ABSTRACT

Title of Dissertation: Soil microbial processes and community structure in natural and restored tidal freshwater wetlands of the Chesapeake Bay, Maryland, USA

Christine E. Maietta, Doctor of Philosophy, 2017

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Tidal freshwater wetlands are integral to downstream water quality because they capture, store, and transform nutrients. Unfortunately, anthropogenic stressors are negatively impacting these habitats. While wetland restoration is helping to reinstate their presence in the landscape, restored wetlands frequently differ physically, chemically, and biologically from their natural counterparts. This research examined plant, soil, and microbe relationships and how their interactions affect soil carbon (C) storage and cycling in natural and restored tidal freshwater wetlands of the Chesapeake Bay, MD, USA.

This research yielded important findings regarding differences between natural and restored habitats. First, we discovered soil microbial community composition of an urban tidal freshwater wetland retained similar composition as their less disturbed, suburban counterpart, and wetland sites constructed using similar restoration methodology produced similar microbial community structure and soil function. Additional research revealed that a natural and a restored wetland store soil C quite differently: A majority of soil C in the natural site was associated with large macroaggregates ($\geq$ 2000 μm) whereas most soil C in the restored site was associated

with smaller macroaggregates (≥ 250 to < 2000 μm). The distributions of six chemical compound classes (i.e., carboxylics, cyclics, aliphatics, lignin derivatives, carbohydrates derivatives, N-containing compounds) were relatively similar across the five soil fractions from both sites, however. In the final study, anaerobic laboratory mesocosms were used to evaluate the effects of clay content (%) and leaf litter quality on soil C cycling processes over time. This study found restored soils, regardless of clay content, mineralized more C as carbon dioxide (CO_2) and methane (CH_4) compared to natural wetland soils. Natural soils respired approximately half the volume of gas as restored soils, suggesting the addition of high- or low-quality C substrates to low C systems elicit a greater response from the heterotrophic microbial community.

The results of these three studies suggest site history and edaphic features of restored wetlands are important drivers of microbial communities and their function. We propose that practitioners and researchers work together to identify practices that will enhance soil functions, particularly C storage, in tidal freshwater wetlands of the Chesapeake Bay region.

SOIL MICROBIAL PROCESSES AND COMMUNITY STRUCTURE IN NATURAL AND RESTORED TIDAL FRESHWATER WETLANDS OF THE CHESAPEAKE BAY, MARYLAND, USA

By

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Dedication

I dedicate this dissertation to my parents without whom none of my success would be possible. I would also like to dedicate this dissertation to my husband. Thank you for your unwavering support and unending love.

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Chapter 1: Soil carbon cycling in tidal freshwater wetlands: A review of processes and controls

Abstract

Situated in the upper, freshwater portion of the estuary, tidal freshwater wetlands form a nexus between terrestrial and aquatic habitats. These diverse habitats support a variety of life and ecosystem functions. For example, tidal freshwater wetlands aid in protecting downstream water quality by capturing, storing, and transforming excess nutrients. Tidal freshwater wetlands also sequester and store large quantities of carbon (C) in their soils. Interest in understanding the characteristics and functions of tidal freshwater wetlands dates back to the early 1950s, yet there is still so much more to figure out about these habitats. For example, we know little about the national and international status and trends of tidal freshwater wetlands, the plants-soil-microbes relationships, and how microbial community composition relates to function at multiple spatiotemporal scales.

Because of their location in the landscape, urbanization, nutrient pollution, and salinization are particular impactful on the physical, chemical, and functional properties of tidal freshwater wetlands. Because these habitats are vital in protecting downstream water quality, efforts to restore tidal freshwater wetlands are widespread along the east coast of the United States. However, it is unknown how disturbance and restoration influence nutrient cycling, greenhouse gas emissions, and long-term storage of soil C. This review summarizes the current knowledge of three major drivers – wetland vegetation, soil physical and chemical properties, and soil microbial communities – on

soil C storage and cycling in tidal freshwater wetlands. Furthermore, this review summarizes our current understanding of the impacts of disturbance and wetland restoration on soil functions and potential new avenues of research.

Introduction

Carbon (C) is an essential element of life and an important factor affecting Earth's global temperature and climate. Carbon occurs in many different forms, including both organic (e.g., carbohydrates, proteins, nucleic acids, lipids, etc.) and inorganic (e.g., carbonates, carbon dioxide, methane, etc.) species. These species move among three major reservoirs - the atmosphere, the terrestrial biosphere, and the oceans - through a series of geological (i.e., uplift, erosion, weathering, etc.) and biological (i.e., photosynthesis and respiration) processes (**Figure 1**). Before the industrial revolution, global C cycling was relatively balanced; however, since the industrial revolution, fossil fuel burning, land conversion, and soil erosion have significantly altered exchange rates and increased flux rates of greenhouse gases to the atmosphere (1–4). Two gaseous species, carbon dioxide (CO_2) and methane (CH_4), play a significant role in global climate change. Both CO_2 and CH_4 absorb infrared radiation and increase global atmospheric temperatures. Methane is an especially harmful greenhouse gas because its global warming potential (GWP) is about 30X more effective at trapping and storing heat than CO_2 (5, 6). Efforts to mitigate global climate change are focused on improving C sequestration, preserving soil C pools, and reducing greenhouse gas emission.

Tidal freshwater wetlands are an important reservoir and transformer of C in the terrestrial biosphere. Tidal freshwater wetlands are found at the interphase between aquatic and terrestrial habitats. These marshes have one of the most diverse and

productive wetland plant communities. In fact, tidal freshwater marshes are estimated to have some of the highest C sequestration rates (~140 g C m^{-2} y^{-1}) (7). While some of the fixed C is exported into the adjacent river habitat, a majority of the C is preserved in the soil as soil organic matter (SOM). Soil organic matter makes up a relatively high fraction of the soil mass in tidal freshwater wetlands (~20-70%) although these pools can be quite variable within and between sites (8). Soil organic matter is essential to site productivity because it supplies nutrient for the overlying plant community. It also fuels microbially mediated processes like denitrification and methanogenesis. Methanogenesis, or the production of CH_4, is prevalent in anoxic soils of tidal freshwater wetlands because C availability is high, soils are reduced, and pore water sulfate (SO_4^{-2}) concentrations are low (< 1 ppm). However, investigators have reported relatively low CH_4 emissions (~32 ± 37 g CH_4 m^{-2} yr^{-1}) compared to other freshwater wetland habitats (92 to 237 Tg CH_4 yr^{-1}) (9–11).

Tidal freshwater wetlands are vulnerable to urbanization and anthropogenic stressors. For example, historical development in the Washington, D.C. metropolitan region has significantly reduced tidal freshwater wetland coverage along the Anacostia River by 96% (12). This kind of disturbance is not limited to the Washington, D.C. region. Poor soil management, river engineering, and urbanization are impacting the coverage of tidal freshwater wetlands in many other parts of the United States today (13, 14). Tidal freshwater wetlands are sensitive to changes in the surrounding watershed because sediment and freshwater supply are master variables maintaining the marsh's physical, chemical, and biological properties. Alterations in either of these two master

variables can ultimately affect the marsh's function, including C sequestration, C storage, and C processing by the soil microbial community.

Given that tidal freshwater wetlands serve an important role in protecting downstream water quality, efforts to restore these habitats are increasing; however, wetland restoration practices are imperfect. Research has shown that plant coverage and community composition can be quite different between restored and natural wetlands (15). Researchers have also documented restored wetlands store less SOM compared to their natural counterparts (16–18) and discharge larger quantities of potent greenhouse gases (19). There is little known about how wetland restoration affects fundamental soil properties in tidal freshwater wetlands, and even less is known about the long-term impacts of restoration on soil C storage and respiration.

The overall goal of this review is to summarize the current literature on tidal freshwater wetlands, highlight gaps, and propose new avenues of research. The review begins with a historical perspective and then discusses some common characteristics and current hot topics in tidal freshwater wetland research. The second section follows up with a more detailed discussion about three major variables – the plant community, the soil habitat, and the soil microbial community – and their role in soil C cycling. And lastly, the review finishes with a summary of the benefits and drawbacks of wetland restoration on soil function.

Part 1: Historical Context and Distribution of Tidal Freshwater Wetlands

The first series of papers concerning tidal freshwater wetlands were published in the early 1950s. These studies primarily focused on describing the diverse waterfowl population (20–22). Beginning in the 1970s through the 1980s, investigators extended

their research to characterize marsh ecology and nutrient cycling (23–26). More recent publications (the 1990s to present) are examining the various drivers as well as the effects of disturbance on the function and resilience of these habitats. These studies include but are not limited to hydrology (27–29), sedimentation patterns (30–32), plant-soil function relationships (33, 34), and variation in biogeochemical cycling (35–38). Although there are currently over 450 publications on tidal freshwater wetlands (Web of Science search using the terms "tidal freshwater wetland*" and "freshwater tidal wetland*"), there lacks a general understanding of the status and trends of natural tidal freshwater wetlands at the national and global scale.

The first national inventory of tidal freshwater wetlands was published in 1984. In this paper, Odum (26) estimated a total of 164,000 ha along the east coast of the United States. The 1984 inventory remains the most comprehensive record of tidal freshwater wetlands in the United States because national surveys do not separately catalog tidal and non-tidal freshwater wetland habitats. Furthermore, many studies are conducted in previously established study sites along the east coast (e.g., Sweet Hall Marsh, Jug Bay Wetland Sanctuary, Tinicum Marsh, etc.). New case studies of these marshes, like Elsey-Quirk (13) and Wilson et al. (39), showcase development is impacting their structure and function. The national wetlands inventory should consider documenting the coverage of tidal freshwater wetlands and monitor the loss and conversion rate of these habitats.

At the international scale, tidal freshwater wetlands are even less well characterized. In fact, only a handful of international studies were identified while reviewing the current literature (28, 40–43). A majority of the identified investigations were conducted in Tielrode Marsh, a 100,000 m^2 marsh located along the Scheldt and

Durme Rivers in Belgium. It is likely that many more marshes exist. In fact, Barendregt et al. (44) evaluated river discharge and sediment data and predicted possible locations along 29 rivers in both the Northern and Southern hemisphere. Language barriers and different wetland classification systems limit my assessment of tidal freshwater wetlands at the global scale.

Part 2. General Characteristics and Natural Variability of Tidal Freshwater Wetlands

Tidal freshwater wetlands occur along the floodplains in the upper reaches of estuaries where sediment and freshwater inputs support soil formation and freshwater wetland plant communities. High sedimentation rates of riverine and terrestrial derived clay, silt, and organic matter form mud flats along the river-terrestrial interface (45). Once vegetation colonizes these mud flats, soil formation accelerates. Plants are essential to soil formation because they slow tidal floodwater velocity, which increases sedimentation rates and enhances vertical accretion (31, 46). Marsh hydrology is primarily fed by tidal river water, but precipitation, surface overflow, and groundwater discharge are also an important component of the marsh's water budget (47). Salinity values typically range between 0.0 and 0.5 ppt; however, it is not uncommon for these values to reach oligohaline levels (0.5 – 5.0 ppt) during low flow conditions or extreme weather events (48). Most tidal freshwater wetlands experience two tidal cycles each day although there are also exceptions to this rule too. For example, marshes along the Newport River in eastern North Carolina experience a diurnal tide (32). Sedimentation rates, tidal water chemistry, and tidal floodwater amplitude, duration, and frequency are

influenced by the marsh's location along the riverine continuum, the river basin's characteristics, and connectivity to the surrounding watershed.

Plant composition and phenology have been the focus of studies for many years. A detailed account of plant community composition will not be addressed in this review because they have been covered extensively in these case studies and reviews (24, 26, 49–52). Briefly, plant communities in tidal freshwater wetlands are both diverse and spatiotemporally variable. Unlike tidal saline marshes, zonation is not pronounced. Instead, the composition of the plant community gradually shifts along the interior elevation gradient (8, 50). During the growing season, exposed mudflats along the river, stream edges, and low-lying areas are frequently colonized by submerged aquatic vegetation (SAV) and emergent vegetation like arrow arum (*Peltandra virginica* (L.) and spatterdock (*Nuphar lutea* (L.) Sm.). The high marsh region is commonly colonized by a dozen or more plant species. Some common species include broadleaf cattail (*Typha latifolia*), wild rice (*Zizania aquatic*), and common reed (*Phragmites australis*) (8, 53). However, inter- and intra-variability in plant community structure can vary quite dramatically from one marsh to the next (8, 49). A mix of herbaceous plants and flood-tolerant woody shrubs and trees (e.g., bald cypress (*Taxodium distichum*), gums (*Nyssa sylvantica* var. *biflora* and *N. aquatica*), Atlantic white cedar (*Chaemaecyparis thyoides*), etc.) often occupy the marsh-upland fringe area.

Tidal freshwater wetland habitats support a range of biota. The most common zooplankton, macrofauna, and invertebrate observed species are described in Odum (26) and Perry et al. (53). Zooplankton, benthic macrofauna, and invertebrates are abundant, but their biodiversity is considerably low. Amphibian and reptile biodiversity is rich.

Frogs, salamanders, snakes, and turtles are just some of the most common organisms found in these habitats (53, 54). Tidal freshwater wetlands are also important spawning grounds for freshwater, oligohaline, and anadromous fish (8). Birds populations of tidal freshwater wetlands are one the largest and most diverse of any wetland type (8, 20, 21). For example, bird surveys conducted at the Jug Bay Wetland Sanctuary and the John Heinz National Wildlife Refuge at Tinicum documented over 300 bird species (55). Only a small portion of these bird population use tidal freshwater wetlands as their permanent refuge; instead, these are temporary habitats during breeding and migration seasons (54). In addition to supporting a diverse bird population, tidal freshwater wetlands provide refuge for many other organisms such as otters, muskrats, nutria, raccoons, and deer.

Because these habitats are situated at the aquatic-terrestrial interface, tidal freshwater wetlands act as a filter for many different types of pollutants. Trash is a major pollutant in urban marshes. There are currently no scientific studies evaluating the quantity of trash captured by these habitats; however, Anacostia Watershed Society removed 1000+ tons of trash during cleanup efforts in 2013-2014 (56). Nitrogen is also a major pollutant in these habitats. Nitrogen pollution has more than doubled in our nation's waterways since the invention of fertilizer. Nitrogen is an especially devastating pollutant because it leads to eutrophic conditions and weedy plant species (57, 58, 34). Denitrification stops the cascading effects of N through aquatic ecosystems by reducing mobile nitrate (NO_3^-) into dinitrogen (N_2) gas. Denitrification is one of the most widely studied biogeochemical cycles in tidal freshwater wetlands. A few thorough reviews and case studies are available here: Bowden et al. (59) and (60), Elsey-Quirk (13), Findlay and Fisher (61), Greene (62), Hopfensperger (63), and Megonigal et al. (10).

Soil C cycling emerges from the interactions of many complex factors in tidal freshwater wetlands. The next section attempts to separately analyze the effects of three major variables – vegetation, soils, and microbes – on soil C cycling in these habitats.

Part 3: Major Mechanisms Shaping Soil C Cycling in Tidal Freshwater Wetlands

3a. Wetland Vegetation – C cycling and modifications

Plants are fundamental to wetland soil development and soil C processes; a brief overview is presented in **Figure 2**. This section discusses the effects of vegetation and provides specific examples of how plants impact soil function in tidal freshwater wetland habitats.

The mere presence of plants affects many aspects of soil C cycling in tidal freshwater wetlands. First, plants are essential to soil formation. Wetland plants enhance marsh formation by trapping sediment and stabilizing soils. In fact, studies have shown sedimentation rates are greatest during the growing season and affected by plant community characteristics like plant density and height (30, 31, 64, 65). These sediments are not only essential to marsh formation, they also contribute to the soil C pool and fuel soil microbial activity (10, 64, 66). Second, plant-mediated photosynthesis is the primary mechanism sequestering atmospheric CO_2. In a C gas flux study conducted by Neubauer and colleagues (36), *in-situ* plant photosynthesis accounted for more than 90% of the assimilated CO_2; sediment microalgae fixed the remaining portion of assimilated organic matter. Some have proposed chemosynthetic C production may fix a small portion of C, but the amount and role of this pathway have not been investigated in tidal freshwater wetlands (66). And lastly, plant-derived organic matter also accelerates marsh accretion (46) and serves as the primary source of organic C entering the SOM pool.

The plant's root system also creates a dynamic soil environment that influences soil C in tidal freshwater wetlands (**Figure 2**). Plant roots secrete a variety of C-based substances, which includes plant-derived secondary metabolites, C-rich photosynthates, mucilage, and decaying root cells (67, 68). These C-rich compounds increase soil microbial growth and heterotrophic decomposition. For example, Ström and colleagues (69) correlated higher concentrations of acetate in the root vicinity of *Eriophorum scheuchzeri* with higher production of CH_4. Roots also release hydrogen (H^+) and bicarbonate (HCO_3^-) ions to aid with nutrient uptake from the adjacent soil environment. The quantity of H^+ and HCO_3^- ions leaked near the root surface effectively creates a soil pH gradient between the surface of the plant root structure, the rhizoplane, and the bulk soil (70, 71). Because soil pH is a major driver of soil microbial composition (72, 73), small shifts in soil pH between the rhizosphere and bulk soils may affect small-scale soil C processing and heterotrophic decomposition.

Due to the soil saturation in wetland habitats, dissolved oxygen (O_2) concentrations are often reduced and cannot support aerobic respiration in plant root cells. Roots can carry out fermentation, but long-term fermentation will lead to cellular acidosis and eventual cell death. To overcome this challenge, most emergent wetland plant species are composed of aerenchyma tissue. Aerenchyma tissue enables plants to translocate O_2 from aboveground leaf tissue to belowground root structures (74). By translocating O_2 from above- to belowground root structures, root cells can aerobically respire; however, this has several indirect consequences on the surrounding soil environment. First, rhizosphere oxidation can significantly influence microbial community composition and activity. For example, O_2-rich microsites enable obligate

aerobes, such as methane-oxidizing bacteria, to flourish and function in a largely anoxic soil environment (75, 76). Second, rhizosphere oxidation auto-oxidizes reduced metals like iron (Fe^{+2}) and manganese (Mn^{+2}). This reduces the toxicity of these metals to the plant and regenerates thermodynamically favorable electron acceptors and suppresses CH_4 production in soils (11, 77, 78). And lastly, the same aerenchyma tissue that facilitates O_2 transport from above- to belowground structures also facilitates CH_4 transport from the soil environment to the atmosphere. In fact, plant-mediated CH_4 ventilation has been shown to be more important than passive CH_4 diffusion and ebullition from soils (11, 35, 79, 80).

Given the critical role of wetland vegetation in C sequestration and soil C processing, there are surprisingly few studies evaluating the effects of individual plants species on soil physiochemical properties, soil microbial ecology, and soil C function in tidal freshwater wetlands. The literature that is available has produced mixed results. For example, van der Nat and colleagues (81) documented a significant increase in CH_4 production associated with *Phragmites australis* compared to *Scirpus lacustris*. However, Keller and colleagues (78) completed a plant removal experiment and did not detect any significant differences in microbially mediated organic matter mineralization between plots with and without vegetation. It is likely that these results are mixed since plant species-specific traits, such as above- and belowground structures and chemical composition, vary widely. Additionally, the plant communities of tidal freshwater wetlands are highly diverse, complex assemblages that change over both space and time. The interaction and competition of these plants, as well as SOM quality and O_2

concentrations, complicate the issue in understanding the relationship between plant species and soil function.

Invasive plant species are commonly investigated because they have substantial economic and ecological impacts on marsh function. One particular plant species, *Phragmites australis* (Cav.) Trin. ex Steud. (common reed), is an aggressive invasive that has colonized tidal freshwater marshes (82). This particular species can dramatically reshape soil C cycling. First, the invasive plant often outcompetes other plant species and decreases overall plant biodiversity. Studies by Zak and colleagues (83) positively correlated plant biodiversity with overall plant productivity and microbially-mediated nutrient processing. Second, *P. australis* in brackish marshes have been found to have deeper rooting depth (> 40 cm) than native species (84). These deeper rooting structures may increase methane oxidation and suppression of methanogenesis through iron-cycling, but *P. australis* has been shown to stimulate methanogenesis as well as transport more CH_4 than other plant species (81, 85, 86). Third, *P. australis* sequesters more N from soils compared to the native plant species *Typha angustifolia* (87). These kinds of changes in the soil N cycle can create competition between plant and soil microbes and intensify SOM decomposition. And lastly, the hollow stems or culms of *P. australis* are excellent channels for CH_4 to escape from the soil to the atmosphere.

In conclusion, the plant communities of tidal freshwater wetlands are critical in shaping soil C biogeochemistry for three reasons: (1) they are the primary mechanism sequestering CO_2 from the atmosphere; (2) they contribute a significant amount of organic matter to the SOM pool; (3) and lastly they modify the soil's physical, chemical, and biological characteristics.

3b. Soils and Soil C Cycling

Soils of tidal freshwater wetlands are hydric, meaning that these soils are "formed under conditions of saturation, flooding, or ponding long enough during the growing season to develop anaerobic conditions in the upper part (88, 89)." The process of soil formation in tidal freshwater wetlands was discussed in previous sections. This section examines SOM formation and preservation in tidal freshwater wetlands.

Soils are the largest active reservoir of C. According to global estimates, soils store approximately 60-80% of the total C sequestered in the terrestrial biome (~2500 Pg of C) (90). The majority of this C is stored in the top 1 m of soils as SOM. Soil organic matter is "the sum of all natural and thermally altered biologically derived organic materials found in the soil or on the soil surface irrespective of its source, whether it is living or dead, or stage of decomposition, but excluding the aboveground portion of living plants (91)." Soil organic matter affects many characteristics of the soil habitat. For example, SOM modifies soil color and texture, decreases bulk density, and increases buffering, water-holding, and cation exchange capacity (74, 92, 91, 93). It is also a major nutrient reservoir for N, P, and S, and serves as the major electron donor and acceptor in anaerobic respiration (91, 93). Soil organic matter pools in non-wetland habitats typically range from 1% to 5% of the soil mass. Unlike these habitats, SOM in wetland soil mass is usually a dominant feature and one of the largest global C sinks (90).

Soil organic matter pools in tidal freshwater wetlands are elevated but extremely variable. Soil organic matter pools range between 20% and 70% (8). This value is variable because the site's plant productivity and geomorphological characteristics (i.e., connectivity, slope, and microtopography) can significantly influence SOM accumulation

as well as retention within the site (45). In addition to inter-site variability, intra-site variability is high. The SOM pool in the high marsh zone is approximately two to three times greater than the low marsh zone (~30 to 45% and 10 to 15%, respectively) (54). Soil organic matter accumulation is elevated in the high marsh area because plant productivity is high and sediment export rates are low. Additionally, differences in the quality of plant-derived organic matter have also been shown to influence the long-term persistence of organic matter in soils. For example, vegetation colonizing the low marsh area, like *Nuphar* and *Peltandra*, typically have a higher nutrient content (low C:N:P:S) and lower fiber content (e.g., cellulose, hemicellulose, and lignin) compared to plants in the high marsh zone (24, 26)(8, 94). As a result, low marsh plant material degrades in about 4-weeks whereas high marsh plant material persists season-to-season (45).

Soil organic matter is studied across many soil habitats; yet, its composition and stability are still elusive to the researcher community. Soil organic matter is difficult to study because it is a chemically diverse; additionally, there are many different approaches to study and describe it. Some of the most comprehensive reviews of SOM and different methodologies used to characterize SOM include Baldock and Broos (91), Baldock and Nelson (95), and Wander (93). A majority of the published investigations examining SOM formation and persistence associated with non-wetland habitats, particularly agriculture and grassland habitats. In these non-wetland habitats, there are two theories of SOM formation and persistence (96).

The first theory proposes that SOM persists in soils because microorganisms preferentially consume easily digestible products and leave behind more chemically complex and novel substrates. This humification theory, was supported by results

obtained from a traditional chemical extraction method developed over 200 years ago by Achard (97). Researchers have since updated the method (98, 99), but new, more advanced screening techniques (e.g., NMR, infrared spectroscopy, etc.) are proposing a different story. Rather than these complex, unrecognizable C substrates building up in soils, investigators have proposed a second theory suggesting that SOM is a composite of more recognizable organic substrates. Furthermore, investigators believe that these recognizable C substrates persist in soils because environmental factors (e.g., temperature, water content) and physical availability (e.g., soil aggregation, mineral sorption, and organo-metal complexation) restrict heterotrophic decomposition (100–102, 96). Additional evidence has suggested that the size and the composition of the microbial community also influence SOM formation, decomposition, and persistence (103).

New theories of SOM stabilization have not been extended to tidal freshwater wetland soils. In wetland soils, slower soil respiration rates coupled with high deposition rates of plant-derived and sediment-associated detritus is thought to generate greater quantities of soils organic matter. While this is likely the dominant pathway, it is important to examine proposed theories of SOM stabilization, such as soil aggregation, mineral sorption, organo-metal complexing, as well as the soil microbial community aspect, on the effects of C persistence in tidal freshwater wetlands soils.

3c. Soil Microbes and Soil C Cycling

Previous sections of this review have addressed the various factors affecting SOM formation, preservation, and emission of greenhouse products from tidal freshwater wetlands. This section discusses the role of soil microorganisms, specifically bacteria, in soil C processes.

Soil bacteria are central to soil C cycling because they initiate the decomposition process of SOM. The majority of macronutrients necessary to support life (C, N, P, and S) are locked up in the SOM pool (91, 104). Soil microorganisms begin the decomposition process by releasing extracellular enzymes into the soil matrix. These enzymes digest complex polymers into more easily digestible substrates (e.g., acetate, formate, N-containing substrates), which are then consumed to build biomass and generate energy (95, 100–102). The consumed organic C compounds are ultimately mineralized into CO_2, CH_4 or other gaseous products and eventually released back into the atmosphere reservoir. The taxonomic structure and life history strategies of the soil bacterial community are thought to play a significant role in soil C processing; however, there are relatively few studies assessing the soil microbial ecology of tidal freshwater wetlands (e.g., 95–98).

Microbes are microscopic unicellular organisms that span two domains of life: *Bacteria* and *Archaea.* Broad functional processes, like glucose mineralization, can be carried out by a diverse group of organisms, whereas more narrow processes, like methanogenesis, are conserved to a phylogenetically distinct group of organisms (i.e., Euryarchaeota). The taxonomic composition of the soil bacterial community can affect the quantity and quality of respiration processes. For example, Aronson et al. (2013) linked direct changes in the abundance and composition of methane-cycling organisms (i.e., methanogens and methanotrophs) with direct changes in CH_4 flux. Strickland and colleagues also found that the microbial community accounted for ~20% of the variation in total C mineralization (108). However, other investigations have not found a similar association between the soil microbial community (i.e., biomass, composition, enzymes)

and soil C mineralization processes (109). It is likely that these conflicting results are related to the fact that complete heterotrophic decomposition of SOM is the sum of many different metabolic steps, some of which are carried out by a complex assemblage of organisms. Additionally, similar to earlier conclusions regarding the effects of individual plant species, these metabolic processes are complicated by the interactions of the microorganism with the adjacent soil environment and plant community.

Sequencing the soil microbiome is a powerful way to examine microbial community composition and assess linkages between community structure and soil functions. However, a few problems arise when trying to sequence the microbial population. Biodiversity metrics, or the number of individuals occupying the soil environment, are one of the most popular metrics used to evaluate the soil microbial community. High biodiversity represents greater functional complexity, which suggests the soil community and their activity may be resistant to change (110). When only using one descriptive metric, like biodiversity, some important nuances about the microbial community and their decomposition properties can be missed. For example, the activity of the methanogenic archaeal communities all result in CH_4 production, but the specific assemblage of methanogens (i.e., *Methanosarcina*, *Methanocella*, *Methanobacterium*, etc.) can provide deeper insight into the metabolic processes (i.e., hydrogenotrophic, acetoclastic, and methyltrophic methanogenesis) and other soil properties (C, H+, and O_2 availability). Second, differences in sequence methodologies (T-RFLP, Pyrosequencing, Illumina) and phylogenetic analysis (e.g., family vs. species) can lead to different interpretations. Third, many sequencing projects identify new "species" or operational taxonomic units (OTUs), but we know little to nothing about the function of these

organisms (111). Fourth, sequencing projects do not account for the problems of DNA preservation in soils (112). And lastly, ecosystem function is scale dependent. Many projects are restricted to one point in time and space, which makes it difficult to extrapolate the effects of species diversity and richness on soil C cycling functions from the plot to landscape scale.

To truly understand how microbial community structure translates to ecosystem function in tidal freshwater wetlands, we must continue to examine how soil bacterial communities interact with the plant community and soil environment at multiple spatiotemporal scales and taxonomic levels.

Part 4: Restoring Tidal Freshwater Wetlands and Ecosystem Function

Development and land use changes in the surrounding watershed of tidal freshwater wetlands threaten their function and existence. Urbanization, stream/river channel engineering, saltwater intrusion, and shifting water/sediment supplies are just some of the pressures tidal freshwater wetlands are experiencing. If these forces are sustained long enough, the marsh may migrate, erode, or evolve into a new habitat. Because tidal freshwater wetlands are integral in maintaining downstream water quality, efforts to reinstate and preserve these habitats and their functions are occurring nationwide. We know relatively little about the impacts of wetland restoration on soil C cycling in tidal freshwater wetlands. Therefore, this section evaluates the positive, negatives, and unknowns of restoration on wetland physical, chemical, and biological properties, and discusses the possible consequences of restoration on long-term C storage and greenhouse gas production.

Wetland restoration is the "return of a wetland from a disturbed or altered condition caused by human activity to a previously existing state (74)." Because hydrology and vegetation are the master variables shaping both wetland structure and function, most projects focus on restoring these two variables. Wetland hydrology is quite complex and not the focus of this review; Mitsch and Gosselink (74) provide some insight into the complexity of this subject. Restoring wetland plant community is the second most important goal targeted in wetland restoration projects. As previously discussed, wetlands plants provide habitat for organisms and are integral in maintaining soil structure and function. There are many methods of restoring wetland vegetation stands, but most restoration projects implement planting interventions (74). While plant community biomass is often quick to recover in restored marshes, vegetation biomass, species composition, and phenology can be quite different than natural habitats (15, 16, 113).

In addition to shifts in plant community dynamics, restoration is destructive to the soil habitat. As seen in the Ballantine and Schneider (16) large equipment used during restoration compacts soils, which makes it more difficult for plants to penetrate and establish in the marsh. Site construction also designs a more homogenous landscape. For example, Bruland and Richardson (17) documented that restored wetlands have less microtopography as well as more homogenous soil texture. Soil texture is important because it affects the long-term storage of C. For example, coarser soils have higher rates of water conductivity, less organic matter holding capacity, and higher C mineralization rate compared to finer-textured soils (114).

