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Influence of Physical and Biological Factors on Methane Emissions and Organic Carbon Mineralization in a Wet, Tropical Forest Soil

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Influence of Physical and Biological Factors on Methane Emissions and Organic Carbon Mineralization in a Wet, Tropical Forest Soil

by

Dara Bridget Goodheart

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Professor Terry C. Hazen

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Abstract

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Professor Mary K. Firestone, Chair

Organic matter decomposition is a central step in the global carbon cycle. Tropical forests generally have high net primary productivity (NPP) and a large percentage of the global carbon stock, making them an important component of the global carbon budget. Organic matter decomposition ultimately produces carbon dioxide (CO_2) and methane (CH_4), both important greenhouse gases. Rates of organic carbon mineralization are affected by soil physical parameters, such as fluctuating redox conditions, and biological parameters, such as microbial community composition and the microbial genetic potential for organic carbon mineralization. Tropical forest ecosystems are predicted to become warmer and drier due to global climate change; such changes would affect CO_2 and CH_4 efflux from tropical forest soils. Thus, understanding how physical and biological factors sensitive to climate affect organic matter mineralization to CO_2 and CH_4 in these soils is important.

The possible microbial controllers of CH_4 emissions in upland tropical forest soils were evaluated in Chapter 1. Recent evidence has suggested that some upland tropical forest soils may be CH_4 sources. This has important implications for climate models because most models consider tropical forest soils to be CH_4 sinks. Here, two microcosm-based experiments were used to identify possible physical (edaphic) and biological (microbial community size and diversity) factors that could influence CH_4 emissions in three upland tropical forest soils from the Luquillo Experimental Forest in Puerto Rico. Edaphic factors, quantification of functional gene markers by Polymerase Chain Reaction (PCR - mcrA for methanogens, pmoA for CH_4 oxidizers), and community composition (clone libraries of mcrA and pmoA) were correlated to cumulative CH_4 production and oxidation to determine the relative importance of each to CH_4 emissions in these soils. Unsurprisingly, edaphic factors including soil moisture and mean annual precipitation drive CH_4 production in these soils. Results indicate that in soils with high soil moisture, methanogens are more abundant, leading to greater potential CH_4 production. Methanogen community diversity did not significantly affect CH_4 production. However, mcrA sequences representing the methanogen genera, *Methanosaeta* and *Methanosphaera* were associated with high CH_4 production. Potential CH_4 oxidation was unaffected by any edaphic factor tested. However, potential CH_4 oxidation was correlated to the estimated size of the CH_4

oxidizing community (as measured by qPCR of the pmoA gene). In particular, greater potential CH_4 oxidation was observed in soils with greater Type I pmoA gene abundance. Rates of CH_4 oxidation were greatest in soils with the highest rates of CH_4 production. The results presented indicate that conditions conductive to methanogen activity (saturated soils) are key to the CH_4 production rates in these soils and that specific genera of methanogens are associated with the highest rates of production. The size of the CH_4 oxidizing community is associated with greater CH_4 oxidation in these soils and overall, CH_4 oxidation potentials were primarily dependent on the rates of CH_4 production.

The focus of Chapters 2, 3 and 4 shifts to the effects of redox conditions (oxygen availability), community profiles of microbial genes involved in decomposition processes (carbon degradation potential), and bacterial community composition on mineralization of organic matter to CO_2 and CH_4. High rates of plant material decomposition have been measured in the soil of the Luquillo Experimental Forest in Puerto Rico. These high rates have been attributed to the consistently warm and moist conditions that these soils experience. These soils also experience redox fluctuations driven in part by frequent rain events and high biological activity. In Chapters 2 and 3, I investigated the following questions: what is the effect of redox condition on organic carbon mineralization and carbon degradation potential, and are there links between mineralization of organic matter, carbon degradation potential, and bacterial community composition. I hypothesized that under fluctuating redox conditions, both aerobic and anaerobic decomposition processes occur and are synergistic, leading to greater rates of organic carbon mineralization than under static oxic or anoxic conditions. To address these questions and hypothesis, I added ^{13}C-labeled organic carbon (either plant material, *Avena barbata*, or cellulose) as a substrate to soils in two microcosm-based experiments. In these experiments, the soil microcosms were either amended with the ^{13}C-labeled carbon or left unamended and incubated under three redox conditions; oxic, anoxic, or alternating four-day oxic-anoxic fluctuation. The timescale of the two experiments was different; the *A. barbata* litter-amended experiment was 38 days, and the cellulose-amended experiment was 22 days. Headspace gases analyzed over time were used to measure cumulative CO_2 and CH_4 and the production of ^{13}C-labeled CO_2 and CH_4. Soil microcosms were destructively sampled over time to allow determination of microbial community abundance by qPCR. Final time point samples were used to analyze the effect of redox condition on carbon and cellulose degradation potentials (by GeoChip 4) and bacterial community composition (by 454 Pyrosequencing). Degradation potential refers to the composition and abundance of gene probes on the GeoChip 4 microarray; for carbon degradation potential, the carbon degradation and organic remediation gene probes were analyzed and for cellulose degradation potential, the gene probes involved in cellulose degradation were analyzed. In Chapter 4, the results from the *A. barbata* litter and the cellulose addition experiments (Chapters 2 and 3) are compared to determine the importance of redox condition, degradation potential and bacterial community composition as controllers of mineralization of a complex plant litter compared to a purified component of plant litter, cellulose.

The influence of redox conditions on the mineralization of plant litter, microbial community abundance, bacterial composition, and carbon degradation potential in a wet, tropical forest soil is presented in Chapter 2. Redox conditions strongly influenced plant litter mineralization to CO_2 and CH_4. Mineralization of ^{13}C-labeled *A. barbata* litter to CO_2 was greatest under fluctuating redox conditions. However, because substantial CH_4 was produced over 38 days under the anoxic condition, the mineralization of *A. barbata* litter to ^{13}C-gas was not

significantly different under the anoxic and the fluctuating redox conditions. Carbon degradation potential was also influenced by redox condition; the carbon degradation potential from samples under anoxic conditions was distinct from the carbon degradation potential from samples under oxic conditions. However, carbon degradation potential was not correlated to mineralization. Redox conditions did not affect bacterial community composition or microbial community abundances. However, there were substantially more Firmicutes sequences detected under anoxic conditions in the presence of added *A. barbata* litter. In addition, bacterial community composition was correlated to indices of mineralization (cumulative CO_2, cumulative CH_4 and ^{13}C-CO_2 production). The release of soluble sugars from *A. barbata* litter probably enabled the activity of bacteria capable of rapid growth on easily degradable carbon sources, such as Firmicutes, thus affecting the bacterial community composition. Bacterial community composition was not correlated to carbon degradation potential, suggesting that the bacterial community composition is functionally redundant. There appears to be distinguishable aerobic and an anaerobic decomposition potentials which is consistent with a synergy between them under fluctuating redox conditions. Indeed, the ^{13}C-CO_2 produced from the added plant litter was significantly greater under the fluctuating redox condition.

In Chapter 3, the influence of redox condition on the mineralization of cellulose, microbial abundance, bacterial community composition, and cellulose degradation potential is assessed. Cellulose is one of the most abundant biopolymers on earth. It is also considered a relatively recalcitrant component of plant material. There are two main microbial metabolic strategies to degrade cellulose: cell-free extracellular enzymes and cellulosomes, cell-associated supra-molecular structures. Cellulosomes are produced under anoxic conditions by both bacteria and fungi whereas cell-free extracellular cellulose degrading enzymes are produced under both oxic and anoxic conditions by bacteria and fungi. Redox condition influenced cellulose mineralization; cellulose mineralization was significantly greater under the static anoxic condition. This result indicates that anaerobic microbes play an important role in cellulose degradation in this soil. Redox condition also affected the cellulose degradation potential when the static oxic samples were compared to the static anoxic samples. However, when the fluctuating redox samples were included in the statistical analysis, redox condition did not affect the total detected cellulose degradation potential. The majority of the detected cellulose degradation potential gene probes were glycosyl hydrolases which are responsible for degrading cellulose fibers to monomer and dimer sugars. Glycosyl hydrolases are grouped into large families based on sequence homology and not microbial origin or whether they are produced under oxic or anoxic conditions. Also glycosyl hydolases do not require molecular O_2 for function. Therefore, it is not surprising that overall redox condition did not affect the cellulose degradation potential. The organization of glycosyl hydrolase families also explains why carbon degradation potential and bacterial community structure were not correlated. Neither carbon degradation potential nor bacterial community composition were correlated to cellulose mineralization. However, bacterial community composition was correlated to cumulative CO_2 which suggests that in this experiment, bacterial community composition was important to mineralization of other allochthonous carbon sources. Cellulose mineralization and cellulose degradation potential in this soil appears to be largely uncoupled from bacterial community composition. Cellulose degradation in these soils may involve synergy between glycosyl hydrolases, but potential synergy in aerobic and anaerobic cellulose degradation was not supported.

In Chapter 4, the results from plant litter mineralization (Chapter 2) and cellulose mineralization (Chapter 3) are compared to assess differences and similarities in the mineralization and degradation potential of these organic carbon sources. Gas measurements from the *A. barbata* litter amended experiment were recalculated using data taken up to day 22 so that they could be compared to the gas measurements of the 22 day cellulose-amended experiment. GeoChip data from each experiment was re-analyzed so that a carbon degradation and a cellulose degradation potential from each experiment could be compared. Likewise, the bacterial community composition data from the litter-amended experimental samples were rarified to the extent of the samples from the cellulose-amended experiment so that they could be compared. In both experiments, redox conditions affected the mineralization to CO_2 and CH_4. In particular, under the anoxic condition, mineralization of cellulose to CO_2 was greater than under the other conditions tested, and mineralization of *A. barbata* litter to CO_2 was substantial. Only slight mineralization of *A. barbata* litter to CH_4 was observed after 22 days, but the CH_4 observed was greater under the anoxic conditions. CH_4 production was below the limit of detection in the cellulose-amended experiment. The substantial mineralization of both *A. barbata* litter and cellulose to CO_2 suggests that anaerobic microorganisms are crucial in decomposing plant litter and cellulose in this soil. Redox condition also affected the carbon and cellulose degradation potentials found in both experiments. A distinguishable aerobic and anaerobic carbon degradation potential was observed in the *A. barbata* litter- and the cellulose-amended experiments, and a distinguishable aerobic and anaerobic cellulose degradation potential was observed only in the *A. barbata* litter-amended experiment. A distinguishable aerobic and an anaerobic cellulose degradation potential was not observed in the cellulose-amended experiment. The observation of distinguishable aerobic and an aerobic carbon degradation potentials indicates that under fluctuating redox conditions, these degradation potentials could work synergistically.

Table of Contents

List of Figures

List of Tables

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Chapter 1

Activity and Diversity of Methanogens and Methane Oxidizing Bacteria in Wet, Tropical Forest soils

Abstract

Tropical forest soils are globally important sources of methane (CH_4). Wet tropical soils in the Luquillo Experimental Forest in Puerto Rico (LEF) have been reported to support high rates of CH_4 flux. While biogeochemical controls of CH_4 emissions from the LEF have been explored, the diversity and abundance of methanogenic Archaea and CH_4 oxidizing bacteria have not been investigated. Three tropical soils comprising an elevation transect from the LEF were used in microcosm-based studies to identify the microbial community members responsible for CH_4 dynamics in these soils. The studies were designed to link methanogens to potential CH_4 production rates and link CH_4 oxidizing bacteria to rates of potential CH_4 oxidation.

Potential CH_4 production rates from the mid-elevation Colorado forest (lower montane wet forest) and higher-elevation Cloud forest soils (lower montane rain forest) followed different patterns. The CH_4 production rates from the Cloud forest soils increased rapidly for two days, and then began to plateau while the CH_4 production rates from the Colorado forest soils continued to increase over the course of the ten-day experiment. The highest initial CH_4 production rates were from the lower montane rain forest soils (Cloud). The size of the active standing methanogen population appeared to drive CH_4 production rates. Soil moisture, mean annual precipitation and percent C and N correlated with high CH_4 production rates and the abundance of the functional gene marker for methanogenesis (mcrA). Analysis of mcrA clone libraries indicated that taxa with mcrA sequences similar to *Methanosaeta* and *Methanosphaera* were more abundant in sites with higher CH_4 production. In the Cloud forest, the CH_4 production rates appeared to reflect the observed mcrA community composition and abundance.

Potential CH_4 oxidation rates were greater in the soils with high potential CH_4 production rates. The highest initial CH_4 oxidation rates were from the Cloud forest soils. The population size of the CH_4 oxidizing bacteria appears to drive CH_4 oxidation rates. No edaphic factors were found to be correlated with the abundance of a functional gene marker for CH_4 oxidation (pmoA). The Type I CH_4 oxidizing bacteria were more abundant in soils with greater CH_4 oxidation rates. The ratio of pmoA:mcrA gene copies tended to be greater in the soils with low CH_4 oxidation and low CH_4 production rates.

Similar to other saturated environments, such as rice paddy soils, potential CH_4 oxidation rates in these upland tropical soils appears to be driven by the potential for CH_4 production. Thus, methanogens are primary drivers of CH_4 emissions from these upland tropical forest sites.

Introduction

Upland humid, tropical forest soils, such as those found in the Luquillo Experimental Forest (LEF) in Puerto Rico, are a globally important source of CH_4 (Teh et al 2005, Keller 1986), a greenhouse gas capable of 72 times the radiative forcing of CO_2 over a 25 year period (IPCC 2007). Although the biogeochemical controls of CH_4 dynamics in LEF soils have been relatively well studied (Silver et al 1999, Teh et al 2005, Teh et al 2006b), the importance of methanogen and methanotroph community structure to CH_4 emissions in this system is not known.

CH_4 emissions from upland tropical forests are important in the global CH_4 cycle; tropical forests are estimated to be responsible for approximately 17% of global CH_4 emissions (Houweling et al 1999). The upland tropical forest soils in the LEF can produce large amounts of CH_4, up to 22-101 nmol CH_4 m^{-2} s^{-1}, rates similar to some natural wetlands and rice paddy soils (Teh et al 2005, Bartlett and Harriss 1993, Sass and Fisher 1997, Mosier et al 1998). CH_4e emissions from tropical forest soils are influenced by CH_4e oxidation prior to efflux as well as climate and soil properties, particularly soil moisture, an important driver of redox dynamics (Keller 1986, Keller and Reiners 1994, Bustamante et al 2009, Neto et al 2011, Silver et al 1999, Liptzin et al 2011). Forest soils from the LEF have high soil moisture and microbial activity and have large potentials for CH_4 production and oxidation activities (Silver et al 1999, Teh et al 2005). Factors such as soil depth (Lin et al 2012) and water saturation (Yavitt et al 2006) have been shown to influence CH_4 emissions in wet, acidic environments, and could be controlling factors in CH_4 emissions from upland tropical forest soils as well. Methanogen diversity has also been shown to influence CH_4 production rates, with higher CH_4 production occurring in locations where methanogen diversity is greater (Yavitt et al 2012). While upland tropical forests play a critical role in the global CH_4 cycle, the microbial communities responsible for CH_4 production and oxidation in these soils have not been thoroughly investigated.

Methanogenic Archaea and methanotrophic bacteria both play key roles in CH_4 emission. Methanogens are obligate anaerobes that catalyze the last step in organic matter decomposition (Conrad 1996, Ferry 1999). There are two main substrates used by methanogenic Archaea, H_2/CO_2 (hydrogenotrophic) and acetate (acetoclastic), though other one carbon (C1) compounds can also be used. Acetoclastic methanogenesis accounts for approximately two thirds of terrestrial biogenic CH_4 production (White 2000). However, in saturated and in acidic environments, hydrogenotrophic methanogenesis generally predominates (Parkes et al 2007, Kotsyurbenko et al 2007, Horn et al 2003). Most methanogens can use H_2/CO_2 as a substrate; only taxa from the genera *Methanosaeta* and some *Methanosarcina* are known to use acetate. The terminal step in converting a methanogenic substrate to CH_4 is carried out by methyl-coenzyme M reductase (mcrA). This enzyme is composed of several subunits; the mcrA gene encodes the active site. The phylogeny of mcrA is congruent with 16S rRNA phylogeny, making mcrA an ideal gene to survey methanogen diversity (Luton et al 2002). In addition, the abundance of mcrA has been shown to influence potential CH_4 production rates (Wilms et al 2007).

There are two groups of methanotrophs, Type I methanotrophs (Gammaproteobacteria) and Type II methanotrophs (Alphaproteobacteria), both of which use methane monooxygenase to catalyze the first step in CH_4 oxidation. The particulate form of methane monooxygenase (pmoA) is present in all but one genus of methanotrophic bacteria. As with mcrA, pmoA phylogeny is

congruent with 16S rRNA phylogeny, making pmoA useful in surveying methanotrophic bacteria diversity (Kolb et al 2003). While the methanotroph community has been investigated in some tropical forests (Holmes et al 1999, Roslev and Iversen 1999, Knief et al 2003, Knief et al 2005), to our knowledge, no studies have examined the structure of both the methanogen and methanotroph communities and how these communities affect CH_4 emissions in upland tropical forests.

The composition and abundance of methanogens and methanotrophic bacteria may influence CH_4 emissions from upland tropical forest soils. The CH_4 dynamics from each of the three distinct forest types in the LEF have been studied. In previous work, CH_4 efflux was found to be greatest from the Cloud forest soils (97 ± 49.78 mg CH_4 m^{-2} d^{-1}), followed by the Colorado (0.32 ± 0.24 mg CH_4 m^{-2} d^{-1}) and Tabanuco forest soils (-0.48 ± 0.07 mg CH_4 m^{-2} d^{-1}) (Silver et al 1999). Up to 78% of the CH_4 emitted from the soils at these sites appeared to be hydrogenotrophic (Teh et al 2006b). The Type I methanotrophic bacteria have been quantified in these soils using PLFA (Teh 2005); the abundance of Type II methanotrophic bacteria has not been quantified. Potential CH_4 production and oxidation rates from soils in the LEF have been reported to be greater in the surface soils, from 0-15 cm (Teh et al 2005). CH_4 fluxes from these tropical soils are likely to be impacted by predicted global/environmental changes, such as increased temperature and decreased precipitation (Wood and Silver 2012, Malhi and Phillips 2004).

In this study, I determined potential rates of methanogenesis and CH_4 oxidation and investigated methanogen and methanotroph community characteristics. Soils were sampled from two depths of three forest types occurring along an elevation gradient encompassing a range of climatic conditions and soil properties. These soils were used to 1) measure the abundance of mcrA and pmoA genes, 2) identify the microbial communities producing and oxidizing CH_4, 3) link microbial community characteristics to potential CH_4 production and oxidation rates and 4) determine the influence of climate and edaphic soil factors on the abundance and diversity of methanogens and methanotrophs.

Materials and Methods

Study Sites

Upland tropical forest soils were collected from three forest types (Tabanuco, Colorado, Cloud) spanning an elevation and rainfall gradient in the Luquillo Experimental Forest (LEF), a Long-Term Ecological Research site in Puerto Rico, USA (18°18'N, 65°50' W). Each forest type represents a distinct life zone and is distinguished by dominant vegetation and edaphic properties (Holdridge 1947) (Table 1). In general, there is little seasonality in rainfall or temperature with an average relative humidity of 98 % (Weaver 1994). Soils are acidic clay-loam utlisols derived from volcanoclastic sediments and have little litter accumulation (Silver et al 1999, Beinroth 1982). The Tabanuco forest type is a subtropical wet forest dominated by the Tabanuco tree (*Dacrodes excelsa*). This forest type occurs on lower elevation slopes and valleys in the Luquillo Mountains; soils for this study were collected from a ridge site. Tabanuco forest ridge soils are well aerated, with bulk soil O2 concentrations rarely below 18% (Silver et al 1999).

The mid-elevation Colorado forest type is a lower montane wet forest dominated by the Colorado tree (*Cyrilla racemiflora*). The soils in this forest type are derived from quartz diorite (Murphy et al 1998). The average bulk soil O2 concentration is 13 ± 0.2% at 0-10 cm, but it can vary from 21% to below 10% on the order of days to weeks driven by frequent rain events (Silver et al 1999). The Cloud forest type occurs at the highest elevations in the Luquillo Mountains and is characterized by dense, epiphyte-covered vegetation typically under 3 m. These soils are humic rich and highly saturated, with bulk soil oxygen concentrations of 8 ± 0.2% at 0-10 cm (Silver et al 1999).

Soil Sampling

Five replicate 10 cm diameter cores were sampled to 20 cm depth from three parallel transects in each of the three tropical forest types. Each transect was one meter apart, and the replicate cores of each transect were one meter apart. Prior to collection, surface plant litter was removed. For each replicate core, the 0-10 cm section was taken and sealed in a whirl-pac bag (Nasco, Modesto, CA), then the trowel or augur was wiped off and the 10-20 cm core was taken and sealed in a separate whirl-pac bag. Within 12 hours of collection, all 0-10 cm cores from an individual transect were combined (total n = 5) and all 10-20 cm cores from an individual transect were combined. Soil samples were shipped in a room temperature cooler to UC Berkeley. Plant litter and root material were removed from the soil within two days after arrival at UC Berkeley. The soils remained at room temperature until the microcosm experiments started (within one week). Immediately prior to the microcosm experiments, the soil was homogenized by hand.

Soil Moisture

Approximately 5 g soil (wet weight) from each depth of each site was weighed into aluminum trays in triplicate and dried at 105 ˚C until the soil reached a constant weight. Soil moisture was calculated using the following formula: Soil Moisture = (initial wet soil weight / (initial wet soil weight – final dry soil weight)) * 100.

Microcosm Incubation Conditions

Potential CH_4 Production

To determine potential CH_4 production rates from each forest type and depth, headspace gas samples were taken over time from soil microcosms. Each soil microcosm contained 20 g (wet weight) soil in 250 ml mason jars fitted with Hungate stoppers as gas sampling ports. At the start, the microcosms were flushed with a $N_2/CO_2/H_2$ gas mix and confirmed to be anoxic using a Model 52 Dissolved Oxygen Meter (YSI Incorporated, Yellow Springs, OH). CH_4e production was determined from four treatments (three replicates each); 1) addition of 500 ppmv difluoromethane (DFM) to inhibit CH_4e oxidation, 2) addition of 30 mM 2-Bromoethane sulphonate (BES), final concentration in the pore water, to inhibit CH_4 production, 3) addition of both 500 ppmv DFM and 30 mM BES and 4) No Addition (control). The 30 mM BES concentration was chosen because it had been found to be the lowest concentration at which CH_4 production was completely inhibited over 20 days in each forest soil in a preliminary experiment (data not shown). The BES was added in 1 ml of sterile water. Sterile water (1 ml) was added to each replicate of the other three treatments as well. The microcosms were incubated at room

temperature. Every two days, 2 ml of headspace gas was sampled into 10 ml N_2-flushed, evacuated serum vials. An equal volume of N_2 was added to maintain the headspace and atmospheric pressure. At the end of the experiment, approximately 5 g of soil (wet weight) from each forest type and depth from the DFM treatment was stored at -80 ˚C.

Potential CH_4 Oxidation Activity

Rates of potential CH_4 oxidation were determined from each forest type and depth by analyzing headspace samples from a separate set of soil microcosms set up as described above. The headspace in these microcosms was room air. CH_4 oxidation was determined from four treatments (3 replicates each); 1) addition of 500 ppmv DFM, 2) addition of 30 mM BES, final concentration in the pore water, 3) addition of both 500 ppmv DFM and 30 mM BES and 4) No Addition. To each sealed microcosm, 400 ppm CH_4 was added, and headspace gas was immediately sampled. After 8 days, headspace gas was sampled from all microcosms, then the microcosms were opened and flushed with room air. The microcosms were then closed, 400 ppm CH_4 was added and immediately sampled again. This procedure was repeated on days 12, 16 and 20. Headspace samples (2 ml) were taken after eight days, then every four days for 12 days (20 days total). The amount of CH_4e oxidized was calculated by subtracting the T_{final} from the $T_{initial}$ headspace samples for each timepoint. At the end of the experiment, approximately 5 g of soil (wet weight) from each forest type and depth from the BES treatment was stored at -80 ˚C.

Gas sampling

CH_4 concentration in the headspace samples was measured with a HP6890 Shimadzu Gas Chromatograph with a Flame Ionization Detector (Shimadzu, Columbia, MD). Standards were made by diluting CH_4 to 10,000, 4,000, 2,000, and 1,000 ppm in a N_2 background and run in triplicate.

DNA extraction and cDNA Synthesis

Total DNA and RNA were extracted using a Phenol:Chloroform method from 3.5 g of soil saved at -80 ˚C as in Placella et al. (2012). Briefly, 350 µl 0.1 M aluminum ammonium sulfate and 350 µl each of CTAB (0.5 M phosphate buffer, pH 8, 5% hexadecyltrimethylammonium bromide, 1 M NaCl) and Phenol:Chloroform:Isoamyl alcohol (25:24:1) were added to 15 ml Lysing Matrix E (MP Biomedicals, Solon, OH) containing 3.5 g frozen, pulverized soil and bead-beaten at 5.5 m/s, for 30 seconds (MP Biomedicals, Solon, OH). The tubes were incubated on ice for 5 minutes before centrifugation at 5,500 rcf for 5 minutes at 4 ˚C. The aqueous phase was transferred to a Phase lock gel tube (5 PRIME, Inc., Gaithersburg, MD), mixed and then centrifuged at 8,000 rcf for 3 minutes at 4 ˚C. The aqueous phase was then transferred to another tube. The soil was then re-extracted with an additional 350 µl of CTAB, and the resulting aqueous phase was pooled. DNA was precipitated at room temperature for at least three hours using two volumes of PEG/1.6M NaCl and 2 µl of 5 mg/ml linear acrylamide (Life Technologies Ambion, Grand Island, New York). DNA was recovered by centrifugation at 16,000 rcf for 10 minutes, washed twice with 70% ethanol and resuspended in 100 µl RNase-free 100 mM Tris buffer. The DNA and RNA were separated using an All-Prep kit (Qiagen, Valencia, CA) and stored at -80 ˚C. The Quantitect kit (Qiagen, Valencia, CA) was used to produce cDNA from up to 1 µg of extracted RNA using the random hexamer primers provided by the manufacturer.

Quantitative PCR (qPCR)

Quantification of Bacterial and Archaeal 16S rDNA, mcrA and pmoA genes was done using qPCR (iCycler, BioRad, Hercules, CA). Transcripts for mcrA and pmoA were also quantified using qPCR. The Eub338F/Eub518R (Fierer et al 2005) and 364F/946R (Kemnitz et al 2005) primers were used for the Bacterial and Archaeal 16S rDNA, respectively. Each 20 µl qPCR PCR reactions contained 1 µl template DNA (~50 ng) or cDNA, 10 µl 2X SsoFast™ EvaGreen Supermix® (BioRad, Hercules, CA), and 1 µl each of 10 µM forward and 10 µM reverse primers. The two-step protocol to amplify Bacterial 16S rDNA was as follows: 95 ˚C for 10 seconds, then 53 ˚C for 20 seconds followed by a melting curve analysis from 53 ˚C to 94 ˚C. The two-step protocol to amplify Archaeal 16S rDNA was the same, except the annealing and extension temperature was 57 ˚C and the starting melting curve temperature was 57 ˚C.

The MLf and MLr primers were used to amplify the mcrA gene (Luton et al 2002) and the II223fwd/II646rev (Type II), A189F/Mcap630 (Type I Methylocapsa), A189F/Mc468R (Type I Methylococcus) primers (Kolb et al 2003) were used to amplify pmoA genes. The two-step qPCR protocol to amplify mcrA was as follows: 2 min at 94 ˚C, 35 cycles of 94 ˚C for 10 seconds, then 50 ˚C for 20 seconds followed by a melting curve analysis from 50 ˚C to 94 ˚C. The qPCR protocol to amplify pmoA was as follows: 2 min at 94 ˚C, 40 cycles of 94 ˚C for 10 seconds, then 55 ˚C for 20 seconds, 72 ˚C for 5 seconds to melt secondary structure and primer dimers prior to measurement, followed by a melting curve analysis from 55 ˚C to 94 ˚C. Sample DNA was diluted to overcome PCR inhibitors. The dilution was chosen for each sample based on serial 1:10 dilutions of DNA in Bacterial or Archaeal 16S rDNA qPCR reactions.

Standards were generated from DNA extracted from Colorado 0-10 cm soils and cloned into pCR2.1 or pCR4 vectors (Invitrogen, Calrsbad, NM). Single clones picked from each clone library were sequenced (UC Berkeley Sequencing Facility, UC Berkeley, CA) and confirmed to be either mcrA or pmoA using the blast too at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmids with confirmed cloned sequences were extracted using a Miniprep Kit (Qiagen, Valencia, CA), quantified on a NanoDrop (NanoDrop Technologies, Wilmington, DE), and diluted to generate a standard curve.

The abundance of genes and transcripts is expressed relative to the amount of 16S rDNA amplified from each site. A relative abundance measure was used because it is less sensitive to differences in DNA extraction efficiency. These soils have different DNA extraction efficiencies. The amount of DNA extracted from the Tabanuco forest site was less than the amount of DNA extracted from either the Colorado or Cloud forest sites at $p < 0.01$. Within each forest site, the effect of depth or transect on the amount of DNA extracted was not significant at $p < 0.01$.

mcrA and pmoA Clone libraries

Equal amounts of DNA (5 ng) from all three transects in each forest type were pooled by depth before PCR amplification. The MLf and MLr primers were used to amplify the mcrA gene (Luton et al 2002) and the II223fwd and II646rev primers (Kolb et al 2003) were used to amplify the Type II pmoA gene. Each PCR mixture contained 1X PCR buffer, 0.16 mM each dNTPs, 3 nM each primer, 5 units of Takara Ex Taq and 1 µl of DNA in a final 25 µl volume. After an initial denaturation for 5 minutes at 94 ˚C, 30 cycles of 94 ˚C for 30 seconds, 50 ˚C for 30

seconds and 72 ˚C for 30 seconds were performed, followed by 10 minutes at 72 ˚C. The protocol was the same for amplification of pmoA, except the annealing temperature was 55 ˚C.

PCR products were purified from a 1X TAE, 1% agarose gel using a Gel Purification Kit (Qiagen, Valencia, CA) and then cloned into the pCR4 or pCR2.1 vector as per the manufacturers' instructions (Invitrogen, Carlsbad, NM). Several PCR and cloning reactions were done to generate the 18 sequences in the pmoA clone libraries. Cloned sequences were amplified with the provided M13 primer set and sequenced on a Hitachi 3730 DNA Analyzer (AB Applied Biosystems, Grand Island, NY) at the Museum of Vertebrate Zoology Sequencing Facility (UC Berkeley). Sequences were BLASTed using the tBLASTn (megablast) tool at NCBI (http://www.ncbi.nlm.nih.gov/).

Phylogenetic trees

Maximum Likelihood phylogenetic trees were constructed with nucleotide sequences of partial mcrA and pmoA sequences using FastTreeMP (version 2.1.3) (Price et al 2010) and visualized using Figtree (version 1.3.1) (http://tree.bio.ed.ac.uk/software/figtree/). Sequences were trimmed to 541 bp for the mcrA sequences and 426 bp for the pmoA sequences and aligned using MUSCLE version 3.8.31 (Edgar 2004) and blocked with Gblocks version 0.91b (Castresana 2000). The original trees contained all available mcrA or pmoA sequences from cultured Archaea or Bacteria in the GenBank database (March, 2013). Selected amoA sequences (for the pmoA tree) and selected mcrA and pmoA environmental clone sequences were also included. Reported subtrees were constructed using 31 (mcrA) and 42 (pmoA) selected GenBank sequences.

Statistical Analysis

All statistical analysis was performed in R (version 2.14.1) unless otherwise specified. Testing for differences between average rates of CH_4 production and oxidation over time and within site depths was done using analysis of variance with Tukeys Honestly Significant Difference analysis. Testing for differences between the amount of DNA extracted and the amount of Archaeal and Bacterial 16S rDNA were also done using analysis of variance withTukeys Honestly Significant Difference analysis.

Gene and transcript abundances below the detection limit were assumed to be 0 copies 16S rDNA gene copies g soil-1. The transcripts and gene copies from the 0-10 cm and 10-20 cm depths of each forest type were combined for the linear correlation analysis to conform to normality.

All linear correlations are reported as significant at $p < 0.001$, unless otherwise specified. The $p < 0.001$ cut-off was chosen to reduce potentially spurious correlations. For the correlation analysis between gene and transcript abundances and rates of CH_4 production and oxidation, gene and transcript abundances were normalized by g soil extracted. Normalizing in this manner allows the comparison of the amount of CH_4 being produced or oxidized by the relevant microbial community without taking into account the percentage of the relevant microbial community in the total microbial community.

For the correlation analysis between gene abundances and edaphic factors, genes (pmoA and mcrA) were normalized by Bacterial or Archaeal 16S rDNA gene copies amplified from each

depth by site. The edaphic factors for each site were measured in 2006 at locations adjacent to the transects used for my study. Since the edaphic factors were measured for soils from 0-10 cm (Dubinsky et al 2010), only the qPCR values from the 0-10 cm soils are included in the linear correlations. Transcript abundances were not correlated to edaphic factors. Because of the time scales involved (10 days for the CH_4 Production Experiment; 20 days for the CH_4 Oxidation Experiment), transcript abundances are probably too transient to reflect methanogenic or methanotrophic activity in response to edaphic factors. Transcript abundance from the initial timepoint of the experiments might better reflect methanogenic or methanotrophic activity in response to an edaphic factor.

Ecological Analyses

Qiime (version 1.5.0) analysis scripts were used to assign OTUs. Rarefaction curves were generated using an R script (http://www.ualberta/ca/~jjacobs/rarefaction.txt) and graphed in Excel (Microsoft Office 2013, Redmond, WA). Detected OTUs were used to determine richness and diversity using richness and diversity functions provided in the vegan package of R (version 2.14.1). The similarity of methanogen community structure was determined using correlation analysis (Pearson) using OTU composition and abundance between each depth in each forest type.

The Canonical Correspondence Analysis was performed using cumulative CH_4 production and a community matrix separated into methanogen families. The mcrA OTUs were assigned to methanogen families based on sequence similarities and nearest cultured neighbors in the phylogenetic tree constructed in this work.

Results

Potential CH_4 Production and Rates

Soils from both depths of the Colorado and Cloud forest soils produced CH_4 under the DFM and No Addition treatments whereas rates of CH_4 production by Tabanuco forest soils were below the limit of detection (Figure 1, No Addition treatment not shown). The CH_4 produced by each depth in each forest type in both the DFM and No Addition treatments were not different at $p < 0.1$, therefore only the DFM treatment is presented. Total CH_4 production in the BES and DFM + BES treatments was on average 0.002 ± 0.001 ppm CH_4^{-1} g $soil^{-1}$ and was not different at $p < 0.05$ between the forest types or depths (data not shown). Cumulative CH_4 produced after ten days in both depths of the Colorado and Cloud forest types were similar in the DFM treatment ($p < 0.05$); the Colorado forest soils produced 463 ± 280 ppm CH_4 hr^{-1} g $soil^{-1}$ (0-10 cm) and 356 ± 179 ppm CH_4 hr^{-1} g $soil^{-1}$ (10-20 cm) and the Cloud forest soils produced 395 ± 10 ppm CH_4 hr^{-1} g $soil^{-1}$ (0-10 cm) and 444 ± 150 ppm CH_4 hr^{-1} g $soil^{-1}$ (10-20 cm) (Figure 1).

Initial rates of CH_4e production were greatest in the 0-10 cm Cloud forest soils (Day 0-6, 0.154 ± 0.015 ppm CH_4 hr^{-1} g $soil^{-1}$) (Figure 2). At the later timepoints, CH_4 production rates decreased from both depths of the Cloud forest. In contrast, CH_4 production rates for both depths of the Colorado forest increased over the course of the experiment (Colorado 0-10 cm; from Day 0-2,

0.029 ± 0.018 to Day 8-10, 0.149 ± 0.090 ppm CH_4 hr^{-1} g $soil^{-1}$; Colorado 10-20 cm; from Day 0-2, 0.015 ± 0.008 to Day 8-10, 0.137 ± 0.082 ppm CH_4 hr^{-1} g $soil^{-1}$).

Correlations between CH_4 production and edaphic factors

Cumulative CH_4 production was correlated with MAP ($r = 0.641$, $p = 0.062$) and soil moisture ($r = 0.527$, $p = 0.024$) (Table 2). Cumulative CH_4 production was positively correlated to percent sand ($r = 0.692$, $p = 0.038$), and negatively correlated to percent clay ($r = -0.833$, $p = 0.005$).

Quantification of Archaeal and Bacterial 16S gene copies

The abundance of Archaeal 16S gene copies differed between forest types and depths. The amount of Archaeal 16S rDNA amplified from the Cloud forest soil was greater than either the Tabanuco or Colorado forest soils at $p < 0.01$. The amount of Archaeal 16S rDNA amplified from the 0-10 and 10-20 cm depths of the Cloud forest soil was significantly different at $p < 0.01$ (Table 3A).

The amount of Bacterial 16S rDNA amplified was greatest in the Cloud forest soil, followed by the Colorado and then the Tabanuco soils at $p < 0.01$ (Table 3A). The amount of Bacterial 16S rDNA amplified was not different from the different depths of any forest site at $p < 0.01$.

mcrA gene copy and transcript abundance

Gene copies and transcript abundance of mcrA were determined using DNA and RNA extracted from the DFM treated soils of the Potential CH_4 Production experiment presented above. The mcrA gene copy and transcript abundance are expressed as a proportion of Archaeal 16S rDNA amplified from each site and depth (Figure 3A and 3B). The proportion of mcrA gene copies was greater in the Cloud forest soils than the Colorado forest soils (by 70%) and greater than the Tabanuco forest soils (by 92%), though only the difference between the Cloud 0-10 cm and Colorado and Tabanuco soils is significant ($p < 0.01$) (Figure 3B). The proportion of mcrA gene copies was similar between the 0-10 cm and 10-20 cm depths within each forest type ($p < 0.01$) (Figure 3B, Table 3A).

In addition to quantifying gene copies, I attempted to quantify gene transcripts to assess whether new enzyme was being produced. Only the 10-20 cm Colorado forest soils produced mcrA transcripts at the final timepoint (Figure 3A, Table 3B). The amplification of mcrA transcripts was at the limit of detection of the qPCR PCR method in all other forest soils.

Correlations of mcrA gene copies and transcripts with CH_4 production rates and edaphic factors

The abundance of mcrA gene copies was positively correlated to the rate of CH_4 production ($r = 0.493$), whereas mcrA transcript abundance was not ($r = 0.050$, $p = 0.720$) (Table 4). The abundance of mcrA gene copies was positively correlated to soil moisture and mean annual precipitation (MAP) and soil moisture ($r = 0.583$; $r = 0.502$, $p = 0.007$, respectively) (Table 5). The abundance of mcrA gene copies was also positively correlated to soil carbon (C) and nitrogen (N) characteristics; percent C ($r = 0.649$), percent N ($r = 0.686$) and the C:N ratio

(r = 0.627). The abundance of mcrA gene copies was positively correlated to the soil property percent sand (r = 0.619).

mcrA clone libraries

Overall, 128 unique sequences were recovered from two of our three sites, which formed 33 OTUs at the 88% identity level (Table 6). The Tabanuco 0-10 cm and 10-20 cm mcrA clone libraries were excluded from further analysis because they contained one and no mcrA sequences, respectively. Rarefaction analysis showed that the number of OTUs detected is beginning to plateau at the 88% identity level for each depth of the Colorado and Cloud forest soils, suggesting the mcrA diversity was sufficiently sampled in these soils (Figure 4). About 16 OTUs remain undetected in these soils, as estimated using the method in Chao et al. (2009).

The Colorado mcrA rank abundance curves were uneven (Pielou's evenness index: 0-10 cm; 0.025, 10-20 cm, 0.024); two OTUs dominate the Colorado forest soil (Figure 5). The Cloud 10-20 cm mcrA rank abundance curves were more even than the Colorado forest curves (Pielou's evenness index: 0-10 cm; 0.029, 10-20 cm; 0.053) (Figure 5), perhaps indicating a more varied niche space.

There was some overlap between the OTUs detected in each depth of each site, but only OTU33, representing sequences 91-98% similar to sequences found in association with rice roots, was detected in all four mcrA libraries (Figure 6). Of the OTUs detected in both the Colorado and Cloud forest soils, 11 were unique to the Colorado forest and 15 were unique to the Cloud forest. In the Colorado soils, the OTUs separated into three main groups; sequences similar to sequences found in 1) fen soils (86-91% identity), 2) rice field soil or associated with rice roots (90-98% identity), and 3) saturated soils (91-97% identity). In the Cloud forest soils, the OTUs separated into the same three main groups; sequences similar to sequences found in 1) fen soils (90-98% identity), 2) rice field soil or associated with rice roots (91-99% identity) and 3) saturated soils (92-97% identity).

The recovered OTU richness was greatest from the Cloud 0-10 cm soil library (Table 6). The statistical estimators Chao1 and ACE predicted richness from 12 to 62 (Colorado) and 28 to 92 (Cloud) OTUs (Table 6). Thus, sampling achieved from 14 % to 83% of the predicted richness for the Colorado and Cloud forest soils, respectively. The Colorado 10-20 cm library had the least diverse mcrA OTUs, while the mcrA OTU diversity of the Colorado 0-10 cm and both depths of the Cloud forest soil were similar, as measured by both Shannon and Simpson's diversity indices (Table 6). As expected, the mcrA OTU composition and abundance in the different depths of the Colorado forest were similar (r = 0.58) as were the different depths of the Cloud forest (r = 0.40, $p < 0.05$) (Table 7). Unexpectedly, the mcrA OTU composition and abundance in Colorado 0-10 cm soils and the Cloud 0-10 cm soils were also similar (r = 0.37, $p < 0.05$) (Table 7).

A metagenome was constructed by DeAngelis et al. (2010) using soils from the Bisley watershed in the Tabanuco forest of the LEF (MG-RAST ID 4446153.3). To assess whether I had captured the dominant mcrA OTUs in my clone libraries, I searched the Bisley metagenome for mcrA sequences. However, no mcrA sequences were annotated as either methyl coenzyme M reductase or coenzyme-B sulfoethylthiotransferase. The metagenomic data could thus not be used to assess the abundance or representativeness of the mcrA OTUs detected in the clone

libraries. However, the shape of the mcrA rarefaction curves does indicate that the most abundant mcrA sequences were recovered.

mcrA Maximum Likelihood Tree

The mcrA OTUs recovered do not cluster by site or by depth on the phylogenetic tree (Figure 7). Most OTUs did not cluster with mcrA sequences from cultured methanogens and instead clustered with sequences recovered directly from the environment. A group of OTUs, (OTU6, 13, 20, 21), are clustered with *Methanomasiliicoccus luminyensis*, a methanogen isolated from the human gastrointestinal tract.

