THE EFFECT OF AN EXTREME RESTORATION APPROACH ON MICROBIAL CARBON CYCLING IN A RESTORED SUBTROPICAL WETLAND

By

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To my family for unconditional love and support

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LIST OF ABBREVIATIONS

ACD	Aerobic CO_2
ANCD	Anaerobic CO_2
BGA	β-glucosidase
CSR	Complete soil removal
C	Carbon
CBH	Cellobiohydrolase
CH_4	Methane
CO_2	Carbon dioxide
DDI	Distilled deionized water
DOC	Dissolved organic carbon
ENP	Everglades National Park
HID	Hole-in-the-Donut
LOI	Loss on ignition
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
MBP	Microbial biomass phosphorus
MET	Methanogenesis
MUF	Methylumbelliferone
MUF P	Phosphatase activity
N	Nitrogen
OC	Organic carbon
OM	Organic matter
P	Phosphorus
Pi	Bioavailable inorganic phosphorus

P_o	Bioavailable organic phosphorus
PPM	Parts per million
TC	Total carbon
TKN	Total kjeldahl nitrogen
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
Res00	Restored in 2000 site
Res03	Restored in 2003 site
SE	Standard Error

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Wetlands are important in global carbon (C) cycling with a high potential for C storage while simultaneously serving as the largest single CH_4 source. Consequently, understanding C cycling in wetlands is crucial, especially in systems impacted by disturbances such as soil alteration, nutrient addition or fire. This study investigated factors regulating microbial C processing in subtropical wetlands in the context of both nutrient levels (phosphorus (P)) and fire.

The study site was the Hole-in-the-Donut (Everglades National Park, Florida), a fire-adapted ecosystem undergoing restoration from agricultural impact. Following restoration (complete soil and vegetation removal), C cycling was elevated and methanogenesis was C-limited. In native sites, methanogenesis was stimulated by C and P, however, a greater response to P was observed. Amending soils with various C-sources indicated that hydrogenotrophic methanogens were dominant and substrate-limited, while fermentation appeared to be P-limited. Thus, nutrients appeared to indirectly regulate CH_4 production by affecting substrate availability.

Fire is also an ecological driver affecting both C and P forms and availability. In a field fire experiment, C cycling was stimulated initially; however, minimal heat transfer coupled with constant microbial biomass suggests nutrients were a regulating factor. Incubation of soils with fire residues demonstrated that char stimulated gas production in all soils, while ash stimulated production only in native soils. Comparison of these results with direct C and P additions, suggests char acts as a source of C and P while ash simulates an addition of P.

This work highlights the role of disturbance as a determinant of wetland greenhouse gas (GHG) emissions. During succession at restored sites, decreasing P availability with time will reduce gas production leading to increased C storage. Contrasting effects of fire residues indicates that residue proportion (determined by fire frequency and intensity) will determine net C cycling, where C sequestered as char could be offset by elevated GHG production within 1.5 and 7 months in native and restored sites, respectively. These results demonstrate a complex relationship between GHG production, fire, and the alteration of soil nutrient availability, and the need for further investigation to better manage these systems C cycling for GHG mitigation.

CHAPTER 1
INTRODUCTION

In wetland ecosystems understanding the soil carbon (C) cycle is of extreme importance because these systems regulate the balance between C storage and C loss. One factor which governs this balance is the shift between dry and flooded conditions (Wright and Reddy 2001). Under dry conditions microbial decomposition can proceed at an accelerated rate in response to the electron acceptors associated with aerobic conditions (D'Angelo and Reddy 1999). In contrast, under flooded conditions soil pores become water saturated inducing anaerobic conditions. Because oxygen is removed, less efficient electron acceptors are used for respiration and increased reliance on fermentation pathways is observed. This results in decreased rates of organic matter (OM) decomposition and elevated C storage.

Through decomposition processes C is lost from the system as either CO_2 or CH_4. Under aerobic conditions, CO_2 is produced whereas both CO_2 and CH_4 can be produced under anaerobic conditions. These gases are referred to as greenhouse gases meaning they have the ability to absorb infrared radiation and trap heat potentially accelerating climate change (*reviewed by* Whalen 2005). While the abundance of CO_2 is much greater than CH_4 (380 and 1.8 ppm, respectively), CH_4 has a greater global warming potential relative to CO_2 suggesting it is more potent as a greenhouse gas (IPCC 2007).

When interested in studying CO_2 and CH_4production wetlands are often ecosystems of focus. The reason the relationship between wetlands and C gas production is studied in detail is because these systems produce large quantities of CO_2 and CH_4. Prevalence of production (i.e. greater CO_2 or CH_4 production) will depend on

multiple environmental factors. Furthermore, the largest single source of CH_4 originates from wetland ecosystems (IPCC 2007). For this reason, understanding the factors regulating production of this greenhouse gas is crucial. Under anaerobic conditions, specialized groups of Archaea are responsible for producing CH_4 through respiration and fermentation pathways (i.e. methanogenesis). The focus of my work was to investigate different factors which may regulate C cycling including soil disturbance (restoration), nutrient limitation, substrate type/availability, and fire.

Nutrient limitation can have drastic effects on microbial decomposition and activity in wetlands. For example, soil OM decomposition can be regulated by P availability (Cleveland et al. 2002). Makino et al. (2003) correlated higher microbial growth rates with elevated bacterial P concentrations. Limitation of P is frequently evaluated by assessing ratios of soil C:N:P and ratios of microbial biomass (C:N:P). A compilation of 186 observations suggests an average soil C:N:P ratio of 186:13:1 and a microbial biomass ratio of 60:7:1 (Cleveland and Liptzin 2007). Wetlands containing a higher soil N:P ratio, indicative of P limitation, have been associated with lower methanogenic populations (Chauhan et al. 2004) and lower CH_4 production rates (Smith et al. 2007) relative to soils with a lower N:P ratio. While decreased methanogenesis in P-limited systems has been observed (Pivničková et al. 2010; Smith et al. 2007) higher P concentrations have been shown to inhibit methanogenesis (Conrad et al. 2000) suggesting the relationship between P and CH_4 production is complex.

Understanding the interactions between P and C is also important because P-limitation can regulate C mineralization (Cleveland et al. 2002). Cleveland et al. (2002) investigated the relationship between P and C in a low-P tropical forest concluding

addition of C resulted in a greater production of CO_2 when amended with P. This suggests, in the tropical forest ecosystem P was regulating decomposition and C gas production. In general, methanogens are separated into two categories (acetoclastic or hydrogenotrophic) based on metabolic pathways (Whalen 2005). Through acetoclastic methanogenesis CH_4 is produced following acetate fermentation. In contrast, hydrogenotrophic methanogens depend on respiratory pathways in which CO_2 (e- acceptor) and H_2 (e- donor) are necessary precursors. Methanogens are unable to use complex molecules (Ferry 1993) thus reliance on higher trophic level organisms for OM decomposition is crucial. Because multiple methanogenic pathways are known and reliance on higher trophic level microbes is plausible, soils amended with varying C sources may directly (acetate and formate) or indirectly (butyrate and glucose) increase methanogenic substrate availability resulting in elevated CH_4 production if the methanogens were substrate limited.

To determine if methanogens were C or P-limited concurrent experiments with soils amended with both substrate (*see above*) and P were conducted. Analyzing gaseous decomposition end products (CO_2 and CH_4) following addition of P, varying substrates, and concurrent substrate and P additions, can provide an opportunity to decipher processing pathways and determine if different anaerobic microbial groups (fermenters and methanogens) are limited by either of these factors. This knowledge can be applied to enhance our understanding of how natural events which may alter soil nutrient concentrations can affect anaerobic OM decomposition and regulate CO_2 and CH_4 production.

Another factor which may alter nutrient concentrations and C cycling is fire (natural and prescribed) in response to OM oxidation and fire residue addition. Variation in nutrient concentrations and availability is largely controlled by fire temperatures. Fire temperature can reach >1500°C (*reviewed by* Neary et al. 1999) thus, variable effects on microbial activity and soil nutrient concentrations are common. Nutrients (post fire) have one of three fates; to (i.) remain incompletely combusted as OM (char), (ii.) be lost to the atmosphere or (iii.) be re-deposited as completely combusted OM (ash) (Boerner 1982). Furthermore, nutrients can respond differently to varying fire temperatures. For example, as temperatures near 500°C, N loss will approach 50% (Neary et al. 1999) however, temperatures in excess of 500°C are necessary for changes in P to be evident (White et al. 1973) although the availability of P can be altered (Smith et al. 2001). These changes in soil nutrients post fire can have a direct effect on microbial activity.

Investigating the effect of fire on biogeochemical processing in forested (Covington et al. 1992; Deluca and Sala 2006; Allison et al. 2010; White 1986) and grassland ecosystems (Toma et al. 2010; Knapp et al. 1998; Xu and Wan 2008) has been studied in detail however, limited data in wetlands have been compiled (Levine et al. 1990; Nakano et al. 2006; Smith et al. 2001).

Research linking fire residues (nutrient source) and the associated effect on soil biogeochemical processing are minimal. Production of char, post fire, is common in forest and prairie ecosystems (Hart et al. 2005) and can increase aerobic conditions by increasing soil porosity (Oguntunde et al. 2008). This residue can increase soil pH, soil organic carbon (OC) concentrations, and total N concentrations (Zhang et al. 2010)

thus, altering soil biogeochemical processing. In addition, this residue can serve as a substrate rich habitat for some microbial communities (Pietikäinen et al. 2000).

Decreased anaerobic conditions following char addition (increased soil porosity) may reduce CH_4 production resulting in lowered CO_2 and CH_4 emissions however published results are highly variable. Char addition resulted in elevated CH_4 emissions in rice paddies (Zhang et al. 2010) although, trends in decreasing CH_4 production were observed in agricultural fields and tropical savannas (Karhu et al. 2011; Rondon et al. 2006). This variation likely originated from differences in the biochar material and charring process.

When comparing fire residues, ash differs from char in nutrient composition. Ash is produced through complete combustion (high temperatures) which results in a C and N limited material which remains elevated in P concentrations (Hogue and Inglett 2012; Qian et al. 2009). Studies have determined the lability of P in the ash may be dependent on the initial vegetation (Hogue and Inglett, 2012; Qian et al. 2009) suggesting nutrients in each ashed material may differ. Thus, in ecosystems which are P-limited, addition of ash may alter microbial activity resulting in elevated decomposition rates and subsequently elevated C gas production. While researchers have quantified gaseous flux post fire (*see above*) few studies have investigated the relationship between nutrient additions, originating from fire residues, and the importance these additional nutrients may have in fueling microbial processing in low- and high-P fire adapted ecosystems.

Case Study: The Hole-in-the-Donut Region of Everglades National Park (FL.)

Close to 50% of wetlands worldwide have been degraded or lost (Zedler and Kercher 2005). In response, wetland restoration has become a crucial mechanism to

reestablish wetland function. Restoration methods are highly variable including less invasive techniques such as vegetation manipulation (Smith et al. 2011, Andersen et al. 2006) or more rigorous procedures such as restoring hydrology (Acreman et al. 2007) or introducing prescribed fire (Toma et al. 2010; Hamman et al. 2008). When these techniques are unsuccessful in meeting restoration goals, extreme restoration strategies such as topsoil removal (Verhagen et al. 2001) or even complete soil removal (Dalrymple et al. 2003) are employed.

The Hole-in-the-Donut (HID) is located within Miami Dade County, Florida within Everglades National Park (ENP). Unlike the surrounding regions of ENP, the HID was heavily farmed prior to 1970. By 1975 all of the farmed land was purchased by ENP. After the farming ceased, colonization of an invasive vegetation (*Schinus terebinthifolius*) resulted in a reduction of native *Muhlenbergia capillaris* and sawgrass vegetation (*Cladium jamaicense* Crantz) due to out-competition. Unlike *Muhlenbergia* and the sawgrass sedge, *Schinus* is a tree with heights that can reach 12 m (Dalrymple et al. 2003). To remove the *Schinus*, multiple methods were used including addition of herbicides, mowing, cutting, burning etc. However, these methods of removal were unsuccessful and the *Schinus* vegetation would recolonize and dominate the ecosystem again (*reviewed by* Smith et al. 2011).

Upon closer examination, the soil associated with the dense *Schinus* stands was elevated in nutrient concentrations relative to the surrounding unfarmed soil (*Cladium* dominated) with 600% greater P concentrations (Orth and Conover 1975). Researchers wanted to investigate the relationship between the soil substrate and the proliferation of *Schinus.* Dalrymple et al. (2003) tried a unique approach to eliminate the *Schinus* and

discourage regrowth which included complete removal of both vegetation and the underlying soil. This approach was successful in removal of *Schinus*.

Following this restoration technique, the National Park began to scrape portions of the HID to bedrock (complete soil and vegetation removal) in a systematic approach. Once the land was scraped, it was abandoned and began to accumulate soil and re-vegetate naturally. This extreme restoration approach began in 1989 and is still continued today. Because the land was scraped at different intervals a mosaic of lands varying in restoration age and soil nutrient content are present.

Removal of the soil substrate affects both microbial and vegetation communities by eliminating the initial nutrient sources necessary for survival and re-introducing the concept of competition. Development of soil and re-vegetation is hypothesized to follow a distinct trajectory via primary succession (*reviewed by* Smith et al. 2011). Newly formed soils are hypothesized to be rich in available P and low in soil N concentrations. In contrast, older soils are typically limited by P (Walker and Syers 1976). This shift from N to P limitation with time is in response to the quantity and availability of these nutrients. The main source of P in soil originates from the degradation of rocks and minerals with little external input (Walker and Syers 1976). For this reason, a finite amount of P is available at any given site however, through microbial transformations changes in P availability will occur.

Based on soil differences following the extreme restoration tactic, the HID ecosystem is an ideal location to investigate factors which may regulate C gas production for several reasons, including:

1. Newly restored sites have high soil P concentrations relative to surrounding reference wetlands.

2. Restored sites are dominated by more woody vegetation such as *Baccharis halimifolia* when compared to reference wetlands which are dominated by *Muhlenbergia* and *Cladium* (sawgrass). This may have an effect on C quality and availability in these sites.

This work was focused in two restored (restored in 2000 and 2003) and a reference site. To investigate the effect of extreme restoration, sites which were recently restored were chosen as opposed to sites restored earlier (1989) to highlight initial differences following restoration (soil nutrient and microbial) with the goal of investigating the following objectives:

Dissertation Objectives

Objective 1. Quantify the effect of extreme restoration on C gas production (Chapter 2).

Hypothesis: Restored soils would be elevated in C gas production relative to production from reference sites. Elevated decomposition would be related to C and P availability in the restored sites.

Objective 2. Investigate the influence of P on different metabolic pathways involved in anaerobic decomposition in low-P (reference) soils. Through simultaneous addition of C and P we can determine the direct and indirect stimulation resulting in CH_4 production (Chapter 3).

- Hypothesis$_1$: Addition of P will stimulate anaerobic C gas production through direct stimulation of methanogens.
- Hypothesis$_2$: Addition of P will stimulate anaerobic C gas production though indirect stimulation of higher trophic level organisms (i.e. elevated substrate availability).

Objective 3. Determine if C gas production is regulated by fire in a fire adapted ecosystem (Chapter 4).

- Hypothesis$_1$: OM decomposition (CO_2 and CH_4 production) will increase in low-P (reference) soils in response to fire.

- Hypothesis$_2$: OM decomposition (CO_2 and CH_4 production) will not be affected in high-P (restored) soils.

Objective 4. Investigate the role of fire residues (ash and char) on C gas production (Chapter 5).

- Hypothesis$_1$: Decomposition would be stimulated by ash (P addition) in the low-P (reference) soil although no response would be evident in the high-P (restored) soil.

- Hypothesis$_2$: In response to char (carbon addition), decomposition would be stimulated regardless of initial soil P content.

CHAPTER 2
PATTERS AND CONTROLS OF ANAEROBIC SOIL RESPIRATION AND METHANOGENESIS FOLLOWING EXTREME RESTORATION OF CALCAREOUS SUBTROPICAL WETLANDS

Carbon Cycling Following Wetland Restoration

Worldwide, wetlands have decreased by approximately 50% (Zedler and Kercher 2005). Subsequently, restoration efforts have increased to both reestablish essential wetland functions and achieve a “no net loss” standard. An assortment of restoration efforts have been tested and vary depending on site characteristics and restoration goals. These approaches range from less invasive techniques such as manipulating vegetation (Andersen et al. 2006; Smith et al. 2011), to more intensive techniques such as restoring hydrology (Acreman et al. 2007) or introducing prescribed fire (Doren et al. 1991; Hamman et al. 2008; Toma et al. 2010). Wetlands which have accumulated excess nutrients, such as agricultural lands, are commonly targeted for mitigation (Van Dijk et al. 2004; Aldous et al. 2007). However, excessive fertilization can result in residual nutrients bound within (Orth and Conover 1975) or released from soil (Van Dijk et al. 2004; Aldous et al. 2007). One restoration technique to remove excess nutrients is topsoil removal (Klimkowska et al. 2007; Tallowin and Smith 2001; Verhagen et al. 2001; Ross et al. 1982; Aerts et al. 1995) which can include the extreme measure of complete soil removal (CSR) to bedrock (Dalrymple et al. 2003).

Soil disturbance, following this restoration technique, can have dramatic effects on ecosystem function. Changes in vegetation (composition and abundance) or organic matter (OM) structure during soil disturbance may disrupt the ecosystem C balance (source/sink) (*reviewed by* Kimmel and Mander 2010) altering greenhouse gas emissions (Andersen et al. 2006; Bortoluzzi et al. 2006; Jauhiainen et al. 2008).

Furthermore, soil disturbance may encourage anaerobic conditions in addition to increasing C lability resulting in wetland environments primed for methanogenic processing (i.e. CH_4 production) (Höper et al. 2008). In addition, nutrient availability can also alter CH_4 production (Wright and Reddy 2001; Smith et al. 2007) suggesting changes in nutrient content following soil disturbance can alter these processes. With concentrations of atmospheric CO_2 and CH_4 currently surpassing 380 and 1.7 ppm, respectively (IPCC 2007) understanding controls on C sources and sinks is crucial. Wetlands emit the largest single source of CH_4 (Lelieveld et al. 1998) furthermore tropical and southern regions account for more than 70% of the total CH_4 produced from these ecosystems (IPCC 2007). Methane fluxes from tropical wetlands (Costa Rica) range from 33-263 g-C $m^{-2}y^{-1}$ which is substantially higher than fluxes from temperate (Ohio) wetlands (Mitsch et al. 2012).

Because soil disturbance can effect C gas emissions and southern regions are important CH_4 sources, understanding the relationship between C cycling and restoration in these regions is essential. An extreme example of the top soil removal technique is CSR, a restoration method used in the Hole-in-the-Donut (HID) region of Everglades National Park (Florida, U.S.A., Dalrymple et al. 2003). The HID contains calcareous marl prairies (wetlands) which were heavily farmed from the early 1900's through 1975 (Smith et al. 2011). Continuous fertilization and soil disturbance led to invasion by *Schinus terebinthifolius* Raddi after farming ceased (*as described in detail by* Ewel et al. 1982). Mechanically-cleared sites were allowed to revegetate naturally through primary succession involving a reduction in the level of available P and a shift from nitrogen (N) to P limitation when approaching the native/reference status (Smith et

al. 2011). Previous work by Smith et al. (2007) investigated the patterns of soil CH_4 production following CSR in the HID, concluding there was an increase in CH_4 production in restored soils compared to those of undisturbed reference areas. This suggests CH_4 production in restored sites may be tightly coupled to soil P availability in this ecosystem. While Smith et al. (2007) had a greater focus on methanogenic composition, our study focused on investigating relationships between CO_2 and CH_4 production and available inorganic P, available C, C enzyme activity (substrate liberation), and differences in microbial biomass carbon (MBC).

Differences in restored and reference site vegetation (*see site description*) suggest C quality may also regulate CH_4 production in addition to P. Vegetation type and C quality has been associated with differing CH_4 production in Blue Cypress Marsh (Inglett et al. 2011) and may be a regulator in the HID system. In wetland soils, methanogens rely on usable C substrates released from respiring or fermenting microbes (Conrad 1999) which decompose complex OM through enzyme activity (Sinsabaugh 1993). However, nutrient availability can also alter microbial decomposition (Hogg et al. 1994), and as a result, OM accumulation and P level could be primary factors leading to the increased methanogenic potentials observed by Smith et al. (2007). Addition of P can result in elevated CH_4 production (Pivničková et al. 2010); however, other studies have observed decreased production (Drake et al. 1996) or no effect (Bridgham and Richardson 1992) on this process. This suggests the effect of P on CH_4 production may depend on initial soil P concentrations and soil OM content.

The variable results of these studies suggest the relationship between anaerobic C processing and P is unclear, but the potential coupling of P biogeochemistry and C

dynamics has important implications for the ability of these restored systems to function as a sink/source of greenhouse gases. On a microbial level, it has been hypothesized high P concentrations are necessary to maintain elevated growth rates (Sterner and Elser 2002; Makino et. al. 2003). This suggests that if high soil P concentrations correlate with high CH_4 production in restored sites a decrease in available P with restoration age may result in decreasing CH_4 emission with time.

Concentrations of N in restored soils are more similar to reference concentrations when compared to the large discrepancy between restored and reference soil P (Inglett et al. 2011b). This suggests the main nutrient regulator of these processes may be P. For this reason, we conducted the following study to quantify the effect of extreme restoration on anaerobic C gas production from restored versus reference HID soils during the wet season. In addition, we quantified soil organic carbon (OC) content, MBC, and β-glucosidase enzyme activity (BGA) to investigate the relationship between soil C parameters and P content. In two separate microcosm experiments, soils were incubated with C or P to assess the relationship between anaerobic C gas production and nutrient availability.

Methods

Site Description

The Hole-in-the-Donut (HID) region of Everglades National Park (Miami Dade County, Florida, U.S.A.) consists of abandoned crop fields overlying a marl prairie wetland ecosystem (Figure 2-1). These soils are Entisols which consist of Biscayne and Perrine marl with poor to poorly drained characteristics (USDA 1996). Restoration of this area began in 1989 with mechanical removal of invasive vegetation followed by complete soil removal to bedrock (Smith et al. 2011; Dalrymple et al. 2003). In the

current study, plots restored in years 2000 (Res00) and 2003 (Res03) were compared to a reference (never farmed) marl prairie wetland located within the HID vicinity. Based on restoration time and prior fertilization history each site varied in biogeochemical properties including soil nutrients, soil depth, and microbial parameters (Table 2-1). Vegetation in the reference site is typical of oligotrophic Everglades dominated by *Cladium jamaicense* Crantz (Loveless 1959) with *Muhlenbergia capillaris* and *Andropogon* and *Schoenus* spp. In contrast, restored sites are dominated by a mixture of invasive and undesirable vegetation including *Baccharis halimifolia* and to a lesser extent *Ludwigia* spp., *Salix* sp. and *Typha domingenesis*.

Soil Sampling

To encompass elevation variability, five approximately equidistant stations were selected along a 1.5 km transect within each of Res00, Res03, and reference wetlands (Figure 1; Liao and Inglett 2012). Soil depth at each site was measured with a soil probe (30 total readings per sampling location). In October 2009 (wet season), three composite samples (5 grabs each) from 0-5 cm depth (or until bedrock) were collected using a spatula from each transect sampling location (15 composite samples per site). To estimate bulk density additional samples (n=3) were taken using a sharpened steel tube. Soils were placed on ice and returned to the University of Florida for processing within two days of collection.

Soil samples were sieved through a 2 mm mesh to remove rocks and roots. Representative aliquots of field-moist soil were removed from each sample and analyzed for multiple microbial properties including microbial biomass carbon (MBC, N and P), ß-glucosidase enzyme activity (BGA), anaerobic respiration (CO_2) and methanogenic potentials (CH_4). Separate soil aliquots were dried at 105°C for three

days, hand ground with a mortar and pestle and used for determination of total carbon, nitrogen, and phosphorus (TC/TN/TP).

Soil Nutrients and Microbial Biomass

Soil nutrient analyses were conducted by the Wetland Biogeochemistry laboratory at the University of Florida, Gainesville, FL. Microbial biomass C/N/P was analyzed by extraction following chloroform fumigation (Vance et al. 1987). Briefly, soil samples were extracted with 0.5 M K_2SO_4 (MBC and N) or 0.5 M $NaHCO_3$ (MBP) after being incubated with (fumigated) and without (control) chloroform. Filtered (0.2 µm) extracts were analyzed for total extractable organic carbon (TOC) using a 5050A TOC auto-analyzer (Shimadzu Corp., Columbia, MD; EPA method 415.1). The difference in extractable TOC between the fumigated and non-fumigated samples was considered MBC following correction with an extraction efficiency (K_{EF}) of 0.37 (Sparling et al. 1990). For MBN determination, extracts were analyzed following Kjeldahl digestion on a Technicon auto-analyzer (EPA method 351.2). The difference between fumigated and non-fumigated TKN was considered MBN following correction with an extraction efficiency of (K_{EF}) of 0.54 (Brookes et al. 1985). Extracts were analyzed on a Technicon auto-analyzer (EPA method 365.1).

Soil TN and TC were analyzed using a Thermo FlashEA 1112 series NC soil analyzer (Thermo Fisher Scientific, Inc., Waltham, MA). Loss on ignition (% LOI) of soil samples was determined after combustion at 550°C for 3-4 hours. Soil OC was estimated using LOI assuming 45% OC content factor derived from total OM (Wright et al. 2008). Soil TP was determined following sequential combustion at 550°C for a 4 hour period and dissolution of remaining ash with 6 M HCl (Anderson 1976). Extracts were analyzed colorimetrically for reactive P using a Technicon™ Autoanalyzer III

(SEAL Analytical, Mequon, WI) (EPA method 365.1). Extractable inorganic P (P_i) was determined in soil samples using 0.5 M $NaHCO_3$ using EPA method 365.1 (Kuo 1996).

Anaerobic Respiration and Methanogenesis

Anaerobic CO_2 and CH_4 potentials were quantified during laboratory incubations. Unfortunately, due to shallow soil depth in restored sites field gas measurements were unfeasible in these study sites. Approximately 2 grams dry weight soil and 10 mL of double deionized water (DDI) were combined in 30 mL serum tubes (n=15 per site), sealed with butyl rubber stoppers and aluminum crimp tops, flushed with oxygen free N_2 to ensure anaerobic conditions and stored in the dark. The bottles remained sealed throughout the incubation and gas headspace (100 µL) was periodically measured (every 2 days prior to day 8 followed by weekly measurements) for CO_2 and CH_4 concentrations for 4 weeks.

Experimental C and P Addition

Additional bulk soils were collected from one sampling station at each site during February 2010 (C addition) and 2011 (P addition) for independent experiments to determine the effect of C and P availability on CO_2 and CH_4 production in soils of the three sites. These soils were processed as described previously and stored at 4˚C prior to initiating the experiment. To test C limitation, soils were amended with 10 mL of a 3.3 mM glucose solution prior to anaerobic incubation in the dark at 25°C. To test for P limitation, a 1 mM NaH_2PO_4 solution was added to lower the soil C:P ratio. In both tests, anaerobic respiration and methanogenic potentials of amended soils were compared with those of control soils incubated at the same time without added amendments. We acknowledge the production cannot be compared directly between

treatments; however, the purpose of these individual additions was to determine how they (C and P independently) affect CO_2 and CH_4 production.

Gas Analysis

Headspace CH_4 from experimental tube incubations was detected using a Shimadzu gas chromatograph-8a with a flame ionization detector (160°C detector, 110°C injection) using N_2 as the carrier gas and a 1.6 m (45/60) Carboxyn 1000 column (Supelco Inc., Bellefonte, PA). Headspace CO_2 was measured using a Shimadzu gas chromatograph-8a equipped with a thermal conductivity detector (120°C injection, 40°C detector), He as the carrier gas and a 1.9 m (80/100) Porapak-N column (Supelco Inc., Bellefonte, PA). Calibration curves were determined via standard gas mixtures (Scott Specialty Gases, Plumsteadville, PA) multiple times throughout each sampling event. Concentrations of sample CO_2 and CH_4 were determined based on calibration curves accounting for possible dissolved inorganic carbon in the calculations. Final values reported represent CO_2 or CH_4 accumulated over a four week period as mg C kg^{-1} soil. Methane production from surrounding marl Everglades soils was concentrated in the top 0-2 cm with negligible production deeper than 4 cm (Bachoon and Jones 1992). For that reason reporting gas production per gram soil as opposed to reporting gas flux (areal basis) would be more representative given the deeper soil depths in reference wetlands.

Enzyme Activity

Soil enzyme activity of β-glucosidase was measured fluorometrically with methylumbelliferone fluorophore on a Bio-tek® model FL600 plate reader (Biotek Instruments, Inc. Winooski, VT) following 100 fold soil dilution with DDI similar to Inglett et al. (2011b). Briefly, 500 µM substrate-fluorophore solution was incubated with soil for

2 hours in the dark. After incubation, fluorescence was measured at an excitation of 350 nm and emission of 450 nm. To account for any soil quenching, quenching curves were prepared separately for each sample. Enzyme activity was determined as fluorescence based on methylumbelliferone standard curves. Results were reported as nmoles MUF g^{-1} dw soil h^{-1}.

Data Analysis

All statistical analyses were performed in JMP vs. 7.0 (SAS institute, Cary NC). Site differences (microbial respiration and CH_4 production, BGA, and soil and microbial parameters or concentrations) were determined via one way analysis of variance (ANOVA). Similarly, the difference in gaseous production following amendment (C or P) was analyzed via one way ANOVA. In addition, differences in parameters within sites at different elevations were compared via one way ANOVA. Differences between means ($\alpha<0.05$) were assessed via Tukey-Kramer means comparison. Regression analysis with Pearson correlation coefficient was used to evaluate relationships between anaerobic processing (CO_2/CH_4 production and BGA) and both soil P (total and extractable) and soil C (organic C and extractable C).

Results

Soil Nutrients

No significant differences were detected in measured parameters between different elevation sampling stations, therefore all five transect locations from each site were pooled for analyses. Restored and reference soils varied greatly in basic characteristics including soil depth, pH, OC, TP, and P_i (Table 2-1). As a result of CSR restored sites accumulated an average of 2 cm soil at the time of sampling in comparison to an average 11.5 cm in reference wetlands. However, no difference in

bulk densities were observed between restored and reference sites. Reference soil pH averaged 8.0 in comparison to a more neutral (7.6) pH observed in restored wetlands. Soil OC in restored sites averaged 80 g kg^{-1} soil, 20 g kg^{-1} greater than OC in reference soils. Soil TN was lowest in Res00 when compared to Res03 and reference soils on average although this was not significant. Differences in site TP resulted in a soil P gradient from Res03 (972±83 mg kg^{-1} soil) to Res00 (638±45 mg kg^{-1} soil) and reference (143±9 mg kg^{-1} soil) wetlands. Similar to soil TP, $NaHCO_3$ extractable P_i was greatest in restored wetlands following the P concentration gradient Res03 (14.4 mg kg^{1} soil) >Res00 (8.6 mg kg^{1} soil) >reference (1.6 mg kg^{1} soil) wetlands (Table 2-1). Ratios of OC:TP and OC:Pi were 3-5 times higher in reference when compared to restored soils.

Microbial Parameters

Microbial biomass C from reference soils was similar to both restored sites, but when comparing restored sites, MBC in Res00 was greater than Res03 (Table 2-1). Microbial biomass N was elevated in restored wetlands compared to reference conditions (p<0.01; Table 2-1). Microbial biomass P was greatest in Res03 compared to Res00 and reference soils (p<0.05; Table 2-1). Similar to trends in MBP, BGA was greater from Res03 (262 nmol MUF g^{-1} dw soil h^{-1}) when compared to Res00 (161 nmol MUF g^{-1} dw soil h^{-1}) and reference (112 nmol MUF g^{-1} dw soil h^{-1}) soils.

