LAND MANAGEMENT, CARBON CYCLING, AND MICROBIAL DYNAMICS IN PACIFIC NORTHWEST WETLANDS

by

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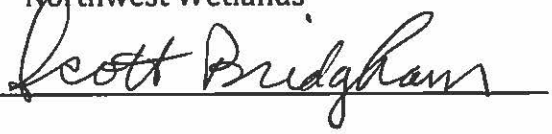
Approved: 

Scott Bridgham

Wetland ecosystems are key players in the global carbon cycle. Understanding the effects of land management, degradation and restoration, on these systems is critical to developing efficient and effective land management practices. Monitoring should be extended to ecosystem functions in order to determine if mitigation results in a no-net loss of wetland function. Our specific objective was to explore microbial function as a mechanism behind the shift in carbon cycling after land management treatment. We sampled two marsh case studies, a saltmarsh freshwater complex, each with a reference, restored, and disturbed site, along the Oregon coast. We calculated soil carbon stocks, and measured CO_2 and CH_4 production. Microbial function was measured by performing an extracellular enzyme assay and a catabolic profile. Our results suggest that restoration in each case study achieved only partial return of soil carbon function, but the freshwater restoration was closer to the reference condition. These findings reflect that the freshwater restoration hydrology and plant community more closely matched the reference condition than in the salt marsh restoration.

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INTRODUCTION

Wetlands and ecosystem services

Human activities over the past century have impacted many facets of global biogeochemistry. Anthropogenic emissions have increased atmospheric concentrations of the greenhouse gases carbon dioxide, methane, and nitrous oxide emissions (Canadell et al. 2007, Scheehle and Kruger 2006). The increase in greenhouse gases, such as carbon dioxide and methane, have led and will continue to lead to shifts in climate (Bridgham et al. 2013). The state and management of carbon-sequestering ecosystems is ever more crucial with this imbalance in the natural carbon cycle. The carbon cycle involves net primary productivity, degradation of organic matter into particulate organic matter, stabilization of soil carbon through a variety of mechanisms, and mineralization of available carbon substrates into carbon dioxide and methane (Reddy and Delaune 2008). In wetland ecosystems, organic matter enters the system through both autochthonous, generated within the system, and allochthonous, generated outside the system, sources (Kellerman et al. 2014).

Wetlands act as a significant carbon sink, which is one of their many ecosystem services (Reddy and DeLaune 2008). An ecosystem service is a natural process that provides a benefit to human society. Wetlands also have a substantial impact on regional water quality, carbon storage and processing, and biodiversity (Reddy and DeLaune 2008). Nitrogen, phosphorous and carbon compounds are stored in soils and may be absorbed into plant structures (Reddy and DeLaune 2008). Decomposition in wetlands is relatively slow, driving accumulation of

organic matter (Reddy and Delaune 2008). Wetland ecosystems are key players in carbon dioxide sequestration, despite their relatively small area (Balser et al 2010). Although wetlands account for only 10 % of terrestrial land area, they store 45-70 % of terrestrial carbon (Morrissey et al. 2014). Carbon is mineralized in two molecular forms: CO_2 and CH_4. The consumption and production of both carbon dioxide and methane must be taken into account in order to analyze the warming and cooling effects of wetlands (Neubauer and Megonigal 2015).

The radiative forcing of carbon cycling in wetland ecosystems depends on the rate of carbon sequestration and methane production. Freshwater wetlands account for one quarter to one third of global methane emissions (Bridgham et al. 2013). The sustained-flux global warming potential of methane over 100 years is forty-five times that of carbon dioxide (Neubauer and Megonigal 2015). Wetland net radiative forcing changes from a positive warming to a net cooling depending on the peaks and decline of methane production (Neubauer 2014). Although most freshwater wetlands initially seem to have a net warming effect, they will eventually have a net cooling effect (Neubaurer 2014). Saltmarshes have been found to have significantly lower rates of methane production in comparison to freshwater wetlands (Lovely and Phillips 1987, Poffenbarger et al. 2011). The tidal environment provides an abundance of sulfate allowing certain sulfate-reducing microbes to outcompete methanogens for electron donors (Poffenbarger et al. 2011)

The initiative to restore wetland ecosystems began with the no-net-loss policy under section 404 of the Clean Water Act. This executive order mandated a

compensatory mitigation for wetland destruction. Although the majority of wetlands are restored or created based on area, this policy specifically refers to a maintenance of wetland "natural systems" (Carter 1977). The Mangan saltmarsh, one of the two marsh case studies in this research project, is an example of compensatory wetland mitigation.

Landscape-scale changes in these ecosystems may cause a significant shift in the carbon flux rates. It is imperative to build on the current knowledge and understanding of wetland soil ecology to aid wetland ecosystem management and ultimately carbon sequestration. My objective was to establish a mechanistic foundation for understanding the effects of wetland restoration on carbon sequestration and methane fluxes in a set of estuarine and freshwater wetlands on the Oregon coast.

Carbon cycling: A microbial function

Organic matter degradation by microorganisms is an important ecosystem function. Soil microorganisms break down organic matter, and release carbon dioxide and methane through metabolic processes (Williams et al. 2010). Soil microbial communities are interconnected webs, which shift in response to landscape change (Morrissey et al. 2014). A change in the microbial community through land management can alter current ecosystem functions. Molecular techniques enable an in situ characterization of microbial community structure. However, linking the community profile and ecosystem function tends to be difficult (Torsvik and Øvreås 2002).

Microbes and enzyme activity

Although microorganisms are a fraction of the soil organic matter, they link organic matter to other organisms in the ecosystem (Reddy and Delaune 2008). Nutrient and energy cycling is critical for the wetland energy food web and for releasing nutrients for plant growth. Extracellular enzymes break down complex organic matter into simpler compounds, which then can be taken up by microorganisms and plants in the ecosystem (Reddy and Delaune 2008). These enzymes are exuded by microorganisms and diffuse away from the parent cell (Burns et al. 2013). The activity of these exoenzymes is often the rate-limiting step in organic carbon decomposition (Morrissey et al. 2014). They enable microorganisms to utilize resources from complex organic molecules and catalyze this step in soil carbon and nutrient cycling. Soil enzyme concentration relies on a balance between enzyme production and degradation. Abundant substrates and scarce products lead to a regulatory increase in enzyme production (Allison and Martiny 2010, Burns et al. 2013). A 2% minimum of microbial assimilated carbon is dedicated to enzyme production in order to sustain biomass and avoid starvation (Burns et al. 2013). Enzyme producers have been shown to allocate more resources toward limiting nutrient enzymes. These allocation patterns link ecological and evolutionary constraints with ecosystem-level processes at the organismal level (Allison and Martiny 2010).

Catabolic profile: Microbial response to carbon substrate addition

A catabolic profile captures a snapshot of the microbial ability to degrade specific carbon compounds at a given point in time (Garland 1997).

Microorganisms utilize various carbon substrates, from simple compounds such as glucose, to more complex molecules like cellulose and lignin. The pattern with which microorganisms utilize certain carbon substrates can be a helpful tool in determining the effects of landscape change on microbial community function (Ritz et al. 2006, Balser et al 2010). Soil structure, oxygen availability, nutrient availability, and moisture all affect the soil profile, and influence which microbes are able to thrive and utilize specific types of available carbon substrates (Balser et al. 2010). Land use alters the organic matter composition, consequently changing the available nutrients and carbon compounds (Portnoy and Giblin 1997). For example, microbial community structure and function are adversely affected by the conversion of ecosystems to agricultural uses (Balser et al. 2010). Analysis of both microbial carbon substrate utilization and enzyme activity provides a robust assessment of microbial function after land management events.

Wetland management effects on microbial function

Wetland degradation is driven primarily by land conversion for agriculture, especially in the Pacific Northwest (Balser et al. 2010). Wetland conversion for agricultural use dramatically increased following the federal Swamplands Acts in the mid 19th century (Boule and Bierly 1986). Northwest marshes and estuaries suffered conversion losses through the early 20th century (Boule and Bierly 1986). Salt marsh diking causes an immediate aeration and desalinization of wetland soil (Portnoy 1999). These sites have altered nutrient availability and abiotic factors such as increased pH and decreased organic content (Portnoy 1999). These physical changes, which follow disturbance events, may have long lasting effects

on microbial communities in both community composition and function (Bernhard et al. 2012). Diking and subsequent hydrologic restoration processes may significantly affect enzyme activity. Hydrolytic enzymes including β 1,4-glucosidase, β 1,4-cellobiosidase, and β D-xylosidase increase in activity with increasing amounts of organic matter and plant cover (Morrissey et al. 2014, Duarte et al. 2012). However, there is a large discrepancy as to how many years are required to return microbial function, measured through microbial enzyme activity and substrate utilization (Duarte et al. 2012, 2008).

Carbon dioxide and methane emissions are linked directly to microbial respiration and ability to break down and utilize carbon compounds. Carbon mineralization is a key piece in the carbon cycle, and affected by landscape scale changes, such as moisture content and plant community structure. Carbon flux rates are useful in characterizing a microbial response to land management events. However, current research has shown that this response is complex (Portnoy 1999). Carbon mineralization was found to increase with decreasing inundation (Sasaki et al. 2009, Freeman et al. 1997), but also increase with re-inundation of previously diked wetlands (Altor and Mische 2008).

Net methane emissions depend on the balance between production, or methanogenesis, and oxidation, or methanotrophy (Reddy and Delune 2008). These processes depend on both microorganism and plant communities. Before methanogens are able to utilize carbon substrates acetate and H_2, exoenzymes must first break down organic matter (Bridgham et al 2013). Consequently, exoenzyme activity can be a limiting factor in methanogenesis (Morrissey et al.

2014). Methane production correlates with shifts in microbial community structure and enzyme kinetics for hydrolytic enzymes (Morrissey et al. 2014, Freeman et al. 1997). In recently restored sites, methane production is relatively low due to reduced organic matter availability (Altor and Mische 2008).