Canonical Correspondence Analysis

The CCA analysis in Figure 8 shows an ordination of mcrA OTUs constrained by CH_4 production. A matrix consisting of the number of detected mcrA OTUs from each site grouped by methanogen genera was used as the community matrix. A matrix of the average CH_4 production by site was used as the environmental factor matrix. The eigenvalue of the x axis, 0.164, represents the correlation between the OTU scores and sample scores. The scores for the x axis are constrained by cumulative CH_4 production. The eigenvalue of the y axis, 0.125, represents the correlation between the OTU scores and sample scores (Figure 8). Some genera, such as *Methanosaeta* and *Methanosphaera*, appear to be associated with higher CH_4 production.

Potential CH_4 Oxidation and Rates

Only the Cloud forest soils oxidized CH_4 in the BES treatment (Figure 9A). The amount of CH_4 oxidized in the 0-10 cm and the 10-20 cm Cloud forest soils increased over time by 2.1 and 16 fold, respectively (Figure 9A). The 0-10 cm Cloud forest soils consistently oxidized more CH_4 than in the 10-20 cm Cloud forest soils, however, this trend was not significant at $p < 0.05$ (Figure 9A). No measureable CH_4 oxidation occurred in the DFM or the DFM + BES treatments (data not shown).

In general, the amount of CH_4 oxidized in each depth of each forest soil was greater in the No Addition treatment than the BES treatment (Figure 9A and 9B). The Tabanuco 0-10 cm soils oxidized more CH_4 in the No Addition treatment at Day 0-8 and the Tabanuco 10-20 cm forest soils oxidized more CH_4 at Day 0-8 and Day 16-20 ($p < 0.05$). The Colorado and Cloud forest soils from each depth oxidized more CH_4 in the No Addition treatment ($p < 0.05$), except the Colorado 10-20 cm soil. The CH_4 oxidation rates in the No Addition treatment in the Colorado 0-10 cm forest soils and both depths of the Cloud forest soils were not different at each timepoint ($p < 0.05$). The CH_4 oxidation rate from the Colorado 10-20 cm soils was lower than the rates from both depths of the Cloud forest soils at Day 12-16 and also lower than the rate from the Cloud 0-10 cm soils at Day 16-20 ($p < 0.05$).

The BES treatment was designed to measure potential CH_4 oxidation rates without the confounding effect of native CH_4 production from these soils. By using BES to inhibit native CH_4 production from these soils, the only CH_4 in the sample jars should be what was manually added at the beginning of each timepoint. Therefore, CH_4 oxidation rates could be calculated without having to account for native CH_4 production. However, the addition of 30 mM BES inhibited aerobic CH_4 oxidation in soils from all three forest types. With the addition of 30 mM

BES, CH_4 oxidation decreased on average by 59 ± 43% (0-10 cm) and 64 ± 51% (10-20 cm) in the Tabanuco forest soils, 97 ± 3% (0-10 cm) and 90 ± 25% (10-20 cm) in the Colorado forest soils and by 58 ± 16% (0-10 cm) and 87 ± 11% (10-20 cm) in the Cloud forest soils (Figure 9B).

Correlations between CH_4 oxidation and edaphic factors

Both soil moisture and MAP were correlated with cumulative CH_4 oxidized in the No Addition treatment ($r = 0.597$, $p = 0.008$; $r = 0.903$, respectively) (Table 2). Cumulative CH_4 oxidized in this treatment was also correlated to percent C ($r = 0.903$) and the C:N ratio ($r = 0.932$). The percent sand was positively correlated to cumulative CH_4 oxidized ($r = 0.940$), whereas the percent clay was negatively correlated ($r = -0.980$). In the BES treatment, soil moisture was positively correlated with cumulative CH_4 oxidized ($r = 0.707$, $p = 0.001$).

pmoA gene copy and transcript abundance

Gene copies and transcript abundance of pmoA genes were analyzed from DNA and RNA extracted from BES treatment soils of the potential CH_4 oxidation experiment and are expressed as a proportion of Bacterial 16S rDNA gene copies amplified via qPCR from each forest site and depth. For each of the pmoA genes, the ratios of CH_4 oxidizer gene copies to total bacterial 16S gene copies tended to be higher in the Colorado soils, followed by the Tabanuco soils, and then the Cloud soils (Figure 10). The ratio of pmoA gene copies to total bacterial 16S gene copies in the Colorado 10-20 cm soils are 1.3-3 fold greater than the other forest soils and depths, though the difference is significant only for the Cloud forest soils (0-10 cm, 3 fold, 10-20 cm 2.6 fold, $p < 0.01$) and the Tabanuco 0-10 cm soils (2.4 fold, $p < 0.01$) (Figure 10B, D, F). Transcripts for Type II pmoA are more abundant in the Tabanuco forest, though this trend is only significant for the Tabanuco 0-10 cm soils and either depth of the Cloud forest soils ($p < 0.01$) (Figure 10A). Type I Methylocapsa pmoA transcripts are more abundant in the Cloud forest soils, though only the Cloud 10-20 cm forest are significantly greater than the 10-20 cm depths of the Tabanuco and Colorado forest soils ($p < 0.01$) (Figure 10C). Transcripts for Type I Methylococcus pmoA were more abundant in the Colorado and Cloud forest soils than the Tabanuco forest soils, though only transcript abundance in the 0-10 cm depth of the Colorado and Cloud forest soils are significantly greater than either depth of the Tabanuco forest soils at $p < 0.01$ (Figure 10E).

Linear correlations: pmoA genes

The abundance of each of the pmoA genes positively correlated to the rate of CH_4 oxidization (Type II, $r = 0.468$; Methylocapsa Type 1, $r = 0.571$; Methylococcus Type 1 $r = 0.628$) (Table 4). Transcript abundance of the Type I Methylococcus pmoA was strongly correlated to potential CH_4oxidation ($r = 0.797$), whereas the Type I Methylocapsa and Type II were weakly correlated (Type II, $r = -0.346$, $p = 0.020$; Type I Methylocapa, $r = 0.357$, $p = 0.016$) (Table 4). There was not a pattern in the correlations observed between pmoA gene abundance and any edaphic factor (Table 5). However, the abundance of Type II pmoA gene copies was negatively correlated to percent silt ($r = -0.586$, $p = 0.001$) and Type I Methylocapsa gene copies did correlate with soil pH ($r = 0.577$, $p = 0.001$) (Table 5).

pmoA clone libraries

As with the mcrA clone libraries, pmoA sequences were recovered from both depths of the Colorado and Cloud forests. No pmoA sequences were recovered from the Tabanuco clone libraries. Overall, 18 unique sequences were recovered from the sites, which formed 15 OTUs at the 92% identity level. Rarefaction curves of the pmoA clone libraries do not plateau, suggesting that the pmoA diversity was not sufficiently sampled (Figure 11). Because the pmoA clone libraries contained so few sequences, and only two OTUs contained more than one member sequence, diversity was not calculated. Several attempts to construct Type II pmoA clone libraries were unsuccessful.

The detected pmoA OTUs fell into three groups, 1) sequences found associated with rice roots (92-98% identity), 2) sequences found in soils, such as upland tropical soil, acidic temperate forest soil and soil above a landfill (86-98% identity), and 3) sequences found in wetland soils (90-99% identity). Because so few sequences were recovered from the pmoA clone libraries, community composition comparisons were not done.

One pmoA sequence was recovered in the MG-RAST automated annotation of the Bisley metagenome (MG-RAST 446153.3) from the Tabanuco forest site. This sequence did not cluster with the OTUs defined from the clone library. This was not unexpected; so few sequences were recovered from the pmoA clone libraries, it is unlikely that any pmoA sequences obtained from another soil sample from these sites (and using a different method) would belong to the OTUs detected in the clone libraries.

pmoA Maximum Likelihood Tree

Like the mcrA sequences recovered from the clone libraries, the recovered pmoA sequences do not cluster by site or by depth on the phylogenetic tree (Figure 12). Most OTUs did not cluster with pmoA sequences from cultured CH_4 oxidizers and instead clustered with sequences recovered directly from the environment.

Discussion

Previous studies investigating the microbes responsible for CH_4 fluxes in tropical forest soils have focused on methanotrophic bacterial diversity (Knief et al 2003, Knief et al 2005). My study investigated both methanogen and CH_4 oxidizer communities to assess the importance of the abundance of each, relative abundance of the communities, and community composition in controlling potential CH_4 emissions in upland tropical soils.

The initial rate of CH_4 production was highest in the Cloud 0-10 cm depth of the Cloud forest soils. In general, the initial rates of CH_4 production in the 10-20 cm depth of Cloud forest soils were similar to both depths of the Colorado soils; however in the Colorado soils, the rates continued to increase over the course of the incubations. The Cloud forest soils produced CH_4 faster than the Colorado forest soils, and the Colorado forest soils emitted CH_4 faster than the Tabanuco forest soils. These results agree with both previous microcosm studies and field measurements (Teh et al 2005, Silver et al 1999, Liptzin et al 2011, Keller 1986). The observed

initial rates of CH_4 production may be influenced by methanogen abundance or diversity or environmental factors; these possibilities are discussed below.

The abundance of methanogens appeared to be an important factor in the production of CH_4at two of the sites, the Colorado and Cloud forests. The abundances of mcrA gene copies in the Colorado and Cloud forest soils were significantly correlated to the initial CH_4 production rates. High CH_4 production due to methanogen abundance has been observed in marine sediments (Wilms et al 2007). Assuming one mcrA gene copy per methanogen, there were more methanogens in the Cloud forest 0-10 cm soil followed by the Colorado and lastly by the Tabanuco soils. The Cloud forest 0-10 cm soils contained 12.9% carbon, while the Colorado 0-10 cm soils contained 7.4% carbon (Dubinsky et al 2010). This difference in C content may partially explain the larger methanogen population and the rapid initial CH_4 production rates in the Cloud 0-10 cm soils compared the other soils. Correlation between mcrA abundance and CH_4 production rates has been observed in rice paddy soils and biogas reactors (Liu et al 2012, Traversi et al 2012). In these systems, C was supplied to the methanogens by rice straw decomposition and waste-water sludge, respectively. The amount of C may be a factor in maintaining a large, active methanogen population. In some peatlands, where decomposition occurs slowly (Yavitt et al 2005), there is a lag period (10-12 days) between initiation of anoxic incubation conditions and CH_4 production (Yavitt et al 2006). The authors speculate that the lag may have been caused by a small methanogen population (Yavitt et al 2006).

The diversity of mcrA OTUs was weakly correlated with CH_4 production rates, and no individual mcrA OTU was correlated with CH_4 production rate. Methanogen diversity has been shown to influence the rate of methanogenesis in peatlands (Yavitt et al 2012, Sun et al 2012), but not in marine sediments (Wilms et al 2007). The mcrA community composition and abundance in the 0-10 and 10-20 cm Colorado soils was similar (Table 7). CH_4 production rates from both depths of the Colorado soils followed a similar pattern over the course of the experiment. The parallel CH_4 production rates in the Colorado forest soils may result from similar community composition and abundance. Likewise, in the Cloud forest soils, the CH_4 production rates in the two depths followed a similar pattern and the community composition and abundance in both depths of the Cloud forest was similar. On a site-specific level, the mcrA OTU abundance was more even in the Cloud forest soils, suggesting these soils have more heterogeneous niche space and can support a more diverse methanogen community (Tilman 1982).

Based on the recovered clone library sequences, the three most abundant mcrA OTUs were *Methanobacter* (29%), *Methanocella*, also known as Rice Cluster I (Sakai et al 2008), (29%) and *Methanosarcina* (19%). The next most abundant genera had abundances at or below 3%, *Methanoregula* (3%), *Methanothermus*-like (3%), and *Methanosaeta* (1%). Members of the *Methanobacteriales* and *Methanocellales* are hydrogenotrophic or use C1 compounds to produce CH_4. *Methanosarcinales* are versatile in terms of the carbon compounds they can use; they use H_2/CO_2, C1 compounds and acetate. Since 58-77% of the detected OTUs were most closely related to hydrogenotrophic methanogens, the majority of CH_4 produced in the 0-20 cm depth of the LEF is most likely hydrogenotrophic. This is supported by the work of Teh et al. (2005), wherein the isotopic measurements indicated that CO_2 was likely the origin of emitted CH_4. However, acetate cannot be discounted as an important methanogenic substrate. In addition to the *Methanosaeta*-like OTUs, the *Methanosarcinales*-like OTUs could be using acetate in acetate-containing soil microsites.

Based on the CH_4 production-constrained CCA, a few methanogen genera appear to be associated with higher rates of CH_4 production (Figure 8). The mcrA OTUs related to *Methanosaeta* and *Methanosphaera* were present only in sites with high CH_4 production (Cloud forest, both depths). The *Methanosaeta* are obligate acetoclastic methanogens, whereas the only other acetoclastic methanogen genera, the *Methanosarcina*, are more functionally robust and can use acetate, H_2/CO_2 and C1 compounds. *Methanosaeta*-like OTUs were recovered only from the Cloud forest soils, and *Methanosarcina*-like OTUs were only recovered from the Colorado forest soils. *Methanosaeta* and *Methanosarcina* have different Kms for acetate; the Km for acetate is higher for the *Methanosarcina* than for the *Methanosaeta*. This suggests that the magnitude of the acetate pool is perhaps larger in the Colorado forest soils and that overall, acetate may be a small, but important methanogenic substrate in both these sites. A previous study, using isotope fractionation, found that only a small percentage of emitted CH_4 was produced from acetate (Teh et al 2005). The previous study reported that the bulk of emitted CH_4 (up to 78%) from the Colorado and Cloud forest soils was hydrogenotrophic in origin.

Methanosphaera were also associated with high CH_4 production rates in these sites. Many cultured representatives of *Methanosphaera* have been isolated from intestinal tracts and feces (Biavati et al 1988, Miller and Wolin 1986). The *Methanomassiliicoccaceae* is also comprised of mcrA sequences recovered from fecal material (Dridi et al 2012). In the LEF, rats are among the largest, most common mammals, along with bats and the mongoose. While *Methanobrevibacter* species are the predominant methanogen isolated from rat intestinal tracts (Maczulak et al 1989), both *Methanosphaera* and *Methanobrevibacter* species are commonly recovered from mammalian intestinal tracts (Dridi et al 2009, Whitford et al 2001, Miller and Wolin 1986, Miller and Wolin 1985). The mcrA sequences related to *Methanosphaera* and *Methanomassiliicoccaceae* recovered from the LEF may be fecal in origin. If this is the case, they likely only play a role in the surface of the soil and may or may not persist in the soil microbial community. Approximately 80% of the *Methanomassiliicocci*-like OTUs were recovered from the 0-10 cm soils. The *Methanosphaera*-like OTUs were recovered only from the Cloud forest soils and only 45% of these were for the 0-10 cm soils. Because the Cloud forest soils have considerable root biomass and are frequently saturated, any fecal material deposited on the surface could follow root channels to lower soil horizons. Soils receiving fecal deposition may be hot spots of CH_4 production. Rain would likely allow the fecal organisms to permeate the surrounding soil.

In these tropical forest soils, CH_4 production was correlated to both MAP and soil moisture, as was the mcrA gene abundance. This was expected because high MAP and soil moisture would provide anoxic conditions for methanogens. Rates of methanogenesis have been demonstrated to be influenced by environmental conditions and edaphic factors, especially rainfall and moisture (Bachoon and Jones 1992, White et al 2008). Because CH_4 production was positively correlated with soil moisture, I expected that CH_4 production and mcrA gene abundance would be positively correlated to soil properties that are associated with anaerobiosis, such as small soil particle size. This was not the case. CH_4 production was positively correlated to percent sand. While this correlation may be coincidental, previous research has shown that CH_4 production occurs under an oxic headspace, probably in anoxic microsites (Teh et al 2005). The frequency and amount of rain may be more important to the maintenance of anoxic microsites than soil particle size distribution. Detecting relationships between soil particle size distribution at the scale of forest type is problematic.

The abundance of mcrA gene was also correlated to carbon and C:N ratio. The amount of carbon was significantly related to CH_4 production rates in these soils as was mcrA gene abundance. The correlation may be driven by the amount of a specific type of organic carbon pool; however, different types of carbon pools were not measured in this study. In addition, the amount of nitrogen was also significantly related both CH_4 production rates in these soils and mcrA gene abundance.

There were distinct patterns of CH_4 production in the Cloud and Colorado forest soils. The rate of CH_4 production in the Cloud forest soils was initially rapid, and then plateaued during the later timepoints. The CH_4 production rate in the Colorado forest soils was initially low, and continued to increase throughout the experiment. This pattern could be due to biological factors, such as methanogen abundance and growth or use of more energetically favorable electron acceptors, during the earlier times of incubation, or to physical factors, such as physical disturbance and the size of the accessible substrate pool. These possibilities are discussed below.

The overall pattern of CH_4 production rates may indicate the status of the accessible substrate pool for methanogens, such as acetate, CO_2, or C1compounds such as formate and methanol. A rapid initial rate of CH_4 production, as seen from the Cloud forest soils, is consistent with a population of existing, active methanogens using an existing substrate pool (Teh and Silver 2006a). The Cloud forest soils have more methanogens per gram soil than either the Colorado or Tabanuco forest soils (Table 3A). The size of the methanogen population may have contributed to the rapid CH_4 production rate in the Cloud forest soils, but only if the methanogen population was active. A similar CH_4 production curve was observed in Cloud forest soils microcosms when acetate was added (Teh and Silver 2006a). In that work, there was an initial lag of a few days, then a rapid increase in CH_4, followed by a plateau in CH_4 production. That pattern of CH_4 production closely resembles CH_4 production from Cloud forest soil microcosms in this experiment, suggesting that methanogens in these soils were using an existing soil C pool. In addition, in that work, both water and acetate were added to an additional set of microcosms. This resulted in CH_4 production rates that surpassed those of the acetate added microcosms. The addition of water presumably filled the pore water space, connecting substrate pools to methanogens enabling methanogens to access more of the added acetate. Thus, the plateau in CH_4 production may be a result of localized substrate depletion.

A slower initial rate of CH_4 production followed by a steady increase in the rates, such as that seen in the Colorado forest soils, could indicate that the physical disturbance of setting up the microcosm disrupted anoxic microsites and syntrophic partnerships. When the Cloud forest soils were disturbed, such as by slurrying, less CH_4 was produced (Teh and Silver 2006a). The magnitude of the decrease in CH_4 production after a disturbance may depend on soil properties. Thus soils with different properties, such as the Cloud and Colorado forest soils, would have different responses to a disturbance.

The lag in methanogenesis from the Colorado soils may be due to other more energetically favorable electron acceptors being used. Iron reduction was shown to reduce methanogenesis in the Cloud forest soils, anoxic paddy soils and wetland soils (Teh et al 2008, Glissmann and Conrad 2000, Jerman et al 2009). The microbial community in the Colorado soils using more energetically favorable electron acceptors would outcompete methanogens for carbon. Previous research using anoxic paddy soils has demonstrated that a lag in demonstrable methanogenesis was because ferric iron and sulfate were used as electron acceptors and may have suppressed

methanogenesis (Jerman et al 2009, Chidthaisong and Conrad 2000, Glissmann and Conrad 1999, Estop-Aragones et al 2013). Thus, detectable methanogenesis would occur only after other electron acceptors were depleted or decreased in availability.

The size of the available substrate pool may have been a factor in the pattern of methanogenesis from the Colorado soils. Because the rates of methanogenesis in the Colorado forest soils continued to increase over the course of the incubation, a substrate pool used may have been generated during the initial timepoints and was not depleted by the final timepoints. The Colorado forest soils are reported to have very high decomposition rates (Parton and Silver et al 2007). Through processes such as fermentation and homoacetogenesis, additional accessible methanogenic substrate pools may have be created during the course of the experiment (Capone and Kiene 1988, Glissmann and Conrad 2000, Stams 1994). In the Colorado soils, decomposition may have produced accessible carbon pools over the course of the experiment.

The increasing CH_4 production rates from the Colorado forest soils also suggest that the size of the substrate pool generated in the Colorado forest soils was larger than the existing carbon pool in the Cloud forest soils. The Cloud forest soils had more fine roots than either the Colorado or Tabanuco forest soils (root diameter and biomass were not measured). Much of the root material was removed prior to setting up the microcosms. The methanogens in the Cloud forest may have been using C derived from root exudation or decomposition, and because the roots were removed this pool of C was not replenished.

It is possible that the increase in CH_4 production in the Colorado soils was due to an increase in the number of methanogens. The rate of CH_4 production in the Colorado soils follows an almost exponential curve, which is consistent with methanogens growing during the course of the experiment. In general, methanogens double at a rate of one to several days (Zinder 1993). Assuming that methanogens were growing over the course of this 10 day experiment, and that the maximum doubling time was four days, the methanogen population would have undergone 1-2.5 doublings. Provided that all the methanogens were producing CH_4 at the same rate over the course of the experiment, these doublings would have caused an increase in CH_4 1-2.5 times the amount measured at Day 0-2. The increase in CH_4 from Day 0-2 to Day 8-10 is 18.8 ± 3.2X, 29.5 ± 4.4X for the Colorado 0-10 cm and 10-20 cm soils, respectively, which is substantially greater than potential CH_4 that could have been produced solely due to an increase in the methanogen population. However, the mcrA transcripts were much greater in the Colorado 10-20 cm soils than in the other forest types and depths. This indicates that methanogens in this soil were either up-regulating MCRA or that they were growing during the course of the experiment.

BES inhibited CH_4 oxidation activity. The amount of CH_4 oxidized in these soils was 58-97% greater in the No Addition than in the BES treatment. The amount of BES used (30 mM) was chosen because in initial experiments this concentration in the pore water was just sufficient to inhibit CH_4 production for twenty days in these soils (data not shown). Previous experiments using these soils used 10 mM BES to inhibit CH_4 production (Teh et al 2006b). BES causes a decrease in pH. While methanotrophs can function over a wide range of pHs (3.5 to 8) (Born et al 1990), a recent study has shown that the methanotrophic population structure changes in response to changes in pH (Kolb 2009). A shift in the methanotrophic bacteria community structure may influence the rate of CH_4 oxidization. However, both 10 mM and 30mM BES decrease pH by a similar magnitude (data not shown). Thus a decrease in pH does not explain the inhibition of CH_4 oxidation in this experiment.

BES may have caused a shift in the microbial community such that particular members increased, drawing resources and nutrients away from methanotrophic bacteria. BES has been shown to alter microbial community structure in trichloroethene-dechlorinating and in chloroethene-dechlorinating enrichment cultures (Chiu and Lee 2001, Loffler et al 1997). In the trichloroethene-dechlorinating enrichment culture, some members decreased and another increased in response to BES. BES could be affecting community structure by differentially suppressing bacteria by an unknown mechanism. Differential sensitivity to BES has been demonstrated for methanogens and chloroethene-dechlorinating cultures (Xu et al 2010, Loffler et al 1997). The sensitivity to BES of other microbes has not been studied, to my knowledge. Differential sensitivity to BES may extend beyond methanogens and chloroethene-dechlorinating cultures to microorganisms in general. Methanotrophic bacteria may be sensitive to BES, and the addition of BES inhibited their activity or made it such that they were ineffective at competing for resources. Alternatively, the activity of other bacteria that may support methanotrophic bacteria, e.g. by providing nutrients or other resources, could have been decreased by the addition of BES.

Because BES inhibited CH_4 oxidation in my study, the No Addition treatment was a more accurate estimate of potential CH_4 oxidation. However, the total amount of CH_4 oxidized in the No Addition treatment cannot be accurately calculated because CH_4 was probably being produced and oxidized (Teh et al 2005). The CH_4 oxidation measured in the No Addition treatment may reflect oxidation of the added CH_4 and native CH_4 production. The amount of CH_4 oxidation measured in the No Addition treatment was as accurate as the treatments and techniques used would allow.

The high initial rates of CH_4 oxidation in the Cloud 0-10 cm soils was not simply a result of the size of the standing population of methanotrophic bacteria. The initial rates of CH_4 oxidation in both the BES and No Addition treatment was greatest in the Cloud forest soils. Initial CH_4 oxidation rates in the BES treatment were positively correlated with gene copy number of all pmoA genes assayed. Assuming two copies of pmoA per CH_4 oxidizing bacterium, there were more methanotrophic bacteria in the Colorado forest soils (Type II, Type I Methylocapsa and Type I Methylococcus). The abundance of pmoA gene copies can indicate the size of the potential standing population of methanotrophic bacteria, but it does not indicate if they were oxidizing CH_4. The Colorado forest soils may have a larger methanotrophic bacterial population overall, but the Cloud forest soils appear to have the larger active methanotrophic bacterial population.

The ratio of the proportion of Type II to Type I methanotrophic bacteria does not appear to play a role in CH_4 oxidation. The proportion of Type II and Type I methanotrophic bacteria in the bacterial population followed the same pattern in each forest type. In each forest soil, the proportionally most abundant methanotrophic bacteria was the Type I Methylocapsa, followed by the Type II and then the Type I Methylococcus bacteria. Previous enumeration of methanotrophic bacteria from the Cloud forest soil quantified the Type I methanotrophic bacteria and indicated the presence of Type II methanotrophic bacteria (Teh 2005). Type I methanotrophs were more abundant and more diverse than Type II methanotrophs in rice field, wetland, permafrost, and some wet meadow soils (Qiu et al 2008, Yun et al 2010, Liebner and Wagner 2007, Zheng et al 2011). Regardless of whether they are numerically dominant, the Type I methanotrophs were also more active in rice field and landfill soils (Shrestha et al 2007, Qiu et al 2008, Cebron et al 2007), incorporating more labeled substrate into PLFAs and DNA

than Type II methanotrophs. Type II methanotrophs may be more abundant in temperate forest soils (Horz et al 2002, Singh et al 2009, Tate et al 2012). In the LEF tropical forest soils, it appears that the Type I methanotrophs are more abundant, similar to the wetland and rice field soils.

CH_4 production appears to drive CH_4 oxidation in these soils. This is consistent with rice field soils and some temperate forest and prairie soils (Gilbert and Frenzel 1995, Chan and Parkin 2001). Initial CH_4 production was highest in the Cloud forest soils and higher rates of CH_4 oxidation were observed in the Cloud forest. Previous research conducted at these sites and in microcosms studies observed the same pattern (Silver et al 1999, Keller 1986, Teh et al 2005, Teh et al 2006b). In sites where CH_4 oxidation is driven by CH_4 production, the expectation would be that few methanotrophic bacteria would be recovered from locations with low CH_4 emission or production potential, which was observed in this study.

Because CH_4 production drives CH_4 oxidation in these soils, factors that would increase CH_4 production were also correlated with CH_4 oxidation. Cumulative CH_4 oxidation in both the BES and No Addition treatments were positively correlated with MAP and soil moisture (Table 2). As expected, cumulative CH_4 oxidation for both the BES and No Addition treatments was positively correlated to percent sand, and negatively correlated to percent silt. A greater percentage of sand would allow for greater diffusion of oxygen into the bulk soil, whereas soils with more silt would tend to hold water, slowing the diffusion of oxygen into the soil. Since both the Colorado and Cloud forest soils contain similar percentages of silt, the negative correlation between cumulative CH_4 oxidation and percent silt may be driven by the large percentage of silt in the Tabanuco forest soils, which did not measurably oxidize CH_4 during the course of this experiment.

Other factors, such as rainfall frequency, may play an important role in CH_4 flux in these soils. Both CH_4 production and oxidation are correlated to percent sand. While a positive correlation was expected between CH_4 oxidation and percent sand for the reasons mentioned above, a correlation between CH_4 production and percent sand was not. More frequent or saturating rain events may be the reason why soils with high percent sand, such as the Colorado forest soils, also have such high CH_4 production potential.

Conclusions

My study considered the microbes responsible for both CH_4 production and oxidation and explored the relationship between the abundance and diversity of both methanogens and methanotrophic bacteria to identify possible underlying drivers of CH_4 emissions in upland tropical soils. In these sites, CH_4 production rates are influenced by the standing methanogen population, possible available C pools and perhaps the frequency of rain events. While mcrA diversity was not a significant driver of CH_4 production in all soils, a few mcrA OTUs were more abundant or were only present in sites with higher CH_4 production. As expected, soil moisture and MAP drive CH_4 production. CH_4 oxidation did not appear to be related to the size of the methanotrophic bacterial community. Rather, CH_4 oxidation appears to be driven by CH_4 production. In this way, these upland tropical forest soils behave similarly to other saturated soils.

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Table 1-1. Site characteristics for the Subtropical wet forest (SWF, Tabanuco), Montane wet forest (MWF, Colorado) and Montane rain forest (MRF, Cloud). Standard errors are in parentheses.

Site	Life Zone	Elev. (masl)	Soil Moisture[1]	MAP[2]	MAT[2]	pH[2]	%C[3]	%N[3]	C:N[2]	%sand[3]	%silt[3]	%clay[3]
Tabanuco	SWF	350	54.5	3500	22	4.8 (0.1)	2.6 (0.3)	0.21 (0.01)	12.8 (0.77)	14	35	52
Colorado	MWF	780	59.5	4200	19.5	5.1 (0.2)	7.4 (2.0)	0.31 (0.06)	18.6 (0.76)	63	18	18
Cloud	MRF	950	140.2	500	18.5	5.0 (0.2)	12.9 (0.8)	0.54 (0.04)	23.7 (1.15)	29	51	19

[1]This study.
[2]Dubinsky, E.A. 2008. UCB Thesis.
[3]Dubinsky E.A., Silver, W.L., and M.K. Firestone. 2010. Ecology. 91(9): 2604-2612.

Table 1-2. Pearson's correlations between edaphic factors and cumulative CH_4 production from the DFM treatment or cumulative CH_4 oxidation from the BES and No Addition treatments. Because the edaphic factors were measured for 0-10 cm soil, the correlations include only the 0-10 cm samples. The correlations between samples and soil moisture included both depths because soil moisture was measured for both depths.

	Elev. (masl)		Soil Moisture		MAP		MAT		pH		% Carbon		% Nitrogen		C:N		% sand		% silt		% clay	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Cumulative CH_4 production	0.767	0.015	0.527	0.024	0.641	0.062	-0.766	0.016	0.822	0.006	0.641	0.062	0.531	0.141	0.680	0.043	0.692	0.038	-0.129	0.739	-0.833	0.005
Cumulative CH_4 oxidation (BES treatment)	0.675	0.045	0.707	0.001	0.792	0.010	-0.677	0.045	0.258	0.501	0.792	0.010	0.834	0.005	0.767	0.015	0.758	0.017	0.669	0.048	-0.491	0.179
Cumulative CH_4 oxidation (No Addition treatment)	**0.983**	**<0.001**	0.579	0.008	**0.903**	**<0.001**	**-0.982**	**<0.001**	0.886	0.001	**0.903**	**<0.001**	0.812	0.007	**0.932**	**<0.001**	**0.940**	**<0.001**	0.115	0.766	**-0.980**	**<0.001**

Table 1-3. Abundance of genes and transcripts measured by qPCR. Standard deviation between three biological replicates are included in parentheses; letters denote significant difference at $p < 0.01$ using TukeyHSD test.

A. Gene Abundance

Site	Depth	Archaeal 16S g soil^{-1} (10^{12})	Bacterial 16S g soil^{-1} (10^{12})	mcrA g soil^{-1} (10^{4})	Archaeal16S^{-1} (10^{-7})	pmoA II g soil^{-1} (10^{8})	Bacterial 16S^{-1} (10^{-3})	MCAP g soil^{-1} (10^{8})	Bacterial 16S^{-1} (10^{-4})	MCOC g soil^{-1} (10^{2})	Bacterial 16S^{-1} (10^{-10})
Tabanuco	0-10 cm	4.8 (0.1) a	5.5 (1.5) a	5.4 (0.9) a	1.0 (0.2) a	4.4 (2.8) ab	2.8 (1.8) a	10.1 (5.6) a	6.4 (3.5) a	2.1 (1.2) ab	1.3 (0.8) b
	10-20 cm	1.9 (0.8) a	5.5 (1.3) a	3.4 (2.0) a	0.5 (0.3) a	3.1 (1.8) a	4.6 (2.3) ab	8.1 (4.3) a	12.0 (5.5) ab	1.6 (1.1) ab	2.4 (1.4) a
Colorado	0-10 cm	2.5 (0.7 a	4.9 (3.2) ab	14.2 (4.6) ab	2.0 (0.6) a	7.2 (3.0) ab	5.3 (2.3) ab	17.5 (6.1) ab	12.9 (4.6) ab	3.1 (1.2) a	2.3 (0.9) a
	10-20 cm	2.5 (0.9) a	4.1 (0.8) bd	28.1 (20.6) a	3.9 (2.9) ab	8.2 (5.0) b	7.0 (4.6) b	20.8 (7.2) b	17.4 (7.1) b	3.9 (2.2) b	3.3 (2.1) a
Cloud	0-10 cm	2.4 (1.5) a	15.1 (4.3) c	69.2 (49.5) bc	9.9 (7.4) b	11.2 (2.2) c	2.3 (0.8) a	34.8 (6.2) c	8.2 (1.4) a	7.3 (1.5) c	1.6 (0.6) a
	10-20 cm	4.3 (0.8) b	12.6 (1.7) cd	75.8 (61.4) c	6.2 (5.0) a	10.4 (1.6) c	2.6 (0.4) a	39.3 (6.3) c	9.0 (3.4) a	7.2 (1.2) c	1.8 (0.2) a

B. Transcript Abundance

Site	Depth	mcrA g soil^{-1} (10^{0})	Archaeal16S^{-1} (10^{-11})	pmoA II g soil^{-1} (10^{3})	Bacterial 16S^{-1} (10^{-8})	MCAP g soil^{-1} (10^{2})	Bacterial 16S^{-1} (10^{-10})	MCOC g soil^{-1} (10^{2})	Bacterial 16S^{-1} (10^{-10})
Tabanuco	0-10 cm	0.4 (0.3) a	7.4 (5.9) a	2.7 (0.5) a	6.0 (4.2) a	8.1 (8.2) a	5.1 (5.2) ab	1.2 (0.9) ab	0.8 (0.6) a
	10-20 cm	0.2 (0.3) a	3.0 (4.5) a	4.2 (0.7) a	9.5 (3.9) a	1.8 (3.5) a	2.7 (5.4) a	0.4 (0.6) ab	0.4 (0.8) a
Colorado	0-10 cm	0.0 ab	0.0 a	7.7 (3.4) a	5.7 (2.5) a	7.2 (3.6) a	5.3 (2.7) ab	7.2 (2.3) b	5.2 (1.6) b
	10-20 cm	5.6 (3.7) b	50.9 (54.1) b	3.0 (3.1) a	2.2 (2.5) ab	5.0 (9.5) a	4.2 (8.1) a	4.5 (4.7) ab	3.7 (4.1) a
Cloud	0-10 cm	0.0 a	0.0 a	3.0 (1.5) a	0.7 (0.4) c	59.3 (81.0) ab	14.0 (19.1) ab	24.5 (11.4) c	5.8 (2.7) b
	10-20 cm	0.3 (0.3) a	2.9 (2.5) a	10.4 (10.4) a	2.5 (2.6) ab	99.5 (77.0) b	14.2 (18.2) b	16.4 (12.1) bc	4.0 (2.8) a

Table 1-4. Pearson's correlations between mcrA gene copies and transcripts and CH_4 production rate and pmoA gene copies and transcripts and CH_4 oxidation rate. Gene copies and transcripts are normalized by g soil extracted.

		Gene		Day	r	p
CH_4 production rate	Gene copies	mcrA		0-6	**0.493**	**<0.001**
	Transcripts	mcrA		8-10	0.050	0.050
CH_4 oxidation rate	Gene copies	pmoA	II	0-12	**0.468**	**0.468**
		pmoA	MCAP	0-12	**0.571**	**0.571**
		pmoA	MCOC	0-12	**0.628**	**0.628**
	Transcripts	pmoA	II	16-20	-0.346	-0.346
		pmoA	MCAP	16-20	0.357	0.357
		pmoA	MCOC	16-20	**0.797**	**0.797**

Table 1-5. Pearson's correlations between gene copies and edaphic factors. All correlations, except the correlation with soil moisture, include only the 0-10 cm samples because the edaphic factors were measured from 0-10 cm soils. Correlation significant at $p < 0.001$ are in bold.

		Elev. (masl)		Soil Moisture		MAP		MAT		pH		% Carbon		% Nitrogen		C:N		% sand		% silt		% clay	
		r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
mcrA		0.549	0.003	**0.583**	**<0.001**	0.502	0.007	-0.550	0.002	0.200	0.316	**0.649**	**<0.001**	**0.686**	**<0.001**	**0.627**	**<0.001**	**0.619**	**<0.001**	0.561	0.002	-0.393	0.042
pmoA	II	0.044	0.823	-0.360	0.007	-0.085	0.671	-0.043	0.830	0.392	0.043	-0.130	0.516	-0.239	0.229	-0.083	0.677	-0.069	0.731	-0.586	0.001	-0.228	0.252
	MCAP	0.314	0.110	-0.272	0.046	0.169	0.397	-0.313	0.111	0.577	0.001	0.145	0.469	0.028	0.887	0.192	0.336	0.206	0.300	-0.457	0.016	-0.467	0.014
	MCOC	0.221	0.266	-0.176	0.202	0.061	0.759	-0.220	0.268	0.425	0.027	0.094	0.641	0.006	0.975	0.129	0.520	0.140	0.485	-0.352	0.071	-0.338	0.084

Table 1-6. Richness and diversity indices of mcrA clone libraries from each forest type and depth.

Site	Depth	No. of sequences	No. OTUs[1] observed	Chao1	ACE	Shannon H′	Simpson's 1/D
Tabanuco	0-10 cm	0	0	nd	nd	nd	nd
	10-20 cm	0	0	nd	nd	nd	nd
Colorado	0-10 cm	35	35	36.2	61.1	2.8	0.91
	10-20 cm	35	35	12	15.8	1.9	0.80
Cloud	0-10 cm	38	38	29	28	2.6	0.91
	10-20 cm	20	20	61.5	92.4	2.6	0.92

[1]OTUs at 88% identity
nd; not done

Table 1-7. Pearson's correlations of the composition and abundance between mcrA OTUs recovered from both depths of the Colorado and Cloud forest soils. Bold values indicate significance at $p < 0.05$.

		Colordo 0-10 cm		Colordo 10-20 cm		Cloud 0-10 cm		Cloud 10-20 cm	
Site	Depth	r	p	r	p	r	p	r	p
Colorado	0-10 cm								
	10-20 cm	**0.58**	**< 0.001**						
Cloud	0-10 cm	**0.37**	**0.032**	0.21	0.237				
	10-20 cm	-0.19	0.285	0.05	0.776	0.40	0.020		

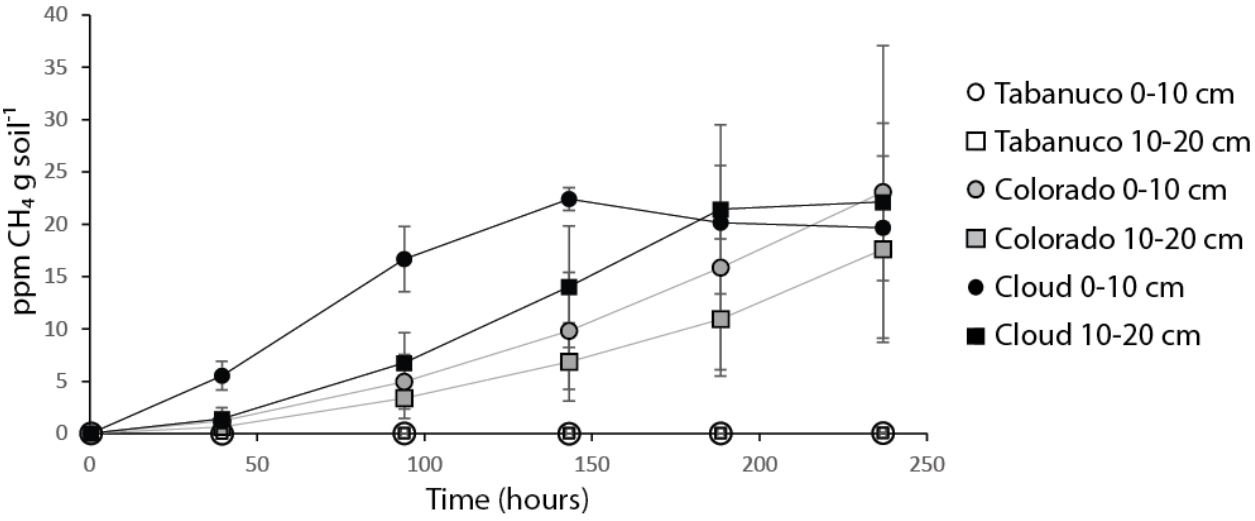

Figure 1-1. Cumulative CH_4 production from the DFM treatment of both depths of the three forest types.

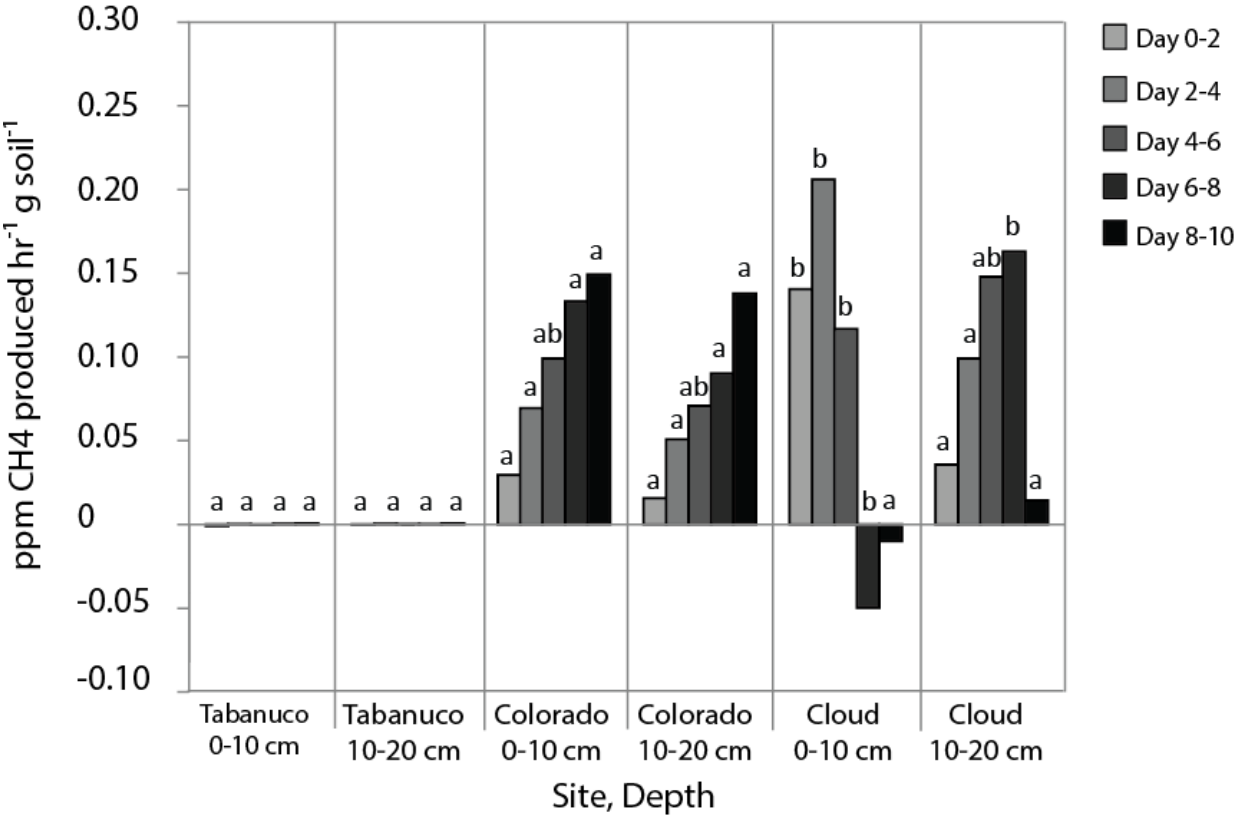

Figure 1-2. Average CH_4 production rates presented here were calculated from data collected from the DFM treatment of soils from both depths of the three forest types. Rates were calculated over 48 hour intervals and are presented as the average biological replicates (n = 3). Letters denote significant differences between the CH_4 production rate of the different forest types and depth within a 48 hour period at $p < 0.001$ using TukeyHSD.