Anaerobic C Loss (CO_2 and CH_4)

Both CO_2 and CH_4 production were greater from restored relative to reference soils (p<0.0001; Figure 2-2). Production of CO_2 averaged 2025 mg C kg^{-1} soil in restored sites in comparison to 430 mg C kg^{-1} soil from reference wetlands after 28 days (Figure 2-2). Regardless of restoration age, CH_4 production averaged 450 mg CH_4-C

kg^{-1} soil from restored wetlands in comparison to 90 mg CH_4-C kg^{-1} soil from reference wetlands after 28 day incubation (Figure 2-2). After 28 days CO_2-C:CH_4-C ratio from restored soils (4.9) were higher than ratios from reference wetlands (3.8) although this was not statistically significant (Figure 2-2).

Microbial and Soil Nutrient Correlations

Correlations were analyzed to determine the relationship of P to microbial C parameters. ß-glucosidase enzyme activity was positively correlated with both anaerobic CO_2 and CH_4 production (p<0.001 and p<0.0001, respectively). Strong positive correlations were observed between BGA and both TP and extractable inorganic P (P_i) (p<0.0001; Figure 2-3A, data not shown). Similar to observed trends between soil P and BGA, anaerobic CO_2 production was correlated with both soil P parameters (p<0.0001;Figure 2-3B, data not shown), however extractable P_i better explained trends in CO_2 production (TP and CO_2, R^2=0.40;P_i and CO_2, R^2=0.49). Similar to trends observed with BGA, positive correlations between CH_4 production and both soil P parameters were observed (p<0.0001; Figure 2-3C, data not shown).

Correlations between soil C parameters and BGA, anaerobic respiration, and methanogenesis were weaker than those observed with soil P parameters although all correlations were significant. Similar to soil P trends, BGA was positively correlated with both soil total and extractable OC (p<0.0001; Figure 2-3D, data not shown). Weak positive correlations were observed between anaerobic respiration potentials and soil C parameters (p<0.01; Figure 2-3E). Although the relationship between extractable OC and methanogenic potential was weak, strong correlations with soil OC (p<0.0001, R^2=0.41) suggests this parameter may be an important predictor of methanogenic trends (Figure 2-3F).

Effects of C and P Amendments

Glucose stimulated anaerobic respiration and methanogenic potentials from all sites (Figure 2-4). Production of CO_2 in glucose amended soils increased by 500 mg C kg^{-1} soil in restored soils and 700 mg C kg^{-1} soil in reference soils after 12 day incubation (Figure 2-4B). In contrast, a 10 and 3.5 fold increase in CH_4 production was observed from restored (Res00 and Res03) and reference soils, respectively (Figure 2-4A). As a result to C addition, the ratio of CO_2-C:CH_4-C decreased from 43 to 6 in Res00 and from 35 to 5 in Res03 (p<0.0001) while increasing in reference soils from 66 to 130 (Figure 2-4C).

When P was added to restored soils, there was no significant effect on anaerobic respiration or methanogenesis (Figure 2-5A,B). In contrast, both anaerobic respiration and methanogenic potentials were stimulated by P in reference soils, although the difference was only significant for methanogenesis (p<0.05; Figure 2-5A). Production of CH_4 increased from 2 to 6 mg CH_4-C kg^{-1} soil when amended with P in reference wetlands (p<0.001; Figure 2-5A). Ratios of CO_2-C:CH_4-C decreased from 222 to 116 in reference soils amended with P although no significant difference in ratios were observed from restored sites (Figure 2-5C). Ratios differed between restored sites (48 to 35 in Res00 and 8 to 5 in Res03); however, the reduction was uniform.

Discussion

Among the key aspects of restoration is the initial loss of nutrients and organic matter contained in the soil (Badiou et al. 2011, Ross et al. 1982). In contrast, soils of the restored HID areas in this study are rich in nutrients (residual un-removed P) with high productivity of pioneer species resulting in enhanced OM accumulation in the shallow young soils (Table 2-1) (Smith et al.2011; Inglett et al. 2011b; Smith et al.

2007). Site differences in OC and TP resulted in OC:TP ratios of 515 and 150 in the reference and restored soils, respectively. These values suggest the reference site is P-limited while the restored sites are not (McGill and Cole 1981). Additional support for nutrient limitation patterns includes the TN:TP ratio, which based on the global analysis by Cleveland and Liptzin (2007) suggest primarily N limitation in the Res03 site (N:P=15), co-limitation of N and P in the Res00 site (N:P=26), and strong limitation by P in the reference site (N:P=108) (Smith et al. 2011; Liao and Inglett 2012).

Nutrient limitation can result in alteration of microbial activity such as enzyme expression, anaerobic CO_2 production (Aerts and Toet 1997), and methanogenic processing (Bridgham and Richardson 1992; Adhya et al. 1998; Aerts and Toet 1997). For example, soil P concentrations have been shown to limit C mineralization in low-P peat soils (Amador and Jones 1995; Wright and Reddy 2001). In this study, BGA was significantly greater in restored (high-P) relative to reference (low-P) sites ($p<0.05$; Table 2-1), a trend which has been observed previously within the HID ecosystem (Inglett et al. 2011a). Enzyme activities for all sites were similar to those reported by Inglett et al. (2011a), and were similar to activities from surrounding freshwater subtropical marshes (Corstanje et al. 2007) Regulation of decomposition by enzyme activity is common (Sinsabaugh et al. 1992). Through hydrolysis, β-glucosidase cleaves the 1, 4 beta-linkage in cellobiose releasing glucose, an available simple sugar crucial to microbial processing, thus providing usable substrates for microbial degradation which could fuel aerobic, anaerobic, and methanogenic processing. Elevated BGA in the Res00 and Res03 soils suggests these sites may contain a greater labile C pool which is supported by a significant relationship between BGA and soil OC

(Figure 2-3D) (Sinsabaugh et al. 2008). However, OC content does not fully explain the difference in BGA between the soils of Res03 and Res00 where OC contents were similar (Table 2-1). One explanation for this could include differences in C forms or quality (e.g., lignin and cellulose) (DeBusk and Reddy 1998, 2005). Also, high soil nutrient concentrations have been shown to correlate with BGA (Eivazi and Zakaria 1993). For example, in subtropical wetlands, Prenger and Reddy (2004) observed positive correlations between BGA and both soil extractable P_i and TP in a Florida freshwater marsh, as did Penton and Newman (2007) in other areas of the Everglades. Similarly, in our study we observed a strong correlation between BGA and both soil TP and extractable P_i (p<0.0001; Figure 2-3A, *data not shown*) suggesting P availability may control BGA and subsequent decomposition rates.

Increased C availability in response to elevated BGA should ultimately result in elevated CO_2 and/or CH_4 potentials (Freeman et al. 1997). In support of this hypothesis, we observed greater anaerobic CO_2 and CH_4 production in restored site soils (p<0.01; Figure 2-2), as well as positive correlations between BGA and CO_2/CH_4 production (p<0.0001, *data not shown*). The rates of methane production in the reference area soils in this study were greater than those reported for low-nutrient soils in the HID (Smith et al. 2007), low-nutrient tropical marshes (Pivinčková et al. 2010), and other temperate wetlands soils (D'Angelo and Reddy 1999), but were similar to the ranges reported for low-nutrient northern Everglades wetlands (Wright and Reddy 2001) and Florida marshes dominated by *Cladium* (Inglett et al. 2011b). In contrast, CH_4 production in restored site soils were greater than those of restored HID soils reported

by Smith et al. (2007), and similar to the rates of the nutrient-impacted northern Everglades (Wright and Reddy 2001).

Trends in CO_2-C:CH_4-C production (Figure 2-2) suggest CO_2 was being utilized for hydrogenotrophic methanogenesis, a process noted in this and other Everglades ecosystems (Ogram et al. 2011; Smith et al. 2007; Chauhan et al. 2004). For example, Chauhan et al. (2004) reported an approximate 100 fold greater population of H_2/CO_2 methanogens when compared to acetoclasts in oligotrophic Everglades peat soil. Based on positive correlations between microbial activities (BGA, CO_2, and CH_4) and soil TP (p<0.0001; Figure 2-3A,B,C) and P_i (p<0.0001;*data not shown*), we conclude P availability is a dominant control on these processes. However, significant correlations between soil OC and BGA/CO_2/CH_4 (p<0.01; Figure 2-3D,E,F) and extractable OC and BGA/CO_2 (p<0.01;*data not shown*) suggest these parameters may also be important controls on these processes to a lesser extent than P.

Phosphorus has been shown to limit decomposition in bog ecosystems (Hogg et al. 1994), therefore we assessed limitation of anaerobic C processing by amending high- (restored sites) and low-P (reference site) soils with P. Stimulation of CO_2 production occurred in the reference (40% increase), but not in the restored soils, while CH_4 production increased in response to P addition in all soils, but was much higher in the reference soils (190% increase; p<0.0001; Figure 2-5). Decreases in ratios of CO_2-C:CH_4-C in P-amended reference soils (Figure 2-5C) further suggest methanogens in these sites may be directly limited by P and an increase in hydrogenotrophy is possible (Smith et al. 2007).

Previous studies conducted in both elevated- and low-P Everglades peat soils observed a negligible effect of P addition on CO_2 (Amador and Jones 1993) and CH_4 production (Drake et al. 1996) indicating P was not limiting anaerobic respiration in the short-term. Similar results in CH_4 production were observed by Bachoon and Jones (1992) in low-P marl soils, however in the marl soils of this study, we found P significantly enhanced CH_4 production during short-term incubations (Figure 2-5). This suggests another factor, such as C quality, may have been regulating these processes. In contrast, insignificant relationships observed between CH_4 and P addition in our high-P restored soils was consistent with short-term results from impacted Everglades wetlands investigated by Drake et al. (1996) likely suggesting methanogenesis is not P-limited in high-P Everglades soils. This suggests that anaerobic C processing is more strongly limited by P in the reference soils, but less so in the higher-P soils of the restored sites.

While correlations suggest soil P was driving site differences in anaerobic respiration and methanogenesis, soil C also appeared to explain site differences to a lesser extent. Similar to previous studies (Amador and Jones 1995; Bachoon and Jones 1992), both CO_2 and CH_4 production were stimulated by C addition (glucose) suggesting decomposition may be limited by C quality. In contrast with the P addition experiment where methane increased dramatically, the greatest response to glucose addition was observed for CO_2 (220% increase; Figure 2-4B) in reference soils. Also, increased processing through primary fermentation pathways is possibly evidenced by elevated CO_2-C:CH_4-C ratios in amended reference soils relative to controls. When glucose was amended to restored soils (both Res00 and Res03) a stronger response

was observed in CH_4 production (7 and 14 times the control in the Res00 and Res03, respectively) (Figure 2-4A) although stimulation of CO_2 production was also significantly enhanced (2.5 times the control in both sites).

The contrasting results in stimulation of CO_2 versus CH_4 production after addition of P and glucose indicates that P limitation dominates anaerobic C processing in the reference sites, while C availability limits CO_2 and CH_4 production in restored soils. It has been hypothesized high-P soils are limited to a greater extent by C substrates relative to low-P soils in Florida Everglades wetlands (Wright et al. 2009) suggesting anaerobic processing in restored (high-P) soils may be ultimately limited by C quality within the HID. The similar response of both CO_2 and CH_4 to either C or P addition in the reference soils also suggest that C substrate availability for methanogenesis (through fermentation) is limited by P.

Conclusions

Nutrient availability is a key regulator of decomposition and thus a key determinant of C greenhouse gas production in wetlands. Methane flux from restored sites, calculated using bulk density and soil depth, averaged 116 (10) g CH_4-C $m^{-2}y^{-1}$ which is within the reported range of emissions from tropical wetlands (Mitsch et al. 2012). Combined with the observation of negligible methane oxidation rates in marl Everglades ecosystems (King et al. 1990), potential rates may better resemble emission rates in the HID than other ecosystems. This study suggests P is a key regulator of CH_4 production in this ecosystem. As a result of CSR wetlands are hypothesized to shift from soil N to P limitation with restoration time.

Thus, if wetlands in this ecosystem follow this restoration trajectory, high CO_2 and CH_4 emissions from restored sites will likely decrease with time. In order to better

understand the hypothesized shift from C source to sink with age (Whiting and Chanton 2001) additional studies in this ecosystem are necessary to target CH_4 production pathways and C utilization to understand the interactions between soil P availability and C substrate acquisition.

Table 2-1. Select soil parameters from the Res00, Res03, and reference site within the Hole-in-the-Donut region of Everglades National Park during the 2009 sampling season

Study site		Res00	Res03	Reference
Lat./Long.		N25.379° W80.675	N25.383° W80.700	N25.376° W80.672
Soil Depth	cm	2.2(0.2)b	1.8(0.1)b	11.7(0.6)a
Bulk Density	$g\ cm^{3}$	0.5(0.0)a	0.6(0.1)a	0.5(0.0)a
pH		7.6(0.0)b	7.6(0.0)b	8.0(0.0)a
TN	$g\ kg^{-1}$	7.4(0.5)a	6.0(1.4)b	6.9(0.3)ab
OC*	$g\ kg^{-1}$	80(3.0)a	80(3.0)a	61.0(3.0)b
TP	$mg\ kg^{-1}$	638(45.0)b	972(83.0)a	143.0(9.0)c
Pi	$mg\ kg^{-1}$	8.6(0.6)b	14.4(1.3)a	1.6(0.1)c
MBC	$mg\ kg^{-1}$	3388(222.0)a	2850(172.0)b	2973(121.0)ab
MBN	$mg\ kg^{-1}$	285(22.0)a	296.0(16.0)a	208.0(15.0)b
MBP	$mg\ kg^{-1}$	22.4(2.4)b	30.4(1.5)a	16.8(1.2)b
BGA**		161.0(15.0)b	262.0 (28.0)a	112(10)b

Soil Depth range Res00 (0-5 cm), Res03 (0-4.5 cm) and reference (2-22.5 cm)
*OC calculate from LOI with a 45% OC from OM factor
**β-glucosidase enzyme activity (nmoles g^{-1} dw soil h^{-1})

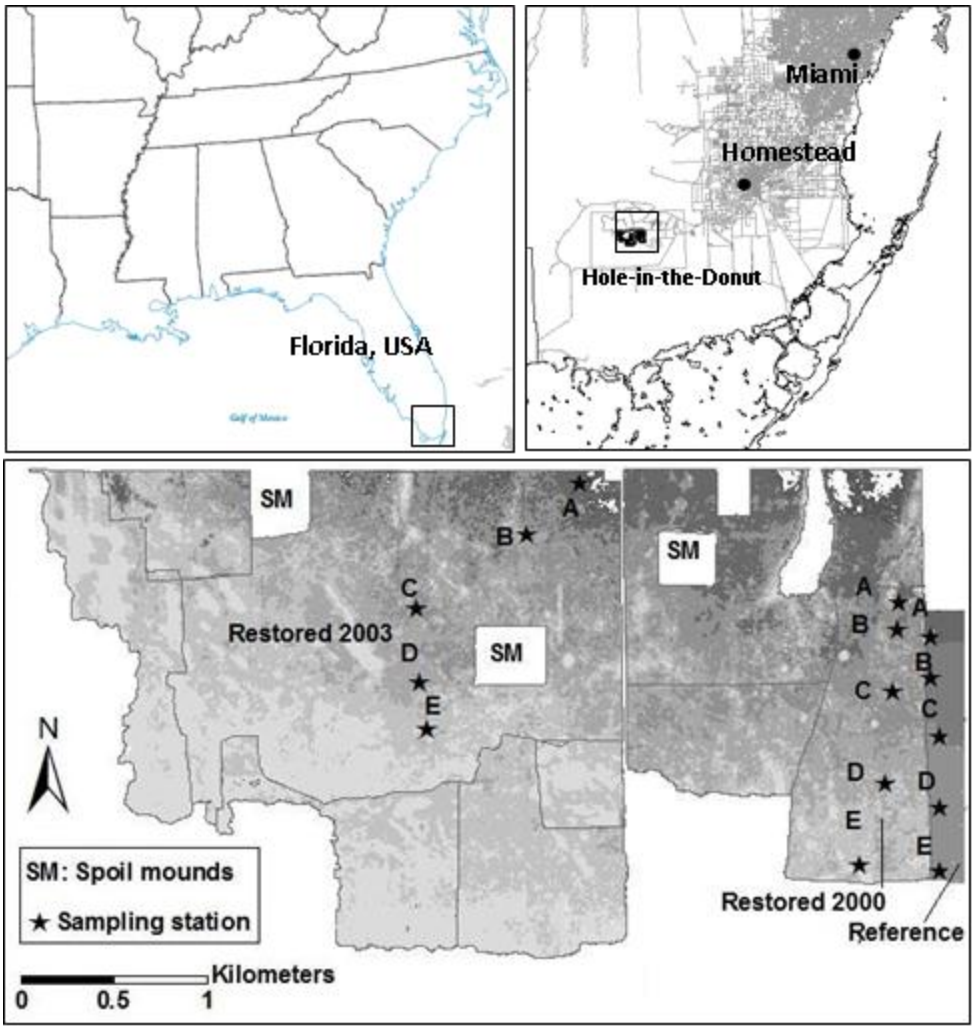

Figure 2-1. Hole-in-the-Donut sampling transect map for Res00, Res03 and reference sites (figure from Liao and Inglett 2011)

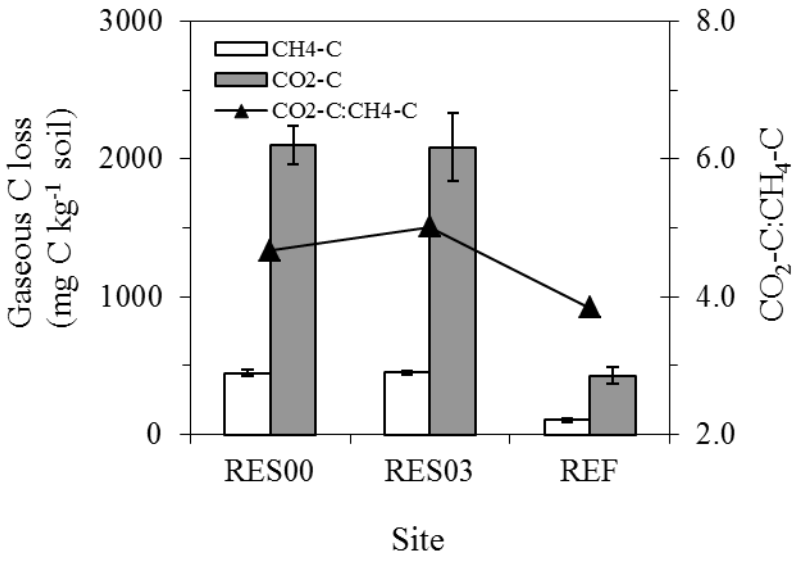

Figure 2-2. Production of CO_2, CH_4, and CO_2-C:CH_4-C ratios following 28 day incubation from Res00, Res03, and reference wetlands within the HID region of Everglades National Park during the 2009 sampling season

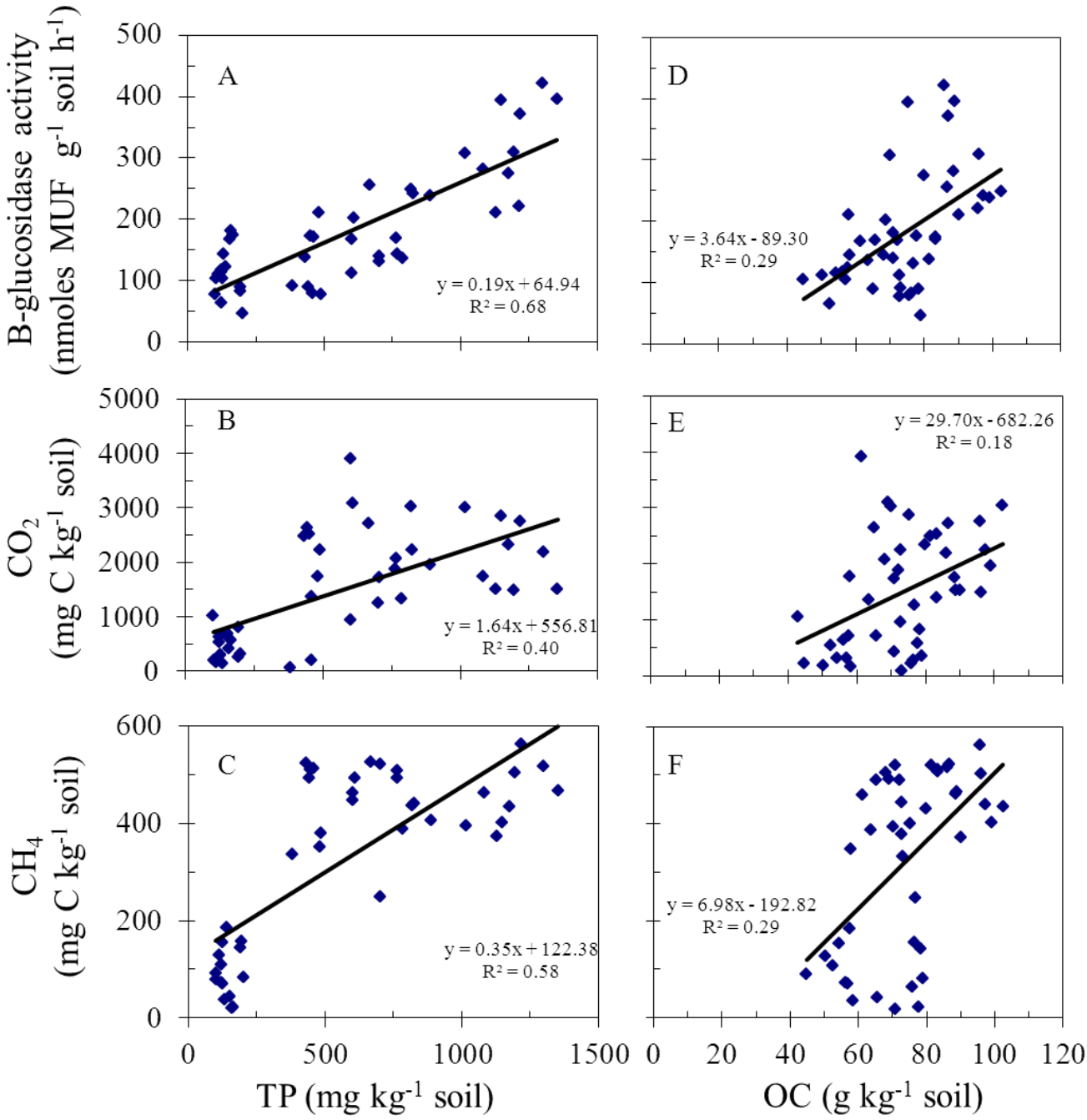

Figure 2-3. Investigation of the effect of soil C and P on BGA, CO_2, and CH_4 production. Correlations between β-glucosidase enzyme activity,anaerobic respiration, methanogenesis and either soil total P or soil organic C. Correlations between A) β-glucosidase enzyme activity, B) anaerobic respiration, C) methanogenesis and soil total P (left side). Correlations between D) β-glucosidase enzyme activity, E) anaerobic respiration, and F)

methanogenesis and soil organic carbon (right side) from restored and reference soils

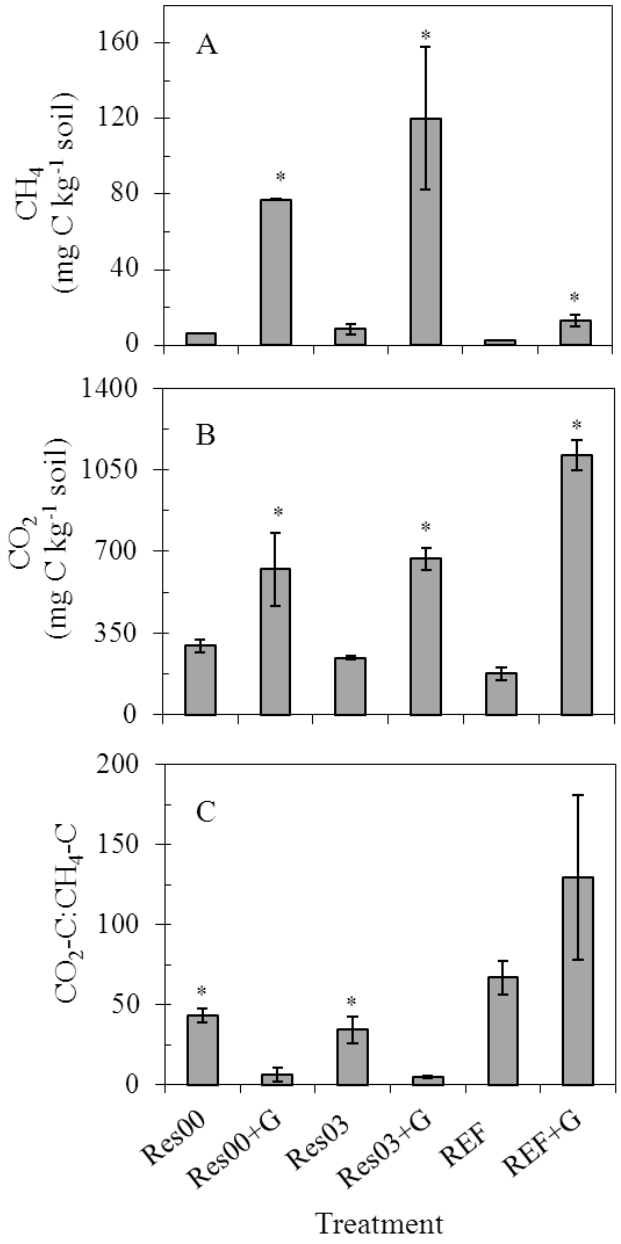

Figure 2-4. The effect of glucose addition on C gas production. Methanogenesis (A), anaerobic respiration (B), and CO_2-C:CH_4-C (C) from Res00, Res03, and reference wetlands within the HID region of Everglades National Park during the dry following glucose addition after 12 day incubation

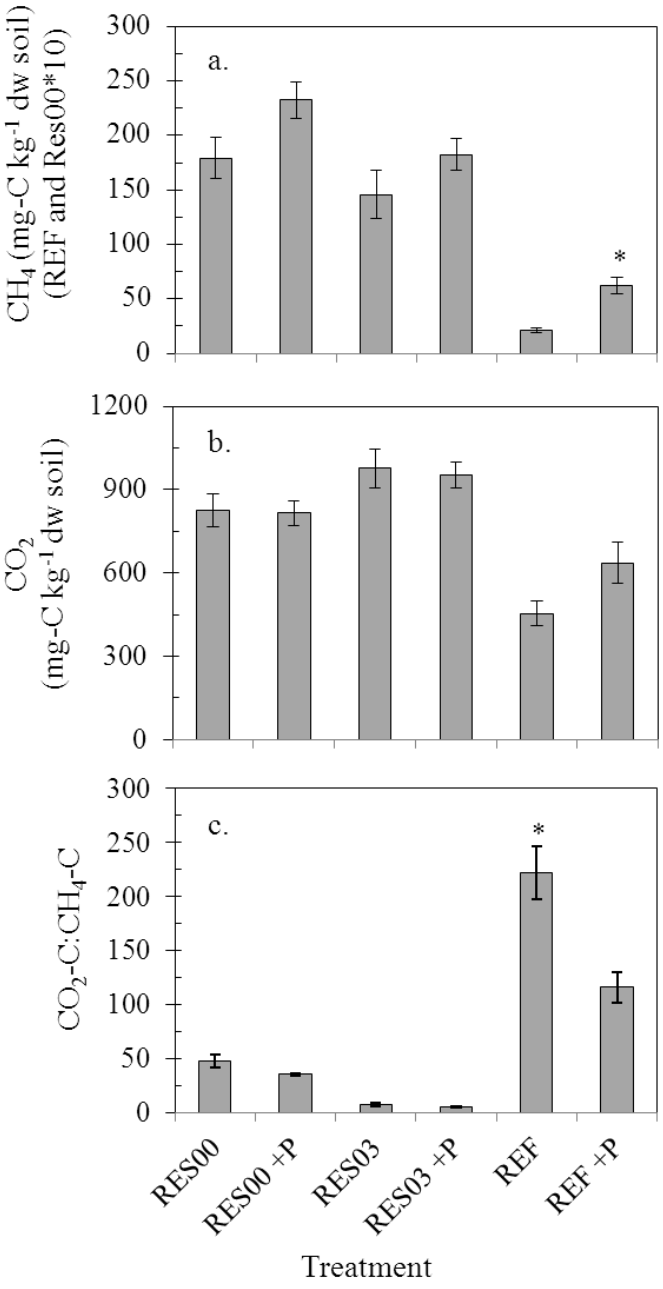

Figure 2-5. The effect of phosphorus addition on C gas production. Methanogenesis (A), anaerobic respiration (B), and CO_2-C:CH_4-C (C) from Res00, Res03, and reference wetlands within the HID region of Everglades National Park during the dry following phosphorus addition after 13 day incubation. Concentrations of methane production were much lower in Res00 and reference soils relative to Res03. Res00 and reference concentrations were multiplied by 10

CHAPTER 3
EVALUATION OF DIRECT AND INDIRECT PHOSPHORUS LIMITATION OF METHANOGENIC PATHWAYS IN CALCAREOUS SUBTROPICAL WETLAND SOILS

Regulators of Methanogenesis

Wetlands are an integral component of the carbon (C) cycle regulating C storage by serving as a major C greenhouse gas (CO_2 and CH_4) source. The balance between C storage and loss is regulated largely by soil organic matter (SOM) decomposition and the shift between aerobic and anaerobic conditions. Under anaerobic conditions, wetlands serve as the largest single source of CH_4 (Lelieveld et al. 1998) thus understanding controls on methanogenic processing from wetlands is crucial.

Common regulators of methanogenesis such as temperature (Conrad 2002; Inglett et al. 2011a; Glissmann et al. 2004), redox/pH (Wang et al. 1993), and electron acceptors (D'Angelo and Reddy 1998) have been studied in detail. However, few studies have focused on nutrient limitation and the associated effects on anaerobic microbial C-processing. Of the three key nutrients, the effect of C and nitrogen (N) on SOM decomposition has been thoroughly investigated. Studies have shown N to have a minimal effect on CH_4 production (Bridgham and Richardson 1992; Williams and Crawford 1984; Bachoon and Jones 1992). However, the effect of P limitation on anaerobic C processing is not clear.

Phosphorus is an important nutrient for the microbial processes involved in SOM decomposition (Cleveland et al. 2002). Furthermore, elevated microbial growth rates have been associated with increasing bacterial P concentrations (Makino et al. 2003). The relationship between P concentration and microbial growth rate has been hypothesized to originate from the high-P concentrations necessary for additional ribosome production to maintain elevated growth rates (Sterner and Elser 2002; Makino

et. al. 2003). Although several studies have shown lowered methanogenesis in P-limited systems (Pivničková et al. 2010; Smith et al. 2007) there are studies that have shown that higher P concentrations can inhibit methanogenesis (Conrad et al. 2000) further suggesting the relationship between P and CH_4 production is complex. In response to P addition, CH_4 production was stimulated in a tropical marsh (Pivničková et al. 2010), inhibited in a flooded paddy soil (Aerts and Toet 1997), and was not affected in North Carolina peatlands (Bridgham and Richardson 1992) or Florida marshes (Bachoon and Jones 1992).

