at low speed for one hour, centrifuged for 10 minutes at 6000 rpm, and then filtered via vacuum filtration using Whatman #41 filter paper. Extracts were acidified with one drop of concentrated sulfuric acid then analyzed for DOC and TDN analysis using an automated TOC-L Total Organic Carbon Analyzer coupled with a TNM-L Total Nitrogen Measuring Unit and ASI-L Auto sampler (Shimadzu Scientific Instruments Inc., Columbia, MD) according to methods ASTM D7573 (ASTM, 2009) and ASTM WK46665 (ASTM, work item), respectively.

Soil total phosphorus (TP) was determined in dried soil by combustion at 550°C for four hours. Ashed samples were dissolved in 6 M HCl (Anderson, 1976) and analyzed using an automated ascorbic acid method according to US EPA Method 365.1 (US EPA, 1993). Soil organic matter (SOM) was estimated via loss-on-ignition (LOI) according to Jackson (1985) by calculating the weight difference in the dried and combusted soil samples.

Pore-water sulfate (SO_4^{2-}) measurements were obtained by centrifuging approximately 20 g of wet soil in 50 mL centrifuge tubes at 12000 rpm for 15 minutes. Recovered pore water was filtered with Whatman #41 filter paper, bubbled with N_2 gas to remove sulfide, and shipped to the U.S. Geological Survey, Reston, Va., for analysis by Ion Chromatography according to USGS Method EEGL IC-A-01.00.

Aerobic methane oxidation

A laboratory manipulation study was performed to determine the potential rates of CH₄ oxidation under aerobic conditions for soils from three sites (F1, U3S, and U3R) at three different depths (0-5 cm, 5-10 cm, and 10-20 cm). A total of 27 samples were used to prepare soil slurries in 30 mL serum bottles using 5 g wet soil weight to 5 mL sterile DDI water. These aerobic microcosms were pre-incubated in the dark at 25°C for 24 hours to acclimate the samples to the experimental temperature. After the pre-incubation period, the soil slurries were shaken for approximately 2 hours on an orbital shaker at a low speed to release any trapped methane. Samples were then sealed with thick butyl rubber stoppers and aluminum crimp tops, fitted with stopcocks, then purged with CO₂-free air for approximately 15 minutes to remove remnant CH₄. The pressure of each microcosm was adjusted to approximately 0.35 bar.

Enriched 99% atom ¹³C-CH₄ (Sigma-Aldrich, St. Louis, MO) was added at varying concentrations to the microcosms. Samples were incubated in the dark at 25°C on an orbital shaker set at low speed for the duration of the experiment. Headspace methane concentrations in each microcosm were measured within one hour after CH₄ addition and periodically over a three day period using a Shimadzu GC-8A gas chromatogram (GC) fitted with a 1.6 m (45/60 mesh) Carboxen-1000 column (160°C; Supelco Inc., Bellefonte, PA) coupled with a flame ionization detector (FID; injection and detector temperatures 110°C; Shimadzu Scientific Instruments Inc., Columbia, MD) to determine the potential rate of CH₄ oxidation. Additionally, CO₂ concentrations in each microcosm were

measured to determine correlations between CO₂ respiration and CH₄ oxidation of the enriched CH₄ based on atom percent. Carbon dioxide measurements were taken using a GC-8A gas chromatograph fitted with a 1.83 m (80/100 mesh) Porapak-N column (40°C; Supelco Inc., Bellefonte, PA) and a thermal conductivity detector (TCD; injection temperature 120°C Shimadzu Scientific Instruments Inc., Columbia, MD). Calibration curves for CH₄ and CO₂ gases were prepared using external standard gas mixtures (Scotty Specialty Gases, Plumsteadville, PA). The Ideal Gas Law was used to determine the concentration of gas in the microcosm headspace, while the quantity of gas dissolved in the slurries was calculated using Henry's Law. Summation of the concentrations of each respective gas calculated in the headspace and slurry determined the total gas in each microcosm. The kinetics of CH₄ oxidation were determined according to the Michaelis –Menton kinetic model (Fig. 2). To determine the kinetic parameters of CH₄ oxidation, the linear Lineweaver-Burk equation was used.

To confirm the oxidation of CH₄, 20 µL CO₂ equivalent volume of headspace gas from each microcosm was transferred to helium purged 12 mL exetainer tubes and analyzed for ¹³C-CO₂ isotopic enrichment using a Delta Plus XL Isotope Ratio Mass Spectrometer (IRMS) (Thermo Finnigan, San Jose, CA) interfaced via a Conflo-III device (Thermo Scientific, Waltham, MA) to a GasBench III (Thermo Finnigan, San Jose, CA) at the Stable Isotope Mass Spectrometry Laboratory, University of Florida, Gainesville, FL.

Aerobic methane oxidation with ammonium (NH₄⁺)

A laboratory manipulation study was performed to determine the effect of NH₄⁺ on CH₄ oxidation in soils. Aerobic microcosms were prepared with soils from three sites (F1, U3S, and U3R) at three different depths (0-5 cm, 5-10 cm, and 10-20 cm). Soil slurries were prepared in 30 mL serum bottles using 1:10 fresh weight soils (1-2 g dry weight equivalent) and sterile double deionized (DDI) water and were pre-incubated at 25°C for 24 hours to acclimate the samples to the experimental temperature. After the pre-incubation period, the soil slurries were shaken for approximately 2 hours on an orbital shaker at a low speed to release any trapped methane. To study the effect of NH₄⁺, varying concentrations of NH₄Cl -N (0, 0.2, 0.3, 0.5, 1.6, or 2.7 µg N mL⁻¹) were added to six replicate microcosms per site and depth. Microcosms were then sealed with thick butyl rubber stoppers and aluminum crimp tops, fitted with stopcocks, then purged with CO₂-free air for approximately 15 minutes to purge the headspace and maintain a similar gas pressure in the bottles.

Enriched 99% atom ¹³C-CH₄ (Sigma-Aldrich, St. Louis, MO) was added to the microcosms at 10% (1156 ± 579 µg CH₄ g⁻¹) of the serum bottle volume.

Samples were incubated at 25°C on an orbital shaker set at low speed for the duration of the experiment. An aliquot of headspace gas in each microcosm was measured within the first hour of CH₄ addition to determine the initial methane concentrations, followed by periodic measurements over a three day period as previously described in this report.