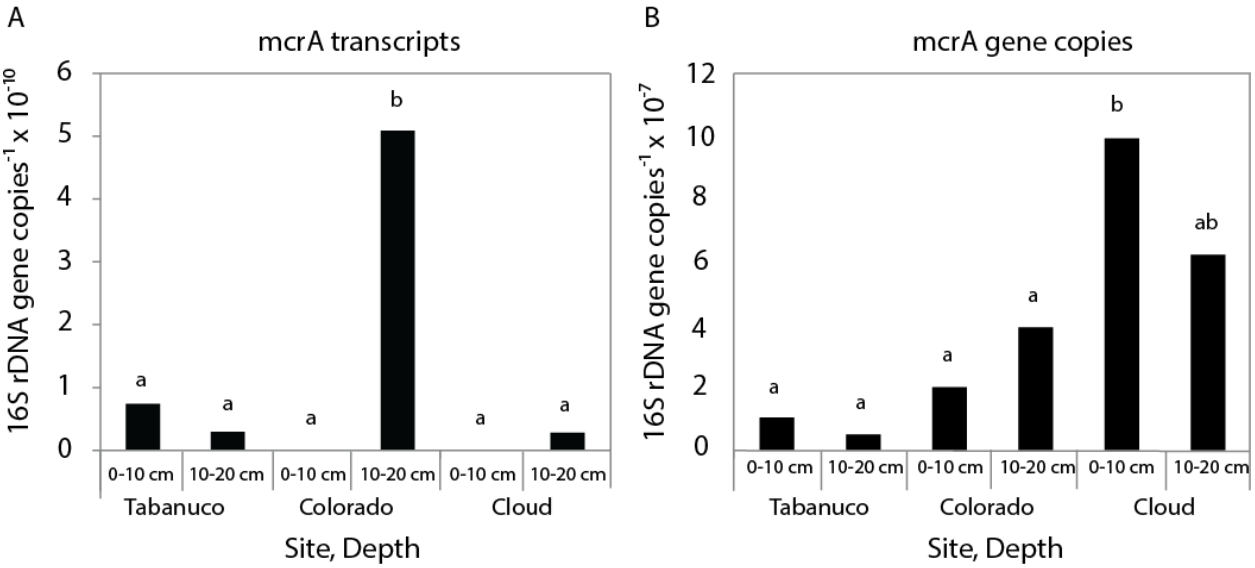

Figure 1-3. Abundance of mcrA gene copy number A) and transcripts B) in each depth of each forest type at Day 10. Gene copies and transcripts are presented as a proportion of Archaeal 16S gene copies amplified from DNA extracted from each forest type. Letters denote statistical significance across the different forest types and depth at $p < 0.01$ using TukeyHSD.

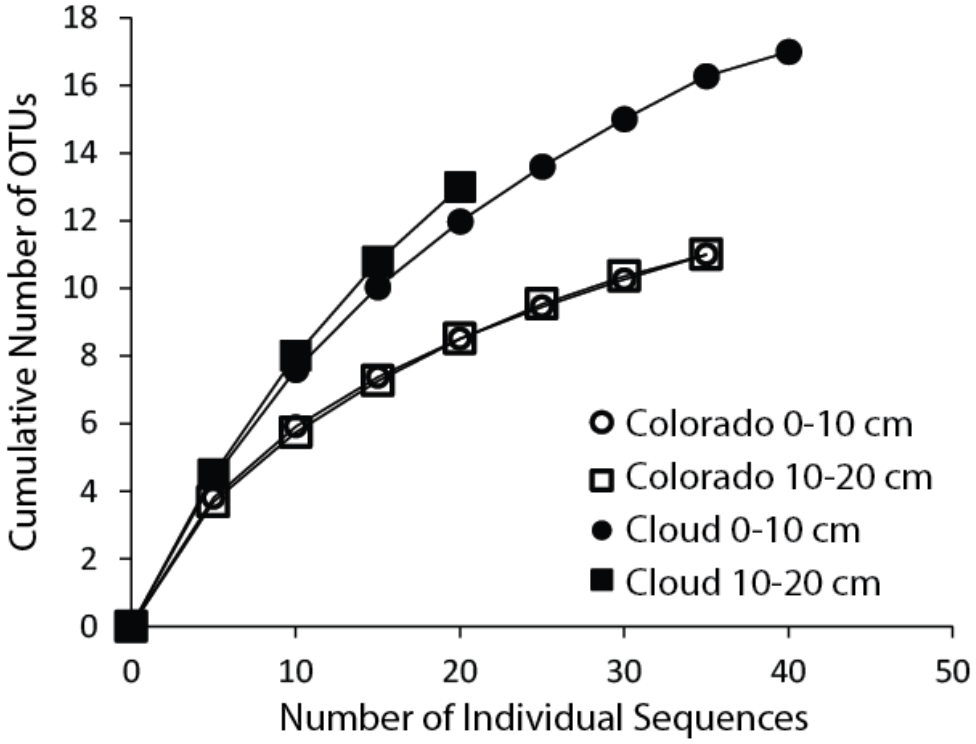

Figure 1-4. Rarefaction curve of mcrA clone libraries by site and depth for the Colorado and Cloud forest types. No mcrA sequences were recovered from the Tabanuco forest site.

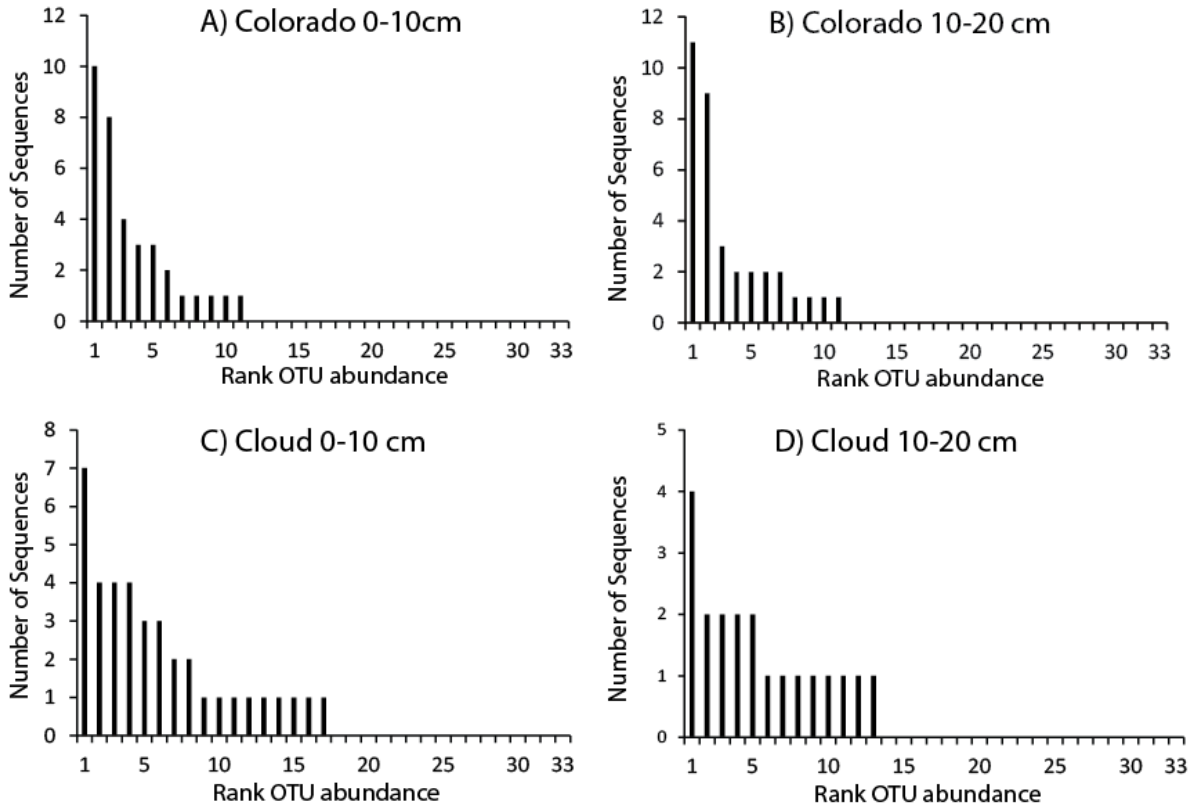

Figure 1-5. Rank abundance curves of mcrA OTUs from A) Colorado 0-10 cm soil, B) Colorado 10-20 cm soil, C) Cloud 0-10 cm soil and D) Cloud 10-20 cm soil.

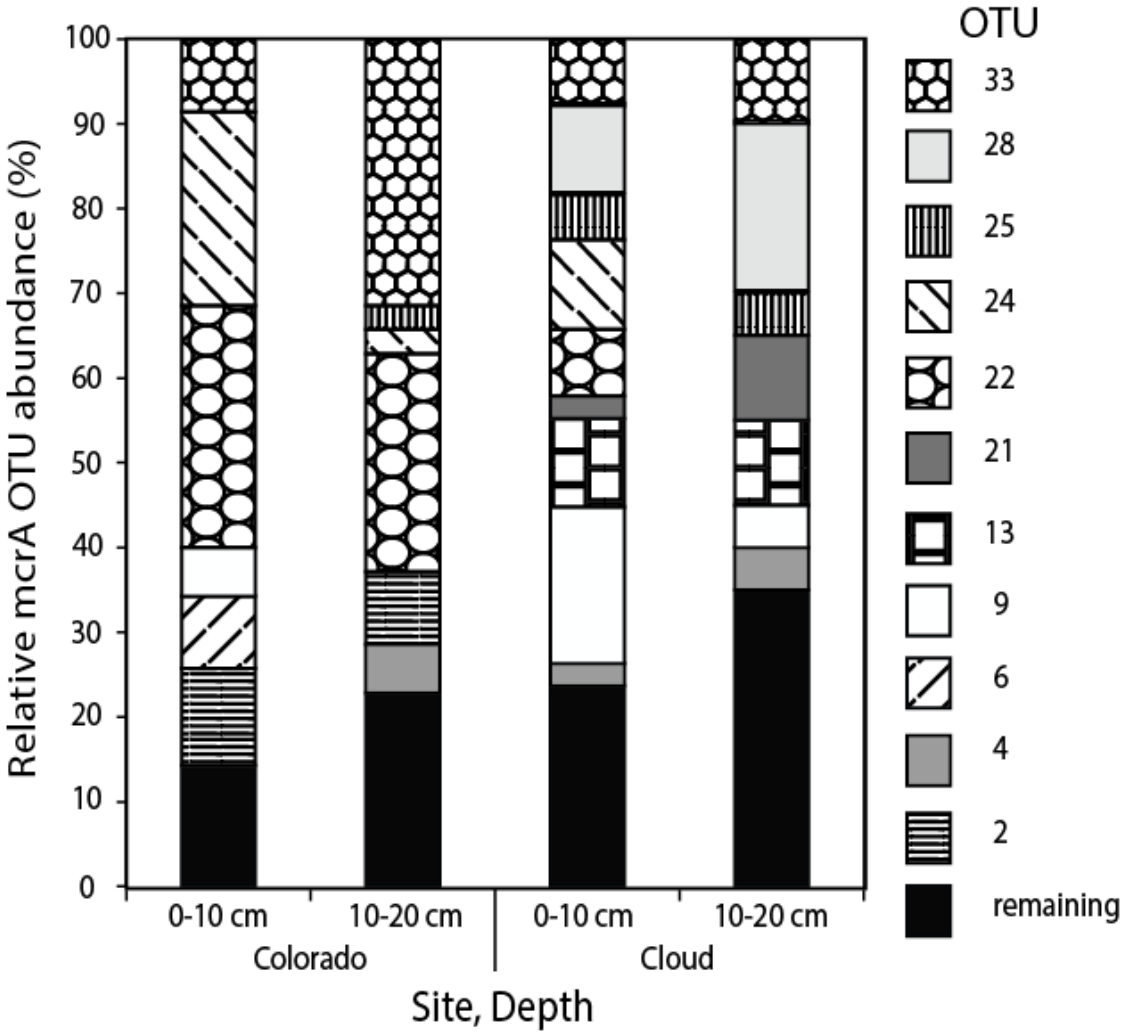

Figure 1-6. The relative abundance of mcrA OTUs recovered from all sites and depths. The 11 most abundant mcrA OTUs are shown individually, with the other 22 OTUs represented as 'remaining'.

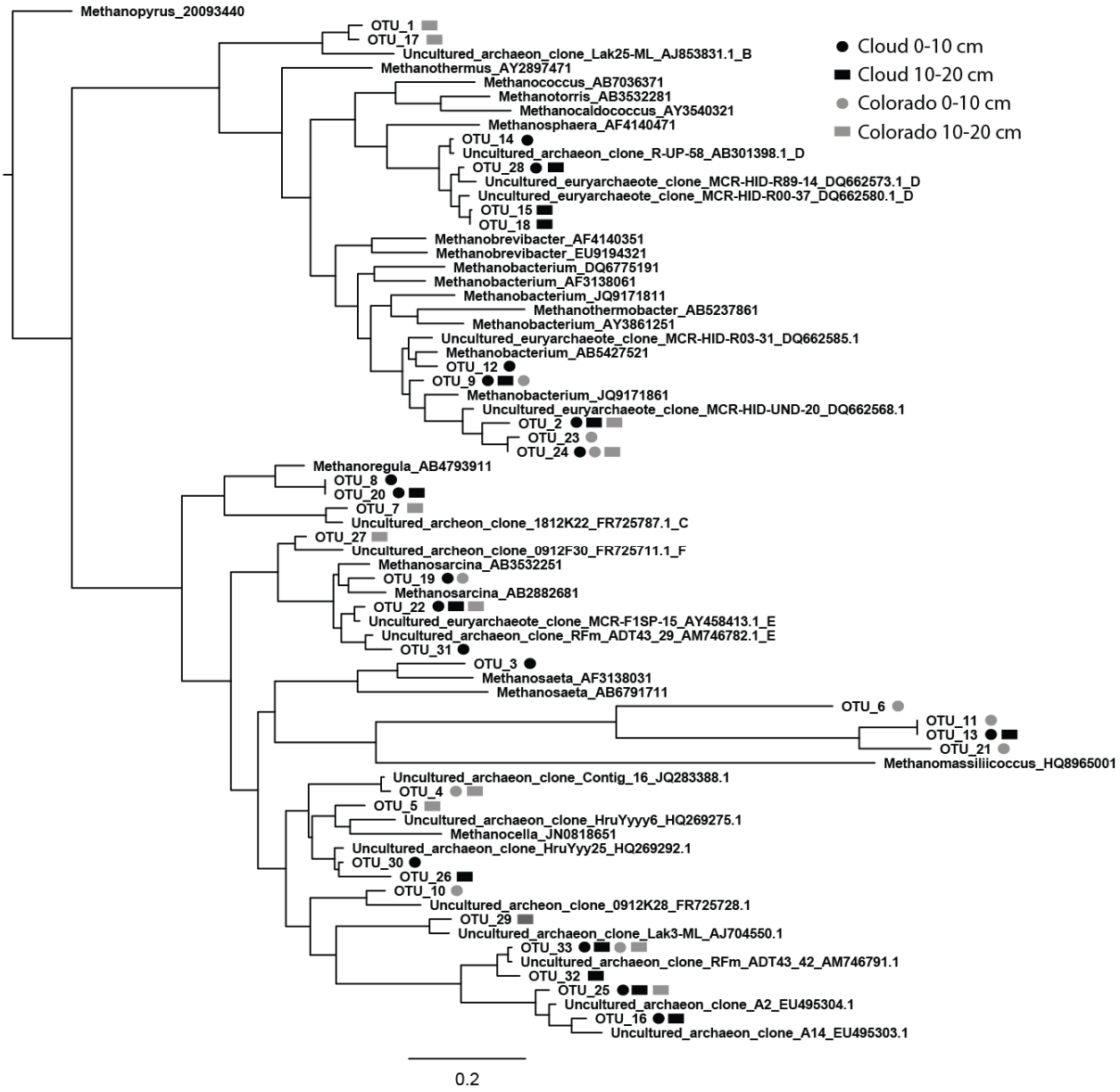

Figure 1-7. Maximum Likelihood phylogenetic tree of mcrA sequences representing each mcrA OTU recovered from the clone library. The NCBI accession number for the sequences from the cultured and non-cultured database are appended to the sequence name.

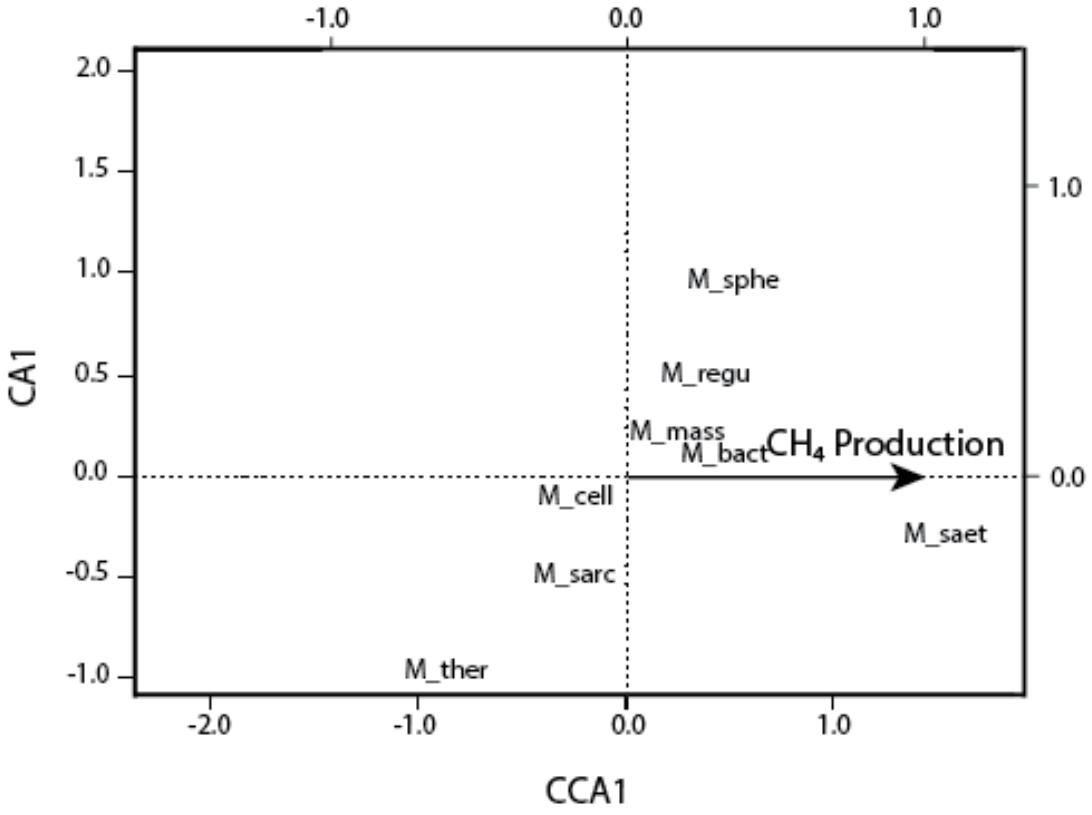

Figure 1-8. Canonical Correspondence Analysis showing the abundance of OTUs similar to known methanogen genera at each site and depth constrained using an environmental matrix consisting of cumulative CH_4 production at each site and depth. OTU labels refer to the following genera: M_ther; *Methanothermus*, M_sarc; *Methanosarcina*, M_cell; *Methanocella*, M_bact; *Methanobacterium*, M_mass; *Methanomassiliicoccus*, M_reg; *Methanoregula*, M_sphe; *Methanospherula*, M_saet; *Methanosaeta*. The arrow depicts the magnitude of cumulative CH_4 production.

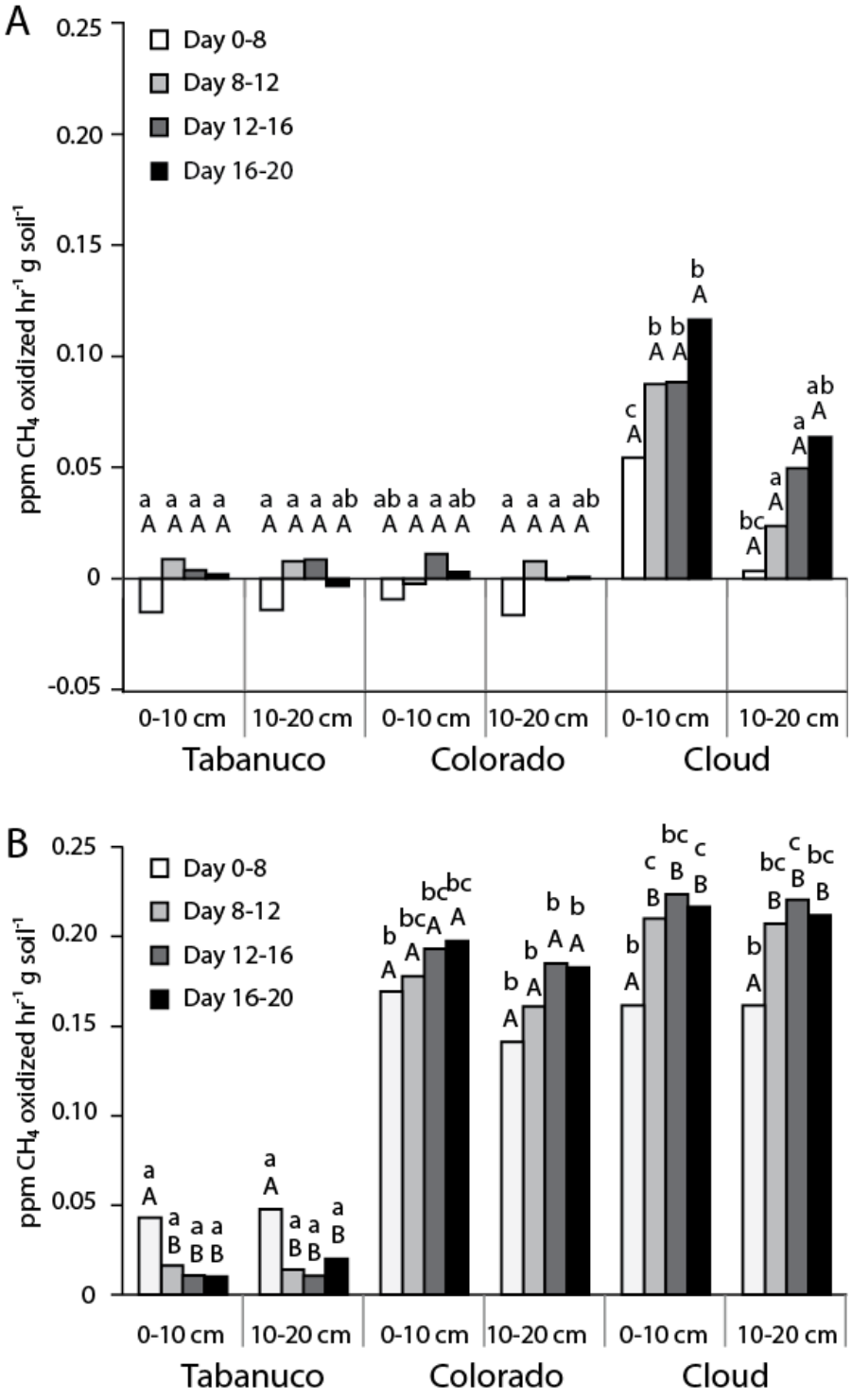

Figure 1-9. CH_4 oxidation rates from the A) BES treatment and B) the No Addition treatment of both depths of the different forest types. Averages are the mean of three biological replicates. Lowercase letters denote a difference between the timepoints from a forest type and depth at $p < 0.05$ using TukeyHSD. Uppercase letters denote a difference between the timepoint across forest types and depths at $p < 0.05$ using TukeyHSD.

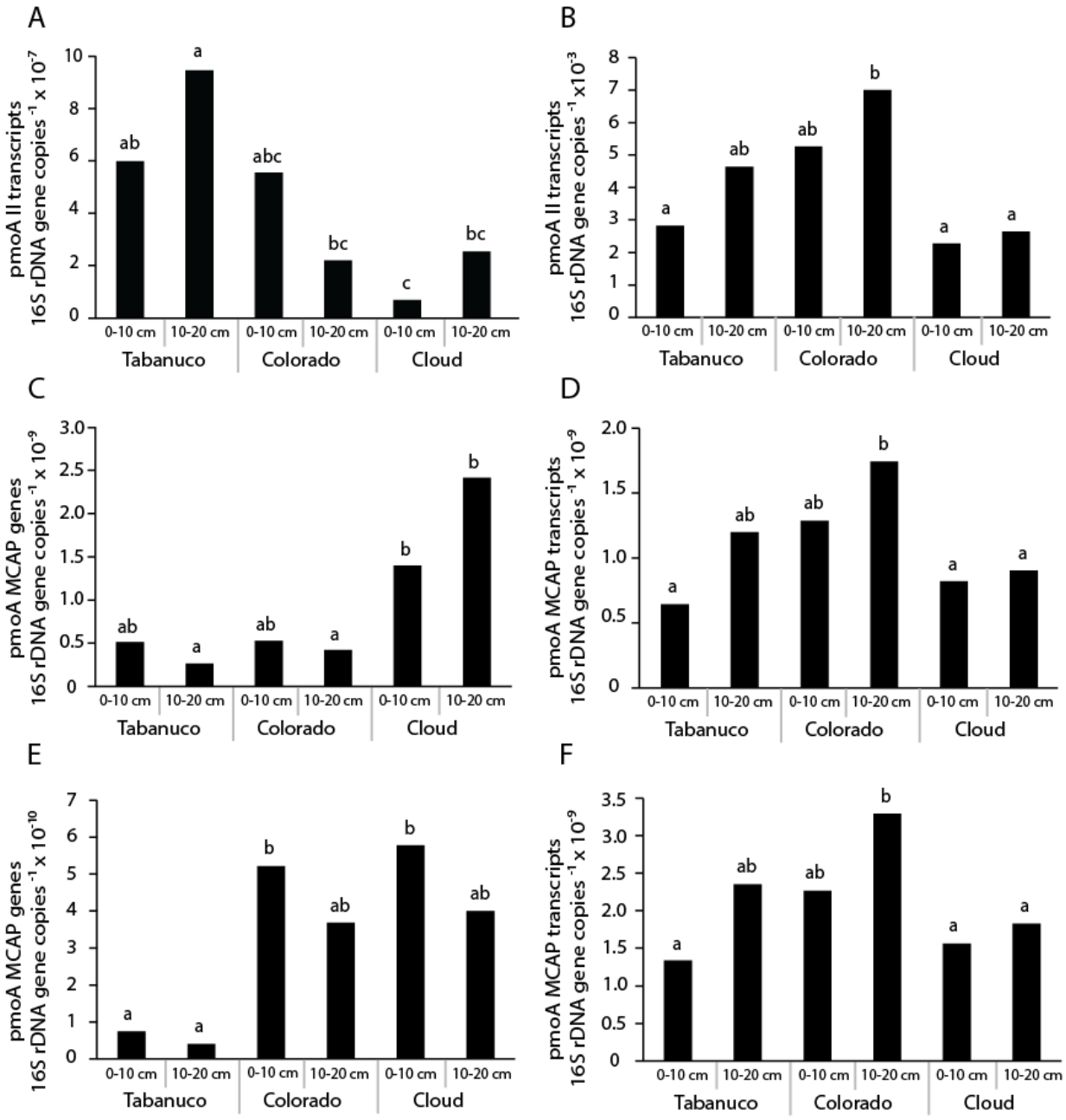

Figure 1-10. Abundance of A) Type II pmoA transcripts and B) gene copy number, C) Type I Methylocapsa pmoA transcripts and D) gene copy number and E) Type I Methylococcus pmoA transcripts and F) gene copy number F) in each depth of each forest type at Day 20. Gene copies and transcripts are expressed as a proportion of Bacterial 16S gene copies amplified from DNA extracted from each forest type. Letters denote a difference across the different forest types and depth at $p < 0.01$ using TukeyHSD.

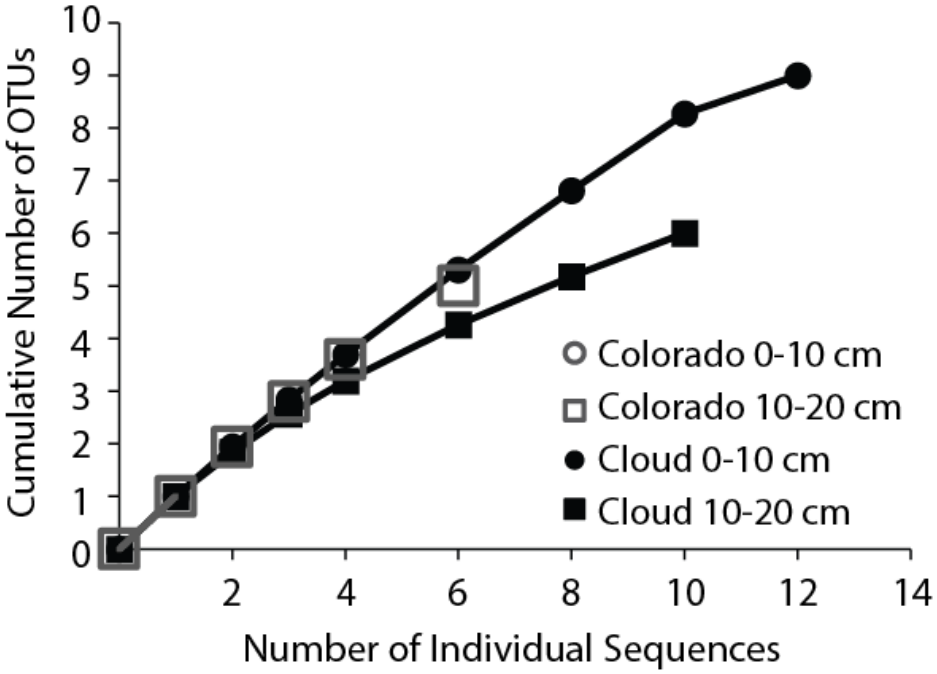

Figure 1-11. Rarefaction curve of pmoA clone libraries by site and depth. No pmoA sequences were recovered from the Tabanuco forest soils.

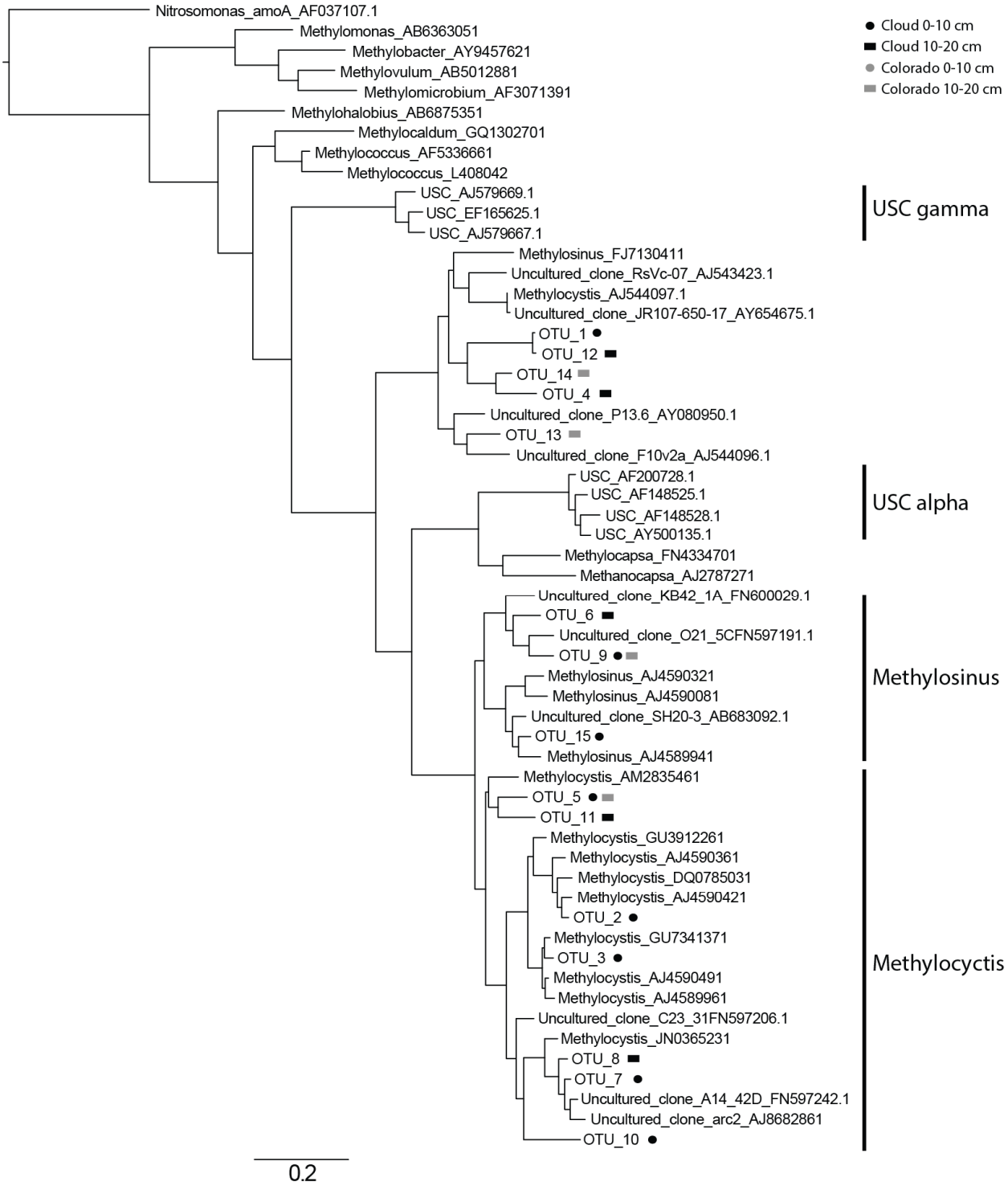

Figure 1-12. Maximum Likelihood phylogenetic tree of Type II pmoA sequences recovered from the clone library. The NCBI accession number for the sequences from the cultured and non-cultured database are appended to the sequence name.

Chapter 2

Deconstructing microbial mineralization of plant material in a wet tropical forest soil

Abstract

The wet tropical soils in the Luquillo Experimental Forest in Puerto Rico (LEF) have been reported to have extremely high rates of litter decomposition. The warm and moist conditions experienced by these soils are thought to enable these high decomposition rates. However, soils in the LEF are also characterized by fluctuating soil oxygen concentration to which the indigenous decomposing microbial communities are likely adapted. This study was designed to determine the effect of fluctuating redox conditions on the mineralization of plant material and to determine if rates of plant litter decomposition could be linked to microbial community composition and function (carbon degradation potential). Soil sampled from the palo colorado forest type in the LEF was amended with ^{13}C-labeled plant material (*Avena barbata*) and subjected to three redox conditions: oxic, anoxic, and fluctuating oxic-anoxic, in a microcosm-based experiment.

Mineralization of plant litter to carbon dioxide (CO_2) and methane (CH_4) was affected by the soil redox condition. Under fluctuating redox conditions, litter mineralization to CO_2 was significantly greater than under static conditions. Plant litter mineralization to CH_4 was significantly greater under the static low redox condition than under oxic or fluctuating condition. However, total litter mineralization (to CO_2 and CH_4) was highest under anoxic and fluctuating and not significantly different between the two redox conditions.

Soil redox condition affected the community gene profile for decomposition (carbon degradation potential as measured by the GeoChip 4). Carbon degradation potential of oxic condition samples clustered separately from carbon degradation potential of anoxic condition samples. However, carbon degradation potential was not correlated to either bacterial community composition or plant material mineralization.

The overall bacterial community composition (by 454 Pyrosequencing) was significantly affected by the presence of plant material, but not by soil redox condition. The bacterial community composition was significantly correlated with rates of plant material mineralization and the production of CO_2 and CH_4. Although bacterial community composition was not affected by redox condition, there was one notable difference in the phyla detected under the oxic and anoxic conditions. Observations of Firmicutes phyla were significantly more abundant in the amended low redox condition and were correlated with ^{13}C-CH_4 production.

Fluctuating redox conditions appear to increase rates of plant material mineralization in the LEF soil tested. The overall composition and abundance of the bacterial community correlated with plant litter material mineralization. In particular, abundance of Firmicutes was associated with

litter material mineralization to CH_4 in these soils. These data suggest both the fluctuating redox conditions and bacterial community composition play important roles of plant materials mineralization in the LEF soils.

Introduction

The upland tropical forest soils in the Luquillo Experimental Forest (LEF) in Puerto Rico have among the highest rates of plant litter decomposition known (Parton and Silver et al 2007). These high rates of decomposition are commonly attributed to the almost constant warm, moist conditions (Moorhead et al 1999). The soils present in the LEF, however, commonly have two additional features that could also contribute to the rapid decomposition rates observed. The soils are characterized by persistent fluctuations in soil oxygen concentration (Silver et al 1999) and the soil microbial community present appears to be adapted to fluctuating redox conditions (Pett-Ridge and Firestone 2005). This upland tropical forest soil presents a unique opportunity to study the influence of redox dynamics on microbial community composition, function, and mediation of a globally important process, litter decomposition.

Litter decomposition is an important component of nutrient cycling. There are three general stages of litter decomposition (Berg and McClaugherty 2008, McInerny and Bryant 1981). In the first stage, water soluble sugars are degraded (Aneja et al 2006. Tenney and Waksman 1929). The length of this stage depends on the litter begin decomposed. Some plant materials, such as grass shoots, can have a protracted first stage, whereas pine needles have an almost undetectable first stage (Berg and McClaugherty 2008). In the second stage, somewhat recalcitrant carbon, such as cellulose, is degraded; in the third stage, even more recalcitrant carbon, such as lignin, is degraded. Rates of decomposition of plant material can be affected by litter quality, the physical environment, and the activity of decomposer organisms.

Redox potential is a key factor in soil processes; it controls microbial respiration activity and thereby affects nutrient cycles (Luedemann et al 2000). Redox potential is a measured index of available electrons in a system (DeLaune and Reddy 2013). Soil redox potential is considered indicative of whether oxygen or other less energetically favorable terminal electron acceptors are used by microorganisms (DeLaune and Reddy 2005). When soil redox potential decreases, microbial respiration shifts from using oxygen, an energetically favorable terminal electron acceptor, to less energetically favorable terminal electron acceptors, such as nitrate and ferric iron. Here I define conditions under which low redox conditions prevail as an anoxic condition. Samples maintained with a headspace gas that includes atmospheric concentrations of oxygen are considered to be under oxic conditions. Hereafter, I refer to the alternative oxic-anoxic conditions as fluctuating redox conditions.

Redox conditions affect decomposition rates. Organic matter breakdown is generally thought to be slower under anoxic conditions than oxic conditions (Acharya 1935, Tenney and Waksman 1929). Thus, it is under oxic conditions that the bulk of litter decomposition is thought to occur. However, decomposition rates have been shown to increase under alternating wet oxic and anoxic periods compared to under either static condition (Reddy and Patrick Jr 1974). Rates of decomposition under the fluctuating redox conditions characteristic of many of the LEF soils

may be the simple sum of oxic and anoxic decomposition. Alternatively, the decomposition rates under fluctuating redox conditions may be higher than that which would be predicted to result from rates under either static condition. That is, fluctuating redox conditions may have a synergistic effect on decomposition rates thus potentially contributing to the rapid decomposition rates characteristic of the LEF systems.

Bacterial community composition in LEF soils is affected by the fluctuating redox conditions. The soil bacterial community structure in LEF soil was affected by the length of oxic and anoxic periods of the fluctuating condition (Pett-Ridge and Firestone 2005). The longer the period of static oxic and anoxic conditions, the more the bacterial community diverged from the native the bacterial community (Pett-Ridge and Firestone 2005). Indeed, under static oxic or anoxic conditions, the LEF soil bacterial community appeared less diverse by T-RFLP (Pett-Ridge and Firestone 2005). The fluctuating redox conditions in the LEF appear to have selected for a phylogenetically diverse bacterial community that is not maintained under static conditions (Pett-Ridge and Firestone 2005). The fluctuating conditions also appear to support the activity of more phyla than static oxic or anoxic conditions (DeAngelis et al 2010). Thus fluctuating conditions may affect decomposition rates by maintaining a microbial community with diverse decomposition capabilities.

Links between microbial community composition and function have been difficult to establish (Grant et al 1993, Sugai and Schimel 1993). A few studies in soils have successfully linked shifts in microbial community composition with addition of organic carbon (Pankratov et al 2011, Cleveland et al 2007, Nemergut et al 2010). However, conclusive links between microbial community composition and function are difficult to demonstrate (Strickland et al 2009, Kemmitt et al 2008). The difficulty lies in part with the apparent functional redundancy of microbes.

Current sequencing and microarray technologies make questions regarding microbial community composition and function more tractable to explore in complex systems such as tropical forest soils. Because the 16S rDNA gene contains both rapidly and slowing evolving sequences, it is commonly used to assign identity (Janda and Abbott 2007). High throughput sequencing technologies, such as 454 Pyrosequencing, are capable of generating thousands of sequences that can provide a snapshot of the microbial community composition (Roesch et al 2007).

Generally, an unknown bacterium with a 16S rDNA sequence that is 97% identical to functionally characterized bacteria is commonly assumed to be capable of the same functions. However, functional capacity does not necessarily follow from identity. Bacteria can have very similar 16S rDNA sequences, but differ in their ability to degrade a particular substrate (Achenbach et al 2001) or live at a particular temperature (Hong et al 2005).

To assess microbial organic carbon degradation capacity, a microarray technology such as the GeoChip is advantageous. The GeoChip is a microarray of functional genes considered characteristic of biogeochemical processes, such as carbon degradation and processes involved in the nitrogen cycle, among others (He et al 2012). Both DNA and RNA can be used in GeoChip analysis (Wang et al 2012, Gao et al 2007). DNA represents the potential functional capacity of a community, and RNA is used as a measure of the functional capacity actively expressed by a community. Here, I use the carbon degradation and a subset of the organic remediation gene probes on the GeoChip 4 as an index of the carbon degradation potential of the sampled communities.