Our previous studies with P-limited soils have shown increased anaerobic SOM decomposition in P amended microcosms (Medvedeff et al. *in review*). Stimulated rates of CH_4 production may be a function of increased availability of methanogenic C substrates or may be due to the stimulation of methanogenic activity. The Florida Everglades (U.S.A.) provide an ideal habitat to investigate the link between P availability and anaerobic C processing due to historically low soil P concentrations. Previous studies conducted in Everglades wetlands suggest methanogens may be substrate limited (Smith et al. 2007; Amador and Jones 1995; Bachoon and Jones 1992), however the response varied by study. For example, Bachoon and Jones (1992) determined methanogenesis in peat and marl soils to be stimulated by cellulose and glucose after a 6-8 day incubation period. In comparison, Amador and Jones (1995) observed an increase in CH_4 production rates from low-P soils when amended with acetate, although, no response to cellulose and an inhibition following glucose addition was observed. Amador and Jones (1995) observed a decrease in methanogenesis when both acetate and glucose were added in high-P soils. In contrast, Smith et al.

(2007) observed stimulated CH_4 production in response to formate addition although acetate had no quantifiable effect further suggesting the response of methanogenesis to varying substrates is highly variable within Everglades National Park.

Wetlands in the south eastern region of the U.S.A. (near the Everglades National Park) are characterized with calcareous marl soils that are naturally P-limited with TP concentrations averaging 150 mg kg^{-1} soil (Liao and Inglett 2011). However, as a result of agricultural eutrophication and soil disturbance, P concentrations are highly variable within the Everglades. Furthermore, throughout the Everglades elevated soil P wetlands have been associated with higher CH_4 production rates (Smith et al. 2007; Castro et al. 2004) suggesting P concentrations may influence the production of CH_4. The addition of P may stimulate a suite of fermentation pathways which can elevate the methanogenic substrate availability or P may stimulate the methanogenic archaea directly. Regardless, the result of indirect or direct stimulation of methanogenic processing will result in elevated CH_4 production. Through addition of varying methanogenic substrates limitation can be easily detected and pathways crucial to methanogenesis may be exposed (Figure 3-1).

This study was designed to investigate the influence of P on different metabolic pathways involved in anaerobic decomposition of OM in wetland soils that are typically P-limited. We hypothesize that P addition will stimulate CH_4 production in P-limited soils, (ii.) Methane production response will vary in C amended soils and, (iii.) Phosphorus addition will stimulate CH_4 production differentially according to C substrates.

Materials and Methods

Soil Sampling

Soil samples were obtained from a P-limited wetland located within the Hole-in–the-Donut region of the Everglades National Park, Florida. Soils are characterized as Biscayne and Perrine marl soils with poor to poorly drained characteristics (USDA 1996) (*described by* Smith et al. 2011). Vegetation dominating this region included *Cladium jamaicense* Crantz and *Muhlenbergia capillaris*, typical of the oligotrophic Everglades ecosystem. Soil biogeochemical properties are provided in Table 3-1. To minimize the spatial variability within the soil samples, these experiments were conducted on composite samples that were made with 10 soil samples (0-5 cm) collected randomly within (30 x 30 m) the sampling site. Following sampling, soils were placed on ice and transported to the laboratory and stored at 4˚C until processing. Soil samples were sieved (2 mm mesh) to remove rocks and roots and to ensure additional C substrate addition via root degradation was minimized.

To characterize the soil, basic soil parameters including pH, organic carbon (OC), TN and P were determined (Table 3-1). Soil pH was measured with a soil to water ratio of 1:2 after equilibration for 30 minutes. For nutrient analyses, soil samples were sieved, dried (at 105˚C) and homogenized. Total C and N concentrations were determined with a Thermo Flash EA 1112 series NC soil auto-analyzer (Thermo Fisher Scientific, Inc., Waltham, MA). Soil TP was determined by combusting soils at 550°C, extracting ash with 6 M HCl (Anderson 1976) and analyzing extracts with a colorimetric method using a Technicon^{TM} Autoanalyzer (SEAL Analytical, Mequon, WI) (EPA method 365.1).

Soil OC was estimated using loss on ignition (550°C combustion for 4 hours) assuming a 45% OC of OM content factor (Wright et al. 2008). Microbial biomass carbon (MBC) and nitrogen (MBN) was analyzed by extraction following chloroform fumigation (Vance et al. 1987). Briefly, soil samples were extracted with 0.5 M K_2SO_4 after being incubated with (fumigated) and without (control) chloroform. Filtered (0.2 µm) extracts were analyzed for total extractable organic carbon (TOC) using a 5050A TOC auto-analyzer (Shimadzu Corp., Columbia, MD; EPA method 415.1). The difference in extractable TOC between the fumigated and non-fumigated samples was considered MBC following correction with an extraction efficiency (K_{EF}) of 0.37 (Sparling et al. 1990). For MBN determination, extracts were analyzed following Kjeldahl digestion on a Technicon auto-analyzer (EPA method 351.2). The difference between fumigated and non-fumigated TKN was considered MBN following correction with an extraction efficiency of (K_{EF}) of 0.54 (Brookes et al. 1985).

Anaerobic Microcosms

Anaerobic microcosms were established with soil (2 grams dry weight equivalent) and 10 ml of amendment solutions in tubes that were sealed with butyl rubber stoppers and aluminum crimp tops (Bellco Glass Inc., Vineland, N.J.), and flushed with O_2 free N_2 to ensure anaerobic conditions. The first experiment was set up to determine the short term (4 days) response of CH_4 production in soils to P additions. Three concentrations of P; low-P (1 mM), intermediate-P (10 mM), and high-P (100 mM) were chosen and added in the form of Na_2HPO_4 amendment solution. Control soils received DDI in place of the amendment solution (with no P). Results from this study were used to prepare the second set of microcosms which were used to determine the effect of P on soils that were amended with different C substrates.

For the C amendment study, four C substrates were chosen to stimulate different groups of soil microorganisms. Glucose, a simple monomeric form of C, can be used by several groups of anaerobic bacteria including fermenters and result in methanogenic substrates (acetate, hydrogen and carbon dioxide) and their precursors (butyrate, acetate). Butyrate is a fermentation product that is commonly used by the syntrophic microorganisms resulting in metabolic substrates usable by methanogens. Acetate is commonly used to stimulate the acetoclastic methanogenic pathway by providing a usable C source (*reviewed by* Whalen 2005). Formate stimulates hydrogenotrophic methanogenesis by providing both H_2 and a C source. A conceptual model of C processing under anaerobic conditions is provided (Figure 3-1). Each of the four C substrates (50 µM-C) were added separately to two sets of anaerobic microcosms. One set of the C-amended soil also received P additions. The amount of C added (Bachoon and Jones, 1992; Bridgham and Richardson, 1992) was based on preliminary studies which suggested the substrate would not become limited over the short-incubation. All treatments were replicated (n=3) and included control microcosms (soil with no amendments and no-soil microcosms). Microcosms were incubated in the dark at 25°C without shaking for 10 days and headspace gas was sampled periodically to analyze for CO_2 and CH_4. Trends in gaseous accumulation (4 days; *data not shown*) were similar to rates calculated from 0-10 days. For this reason, we chose to report the rates of production.

Gas Analysis

Methane in the headspace gas was analyzed using a gas chromatograph equipped using a flame ionization detector (Shimadzu-GC8a) and a 1.6 m (45/60) Carboxyn 1000 column (Supelco Inc., Bellefonte, PA). Temperature conditions for CH_4

analysis were set as 160°C for the detector, and 110°C at the injection port and N_2 was used as the carrier gas. For CO_2 analysis, a gas chromatograph equipped with a Porapak-N column (1.9m, 80/100; Supelco Inc., Bellefonte, P.A) and a thermal conductivity detector was used with conditions previously described by Inglett et al. (2011b). Helium was used as a carrier gas. Throughout each sampling event, calibration curves were determined via standard gas mixtures (Scott Specialty Gases, Plumsteadville, PA) and were used to determine concentrations of CO_2 and CH_4 from experimental microcosms. Final concentrations of CO_2 or CH_4 production were reported as mg C kg^{-1} soil d^{-1}.

Statistical Analysis

Before analysis, normality (goodness of fit) of CH_4 and CO_2 production rates were tested ensuring both parameters fell within a normal distribution. For this reason, data were not transformed prior to running analyses. Differences between C substrates, P concentrations and combined treatments were determined by one way analysis of variance (ANOVA) accomplished via student's t-test means comparison at a 95% confidence interval. Least squares regression analysis was used to determine C, P and interactive effects on CO_2 and CH_4 production. All statistical analyses were performed in JMP vs. 8.0 (SAS institute, Cary NC).

Results

Response of Anaerobic Processing to P Amendments

Soils with the high-P amendment exhibited elevated production rates of CH_4 relative to the control soils (no P amendment) within a 4 day period (p<0.0001; Figure 3-2A). However, there was no significant increase in the rates of CH_4 production in soils amended with intermediate and low-P concentrations (Figure 3-2A). Accumulated CH_4

in soils with the high-P amendments was eight and three times greater than that observed in the control and intermediate-P soils, respectively.

Addition of P at all three concentrations also stimulated CO_2 production ($p<0.05$; Figure 3-2B). Rates of CO_2 production were greatest in the intermediate-P treatment followed by high-and low-P treatments. Production of CO_2 after 4 days in the intermediate-P treatment was 1.8 times greater compared to both the low- and high-P treatments. Furthermore, production of CO_2 was 2.8 times greater relative to control soil CO_2 production. The ratio of CO_2-C: CH_4-C in soils exposed to the high-P amendment was 5.4 times lower than the ratio derived from the remaining P treatments and the control. For this reason, we chose the high-P concentration for the C and P combined additions.

Response of Anaerobic Processing to C Substrate Addition

Rates of methane production in all C amended soils were enhanced relative to control soil production (Figure 3-3A). Methane production rates originating from formate amended soils was greater than production rates from all other treatments ($p<0.05$; Figure 3-3A) with twenty eight fold greater production than control soils (0.77 and 0.028 mg CH_4-c kg^{-1} soil d^{-1}, respectively). In addition, an increase in CH_4 production rates was observed when amended with acetate or glucose ($p<0.05$), although no significant response to butyrate addition was detected (Figure 3-3A).

Similar trends were observed in total CH_4 production ten days after substrate addition. Production of CH_4 from formate amended soils was greater than all other treatments ($p<0.001$) with eighteen times greater production than control soils (0.38 and 7.6 mg CH_4-C kg^{-1} soil at day 10). Methane produced from acetate treated soils was

elevated relative to production from control soils (p<0.1). No difference between CH_4 accumulated from control, butyrate, or glucose treated soils was observed at 10 days.

In contrast, rates of CO_2 production were not affected by C source addition with the exception of the glucose amendment which increased production rates from 30 to 60 mg CO_2-C kg^{-1} soil d^{-1} (p<0.05; Figure 3-3B). Although not significant, a slight decrease in CO_2 production rates were observed in formate amended soils relative to control production rates.

Accumulated CO_2 (at 10 days) was also elevated from soils amended with glucose (p<0.001) relative to all other treatments. The production of CO_2 in glucose amended soils was 2.7 times greater than CO_2 accumulated from the control soils. No difference between CO_2 accumulated from any other treatment (acetate, formate, or butyrate) and the control was detected after 10 days.

Response of Anaerobic Processing to Concurrent C and P Addition

When both C and P were added to the soil simultaneously, CH_4 production rates were elevated relative to production from control microcosms in all treatments (p<0.01; Figure 3-3A). Regardless of C substrate added (excluding formate), soils amended with P exhibited higher CH_4 production rates relative to the respective C only treatment (p<0.05, Figure 3-3A). Methane production increased from 0.23 (control production) to 0.48 and from 0.23 to 0.74 mg CH_4-C kg^{-1} soil d^{-1} in acetate and glucose treatments, respectively, when amended in conjunction with P (Figure 3-3A). A 3-fold increase in CH_4 production rates from soils amended with butyrate and P was observed when compared to the butyrate only treatment with concentrations increasing from 0.14 to 0.45 mg C $kg^{-1}d^{-1}$. In contrast, the addition of P had no effect on CH_4 production in formate amended soils.

Patterns of CH_4 produced from soils treated with both C and P following ten day incubation was similar to patterns observed from production rates. Methane accumulated from soils amended with acetate, butyrate, and glucose were further stimulated when supplemented with P (p<0.05). The addition of P to soils amended with formate had no additional effect on CH_4 production.

The effect of P on CO_2 production from soils amended with C was highly variable (Figure 3-3B). Visually, the addition of P stimulated CO_2 production when added in conjunction to formate (Figure 3-3B). In addition, CO_2 production was visually lowered when added with acetate, butyrate, or glucose. Although trends were apparent, no statistical differences in CO_2 production in response to P and C co-addition were observed on either production rates or the accumulated amount of CO_2 following 10 day incubation (Figure 3-3B).

A least squares regression analysis model was used to determine the effect of C and P on anaerobic microbial processing and to investigate a possible interactive effect (C and P). Production of CH_4 was significantly enhanced when amended with both C (p<0.1) and P (p<0.01). Although both parameters had a significant effect on CH_4 production, F-stat values from C (F-stat =4) and P (F-stat=9) parameters suggest the response to P addition was twice as great as the response to C. In contrast, CO_2 production was not affected by either C or P. Regardless of anaerobic gaseous end product, no interactive effect of C and P on CO_2 or CH_4 production was determined.

Variation in CO_2-C:CH_4-C from Amended Soils

Addition of P significantly reduced CO_2-C:CH_4-C ratios from 1304 in control microcosms to 97 (p<0.1; Figure 3-4). Upon addition of C substrates, CO_2-C:CH_4-C ratios decreased from 1304 (control ratios) to 330 in response to butyrate and glucose

addition, and 170 in response to acetate addition. When amended with formate the ratio decreased to 33 (Figure 3-4).

A reduction in CO_2-C:CH_4-C ratios was also observed in C treated soils when amended simultaneously with P (p<0.05) in all treatments except for formate. When soils were amended with acetate and P the ratio of CO_2-C:CH_4-C was reduced by a factor of 2 when compared to ratios in the acetate only treatment (Figure 3-4). A larger reduction in ratios (5-fold) was observed when P was added in addition to butyrate and glucose. Ratios (CO_2-C:CH_4-C) were slightly increased when P and formate were added simultaneously although the difference in ratios was not significant (Figure 3-4).

Discussion

Response to C addition. Under anaerobic conditions mineralization of an experimental substrate can be estimated by quantifying CO_2 and CH_4 production relative to that in the control. Substrate quality may be a primary control on methanogenesis in wetlands (Whalen et al. 2005) hence; addition of formate, butyrate, acetate and glucose were chosen to stimulate all direct and indirect pathways. Formate can be used by <50% of hydrogenotrophic methanogens (Balch et al. 1979), thus, stimulation of this pathway may have been underestimated in the current study. Nevertheless, formate can be used to test hydrogenotrophic activity (Dolfing et al. 1985) and is a common substrate for incubation studies (Chauhan et al. 2004; Smith et al. 2007; Uz et al. 2007). Smith et al. (2007) observed greater CH_4 potentials when soils were amended with formate within the HID sites similar to observations from peat Everglades (Uz et al. 2007; Castro et al. 2004) and flooded rice paddy soils (Frenzel and Bosse 1996) consistent with our results suggesting hydrogenotrophic methanogens were abundant and active. In the current study, formate stimulated methanogenesis to

the greatest extent relative to stimulation from all other C substrates (Figure 3-3A) suggesting CH_4 production was dominated by hydrogenotrophic processing. The response of anaerobic CO_2 production to formate addition had not yet been quantified at the HID site prior to this study and provided important information regarding microbial anaerobic processing. More specifically, no response of CO_2 production to formate addition was detected suggesting formate was processed through the hydrogenotrophic pathway via direct methanogen utilization (Figure 3-3B).

In order for butyrate to stimulate methanogenesis (provide H_2 substrate) initial processing by fermenters (namely syntrophs) is necessary (Conrad 1999) suggesting a response to butyrate addition quantified as elevated CH_4 production is a measurement of indirect pathway stimulation. Within the Florida Everglades, it has been observed that syntrophic associations with methanogens may be more common than previously thought (Chauhan et al. 2004; Ogram et al. 2011). In Everglades oligotrophic peat soils, butyrate-oxidizing syntrophic populations were 3-fold greater relative to acetate-utilizing methanogenic populations (Uz et al. 2007; Chauhan et al. 2004) indicating when stimulated (with butyrate) the increase in CH_4 end products should be greater than products produced from soils amended with acetate. To target syntrophic oxidation substrates such as propionate (Uz et al. 2007; Chauhan et al. 2004) and butyrate (Chauhan and Ogram 2006; Uz et al. 2007; Chauhan et al. 2004; Tabassum and Rajoka 2000) are commonly used. In our study we observed no significant response of CH_4 production to butyrate addition similar to Chauhan et al. (2006b) (Figure 3-3A) possibly as a result of the short incubation time (Chauhan et al. 2004). Through butyrate oxidation, acetate and H_2 substrates are produced both of which can increase

methanogenic activity if methanogens are substrate limited. Since we observed a response in CH_4 production when both formate and acetate were added independently, we concluded the lack of response to butyrate addition was associated with a small active syntrophic population. Similarly, butyrate had no effect on CO_2 production further suggesting a low active population.

Smith et al. (2007) observed a minimal response of methanogens to acetate addition within the HID region possibly due to a smaller population size in comparison to H_2/CO_2 utilizing methanogens, a trend commonly seen throughout the Everglades (Uz et al. 2007). Similar to Smith et al. (2007), Castro et al. (2004) quantified a lower concentration of CH_4 production when soils were amended with acetate over a short-term incubation in the oligotrophic peat Everglades. In contrast, we observed a slight increase in CH_4 production when soils were amended with acetate ($p<0.05$; Figure 3-3A) suggesting acetoclastic methanogens were active in our site. In contrast, we did not observe any response in CO_2 production to acetate addition suggesting acetate was predominantly processed through methanogenic pathways (Figure 3-3B). Drake et al. (1996) observed a simultaneous increase in acetate and CH_4 with decreasing H_2 concentrations when microcosms were spiked with CO_2 further suggesting a lack of acetoclastic processing and illustrating the importance of the hydrogenotrophic pathway in Everglades ecosystems. If the acetoclastic population size was responsible for the limited response in acetate amended soils, an increase in CH_4 would be probable if the experiment was continued for a longer duration. Following soil flooding, a lag phase between 1 and 10 days prior to CH_4 production suggests a low methanogenic population (Conrad 2002); furthermore, stimulation of the hydrogenotrophic pathway

following flooding can be initiated quicker than the acetoclastic pathway (Roy et al. 1997). Due to the short nature of our experiment the response observed likely represented the active methanogenic population present immediately following incubation.

Stimulation of CH_4 following glucose addition is an indirect response originating from elevated activity of higher trophic level microbes suggesting substrate limitation. Because glucose cannot be directly used by methanogens (Ferry, 1993) the monomer must first be processed to yield usable C substrates for methanogenesis to proceed. When amended with glucose, an increase in acetate and H_2 is probable from primary and secondary fermentation further suggesting the associated increase in CH_4 production was in response to alleviated substrate limitation (Figure 3-3A). Glucose can also be used directly by some syntrophic assemblages (Stams and Plugge, 2009) suggesting glucose may have been processed by secondary fermenters thus bypassing initial processing by primary fermenters. However, cultured syntrophs within the Everglades Agricultural Area (Chauhan et al. 2004) were associated with obligate syntrophs (Stams and Plugge, 2009) suggesting hydrogenotrophic methanogen presence was necessary for glucose processing by the dominate syntrophs in this site. Extrapolation of this knowledge to our study site within Everglades National Park must be interpreted with caution; however it suggests this pathway (syntrophy) may not have been important in our study site. The addition of glucose was the only C source that stimulated CO_2 production. This was not surprising as glucose can be used by most microbes and would be expected to stimulate primary fermenters in addition to all other substrate processing pathways. Although trends suggest C source addition decreased

the CO_2-C:CH_4-C ratio (Figure 3-4) significant differences were not observed likely masked by high within treatment variability.

Response to P addition. In addition to investigating the effect of different C substrates on methanogenesis we also investigated the response of CO_2 and CH_4 production to P additions at varying concentrations (1, 10 or 100 mM-P). Research suggests the concentration of P may affect methanogenic activity. A study by Conrad et al. (2000) determined P additions at concentrations ≥ 20 mM may negatively affect acetoclastic methanogens on rice roots following a 3-4 day incubation period. Rath et al. (2005) also observed a negative response in methanogenic activity following P addition postulating the negative response may have resulted from the high concentrations of P added. In P-deficient studies, a negative effect on methanogenesis was only observed when P was added at concentrations in excess of 100 mg P kg^{-1} soil (Adhya et al. 1998) illustrating the inhibitory effect of P on methanogenesis may vary by site characteristics and may not only be in response to P concentration. In the current study, the 100 mM-P addition stimulated CH_4 production to the greatest extent relative to any other P concentration. Due to high within treatment variability, the increase in CH_4 production from the intermediate-P treatment was not significantly different from the control at 4 days (Figure 3-2A). The difference in lag time (days) prior to an observed increase in CH_4 production varied by P concentration. For example, there was no change in CH_4 production from the low-P treatment over the four day period. However, CH_4 production began to increase after one day in the high-P treatment and after four days in the intermediate-P treatment. The opposite trend from CH_4 production was observed in CO_2 production. For example, in the 10 mM-P treatment the lag phase

prior to CO_2 production was one day whereas the lag phase remained until four days in the high-P treatment.

Stimulation of CH_4 in response to P addition can suggest either (i.) the methanogens are P-limited or (ii.) P is stimulating higher trophic level microbes which provide more usable C substrates (fuel methanogenesis, Figure 3-1). Anaerobic CO_2-C:CH_4-C ratios from soils amended with 100 mM-P were greatly reduced relative to control soil ratios ($p<0.05$) suggesting a preferential utilization of P by methanogenic pathways (Figure 3-4). Although studies have investigated the combined response of C substrate on CH_4 production from Everglades low and high-P soils (Amador and Jones 1995) we are unaware of any study which has used formate or butyrate (C substrate) in concert with a P addition with the overarching goal to investigate C and P interaction or co-limitation of anaerobic processing (CH_4 and CO_2) in low-P marl Everglades wetlands. As we observed, formate stimulated methanogenesis to a greater extent than all other C substrates thus, understanding this relationship (formate and P) is crucial when investigating regulators and stimulatory factors on methanogenic processing and understanding possible consequences of increased P loading to oligotrophic Everglades wetlands.

Least squares regression analysis determined CH_4 production was influenced by both C and P availability. In contrast, CO_2 production was not affected by either C or P suggesting P is primarily processed through methanogenic pathways or through direct utilization by methanogens. No C x P interaction was detected regardless of C gaseous product measured (CO_2 or CH_4). Apparently, P availability is driving the observed

response in CH_4 stimulation, thus, an interactive effect would not likely be deciphered if present.

Simultaneous P and C additions stimulated CH_4 production to a greater extent than the C only treatments (excluding formate), signifying P may ultimately limit mineralization of these substrates or C may not be the main factor regulating methanogenesis. No difference in CH_4 production in the formate treatment with or without P suggests hydrogenotrophic methanogens may not be P limited. Hendrickson et al. (2007) concluded hydrogenotrophic activity was not limited by P but was limited by substrate availability (H_2) suggesting P may not be affecting the hydrogenotrophic methanogens directly in agreement with our results. Because formate was provided to the methanogens in excess (C only treatment) increased substrate production in response to P addition (primary or secondary fermentation stimulation) in the C and P treatment would have a minimal effect on the hydrogenotrophic pathway.

Products of butyrate oxidation can include acetate, CO_2, and H_2 (Müller et al. 2010; Ogram et al. 2011) providing substrates to fuel both the acetoclastic and hydrogenotrophic pathways. Methanogens grow at a slower rate than most fermenting organisms (Hawkes et al. 2002) suggesting fermenters may have an initial competitive advantage following nutrient addition. Lin and Lay (2004) determined a decreasing lag phase from H_2 producing fermenters in sewage sludge concluding the reduction in lag phase was in response to increasing the P concentration. Hawkes et al. (2002) determined production of H_2 via fermentative pathways was reduced in P-limited solutions. In agreement, Lin and Lay (2004) observed greater H_2 production when

fermenters were provided additional P. These studies suggest H_2 producing fermenters may respond quicker to P addition enhancing methanogenic substrate production.

Smith et al. (2007) concluded CH_4 production from the HID sites was dominated by hydrogenotrophic methanogens although a small quantity of *Methanosarcina* was cultured from the low-P site. *Methanosarcina* can use both the acetoclastic or hydrogenotrophic pathway. Similarly, Chauhan and Ogram (2006) reported neighboring oligotrophic wetlands were also dominated by *Methanosarcina*. Butyrate oxidizers require environments low in H_2 and acetate (Müller et al. 2010) to proliferate however, *Methanosarcinaceae* are known to be favored at high acetate concentrations (Yu et al. 2005). In nutrient impacted soils (high-P) acetate is mostly consumed by syntrophs, whereas acetate consumption in un-impacted soils (low-P) is dominated by acetoclastic methanogens and sulfate reducing bacteria (Chauhan and Ogram, 2006). This suggests that in low-P soils amended with P acetate consumption may shift pathways. Increased syntrophic activity may decrease overall acetoclastic activity (out-competition) in the P addition treatment. However, when super saturated with both acetate and P, the difference between CH_4 produced in the acetate and acetate with P treatment likely originated from increased substrate availability fueling the hydrogenotrophic pathway.

When soils were amended with glucose and P the increase in CH_4 production was similar to rates observed in the formate and formate with P treatment (Figure 3-3A). Glucose addition would fuel both primary and secondary fermentation directly resulting in elevated substrate availability for acetoclastic and hydrogenotrophic methanogens. It is likely syntrophic utilization of glucose is contingent on hydrogenotrophic methanogen presence suggesting the stimulation originated from primary fermenters. Thus, CH_4

production in response to glucose and P addition may have originated from both pathways through elevated substrate availability.

Although not significant, addition of P to soils amended with acetate, butyrate, and glucose resulted in decreased CO_2 production (Figure 3-3B) relative to the production rate from the corresponding C only treatment. This suggests CO_2 was being consumed in these treatments (i.e. hydrogenotrophic methanogenesis). Acetoclastic methanogenesis results in CO_2 production, further suggesting decreased CO_2 production when amended with P was in response to hydrogenotrophic activity. In contrast to all other C and P treatments, addition of P to formate treated soils resulted in elevated CO_2 production relative to the production from the formate only treatment. This suggests P is being processed through higher trophic level fermentation pathways.

Conclusions

While multiple studies have concluded C substrate limitation on methanogenic processing fewer studies have focused on nutrient limitation of methanogens and C mineralization resulting in a general lack of knowledge with regard to interactive or nutrient co-limitation (C and P) on anaerobic microbial processing. While most studies conclude nutrients (N or P) do not limit methanogenesis, it is clear that P is limiting this process in low-P marl Everglades wetlands. Stimulation of methanogenesis and not anaerobic respiration (CO_2) in response to P addition suggests methanogens may be directly affected by P; however, the large response to formate addition suggests hydrogenotrophic methanogens are substrate, not P, limited in this ecosystem. A closer examination suggests P availability ultimately limits substrate liberation via fermentation reducing methanogenic activity. Our results suggest fermenting bacteria may be controlling the release of substrates in response to severe P limitation. This study

highlights the complexity and interconnectivity of anaerobic microbial processing and resultant greenhouse gas production. While understanding regulators of CH_4 production from wetlands is crucial, considering mechanisms which limit higher level fermentation may be equally if not more important in understanding overall controls on anaerobic C processing and C loss.