Statistical Analyses

In order to assess the effects of site and depth on the kinetic, biogeochemical, and physio-chemical characteristics, we fitted a multivariate analysis of variance (MANOVA) model using the SAS procedure GLM of the SAS statistical software version 9.4 for Windows (SAS Institute, Inc., 2013). This approach also enabled us to identify the (Spearman and Pearson) correlations among the kinetic, biogeochemical, and physio-chemical variables adjusting for the effects of sites and depth. The MANOVA model includes both the main effects as well as the interactions of the factors of site and depth. All tests were considered significant if the corresponding p values were less than the normal type-I error rate of 0.05. Tukey's multiple comparison procedures were performed when the F -tests of the effects were found to be significant in order to identify and quantify the strengths of these significant effects.

In order to assess the effects of NH_4^+ concentration on the rates of CH_4 oxidation, we fitted a Bivariate model using the JMP Pro statistical software version 11.0.0 for Windows (SAS Institute, Inc., 2013). This approach also enabled us to identify the degree and of pattern of the relationships among the NH_4^+ concentration on the rates of CH_4 oxidation adjusting for the effects of sites and depth. The Bivariate model includes both the main effects as well as the interactions of the factors of site and depth. All tests were considered significant if the corresponding p values were less than the normal type-I error rate of 0.05.

Results

Soil Characteristics

Soil BD (Tbl. 2) in the F1 and U3S sites was similar along the depth profile, while BD in the U3R site increased with depth. Soil BD was highest in the U3S site and was found to be statistically different from the F1 and U3R sites to the 10 cm depth. Soil BD at the 10-20 cm depth was similar between the U3R and U3S sites. The soil pH (Tbl. 2) of all sites fell within one unit of each other, however the U3S site was found to be statistically higher than the F1 and U3R sites along the depth profile. Within the U3S site, the 0-5 cm depth was found to be statically higher than the 10-20 cm depth. The LOI (Tbl. 2) was significantly lower in the U3S site than in both the F1 and U3R sites at all depths. Within the U3S site, LOI at the 10-20 cm depth was significantly higher than the upper depths.

Table 2. Physio-chemical soil characteristics of soils at various depths from three study sites in the WCA2A region of the Florida Everglades.

Site	Depth (cm)	BD (g cm ⁻³)	Moisture Content (%)	pH	LOI (%)
Eutrophic F1	0-5	0.05 ± 0.000 ^a	93.4 ± 0.1 ^a	7.05 ± 0.03 ^a	89.8 ± 0.5 ^a
	5-10	0.063 ± 0.006 ^a	92.3 ± 0.6 ^a	7.08 ± 0.03 ^a	89.7 ± 0.2 ^a
	10-20	0.067 ± 0.012 ^a	91.8 ± 1.3 ^a	7.23 ± 0.16 ^a	86.0 ± 1.2 ^a
Oligotrophic U3S	0-5	0.117 ± 0.015 ^a	85.1 ± 1.3 ^c	7.65 ± 0.07 ^a	43.3 ± 15.8 ^b
	5-10	0.110 ± 0.010 ^b	88.0 ± 1.2 ^b	7.61 ± 0.04 ^{ab}	48.0 ± 13.6 ^b
	10-20	0.093 ± 0.012 ^a	90.2 ± 0.9 ^a	7.45 ± 0.11 ^b	74.4 ± 9.3 ^a
Oligotrophic U3R	0-5	0.043 ± 0.006 ^b	92.8 ± 0.3 ^a	6.95 ± 0.09 ^a	87.5 ± 0.4 ^a
	5-10	0.067 ± 0.012 ^{ab}	91.9 ± 0.5 ^a	7.10 ± 0.08 ^a	85.7 ± 0.3 ^a
	10-20	0.077 ± 0.006 ^a	91.0 ± 0.2 ^a	7.04 ± 0.05 ^a	86.5 ± 1.0 ^a

Data represent mean (n=3). Letters represent site specific significant differences among the mean values according to Tukey's test ($\alpha = 0.05$). BD: bulk density; LOI: loss on ignition.

Water extractable P (Tbl. 3) in the U3S site was significantly lower than water extractable P in the F1 and U3R sites down to 10 cm. No significant differences in water extractable P were found among the sites at the 10-20 cm depth. The highest overall extractable NH_4^+ concentration (Tbl. 3) was found in the U3R site, followed by the F1, and U3S sites. No significant differences in extractable NH_4^+ were found among the sites. Nitrate (NO_3^-) (Tbl. 3) was found to be significantly higher in the 0-5 cm depth of the F1 site, followed by the U3R, then U3S sites, with each site being significantly different. No significant differences in the NO_3^- concentrations were observed within the deeper depths among the sites. The DOC (Tbl. 3) content in the U3S site was found to be significantly lower than the F1 and U3R sites at the 0-5 cm depth. No significant differences in DOC were found at the 5-10 cm depth, yet, at the 10-20 cm depth, DOC in the F1 site was significantly higher than the U3S site. Total dissolved nitrogen (TDN; Tbl. 3) was significantly higher in the U3R site than in the U3S site at the 0-5 cm depth. At the 5-10 cm depth, TDN in the U3R site was significantly higher than TDN in the F1 site. At the 10-20 cm depth, TDN in both the U3R and U3S sites were significantly higher than TDN in the F1 site. Pore-water SO_4^{2-} (Tbl. 3) was highest in the U3S site, however no significant differences in the concentrations of pore water SO_4^{2-} were found among the sites at the 0-5 cm and 5-10 cm depths. At the 10-20 cm depth, the concentration of pore water SO_4^{2-} in the U3R site was significantly lower than the pore-water SO_4^{2-} in the F1 and U3S sites.

Table 3. Extractable nutrients in soils at various depths from three study sites in the WCA2A region of the Florida Everglades.