Current literature evaluating the effects of habitat restoration on soil function have shown long-term impacts on SOM pools. Papers by Ballantine and Schneider (16), Bruland and Richardson (17), and Moreno-Mateos et al. (18) have shown SOM pools remained far below expected levels years after restoration. Underdeveloped SOM pools are not an unexpected result given that soils of restored wetlands are young; however, even after plant biomass returned to historical levels, SOM levels remain diminished. Major changes to the site's topography, soil physical properties (both texture and structure), and plant community (phenology, composition, and productivity) may forever change the mechanisms that control SOM formation, stabilization, and long-term storage of C in these habitats. Because SOM is integral to soil function, organic matter amendments are being incorporated into the topsoil during the restoration hoping it will jumpstart ecosystem performance (115–117). There is little information available to users about how to select and apply these organic matter amendments, and even less about how it will affect soil microbial community composition, nutrient cycling, and greenhouse gas production.

The effects of restoration on the soil microbiome are not well understood in any wetland ecosystem because there are a limited number of studies characterizing soil microbial populations in wetland soils. Instead, many studies investigate the impacts on wetland restoration on the microbial community through indirect methods (e.g., gas emissions and nutrient turnover). We do know from other studies in non-wetland habitats that shifts in soil physical and chemical properties influence microbial colonization and composition. For example, limited resource availability can affect the way microbial communities use C (118, 119), can increase competition between organisms (120), and

can reduce the overall performance of critical biogeochemical processes like denitrification (121). What is less understood is how these shifts in the soil microbial community translate to changes in soil C processing at the landscape scale.

Soil C cycling in tidal freshwater wetlands emerges from a symphony of interactions between the plant community, the soil habitat, and the microbial populations. While restored tidal freshwater wetlands provide some ecosystem service, these restored habitats often do not resemble the same physical and chemical properties as their natural counterparts. As a result, soil C cycling may be permanently changed in these restored habitats. The question then becomes, will restored wetlands ever accumulate the same amount of soil C as naturally formed tidal freshwater wetlands, and what will soil C cycling look like in these restored habitats? To answer this question, we must investigate top-down (e.g., landscape, topography, etc.) as well as bottom-up (e.g., microbial species composition, soil physical-chemical properties) controls on soil C cycling in natural and restored tidal freshwater wetlands.

Part 5: Conclusion

In conclusion, tidal freshwater wetlands are diverse ecosystems that provide many valuable ecosystems functions at the local scale. Unlike other wetland types, and do their limited size, tidal freshwater wetlands are not considered a major source of CH_4. However, anthropogenic disturbances, such as global climate change, saltwater intrusion, and land use modification, are negatively impacting tidal freshwater wetland acreage and function (122–124). Not only do we need an updated inventory of tidal freshwater wetlands at the national and international scale, but we also need to continue investigating the effects of primary drivers – wetland vegetation, soils, and microbial

communities, on their function at multiple spatiotemporal scales. We also need to examine how direct and indirect disturbances affect soil C cycling and overall potential for tidal freshwater wetlands to sequester and store soil C.

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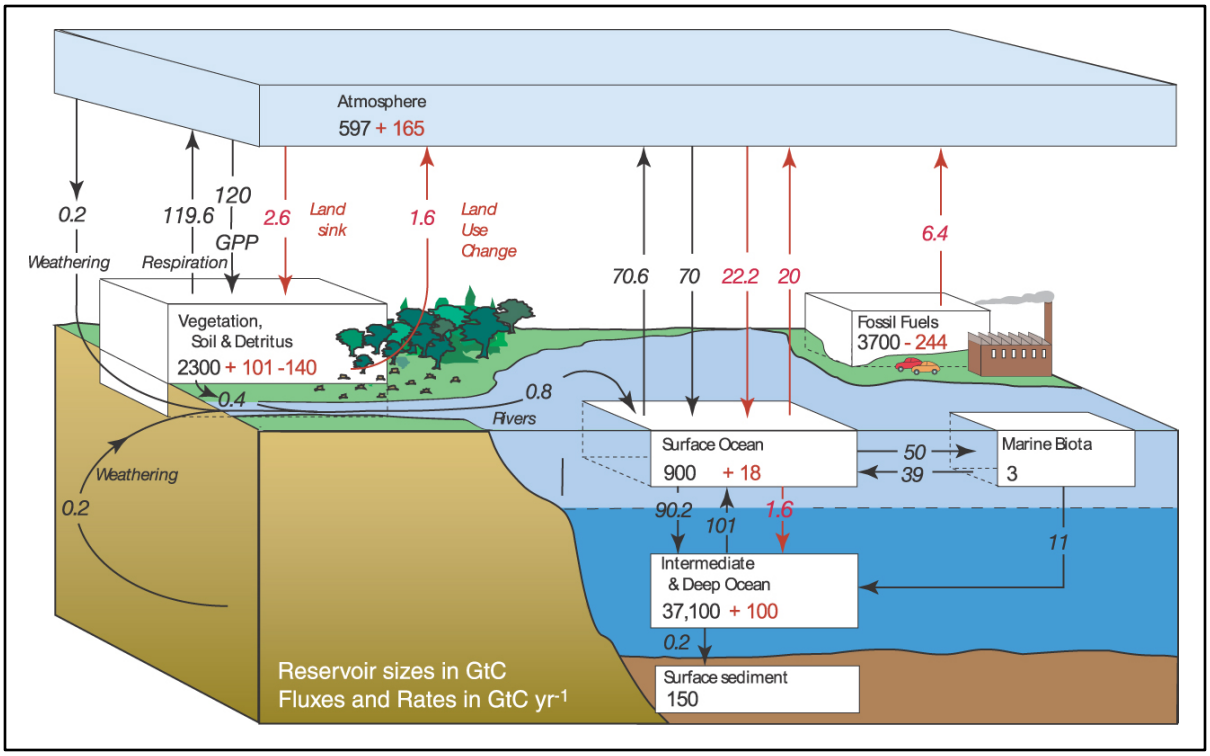

Figure 1. A depiction of global carbon (C) reservoirs (GtC) and annual fluxes (GtC yr^{-1}) in the 1990s. *Reprint from Figure 7.3 in IPCC, 2007 report (125).*

Original Figure Caption (page 515): The global carbon cycle for the 1990s, showing the main annual fluxes in GtC yr^{-1}: pre-industrial 'natural' fluxes in black and 'anthropogenic' fluxes in red (modified from Sarmiento and Gruber, 2006, with changes in pool sizes from Sabine et al., 2004a). The net terrestrial loss of –39 GtC is inferred from cumulative fossil fuel emissions minus atmospheric increase minus ocean storage. The loss of –140 GtC from the 'vegetation, soil and detritus' compartment represents the cumulative emissions from land use change (Houghton, 2003), and requires a terrestrial biosphere sink of 101 GtC (in Sabine et al., given only as ranges of –140 to –80 GtC and 61 to 141 GtC, respectively; other uncertainties given in their Table 1). Net anthropogenic exchanges with the atmosphere are from Column 5 'AR4' in Table 7.1. Gross fluxes generally have uncertainties of more than ±20% but fractional amounts have been retained to achieve overall balance when including estimates in fractions of GtC yr^{-1} for riverine transport, weathering, deep ocean burial, etc. 'GPP' is annual gross (terrestrial) primary production. Atmospheric carbon content and all cumulative fluxes since 1750 are as of end 1994.

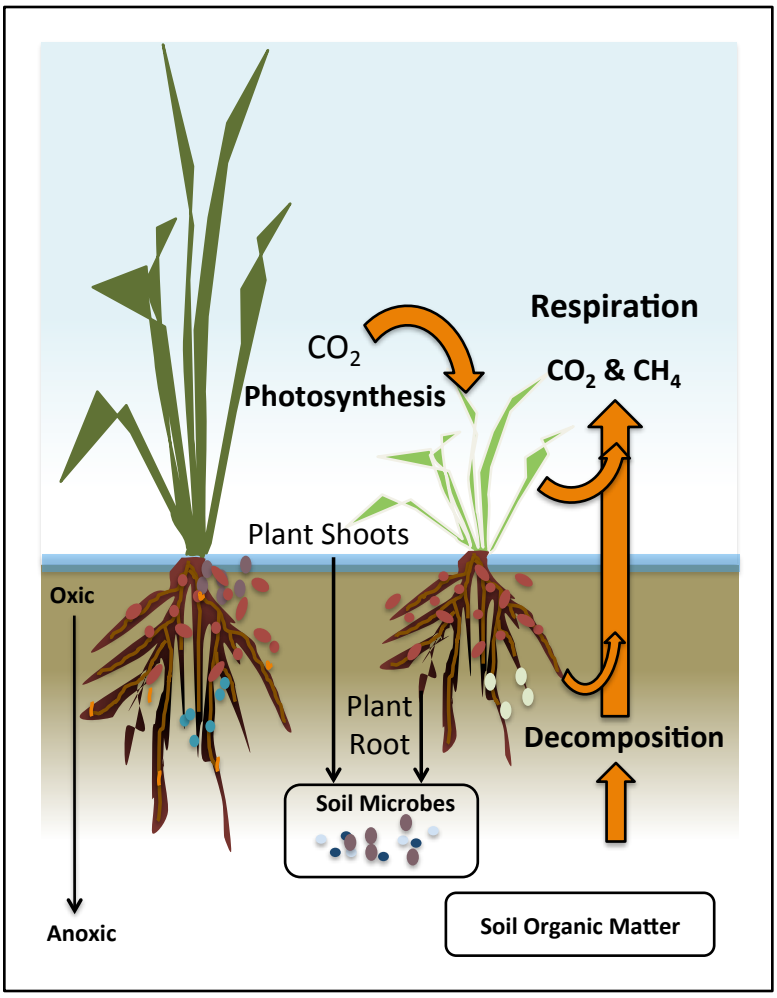

Figure 2. Effects of wetland vegetation and soil microbial communities on soil C cycling and greenhouse gas emissions from tidal freshwater wetland soils. The diverse and highly productive wetland vegetation community fixes a majority of soil organic matter found in tidal freshwater wetlands. Aboveground plant shoot material and belowground root exudates are deposited in the adjacent soil habitat and preserved as soil organic matter, which is eventually metabolized by the soil microbial community. Microbial respiration byproducts include both carbon dioxide (CO_2) and methane (CH_4).
Plant images courtesy of S. Yarwood.

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Chapter 2: Site history and edaphic features override the influence of plant species on microbial communities in restored tidal freshwater wetlands

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Abstract

Restored wetland soils differ significantly in physical and chemical properties from their natural counterparts even when plant community composition is similar, but effects of restoration on microbial community composition and function are not well understood. Here we investigate plant-microbe relationships in restored and natural tidal freshwater wetlands from two subestuaries of the Chesapeake Bay. Soil samples were collected from the root zone of *Typha latifolia*, *Phragmites australis*, *Peltandra virginica*, and *Lythrum salicaria*. Soil microbial composition was assessed using 454 pyrosequencing and genes representing bacteria, archaea, denitrification, methanogenesis, and methane oxidation were quantified. Our analysis revealed variation in some functional gene copy numbers between plant species within sites, but inter-site comparisons did not reveal consistent plant-microbe trends. We observed more microbial variations between plant species in natural wetlands, where plants have been established for a long period of time. In the largest natural wetland site, sequences putatively

matching methanogens accounted for ~17% of all sequences and the same wetland had the highest numbers of genes coding for methane coenzyme A reductase (*mcrA*). Sequences putatively matching aerobic methanotrophic bacteria and anaerobic methane-oxidizing archaea (ANME) were detected in all sites, suggesting that both aerobic and anaerobic methane-oxidation are possible in these systems. Our data suggest that site history and edaphic features override the influence of plant species on microbial communities in restored wetlands.

Introduction

Diverse soil microbial communities, capable of using numerous metabolic processes to generate energy and assimilate nutrients, mediate key wetland functions. Although recent studies have described microbial community composition and functional gene abundance related to land use, vegetation, and environmental factors (1-3), structure-function relationships in freshwater wetland soils are not well understood. Biogeochemical activities are not only regulated by the size of the microbial biomass, but also by the presence, distribution, and abundance of functional guilds (4). Functional gene markers can, therefore, provide valuable insight into key biogeochemical processes and their relationships to site properties (5, 6). Given that the underlying mechanisms of major nutrient cycles are related to microbial taxonomic diversity, it is surprising that relatively few studies have described both microbial composition and functional group abundance in freshwater wetlands, a biogeochemical hotspot of carbon (C) and nitrogen (N) cycling.

Tidal freshwater wetlands (TFWs) are located in the upper reaches of estuaries along the coastlines of the U.S. Atlantic, the Gulf of Mexico, and elsewhere, where

salinity is low (typically <0.5 ppt) (7-9). Unlike saline wetlands that tend to produce large quantities of hydrogen sulfide, the main C mineralization pathways in TFWs include methanogenesis (7, 8, 10) and, depending on mineralogy, iron reduction (11). The global contribution of methane from TFWs is unknown, but it is hypothesized to be negligible because of their limited area and competition with iron reduction (8, 11). However, the contrasting oxic and anoxic environments in TFWs support coupling of nitrification and denitrification, making these habitats important N sinks (12). Only a handful of studies have examined microbial community composition related to these processes in TFWs (10), and to our knowledge no study has compared microbial composition between natural and restored TFWs.

Intense development in coastal zones has reduced TFW acreage and their associated ecosystem functions (7, 9). Efforts to restore these habitats unfortunately often fail to reinstate ecosystem services observed in natural wetlands, likely due to continued differences in abiotic and biotic factors (13, 14). Restoration of tidal wetland hydrology often necessitates lowering surface elevation by removing topsoil or raising it by depositing dredged sediment. These drastic alterations have direct impacts on physiochemical properties such as bulk density, soil organic matter (SOM), and pH. Urban-impacted wetlands are particularly difficult to reestablish because watershed development alters hydrology, nutrient flux, sedimentation pattern, and disturbance regime, impacting the trajectory of plant community and soil development (9). It has become clear that restored wetland soils continue to differ from natural wetlands for decades or even centuries (13-16), but little is known about the effect of restoration on microbial communities and associated biogeochemical functioning in TFW(1, 17).

Wetland vegetation can impact soil microbes directly and indirectly. Microbial biomass and oxygen (O_2)-dependent metabolism are stimulated in the plant rhizosphere, where O_2 and C compounds are increased compared to the surrounding soil (18-20). It has been observed that exotic plant species can significantly alter microbial-mediated function (21, 22). For example, soils under the Eurasian lineage of *Phragmites australis* had nitrification rates three times greater than the native *Spartina patens* in a brackish marsh (23, 24), and *Lythrum salicaria* tissue was observed to have a slower decomposition rate compared to the native *Typha latifolia*, leading to decreased nutrient pools (25, 26). However, other studies investigating plant-microbe dynamics, including in stands of *Phragmites australis,* reported negligible effects of plant species on microbial biomass C and N, soil respiration, denitrification, and potential net N mineralization (27, 28). These mixed results suggest mechanisms controlling microbial composition and by extension the processes they mediate are not well understood.

In the current study, we characterized bacterial and archaeal community composition and functional capacity via functional gene abundance in TFW soils from five locations, including natural and restored wetlands in urban and sub-urban watersheds. We hypothesized that soil properties such as SOM and mineral N concentration would differ between sites and that these differences would correspond to differences in bacterial and archaeal composition and the abundance of functional genes. Furthermore, we tested if wetland microbial community composition and functional capacity would vary between plant species. For each of the five sites, we collected soil samples from the rhizosphere of four plant species: *Typha latifolia* (broad leaf cattail), *Peltandra virginica* (green arrow arum), *Lythrum salicaria* (purple loosestrife), and the

Eurasian lineage of *Phragmites australis* (common reed). We examined the relative abundance of major phylogenetic groups and quantified 16S rRNA gene abundance for bacteria and archaea. In addition, Q-PCR was used to measure functional genes representing denitrification (*nirK, nirS*, *nosZ*), methanogenesis (*mcrA*), and methane oxidation (*pmoA*).

Materials and Methods

Site Description

In July and August 2012, samples were collected from three restored and two natural reference TFWs. One natural (Jug Bay, N38.78580 W76.71308; Soil series: Nanticoke Mannington) and one restored (Wootons Landing, N38.85646 W76.69124; Soil series: Udorthents/water) site were located in the suburban area of central Maryland on the Patuxent subestuary of Chesapeake Bay. In 1992, soils were scraped down at Wootons to restore wetland hydrology (29). The lower Anacostia River is highly urbanized as it enters Washington D.C. from central Maryland. In the Anacostia watershed, a natural remnant wetland (Dueling Creek, N38.92411 W76.94018; Soil series: Zekiah and Issue) was selected along with two restored marshes, one restored in 1992-93 (Kenilworth, N38.91035 W76.94588; no soil data available) a second in 2000 (Kingman, N38.90414 W76.96182; no soil data available). Kenilworth and Kingman sites were restored by raising the elevation with dredged Anacostia river sediments and then contoured with a mud cat (30). Additional detail for these three Anacostia sites is available in Baldwin (9).

Experimental Design and Sample Collection

For each of the sites, three replicated stands of four common plant species were targeted: *Typha latifolia* L., *Peltandra virginica* (L.) Schott, *Lythrum salicaria* L., and *Phragmites australis* (Cav.) Trin. ex Steud. ssp. *australis*. Each site contained areas dominated by these four species, with the exception of *Lythrum*, which was absent in Wootons. This study design resulted in a total of 57 collected samples. Aboveground biomass was clipped at the soil surface from a 625-cm^2 plot using a serrated knife and then placed in a large plastic bag to be later separated by species and dried to determine plant biomass (data not shown). After removing plant biomass, a half circular Russian peat borer (Eijelkamp, Giesbeek, Netherlands) was used to collect two 5.2 x 50-cm cores. In each plot, soils were sampled <1 cm away from the clipped shoots of the species of interest. Cores were described in the field to identify major horizons (data not shown). Oi horizons were not observed in some restored locations and, therefore, were excluded from all samples. Remaining material from both cores was homogenized into a single representative sample and stored on ice until returning to the lab. Soil samples were thoroughly mixed, and ~10 g of soil was removed from each sample and stored at -20^oC until DNA extraction. The remaining soil was stored at 4°C until edaphic features were analyzed the following week.

Soil chemistry

Soil pH was determined using an Accumet 15 plus pH meter on 5:1 water:soil slurries. Soil moisture content was determined by drying ~10 g of field-moist soil to a constant mass at 105°C for 36 h. Soil organic matter was calculated using loss-on-ignition (400°C for 16 h) (31) and total C and N content was determined by combustion

analysis at 950°C on a LECO CHN-2000 analyzer (LECO Corp, St. Joseph, MI)(32) Nitrate (μg NO_3^--N per g dry soil) concentrations were determined by ion chromatography. Briefly, 5 g of soil was shaken in 12.5 ml of 0.1 M KCl for 1 hour before centrifugation to pellet soil. The supernatant was passed through a 0.45 um syringe filter to remove fine particles. The filtrate was stored at 4°C until analysis on an 850 Professional IC Autosampler (Metrohm USA, Inc., Riverview, FL) with an METROSEP A Supp 5-150/4.0 separation column and 20 μL injection. Ammonium (μg NH_4-N per g dry soil) was extracted from 5 g of soil mixed with 2 M KCL and measured colorimetrically from the filtrate using a Multiskan FC spectrophotometer (Thermo Scientific, Waltham, MA) (33). Soil texture was determined using the hydrometer method (34) using composite samples from each site. Textures for each site were relatively similar: Jug Bay, ranged from silt-loam to loam; Dueling, silt loam; Wootons, loam; Kenilworth and Kingman, both loamy sands.

Soil microbial characterization

Total genomic DNA was extracted using a PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's instructions with the exception that soils were homogenized using a FastPrep®-24 (45 sec at 6 m/s; MP Biomedicals, LLC., Solon, OH). All samples were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY).

Quantitative PCR

Quantitative PCR was used to estimate abundance of bacterial and archaeal 16S rRNA genes and seven functional genes: methyl coenzyme M reductase (*mcrA*), particulate methane monoxygenase (*pmoA*), ammonium monooxygenase α-subunit

(*amoA*) for ammonia oxidizing archaea (AOA) and bacteria (AOB), nitric oxide reductase (*nirk* and *nirS*), and nitrous oxide reductase (*nosZ*).

Plasmid standards were constructed by amplifying functional genes from pure culture (**Table S1**). Target genes of interest were amplified using a 20 µl PCR reaction with the following reagent concentrations: 1X GoTaq® Colorless Flexi Buffer (Promega Corporation, Madison, WI), 1.75 mM $MgCl_2$, 0.20 mM dNTPs, 0.50 µM forward primer, 0.5 reverse primer, 0.064% bovine serum albumin (BSA), and 0.025 U/µl GoTaq® Hot Start Polymerase (Promega Corporation, Madison, WI); details regarding primers, thermal cycling conditions, and efficiencies are listed in **Table S1**. Amplified functional gene fragments were subsequently cloned using the Topo TA cloning™ kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Prior to analysis, plasmid standards were linearized using EcoRV (Thermo Scientific, Waltham, MA) and purified using the UltraClean PCR clean-up kit (Mo Bio Laboratories, Carlsbad, CA). Standard plasmid concentrations were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY) and subsequently adjusted to 2.5 ng/µl; this stock solution was then serially diluted 10-fold to 2.5 x 10^{-6} ng/µl. At least three of the six serial diluted standards were used to evaluate amplification efficiency and calculate gene copy numbers for the unknown environmental samples. Because reaction- and sample-specific inhibition can influence gene copy numbers, a soil standard dilution series was prepared to relativize plasmid curves (35). Following a similar procedure outlined in Hargreaves et al. (35), we prepared a soil standard by combining equal amounts of pre-diluted DNA samples. The pooled 2.5 ng/µl soil standard stock was serially diluted 10-fold to 2.5 x 10^{-6} ng/µl.

Soil DNA extracts, plasmid standards, and pooled soil standards were run in triplicate 20 µl reactions with 10.0 µl of KiCqStart® SYBR® Green qPCR ReadyMix™ with ROX (Sigma, St. Louis, MO), 0.5 µM final concentration of each the forward and reverse primer, and 2.5 ng template DNA for community composition or 5 ng of template DNA for functional gene quantification. All reactions were run on the StepOne Plus real-time PCR instrument (Applied Biosystems, Foster City, CA).

Data were extracted from runs with standard curve r^2 values > 0.99, efficiency values between 90% and 110%, and a single dominant peak in dissociation curves (36). To calculate gene abundance for unknown samples, at least three of the six serial diluted plasmid standards were used to evaluate amplification efficiency. Additionally, C_t values were adjusted for differences between plasmid and soil standard efficiency according to equations outlined in Hargreaves et al. (35). Final gene abundance values (genes g^{-1} wet soil) were log-transformed prior to statistical analysis.

Pyrosequencing

Pyrosequencing was used to investigate microbial community structure. PCR reactions were set up using Promega GoTaq® DNA Polymerase (Promega Corporation, Madison, WI) following the protocol described by Bates, et al. (37). Each reaction was set up using primers F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-GGACTACVSGGGTATCTAAT-3') targeting a 291 bp fragment in the V4 and V5 region of 16S rRNA genes (37). This primer set was selected because it provides sufficient resolution for nearly all bacterial and archaeal organisms with few biases or excluded taxa (37). Multiplexing and sequencing of all 57 samples was accomplished using a 10-bp MIDS barcoded F515 primer also containing a Roche 454-A

pyrosequencing adaptor (5'- CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'; Roche Applied Science, Branford, CT, USA) and a "GA" linker sequence.

Target sequences were amplified in a 25 µl PCR reaction. Each reaction contained 0.20 µM forward and reverse primers, 0.20 mM of dNTPs, 1.75 mM $MgCl_2$, 1X GoTaq® Colorless Flexi Buffer (Promega Corporation, Madison, WI) with 1.5 mM $MgCl_2$, 0.064% BSA, and 0.025 Taq U/µl GoTaq® Hot Start Polymerase (Promega Corporation, Madison, WI). PCR reaction conditions began with a 95°C heat activation step for 5 min followed by 30 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension step at 72°C for 60 sec. Post-amplification, each barcoded PCR product was purified following the UltraClean PCR Clean-Up Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) except 4.5X SpinBind solution was mixed with the 25 µl product. Separate sample amplifications were combined in equal amounts (37). The sample was sent to the Institute for Genome Sciences and Policy (Duke University, Durham, NC) and sequenced using titanium chemistry on a Roche 454 GS-FLX (Roche Applied Sciences, Penzberg, Germany).

Data Analysis

Prior to statistical analysis, each parameter was assessed for normality and homogeneity of variance assumptions. All variables except pH were log_{10}-transformed to meet normality assumptions. A split-plot design was analyzed using mixed model ANOVA in the SAS System v. 9.2 (SAS Institute, Cary, NC) to evaluate the effects of site (whole-plot factor), plant species (sub-plot factor), and the plant x site interaction on soil parameters (pH, SOM, Total C and N, NO_3-N, and NH_4-N) and microbial community functional genes (EUB, ARC, *mcrA*, *pmoA*, *nirK*, *nirS*, and *nosZ*) (38). The

effects of plant species within each site (i.e. the simple effects) were included in ANOVA analyses because of significant interaction terms for several of the dependent variables. Pearson's correlation coefficients were calculated between univariate data and permutation tests were used to determine p-values using Microsoft Excel.

Sequence data generated from the 454-sequencing runs were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (39). A full description of scripts and justification for each step is available (**Text S1**). Briefly, sequences were de-multiplexed and trimmed to remove barcodes, linker, and both forward and reverse primer base pairs. Sequences were quality checked using default settings in the split_libraries.py command, except minimum and maximum sequence length, and were adjusted to include a majority of sequences representing the 291 bp region. Samples were not denoised (40). Similar sequences were clustered into operational taxonomic units (OTUs) using Uclust, and a threshold with 97% similar sequence and taxonomy was assigned using the Greengenes database (www.greengenes.lbl.gov). The resulting relative abundances for each soil sample were used for subsequent analysis.

Rarefaction curves did not approach asymptote for all sample units (**Figure S1)**. Due to unequal sampling depth among sample units, a rarified community was generated using the jackknifed_beta_diversity.py workflow script; rarefaction depth was set to the lowest sequence count (1,922 sequences). After rarifying the dataset, unweighted Principle Coordinates Analysis (PCoA) was used. Recently it has been reported that rarefaction removes valuable data and may lead to false conclusions (McMurdie and Holmes, 2013); therefore, we also analyzed total community composition by site and plant species using the full quality-checked dataset using non-metric multidimensional

scaling (NMS). Non-metric multidimensional scaling (NMS) was performed in PC-ORD version 6 (MjM Software, Gleneden Beach, OR) to visualize overall differences in bacterial and archaeal 454 patterns across sites and plant species (41, 42). Analysis was performed using the Sorenson/Bray Curtis distance metric and random starting configurations with 250 runs with real data. Prior to analysis, rare species (less than ten observations) were removed. A two-dimensional NMS with a final stress value of 9.7 was achieved after eight iterations and used for subsequent analysis. Multi-response permutation procedure (MRPP) was used to test for differences between sample units based on within-group similarities (42).

Sequences were submitted to Genbank as a single pooled sample under accession number SRP055495.

Results

Soil characteristics differed significantly among the five sites but varied little between the different plant species (**Tables 1-3**). Among the five sites, Jug Bay soils were more acidic and had higher concentrations of SOM, total C, total N, and NH_4-N (**Table 1**). The most recently restored site, Kingman, had less SOM, total C, total N, and NH_4-N than other locations. Dueling was more similar to the 1992 suburban restored site, Wootons, than to Jug Bay, its natural counterpart in the Patuxent subestuary. The site x plant interaction was significant for pH (**Table 2**) due to significant variation between plant species at the two natural sites, Jug Bay and Dueling (**Table 3**) Across sites and plant species, negative correlations were observed between pH values and SOM ($r = -0.59$, $p < 0.01$), total C ($r = -0.56$, $p < 0.01$), total N ($r = -0.54$, $p < 0.01$), and NH_4-N

concentrations ($r = -0.34$, $p = 0.01$). Ammonium concentrations were positively correlated with SOM ($r = +0.54$, $p < 0.01$).

Pyrosequencing generated a total of 478,143 quality-checked 16S rRNA sequences. Sequence lengths ranged between 166 – 317 bp with the majority of sequences averaging 253 bp. Following the removal of low quality sequences and chimeras, sequence counts ranged from 1,922 to 12,346 with an average sequence count of 8,388 per sample. When sequences were compared to the Greengenes database, 1,038 operational taxonomic units (OTUs, 97% similarity) were represented across all samples. Unclassified sequences were relatively small for all samples (mean = 0.5%).

Ordination of the rarified sequence dataset revealed microbial compositional differences between sites (**Figure 1a**), but not by plant species (**Figure 1b**). NMS ordinations and MRPP analysis of the non-rarified data resulted in a similar pattern, with microbial composition separated by site (**Figure S2a**; MRPP $p < 0.01$) but not by plant species (**Figure S2b**). Microbial community composition correlated with pH ($r = 0.49$, $p < 0.01$) and with NO_3^--N ($r = 0.52$, $p < 0.01$) (**Figure S2a**). Most sequences putatively matched bacteria, averaging 79% of the total sequences per sample. The majority of the bacterial sequences were comprised of twelve phyla (**Figure 2a)**. Forty-eight to seventy-two percent of sequences with each sample matched one of these 12 phyla. The most abundant phylum across all samples was *Proteobacteria* (16%), with a large majority of sequences matching *Delta* (7%), *Beta* (5%), *Alpha* (2%), and *Gamma* (1%) - *proteobacterial* class. *Acidobacteria* tended to make up a large percent abundance in Dueling (14%) and Wootons (13%), but only accounted for 6% of the relative sequence abundance in Jug Bay. The "Other" group in **Figure 2a** refers to 58 additional phyla

(12%) that were found in low abundance and unclassified bacterial sequences (5%). In general, relative proportions of bacteria to archaea were similar among all sites except in Jug Bay, where archaea made up a significant proportion of the microbial community (32%) (**Figure 2**). The relative ratio of *Euryarchaeota* to *Crenarchaeota* was similar in all samples, and only a small percentage of sequences were unclassified archaea (0.5%; not plotted).