Wetland management in the Pacific Northwest

Restoration of disturbed estuaries and marshes in the Pacific Northwest took hold during the late 1970's and 1980's. Oregon introduced statewide planning goals, which included replacement and restoration of lost resources (Boule and Bierly 1986). During the past fifty years, wetland hydrology has been restored to approximately fifty wetlands on the Oregon coast (Adamus et al. 2005). In 1974, NOAA established the South Slough National Estuarine Reserve (NERR) in Coos Bay, Oregon (NOAA 2007). Restoration activities in this wetland reserve have unearthed several important attributes that apply to the restoration of diked marshes. Diked wetlands experience sediment subsidence due to organic matter oxidation and removal of plant material through grazing. Accretion rates in the Pacific Northwest are 2.4-2.8 mm/yr (Cornu 2005). At this rate, previously diked wetlands in the Pacific Northwest would require 20-40 years to reach a sediment level necessary to support the natural vegetation community (Cornu 2005). South Slough watershed marshes were filled with sediment and restored with tidal channels, which resulted in rapid re-colonization of native plant communities and closely resembled neighboring reference wetlands after eight years (Cornu 2005). Tidal channels are also key wetland structural elements. They connect the wetland

to important sources of organic matter, providing structure, nutrients and detritus for the food web (Cornu 2005).

Structural elements are necessary in order for wetlands to return to a reference-level equilibrium. However, the knowledge gap between these visible elements, such as tidal channels, sediment levels, and plant colonization, and their effect on microbial community needs further study. Past research indicates incredible complexity in the relationship between wetland soil microbes, their habitat, and habitat change. Additional understanding in how these shifts in microbial function are affected by wetland disturbance and restoration will increase the ability to effectively manage wetlands in the context of key ecosystem services related to carbon cycling.

In this thesis, I investigated the question, how does land management affect carbon cycling through microbial function in Pacific Northwest tidal and freshwater wetlands?

MATERIALS AND METHODS

Site description

The wetland sites were located in the South Slough Reserve and North Bend, both part of Coos Bay on the Oregon coast. The South Slough watershed encompasses 19,295 acres within the Coos watershed drainage. Sand flats, mud flats, and salt marshes form a mosaic, and constitute 60-70 % of the estuary. About 70 % of the South Slough watershed is privately owned and managed for timber harvest. Rural residences occupy 5 %, while the remaining 25 % of the watershed constitutes the South Slough National Estuarine Research Reserve (NOAA 2007). This study included a total of six sites; two sets of reference, restored and disturbed marshes (table 1).

Three of the six sites chosen were freshwater, located in the South Slough Reserve: Tom's Creek Reference marsh (43°16'44.97"N 124°19'6.41"W); Anderson Restored marsh (43°16'3.78"N, 124°19'26.32"W), Wasson Disturbed marsh (43°16'17.07"N 124°19'22.47"W). Tom's Creek Reference site was dominated by wetland species such as willow (*Salix sp.),* small foot bulrush (*Scirpus microcarpus),* and Lyngbye's sedge (*Carex lyngyei).* The site contained winding channels and several low beaver dams, which have altered the hydrology by increasing inundation. The soil profile was comprised of an organic layer that extended farther than the sampled 30cm. The soil contained dense root systems, both fine and coarse. The Anderson Restored and Wasson Disturbed sites are parallel, narrow wetland drainages, separated by a 0.4-km ridge. The Wasson Disturbed site was ditched and drained for grazing in the early 20th century. It was

abandoned for agriculture in the 1970's, but a deep incised ditch on the north boundary continued to divert the majority of the water away from the marsh. Water in the lower part of the marsh was backed up by a dirt road and was much wetter, with extensive reed canary grass (*Phalaris* arundinaceae) (Turnull and Bridgham 2015). We sampled above the damming effect of the road, where the water table was always greater than 30 cm from the surface, with the assumption that this area best represented the antecedent condition of the Anderson marsh. The dominant vegetation included exotic grass and forb species, and creeping thistle (*Cirsium sp.).* Before restoration, the Anderson site reflected the disturbed, ditched Wasson conditions. This site underwent restoration, which included digging channels and scraping off the top 20 to 60 cm of soil (Cornu 2005). Anderson Restored site reflected the vegetation and channel hydrology of the Tom's Creek Reference site (Cornu 2005). The soil profile included an accreted organic layer that ranged from 6 to 15cm thick. This accreted soil layer was much less root dense than the reference organic layer. A dense, clay layer was directly beneath the accreted layer.

The second set of sites were located in the North Bend area: Mangan Reference marsh (43°27'15.68"N 124°12'26.15"W); Mangan Restored marsh (43°27'21.99"N 124°12'17.67"W); Mangan Disturbed marsh (43°27'18.91"N 124°12'21.02"W). All of these sites are next to each other and formed a contiguous salt marsh until diking for pasture in 1934. Diking and tide gates removed salt water influx and the site became freshwater. These activities were widespread in salt marshes in the early twentieth century (Portnoy 1999). Part of the marsh was

restored in 2006 by removing the dike. During the rainy season, the Mangan Disturbed marsh is typically inundated, but is relatively dry during the summer season. This site experienced significant subsidence, the loss of organic soil volume, after the diking event, which we visually estimated to be at least 1 meter. With the transformation to an aerobic environment during part of the year, microorganisms were able to oxidize organic material at a much higher rate. The Mangan Disturbed site is dominated by reed canary grass (*Phalaris arundinaceae)*. The Mangan Reference site is a relatively high marsh, fringing the seaward side of the dike. It is dominated by pickle weed (*Salicornia sp.).* The soil profile has an organic layer that extends deeper than the 30cm sampling depth. Because of the previous soil subsidence, the Mangan Restored Marsh is a low marsh, mostly devoid of vegetation, with a rapidly accreting silt layer that ranges from 8-12 cm thick. The soil profile included an accreted silt layer that ranges from 8cm to 12 cm. A dense, clay layer underlies the accreted layer, which represents the surface layer of the marsh before the tidal hydrology was restored (Figure 1).

At each site, soil samples were collected at five locations, thirty meters apart, to capture ecological variation. At each location, two samples were cored to capture the shallow (0-15 cm) and deep profile (15-30 cm). However, in both restored sites, the sample depth for the shallow profile was adjusted to capture only the accreted layer. Samples were collected using PVC pipe cores and rubber stoppers to ensure samples from inundated sites were stored anaerobically. Sample cores were placed on ice during transport, stored in a refrigerator, and processed within 5 days.

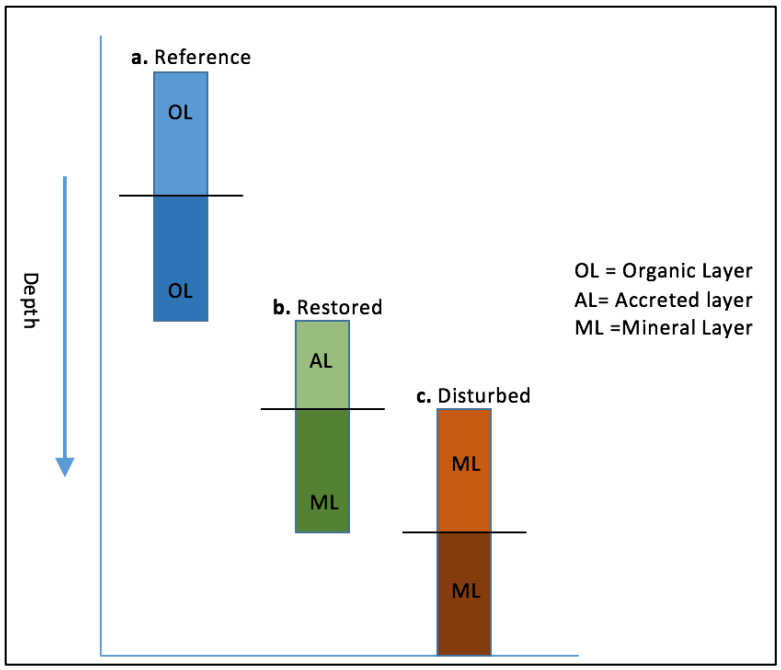


Figure 1: Comparison of the Mangan saltmarsh sites' core profiles. **a.** contains highly organic soil in both soil cores; **b**. represents the accreted layer of soil in the upper core, and the mineral layer below; **c.** represents the mineral layers of both soil cores. The black dividing lines separate the shallow and deep cores.

I compared soil carbon content, carbon dioxide and methane emissions, enzyme activity, and substrate utilization across the three land use treatments, Restored, Reference and Disturbed, and the two depth profiles, shallow and deep.

Table 1: Site information

Site Name	**Treatment**	**Salinity**	**Location**
Mangan Reference	Reference	Salty	North Bend
Mangan Restored	Restored	Salty	North Bend
Mangan Disturbed	Disturbed	Fresh	North Bend
Tom's Creek Reference	Reference	Fresh	South Slough
Anderson Restored	Restored	Fresh	South Slough
Wasson Disturbed	Disturbed	Fresh	South Slough

Sample processing and bulk density

All anaerobic samples were processed and incubated anaerobically using an anaerobic glovebox (Coy laboratory, inc.). Aerobic sites, Wasson Disturbed and Mangan Disturbed, were processed aerobically. All samples were weighed, homogenized, and cleared of roots and wood matter by hand picking. A subset of each sample was weighed, dried, and weighed again to determine percent moisture. Bulk density was determined by dividing the total grams of dried soil by the core volume.

Soil carbon content and carbon flux

Soil organic carbon and nitrogen were determined on duplicate technical replicates from each core increment with a Costech elemental combustion system (ECS4010). Samples were dried for 48 hours at 60 degrees Celsius, and ground and homogenized. Standard curves were generated using acetanilide. San Joaquin soil replicates (NIST Standard Reference Material 2709) with known carbon content were interspersed throughout the sample run to indicate accurate measurement. I calculated carbon stocks from the percent carbon and bulk density data.

CO_2 and CH_4 production were measured using a flame ionization detector (FID) gas chromatograph (SRI 8610C). CO_2 samples were injected, and converted into methane by a methanizer. Samples were prepared aerobically or anaerobically in serum bottles. Anaerobic samples were flushed with N_2 for 15 minutes before incubating at 21 ℃ for an average of 91 hours. Measurements were taken at this single time point and linear gas accumulation was assumed

(Bridgham and Ye 2013). We also calculated the concentration of dissolved CO_2. At a given temperature, the amount of gas dissolved in a liquid is proportional to the partial pressure of the container. When CO_2 dissolves in water, it forms H_2CO_3, HCO_3^-, and CO_3^{-2}. We measured pH to determine the concentration of these molecules and calculated the corresponding concentration of CO_2. For aerobic samples, we measured methane oxidation instead of methane production at four time points over 29 hours.