Site	Depth (cm)	Ext P (mg kg ⁻¹)	Ext NH ₄ -N (mg N kg ⁻¹)	Ext NO ₃ -N (mg N kg ⁻¹)	DOC (mg kg ⁻¹)	TDN (mg kg ⁻¹)	SO ₄ ²⁻ (mg L ⁻¹)
Eutrophic F1	0-5	20.6 ± 7.5 ^a	52.2 ± 14.5 ^a	120.4 ± 7.5 ^{†,a}	740 ± 122 ^{ab}	187 ± 40 ^a	70 ± 5 ^a
	5-10	19.0 ± 0.8 ^a	21.4 ± 3.7 ^a	7.8 ± 5.4 ^b	670 ± 30 ^b	100 ± 17 ^a	106 ± 37 ^a
	10-20	19.1 ± 4.7 ^a	25.1 ± 9.0 ^a	9.7 ± 3.0 ^b	989 ± 96 ^a	103 ± 11 ^a	88 ± 34 ^a
Oligotrophic U3S	0-5	8.0 ± 0.6 ^b	9.1 ± 4.9 ^b	17.6 ± 3.4 ^a	353 ± 125 ^b	91 ± 49 ^b	70 ± 18 ^b
	5-10	10.8 ± 3.0 ^{ab}	6.3 ± 1.8 ^b	19.6 ± 8.7 ^a	485 ± 65 ^{ab}	173 ± 42 ^{ab}	126 ± 46 ^{ab}
	10-20	15.7 ± 3.1 ^a	66.5 ± 52.2 ^a	7.8 ± 6.0 ^a	695 ± 129 ^a	282 ± 89 ^a	137 ± 18 ^a
Oligotrophic U3R	0-5	23.8 ± 0.9 ^a	62.7 ± 28.1 ^a	63.2 ± 11.2 ^a	818 ± 38 ^a	362 ± 55 ^a	112 ± 50 ^a
	5-10	22.9 ± 1.8 ^a	43.8 ± 22.4 ^a	7.2 ± 4.4 ^{†,b}	722 ± 68 ^a	293 ± 10 ^a	118 ± 35 ^a
	10-20	19.3 ± 1.4 ^a	71.5 ± 5.2 ^a	1.2 ± 0.6 ^b	916 ± 104 ^a	362 ± 43 ^a	66 ± 19 ^a

Data represent mean (n=3; where [†], n=2). Letters represent site specific significant differences among the mean values according to Tukey's test ($\alpha = 0.05$). P: extractable phosphorous; NH₄-N: extractable ammonium; NO₃-N: extractable nitrate; DOC: dissolved organic carbon; TDN: total dissolved nitrogen; SO₄²⁻: pore-water sulfate.

The TC content (Tbl. 4) of the U3S site was significantly lower than TC content of the F1 and U3R sites at the 0-5 cm and 5-10 cm depths. No differences in TC content were found among the sites at the 10-20 cm depth. Similar observations were found for TN (Tbl. 4) at the 0-5 cm and 10-20 cm depths. At the 5-10 cm depth, TN of the U3S site was significantly lower than the TN of the F1 site. Total phosphorous (TP; Tbl. 4) of the F1 site was significantly different from the U3R and U3S sites at all depths with the highest values found in the F1 site, followed by the U3R, then U3S sites. At the 0-5 cm and 10-20 cm depths, TP of the U3R and U3S sites were similar. Microbial biomass carbon (MBC; Tbl. 4) in the U3S site was significantly lower than the F1 site at the 0-5 cm depth and significantly lower than the F1 and U3R sites at the 5-10 cm depth. No significant differences were observed among the sites at the 10-20 cm depth. Microbial biomass nitrogen (MBN; Tbl. 4) was found to be significantly lower in the U3S site at the 0-5 cm depth. At the 5-10 cm depth, MBN in the U3R site was significantly higher than in the F1 and U3S sites. At the 10-20 cm depth, MBN in all sites were similar.

Table 4. Biogeochemical soil characteristics of soils at various depths from three study sites in the WCA2A region of the Florida Everglades.

Site	Depth (cm)	TC (g kg ⁻¹)	TN (g kg ⁻¹)	TP (mg kg ⁻¹)	MBC (mg kg ⁻¹)	MBN (mg kg ⁻¹)
Eutrophic F1	0-5	454 ± 3 ^a	28.9 ± 0.5 ^a	1094 ± 30 ^a	5579 ± 456 ^a	947 ± 99 ^a
	5-10	453 ± 5 ^a	29.4 ± 0.5 ^a	1111 ± 54 ^a	3849 ± 1087 ^b	579 ± 110 ^b
	10-20	447 ± 4 ^a	27.5 ± 1.3 ^a	1008 ± 279 ^a	2842 ± 628 ^b	349 ± 40 ^b
Oligotrophic U3S	0-5	231 ± 16 ^b	12.7 ± 1.3 ^c	191 ± 14 ^b ^a	2967 ± 754 ^a	513 ± 110 ^a
	5-10	293 ± 52 ^b	20.0 ± 6.4 ^b	196 ± 17 ^a	2050 ± 1057 ^a	336 ± 122 ^a
	10-20	405 ± 47 ^a	30.6 ± 3.0 ^a	186 ± 28 ^a	1627 ± 594 ^a	268 ± 165 ^a
Oligotrophic U3R	0-5	432 ± 10 ^a	27.2 ± 0.8 ^a	459 ± 28 ^a	5043 ± 563 ^a	1150 ± 214 ^a
	5-10	434 ± 1 ^a	28.0 ± 0.2 ^a	560 ± 59 ^{†,a}	4421 ± 1564 ^a	866 ± 257 ^a
	10-20	451 ± 11 ^a	30.2 ± 1.1 ^a	308 ± 49 ^a	3081 ± 850 ^a	461 ± 171 ^b

Data represent mean (n=3; where [†], n=2). Letters represent site specific significant differences among the mean values according to Tukey's test

($\alpha = 0.05$). TC: total carbon; TN: total nitrogen; TP: total phosphorous; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen.

Kinetic Measurements

V_{\max}

Significant differences in V_{\max} (Tbl. 5) were found among the sites, with the eutrophic F1 site having the highest overall maximal rate of CH_4 oxidation ($39.2 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$), followed by the oligotrophic sites U3R ($26.4 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$) and U3S ($13.8 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$). In the F1 site, V_{\max} of the 0-5 cm depth was significantly different than the 5-10 cm and 10-20 cm depths, with $p = 0.0078$ and $p = 0.0253$, respectively. No significant difference was found in V_{\max} between the 5-10 cm and 10-20 cm depths ($p = 0.3144$). Neither oligotrophic site revealed significant differences in V_{\max} ($p > 0.1$) among all depth comparisons.

When comparing the depth profile of the sites, no significant difference was found in the 0-5 cm depth ($p = 0.7514$) among the sites. In the 5-10 cm depths, there was a significant difference between the V_{\max} value of the F1 site relative to the U3R and U3S sites ($p = 0.0154$ and $p = 0.0023$, respectively). No significant difference was found between the U3R and U3S sites at the 5-10 cm depth ($p = 0.4060$). Similarly, the F1 site differed significantly from the U3S site ($p = 0.0038$) at the 10-20 cm depth. No differences were found between the F1 and U3R sites at the 10-20 cm depth ($p = 0.0970$), however the U3R site was significantly different than the U3S site at this depth ($p = 0.0393$).