Bacterial 16S rRNA gene copy numbers ranged from 2.3×10^8 to 2.1×10^{10} genes g^{-1} wet soil with more bacterial gene copies in Wootons soils (1.1×10^{10} genes g^{-1} wet soil compared to Kingman (3.8×10^9 genes g^{-1} wet soil), with the other sites intermediate (**Figure 3a**). Both at Kenilworth and Kingman bacterial 16S rRNA gene copies were lower in *Peltandra* compared to other plant species, but this trend was not observed at other locations (**Table 3**, **Figure 3a**). Archaeal 16S rRNA ranged from 5.7×10^6 to 2.2×10^9 genes g^{-1} wet soil and were significantly greater in Jug Bay (1.7×10^9 genes g^{-1} wet soil) compared to other locations (**Table 2**, **Figure 3b**). Similar to the bacterial 16S rRNA, plant species differences were observed (with *Peltandra* again having the lowest copy numbers) for archaeal gene copy numbers at Kenilworth and Kingman but were only significant at the 0.1 level (**Table 2, Figure 3b**). When the predicted ratio of archaea to bacteria using sequence data was plotted against the archaea to bacteria 16S rRNA gene copy numbers, the ratios were significantly correlated ($r = 0.92$, $p < 0.01$; **Figure S3a**).

Copy numbers for some functional genes measured by Q-PCR differed between sites and plant species (**Tables 2 and 3**). Interactions between site and plant species were significant (some at the 0.1 level) for five of the seven genes examined, indicating that

plant effects across sites were not uniform, but site effects were stronger than plant effects, based on lower P-values for site than plant main effects (**Table 2**). Within individual sites, plant species related significantly to at least one function gene, with the exception of *mcrA* (**Table 3**, **Figure 3c**).

Minimal plant effects were observed for methanogens (**Tables 2 and 3**, **Figure 3c**), but gene copies of *mcrA* were higher in Jug Bay (9.5×10^8 genes g^{-1} wet soil) compared to the suburban reference site, Dueling (3.3×10^8 genes g^{-1} wet soil), and the three restored sites (**Table 2**). There was a positive correlation observed between *mcrA* and SOM ($r = +0.35$, $p < 0.01$). Examination of the methanogenic sequences revealed three classes of methanogenic *Euryarchaea*: *Methanobacteria, Methanomicrobia, and Thermoplasmata*. Eight families were represented in the sequence libraries: *Methanobacteriaceae, Methanocellaceae, Methanomicrobiaceae, Methanoregulaceae, Methanospirillaceae, ANME-2D, Methanosaetaceae,* and *Methanosarcinaceae*. Examination of the sequences found 93% of the sequences were dominated by four groups: *Methanoplasmatales*, *Methanobacteriaceae*, *Methanoregulacae*, and *Methanosaetacae* (**Figure 4a**). The percentages of sequences putatively identified as methanogens were significantly correlated to the gene copies of the *mcrA* ($r = 0.46$, $p < 0.01$; **Figures S3b).**

Methanotroph *pmoA* gene abundance was greatest in Kenilworth (2.3×10^5 genes g^{-1} wet soil) and Wootons soils (1.6×10^5 genes g^{-1} wet soil) compared to Dueling (9.1×10^4 genes g^{-1} wet soil), Jug Bay (6.4×10^4 genes g^{-1} wet soil), and Kingman (5.2×10^4 genes g^{-1} wet soil) (**Table 2, Figure 3d**). Sequences putatively identified as matching aerobic methanotrophs were present in all samples, including: Type I

Gammaproteobacteria (Order Methylococcales), Type II *Alphaproteobacteria (Families Methylocystaceae and Methylobacteriaceae)*, NC10, and *Verrucomicrobia* (*Class Methylacidiphilae*) (**Figure 4b**). Similar to the methanogens, the percent abundance of sequences matching aerobic methanotrophs and *pmoA* gene copy numbers were significantly correlated ($r = 0.33$, $p = 0.02$; **Figure S3c**). Anaerobic methanotrophs (ANME-2D) were also detected in archaeal sequences across all five tidal freshwater wetland sites (**Figure 4b**).

Ammonia-oxidizing bacteria genes were below detection in all samples and ammonia-oxidizing archaea genes were below detection in most samples; only 30% of the total samples fell within plasmid standard range (data not shown). In general, the effect of plant species on denitrification genes varied between sites (significant site x plant interactions; **Table 2**), but some plant trends emerged. In Jug Bay, *nirS* gene copy numbers were higher under *Phragmites* compared to other plant species (**Table 3**, **Figure 5b**; significant at 0.1 level). The gene copies of *nirK* and *nirS* genes were lower in Jug Bay compared to other sites (**Table 2**, **Figure 5**), and correlated to pH ($r = +0.58$ $p < 0.01$ and $r = +0.56$ $p < 0.01$, respectively) across all sites. Overall, gene copies of nitrous oxide reductase (*nosZ*) were highest in Wootons soils (**Table 2**, **Figure 5c**) and correlated to SOM content ($r = +0.45$, $p < 0.01$), total C ($r = +0.40$, $p < 0.01$), total N ($r = +0.49$, $p < 0.01$), and NH_4-N ($r = +0.39$, $p < 0.01$) across all sites.

Discussion

Microbial community structure significantly differed between the five TFWs studied. Microbial community composition correlated with soil pH and NO_3^--N concentration (**Figure S2**). These findings partially support our hypothesis and

corroborate other studies that have reported soil pH as an important factor shaping soil bacterial composition in many different ecosystems (43), including wetlands (1). Although this is a commonly reported finding, the mechanisms underlying these trends have not been fully explored. For example, Rouske et al. (44) and others presented evidence relating pH effects on microbial community composition; however, they did not find evidence for a link between different composition and C cycling functions (44, 45). Interestingly, pH did significantly vary between plant species in the two natural sites (**Table 3**), suggesting that plants may indirectly shape microbial communities in cases where vegetation has been established for a long period of time.

Significantly lower SOM was observed in the urban and restored wetlands (**Table 1 and 2**). Although we did not measure methane production, SOM content correlated with *mcrA* gene copy numbers ($r = +0.35$, $p < 0.01$), suggesting that there is increased potential for methane production in natural compared to restored sites. Putative hydrogenotrophic methanogens were dominant compared to acetoclastic sequences in all sites (**Figure 4a**). This is in agreement with other studies of freshwater sediments, including peatlands and TFW sediments (10, 46). The most abundant group of methanogens matched a lineage of *Thermoplasmatales* that has been provisionally re-classified as *Methanoplasmatales* (47). These putative methylotrophic methanogens have been identified in many habitats, including another study of Jug Bay soils (10). Although there is not much known about this particular order, recent studies have shown that groups of methanogens vary in their O_2 sensitivity and available metabolic substrate (48-50). Seasonal O_2 penetration is relatively stable in TFWs (51), and therefore could favor methanogen groups more sensitive to O_2. We plan to follow up this work by examining

seasonal methane flux and tracking variations in the methanogen community through time.

We were surprised to find sequences putatively matching anaerobic methanotrophic archaea (ANME-2D) in all five of the wetlands. Anaerobic oxidation of methane (AOM) was first recognized in marine sediments and coupled with sulfate reducing bacteria (52, 53), and we assume low levels of sulfate in all of our sites. However, recent studies have demonstrated the importance of AOM in TFW sediments and mudflats *in situ* (54). Furthermore, microcosm experiments demonstrated sulfate-independent AOM and coupled activity with alternative terminal electron acceptors, including NO_3^-, iron (III) and manganese (IV)(54). Although AOM sequences made up a higher relative abundance in the two natural reference sites, Jug Bay and Dueling, we documented relatively similar aerobic methane oxidizing bacterial diversity (NC10, Type I, Type II, and *Verrucomicrobia*) (Figure 4b). It is important to note that anaerobic methane oxidizing archaea do not contain *pmoA* genes, but instead contain *mcrA*; therefore, our Q-PCR targets do not clearly separate methanogenesis from methane-oxidation. Given the abundance of ANME sequences, we plan to follow up this work to determine the relative contribution of aerobic and anaerobic methane oxidizers within TFWs and to also investigate the role of iron reduction as an alternative to methanogenesis.

Although we originally hypothesized that microbial communities would differ between the four plant species, we observed minimal difference in bacteria and archaea community composition (**Figure 1b**). Some functional gene copy numbers did vary between plant species within sites, but the effect of plant species was not uniform across

site and tended to be weaker than site effects (**Tables 2 and 3**, **Figures 3 and 5**). Other studies have reported similar findings, concluding that edaphic properties and large landscape features may obscure plant-microbe relationships (27, 28). While we made an effort to sample the rhizosphere, the plant-affected area may comprise a small percentage of the soil, and our sampling efforts may have been too expansive to capture plant effects (20). Additionally, DNA analysis methods are limited and cannot capture dynamic changes due to radial oxygen leakage on microbial community composition or function. For example, denitrification genes are carried by numerous bacterial species, some of which may not express these genes if there is ample O_2 for aerobic respiration (59). Although we hypothesized that *P. australis* would support higher populations of aerobic functional groups such as nitrifying archaea and bacteria, we found little evidence for *amoA* genes. These data suggest that even with radial oxygen leakage the soils stay primarily anaerobic.

Conclusions

Both restoration method and site legacy appear to be important factors affecting microbial community parameters. For example, we documented comparable composition and functional gene abundance between Kenilworth and Kingman in spite of the fact that Kenilworth was restored eight years earlier. The similar restoration methods used to restore Kenilworth and Kingman (use of dredged sediment as substrate) may account for a similar and persistent microbial communities. In contrast, composition in Wootons was significantly different, which may be attributed to the years of soil mining and the method of restoration (excavation to create tidal hydrology). Despite significant urbanization surrounding the Dueling site, microbial community composition was more

similar to Jug Bay than to the three restored sites. We are encouraged that the small remnant wetland appears to maintain a similar microbial community to the suburban natural reference wetland, demonstrating the importance of conserving small TFWs in other urban centers. While plant metrics are commonly used as a proxy for wetland restoration success, our data suggest that differences in plant species, including native versus non-native species, do not strongly affect microbial composition or functional potential, especially in restored wetlands. The main drivers of microbial composition and function appear to be related to substrate, surrounding land use, legacy, and restoration method.

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Table 1. Soil characteristics for each of the five tidal freshwater wetland sites (arithmetic mean ± SE).

Parameter	**Jug Bay**	**Dueling**	**Wootons**	**Kenilworth**	**Kingman**
pH	4.6 ± 0.1	6.0 ± 0.2	6.0 ± 0.1	6.4 ± 0.1	6.3 ± 0.1
SOM	15.5 ± 2.3	6.1± 0.6	6.7 ± 0.4	5.0 ± 0.9	2.5 ± 0.6
TOTAL C	7.8 ± 1.4	2.8 ± 0.4	2.9 ± 0.2	3.1 ± 0.4	1.3 ± 0.3
TOTAL N	0.57 ± 0.10	0.19 ± 0.04	0.21 ± 0.01	0.18 ± 0.03	0.05 ± 0.02
NH_4-N	16.2 ± 2.4	8.0 ± 0.9	9.8 ± 0.5	12.0 ± 2.6	5.9 ± 0.9
NO_3-N	1.4 ± 0.1	1.6 ± 0.1	4.9 ± 1.9	1.3 ± 0.1	1.5 ± 0.1

Table 2. Results of ANOVA testing variation in soil characteristics and functional genes among sites, plant species, and the site x plant interaction. A SAS PROC MIXED model was used to evaluate the whole plot completely randomized design. Degrees of freedom for the numerator (ndf) and denominator (ddf) were calculated using the Satterthwaite approximation. Significance indicated as *p ≤ 0.05 or +p ≤ 0.1.

	Site				**Plant**				**Site*Plant**			
Parameter	ndf	ddf	F	p-value	ndf	ddf	F	P	ndf	ddf	F	p-value
Edaphic Characteristics												
pH	4	10.3	47.3	<0. 01*	3	28.3	2.5	0.08+	11	28.3	2.4	0.03*
SOM	4	38.0	17.1	<0.01*	3	38.0	1.6	0.20	11	38.0	0.8	0.66
TOTALC	4	38.0	13.1	<0.01*	3	38.0	1.8	0.16	11	38.0	0.8	0.63
TOTALN	4	38.0	16.0	<0.01*	3	38.0	2.0	0.13	11	38.0	0.8	0.64
NH_4-N	4	10.5	6.8	0.01*	3	28.5	1.7	0.19	11	28.5	0.7	0.76
NO_3-N	4	38.0	10.4	<0.01*	3	38.0	2.2	0.11	11	38.0	1.6	0.16
Functional Genes												
EUB	4	10.4	3.7	0.04*	3	27.7	1.3	0.29	11	27.7	2.6	0.02*
ARC	4	36.0	13.5	<0.01*	3	36.0	1.0	0.40	11	36.0	1.8	0.08+
mcrA	4	10.1	3.8	0.04*	3	27.4	0.5	0.67	11	27.3	0.7	0.71
pmoA	4	35.0	6.0	<0.01*	3	35.0	0.9	0.43	11	35.0	1.4	0.23
nirK	4	10.5	15.4	<0.01*	3	27.8	4.3	0.01*	11	27.8	5.8	<0.01*
nirS	4	10.8	5.4	0.01*	3	28.2	0.7	0.56	11	28.1	2.1	0.06+
nosZ	4	36.0	7.1	<0.01*	3	36.0	0.4	0.76	11	36.0	2.9	0.01*

Table 3. Results of ANOVA simple effects tests of plant species within each site for soil characteristics and functional genes. Degrees of freedom for the numerator (ndf) and denominator (ddf) were calculated using the Satterthwaite approximation. Significance indicated as *p ≤ 0.05 or +p ≤ 0.1.

Site	Jug Bay				Dueling				Wootons				Kenilworth				Kingman			
Parameter	ndf	ddf	F	p-value	ndf	ddf	F	p-value	ndf	ddf	F	p-value	ndf	ddf	F	p-value	ndf	ddf	F	p-value
Soil Characteristics																				
pH	3	28.3	3.0	0.05*	3	28.3	5.0	<0.01*	2	28.3	1.0	0.39	3	28.3	1.7	0.19	3	28.3	0.9	0.45
SOM	3	38.0	0.9	0.44	3	38.0	0.3	0.86	2	38.0	0.1	0.88	3	38.0	1.1	0.35	3	38.0	2.1	0.12
TOTALC	3	38.0	1.3	0.30	3	38.0	0.6	0.64	2	38.0	0.2	0.84	3	38.0	1.0	0.42	3	38.0	1.7	0.19
TOTALN	3	38.0	0.8	0.50	3	38.0	0.6	0.61	2	38.0	0.1	0.91	3	38.0	0.9	0.44	3	38.0	2.5	0.07+
NH_4-N	3	28.5	1.4	0.27	3	28.5	0.5	0.67	2	28.5	0.1	0.91	3	28.5	1.0	0.43	3	28.5	1.2	0.33
NO_3-N	3	38.0	0.4	0.73	3	38.0	0.5	0.72	2	38.0	9.7	<0.01*	3	38.0	0.2	0.90	3	38.0	0.3	0.83
Functional Genes																				
EUB	3	27.4	0.5	0.68	3	27.4	0.7	0.56	2	27.4	2.0	0.16	3	27.4	4.5	0.01*	3	28.4	3.3	0.04*
ARC	3	36.0	0.4	0.74	3	36.0	1.5	0.23	2	36.0	0.2	0.86	3	36.0	2.8	0.06+	3	36.0	2.6	0.07+
mcrA	3	27.1	0.6	0.65	3	27.1	1.5	0.24	2	27.1	1.1	0.34	3	27.1	0.1	0.96	3	28.1	0.3	0.84
pmoA	3	35.0	2.4	0.09+	3	35.0	0.8	0.50	2	35.0	0.4	0.65	3	35.0	1.7	0.18	3	35.0	0.6	0.60
nirK	3	27.6	1.7	0.19	3	27.6	14.3	<0.01*	2	27.6	3.6	0.04*	3	27.6	1.8	0.17	3	28.6	5.3	<0.01*
nirS	3	27.9	2.8	0.06+	3	27.9	1.0	0.42	2	27.9	1.0	0.39	3	27.9	2.3	0.10+	3	28.9	1.5	0.24
nosZ	3	36.0	1.2	0.32	3	36.0	1.4	0.27	2	36.0	3.8	0.03*	3	36.0	2.6	0.07+	3	36.0	3.4	0.03*

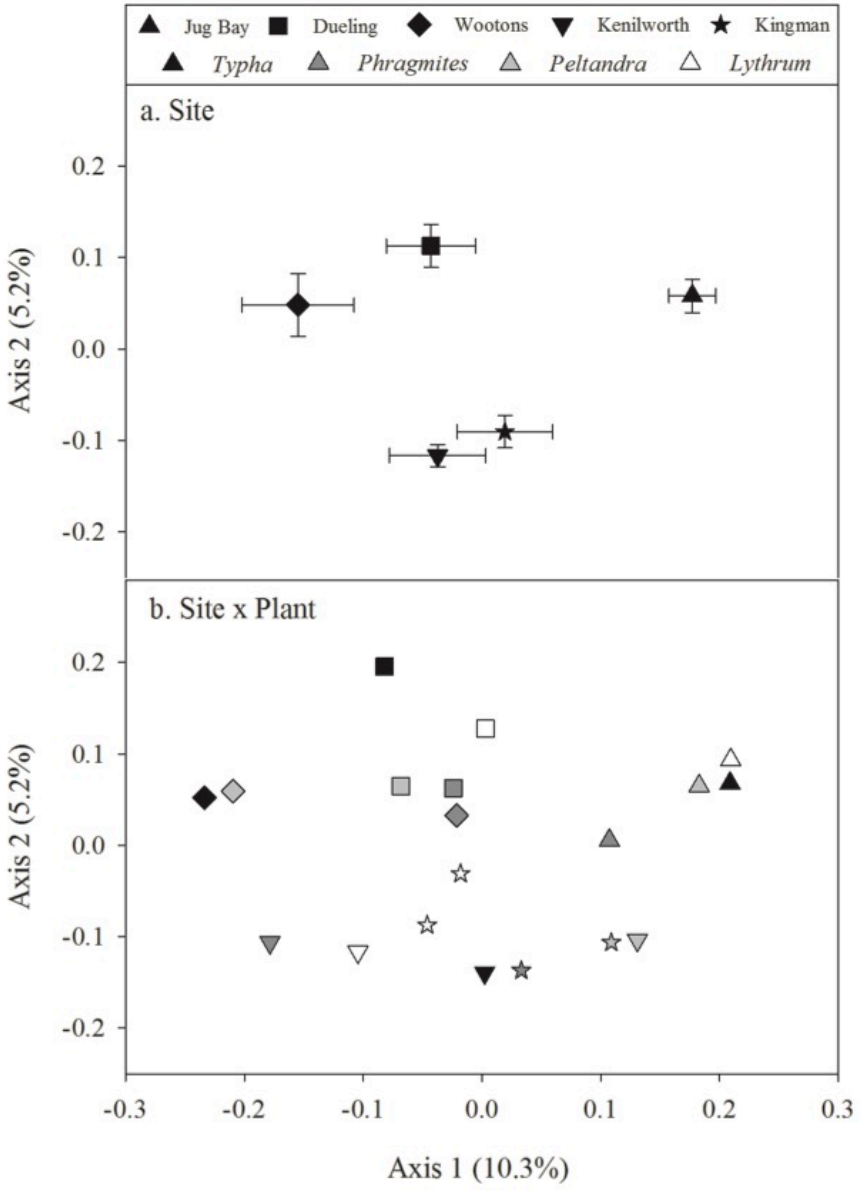

Figure 1. Principle components analysis (PCoA) ordination of the microbial community composition rarified to 1922 sequences per sample. Mean relative abundance±SE is plotted by (a) Site (n =12) and (b) Site by plant species (n=3).

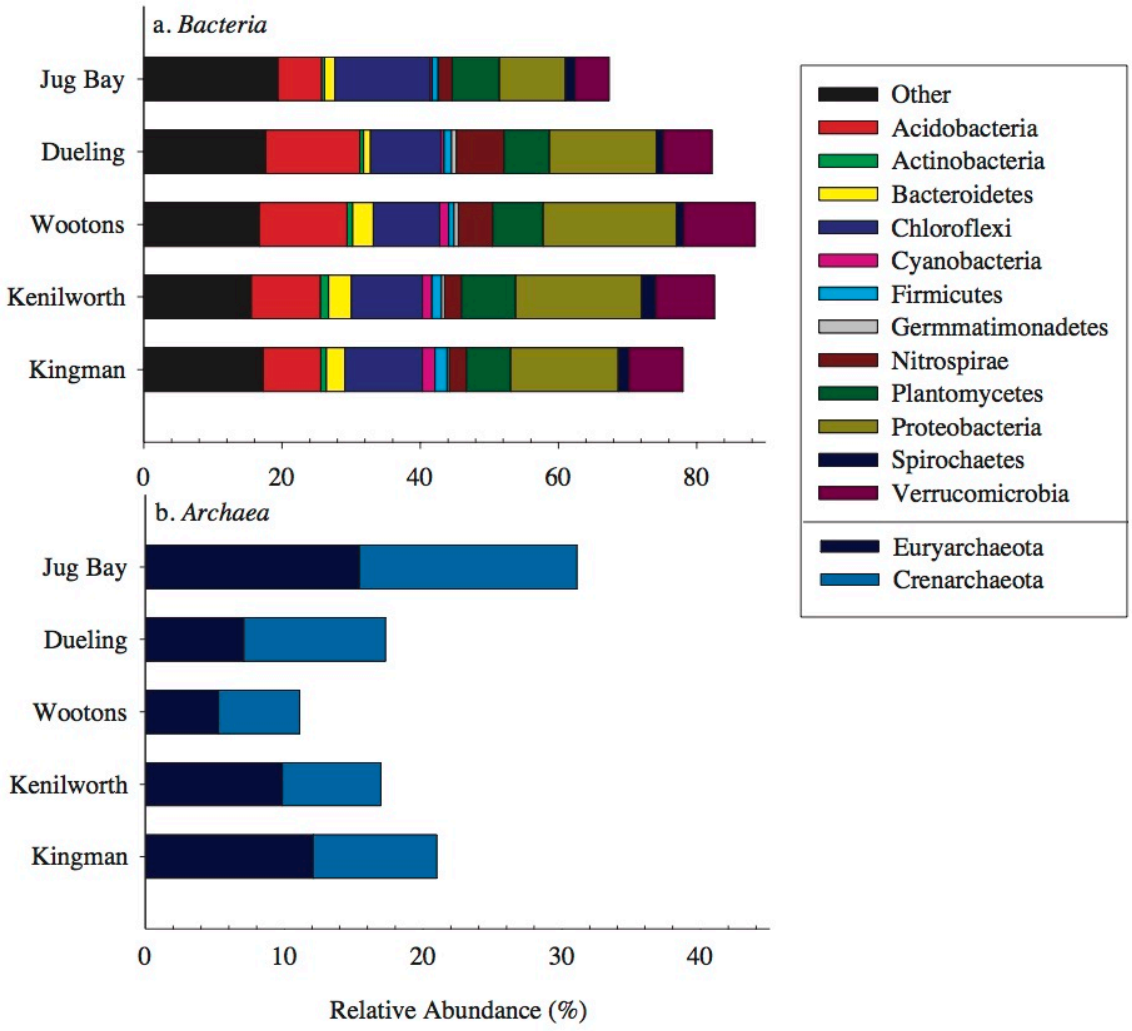

Figure 2. Percent relative abundance of (a) Bacteria and (b) Archaea for five freshwater tidal wetlands (n=12). The top twelve phyla in (a) represent the majority of the total bacterial sequences across all five sites (48-72%). The "Other" category in (a) represents the sum of 59 additional phyla, with 5% of the bar accounting for unclassified bacteria. The two major phyla in (b) represent 99% of the total identified archaeal sequences. Unclassified archaeal sequences are not shown.

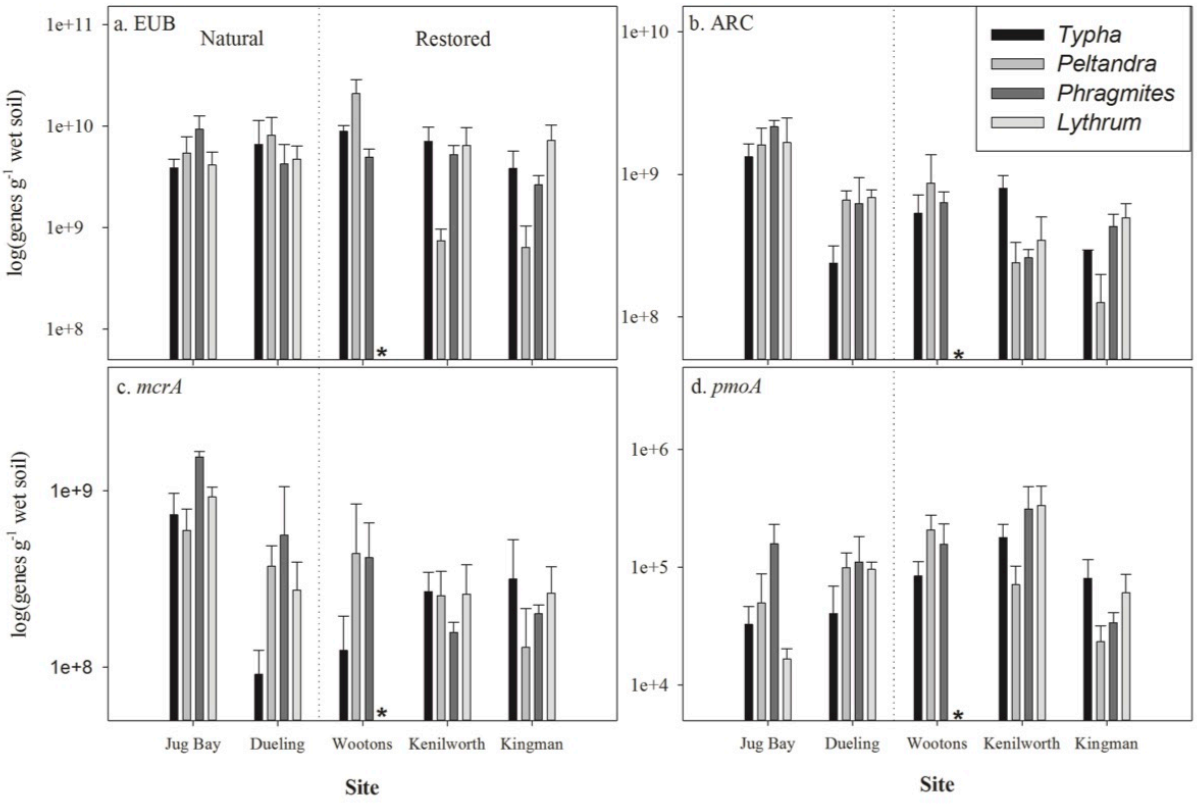

Figure 3. Gene copy numbers g-1 of wet soil for genes targeting (a) Bacterial 16S rRNA, (b) Archaeal 16S rRNA, (c) Methyl coenzyme A reductase (mcrA), and (d) Particulate methane monooxygenase (pmoA). Values were calculated based on a linearized plasmid standard and efficiencies were adjusted with a soil standard to account for inhibition. Each bar represents the mean (n=3) ±SE. Note: panels have different y-axis ranges and stars denote missing Lythrum at Wootons.

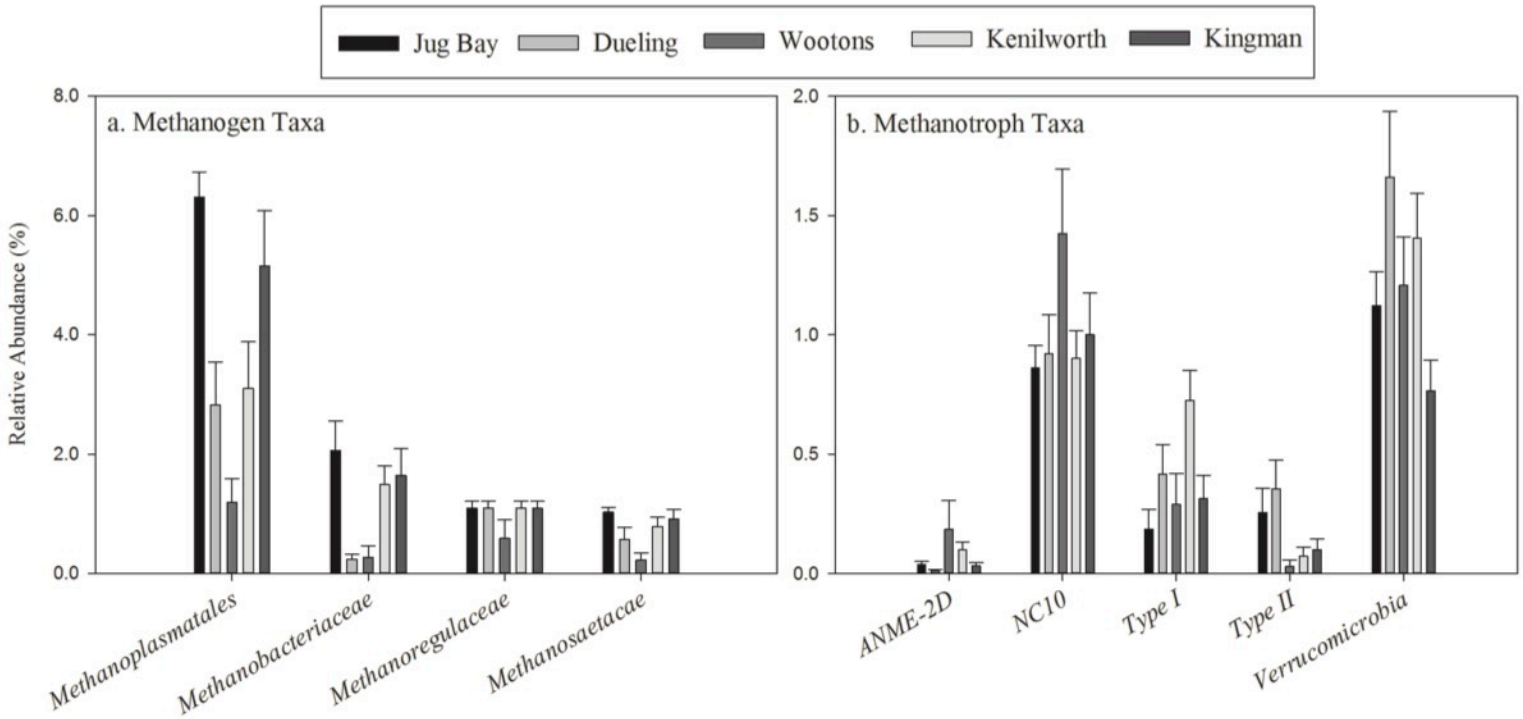

Figure 4. The percent relative abundance of sequences putatively identified as belonging to (a) methanogen and (b) methanotroph taxa; bars represent the site mean (n=12) ±SE. Methanoplasmatales represent the recently reclassified Thermoplasmata (47).