GeoChip probes are predominantly derived from bacterial sequences (He et al 2012). The soil microbial community in the palo colorado forest type in the LEF is predominantly bacterial (Cusack et al 2011), making the GeoChip a reasonable method of measuring potential functional capacity in this soil. Due to the nature of microarray technology, the GeoChip is limited to a snapshot of the functional potential included in the array.

Bacterial and Archaeal 16S gene abundances have been used to measure the abundance of Bacteria and Archaea in complex environments. However, there are caveats that must not be overlooked when using 16S gene abundance as a measure of cell numbers. For one, 16S rRNA that is amplified by the primers used in PCR, may not represent all living organisms. Also, not all Bacteria, or Archaea, contain the same number of 16S rRNA genes. Thus, 16S rRNA qPCR (quantitative PCR) measurements may be equivalent between two samples, but the community from one sample may be much larger or smaller than that in the other sample. Likewise, there are caveats that must be recognized when using quantification of the Internal Transcribed Spacer (ITS) region as a measurement of fungi. As with Bacterial and Archaeal 16S rRNA, amplified ITS DNA can represent both living and deceased fungi. In addition, fungi commonly make multinucleate hyphae and even in an individual cell, copy number can vary. Thus, a measurement of ten ITS sequences may indicate ten fungal cells, one fungal hyphae, or some combination thereof. Despite these caveats, ITS abundance has been used to provide an estimate of the size of a fungal community (Fierer et al 2005, Guo et al 2010, Jiminez-Fernandez et al 2010, Lievens et al 2006).

Mineralization of organic compounds results in the production of CO_2 and CH_4, which are both potent greenhouse gases. If CO_2 is assigned a global warming potential of 1 over 100 years then CH_4 has a global warming potential of 21 over 100 years (IPCC 2007). Current global climate change models predict that tropical forest ecosystems will become warmer and drier, possibly with less frequent, but more intense rainfall events (Christensen et al 2007, Cramer et al 2004, Hulme and Viner 1998). The frequency of rainfall events coupled with potential drying of the tropical forest soils could change the frequency of the fluctuating redox conditions, which may impact the magnitude of CO_2 and CH_4 emissions.

My goal was to determine the influence of redox conditions (specifically the availability of oxygen) on litter decomposition as it is affected by microbial community composition and microbial genetic carbon degradation potential. I hypothesized that there is synergy between the metabolic processes of the oxic and anoxic fluctuating periods, which enables rapid decomposition of plant material in this wet, tropical forest soil. To test this hypothesis, I amended upland tropical forest soils with ^{13}C-labeled plant material and incubated the material under three redox regimes 1) static oxic, 2) static anoxic and 3) fluctuating oxic-anoxic. These soil incubations were used to determine 1) the influence of redox regime on the rates of mineralization of plant material, carbon degradation potential and microbial community composition and 2) the relationship between microbial community taxonomic and functional composition and rates of mineralization to CO_2 and CH_4.

Materials and Methods

Study Sites

Upland tropical forest soil was collected from the palo colorado forest type in the Luquillo Experimental Forest, a Long-Term Ecological Research site in Puerto Rico, USA (18°18'N, 65°50' W). The palo colorado forest type is a lower montane wet forest in the mid-elevations of the LEF, dominated by the Colorado tree (*Cyrilla racemiflora*) (Holdridge 1947, Willig et al 2011). Edaphic properties are presented in Table 1. In general, the climate is aseasonal with an average relative humidity of 98 % (Weaver 1994). Soils are acidic clay-loam utlisols derived from volcanoclastic sediments (Beinroth 1982). On average, soils have approximately 8% organic matter and have little litter accumulation at the surface. The average bulk soil oxygen concentration is 13 ± 0.2% at 0-10 cm, but it can vary from 21% to below 10% on the order of days to weeks driven by frequent rain events and high microbial activity (Silver et al 1999).

Soil Sampling

Five replicate 30 x 10 cm cores (1 m apart) were sampled from a randomly located transect in the palo colorado type forest. Prior to collection, surface plant litter was removed. Cores were transported at room temperature to UC Berkeley. Upon arrival, plant litter and root material in the soil were removed and the 5 cores were combined into a composite sample that was used for further analysis. All replicate samples for microcosm incubations were taken from this one composited soil sample. The soil remained at room temperature until the microcosm experiments started (within one week).

Soil Moisture

Approximately 5 g soil (wet weight) of the composited soil was weighed into aluminum trays in triplicate and dried at 105 ˚C until the soil reached a constant weight. Soil moisture was calculated using the following formula: Soil Moisture = (initial wet soil weight / (initial wet soil weight – final dry soil weight)) * 100.

Microcosm Incubation Conditions

^{13}C-labeled plant material

The ^{13}C-labeled plant material used was shoot material from *Avena barbata*, an annual grass species; native ^{13}C-labeled plant material was not available from LEF native plants. *A. barbata* was labeled to 2 atom percent under controlled conditions in growth chambers (Bird et al 2011) using the method described in Bird et al. (2003). Briefly, *A. barbata* was grown to maturity under temperature controlled conditions in plexiglass growth chambers. CO_2 concentrations were monitored with a LI-6200 infrared gas analyzer (IRGA) (Li-Cor, Lincoln, NE). A ^{13}C-CO_2 generator was connected via a diaphragm pump (GAST, Benton Harbor, MI) to circulate ^{13}C-CO_2 into the chamber. The ^{13}C-CO_2 generator was a vessel containing concentrated H_2SO_4 attached to a reservoir containing 1 M $NaHCO_3$ solution (99.9% atom excess ^{13}C). A computer and data acquisition manager (21X Micrologger, Campbell Scientific, Inc., Logan, UT) controlled temperature and CO_2 concentration level with solenoid actuated valves (Horwath and

Paul 1994). Total organic carbon (C) and nitrogen (N) were determined for shoot material (< 2 mm) after subsampling and homogenizing (using a ball mill roller) using a Roboprep-CN elemental combustion system interfaced to a Europa 20-20 isotope ratio mass spectrometer (PDZ Europa, Cheshire, UK). The C:N ratio of the ^{13}C-labeled *A. barbata* shoot material was 39:1. Using grass shoots as whole plant material allowed me to investigate the initial stages of decomposition: degradation of soluble sugars and perhaps some recalcitrant carbon degradation.

Set-up

Each soil microcosm contained 20 g (wet weight) soil in 250 ml mason jars fitted with Hungate stoppers as gas sampling ports. The microcosms were divided into two treatments, 1) amended with ^{13}C-labeled *Avena barbata* litter, and 2) unamended. The ^{13}C-labeled *Avena barbata* litter had been oven-dried at 25 ˚C and cut into approximately 2 cm lengths. In each treatment, three redox regimes were maintained, 1) oxic, 2) anoxic and 3) fluctuating oxic-anoxic. Four days was chosen as the length of the oxic and the anoxic periods of the fluctuating condition. This frequency of fluctuating conditions maintains a bacterial community structure more similar to the native community than other periodicities previously tested (Pett-Ridge and Firestone 2005). The microcosms in the oxic condition were flushed with medical grade air (Praxair, Inc., Danbury, CT) and initial oxygen concentration was measured with a Model 52 Dissolved Oxygen Meter (YSI Incorporated, Yellow Springs, OH). The initial condition for the microcosms in the fluctuating oxic-anoxic condition was oxic. Hereafter, the fluctuating oxic-anoxic condition will be referred to as the fluctuating condition. The fluctuating condition microcosms were made anoxic when appropriate by flushing the headspace with N_2 and confirmed to be anoxic using the Dissolved Oxygen Meter. To inhibit CH_4 oxidation, 500 ppm Difluoromethane (DFM) was added to each replicate of each treatment (Miller et al 1998).

Sampling

At the end of the first two days, then after every four day period, 2 ml of headspace gas was sampled into 10 ml N_2-flushed, evacuated serum vials. Oxic microcosms and microcosms in the oxic period of the fluctuating condition were destructively sampled on days 2, 18 and 34 (Figure 1). Anoxic microcosms and microcosms in the anoxic period of the fluctuating condition were destructively sampled on days 6, 22 and 38. Destructive sampling of the fluctuating samples occurred at the end of a 4-day period (on days 2, 6, 18, 22, 34 and 38). At each time point, triplicate amended and unamended microcosms from the static and the fluctuating condition were destructively sampled. The destructive sampling consisted of removing 120 ml of headspace gas into 100 ml N_2-flushed, evacuated serum vials, and weighing approximately 5 g of soil for DNA extraction. Soil was stored at -80 ˚C.

On the dates of destructive sampling, the percentage oxygen was measured in all remaining microcosms. After determining oxygen concentration the microcosms they were flushed with the appropriate gas and 500 ppm DFM was again added to inhibit CH_4 oxidation.

Gas sampling

Both the 2 and 120 ml headspace samples were used for gas analysis. Cumulative CO_2 and CH_4 production was determined from analysis of the 2 ml samples. Analyses of the 120 ml samples were used for ^{13}C-gas measurements. The concentration of CO_2 and CH_4 were measured with a HP6890 Shimadzu Gas Chromatograph using a Flame Ionization Detector and a Thermal

Coupled Detector respectively (Columbia, MD). A standard containing known concentrations of CO_2 and CH_4 was used to make a standard curve. Cumulative CO_2 and CH_4 from the microcosms destructively sampled on Day 34 and Day 38 are shown in Figure 2 and 3, respectively.

^{13}C-labeled gas sampling

A Micromass JA Series Isoprime mass spectrometer (Cheadle, UK) was used to quantify ^{13}C-CH_4. An IsoPrime100 mass spectrometer (Cheadle, UK) was used to quantify ^{13}C-CO_2. Both mass spectrometers were at the Lawrence Berkeley National Laboratory Center for Isotope Geochemistry.

The amended and unamended microcosms for each condition were set up in triplicate. However, due to technical issues, eight ^{13}C gas samples were lost. For the samples with only two available replicates, the measurements were averaged and the average value was substituted for the missing value.

The ^{13}C-gas measurements for each replicate of each condition was analyzed separately. The µmol C gas values at each time point were calculated and graphed individually, and the equation that best described the curve of the values over time were determined using Excel (Microsoft Office 2013, Redmond, WA). The µmol C gas produced was calculated by taking the integral of the equation that best described the data using an Integral Calculator (http://www.integral-calculator/#). The percentage of *A. barbata* litter that was mineralized to ^{13}C-gas was determined by dividing the µmol ^{13}C gas by the µmol ^{13}C of *A. barbata* litter. The ^{13}C-CO_2:^{13}C-CH_4 ratio is the average of the ratio calculated for each replicate. For example, the percent CO_2 from ^{13}C-labeled *A. barbata* litter from the Day 6 amended anoxic condition replicate 1 sample was used only in downstream calculations with the percent CH_4 from the Day 6 amended anoxic condition replicate 1 sample. Likewise, the percent ^{13}C-gas values presented are averages of the sum of percent ^{13}C-CH_4 and ^{13}C-CO_2 calculated from each replicate.

DNA extraction

Total DNA and RNA were extracted from 3.5 g of soil (saved at -80 ˚C; Placella et al 2012) using a Phenol:Chloroform method. Briefly, 350 µl 0.1 M aluminum ammonium sulfate and 350 µl each of CTAB (0.5 M phosphate buffer, pH 8, 5% hexadecyltrimethylammonium bromide, 1 M NaCl) and Phenol:Chloroform:Isoamyl alcohol (25:24:1) were added to 15 ml Lysing Matrix E tubes (MP Biomedicals, Solon, OH) containing 3.5 g frozen, pulverized soil and bead-beaten at 5.5 m/s, for 30 seconds (MP Biomedicals, Solon, OH). The tubes were incubated on ice for 5 minutes before centrifugation at 5,500 rcf for 5 minutes at 4 ˚C. The aqueous phase was transferred to a Phase lock gel tube (5 PRIME, Inc., Gaithersburg, MD), mixed and then centrifuged at 8,000 rcf for 3 minutes at 4 ˚C. The aqueous phase was then transferred to another tube. The soil was then re-extracted with an additional 350 µl of CTAB, and the resulting aqueous phase was pooled. DNA was precipitated at room temperature for at least 3 hours using 2 volumes of PEG/1.6M NaCl and 2 µl of 5 mg/ml linear acrylamide (Life Technologies Ambion, Grand Island, New York). DNA was recovered by centrifugation at 16,000 rcf for 10 minutes, washed twice with 70% ethanol and resuspended in 100 µl RNase-free 100 mM Tris buffer. The DNA and RNA were separated using an All-Prep kit (Qiagen, Valencia, CA) and stored at -80 ˚C.

GeoChip

The GeoChip analysis was done at the Institute for Environmental Genomics (University of Oklahoma) using the GeoChip version 4. The GeoChip 4 contains approximately 83,992 gene probes targeting 152,414 genes in 410 functional gene families (Lu et al 2012). For the GeoChip analysis, 500 ng of DNA from triplicate final time point samples from amended and unamended samples from each redox condition were used. Triplicate samples of DNA (500 ng) from the bulk soil collected and stored at -80 ˚C immediately before the experiment began were also analyzed.

Extracted community DNA (no amplification step) was labeled with cyanin-5 (Cy-5) dye. Approximately 500 ng of genomic DNA was denatured for 5 minutes at 99.9 ˚C in solution with random octamer mix (Invitrogen, Carlsbad, CA, USA) and snap chilled on ice. Following denaturation, 2.5 mM dithiothreitol (DTT), 0.25 mM dATP, dCTP and dGTP, 0.125 mM dTTP, 0.125 mM Cy5-dUTP, and 80 U Klenow fragment (Invitrogen, Carlsbad, CA, USA) were added. Reaction mixtures were incubated at 37 ˚C for 3 hours. Labeled target DNA was purified with a QIAquick PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and measured on a NE-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and dried using a speed-vac at 45 ˚C for 45 min.

A sample tracking control solution (2.88 µL, NimbleGen, Madison, WI, USA) was added to each dried sample. The sample tracking control solution and dried DNA mixture was incubated at 50 ˚C for 5 minutes, vortexed for 30 seconds, and then centrifuged to collect all liquid at the bottom of the tube. The collected liquid was resuspended in hybridization buffer (40% formamide, 35% SSC, 1% SDS, 2.24% aligment oligo Cy-3 (NimbleGen, Madison, WI, USA) and 2.8% Cy5-labeled CORS target, vortexed and denatured at 95 ˚C for 5 minutes. Resuspended samples were kept at 42 ˚C until hybridization. The mixture (7.3 µL) was loaded onto arrays (NimbleGen 12-plex arrays) with mixer and hybridizations were performed at 42 ˚C for 17 hours using a MAUI hybridization station (MAUI, BioMicro Systems, Salt Lake City, UT, USA).

After hybridization, the arrays were washed three times with NimbleGen wash buffer, spin dried, and scanned with a NimbleGen MS200 scanner (Madison, WI, USA) at a power setting of 100%. An ImaGene instrument (version 6.1, BioDiscovery, El Segundo, CA, USA) was used to obtain raw data images. The raw data images were uploaded to the Institute for Environmental Genomics microarray data manager (http://ieg.ou.edu/microarray/) for pre-processing. A local background signal for each probe was obtained using a customized void gridding file targeting array positions without probes (Qichao Tu, personal communication). Then, NimbleScan software (version 2.6) was used to obtain the signal intensity for each probe. Each probe was normalized by the mean signal from the added CORS probes (Liang et al 2010).

All readings from probes with a signal intensity less than 2X the average background signal were removed. The signal to noise ratio [SNR = (signal intensity – background mean)/background standard deviation] was adjusted so that thermophile probes were excluded. Since the average temperature of the LEF is approximately 19.5 (Table 1), by removing the thermophile probes a probe set more representative of the microbial community in this relatively aseasonal environment. Probes present in only 1 of 3 samples were removed. Probe signals were then normalized by dividing the probe signal intensity by the average of all probe signal intensities for

the treatment. From this set of gene probes, only the carbon degradation and organic remediation gene probes were used in all further analyses. The organic remediation gene probes were curated into the following categories: Dioxygenases, Glucose oxidases, Monooxygenases, Oxidases, Peroxidases, and Pyruvate formate lyases. Gene probe membership in the curated Organic Remediation gene probe categories was confirmed using NCBI Protein ID information available during August, 2013 (http://ncbi.nlm.nih.gov). Hereafter, I will refer to the composition and abundance of carbon degradation and curated organic remediation gene probes as carbon degradation potential. Organic remediation gene probes were included in the carbon degradation potential because oxygenases are required for complex plant material breakdown (Berg and McClaugherty 2008).

The carbon degradation and curated organic remediation gene probes were ordinated using non-metric multidimensional scaling (NMDS) in R (version 3.0.2) using the vegan package. For all ordinations of GeoChip data, the Jaccard distance method was used.

Gene probes from the amended oxic condition were compared to gene probes from the amended anoxic condition. In addition, gene probes from amended samples from a particular condition were compared to the unamended samples from the same condition. Signals from all probes representing a particular gene were summed to determine the relative signal intensity of the group of gene probes. To determine the treatment specific gene probes from a pairwise comparison, the relative intensity data was first converted to presence absence data. A gene probe was considered specific to a treatment if it was present in 2 of 3 (or 4 of 6 in the case of the fluctuating condition) samples of a particular treatment and if it was not present in the other treatment. All treatment specific gene probes in a gene category were summed for each treatment in a pairwise comparison as well as the non-treatment specific (shared) gene probes.

Estimated alpha diversity (by Shannon and Simpson indices) of each replicate of each set of samples was done using the Institute for Environmental Genomics website tool (http://ieg.ou.edu). Each sample was rarified to 6000 sequences per sample prior to calculating the Shannon and Simpson diversity indices.

Quantitative PCR (qPCR)

Quantification of Bacterial and Archaeal 16S rDNA, and Fungal ITS was done using qPCR. Bacterial and Archaeal 16S rDNA were amplified using Eub318F/Eub518R and 340F/1000R primers, respectively (Fierer et al 2005, Gantner et al 2011). Fungal ITS was amplified using 5.8s/ITSf primers (Fierer et al 2005). Each 20 µl qPCR reactions contained 1 µl template DNA (~50 ng), 10 µl 2X SsoFast™ EvaGreen Supermix® (BioRad, Hercules, CA), and 1 µl each of 10 µM forward and 10 µM reverse primers. The following two step amplification protocol was used for each gene; 95 ˚C for 5 minutes for one cycle, 95 ˚C for 10 seconds, then at 53 ˚C for 20 seconds for 40 cycles, followed by a melting curve analysis from 53 ˚C to 95 ˚C.

Sample DNA was diluted to overcome PCR inhibitors. The dilution was chosen for each sample based on serial 1:10 dilutions of DNA in bacterial 16S rDNA qPCR reactions. Standards were generated from DNA extracted from palo colorado 0-10 cm soils and cloned into pCR2.1 or pCR4 vectors (Invitrogen, Carlsbad, NM). Standards were generated from plasmids extracted using a Miniprep kit (Qiagen, Valencia, CA) from single clones. Single clones picked from each clone library were sequenced (UC Berkeley Sequencing Facility, UC Berkeley, CA) and

confirmed to be a partial Bacterial or Archaeal 16S gene or a Fungal ITS gene using the blast too at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmids with confirmed cloned sequences were extracted using a Miniprep Kit (Qiagen, Valencia, CA), quantified on a NanoDrop (NanoDrop Technologies, Wilmington, DE), and diluted to generate a standard curve.

The abundance of genes are expressed relative to the amount of DNA (ng) extracted from the sample. The redox condition and the combination of redox condition and amendment affected the amount of DNA extracted at $p < 0.05$. A relative abundance measure was used because it is less sensitive to differences in DNA extraction efficiency.

454 Pyrosequencing

The V8 region of the small subunit rRNA genes (16S) was amplified by the 926F/1392R primer set (Kunin et al 2009) in 454 Pyrosequencing reactions (Joint Genome Institute, Walnut Creek, CA). Pyrosequencing was done using the FLX Titanium Platform (Roche, Branford, CT). Template for the reactions was 1 µg DNA from the final time point samples from the amended and unamended samples of each condition. Triplicate samples of DNA (1 µg) from the starting soil collected and stored at -80 ˚C immediately before the experiment began were also included. Pyrosequencing data was analyzed using the pyrosequencing analysis pipeline in Qiime version 1.6.0 (Caporaso et al 2010b). The Qiime workflow was used to denoise the sequences (Reeder and Knight 2010). OTUs were picked (Edgar 2010) and sequences were aligned to the Greengenes core reference alignment using PyNAST (Caporaso et al 2010a, DeSantis et al 2006). Chimeric sequences were identified using Chimera Slayer (Haas et al 2011) and removed. Sequences were assigned taxonomy using the RDP Classifier (version 2.2) and the Greengenes reference taxonomy database (Wang et al 2007, Werner et al 2012, McDonald et al 2012). FastTree was used to make phylogenic tree of the sequences (Price et al 2010). Alpha diversity metrics were also calculated using Qiime scripts. Samples were rarefied to 1130 sequences per sample. Of the triplicate samples from the starting soil, two samples had less than 255 sequences and were removed from further analysis. A weighted unifrac distance matrix was used to compare the composition and abundance of microbial communities from each redox condition and amendment (Lozupone and Knight 2005). Detrended Correspondence Analysis (DCA) was used to ordinate the weighted unifrac distances of samples using the vegan package of R (version 3.0.2). In addition, sequences were sorted by phyla and class. Only sequence observations of a phyla or class that were greater than 1% of the total sequences were compared using a Students T test in the following pairwise comparisons: amended oxic condition and amended anoxic condition samples, amended and unamended oxic condition samples, amended and unamended anoxic condition samples and amended and unamended fluctuating condition samples.

Statistical Analysis

All statistical analysis was performed in R (version 3.0.2) unless otherwise specified.

To determine how the redox condition, time and amendment contributed to any variation among gas production measurements, the adonis function from the vegan package was used. The adonis function from the vegan package of R was also used to determine how the test variables attributed to any variation among abundance measurements from the qPCR analysis, the abundance and diversity of carbon degradation and curated organic remediation probes and the

abundance and diversity of detected OTUs from the 454 Pyrosequencing data. The Bray distance measure was used for all adonis tests.

Analysis of variance, using Tukey's Honestly Significant Difference (TukeyHSD) test as a multiple means comparison test, was used to determine the variation in measurements from the ^{13}C-gas fluxes from ^{13}C-labeled *A. barbata* litter. Analysis of variance and TukeyHSD was also used to determine any difference in DNA extracted from oxic, anoxic and fluctuating samples as well as from amended and unamended samples. Alpha diversity measurements of the carbon degradation and curated organic remediation probes and from the alpha diversity measurements from the 454 Pyrosequencing data were compared using the Students T test. The variation in observations of sequences belonging to a particular phyla or class were also determined using the Students T test.

The large number of comparisons of relative gene probe intensities necessitated the correction for Type I errors. Thus, the Benjamini and Hochberg (BH) correction from the stats package of R was applied to correct for Type I error in p values obtained from Students T test of carbon degradation potential tests.

A Mantel test was used to correlate the gas production, bacterial community composition and the carbon degradation potential data. The microbial community qPCR abundance, 454 Pyrosequencing and carbon degradation potential data was first ordinated using Principal Coordinates Analysis (PCoA). The scores for the first PCoA axis were recorded for each ordination. The cumulative gas data and total ^{13}C-gas data, and the first axis PCoA scores from the qPCR abundance, 454 Pyrosequencing and carbon degradation potential data were correlated using a mantel test (vegan package in R). The Bray distance measure was used for all mantel tests.

Results

Gas Production

Cumulative CO_2 production from the amended oxic and the fluctuating conditions was 57.3 ± 6.8% and 46 ± 4.0% greater than CO_2 produced from the amended anoxic condition, respectively ($p < 0.01$) (Figure 2). Cumulative CO_2 production from the amended oxic condition was slightly greater than from the amended fluctuating condition, but it was not significantly different ($p = 0.128$). Cumulative CO_2 production from the amended samples was significantly greater than the unamended samples over time for each condition at $p < 0.01$ (Figure 2). Individually, both amendment and time explained substantial variation in cumulative CO_2 in each condition (Table 2A). Together, amendment and time were also correlated with cumulative CO_2 production within the amended and unamended samples (Table 2A). Individually, the redox condition and time were correlated with cumulative CO_2 production within the amended and unamended samples (Table 2B). Together, condition and time were also correlated to cumulative CO_2 production, however, this correlation was not as strong as when condition and time are considered individually (Table 2B).

Cumulative CH_4 production from the amended anoxic condition samples were 70.9 ± 21.8% and 72.4 ± 22.4% greater than the amended oxic and amended fluctuating conditions, respectively ($p < 0.05$) (Figure 3). Cumulative CH_4 production was not significantly different in the amended oxic and amended fluctuating conditions ($p = 0.846$). The cumulative CH_4 production was greater in the amended samples compared to the unamended samples in the anoxic condition ($p < 0.01$). Cumulative CH_4 production from the amended samples was not significantly different than from the unamended samples in the oxic and fluctuating conditions (oxic; $p = 0.155$, fluctuating; $p = 0.181$). Individually and together, amendment and time explained a significant but relatively small amount of variation in cumulative CH_4 in the anoxic and the fluctuating conditions (Table 3A). Time was a significant explanatory factor for the variation in cumulative CH_4 production only in the amended samples (Table 3B). Neither redox condition nor the combination of redox condition and time explained the variation in cumulative CH_4 production in the unamended samples (Table 3B).

Fluxes of ^{13}C-labeled CO_2 and CH_4

Under all redox regimes, ^{13}C-labeled *A. barbata* litter was mineralized into both ^{13}C-CO_2 and ^{13}C-CH_4. The percentage of ^{13}C-labeled litter mineralized into ^{13}C-CO_2 and ^{13}C-CH_4 varied under each redox regime (Table 4). The percent of ^{13}C-labeled litter mineralized into ^{13}C-CO_2 was significantly greater under the fluctuating redox condition than under either the oxic or anoxic conditions (oxic: 3.14%, anoxic: 3.33%, fluctuating 5.16%, $p < 0.01$) (Table 4). The percent of ^{13}C-labeled litter mineralized into ^{13}C-CH_4 was significantly greater under the anoxic condition than under either the oxic or fluctuating conditions (oxic: 0.42%, anoxic: 3.30%, fluctuating 0.92%, $p < 0.01$) (Table 4).

The percent of ^{13}C-labeled *A. barbata* litter mineralized to ^{13}C-gases (combined ^{13}C-CO_2 and ^{13}C-CH_4 gases) was not significantly different under the fluctuating and anoxic redox regimes (fluctuating: 6.11%, anoxic: 6.63%, $p < 0.01$). The percent of ^{13}C-labeled litter mineralized to ^{13}C-gases was significantly lower under the oxic redox regime than under either the anoxic or fluctuating redox regimes (oxic: 3.59%, anoxic: 6.63%, fluctuating 6.11%, $p < 0.01$) (Table 4). The ratio of ^{13}C-CO_2:^{13}C-CH_4 was greatest under the oxic condition followed by the fluctuating and the anoxic conditions. However, only the difference between the ^{13}C-CO_2:^{13}C-CH_4 ratios under the oxic and anoxic regimes was significant at $p < 0.01$. The difference between the ^{13}C-CO_2:^{13}C-CH_4 ratios under the oxic and fluctuating regimes was not significant at $p < 0.01$.

Degradative Metabolic Potential (GeoChip)

The carbon degradation potential was assessed using GeoChip analysis of the relative abundances of genes coding for specific enzymes involved in carbon degradation and bioremediation of organic carbon compounds. The preparation and incubation of the soils (putting the soil in microcosms and incubating them for 34 or 38 days), significantly affected the aggregated carbon degradative potential (as compared to non-incubated bulk soil) for the amended and unamended samples in each condition (Figure 4A). Thus, the starting soils samples were not included in further analyses.

Redox condition (oxic vs. anoxic), but not amendment significantly affected the degradative metabolic potential (Figure 4B, 4C, Table 5). On NMDS plots, the amended and unamended oxic samples separate from the amended and unamended anoxic samples (Figure 4B), however,

the 95% confidence intervals of the two clusters overlap (data not shown). Redox condition did significantly affect the degradative metabolic potential (Table 5, Figure 4B, 4C). By the adonis statistic, the separation of the oxic and anoxic samples is significant at $p < 0.1$ ($r^2 = 0.160$, $p = 0.056$). Amendment appeared to have less effect on the degradative metabolic potential of samples in a specific redox condition (Table 5). The combination of redox condition and amendment did affect the degradative metabolic potential, but less than redox condition alone (Table 5). The amended and unamended fluctuating samples in the oxic period of flux appear to loosely cluster with the amended and unamended oxic condition samples. Likewise, the amended and unamended fluctuating samples in the anoxic period of flux appear to cluster with the amended and unamended anoxic condition samples (Figure 4C).

Figure 5 shows pairwise comparisons of the carbon degradation and curated organic remediation gene probe families present in the amended oxic and anoxic samples at the end of the incubation period. There are no significant differences between relative intensities of gene probe families in the amended oxic and anoxic conditions at $p < 0.01$ (Figure 5A). There are also no significant differences observed between relative intensities of gene probe families in the amended and unamended samples of a condition at $p < 0.01$ (Figure 5B, 5C, 5D).

In general, over 50% of the detected gene probes were detected in both sets of samples of each pairwise comparison (Figure 6). The alpha diversity of the carbon degradation and the curated organic remediation gene probes was higher in the unamended compared to the amended anoxic condition samples using the Shannon metric at $p < 0.05$ (Table 6A). There was no difference in the alpha diversity of carbon degradation and curated organic remediation probes of any other comparison at $p < 0.05$ (Table 6).

Abundances of 16S and ITS rRNA genes by qPCR

Under each condition, Bacterial 16S abundance is greater than Archaeal 16S ($p < 0.01$) regardless of amendment (Figure 7). Amendment increased Fungal ITS abundance ($r^2 = 0.383$, $p = 0.001$), but only had a slight (but significant) effect on Bacterial and Archaeal 16S abundances (Table 7). Individually, condition and amendment explained a small amount of variability in ITS, and Bacterial and Archaeal 16S abundance (Table 7). Time did not explain significant variability in measurements of any kingdom (Bacterial, Archaeal, Fungal) (Table 7). The combination of redox condition and amendment had a slight, but significant, effect on Archaeal 16S and ITS abundances. The combination of amendment and time had a slight, but significant, effect on Archaeal 16S abundance (Table 7). The combination of redox condition and time did not significantly affect Bacterial and Archaeal 16S or ITS abundances.

Visually, ITS abundance appears to decrease in the amended anoxic condition and the anoxic periods of the fluctuating condition, regardless of amendment (Figure 7). In the amended anoxic condition, the trend of decreasing ITS abundance over time is significant only from Day 6 to 22 at $p < 0.05$. The trend of decreasing ITS abundance during the anoxic periods of the fluctuating condition, is significant only in the amended samples from Day 34 to 38 and in the unamended samples from Day 18 to 22 and Day 34 to 38 ($p < 0.05$). Archaeal 16S abundance appears to increase in the amended samples during the anoxic periods of the fluctuating condition and decrease in the unamended samples during the oxic periods of the fluctuating condition (Figure 7). However, neither trend in Archaeal abundance is significant at $p < 0.05$.

Taxonomic analysis using 454 Pyrosequencing

After rarefaction to 1130 sequences per sample, pyrosequencing allowed detection of 393 bacterial OTUs in 8 phyla. The number of OTUs detected was similar in each redox condition. However, the number of detected taxa in the unamended samples was greater (by 30-60 taxa) than in the amended samples in all conditions, though the difference was significant only for the anoxic and fluctuating conditions at $p < 0.05$.

By DCA analysis of bacterial community composition, the taxonomic composition and abundance of amended samples was clearly distinct from the composition and abundance of the unamended samples for each redox condition (Figure 8, Table 8). The unamended samples from all conditions and the starting soil sample cluster tightly together; their 95 % confidence intervals overlap. The combination of amendment and redox condition affected the taxonomic distance as well (Table 8). Amended samples from all conditions form distinct clusters (Figure 8). While the combination of amendment and redox condition was significant in explaining the composition, condition alone was not significant in explaining aggregate composition in either the static samples or the fluctuating samples.

Deconstructing the detected taxa by phyla and class, showed that the amended oxic and anoxic samples have distinct patterns of observed phyla (Figure 9). In the amended oxic condition samples, there were 8.0 ± 2.7, 1.7 ± 0.6 and 2.7 ± 0.5 times more sequences observed from the Gemmatimonadetes, Proteobacteria and Verrucomicrobia phyla, respectively (Figure 9). Within the Bacteriodetes, there were 3.6 ± 2.0 times more observed Saprospiracaea in the amended anoxic samples ($p < 0.05$) (Table 9). There were 12.3 ± 1.3 times more observations of Betaproteobacteria in the amended oxic samples than the amended anoxic samples ($p < 0.05$) (Table 9). In the amended anoxic condition, sequence observations of the Firmicutes were 15.8 ± 12.6 times greater than in the amended oxic condition (Figure 10, Table 9). Within the Firmicutes, there were 15.8 ± 3.5 times more observations of Clostridia sequences (Table 9). Sequence observations of Clostridia in amended samples of all conditions were positively correlated with the amount of ^{13}C-CH_4 produced ($r = 0.914$, $p < 0.001$).

The pattern of observed phyla in the amended versus the unamended samples were very similar in all conditions (Figure 10). In the unamended samples, there were more observed sequences in the Acidobacteria, Chloroflexi and Planctomycetes phyla by 1.7 ± 0.5, 2.1 ± 0.6 and 1.8 ± 0.3 times, respectively ($p <0.05$) (Figure 10). However, the difference in sequence observations of Chloroflexi were significant only in the oxic and fluctuating conditions. Also in the unamended samples, sequence observations of the Actinobacteria, Bacteriodetes and Verrucomicrobia were greater. The sequence observations of Actinobacteria were 1.9 ± 0.4 times greater in the oxic and fluctuating conditions ($p < 0.05$). Bacteriodetes sequence observations were 12.6 ± 8.2 times greater in the unamended samples, though this was significant only in the oxic and fluctuating condition samples at $p < 0.05$). Sequence observations of Verrucomicrobia were 3.8 ± 2.4 time greater in the oxic and anoxic conditions at $p < 0.05$.

The similarities in the pattern of observed sequences extended to the Class level (Table 10). Sequence observations of the DA052 Class of the Acidobacteria were more abundant under all conditions at $p < 0.05$. Sequence observations of the Planctomycetia Class of the Planctomycetes were also more abundant in the unamended samples in all conditions ($p < 0.05$). In the unamended samples of the oxic and fluctuating condition samples, sequence observations

of Ktedonobacteria Class of Chloroflexi and Acidimicrobiia Class of the Actinobacteria were more abundant than in the amended samples at $p < 0.05$.

There were also striking differences in the pattern of observed phyla in the amended versus the unamended samples (Figure 10). Sequence observations of Firmicutes in the amended anoxic samples were 10.7 times greater than in the amended oxic and fluctuating conditions. Also for the anoxic amended samples, there were 15.8 ± 12.6 times more sequence observations of Firmicutes than in the anoxic unamended samples ($p < 0.05$). The majority of Firmicutes sequence observations were from the Clostridia Class (96.8 ± 3.2 %). Proteobacteria sequence observations were 1.2 ± 0.1 times greater in the amended fluctuating and oxic conditions than in the respective unamended conditions (Figure 10). In the anoxic condition, Proteobacteria sequence observations were 1.5 ± 0.1 times greater in the unamended samples compared to the amended samples ($p < 0.05$). Even with the difference in sequences observations, within the Proteobacteria there is a similar pattern of sequence observations at the Class level (Table 11). Under all conditions there were 1.5 ± 0.1 times more sequence observations of Alphaproteobacteria in the unamended samples ($p < 0.05$). The differences in the Proteobacterial sequence observations in the amended samples was driven by the relative abundance of sequence observations in of the Beta and Delta Proteobacteria (Table 11). There were 5.9 ± 0.8 times more sequence observations of Betaproteobacteria in the amended oxic and fluctuating condition samples compared to the unamended samples. In the amended fluctuating conditions samples, there were 2.6 ± 0.5 times more sequence observations of Deltaproteobacteria.

The alpha diversity of the unamended samples is greater than the amended samples under all conditions at $p < 0.05$ (Table 11A). The alpha diversity of the amended anoxic samples was greater than the amended oxic samples at $p < 0.05$ only when using the Simpson statistic (Table 11B).

Mantel Correlations

Mantel tests were used to determine whether community abundance (qPCR data) or structure (454 Pyrosequencing data) affected decomposition or carbon degradation potential (Table 12). The community structure was correlated to total ^{13}C-gas produced as well as total CO_2 and CH_4 at $p < 0.05$ (^{13}C-gas, $r = 0.162$; CO_2, $r = 0.950$; CH_4 $r = 0.181$). The carbon degradation potential was correlated to community abundances ($r = 1$, $p = 0.001$). In addition, the amount of ^{13}C-gas produced was positively correlated to total CO_2 and CH_4 at $p < 0.05$ (CO_2, $r = 0.158$; CH_4, $r = 0.687$).

Discussion

The effect of fluctuating redox conditions on the soil microbial community structure and composition in the LEF has been the subject of previous investigation (DeAngelis et al 2010, Pett-Ridge and Firestone 2005). *In situ* litter decomposition in the LEF and the microbial community associated with long-term litter decomposition has also been studied (DeAngelis et al 2013, Cusack et al 2009). My study was designed to investigate the impact of the fluctuating

redox conditions in soil from the LEF on decomposition and the decomposition capabilities of the soil microbial community and determine whether the bacterial community composition under each redox condition could be linked to plant material mineralization.

Carbon Mineralization

Carbon mineralization was affected by the redox condition as evidenced by both the patterns of total CO_2 and CH_4 produced as well as the percent ^{13}C-labeled *A. barbata* litter transformed into ^{13}C-CO_2 and ^{13}C-CH_4. The pattern of total non-labeled CO_2 and CH_4 production was similar under the fluctuating and oxic conditions. The percent ^{13}C-labeled *A. barbata* litter mineralized to ^{13}C-CO_2 and ^{13}C-CH_4 was correlated to cumulative CO_2 produced and to cumulative CH_4 produced. However, the patterns of percent of ^{13}C-CO_2 and ^{13}C-CH_4 produced under each redox regime were markedly different from the total unlabeled CO_2 and CH_4 production produced. The percentage of ^{13}C plant material that was converted to terminal gas product (CO_2 and CH_4) was significantly lower under oxic conditions that under anoxic or fluctuating conditions. The percentage of ^{13}C plant material converted to CO_2 in the fluctuating redox condition may be greater than in the oxic condition because of synergy between aerobic and anaerobic decomposition processes. The majority of the ^{13}C-gas in the oxic and fluctuating condition was CO_2, whereas in the anoxic condition similar percentages of ^{13}C-labeled litter was transformed into ^{13}C-CO_2 and ^{13}C-CH_4. Previous studies have also observed a 1:1 ratio of CO_2 to CH_4 under anoxic conditions (Conrad 1999). The percentage of ^{13}C plant material that was converted to terminal gas product in the anoxic condition was greater than in the oxic condition because of the substantial amount of CH_4 produced.

The pattern of CO_2 accumulation was affected by both the redox condition and the presence of litter. Cumulative CO_2 from the unamended oxic and fluctuating samples were greater than from the unamended anoxic samples. The addition of litter enhanced this pattern. A previous study documented a similar pattern in plant litter amended samples, where cumulative CO_2 in the oxic and fluctuating conditions was greater than the cumulative CO_2 in the anoxic condition (Reddy and Patrick Jr 1974). Since CO_2 is greater in the amended oxic and fluctuating conditions than in the anoxic condition samples, I expected that the oxic and fluctuating conditions would transform similar amounts of ^{13}C-labeled litter into CO_2. However, the final decomposition products of ^{13}C-labeled litter (^{13}C-gases) were markedly different from total unlabeled CO_2.

Under fluctuating conditions, the LEF soils transformed substantially more ^{13}C plant material into $^{13}CO_2$ than under the oxic or anoxic conditions (Table 4). This suggests that the alternating oxic and anoxic condition enhances mineralization, perhaps though synergy of oxic and anoxic decomposition processes. That is, that under fluctuating conditions, the full suite of aerobic and anaerobic (fermentative and respiratory) metabolic pathways can potentially be used. The sum of the µmol ^{13}C-CO_2 produced in the fluctuating condition was approximately twice that produced in either the oxic or anoxic conditions. A period of oxic conditions presents an opportunity for microorganisms to use oxygen-requiring enzymes (primarily oxygenases) to breakdown complex macromolecular structures resulting in the release of smaller molecular weight, potentially more labile carbon compounds. These smaller molecular weight products may then be substrates for microorganisms under both oxic and anoxic conditions. In addition, the aromatic compounds produced as a result of complex macromolecular breakdown may be degraded under anoxic conditions (Donlon et al 2000, Ong et al 2012, Sponza and Isik 2005).

Presumably, obligately anaerobic bacteria would be inhibited under static oxic conditions and the oxic period of the fluctuating condition, precluding substantive microbial production of CO_2 from anaerobic pathways. However, the presence of facultative anaerobic microorganisms may partially explain why the percentage of ^{13}C plant material converted to ^{13}C-CO_2 gas was greater under the fluctuating condition than in the oxic condition. Pett-Ridge and Firestone (2005) postulated seven survival strategies of bacteria in response to the fluctuating conditions in the LEF soils, among them facultative advantage and obligately anaerobic strategies. Facultative advantage refers to organisms that were identified in static aerobic, static anaerobic and in fluctuating conditions. In that work, substantially more facultative advantage ribotypes were identified than obligately anaerobic ribotypes. This suggests that a substantial portion of the microbial community in the LEF soil is facultative, capable of activity under both aerobic and anaerobic conditions.

Approximately half of the ^{13}C gas produced in the anoxic treatment was CH_4, whereas very little ^{13}C-CH_4 was produced in the oxic condition. The sum of ^{13}C-CO_2 and $^{13}CH_4$ produced in the anoxic condition was greater than the sum of ^{13}C-CO_2 and ^{13}C-CH_4 produced in the oxic condition. The cumulative CH_4 as well as the ^{13}C-CH_4 produced in the anoxic condition was likely the result of increased in methanogenic activity due to increased methanogenic substrate availability.

Fermentation pathways generally require small, relatively non-complex compounds as substrates. *A. barbata* litter includes a relatively labile portion of carbon. That is, soluble sugars and other simple compounds are released when *A. barbata* litter comes into contact with water. The availability of soluble sugars and other simple compounds precludes the requirement of aerobic microorganisms to breakdown complex plant structure to release more labile carbon. Over the time course of this experiment, it may be the simple sugars that are the primary compound being broken down over the time course of the experiment. Under static anoxic conditions, anaerobic microorganisms such as Clostridia would not have to compete with aerobic microorganisms for the soluble sugars. In this way, fermentative bacteria would take the soluble sugars, producing one carbon compounds that methanogens could use. Thus, under anoxic conditions, fermentative bacteria and methanogens mineralized ^{13}C plant material such that the total mineralized gas was not significantly different than that produced in the fluctuating condition.