A significant positive correlation ($p = 0.0002$) was determined between V_{\max} and TP. A significant negative correlation ($p = 0.0126$) was found between V_{\max} and NO_3^- . No other soil characteristics were found to be statistically correlated with V_{\max} .

K_m

Significant differences were found along the depth profile within the F1 site. The K_m of the 0-5 cm depth of the F1 site was found to be significantly different from the 5-10 cm depth ($p = 0.0028$). In addition, K_m of the 5- 10 cm depth was found to be significantly different than the K_m of 10-20 cm depth ($p = 0.0348$). However, no significance was established between the K_m values of the 0-5 cm and 10-20 cm depths ($p = 0.0568$). The K_m of the U3R and U3S sites showed no significance with depth.

When comparing the depths among the sites, no significant difference in K_m was found at the 0-5 cm or 10-20 cm depths. The K_m values of the 5-10 cm depths of the F1 and U3S sites were found to be significantly different ($p = 0.0113$).

A significant negative correlation ($p = 0.0045$) was found between NO_3^- and K_m . No other soil characteristics were found to be statistically correlated with K_m .

Table 5. Kinetic parameters for CH₄ oxidation from soils along the depth profile within the three study sites in the WCA2A region of the Florida Everglades.

Site	Depth (cm)	V_{max} (mg CH ₄ g ⁻¹ h ⁻¹)	K_m (mg CH ₄ g ⁻¹)	V_{max} (mg CH ₄ MBC ⁻¹ h ⁻¹)	K_m (mg CH ₄ MBC ⁻¹)
Eutrophic F1	0-5	0.020 ± 0.005 ^b	0.68 ± 0.31 ^b	3.6 ± 0.9 ^b	0.12 ± 0.05 ^b
	5-10	0.053 ± 0.018 ^a	4.25 ± 2.00 ^a	13.7 ± 3.3 ^a	1.11 ± 0.48 ^a
	10-20	0.045 ± 0.011 ^{ab}	2.27 ± 0.88 ^{ab}	16.7 ± 6.4 ^a	0.847 ± 0.43 ^{ab}
Oligotrophic U3S	0-5	0.015 ± 0.005 ^a	1.33 ± 0.30 ^a	5.8 ± 3.7 ^a	0.489 ± 0.25 ^a
	5-10	0.016 ± 0.009 ^a	1.70 ± 1.27 ^a	8.4 ± 3.8 ^a	0.911 ± 0.52 ^a
	10-20	0.011 ± 0.004 ^a	0.66 ± 0.41 ^a	6.9 ± 2.8 ^a	0.419 ± 0.21 ^a
Oligotrophic U3R	0-5	0.031 ± 0.000 ^{†,a}	1.38 ± 0.23 ^{†,a}	5.2 ± 2.1 ^{†,a}	0.214 ± 0.12 ^{†,b}
	5-10	0.022 ± 0.007 ^a	3.12 ± 1.30 ^a	5.9 ± 4.3 ^a	0.854 ± 0.63 ^a
	10-20	0.032 ± 0.014 ^a	2.33 ± 1.51 ^a	11.4 ± 6.6 ^a	0.879 ± 0.67 ^a

Data represent mean (n=3; where [†], n=2). Letters represent site specific significant differences among the mean values according to Tukey's test ($\alpha = 0.05$).

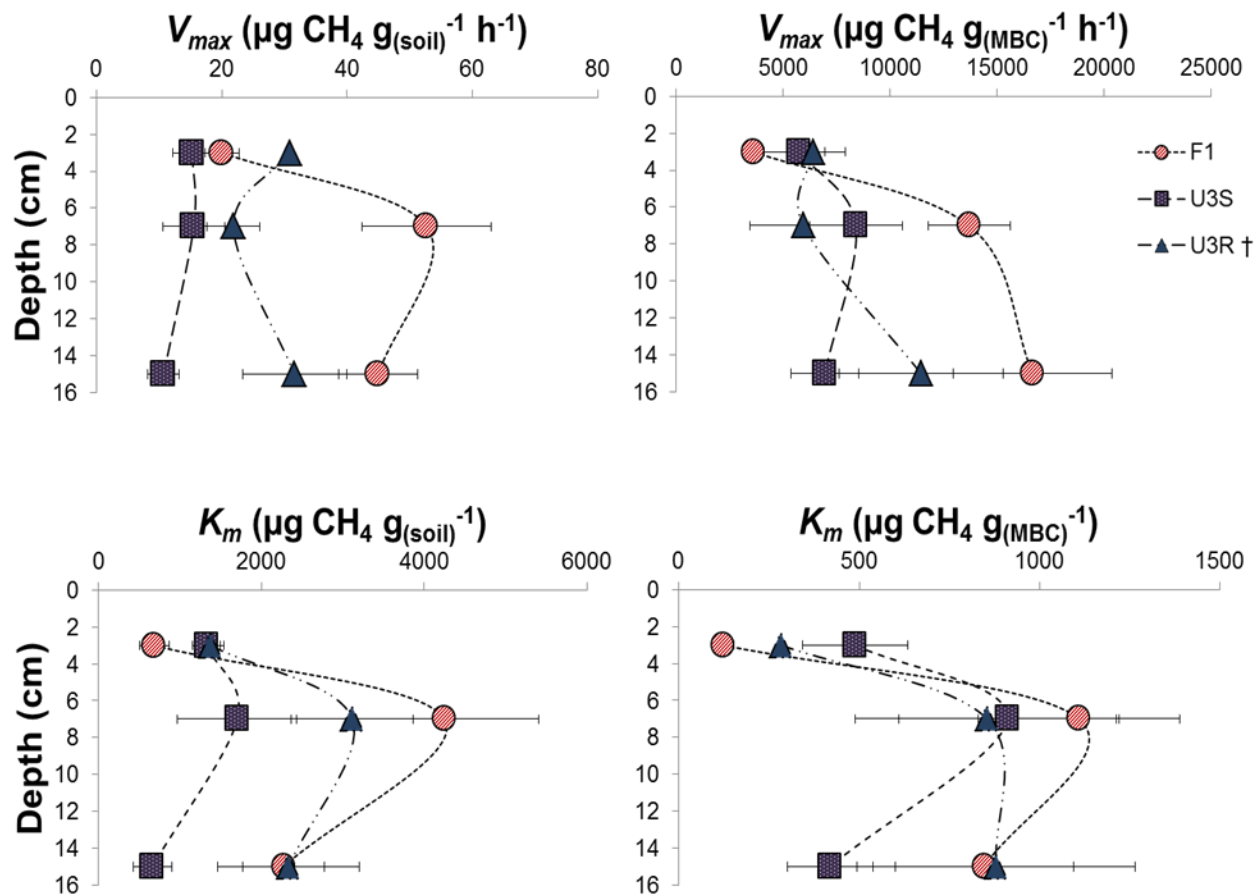


Figure 7. V_{max} and K_m measurements from the F1 (circle), U3R (diamond), and U3S (square) sites after incubation with CH_4 . Mean values ($n=3$; where † , $n=2$) are presented with standard error bars.