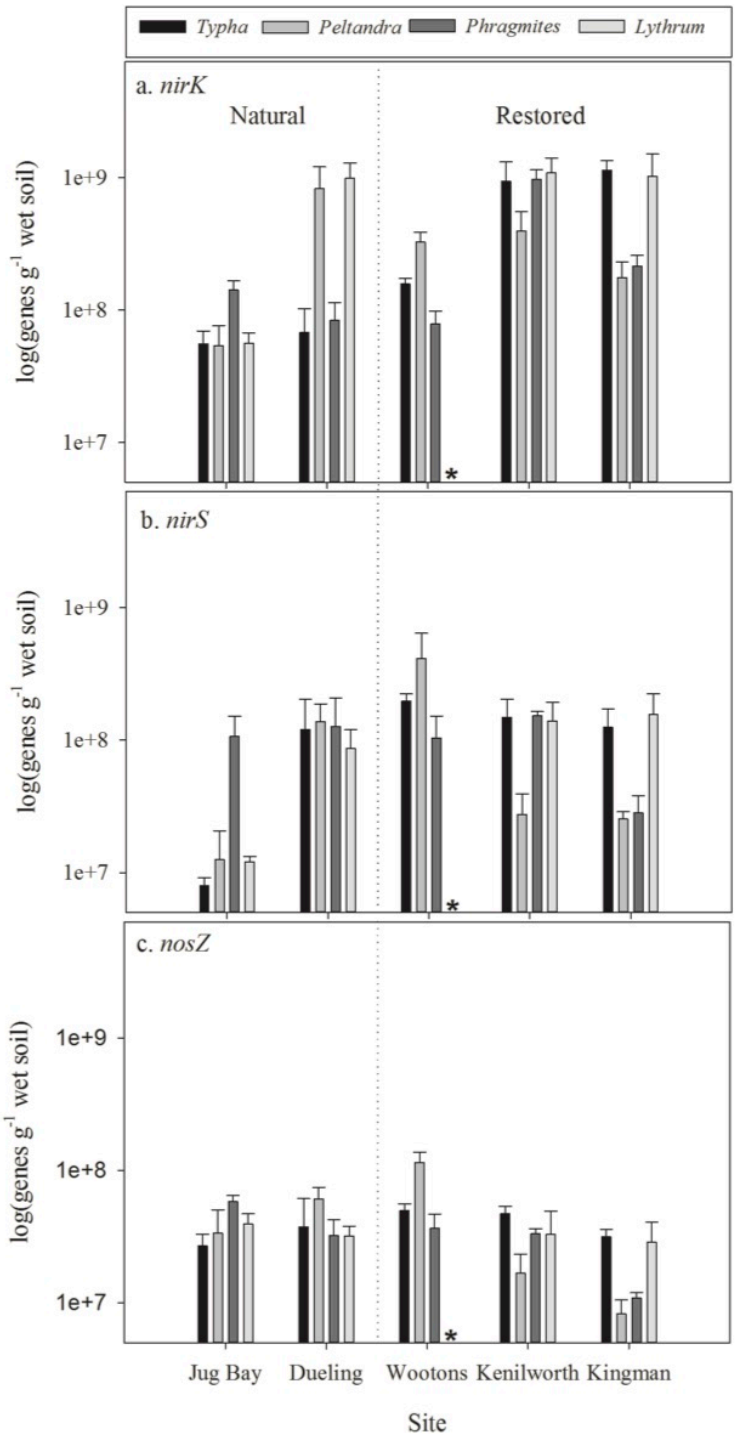

Figure 5. Gene copy numbers g^{-1} of wet soil for genes targeting (a) nitric oxide reductase (nirK), (b) nitric oxide reductase (nirS), and (c) nitrous oxide reductase (nosZ). Values were calculated based on a linearized plasmid standard and efficiencies were adjusted with a soil standard to account for inhibition. Each bar represents the mean (n=3) ±SE. Note: stars denote missing Lythrum at Wootons.

Supplemental Table 1: Conditions for quantifying bacterial and archaeal communities, as well as seven functional genes via quantitative PCR (Q-PCR).

Target	Gene	Pure Culture	Primers & References (Forward/Reverse)	Thermocycler Conditions (Acquisition Step Bolded)	Number of Cycles	Plasmid standard and soil correction efficiency (%) (r^2 values)
EUB	Bacteria 16S rRNA	*Escherichia coli*	EUB338 (1) EUB518 (1)	95°C for 5 min 95°C for 5 s / 55°C for 15 s / **72°C for 10 s**	1 40	97, 97, 98; soil = 95% All $r^2 \geq$ 99%
ARC	Archaeal 16S rRNA	*Sulfolobus solfataricus*	A915-for (2) Arc1059r (2)	95°C for 5 min 95°C for 5 s / 55°C for 15 s / **72°C for 10 s**	1 40	97, 95, 97; soil = 94% All $r^2 \geq$ 99%
mcrA	Methyl coenzyme M reductase	*Methanococcus maripaludis*	mcrA_1035F (3) mcrA_1530R (3)	95°C for 5 min 95°C for 30 s / 56°C for 45 s / 72 °C for 60 s / **80°C for 10 s**	1 40	92, 92, 95; soil = 87% All $r^2 \geq$ 99%
pmoA	Particulate methane monoxygense	*Methylosinus trichosporium* OB3b	pmoA-A189 (4) pmoA-mb661 (4)	95°C for 5 min 95°C for 5 s / 60°C for 15 s / **72°C for 10 s**	1 40	96, 96; soil = 112% All $r^2 \geq$ 99%
AOB	Ammonia oxidizer bacteria *amoA*	*Nitrosomonas europaea*	amoA-1F (5) amoA-2R (5)	95°C for 5 min 95°C for 15 s / 58°C for 30 s / **72°C for 60 s**	1 40	90, 87, 78%; soil = (**BDL**)
AOA	Ammonia oxidizer archaea *amoA*	*Nitrosopumilus maritimus*	CrenamoA23f (6) CrenamoA616r (6)	94°C for 5 min 94°C for 30 s / 55°C for 30 s / 72°C for 60 s / **78°C for 10 s** 94°C for 15 s / 55°C for 15 s / 72°C for 30 s / **78°C for 10 s**	1 5 30	 69, 69, 75%; soil = 87% All $r^2 \geq$ 98%
nosZ2	Nitrous oxide reductase	*Pseudomonas stutzeri*	nosZ2F (7) nosZ2R (7)	95°C for 5 min 95°C for 15 s / 65-61°C for 30 s 95°C for 15 s / **60°C for 30 s**	1 5 (**TD**) 40	95, 96; soil =90% All $r^2 \geq$ 99%
nirK	Nitric oxide reductase	*Sinorhizobium meliloti*	nirk876 (8) nirK1040 (8)	95°C for 5 min 95°C for 15 s / 63-59°C for 60 s 95°C for 15 s / **58°C for 60 s**	1 5 (**TD**) 40	96, 97; soil – 72% All $r^2 \geq$ 99%
nirS	Nitric oxide reductase	*Pseudomonas stutzeri*	nirSCD3aF (9) nirSR3cd (9)	95°C for 5 min 95°C for 15 s / 63-59°C for 75 s 95°C for 15 s / **58°C for 75 s**	1 5 (**TD**) 41	105, 109; soil = 93% All $r^2 \geq$ 99%

BDL = Below Detection Limit; **TD** = Touchdown

Supplemental Table 1 (cont.)

1. **Fierer N, Jackson JA, Vilgalys R, Jackson RB.** 2005. Assessment of Soil Microbial Community Structure by Use of Taxon-Specific Quantitative PCR Assays. Appl Environ Microbiol **71:**4117-4120.

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3. **Pereyra LP, Hiibel SR, Prieto Riquelme MV, Reardon KF, Prudent A.** 2010. Detection and quantification of functional genes of cellulose-degrading, fermentative, and sulfate-reducing bacteria and methanogenic archaea. Appl Environ Microbiol **76:** 2192 -2202.

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5. **Aoi Y, Shiramasa Y, Masaki Y, Tsuneda S, Hirata A, Kitayama A, Nagamune T.** 2004. Expression of *amoA* mRNA in wastewater treatment processes examined by competitive RT-PCR. J Biotechnol **111:**111-120.

6. **Nicol GW, Leininger S, Schleper C, Prosser JI.** 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammoia oxidizing archaea and abcteria. Environ Microbiol **10**(11): 2966-2978.

7. **Henry S, Bru D, Stres B, Hallet S, Philippot L.** 2006. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Appl Environ Microbiol **72:**5181-5189.

8. **Henry S, Baudoin E, Lopez-Gutierrez JC, Martin-Laurent F, Brauman A, Philippot L.** 2004. Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. J Microbiol Meth **59:**327-335.

9. **Kandeler E, Deiglmayr K, Tscherko D, Bru D, Philippot L.** 2006. Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. Appl Environ Microbiol **72:**5957-5962.

QIIME COMMANDS AND RESULTS

1. **File Names -** H4SYGNH02.sff, H4SYGNH02.fna, H4SYGNH02.qual

2. **Sequence File**

   Command: check_id_map.py -m 454_Map_SY2_Reverse.txt -o check_id_map_output

   Result: No errors or warnings were found in mapping file.

3. **Explore Data**

   Command: grep -c ">" H4SYGNH02.fna

   Result: 789013 total raw sequences

4. **Trimming Barcodes**

   Command: split_libraries.py -m 454_Map_SY2_Reverse.txt -b 10 -l 210 -L 370 -f H4SYGNH02.fna -q H4SYGNH02.qual -z truncate_remove -o split_library_output_reverseremoved_210_370

   > Note: I trimmed the length of the sequences to target my targeted sequence (291 bp). Because the length outside bounds is the first trimming step, the min and max length is trimmed including the barcode, linker, forward and reverse primer base pairs (total 51) truncate_only script.

   Number raw input seqs 789013

Length outside bounds of 210 and 370	125647
Num ambiguous bases exceeds limit of 615	
Missing Qual Score	0
Mean qual score below minimum of 25	4687
Max homopolymer run exceeds limit of 6	14613
Num mismatches in primer exceeds limit of 0:	83267

   Number of sequences with identifiable barcode but without identifiable reverse primer: 82604

   -z truncate_remove option enabled; sequences without a discernible reverse primer as well as sequences with a valid barcode not found in the mapping file will not be written.

   Sequence length details for all sequences passing quality filters:
   Raw len min/max/avg 219.0/370.0/306.4

Wrote len min/max/avg 166.0/317.0/253.4

Barcodes corrected/not 3113/37
Uncorrected barcodes will not be written to the output fasta file.
Corrected barcodes will be written with the appropriate barcode category.
Corrected but unassigned sequences will not be written unless --
retain_unassigned_reads is enabled.

Total valid barcodes that are not in mapping file 0
Sequences associated with valid barcodes that are not in the mapping file will not be written.

Barcodes in mapping file
Num Samples 57
Sample ct min/max/mean: 1922 / 12346 / 8388.47

Sample	Sequence Count	Barcode
9	12346	TCTCTATGCG
3	11924	AGACGCACTC
6	11683	ATATCGCGAG
11	11134	CATAGTAGTG
40	11047	TACAGATCGT
57	10954	CGCAGTACGA
49	10907	ACAGTATATA
53	10865	AGTATACATA
34	10859	ATAGAGTACT
47	10766	TGACGTATGT
2	10532	ACGCTCGACA
41	10477	TACGCTGTCT
27	10268	ACATACGCGT
30	10168	ACTGTACAGT
37	9975	CGACGTGACT
36	9932	CAGTAGACGT
4	9820	AGCACTGTAG
59	9773	CGTACAGTCA
52	9720	AGCTCACGTA
7	9555	CGTGTCTCTA
56	9525	CGATCGTATA
48	9445	TGTGAGTAGT
12	9224	CGAGAGATAC
32	9220	AGCGTCGTCT
50	9145	ACGCGATCGA
10	9111	TGATACGTCT
33	9042	AGTACGCTAT
39	8898	TACACGTGAT
58	8876	CGCGTATACA
44	8847	TCGCACTAGT
38	8822	TACACACACT
43	8571	TCGATCACGT
29	8538	ACTACTATGT
42	8459	TAGTGTAGAT
51	8370	ACTAGCAGTA
55	8333	AGTGCTACGA
31	8276	AGACTATACT
60	8241	CGTACTCAGA
46	8148	TCTATACTAT
8	8095	CTCGCGTGTC
1	7483	ACGAGTGCGT
28	7399	ACGCGAGTAT
18	7222	ACGACTACAG
54	6567	AGTCGAGAGA
35	6559	CACGCTACGT
21	6488	TACTCTCGTG
17	6411	TGTACTACTC
5	5784	ATCAGACACG
45	5560	TCTAGCGACT
15	5475	CGTCTAGTAC
19	5169	CGTAGACTAG
26	4615	TCGTCGCTCG
16	4424	TCTACGTAGC
13	3326	ATACGACGTA
20	3297	TACGAGTATG
14	2551	TCACGTACTA
25	1922	TAGAGACGAG

Total number seqs written 478143

5. **Picking OTUS**

Command: pick_otus.py -i split_library_output_reverseremoved_210_370/seqs.fna

Command: pick_rep_set.py -i uclust_picked_otus/seqs_otus.txt -f split_library_output_reverseremoved_210_370/seqs.fna -o rep_set.fna

6. **Taxonomy & OTU Table**

Command: assign_taxonomy.py -i rep_set.fna -o taxonomy_results/

Command: make_otu_table.py -i uclust_picked_otus/seqs_otus.txt -t taxonomy_results/rep_set_tax_assignments.txt -o otu_table.biom

Command: convert_biom.py -i otu_table.biom -o otu_table_tabseparated.txt -b --header_key taxonomy --output_metadata_id "Consensus Lineage"

Command: summarize_taxa.py -i otu_table.biom -o taxonomy_summaries/
Notes #1: Results reported as relative abundance
Notes #2: L2 – Kingdom; L3 = Class; L4 = Order; L5 = Family, L6 = Genus

7. **Alignments & Trees**

Command: align_seqs.py -i rep_set.fna -o alignment/

Command: filter_alignment.py -i alignment/rep_set_aligned.fasta -o alignment/

Command: make_phylogeny.py -i alignment/rep_set_aligned_pfiltered.fasta -o rep_set_tree.tre

8. **Exploring Rarefaction Curves**

Command: alpha_rarefaction.py -i otu_table.biom -o alpharare_500-8000corrected/ -t rep_set_tree.tre -m 454_Map_SY2_Reverse.txt -e 8000 --min_rare_depth 500

- This alpha workflow script produces the rarefaction plots you need to visualize the data's diversity
- --min_rare_depth = 500
- e – max_rare_depth = 8000
    - The resulting file will be use for beta_diversity_through_plots
    - I choose my max_rare_depth based on the mean values reported in the split_library_log.txt
- # of iterations = 10 (default, not specified in code)
- # of steps = calculated by program (min – max/ mean) =750

- Special Note:
    - I did not use the multiple_rarefactions.py script because this step is not necessary needed. The alpha_rarefaction.py workflow script will produce the rarefaction set and the curves you need to evaluate sampling depth.
    - Plotted Observed_SpeciesSite.txt and Observed_SpeciesSite_Plant.txt

9. Beta Diversity

Command: jackknifed_beta_diversity.py -i otu_table.biom -o jackknifed_beta_diversity_1922/ -e **1922** -m 454_Map_SY2_Reverse.txt -t rep_set_tree.tre

Note: The beta_diversity script was set to 1922, the lowest sequence count in my dataset. This value can be found in the split_library_log.txt.

10. Ordination Plots – PCOA Plots

PCoA Plots on Rarified Datasets

Command: beta_diversity_through_plots.py -i otu_table.biom -o bdiv_even1922/ -t rep_set_tree.tre -m 454_Map_SY2_Reverse.txt -e 1922

Note #1: In the bdiv_even1922/ folder, I used the weighted_unifrac_pc.txt file to re-create the PCoA file in Sigma Plot

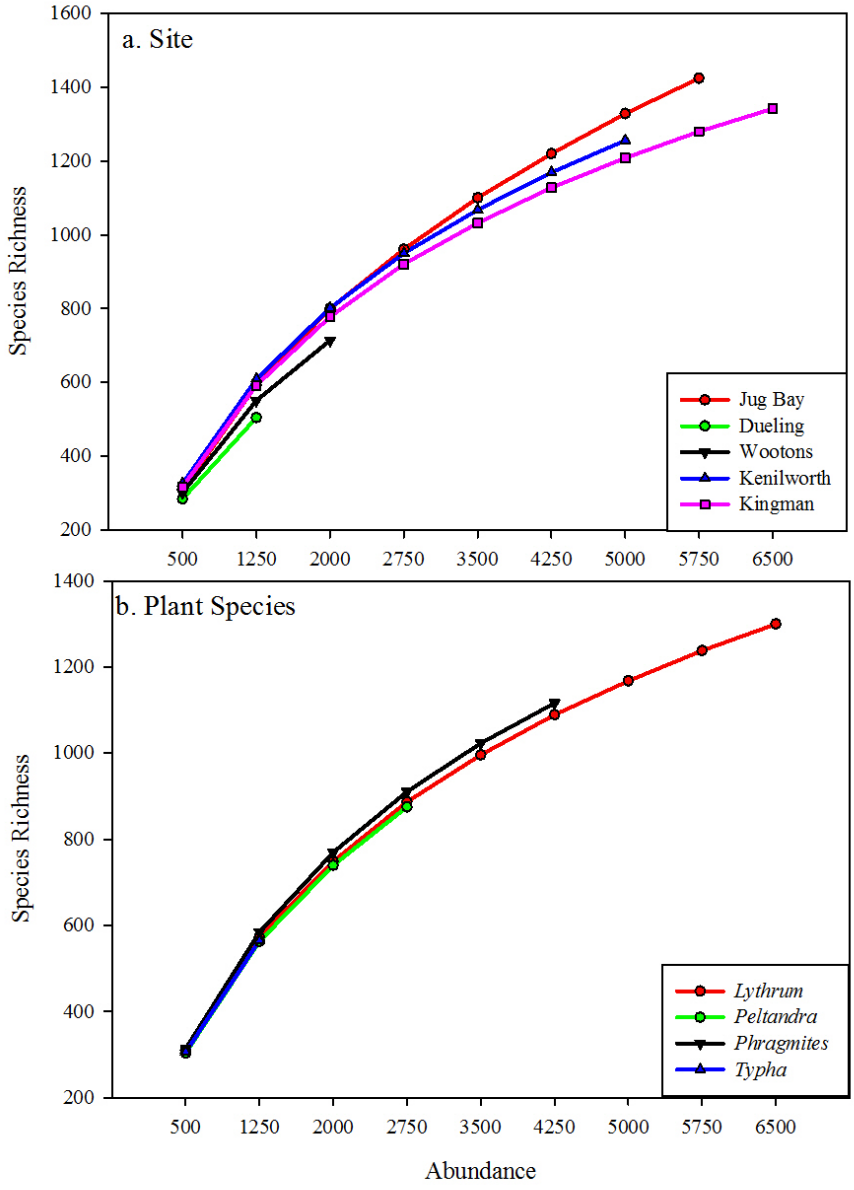

Figure S1. Rarefaction curves of OTU species richness sorted by (a) Site and (b) Plant species.

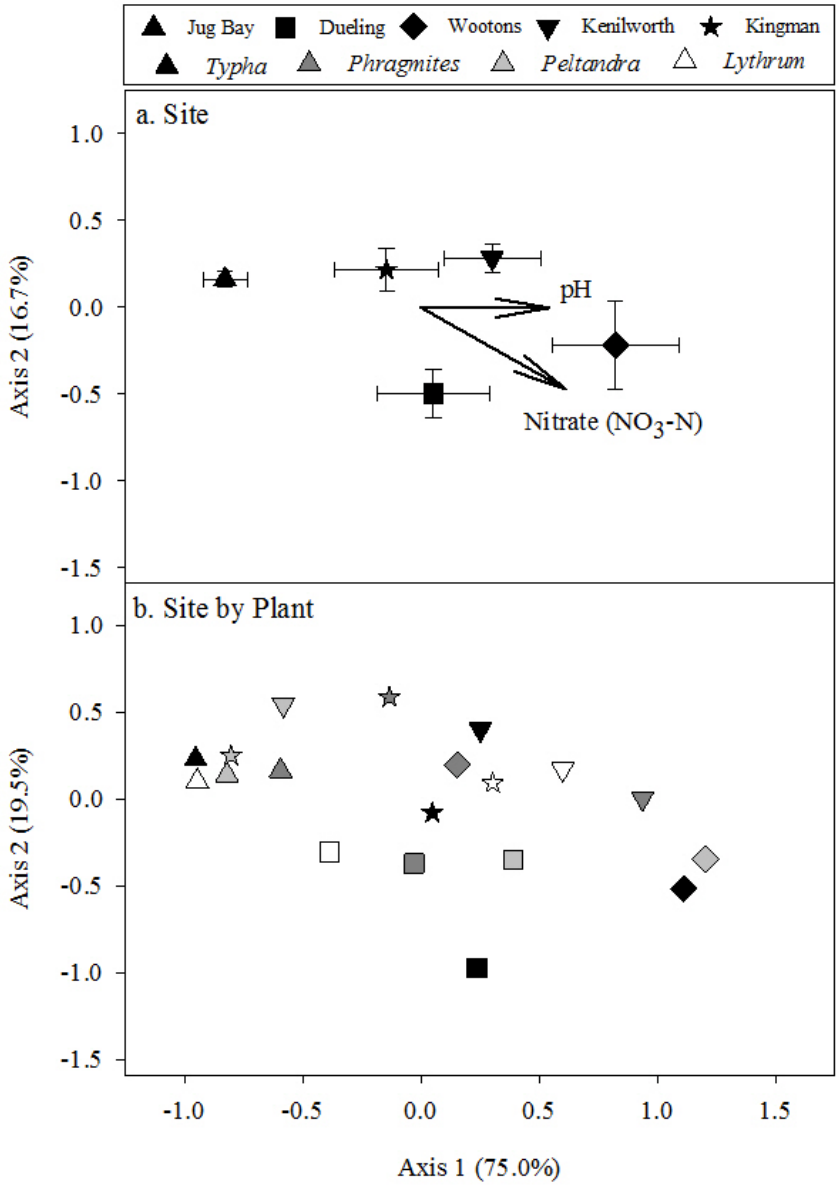

Figure S2. Nonmetric multidimensional scaling (NMS) ordination of the microbial community composition plotted by mean relative abundance ± SE for (a) Site (n = 12) and (b) Site by Plant (n = 3). Multidimensional analysis was performed using a Bray-Curtis distance a Bray-Curtis distance metric using OTUs defined as 97% similarity. Overall stress was 9.7. Vectors represent environmental variable biplots with significant Pearson's correlation coefficients ($\alpha = 0.05$).

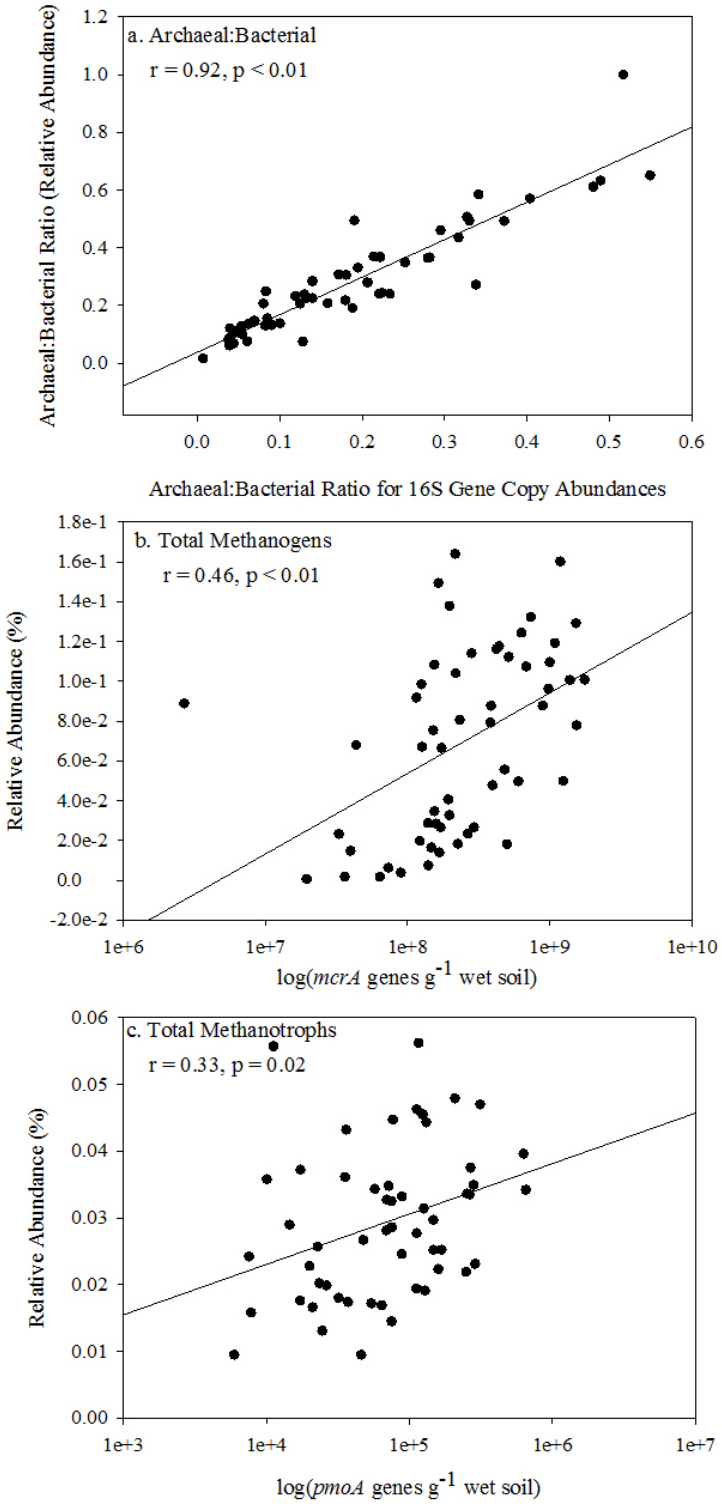

Figure S3. Correlations between Q-PCR gene abundance and 454 sequence data illustrating association between molecular techniques. Each subplot includes Pearson's correlation coefficient and p-values.

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Chapter 3: Distribution of soil carbon associated with water-stable soil aggregates and metals in a natural and restored tidal freshwater wetlands of the Chesapeake Bay region (Maryland, USA)

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Keywords: soil carbon, soil organic matter, chemical fraction, physical fractionation, wet sieving, soil aggregates, organo-metal complexes, stabilization, sequestration, carbon preservation, carbon storage

Abstract

A major goal of wetland restoration focuses on promoting sequestration and long-term storage of carbon (C) as soil organic matter (SOM). Unfortunately, SOM pools in restored wetlands are slow to accumulate even after reestablishing wetland hydrology and plant productivity. This research extends the work of SOM preservation theories in upland terrestrial soils and investigates the association of C with soil aggregates, silt + clay particles, and metal oxides in one natural and one restored tidal freshwater wetland habitat (Anne Arundel County, Maryland). For each site, soils were collected from two soil horizons across three different habitats (low marsh, high marsh, and an adjacent upland region). A wet sieving procedure was used to fractionate bulk soils into five pools: floating particulate organic matter (fPOM), large macroaggregates ($\geq$ 2000 μm),

small macroaggregates (≥ 250 to < 2000 μm), microaggregates (≥ 53 to < 250 μm), and silt + clay minerals (< 53 μm). Total C was quantified for each of the four soil fractions, and the relative abundance of six C compound classes was characterized for all five fractions. A sequential chemical extraction procedure was also used to evaluate the relationship of dissolved organic carbon (DOC) with iron (Fe), aluminum (Al), and manganese (Mn) oxides. The results of this study suggest soils in these two wetlands store soil C differently across habitat and depth. The most striking differences were in the quantity of C associated with each soil aggregate fraction. In soils from the natural site, a majority of the soil C was associated with the large macroaggregate fraction (> 2000 μm), and, in restored soils, most soil C was associated with the small macroaggregate fraction (≥ 250 to < 2000 μm). Organic matter characterization was similar between the two sites with respect to the relative abundance of C compounds across five fractions. For example, a greater abundance of lignin derivatives was associated with fPOM, and more nitrogen-containing compounds were associated with the silt + clay mineral fraction (< 53 μm). We also observed positive associations between extractable DOC, Fe, and Al for both sites, suggesting that DOC-metal complexing may contribute to DOC dynamics in these habitats. This research yielded important findings regarding that macroaggregates are an important mechanisms in soil C formation and preservation. Disruption to the soil profile not only reduces these pools, but also changes the linkages between plant-soil-microbe relationships.

Introduction

Approximately 20 to 25% of the world's organic soil carbon (C) is stored in wetlands as soil organic matter (SOM) (1–3); however, a meta-analysis of 621 wetlands throughout the world revealed restored wetlands store significantly less SOM compared to their natural counterparts (4). The process of decomposition, formation and preservation of SOM is a complicated process regulated by many physical, chemical, and biological factors. For example, plant litter quality, particularly its' nitrogen (N) content, affects SOM mineralization through its' control on carbon use efficiency and allocations of C and N in the soil microbial community (5). Soil microbes are also essential to SOM dynamics because they release extracellular enzymes that catalyze SOM decomposition (6). Limited O_2 availability for microbial respiration has also been linked to lower microbial biomass, enzyme activity, and overall soil C respiration rates (7, 8); regardless of these suppression mechanisms, low O_2 soil conditions do not always reduce enzyme activity or lead to substantial accumulation of SOM (9, 10). New evidence suggests that stabilization of microbial decomposition byproducts on mineral surfaces and storage inside aggregates is the precursor to the long-term stability of SOM (11). Most work examining these mechanisms is conducted in grassland (12, 13), agriculture fields (14–16), and converted ecosystems (17–19). As a result, it is not known if these physical or chemical stabilization mechanisms impact SOM dynamics in natural or restored wetlands.