Teh et al. (2006) demonstrated that CH_4 can be produced from LEF soil under an oxic headspace. Methanogens are considered obligate anaerobes; CH_4 would not be produced unless there was a zone from which oxygen was absent within the soil structure, an anoxic microsite. Anoxic microsites could form due to the biological oxygen demand of aerobic microorganisms. In these pockets of anaerobiosis, anaerobic microorganisms could have been producing CO_2 and CH_4.

The total amount of unlabeled CH_4 and ^{13}C-CH_4 produced was strongly affected by anoxia. Under all amended redox conditions, CH_4 was produced after 18 days and substantial CH_4 was produced in the amended anoxic condition after 30 days. The unamended samples under all conditions did not produce measurable CH_4 over the course of the experiment. The amount of ^{13}C plant material converted to ^{13}C-CH_4 was markedly greater under the anoxic condition than under the oxic and fluctuating conditions. The ^{13}C-CH_4 produced under the anoxic periods of the

fluctuating condition would account for more labeled ^{13}C-CH_4 production occurring in the fluctuating incubations than in the aerobic incubations.

Carbon Degradation Potential

The redox conditions also affected the genetic carbon degradation potential of the microbial community as determined by GeoChip 4 analyses. The carbon degradation and curated organic remediation gene probes tended to cluster into an oxic and an anoxic group, even for the fluctuating samples. The presence and abundance of the curated organic remediation gene probes could reflect the capability of the microbial community to breakdown complex macromolecular structures and recalcitrant carbon. The relative intensity of the organic remediation gene probes was similar between the amended oxic and anoxic condition samples (Figure 5), indicating that the potential ability to degrade macromolecular structure does not change significantly over the 38 days. A previous study showed that potential oxidative enzyme activity remained high in the more anaerobic LEF soils even after 96 weeks (DeAngelis et al 2013). Thus, even when anoxic conditions persist in these soils for long periods of time, they remain poised to breakdown macromolecular structure.

Carbon degradation potential was positively correlated to bacterial and archaeal 16S and ITS abundance. This could reflect the response of all of these values to redox condition or it could result from changing gene profiles with growth of communities.

Bacterial Community Composition

Litter addition appears to be the main driver of microbial community composition. The unamended samples of all conditions, including the initial soil samples, form a tight cluster removed from any of the amended condition sample clusters. The unamended samples are dominated by Proteobacteria (mostly Alphaproteobacteria), Actinobacteria and Acidobacteria. These phyla are commonly found to be the dominant phyla in soil (Janssen 2006, Nemergut et al 2010, Miyashita et al 2013, Summers et al 2013, An et al 2013). The phylogenetic alpha diversity of the unamended samples is greater than the amended samples, suggesting that the presence of litter enables some phyla to become dominant, reducing the evenness of the community sampled. The richness of the unamended samples may appear greater because the detected phyla are more even. The presence of litter decreased the abundance of sequence observations of the phyla dominant in the unamended soil and increased the abundance of less-dominant phyla.

Redox condition did not appear to effect overall bacterial community structure, though the combination of redox condition and presence of litter appears to have had an effect. The effect of oxic and anoxic conditions did however appear to have been significant for the Firmicutes (higher in the anoxic condition) and the Bacteriodetes and Betaproteobacteria (higher in the oxic and fluctuating conditions; Figure 10).

Community structure was positively correlated to mineralization, the amount of ^{13}C-gas produced (Table 12). While it is difficult to assign a function or process to a particular Class of bacteria, there are patterns in this data that are suggestive. For example, under amended anoxic conditions, sequence observations of Firmicutes (Clostridia) increased substantially compared to unamended anoxic condition and amended oxic and fluctuating condition samples. In previous

research, the addition of labile carbon resulted in the increase of Firmicutes (Cleveland et al 2007). In my work, sequence observations of Clostridia in amended samples of all conditions were positively correlated with the amount of ^{13}C-CH_4 produced. As a Class, Clostridia are capable of fermentation which can produce single carbon end products that provide substrates for methanogens. This may in part account for the substantial amount of ^{13}C-CH_4 produced.

The carbon degradation potential of the microbial community in the LEF appears to be robust. Whereas the bacterial community structure is affected by the presence of litter, the carbon degradation potential is only slightly affected. The relative intensity of the carbon degradation gene probes are similar in amended and unamended samples, regardless of condition, and the majority of gene probes are shared between the amended and unamended samples in a particular condition. Even the diversity of the amended and unamended samples was similar under all conditions; only the unamended anoxic samples were statistically different than the amended anoxic samples. The changes in community structure (notably the increase in the sequence observations of Firmicutes) in the amended anoxic condition did not apparently affect the relative abundance or number of shared carbon degradation gene probes. The carbon degradation gene probes are an index for the potential carbon degradation capacity. However they can only assess the capacity that is included on the GeoChip 4, and cannot measure the total functional carbon degradation capacity of the soils. This may be an important reason why carbon degradation potential was not correlated to the amount of ^{13}C-gas produced, or to the community composition.

Conclusion

My study investigated the impact of fluctuating redox conditions on mineralization of soil C and added plant material, carbon degradation potential and whether carbon degradation potential could be linked to microbial community composition. Overall, redox conditions affected the mineralization of *A. barbata* litter and carbon degradation potential, but not bacterial community composition. However, bacterial community composition was positively correlated to C mineralization to CO_2 and CH_4. Carbon degradation potential in this soil is uncoupled from bacterial community composition. The fluctuating redox conditions in this soil appear to be a more important driver of plant material mineralization than bacterial community structure.

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Table 2-1. Site characteristics from where the soil used in this experiment were collected.

Life Zone	Elev. (masl)	Dominant vegetation[2]	Soil Moisture[1]	MAP[2]	MAT[2]	pH[2]	%C[3]	%N[3]	C:N[2]
Montane Wet Forest	780	*Cyrilla racemiflora*	59.5	4200	19.5	5.1 (0.2)	7.4 (2.0)	0.31 (0.06)	18.6 (0.76)

[1]This study.
[2]Dubinsky, E.A. 2008. UCB Thesis.
[3]Dubinsky E.A., Silver, W.L., and M.K. Firestone. 2010. Ecology. 91(9): 2604-2612.

Table 2-2. Effects of condition (redox) and amendment on the variation observed in cumulative CO_2 measurements. Bold values indicate measurements determined to be significant at $p < 0.05$ using the Students T test.

A	Amendment		Time		Amendment*Time	
Condition	r^2	p	r^2	p	r^2	p
Oxic	0.446	**0.001**	0.231	**0.001**	0.182	**0.001**
Anoxic	0.405	**0.001**	0.194	**0.001**	0.183	**0.001**
Fluctuating	0.498	**0.001**	0.201	**0.001**	0.191	**0.001**

B	Condition		Time		Condition*Time	
Amendment	r^2	p	r^2	p	r^2	p
Amended	0.262	**0.001**	0.510	**0.001**	0.080	**0.001**
Unamended	0.131	**0.001**	0.497	**0.001**	0.073	**0.001**

Table 2-3. Effects of condition (redox) and amendment on variation observed from cumulative CH_4 measurements. Bold values indicate measurements significant at $p < 0.05$ using the students T test.

A	Amendment		Time		Amendment*Time	
Condition	r^2	p	r^2	p	r^2	p
Oxic	-0.562	0.947	0.074	0.405	0.080	0.410
Anoxic	0.157	**0.001**	0.086	**0.001**	0.171	**0.001**
Fluctuating	0.089	**0.014**	0.116	**0.005**	0.124	**0.003**

B	Condition		Time		Condition*Time	
Amendment	r^2	p	r^2	p	r^2	p
Amended	0.017	0.490	0.262	**0.001**	0.017	0.488
Unamended	0.990	0.075	0.005	0.262	-0.004	0.681

Table 2-4. ^{13}C-Gas fluxes from amended treatments. Each value is an average of three biological replicates. Significant differences were determined using TukeyHSD at $p < 0.01$.

Condition	% ^{13}C-substrate → CO_2	% ^{13}C-substrate → CH_4	% ^{13}C in gas	^{13}C-CO_2:^{13}C-CH_4
Oxic	3.14 a	0.42 a	3.59 a	8.20 a
Anoxic	3.34 a	3.30 b	6.64 b	1.07 b
Flux	5.16 b	0.92 a	6.11 b	6.14 ab

Table 2-5. Effects of condition and amendment on the variance observed in the carbon degradation and curated organic remediation probes. Bold values indicate measurements significant at $p < 0.05$ using the students T test.

	r^2	p
Condition	0.233	**0.001**
Amendment	0.077	0.059
Condition*Amendment	0.193	**0.004**

Table 2-6. Alpha diversity indices of the carbon degradation and curated organic remediation GeoChip 4 probes of the amended oxic and amended anoxic samples and amended and unamended samples from each condition. P values are from pairwise comparisons (Students T test) of the averages (n = 3) of either the Shannon or the Simpson alpha diversity index. Alpha diversity was determined separately for each biological replicate. Bold values indicate a difference at $p < 0.05$.

A	Shannon Index (H)			Simpson Index (1/D)		
Condition	Amended	Unamended	p value	Amended	Unamended	p value
Oxic	8.07	8.17	0.278	2218.6	2538.2	0.244
Anoxic	8.00	8.09	**0.017**	2049.2	2319.7	0.062
Fluctuating	8.09	8.05	0.363	2279.3	2245.4	0.775

B	Oxic	Anoxic	p value	Oxic	Anoxic	p value
Amended	8.07	8.00	0.065	2218.6	2049.2	0.092

Table 2-7. Effects of condition (redox), amendment and time on the variation observed in Bacterial and Archaeal 16S and ITS abundance measurements (qPCR).

	Bacterial 16S		Archaeal 16S		ITS	
	r^2	p	r^2	p	r^2	p
Condition	0.039	**0.005**	0.080	**0.001**	0.031	**0.021**
Amendment	0.013	**0.023**	0.016	**0.014**	0.383	**0.001**
Time	0.014	0.086	0.030	**0.001**	0.002	0.549
Condition* Amendment	0.098	0.309	0.054	**0.001**	0.058	**0.001**
Condition* Time	0.006	0.494	0.015	0.077	0.013	0.200
Amendment* Time	0.011	0.099	0.020	**0.005**	0.003	0.336

Table 2-8. Effects of condition (redox) and amendment on the observed variation from 454 Pyrosequencing data.

	r^2	p
Condition	0.190	0.084
Amendment	0.282	**0.001**
Condition*Amendment	0.189	**0.001**

Table 2-9. Classes that contain sequences that were observed to be more abundant ($p < 0.05$) in the phyla whose observed sequences were greater (or fewer) in amended oxic versus amended anoxic samples at $p < 0.05$. No listing indicates that the class were not over 1 % of the sequence observations in the phyla or that the sequence observations were not significantly different between the unamended or amended samples at $p < 0.05$.

Phyla	Amended Oxic	Amended Anoxic
Acidobacteria	Solibacteres	
Actinobacteria		Actinobacteria
Bacteriodetes	Sapropiraea	Bacteroidia
Firmicutes		Clostridia
Proteobacteria	Beta	
Verrucomicrobia	Pedosphaerae	

Table 2-10. Classes that contain sequences that were observed to be more abundant ($p < 0.05$) in the phyla whose observed sequences were greater (or fewer) in amended (A) versus unamended (U) samples at $p < 0.05$. No listing indicates that the sequence observations in the class was not over 1 % of the sequence observations in the phyla or that the sequence observations were not significantly different between the unamended or amended samples at $p < 0.05$.

Phylum	Oxic	Anoxic	Fluctuating
Acidobacteria	Acidobacteriia (U) DA052 (U) Solibacteres (U)	DA052 (U) Solibacteres (U)	DA052 (U)
Actinobacteria	Acidimicrobiia (U)		Acidimicrobiia (U) Thermoleophilia (U)
Bacteriodetes	Saprospirae (A)		
Chloroflexi	Ktedonobacteria (U)		Ktedonobacteria (U)
Firmicutes		Clostridia (A)	Clostridia (A)
Planctomycetes	Planctomycetia (U)	Plantomycetia (U)	Planctomycetia (U)
Proteobacteria	Alpha (U) Beta (A)	Alpha (U) Delta (U)	Alpha (U) Beta (A) Delta (A)
Verrucomicrobia	Pedosphaerae (U)	Pedosphaerae (U)	Pedosphaerae (U)

(U) Unamended
(A) Amended

Table 2-11. Alpha diversity table for 454 Pyrosequencing data. The alpha diversity was determined individually for each biological replicate at 1130 sequences per sample.

A

	Shannon (H)			Simpson (1/D)			Chao1		
Condition	Amended	Unamended	p value	Amended	Unamended	p value	Amended	Unamended	p value
Oxic	4.81	5.16	**0.016**	40.45	81.30	**0.024**	762.04	895.28	0.054
Anoxic	5.00	5.19	**0.004**	78.52	88.40	**0.009**	737.52	828.80	0.254
Fluctuating	4.73	5.21	**0.001**	41.36	84.44	**<0.001**	675.30	919.07	**0.007**

B

	Oxic	Anoxic	p value	Oxic	Anoxic	p value	Oxic	Anoxic	p value
Amended	4.81	5.00	0.052	40.45	78.52	**0.013**	762.04	737.52	0.292

Table 2-12. Mantel tests correlating gas data, community data and carbon degradation potential data. The first axis PCoA scores from the community abundance data (qPCR), community structure (454 Pyrosequencing) and carbon degradation potential (GeoChip 4 gene probes) were used to correlate to the gas data. Bold value indicate significance at $p < 0.05$.

	^{13}C-gas		total CO_2		total CH_4		Community abundances		Community composition	
	r	p	r	p	r	p	r	p	r	p
^{13}C-gas										
total CO_2	0.158	**0.035**								
total CH_4	0.687	**0.001**	0.120	**0.047**						
Community abundances	0.319	0.062	0.000	0.424	0.346	0.052				
Community composition	0.162	**0.042**	0.950	**0.001**	0.181	**0.032**	0.023	0.305		
Carbon degradation potential	0.316	0.051	0.000	0.437	0.346	0.050	1	**0.001**	0.023	0.334

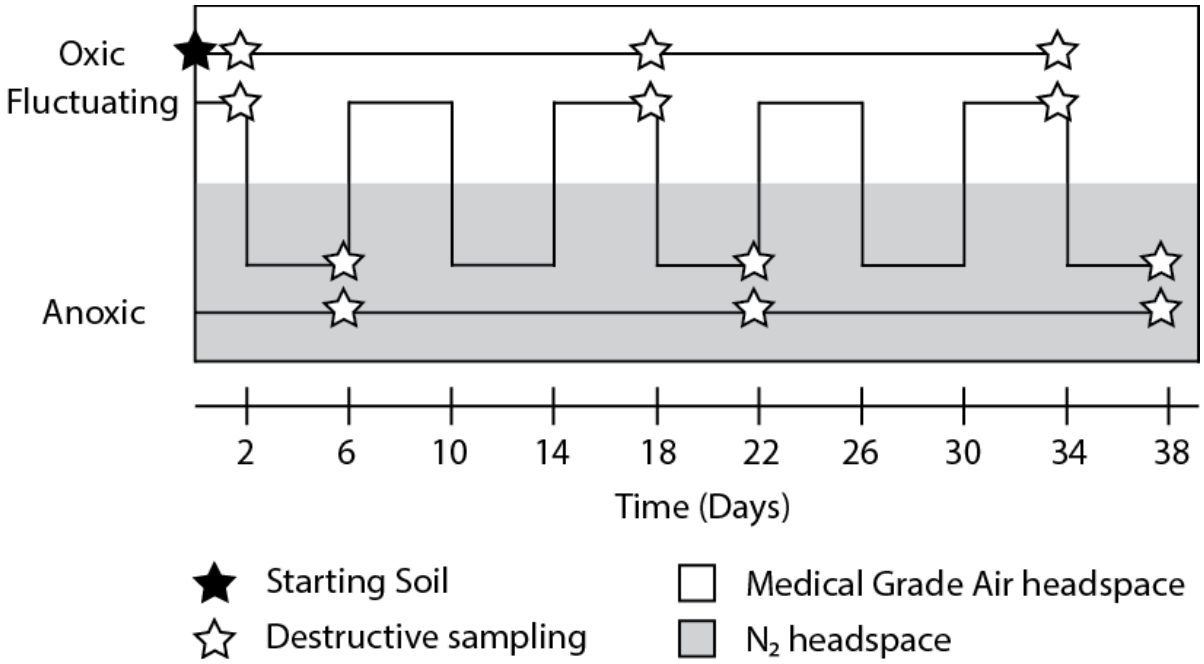

Figure 2-1. Schematic of the experimental design.

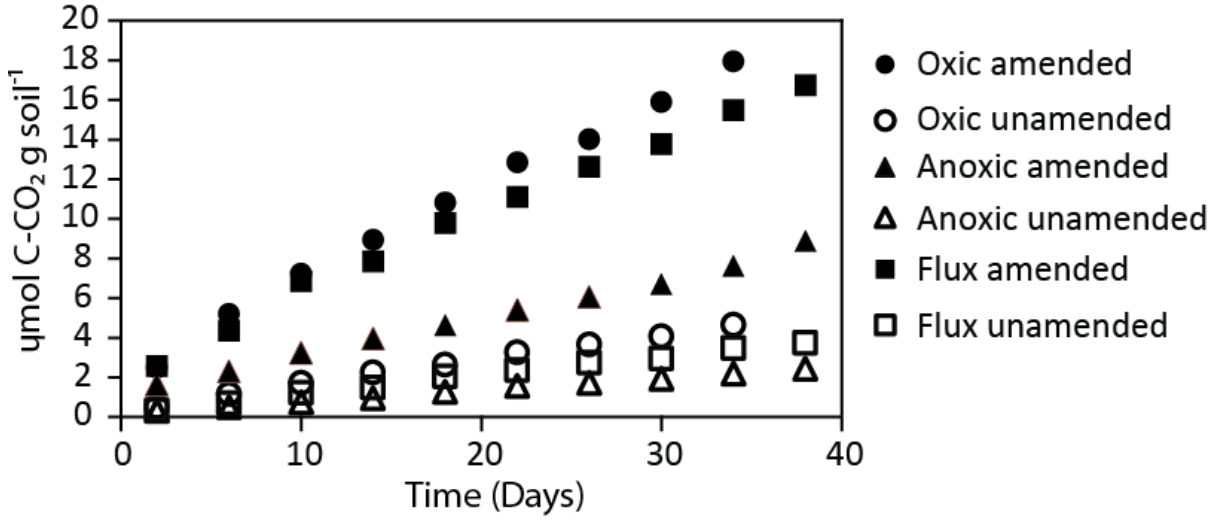

Figure 2-2. CO_2 produced over time from each treatment. Data are from measurements from samples destructively sampled on Day 34 and Day 38. Each point is the average of three replicates.

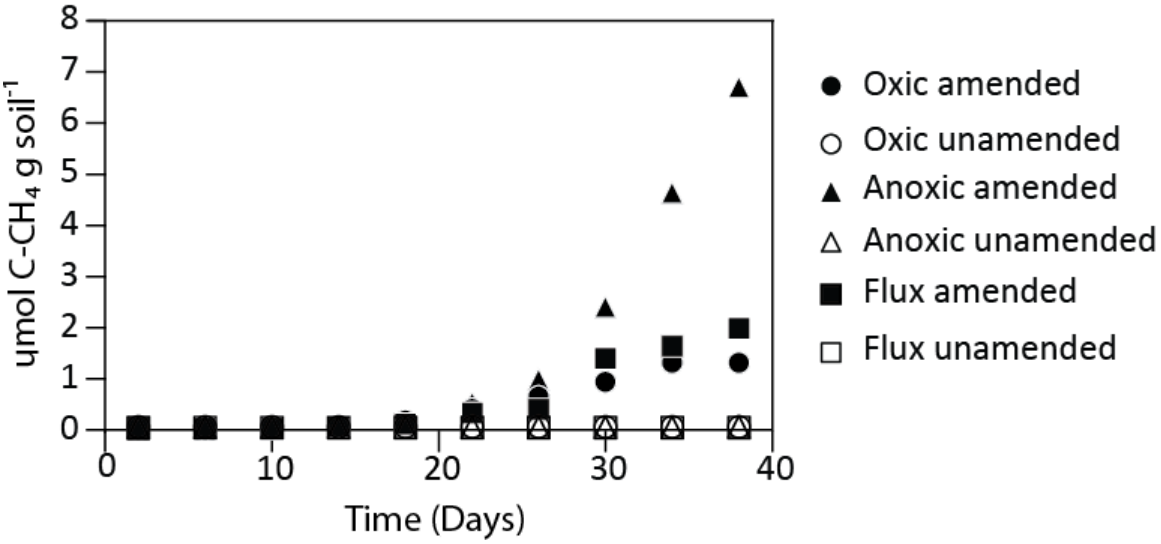

Figure 2-3. CH_4 produced over time from each treatment. Data are from measurements from samples destructively sampled on Day 34 and Day 38. DFM was added to all treatments to inhibit CH_4 oxidation. Each point is the average of three replicates.

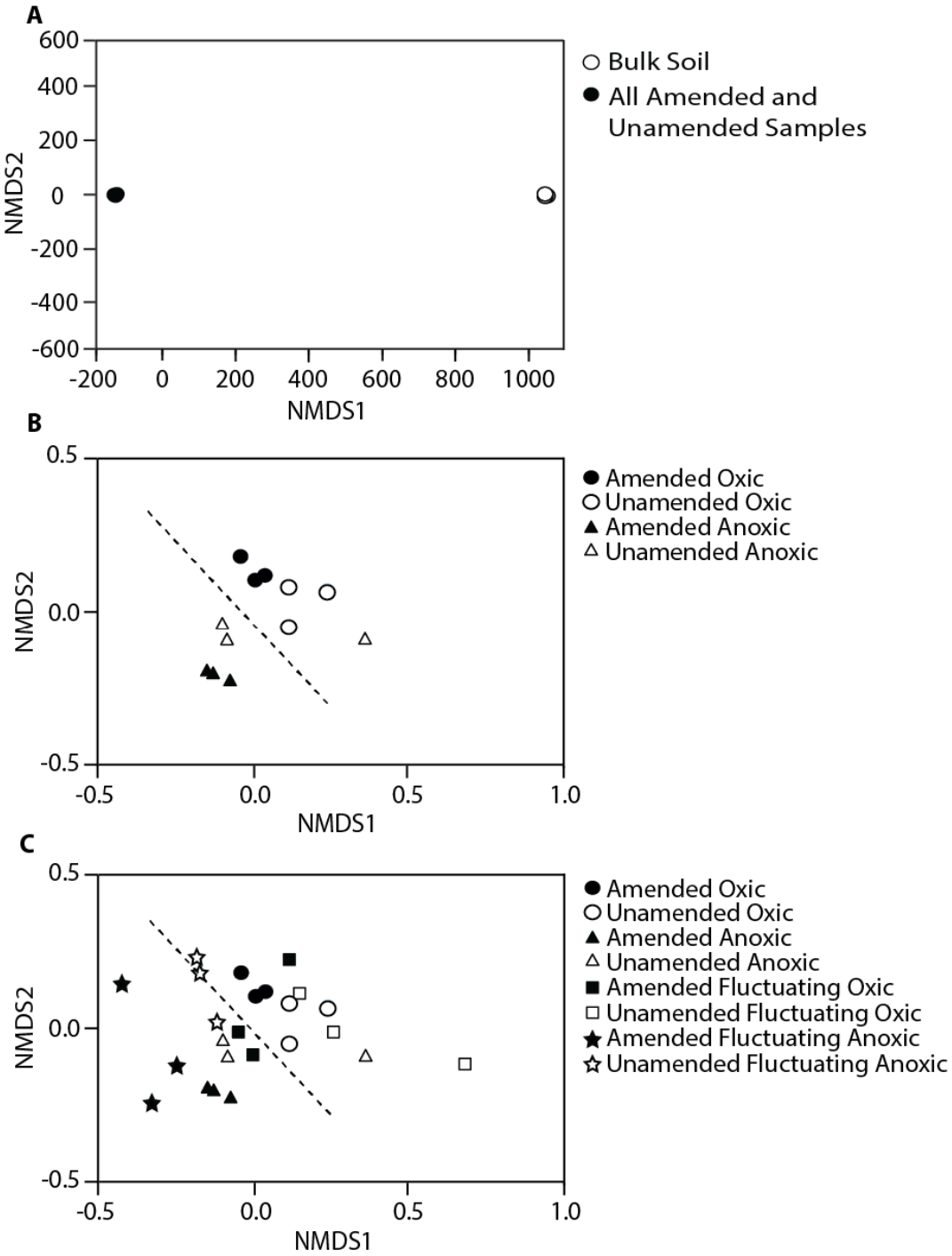

Figure 2-4. NMDS of Carbon Degradation and curated Organic Remediation gene probes of all final time point samples, including bulk soil samples taken immediately prior to the start of the experiment (A), only the amended and unamended samples of the oxic and anoxic conditions (B) and only the amended and unamended samples of all conditions without bulk soil samples (C). Dotted line indicates a plane of separation between oxic and anoxic samples from the static and fluctuating conditions.

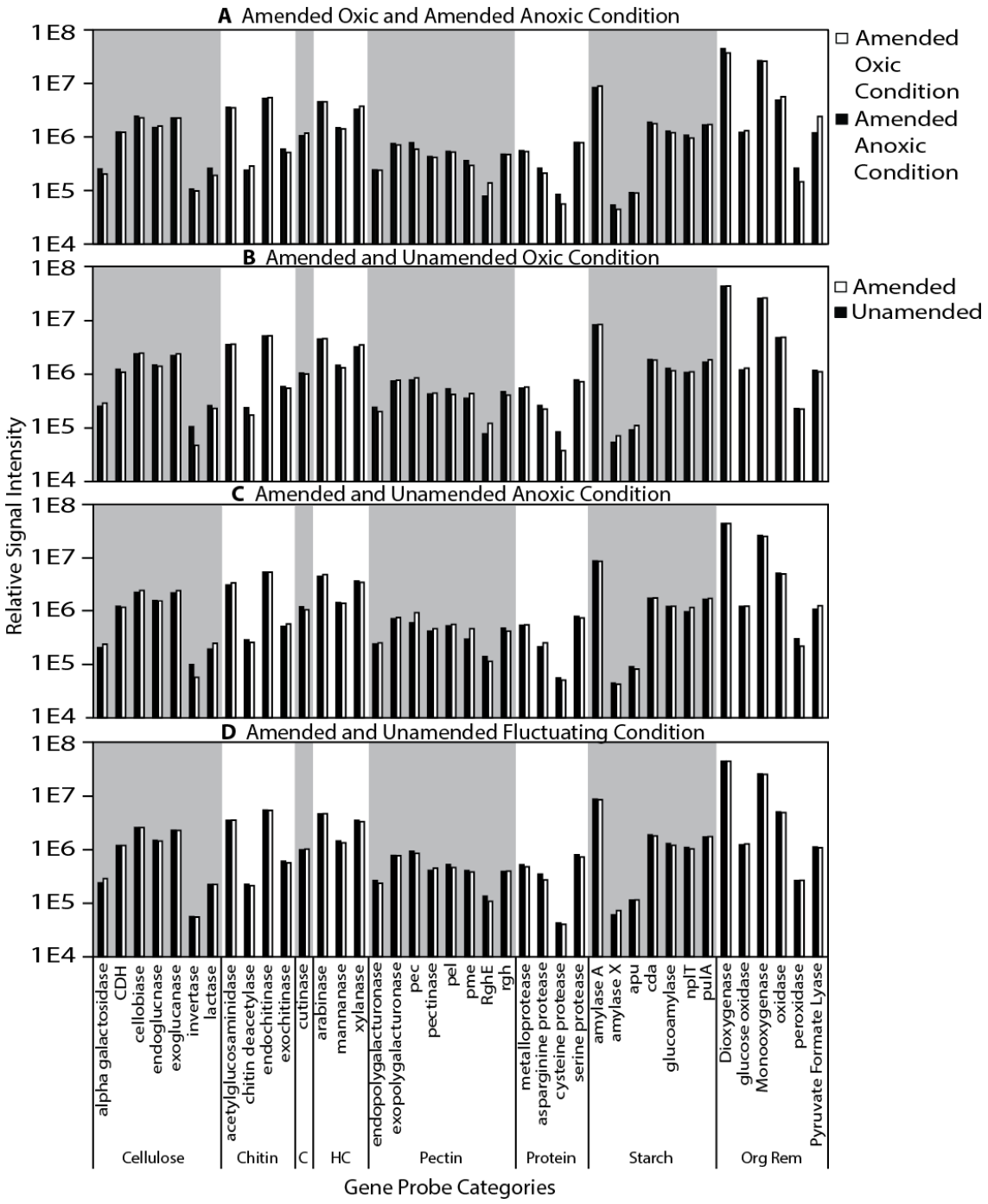

Figure 2-5. Pairwise comparison of the relative intensity of Carbon Degradation and curated Organic Remediation gene probes of the amended oxic and amended anoxic samples (A), the amended and unamended oxic (B), anoxic (C) and fluctuating (D) samples. There are no significant differences in relative abundance between probes ($p < 0.05$). Significance was determined by the Students T test and corrected for false positives using Benjamini-Hochberg correction. Gene probe categories: C, Chitin; HC, Hemicellulose; Org Rem, Organic Remediation.

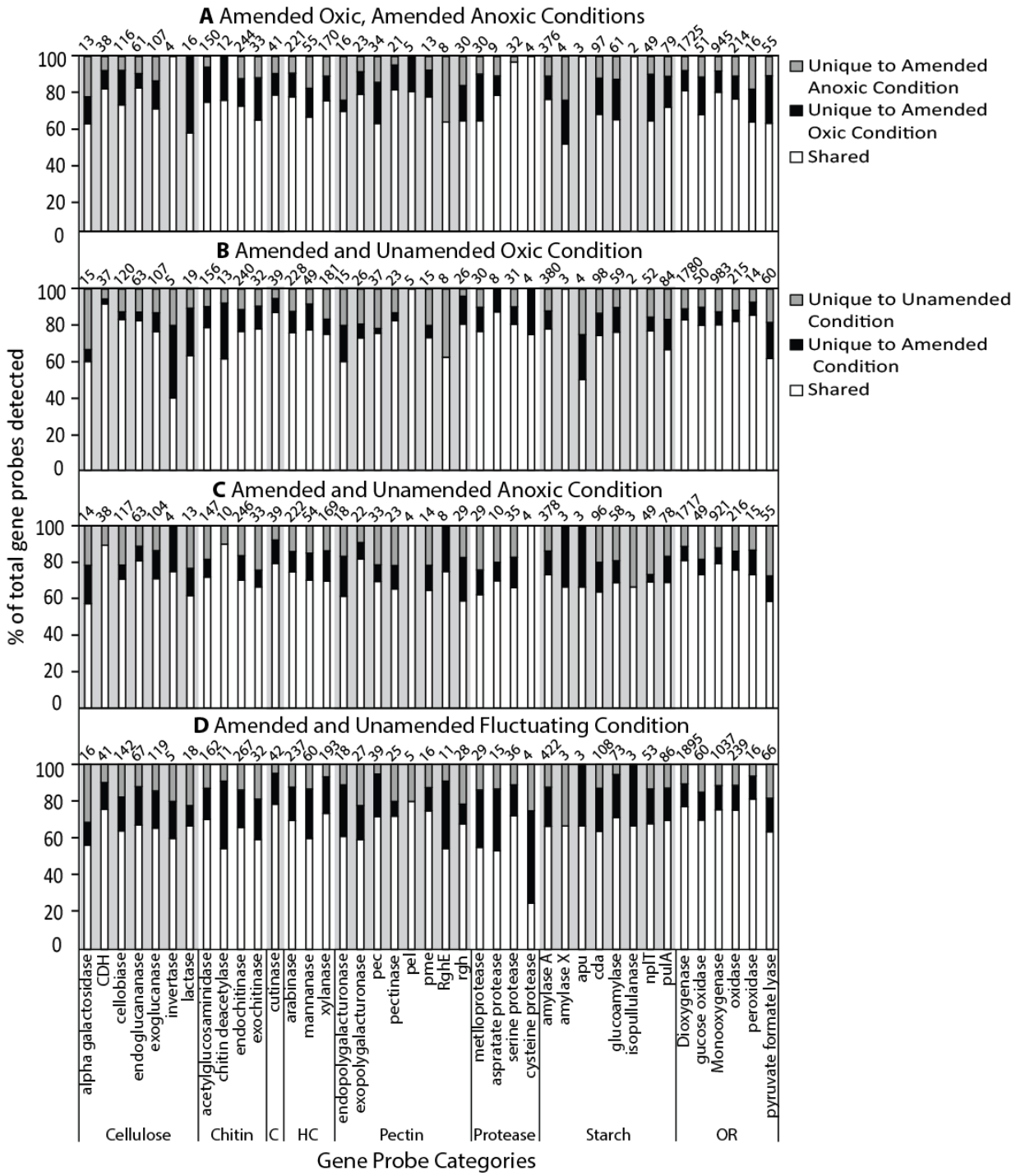

Figure 2-6. Pairwise comparison of treatment specific and shared carbon degradation and curated organic remediation gene probes of the amended oxic and amended anoxic samples (A), amended and unamended oxic (B), anoxic (C), and fluctuating (D) samples. Numbers above each bar represent the number of detected gene probes in the comparison. Gene probe categories: C, Chitin; HC, Hemicellulose; OR, Organic Remediation.

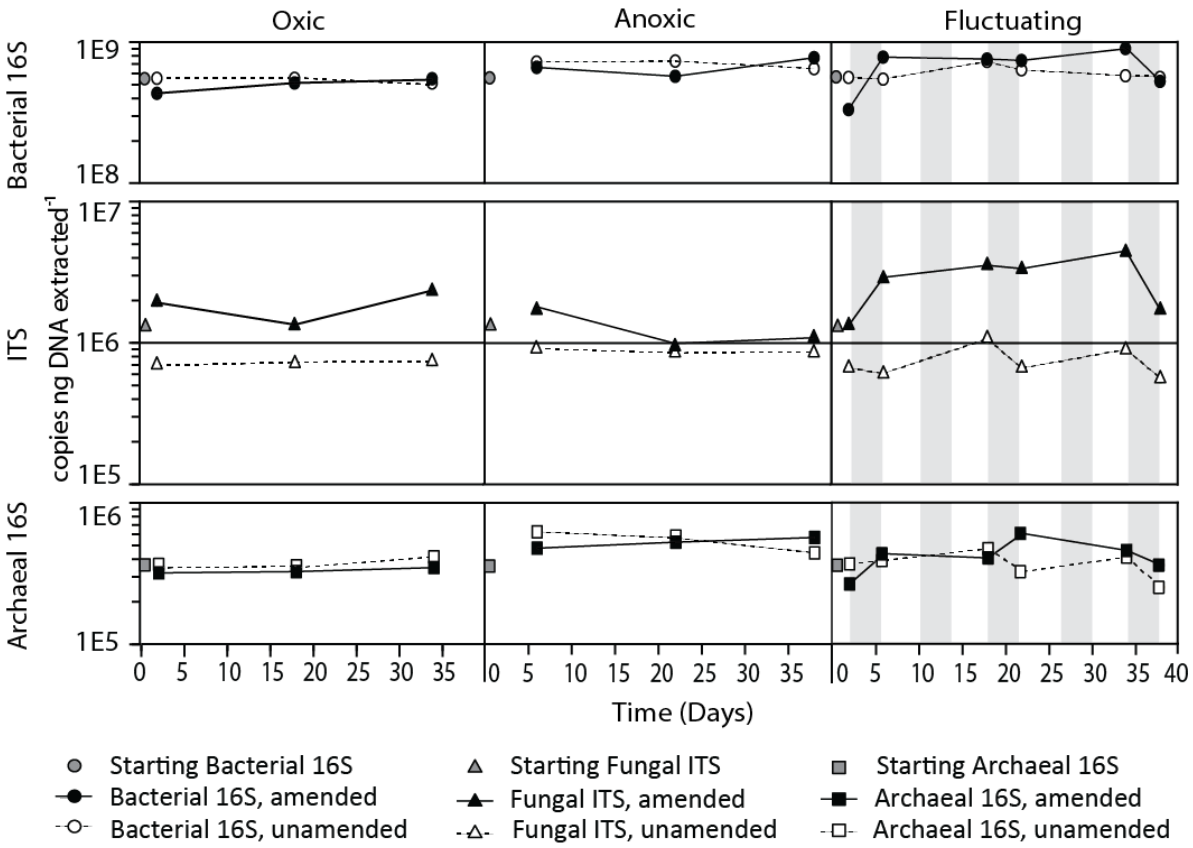

Figure 2-7. Bacterial 16S, Archaeal 16S and Fungal ITS abundances as measured by qPCR in the oxic, anoxic and fluctuating conditions. Grey bars indicate the anoxic periods of the fluctuating condition. Each point is the average of three technical replicates for each of three biological replicates (n = 9).

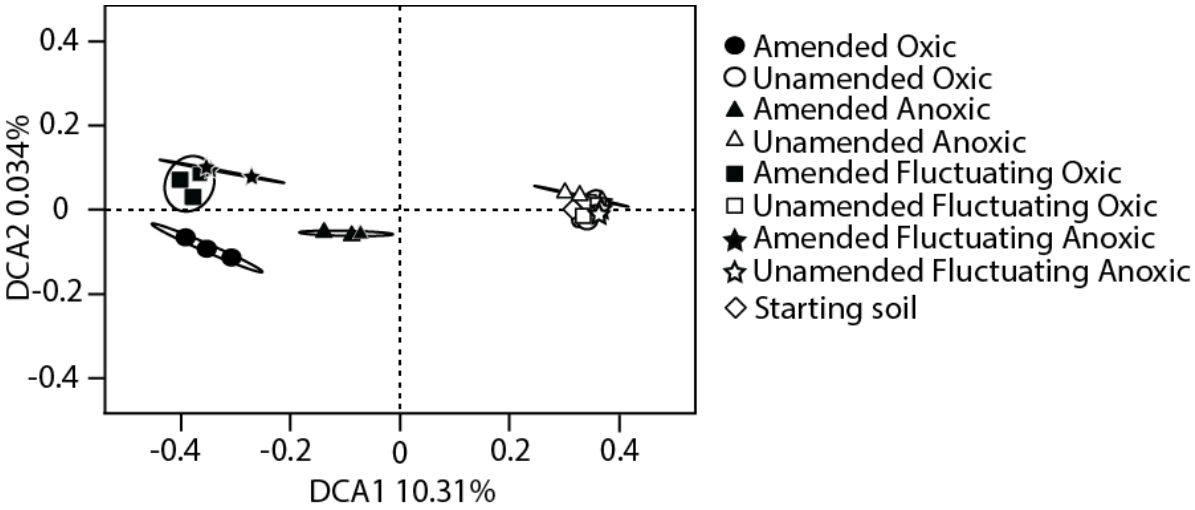

Figure 2-8. Detrended correspondence analysis of weighted unifrac distances of OTUs from the final timepoint samples and the starting soil rarefied to 1130 sequences per sample. Ellipses are 95% confidence intervals.

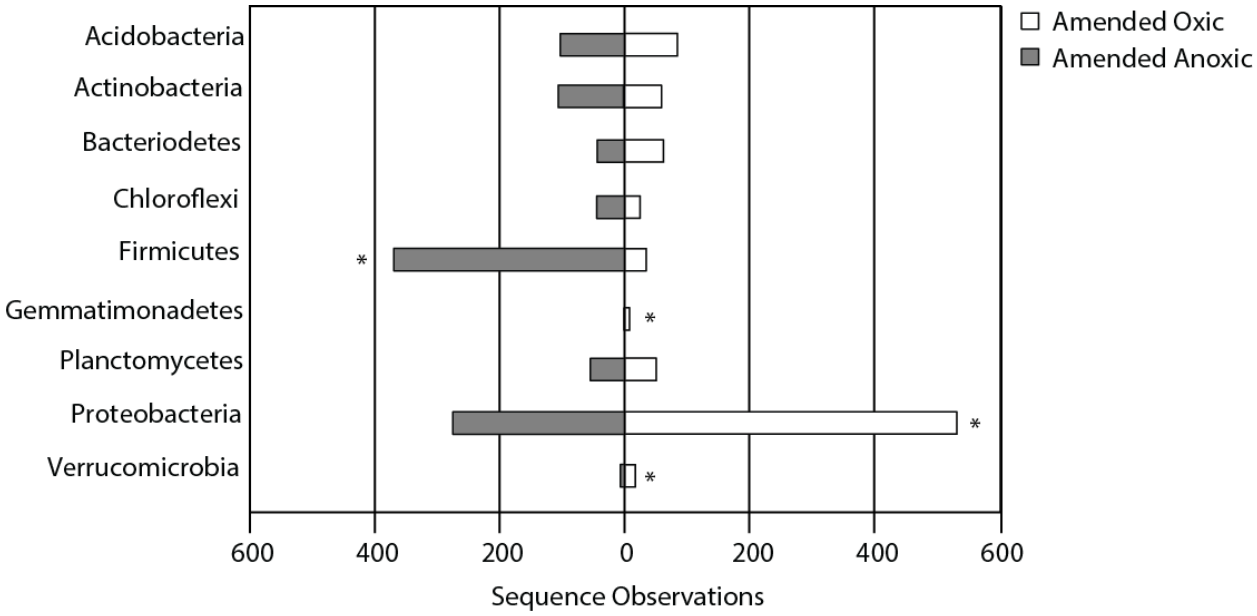

Figure 2-9. Sequence abundance of detected phyla in samples rarefied to 1130 sequences per sample. Asterisks indicate the observation of sequences belonging to a particular phyla are significantly greater at $p < 0.05$ using the Students T test.

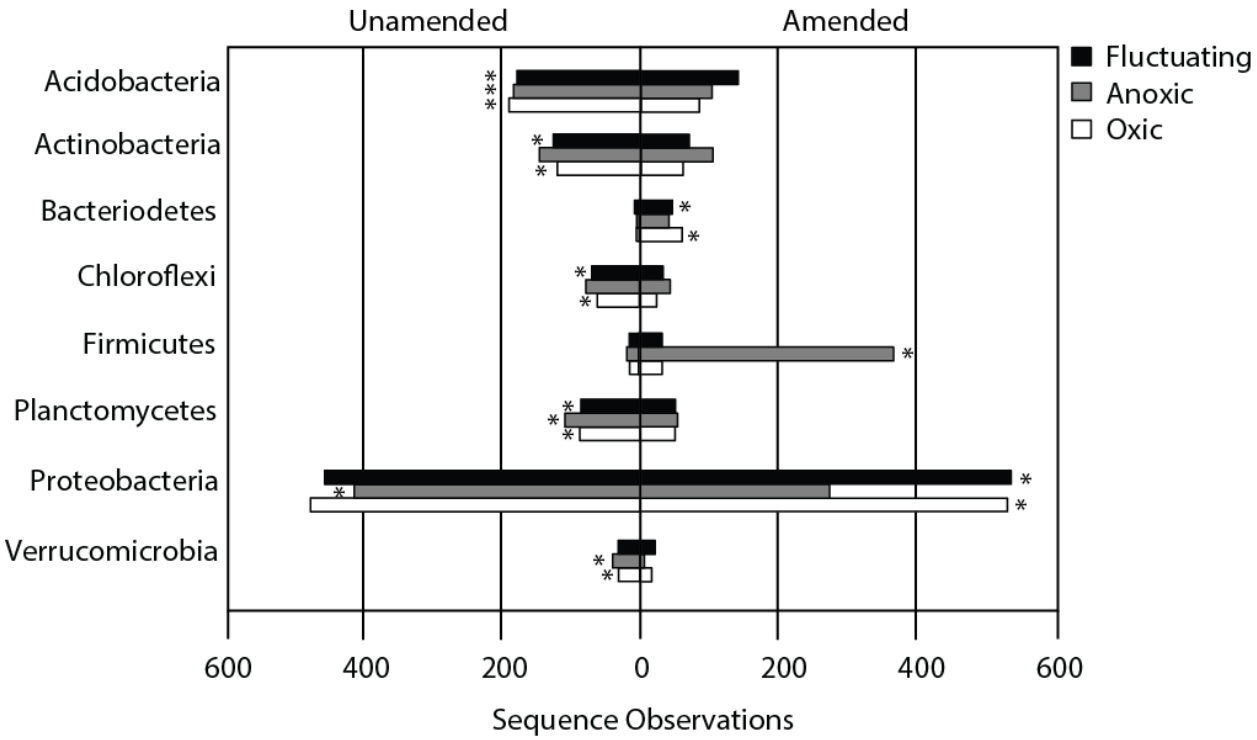

Figure 2-10. Sequence abundance of detected phyla in samples rarefied to 1130 sequences per sample. Asterisks indicate the observation of sequences belonging to a particular phyla are significantly greater at $p < 0.05$ using the Students T test.