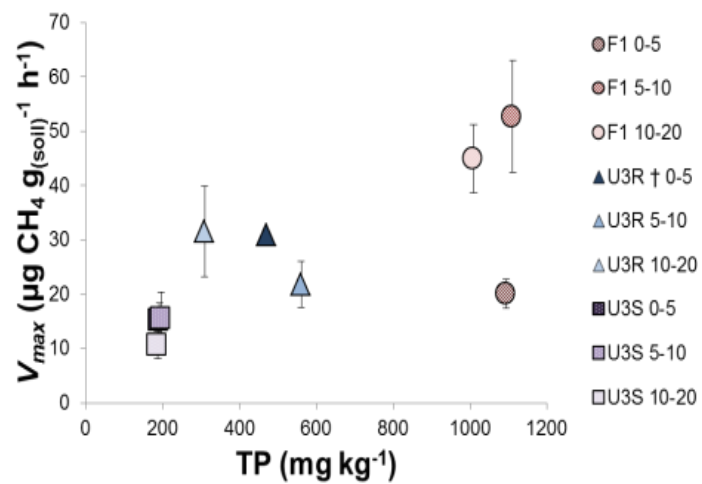
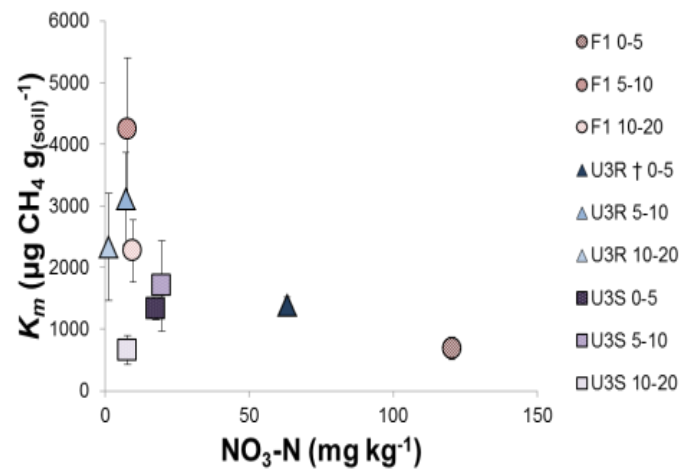
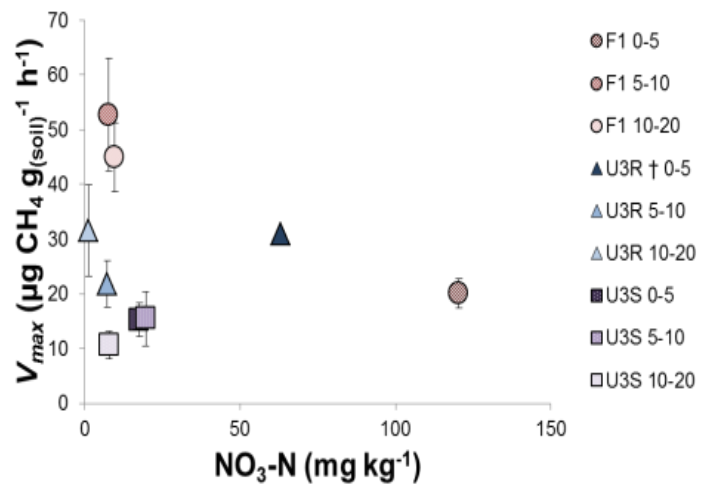


Figure 8. Significant positive correlations between V_{max} and TP; negative correlations between V_{max} and NO_3-N . Significant negative correlations between K_m and NO_3-N .

Effect of $[\text{NH}_4^+]$ on the rate of CH_4 Oxidation

No significant correlation was found when comparing the concentration of NH_4^+ with the rate of CH_4 oxidation overall among all sites and depths. The concentration of NH_4^+ did not appear to be significant within each site or at the depth intervals among the sites. When comparing the concentration of NH_4^+ with the rate of CH_4 oxidation of each site along with the depth increments (Tbl. 6; Fig. 9), increases in $[\text{NH}_4^+]$ were negatively correlated with the rates of CH_4 oxidation within the F1 ($p = 0.0011$) and U3S ($p = 0.0078$) sites at the 0-5 cm depth (Tbl. 7). Significant effects were not found in the deeper depths of the F1 and U3S sites. No significant correlation was found with depth within the U3R site.