Catabolic profile methodology

We used MicroResp™ equipment and protocol to perform a catabolic profile. The protocol involves the addition of specific carbon substrates to soil samples. We loaded six deep 96-well plates with soil samples using the Microresp plate loaded. The plate loader ensures a constant volume of soil is added to each well. Individual soil weights were recorded to determine the respiration response per gram soil. After substrate addition, the plates were sealed with MicroResp rubber mats and cresol red agar indicator plates. The special seal allows CO_2 to interact with the respective indicator well. The color change of the agar indicates the concentration of CO_2 present in the well. Standards were determined by injecting known concentrations of CO_2 into sealed serum vials with single agar-filled wells.

Average sample percent moisture was used to calculate the concentration of each substrate solution added to all samples. Our catabolic profile included eight substrates: glucose, fructose, lignin, cellulose, asparagine, histidine, ∝ ketoglutaric acid, and malic acid. Deionized water was added to account for the background

respiration rate. Each sample was tested with two technical replicates per substrate. Plates were incubated at 25^{o}C for 6 hours, and read using a colorimetric plate reader (Magellan™, Tecan) at 570 nm.

Enzyme activity methodology and development

We developed the extracellular enzyme assay used in this experiment during June and July 2015 based on Sinsabaugh et al. 2003 and German et al. 2011. The enzymes targeted included both hydrolytic and oxidative enzymes. Enzyme activity assays have not previously been use in Pacific Northwest wetlands to my knowledge. I worked to develop the optimal assay conditions including incubation time, the effect of anaerobic and aerobic incubation conditions, and hydrolytic assay Vmax (results not shown). The Vmax is a value that indicates the maximum rate of an enzyme, with a given threshold concentration of substrate. A two-hour incubation time was sufficient for consistent results. Any additional time did not increase, and even decreased precision. Aerobic and anaerobic conditions did not affect hydrolytic enzyme activity. As a result, all hydrolytic assays were performed in aerobic conditions. The Vmax substrate concentration of 200 μmol is accepted across all the reviewed enzyme assay literature for hydrolytic enzymes (Sinsabaugh et al. 2003, German et al. 2011, Saiya-Cork et al. 2002). I assayed soil samples across all enzymes at both 150 and 200 μmol of substrate. 150 μmol was not sufficient to bring enzymes to Vmax, and consequently, 200 μmol was used in enzyme assays. Because soil pH affects enzyme performance (German et al. 2011), we conducted all assays at *in situ* pH in order to maintain field conditions. Instead of using a buffer solution to create the same pH across all samples, we used distilled (DI) water as the soil slurry base to ensure field pH was maintained.

The hydrolytic enzymes assayed in this experiment were β 1,4-glucosidase, α 1,4-glucosidase, β -^{D}-1,4-cellobiosidase, and β- 1,4-xylosidase. These enzymes

hydrolyze glucoside isomers, cellulose, and hemicellulose respectively. Synthetic substrates with the fluorescent tag, methylumbeliferone (MUB), were used to quantify the activity of each enzyme. Assays were run on microplates along with soil and substrate controls. The fluorescence MUB standard quench control was run with MUB quench standards. The quench standards consisted of the MUB standard concentrations in soil slurry to control for absorbance of signal by soil particles. Approximately 1.0 gram of soil was slurried with DI water for one minute using a milk frother. 50 μl of substrate added to the appropriate well, along with 200 μl of soil slurry (or 200 μl DI water for the substrate control). In addition, soil slurries were run with 50 μl DI water to account for the background soil absorption. The MUB standard curve solutions, between 0 and 100 μmol, were added to wells, along with 200 μl soil slurry for each sample. An additional curve was run with DI water to obtain the fluorescence standard. Each substrate was assayed with four technical replicates for each sample. After the two hours of incubation, 20 μl of 0.5 M NAOH was added to increase the fluorescence and decrease the background quench values. The microplate wells were read by a Victor^3 plate reader (PerkinElmer, Waltham, MA), at 254 nm excitation and 450 nm absorbance at 0.1 seconds per well. Enzyme activity was calculated using the calculations outlined in German et al. 2011.

We optimized a colorimetric assay to quantify the oxidative enzyme activity of phenol oxidase and peroxidase, both of which target phenolic compounds. L-dihydroxyphenylanlanine (LDOPA) is a chemical used to indicate oxidative enzyme activity. LDOPA is oxidized to dopachrome, an indicator pigment which absorbs

light. Assay wells received 200 μl of soil slurry and 50 μl of 25 mM LDOPA. Negative control wells were received the 200 μl of soil slurry, along with 50 μl DI water. The blank control wells received 50 μl of DLOPA with 200 μl DI water. Peroxidase assay and control wells also received 20 μl of 0.3% H_2O_2. Plates were incubated for 18 hours in the dark and read at 450 nm absorbance on the Victor^3 plate reader. Peroxidase activity was determined by subtracting phenol oxidase activity from the overall peroxidase and phenol oxidase activity (wells with added H_2O_2).

Statstics

We analyzed the data using the statistical package R version 3.2.1 (06/2015). We compared treatment, depth, and enzyme or substrate, when appropriate, using 2-way or 3-way ANOVAs and Tukey's HSD.

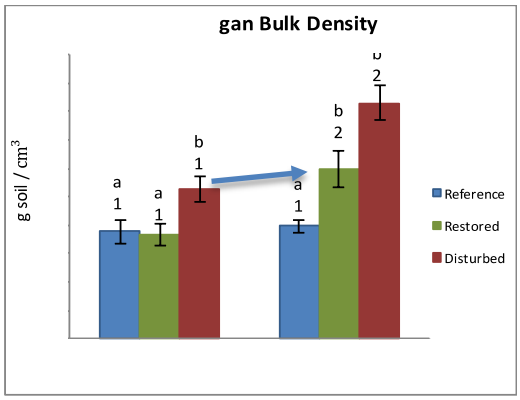
RESULTS

Mangan bulk density and carbon stocks

Treatment significantly affected bulk density, but varied by depth (p=0.004). Restored bulk density significantly differed between the shallow and deep profiles (Figure 2a, p=0.00168). It was important to compare the deep Restored depth increment to the shallow Disturbed increment because these layers were similar in terms of treatment (p=0.1). The Mangan carbon stocks were highest in the shallow core of the disturbed site, and the deep core of the restored site (Figure 2b). The shallow Restored and Disturbed carbon stocks were significantly different, indicating a considerable loss of organic matter with

restoration (p<0.0001). The deep Restored and Shallow disturbed depth increments are also statistically similar (p=0.0019). The carbon stock calculation depended on the bulk density and percent soil carbon from each core. We also calculated the overall carbon (per m^2). The Reference (8.2 SE +/- 0.4) and Restored (7.3 SE +/- 0.4) sites were significantly lower than the Disturbed site (10.0 SE +/- 0.3) (p<0.001).

a)¶



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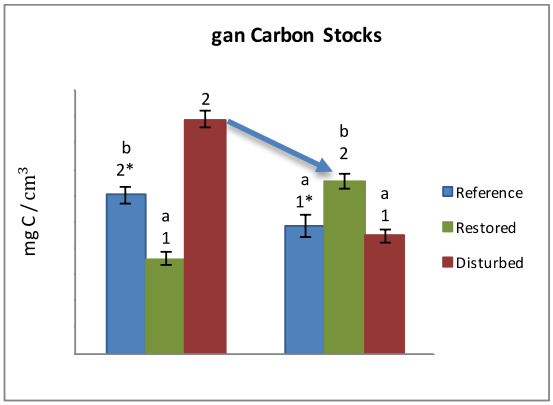

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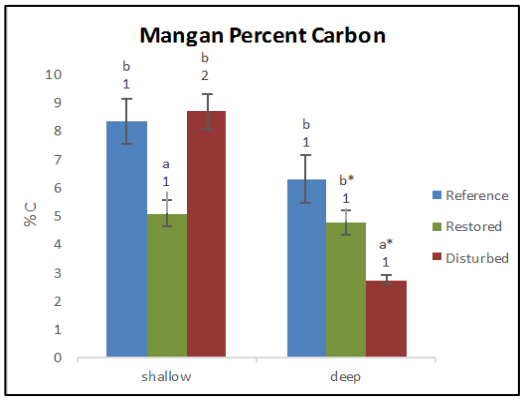
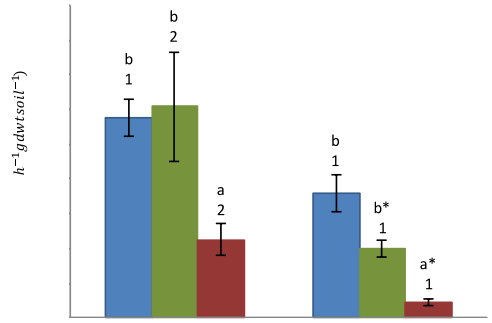


Figures 2a Bulk Density, 2b Carbon Stocks, 2c Percent Carbon: Different letters denote treatment differences within each depth, where a represents the lowest value. Numbers show differences between depths within a treatment. The asterisks represent a moderately significant effect. The arrows highlight the comparison between the shallow Disturbed and deep Restored layers. Bulk density showed a return to reference-level in the shallow core, and a partial return in the deep core after restoration. Carbon stocks and percent carbon showed a loss of carbon in the deep Restored layer after the restoration event. However, the shallow Restored layer indicated an ongoing accumulation of carbon through sediment deposits. It is worth noting the high percent carbon in the Reference site across depth, in comparison to Disturbed site's decrease in percent carbon with depth. In addition, the Restored organic layer extended past the depth measured in this study.