Chapter 3

Cellulose decomposition in a wet tropical forest soil

Abstract

Cellulose is one of the most abundant components of plant material on earth. Decomposition of cellulose is a critical part of the carbon cycle in forest soils. In the Luquillo Experimental Forest in Puerto Rico (LEF), wet tropical soils from lower elevation sites have been reported to have extremely high rates of litter decomposition. Thus, cellulose should also be degraded rapidly in LEF soils. These rapid decomposition rates are thought to be enabled by the almost constant warm, moist conditions experienced by these soils. These soils in the LEF are also characterized by fluctuating soil oxygen concentrations to which the indigenous decomposing microbial communities are likely adapted. This study was designed to determine the effect of fluctuating redox conditions on cellulose decomposition and to determine if a link between cellulose decomposition and microbial community composition and function (cellulose degradation potential) could be made. Soil sampled from the palo colorado forest type in the LEF was amended with ^{13}C-labeled cellulose (*Zea mays*) and subjected to three redox conditions: oxic, anoxic, and fluctuating oxic-anoxic, in a microcosm-based experiment.

Cellulose decomposition to CO_2 was affected by the soil redox condition. Under anoxic conditions, cellulose decomposition to CO_2 was significantly greater than under oxic or fluctuating redox conditions. Cellulose decomposition to CH_4 was not observed.

Soil redox condition affected the cellulose degradation potential (as measured by the GeoChip 4); cellulose degradation potential of samples under oxic conditions clustered separately from cellulose degradation potential of samples under anoxic conditions. However, cellulose degradation potential was not correlated to either cellulose decomposition or bacterial community composition.

The overall bacterial community composition (by 454 Pyrosequencing) was not significantly affected by the soil redox condition or the presence of cellulose. The bacterial community composition was significantly correlated with cellulose decomposition. However, there were few notable differences between sequence observations in the phyla detected under any condition. In particular, the abundance of Proteobacteria was greater in the amended oxic condition, compared to the amended anoxic condition.

Over the timescale tested, cellulose degradation was greater under anoxic conditions, indicating that anaerobic microorganisms play a role in the initial stage of cellulose degradation in the LEF soil. The overall composition and abundance of the bacterial community correlated with rates of cellulose decomposition. These data suggest both the fluctuating redox conditions and bacterial community composition play important roles in cellulose decomposition in the LEF soils.

Introduction

Cellulose, a principal constituent of plant cell wall material, is one of the most abundant carbon biopolymers on earth (Swift et al 1979). Plants produce approximately 10^{11} tons of cellulose each year (Malhi 2002). Decomposition of plant cell wall material, such as cellulose, is an important part of the carbon cycle. Plant cellulose is difficult to degrade due its insolubility and its crystalline structure that restricts access to the polymer.

Cellulose degradation by fungi and bacteria involves the synergistic action of three types of cellulose degrading enzymes 1) endoglucanases, 2) exoglucanases and 3) beta-glucosidases (Atlas and Bartha 1998). Endoglucanases attack beta-1,4 glycosidic linkages in the interior of the cellulose polymer, whereas exoglucanases degrade the cellulose polymer from the ends (Beguin 1990, Wilson 2008). The action of both types of cellulases produce soluble sugars. Beta-glucosidases produce glucose from the soluble sugars released from the cellulose polymer by the endo- and exoglucanases. Synergized cellulose degradation occurs when, by the action of internal cleavage of the cellulose polymer, endoglucanases provide polymer ends that exoglucanase can then degrade.

There are ten families of glycosyl hydrolases (GHs), enzymes that cleave glycosyl bonds in cellulose, listed in the Carbohydrate-Active enZyme (CAZy) database (http://www.cazy.org/Glycoside-Hydrolases.html). GHs can vary in their substrate specificity, activity and mode of action. Since GH families are sequence-based (Henrissat and Davies 1997), GH families contain GH sequences from both bacteria and fungi and from aerobic and anaerobic microorganisms (Beguin 1990). The ten GH families are not represented equally in bacteria or fungi, or even in aerobic and anaerobic microbes (Berlemont and Murphy 2013, Murphy et al 2011).

Cellulose degradation occurs by two main routes, degradation by extracellular enzymes or degradation by cellulosomes, which are cell-associated enzyme complexes. Because cellulose cannot pass through microbial cell membranes, extracellular enzymes are used to degrade cellulose. Both bacteria and fungi degrade cellulose by secreting a variety of endo-, and exoglucanases into their environment (Atlas and Bartha 1998). The soluble sugars produced from the action of these cellulases diffuse or can be transported into the cell to be degraded into glucose by internal beta-glucosidases.

Cellulosomes are cell associated multi-enzyme complexes produced by anaerobic bacteria and fungi to degrade cellulose (Berg and McClaugherty 2008, Wilson 2008, Ljungdahl 2008). Overall, cellulosomes produced by anaerobic bacteria and fungi have a similar basic structure, however, the enzymatic components are heterogeneous and vary between organisms (Bayer et al 2004, Wilson 2008, Flint and Bayer 2008). Cellulosomes are attached to the cell surface by way of an anchoring protein. They are comprised of several catalytic (enzymatic) and non-catalytic (structural) proteins attached together by specialized domains. A scaffold protein, “scaffoldin”, is the non-catalytic backbone of a cellulosome. Catalytic components, such as endo- and exoglucanases and xylanases, attach to the dockerin domain of the scaffoldin protein by way of a cohesion domain. Cellulosomal enzymes work synergistically to degrade cellulose under anaerobic conditions.

The LEF soil has two features that could be important to cellulose degradation, 1) it is characterized by fluctuating redox (Liptzin et al 2011, Silver et al 1999) and 2) there are more active bacterial taxa under fluctuating conditions than static oxic or low redox conditions (DeAngelis et al 2010). Soils in the LEF fluctuate from oxic to low redox conditions in a period of days to weeks (Silver et al 1999). This chapter explores the potentially synergistic relationship between aerobic and anaerobic cellulose degradation in this soil. This upland tropical forest soil presents a unique opportunity to study the influence of redox conditions, and bacterial community composition on a critical step in the carbon cycle, cellulose degradation.

Redox potential is a key factor in soil processes; it controls microbial respiration activity and thereby affects nutrient cycles (Luedemann et al 2000). Redox potential is the measurement of available electrons in a system (DeLaune and Reddy 2013). Soil redox potential is considered indicative of whether oxygen or other less energetically favorable terminal electron acceptors are used by microorganisms (DeLaune and Reddy 2005). When soil redox potential decreases, microbial respiration shifts from using oxygen, a highly energetically favorable terminal electron acceptor, to less energetically favorable terminal electron acceptors, such as nitrate and ferric iron.

My goal was to determine the influence of redox condition on cellulose degradation and the relationship between microbial community composition and cellulose degradation. To achieve this goal, I amended upland tropical forest soil microcosms with a small amount of ^{13}C-labeled cellulose and incubated them under three redox regimes 1) static oxic, 2) static anoxic and 3) fluctuating oxic-anoxic. By adding a trace amount of cellulose, cellulose degradation was investigated without significantly altering the microbial community. Microbial community composition, abundance and cellulose degradation potential were correlated to cumulative ^{13}C-gases to determine their influence on cellulose decomposition.

Materials and Methods

Study Sites

Soil was collected from the palo colorado forest type, an upland tropical forest, from the Luquillo Experimental Forest (LEF), a Long-Term Ecological Research site in Puerto Rico, USA (18°18' N, 65°50' W). The annual amount of precipitation (4200 mm) and the temperature (95 ˚C) define the palo colorado forest type as a lower montane wet forest by the Holdridge system (Table1) (Brown et al 1983, Holdridge 1947). In general, the temperature and the precipitation events remain relatively constant throughout the year (Silver et al 1999, Weaver 1994). The palo colorado forest type is named after the dominant vegetation, the *Cyrilla racemiflora* tree, and is generally located in the mid-elevations of the LEF (Willig et al 2011). Soils are highly weathered, derived mainly from volcanoclastic sediments and as such are acidic (Beinroth 1982). Because of the frequent rain events and high biological activity, the average bulk soil oxygen concentration can vary from 21% to under 10%. Soils typically do not have large surface litter accumulation.

Soil Sampling

Soils from five 30 x 10 cm cores were collected from a randomly-located, 4 meter transect in the palo colorado forest type in the LEF and combined into a composite sample in the field (n = 5). Each core was 1 m apart. Surface plant litter was removed from the soil surface before soils were collected. The soil was transported to UC Berkeley at room temperature and any remaining plant litter as well as root material in the soil were removed by hand. The microcosm experiment began ten days after sampling, during which time the soil remained at room temperature.

Soil Moisture

Soil moisture was measured from three, ~ 5 g soil aliquots (wet weight). The weight of the soil measured and then the soil was dried at 105 ˚C until the soil reached a constant weight. The following formula was used to calculate soil moisture: Soil Moisture = (initial wet soil weight / (initial wet soil weight – final dry soil weight)) * 100.

Microcosm Incubation Conditions

^{13}C-labeled cellulose

^{13}C-labeled cellulose from *Zea mays* was obtained from IsoLife (Wageningen, The Netherlands). The ^{13}C-labeled cellulose was universally labeled at 97 atom percent.

Set-up

Upland tropical forest soil was incubated in a microcosm study under three redox conditions, oxic, low redox (anoxic) and a four day fluctuating redox condition. Here I define conditions under which low redox conditions prevail as an anoxic redox condition. Samples maintained with a headspace gas that includes atmospheric concentrations of oxygen are defined as oxic redox conditions. Hereafter, I refer to the repeated 4-day periods of anoxic and oxic conditions as fluctuating redox conditions.

The experimental design for the cellulose incubations was the same as the experimental design presented in Chapter 2, with two exceptions, the length of the experiment and the identity and quantity of substrate added. The labeled substrate, 0.1 g ^{13}C-labeled cellulose was added to 20 g wet weight of soil (approximately 11 g dry weight soil). Soil for the microcosms was taken from the composited soil cores. The length of the experiment (22 days) was shorter than the 38-day incubations used for the plant litter. The shorter timescale was chosen because previous research had demonstrated that this length of time was sufficient for cellulose degradation (Desvaux et al 2000, Jung et al 2004, Morris and Cole 1987).

Each soil microcosm contained 20 g (wet weight) soil in 250 ml mason jars fitted with Hungate stoppers as gas sampling ports. The microcosms were divided into two treatments, 1) amended with ^{13}C-labeled cellulose, and 2) unamended. For each treatment, three redox conditions were maintained, 1) oxic, 2) anoxic and 3) fluctuating oxic-anoxic. Four days was chosen as the length of the oxic and the anoxic periods of the fluctuating condition. This frequency of fluctuating conditions had been reported to maintain a bacterial community composition similar to the native community (Pett-Ridge and Firestone 2005). The microcosms in the oxic condition were flushed with medical grade air (Praxair, Inc., Danbury, CT) and initial oxygen concentration was measured with a Model 52 Dissolved Oxygen Meter (YSI Incorporated,

Yellow Springs, OH). The initial condition for the microcosms in the fluctuating oxic-anoxic condition was oxic. Hereafter, the fluctuating oxic-anoxic condition will be referred to as the fluctuating condition. The fluctuating condition microcosms were made anoxic when appropriate by flushing the headspace with N_2 and confirmed to be anoxic using the Dissolved Oxygen Meter. To inhibit CH_4 oxidation, 500 ppm Difluoromethane (DFM) was added to each replicate of each treatment.

Sampling

Sampling was done at the end of the first 2 days (fluctuating and oxic conditions), then at the end of every 4 (fluctuating condition) or every other 4 days (oxic and anoxic conditions). At each sampling time, 2 ml of headspace gas was transferred to 10 ml N_2-flushed, evacuated serum vials. Triplicate oxic microcosms and microcosms in the oxic period of the fluctuating condition were destructively sampled on days 2, 10 and 18. Triplicate anoxic microcosms and microcosms in the anoxic period of the fluctuating condition were destructively sampled on days 6, 14 and 22. At each time point, triplicate amended and unamended microcosms from the fluctuating condition and either the oxic or anoxic condition were destructively sampled. The destructive sampling consisted of transferring 120 ml of headspace gas into 100 ml N_2-flushed, evacuated serum vials, and weighing approximately 5 g of soil for DNA extraction. Soil was stored at -80 ˚C.

At each sampling time, the percentage oxygen was measure in all remaining microcosms using a Model 52 Dissolved Oxygen Meter, after which, all microcosms were flushed with the appropriate gas and 500 ppm DFM was added to inhibit CH_4 oxidation (Miller et al 1998).

Total (^{12}C and ^{13}C) Gas and ^{13}C-labeled gas sampling

CO_2 and CH_4 were measured from 2 ml gas samples and used to calculate the cumulative gas on a HP6890 Shimadzu Gas Chromatograph (Columbia, MD). A Flame Ionization Detector was used to measure CH_4 and a Thermal Coupled Detector was used to measure CO_2. Total gas was also measured from the 120 ml gas samples to confirm the dilution, if any, necessary to measure ^{13}C-gases on an IsoPrime100 mass spectrometer (Cheadle, UK). Since CH_4 measurements were at the limit of detection by FID, no analyses of ^{13}C- CH_4 were attempted. In addition, the ^{13}C signature of the soil was tested to confirm background natural abundance.

The ^{13}C-gas measurements for this experiment were analyzed exactly as the ^{13}C-gas measurements for experiments presented in Chapter 2. Briefly, the equation that best described the µmol carbon data from each replicate of each condition were integrated separately (Excel, Microsoft Office 2013, Redmond, WA and http://www.integral-calculator.com/#). Since CH_4 was below detection in all samples, ^{13}C-CH_4 measurements were not done and are assumed to be zero.

DNA extraction

Total DNA and RNA were extracted using a Phenol:Chloroform method from 3.5 g of soil saved at -80 ˚C as in Placella et al. (2012). Briefly, 350 µl 0.1 M aluminum ammonium sulfate and 350 µl each of CTAB (0.5 M phosphate buffer, pH 8, 5% hexadecyltrimethylammonium bromide, 1 M NaCl) and Phenol:Chloroform:Isoamyl alcohol (25:24:1) were added to 15 ml Lysing Matrix E tube (MP Biomedicals, Solon, OH) containing 3.5 g frozen, pulverized soil and

bead-beaten at 5.5 m/s, for 30 seconds (MP Biomedicals, Solon, OH). The tubes were incubated on ice for 5 minutes before centrifugation at 5,500 rcf for 5 minutes at 4 ˚C. The aqueous phase was transferred to a Phase lock gel tube (5 PRIME, Inc., Gaithersburg, MD), mixed and then centrifuged at 8,000 rcf for 3 minutes at 4 ˚C. The aqueous phase was then transferred to another tube. The soil was then re-extracted with an additional 350 µl of CTAB, and the resulting aqueous phase was pooled. DNA was precipitated at room temperature for at least 3 hours using 2 volumes of PEG/1.6M NaCl and 2 µl of 5 mg/ml linear acrylamide (Life Technologies Ambion, Grand Island, New York). DNA was recovered by centrifugation at 16,000 rcf for 10 minutes, washed twice with 70% ethanol and resuspended in 100 µl RNase-free 100 mM Tris buffer. The DNA and RNA were separated using an All-Prep kit (Qiagen, Valencia, CA) and stored at -80 ˚C.

GeoChip

The GeoChip version 4 was used to analyze the carbon degradation potential of the samples (Institute for Environmental Genomics, University of Oklahoma, Norman, Oklahoma). The GeoChip 4 contains more than 80,000 probes targeting coding sequences in 410 functional gene families. While GeoChip probes are predominantly derived from bacterial sequences (He et al 2012), the majority of cellulose degradation gene probes detected in this experiment were of fungal origin. The majority of the detected cellulose degradation gene probes were glycosyl hydrolase (GH) enzymes. Since GH families are sequence homology-based (Henrissat and Davies 1997), a particular GH family can contain sequences from bacteria and fungi (Beguin 1990). Thus, the GeoChip GH probes of fungal origin may not discriminate against bacterial GH DNA.

For the GeoChip analysis, 500 ng of DNA from triplicate final time point samples from amended and unamended samples from each redox condition were used. In addition, 500 ng DNA from three soil subsamples that were taken immediately prior to the start of the experiment were also used in GeoChip 4 analysis.

Extracted community DNA (no amplification step) was labeled with cyanin-5(Cy-5) dye. Briefly, approximately 500 ng of genomic DNA was denatured for 5 minutes at 99.9 ˚C in solution with random octamer mix (Invitrogen, Carlsbad, CA, USA) and snap chilled on ice. Following denaturation, 2.5 mM dithiothreitol (DTT), 0.25 mM dATP, dCTP and dGTP, 0.125 mM dTTP, 0.125 mM Cy5-dUTP, and 80 U Klenow fragment (Invitrogen, Carlsbad, CA, USA) were added. Reaction mixtures were incubated at 37 ˚C for 3 hours. Labeled target DNA was purified with a QIAquick PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and measured on a NE-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and dried using a speed-vac at 45 ˚C for 45 min.

A sample tracking control solution (2.88 µL, NimbleGen, Madison, WI, USA) was added to each dried sample. The sample tracking control solution and dried DNA mixture was incubated at 50 ˚C for 5 minutes, vortexed for 30 seconds, and then centrifuged to collect all liquid at the bottom of the tube. The collected liquid was resuspended in hybridization buffer (40% formamide, 35% SSC, 1% SDS, 2.24% alignment oligo Cy-3 (NimbleGen) and 2.8% Cy5-labeled CORS target), vortexed and denatured at 95 ˚C for 5 minutes. Resuspended samples were kept at 42 ˚C until hybridization. The mixture (7.3 µL) was loaded onto arrays

(NimbleGen 12-plex arrays) with mixer and hybridizations were performed at 42 ˚C for 17 hours using a MAUI hybridization station (MAUI, BioMicro Systems, Salt Lake City, UT, USA).

After hybridization, the arrays were washed three times with NimbleGen wash buffer, spin dried, and scanned with a NimbleGen MS200 scanner (Madison, WI, USA) at a power setting of 100%. An ImaGene instrument (version 6.1, BioDiscovery, El Segundo, CA, USA) was used to obtain raw data images. The raw data images were uploaded to the Institute for Environmental Genomics microarray data manager (http://ieg.ou.edu/microarray/) for pre-processing. A local background signal for each probe was obtained using a customized void gridding file targeting array positions without probes (Qichao Tu, personal communication). Then, NimbleScan software (version 2.6) was used to obtain the signal intensity for each probe. Each probe was normalized by the mean signal from the added CORS probes (Liang et al 2010).

All probes with a signal intensity less than 2X the average background signal were removed. The signal to noise ratio [SNR = (signal intensity – background mean)/background standard deviation] was adjusted so that thermophile probes were excluded. Since the average temperature of the LEF is approximately 19.5 (Table 1), by removing the thermophile probes a probe set more representative of the microbial community in this relatively aseasonal environment. Probes present in one of three samples were removed. Probe signals were then normalized by dividing the probe signal intensity by the average of all probe signal intensities for the treatment. From this set of gene probes, only the gene probes in the carbon degradation category involved in cellulose degradation (439) were selected for further analysis. Hereafter cellulose degradation potential will be used to refer to the composition and abundance of the detected cellulose degradation gene probes. The majority of the cellulose degradation gene probes (61%) detected in this experiment were designed from fungal genes. The gene probes related to cellulose degradation are as follows: alpha galactosidase, cellobiose dehydrogenase (CDH), cellobiase, endoglucanase, exoglucanase, invertase and lactase.

The cellulose degradation gene probes were ordinated in non-metric multidimensional space using the metaMDS function in the vegan package of R (version 3.0.2). The same package was used to calculate and visualize 95% confidence intervals of the samples by redox condition and substrate addition. The 95% confidence intervals are shown when any overlap does not occlude the visualization of the ordinated data.

The relative intensities of the cellulose degradation gene probes were compared in the following pairwise comparisons: amended oxic and amended anoxic, amended and unamended oxic, amended and unamended anoxic, amended and unamended fluctuating condition samples. Shared and treatment-specific gene probes were also determined. The relative intensity data of each gene probe was first converted to presence absence data. A gene probe was considered specific to a treatment if 1) it was present in 2 of 3 (or 4 of 6 in the case of the fluctuating condition) samples of a particular treatment and 2) if it was not present in the other treatment. The data is presented as the percentage of shared and treatment-specific gene probes from the following pairwise comparisons: amended oxic and amended anoxic, amended and unamended oxic, amended and unamended anoxic, amended and unamended fluctuating condition samples.

I determined the diversity (Shannon and Simpson Indices) of cellulose degradation gene probes using the Institute for Environmental Genomics website tool for each replicate of each sample (http://ieg.ou.edu). Diversity indices were determined individually for each triplicate endpoint

samples from a treatment (either amended or unamended) of a redox condition (oxic, anoxic, fluctuating).

Quantitative PCR (qPCR)

Quantitative PCR was used to quantify Bacterial and Archaeal 16S rDNA, and Fungal ITS using an iCycler (BioRad, Hercules, CA). The following primers were used: Eub318F/Eub518R, Bacterial 16S; 340R/1000R, Archaeal 16S; 5.8s/ITSf, ITS (Fierer et al 2005, Gantner et al 2011). Each 20 µl qPCR reaction to amplify Bacterial 16S, Archaeal 16S or ITS contained 1 µl template DNA (~50 ng), 10 µl 2X SsoFast™ EvaGreen Supermix® (BioRad, Hercules, CA), and 1 µl each of 10 µM forward and 10 µM reverse primers. The optimal annealing temperature for each primer set was determined to be 53 ˚C. Thus all genes were amplified in the following two step amplification protocol; one cycle of 95 ˚C for 5 minutes, 40 cycles of 95 ˚C for 10 seconds, then at 53 ˚C for 20 seconds. Immediately after the last amplification cycle, a melting curve analysis was done, starting from the annealing temperature, 53 ˚C and ending at 95 ˚C. The melting temperature of the Bacterial and Archaeal 16S standard and sample reactions were similar. The melting temperature of the ITS standard and sample reactions were within a few degrees.

Initial PCR tests of the extracted DNA indicated the presence of PCR inhibitors. To overcome the amplification inhibition, sample DNA was diluted in a 1:10 serial dilution. The sample dilution used in further analysis was chosen based of the results of Bacterial 16S qPCR reactions using the serially diluted DNA as template. Standards for qPCR were made by cloning the results of PCR using the above Bacterial 16S, Archaeal 16S and ITS primers and using DNA extracted from palo colorado soil taken from a depth of 1-10 cm into pCR2.1 or pCR4 vectors (Invitrogen, Carlsbad, NM). Single clones were picked from each clone library and sequenced at the UC Berkeley Sequencing Facility. Sequences were confirmed to be Bacterial, Archaeal or Fungal using the blast tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Clones with confirmed sequences were grown overnight as per manufacturer's instructions and plasmids were extracted using a Miniprep Kit (Qiagen, Valencia, CA). Plasmids were then quantified on a NanoDrop (NanoDrop Technologies, Wilmington, DE), and diluted to an appropriate standard curve for qPCR reactions.

Quantitative PCR results are expressed as the abundance relative to the amount of ng DNA extracted. Redox conditions and the presence or absence of substrate was determined to affect the amount of DNA extracted at $p < 0.05$. Thus, I expressed the qPCR results as abundance relative to the amount of ng DNA extracted.

454 Pyrosequencing

Pyrosequencing (454 FLX Titanium Platform, Roche, Zurich, Switzerland) was used to sequence the small subunit rRNA genes (16S) from 1 µg DNA extracted from the final time point samples from the amended and unamended samples of each condition (Joint Genome Institute, Walnut Creek, CA). As for the GeoChip 4 analysis, DNA (1 ug) of three subsamples of soil collected immediately prior to the start of the experiment were also included. The V8 hyper variable region of the 16S small subunit rRNA gene from bacteria was amplified from the samples using the 926F/1392R primer set (Kunin et al 2009).

The Qiime (version 1.6.0) analysis pipeline was used to analyze the 454 Pyrosequencing data, from denoising, picking and aligning OTUs, removing chimeric sequences, and assigning taxonomy (Caporaso et al 2010b, Caporaso et al 2010a, Reeder and Knight 2010, Edgar 2010, DeSantis et al 2006, Haas et al 2011, Wang et al 2007, Werner et al 2012, McDonald et al 2012). Samples were rarefied to 710 sequences/sample. However, two samples were removed because they did not contain at least 710 sequences after curation through the Qiime pipeline, both were unamended samples from the anoxic period of the fluctuating condition. A tree constructed using the FastTree algorithm in Qiime was used in conjunction with the constructed OTU table to calculate a matrix of weighted unifrac distances (Price et al 2010, Lozupone and Knight 2005). The weighted unifrac distance matrix was used to compare the samples in ordination space using Detrended Correspondence analysis (DCA) in the vegan package of R (version 3.0.2). The observations of sequences belonging to a particular phyla were calculated and used in the following comparisons: amended oxic and amended anoxic, amended and unamended oxic, amended and unamended anoxic and amended and unamended fluctuating condition samples.

Statistical Analyses

Unless otherwise specified, all statistical analysis was performed in R using the native build packages, the stats package or the vegan package (R version 3.0.2). Analysis of variance, using Tukey's Honestly Significant Difference (TukeyHSD) test as a multiple means comparison test, was used to test for significant changes in ^{13}C-CO_2 fluxes across redox conditions and differences related to redox condition and amendment between the amounts of DNA extracted.

The adonis function from the vegan package of R was used to determine how redox condition and amendment affected the cumulative CO_2, carbon degradation potential, qPCR data and 454 Pyrosequencing results. Time was included as a factor when testing the cumulative CO_2 and qPCR data with the adonis function. To determine how the redox condition, time and amendment contributed to variation among gas production measurements, the adonis function was used. The adonis function was also used to determine how the conditions and amendment contributed to any variation among abundance measurements from the qPCR analysis, the abundance and diversity of cellulose degradation gene probes and the abundance and diversity of OTUs detected from the 454 Pyrosequencing data. The Bray distance measure was used for all adonis tests.

Difference between alpha diversity index measurements for the cellulose degradation potential and 454 Pyrosequencing data between samples was tested using the Students T test in Excel (Microsoft Office 2013, Redmond, WA). The Students T test was also used to test the difference in the sequence observations of a particular phyla or class between samples.

The large number of comparisons of relative gene probe intensities necessitated a correction for Type I errors. Thus, the Benjamini and Hochberg (BH) correction from the stats package of R was applied to correct for Type I error in p values obtained from Students T test of carbon degradation potential tests.

Results

Gas Production

Cumulative CO_2 production from the amended and unamended samples was very similar ($p > 0.05$); amendment did not explain significant variation in cumulative CO_2 produced (Figure 1, Table 2). Time was the single greatest explanatory variable for cumulative CO_2 production (Table 2). Cumulative CO_2 production from the oxic and the fluctuating condition samples were significantly greater (1.63 ± 0.07 and 1.29 ± 0.04 times) than CO_2 produced from the anoxic condition samples, respectively ($p < 0.001$) (Figure 1). Cumulative CO_2 production from the oxic condition samples was significantly (1.20 ± 0.03 times) greater than from the fluctuating condition samples ($p < 0.001$). Difluoromethane was added to each microcosm after flushing with the appropriate gas to inhibit CH_4 oxidation. However, cumulative CH_4 production was below the limit of detection in each redox condition regardless of amendment after 18 and 22 days (data not shown).

Gas Fluxes

Under anoxic conditions, 0.20 ± 0.0004 % of ^{13}C-labeled cellulose was transformed into ^{13}C-CO_2 ($n = 3$) (Table 3). Similar percentages of ^{13}C-labeled cellulose was transformed into ^{13}C-CO_2 under the oxic and fluctuating condition (oxic: 0.015 ± 0.001 %, fluctuating 0.15 ± 0.002 %, $p < 0.01$, $n = 3$ for each condition). The percentage of ^{13}C-labeled cellulose transformed into ^{13}C-CO_2 was significantly greater under the anoxic condition than in either the oxic or fluctuating conditions ($p < 0.01$) (Table 3).

Degradative Metabolic Potential of 439 Detected Cellulose-related Genes (GeoChip)

In NMDS ordination space, the degradative gene potentials of the soil sampled before incubation were dramatically different from the samples taken after incubation. The 22 day incubation caused a substantial shift in the carbon degradation potential from the native (ie, not incubated in a microcosm) soil community (Figure 2A). Because the carbon degradation potential of the native soil was so different than the experimental samples, it was not included in further analyses.

The unamended and amended oxic samples tended to separate from the unamended and amended anoxic samples (Figure 2B); however, the 95% CIs overlap. Static oxic and anoxic conditions explained a small amount of the variation in cellulose decomposing enzymes (Table 4). Amendment did not have a significant effect on the abundance and composition of carbon degradation potential (Figure 2B, 3C, Table 4).

The comparisons of relative intensities of gene probe families yielded no significant differences at $p < 0.05$, regardless of comparison (Figure 3). The amended oxic condition samples contained more treatment-specific probes than the amended anoxic condition samples (Figure 4A) and the carbon degradation gene probes in the amended oxic samples were more diverse (Table 5A). I compared the proportion of total relative intensity of each cellulose gene probe category (endoglucanase, cellobiase, etc) specific to the amended oxic conditions samples to that shared between the amended oxic and anoxic samples. The gene probes unique to the amended oxic

condition in the alpha galactosidase, exoglucanase, invertase and lactase sub-categories made up from 53 to 91 % of the total relative intensity for those sub-categories. The relative intensity of the remainder of the sub-categories, cellobiose dehydrogenase (CDH), cellobiase and endoglucanase, were from 31 to 49 % of the total relative intensity for the sub-categories. The majority of gene probes were shared between the amended and unamended samples for the oxic, anoxic and fluctuating condition samples (Figure 4B, 4C, 4D).

rRNA gene abundances

Redox condition significantly affected the abundance of Bacterial 16S, Archaeal 16S, and ITS genes (Table 6). Under the amended anoxic condition, the abundance of bacterial 16S and fungal ITS genes appear to decrease (Figure 5, Table 6). ITS abundance also appears to decrease during the anoxic periods of the fluctuating condition, but no significant trends were detectable in the data from the fluctuating conditions.

454 Pyrosequencing

Neither redox condition nor amendment affected the overall taxonomic composition of the bacteria in the samples (Figure 6, Table 7). The 95% CI overlap and are not shown so that the data is more easily visualized. No distinct or loosely associated clusters of samples from a redox condition or type of amendment (amended or unamended) were apparent.

In general, the pattern of observed phyla was similar in the amended oxic and anoxic samples (Figure 7). There was one difference, the sequence observations of Proteobacteria were 1.2 ± 0.1 times more abundant in the oxic amended samples than in the anoxic amended samples. None of the Proteobacterial classes detected were significantly more or less abundant in the amended oxic compared to the amended anoxic condition samples.

There are two significant differences (at $p < 0.05$) in the pattern of observed phyla in the amended versus the unamended samples, both from the anoxic condition samples (Figure 8). The number of sequence observations of the Acidobacteria were greater in the amended anoxic samples than in the unamended anoxic samples. Proteobacterial sequence observations were greater in the unamended anoxic samples. However, the sequence observations of classes detected in the Acidobacteria and Proteobacteria were not significantly different between the amended and unamended anoxic condition samples.

Alpha diversity indices were not consistently significant (Table 8). Using the Simpson statistic, unamended samples were more diverse than amended samples in the anoxic condition and amended anoxic samples are more diverse than amended oxic samples at $p < 0.05$. Using the Chao1 statistic, unamended samples were more diverse than amended samples in the fluctuating condition at $p < 0.05$.

Mantel tests were used to assess the relationships between measures of decomposition (CO_2 and $^{13}CO_2$), community composition (454 Pyrosequencing data), abundance (qPCR), and function (GeoChip 4). The community composition was positively correlated with cumulative CO_2 produced ($r = 0.187$, $p = 0.012$) (Table 9). No other correlation was significant at $p < 0.05$.

Discussion

Cellulose is a major component of plant material. Both fungi and bacteria can degrade cellulose under both oxic and anoxic conditions (Wilson 2008, Tomme et al 1995, Atlas and Bartha 1998). However, few studies have investigated cellulose degradation under fluctuating redox conditions (Schellenberger et al 2011). My study was designed to investigate the impact of the fluctuating redox conditions in the LEF soils on cellulose decomposition and the cellulose degradation potential of the soil microbial community and determine whether links between the bacterial community composition or functional profiles and cellulose degradation could be made. Since the duration of this study was relatively short, this discussion is limited to initial cellulose degradation and may not be indicative of the long term rates of cellulose degradation in this soil.

Patterns of Total CO_2 Production

The rates of total CO_2 production were greatest under oxic conditions, followed by fluctuating conditions, with anoxic condition slightly but significantly lower than the fluctuating conditions. The rates of CO_2 production were not statistically affected by the cellulose amendment. The relatively small amount of cellulose added as an amendment (0.1 g per 20 g wet weight soil) may have made detection of any cellulose growth-dependent responses difficult. Using a small amount of substrate has the advantage of not altering the microbial community by promoting growth. In this way, cellulose degradation by a microbial community representing a more natural microbial community can be investigated.

Cellulose Decomposition

Cellulose decomposition, as measured by ^{13}C-CO_2 production over 22 days, was greatest in the anoxic condition. More ^{13}C-CO_2 was produced in the amended anoxic condition than in the oxic or fluctuating conditions (Table 3). This was unexpected, even at this time scale. However, possible differences in cellulose degradation strategies, or the preferential degradation of other carbon pools in the other redox conditions could help explain why more cellulose was apparently initially degraded under the anoxic condition.

I expected that cellulose decomposition would be more rapid under fluctuating conditions because aerobic and anaerobic cellulose degradation processes could be used. Cellulose degradation enzymes do not require oxygen; enzymes produced under aerobic conditions can still function under anaerobic conditions. Thus, cellulose degradation enzymes produced under either condition would be able to degrade cellulose regardless of the redox state. In addition, soluble sugars produced as a result of cellulose degrading enzymes produced in either condition could induce the production of more cellulose degrading enzymes. However, increased cellulose decomposition in the fluctuating condition was not observed.

^{13}C-CO_2 production was highest under anoxic conditions. This suggests that the primary initial cellulose decomposers in this wet tropical soil were anaerobic bacteria. It is commonly assumed that in most upland, dominantly aerobic soils, the primary cellulose decomposers are aerobic fungi (Berg and McClaugherty 2008). The common occurrence and in some cases predominance of anoxic conditions in the soils used here likely result in substantial reduction of fungal abundance in the wet mineral soil. Indeed the abundance of ITS genes in these soils appear to be

about three orders of magnitude less than in upland CA soils that have been analyzed (Blazewicz et al 2014). However, because of potentially marked differences in extraction efficiencies and the multinucleate nature of fungi, direct comparisons of qPCR quantification of ITS genes is problematic.

It may be that over a longer timescale, a different pattern of overall ^{13}C-CO_2 production would occur and that aerobic microorganisms would become the primary cellulose decomposers. Previous research has demonstrated that microorganisms with surface associated glycosyl hydrolase enzymes, such as cellulosomes, may benefit (in terms of biomass increase) from cellulose degradation before microorganisms using extracellular enzymes (Resat et al 2012). The increase in biomass in the experiment by Resat et al. (2012) is presumably due to microorganisms with surface associated glyosyl hydrolase enzymes obtaining energy from cellulose degradation. The results of Resat et al. (2012), indicate that over a short time scale, microorganisms with cellulosomes may be transiently more active than microorganisms using extracellular enzymes to degrade cellulose.

Alternatively, the greater cellulose decomposition observed in the anoxic condition could be due to the action of anaerobic fungi. The majority of known cellulose-degrading anaerobic fungi exist in the mammalian digestive tract (Milne et al 1989, Trinci et al 1988). However, recent studies have demonstrated the cellulose-degrading activity of anaerobic fungi in forest soils (Stursova et al 2012, DeAngelis et al 2013).

Cellulosomes have been described as an efficient way for anaerobic bacteria to degrade cellulose (Shoham et al 1999). The efficiency could be due to the cellulosome structure, which keeps cellulose degrading enzymes together, perhaps enabling a 'stronger synergy' (Shoham et al 1999). Because the cellulose is attached to the cell wall, the soluble sugars produced as a result of cellulose degradation are more likely to be used by the organism that produced the cellulosome. In contrast, extracellular cellulose degrading enzymes that are generally produced under aerobic conditions, can diffuse away from the cell. Thus, the products of an extracellular cellulose degrading enzyme may not be concentrated around the cell and hence could be used by other organisms that are closer to the extracellular enzyme. This has the potential to decouple cellulose enzyme production from growth on the products of decomposition. Thus anaerobic bacteria producing cellulosomes may have a competitive advantage initially in a fluctuating redox soil. However, as a process, aerobic cellulose degradation is considered more efficient than anaerobic cellulose degradation (Gu et al 1993). In light of this, cellulose degradation may be primarily degraded under aerobic conditions in this soil when longer time scales are considered.

Cellulose Degradation Potential

The cellulose degradation potential (as quantified by the relative abundances of the 439 cellulose-involved genes detected by GeoChip 4) in the LEF soil was statistically affected by redox condition. However, the effect of redox condition on the cellulose degradation potential was significant only when the static oxic and anoxic condition samples were compared. In the NMDS ordination, the cellulose degradation potential in the samples under static oxic conditions tended to separate from the samples under static anoxic conditions (Figure 2B, Table 4).

Extracellular cellulose degrading enzymes produced in the oxic condition can function and provide soluble sugars that may induce cellulose degradation by anaerobes in the anoxic condition, and vice versa. In addition, enzymes produced in the anoxic (or oxic) period of the fluctuating redox condition could degrade cellulose under both the oxic and anoxic periods of the fluctuating redox condition. This may account for the lack of clustering of the samples from the oxic or anoxic period of flux with the corresponding static condition samples.

Cellulose, in combination with redox condition affected the diversity of cellulose degradation potential. The amended oxic samples are more diverse than the amended anoxic condition samples (Figure 4, Table 5). However, the anoxic condition samples produced more ^{13}C-CO_2 than the oxic condition samples. Taken together, this data suggests that the diversity of cellulose degradation gene probes did not simply control the initial ability to degrade cellulose.

This result could suggest that the time period over which this experiment was run did not allow diversification of cellulose degradation potential in the anoxic condition. This could be the result of organisms stimulated by cellulose that have diverse cellulose degrading genes. The cellulose degradation gene probes specific to the amended oxic condition samples (when compared to the amended anoxic condition samples), comprise the majority of the relative intensity signal. This suggests that not only were the cellulose degradation gene probes more diverse in the amended oxic samples, they were also more abundant by the relative intensity measure. This could happen because the organisms with genes that matched the gene probes increased in abundance. Alternatively, there could be genetic cellulose degradation potential in the LEF that is not included on the GeoChip 4. The results presented suggest that the genetic diversity of functional genes does not directly impact the potential to perform the function.

Bacterial Community Composition

The bacterial community structure was largely unaffected by redox condition (Figure 6, 7, 8). This result contrasts with previous studies that have investigated the bacterial community structure in LEF soil, but is similar to what I report in Chapter 2. Previous research found that the fluctuating redox affected the community composition and activity (Pett-Ridge and Firestone 2005, DeAngelis et al 2010). The differences in results may be due to use of different soils, experimental design or the techniques used to assay microbial community structure.

Both earlier studies that observed a difference in microbial community structure due to redox condition used soil from a different site than my study. The sites are located in the same upland tropical forest, but they are not proximally located. Previous research has found that community structure varies depending on the site (Costa et al 2006). Thus, the lack of effect of condition on microbial community structure in the NMDS ordination could be due to site differences in the microbial community.

The experiment by Pett-Ridge and Firestone (2005) was an incubation of soil that was mixed and incubated under a continuous flow of hydrated gas for 22 days. The experiment by DeAngelis et al. (2010) was an incubation of intact soil cores under a flow of hydrated gas for 32 days. In contrast, I incubated my soil microcosms under static headspace for 22 days.

One study used T-RFLP, an enzyme-based fingerprinting technique, to assay the microbial community structure (Pett-Ridge and Firestone 2005). Pyrosequencing provides more resolution than T-RFLP (Pilloni et al 2012, Lee et al 2010). In the study by Pett-Ridge and Firestone

(2005), approximately 130, 95 and 60 ribotypes were detected by T-RFLP in the fluctuating, oxic and anoxic condition samples. When my samples are rarefied to 710 sequences per sample, 459, 425, and 439 OTUs are detected in the fluctuating, oxic and anoxic condition samples.

The other study used the PhyloChip, a 16S gene microarray to measure taxa in the LEF (DeAngelis et al 2010). The Phylochip is advantageous because it assays the bacterial community structure from any sample based on the same metric. That is, only the bacterial 16S rRNA genes present on the Phylochip can be measured. In contrast, 454 Pyrosequencing technology is not constrained to a predetermined set of 16S rRNA bacterial genes. Using the PhyloChip, DeAngelis et al. (2010) detected 2489 bacterial taxa at the species level, in 29 bacterial phyla. In my study, when the samples were rarefied to 710 sequences/sample, 466 bacterial taxa in 8 bacterial phyla were detected. The results of Pett-Ridge and Firestone (2005) and DeAngelis et al (2010) both show similar effects of condition on the microbial community structure, even with different numbers of OTUs and detected taxa. It is therefore unlikely that the lack of effect of redox on microbial community structure in my study is due to a difference in the number of taxa or OTUs detected.

I expected to observe an increase in sequences representing bacteria known to degrade cellulose, such as those from the phyla Firmicutes and perhaps Bacteriodetes, in the amended samples regardless of condition. This was not the case. The bacteria present could already be poised to degrade cellulose, so no change in bacterial community structure would be observed. Because cellulose is the most abundant plant polymer and plant material is degraded rapidly in this soil, bacteria in this soil may frequently encounter cellulose. With this in mind, it would not be likely that the bacterial community structure would be affected unless substantial cellulose was added.