Stabilization of microbial byproducts in soils depends on particle size distribution, mineralogy, and Fe-, Al-, and Mn-oxide coatings on mineral surfaces. The proportion of primary particles, sand, silt, and clay, affect SOM stabilization in soils. Finer texture

soils, like clay and silt minerals, have a high surface area and provide more reactive surfaces for organic matter to bind via ligand and polyvalent cation bridges (20). Finer textured soils also promote more microbial biomass (21). As a result, most organic matter associated with clay surfaces are the products of microbial decomposition (5, 22, 23); however, the quantity of stabilized organic matter depends on the proportion of clay and its' mineralogy (24). Amorphous and crystalline Fe-, Al-, and Mn-oxide coatings on mineral surfaces can enhance the stability of SOM, even in sandy textured soils (23, 25, 26). The role of these sesquioxides, particularly Fe, is thought to be one of the many mechanisms contributing to SOM stability in mineral soils. Since concentrations of organic matter often exceed the concentration of these sesquioxides, other mechanisms, like soil aggregation, are probably more influential in the long-term stability of SOM.

Soil aggregates are primary particles bound together by polysaccharides, bacteria, and plant debris. The hierarchical formation of aggregates proposed by Tisdale and Oades (27) suggests that organic matter, mucilage, and polyvalent cations bind clay and silt mineral particles into aggregates. Different size aggregates have been shown to stabilize different types of organic matter. For example, C associated with macroaggregates (> 250 μm) resemble plant detritus, and C associated with microaggregates (< 250 μm) tend to be older (100 – 300 y) and highly decomposed (20, 22, 28). Additionally, the quality of C decreases through the different aggregate size fractions – larger aggregates have a higher C: N ratio (~20) compared to smaller aggregates (~8) (20). In natural non-wetland systems, macroaggregates (> 250 μm) make up a majority of the soil matrix and contain more C than other fractions (29, 30). Macroaggregates are a relatively dynamic fraction and sensitive to change; studies have

shown that disturbance, like cultivation, significantly reduces the distribution and associated C within this fraction (29, 30). Some contrasting views on the hierarchical theory suggest that microaggregates form macroaggregates (22). If this is the primary mechanism of microaggregates formation, loss of macroaggregates will also diminish organic matter stability across multiple aggregate pools.

Soil aggregates and organic matter complexes with mineral surfaces and metals (Fe-, Al-, and Mn-oxides) are thought to be negligible in wetland soils. However, a study investigating aggregate-associated C in a drained and converted Histosol soil (85% SOM) demonstrated more C was associated with the macroaggregate pool (30). Additionally, a study investigating SOM stabilization across eight of the twelve soil orders (i.e., Entisols, Inceptisols, Alfisols, Mollisol, Ultisols, Spodosols, Andisols, and Oxisols) found organo-Fe complexes were particularly important in low pH, organic-rich soils (31). Given that many freshwater wetland soils are organic-rich and mildly acidic, organo-Fe complexes may be a dominant stabilizing mechanism.

This project examined the quantity of C and the chemical composition of organic matter associated with soil aggregates, reactive mineral particles (i.e., silt and clay), and metal oxides in contrasting tidal freshwater wetlands. Tidal freshwater wetlands were selected for this study because soils in these habitats are formed by clay, silt, and organic matter deposits along the banks of rivers (32) and thus should meet the minimum criteria for aggregate formation (28). Research plots were established in the low and high marsh habitats of one restored and one natural tidal freshwater wetland. Plots were established across the marsh habitat to account for differences in plant community composition and observed levels of soil C. Soils were also collected from research plots established in an

adjacent upland habitat to validate the findings of this study with other published literature. We hypothesized soils with higher levels of soil C would have more macroaggregates and greater associations with Fe-, Al-, and Mn-oxides. We also expected to find a greater abundance of N-containing compounds associated with the mineral fraction. And lastly, we expected less water-stable macroaggregates in restored tidal freshwater soils compared to its' natural counterpart.

Materials and Methods

Experimental Design

Research plots were established in contrasting tidal freshwater wetlands: Patuxent Wetlands Park ("Pax Park") and Wootons Landing Wetland Park ("Wootons"). Within each field site, three replicate plots were established in three distinct habitats: low marsh (LM), high marsh (HM), and an adjacent upland area (UP). In each subplot, the duff layer was removed, and two soil biscuits were collected with a 40 cm spade, except soils from all six UP plots were collected with a bucket auger. Soil horizons were identified based on soil color, texture, and redoximorphic features. Subsamples were collected from the top two dominant soil horizons and separately composited into "upper" and "lower" samples; transition layers were excluded. A total of 36 soil samples (2 sites × 3 habitats × 2 horizons × 3 replicate plots) were collected and stored at 4°C until further analysis.

Site Description

Patuxent Wetlands Park ("Pax Park") and Wootons Landing Wetland Park ("Wootons") are located along the Patuxent River in Anne Arundel County, Maryland (**Figure 1**). Pax Park (N 38°51' 20.9" N, 76°41'27.3" W) is a naturally occurring tidal

freshwater wetland that shares many common characteristics of east coast tidal freshwater wetlands including the nearby and highly characterized Jug Bay Wetlands Sanctuary (32, 33). Wootons (38°51’ 20.9” N, 76°41’27.3” W) is located approximately four nautical miles north of Pax Park. Wootons is a restored marsh primarily composed of non-tidal forested wetlands, except a small section of tidal freshwater wetlands (~2.4 acres) is located near the southwest portion of the marsh (34). Before restoration, the site was mined for sand and gravel until it was exhausted in 1973. The site was turned over to Anne Arundel County, MD and unmanaged for approximately two decades until the Maryland State Highway began restoration in 1992 (34–36).

Soils in Wootons are shallow and primarily composed of “greensands”, or glauconite pellets; fossilized oyster shells and large rocks are also deposited throughout the soil profile. Marsh soils in Wootons (i.e., LM and HM) have not been classified by the USDA-NRCS, but the UP site was classified as Udorthents (UpB) (37). Marsh soils in Pax Park (i.e., LM and HM) occurred in delineations of soil map units named for Mispillion and Transquaking (MZA) series, and UP soils named for the Collington, Wist, and Westphalia (CSE) complex (37). Representative soil samples from each site and habitat were collected with a bucket auger to corroborate USDA-NRCS soil profile descriptions (**Supplemental Table 1 and Supplemental Figure 1**).

Soil Physicochemical Properties

Gravimetric moisture content (W_d) was determined by drying 20 to 50 g of wet soil at 105°C for 36 h; triplicate analytical replicates were prepared for each soil sample. Gravimetric moisture content was calculated using the following formula: Wet Soil Weight (g) − Dry Soil Weight (g) ÷ Wet Soil Weight (g) × 100.

Soil organic matter content was determined via loss on ignition (38). Briefly, subsamples from the moisture analysis were ground to pass through a 0.45 mm sieve; sieving removed large pieces of particulate organic matter (POM), roots, and rocks. Approximately 2.0 g of air-dried soils were placed into clean, pre-weighed porcelain crucibles, oven-dried at 70°C for 24 h, and then cooled to room temperature in a desiccator with fresh desiccant; crucibles containing soils were weighed to the nearest 0.0001 g. Samples were baked at 400°C for 16 h in a programmable Isotemp muffle furnace (Model 550-58, Fisher Scientific, Pittsburg, PA) and then cooled to 70°C. Baked samples were removed from 70°C and then stored in a desiccator with fresh desiccant for 24 h. After cooling samples to room temperature, baked soil samples were re-weighed to the nearest 0.0001 g. Soil organic matter (%, w/w) was calculated using the following formula: Dry Soil Weight (g) − Ash Soil Weight (g) ÷ Dry Soil Weight (g) × 100).

Total C and N for bulk soil samples were determined by combusting 200 ± 5 mg of ground soil at 950°C on a LECO CHN-2000 analyzer (LECO Corp, St. Joseph, MI). Two Leco standards – barley flour (% C content: 44.66 ± 0.40; %N: 1.74 ± 0.02) and soil mineral #309 (%C: 8.92 ± 0.08; %N: 0.77 ± 0.024) – were used to extrapolate total C and N content for unknown soil samples. An additional in-house standard (Othello silt loam, <60 mesh, 1.27% C, and 0.1100% N) was used to verify the quality of each run. Nitrogen values falling below the machine's detection limit were reported as 0.01%.

Following procedures outlined in Howard et al. (39), total C values were averaged for the top 40 cm of the LM and HM habitats and used to estimate soil C stocks for the tidal freshwater wetland region of each park. In 2015, five soil cores were collected from the high marsh zone and used to estimate bulk density for Wootons; bulk density for this

region was 0.45 ± 0.16 g cm^{-3}. High fibrous content and high water table made it difficult to collect soils cores from Pax Park; therefore, using the reported bulk density values from the National Resources Conservation Service for muck soil textures, a bulk density value of 0.20 g cm^{-3} was used to calculate soil C stocks for Pax Park (40). Web Soil Survey was used to estimate the contiguous sample area of Pax Park; this area was ~35 ha (40).

Soil pH was determined by mixing 1 part field-moist soil with 2 parts 0.01 M calcium chloride ($CaCl_2$). Soil slurries were mixed with a metal spatula for 30 s and then left undisturbed for 7.5 min. After 7.5 min, a double-junction combination pH electrode probe (Fisher Scientific™, Waltham, MA) was submerged into the upper aqueous layer. Soil pH values were recorded once readings stabilized or the total settling time reached 10 min whichever came first. This soil pH procedure was selected because it provided stable and reproducible results (**Supplemental Figure 2**).

Aggregate-size distribution and Aggregate-associated C

Soils were fractionated following the wet sieving protocol outline in Six et al. (29, 41). Briefly, 200 g of wet soils were air-dried for 7 d and then passed through a 4.75 mm sieve. Eighty grams of air-dried soil were placed on top of a 2000 μm sieve and then submerged in distilled water for 5 min. After slaking, aggregates were separated by moving the sieve up and down through the distilled water column (50 repetitions over 2 min). Soils remaining on top of the 2000 μm sieve were transferred to a pre-weighed aluminum tin; floating POM (fPOM) was skimmed from the water surface and stored in a separate pre-weighed tin. Material that passed through the 2000 μm sieve was sequentially passed through 250 μm and 53 μm sieves. All five fractions were air-dried

for 7 d, or until the water completely evaporated from the pan, and then oven dried at 65°C for 3 d. Samples were removed from the oven and immediately weighed to the nearest 0.01 g. The sieving procedure produced five fractions: (1) fPOM, (2) large macroaggreates (≥ 2000 μm), (3) small macroaggregates (≥ 250 to < 2000 μm), (4) microaggregates (≥ 53 to < 250 μm), and (5) silt + clay minerals (< 53 μm).

In addition to the above procedure, a separate supplemental test was conducted to ensure that the mass of recovered macroaggregate fraction was not inflated due to insufficient slaking time. To test the effect of slaking time on recovered macroaggregate mass, three replicate samples from each site and habitat were processed following the wet sieving procedure except soils were incubated in distilled water for 5, 10, or 20 min (n = 54). Soils passing through the 2000 μm sieve were discarded. Recovered large macroaggregates (≥ 2000 μm) were processed following the drying and weighing procedure outlined above (**Supplemental Table 2**).

Total C content was quantified for each of the four individual soil fractions (g C g^{-1} soil). To quantify total C for all soil samples (n = 144), SOM content was determined following loss on ignition procedures. Before combusting samples, soils were ground to pass through a 0.45 mm sieve to remove rocks and POM from the soil matrix; total weight of removed rocks and POM (mostly a few small, dried roots) was subtracted from the starting soil weight (80 g). In addition to the SOM analysis, sixteen ground soil samples were selected and processed on the LECO CHN-2000 analyzer (see *Edaphic Analysis*). These sixteen samples were selected because they represented the entire range of observed SOM values. Soil organic matter and total C values for the sixteen samples

were used to produce a predictive linear equation and used to extrapolate total C content for the remaining 128 samples (**Supplemental Figure 3**).

Organic matter chemical composition was determined for all five fractions using pyrolysis-gas chromatography coupled with mass spectrometry (pyrolysis GC/MS) following procedures outlined in Spargo et al. (15). Starting soil weights for each fraction were adjusted to minimize background noise and produce a satisfactory signal.

Organo-metal complexes

In addition to the physical fractionation procedure, finely ground bulk soil samples from Pax Park and Wootons were chemically fractionated to assess the association of DOC with Al, Fe, and Mn metals. The following procedure was developed from a series of published protocols and modified to address the pros and cons of available techniques (26, 42, 43). To verify the reliability of this procedure, soils from an Atsion Bh (44) and a Christiana Bs (45) were concurrently extracted (data not shown). All solutions were prepared with nanopure water (18.2 MΩ × cm).

Soils were chemically fractionated by first mixing 0.5 g of air-dried soil with 25 mL of 1.0 M potassium chloride (KCl). The KCl extraction removes easily exchangeable DOC and metals thus reducing overestimation of DOC and other metals in successive pools. Soil-KCl slurries were shaken on an Eberbach™ Corp 115V, 60 cycles reciprocal shaker table (Model E6000, Eberbach Corporation, Ann Arbor, MI) for 16 h at 47 opm. Samples were then centrifuged for 5 min at 16,000 rpm in a Sorvall RC-5B refrigerated super speed centrifuge (GMI, Inc, Ramnsey, MN); temperatures were held between 18°C and 25°C. The resulting supernatant was transferred to a 120 mL graduated sterile specimen cup (Fisher Scientific™, Waltham, MA). The remaining soil pellet was

extracted one additional time with 1.0 M KCl except soil-KCl slurries were mixed for 1 h rather than 16 h. The resulting supernatant was mixed with the first extraction and the final volume was recorded to the nearest ± 1.0 mL. The soil pellet was resuspended in 25 mL of nanopure water and then shaken for 15 min to assist in removing residual salts. To maximize soil recovery, the soil-water mixture was centrifuged for 5 min at 18,000 rpm. The washing procedure was repeated two additional times before proceeding to the next extraction step.

Following the washing procedure, wet soil pellets were resuspended in 0.1 M sodium pyrophosphate ($Na_4P_2O_7$ □ 10 H_2O, pH 10; "Pyro"), a chelating agent that solubilizes organic matter complexed with metal sesquioxides (46). The extraction procedure followed similar steps as previously described except soils were extracted three times with 0.1 M $Na_4P_2O_7$ □ 10 H_2O. Following the Pyro extraction and washing procedure, soils were extracted one additional time with 25 ml of 0.05 M sodium dithionite ($Na_2S_2O_4$) for 16 h at room temperature. Sodium dithionite ("Dit") has been demonstrated to extract amorphous to strongly crystalline Fe and Al sesquioxides (26). Following extraction, soil pellets were resuspended in 25 mL of 0.05 M hydrochloric acid (HCl) and mixed for 1 h. Supernatant pH values were recorded prior to and post-mixing Dit with HCl extractions (data not shown).

The final soil pellet was washed three times with nanopure water and then transferred to a pre-weighed aluminum tin. Pellets were oven dried for 36 h at 105°C and re-weighed to the nearest 0.0001 g. Total C and N content for the final pellet was determined following CHN procedures outline in *Edaphic Analysis*. All extracts were

filtered sterilized using a 0.2 μm nylon syringe filter (Fisher Scientific™, Waltham, MA) and then stored at 4°C until analysis.

Each solution was measured for DOC as well as the concentration of three metals (Al, Fe, and Mn). A TOC/TN Analyzer (Shimadzu Corporation, Kyoto, Japan) was used to quantify DOC content. Dextrose was used to generate DOC standards curves (0.1 – 100 mg L^{-1}), and nanopure water served as a blank. Freshly prepared solutions of KCl, Pyro, Dit, and HCl were also run to quantify C associated with the matrix solution. Metals were analyzed on a Perkin Elmer AAnalsyt 400 Atomic Absorption Spectroscopy (AAS; Waltham, MA). Alfa Aesar Fe (#88073), Mn (#88078), and Al (#33557) standards (0.1 – 50 mg L^{-1}) were prepared in corresponding matrix solution, and the appropriate matrix solution served as the blank (0.0 mg L^{-1}). If necessary, samples were diluted with nanopure water to fall within the standard range. Standard curves were analyzed before running samples and checked throughout the procedure to ensure accuracy; all R^2 values exceeded 99.8%. Final undiluted values were corrected for solution background and then converted to represent total DOC or metal concentration g^{-1} soil.

Statistical Analysis

Data exploration and statistical analysis were completed using R for Mac OS X version 3.3.2 (47) and JMP® Pro 12.2.0 (48). Figures were generated in SigmaPlot for Windows version 10.0.0.54 (Systat Software, Inc., San Jose, CA).

A generalized linear mixed-effects model (GLMM) accounting for horizon nested in habitat and habitat nested within site would have been the most appropriate model to evaluate this dataset; however, this was an exploratory study, and a limited number of samples were collected from the field. As a result, there was not sufficient replication to

analyze this dataset using the full GLMM model. Given that these two sites were selected based on the known difference in soil parameters, the dataset was split by site, and a mixed-effects repeated measures analysis of variance (ANOVA) model was used to assess mean differences by habitat, horizon, fraction and their interactions ($\alpha = 0.05$). Additionally, a two-way ANOVA was used to evaluate mean differences in soil physicochemical parameters (i.e., soil pH, SOM, total C, and total N).

Before statistical analysis, all continuous variables were screened for normality assumptions and, if necessary, were $\log_{10}$ transformed. In cases where response variables included a value of 0, a $\log_{10}(Y + 1)$ was applied. Outliers that resulted in methodological error were removed from the dataset. Missing values were not imputed because mixed-effects repeated measures models can correctly compute statistics with unbalanced datasets. Differences for $\log_{10}$-transformed datasets were calculated using back-transformed least square means. In cases of a significant model, Tukey's honest significant differences (HSD) post hoc test was used to evaluate mean differences. Given the large number of comparisons associated with a significant three-way interaction (i.e., habitat × horizon × fraction), least squares (ls) means contrast with a Bonferroni corrected alpha was used to reduce Type I and Type II errors.

To visualize differences in the data generated by pyrolysis GC-MS, a nonmetric multidimensional scaling (NMS) was performed in PC-ORD version 5.10 (49) using the relative abundance of six chemical compound classes: (1) carboxylics, (2) cyclics, (3) aliphatics, (4) lignin derivatives, (5) carbohydrates derivatives, and (6) N-containing compounds. The analysis was performed using the Sorenson/Bray Curtis distance metric and random starting configurations with 250 runs with real data. For Pax Park and

Wootons, a final two-dimensional solution with a stress value of 13.3 (mean = 15.9) and 11.0 (mean = 13.5), respectively, was used for subsequent analysis. For each site set, a separate multi-response permutation procedure (MRPP) was used to test for difference between sample units based on within-group similarities (50).

Results

A summary of the model statistics (i.e., percent variance explained, F-statistics, degrees of freedom, and p-values) is presented in **Table 1** and **Supplemental Table 3**.

Soil Physicochemical Properties

Soils from Pax Park were slightly acidic and similar across habitats and horizons (**Table 2**). Unlike soil pH, mean SOM, total C, and total N content decreased significantly in the order of HM > LM > UP, and nearly twice as much SOM, total C, and total N were observed in upper soil horizons compared to lower soil horizons. For Wootons, soil pH, SOM, total C, and total N content were similar between the two marsh habitats but significantly lower in the adjacent UP habitat. Across the three habitats in Wootons, upper soil horizons had a significantly higher mean concentration of SOM and total C compared to lower soil horizons. Mean total N was significantly greater in HM upper soil horizons compared to soils in the lower HM and upper UP soil horizons. For both sites, soil C: N ratios were similar across habitats but slightly elevated in the upper soil horizon.

Total soil C averages across marsh habitats for Pax Park and Wootons Landing was 10.9% and 3.2%, respectively. Total soil C stocks for the upper 40 cm of Pax Park and Wootons was estimated to be 57 and 87 Mg C ha^{-1}. Based on C stock estimates per hectare, Pax Park has approximately 30 Mg C ha^{-1} more soil C than Wootons. When

accounting for the represented sample area (ha), Pax Park and Wootons soils hold 3052 and 138 Mg C, respectively. Although, these values likely underestimate total soil C stocks because the Oa horizon extends to 2 m in Pax Park whereas Wootons soil profile abruptly ends around 40 cm. If we assume total soil C is similar throughout the top 2 m of Pax Park, then total soil C stocks for Pax Park is 436 Mg C ha^{-1} or 15, 260 Mg C for the whole representative marsh area.

Aggregate-size distribution

At both sites, fPOM isolated from the large macroaggregate fraction (≥ 2000 μm) made up a small proportion of the soil mass, and the log_{10}-mean distribution was relatively similar across the habitats and horizons for each park (≤ 2.0% w/w) (**Table 3 and 4**). Rocks made up 15% to 17% of the UP soil sample and less than 2% of the soil matrix in the LM and HM habitats (**Table 3 and 4**). For both sites, increasing slaking time did not affect the mass of macroaggreates (**Supplemental Table 2**).

Of the four soil aggregate fractions in Pax Park, approximately 40% to 60% of the LM and HM soil separated into the large macroaggregate size fraction (> 2000 μm) (**Table 3**). In contrast, soils from the UP habitat had a greater mass of small macroaggregates (≥ 250 to < 2000 μm). The distribution of the microaggregates (≥ 53 μm to < 250 μm) was similar across the two horizons and three habitats except the lower soil horizons in the UP and LM habitats had approximately two times more mass than upper HM soil horizons. Soil mass in the silt + clay mineral fraction (< 53 um) were similar between habitats and horizons; however, approximately two times more silt + clay was found in the lower soil horizons in the UP, HM and LM habitats compared to the HM and UP upper soil horizons.

Unlike the Pax Park, $\log_{10}$-mean distribution of fPOM, large macroaggregates (≥ 2000 μm), small macroaggregates (≥ 250 to < 2000 μm), and microaggregates (≥ 53 to < 250 μm) were similar across habitats and horizons in Wootons with a few minor exceptions (**Table 4**). For example, soils found in the LM habitat had a significantly higher mass of small macroaggregates (≥ 250 to < 2000 μm) and silt + clay minerals compared to soils in the UP and HM habitats. Overall, the majority of Wootons' soil fractionated into the small macroaggregates (≥ 250 to < 2000 μm) and microaggregates (≥ 53 to < 250 μm) fractions (**Table 4**).

Aggregate-associated C

In Pax Park site, C concentrations (g of C g^{-1} air-dried soil) differed by habitat, horizon, and fraction for larger macroaggregates but not for smaller fractions (**Table 3**). For example, macroaggregate-C concentrations varied across all habitats and horizons except a similar concentration of macroaggregate-C was documented between LM-upper and HM-lower soil horizons. Unlike the macroaggregate fraction, soil C concentration associated with the small macroaggregates (≥ 250 to < 2000 μm) fraction was similar across all habitats, except soil C was lower in UP-lower soil horizon. Mean aggregate-C concentration associated with the microaggregate (≥ 53 to < 250 μm) was also lower in both UP soil horizons compared to tested soil horizons in the marsh. Carbon concentration associated with the silt + clay (< 53 μm) fraction were similar across all habitats and horizons. In Wootons, the distribution of C was similar across the three habitats, two horizons, and four aggregate fractions (**Table 4**).

Looking across the five fractions, a majority of the soil C in Pax Park marsh soils (i.e., LM and HM) was associated with the macroaggregate-size fraction, and, in

comparison, very little soil C was stored UP habitat. The majority of the soil C in Wootons was linked to small macroaggregates (≥ 250 to < 2000 μm) (**Table 4**).

Chemical Composition of Organic Matter

Preliminary analysis revealed organic matter chemistry was similar between the upper and lower soil samples (data not shown); therefore, lower samples were excluded from analysis. The median (± SD) soil weight of extracted data (upper horizon only) was 3.0 ± 0.7 mg for fPOM, 6.7 ± 1.3 mg for large macroaggregates (> 2000 μm), 8.6 ± 5.6 mg for small macroaggregates (≥ 250 to < 2000 μm), 9.5 ± 5.8 mg for microaggregates (≥ 53 to < 250 μm), and 15.3 ± 2.9 mg for the silt + clay mineral fraction (< 53 μm). Slightly more mass was required to produce adequate data for Wootons samples.

An ordination of the six chemical compounds classes revealed strong separation across the five fractions and not by habitat or the horizon (**Figure 2**). The strongest separation was observed between fPOM and the four aggregate size classes. Floating POM samples had a higher abundance of cyclic (~25%) and lignin derivatives (~50%) compounds, and relatively equal abundance of carboxylics, aliphatics, and carbohydrate derivatives (~10% to 20%); the relative abundance of N-containing compounds was quite low (< 2%, **Figure 3A**). Trends across the six chemical compound classes were similar among the four soil fractions; this is likely due to the similar abundance carbohydrate derivatives (~10%), cyclics (~30%), and aliphatic (20 to 30%) across the soil fractions (**Figures 3A – 3F**). In addition to within fraction variability, there were some trends across the five fractions. For example, the abundance of lignin derived compounds decreases from approximately 40% in the fPOM fraction to less than 20% in the silt + clay mineral fraction (**Figures 3A – 3F**). The relative abundance of N-containing

compounds increases from < 2% in the fPOM fraction to 20% in the < 53 um fraction (**Figures 3A – 3F**).

The ordination for Wootons revealed a similar separation between fPOM and soil aggregates (**Figure 2).** Similar to the observations in Pax Park, fPOM samples contained a high abundance of cyclic (~25%) and lignin derivatives (~50%), similar quantities of carbohydrate derivatives, carboxylics, aliphatics, and, and very low abundance of N-containing compounds (< 2%, **Figure 3G – 3L**). Unlike Pax Park, the four soil aggregate-size fractions in Wootons separated into two distinct clusters. The first cluster included the large macroaggregate (≥ 2000 μm) and small macroaggregates (≥ 250 to < 2000 μm) soil fractions. These two soil fractions had relatively equal proportions of cyclics, aliphatics, and lignin-derived compounds (~25%). The second cluster contained the microaggregate (≥ 53 to < 250 μm) and the silt + clay mineral (< 53 μm) soil fractions. These two smaller soil fractions had a relatively equal abundance of cyclics and aliphatics compounds and less than 10% of the sample included lignin derivatives. Similar to the Pax Park site, the relative abundance of lignin derivatives decreased, and N-containing compounds increased as aggregate-size class decreased (**Figure 3G – L)**.

Organo-metal complexes

The three-part extraction procedure removed approximately 1.2 and 0.5 g C per mg soil for Pax Park and Wootons, respectively. For each site, concentrations of DOC (mg C g^{-1} soil), iron (mg Fe g^{-1} soil), and aluminum (mg Al g^{-1} soil) varied by habitat, horizon, and extracting solution (**Table 5**). Due to low concentrations and multiple outliers in the Mn dataset, assumptions of normality were violated, and the dataset was not statistically analyzed.

In Pax Park, extractable DOC was similar across habitats, horizons, and extracting solution; however, soils in the UP lower horizon had less extractable DOC compared to all other habitats. Extractable Fe was similar across habitats and horizons for KCl extractions, but not for Pyro and Dit extractions. Fe_{Pyro} concentrations were nearly 10X more concentrated in both UP soil horizons compared to marsh soil horizons. Concentrations of extractable Fe_{Pyro} were approximately double the concentration in upper soil horizons of the LM habitat compared to the lower soil horizons of the LM habitat and both soil horizons in the HM habitat. Dithionite extractable Fe followed a similar pattern as Fe_{Pyro}, but total concentrations were significantly lower. Extractable Al was variable across the three habitats, two horizons, and three extracting solutions with no obvious trends in the data. Even though there was a lot of variability in Mn dataset, Mn concentration tended to be lower in UP lower soil horizons compared to all other analyzed samples. Total extractable DOC in Pax Park positively correlated with total extractable Fe but not Al (**Table 6**). The association between Pyro and Dit extractable DOC and Fe explained most of the variance. There was also a significant positively association between Dit extractable Al and DOC.

In Wootons, extractable DOC and Fe concentrations were similar between habitats and horizons. Dithionite extracted significantly more Fe from upper marsh soil horizons compared to lower marsh soil horizons and UP soil horizons. According to Tukey's HSD, soils from the upper HM horizon (0.91 ± 0.22) had nearly three times more extractable Al compared to HM lower soil horizons (0.26 ± 0.04); all other mean comparisons were not statistically significant. While there were separate significant interactions for Al across habitat × extracting solution and horizon × extracting solution

(**Table 1**), there were no clear patterns worth noting (**Table 5**). Total extractable DOC in Wootons positively correlated with total extractable Fe and Al (**Table 6**). Again, the most significant relationships were found between Pyro and Dit extractable DOC, Fe, and Al.

Discussion

This project examined the distribution of C associated with aggregates, minerals, and metals in one natural and one restored tidal freshwater wetland. The results of this study found differences in C quantity and organic matter chemical composition associated with different aggregate-size fractions, fPOM, and minerals. Additionally, we identified a significant positive relationship between extractable DOC, Fe, and Al metals.

In Pax Park, the macroaggregate fraction was the most abundant aggregate-size fraction and stored substantially more soil C compared to all other fractions in the natural soil habitats (**Table 3**). These results are supported by previous findings showing that large macroaggregates ($\geq$ 2000 μm) are the largest pools in natural habitats and store more C compared to small aggregates (29, 30). In contrast, soil aggregates in Wootons were mainly distributed between the smaller macroaggregate fraction sizes ($\geq$ 250 to < 2000 μm) and microaggregates ($\geq$ 53 to < 250 μm), and the majority of the C was associated with smaller macroaggregates ($\geq$ 250 to < 2000 μm) (**Table 4**). These results are also supported by previous findings, showing that disturbed soils had a larger proportion of microaggregates (< 250 μm) and store less soil C (29). Additionally, the soil parent material in Wootons is unlike what is typically found in natural tidal freshwater wetlands. The Wootons parent material is primarily greensands or glauconite pellets (**Supplemental Table 1** and **Supplemental Figure 1**). Given the high quantity of these sand-sized grains and low C quantity, it is perhaps not surprising that aggregate

distribution is dominated by microaggregates (≥ 53 to < 250 μm) across the marsh habitat.

Total C content differed between the low and high marsh habitat for Pax Park, but not for Wootons (**Tables 3 and 4**). It was expected that there would be differences between the two marsh habitats in Pax Park because erosion and POM export are greater in the LM habitat (51, 52). Additionally, LM habitats are colonized by plant species with lower plant C: N ratios (e.g., *Nuphar lutea* (L.) and *Peltandra virginica* (L.)) that decompose seasonally, in contrast to the mixed plant community in the HM zone (e.g., *Typha latifolia*, *Zizania aquatica*, *Phragmites australis*) that persist season-to-season (33, 53, 54). In Wootons, total C concentrations did not differ between the two marsh habitats and showed similar distribution trends across the soil aggregate fractions (**Table 4**). This was unexpected because there were differences in the plant community composition between the two habitats. In Wootons, the HM habitat was primarily colonized by the nonnative, common reed (*Phragmites australis*), and the LM habitat was primarily colonized by pickerelweed (*Pontederia cordata*). In a previous study (55), we found that microbial communities differed between plant species in natural wetlands, but not in restored wetlands. While these relationships take time to develop, perhaps the soil conditions at Wootons played a larger role than plant communities.