Table 3-1. Basic chemical and microbial properties (average ± SE) of study soils from low-P marl soils within the HID region of Everglades National Park

Chemical and microbial properties	
pH	8.3±0.1
OC* (g kg^{-1})	54±4.2
N (g kg^{-1})	5.8±0.2
P (mg kg^{-1})	128±8.1
TN:TP	45±2.4
OC:P	420±8.1
MBC (mg kg^{-1})	3807±322
MBN (mg kg^{-1})	345±32
MBP (mg kg^{-1})	29±2

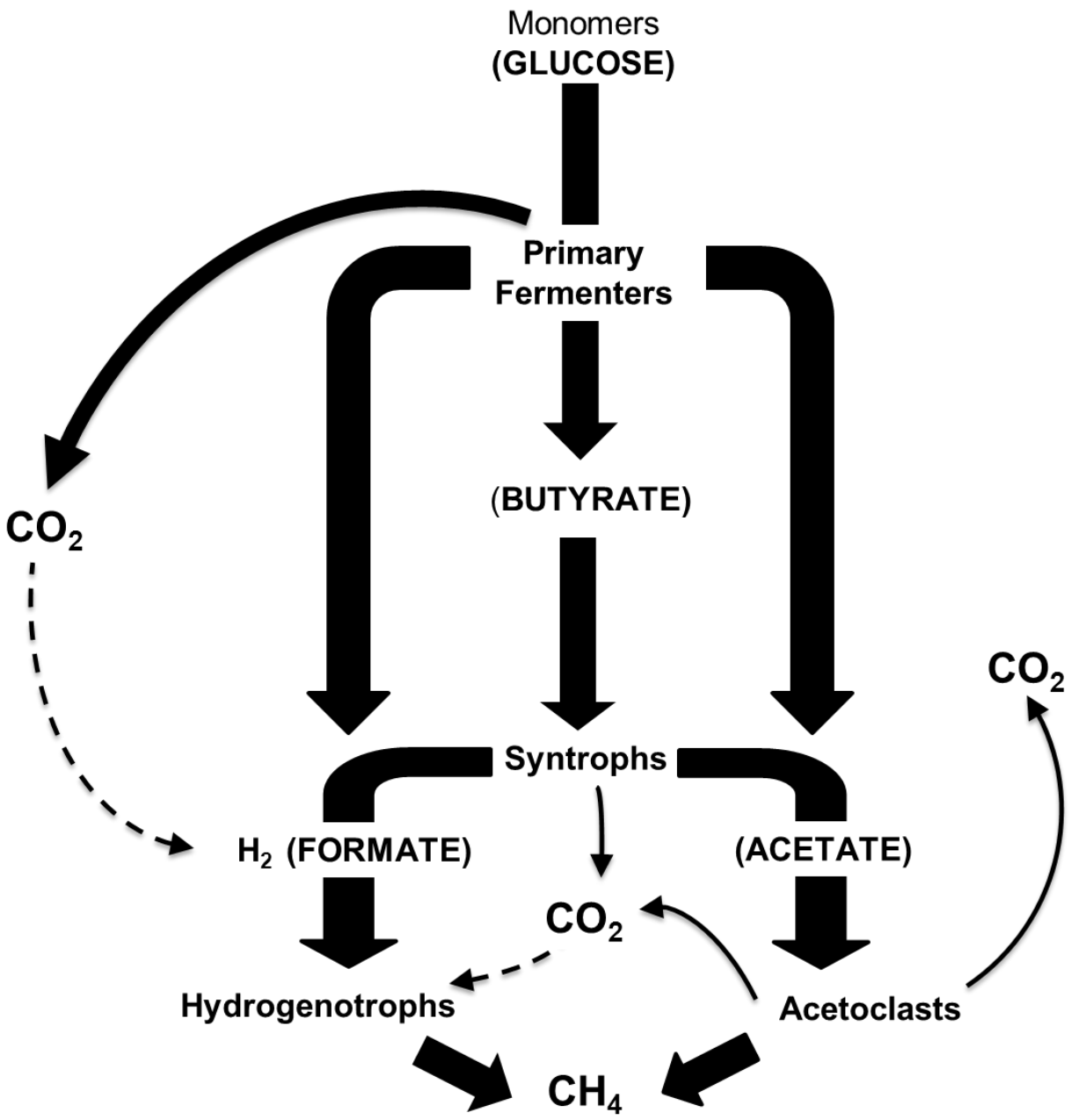

Figure 3-1. Conceptual model of anaerobic processing pathway response to carbon (acetate, butyrate, formate, or glucose) and phosphorus addition

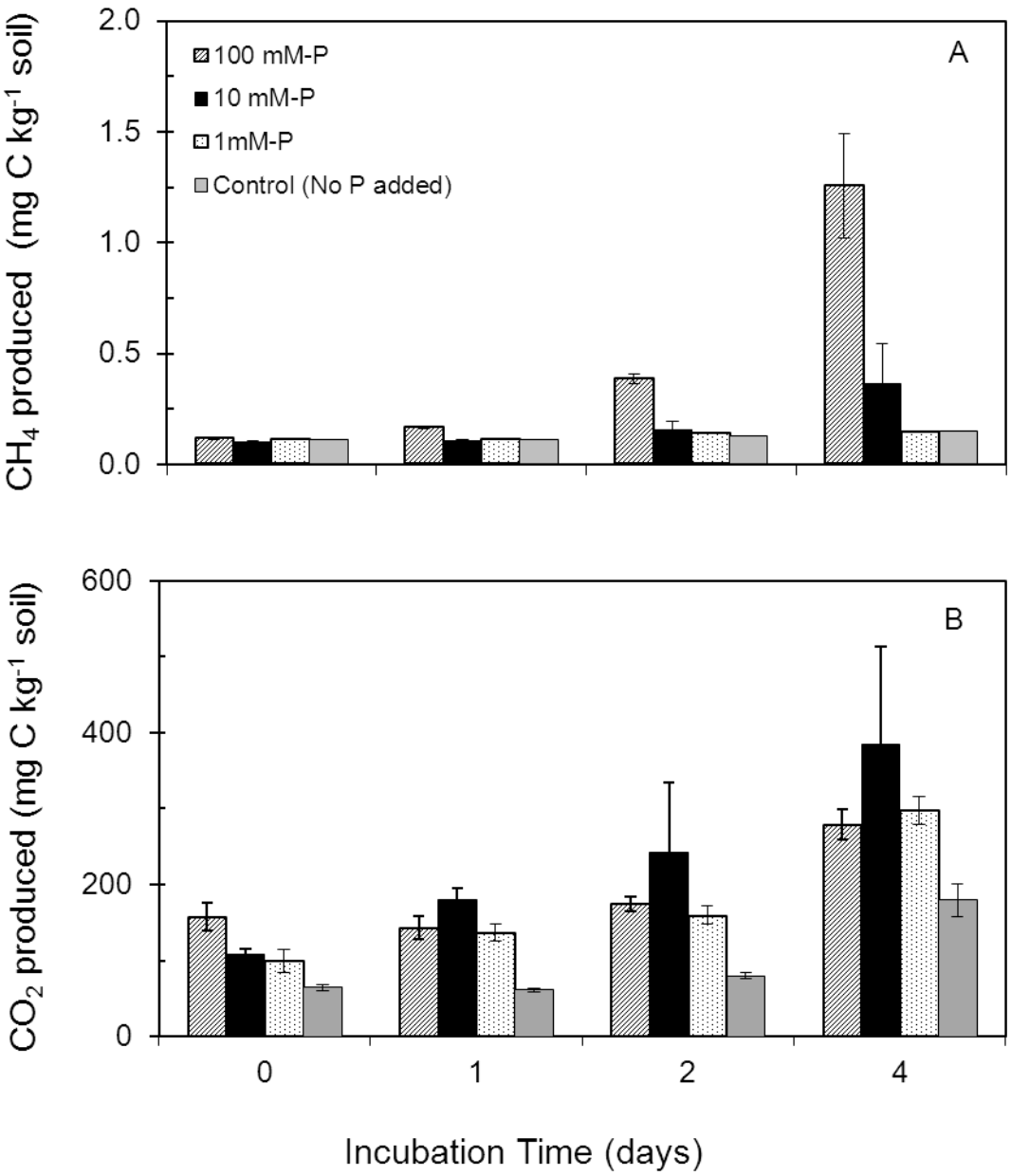

Figure 3-2. The effect of different P concentrations on C gas production. Response of A) methanogenesis (production ± SE, n=3) and B) CO_2 production (production ± SE, n=3) to low (1 mM) and high (10 and 100 mM) P additions in P-limited marl subtropical wetlands within the HID region of Everglades National Park

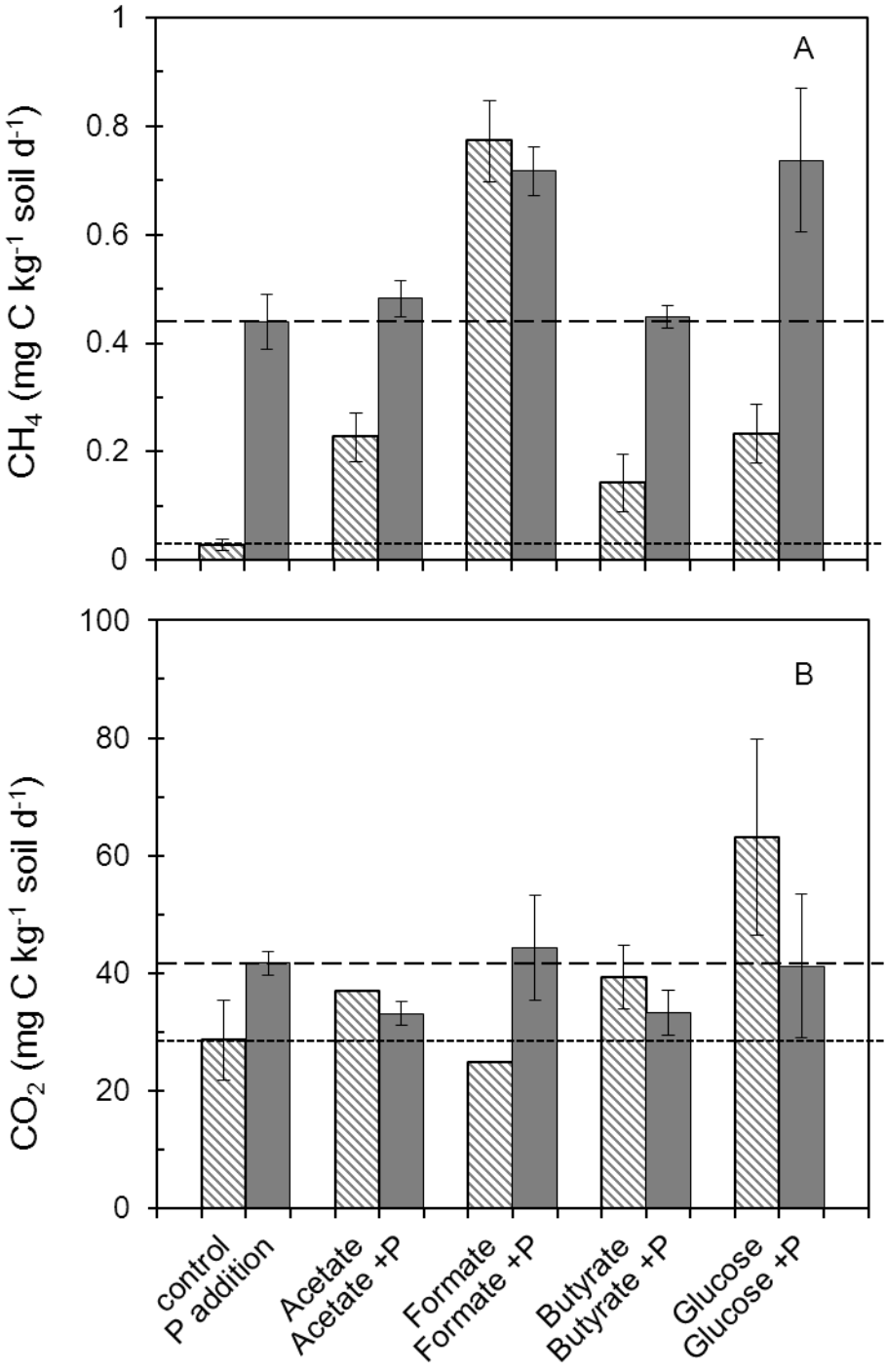

Figure 3-3. Effect of C, P, and C+P on CO_2 and CH_4 production from low-P marl subtropical wetland soils within the HID region of Everglades National Park. Influence of 0.05 mM-C (acetate, butyrate, formate, or glucose), 100 mM-P, and combined C substrate and P amendments on A) methanogenesis and B) anaerobic respiration rates. Gas production rates ± SE, n=3. Solid gray=with P, stripes=without P

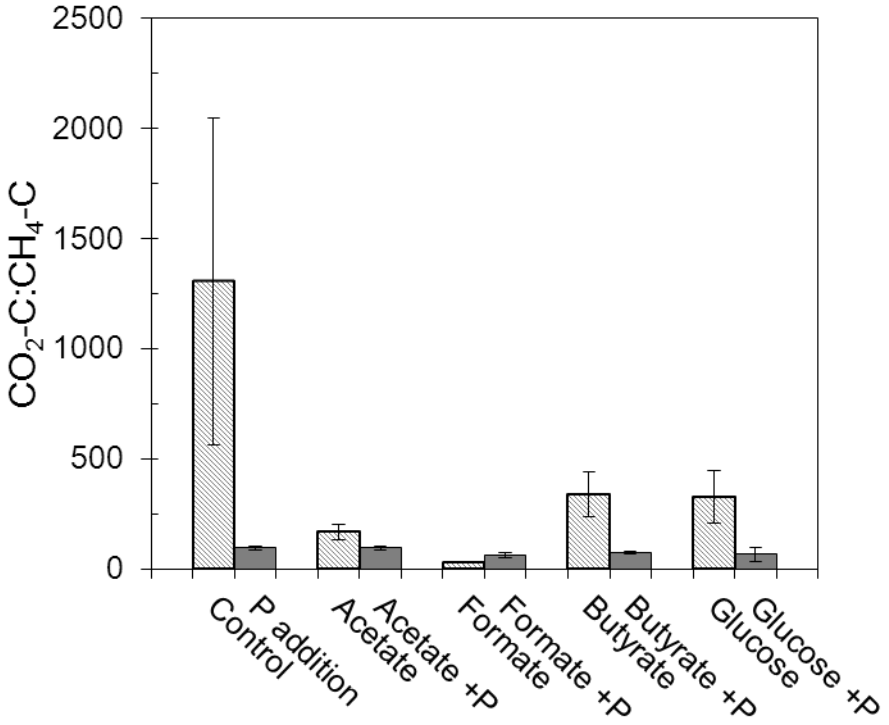

Figure 3-4. The influence of C and combined C (0.05 mM-C) and P (100 mM-P) additions on anaerobic CO_2:CH_4 (gas production ± SE, n=3) from low-P marl wetlands within the HID region of Everglades National Park. Solid gray=with P, stripes=without P

CHAPTER 4
IMPACTS OF FIRE ON MICROBIAL CARBON PROCESSING IN SUBTROPICAL WETLAND ECOSYSTEMS

Field Fire Introduction

Fires (natural and prescribed) can drastically alter ecosystem function by affecting soil nutrients, microbial activity/community, and organic matter (fire residue) inputs. The extent to which an ecosystem is altered (post fire) is controlled largely by fire intensity, severity, and type. Fire intensity is defined as the energy released by the combustion of fuel loads whereas fire severity is a measure of above and below ground biomass change. These two factors along with post-fire climate and additional disturbance contribute greatly to the ecosystem response (Keeley 2009) and are crucial when evaluating and/or predicting fire effects.

Most fire data has been compiled from forested (Covington et al. 1992; Deluca and Sala 2006; Allison et al. 2010; White 1986) and grassland ecosystems (Toma et al. 2010; Knapp et al. 1998; Xu and Wan 2008) however limited data on the biogeochemical response to fire in wetlands has been documented (Levine et al. 1990; Nakano et al. 2006; Smith at al. 2001). Furthermore, limited studies have quantified the effect of fire on anaerobic and methanogenic processing coupled with fire characteristic data (i.e. temperature, residence time) greatly decreasing the ability to report concrete conclusions with the inability to model the data or extrapolate. Without knowing fire characteristic data we are making an assumption that each fire is identical when comparing the fire response which is likely incorrect. A knowledge gap relating biogeochemical carbon (C) processing and fire characteristics such as temperature (air and soil) is evident from literature and short and long-term monitoring of enzyme activity and decomposition processes post burn is minimal.

Monitoring the fire temperature is important because variation in fire intensity can result in alteration of soil nutrients through removal, volatilization, and/or deposition. With temperatures ranging from 50 to >1500°C heat release will vary greatly (*reviewed by* Neary et al. 1999) and alter microbial and soil characteristics differentially. During a fire, nutrients have one of three fates; to (i.) remain as incompletely burned organic matter (char), (ii.) be lost to the atmosphere, or (iii.) redeposit as organic matter derived ash (Boerner 1982). White et al. (1973) found nitrogen (N) volatilization to occur at fire temperatures greater than 200°C, although, N loss has been reported at much lower temperatures closer to 120°C (Hart et al. 2005) suggesting volatilization may be influenced by site and vegetation characteristics. Regardless of the temperature in which N volatilization begins, if temperatures approach 500°C close to 50% of the N will be lost (Neary et al. 1999). In contrast, soil phosphorus (P) responds differently to fire when compared to N because there is no appreciable volatilization reported, thus P integrity is preserved at high temperatures (≥500˚C) (White et al. 1973) although the form may be altered (Smith et al. 2001). A direct fire response may result in alteration of nutrient concentrations and availability directly affecting microbial survivorship.

One parameter which may affect microbial activity is fire residue deposition (char/ash) produced from complete or incomplete combustion of organic matter (OM). Char is commonly produced during most natural fires (Hart et al. 2005) and affects not only nutrient availability but alters soil properties. Vegetation derived “biochar” can increase water and nutrient holding capacity (Glaser et al. 2002) and has been hypothesized to adsorb organic compounds such as carbohydrates and amino acids (Rodman et al. 1987). Furthermore, the structural integrity of char can result in

microbial adsorption to external surfaces (De Latt et al. 1985) while serving as a habitat and substrate source (Pietikäinen et al. 2000). The functionality of char differs by vegetation type and the charring process (low vs. high temperature creation) resulting in variable effects on microbial activity.

The relationship between char and microbial decomposition (aerobic, anaerobic, and methanogenic) is unclear. Few studies have been conducted thus far relating these processes resulting in varying conclusions. For example, CH_4 stimulation has been reported (Karhu et al. 2011; Rondon et al. 2006) while other studies have observed an overall suppression (Zhang et al. 2010; Knoblauch et al. 2008) in response to fire. One plausible explanation for decreased CH_4 production in response to char addition originates from the char structure. The char structure can increase soil aeration discouraging environmental conditions necessary for methanogenesis while possibly preferentially stimulating methanotrophic activity (Karhu et al. 2011). Karhu et al. (2011) found no effect of char on CO_2 production. Stimulation of C gas production following fire has been observed in wetlands previously (Levine et al. 1990; Nakano et al. 2006). In grassland ecosystems, Toma et al. (2010) found no response in CO_2 and CH_4 production following fire whereas others observed a shift from net CH_4 source to sink (Castaldi et al. 2010).

While multiple studies have investigated the relationship between char and C gas production, limited research has focused on the effect of ash addition to these processes. In contrast to char (incomplete combustion), ash residues are created following complete combustion of OM. During high intensity fires, both N and C are lost from the system while P can become concentrated into vegetation derived ash. This

ash which is typically P rich (Raison et al. 1985) is deposited on the soil surface and may serve as a nutrient enrichment to P-limited soils.

A critical need to better understand the effect of fire on C loss processes is necessary especially in fire adapted wetland ecosystems. The inability to compare biogeochemical fire responses is apparent largely because fire characteristic data is seldom quantified. The objective of this study was to investigate the effect of fire on soil C processing from two fire adapted subtropical wetlands which vary in soil nutrient concentrations. One of these wetlands was rich in soil P whereas the other was P-limited. Addition of P following fire is likely, thus; we hypothesized that ecosystems which vary in soil P concentrations would respond differently to fire. More specifically, a greater stimulation in C gas production in low relative to high-P soils would be in response to decreased microbial P limitation (i.e. elevated C cycling).

Methods

Site Characteristics

The effect of prescribed burning on soil biogeochemical processing was tested in the Hole-in-the-Donut (HID) region of Everglades National Park of Southern Florida, USA. Soils in this region belong to the Perrine and Biscayne soil class and are calcareous subtropical wetlands with poor to very poor draining characteristics (USDA 1996). This land was heavily farmed until 1975 when the park purchased the land resulting in farm abandonment. In response to land use change, soil disturbance, and nutrient addition (fertilization) the land was overgrown by the invasive vegetation *Schinus terebinthifolius* (Smith et al. 2011). Through wetland mitigation, the land was scraped to bedrock (complete removal of soil and vegetation) exemplifying an extreme restoration approach. Scraping of the soils (and vegetation removal) began in 1989 and

is still continued today with the overarching restoration goal to remove the invasive vegetation, *Schinus*, and discourage regrowth (Smith et al. 2011).

In the current study, soils restored by extreme restoration (*see above*) in 2000 (site referred to as Res00) and a native reference site (never farmed or fertilized) were chosen to investigate the effects of fire on C cycling. Two main differences between the restored and reference site are the (i.) dominant vegetation and (ii.) soil P concentrations. Vegetation from Res00 consists of *Baccharis halimifolia* and *Muhlenbergia capillaris* with *Cladium* and *Typha* (present to a lesser extent). In contrast, the reference site is dominated by sedges and grasses (*Cladium*, *Muhlenbergia*, respectively) similar to oligotrophic wetlands within the greater Everglades. Soils from Res00 contain high concentrations of soil P (Table 4-1). In contrast, the reference soils contain very low soil P concentrations (150 mg kg^{-1} soil).

Fire Characteristics

According to conditions provided by the prescribed burn manager (Everglades National Park) the Res00 and reference sites were burned on May 4th 2010. Field sites received 1.27 mm of precipitation four days prior to the burn. All desired conditions were met including a dry bulb temperature ranging from 86-89°C, relative humidity between 55-75%, a cloud cover of 30-50% with wind speed between 2 and 12 mph, and fine fuel moisture averaging 8%. Wind direction was predominately S or S SE. The fire was completed through manual and aerial ignition and was classified as a head fire with a rate of spread between 15 and 30 chains hr^{-1}(1 chain=66 feet). Fire temperatures in Res00 reached 190°C and 140°C at high and low elevations, respectively. However, greater increases in temperature were reported from reference wetlands with temperatures reaching 425°C and 190°C at high and low elevations, respectively.

Experimental Design

Two adjacent 30 x 30 m plots were established at the Res00 and reference sites at both a high (N25.382°W80.674, N25.381°W80.672, respectively) and low elevation (N25.372°W80.674, N25.372°W80.672, respectively). One 30 x 30 m plot was protected from fire with a 2 m buffer strip which was cut on April 8-10th 2010 (Figure 4-1). At the time of plot preparation soil samples were collected to determine baseline conditions from all designated burn and non-burn plots.

Each 30 x 30 m plot was evenly divided to represent three replicates. Each replicate plot was further divided into 75 (2 x 2 m) sub plots which were sampled at random as chosen through random number generation. In total, we sampled a burn and control plot from two sites (Res00 and reference) at two elevations (high and low). To monitor the soil and fire temperature, six thermocouples were placed on the soil surface and installed at a 1 cm soil depth, where the majority of microbes reside (Neary 1999).

Temperature data were recorded every second for a month prior to and after the fire to ensure temperature data was complete. The prescribed fire occurred on May 4th-5th 2010 and our post fire sampling began on the 6th-7th of May. Additional samples were retrieved July 6th 2010, September 15th 2010, and May 25th 2011. Multiple collections (soils, *see below*) gave us an opportunity to study the effect of fire on select parameters on a short (2 day, 1 month), intermediate (3 months), and longer (1 year) term basis.

Soil Sampling and Analysis

Soils were collected from 0-5 cm depths (or until bedrock) using a spatula, transferred to a bag and placed on ice, and transported to the University of Florida

Wetland Biogeochemistry Laboratory for processing within 1-2 days of sampling. Soil samples were sieved through a 2 mm mesh to remove rocks and roots. Representative aliquots were removed from each sample (n=3 per site) and analyzed for soil nutrients (C/N/P), MBC, ß-glucosidase enzyme activity (BGA), respiration potentials (aerobic/anaerobic CO_2) and methanogenesis.

Microbial biomass C was analyzed by 0.5 M K_2SO_4 extraction following chloroform fumigation (Vance et al. 1987). Extracted samples were filtered (0.2 µm filter) and analyzed for total extractable organic carbon (TOC) on a Shimadzu TOC 5050A auto-analyzer. The difference between the TOC from the fumigated and un-fumigated sample was considered MBC. To quantify loss on ignition (LOI %) soil samples were combusted at 550°C for 3-4 hours. This value was used to estimate the organic C content (OC) with a 45% OC from total OM factor.

Soils were dried at 105°C for 2 days followed by hand grinding with a mortar and pestle in preparation for additional analyses. Soil TN/TC was determined using the EPA method 3010 (EPA, 1993) and analyzed on a Costech Model 4010 Elemental Analyzer (Costech Analytical Industries, Inc., Valencia, CA). For TP analysis, samples were combusted at 550°C for a 4 hour period followed by dissolution of remaining ash with 6 M HCl (Anderson 1976). Bioavailable phosphorus (P_i) was determined via $NaHCO_3^-$ extraction concurrent with acquisition of microbial biomass phosphorus (MBP) via chloroform fumigation. All above analyses were conducted by the University of Florida Wetland Biogeochemistry certified laboratory (Gainesville, Florida).

Aboveground Biomass

To determine severity, we collected vegetation and litter material from a 1x1 m subplot (n=4) from Res00 and reference-high and low elevations on the April and May

sampling dates. Following collection, materials were separated into standing live vegetation, dead vegetation, and litter prior to being dried at 60˚C for three days.

Basal Microbial Respiration and Methanogenesis

Aerobic soil respiration was measured by using 2 g dry weight soil aliquots in sealed 120 mL pyrex glass bottles using a 0.5 M NaOH trap base. Traps were changed on days 1, 2, 3, and 4 where values for each time interval were summed to calculate cumulative respiration. Soil free controls were used to account for background CO_2 levels and subtracted from soil respiration determinations. Upon removal, the base trapped CO_2 was released by acidifying the solution (3 M HCl) and analyzing the released CO_2 by gas chromatography.

To quantify anaerobic C loss potentials (CO_2 and CH_4) 2 grams dry weight soil and 10 mL of DDI were combined in 30 mL conical tubes (n=3 per site), flushed with oxygen free N_2 to ensure anaerobic conditions and stored in the dark. After a 24 hour incubation period gas headspace was measured and periodically re-measured up to 30 days on a gas chromatograph to obtain a linear phase of gas production.

Gas Analysis

Methane headspace was detected by a Shimadzu gas chromatograph-8a fitted with a flame ionization detector (160°C, injection temperature 110°C), N_2 as the carrier gas and a 5.25 ft. (45/60) Carboxen-1000 column (Supelco Inc., Bellefonte, PA). A Shimadzu gas chromatograph-8a fitted with a thermal conductivity detector (column injection 120°C, 40°C oven temperature), He as the carrier gas and a 6 and 1/8 ft. (80/100) Porapak-N column (Supelco Inc., Bellefonte, P.A) was utilized to analyze CO_2 headspace. Calibration curves were determined via standard gas mixtures (Scotty Specialty Gases, Plumsteadville, PA) multiple times throughout each sampling event.

Enzyme Activity

Soil enzyme activity of cellobiohydrolase (CBH) and β-glucosidase (BGA) were measured fluorometrically with methylumbelliferone fluorophore on a Bio-tek® model Fl600 plate reader (Biotek instruments, Inc. Winooski, VT) following 100 fold soil dilution with DDI similar to Inglett et al. (2011b). Briefly, a 500 µM substrate-fluorophore solution was added to the soil solution and incubated in the dark for 2 hours. Following the incubation, fluorescence was measured at an excitation of 350 nm and emission of 450 nm. Soil quenching curves were prepared to detect any possible quenching which may have occurred throughout the incubation. Methylumbelliferone standard curves were prepared and used to determine enzyme activity from the fluorescence. Activities were reported as nmoles MUF g^{-1} MBC h^{-1}.

Data Analysis

All statistical analyses were performed in JMP vs. 8.0 (SAS institute, Cary NC). Initial differences in soil nutrient concentrations and microbial parameters between pre-fire burned plots and control plots were determined via one way analysis of variance (ANOVA). Similarly, the fire effect on soil nutrients and microbial parameters were investigated via one way ANOVA. In addition, differences in parameters within sites at different elevations were compared via one way ANOVA. Differences between means ($\alpha<0.05$) were assessed via Tukey-Kramer means comparison. Parameters were compared at each sampling time thus each sampling event was analyzed independently.

Results

Pre Burn Analyses

Prior to the fire (April), soil samples were collected from pre-fire burned and control plots to determine within site variability in C parameters (Table 4-1 and 4-2). In Res00-low elevation wetlands aerobic CO_2 production was 50% greater in the pre-fire burned relative to the control plots. In high elevation wetlands BGA was approximately 50% greater from the control plot relative to the pre-fire burned plot ($p<0.05$; Table 4-2). No other differences were distinguished.

Short-Term Effects (2 Days)

Aboveground biomass was quantified pre and post fire to determine the overall severity. A greater mass of vegetation was present at high relative to low elevations (Table 4-3). There was more than double the biomass in the high relative to low Res00 site. Reference site severity was greater than observed at the Res00 site. The severity was 36% and 89% in the Res00-low and high elevation site, respectively. In the reference site severity ranged from 93-96% from the low and high site, respectively.

Temperatures at 1 cm soil depths remained elevated at 50°C or greater for less than 2 minutes. Two days following the fire, temperatures had returned to natural *in situ* temperatures. At this time (2 days), differences in soil and microbial parameters were observed.

Res00

High elevation response. Immediately following the fire no difference in soil OC from burned and control plots was evident (Figure 4-2). The response of C enzyme activity to fire varied by enzyme. There was no difference between CBH activity from the burned and control plot (Figure 4-3A). In contrast, BGA was enhanced (27%)

following exposure to fire (Figure 4-3B). Similar to the observed response of BGA to fire, TOC was elevated by 22% in the burned soils (Figure 4-4). This result was consistent with stimulated aerobic decomposition (CO_2) which was increased from 390 to 565 mg CO_2-C g MBC d^{-1} (a 31% increase) in the burned relative to control plots, respectively (Figure 4-5). The fire had no significant effect on anaerobic processing evident by no change in CO_2 or CH_4 production when compared to control production rates (Figure 4-6).

One month after the fire there was no change in soil OC (Figure 4-2). Activity of C enzymes (CBH and BGA) differed from changes detected immediately following the fire. Activity of CBH was decreased in burned plots by 32% when compared to activity in control plots (Figure 4-3A). In comparison, no effect of fire on BGA or TOC was detected (Figure 4-3B and 4-4). Decreased respiration in burned plots was evident regardless of aerobic or anaerobic conditions. To be more specific, a decrease of 49, 61, and 83% was detected in burned relative to control plots for aerobic CO_2, anaerobic CO_2, and CH_4 production, respectively (Figure 4-5 and 4-6).

Low elevation response. While fire had a stimulatory effect on select C parameters at high elevations a minimal affect was observed at low elevations. Following the fire, no effect on OC, C enzyme activity (CBH or BGA) or TOC was detected (Figure 4-7,8,9). While a 50% increase in aerobic CO_2 production in the burned plot was quantified this difference was similar to the initial difference (pre-burn) in production rates between plots (Figure 4-10). Thus, there was no actual burn effect on this parameter. Under anaerobic conditions, there was no difference in either CO_2 or CH_4 production (Figure 4-11).

Similar to the response at 2 days, there was no difference in soil OC, CBH, TOC, ACD, or MET between control and burned plots (Figure 4-7, 4-8A,4-9,4-10,4-11B) after one month. Activity of BGA was 28 nmoles g^{-1} MBC h^{-1} higher in burned relative to control plots equating to a 47% increase in activity (Figure 4-8B). There was also a 36% decrease in anaerobic CO_2 production from burned plots (Figure 4-11A).

Res00 high and low elevation response after three months. After three months, differences between C parameters were similar at high and low elevations. No change in soil OC, CBH activity, or anaerobic decomposition (CO_2 or CH_4) was detected (Figure 4-2, 4-3A, 4-6:8A, 4-10). Elevation specific responses were detected in BGA, TOC, and ACD. At high elevations, a 40% reduction in CO_2 production rates from burned plots was observed (Figure 4-5). At low elevations, BGA remained elevated by 40% ($p<0.1$), similar to the trend observed after one month (Figure 4-8B). There was also a slight reduction in TOC from burned plots at the low elevation ($p<0.1$) (Figure 4-9).

Differences in C processing were still quantified one year after the fire in Res00. Regardless of elevation, no effect of fire was detected on soil OC, C enzyme activity (CBH and BGA), or ACD (Figure 4-3,4-5,4-7,4-8,4-10). There was still a fire effect on TOC and anaerobic processing (CO_2 and CH_4 production) which differed by elevation. At the high elevation site increased TOC in the burned plot was accompanied by a 156% increase in methanogenic processing (Figure 4-4 and 4-6B). In contrast, soil TOC at the lower elevation was decreased in burned plots. Decreased TOC at the burned plots (14%) was accompanied by decreased anaerobic CO_2 and CH_4 (Figure 4-9 and 4-11).

Reference

High elevation response. There was no change between soil OC content in burned and control plots (Figure 4-12). The fire effected C enzymes differently. In burned plots, CBH decreased from 6.3 to 0.4 nmoles g^{-1} MBC h^{-1} resulting in a 94% reduction in activity (Figure 4-13A). In contrast, there was no effect on BGA (Figure 4-13B). However, according to pre burn data retrieved in April high activity originated from the control soils (relative to the future "burn" plot). Two days following the fire, BGA was greater in the burned plots although not significant suggesting a positive effect may have been masked by the initial discrepancy in activity rates. There was an 18% increase in TOC following the fire resulting in an additional 83 mg TOC kg^{-1} soil in burned plots (Figure 4-14). Regardless of aerobic or anaerobic conditions no change in CO_2 was detected (Figure 4-15 and 4-16A). In contrast, a 54% increase in CH_4 production was detected (Figure 4-16B).

Low elevation response. Similar to Res00, a lesser effect of fire was evident on C parameters at the low relative to high elevation site. There was no effect of fire on OC or C enzyme activity (CBH or BGA) (Figure 4-17 and 4-18). The concentration of TOC was increased by 12% in burned plots (Figure 4-19). This equates to an increase in soil TOC of 28 mg kg^{-1}soil, an addition almost 3-fold lower than the amount added to burned plots at the corresponding high elevation. No difference in aerobic CO_2 production between burned and control soils were detected (Figure 4-20). In contrast to high elevations, under anaerobic conditions a slight increase in CO_2 production was observed (p<0.1) although no difference in MET was detected (Figure 4-21).