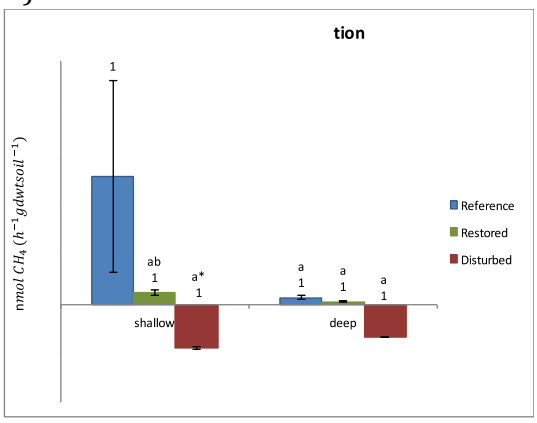
Mangan saltmarsh complex CO_2 and CH_4 production

Treatment and depth had significant effects on CO_2 production (Figure 3a, $p<0.0001$, $p<0.0001$). Carbon dioxide production rates in Mangan saltmarsh sites reflected a return to reference level with restoration in the shallow layer. The Restored site was similar to the Refernce site ($p=0.99$), and dissimilar from However, the difference between the restored and disturbed treatment in the deep core was marginally significant ($p=0.058$). For CH_4 production rates, the shallow Reference was significantly different from the shallow Disturbed (Figure 3b, $p<0.01$). However, CH_4 production rates in the Restored site were statistically similar to the Disturbed site across depth (Figure 3b). The overall CH_4 production was very low, and expressed in nmol while CO_2 production was expressed in μmol.

a)



b)



Figures 3a CO_2 and 3b CH_4: Treatment is compared within each depth. Depths are compared between treatments. Different letters denote treatment differences within each depth, where a represents the lowest value. Numbers show differences between depths within a treatment. The asterisks represent a moderately significant effect. CO_2 production indicated a return to reference-level with restoration in the shallow layer, and a partial restoration in the deep layer. CH_4 production showed an oxidation of methane in the Disturbed site, and a slight production in the Restored and Reference sites.

Mangan enzyme activity

The Reference site was significantly different from the Disturbed site, indicating that disturbance had a significant effect on enzyme activity across β glucosidase ($p<<0.0001$), α glucosidase ($p<0.01$), cellobiosidase ($p<<0.0001$), and peroxidase ($p<<0.0001$ (figures 9a-9f supplementary section). A non-metric multi dimensional scaling (NMDS) plot illustrates the similarity between the Restored and Disturbed site (Figure 4). β glucosidase, cellobiosidase, and peroxidase separated the Reference site from the Restored and Disturbed site.

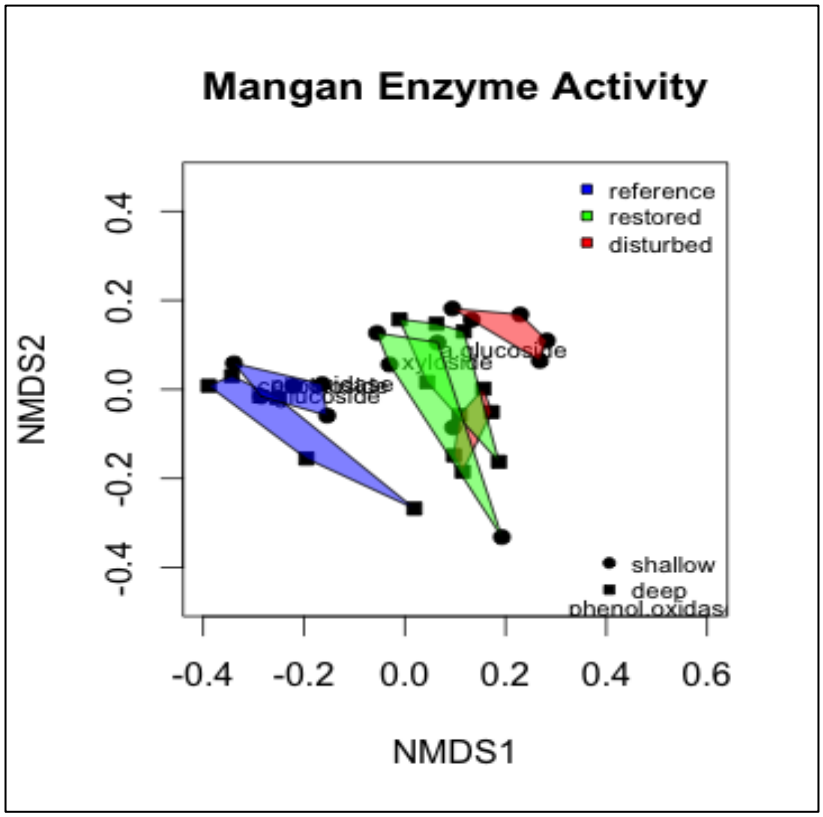


Figure 4: Polygons represent a set of shallow cores (circle points) or deep cores (square points) per treatment. Enzyme activity is significantly affected by treatment and depth (p=0.001, p=0.001). Restoration did not return enzyme activity to reference-level. The overlap of the green and red polygons indicate that the Restored site is more similar to the Disturbed site than the Reference site.

Mangan catabolic profile

Treatment significantly affected catabolic responses for glucose ($p<<0.0001$), fructose ($p<< 0.0001$), asparagine ($p=0.0095$), α ketoglutaric acid ($p=0.005$), and malic acid ($p<0.001$) responses (Figure 11a and 11b supplementary section). Cellulose produced a moderately significant response ($p=0.10$). Treatment response varied between deep and shallow profiles for lignin

(p=0.01) and histidine (p=0.08). An NMDS plot illustrates a similarity between the Restored and Reference sites, which split away from the Disturbed site (Figure 5). The shallow layer responses were much more variable than the deep layer. The Disturbed deep cores had a relatively low response to any added substrate. These samples may have been moisture limited. The Reference site expressed a significantly higher response to added substrates than the Disturbed site across all substrates in the deep layer. The Restored site expressed reference-level response across glucose and fructose in the shallow layer (p= 1.0E-06, p=1.0E-07), while the other six substrates expressed insignificant differences.

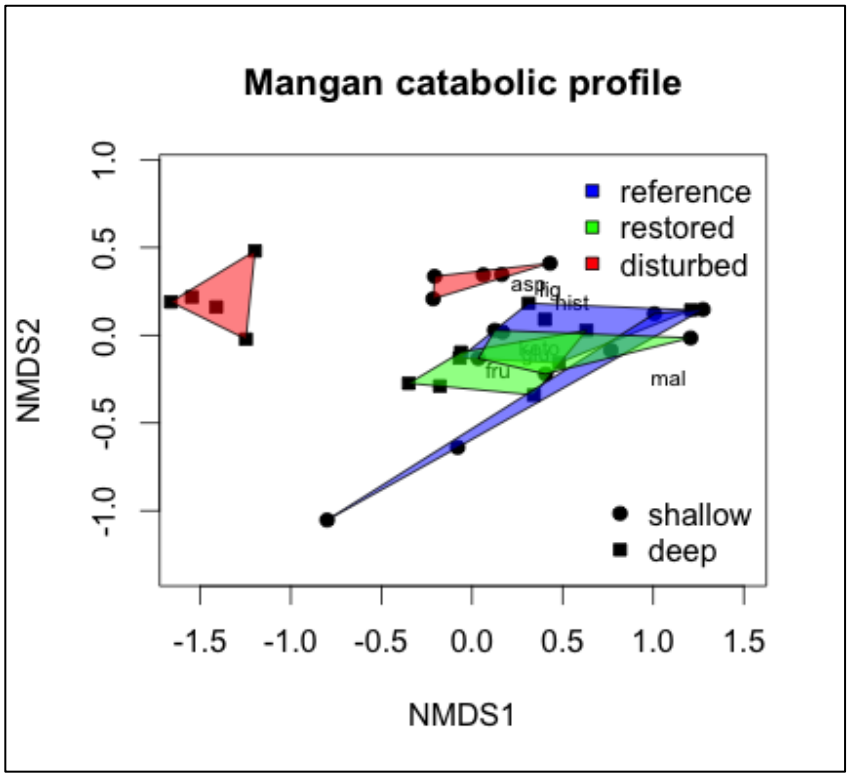
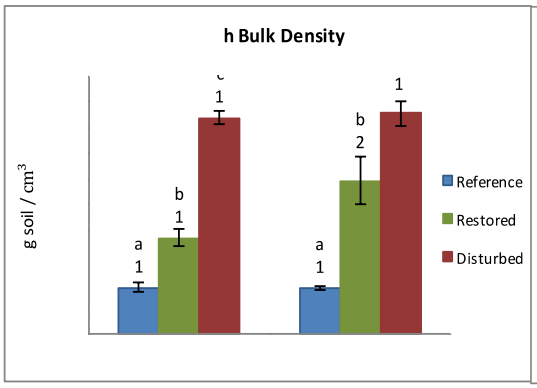


Figure 5: Polygons represent a set of shallow cores (circle points) or deep cores (square points) per treatment. Treatment and depth have significant effects (p=0.001, p=0.009). The overlap of the green (Restored) and blue (Reference) polygons indicate that restoration returned substrate utilization to reference-level.