The community composition was correlated to the cumulative CO_2 produced, but not to the ^{13}C-CO_2 produced. CO_2 production from soils has been called a 'broad' process (Schimel 1995). As a process, CO_2 production is not limited to a particular group of microorganisms, and it is a result of many different processes and metabolisms. In previous research, after addition of a labile carbon source, shifts in microbial community have been observed concomitant with increased CO_2 production (Cleveland et al 2007). However, other research has demonstrated that even though CO_2 is a microbially mediated process, microbial composition has very little influence over CO_2 production (Kemmitt et al 2008). Very few statistically significant differences in detected taxa at the phyla and Class level were observed in my study. However, these shifts in the bacterial community composition could have affected cumulative CO_2 production.

A relationship between bacterial community composition and cellulose decomposition was not supported. Previous research has demonstrated that microbial composition can be linked to cellulose addition (Pankratov et al 2011). However, definitive links between microbial community composition and function have been a challenge to establish (Grant et al 1993, Sugai and Schimel 1993, Strickland et al 2009, Kemmitt et al 2008). Many soil bacteria are functionally redundant in terms of the ability to use soluble sugars produced by the action of endo- and exoglucanases on cellulose (Berlemont and Murphy 2013). Bacteria in the LEF soils are perhaps also functionally redundant in this manner and as such, no correlation between community composition and cellulose decomposition was observed.

Conclusion

My study investigated the impact of fluctuating redox conditions on cellulose decomposition and cellulose degradation potential. Cellulose decomposition was affected by redox condition, although cellulose degradation potential was not. After 22 days, ^{13}C-CO_2 production was greatest in the anoxic condition, which suggested that anaerobic microbes were active cellulose degraders in the initial stage of cellulose degradation. However, a link between cellulose decomposition and cellulose degradation potential to microbial community composition could not be established. At this early stage, cellulose decomposition and cellulose degradation potential in this soil appears to be uncoupled from bacterial community composition.

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Table 3-1. Site characteristics from where the soil used in this experiment were collected.

Life Zone	Elev. (masl)	Dominant vegetation[2]	Soil Moisture[1]	MAP[2]	MAT[2]	pH[2]	%C[3]	%N[3]	C:N[2]
Montane Wet Forest	780	*Cyrilla racemiflora*	59.5	4200	19.5	5.1 (0.2)	7.4 (2.0)	0.31 (0.06)	18.6 (0.76)

[1]This study.
[2]Dubinsky, E.A. 2008. UCB Thesis.
[3]Dubinsky E.A., Silver, W.L., and M.K. Firestone. 2010. Ecology. 91(9): 2604-2612.

Table 3-2. Effect of redox condition, amendment and time on the variation observed from cumulative carbon dioxide measurements. Bold values indicate factors significant at $p < 0.05$.

A

Condition	Amendment		Time		Amendment*Time	
	r^2	p	r^2	p	r^2	p
Oxic	0.00008	0.942	0.821	**0.001**	0.00009	0.981
Anoxic	0.0005	0.856	0.782	**0.001**	0.0003	0.932
Fluctuating	0.00004	0.976	0.807	**0.001**	0.00001	1.000

B

Amendment	Condition		Time		Condition*Time	
	r^2	p	r^2	p	r^2	p
Amended	0.363	**0.016**	0.740	**0.001**	0.033	**0.013**
Unamended	0.046	**0.005**	0.731	**0.001**	0.374	**0.012**

Table 3-3. ^{13}C-CO_2 produced from ^{13}C-cellulose in amended samples. Each value is an average of three biological replicates. Significant differences were determined using TukeyHSD at $p < 0.01$.

Substrate	Condition	% ^{13}C-substrate -> CO_2
Cellulose from *Zea mayes*	Oxic	0.15 a
	Anoxic	0.20 b
	Flux	0.15 a

Table 3-4. Effects of condition (redox) and amendment on the observed variance in the abundance and composition of cellulose degradation genes. Bold values indicate factors significant at $p < 0.05$ using the Adonis test.

	All Conditions		Oxic and Anoxic Conditions	
	r^2	p	r^2	p
Condition	0.177	0.098	0.205	**0.014**
Amendment	0.045	0.384	0.095	0.425
Condition*Amendment	0.151	**0.013**	0.203	**0.001**

Table 3-5. Shannon and Simpson diversity indices of carbon degradation and curated organic remediation probes from pairwise comparisons. The Students T test was used to determine significance. Bold values indicate significance at $p < 0.05$.

A

	Shannon Index (H)			Simpson Index (1/D)		
	Oxic	Anoxic	p value	Oxic	Anoxic	p value
Amended	8.22	7.87	**0.003**	2548.7	1825.7	**0.007**

B

	Amended	Unamended	p value	Amended	Unamended	p value
Oxic	8.22	8.18	0.058	2548.7	2462.4	0.251
Anoxic	7.87	8.05	**0.037**	1825.7	2168.2	0.072
Fluctuating	8.10	8.12	0.878	2298.0	2315.3	0.938

Table 3-6. Effect of condition (redox), amendment and time on the variance observed from Bacterial and Archaeal 16S and ITS abundance qPCR measurements. Values in bold indicate significance at $p < 0.05$.

	Bacterial 16S		Archaeal 16S		ITS	
	r^2	p	r^2	p	r^2	p
Condition	0.207	**0.001**	0.043	**0.007**	0.347	**0.001**
Amendment	0.0009	0.948	0.003	0.389	0.004	0.373
Time	0.015	**0.043**	0.015	0.050	0.006	0.232
Condition* Amendment	0.006	0.467	0.013	0.206	0.031	**0.008**
Condition* Time	0.019	0.052	0.012	0.205	0.035	**0.003**
Amendment* Time	0.041	**0.001**	0.002	0.466	0.026	**0.007**

Table 3-7. Effect of condition (redox) and amendment on the variance observed in the composition and abundance of the 454 Pyrosequencing results rarefied to 710 sequences per sample.

	r^2	p
Condition	0.155	0.200
Amendment	0.137	0.076
Condition*Amendment	0.553	0.770

Table 3-8. Shannon, Simpson and Chao1 diversity indicies of the 454 Pyrosequencing data (rarefied to 710 sequences per sample). The alpha diversity was determined individually for each biological replicate rarefied to 710 sequences per sample. Bold values indicated comparisons significantly different at $p < 0.05$.

A

	Shannon (H)			Simpson (1/D)			Chao1		
Condition	Amended	Unamended	p value	Amended	Unamended	p value	Amended	Unamended	p value
Oxic	5.14	5.08	0.274	74.09	72.90	0.706	540.46	536.25	0.873
Anoxic	5.27	5.11	0.249	91.65	78.32	**0.018**	614.25	566.31	0.118
Fluctuating	5.16	5.01	0.302	79.39	78.37	0.835	545.59	575.64	**0.029**

B

	Oxic	Anoxic	p value	Oxic	Anoxic	p value	Oxic	Anoxic	p value
Amended	5.14	5.27	0.369	74.09	91.65	**0.048**	540.46	614.25	0.873

Table 3-9. Mantel tests correlating gas data, community data and cellulose degradation potential data. The first axis PCoA scores from the community abundance data (qPCR), community structure (454 Pyrosequencing) and cellulose degradation potential (GeoChip 4) were used to correlate to the gas data. Bold values indicate significance at $p < 0.05$.

	^{13}C-gas		total CO_2		Community abundances		Community composition	
	r	p	r	p	r	p	r	p
^{13}C-gas								
total CO_2	-0.032	0.709						
Community abundances	0.016	0.357	-0.043	0.789				
Community composition	0.055	0.141	0.187	**0.012**	-0.006	0.510		
Cellulose degradation potential	0.108	0.058	-0.053	0.846	0.004	0.359	0.027	0.374

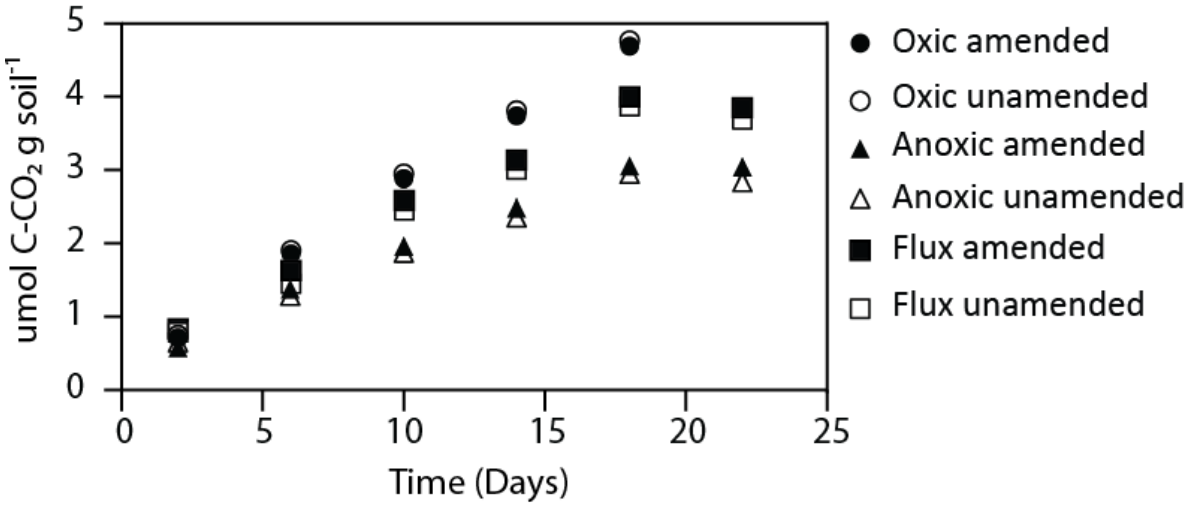

Figure 3-1. CO_2 production over time from each treatment. Data are from measurements from samples destructively sampled on Day 18 and Day 22. Each point is the average of three biological replicates.

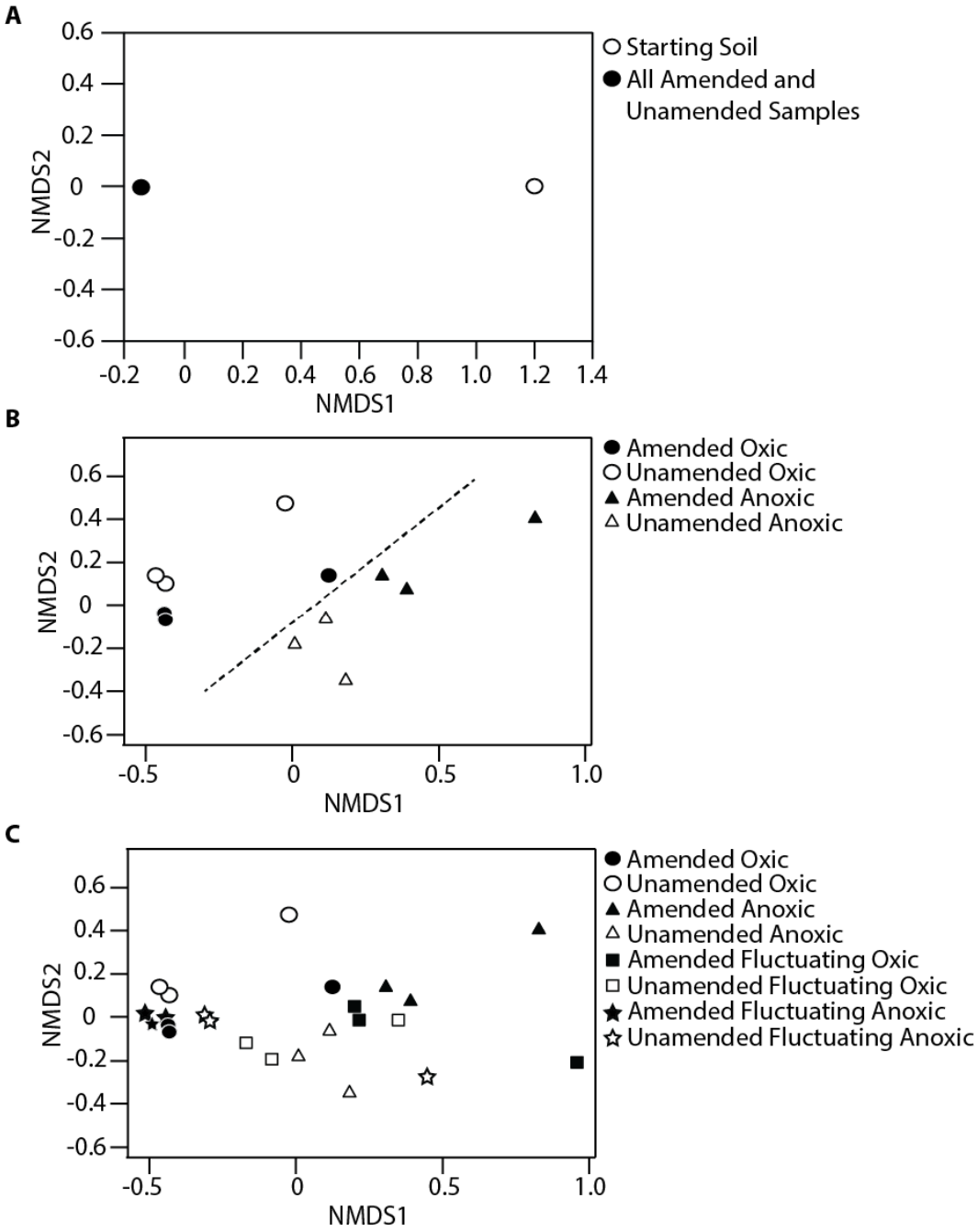

Figure 3-2. NMDS of cellulose degradation gene probes of all final time point samples, including bulk soil samples taken immediately prior to the start of the experiment (A), only the amended and unamended samples of the oxic and anoxic conditions (B) and only amended and unamended samples of all conditions without bulk soil samples (C). Dotted line indicates a plane of separation between oxic and anoxic samples from the static and fluctuating conditions.

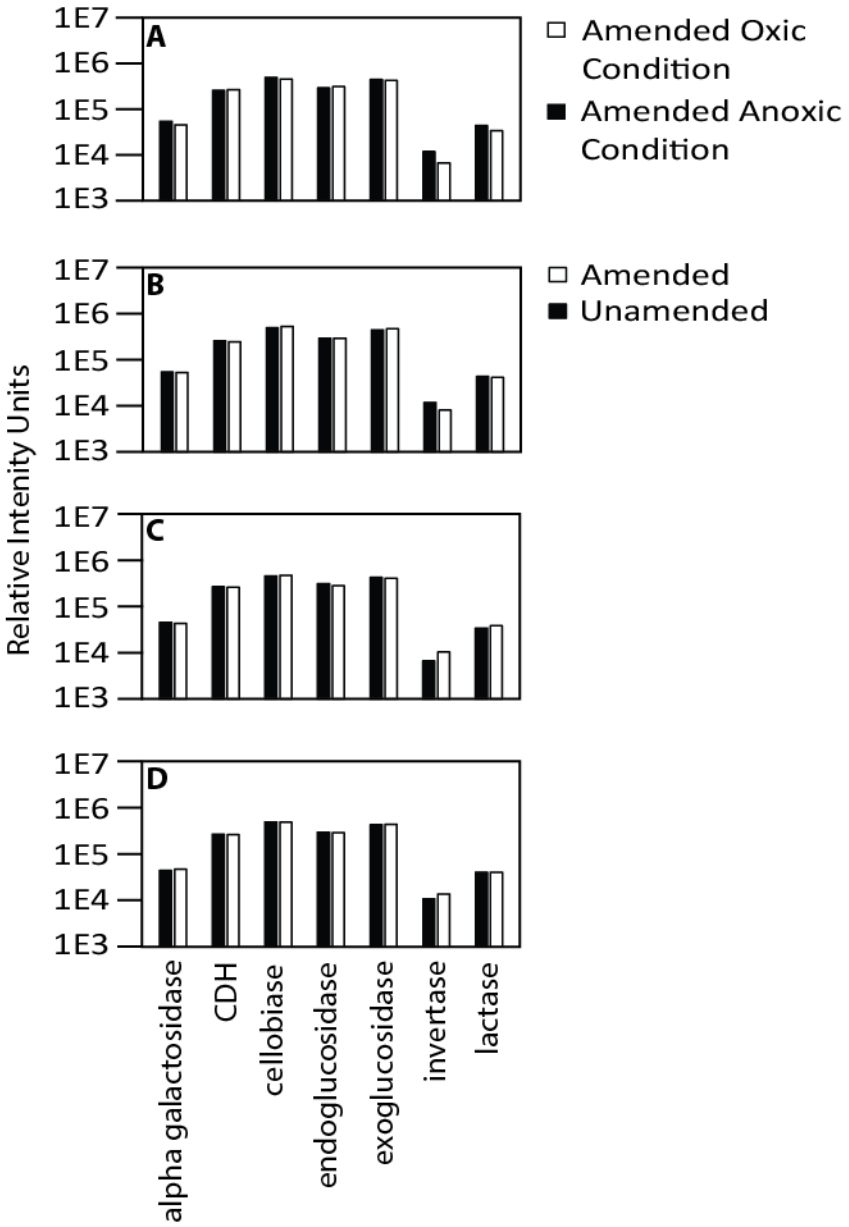

Figure 3-3. Relative intensity of cellulose degradation gene probes compared in the following comparisons, amended oxic and amended anoxic samples (A), the amended and unamended oxic (B), anoxic (C), and fluctuating (D) samples.

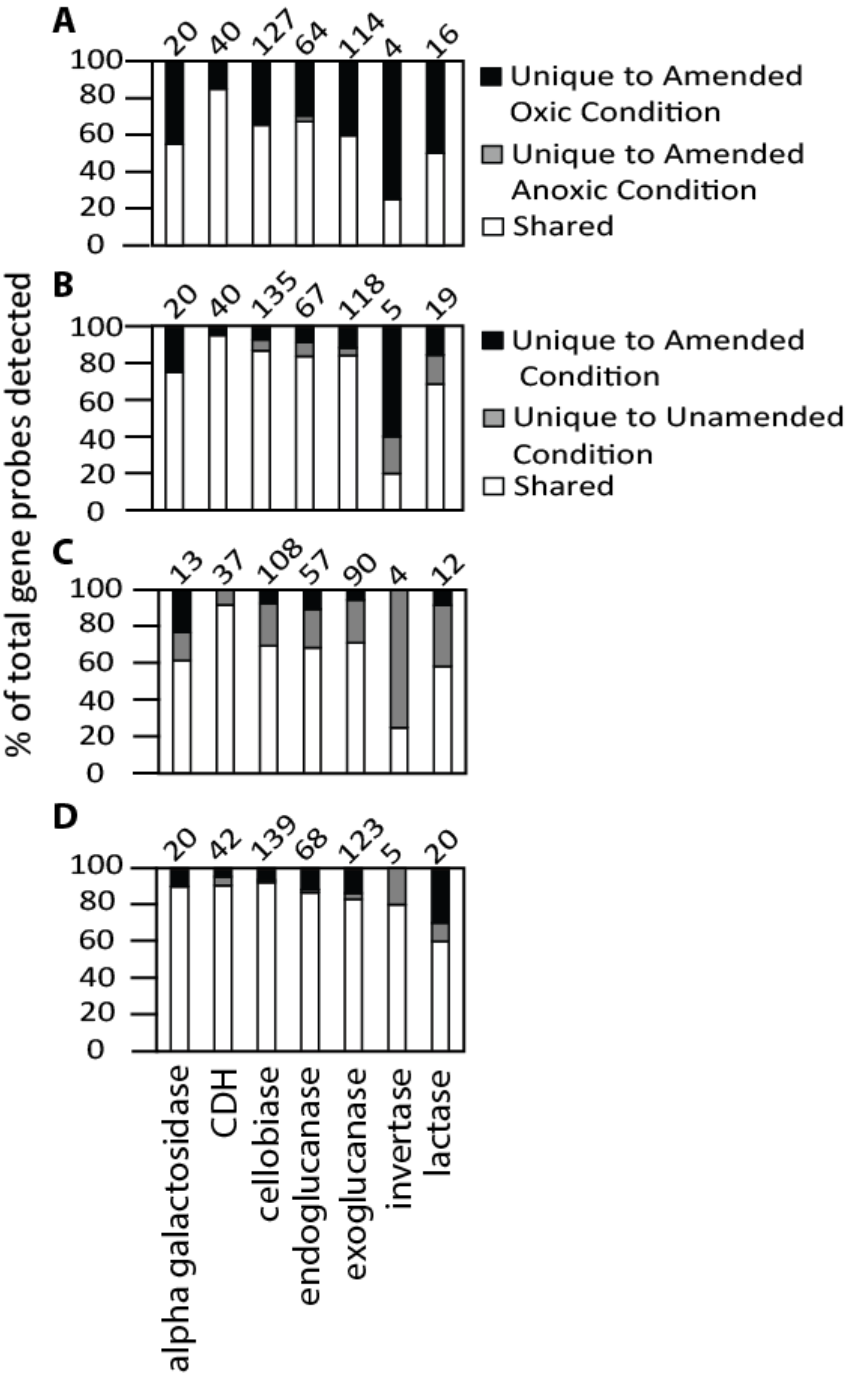

Figure 3-4. Treatment specific and shared cellulose degradation gene probes of the amended oxic and amended anoxic samples (A), amended and unamended oxic (B), anoxic (C), and fluctuating (D) samples. The umber of detected gene probes in a comparison is listed above each bar.

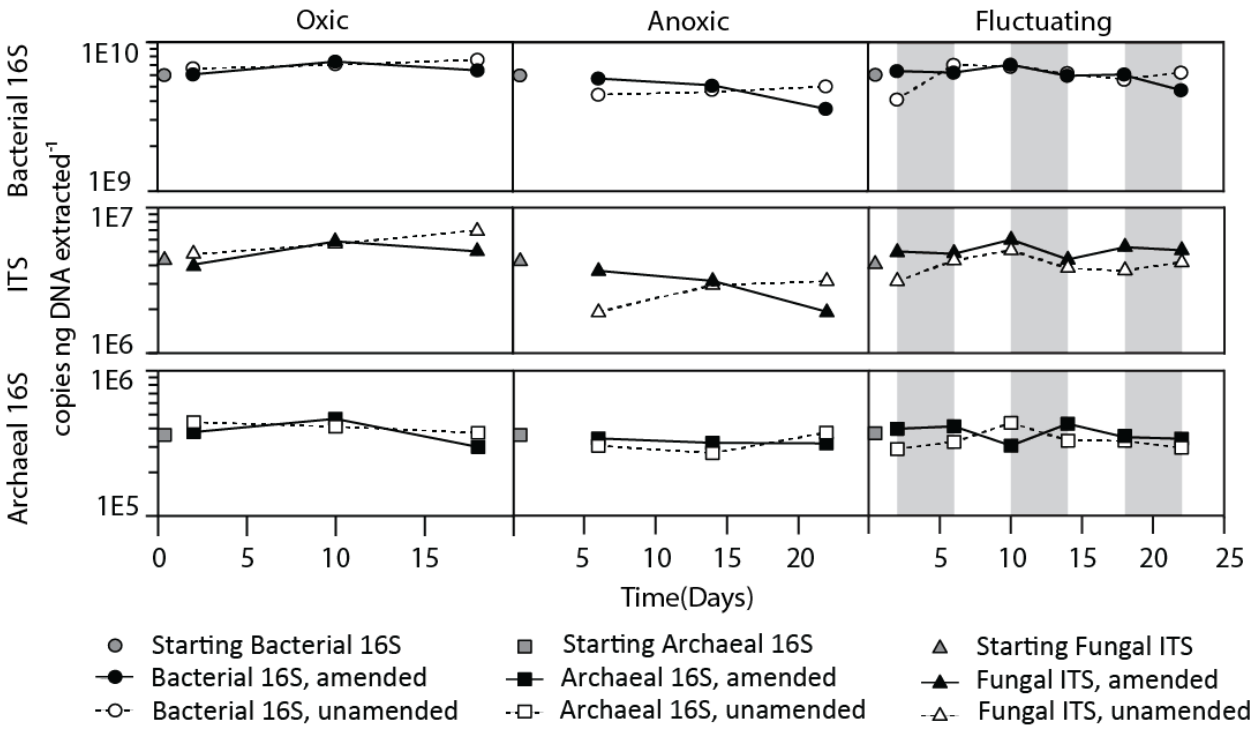

Figure 3-5. Abundance of Bacterial 16S, Archaeal 16S and Fungal ITS as measured by qPCR. Each point represents the average of three technical replicates of the three biological replicates (n = 9).

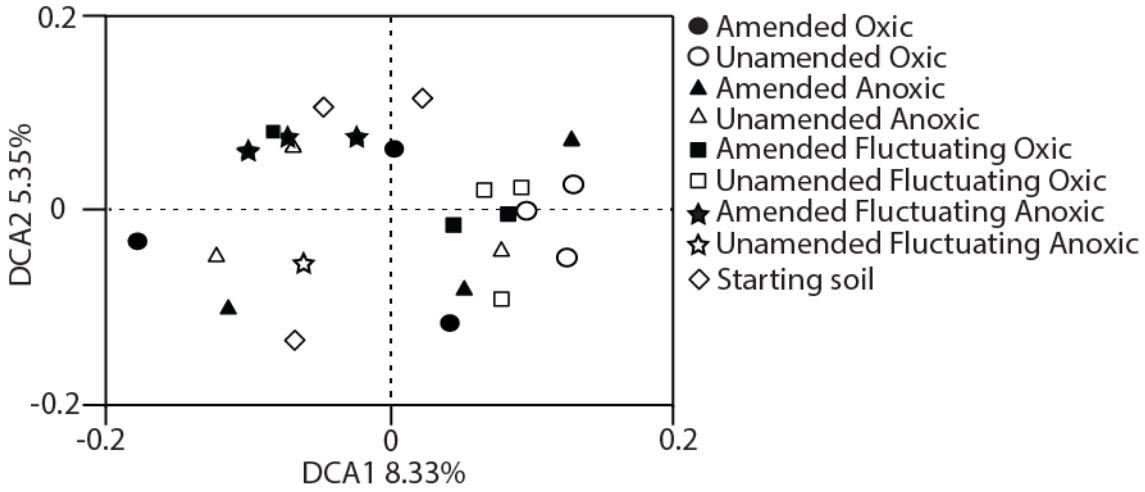

Figure 3-6. Detrended correspondence analysis of weighted unifrac distances of OTUs from the final timepoint samples and the starting soil rarefied to 710 sequences per sample.

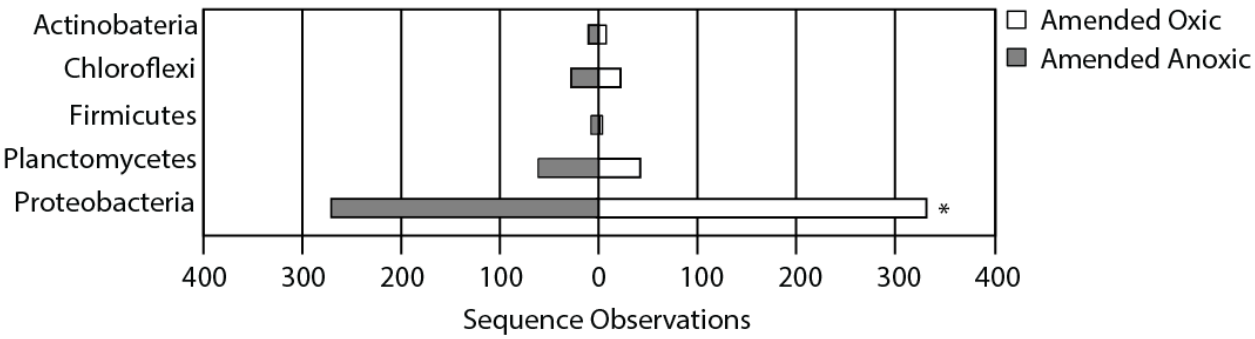

Figure 3-7. Sequence observations belonging to detected phyla compared between amended oxic and amended anoxic samples. Samples are rarefied to 710 sequences per sample. Asterisks indicate the observation of sequences belonging to a particular phyla are significantly greater at $p < 0.05$ using the Students T test.

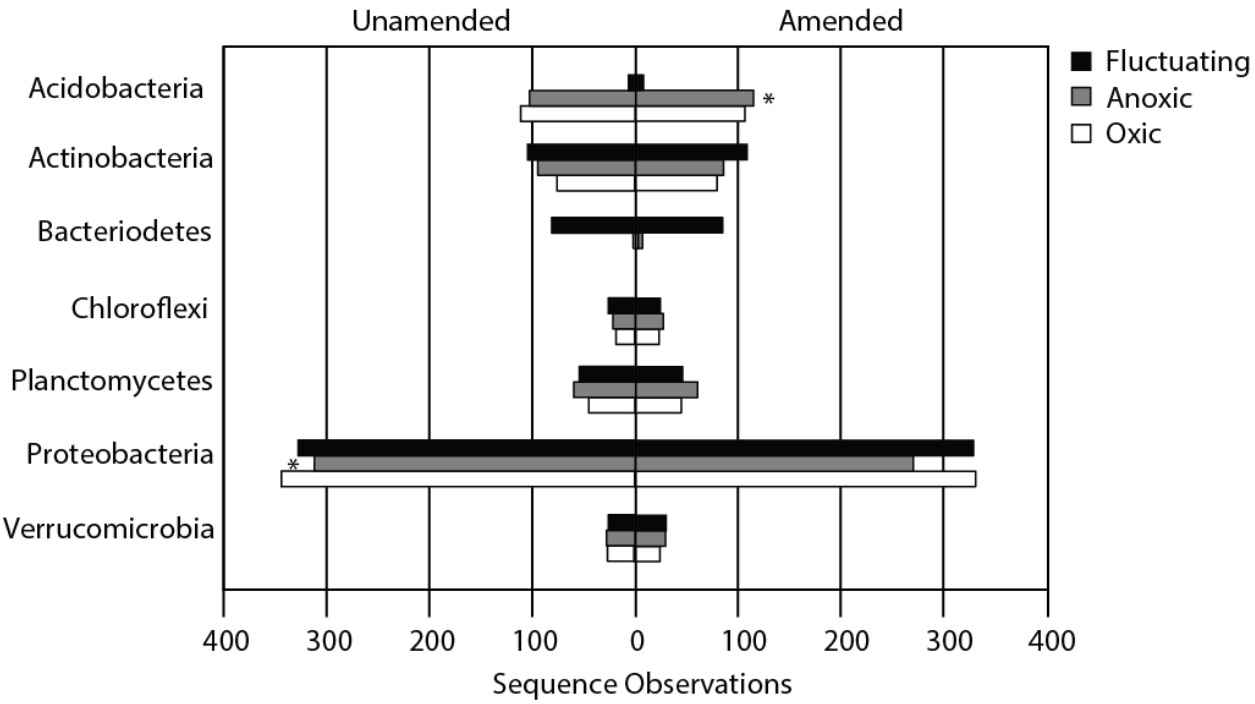

Figure 3-8. Sequence observations of detected phyla compared between the amended and unamended samples of each redox condition. Samples were rarefied to 710 sequences per sample. Asterisks indicate the observation of sequences belonging to a particular phyla are significantly greater at $p < 0.05$ using the Students T test.

Chapter 4

The influence of redox conditions on organic carbon mineralization, carbon degradation potential and bacterial community composition in a wet tropical forest soil

Abstract

High rates of litter decomposition have been measured in the wet tropical soils in the Luquillo Experimental Forest in Puerto Rico (LEF). These high decomposition rates are attributed to the warm, moist conditions experienced by these soils. However, soils in the LEF are also characterized by fluctuating soil oxygen concentration which may also enable high decomposition rates in these soils by coupling aerobic and anaerobic decomposition processes. Two studies were designed to determine the effect of fluctuating redox conditions on the mineralization of plant material and cellulose, one of the main components of plant material. These studies were also designed to determine if mineralization of organic carbon could be linked to microbial community composition or function (carbon degradation potential). Soil sampled from the Colorado forest type in the LEF was amended with either ^{13}C-labeled plant material (*Avena barbata*) or ^{13}C-labeled cellulose and subjected to three redox conditions: oxic, anoxic, and fluctuating oxic-anoxic, in a microcosm-based experiment.

Redox condition had different effects on the mineralization of *A. barbata* litter and cellulose to CO_2 and CH_4. The mineralization of *A. barbata* litter to CO_2 was greatest in the fluctuating redox condition, but not significantly different from the anoxic condition. Cellulose mineralization to ^{13}C-CO_2 was greatest under the anoxic condition. The production of CH_4 in the *A. barbata* litter-amended experiment was greatest under fluctuating redox conditions, but below the limit of detection in the cellulose-amended experiment.

Both the carbon and cellulose degradation potentials (as measured by the GeoChip 4) were affected by soil redox condition. Carbon degradation potentials of samples under the oxic condition were distinct from carbon degradation potentials of samples under the anoxic condition in both experiments. However, the separation of aerobic and anaerobic cellulose degradation potentials in ordination space was observed only in the *A. barbata* litter-amended experiment.

The overall bacterial community composition (by 454 Pyrosequencing) was affected by redox condition and by the presence of plant material in the litter-amended experiment. Neither redox condition nor the presence of cellulose affected bacterial community composition in the cellulose-amended experiment.

Correlations between mineralization measurements and degradation potential and bacterial community structure were inconsistent between the two experiments. In the cellulose-amended

experiment, carbon and cellulose degradation potentials were not correlated to measures of mineralization (production of ^{13}C-CO_2, or CO_2). However, in the *A. barbata* litter-amended experiment, both carbon and cellulose degradation potentials were correlated to CH_4 production. Bacterial community composition was significantly correlated to ^{13}C-gas, CO_2 and CH_4 production in the litter-amended experiment. However, in the cellulose-amended experiment, bacterial community composition was correlated to CO_2 production but not to ^{13}C-gas production.

Redox conditions in this soil affect plant litter and cellulose mineralization and carbon (and cellulose) degradation potential. The pattern of aerobic and anaerobic carbon (and cellulose) degradation potentials in ordination space suggests that there are either distinguishable aerobic and anaerobic decomposition processes or distinguishable differences in the relative abundance of the same carbon (and cellulose) degradation genes in this soil. Synergy between the aerobic and anaerobic processes appears likely in this soil, but is apparent after a period of several weeks.

Introduction

In this chapter, I compare results from experiments following the mineralization of *A. barbata* litter (Chapter 2) and cellulose (Chapter 3) by a soil from the mid-elevation Colorado type forest in the Luquillo Experimental Forest (LEF) in Puerto Rico. In particular, the effect of redox conditions on carbon mineralization, carbon and cellulose degradation potential and bacterial community composition are compared. Correlations between cumulative gases, ^{13}C-gases from the labeled carbon (C) added, degradation potential and community composition from the two experiments are also contrasted.

Tropical forests generally have high net primary productivity (NPP) and a large percentage of the global carbon stock, making them important in global carbon cycling. The NPP in tropical forests is estimated to account for approximately one fourth of the global net primary productivity (Hanson et al 2008). In addition, tropical forests make up approximately 20% of the terrestrial carbon stock (Dixon et al 1994); approximately 9% of terrestrial organic carbon stocks are stored in tropical forest soils (IPCC 2000). Tropical forests are expected to become warmer and drier due to global climate change (Christensen et al 2007). Because climate is changing, understanding how factors sensitive to climate affect decomposition in these soils is important. The Luquillo Experimental Forest (LEF) in Puerto Rico has very high rates of litter decomposition (Parton and Silver et al 2007). The overall objective of the research that I present in Chapters 2 and 3 is to determine the effect of redox conditions on organic C mineralization in a wet tropical LEF soil.

Rates of organic C mineralization are affected by physical parameters, such as fluctuating redox conditions (Reddy and Patrick, Jr 1974, Delaune et al 1981, Hulthe et al 1998), the quality of the substrate (Berg and McClaugherty 2008), and the diversity of the C source (Orwin et al 2006). The LEF upland tropical forest soils are characterized by fluctuating redox conditions driven by frequent rainfall events and high biological activity (Silver et al 1999). Soil redox potential is commonly considered indicative of which terminal electron acceptor can be used in microbial

respiration (DeLaune and Reddy 2013). It is generally accepted that when soil redox is high (over 300 mV), the bulk soil is considered oxic and oxygen is used as the dominant terminal electron accepting process (DeLaune and Reddy 2013). As soil redox decreases, microbial respiration shifts from using oxygen, an energetically favorable terminal electron acceptor, to less energetically favorable terminal electron acceptors, such as nitrate and ferric iron. For these experiments, low redox conditions are defined as anoxic conditions. Oxic conditions were considered to prevail when headspace gas includes atmospheric concentrations of oxygen.

I designed a series of experiments to determine whether fluctuating redox conditions affected carbon mineralization and carbon degradation potential. In these experiments, I define carbon (and cellulose) degradation potential as the composition and abundance of carbon (and cellulose) degradation gene probes detected on the GeoChip 4. I hypothesized that the fluctuating redox conditions in the LEF provide a situation where both aerobic and anaerobic decomposition processes can occur synergistically. This potential synergy between aerobic and anaerobic decomposition processes may be a factor underlying the rapid decomposition rates reported for these soils.

In some instances, microbial community composition has been linked to enzyme activities and substrate degradation (Waldrop and Firestone 2006, Pankratov et al 2011). However, unequivocal links between microbial community composition and biochemical processes are difficult to demonstrate (Frossard et al 2012, Langenheder et al 2005). In Chapters 2 and 3, I determine bacterial community composition using a high throughput sequencing approach and evaluate the relationship between community composition and decomposition/mineralization processes. Previous work has reported that the bacterial community composition in soils from the LEF reflects adaption to fluctuating redox conditions (Pett-Ridge and Firestone 2005, DeAngelis et al 2010). I hypothesized that there would be links between community composition and carbon mineralization and carbon degradation potential. In the following sections, I will outline the overall questions the experiments were designed to answer and present a comparison of the results of the experiments.

Questions

Question 1: Does the soil redox condition affect organic carbon mineralization? If so, does the soil redox condition affect mineralization of distinct organic substrates differently?

Redox condition, that is, whether low redox (anoxic) or oxic conditions prevail, has been shown to affect organic matter decomposition (Reddy and Patrick 1974, Hulthe et al 1998). In general, organic matter is considered to be more rapidly and more completely decomposed in oxic conditions than under anoxic conditions (Berg and McClaugherty 2008, Acharya 1935, DeLaune et al 1981).

Litter decomposition can be simply grouped into a series of successive steps. Depending on the plant material, the initial stage of decomposition is the consumption of labile carbon. During this stage, the plant material is colonized by diverse fungi and bacteria capable of degrading soluble sugars and other low molecular weight compounds. For recalcitrant substrates such as pine needles, this stage is very brief or non-existent (Berg and McClaugherty 2008). For more labile

substrates, such as *A. barbata* (a wild oat sp.) litter, this step is more pronounced. The second stage involves the breakdown of complex macromolecular structure, such as plant primary and secondary cell walls. Plant cell walls are composed of dense cellulose fibrils coated in a mesh of hemicellulose and pectin interspersed with lignin. During this stage, microbial community tends to shift from bacteria and fungi that specialize in consumption of easily degraded substrates to microbes that can degrade more complex plant components (Rui et al 2009, Pascault et al 2013, Lunghini et al 2013, Voriskova and Baldrian 2013). At this stage, plant components, such as cellulose and hemicellulose, are degraded (Berg and McClaugherty 2008). Soft, brown and white rot fungi, as well as bacteria, degrade cellulose and hemicellulose (Hatakka and Hammel 2010, Flint and Bayer 2008). The more recalcitrant plant material, such as lignin, is degraded in a third stage. During this stage, white rot fungi degrade lignin through the use of laccases and various peroxidases (Palmer and Evans 1983, Berg and McClaugherty 2008). Fungi are the primary lignin degraders, however, bacteria can also contribute to lignin degradation (Zimmerman 1990).

I used a whole plant tissue (*A. barbata* shoots) and cellulose as substrates under the same experimental conditions; static oxic, static anoxic and four day fluctuating oxic-anoxic conditions. This design was intended to assess the effect of the fluctuating redox conditions on plant material and cellulose mineralization. I chose cellulose as a substrate because it is a major component of plant material (Malhi 2002). I intended to determine if fluctuating redox conditions had similar effects on plant material and cellulose mineralization.

Question 2: Does the redox condition effect carbon degradation potential, as measured by the GeoChip 4?

I hypothesize that under fluctuating conditions, the full carbon degradation potential of the soil microbial community can be used. Under oxic conditions, aerobic and facultative anaerobic organisms use a suite of enzymes to attack and consume organic matter. Likewise, under anoxic conditions, anaerobes use a suite of enzymes to decompose organic matter. The suite of enzymes used under oxic and anoxic conditions are not mutually exclusive. However, there may exist a synergy between the suites of enzymes used to consume organic matter under alternating oxic and anoxic conditions. This synergy may be one factor enabling the rapid rate of decomposition in the LEF.

I asked whether the potential carbon degradation capabilities were distinct in the static oxic and anoxic conditions. Distinct potential carbon degradation capabilities under the different static conditions would suggest that carbon degradation in the fluctuating redox condition could be a result of synergy between these capabilities.

Question 3: Are there links between bacterial community composition, redox condition and carbon degradation potential?

Fungi and bacteria are key players in litter decomposition in forest soils (Hattenschwiler et al 2005). Indeed, fungi degrade the principal structural polymers of plants; cellulose, hemicellulose and lignin (Berg et al 2001, Blanchette 1995, Palmer and Evans 1983). Bacteria can also degrade cellulose and hemicellulose (Flint and Bayer 2008). However, bacteria are generally thought to play a very minor role in lignin degradation (Zimmerman 1990, Berg and McClaugherty 2008). However, bacteria may play a larger role in lignin degradation in termite guts (Kato et al 1998).

Measurements of microbial biomass have demonstrated that fungi dominate forest soil microbial biomass (Joergensen and Wichern 2008). Soils in the LEF experience frequent periods of low oxygen availability (Silver et al 1999). Soft, brown, and white rot fungi can degrade plant material under oxic, and microaerophilic conditions (Pavarina and Durrant 2002, Lynd et al 2002). Fungi capable of cellulose degradation under anoxic conditions have been isolated from soil (Durrant et al 1995). However, soil fungi have been demonstrated to contribute less to decomposition under low redox conditions (Seo and DeLaune 2010). A previous study has demonstrated that there is more bacterial than fungal biomass (by PLFA analysis) in some LEF soils (Cusack et al 2011). This and the frequent periods of low redox conditions suggest that in some LEF soils, bacteria may dominate the composition of the soil microbial community.

The bacterial community in some LEF soils appears to be adapted to fluctuating redox conditions (DeAngelis et al 2010, Pett-Ridge and Firestone 2005). Under static oxic or anoxic conditions fewer ribotypes were detected than under four day fluctuating conditions (Pett-Ridge and Firestone 2005). Also, under fluctuating redox conditions, more taxa were active than under static oxic or anoxic conditions (DeAngelis et al 2010). These results demonstrate that bacterial community composition and activity in some LEF soil is affected by redox conditions.