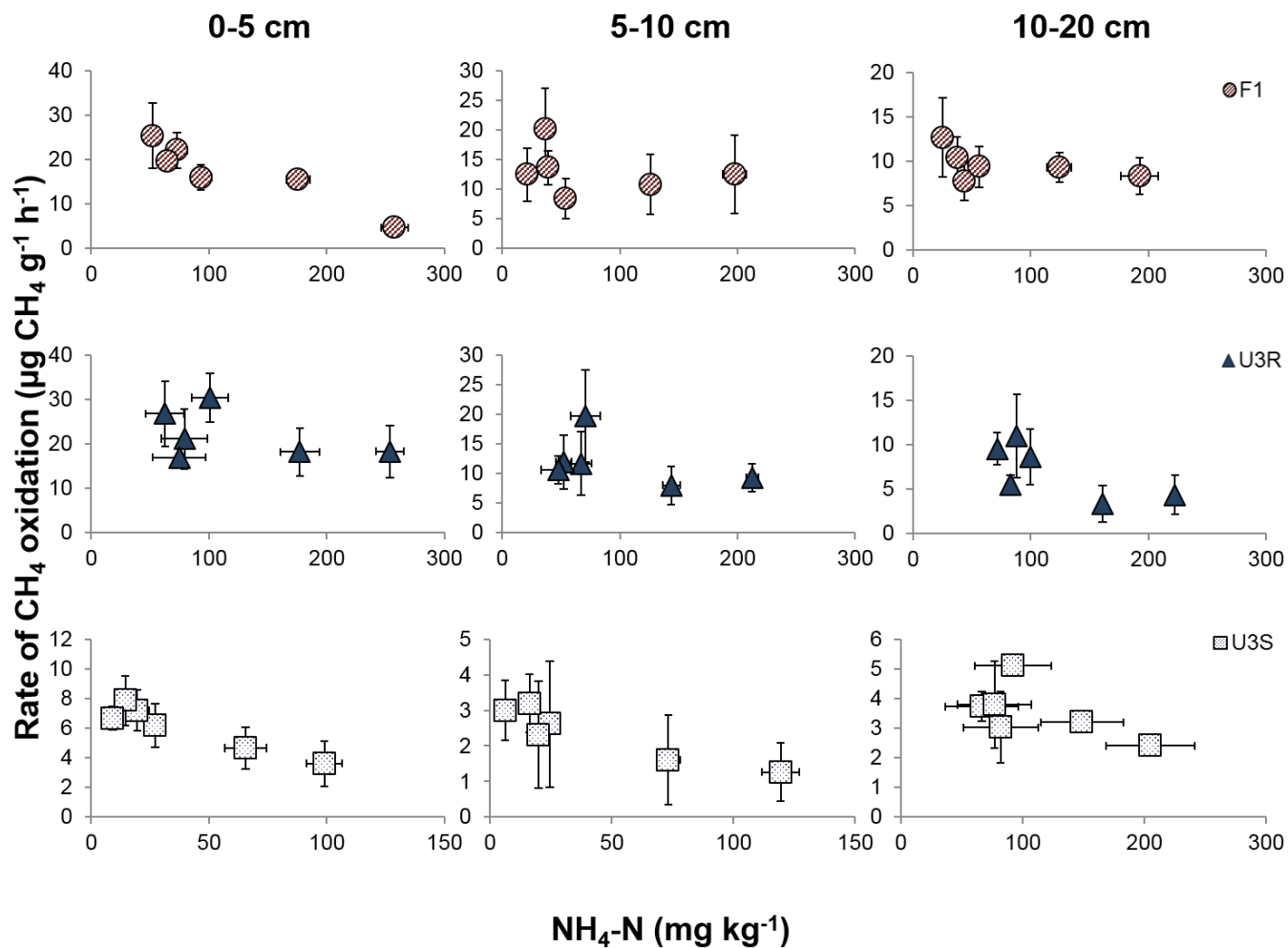


Figure 9. Increases in $[\text{NH}_4^+]$ were negatively correlated with the rates of CH_4 oxidation within the F1 ($p = 0.0011$) and U3S ($p = 0.0078$) sites at the 0-5 cm depth. Significant effects were not found in the deeper depths of the F1 and U3S sites. No significant correlation was found with depth within the U3R site.

Effect of [CH₄]:[NH₄⁺] on the rate of CH₄ Oxidation

There was no significant correlation between the rate of CH₄ oxidation and the ratio of [CH₄]:[NH₄⁺] when comparing all sites and depths. Significant correlations in this relationship were found between the rate of CH₄ oxidation and the ratio of [CH₄]:[NH₄⁺] within each site at each depth increment (Tbl. 6), however. At the 0-5 cm depth, an increased [CH₄]:[NH₄⁺] was positively correlated with increased rates of CH₄ oxidation within the F1 site ($p = 0.0028$; Tbl.7). There was a slight positive correlation in this relationship within the U3S site ($p = 0.0793$; Tbl. 7). No significant correlation was found at this depth within the U3R site. At the deeper depths, no further significant correlations were found within the F1 and U3S sites. However, significance in this relationship was found within the U3R site at both the 5-10 cm ($p = 0.0354$) and 10-20 cm (0.0001) depths.

Effect of [CH₄]:[NH₄⁺] on the rate of CO₂ production

No correlation was found in the [CH₄]:[NH₄⁺] and CO₂ production when doing an overall comparison or within each site. Negative correlations were found among the sites at the 0-5 cm and 5-10 cm depths ($p = 0.0005$ and $p = 0.0093$, respectively). A positive correlation ($p = 0.0071$) was found at the 10-20 cm depth, however. When comparing the [CH₄]:[NH₄⁺] with the rate of CO₂ production for each site at each depth increment (Tbl.6), an increased rate of CO₂ production was positively correlated with increased rates of CH₄ oxidation within the F1 ($p = 0.0372$) and U3S ($p = 0.0153$) sites at the 0-5 cm depth (Tbl. 7). No significant correlation was found at this depth within the

U3R site. At the 5-10 cm depth, a significant positive correlation was found with the U3S soils ($p = 0.0020$) only (Tbl.7).

Effect of $[\text{NH}_4^+]$ on the rate of CO_2 production

No significant correlation was found when comparing the $[\text{NH}_4^+]$ with the rate of CO_2 production overall or within each site. Correlations were found when comparing the $[\text{NH}_4^+]$ with the rate of CO_2 production (Tbl.6) among sites at each depth increment. At the 0-5 cm and 5-10 cm depths ($p = <0.0001$ and $p = 0.0007$, respectively), positive correlations were found. A negative correlation ($p = 0.0048$) was found at the 10-20 cm depth, however. At the 0-5 cm depth, increases in $[\text{NH}_4^+]$ were positively correlated with the rate of CO_2 oxidation for each site. No significant correlations were found within the three sites at the 5-10 cm depth. At the 10-20 cm depth, soils from the F1 ($p = 0.0346$) and U3S ($p = 0.0021$) sites were significantly negatively correlated with the $[\text{NH}_4^+]$ (Tbl. 7). No significant effect of $[\text{NH}_4^+]$ was found at the 10-20 cm depth within the U3R site.

Rate of CH_4 oxidation vs rate of CO_2 production

An overall positive correlation ($p = 0.0008$) was found when comparing the rate of CH_4 oxidation with the rate of CO_2 production (Tbl.6). No significant correlations were found overall within each site. Among the sites at each depth increment, significant positive correlations were found at the 5-10 cm ($p = 0.0012$) and the 10-20 cm ($p = 0.0031$) depths. When comparing each site individually at each depth, no significant correlations were found.

Table 6. Ranges ammonium, methane and carbon dioxide concentrations, the ratio of methane concentrations to ammonium concentrations and the rates of methane and carbon dioxide production from soils along the depth profile within the three study sites in the WCA-2A region of the Florida Everglades.