Freshwater bulk density and carbon stocks

There was a significant interaction between treatment and depth for both soil bulk density ($p=0.005$) and carbon stocks ($p=0.0029$). Soil bulk density was significantly higher in the Disturbed site across depth (Figure 6a). The Restored site was significantly lower than the Disturbed, but not yet to reference-level soil bulk density in the shallow ($p<0.0001$) and deep ($p<0.0001$). The deep Restored layer had a higher bulk density than the shallow Restored layer ($p=0.0077$). Treatment significantly affected carbon stocks, but differently between the shallow and deep profile ($p=0.0029$) (Figure 6b). Shallow Restored had moderately higher carbon stocks than the deep Restored ($p=0.1$). The Shallow Disturbed contained higher carbon stocks than the deep Disturbed ($p<0.0001$). The Disturbed shallow layer had a higher carbon content than both the Reference and Restored shallow layers ($p=0.1$, $p=0.025$). The shallow mineral layer had 42.5 mg C cm^{-3}. The Restored site had accreted sediment in addition to the mineral layer, yielding 51.4 mg C cm^{-3}. The Disturbed site was compacted and highly oxidized, and lost a significant amount of soil organic matter in the disturbance process.

a)



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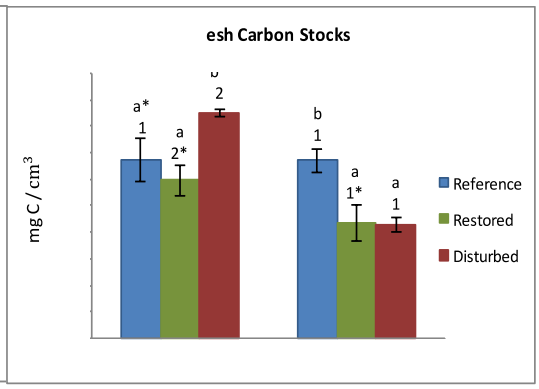
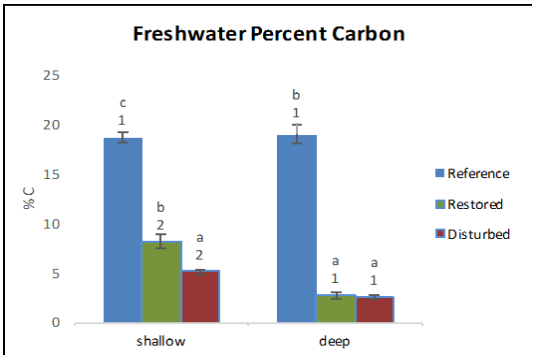


c)



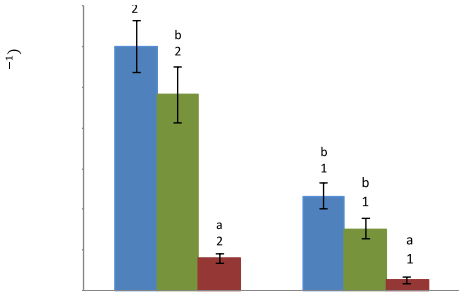
Figures 6a Bulk Density, 6b Carbon Stocks, 6c Percent Carbon: Different letters denote treatment differences within each depth, where a represents the lowest value. Numbers show differences between depths within a treatment. The asterisks represent a moderately significant effect. Bulk density showed a partial return to reference-level in the shallow layer, but not in the deep layer. Carbon stocks indicated a loss of carbon in the deep Restored layer, but also a continual accumulation of carbon in the shallow Restored layer after the restoration event. The Reference site had a high percent carbon, but carbon stocks similar to the Disturbed site due to a relatively low bulk density. Carbon stocks and percent carbon also showed an increase in carbon in the shallow Restored layer after the restoration event.

Freshwater CO_2 and CH_4 production

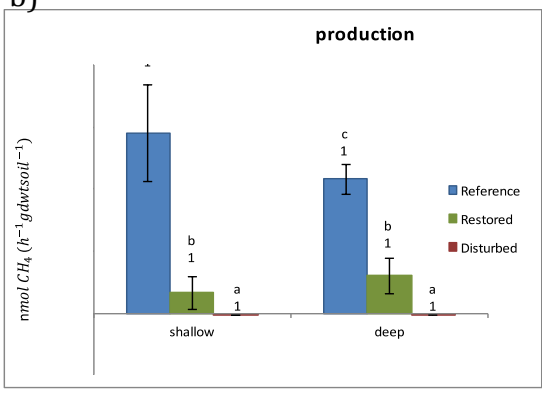
Treatment had a significant effect on both CO_2 and CH_4 gas production levels ($p<0.0001$, $p< 0.0001$). Specifically, Disturbance significantly reduced both CO_2 and CH_4 production rates across depth (Figures 7a and 7b). Methanogenesis requires anaerobic conditions. Because the water table in the Disturbed site was so low, CH_4 was actually consumed through methanotrophy instead of produced.

Although restoration significantly increased the CH_4 production, rates are still well below reference-level. In general, CH_4 rates were low in comparison to other freshwater environments (Bridgham et al. 2013). Restored CO_2 production was similar to the Reference site for the shallow core (p=0.3), but moderately different in the deep core (p=0.0757).

a)



b)



Figures 7a CO_2 production and 7b CH_4 production: Treatment is compared within each depth. Depths are compared between treatments. Different letters denote treatment differences within each depth, where a represents the lowest value. Numbers show differences between depths within a treatment. CO_2 production in the Restored site indicated a return to reference-level production across depth. CH_4 production showed that methane was consumed in the Disturbed site, and produced in the Reference and Restored site. The Restored site showed an intermediary return to reference-level production. Note that CH_4 production is still relatively low in comparison to CO_2 production.

Fresh enzyme activity

Disturbance significantly decreased enzyme activity across all enzymes (all p values <0.0001) (Figures 12a-12f). Restored hydrolytic enzyme activity significantly increased in enzyme activity across depth (all enzyme p values < 0.0001). The shallow layer in Restored site is more similar to the Reference site than the deep Restored layer (Figure 8). This general trend is more defined using pair-wise comparisons. The Restored shallow layer showed a significant return to

reference level, while the deep layer showed an intermediate restoration (Figures 12a-12f). The oxidative enzyme activities illustrate a similar trend. Phenol oxidase activity has returned to reference-level across depth, while peroxidase activity was restored to an intermediary level only in the shallow layer (Figures 12e and 12f).

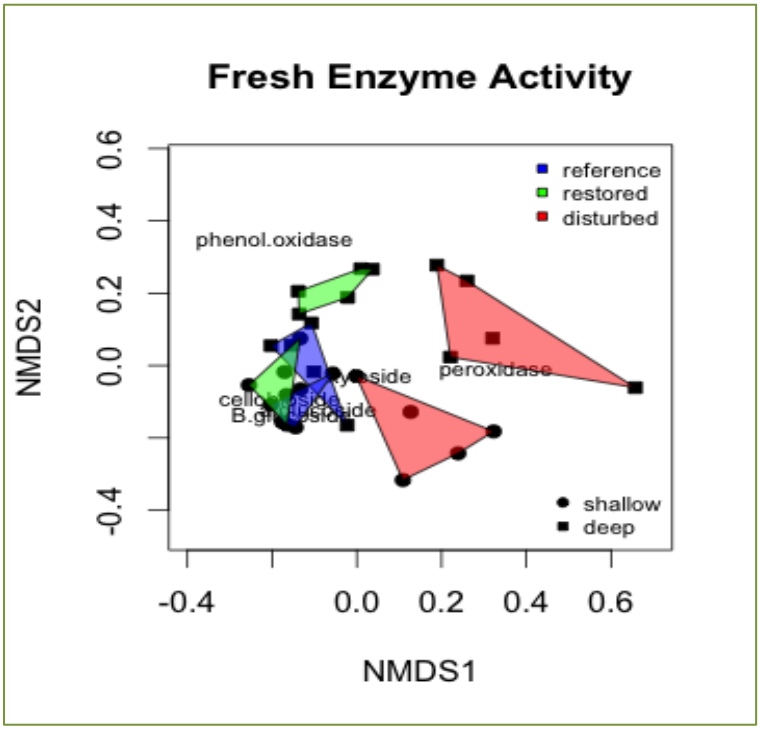


Figure 8: Polygons represent a set of shallow cores (circle points) or deep cores (square points) per treatment. Treatment and depth have significant effects (p=0.001, p=0.003). The overlap of the blue (Reference) and green (Restored) polygons indicated a return to reference-level activity with restoration.

Freshwater catabolic profile

The significant effect of treatment varied between the shallow and deep profiles ($p<0.0001$). Disturbance significantly decreased response to added carbon substrate across all substrates in the shallow layer, and most substrates in the deep layer (Figures 12a and 12 b). The restoration event significantly shifted response away from disturbed-level response, toward reference-level response (Figure 9). The shallow Restored layer was more similar to the Reference site than

the deep Restored layer. In the shallow layer, Restoration returned catabolic response to reference-level across the simple sugars, amino acids, and lignin. The addition of cellulose and carboxylic acids produced a response in the Restored site significantly greater than the reference-level response (Figure 13a). In the deep Restored layer, addition of simple sugars and complex carbon substrates produced an intermediary response, in between the disturbed and reference-level response. Addition of amino acids and α ketoglutaric acid indicated a similar catabolic response across treatment. Only the addition of malic acid produced a significantly higher response in the Restored site (Figure 13b).

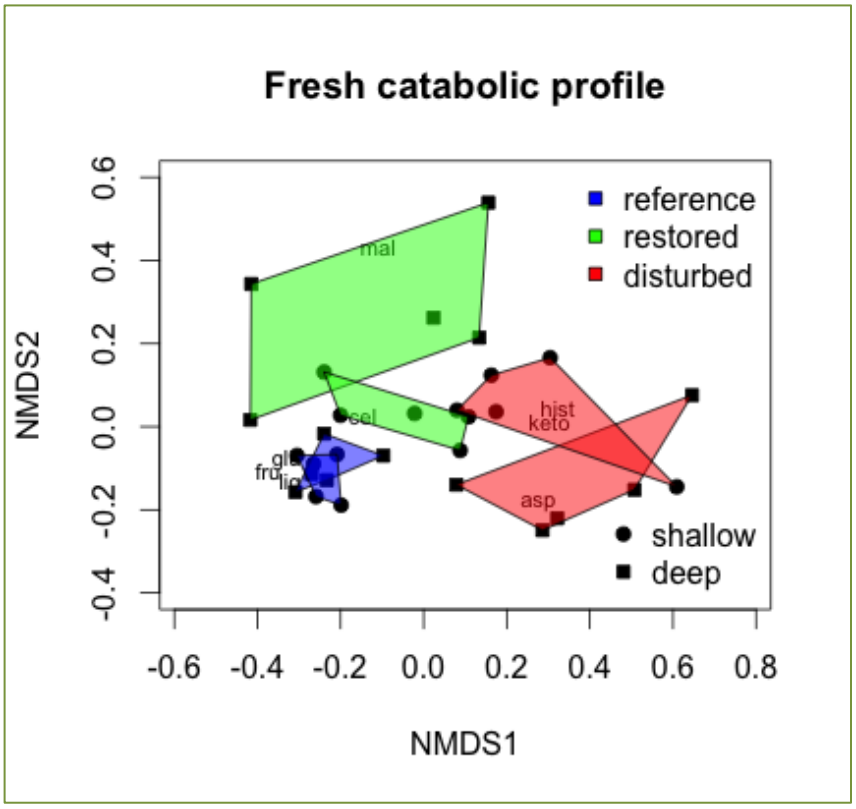


Figure 9: Polygons represent a set of shallow cores (circle points) or deep cores (square points) per treatment. Treatment and depth have significant effects (p=0.001, p=0.003). The shallow Restored layer was closer to the Reference site than the deep Restored layer, which indicated a partial return to reference-level substrate utilization after restoration.