Soils from the two UP sites fell within expected ranges of typical upland habitats (1 to 5% SOM). Soil organic matter content in the upper horizons of Pax Park was greater compared to Wootons, but in both sites, most aggregates and soil C in the UP site were small macroaggregates (≥ 250 to < 2000 μm) (**Tables 3 and 4**). In Wootons, soil C was relatively similar across the five different fractions (**Tables 3 and 4**). Floating POM

mass of fPOM was similar between the UP and LM habitats, but significantly lower compared to the HM habitat (**Table 3 and 4**). The mechanisms that control the incorporation of fPOM into the soil matrix and aggregates are probably quite different between the UP and LM habitats. In UP habitats, fPOM deposited on the soil surface is decomposed rapidly, but in LM habitats, high export rates reduce incorporation of fPOM into the soil matrix.

For both parks, a higher mass of silt + clay minerals was observed in the LM soil habitat. This is due to high deposition of minerals along the marsh's shoreline (52). Even though there were slight differences in the mass, C associated with the silt + clay mineral fraction (< 53 μm) were relatively similar across the different habitats and depth (**Table 3 and 4**). The large variance in the values is likely due to the low recovery of soils in this pool (sometimes < 2.0 g of soil), which also contributed to a lack of statistical differences. However, these results are not totally unexpected. Studies suggest that clay + silt mineral fractions have a maximum sorption limit (24); therefore, relatively similar amounts of C associated with the silt + clay mineral fraction suggests mineral sorption may be similar at both sites. It would be interesting to study and quantify the age of C associated with this fraction.

Relative abundance of the six classes of C compounds changed in a similar manner for both Pax Park and Wootons Landing (**Figures 2 and 3**). The relative abundance of N-containing compounds was lowest in the fPOM fraction and increased as soil aggregate size decreased. Finding a greater abundance of N-containing compound associated with the silt + clay fraction was not unexpected because previous research has shown that finer textured soils have a lower C: N ratio (20). We also see that lignin

derivatives have a greater abundance in the fPOM and macroaggregate fractions compared to smaller fractions. Again, this is not unexpected because previous research has shown that C derivatives associated with larger aggregate fractions more closely resemble the chemistry of the plant detritus (28). Lignin is also a more recalcitrant compound, and this material persists in the soil profile longer than other C compounds. It is interesting that Wootons had a greater abundance of lignin derivatives associated with all three aggregate-size fractions (**Figure 3A – 3H**). This region of the marsh is almost entirely colonized by a single plant species, the nonnative lineage *P. australis*. The plant tissue of *P. australis* has a relatively high abundance of recalcitrant C, and is thus driving the C-chemistry in the soil profile.

Only a small proportion of total C was removed with the sequential extraction procedure (15% to 21%). Across the three extracting solutions, Pyro desorbed more DOC compared to KCl and Dit. Similar findings were reported in Lopez-Sangil and Rovira (42) suggesting that DOC is weakly associated with the mineral matrix. While there was less DOC, Fe, and Al isolated with Dit, there was a positive association between these variables (**Table 6**). Previous reports have shown a similar relationship between crystalline oxides and C compounds, but they also observed that these relationships are not the result of sorption; instead, C is serving as a nucleation point for crystalline structures (31). Mn-oxide concentrations were low across all sites and probably not important in DOC stabilization. Association of DOC with Fe and Al may regulate the bioavailability of these substrates for microbial decomposition. However, these are just correlations and do not point to specific mechanisms. Previous work has suggested that chemical stabilization plays a minor role in stabilizing soil C because SOM often exceeds

the concentrations sesquioxides in soils (31). More work related to the organic matter chemistry and age of C extracted with each fraction might elucidate the role of these associations in SOM stabilization.

A major oversight of this work was the failure to separate and quantify sand particles in the soil aggregate fractions. It is likely the mass associated with the small macroaggregate ($\geq$ 250 to < 2000 μm) and microaggregate ($\geq$ 53 to < 250 μm) fractions in Wootons were inflated due to the presence of greensands (i.e., glauconite pellets); therefore, we intend to reprocess samples from this site and update reported aggregate mass and C values. In addition to this oversight, we did not remove calcium carbonate ($CaCO_3$) before quantifying soil C via LOI or CHN. The calcite layer, initially discovered during mitigation, suggests that the formation of $CaCO_3$ may be an important component of soil C in this restored habitat. Because soils are mildly acidic, the contribution of $CaCO_3$ is likely low; however, it would be worth testing for their presence and possibly monitoring their abundance over time since it is a less stable way of storing C in the soil matrix.

Conclusions

Based on the evidence from this project, it appears that the two wetlands store soil C quite differently. A majority of soil C in the Natural site was associated with large macroaggregates ($\geq$ 2000 μm) whereas most soil C in the Wootons site was associated with smaller macroaggregates ($\geq$ 250 to < 2000 μm). Regardless of these differences in C storage, the distributions of six C compound classes were relatively similar across the five fractions for both sites. We identified a significant positive correlation between DOC and Fe and Al metals, but the contribution of these are likely minimal since it only

accounted for a small proportion of total C in the system (15 to 21%). We cannot comment on how much soil C has been added (or lost) since the restoration of Wootons because we do not have data dating back to 1992. Results from our previous study in 2012 (55) reveal similar levels of soil C (6.7% ± 0.70% and 7.5% ± 1.5%, respectively). Soil organic matter accumulation is a slow process, and it may take decades to centuries for restored wetlands to reach levels observed in natural habitats. Change to the soil habitat, specifically soil texture and mineralogy, could affect the long-term trajectories of SOM dynamics by diminishing the mechanisms that stabilize soil C in these habitats. While the results of this study need to be corroborated with additional research, these data suggest that practitioners should carefully select topsoil amendments that are representative of soil textures in natural systems of that region.

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Table 1. Summary statistics for mixed-effects repeated measures ANOVA. Significant models are indicated with an asterisk (*). All variables were log_{10}-transformed prior to statistical analysis except the Aluminum model for Wootons Landing Wetland Park.

Parameters	F Statistic (df_{num}, df_{den})	Aggregate Analysis		Chemical Fractionation Procedure		
		Mass	Carbon	DOC	Iron	Aluminum
Patuxent Wetlands Park						
% Variance Explained		98%	99%	99%	89%	84%
Habitat	$F_{(2, 12)}$ =	110, $p < 0.01$*	298, $p < 0.01$*	27.4, $p < 0.01$*	24.0, $p < 0.01$*	77.7, $p < 0.01$*
Horizon	$F_{(1, 12)}$ =	0.51, $p = 0.49$	84.7, $p < 0.01$*	22.9, $p < 0.01$*	8.18, $p = 0.01$*	18.3, $p < 0.01$*
Habitat × Horizon	$F_{(2, 12)}$ =	17.2, $p < 0.01$*	11.4, $p < 0.01$*	1.10, $p = 0.36$	5.11, $p = 0.02$*	6.68, $p = 0.01$*
Fraction	$F_{(2, 24)}$ =	601, $p < 0.01$*	453, $p < 0.01$*	940, $p < 0.01$*	54.3, $p < 0.01$*	42.2, $p < 0.01$*
Habitat × Fraction	$F_{(4, 24)}$ =	88.4, $p < 0.01$*	99.6, $p < 0.01$*	15.2, $p < 0.01$*	5.82, $p < 0.01$*	1.50, $p = 0.23$
Horizon × Fraction	$F_{(2, 24)}$ =	44.3, $p < 0.01$*	28.3, $p < 0.01$*	3.13, $p = 0.06$	10.1, $p < 0.01$*	12.5, $p < 0.01$*
Habitat × Horizon × Fraction	$F_{(4, 24)}$ =	12.8, $p < 0.01$*	10.7, $p < 0.01$*	5.46, $p < 0.01$*	4.96, $p < 0.01$*	3.78, $p = 0.02$*
Wootons Landing Wetlands Park (rest. 1992)						
% Variance Explained		89%	83%	89%	82%	74%
Habitat	$F_{(2, 12)}$ =	10.9, $p < 0.01$*	5.29, $p = 0.02$*	1.52, $p = 0.26$	4.86, $p = 0.03$*	1.02, $p = 0.39$
Horizon	$F_{(1, 12)}$ =	8.09, $p = 0.01$*	11.7, $p = 0.01$*	13.7, $p < 0.01$*	7.89, $p = 0.02$*	4.69, $p = 0.05$
Habitat × Horizon	$F_{(2, 12)}$ =	0.83, $p = 0.46$	0.16, $p = 0.86$	4.57, $p = 0.03$*	5.20, $p = 0.02$*	4.71, $p = 0.03$*
Fraction	$F_{(2, 24)}$ =	126, $p < 0.01$*	12.3, $p < 0.01$*	89.4, $p < 0.01$*	36.9, $p < 0.01$*	5.61, $p = 0.01$*
Habitat × Fraction	$F_{(4, 24)}$ =	2.50, $p = 0.02$*	2.45, $p = 0.04$*	0.61, $p = 0.66$	2.59, $p = 0.06$	6.17, $p < 0.01$*
Horizon × Fraction	$F_{(2, 24)}$ =	5.41, $p < 0.01$*	7.04, $p < 0.01$*	0.73, $p = 0.49$	3.90, $p = 0.03$*	5.88, $p = 0.01$*
Habitat × Horizon × Fraction	$F_{(4, 24)}$ =	0.84, $p = 0.57$	2.03, $P = 0.09$	1.97, $p = 0.13$	4.12, $p = 0.01$*	2.73, $p = 0.05$

Table 2. Edaphic properties for marsh soils in two tidal freshwater wetlands and an adjacent upland habitat located along the Patuxent River, MD. Patuxent Wetlands Park is made up of a series of natural tidal freshwater wetlands, and the ~2.4 acres of tidal freshwater wetlands in Wootons Landing Wetland Park was restored in 1992. Soils were collected from three habitats and two soil horizons at each park (n = 3, mean ± SEM).

Habitat	**Low Marsh**		**High Marsh**		**Upland**	
Horizon	Upper	Lower	Upper	Lower	Upper	Lower
Patuxent Wetlands Park						
Soil pH_{CaCl2}	5.9 ± 0.1	5.1 ± 0.3	5.4 ± 0.1	4.8 ± 0.1	5.5 ± 0.6	5.3 ± 0.4
SOM (w/w, %)	18.8 ± 0.8	11.5 ± 0.6	36.1 ± 2.5	20.4 ± 3.2	9.7 ± 1.0	1.8 ± 0.3
Total C (w/w, %)	9.0 ± 0.4	5.7 ± 0.2	18.2 ± 1.5	10.7 ± 2.0	4.7 ± 0.7	0.8 ± 0.2
Total N (w/w, %)	0.77 ± 0.04	0.44 ± 0.01	1.39 ± 0.10	0.79 ± 0.13	0.28 ± 0.03	0.03 ± 0.01
C: N	13.4 ± 0.6	13.1 ± 0.3	12.9 ± 0.26	11.7 ± 0.2	28.7 ± 9.0	17.0 ± 0.6
Wootons Landing Wetland Park (rest. 1992)						
Soil pH_{CaCl2}	6.0 ± 0.3	5.5 ± 0.7	6.3 ± 0.2	5.9 ± 0.5	4.5 ± 0.2	4.3 ± 0.2
SOM (w/w, %)	10.4 ± 2.7	5.4 ± 2.2	12.6 ± 0.4	1.6 ± 0.3	3.0 ± 0.8	1.7 ± 0.3
Total C (w/w, %)	4.3 ± 1.1	2.4 ± 1.2	5.4 ± 0.2	0.5 ± 0.2	1.7 ± 0.2	0.8 ± 0.1
Total N (w/w, %)	0.30 ± 0.07	0.14 ± 0.07	0.40 ± 0.01	0.02 ± 0.01	0.13 ± 0.03	0.04 ± 0.02
C: N	35.7 ± 13.8	13.6 ± 0.4	19.1 ± 2.5	14.7 ± 0.8	22.4 ± 3.7	14.2 ± 1.3

Table 3. Aggregate-size distribution (g of air-dried soil) and aggregate-associated C (g C g^{-1} soil) for Patuxent Wetlands Park (n = 3, median ± SD). All values were log_{10}-transformed prior to statistical analysis. Different upper case letters signify statistically significant differences among habitat type and horizon within aggregate-size class or aggregate associated C level. Lower case letters signify differences among aggregate-size class or aggregate-associated C within habitat and horizon.

Aggregate-size class	**Low Marsh**		**High Marsh**		**Upland**	
(Bonferroni α = 0.0004)	Upper	Lower	Upper	Lower	Upper	Lower
Rocks	0.00 ± 3.44	0.25 ± 0.74	0.00 ± 0.00	0.00 ± 0.29	9.08 ± 4.50	15.9 ± 3.02
fPOM	0.60 ± 0.12 ^{ABd}	0.24 ± 0.17 ^{Bc}	1.11 ± 0.32 ^{Ad}	0.39 ± 0.29 ^{Abd}	0.21 ± 0.29 ^{Abd}	0.09 ± 0.05 ^{Bd}
Large Macro (≥ 2000 μm)	44.8 ± 0.77 ^{Aa}	33.5 ± 2.47 ^{Aa}	47.4 ± 1.53 ^{Aa}	41.0 ± 2.96 ^{Aa}	10.3 ± 4.61 ^{Bb}	0.06 ± 0.23 ^{Cd}
Small Macro (≥ 250 to < 2000)	17.2 ± 3.51 ^{Bb}	24.5 ± 0.73 ^{Ba}	17.7 ± 0.77 ^{Bb}	18.9 ± 0.83 ^{Bb}	48.1 ± 5.64 ^{Aa}	39.0 ± 4.84 ^{Aa}
Micro (≥ 53 to < 250)	6.52 ± 0.25 ^{BCc}	10.7 ± 1.59 ^{Abb}	4.65 ± 0.80 ^{Cc}	8.71 ± 1.23 ^{Bc}	6.85 ± 1.90 ^{BCb}	17.5 ± 4.04 ^{Ab}
Silt + Clay Mineral (< 53 μm)	6.05 ± 0.31 ^{Ac}	7.88 ± 0.89 ^{Ab}	3.12 ± 0.75 ^{Bd}	6.32 ± 0.71 ^{Ac}	2.05 ± 0.78 ^{Bc}	8.04 ± 3.43 ^{Ac}
Aggregate-associated C	**Low Marsh**		**High Marsh**		**Upland**	
(Bonferroni α = 0.0005)	Upper	Lower	Upper	Lower	Upper	Lower
Large Macro (≥ 2000 μm)	3.82 ± 0.33 ^{Ba}	2.21 ± 0.18 ^{Ca}	7.39 ± 1.30 ^{Aa}	4.12 ± 0.67 ^{Ba}	0.52 ± 0.31 ^{Db}	0.00 ± 0.00 ^{Ea}
Small Macro (≥ 250 to < 2000)	1.68 ± 0.44 ^{Bb}	1.53 ± 0.26 ^{Ba}	2.97 ± 0.29 ^{Ab}	2.01 ± 0.28 ^{Abb}	1.47 ± 0.18 ^{Ba}	0.00 ± 0.12 ^{Ca}
Micro (≥ 53 to < 250)	0.57 ± 0.04 ^{Ac}	0.60 ± 0.12 ^{Ab}	0.79 ± 0.08 ^{Ac}	0.65 ± 0.08 ^{Ac}	0.38 ± 0.12 ^{Abbc}	0.17 ± 0.12 ^{Ba}
Silt + Clay Mineral (< 53 μm)	0.31 ± 0.02 ^{Ac}	0.28 ± 0.03 ^{Ab}	0.31 ± 0.05 ^{Ad}	0.29 ± 0.03 ^{Ac}	0.17 ± 0.05 ^{Ac}	0.11 ± 0.11 ^{Aa}

Table 4. Aggregate-size distribution (g of air-dried soil) and aggregate associated C (g C g^{-1} soil) for Wootons Landing Wetland Park (n = 3, median ± SD). All values were log_{10}-transformed prior to statistical analysis. Different upper case letters signify statistically significant differences among habitat type or horizon within aggregate-size class or aggregate associated C level. Lower case letters signify differences among aggregate-size class or aggregate-associated C within habitat or horizon.

Aggregate-size class	**Habitat** (n = 6) (Bonferroni α = 0.001)			**Horizon** (n = 9) (Bonferroni α = 0.002)	
	Low Marsh	High Marsh	Upland	Top	Bottom
Rocks	0.54 ± 0.77	0.53 ± 0.57	9.51 ± 16.7	1.01 ± 4.47	1.00 ± 15.3
fPOM	0.38 ± 0.30 ^{Ac}	0.28 ± 0.34 ^{Ad}	0.06 ± 0.10 ^{Ad}	0.29 ± 0.34 ^{Ad}	0.07 ± 0.18 ^{Ac}
Large Macro (≥ 2000 µm)	3.81 ± 9.61 ^{Ab}	1.26 ± 14.9 ^{Bb}	0.44 ± 1.39 ^{Bd}	3.69 ± 13.0 ^{Ab}	0.39 ± 1.85 ^{Bc}
Small Macro (≥ 250 to < 2000)	34.4 ± 3.25 ^{Aa}	55.9 ± 14.2 ^{Aa}	45.4 ± 13.2 ^{Aa}	43.1 ± 11.1 ^{Aa}	40.7 ± 13.9 ^{Aa}
Micro (≥ 53 to < 250)	26.2 ± 7.4 ^{Aab}	17.11 ± 3.50 ^{Aac}	15.5 ± 10.6 ^{Aac}	19.7 ± 6.51 ^{Ac}	19.1 ± 9.10 ^{Aa}
Silt + Clay Mineral (< 53 µm)	9.65 ± 3.33 ^{Ab}	1.75 ± 1.33 ^{Bbd}	2.09 ± 1.04 ^{Bbd}	3.26 ± 4.18 ^{Ab}	2.37 ± 3.68 ^{Ab}
Aggregate-associated C	**Habitat** (n = 6) (Bonferroni α = 0.002)			**Horizon** (n = 9) (Bonferroni α = 0.003)	
	Low Marsh	High Marsh	Upland	Top	Bottom
Large Macro (≥ 2000 µm)	0.19 ± 0.71 ^{Ab}	0.02 ± 1.06 ^{Aa}	0.01 ± 0.06 ^{Aa}	0.30 ± 0.93 ^{Aa}	0.00 ± 0.02 ^{Ba}
Small Macro (≥ 250 to < 2000)	1.34 ± 0.60 ^{Aa}	0.25 ± 0.56 ^{Ba}	0.28 ± 0.39 ^{Ba}	0.93 ± 0.57 ^{Aa}	0.04 ± 0.51 ^{Ba}
Micro (≥ 53 to < 250)	0.47 ± 0.16 ^{Ab}	0.09 ± 0.13 ^{Aa}	0.14 ± 0.15 ^{Aa}	0.28 ± 0.15 ^{Ab}	0.08 ± 0.21 ^{Aa}
Silt + Clay Mineral (< 53 µm)	0.24 ± 0.12 ^{Ab}	0.05 ± 0.07 ^{Aa}	0.09 ± 0.06 ^{Aa}	0.13 ± 0.06 ^{Ab}	0.05 ± 0.16 ^{Aa}

Table 5. Chemically extractable dissolved organic carbon (mg C g^{-1} soil) and metals (mg Fe, Al, or Mn g^{-1} soil) for soils collected from two tidal freshwater wetlands located along the Patuxent River in Anne Arundel County, MD. All values (except the Wootons-AL dataset) were log_{10} transformed prior to statistical analysis and values reported here represent median ± SD (n = 3). Results for significant three-way interactions are denoted as letters below. Different upper case letters signify statistically significant differences among habitat type and horizon within each extracting solution. Lower case letters signify differences among extracting solutions within each habitat and horizon.

Fraction / Extracting Solution	Low Marsh		High Marsh		Upland	
	Upper	Lower	Upper	Lower	Upper	Lower
Patuxent Wetlands Park						
Dissolved Organic Carbon						
1.0 M KCl	1.59 ± 0.36 ^{Ab}	1.09 ± 0.02 ^{Ab}	2.14 ± 0.77 ^{Ab}	1.66 ± 0.83 ^{Ab}	1.38 ± 0.31 ^{Abb}	0.38 ± 0.13 ^{Bb}
0.1 M Pyro	6.49 ± 0.68 ^{Aba}	4.98 ± 0.46 ^{Aba}	9.43 ± 1.03 ^{Aa}	6.30 ± 3.42 ^{Aba}	3.48 ± 1.84 ^{Ba}	1.41 ± 0.44 ^{Ca}
50 mM Dit	0.60 ± 0.19 ^{ABc}	0.13 ± 0.02 ^{ABc}	0.76 ± 0.04 ^{Ac}	0.41 ± 0.22 ^{ABc}	0.14 ± 0.07 ^{ABc}	0.00 ± 0.02 ^{Bc}
Iron						
1.0 M KCl	0.06 ± 0.16 ^{Ab}	0.49 ± 0.12 ^{Ab}	0.07 ± 0.09 ^{Ab}	0.28 ± 0.05 ^{Ab}	0.02 ± 0.02 ^{Aa}	0.01 ± 0.02 ^{Aa}
0.1 M Pyro	10.8 ± 1.82 ^{Aa}	5.81 ± 0.76 ^{ABCa}	6.09 ± 2.43 ^{Aa}	4.51 ± 1.89 ^{ABCa}	0.79 ± 0.29 ^{Ca}	1.44 ± 0.50 ^{Bca}
50 mM Dit	22.1 ± 3.97 ^{Aa}	0.41 ± 0.12 ^{Cb}	12.2 ± 11.7 ^{ABa}	6.59 ± 7.24 ^{Ba}	0.56 ± 0.24 ^{Ca}	0.08 ± 0.05 ^{Ca}
Aluminum						
1.0 M KCl	0.17 ± 0.18 ^{Cb}	1.79 ± 0.45 ^{Aa}	0.49 ± 0.34 ^{BCb}	1.25 ± 0.72 ^{Ab}	0.18 ± 0.37 ^{Bca}	0.18 ± 1.22 ^{Ca}
0.1 M Pyro	1.76 ± 0.34 ^{Ba}	3.53 ± 0.65 ^{Aa}	1.88 ± 0.10 ^{ABCa}	2.80 ± 0.67 ^{Aa}	0.64 ± 0.17 ^{Da}	0.84 ± 0.50 ^{Cda}
50 mM Dit	1.53 ± 0.16 ^{Aa}	0.66 ± 0.17 ^{ABb}	1.21 ± 0.21 ^{ABab}	1.18 ± 0.25 ^{ABab}	0.44 ± 0.18 ^{BCa}	0.17 ± 0.00 ^{Ca}
Manganese						
1.0 M KCl	0.64 ± 0.32	0.31 ± 0.03	1.35 ± 0.16	0.36 ± 0.07	0.05 ± 0.01	0.00 ± 0.00
0.1 M Pyro	0.07 ± 0.02	0.02 ± 0.00	0.15 ± 0.37	0.02 ± 0.01	0.02 ± 0.01	0.00 ± 0.00
50 mM Dit	0.38 ± 0.29	0.03 ± 0.02	0.28 ± 0.41	0.03 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
Wootons Landing Wetland Park						
Dissolved Organic Carbon						
$DOC\text{-}_{KCl}$	1.10 ± 0.59	0.63 ± 0.26	1.26 ± 0.24	0.20 ± 0.08	0.62 ± 0.17	0.38 ± 0.15
$DOC\text{-}_{Pyro}$	2.42 ± 0.84	4.33 ± 2.77	4.13 ± 1.84	0.74 ± 0.09	2.42 ± 0.74	1.09 ± 1.41
$DOC\text{-}_{Dit}$	0.39 ± 0.15	0.10 ± 0.09	0.59 ± 0.20	0.07 ± 0.04	0.17 ± 0.06	0.01 ± 0.04
Iron						
$Fe\text{-}_{KCl}$	0.00 ± 0.02 ^{Ab}	0.13 ± 0.11 ^{Aa}	0.00 ± 0.00 ^{Ab}	0.00 ± 0.00 ^{Aa}	0.02 ± 0.02 ^{Aa}	0.01 ± 0.02 ^{Aa}
$Fe\text{-}_{Pyro}$	3.88 ± 1.45 ^{Aa}	3.84 ± 1.20 ^{Aa}	5.23 ± 1.00 ^{Aa}	0.95 ± 0.30 ^{Aa}	2.56 ± 1.08 ^{Aa}	1.43 ± 0.69 ^{Aa}
$Fe\text{-}_{Dit}$	19.9 ± 6.48 ^{ABa}	3.21 ± 3.19 ^{BCa}	19.3 ± 11.6 ^{Ba}	0.41 ± 0.23 ^{Ca}	0.28 ± 0.22 ^{Ca}	0.36 ± 8.96 ^{Ca}
Aluminum						
$Al\text{-}_{KCl}$	0.18 ± 0.21	0.80 ± 0.38	0.16 ± 0.01	0.16 ± 0.00	0.80 ± 0.3	0.80 ± 0.49
$Al\text{-}_{Pyro}$	1.05 ± 0.47	1.18 ± 0.45	0.96 ± 0.35	0.27 ± 0.03	0.94 ± 0.34	0.48 ± 0.25
$Al\text{-}_{Dit}$	1.33 ± 0.38	0.64 ± 0.43	1.67 ± 0.14	0.4 ± 0.15	0.31 ± 0.3	0.59 ± 0.48
Manganese						
$Mn\text{-}_{KCl}$	0.12 ± 0.13	0.07 ± 0.06	0.21 ± 0.09	0.00 ± 0.01	0.05 ± 0.02	0.01 ± 0.01
$Mn\text{-}_{Pyro}$	0.15 ± 0.08	0.01 ± 0.01	0.15 ± 0.15	0.00 ± 0.01	0.02 ± 0.01	0.01 ± 0.00
$Mn\text{-}_{Dit}$	0.23 ± 0.24	0.01 ± 0.03	0.38 ± 0.24	0.00 ± 0.01	0.03 ± 0.02	0.01 ± 0.02

Table 6. Pearson's correlation coefficient (r) and p-values between DOC, Fe, and Al extracted with 1.0 M potassium chloride (KCl), 0.1 M sodium pyrophosphate ("Pyro"), and 0.05 M dithionite-hydrochloric acid (Dit-HCl) (n = 18). Parameters that were $\log_{10}$-transformed prior to statistical analysis are marked with a hash tag (#). Significant correlations ($p < 0.05$) are designated with an asterisk (*).

Parameters	Extractable Dissolved Organic Carbon			
	1.0 M KCl	0.1 M Pyro, pH 10	0.05 M Dit-HCl	Total DOC Extracted
Patuxent Wetlands Park				
#Fe_{KCl}	+ 0.21, p = 0.40			
#Al_{KCl}	+ 0.12, p = 0.63			
Fe_{Pryo}		+ 0.72, p < 0.01*		
Al_{Pyro}		+ 0.29, p = 0.24		
#Fe_{Dit}			+ 0.79, p < 0.01*	
Al_{Dit}			+ 0.81, p < 0.01*	
#Total Extracted Fe				+0.75, p < 0.01*
Total Extracted Al				0.38, p = 0.11
Wootons Landing Wetland Park (rest. 1992)				
#Fe_{KCl}	- 0.04, p = 0.88			
#Al_{KCl}	-0.32, p = 0.20			
Fe_{Pryo}		+ 0.81, p < 0.01*		
Al_{Pyro}		+ 0.75, p < 0.01*		
#Fe_{Dit}			+ 0.54, p = 0.02*	
Al_{Dit}			+ 0.82, p < 0.01*	
#Total Extracted Fe				+ 0.62, p < 0.01*
Total Extracted Al				+ 0.74, p < 0.01*

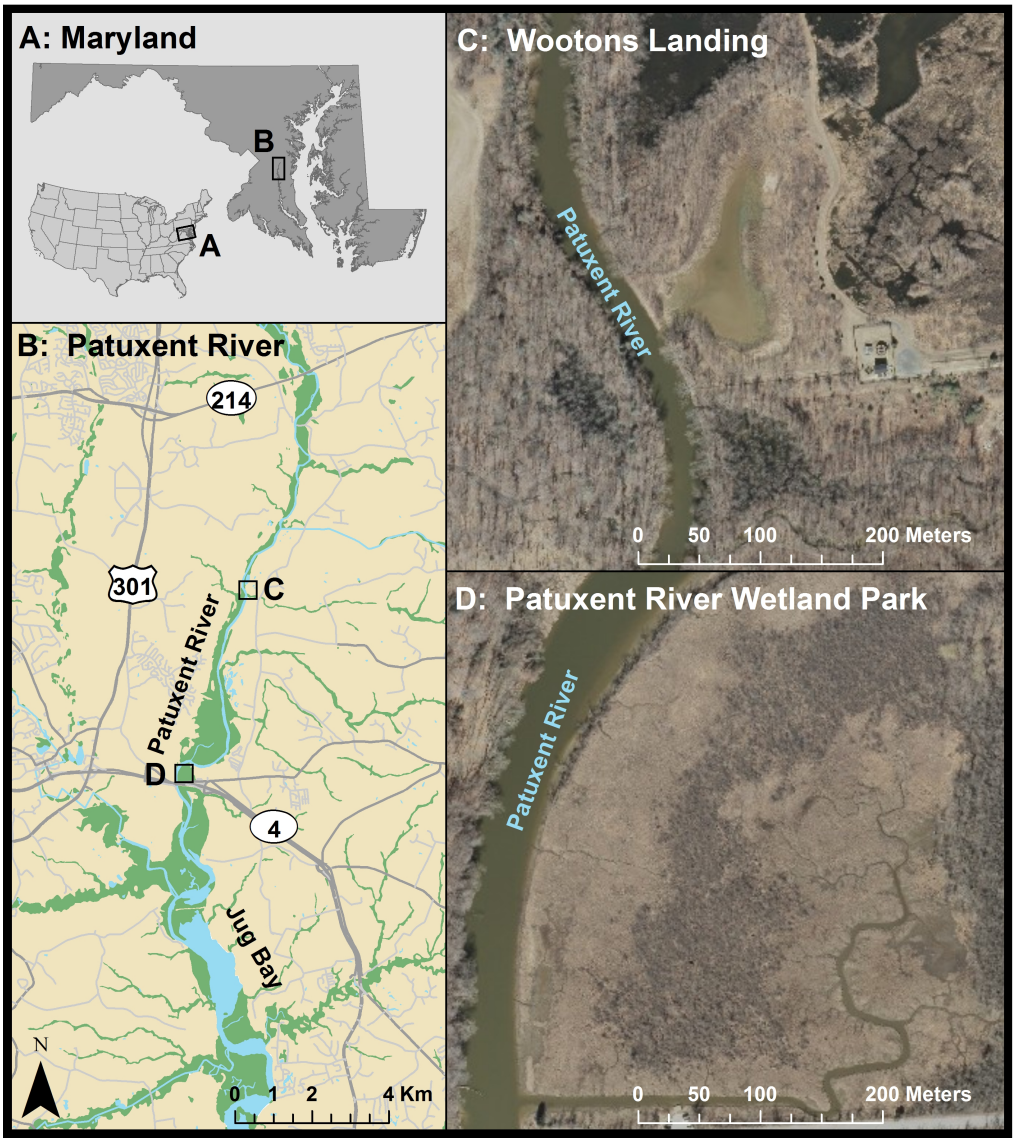


Figure 1. Soils were collected from two tidal freshwater wetlands located along the Patuxent River in Anne Arundel County, MD. Wootons Landing Wetland Park ("Wootons"; C) is a restored marsh, and Patuxent Wetlands Park ("Pax Park"; D) is a natural site. *Image courtesy of Matt Spielman*.