Site	Depth (cm)	[NH ₄ ⁺] (mg N kg ⁻¹)	[CH ₄] (mg kg ⁻¹)	[CO ₂] (mg kg ⁻¹)	[CH ₄]:[NH ₄ ⁺]	Rate of CH ₄ oxidation (mg CH ₄ kg ⁻¹ hr ⁻¹)	Rate of CO ₂ production (mg CO ₂ kg ⁻¹ hr ⁻¹)
Eutrophic F1	0-5	52-258	1506-1985	1655-4981	6.2-32.9	4.6-25.3	21-80
	5-10	21-198	1232-1592	4275-8686	6.6-63.7	8.4-20.1	16-31
	10-20	25-192	974-1397	1236-2350	5.1-63.0	7.7-12.7	12-40
Oligotrophic U3S	0-5	9-99	695-1096	1028-1685	7.1-111.7	3.6-7.9	1-24
	5-10	6-119	454-718	1418-3931	3.8-120.6	1.3-3.2	5-16
	10-20	67-205	397-664	375-498	2.1-12.8	2.4-3.8	2-8
Oligotrophic U3R	0-5	63-254	1332-2556	1548-4310	7.0-37.7	16.9-30.4	12-73
	5-10	48-212	1132-2180	2722-6883	5.8-41.4	8.0-19.7	15-34
	10-20	72-223	520-1176	647-1815	2.7-16.5	4.4-11.0	9-33

Data represent mean (n=3).

Table 7. p values showing the significance of the relationships between the rates of methane oxidation and carbon dioxide production, and these rates against ammonium concentration and the ratio of the concentrations of methane and ammonium per site at each depth increment.

Site	Depth (cm)	Rate of CH ₄ Oxidation (mg CH ₄ kg ⁻¹ hr ⁻¹) vs [NH ₄ ⁺] (mg N kg ⁻¹)	Rate of CH ₄ Oxidation (mg CH ₄ kg ⁻¹ hr ⁻¹) vs [CH ₄]:[NH ₄ ⁺]	Rate of CO ₂ Production (mg CO ₂ kg ⁻¹ hr ⁻¹) vs [NH ₄ ⁺] (mg N kg ⁻¹)	Rate of CO ₂ Production (mg CO ₂ kg ⁻¹ hr ⁻¹) vs [CH ₄]:[NH ₄ ⁺]	Rate of CH ₄ Oxidation (mg CH ₄ kg ⁻¹ hr ⁻¹) vs Rate of CO ₂ Production (mg CO ₂ kg ⁻¹ hr ⁻¹)
Eutrophic F1	0-5	0.0011	0.0028	0.0091	0.0372	0.0532
	5-10	0.5342	0.2322	0.4198	0.2890	0.9802
	10-20	0.5355	0.1120	0.0346	0.9445	0.2175
Oligotrophic U3S	0-5	0.0078	0.0793	0.0181	0.0153	0.0311
	5-10	0.3578	0.6048	0.1189	0.0020	0.9713
	10-20	0.5436	0.7785	0.0021	0.5455	0.0880
Oligotrophic U3R	0-5	0.5633	0.1676	0.0005	0.0670	0.7380
	5-10	0.4439	0.0354	0.8036	0.4175	0.0408
	10-20	0.1331	0.0001	0.3015	0.0020	0.1208

Significant values are shown in bolded text.

Discussion

Methane and oxygen availability

The availability of CH₄, the nutrient status of the system, dominant vegetation present, as well as the composition of the microbial community appear to be major driving factors potentially influencing the observed V_{\max} and K_m of these soils. The overall V_{\max} of the F1 soils was nearly two-fold higher than the V_{\max} of the U3R soils and nearly four-fold higher than the V_{\max} of the U3S soils. Previous studies have shown that CH₄ production is higher in the eutrophic region of WCA-2A (Wright and Reddy, 2001; Chauhan et al., 2011). This overall increased substrate availability may allow for the potential of a higher rate of activity and growth of methanotrophic bacteria, resulting in the observed overall higher V_{\max} of the F1 site.

While depth did not appear to affect the rates of CH₄ oxidation in the oligotrophic sites, a significant increase in the V_{\max} along the depth profile of the eutrophic site was observed. The V_{\max} of the surface layer of the F1 site was significantly lower than the 5-10 cm and 10-20 cm depths, with the 5-10 cm depth exhibiting the highest rates of CH₄ oxidation. When calculating V_{\max} and K_m based on MBC, both the V_{\max} and K_m of the 0-5 cm depth within the F1 site was found to be significantly lower than the 5-10 cm ($p = 0.0057$ and $p = 0.0037$, respectively) and 10-20 cm ($p = 0.0010$ and $p = 0.0211$, respectively) depths within this site.

According to Bender and Conrad (1992), the transition from higher affinity oxidation capability to low affinity oxidation may occur between 100 and 1000 $\mu\text{L CH}_4 \text{ L}^{-1}$, with both types of oxidation occurring in the soil. In WCA-2A soils, the K_m of the 0-5 cm

depth in the F1 site was approximately six times lower than the K_m of the 5-10 cm depth and three times lower than the K_m of the 10-20 cm depth within the F1 site. Evidence of lower CH_4 availability within the 0-5 cm depth of the F1 site may be shown by the higher affinity for CH_4 as determined by K_m .

Wetland plants influence the growth of methanotrophic bacterial populations due to their ability to transport O_2 into the soil via aerenchyma tissue. As O_2 is emitted from the plant roots, oxic zones form along the plant rhizosphere allowing for significant oxidation of CH_4 to occur. The quantity of O_2 supplied to this zone is dependent upon the plant species. It has been shown that rice cultivars differ in their ability to supply O_2 to plant rhizospheres and thereby affect methanotrophic population growth. Rice plant species which have low gas conductance have a negligible effect on the methanotrophic population density as observed in a study which compared the methanotrophic population densities within the rhizosphere of different plant species and in bare rice plots (Macalady et al., 2006). Under flooded conditions, *Typha dom.* was shown to have the capability to release a greater concentration of O_2 into the soil layers than *Cladium Jam.* due to higher porosity of *Typha* roots (Chabbi et al., 2000). This difference in vegetation type and the variation in O_2 transport via plant species may contribute to the ability of increased CH_4 oxidation capability found within the F1 site when compared to the U3R and U3S sites. In contrast, increased O_2 availability may also act to suppress CH_4 oxidation activity indirectly through inhibition of CH_4 production. Lowered CH_4 production may occur in the 0-5 cm depth of the F1 site due to a higher concentration of O_2 present which can act to inhibit methane production by suppressing the reduced

conditions required for CH₄ production. This relative reduction in CH₄ concentration may be shown by the significantly lower K_m at this depth.