DISCUSSION

Mangan saltmarsh sites

Microbial function was altered by land management treatment within the Mangan saltmarsh sites. Land management treatment had a profound effect on wetland function including bulk density and carbon stock properties, CO_2 and CH_4 production, enzyme activity, and the microbial catabolic profile. The carbon stock calculations illustrate the complex events following disturbance and restoration treatment.

Carbon stocks over the entire 30 cm profile were similar between the Restored and Reference sites, and slightly greater in the shallow disturbed (Figure 2b). The Disturbed site had a thick A horizon, reflecting the dense freshwater vegetation. Although the data reflect a higher carbon stock in the disturbed site, this comparison only takes into account the top 30 cm of the soil profile. The Reference site contained organic soil throughout the top 30 cm, and extended to an unknown extent deeper into the soil profile. The amount of carbon in the Disturbed site decreased even after 15 cm (Figure 2b). The depth of the organic layer in the reference site must be known to gain an overall picture of the amount of stored carbon in comparison to the restored and degraded site. The restored site is split into two distinctly different layers: the surface layer of accreted sediment from the tide, and the compact, mineral layer from the previously diked conditions. Although it may appear that disturbance increases carbon stocks, this site actually lost a substantial amount of carbon to subsidence, while the restored site has accumulated carbon. The combined carbon carbon stocks from Restored

deep mineral and accumulated shallow layer were 50.6 mg C cm^{-3}, and the carbon stocks from the shallow Disturbed layer were 44.5 mg C cm^{-3}. The carbon in the Restored surface layer was supplied by the tidal flow. The Restored marsh is not yet high enough to support the plant community of the high, reference marsh. Consequently, there is no accumulation of autochthonous, or in-site produced, organic matter. The Restored site may have also lost carbon, through decomposition or erosion, in its surface A horizon after the restoration event, assuming that it was similar to the current Disturbed site. While the Disturbed site almost certainly has a high net primary productivity and continues to add some organic matter from cow manure and grass roots, the deep core from the Restored marsh was inundated, and covered with an increasing amount of tidal sediment. Carbon stocks are calculated using soil bulk density and percent carbon. Soil carbon stocks reflect the actual amount of carbon present, and increase with increasing soil bulk density and percent carbon. The Disturbed site had a much higher bulk density, and consequently higher carbon stocks in sampled soil layer.

This treatment effect across the three sites is reflected in the CO_2 and CH_4 production. Restoration has not yet returned soil respiration production to reference-level activity (Figures 3a and 3b). The CH_4 production rates were extremely low across all Mangan sites. The Reference and Restored sites are saline, and reflect microbial communities that may be dominated by sulfate-reducing bacteria instead of methanogens (Poffenbarger et al. 2011). Microbial activity in the Disturbed site may have been seasonally limited by moisture. These samples were taken in the summer and the Disturbed site was very dry relative to the

Reference and Restored site. I expect production rates to increase in the Disturbed site after the winter rains transform the site into a freshwater, anaerobic environment. It is imperative to capture the seasonality and measure gas production rates during the winter rains and the spring post-wetting period.

The Mangan saltmarsh microbial function was partially restored by restoration. Enzyme activity reflected only a partial return to reference-level activity with restoration (Figure 4), but the catabolic profile indicates a similar substrate utilization between the Reference and Restored site (Figure 5). Enzymes β glucosidase and cellobiosidase activity in the Reference site were significantly different from activity in the Restored and Disturbed site across depth (Figures 10a and 10c supplementary section). This trend is very similar to the other enzymes and significantly so in the surface layer. α glucosidase and β glucosidase are isomers. The α isomer is much less prevalent than the β isomer due to structural favorability. This difference could explain the more homogenous expression across treatment in comparison to the β glucosidase activity. Reference peroxidase activity was also significantly different from activity in the Restored and Disturbed site (Figure 10e supplementary section). The other oxidative enzyme, phenol oxidase, had very low activity with high variation across depth and treatment (Figure 10f supplementary section). These collective enzyme activities indicate that the restoration treatment was insufficient to return enzyme activity to reference level. Although enzymes persist in soil environments after they are excreted by microorganisms, the amount of time since restoration may not have affected accounted for a difference between treatment.

The catabolic profile indicated a restoration of substrate utilization in the Mangan site. The catabolic profile indicates the types of soil microorganisms present at a given point in time because it quantifies the types of carbon substrate use. The production of CO_2 with added carbon substrates indicates groups of microbes actively breaking down and metabolizing substrates. Substrate response was more similar across treatment in shallow cores than deep cores in the Mangan sites (Figure 5). The shallow depth from the Mangan sites indicated a return to reference-level substrate response with glucose and fructose and malic acid. Substrate response was similar across treatment for the other six substrates (Figure 11a supplementary section). The deep profile illustrated a significant difference between the Reference and Disturbed site across all substrates, indicating a severe loss of microbial function with diking disturbance (Figure 11b supplementary section). The deep Restored site also showed an intermediate increase in response with the addition simple sugars, complex carbon molecules and carboxylic acids, indicating a general restoration of the microbial community (Figure 11b supplementary section).

The functional ability to utilize simple sugars and complex carbon substrates was restored with the re-introduction of the tide. However, the enzyme activity has been only partially restored. This temporal discrepancy may reflect the fact that the Restored site has not yet accreted enough sediment to reflect the Restored high marsh habitat type. With time, both habitat type and enzyme microbial function in the shallow Restored profile may be fully restored. However, the time needed to restore habitat type and increase the carbon stocks to reference

level is limited by the sediment accretion rate. The microbial function measurements captured a snapshot in time, while the measure of carbon stocks represented the replete carbon since the restoration. The Mangan carbon accumulation rate is 1.8 mg C cm^3 yr^{-1}, and it may take many more years for reference-level carbon stocks to accumulate in the Restored site.

Freshwater sites

The freshwater restored site reflected restoration of microbial function, and a partial restoration of bulk density and carbon stocks in comparison to the Reference site. The bulk density was partially returned to reference-level with restoration (Figure 6a), and indicates subsidence and compaction in the prior disturbed condition. Grazing has been shown to compact soil (Portnoy and Giblin 1987). After the Restored site was originally ditched and drained, it was used as grazing land for many decades before abandonment. The carbon stocks indicated a partial return to reference level with restoration (Figure 6b). The Disturbed marsh contained soil high in carbon stocks, primarily due to the high bulk density. The high carbon stocks in the Disturbed site were most likely due to the input of organic matter from grass vegetation. Although it may appear that disturbance increases carbon stocks, this site actually lost a substantial amount of carbon, most likely to subsidence, while the Restored site has accumulated carbon at an estimated rate of 2.1 mg C cm^{-3} yr^{-1} since the restoration event. The carbon stocks from the Restored deep mineral and accumulated shallow layer were 51.4 mg C cm^{-3}, and the carbon stocks from the Disturbed shallow mineral layer were 42.5 mg C cm^{-3}.

The Restored site was split between two layers: a surface layer consisting of accumulated allochthonous and autochthonous organic matter, and a deep layer that existed prior to restoration. During restoration, the Restored marsh was physically altered to reflect historic tidal channels and elevation. A layer of soil was scraped from the mineral surface layer. This deeper layer was missing a significant part of the relatively carbon-rich surface layer reflected in the Disturbed marsh. Carbon stocks illustrated a significant difference between the Restored shallow and deep profile (Figure 6b). While the shallow profile indicated similar carbon stocks between the Restored and Reference site, the deep layer is significantly different. Although the shallow profile in both the Reference and Restored sites was similar, the Reference site organic layer extended past the total 30 cm sampled. The Restored site contained 8 to 15 cm of sediment in the shallow accreted layer. It may take a significant amount of time for the total reference level carbon stocks to accumulate in the Restored site.

This structure is reflected across the different cores, where the shallow core indicated a return to reference level microbial function, and the deep core has not yet returned to reference level. The Restored site reflected the Reference hydrology and habitat type, and this restoration treatment seems to have catalyzed a rapid return of soil carbon functionality. Although active restoration may have scraped a layer of soil from the Restored site, the accreted shallow layer demonstrated reference-level microbial function across gas fluxes, enzyme activity, and substrate utilization.

Disturbance significantly decreased both CH_4 and CO_2 production rates. Restoration returned CO_2 production rates to reference-level across depth, while CH_4 production rates increased to an intermediary level between disturbance and reference-level gas flux rates (figures 8 and 9). CH_4 production was low across all treatments. Anaerobic, freshwater soil environments have been found to produce a significant portion of CH_4 in the global cycle (Bridgham et al. 2013). These sites may contain notable amounts of sulfate because of their proximity to marine sources. The disturbed site was ditched and drained, and the microbial activity may have been limited by moisture. Although the Restored and Reference sites were anaerobic, they expressed much higher activities than the moisture-limited Disturbed site. In order to fully capture the relative effects of disturbance and restoration on microbial function, gas flux rates must be measured after the Disturbed site has been wetted by the winter rains.

Enzyme activity was returned to reference level with restoration. Disturbance had a profound effect on microbial function: enzyme activity was significantly lower than Reference enzyme activity (Figure 8). Restoration increased activity level across all hydrolytic enzymes and oxidative enzymes in the shallow profile (figures 12a-12f supplementary section). The Restored deep profile also reflected an increase in enzyme activity from disturbance-level, but was still significantly lower than reference-level activity. This pattern is demonstrated across all enzymes with the exception of peroxidase (figure 12e), and demonstrates the restoration of the microbial ability to degrade plant materials

such as cellulose, hemicellulose, simple sugars, and phenolic compounds such as lignin.