Reference high and low elevation response. One month following the fire, the response of C parameters was similar regardless of elevation. No difference in soil OC,

C enzyme activity (CBH and BGA), TOC, ACD, or MET was detected between burned and control plots (Figure 4-12:15,4-16B,4-17:20,4-21B). However, a different response in anaerobic CO_2 production was observed at both elevations. At high elevations, anaerobic decomposition was decreased by 69% from 53 to 16 mg CO_2-C g^{-1} MBC d^{-1} in control and burn plots, respectively (Figure 4-16A). In contrast, a 94% increase in burned plots at the low elevation was detected increasing CO_2 production from 23 (control) to 44 mg CO_2-C g^{-1} MBC d^{-1}(burned) (Figure 4-21A).

At three months an overall decrease in C processing was observed from reference-high elevation burned soils. While there was no change in C enzyme activity or methanogenesis between burned and control plots a 18, 33, and 46% reduction in soil TOC, anaerobic, and aerobic CO_2 production was observed (Figure 4-14,4-15,4-15A). At low elevations, no effect of fire on C processing was observed (Figure 4-17:21).

By one year, there were no differences in C processing in burned and control plots at high or low elevation reference sites (Figure 4-12:21).

Trends in C Parameters

In response to high within treatment variability, clarification of long-term effects of fire on C parameters can be deciphered through interpretation of burn:control ratios. While some trends did not appear consistent others provided additional insight to the effect of fire on these parameters and the trajectory these differences with time.

Res00

High elevation response. The burn:control ratio of CBH activity was always below 1 throughout the year following the fire (Figure 4-3A). The ratio decreased for all C parameters between the two day and one month sampling. However, between one

month and one year the ratio of soil TOC, aerobic (CO_2), and anaerobic decomposition increased (Figure 4-4:6).

Low elevation response. The burn:control ratio of soil TOC was consistently below 1 throughout the year (Figure 4-9). In contrast, the ratio of BGA was always above 1 (Figure 4-8B) as was the ratio of CBH after day 2 (Figure 4-8A). An increasing ratio after two days was also detected for ACD (Figure 4-10). In contrast, a decreasing trend in the burn:control ratio was evident with time in TOC, and anaerobic decomposition (Figure 4-9 and 4-11).

Reference

High and low elevation response. Trends in the burn:control ratio were not as evident in reference wetlands when compared to trends from Res00. At the high elevation site, the burn:control ratio of CBH activity increased with time (Figure 4-13A). In contrast, there was a decreasing ratio of BGA with time (Figure 4-13B). The decreasing ratio of BGA was also observed at the low elevations (Figure 4-18B).

Discussion

Immediate Response of Microbial and Soil Parameters to Fire

Close to 77 and 94% of the vegetation and litter was removed from Res00 and reference wetlands, respectively. This suggests a more complete burn in the reference relative to Res00 site. Variation in the fire can affect microbial activities. In most grassland fires burn intensity can approach 300°C on the soil surface; however at 1 cm depths, where most microbes reside (Neary 1999), temperatures spike to ~60°C gradually decreasing with time (Ryan 2002) suggesting microbial mortality in low intensity grassland fires is minimal (Neary 1999; Hart et al. 1995; Raison 1979). In mineral soils, Raison et al. (1986) observed less than a 15% transfer of aboveground

heat belowground. Nevertheless, in response to fire most soils increase in temperature although the amplitude of this temperature change can vary immensely. Increasing soil temperatures in response to fire can result in a reduction of the soil moisture content (Tix et al. 2006) further inhibiting survival of some microbial communities. This change in temperature and moisture content may provide a competitive advantage to microbes which flourish or can withstand high temperature and desiccation. There was a reduction in the soil moisture content from the burned relative to the control Res00 plot at the high elevation ($p<0.05$*, data not shown*) although there were no other differences in soil moisture content detected. As the soil begins to warm, the active sites of microbial derived enzymes begin to lose function prior to the protein denaturing (Wallenstein et al. 2011) suggesting short temperature variation may reduce active site function although temperatures (in this case) may not be extreme enough to denature the protein. An elevated temperature post fire may persist for months or even years (Neary 1999) mostly due to increased heat absorption from minimal vegetation shading (Rivard and Woodward 1989).

In our study sites, temperature transfer would likely be more extreme in Res00 due to shallower soil depths relative to the deeper reference soils. For example, in Res00 soil depths average 1.7 cm thus 60% of the soil collected was likely exposed to fire whereas closer to 20% of soil collected from reference wetlands (0-5 cm) would come in contact with temperature fluctuation from the fire assuming a heat penetration depth of 1 cm. Temperatures within the HID soils fluctuate largely at 1 cm depths extending from 20 to 40°C in both sites across spring and summer months. Furthermore, soil

temperatures did not exceed 70°C at 1cm depths (*data not shown*) during the fire event suggesting microbial activity would be minimally affected (Ryan 2002).

Although environmental conditions were uniform while burning (air temperature, humidity etc.), variation in site vegetation and production of associated fire residues (ash/char) may explain the differences in microbial and soil C parameters post fire in the different sites. Wind-driven surface fires, especially those burning through grass-dominated fuelbeds are expected to have a minimal effect on soil OM (DeBano 1988) as observed in Res00 and reference wetlands likely due to an accelerated rate of spread and reduced residence time of the fire. In addition, low OM at these sites (10-20%) may discourage high intensity burns, thus decreasing heat transfer to the soil in response to minimal soil based fuel.

The highest proportion of microbial activity is typically within the top 1 cm of soil (Neary 1999). At depths greater than 2-3 cm the temperature post fire can be similar to ambient temperatures (González-Pérez et. al. 2004). For this reason, fire characterization data such as temperature and residence time is important when postulating the effect of fire on microbial biomass and associated parameters. It is common to observe no stimulation in soil MBC post fire in prairie (Ajwa et al. 1999) and forested ecosystems (Prieto–Fernández et al. 1998). In agreement, no difference in MBC in Res00 or reference burned sites was detected immediately following the fire possibly due to the low fire intensity and minimal heat transfer to the soil.

Following a fire, ecosystems with no change in MBC coupled with complete vegetation removal (substrate reduction) would suggest C enzyme activity would be minimally affected. However, activity of C enzymes are considered to be a main

regulator of decomposition (Sinsabaugh et al. 1992), which is why we quantified the activities of two C enzymes in the current study. One of these enzymes (CBH) targets the degradation of more complex OM whereas the other C enzyme (BGA) can only degrade more decomposed OM. Following fire many studies have quantified BGA activity with varying results (Ajwa et al. 1999; Boerner and Brinkman 2003; Gutknecht et al. 2010); however, limited data on CBH has been reported (Gutknecht et al. 2010). Immediately following the fire, no response in CBH was observed from Res00 (Figure 4-3A and 4-8A). It is plausible a reduction in vegetation derived complex C inputs reduced the substrate availability necessary to stimulate CBH production. The products of CBH activity provide the substrates necessary to fuel BGA activity. For this reason, the production of BGA is typically related to CBH activity. Because CBH (g^{-1} MBC) was not stimulated post fire an increase in substrates fueling BGA was not expected. However, at high elevations stimulation of BGA was evident (p<0.05; Figure 4-3B). Elevated BGA activity suggests an increased proportion of degraded OM compounds (substrates) were present at the burned relative to the control plot. An increase in more decomposed OM may be in response to the addition of incompletely combusted OM and litter material post fire. It may have also originated from relocation of C within the vegetation during the fire which may have increased the proportion exuded from the roots.

In contrast to results from Res00, CBH (g^{-1} MBC) was suppressed in high elevation reference wetlands (p<0.05; Figure 4-13A) immediately following the fire likely due to decreased complexity of the remaining OM. When taking into account the higher activity from control plots quantified pre-burn the stimulation of BGA was likely masked

although variation in BGA is not commonly reported post fire (Gutknecht et al. 2010; Boerner et al. 2005). This suggests a tight coupling between C enzyme activities in the reference site.

Elevated extractable TOC may be observed post fire in response to an increase in BGA, degradation of microbial biomass, or as a leachate from incompletely combusted OM. Anderson et al. (2004) observed elevated TOC from burned relative to control soils for 90 days following a fire. In the current study, there was stimulation of extractable TOC at high elevation wetlands regardless of site and at the low elevation reference wetland (p<0.05; Figure 4-4,4-9,4-19). Increased TOC initially following a fire may stimulate microbial decomposition processes (Anderson et al. 2004).

Aerobic decomposition (CO_2) was stimulated in Res00 burned plots regardless of elevation (Figure 4-5 and 4-10). However, the stimulation at the low elevation site may be misleading because higher production originated from the burned plot prior to initiation of the experiment. Furthermore, the stimulation pre and 2 days post fire was equivalent (~50%). The increase in BGA, TOC, and ACD immediately following the fire at the high elevation site suggests an increased pulse of TOC was delivered to the burned plots which may have been used to fuel aerobic processing. Furthermore, at the high elevation site there were positive correlations between TOC and BGA (p<0.01) as well as TOC and ACD to a lesser extent (p<0.1). It has been hypothesized that the attack of BGA on organic matter would likely release TOC as a decomposition product (Freeman et al. 1997). A lack of stimulation in ACD from the reference sites coupled with enhanced TOC concentrations suggests C substrate availability was not the main regulator of this process in reference wetlands.

Under anaerobic conditions, the fire initially affected CH_4 production (in some locations) while having a limited effect on CO_2 production. A lack of stimulation in anaerobic CO_2 production was observed at Res00 sites, a result which has been reported previously on net soil CO_2 flux (Toma et al. 2010). We are unaware of any study which has quantified the effect of fire on anaerobic CO_2 production.

Toma et al. (2010) concluded there was no effect of burning on CH_4 production in a grassland ecosystem. This is in agreement with results from the Res00 site suggesting fire initially had a greater effect on aerobic as opposed to anaerobic processing. In reference wetlands, MET production was stimulated by the fire at the high elevation site (p<0.05) similar to Nakano et al. (2006). No difference in CH_4 production was detected between burn and control plots at the lower elevation. Levine et al. (1990) found CH_4 production to decrease immediately following fire, although, a delayed stimulation (day 3-9) was observed illustrating how important multiple collection and analyses of soils are post fire. We sampled the soil two days following the fire thus our results in the reference site (stimulation) was similar (time) to those of Levine et al. (1990). A change in C quality or availability may be fueling differences in anaerobic decomposition (Ajwa et al. 1999; Boerner et al. 2000). However, it is plausible a change in soil P availability may have stimulated anaerobic decomposition in our low-P reference site. Although an initial increase in available P was not observed at the high elevation reference site, a significant decrease in the MBC:MBP ratio suggests a decrease in microbial P limitation (p<0.05) initially post fire.

Monitoring from One Month to One Year Post Fire

We conclude stimulation of C parameters in response to fire were the greatest 2 days post fire regardless of site. Monitoring soil and microbial C parameters for a year

following the fire provided a unique opportunity to conclude that each wetland responded differently to the fire. For example, at high elevations C parameters quantifying decomposition (CO_2 and/or CH_4) were suppressed after a different length of time depending on the site. In Res00 wetlands, decreased CO_2 and CH_4 production was observed at the one month mark. In comparison, C parameters including TOC and CO_2 production (aerobic and anaerobic) were decreased in the reference-high elevation site after three months. This suggests these processes may be controlled by different factors at each site. At the three month mark, available P was significantly lower than quantified at 2d, 1mo, or 1 year at the high elevation reference site which suggests these processes may be regulated by P availability. After one year, the response of C parameters to fire varied greatly between sites and elevations. The results at one year illustrate the necessity of intensive sampling. Unfortunately, due to difficultly in acquiring site access (travel) and the long duration for laboratory incubations more intensive sampling was not feasible in the current study.

One year post fire the effect on C parameters still persisted at some burned sites. Ecosystem recovery (post fire) is largely dependent on vegetation regrowth (species and time) (Hart et al. 2005). In our study sites which are dominated by grasses, vegetation regrowth was quick compared to ecosystems dominated by woody vegetation such as forests. Two days post fire grasses were already visible in the reference site. Quick regrowth of vegetation will negate many fire responses such as elevated soil temperature, decreased moisture content, and reduced C substrate availability. Furthermore, this study suggests fire residues may have been the driving factor explaining differences in C parameters as opposed to heat transfer largely

because MBC was not stimulated in burned plots (relative to the control) and heightened soil temperature did not persist.

Many studies have investigated the response of soil and vegetation C parameters to fire in multiple ecosystems (Allison et al. 2010; Toma et al. 2010; Nakano et al 2006). Understanding the mechanisms that govern observed differences is difficult because fire characteristic and long-term data is rarely collected. As seen in the current study, most of the fire effect on microbial C processing was transient (2d) suggesting the timing of sampling is crucial. In grassland ecosystems, similar to the HID condition prior to burning, fire is capable of oxidizing a large proportion of OM. Overall, oxidation of OM was more complete in reference relative to Res00 wetlands. Biomass removal was also greater from high relative to low elevation plots. Thus, these differences may have altered the proportion of ash and char produced at each site and associated elevation which may have explained the initial stimulation of C processing.

Conclusions

The response of soil and microbial C parameters to fire varied at each site. A minimal transfer of aboveground heat to the soil and no change in microbial biomass suggests differences in C cycling post fire may have been in response to initial site differences. Our results suggest C cycling in the HID was stimulated immediately following fire, however, stimulation of microbial processes (CO_2/CH_4 production) were accentuated in reference (low-P) soils. Regardless of site, no loss in soil OC was detected however, close to 100% of the aboveground biomass (vegetation and litter) was removed. Following one year, the effect of fire was not detected on C parameters in the reference wetland suggesting these processes recovered quickly following fire. In contrast, differences in C parameters from burned and control plots were still present

one year post fire in restored soils. This suggests the microbial communities may respond differently in this disturbed, high-P wetland. Differences may have been in response to woody vegetation present in the restored site which may have altered fire residue production and subsequent nutrient addition.

Future studies are necessary to investigate if fire residue addition (nutrients) can explain differences in soil TOC, C enzyme activity, and aerobic and anaerobic decomposition processes observed in this study.

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Table 4-1. Basic soil nutrient parameters from control and burned plots from res00 and reference areas measured prior to the fire (4/8/10)

Site	Transect	Treatment	pH	MC	LOI	TN	TC	Ext. TOC	TP	Ext-NOx	Ext-NH_3
				%		g kg^{-1}			mg kg^{-1}		
Res2000	High	Burn	8.2 (0)	44.2 (0.5)	20.1 (2.1)	7.1 (0.5)	159 (4)	302 (27)	755 (77)	19.2 (4)	58.4 (6.2)
		Control	8.2 (0)	43.6 (1.6)	17.9 (2.2)	7.6 (0.5)	165 (7)	299 (22)	700 (7)	16.3 (0.7)	56.2 (14)
	Low	Burn	8.1 (0)	44.8 (0.7)	21 (0.5)	7.3 (0.4)	154 (3)	298 (18)	492 (38)	29.1 (2.1)	55 (11.9)
		Control	8.2 (0)	47.9 (2)	20 (1.6)	7.7 (0.6)	161 (3)	310 (29)	456 (10)	21.7 (2.3)	60 (13.8)
Reference	High	Burn	8.4 (0)	37.6 (2.5)	11 (0.9)	6.4 (0.5)	157 (2)	258 (4)	140 (6)	4.5 (0.3)	25.8 (4.5)
		Control	8.4 (0)	37.9 (0.6)	10.5 (1.3)	7 (0.1)	158 (2)	304 (27)	119 (3)	2.4 (0.4)	38.8 (5)
	Low	Burn	8.3 (0)	42.5 (1.3)	13.2 (0.7)	7.5 (0.3)	157 (2)	292 (7)	140 (11)	5.1 (0.9)	35 (0.6)
		Control	8.3 (0)	41.5 (0.8)	13.2 (1.2)	7.3 (0.1)	156 (3)	250 (18)	134 (5)	5.1 (0.2)	31.4 (4.9)

LOI=loss on ignition

Table 4-2. Basic soil microbial parameters from control and burned plots from res00 and reference areas measured prior to the fire (4/8/10)

Site	Transect	Treatment	MBN	MBC	MBP	CBH	BGA	ACD	ANCD	MET
				mg kg^{-1}		nmoles MUF g^{-1} soil h^{-1}		mg gaseous-C kg^{-1} soil d^{-1}		
Res2000	High	Burn	364 (18)	3187 (83)	40.1 (3)	3.5 (1.0)	17.9 (2.1)^{B}	1229 (148)	74 (10)	0.61 (0.21)
		Control	438 (60)	3800 (290)	44.5 (5)	5.9 (2.0)	28.5 (2.2)^{A}	1622 (114)	144 (38)	0.34 (0.33)
	Low	Burn	265 (19)	3164 (237)	29.3 (1)	8.0 (0)	35.4 (4.3)	2623 (15)^{A}	114 (43)	1.78 (0.24)
		Control	320 (57)	3445 (259)	37 (6)	9.4 (3.4)	37.2 (0.4)	1614 (218)^{B}	120 (30)	1.02 (0.10)
Reference	High	Burn	185 (29)	2568 (233)	13.1 (1)	1.7 (0.2)	5.6 (0.3)^{B}	1048 (179)	100 (13)	0.042 (0.034)
		Control	138 (8)	2445 (176)	14 (1)	1.8 (0.7)	11.5 (2.1)^{A}	1237 (388)	145 (43)	0.021 (0.009)
	Low	Burn	148 (3)	2532 (50)	15.3 (1)	2.1 (0.2)	12.3 (4.3)	800 (6)	62 (10)	0.008 (0.002)
		Control	142 (26)	2412 (283)	14 (2)	2.2 (0.8)	12.1 (3.3)	1084 (93)	56 (3)	0.011 (0.002)

Abbreviations: MBN/C/P=Microbial biomass nitrogen, carbon, phosphorus, CBH=Cellobiohydrolase enzyme activity, BGA=β-glucosidase enzyme activity, ACD=aerobic CO_2 production, ANCD=anaerobic CO_2 production, MET=CH_4 production

Table 4-3. Changes in biomass before and after the fire in different component (g biomass m^{-2}) calculated from 1m×1m biomass plots

		Plants-dead			Plants-live			Litter		
		biomass (g m^{-2})			biomass (g m^{-2})			biomass (g m^{-2})		
Site	Plot	pre-fire	post-fire	% loss	pre-fire	post-fire	% loss	pre-fire	post-fire	% loss
Res2000	High	133 (48)	22 (6)	84	40 (22)	0 (0.9)	100	196 (23)	19 (3)	90
	Low	98 (12)	29 (9)	70	19 (3)	7 (4)	61	29 (7)	16 (5)	45
Reference	High	164 (27)	5.7 (0.2)	97	92 (12)	0 (0.1)	100	47 (20)	7 (3)	85
	Low	160 (21)	4.7 (0.5)	97	65 (11)	0 (0.3)	100	19 (6)	10 (3)	49

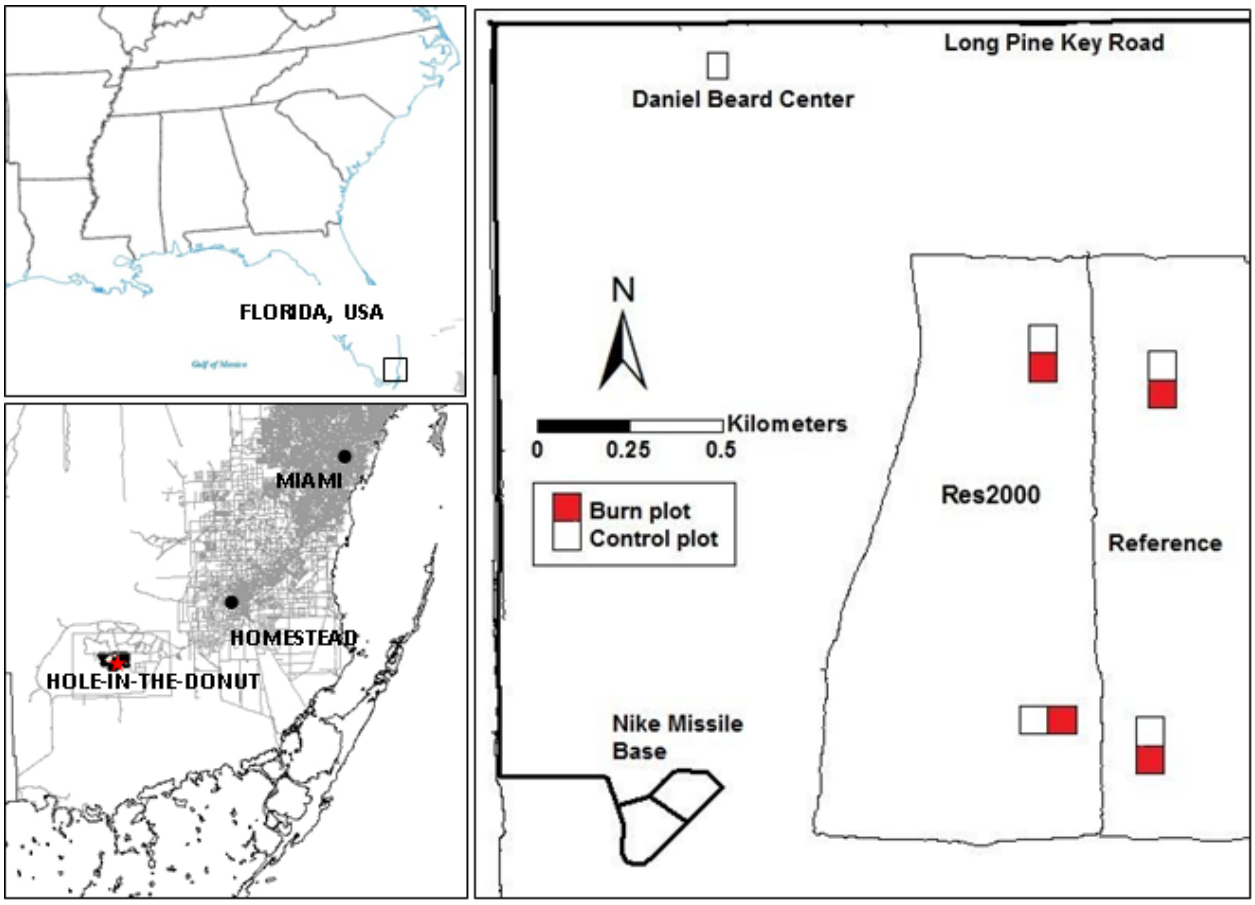
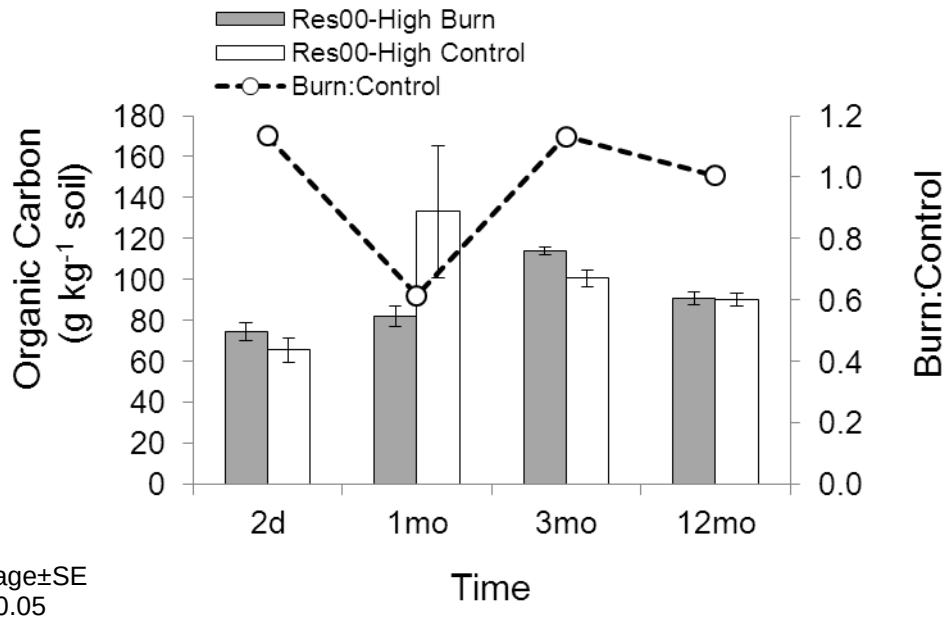


Figure 4-1. Schematic of experimental design prior to the prescribed fire with the Hole-in-the-Donut (Everglades National Park) (Figure from Liao, PhD. Dissertation 2012)



Average±SE
*=p<0.05
**=p<0.1

Figure 4-2. Effect of fire on soil organic carbon concentrations 2 days, 1 month, 3 months, and 12 months post fire in the Res00-high elevation site

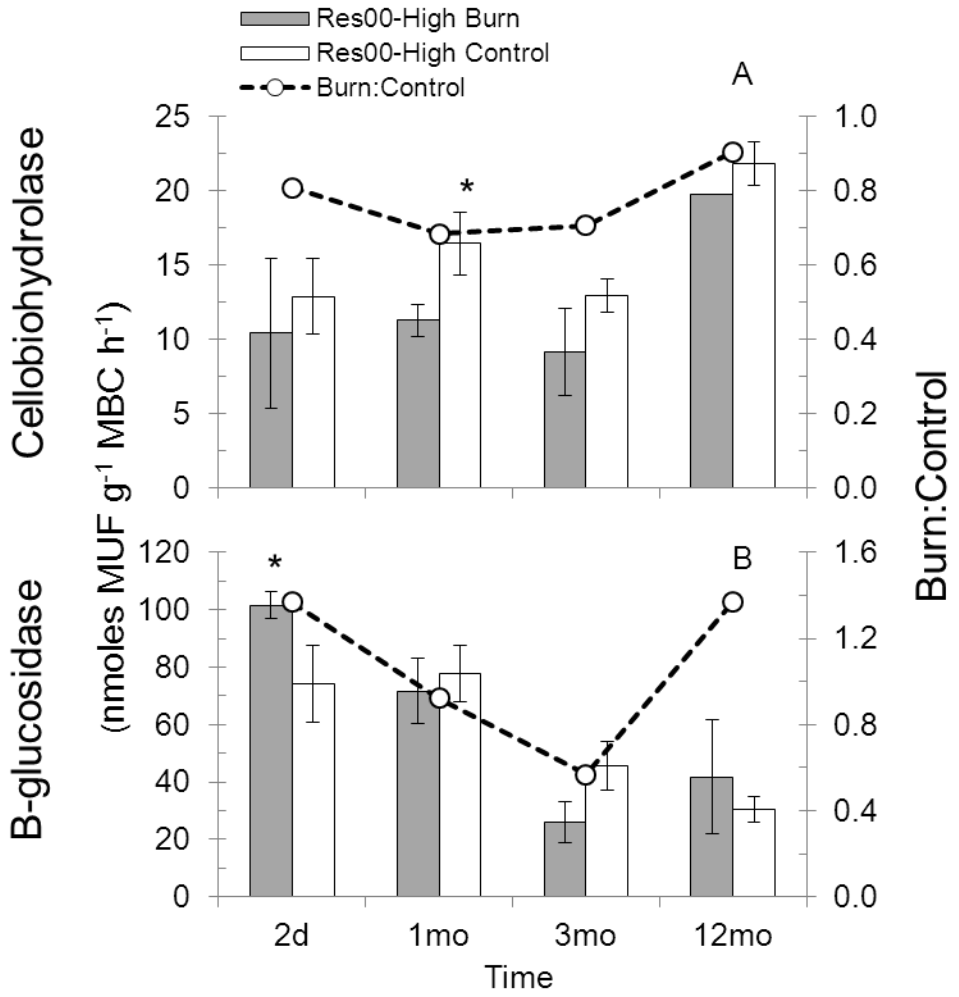


Figure 4-3. This figure depicts the effect of fire on C enzyme activity from Res00-high elevation sites. Data shows the effect of fire on A) cellobiohydrolase and B) β-glucosidase C enzyme activity 2 days, 1 month, 3 months, and 12 months post fire

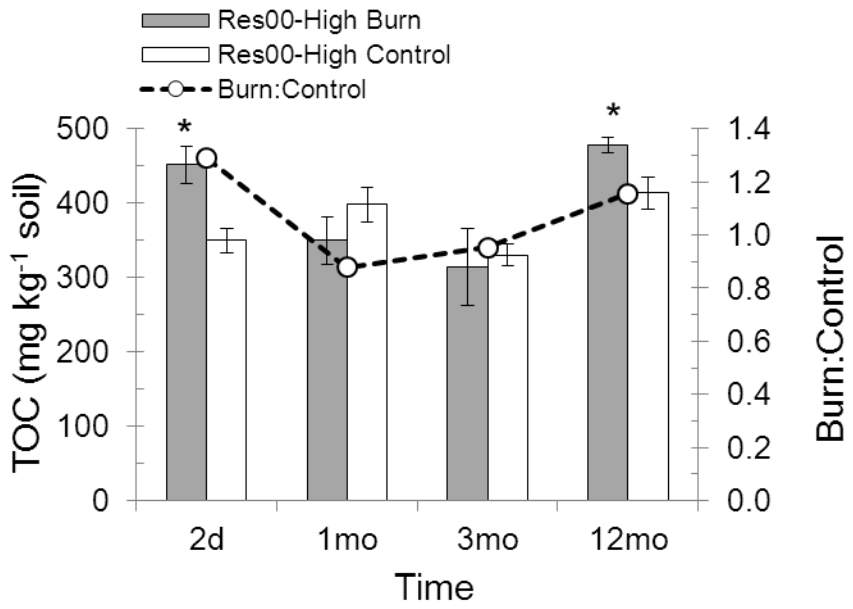


Figure 4-4. Effect of fire on soil total extractable organic carbon concentrations 2 days, 1 month, 3 months, and 12 months post fire in the Res00-high elevation site

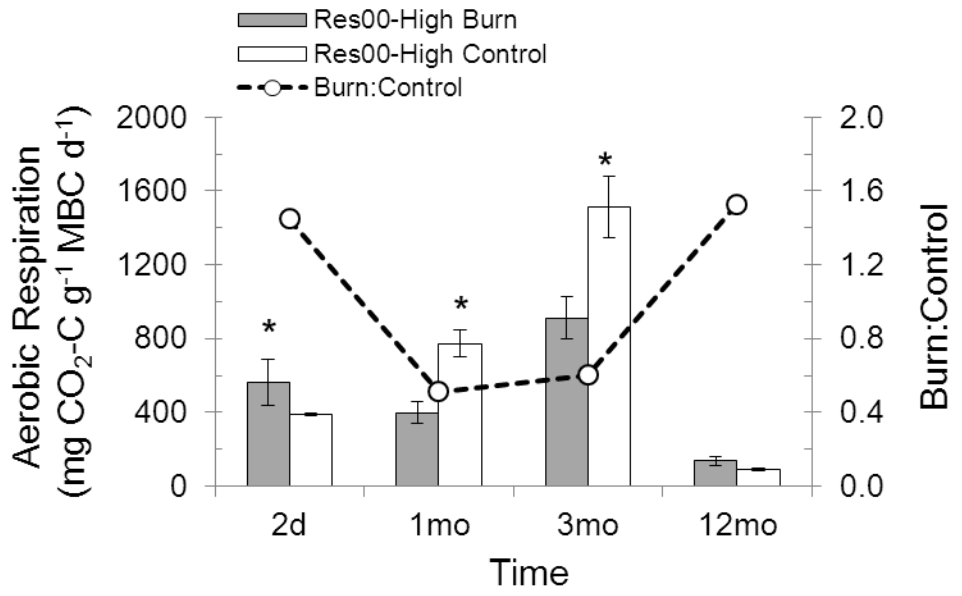


Figure 4-5. Effect of fire on aerobic respiration potentials 2 days, 1 month, 3 months, and 12 months post fire in the Res00-high elevation site