My goal was to determine if bacterial community composition could be linked to either carbon mineralization or carbon degradation potential. Biochemical functions have been linked to microbial community composition (Waldrop and Firestone 2006) and shifts in microbial community composition have been associated with organic carbon additions (Pankratov et al 2011). However, linking community composition to function is a non-trivial undertaking and frequently such links are unsupported (Strickland et al 2009, Kemmitt et al 2008). Microbial functional redundancy may make conclusive links between community composition and function problematic.

Materials and Methods

The site information, experimental set-up and material and methods used to generate and analyze the data presented here are the same as in Chapters 2 and 3. However, some data were re-analyzed so that they could be compared. In this section, I detail how the ^{13}C-gas measurements, the GeoChip 4 data and the 454 Pyrosequencing data were analyzed to generate the data presented here.

Microcosm Incubation Conditions

^{13}C-labeled plant material

In one experiment, ^{13}C-labeled *Avena barbata* shoot material was used. *A. barbata*, a wild annual oat species, was used because native ^{13}C-labeled LEF plant material was not available. *A. barbata* was uniformly labeled to 2 atom percent (Bird et al 2011) using the method described in Bird et al (2003). The C:N ratio of the *A. barbata* shoots used in this experiment was 39:1.

In the second experiment, ^{13}C-universally labeled cellulose from *Zea mays* was used. The cellulose was labeled to 97 atom percent by IsoLife (Wageningen, The Netherlands). In this experiment, a tracer approach was used; a small amount of cellulose was added so that cellulose degradation could be studied without altering the microbial community.

Total (^{12}C and ^{13}C) Gas and ^{13}C-labeled gas sampling

The cellulose experiment was terminated at 22 days. Thus, the ^{13}C-gas data from the cellulose-amended experiment was calculated from 0 to 22 days. The timescale of the *A. barbata* litter-amended experiment was 38 days. To compare the ^{13}C-gases produced in the *A. barbata* litter-amended and cellulose-amended experiment, the µmol C gas values for each replicate of the *A. barbata* litter-amended samples were calculated using measurements taken up to day 22.

GeoChip

I compared the carbon degradation potential of the *A. barbata* litter- and cellulose-amended experiments separately from the cellulose degradation potential of the two experiments. Since only the gene probes involved in cellulose degradation had been analyzed in the cellulose-amended experiment, the gene probes were re-analyzed to include carbon degradation and organic remediation gene probes. The organic remediation gene probes detected in the cellulose experiment were curated using the same groups used to curate organic remediation gene probes detected in the *A. barbata* litter-amended experiment. Thus, the carbon degradation and organic remediation gene probes detected in the *A. barbata* litter-amended experiment were compared to the carbon degradation and organic remediation gene probes detected in the cellulose-amended experiment. Hereafter, the composition and abundance of detected carbon degradation and curated organic remediation gene probes will be referred to as carbon degradation potential.

In the *A. barbata* litter-amended experiment, the carbon degradation (including the gene probes involved in cellulose degradation) and organic remediation gene probes had been analyzed. From this data set, a sub-dataset was created, containing only the gene probes involved in cellulose degradation. This data subset from the *A. barbata* litter-amended experiment was compared to the detected gene probes involved in cellulose degradation detected in the cellulose-amended experiment. Hereafter, the composition and abundance of detected cellulose degradation gene probes will be referred to as cellulose degradation potential. All GeoChip 4 data presented here were ordinated using non-metric multidimensional scaling (NMDS) in R (version 3.0.2) using the vegan package.

454 Pyrosequencing

In the cellulose experiment, to include the majority of samples, the 454 Pyrosequencing data generated from the samples were rarefied to 710 sequences per sample. To compare the 454 Pyrosequencing data between the *A. barbata* litter-amended and cellulose-amended experiments, the *A. barbata* litter-amended experiment samples were also rarefied to 710 sequences per sample. Detrended Correspondence Analysis (DCA) was used to ordinate the weighted unifrac distances of samples using the vegan package of R (version 3.0.2).

Statistical Analysis

All statistical analysis was performed using the native build packages and the vegan package in R (version 3.0.2) unless otherwise specified. The adonis function was used to determine the effect of redox condition and amendment on the variation observed in measurements from the composition and abundance of GeoChip 4 gene probes and the composition and abundance of detected OTUs. The Bray distance measure was used for all adonis tests. Students T tests and analysis of variance using Tukeys Honestly Significant Difference as a multiple means comparison were used to determine the variation in measurements from the ^{13}C-gas measurements. Analysis of variance, using Tukey's Honestly Significant Difference (TukeyHSD) test as a multiple means comparison test,

A Mantel test was used to correlate the cumulative gases, ^{13}C-gases, and the first axis scores from Principal Coordinates Analysis (PCoA) of the microbial community and GeoChip 4 data. For the microbial community abundance data, only the qPCR measurements taken up to day 22 were used. As a technique, PCoA analysis determines the main linear trends in data such as the composition and abundance of GeoChip probes. The PCoA scores generated can be used to order the samples. The PCoA scores for the first axis represent an ordering of the samples that explains the most variation in the data. When using PCoA scores in a correlation between GeoChip data (composition and abundance) and cumulative CO_2 production, it is the dominant linear trend in the composition and abundance of GeoChip probes that is being correlated to cumulative CO_2 production. Microbial community qPCR abundance, weighted unifrac distances of OTUs detected by 454 Pyrosequencing and carbon degradation potential data was first ordinated using Principal Coordinates Analysis (PCoA), and the scores for the first PCoA axis were recorded. Weighted unifrac distances of the OTUs detected in the 454 Pyrosequencing data are hereafter referred to as weighted unifrac distances. The cumulative gas data and total ^{13}C-gas data, and the first axis PCoA scores from the qPCR abundance, 454 Pyrosequencing and carbon degradation potential data were correlated using the mantel test in the vegan package of R. For the correlation between the carbon degradation and cellulose degradation potentials, the detected cellulose degradation gene probes were removed from the carbon degradation gene probe set. The cellulose degradation gene probes are a subset, comprising approximately 6%, of the carbon degradation gene probes detected in each experiment. The cellulose degradation gene probes were removed so that the carbon degradation and cellulose degradation potentials would be independent data sets. The Bray distance measure was used for all mantel tests.

Results and Discussion

Effect of redox condition on organic carbon mineralization

In this section, I will discuss the effects of redox condition on *A. barbata* litter and cellulose mineralization to CO_2, CH_4 and ^{13}C-gases.

Redox condition appeared to be a controlling factor in cumulative CO_2 production. The pattern of cumulative CO_2 was similar regardless of whether *A. barbata* litter or cellulose were added (Figure 1, Table 1A). More CO_2 was produced in the oxic condition, followed by the fluctuating

redox condition and the anoxic condition. The oxic condition produced significantly more CO_2 than the fluctuating redox condition only in the cellulose-amended experiment. If there was an overall synergy between oxic and anoxic periods, I would expect to have observed greater CO_2 production in the fluctuating redox condition. The idea that the fluctuating redox condition is the simple sum of aerobic and anaerobic process is not supported by this data. Otherwise, the CO_2 produced under the fluctuating redox condition would be approximately half that of the oxic condition in both experiments.

Amendment affected CO_2 production only in the *A. barbata* litter-amended experiment (Table 1B). Amendment of soil samples with *A. barbata* litter resulted in a substantial increase in cumulative CO_2 over the unamended samples. However, the pattern of CO_2 production in the *A. barbata* litter-amended samples was the same as the unamended samples. That is, more CO_2 was produced in the oxic condition, followed by the fluctuating condition and the anoxic condition.

In the fluctuating redox condition, a higher percentage of *A. barbata* litter was mineralized to ^{13}C-CO_2 than cellulose ($p < 0.05$) (Table 2). Similar percentages of *A. barbata* litter and cellulose were mineralized to ^{13}C-CO_2 over 22 days under the static conditions ($p < 0.05$). The percent of ^{13}C-labeled carbon mineralized to ^{13}C-CO_2 does not appear to be affected by the amount of carbon added in these two experiments or the quality (C:N ratio) of the added material.

The amount of substrate added was 0.5 g (16.6 mmol carbon (C)) per 8 g dry weight equivalent of soil in the *A. barbata* litter-amended experiment and 0.1 g (3.5 mmol C) per 8 g dry weight equivalent of soil in the cellulose-amended experiment. According to Singh et al. (2012), approximately 30% of the dry weight of an *Avena* sp. is cellulose. Thus, of the 16.6 mmol of *A. barbata* C added, approximately 5.4 mmols C was cellulose. Different amounts of material were added in the initial setup in part because the ^{13}C enrichment was different (*A. barbata*, 2 atom percent; cellulose, 97 atom percent).

In contrast to cellulose, *A. barbata* litter was a source of carbon and nitrogen. The C:N ratio of organic matter has long been used as a measure of substrate quality. In general, as the C:N ratio of a substrate decreases, the degradability increases. Indeed, N additions have been shown to increase the rate of cellulose degradation (Pankratov et al 2011). Exogenous N was not added to the cellulose-amended experiment. The pattern and percentage of substrate mineralized to CO_2 are not significantly greater in the *A. barbata* litter-amended experiment, except under the fluctuating condition. Thus the addition of N in *A. barbata* litter did not appear to simply explain the pattern of CO_2 mineralization observed.

The pattern of mineralization of added organic C was affected differently by redox condition in each experiment (Table 2). In the *A. barbata* litter-amended experiment, more labeled *A. barbata* litter was mineralized to CO_2 in the fluctuating redox condition. However, this result is significant at $p < 0.05$ only compared to the oxic condition (Table 2); there was no statistical difference between the plant material mineralized to ^{13}C-CO_2 in the anoxic and fluctuating redox conditions. In the cellulose-amended experiment, more ^{13}C-cellulose was mineralized to ^{13}C-CO_2 under the anoxic condition. The percent cellulose mineralized to ^{13}C-CO_2 was not statistically different at $p < 0.05$ under oxic and fluctuating redox conditions.

Mineralization of added organic carbon to ^{13}C-CO_2 was observed under the anoxic condition in both experiments at 22 days. This suggests that anaerobic microorganisms are active during the initial stage of organic carbon mineralization in these soils. Previous research has also suggested that anaerobic decomposition in LEF soil is important (DeAngelis et al 13). In the cellulose-amended experiment, greater cellulose mineralization to CO_2 may be due to efficient cellulose degradation by cellulosomes, supra-molecular, cell-associated structures produced by anaerobic microorganisms. Greater mineralization *of A. barbata* litter to CO_2 in the anoxic condition may also be due to enhanced decomposition of the cellulose component of the litter. In addition, both cumulative CH_4 and the percent ^{13}C-CH_4 from *A. barbata* litter was substantially greater in the anoxic condition. CH_4 was not detected in the cellulose-amended experiment and so ^{13}C-CH_4 was assumed to be zero.

These experiments suggest that anaerobic microorganisms are active during the initial stage of decomposition in this soil. In addition to the percent of ^{13}C-labeled *A. barbata* litter and cellulose mineralized to CO_2 in the anoxic condition, CH_4 was produced under oxic conditions in the *A. barbata* litter-amended experiment. This suggests that anaerobic decomposition processes occurred under an oxic headspace. Previous research has demonstrated that methanogenesis, a strict anaerobic process, occurred under an oxic headspace in LEF soils (Teh et al 2005), probably in anoxic microsites in the soil.

CH_4 production was observed only in the *A. barbata* litter-amended experiment. The cellulose-amended experiment may have been terminated too early to observe significant production of CH_4. Because a larger amount of C (mmol) was added in the *A. barbata* litter-amended experiment, the demand for terminal electron acceptors was probably greater than in the cellulose experiment. Thus, methanogenesis may have become a favorable process earlier in the *A. barbata* litter-amended experiment due to the exhaustion of other terminal electron accepting processes.

The ^{13}C-CO_2 produced in the *A. barbata* litter-amended experiment was positively correlated with the cumulative production of CO_2 and CH_4 (Table 5). However, the ^{13}C-CO_2 produced in the cellulose-amended experiment was not correlated with cumulative CO_2 production. This suggests that in the *A. barbata* litter-amended experiment, *A. barbata* litter was one of the main sources of carbon mineralized. While in the cellulose-amended experiment, other autochthonous carbon pools were the major sources of carbon for mineralization (see also Fig 1B).

The effect of redox condition on carbon and cellulose degradation potential

In the previous section, the effect of redox condition on organic carbon mineralization was discussed. In this section, I will expand the discussion of the effect of redox condition to carbon and cellulose degradation potentials as measured by the GeoChip 4.

Carbon degradation potential was significantly affected by redox condition when either *A. barbata* litter or cellulose was added (Figure 2, Table 3). In NMDS ordinations of the composition and abundance of carbon degradation gene probes, two loose clusters are evident; one aerobic and one anaerobic (Figure 2). These observations support the idea that there was a discernable difference in the carbon degradation potentials under oxic and anoxic conditions.

Carbon degradation potential was not consistently correlated to indices of carbon mineralization. In the *A. barbata* litter-amended experiment, carbon degradation potential was correlated to

cumulative CH_4 production (Table 5). However, GeoChip 4 gene probes involved in methanogenesis were not included in this analysis. This correlation suggests that there was carbon degradation potential present in the anoxic condition that enhances methanogenic activity. The carbon degradation potential (gene probes) measured could represent genes encoding for enzymes that produce simple carbon compounds that methanogens can use.

There was no correlation between carbon degradation potential and the ^{13}C-CO_2 or CO_2 produced in the *A. barbata* litter- or cellulose-amended experimental samples (Table 5). Production of CO_2 is considered to be a 'broad' process (Schimel 1995). That is, there are many enzymatic pathways involved in the production of CO_2. The carbon degradation potential is a relative measure of carbon degradation genes contained on the GeoChip; the GeoChip provides no quantitative information on the absolute number of degradative genes present. Since the GeoChip provides an assessment of the relative presence of different decomposition genes but not of the abundance of the genes, it may not be surprising that carbon degradation potential, as used here, was not correlated with CO_2 mineralization.

The cellulose degradation potential in the *A. barbata* litter-amended experiment was significantly affected by redox condition (Figure3A, Table 3). The pattern of cellulose degradation potential in NMDS ordination space of the *A. barbata* litter experiment was similar to the pattern of carbon degradation potential in the *A. barbata* litter experiment; the cellulose degradation genes separated into two loose groups, one aerobic and the other anaerobic. In the cellulose-amended experiment, cellulose degradation potential was also significantly affected by redox condition (Table 3). However, no pattern based on redox condition was apparent in NMDS ordination space (Figure 3B).

The majority of the cellulose degradation potential detected in both experiments resulted from glycosyl hydrolase (GH) gene probes (*A. barbata*; 85%, cellulose; 80%). GHs are grouped into families based on sequence homology (Henrissat and Davies 1997). That is, a GH from an aerobic fungi, an aerobic bacterium and an anaerobic bacterium can all belong to the same family. The most abundant GH gene probes detected belonged to GH family 1, 5, and 9. Both bacterial and fungal GH sequences are represented in these very common GH families. Because of this, it is unlikely that an aerobic or anaerobic degradation potential signature would be detected in ordination space.

Cellulose degradation potential measured in the *A. barbata* litter-amended experiment was correlated to cumulative CH_4 production (Table 5). However, in the cellulose-amended experiment, neither carbon or cellulose degradation potential were correlated to indices of mineralization, (^{13}C-CO_2 production, cumulative CO_2 or CH_4 production).

Cellulose degradation potential was correlated to carbon degradation potential in both experiments (Table 5). Recall that the cellulose degradation gene probes were removed from the carbon degradation gene probe set prior to using the correlation statistic. Redox condition explained a significant amount of variation in the composition and abundance of carbon degradation and cellulose degradation potentials. Redox condition may have been the dominant factor ordering both the carbon and cellulose degradation gene probes in the samples. This would explain the strong significant correlation between carbon and cellulose degradation potential in both the *A. barbata* litter- and cellulose-amended experiments.

Bacterial community composition

In the previous sections, the effect of redox conditions on organic carbon mineralization and carbon degradation potential was examined. In this section, the effect of redox condition on bacterial community composition will be discussed, as well as links between bacterial community composition, organic carbon mineralization and carbon degradation potential.

In most upland soils, the predominant decomposers of plant material are fungi (Berg and McClaugherty 2008). In the upland tropical forest soils used in these experiments, the frequency and duration of anoxic or low redox conditions in the bulk soil may result in a decrease in fungal abundance compared to other soils (Blazewicz et al 2014). In some sites in the LEF, bacteria have been observed to be a dominant component of the microbial community using PLFA (Cusack et al 2011). Thus, for these experiments, I focused on bacterial community composition.

The effect of redox condition on bacterial community composition depended on the substrate added. Redox condition affected bacterial community composition in the *A. barbata* litter-amended experiments (Table 4). However, redox conditions did not affect the bacterial community composition in the cellulose-amended experiment (Figure 4B, Table 4).

The difference in the effects of redox conditions on bacterial community composition could be due to the length of time each experiment was run. At 38 days, the *A. barbata* litter-amended experiment was 16 days (1.4 times) longer than the cellulose-amended experiment (22 days).

Amendment affected the bacterial community composition in the *A. barbata* litter-amended experiment, but not in the cellulose-amended experiment (Figure 4, Table 4). This likely reflects the differences in the C-sources added, namely differences in the composition and in the ease of degradation.

Comparatively, *A. barbata* litter is a more diverse carbon substrate than cellulose. As a substrate, *A. barbata* litter is composed of several pools of carbon, some probably more easily degradable than cellulose. Different carbon pools could represent different bacterial substrate niches. Thus, a substrate with several different pools of carbon, such as *A. barbata* litter, could probably support a more diverse community than cellulose alone.

Cellulose is considered a relatively recalcitrant component of plant material (Berg and McClaugherty 2008). The rate limiting step in cellulose degradation is breaking down the cellulose fibers into usable sugars (Gallert and Winter 2005, Noike et al 1985). If cellulosomes were the primary means of degrading cellulose in the cellulose-amended experiment, then the sugars produced would have probably been concentrated near the cellulosome-producing bacteria and would have been less available to other bacteria. Thus, only a very limited portion of the bacterial community composition would be affected by cellulose-amendment, also potentially contributing to the lack of correlation between cellulose-amendment and bacterial community composition.

Bacterial community composition was significantly correlated to $^{13}C\text{-}CO_2$ in the *A. barbata* litter-amended experiment. This correlation suggests that the bacterial community had a dramatic response to the presence of *A. barbata* litter, but not to cellulose. This could be because cellulose, as a main constituent of plant material, is a frequent carbon source in this soil. *A. barbata* litter is uncommon in the LEF, and as such it represents a somewhat exotic, labile

carbon source not usually encountered by the bacterial community. Bacterial members specializing in degradation of the carbon sources released during the initial phase of decomposition probably contributed to the correlation of the bacterial community composition to ^{13}C-CO_2.

Bacterial community composition was significantly correlated to cumulative CO_2 production in both experiments (Table 5). Because the amount of cumulative CO_2 was significantly affected by redox condition and bacterial community composition was correlated to cumulative CO_2, the dominant linear trend in the composition and abundance of bacteria in both experiments was probably redox condition.

Bacterial community composition was also significantly correlated to cumulative CH_4 in the *A. barbata* litter-amended experiment. This further underlines the potential importance of compositional and abundance changes in the anaerobic bacterial community in this soil.

Bacterial community composition was not correlated to carbon or cellulose degradation potential in either experiment (Table 5). The lack of correlation between bacterial community composition and carbon (or cellulose) degradation potential was probably due to the functional redundancy of carbon and cellulose degradation in the bacterial community.

An alternative explanation for the lack of correlation between bacterial community composition and carbon (or cellulose) degradation is that the bacterial community was only part of the microbial community capable of carbon and cellulose degradation in soil. The GeoChip contains gene probes that originated from bacterial, fungal and archaeal sequences (He et al 2012). Fungal community composition was not determined in these experiments. Perhaps carbon (and cellulose) degradation potential would have a stronger correlation to the combination of fungal and bacterial community composition.

Conclusion

I investigated the impacts of static oxic, static anoxic, and fluctuating redox conditions on the mineralization of cellulose and whole plant tissue as well as the decomposition gene profiles representing the carbon degradation potential. The questions that these experiments were designed to answer are:

Question 1: Does the soil redox condition affect organic carbon mineralization? If so, does the soil redox condition affect mineralization of distinct organic matter pools differently?

Question 2: Does the redox condition effect carbon degradation potential, as measured by the GeoChip 4?

Question 3: Are there links between bacterial community composition, redox condition and carbon degradation potential?

Redox condition affected the mineralization of distinct organic substrates, *A. barbata* litter and cellulose, differently. However, under anoxic conditions, mineralization to CO_2 was observed for each substrate, which indicates that anaerobic microbes play an important role in plant material and cellulose degradation when the LEF soils experience anoxic periods.

Redox conditions also affected carbon and cellulose degradation potentials. The effect of redox condition on carbon and cellulose degradation potential in the *A. barbata* litter- and cellulose-

amended experiments were similar. The carbon and cellulose degradation potential results from both experiments suggest that an aerobic and an anaerobic suite of genes comprising the carbon degradation potential differ in abundance or composition. Carbon degradation potential from the *A. barbata* litter, but not the cellulose-amended experiment, was correlated with ^{13}C-CH_4, again indicating that anaerobic microbes are important in plant material and cellulose degradation in these soils.

Redox condition affected the composition and abundance of the taxa detected in the *A. barbata* litter, but not the cellulose-amended experiment. In addition, *A. barbata* litter mineralization was coupled to bacterial community composition. The correlations between *A. barbata* litter mineralization and bacterial community composition may be due in part to the bacterial community responding to the release of soluble sugars from decomposing *A. barbata* litter. Cellulose mineralization and cellulose degradation potential in this soil appear to be independent of bacterial community composition. This is probably due to the microbial and redox independent, sequence-based organization of the cellulose degrading enzymes, glycosyl hydrolases. A relationship between carbon (and cellulose) degradation potential and bacterial community composition was not supported in either experiment. This suggests two possibilities, neither of which can be distinguished from the data. First, in terms of carbon and cellulose degradation, the bacterial community was functionally redundant. Or, because the bacterial community was a subset of the total microbial community, a more comprehensive comparison would be between the microbial (bacterial and fungal) community composition and the carbon and cellulose degradation potential.

Overall, redox condition affects carbon and cellulose degradation potentials in the LEF soils. Greater mineralization of organic matter into ^{13}C-gases in the fluctuating redox condition in both experiments would also indicate synergy between aerobic and anaerobic processes. The ^{13}C-gases produced in the fluctuating redox condition in the *A. barbata* litter-amended experiment were not significantly greater than those produced in the anoxic condition at the 22 day time point analyzed in this chapter. Although, after 38 days, the ^{13}C-gases produced in the fluctuating redox condition in the *A. barbata* litter-amended experiment were significantly greater than in the oxic and anoxic conditions (Chapter 2). Thus, it is likely that synergy between the aerobic and anaerobic carbon and cellulose degradation potentials does occur in this soil, but it is not apparent within the first few weeks of decomposition.

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Table 4-1. Effect of A) redox condition and B) amendment on variation observed in cumulative CO_2 measurements from the *A. barbata* litter-amended experiment and cellulose-amended experiment.

A

	Condition			
	A. barbata		Cellulose	
Amendment	r^2	p	r^2	p
Amended	0.262	**0.001**	0.363	**0.016**
Unamended	0.131	**0.001**	0.046	**0.005**

B

	Amendment			
	A. barbata		Cellulose	
Condition	r^2	p	r^2	p
Oxic	0.446	**0.001**	0.00008	0.942
Anoxic	0.405	**0.001**	0.0005	0.856
Fluctuating	0.498	**0.001**	0.00004	0.976

Table 4-2. ^{13}C-Gas fluxes from amended samples from experiments with added ^{13}C-labeled *A. barbata* litter and ^{13}C-labeled cellulose. Each value is an average of three biological replicates. The values are calculated from ^{13}C-gas measurements taken through day 22 for both experiments. Lowercase letters denote significant differences between measurements within an experiment using a TukeysHSD test ($p < 0.05$). Uppercase letters denote differences between values of both experiments in one redox condition using a Student's T test at $p < 0.05$.

Substrate	Condition	% ^{13}C-substrate → CO_2	% ^{13}C-substrate → CH_4 ‡	% ^{13}C in gas	^{13}C-CO_2:^{13}C-CH_4
Avena barbata shoots (2 ap)	Oxic	0.20 a A	0.67 h	0.19 s	28.62 j
	Anoxic	0.21 ab A	5.24 i	0.26 st	4.05 k
	Flux	0.33 b B	1.89 h	0.35 t	17.81 jk
Cellulose from *Zea mayes* (97 ap)	Oxic	0.15 x A	——	0.15 d	——
	Anoxic	0.20 y A		0.20 e	
	Flux	0.15 x A		0.15 d	

‡ values * 10^{-3}
ap; atom percent

Table 4-3. Effect of redox condition and amendment on the variation observed in the composition and abundance of carbon degradation and cellulose GeoChip 4 gene probes in the *A. barbata* litter- and cellulose-amended experiments.

Substrate	Carbon degradation potential				Cellulose degradation potential			
	A. barbata		Cellulose		*A. barbata*		Cellulose	
	r^2	p	r^2	p	r^2	p	r^2	p
Condition	0.233	**0.001**	0.160	**0.014**	0.146	**0.024**	0.151	**0.038**
Amendment	0.077	0.059	0.045	0.373	0.071	0.069	0.047	0.366
Condition*Amendment	0.193	**0.004**	0.120	**0.017**	0.119	0.064	0.150	**0.013**

Table 4-4. Effect of redox condition and amendment on the variation observed in composition and abundance of OTUs detected in the *A. barbata* litter- and the cellulose-amended experiments. The samples in both experiments were rarefied to 710 sequences per sample.

	A. barbata		Cellulose	
	r^2	p	r^2	p
Condition	0.172	**0.028**	0.155	0.200
Amendment	0.324	**0.001**	0.137	0.076
Condition*Amendment	0.167	**0.002**	0.553	0.770

Table 4-5. Mantel tests correlating gas data, microbial community data and GeoChip 4 data. The first axis PCoA scores from the community abundance data (qPCR), community composition (weighted unifrac distances) and carbon and cellulose degradation potential (GeoChip 4 gene probes) were used in correlations with the gas data. Bold values indicate significance at $p < 0.05$.

A. *A. barbata*

	^{13}C-gas		total CO_2		total CH_4		Community abundances		Community composition		Carbon degradation potential	
	r	p	r	p	r	p	r	p	r	p	r	p
^{13}C-gas												
total CO_2	0.740	**0.001**										
total CH_4	0.386	**0.001**	0.176	**0.027**								
Community abundances	0.006	0.336	-0.063	0.902	0.086	0.186						
Community composition	0.728	**0.001**	0.947	**0.001**	0.166	**0.031**	-0.032	0.623				
Carbon degradation potential	0.089	0.091	-0.005	0.452	0.624	**0.001**	0.024	0.385	0.008	0.385		
Cellulose degradation potential	0.026	0.298	-0.047	0.719	0.551	**0.004**	0.010	0.422	-0.013	0.498	0.954	**0.001**

B. Cellulose

	^{13}C-gas		total CO_2		Community abundances		Community composition		Carbon degradation potential	
	r	p	r	p	r	p	r	p	r	p
^{13}C-gas										
total CO_2	-0.032	0.709								
Community abundances	0.016	0.357	-0.043	0.789						
Community composition	0.055	0.141	0.187	**0.012**	-0.006	0.510				
Carbon degradation potential	0.042	0.183	-0.033	0.846	0.004	0.359	0.078	0.133		
Cellulose degradation potential	0.040	0.057	0.019	0.926	0.026	0.902	0.088	0.681	0.706	**0.001**

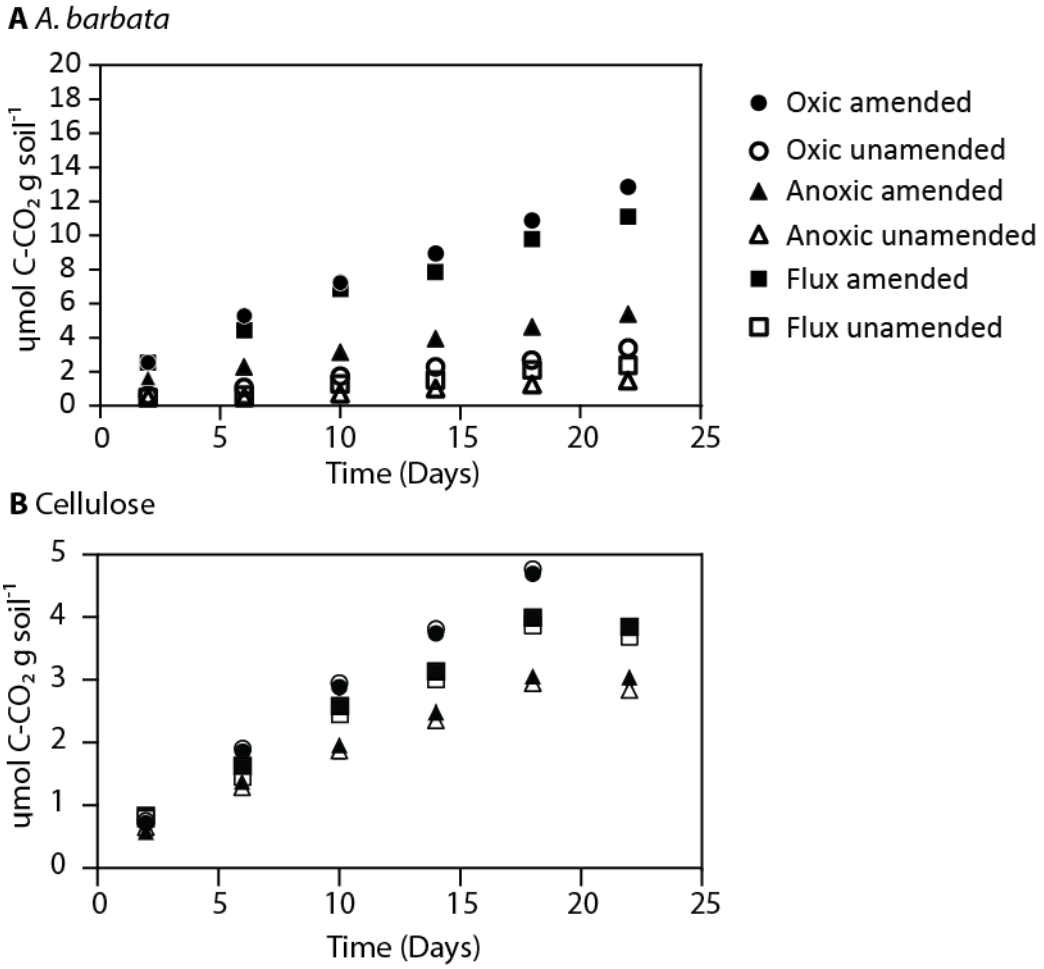

Figure 4-1. CO_2 produced over time in the A) *A. barbata* litter-amended and the B) cellulose-amended experiments.

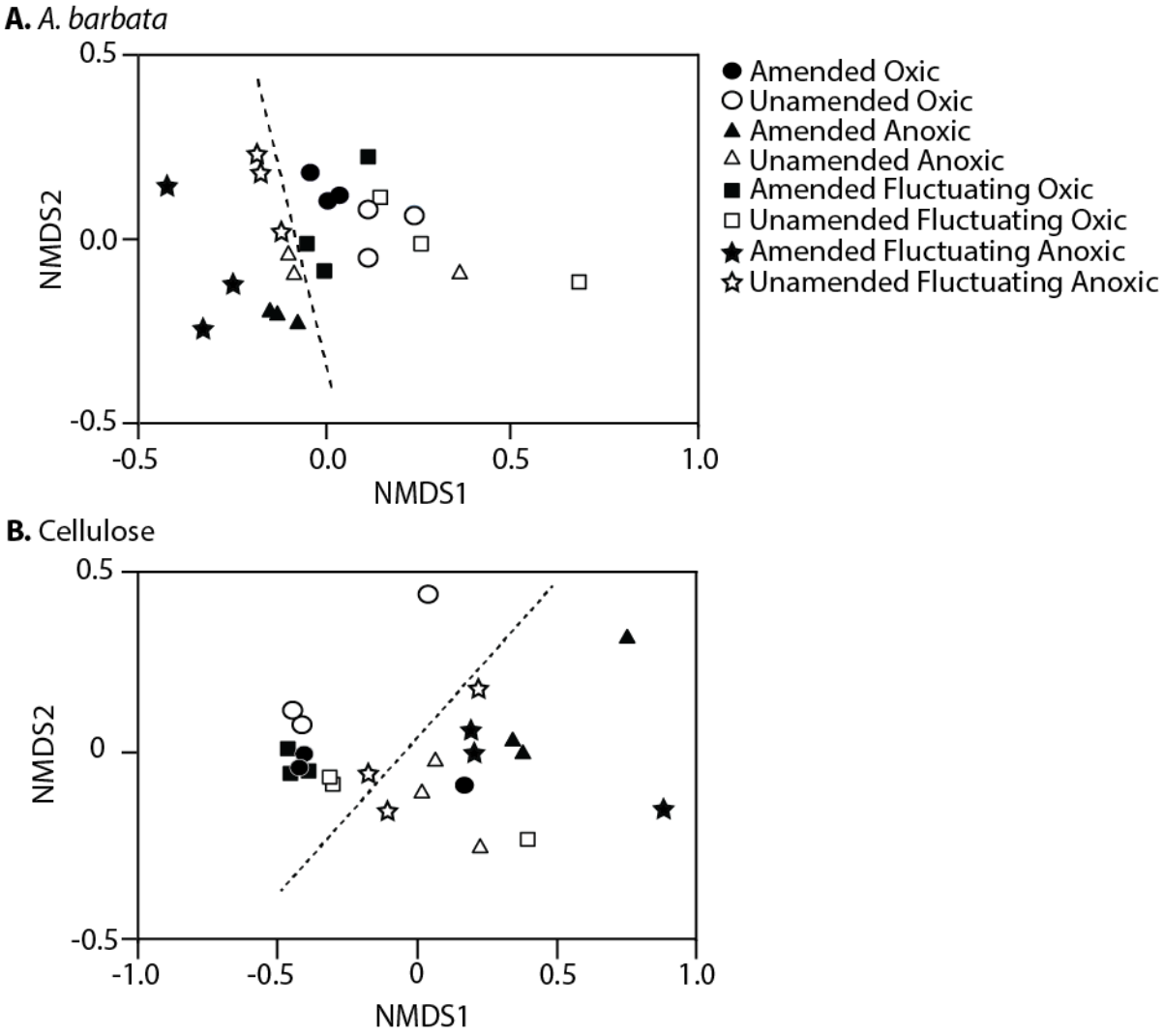

Figure 4-2. NMDS of carbon degradation gene probes of final time point samples from experiments amended with (A) ^{13}C-labeled *A. barbata* litter and (B) ^{13}C-labeled cellulose. Carbon degradation potentials from samples under the oxic condition are separated from carbon degradation potentials from samples under the anoxic condition by a dashed line.

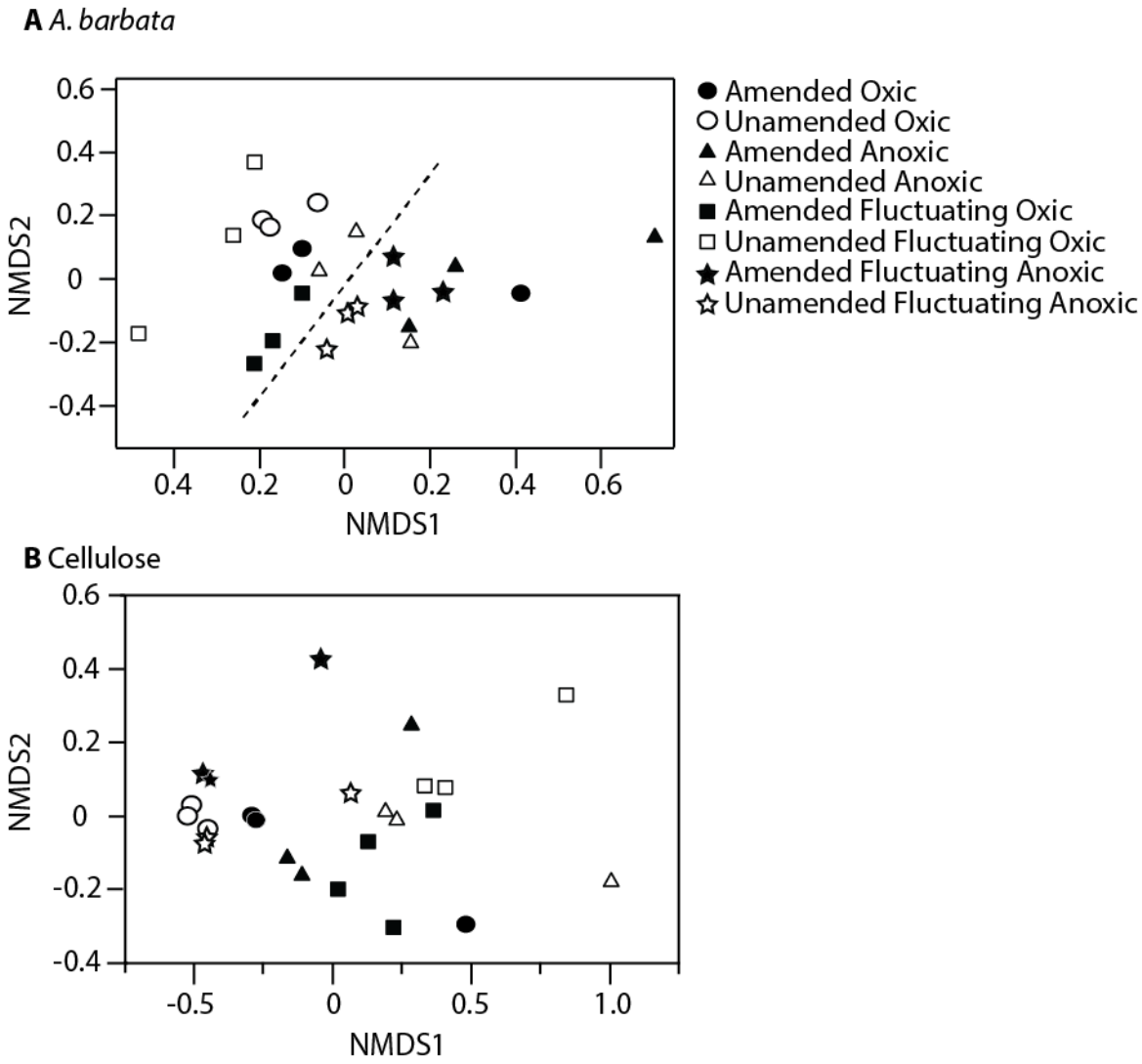

Figure 4-3. NMDS of cellulose degradation gene probes of final time point samples from experiments amended with (A) *A. barbata* litter and (B) cellulose. Dashed line in (A) separates the cellulose degradation potential recovered from samples under oxic conditions from samples under anoxic conditions.

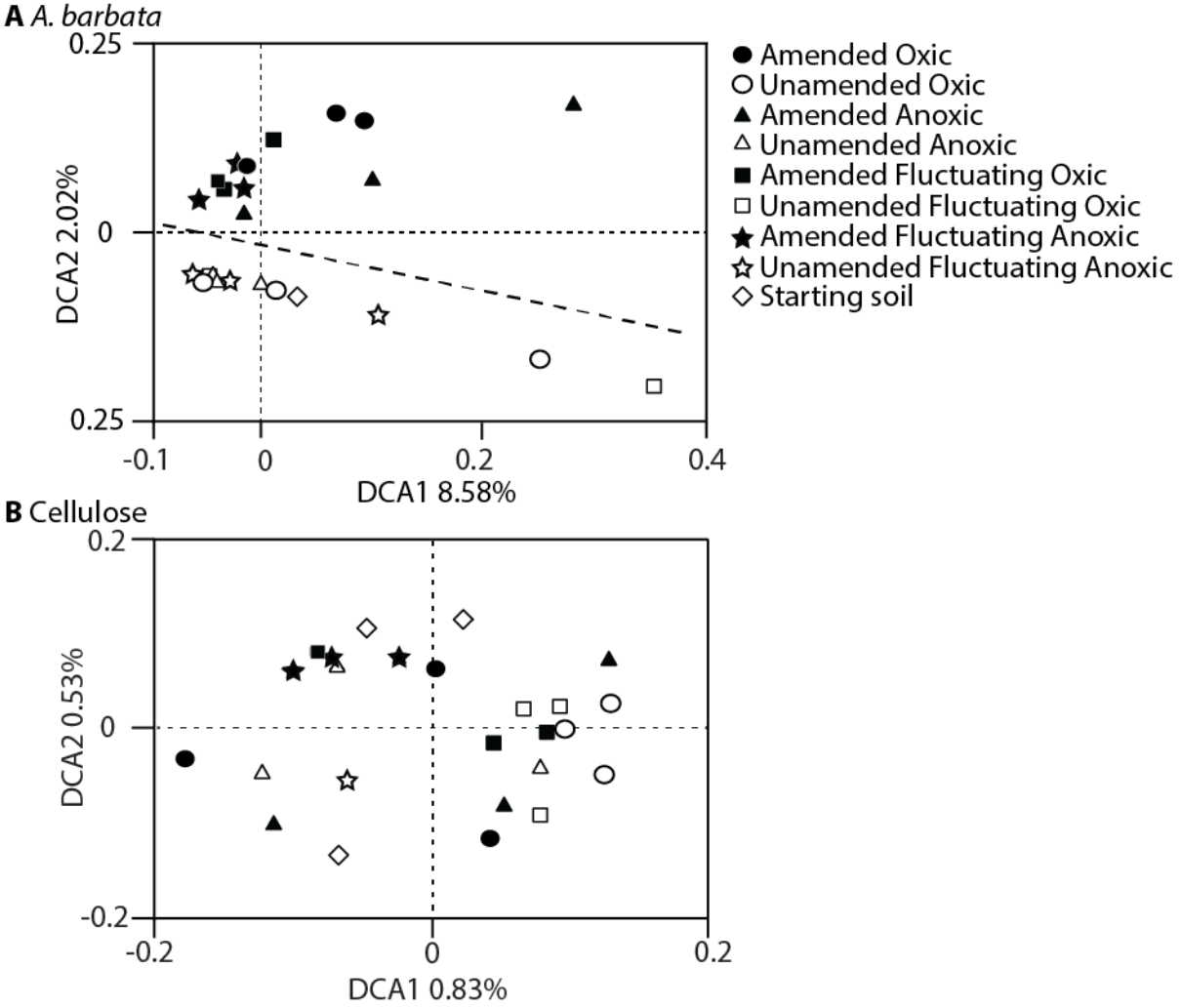

Figure 4-4. Detrended Correspondence Analysis of weighted unifrac distances of OTUs from the final time point samples from the (A) *A. barbata* litter- amended experiment and the (B) cellulose-amended experiment. All samples were rarefied to 710 sequences per sample. Dashed line in (A) separates the samples into amended and unamended groups.