The catabolic profile illustrated a partial return of substrate utilization to reference level. There was a significant difference in response between the Restored depth profiles. The Restored shallow layer is more similar to the Reference site than the deep layer (Figure 9). The shallow layer demonstrated a significant decrease in microbial catabolic response with disturbance (Figure 13a). Restoration returned catabolic response to, and even increased beyond reference-level across all substrates in the Restored shallow profile (Figure 13a). The deep layer demonstrated a significant decrease in catabolic response with disturbance, and a subsequent increase in catabolic response after restoration across the simple sugars, complex carbon molecules, and malic acid (Figure 13b). Overall, restoration catalyzed a shift in catabolic response to reference levels in the shallow layer more so than the deep layer (Figure 9), indicating partial restoration of microbial function.

Anderson Creek, the freshwater Restored site, was restored in 2002, four years before the Mangan Restored site. The historical tidal channels were re-opened to restore hydrology, sediment was scraped to restore the high water table, and native plant species were re-introduced. This active restoration regime in the freshwater Restored site may have made all the difference in speeding the return to reference-level microbial function. The shallow layer in the freshwater Restored site has effectively been reverted to reference-like microbial function. The restoration of both enzyme activity and the majority of substrate utilization

indicated similar microbial communities between the Restored and Reference site at that period in time. These microbial functions were mirrored by the CO_2 and CH_4 production rates. The deep Restored layer in the freshwater complex also reflected some movement away from disturbed-level and toward reference-level microbial function. Although the mineral soil was initially scraped away, the fourteen years since the restoration event re-instated an intermediate level of microbial function. The type of restoration completed in this freshwater complex may be a key component to the speeding of recovery for the deep Restored layer. Opening historic tidal channels and actively introducing the reference-like plant community seems to have sped the recovery of the denser deep layer.

Implications for future management

The Mangan saline complex and freshwater complex case studies involved disturbance events in the early 20th century, and restoration of part of the disturbed marsh in the early 2000's. However, the two case studies are characterized by two key differences: they are different ecosystem types, and experienced different restoration techniques. The active restoration in the freshwater Restored site established hydrology and plant community that reflected the Reference site. This macro change may have sped the microbial function recovery. The tidal flow was reestablished in the Mangan Restored site. Sediment accreted with the incoming tide over the course of ten years, building a layer over the previously disturbed soil surface layer. The restored site is a low marsh and unable to support the vegetation present in the Mangan Reference site. Return to the reference-like habitat is a continual process with daily sediment

accretion. Microbial function also seemed to reflect the recovering habitat structure. Enzyme activity was restored, indicating a difference in the microbial ability to degrade carbon compounds. The Restored site lacks vegetation, and the resulting input of plant matter. Consequently, the carbon content of the shallow layer was significantly lower than the restored layer. This difference in carbon input may have altered the structure of microbial enzyme secretion. It is worthy to note the drastic shift in salinity during the Mangan restoration event. The previously diked site was cut off from the tide, and essentially freshwater. Upon restoration, the soil environment was exposed to saline conditions. The freshwater Restored site underwent considerable structural change, but this change retained a fresh gradient.

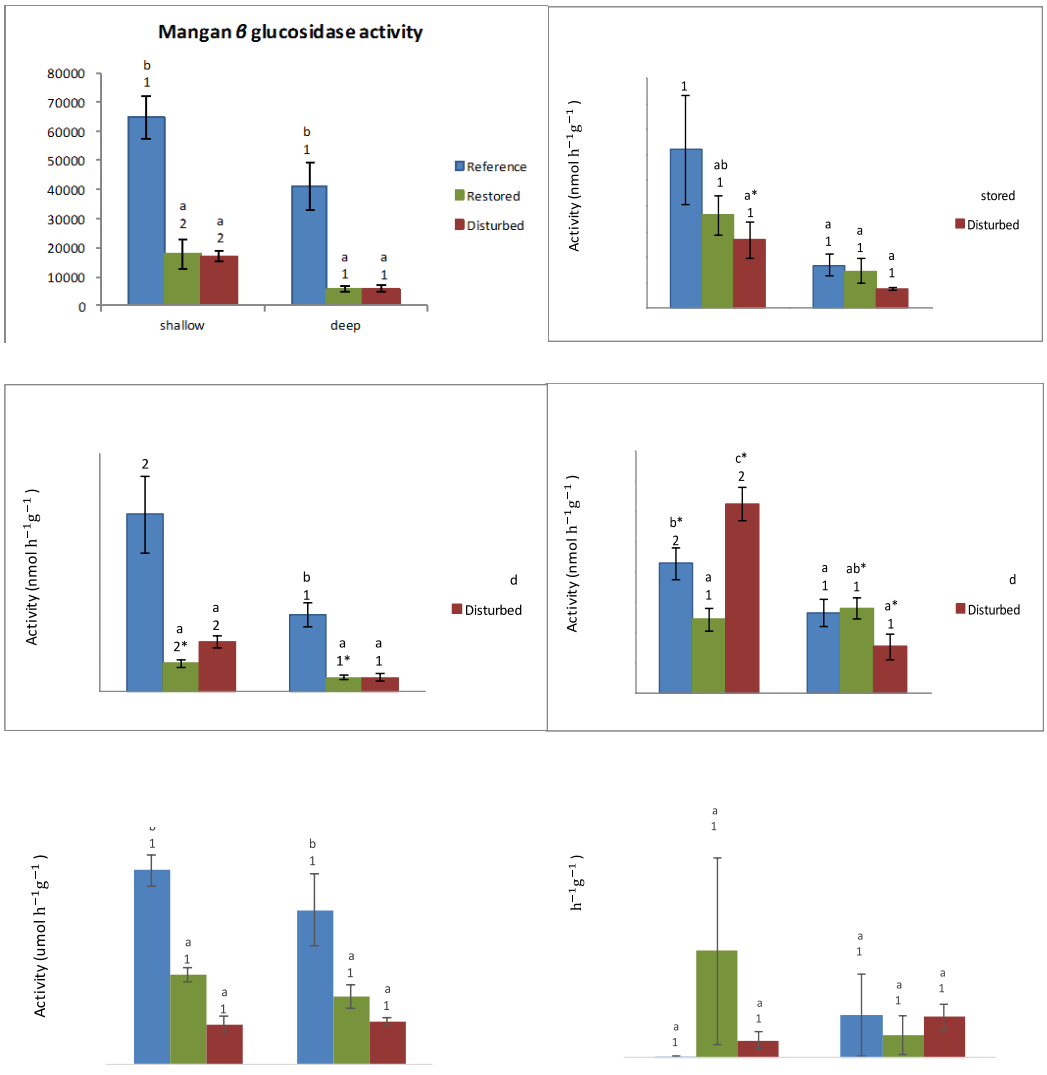
Land management had significant effects on both tidal and freshwater wetland ecosystems. Carbon cycling is a wetland ecosystem function which directly depends on the microbial community function. This study indicated shifts in gas fluxes, loss of organic matter, and shifts in both enzyme activity and the microbial community composition after a disturbance event. The results also illustrate key differences in restoration techniques and the speed of recovery. Active restoration in the freshwater marsh catalyzed the return of reference-like habitat. This similarity between the Reference and Restored freshwater site was reflected across gas fluxes, and in general, across the microbial metrics. The Mangan Restored site experienced restoration in which the accreted sediment is not able to support the vegetation of the Reference site. Gas fluxes and in part the microbial function reflect this habitat distinction. This site may take several more

years to fully support the necessary vegetation, and return microbial function to reference-level.

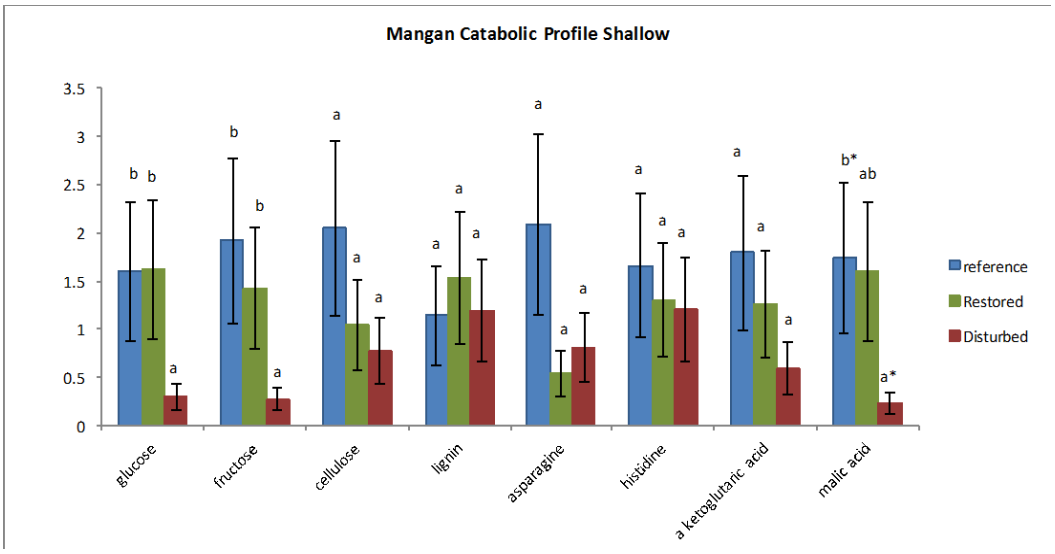
This study is in the process of capturing the seasonality of these wetland sites. In order to fully characterize the effect of land management on microbial function, the microbial parameters and gas fluxes will be measured during the winter and later spring seasons.