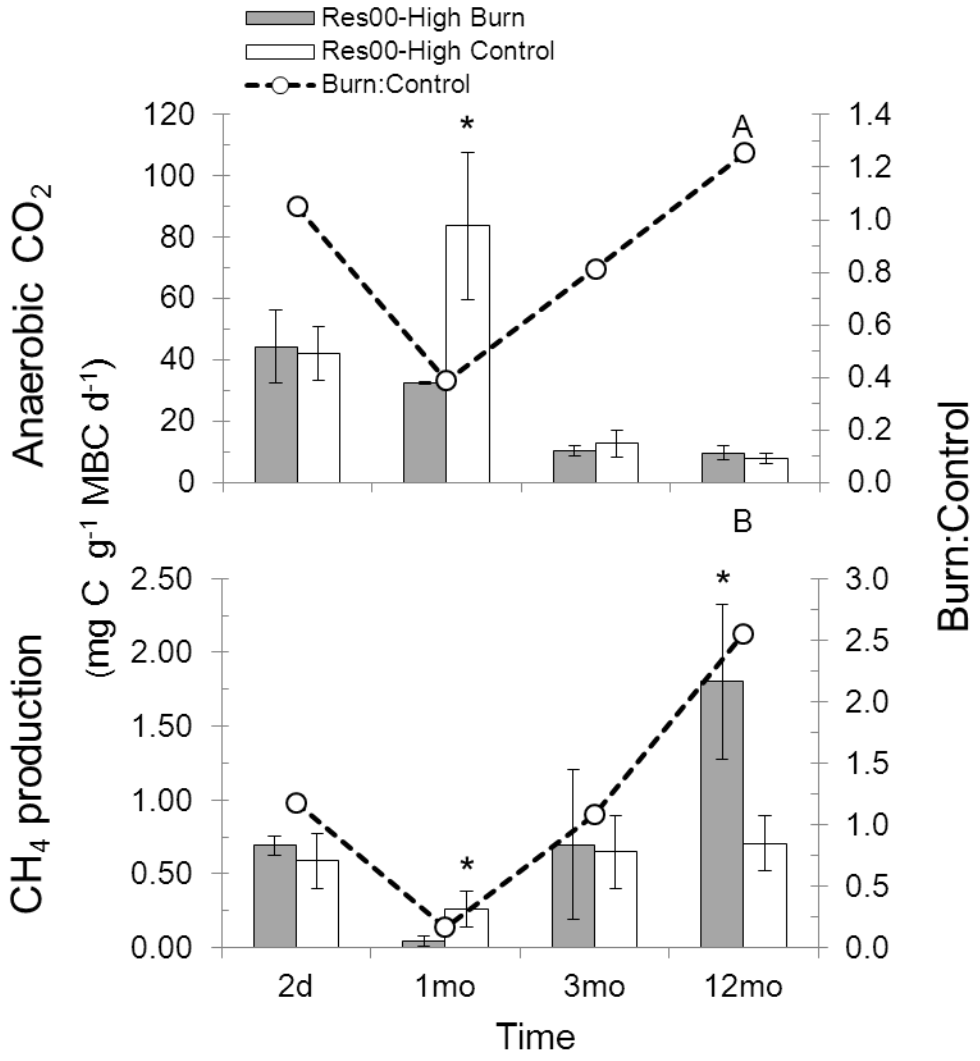


Figure 4-6. This figure depicts the effect of fire on anaerobic respiration from Res00-high elevation sites. Data shows the effect of fire on A) CO_2 and B) CH_4 production 2 days, 1 month, 3 months, and 12 months post fire

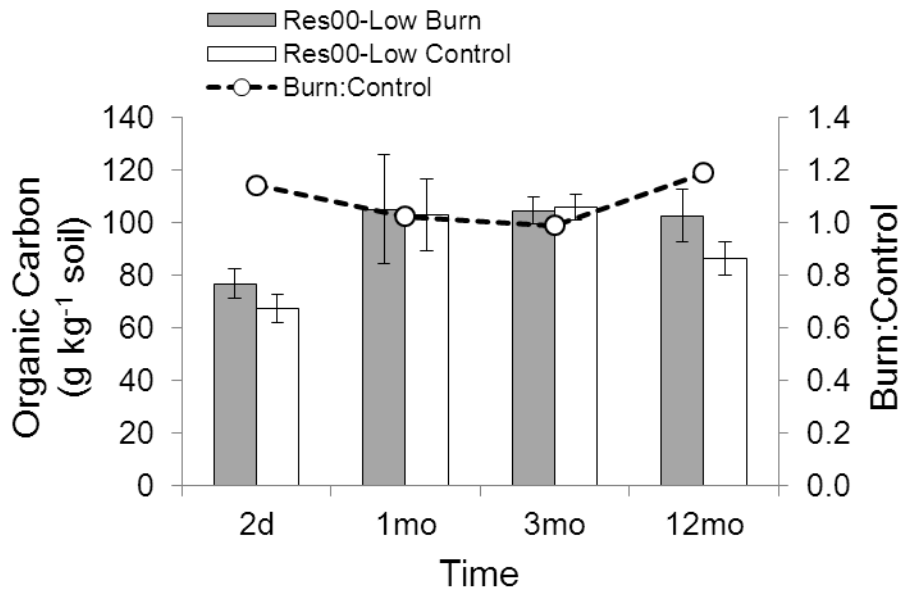


Figure 4-7. Effect of fire on soil organic carbon concentrations 2 days, 1 month, 3 months, and 12 months post fire in the Res00-low elevation site

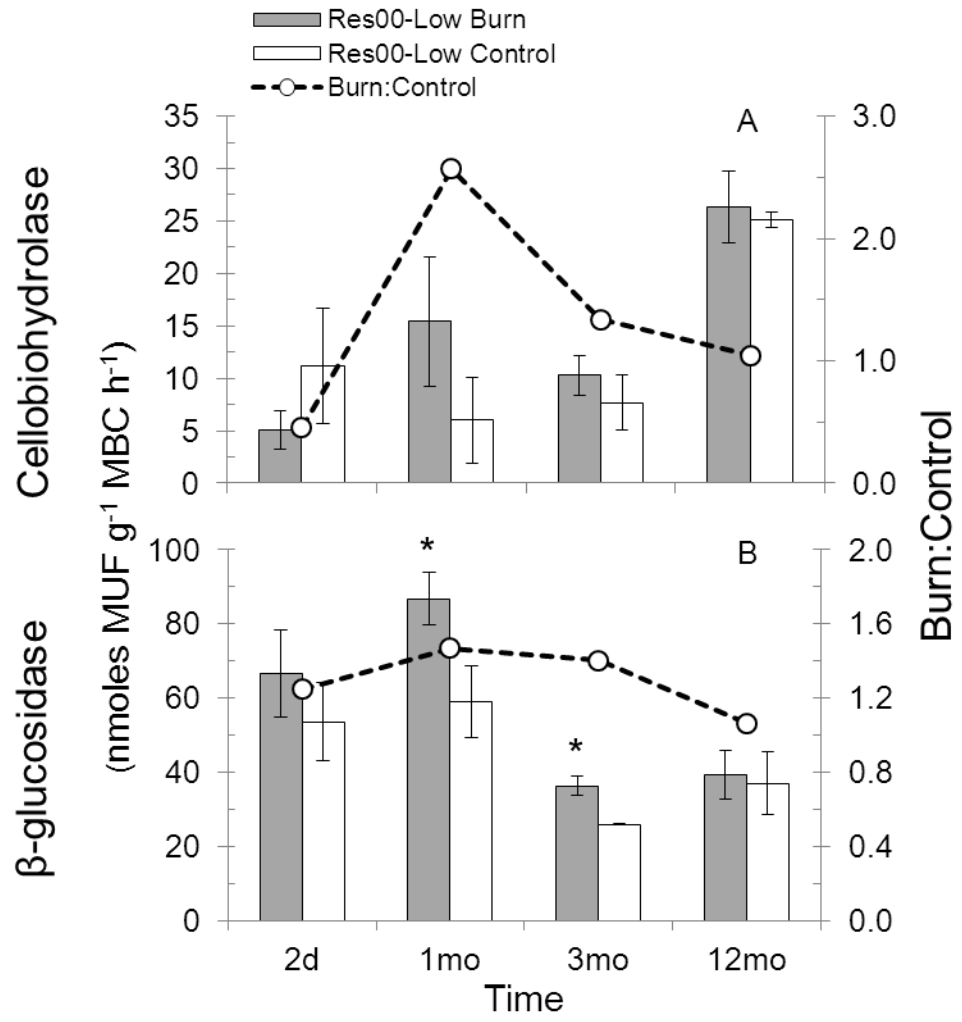


Figure 4-8. This figure depicts the effect of fire on C enzyme activity from Res00-low elevation sites. Data shows the effect of fire on A) cellobiohydrolase and B) β-glucosidase C enzyme activity 2 days, 1 month, 3 months, and 12 months post fire

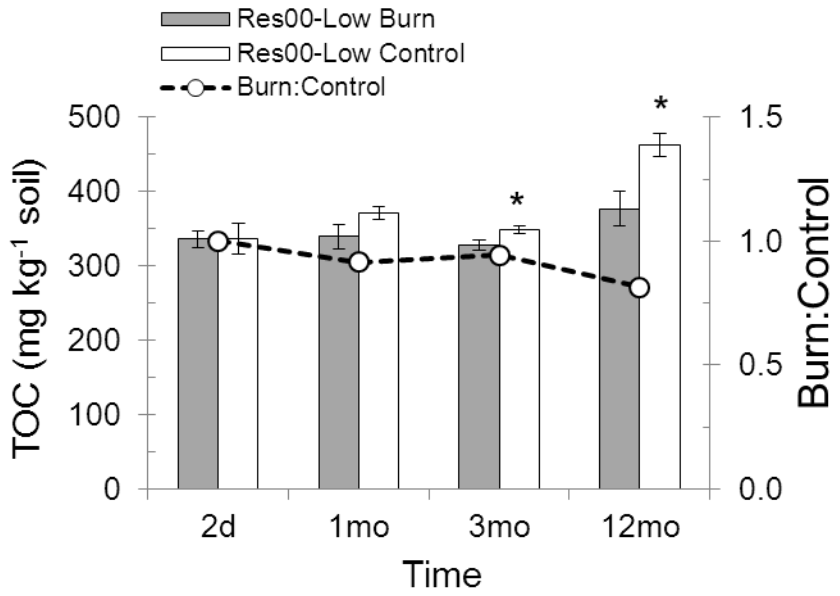


Figure 4-9. Effect of fire on soil total extractable organic carbon 2 days, 1 month, 3 months, and 12 months post fire in the Res00-low elevation site

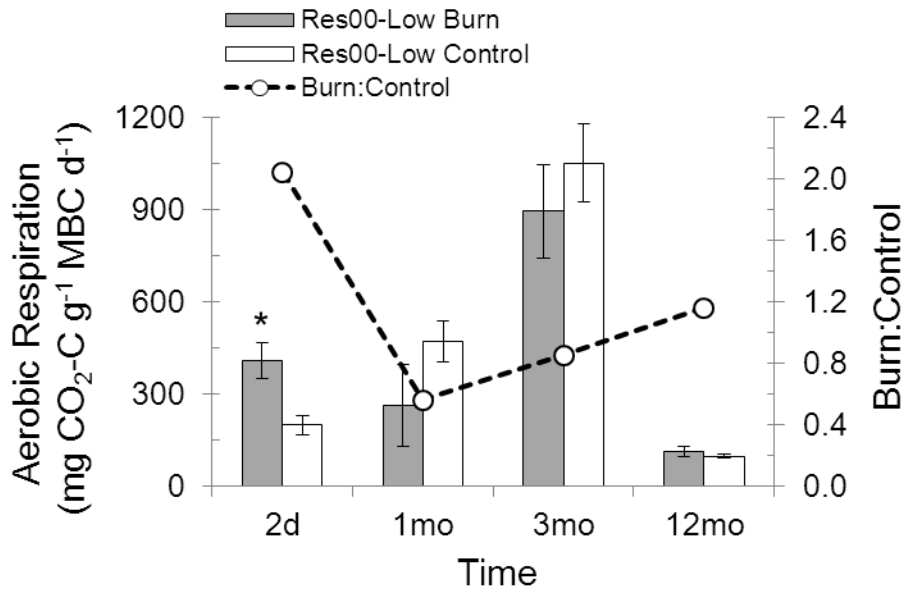


Figure 4-10. Effect of fire on aerobic respiration potentials 2 days, 1 month, 3 months, and 12 months post fire in the Res00-low elevation site

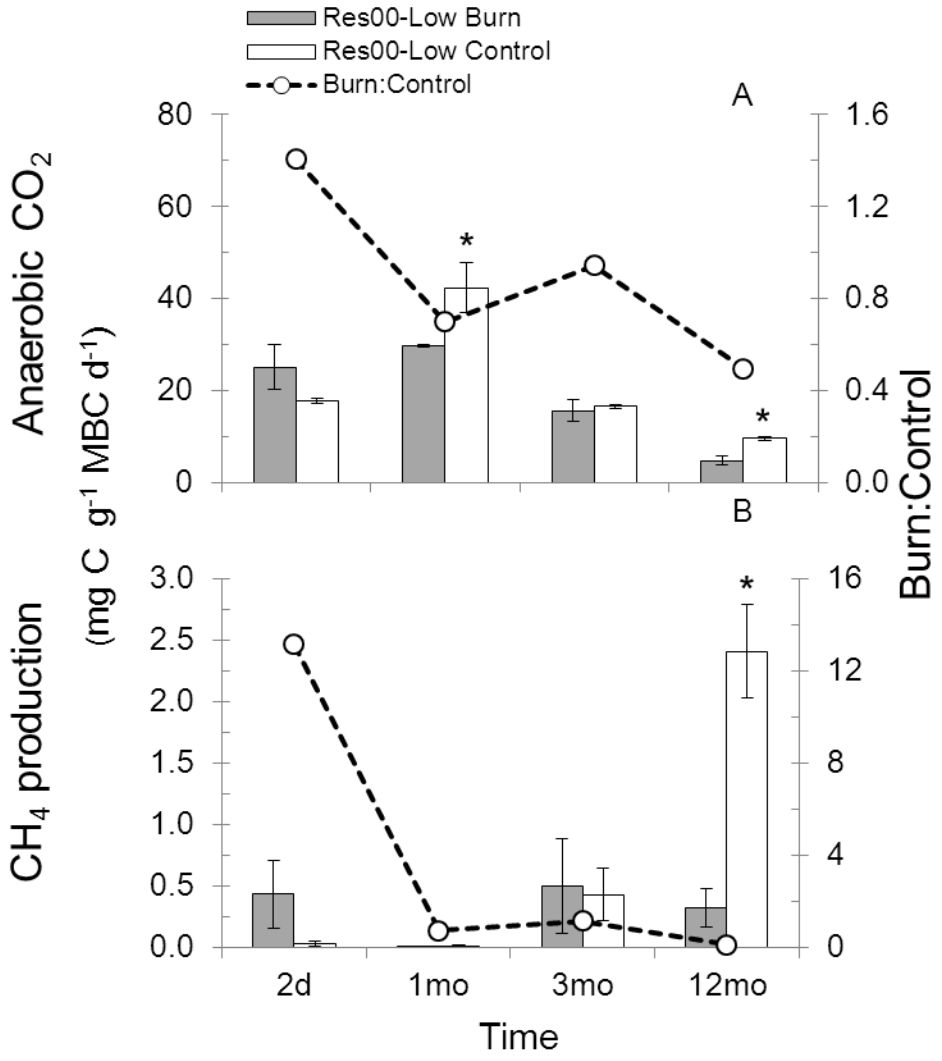


Figure 4-11. This figure depicts the effect of fire on anaerobic respiration potentials from Res00-low elevation sites. Data shows the effect of fire on A) CO_2 and B) CH_4 production 2 days, 1 month, 3 months, and 12 months post fire

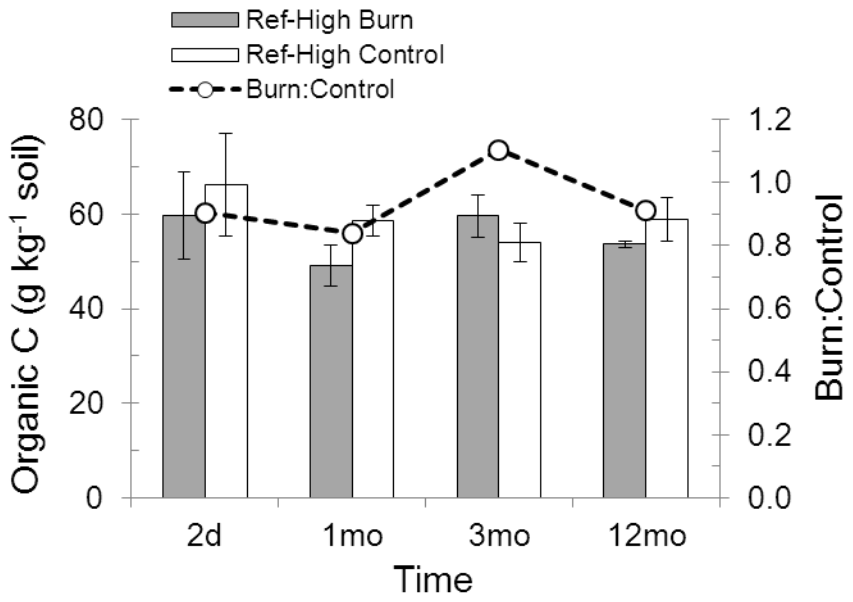


Figure 4-12. Effect of fire on soil organic carbon 2 days, 1 month, 3 months, and 12 months post fire in the reference-high elevation site

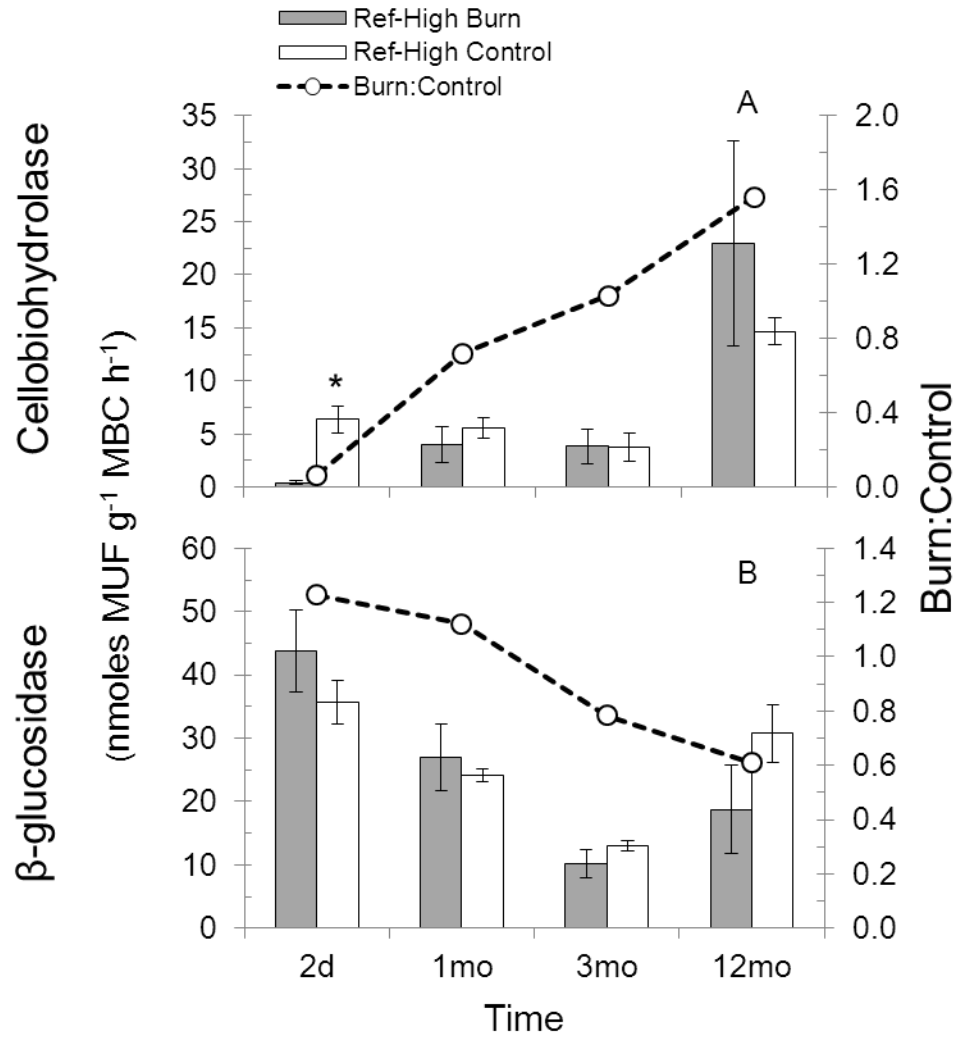


Figure 4-13. This figure depicts the effect of fire on C enzyme activity from Ref-high elevation sites. Data shows the effect of fire on A) cellobiohydrolase and B) β-glucosidase C enzyme activity 2 days, 1 month, 3 months, and 12 months post fire

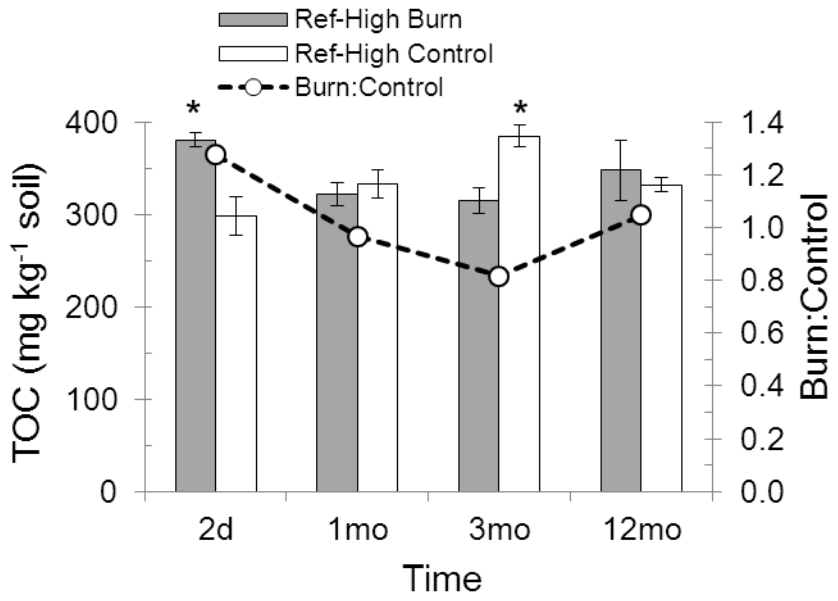


Figure 4-14. Effect of fire on soil total extractable organic carbon 2 days, 1 month, 3 months, and 12 months post fire in the reference-high elevation site

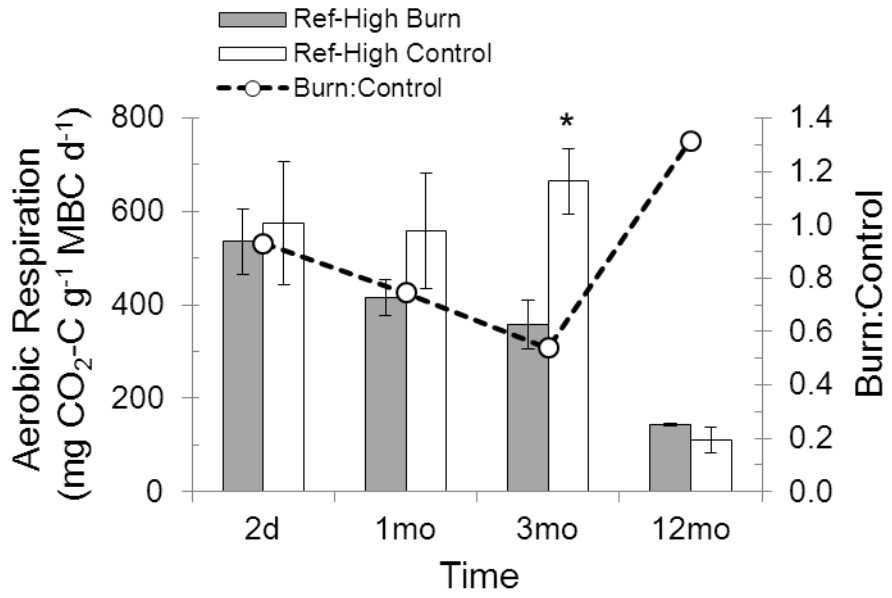


Figure 4-15. Effect of fire on aerobic respiration potentials 2 days, 1 month, 3 months, and 12 months post fire in the reference-high elevation site

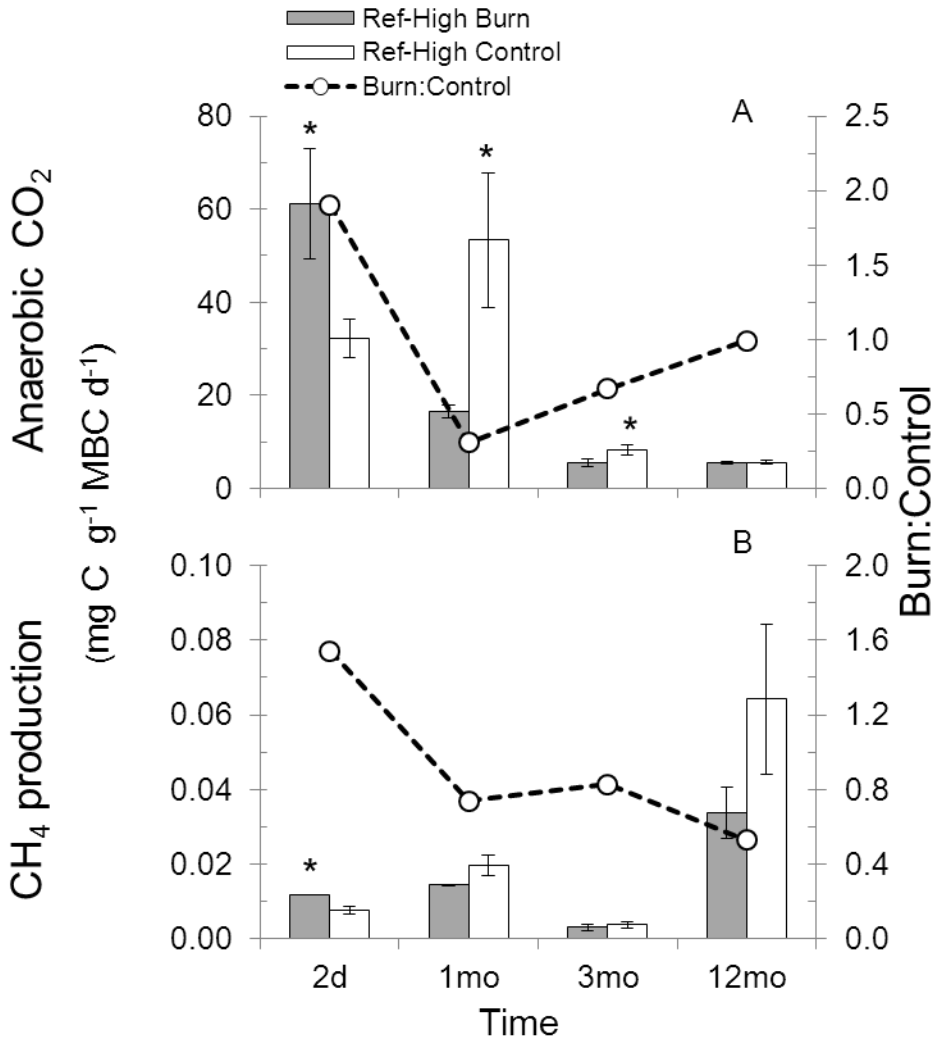


Figure 4-16. This figure depicts the effect of fire on anaerobic respiration from reference-high elevation sites. Data shows the effect of fire on A) CO_2 and B) CH_4 production 2 days, 1 month, 3 months, and 12 months post fire

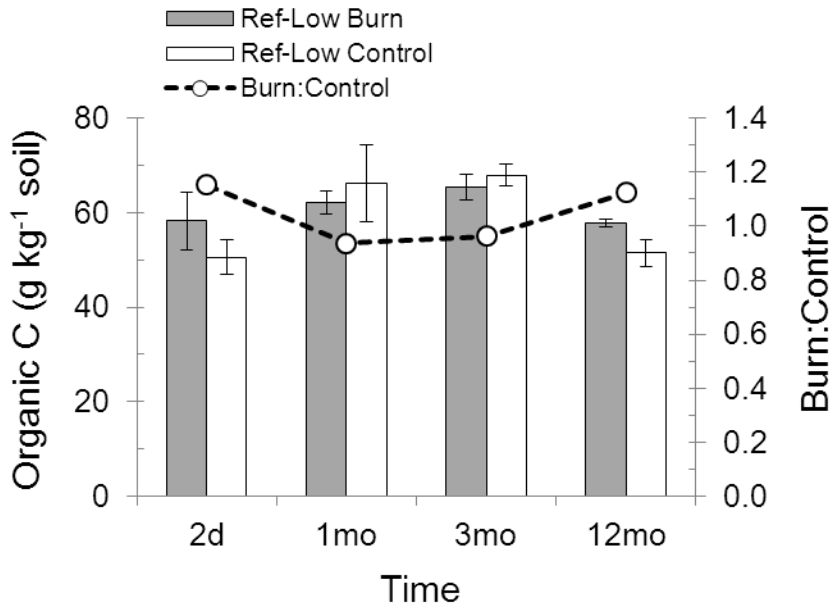


Figure 4-17. Effect of fire on soil organic carbon concentrations 2 days, 1 month, 3 months, and 12 months post fire in the reference-low elevation site