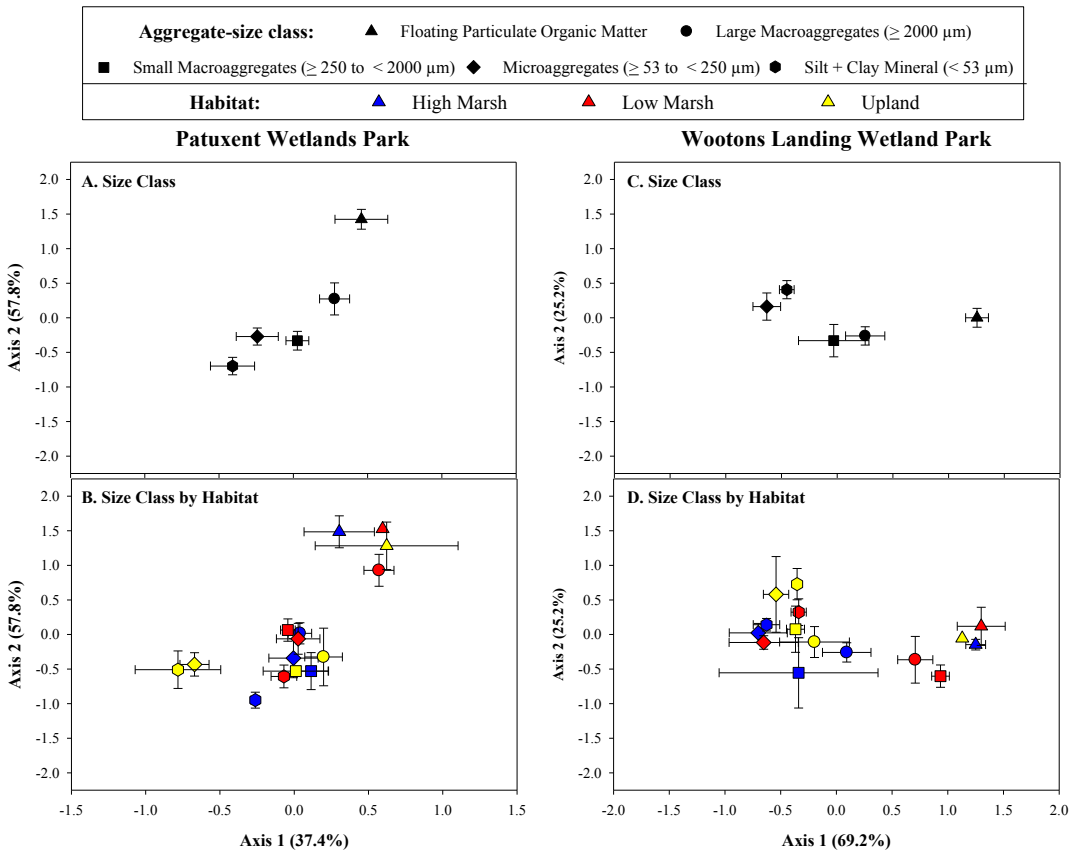


Figure 2. Ordination of the relative abundance of six chemical compound classes generated by pyrolysis GC-MS. Soils were collected from the upper soil horizon of the low and high marsh zone of two tidal freshwater wetlands (i.e., Patuxent Wetlands Park and Wootons Landing Wetlands Park) as well as an adjacent upland site. These data are plotted by aggregate-size class (A, C) and aggregate-size class by habitat (B, D).

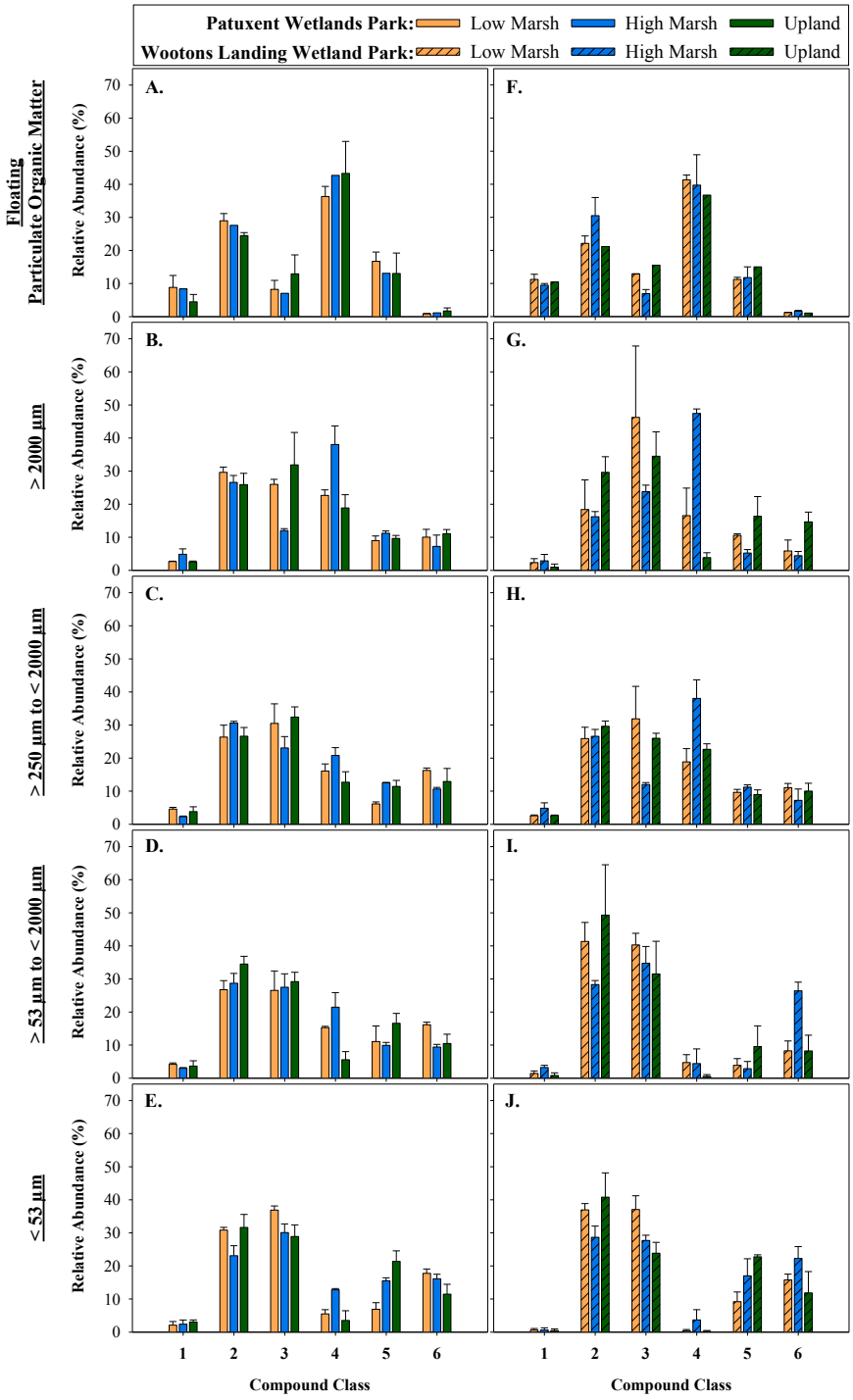


Figure 3. Assessment of soil organic matter chemical composition associated with POM and the four aggregate-size classes for Patuxent Wetlands Park (A-E) and Wootons Landing Wetland Park (F-J). Each bar represents the mean ± SEM relative abundance (%) for one of six compound classes: (1) carboxylics, (2) cyclics, (3) aliphatics, (4) lignin derivatives, (5) carbohydrate derivatives, and (6) N-containing compounds.

Supplemental Table 1. Soil profile descriptions from six representative subplots established in two tidal freshwater wetlands parks located along the Patuxent River, Anne Arundel County, MD.

Horizon (cm)	Horizon	Matrix Color	Texture (est. clay, %)	Redox Conc.	Redox Depletion
Patuxent Wetlands Park – Low Marsh (Subplot #3; Image Not Available)					
0-31*	Oa1	2.5Y 4/1	Muck (+roots)		
31-44**	Oa2	2.5Y 5/1	Muck		
44-200	Oa3	5Y 3/1	Muck		
Patuxent Wetlands Park – High Marsh (Subplot #5; Supplemental Figure 1A)					
0-17*	Oe	2.5Y 3/2	Muck (+roots)		
17-81**	Oa1	2.5Y 4/1	Muck		
81-200	Oa2	2.5Y 3/1	Muck		
Patuxent Wetlands Park – Upland (Subplot #7; Supplemental Figure 1B)					
0-11*	Oa	10YR 3/2	Muck		
11-23*	Ag	10YR 5/2	SL (4)	Distinct 7.5 YR 4/6 10-15%	
23-53**	BEg	10YR 6/2	SL (3)	Distinct 7.5YR 5/6 15-20%	
53-72	Bt1	10YR 6/3	L (24)	Prom. 7.5YR 5/6 30%	Distinct 10YR 6/1 5%
72-87	Bt2	10YR 6/4	CL (30)	Prom. 7.5YR 5/6 40%	Distinct 10YR 6/1 10%
Patuxent Wetlands Park – Upland (Subplot #8; Supplemental Figure 1C)					
0-12*	A	10YR 3/2	L (<5)		
12-21	AB	10YR 5/4	L (5)		
21-42**	Bt1	10YR 6/4	CL	Distinct 10YR 5/6 30%	Distinct 10YR 6/1 ~10%
42-51	Bt2	10YR 6/4	CL	Distinct 7.5YR 5/6 ~40-45%	Distinct 10YR 6/1 ~10%
51-60	BC	10YR 6/6	SCL	Faint & Distinct 7.5 YR 5/6 10%	Faint 10YR 6/2 5%

Sampled upper (*) and lower (**) horizons

Supplemental Table 1 (cont.)

Horizon (cm)	Horizon	Matrix Color	Texture (est. clay, %)	Redox Conc.	Redox Depletion
Wootons Landing Wetland Park –Low Marsh (Subplot #11; Supplemental Figure 1D)					
0-14*	A	10YR 3/1	Sand		
14-67* *	Oa	2.5Y 5/1	Muck		
Wootons Landing Wetland Park – Low Marsh (Subplot #12; Image Not Available)					
0-17*	Oa1	2.5Y 4/1	Muck (+roots)		
17-40**	Oa2	2.5Y 3/1	Muck		
+40	R		Cobbles		
Wootons Landing Wetland Park – High Marsh (Subplot #14; Supplemental Figure 1E)					
0-10*	Oa	10Y 2.5/1	Muck		
10-24*	A	N 2.5	Mucky S		
24-37**	C1	5Y 6/1	S		
37-91	C2	5Y 4/2	S		
Wootons Landing Wetland Park – Upland (Subplot #17; Supplemental Figure 1F)					
0-10*	A1	10YR 3/2	L (10)		
10-16*	A2	10YR 4/2	SL (12)		
16-40**	Bw1	10YR 5/4	S (1)		
40-51	Bw2	7.5 YR 4/6	SL (4)		
51-83	C1	5Y 4/2	SL (5)	Distinct 7.5 YR 5/6 (carry over?)	
83-11	C2	5G 4/1	LS (2-3)	Distinct 7.5 YR 5/6 12%	
113-134	C3 (mixed)	5YR 3/4 & 10YR 6/6	SL (8)		

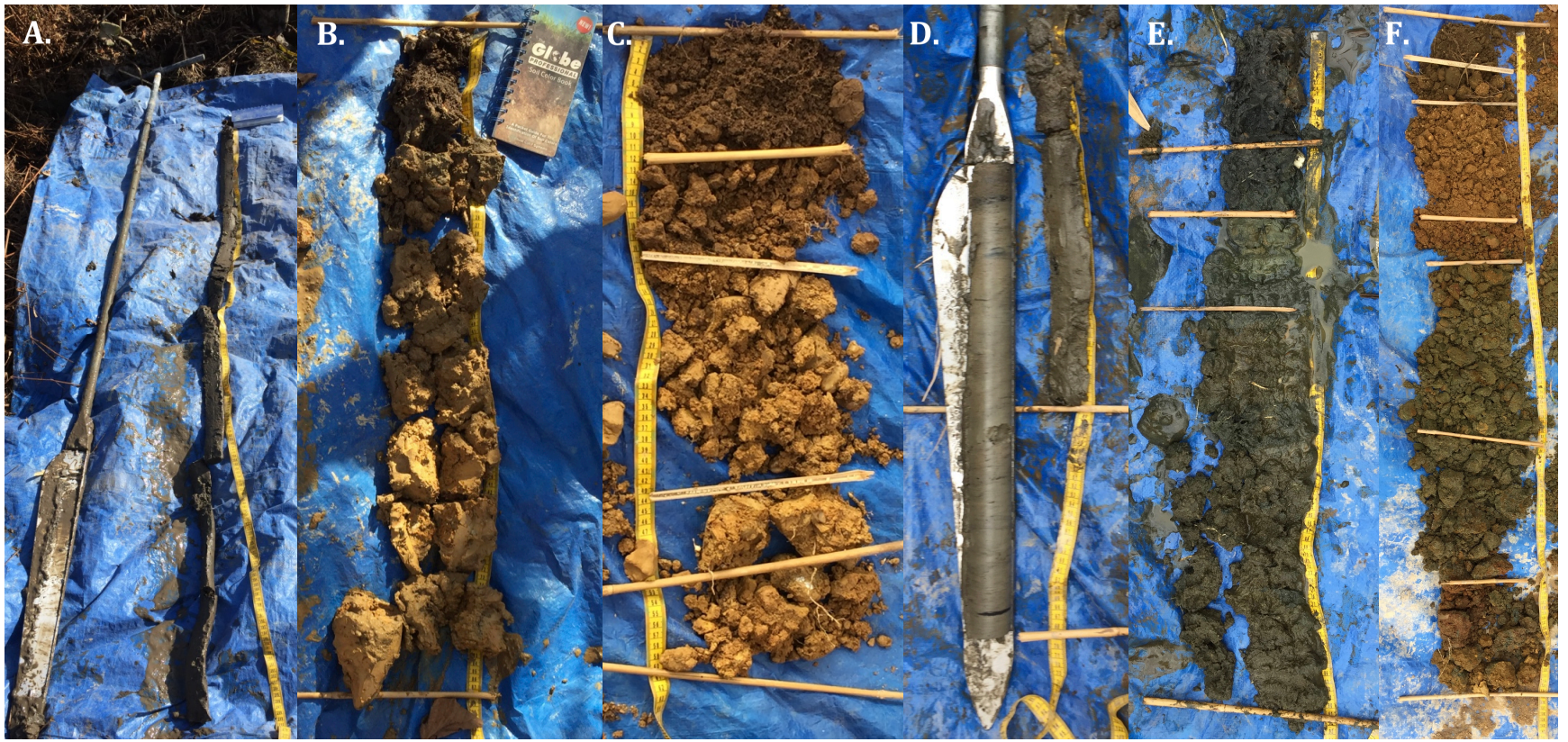
Sampled upper (*) and lower (**) horizons

Supplemental Table 2. Slaking test results. Slaking time was tested by submerging 80 g of air-dried soil on a 2000 μm sieve in distilled water for a total of 5, 10, or 20 minutes. Three replicates were run independently for each time treatment and soil type (n = 54). A one-way analysis of variance (ANOVA) was used to model mean differences in recovered soils ≥ 2000 μm by slaking time; soil type was not included in the model.

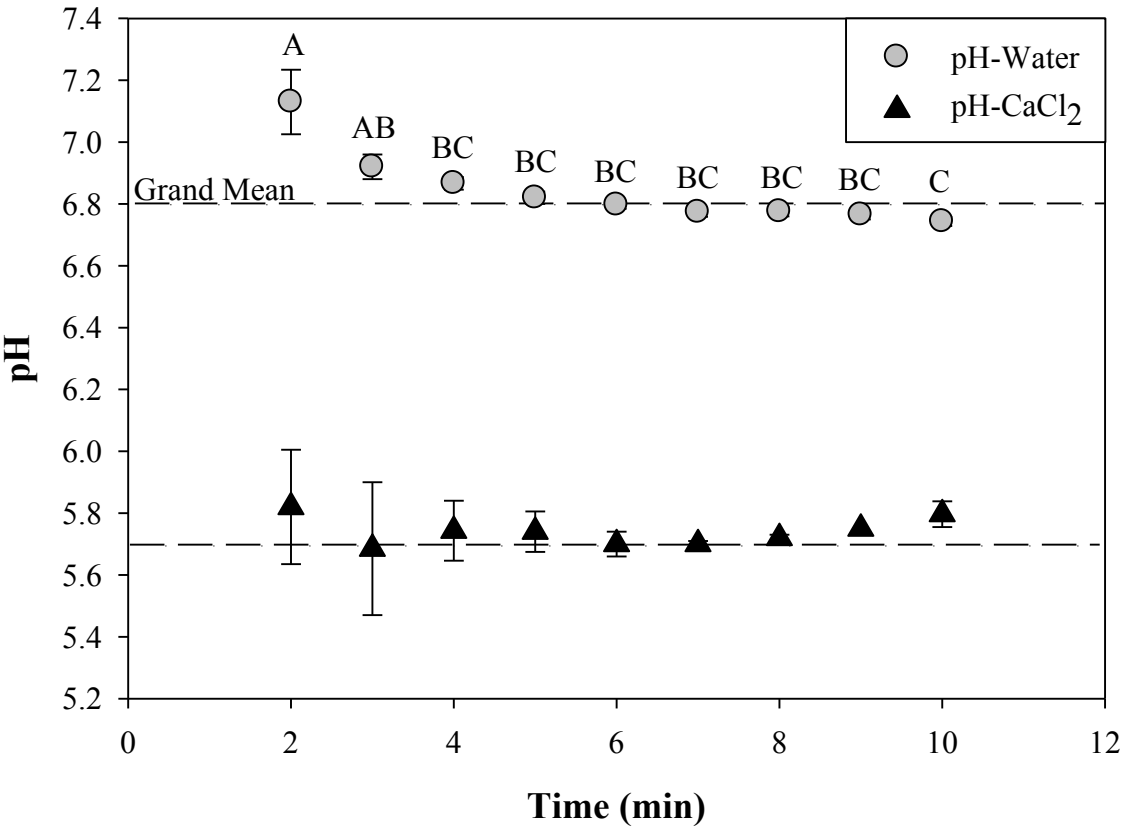
			Slaking Time		
Soil Type	Sample Size (n)	Model Statistics	5 minutes	10 minutes	20 minutes
Patuxent Wetlands Park					
Low Marsh	9	$F_{(2,6)} = 0.91$, $p = 0.45$	46.2 ± 2.84	41.6 ± 1.29	44.6 ± 2.92
High Marsh	9	$F_{(2,6)} = 0.14$, $p = 0.87$	52.3 ± 2.15	53.6 ± 4.58	55.0 ± 4.02
Upland	9	$F_{(2,6)} = 1.38$, $p = 0.32$	23.1 ± 1.92	28.3 ± 4.17	22.7 ± 0.29
Wootons Landing Wetland Park					
Low Marsh	9	$F_{(2,6)} = 1.65$, $p = 0.27$	36.4 ± 7.61	23.7 ± 2.75	28.8 ± 3.03
High Marsh	9	$F_{(2,6)} = 0.62$, $p = 0.57$	20.0 ± 1.71	24.0 ± 4.94	26.9 ± 5.46
Upland	9	$F_{(2,6)} = 0.84$, $p = 0.48$	23.8 ± 0.94	17.6 ± 2.83	21.8 ± 5.26

Supplemental Table 3. Summary statistics for soil physicochemical properties. A two-way analysis of variance (ANOVA) was used to inspect mean difference among the three habitats (LM, HM, and UP) and horizon (upper/lower) for Patuxent Wetlands Park (Pax Park) and Wootons Landing Wetland Park (Wootons). Parameters that were log_{10}-transformed prior to statistical analysis are marked with a hash tag (#).

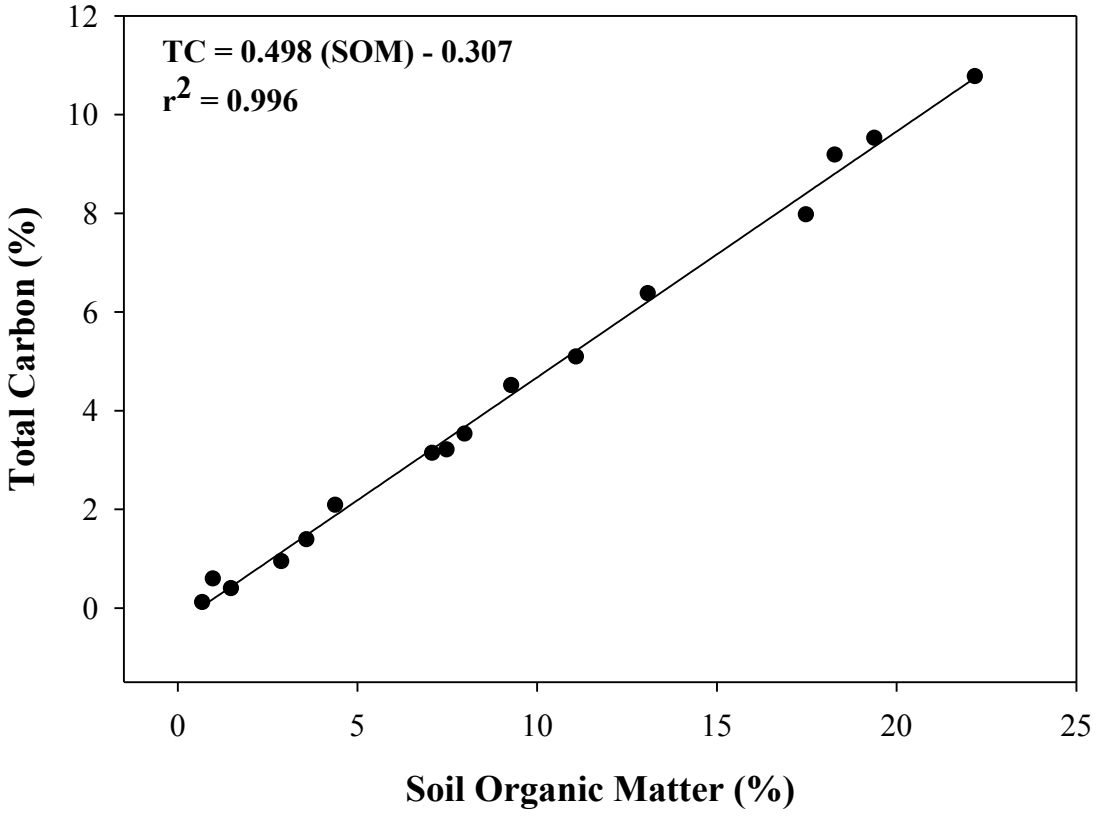
Parameters	Model	% Variance Explained	Habitat	Horizon	Habitat × Horizon
Soil pH					
Pax Park	$F_{(5,12)} = 1.41$, $p = 0.29$	37%	--	--	--
Wootons	$F_{(5,12)} = 4.56$, $p = 0.01$*	66%	$F_{(2,12)} = 10.48$, $p < 0.01$*	$F_{(1, 12)} = 1.73$, $p = 0.21$	$F_{(2, 12)} = 0.06$, $p = 0.94$
Soil Organic Matter (%, g of OM per 100 g^{-1} soil)					
Pax Park	$F_{(5,12)} = 44.33$, $p < 0.01$*	95%	$F_{(2,12)} = 81.76$, $p < 0.01$*	$F_{(1, 12)} = 51.03$, $p < 0.01$*	$F_{(2, 12)} = 3.54$, $p = 0.06$
#Wootons	$F_{(5,12)} = 7.67$, $p < 0.01$*	76%	$F_{(2,12)} = 6.13$, $p = 0.01$*	$F_{(1,12)} = 19.37$, $p < 0.01$*	$F_{(2, 12)} = 3.35$, $p = 0.07$
Total Organic Carbon (%, w/w)					
Pax Park	$F_{(5,12)} = 30.44$, $p < 0.01$*	93%	$F_{(2,12)} = 58.83$, $p < 0.01$*	$F_{(1, 12)} = 30.26$, $p < 0.01$*	$F_{(2, 12)} = 2.14$, $p = 0.16$
#Wootons	$F_{(5,12)} = 4.57$, $p = 0.01$*	66%	$F_{(2,12)} = 1.12$, $p = 0.36$	$F_{(1, 12)} = 16.12$, $p < 0.01$*	$F_{(2, 12)} = 2.24$, $p = 0.15$
Total Organic Nitrogen (%, w/w)					
Pax Park	$F_{(5,12)} = 45.66$, $p < 0.01$*	95%	$F_{(2,12)} = 87.75$, $p < 0.01$*	$F_{(1, 12)} = 45.96$, $p < 0.01$*	$F_{(2, 12)} = 3.43$, $p = 0.07$
Wootons	$F_{(5,12)} = 12.34$, $p < 0.01$*	84%	$F_{(2,12)} = 6.23$, $p = 0.01$*	$F_{(1, 12)} = 36.03$, $p < 0.01$*	$F_{(2, 12)} = 6.62$, $p = 0.01$*



Supplemental Figure 1. Six images representing soil profiles from Supplemental Table 1. Soils were collected from the low marsh (LM), high marsh (HM), and upland (UP) habitats in Patuxent Wetlands Park (Pax Park) and Wootons Landing Wetland Park (Wootons). The first three images represent soils from Pax Park (A) subplot #5 (HM), (B) subplot #7 (UP), and (C) subplot #8 (UP). The last three images represent soil profiles at Wootons (D) subplot #12 (LM), (E) subplot #14 (HM), (F) subplot #17(UP).



Supplemental Figure 2. Soil pH procedure test. Mean soil pH (± SEM) was determined for one soil sample collected from the Patuxent Wetlands Park upland subplot (#7B). Samples were prepared by mixing 1 part soil to 2 parts 18 MΩ water or 0.01 M calcium chloride ($CaCl_2$) for 30 s with a metal spatula. Soils were left undisturbed for 1.5 minutes and then a pH probe was inserted into the upper aqueous layer. Soil pH values were recorded every minute from 2 to 10 min. A one-way repeated measures analysis of variances (ANOVA) was used to separately evaluate changes in soil pH associated with 18 MΩ water or $CaCl_2$ over time. The model detected mean differences in soil pH over time for soils prepared in 18 MΩ water ($F_{8, 8} = 13.0$, $p < 0.01$) but not for $CaCl_2$ ($F_{8, 8} = 0.19$, $p = 0.99$). Tukey's HSD post hoc test was used to evaluate changes in soil pH associated with 18 MΩ water; those differences are indicated with Tukey's letters in the figure above. Due to limited sample size, an independent t-test was used to evaluate differences between the two grand means (dashed line). Using a parametric t-test, it was found that mean soil pH prepared in 18 MΩ water (6.8 ± 0.03) was significantly higher compared to soils prepared in 0.1 M $CaCl_2$ (5. 7 ± 0.03; t-ratio = 28.5, df = 31.3, $p < 0.01$); a Wilcoxin's non-parametric ranks sums t-test confirmed the statistical differences between the two grand means ($Z = 5.23$, $p < 0.05$). In conclusion, soils were prepared in a 0.01 M $CaCl_2$ matrix solution because we did not detect differences in soil pH over time. Furthermore, $CaCl_2$ flocculates suspended material, which produces a clear supernatant layer for easy measuring, normalizes ionic strength, and thus standardizes comparisons across multiple soils types.



Supplemental Figure 3. Pairwise comparison of soil organic matter content (w/w, %) and total C (w/w, %) for sixteen representative soil samples. The linear relationship was used to extrapolate total C values for the four soil fractions.

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Chapter 4: Leaf quality and not clay content drives soil respiration and microbial community composition patterns in wetland soil mesocosm experiment

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Keywords: Carbon Biogeochemistry, Greenhouse Gases, Soil Organic Matter, Tidal Freshwater Wetlands, Soil Respiration, Soil Microbial Ecology

Abstract

When compared to their natural counterparts, restored tidal freshwater wetlands store less soil C and mineralize more C as carbon dioxide (CO_2) and methane (CH_4). The reasons for these discrepancies are not clear, but may in part be due to the difference in soil texture. To test this idea, anaerobic laboratory mesocosms were established to evaluate effects of % clay concentrations and leaf litter quality on soil C cycling processes over time. We expected increasing clay concentration in a primarily sandy restored wetland soil would protect C from microbial attack, decreasing soil C

mineralization and greenhouse gas production. We also expected soils treated with low-quality organic amendments would lower microbial carbon use efficiency and increase soil respiration. Representative soils were collected from contrasting marshes – one natural and one restored tidal freshwater wetland – located along the Patuxent River in Anne Arundel County, Maryland. Total soil respiration in natural soils was approximately half of what was observed in restored soils. Surprisingly, increasing clay concentration in restored soils from 13% to 20% and 30% did not reduce soil respiration. We also found that total soil respiration was greater in restored soils treated with high-quality C substrates compared to low-quality C substrates, but natural soils were not affected by the quality of the C amendment. In addition to observing greater total soil respiration in restored soils, restored soils treated with organic amendments leached more dissolved organic carbon to the adjacent water column. More C was partitioned as CH_4 compared to CO_2, but the mass ratio of CO_2 to CH_4 was similar across all substrate treatments. Even after adjusting for potential CH_4 oxidation, soils from the restored wetland emitted more greenhouse gases compared to their natural counterpart. It appears that soil C cycling is quite different between these two wetlands, and the addition of high and low-quality C substrates to low C systems elicit a greater response from the heterotrophic microbial community. Therefore, we would suggest that the quantity and lability of organic matter amendments be carefully considered prior to their addition to low C soil systems.