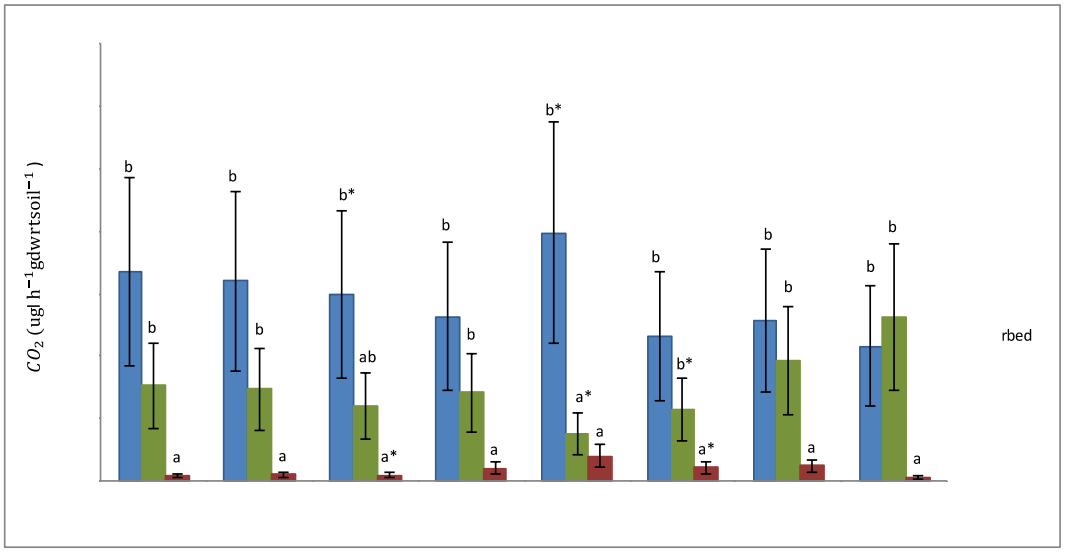
Research in microbial function is necessary for an in-depth analysis of wetland restoration. The compensatory mitigation regulations of wetlands include the restoration, establishment, enhancement, and in some circumstances, preservation of wetland habitats (Carter 1977). Under section 404 of the Clean Water Act, agencies must maintain natural wetland systems, including habitat diversity and stability. Natural wetland systems have ecosystem functions which are critical to overall habitat stability. Wetland soil carbon cycling is an ecosystem function which plays a critical role in carbon sequestration. Measurement of microbial function is an effective way to fully characterize the effects of restoration on wetland carbon cycling. These methods allow an indication of specific function trajectories, and quantify the level in which restoration has returned function to reference level.

SUPPLEMENTARY FIGURES

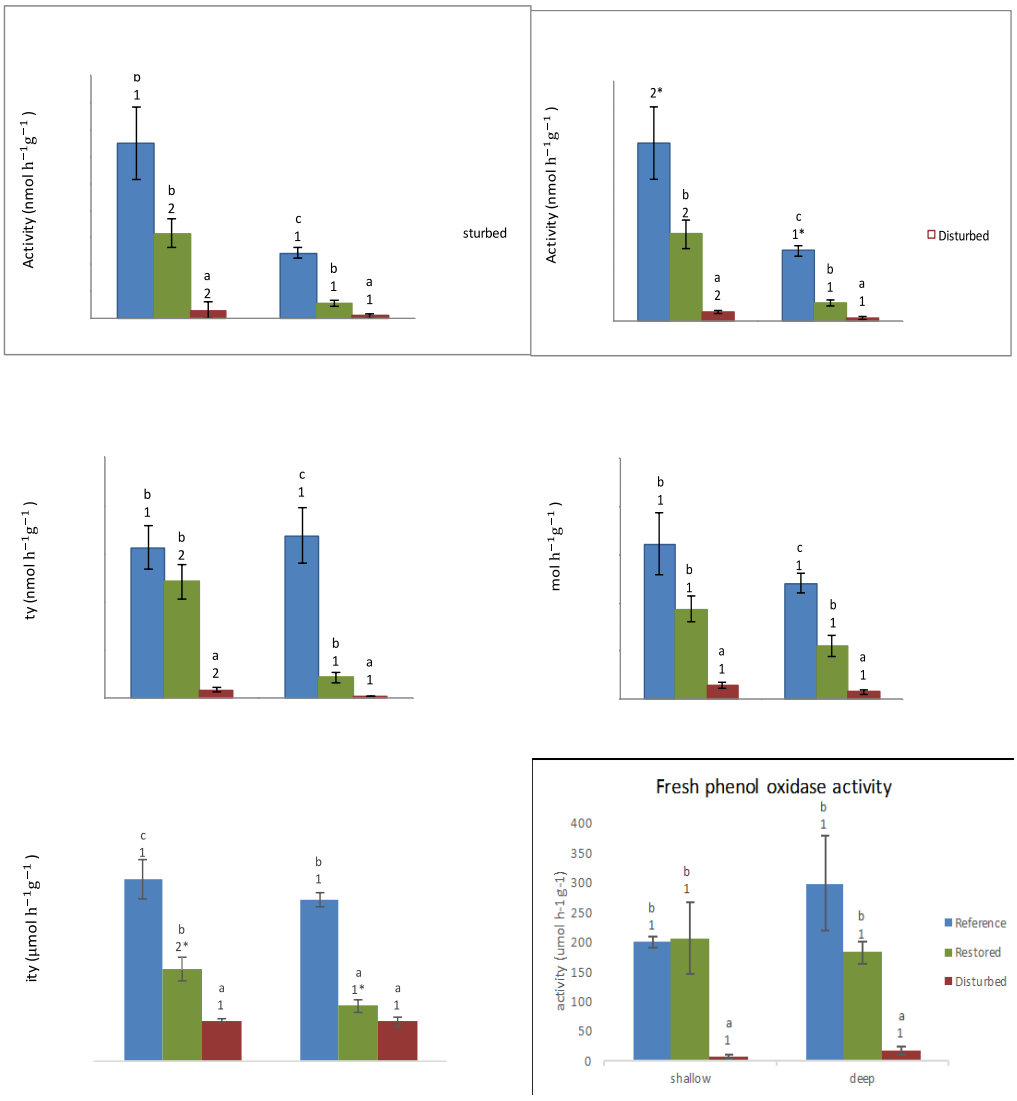


Figures 10a *β* glucosidase, 10b *α* glucosidase, 10c cellobiosidase, 10d xylosidase, 10e peroxidase, 10f phenol oxidase: Treatments are compared within each depth. Letters indicate significance. Depth is compared within each of the three treatments. Numbers indicate significance.

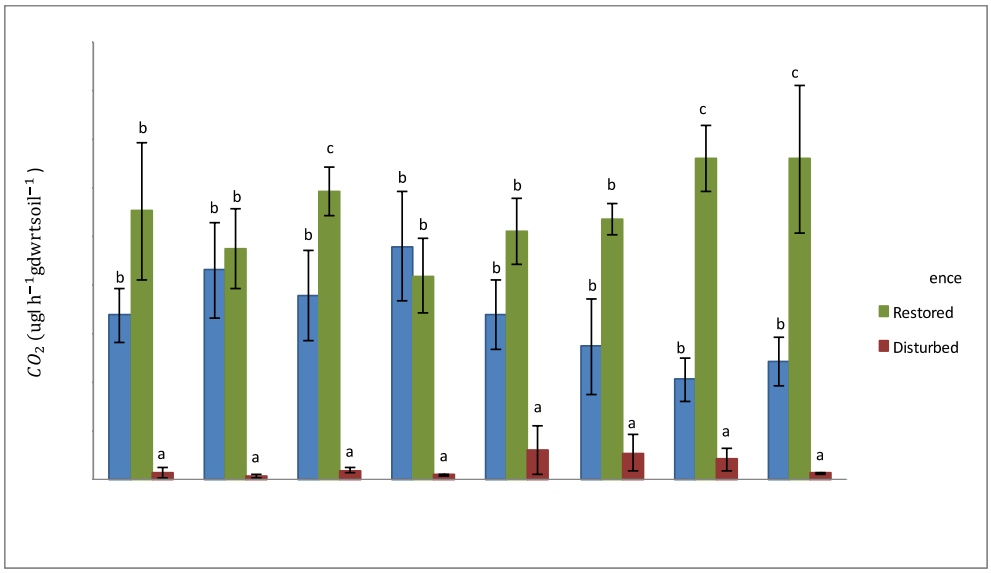




Figures 11a Shallow, 11b Deep: Treatment is compared within each substrate. Letters indicate significance between treatments, where a represents the lowest value. A pair of letters with asterisks indicates a moderately significant difference.



Figures 12a *β* glucosidase, 12b *α* glucosidase, 12c cellobiosidase, 12d xylosidase, 12e peroxidase, 12f phenol oxidase: Treatment is compared within each depth. Depths are compared between treatments. Different letters denote treatment differences within each depth, where a represents the lowest value.



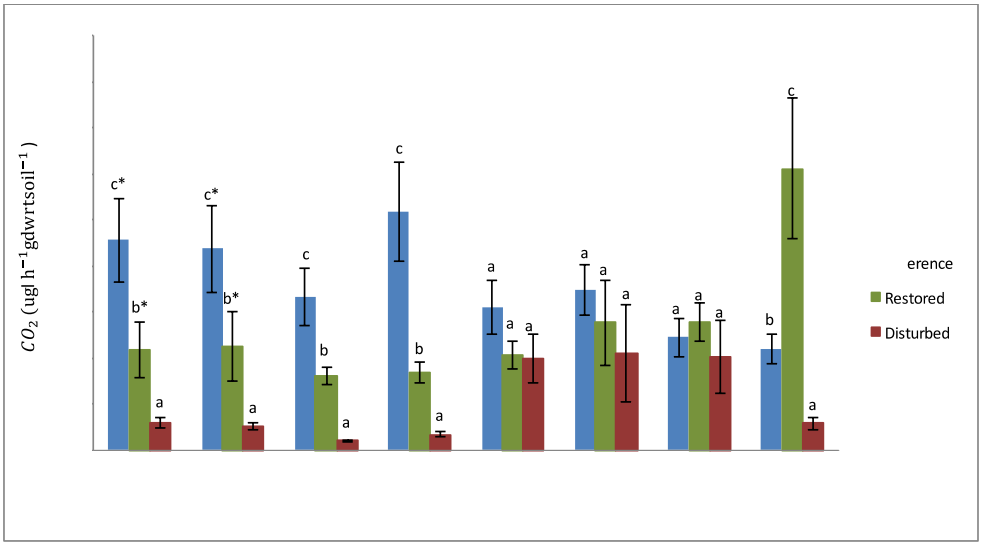


Figure 13a Shallow, 13b Deep: Treatment is compared within each substrate. Letters indicate significance between treatments, where a represents the lowest value. A pair of letters with asterisks indicates a moderately significant difference.

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