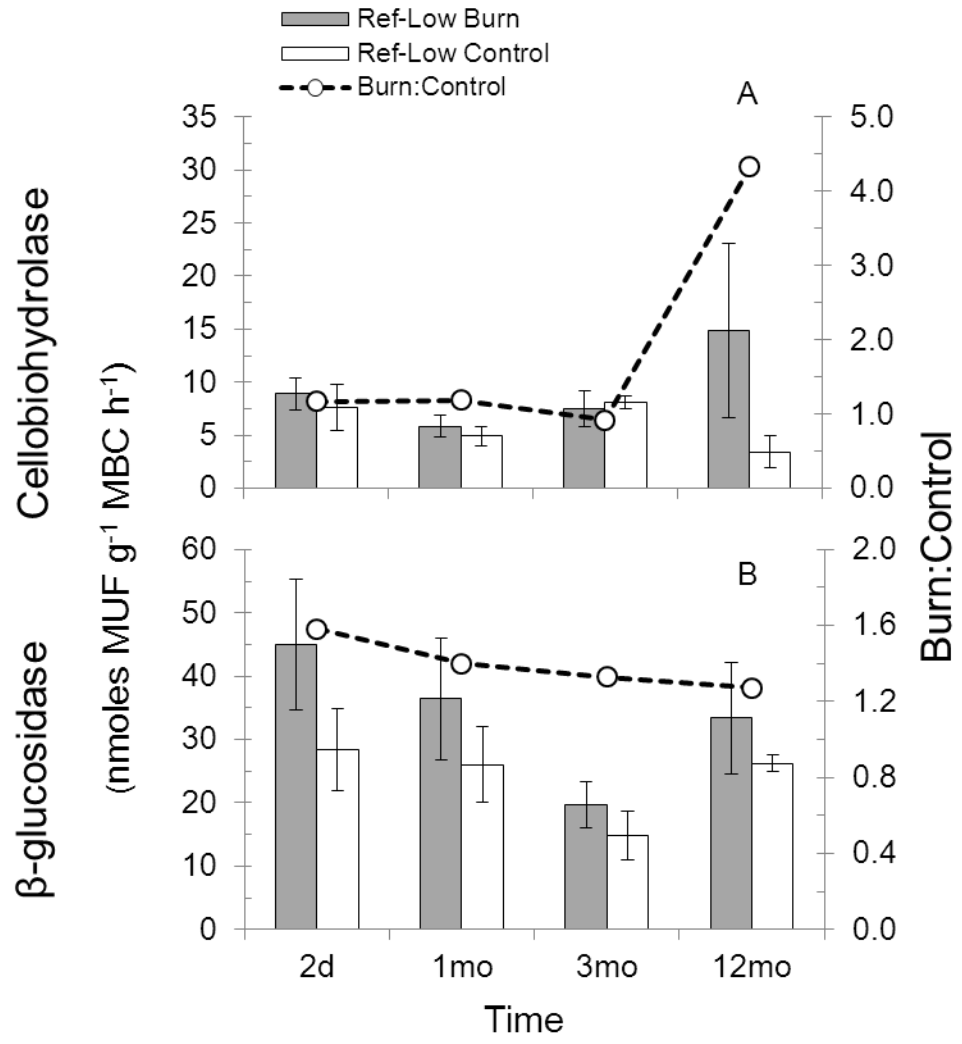


Figure 4-18. This figure depicts the effect of fire on C enzyme activity from reference-low elevation sites. Data shows the effect of fire on A) cellobiohydrolase and B) β-glucosidase C enzyme activity 2 days, 1 month, 3 months, and 12 months post fire

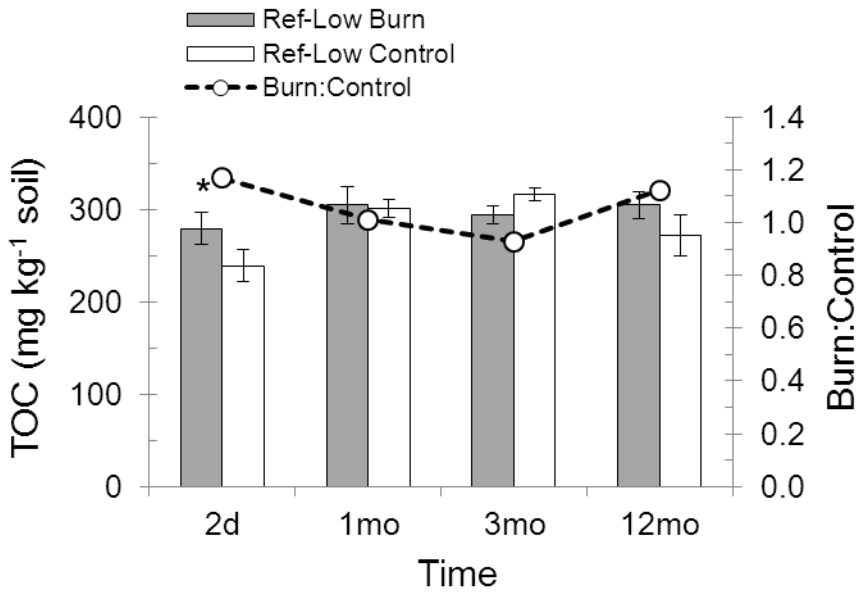


Figure 4-19. Effect of fire on soil total extractable organic carbon 2 days, 1 month, 3 months, and 12 months post fire in the reference-low elevation site

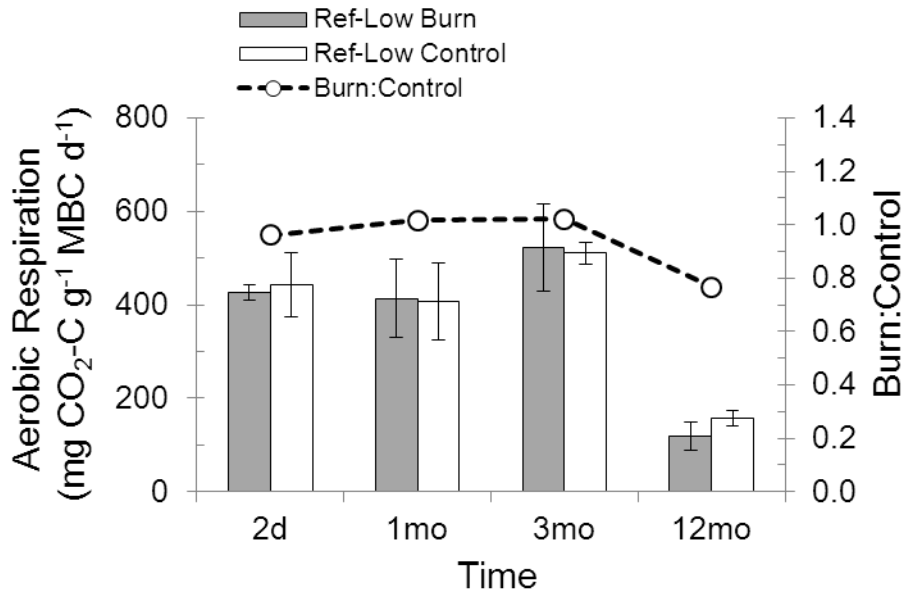


Figure 4-20. Effect of fire on aerobic respiration potentials 2 days, 1 month, 3 months, and 12 months post fire in the reference-low elevation site

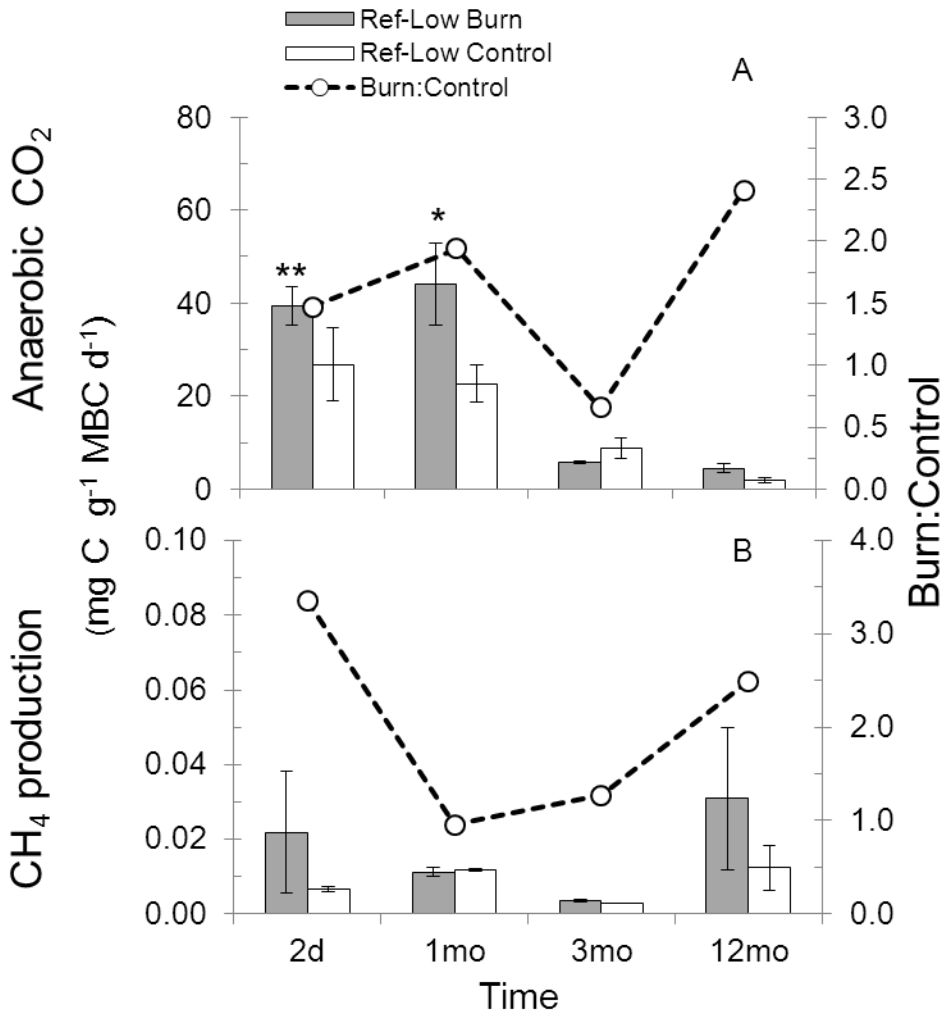


Figure 4-21. This figure depicts the effect of fire on C enzyme activity from reference-low elevation sites. Data shows the effect of fire on A) CO_2 and B) CH_4 production 2 days, 1 month, 3 months, and 12 months post fire

CHAPTER 5
RESPONSE OF MICROBIAL RESPIRATION AND METHANOGENESIS TO FIRE RESIDUES (ASH AND CHAR) IN TWO CONTRASTING SUBTROPICAL WETLAND SOILS

Impacts of Fire Residues on Carbon Cycling

During a fire, vegetation is decomposed and volatiles are combusted reducing plant biomass by oxidation to CO_2. In the process of biomass oxidation, fire residues such as char and ash are produced dependent on combustion completeness. Through incomplete combustion, char can be produced and dispersed on the soil surface. In forest and prairie ecosystems char is commonly produced by fire (Hart et al. 2005) and is known to increase plant growth through improvement of soil properties such as soil porosity (Oguntunde et al. 2008) which may increase the duration of aerobic conditions. Char has been hypothesized to retain and slowly release plant available nutrients (*Reviewed by* Lehmann et al. 2006; Glaser et al. 2002). "Biochar" has been documented to increase soil pH, soil organic carbon (OC), and soil nitrogen (N) (Zhang et al. 2010) which can alter biogeochemical processing. Char may provide microbes a new habitat with ample substrates thus, altering microbial populations and activity (Pietikäinen et al. 2000).

Although char may have a limited influence on total microbial biomass, specific microbial groups may be affected more than others (Pietikäinen et al. 2000). Few studies have quantified the effect of vegetation derived biochar on microbial decomposition processes under aerobic and anaerobic conditions quantified by aerobic and anaerobic respiration (CO_2) and methanogenesis (CH_4 production). Because char can increase soil porosity (Oguntunde et al. 2008) it has been hypothesized anaerobic conditions would decrease thus reducing anaerobic processes including

methanogenesis. This suggests char may decrease C gas emissions. However, previous studies observed an increase in CH_4 emissions following char addition (Zhang et al. 2010) while others reported decreased CH_4 emissions (Karhu et al. 2011; Rondon et al. 2006) coupled with elevated CO_2 production (Karhu et al. 2011). Variation in the response of methanogenesis to biochar addition can be partially explained by the original biochar material (vegetation) and the associated effect on soil properties. Increased fire frequency with global climate change has been hypothesized (Westerling et al. 2006; Krawchuck et al. 2009). Thus, understanding the effect of char on these processes is crucial.

If combustion is complete, fire can yield vegetation derived ash. Ash retained from temperatures above 500°C is typically carbon (C) and N poor, while P concentrations accumulate with increasing burn temperature (Qian et al. 2009). However, the quality of P retained in ash may be dependent on the associated vegetation (Qian et al. 2009). More labile P fractions have been reported in *Cladium* ash when compared to ash derived from *Typha* vegetation (Qian et al. 2009) suggesting ash deposits may vary across an ecosystem. Inputs of ash to P-limited soils may result in a pulse of microbial activity in response to elevated available P deposited post fire and decreased microbial P limitation.

Although certain elements may increase in ash material, it is important to note that both macro and microelement concentrations are highly variable (Demeyer et al. 2001; Someshwar 1996). Liu et al. (2010) measured nutrient concentrations in ash derived from a cattail dominated ecosystem within the Florida Everglades concluding TP in ash

was much higher than found in pre-burn biomass. The ash derived TP pulse could be measured in the surrounding water column.

Fires are known to alter soil nutrient forms and availability. An increase in available inorganic P post fire is common (Smith et al. 2001; Wilbur and Christensen 1983), although this is not the case in all soils (Ahlgreen and Ahlgreen 1960) which may be due to P binding. The role ash may play as a bioavailable P source is of extreme importance in P-limited ecosystems; however, the variation of P released is further complicated by P fractions which are dependent on vegetation type (Qian et al. 2009). An increase in microbial respiration post fire may be in response to fire residues (ash and/or char), elevated soil temperatures (microbial stimulation), increased available C substrates (microbial death and lysis), or a combination of parameters listed above. However, limited studies have isolated the effect of fire residues on microbial processing independent from other possible factors (*listed above*).

The hypothesis for this study was based on the premise that fire residues may act as a nutrient source resulting in short-term soil fertilization. The objective of this study was to determine the effect of vegetation derived ash and char on (i.) aerobic respiration, (ii.) anaerobic respiration, and (iii.) methanogenic processing in a low- and high-P wetland. This study has implications for understanding the effect ash and char deposition post fire may have on C microbial parameters across a soil P gradient.

Materials and Methods

Study Site

The Hole-in-the-Donut (HID) region of Everglades National Park consists of Biscayne and Perrine marl wetlands (USDA 1996). Soils were heavily farmed until 1975 followed by land use abandonment. High nutrient contents post farming

encouraged the colonization of the invasive vegetation *Schinus*. To remove the *Schinus* stands, Everglades National Park staff embarked on a restoration effort which included complete soil and vegetation removal to bedrock (Smith et al. 2011). In this study we focused on two wetlands within the HID, one which was restored in 2000 (Res00) and the other which served as a reference (never farmed) site.

In the following study we burned litter obtained from Res00 and reference wetlands in a controlled laboratory setting. Differences in initial basic soil and microbial properties from the two sites were observed (Table 5-1) as were differences in the litter nutrient content prior to burning (Table 5-2). Prior to burning the vegetation, litter moisture content was normalized to 7%, well within the acceptable range for prescribed fires in our site. Twenty five grams of litter material from each site was placed in triplicate aluminum pans. This amount of litter was chosen to ensure production of ample residues for the microcosm studies. To limit airflow, aluminum walls (~50 cm high) were placed around three sides of the pan following which the material was lit with a portable propane gas tank. Following the fire, charred fragments were handpicked from the resultant mixture providing a “charred amendment”. Ash could not be separated from the char thus; we used a muffle furnace at 550°C for 2 hours to completely combust the vegetation providing an “ash amendment”. Once all treatment materials were produced, we weighed approximately 80 g wet weight soil into 120 mL specimen cups following which we added an appropriate aliquot of ash or char in triplicate and monitored varying parameters over a 28 day period. Based on combustion efficiency calculated from biomass plots (1 m^2) in a previous prescribed fire within the HID, estimates of ash or char produced were determined assuming 100%

efficiency. When scaled to our 80 g soil (wet weight) microcosms this corresponded to an addition of 0.13 and 0.05 g of ash added to the Res00 and reference soil, respectively. In comparison, 0.5 and 0.44 g of char was introduced to the Res00 and reference microcosms, respectively. Soil controls (un-amended) were analyzed concurrently (n=3) to decipher the role individual fire residues may have on various soil and microbial parameters.

Fire Residue Analyses

The following analyses were completed on the initial biomass material and fire residues (ash and char). Total P was determined following complete dissolution of ash (550°C for 4 hours) with 6 M HCl (Anderson 1976). Available P was analyzed as water extractable (DDI) P (1:10 dilution) (Hedley et al. 1982). Total C and N were determined using EPA method 3010 (EPA 1993) and analyzed on a Costech Model 4010 Elemental Analyzer (Costech Analytical Industries, Inc., Valencia, CA).. Extractable N (NO_3 and NH_4) and C were determined on 1 M KCl extracts and analyzed on an auto-analyzer.

Soil Analyses

Soil microcosms consist of composite samples made from 10 soil samples (0-2 cm) which were randomly collected within (30 m^2) the Res00 and reference site in May 2011. Following collection, soils were sieved (2 mm mesh) and aliquots were used to determine initial microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), and microbial biomass phosphorus (MBP) concentrations. Separate soil aliquots were dried at 105˚C for three days, hand ground with a mortar and pestle and used in total C/N/P determination.

Microbial biomass C and N was measured at day 0 and 28 by 0.5 M K_2SO_4 extraction following chloroform fumigation (Vance et al. 1987). Extracts were filtered

(0.2 µm) and analyzed for total organic carbon (TOC) using a 5050-A TOC auto-analyzer (Shimadzu Corp., Columbia, MD; EPA method 415.1). The difference between extractable TOC from the fumigated and non-fumigated sample was considered MBC. Reported MBC values were corrected with an extraction efficiency (K_{EF}) of 0.37 (Sparling et al. 1990). Microbial biomass N extracts were analyzed following Kjeldahl digestion on a Technicon auto-analyzer (EPA method 351.2). An extraction efficiency (K_{EF}) of 0.54 was applied to reported MBN values (Brookes et al. 1985). Microbial biomass P was measured at the start of the experiment by 0.5 M $NaHCO_3$ extraction following fumigation (Brookes et al. 1982). Extracts were analyzed on a Technicon auto-analyzer (EPA method 365.1). Unfortunately, not enough soil was remaining after 28 days to determine final MBP values.

Water Extractable Organic Carbon

Water extractable C was determined over the 28 day incubation period. Briefly, air dried soil (1 soil:100 DDI) was shaken for one hour and filtered through a Whatman # 41 filter (Vanhala et al. 2008). Extracts were analyzed for TOC using a 5050-A TOC auto-analyzer (Shimadzu Corp., Columbia, MD). Water extractable TOC was quantified following aerobic incubation experiments (*see below*)

Basal Microbial Respiration and Methanogenesis

Aerobic soil respiration was measured by using 2 g dry weight equivalent soil aliquots in 120 mL sealed pyrex glass media bottles using a 0.5 M NaOH base trap (modified from Coleman 1973). Traps were changed on days 1, 2, 3, and 4 where values for each time interval were summed to calculate cumulative respiration. Soil free controls were used to account for background CO_2 levels and subtracted from soil respiration determinations. Upon removal, the base trapped CO_2 was released by

acidifying the solution (3 M HCl) and analyzing the released CO_2 by gas chromatography. This analysis was conducted over 5-10, 10-15, and 15-20 day time periods.

To quantify anaerobic C loss potentials (CO_2 and CH_4) 2 grams dry weight equivalent soil and 10 mL of DDI were combined in 30 mL conical tubes (n=3 for each site), flushed with oxygen free N_2 to ensure anaerobic conditions and stored in the dark. After a 24 hour incubation period gas headspace was measured and periodically re-measured up to 28 days to obtain a linear phase via gas chromatograph.

Gas Analysis

Methane headspace was detected by a Shimadzu gas chromatograph-8a fitted with a flame ionization detector (160°C, injection temperature 110°C), N_2 as the carrier gas and a 5.25 ft (45/60) Carboxen-1000 column (Supelco Inc., Bellefonte, PA). A Shimadzu gas chromatograph-8a fitted with a thermal conductivity detector (column injection 120°C, 40°C oven temperature), He as the carrier gas and a 6 and 1/8 ft. (80/100) Porapak-N column (Supelco Inc., Bellefonte, PA) was utilized to analyze CO_2 headspace. Calibration curves were determined via standard gas mixtures (Scott Specialty Gases, Plumsteadville, PA) multiple times throughout each sampling event. Standard curves were used to calculate gaseous production. Rates were reported as mg gaseous-C g^{-1} MBC d^{-1}.

Data Analysis

The fire residue treatment effect was calculated as the percent difference between each treatment (ash or char amended) value and the average control value. The response of soil and microbial parameters to fire residues was analyzed via Tukey-

Kramer means comparison between both sites and treatments ($\alpha<0.05$, unless otherwise stated). All statistics were run on JMP vs. 8.0 (SAS institute, Cary NC).

Results

Fire Residue Nutrient Concentrations

Although concentrations of fire residue nutrients differed between restored and reference sites the trends were similar. The ash residue contained a greater concentration of TP relative to the char treatment. However, the char residue contained a greater concentration of available P relative to the ash residue. In addition, the char residue contained higher total and extractable N and C ($p<0.01$; Table 5-3).

Fire Residue Effect on Basic Soil and Microbial Parameters

After the 28 day incubation, residue addition had no effect on MBC, TOC, or available P in the high-P soil (Table 5-2). However, MBN was decreased in treated soils although only significant in the char treatment ($p<0.05$). Similarly, residue addition had no effect on soil TOC concentrations or available P in the low-P soil after 29 days. However, MBC in residue treated reference soils was much lower than in control soils ($p<0.05$). In addition, MBN in ash treated reference soils was significantly reduced relative to control MBN ($p<0.05$).

Aerobic Respiration Potentials

Production of CO_2 (aerobic conditions) from un-amended soils was initially (≤10 days) greater from high-P relative to low-P soils ($p<0.01$, *data not shown*). After the microcosms were incubated for 10 days there was no difference in CO_2 production between sites. Trends in CO_2 production relative to the control were similar regardless of fire residue added although trends varied by site. In high-P soils, aerobic respiration was initially suppressed when treated with ash (46%) and char (18%), respectively

(Figure 5-1A,B). With time, soils treated with fire residues were stimulated (elevated CO_2 production) averaging a 34% increase when treated with ash and 28% increase when treated with char.

In contrast to increasing trends in aerobic CO_2 production in amended high-P soils, there was a decreasing trend in CO_2 production from low-P soils (Figure 5-1C,D). Initially, both fire residues elevated aerobic CO_2 production relative to the control. A much larger response (179%) was observed when amended with char as opposed to ash. With time, CO_2 production was suppressed relative to the control resulting in a 30% suppression in treated soils at the end of the experiment (Figure 5-1C,D).

Extractable Organic Carbon

The trends in DDI extractable OC differed by site. In the high-P soil, DDI extractable C was initially lower in the treated soils. However, after day 10 the treated soils contained elevated available OC relative to the control (Figure 5-2A,B). In low-P soils, available C was initially elevated in treated soils. Furthermore, the response in char amended soils was 2-fold greater than in ash treated soils. However, by the end of the experiment, available C was lower in the treated soils (p<0.05; Figure 5-2C,D).

Anaerobic Respiration Potentials

Carbon dioxide production

Under anaerobic conditions, CO_2 production was greater from the high-P soil relative to rates from the low-P site (p<0.01; Table 5-4). In high-P wetlands, anaerobic processing differed in response to ash or char addition indicated by a variable CO_2 production response (Figure 5-3A). When amended with ash, anaerobic CO_2 production was suppressed by 24% relative to control production rates. Following char

addition, CO_2 production was stimulated by 28% resulting in an increase of 15 mg CO_2-C g^{-1} MBC d^{-1} relative to control production.

In the low-P wetland, trends in anaerobic CO_2 production were similar regardless of the fire residue (ash or char) added (Figure 5-3B). There was a 44% increase in production rates following ash addition. While CO_2 was also stimulated by char, the magnitude was much larger with a 113% increase relative to control production.

Methane production

Production of CH_4 was close to 40 times greater from high-P soils relative to low-P soils under ambient conditions (p<0.0001; Table 5-4)**.** A similar site specific response as observed in anaerobic CO_2 production following ash and char addition was seen in CH_4 production rates (Figure 5-4A). Production of CH_4 was suppressed in ash amended high-P soils relative to control production rates however; production was suppressed to a lesser extent than anaerobic CO_2 production (6% vs. 24%). Relative to control production rates, there was an increase of 0.16 mg CH_4-C g^{-1} MBC d^{-1} when amended with char equating to a 70% increase in production relative to the control (Figure 5-4A).

In low-P soils, both ash and char stimulated CH_4 production relative to the control. Trends suggest char stimulated CH_4 production to a greater extent than ash (74 vs. 47%, respectively) however, due to high variability; observed differences were not statistically significant (Figure 5-4B).

Discussion

Microbial Response to Fire Residues

Aerobic processing

High phosphorus soil. Fire residues can affect nutrient concentrations and availability (Hogue and Inglett 2012) in addition to microbial composition and activity. Alteration of these parameters may ultimately affect aerobic decomposition processes. The response in aerobic microbial respiration was similar regardless of fire residue added in high-P soils. Initially, ash and char suppressed CO_2 production relative to the control. This suggests the fire residues may not have been immediately available for microbial degradation/uptake.

Decreased CO_2 production following fire has been detected in many field experiments (Rondon et al. 2005 and 2006; Yanai et al. 2007; Spokas et al. 2009; Spokas and Reicosky 2009) suggesting the initial response was not unique. However, in field studies many other mechanisms may be confounding the results. For example, a fire response cannot differentiate between direct fire effects (i.e. changes in soil temperature or biomass removal) and indirect fire effects (fire residue addition) on C processing. In the current study, we have eliminated any "direct" fire effect thus; our focus was solely on the microbial response to fire residue (nutrient) addition. Wong and Wong (1986) observed an inverse relationship between ash concentrations and CO_2 production in a sandy soil. They concluded the initial inhibition of CO_2 production following the ash amendment may have resulted from increased toxicity from the ash material (Wong and Wong 1986) similar to conclusions by Pitchel and Hayes (1990). Unfortunately, in the current study metals were not analyzed.

There was an initial suppression in aerobic CO_2 production when high-P soils were amended with char, similar to the response following ash addition. Research suggests OM may bind to the char surface reducing the char availability for microbial degradation (Zimmerman et al. 2011). Char can also block enzyme active sites (Bailey et al. 2011) which can decrease the microbial degradation of OM resulting in an initial decrease in aerobic respiration (decreased substrate availability) as seen in the current study. Luo et al. (2011) determined a loss of C averaging 319 mg CO_2 kg^{-1} soil when amended with char which was a much greater C loss than the 92 mg CO_2 kg^{-1} soil loss observed in high-P char treated soils.

As time progressed, respiration in the treated soils (ash and char) began to increase. An increase in aerobic respiration with time suggests the microbial communities may be adapting to less than optimal conditions (Pitchel and Hayes 1990; Cornfield 1977) possibly introduced following residue addition. Perkiomaki and Fritze (2002) found CO_2 production to increase from a Scots pine forest in addition to a shift in microbial communities although no change in microbial biomass was detected. This suggests a shift in microbial communities may have occurred however, we were unable to detect this change by monitoring microbial biomass carbon alone. The similar trend (with time) in aerobic CO_2 production suggests the response to both fire residues was constant. Thus, the fire residues directly affected microbial processing as opposed to the variation in CO_2 production originating from changes in soil properties as hypothesized in many field studies. Increasing available DOC with time suggests the extra available C pulse from the fire residues may have been processed by the microbes explaining the concurrent increase in aerobic CO_2 production.

Low phosphorus soils. Trends in aerobic decomposition (CO_2 production) following fire residue addition differed between high and low-P soils. Ash and char fire residues initially stimulated CO_2 production relative to the control (NS, $p<0.05$, respectively). Char addition had the largest effect on aerobic CO_2 production suggesting the increase may have resulted from the elevated nutrient concentrations (C and P) from the residue. Smith et al. (2010) observed a positive relationship between CO_2 production and increasing char application. Through stable isotope analysis of the $^{13}CO_2$ they were able to correlate the respired CO_2 value to that of char. Rapid use of labile C from the biochar has been reported previously (Luo et al. 2011; Hilscher et al. 2009; Zimmermann and Frey 2002) and may explain the immediate response in our study.

Patterns of ext. available DOC was similar to trends in aerobic CO_2 production with time. Concentrations of available DOC were elevated in the treated soils initially relative to the control. Kuzyakov et al. (2009) suggests that an initial preferential use of select compounds may result in a decreased decomposition rate with time which could explain trends in our low-P soil. However, in order for the additional available C to be processed increased soil P concentrations are necessary to overcome the microbial P limitation in this site (Table 5-1). Because the concentration of P in the ash residue was significantly smaller than in the char residue (Table 5-3; although still an increase relative to background soil concentrations) we hypothesized the response in CO_2 production would be minimal in the ash relative to the char treatment as seen in the current study. The ratio of available C:P decreased by 124 and 188 relative to the control in the ash and char treatment, respectively after 7 days (Figure 5-5). A ratio

reduction suggests increased availability of P for microbes thus providing an explanation for the initial stimulation of the process.

As the incubation progressed, aerobic CO_2 became suppressed from the low-P treated soils. This trend was inverse to time dependent patterns in CO_2 production following residue addition in high-P soils. This suggests microbial decomposition became limited by some factor (nutrients or substrates) in the low-P soil. Based on available C data (Figure 5-2) we conclude this limitation could not have likely originated from low C availability suggesting another element may have become limited. The available C:P ratio increased relative to the control after 7 days suggesting in response to a reduction in P_i (Figure 5-5, *data not shown*). There was a similar decreasing CO_2 trend in high pH soils (Luo et al. 2011). Luo et al. (2011) concluded most of the char derived priming effect was eliminated 13 days following addition in agreement with decreasing C availability with time.

Anaerobic respiration

Methanogenesis. Many studies that focus on the effect of fire residues on CO_2 and CH_4production are field based studies (Karhu et al. 2011). These studies conclude through chamber measurements that CH_4 emissions were decreased immediately following a fire likely due to elevated CH_4 oxidation (Kim et al. 2011; Karhu et al 2011). When separated into fire residue effects, these results become highly variable which would be expected considering biochar and ash in these studies originate from very different vegetation sources. Furthermore, most studies investigating the effect of biochar on microbial processing produce biochar via pyrolysis with differing combustion temperatures which can affect the biochar properties (Luo et al. 2011). In comparison, char created for this study was produced following a live combustion in a laboratory

setting (*see Hogue and Inglett 2012*). For this reason, it is hard to compare results from multiple studies thus caution must be used when interpreting data.

For example, the addition of biochar has been reported to increase CH_4 production in some studies (Zhang et al. 2010) while other research groups have concluded char to have no clear effect on the production of CH_4 (Castaldi et al. 2011). These studies added char directly to field plots however; few studies have isolated the effect fire residues have on process level mechanisms. Yoo and Kang (2011) conducted short-term laboratory incubation studies in aerated glass jars and observed a decreased in CH_4 production following char addition. They were unable to distinguish if methanotrophic bacteria were stimulated by the char or if the char was inhibiting the methanogens.

This study is one of the first to investigate the response of anaerobic microbial processing to an isolated fire residue addition (Knoblauch et al. 2011). We observed a stimulatory effect of char on CH_4 production in both soils. Because our incubations were anaerobic and the soil was flooded we were able to rule out possible factors which are present in the field experiments such as alteration of methanotrophic activity and increased porosity, which are both common rationales for understanding decreased CH_4 production following a fire. Based on fire residue characteristics, we know char increased both the available P and C pool following residue addition (Table 5-3). In our previous study (Medvedeff et al. *in review*), direct C (glucose) addition increased CH_4 production in both soils while addition of P only increased CH_4 production in low-P soils. Therefore, it is plausible the increase in CH_4 production in the high-P soils was in response to increased C substrate availability. In contrast, both C and P increased CH_4

production in low-P soils thus we cannot differentiate between the effect of these nutrients on CH_4 production following char addition. However, the response in CH_4 production to these direct additions (C or P) (Medvedeff et al. *in review*) resulted in a higher increase when amended with P suggesting the response may have been driven largely by the P addition.

While literature is available on the effect of char on CO_2 and CH_4 production (*see discussion above*), minimal studies have investigated the effect of ash on process level microbial activities (Björk et al. 2010; Galand et al. 2005; Zimmermann and Frey 2002). Based on the limited data available, no effect of ash on CH_4 production has been observed in either a drained forested peatland (Björk et al. 2010) or a drained bog (Galand et al. 2005). Galand et al. (2005) used PCR magnification to conclude no changes in dominate methanogens between an ash treated and control soil. Interestingly, additional methanogenic groups were detected from the ash fertilized soils which were absent from control soils suggesting a possible change in microbial community.