An additional explanation for lower CH₄ concentrations in the surface depth may be based on the high potential CH₄ oxidation rates of the soils below 5 cm. It is likely much of the CH₄ produced deeper within the soil profile is consumed prior to reaching the surface layer, as evidenced by the relatively high K_m values found in the 5-10 and 10-20 cm depths. Methane produced in deeper depths may also be transported from the soil via the aerenchyma tissues in wetland plants, contributing to the limited CH₄ availability within the surface depth.

Nitrogen availability and kinetics

Alam and Jia (2012) studied the inhibitory effect of N-based fertilization on CH₄ oxidation in paddy soils and showed high nitrification activity occurred under treatments of urea and of ammonium sulfate ((NH₄)SO₄). Significant inhibition of oxidation activity was found in soils treated with N concentrations above 200 µg N g⁻¹, regardless of whether urea or (NH₄)SO₄ was applied, while concentrations of 100 µg N g⁻¹ or less were found to have no significant impact on oxidation activity. When NH₄⁺ concentrations exceeded 200 µg N g⁻¹, it was thought that competition between NH₄⁺ and CH₄ for the active site of MMO was a potential mechanism of inhibition of CH₄ oxidation activity in these soils. During the process of nitrification, NH₄⁺ is converted to NO₃⁻ in the presence of O₂. Nitrate strongly inhibited CH₄ oxidation in oak soil at high (5 mL L⁻¹) and low (10 µL L⁻¹) CH₄ concentrations (Reay and Nedwell, 2004). The potential for high nitrification activity within the 0-5 cm depth of the F1 site may contribute to the

lower V_{\max} due to competition for O_2 . Relatively higher NO_3^- concentrations are present within the 0-5 cm depth of the F1 site. As an available electron acceptor, relatively higher NO_3^- concentrations within the surface depth may also act to suppress CH_4 production within the 0-5 cm depth of the F1 site. The significant increase in the quantity of NO_3^- in the 0-5 cm depths of the F1 site relative to the 5-10 cm and 10-20 cm depths may additionally be evident of greater O_2 availability in the surface layer, resulting in less CH_4 production and availability. This increased O_2 availability and increased alternate electron acceptor (i.e., NO_3^-) availability may result in a decrease in CH_4 production and availability.

In contrast, N additions have been shown to increase the rate of microbial CH_4 oxidation by reducing competition for N between the plant and the microbial populations in areas of high root density (Bodelier et al., 2000). In soils under N-limitation, it is believed the addition of N-fertilizers may act to stimulate CH_4 oxidation activity by providing a necessary nutrient to the microbial population. The lower concentrations of NO_3^- found within the deeper depths of the F1 site and in the oligotrophic sites may be stimulatory to CH_4 oxidation. Further research is needed to determine how the concentration of inorganic N impacts CH_4 production and oxidation.

The V_{\max} of U3S soils were considerably lower than the F1 and U3R sites. This may be due to the marl soil present along the depth profile of this site. In the U3S site, marl soil was dominant down to a depth of 10 cm. King et al. (1990) found no evidence of CH_4 oxidation in the plant rhizosphere of marl soils. While the soil at the 10-20 cm depth of the U3S site has more carbon availability, the limitation of O_2 may explain the low CH_4 oxidation potential. King et al. (1990) speculated that the lower CH_4 oxidation

activity in marl soils may be due to differences in the alkalinity of marl soils, however the measured pH values among the peat and marl sites fell within one unit. Similar pH differences were measured between the peat and marl soils of this study. Therefore, as concluded in King et al. (1990), it is unlikely that pH is a contributing factor in the reduced rates of CH₄ oxidation found in U3S soils.

Ammonium influence on methane oxidation

The degree of influence NH₄⁺ has on the rates of CH₄ oxidation may depend on the concentration of NH₄⁺, as well as the metabolic features of the microbial populations within the soil. One suspected mechanism of competitive substrate inhibition by NH₄⁺ is believed to be due to the ability of NH₄⁺ to block the active site of the MMO enzyme. Another mechanism of inhibition may be due to the production of toxic NH₄⁺ oxidation products (e.g., hydroxylamine and NO₂⁻). Substrate competition is believed to have a greater affect in short term studies, while inhibition by toxicity is suspected to be a more dominant inhibition process in long term incubation studies. The genetic potential of methanotrophs to function in the presence of and metabolize NH₄⁺ may be key to determining if the effect of NH₄⁺ will be stimulatory it inhibitory.

As previously stated, increasing concentrations of NH₄⁺ were negatively correlated with the rates of CH₄ oxidation in the F1 and U3S sites at the 0-5 cm depths. This may signify that NH₄⁺ availability may be the driving factor influencing the rate of CH₄ oxidation within these sites at this depth. A likely explanation is that the microbial population, within these sites, at this depth, may be sensitive to shifts in N and CH₄ availability and that methanotrophic activity within the F1 and U3S sites are N-

dependent at the 0-5 cm depth. Type I methanotrophs tend to experience a faster growth rate and be dominant under conditions in which N is not limiting. Type II methanotrophs have the ability to fix N_2 and are therefore not affected under N limited conditions as Type I methanotrophs may be. Type II methanotrophs tend to be dominant under conditions of high $[CH_4]$. In a study by De Visscher et al. (2003), it was shown that differences in methanotrophic populations may be shown by the degree of inhibition of CH_4 oxidation by NH_4^+ in landfill cover soils, with Type I methanotrophs dominating during phases of N dependence and Type II methanotrophs dominating under conditions of N limitation.

Conclusions

Increased eutrophication status appears to result in higher rates of CH_4 oxidation and lowered affinity for CH_4 . Significant positive correlations with TP suggest increased TP may influence the rates of CH_4 oxidation directly and indirectly. Increased TP is known to result in greater ecosystem productivity and may stimulate CH_4 production, thereby increasing substrate availability and overall CH_4 oxidation activity. Overall negative correlations with NO_3-N suggest the presence of inorganic N may be inhibitory to CH_4 oxidation. The degree of influence of NH_4^+ concentration on the rate of CH_4 oxidation may be dependent on the microbial population present and the level of sensitivity to shifts in N and CH_4 availability of this population. Further research is needed to determine the effect of N limitation and NH_4^+ concentration on CH_4 oxidation activity.

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