In our previous studies (Medvedeff et al. *in review*) we investigated the response in CH_4 production to P additions in low-and high-P soils. It was apparent that P addition had no effect on CH_4 production from high-P wetlands although stimulation was evident in the low-P site. Based on fire residue characterization we know ash from our site is a P source and has negligible N and C components (Table 5-3). Based on our previous studies, we postulate the stimulation of CH_4 when amended with ash would yield a similar response to CO_2 and CH_4 production from the previous P addition study because ash would increase the available P concentrations. In comparison, char increased both

P and C availability thus we could isolate the P affect in this treatment. In our study, we observed a negative and positive response to ash addition on CH_4 production in high and low-P soils, respectively. The negative response to ash in high-P soils was consistent with the lack of response when amended with available P. This serves as an additional line of evidence that methanogenic activity in high-P soils is not limited by P. In contrast, stimulation of CH_4 in response to ash in low-P soils supports our hypothesis that the process of methanogenesis was ultimately P-limited. Zimmermann and Frey (2002) added wood ash to soil from a forested ecosystem and observed elevated CO_2 production in the treated soil both *in situ* and in laboratory incubations. They concluded increased activity in the treated soils may have originated from increased activity from microbes already present in the soil but inactive prior to the ash addition. This suggests ash may preferentially stimulate select microbes similar to findings from Galand et al. (2005) under anaerobic cultures.

Carbon dioxide production. As mentioned above, we were unable to find any studies that monitored the effect of vegetation derived ash on process level anaerobic CO_2 production. The ash amendment stimulated anaerobic CO_2 production in low-P soils while suppressing it in high-P soils. A decrease in CO_2 concentration would reduce the electron acceptor necessary to complete hydrogenotrophic processing thus, the suppressed response in CH_4 production (high-P soils) may have been dependent on the relationship between ash and CO_2 production. As discussed previously, the reason for decreased CO_2 production following ash addition is unknown. One possible reason may have originated from the high pH of the ash material resulting in an immediate conversion to apatite as observed in our site (P. Inglett, *personal communication*). In

the current study, no difference in soil pH was detected following fire residue addition likely in response to the low concentrations of material added. However, ash produced from the high-P vegetation (high-P site) was elevated in pH relative to the low-P ash (11.5 and 11.2, respectively, $p<0.05$). Ohlsson (2000) determined a large amount of CO_2 can be removed via carbonation of ash granules which may have also reduced CO_2 concentrations under our closed system incubations.

Production of CO_2 was stimulated in char amended soils regardless of initial soil P concentrations (high or low-P site). Char composition contained both labile C and P (Table 5-3) thus the similar response may have originated from different mechanisms. In our previous studies (Medvedeff et al. *in review*), an increase in anaerobic CO_2 production following glucose addition in both high and low-P soils suggests the response following char addition could be explained by increasing C availability.

Conclusions

The response of fire residues to C greenhouse gas production varied based on aerobic or anaerobic conditions and initial soil P content. Under aerobic conditions, fire residues suppressed CO_2 production initially although with time activity was stimulated in high-P soils. In contrast, processing became increasingly suppressed in low-P soils suggesting possible nutrient or substrate limitation.

Under anaerobic conditions, ash suppressed CO_2 and CH_4 production in high-P soils. In contrast, when treated with char C gas production in the high-P soil was stimulated. In low-P soils, both fire residues stimulated CH_4 and CO_2 production. This study suggests the production of C gases post fire maybe dependent on the proportion of fire residue (ash or char) produced and the associated nutrient concentration of the vegetation. The addition of char to soil microcosms stimulated C gas production to a

greater extent than the ash residue in both nutrient impacted and un-impacted sites. Understanding the effects of these residues on C gas production is crucial when investigating the effect of fire on C cycling in fire adapted ecosystems.

Table 5-1. Basic soil characteristics from Res00 and reference wetlands within the Hole in the Donut region of ENP (May 2011)

soil parameter		Res2000	Reference
pH		8.2 (0)^{B}	8.7 (0.2)^{A}
MC	%	46 (2)^{A}	37.6 (0.2)^{B}
LOI	%	23 (2)^{A}	15(1)^{B}
TN	g kg^{-1}	9.7 (0.3)^{A}	7.1 (0.3)^{B}
OC	g kg^{-1}	103 (7)^{A}	67 (4)^{B}
TP	mg kg^{-1}	689 (44)^{A}	140 (8)^{B}
OC:TP		153 (19)^{B}	480 (12)^{A}
TN:TP		14 (1)^{B}	51 (4)^{A}
NOx	mg kg^{-1}	7.2 (2.1)^{A}	5.6 (0.2)^{A}
NH_3	mg kg^{-1}	65.4 (2.5)^{A}	52.2 (0.2)^{B}
Pi	mg kg^{-1}	9.1 (1.8)^{A}	1.4(0.4)^{B}
TOC	mg kg^{-1}	242 (14)^{B}	409 (4)^{A}
TOC*	mg kg^{-1}	222 (12)^{B}	342 (8)^{A}
MBN	mg kg^{-1}	291 (2)^{A}	179 (7)^{B}
MBC	mg kg^{-1}	3039 (138)^{A}	2442 (2)^{B}
MBP	mg kg^{-1}	28 (0)^{A}	19 (1)^{B}

Table 5-2. Basic soil and microbial parameters analyzed following 28 day incubation from soils (Res00 and reference) incubated with no addition, ash, or char

$mg\ kg^{-1}$	High-P Soil 28 days			Low-P Soil 28 days		
	Control	Ash	Char	Control	Ash	Char
MBC	3553 (86)	3370 (83)	3219.2 (103)	2664 (33)^{A}	2428 (32)^{B}	2429 (34)^{B}
MBN	472.4 (9.1)^{A}	452.8 (11.6^{AB})	433.7 (9.0)^{B}	257.6 (3.8)^{A}	231.7 (5.2)^{B}	242.5 (4.2)^{AB}
TOC	274 (14.2)	257.6 (2.7)	276.4 (6.1)	295.6 (4.9)	291.4 (12.4)	330.5 (16.5)
$NaHCO_3$-Pi	9.1 (1.1)	8.9 (0.5)	9.9 (0.4)	1.3 (0.3)	2.1 (0.3)	1.9 (0.2)

Table 5-3. Basic fire residue characterization from Res00 and reference vegetation

	Restored		Reference	
	ash	char	ash	char
TP (mg kg^{-1})	2947 (77)	250 (38)	2996 (80)	1042 (270)
DDI ext P_i (mg kg^{-1})	2.3 (0.8)	115 (42)	7.9 (2.3)	46 (6.6)
TN (g kg^{-1})	BDL	15 (1)	BDL	12 (0.4)
NO_3 (mg kg^{-1})	3.7 (1.6)	20.3 (2.2)	1.5 (0.4)	2.3 (0.9)
NH_4 (mg kg^{-1})	3.6 (1.8)	37.6 (14.1)	2.8 (1.1)	58.1 (14.5)
TC (g kg^{-1})	81 (8)	590 (4)	30 (1)	540 (3)
KCl ext. TOC (g kg^{-1})	BDL	7.0 (0.3)	BDL	5.9 (0.3)

Table 5-4. Gaseous C production rates for each amendment (ash or char) and un-amended soil. Rates taken between 1 and 12 days; significance determined for each site individually

	High-P Soil mg C kg^{-1} soil d^{-1}			Low-P Soil mg C kg^{-1} soil d^{-1}		
	Control	Ash	Char	Control	Ash	Char
AN-CO_2-C	122.1 (9.9)^{A}	88.5 (11.8)^{B}	134.9 (9.1)^{A*}	59.3 (9.1)^{B}	80.0 (4.9)^{AB}	121.9 (13.9)^{A*}
CH_4-C	1.24 (0.35)^{B}	0.8 (0.03)^{B}	2.15 (0.08)^{A*}	0.031 (0.003)^{B}	0.047 (0.007)^{A}	0.049 (0.003)^{A}

*=$p<0.1$

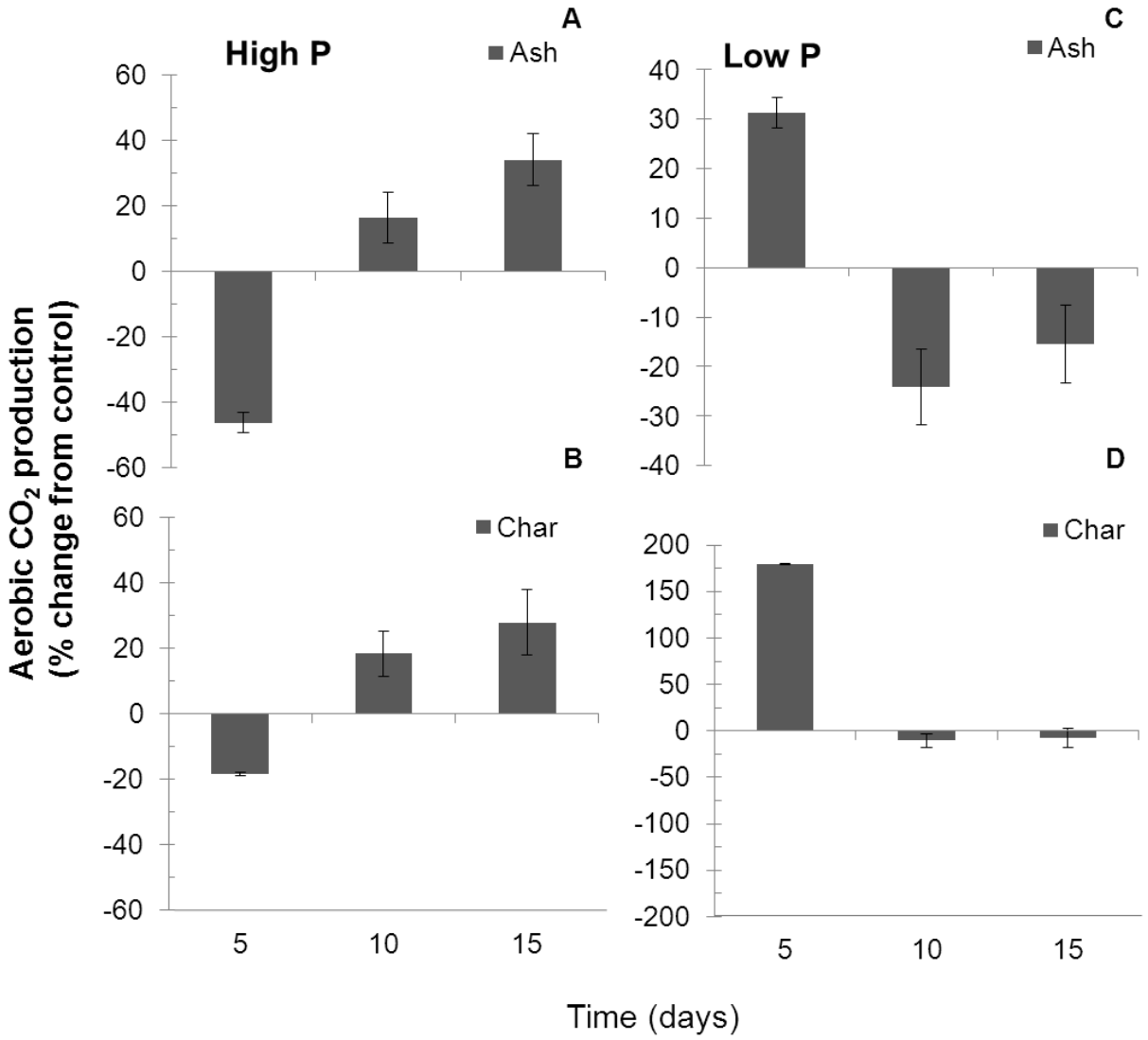


Figure 5-1. The effect of fire residues (ash and char) on aerobic CO_2 production. In high-P soils the effect on aerobic CO_2 production following A) ash and B) char addition. In low-P soils the effect on aerobic CO_2 production following C) ash and D) char addition. Aerobic respiration quantified starting at day 5, 10, and 15. Rates were cumulative over a 5 day period and are reported as percent change relative to control production

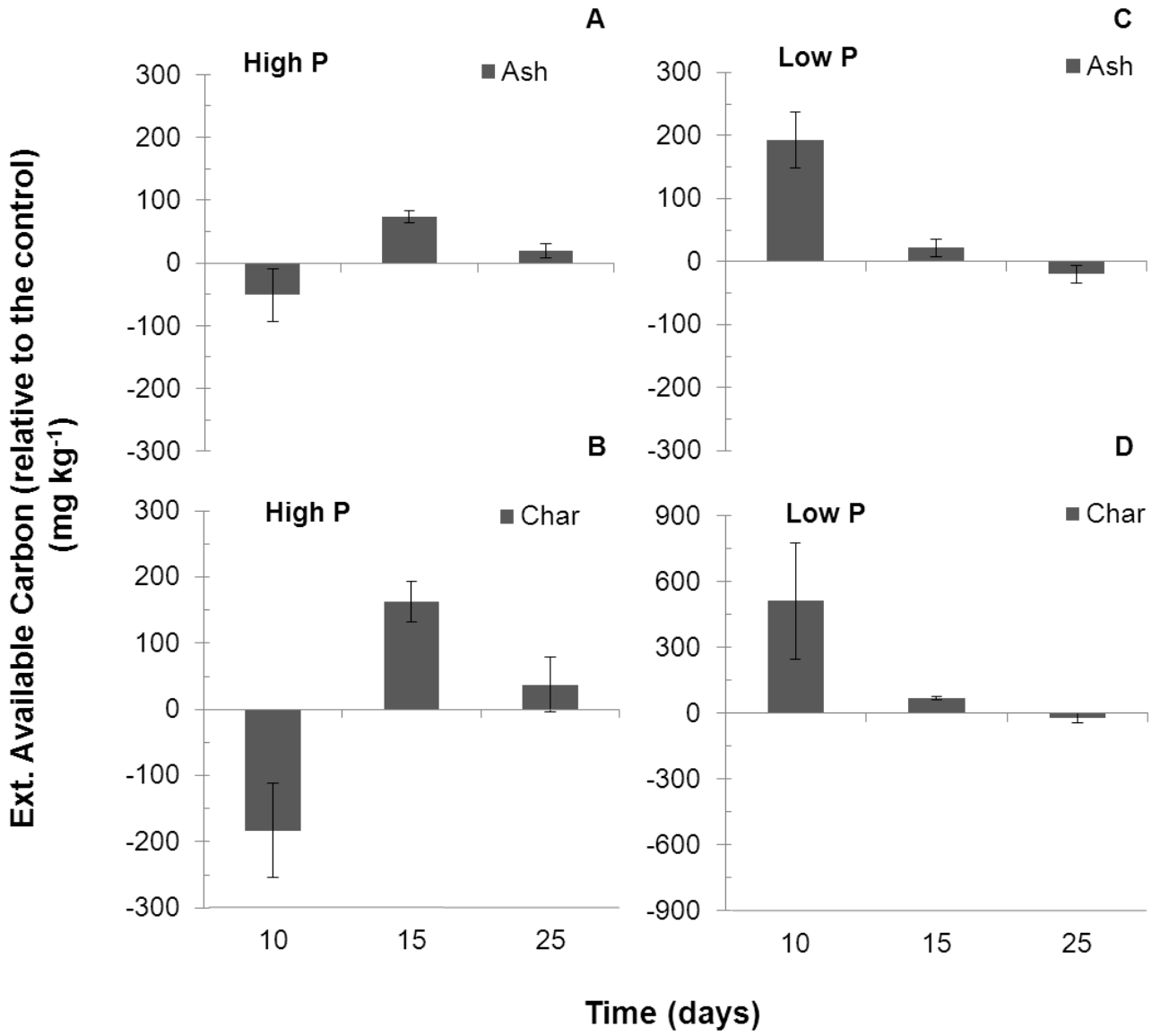


Figure 5-2. The effect of fire residues (ash and char) on extractable C availability. In high-P soils the effect on extractable C following A) ash and B) char addition. In low-P soils the effect on extractable C following C) ash and D) char addition. Soils were extracted following aerobic incubation. Values are reported as concentrations relative to the control

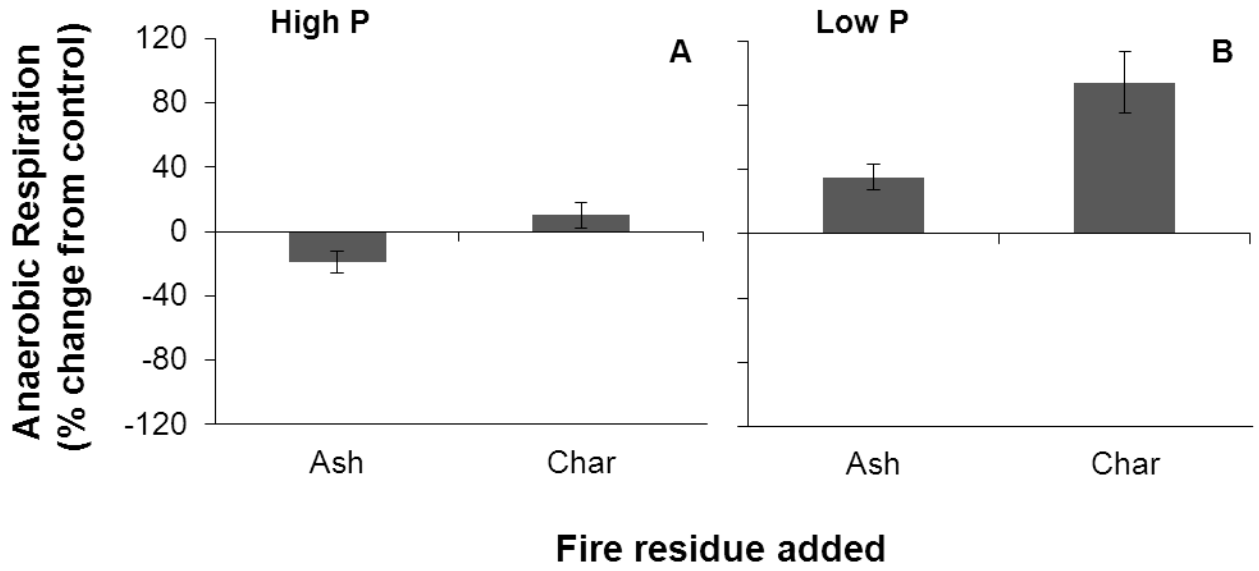


Figure 5-3. This figure depicts the effect on fire residues on anaerobic CO_2 production. Data shows the effect of ash and char fire residues on anaerobic CO_2 production in A) high and B) low-P soils. Percent change is relative to control production

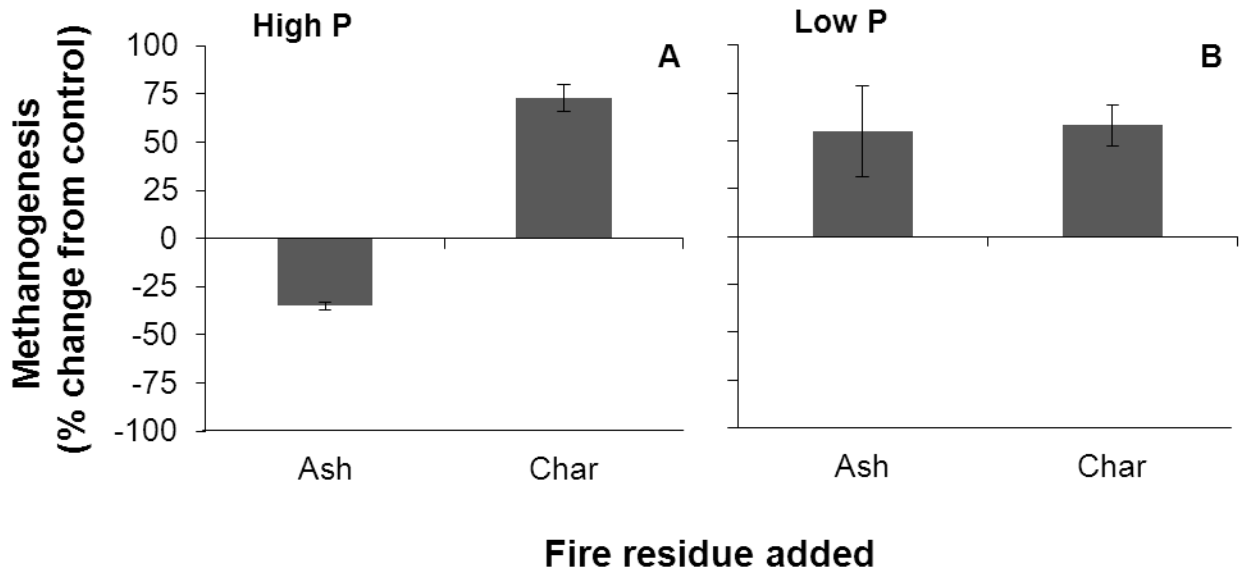


Figure 5-4. This figure depicts the effect on fire residues on anaerobic CH_4 production. Data shows the effect of ash and char fire residues on CH_4 production in A) high and B) low-P soils. Percent change is relative to control production

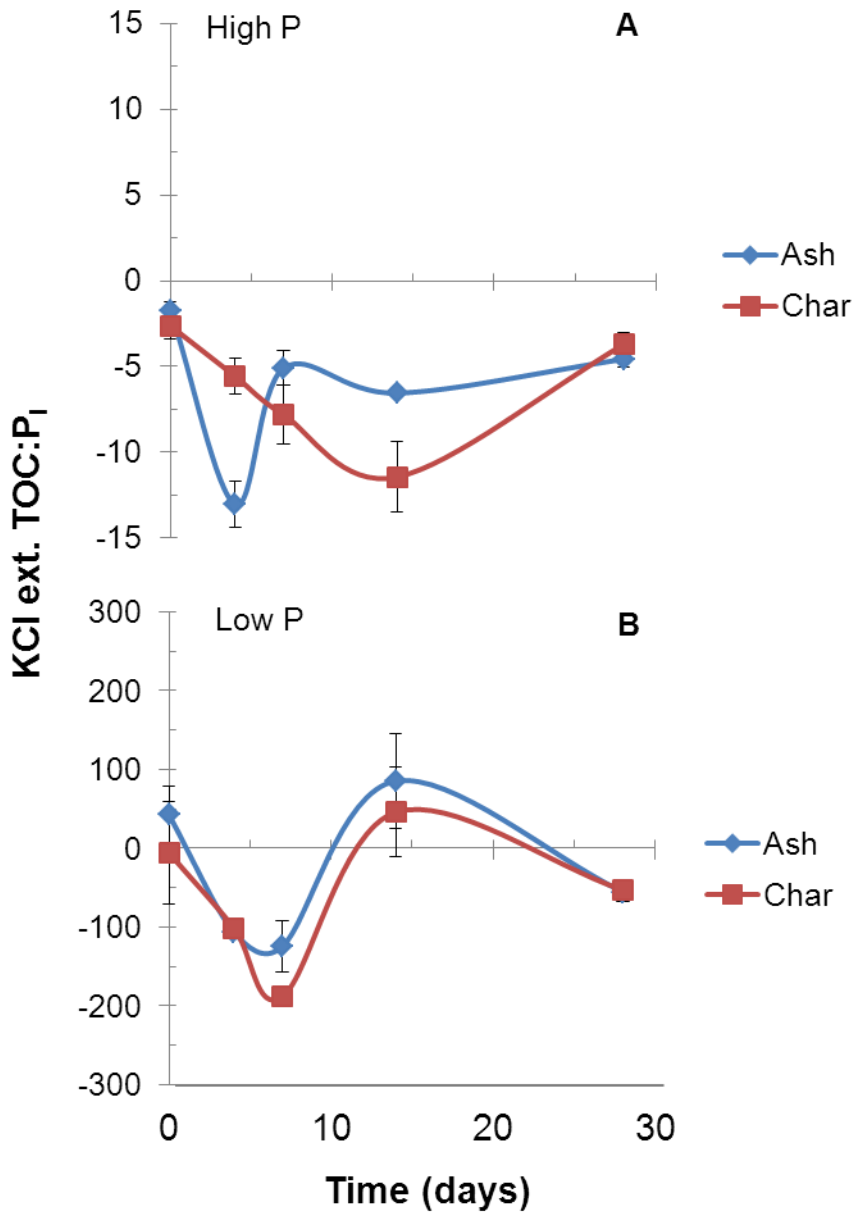


Figure 5-5. This figure depicts the effect on fire residues TOC:available P. Data shows the variation in total extractable organic carbon:available P with incubation time in the A) high and B) low-P soils.

CHAPTER 6
SYNTHESIS AND CONCLUSIONS

Synthesis

Elevated soil P concentrations can affect C cycling in subtropical calcareous wetlands (Chapter 2) evidenced by elevated C enzyme activity and CO_2 and CH_4 production relative to undisturbed soil. Although soil P was reduced following restoration, elevated concentrations persisted resulting in a high and low nutrient (P) site. Regression analysis indicated C cycling was tightly coupled with soil P concentrations (Chapter 2). Based on these correlations, possible C and P controls on C cycling were investigated. Results following C or P addition to soils indicated methanogenesis in restored sites was limited by C availability or C quality.

To investigate the role of P in regulating methanogenic processing, laboratory soil manipulations were conducted to determine if an increase in CH_4 production was in response to direct methanogen P limitation or indirect P limitation of higher trophic level microbes (increased methanogenic substrate availability) (Chapter 3). Results suggest hydrogenotrophic methanogens were dominant at the low-P reference site. Based on stimulation patterns of CO_2 and CH_4 production in response to joint C and P additions it appeared that fermenters, not methanogens, were P-limited. Hydrogen, a substrate for hydrogenotrophic methanogens, can be produced by fermenters and is always produced through obligate syntrophic associations (Conrad 1999) indicating stimulation of fermentation would elevate substrates to fuel hydrogenotrophic methanogenesis. Furthermore, increased substrate availability following stimulation of fermentation with P addition resulted in elevated CH_4 production rates (Chapter 3).

The Everglades are an example of a fire adapted ecosystem which varies greatly in soil P concentrations. One goal of this research was to apply the overall knowledge of nutrient controls on methanogenic processing (from Chapter 2 and 3) to evaluate *in situ* fire effects involving nutrient addition or removal. To achieve this goal, the short- and long-term effect of fire on C processing (enzyme activity, aerobic (CO_2) and anaerobic CO_2 and CH_4 production in restored (high-P) and reference (low-P) wetlands was monitored. The response of C cycling to fire appeared to differ by site and elevation initially (2 days). However, the difference in response between high and low elevations was likely driven by the more complete burn at the high relative to low elevation site and subsequent difference in fire residues (ash and char) proportion and quantity produced. For example, in Res00-high elevation plots C cycling was stimulated evidenced by higher TOC and BGA from burned sites. However, this trend was not observed at the lower elevation suggesting C cycling was affected differentially.

The difference in high and low elevation C cycling post fire was not as apparent in reference wetlands likely in response to the *Muhlenbergia* and sawgrass dominated land cover resulting in a more complete burn than restored sites (woody vegetation). Stimulation of C cycling (TOC and ANCD) in reference soils was evident regardless of elevation; however, stimulation of methanogenesis was only observed at the higher elevation. Initial stimulation was only observed at 2 days and long-term trends were not consistent suggesting the initial stimulation may be in response to a short-lived nutrients (P) pulse. Furthermore, the stimulation of C cycling could not be explained by changes in MBC or high soil temperature fluctuation (Chapter 4).

To confirm the stimulation of C processing was in response to a change in soil nutrient content or availability the research focus was narrowed to directly investigate the effect of fire residues (ash and char) on CO_2 and CH_4 production. By amending soils with these residues, confounding factors affecting these processes in the field were reduced. Results indicate that char was a source of both available C and P whereas ash served solely as a P source (Chapter 5). Following amendment, the response to aerobic and anaerobic decomposition processes differed by site. The addition of both residues initially increased aerobic CO_2 production in reference wetlands although this trend was short-lived and decreased quickly with time. In contrast, residue addition initially suppressed aerobic CO_2 production from restored wetlands however, with time, aerobic CO_2 production was stimulated. Stimulation of anaerobic CO_2 production and methanogenesis was observed when amended with char regardless of site. However, only reference soils responded favorably to ash. These results were similar to trends observed following nutrient addition (Chapters 2 and 3).

Conclusions

From the current study, it appears that the main regulator on methanogenesis in the HID site is substrate availability. However, P availability to higher trophic level microbes was the ultimate control (increased substrate availability). Both CO_2 and CH_4 production were elevated from restored relative to reference wetlands. When taken together with observed nutrient correlations, results suggest production of C greenhouse gases will likely decrease with restoration time as P availability is reduced. This has implications for soil removal restoration tactics and the associated C balance suggesting elevated C greenhouse gas production post restoration may be transitory. This warrants further investigation which is necessary to determine if these sites may

shift from a C source to sink with restoration time. Monitoring CO_2 and CH_4 produced from older restored sites and bi-annual monitoring (wet and dry season) in the current sites would be necessary to better understand changes in these patterns with restoration time.

Results linking soil P concentrations and anaerobic CO_2 and CH_4 production can be applied to other disturbance events which naturally alter soil nutrient concentrations such as fires (natural and prescribed), a disturbance which is common across Everglades wetlands. While many studies quantify CO_2 and CH_4 production post fire, limited studies have focused on the short- and long-term effect of fire on these processes with initial (pre-burn) soil and microbial data collected from an adjacent control (un-burned) site for comparison across time. From this study (Chapter 4), we learned the importance of quantifying these parameters on both time scales (short- and long-term). For example, soil was not analyzed 2 days post fire the initial stimulation of C cycling would not have been captured. This stimulation was eliminated one month after the fire.

Data suggests the short-lived fire affect was in response to an additional pulse of available nutrients derived from OM burning (i.e. ash and char production). Based on fire intensity at each elevation and site, the proportion of ash and char produced would vary. Although both residues serve as a P source, char contained a greater proportion of available P and provided a pulse of available C which also limits decomposition in the HID sites. Minimal information investigating the link between fire residues and anaerobic CO_2 and CH_4 production is available. While char is currently being investigated as a C sequestering agent, the offset of this addition under anaerobic

conditions in the HID site was estimated at 1.5 months in the reference site and 6-9 months in the restored sites. This suggests the effect of char may have a transitory effect on increasing soil C sequestered post fire in response to elevated C loss (CO_2 and CH_4) especially in the low-P soils. The investigation of fire residues increased our understanding of how fire can affect CO_2 and CH_4 production in soils which differ in nutrient concentrations (Chapter 5). In addition, results highlight nutrient differences associated with each residue and how these residues can affect CO_2 and CH_4 production differently providing a plausible explanation for the variable response (increase/decrease/no change) in C gaseous production following fire as reported in the literature and seen in this study.

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BIOGRAPHICAL SKETCH

Cassandra Anne Medvedeff was born in Akron, Ohio but was raised in Madison, Connecticut. Prior to completing a Bachelor of Science degree in Biology (University of North Carolina at Greensboro) she began to work in Dr. Anne Hershey's aquatic ecology lab. Dr. Hershey gave her an opportunity to conduct research in Arctic Alaska as an undergraduate. After the first sampling season she knew she has found her passion, research. Cassandra enjoyed the experience so much that she pursued a Master of Science degree (Biology) with Dr. Hershey and expanded upon her previous work in arctic lakes. Following this experience, she decided to continue her education and love of research and was offered an opportunity in the Soil and Water Science Department at the University of Florida.