Introduction

Wetlands are a critical global carbon (C) sink (1–3), but they are also a significant source of methane (CH_4), discharging 100 to 300 Tg CH_4 yr^{-1} to the atmosphere (1, 4). Methane (CH_4) is a potent greenhouse gas with a global warming potential 28 times greater than carbon dioxide (CO_2) (5). Unlike non-tidal freshwater wetlands, reported annual CH_4 emissions are relatively low for tidal freshwater wetlands (32 ± 37 g CH_4 m^{-2} yr^{-1}, respectively) (3, 6). Tidal freshwater wetlands are found in the upper freshwater, tidal portion of the estuary, wedged between the aquatic and terrestrial habitat (7). Because of their location, tidal freshwater wetlands are subjected to the adverse effects of urbanization, stormwater management, and river channelization (8–10). In the last two decades efforts to restore wetlands, including tidal freshwater wetlands, and their ecosystem services have increased nationwide. However, research has demonstrated that restored wetlands store less soil C (11–13) and emit more greenhouse gases (14, 15) than their natural counterparts. Little is known about the effects of restoration, specifically changes to the soil microbial community, C quality, and soil texture, on soil C processing and subsequent greenhouse gas emission in these habitats.

Soil C cycling in natural tidal freshwater wetlands is similar to natural non-tidal freshwater wetlands (6, 16, 17). Carbon sequestration is primarily driven by plant-mediated photosynthesis. After capturing and storing CO_2 into organic compounds, plant detritus is eventually deposited on the soil surface. While some plant particulate organic matter is exported, most of plant detritus and root exudates are retained in the soil habitat, resulting in a significant accumulation of soil C as soil organic matter (20 to 70%) (7). Soil organic matter decomposition is primarily anaerobic because oxygen (O_2) diffusion

rates are 10,000 times slower in water-filled pore spaces than air (18). As a result of limited O_2 availability, soil microbes utilize alternative terminal electron acceptors in the following thermodynamically favorable order: NO_3^- (denitrification), $Fe^{(+III)}$ (iron reduction), $Mn^{(+III/+IV)}$ (manganese reduction), $SO_4^{(-II)}$ (sulfate reduction), and CO_2 (methanogenesis). Because pore water $SO_4^{(-II)}$ concentrations are low (< 1 ppm), methanogenesis is a dominant anaerobic decomposition pathway in tidal freshwater wetlands (19).

Soil microorganisms are central to SOM mineralization to CO_2 and CH_4; therefore, modifications to the microbial population can significantly impact soil C processing and greenhouse gas emissions. Research has demonstrated that soil disturbance and land management affects microbial community composition (20–22), but changes in community structure are not always straightforward (23). For example, studies investigating soil C mineralization did not detect changes in process rates even after changes in microbial structure or the loss of 90% of the soil microbial biomass (24). Strickland and colleagues reported that only ~20% of the variance in C mineralization was accounted for in the microbial community and land-use was a stronger predictor of C mineralization (25, 26). C mineralization is a broad process that results from many sequential steps carried out by a consortium of decomposers; functional redundancy across many different types of organisms can, therefore, make up for loss of species diversity (27). While some functions, like soil C mineralization, may be unaffected, other metabolic processes, like methanogenesis and methane oxidation, are sensitive to changes in the soil habitat (28).

Methane emission is controlled by two groups of organisms. Methanogens are specialized microorganisms conserved to the phylogenetic lineage Euryarchaeota (29). While methanogens are solely responsible for methanogenesis (4, 30, 31), their activity is dictated by a sufficient supply of low molecular weight C substrates. These labile C substrates are limited in the soil system (17); therefore, methanogens rely on the C waste byproducts of hydrolytic and fermentative bacteria. Changes in the activity of hydrolytic and fermentative bacteria can affect CH_4 production (14). Emission of CH_4 is not only affected by the methanogen population, but also by the oxidation of CH_4. Chemoautotrophic methanotrophs reside in the oxic microsites of the soil habitat (32). These microorganisms are essential to CH_4 cycling because methanotrophy, along with autooxidation, converts 20 to 40% of CH_4 to CO_2 (33). These two populations are integral to CH_4 cycling so that direct changes in composition have been found to modify CH_4 flux (28).

Plant litter quality, particularly the litter C: N ratio, is also influential on soil C processes because it affects the carbon use efficiency (CUE) of the microbial community. Carbon use efficiency is the ratio of C incorporated into microbial biomass to C lost via respiration (34–36). Carbon use efficiency ratios are variable but typically range from 0.2 to 0.3 for aquatic and terrestrial systems (34, 35). In the eastern United States, the nonnative lineage of common reed, *Phragmites australis*, has expanded into disturbed coastal marshes. This plant species has a high plant C: N (37) which can lower CUE. Additionally, the nonnative lineage of *P. australis* has been shown to change the soil microbial community composition (38). Shifts in microbial composition, especially from oligotrophs to copiotrophs, can further lower CUE. Copiotrophs are typically dominant in

organic-rich soils and have fluctuating populations with high respiration rates. In contrast, oligotrophs are slower growing organisms that tend to inhabit low C systems and (39).

Soil texture is another important variable affecting soil C processes. Texture indirectly modifies many properties of the soil, including water holding capacity (40) and nutrient availability (41, 42), which have direct and indirect effects on soil microbial populations and subsequent activity (21, 43). Soils of natural tidal freshwater wetlands are formed primarily from sediment deposits of clay, silt, and organic matter (44). Habitat restoration changes many soil properties. For example, in a comparative analysis of wetlands in the North Carolina Coastal Plain, Bruland and Richardson (13) documented restored wetland topography was more homogenous and soil sand content was increased by approximately 20%. Since coarser soil textures, like sand, have less capacity to store and protect soil C from microbial decomposition (43), increasing sand content can change the long-term trajectory of soil C processes. Studies found the addition of medium size clay peds (< 2 mm) to sandy soils can reduce cumulative soil C respiration. (45, 46). The predictability of clay on soil C respiration is not always clear because studies have also reported positive correlations between clay concentrations and soil C respiration rates (41, 47, 48) and CH_4 production (30).

This study investigated the effects of clay, leaf litter quality, and their interaction on C mineralization and greenhouse gas production in soils collected from one natural and one restored tidal freshwater wetland. We hypothesized that increasing clay content would reduce soil respiration and lead to an increased ratio of CO_2: CH_4. We further hypothesized soils treated with high-quality organic amendments (i.e., low C: N ratios)

would have lower soil respiration compared to low-quality organic amendments (i.e., high C: N) due to higher CUE. And lastly, we expected to find a positive correlation between the archaea population and CH_4 production.

Methods and Materials

Experimental Design

Laboratory mesocosms were independently assembled and randomly assigned to receive one of four soil types (Natural, Restored, Restored + 7% clay, or Restored + 17% clay) and one of three C amendments (+*Peltandra virginica* leaf material ("High Quality"), + *Phragmites australis* leaf material ("Low Quality), or no additional C amendment ("control")). A total of three replicates were assembled for each of the 12 treatments resulting in a total of 36 mesocosms per set. Three replicate mesocosm sets were assembled, and each set was destructively sampled at different time points related to changes in soil respiration: post-lag (incubation day 14), peak respiration (incubation day 34), and stationary phase (incubation day 62). Mesocosm sets were incubated separately up to 9 wk under anaerobic conditions. For each destructive day, soil and water samples were collected to evaluate physiochemical characteristics and microbial community composition. Total soil respiration and gas composition (CO_2 and CH_4) were also monitored for 9 wk.

Site History

Soils were collected from the high marsh zone of two tidal freshwater wetlands: Patuxent Wetlands Park (38° 48' 40.8" N, 76° 42' 38.0 W) and Wootons Landing Wetland Park (38° 51' 20.9" N, 76° 41' 27.3" W). Both tidal freshwater wetlands are

located along the Patuxent River in Anne Arundel County, Maryland. Wetlands of Patuxent Wetlands Park share many of the traditional characteristics of tidal freshwater wetlands found along the East coast (7). Wootons Landing Wetland Park consists of 140-acres of non-tidal forested and tidal freshwater wetlands and. The park is located approximately four miles up river of Patuxent Wetlands Park and has a unique history.

According to construction and summary reports, Wootons Landing Wetland Park served as a sand and gravel mining site until 1973. In 1973, mining at the site was exhausted, and the land was turned over to Anne Arundel County, Maryland. For several years the site served as an unofficial dumping ground for trash, appliances, and cars as well as an informal recreation dirt bike park (49, 50). In 1992, the Maryland State Highway Administration restored 73 acres of wetlands to compensate for impacts to other off-site wetland habitats (49–51). The top portion of the soil profile was removed to reinstate wetland hydrology. During construction, a thin calcite layer was discovered and subsequently broken up; evidence of this is still present as shells are distributed throughout the soil profile. After restoring the hydrology, a layer of topsoil amended with composted woody material was spread across the marsh (50). Today, a small tidal freshwater wetland (~2.42 acres) composed of *Nuphar lutea, Typha latifolia,* and the nonnative lineage *Phragmites australis* (Cav.) Trin. ex Steud is present in the marsh.

Soils of the two parks were previously characterized in Maietta et al., *in prep*; a summary is presented in **Table 1**. Briefly, soils in Patuxent Wetlands Park are classified as Mispillion and Transquaking and have a high SOM content (52). Soils in Wootons Landing Wetland Park are underdeveloped and primarily composed of sand (~76%). Upon closer inspection, the greenish sand-size deposits were identified as glauconite

pellets. Sometimes referred to as "greensands," glauconite pellets are small, spherical pellets that behave like sand grains within the soil profile (53–55). Greensands are also a rich source of iron, which can affect soil oxidation potential and chemical transformations (56).

Patuxent Wetlands Park and Wootons Landing Wetland Park will be abbreviated "Natural" and "Restored," respectively, for the remainder of the paper.

Soil Collection and Manipulation

Five 1 m^2 plots were established in the high marsh zone of each park. In each plot, the standing vegetation and the duff layer were removed, and five 40 cm soil biscuits were collected with a 40 cm spade. Large roots, rhizomes, and living organisms were removed by hand, and the remaining soil was transferred to several 5 gals buckets. Upon returning to the lab, soils were passed through a 9.0 mm and 4.5 mm sieve, effectively creating one representative soil mixture per site. Homogenized soils were stored in 5 gals buckets lined with two plastic bags. Three representative subsamples were collected from each soil type and analyzed for SOM content, soil pH, and texture. Plastic bags were loosely tied, and several wet paper towels were placed on top to help maintain field conditions. After loosely securing a lid to the top of each bucket, buckets were stored at 4°C until mesocosms were assembled.

Clay Amendment & Isolation Procedure

Approximately 15 kg of soil was collected from the Btg-2 and Btg-3 horizons of an Elkton series soil pit located in Beltsville, Maryland (39° 00' 28.7" N, 76° 50' 49.6" W). Soils of the Elkton series are classified as a fine-silty, mixed, active, mesic Typic Endoaquults and consists of a very deep, poorly drained soil often found in smooth,

nearly level sloping (0 to 2%) woodland areas (57). The Btg-2 and Btg-3 horizons were targeted because soils from these horizons had a high concentration of clay (~35 to 45%) and similar clay mineralogy as the Natural site (i.e., kaolinites, vermiculite, and illite; data not shown).

Soils were spread across a tarp and, with the help of two oscillating fans, air-dried for three days. Air-dried soils were ground to pass through a 2 mm sieve using a custom-built mechanical belt grinder. Two kg of air-dried, ground soils were suspended in 18 L of a 5% (w/v) sodium hexametaphosphate ($Na_6P_6O_{18}$) solution. The soil-water-dispersing agent mixture was stirred for 10 min and, based on Stoke's law, left undisturbed for 24 h. After 24 h, suspended clay particles in the top 20 cm of the water column were transferred to a separate 5 gals bucket and flocculated with 50 g of magnesium chloride ($MgCl_2 \bullet 6\ H_2O$). A siphon was used to remove the upper aqueous layer, leaving behind a semi-flocculated clay pellet. This process was repeated until a total of 15 gals of flocculated clay was collected.

Following initial isolation, Mg-saturated clay was condensed into a firm pellet, washed, dried, and then ground into a fine powder-like product. This was accomplished by centrifuging ~ 200 mL of Mg-saturated clay on a model K size 2 International Centrifuge (Needham HTS, Mass) for 5 min at 1,500 rpm. The aqueous layer was poured off, and an additional 200 mL of Mg-saturated clay was mixed in with the clay pellet. This procedure was repeated 3 to 5 times until a semi-firm clay pellet filled ¾ of the 250 mL Nalgene™ bottle. Following the final centrifugation, 1 to 3 mL of a strong flocculating agent, hexaaqua-aluminum chloride ($AlCl_3 \bullet 6\ H_2O$), was added to further condense the clay pellet. The Al-clay suspension was mixed on a mechanical shaker for

15 min and then centrifuged for 10 min at 2,000 rpm. The supernatant was poured off and the final firm pellet was centrifuged washed with nanopure water (18 mΩ) until the pellet began to disperse (~2 to 3 washes). The product was stored at -20°C for 24 h and lyophilized 3 to 5 d.

A total of 2.7 kg of dried clay was ground into a fine powder using a quartz mortar and pestle. The final product was homogenized and stored in the dark at room temperature until the experiment was assembled.

Leaf Tissue Collection and Carbon Amendment

Leaf tissue collected from green arrow arum (*Peltandra virginica*) and common reed (*Phragmites australis*) plant stands were used for the C amendment. These two plant species were selected because *P. virginica* has a lower C: N ratio compared to *P. australis* (37, 58). Fresh leaf material was washed thoroughly with tap water, patted dry, and then dried at 60°C for 3 days. Dried plant material was ground to pass through a 2 mm sieve using a Wiley Mill (Standard #3, Arthur H. Thomas Co. Philadelphia, PA, USA). After grinding, three subsamples were collected and then the remaining fraction was dried at 60°C for an additional 24 h. All dried leaf material was stored with desiccant at room temperature in the dark until the experiment began.

Three subsamples of air-dried plant tissue were run on the LECO CHN. In addition to determining total C and N content, subsamples were extracted with tap water, nanopure water (18 MΩ), and 1.0 M KCl to quantify the potential range of leachable dissolved organic carbon (DOC) and total nitrogen (TN). Dried leaf tissue C: N ratios for *P. virginica* and *P. australis* were 11.6 (46.4 ± 0.1% C and 4.0 ± 0.0% N) and 16.1 (45.1 ± 0.1% C and 2.8 ± 0.0% N), respectively. The range of total extractable DOC and TN

were similar for both *P. virginica* (189 to 258 mg C L^{-1} and 19 to 35 mg N L^{-1}) and *P. australis* (188 to 260 mg C L^{-1} and 10 to 24 mg N L^{-1}).

Mesocosm Design and Assembly

Mesocosms were built based on the original design presented in Updegraff et al. (59); however, due to a few unavailable products, the mesocosm design was modified (**Supplemental Figure 1**). Laboratory mesocosms were assembled using 1000 mL Nalgene™ Straight-Sided Wide-Mouth polymethypentene jars with polypropylene lids ("mesocosm chambers"; Thermo Fisher Scientific) and Zap Cap-CR Non-Sterile Bottle-Top Filters ("soil sample cups"; Maine Manufacturing, Sanford, ME). Before assembling mesocosms, the filter was removed from the soil sample cup. Additionally, four holes were drilled into the soil sample cup stand. These holes reduced air bubbles from accumulating around the base and facilitated water movement between the mesocosm chamber and soil sample cup. Additionally, two gas ports, 5-mm and 20-mm in diameter, were drilled into the mesocosm chamber lid. Finally, all materials were autoclaved at 121°C at 15 psi for 15 min and stored in two plastic bags until mesocosms were assembled.

Soil slurries were prepared by mixing an appropriate amount of soil (Natural or Restored), clay (+ 0%, + 7%, or + 17%), and leaf tissue (+ 0% C amendment, + 2.6% C of high quality, or +2.6% low quality) in a DI washed 600 mL borosilicate beaker; specific values for each treatment are summarized in **Supplemental Table 1**. Soil slurries were mixed with 10 mL of sterile tap water supplemented with filter sterilized $CaCl_2$ (final concentration = 1mM $CaCl_2$, pH = 6.8 ± 0.1) for at least 2 min or until the clay was thoroughly incorporated into the soil matrix. Tap water was supplemented with $CaCl_2$ to

reduce clay from resuspending during initial prep and throughout the experiment (**Supplemental Figure 2**). One additional sample per treatment (n=12) was prepared and immediately subsampled to characterize initial soil pH and microbial gene abundance.

To assemble mesocosms, homogenized soil slurries were transferred to a sterile soil sample cup lined with one sterile 85-mm diameter A/E glass fiber disc (1 μm pore size; Pall Corporation). Soil sample cups were gently tapped on the bench top to transfer soil to the bottom of the container and remove large air pockets. Filled soil sample cups were placed into a sterile 1,000 mL mesocosm chamber and then flooded to the soil surface from the bottom up with sterile tap water supplemented with $CaCl_2$. A layer of 100% type II silicone was placed around the edges of the two gas ports and then plugged with one 5 mm and one 20 mm butyl rubber stopper. After the silicone had cured (~30 mn), lids were threaded onto each mesocosm and stored at 4°C overnight. After 24 h, water levels were adjusted to the container's maximum volume line, and O_2 was purged from each mesocosm.

To displace O_2 in the water column and headspace, pure nitrogen (N_2) gas (99.9999%) was bubbled through the free flowing water in the mesocosm chamber for 1 min. A lid lined with a thick layer of 100% type II silicone was then threaded onto each sample chamber, and N_2 was continuously pumped through the headspace for 30 s. After the silicone had cured (~30 min), N_2 was pumped through the headspace for an additional 30 s. The venting needle was removed, and an additional volume of N_2 was injected into the mesocosm chamber to increase the internal pressure. After confirming mesocosms were sealed, containers were vented to atmospheric pressure. Mesocosms were stored at

4°C until every mesocosm was properly sealed and then an additional 24 h to ensure similar starting conditions.

Incubation, Respiration Monitoring, and Destructive Sampling

Due to limited space and access to equipment, each mesocosm set (n = 36) was separately incubated in one of three incubator chambers. Incubators were set to maintain an internal temperature of 20.5°C. Internal temperatures were recorded every 15-min using an iButton® temperature data logger (± 0.5°C, Maximum Integrated, San Jose, California). Individual mesocosms were randomly assigned to one of five shelves and reassigned after gas measurements were collected.

Unfortunately, unexpected soil expansion clogged gas ports and inhibited gas collection; therefore, a smaller mesocosm experiment was deployed in 250 mL glass containers to accurately quantify total gas production and gas composition (**Supplemental Figure 3**). Total gas production was continuously monitored for the full-scale mesocosm experiment (data not shown), and total gas production, as well as gas composition, was collected for the smaller mesocosm experiment. Room temperature (°C) was recorded using three Fisherbrand™ general-purpose thermometers (± 1.5°C), and silicone was applied to the punctured gas port before placing mesocosms back in the incubator.

Soil respiration (mL) was measured every 2-3 days using a borosilicate glass, gas-tight 50 mL (± 1 mL) syringe (Poulten and Graf Fortuna™, Air-Tite) equilibrated to atmospheric pressure. After venting mesocosms to atmospheric pressure, a representative 0.5 mL gas sample was collected from the headspace using a Valco® Precision Sampling syringe (series A-2) with a removable point style 2 bevel tip (Sigma-Aldrich Co. LLC, St.

Louis, MO). The 0.5 mL gas sample was analyzed using gas chromatography (Agilent Technologies, Inc. Shanghai China; model 7890A). The thermal conductivity detector was set at 250°C and equipped with a HP-Plot Q capillary column (Agilent J&W; USA). The carrier gas, helium (He), was dispensed at 8.6 ml min^{-1} and the oven operated at 60°C for 2 min and subsequently ramped at 30°C min^{-1} to 240°C. Before each sample run, a 50%:50% CO_2: CH_4 standard gas mixture was used to produced a standard calibration curve (Industrial Safety Equipment, LLC); calibrations were acceptable if the R^2 value exceeded 99.9%. Hydrogen sulfide (H_2S) was randomly spot checked for 28 d; the practice was discontinued after 28 d since there was no evidence of sulfate reduction. Gas composition (% CO_2 and % CH_4) was converted to parts per million (50% = 50,000 PPM) and then used to calculate mg of C partitioned as CO_2 or CH_4 (mg C-CO_2 or C-CH_4 g^{-1} soil).

Based on a preliminary gas sampling experiments, mesocosms were destructively sampled on incubation day 14 (post-lag phase), 34 (peak respiration), and 62 (stationary phase; **Supplemental Figure 4**). On destructive sampling days, a final gas measurement was collected and then two wrench straps were used to remove the sealed lids from the mesocosm container (**Supplemental Figure 1C**). Each soil sample cup was removed from the mesocosm chamber, and soil redox was immediately measured.

Five soil redox probes were used to estimate soil redox potential. Platinum electrodes were prepared following instructions outlined in Megonigal and Rabenhorst (60), and a modified multimeter was prepared following instructions developed by Rabenhorst (61). Soil redox potentials were measured following similar procedures described in Rabenhorst (62). All redox electrodes were tested using the oxidation-

reduction potential (ORP) standard, Light's solution (Ricca Chemical Company, CAS# 7732-1805), and a silver/silver chloride ($Ag/AgCl_2$) single junction half-cell reference electrode (ORION® Sure-Flow®; correction factor of +222 mV). After confirming similar ORP values among the five replicate probes, five platinum (Pt) electrodes and one reference electrode were inserted to a depth of 5 cm from the soil surface. Raw ORP values were recorded once voltages (V) stabilized. Raw ORP values were converted to mV and then corrected for the $Ag/AgCl_2$ reference electrode (+222 mV) and the multimeter (+2 to +5 mV) offset.

Soils were then removed from the soil sample cup, homogenized, and then four soil subsamples (~20 g each) were placed in 100 mL Whirl-pack® Stand-Up Thio-bags®; samples were stored at -40°C and -80°C for DNA extraction, nutrient analysis, and metabolomics analysis. The remaining fresh sample was analyzed for soil pH following procedures outlined in Maietta et al. (*in prep*). Briefly, a 1:2 mixture of wet soil to 0.01 M $CaCl_2$ was mixed with a spatula for 30 seconds and then left undisturbed for 7.5 min. After 7.5 min, a standardized double-junction combination pH probe (Fisher Scientific™, Hampton, NH) was submerged into the clear supernatant layer. Soil pH values were recorded once readings stabilized or a total settling time reached 10 min.

Dissolved oxygen (DO) concentrations of the freestanding water in the mesocosm chamber were promptly measured. Dissolved oxygen concentrations (mg of O_2 L^{-1}) were quantified using a YSI 55 DO meter (YSI Inc., Yellow Springs, Ohio). After removing the soil sample cup, the remaining standing water was gently swirled three full times, and the calibrated YSI probe was suspended in the middle of the water column. Dissolved oxygen concentrations were recorded once readings stabilized or the 5 min incubation

maximum was reached whichever came first. Four representative water samples (~45 mL) were transferred to 50-mL Falcon™ conical centrifuge tubes and stored at 4°C and -80°C; the remaining solution was discarded.

Microbial Community Analysis

Total genomic DNA was extracted from soils using a PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) following standard protocol with a few minor modifications: soils were homogenized for 45 sec at 4 m/s using a FastPrep®-24 (MP Biomedicals LLC., Solon, OH), centrifuged at 10,000 g for 3 min, and the final DNA product was removed from the spin column using 50 µl of elution buffer. Total genomic DNA concentrations were quantified using a Qubit Fluorometer (Life Technologies, Grand Island, NY).

Quantitative PCR was used to estimate the population size of bacteria and archaea. A complete description of plasmid standard construction was previously described in Prasse et al. (63). All reactions were run in triplicate 20 µl reactions containing 10.0 µl of SybrGreen qPCR readymix with ROX (Sigma, St. Louis, MO), 0.5 µM final concentration of each forward and reverse primer, and 2.0 µl of template DNA. Template DNA concentrations for assessing bacterial and archaeal 16S rRNA gene was determined using 2.5 ng and 5.0 ng, respectively. All reactions were run on the StepOne Plus real-time PCR instrument (Applied Biosystems, Foster City, CA).

Raw CT values were extracted from runs with a standard curve R^2 values exceeding 95%, amplification efficiency between 95% and 105%, and a single dominant peak in the dissociation curve analysis. A soil standard dilution series was used to relativize standard plasmid curves for sample-specific inhibition (64). A soil standard

dilution series was constructed by combining equal molar ratios of one randomly selected replicate from each treatment and destructive sampling period (n = 36). The representative mixture was diluted to 2.5 ng/ul or 1.25 ng/ul, and a 10-fold dilution series was run following the gene specific conditions. **Table 2** summarizes the primers, run method, and amplification efficiencies for each gene used.

Statistical Analysis

Data exploration and statistical analysis were completed using R version 3.3.2 (65) and JMP® Pro 12.2.0 (66). All figures were generated in SigmaPlot for Windows version 10.0.0.54 (Systat Software, Inc., San Jose, CA).

All generated datasets were inspected for normality assumptions before statistical analysis. If experimental error resulted in an outlier or influential data point, those values were removed. In cases of random missing values or justifiably removed outliers, multivariate imputation by chained equations ("mice") was used to impute missing data (m = 5). Each dataset with imputed values was assessed for normality and then fit with the correct linear model. The results from each of the five models were compared using Akaikie's 'An Information Criterion' (AIC). The linear model generating the lowest AIC value was used for all future statistical analysis, figures, and tables.

Gas measurements from the smaller-scale mesocosm experiment were used to assess mean differences in soil respiration. Cumulative respiration values (mL of gas g^{-1} SOC) from days 0, 7, 14, 21, 28, 35, 42, 49, 56, and 63 were used to show changes in respiration over time. A mixed-effect repeated measures analysis of variance (ANOVA) model was not used to analyze mean differences in soil respiration over time because soil respiration was expected to change over time. Instead, soil respiration data (days 14, 35,

and 63) corresponding to destructive sampling days (incubation days 14, 34, and 62) were used for statistical analysis.

A two-way ANOVA was used to model mean group differences by soil type, C amendment, and soil type × C amendment. After running the initial linear model, residuals were inspected for normality and variances were inspected for homoscedasticity. Significant linear models ($p < 0.05$) were further assessed for significant main and interaction effects. Tukey's honest significant difference (HSD) post hoc test was used to identify significant mean differences among the various treatment combinations. If a continuous data measurement was $\log_{10}$ transformed to meet normality assumptions, mean differences for the *Results* section were calculated by taking the differences between back-transformed $\log_{10}$ least squares mean values. A summary of ANOVA F-statistics, degrees of freedoms, and p-values are presented in **Table 4**.

Results

Incubator Temperature

Temperature data loggers recorded 96 measurements per day for up to 9 wk (**Supplemental Figure 5**). For the duration of the experiment, incubators maintained an internal median temperature of 20.6 ± 0.61 (day 14; n = 1575), 20.6 ± 0.39 (day 34, n = 3696) and 20.6 ± 0.46 (day 62, n = 6187), respectively. Two temperature data logs were used to monitor temperature for the smaller scale soil mesocosm; the grand average internal temperature was 20.6 ± 0.45 (n = 11, 894).

Soil Respiration

Total soil respiration (mL g^{-1} SOC) changed over time for the entire duration of the experiment (**Figure 1**). Less than 50 mL of total gas was produced in the first 14 days. Gas production nearly doubled every 7 days from incubation day 14 to incubation day 35. Total soil respiration continued to increase after day 35, but the rate of production slowed from approximately 50 mL to 20 mL every 7 days. Control mesocosms produced less than 2 mL of total gas for the entire duration of the experiment.

All soils treated with an amendment respired significantly more than control treatments (**Figure 1**). All Restored treatments (+0, +7, and +17% clay) amended with high-quality C produced considerably more gas than all Restored treatments amended with low C substrates. It also appears Restored soil types produced more gas than Restored +7%, and Restored +17%; however, statistical analysis did not detect mean differences in total gas produced among these three Restored soil types. Total cumulative gas on incubation day 63 was similar between the two C treatments for Natural soil types, but not for Restored soils.

Mesocosm Analysis

Measured DO concentrations (mg O_2 L^{-1}) remained significantly higher in control mesocosm compared to soils treated with either high or low-quality C amendment for the entire duration of the incubation (**Table 5**). On day 34, mean DO concentration in all control treatments were significantly higher compared to amended soils. But, by day 62, mean DO did not statistical differ between C treatments.

Dissolved organic C (mg C L^{-1}) varied across soil type, C amendment, and soil type × C amendment treatments and the trends were different for each destructive

sampling date (**Table 5**). On day 14, DOC values were lower in all Restored soil types compared to other treatments. By day 34, the main effects for soil and C amendment were individually significant. According to Tukey's HSD, Natural and control treatments had the lowest DOC concentration. DOC trends for day 62 were drastically different than initially observed on days 14 and 34. Overall, DOC concentration tended to be lower in Natural and controls soils and similar between Restored, Restored +7%, and Restored +17% soils amended with high or low C amendment.

Mean water pH was relatively stable over time, although there were slight differences in mean by soil, C amendment, and soil × C amendment type for each destructive sampling date (**Table 5**). For day 14, water pH was more acidic in Restored +17% soil types than Natural (-0.13) and Restored (-0.14) soil types. Control C amendments had a neutral water pH (6.6 ± 0.02) compared to soils treated with high (-0.33) and low (-0.21) quality substrates. Soils treated with high-quality C substrates were more acidic than low-quality substrates (-0.13). Water pH for day 32 ranged from 6.3 to 6.7. Natural × control treatment mesocosm had a significantly lower mean water pH compared to all control treatments (diff for each = -0.47). Restored, Restored + 7%, and Restored + 17% soils amended with low-quality amendments had a significantly lower mean water pH compared to Restored, Restored 7% and Restored 17% × control treatments. Mean water pH for mesocosms treated with high and low-quality C amendments did not significantly differ for each soil type. On incubation day 62, mean water pH was considerably lower in Natural (6.5 ± 0.04) and Restored +17% (6.5 ± 0.04) soil types compared to Restored (6.7 ± 0.05) and Restored +7% (6.7 ± 0.03) soil types. Mean water pH did not differ significantly between Natural and Restored 30% soil types

or between Restored and Restored 20% soil types, but it varied by C amendment. Soils treated with low-quality tissue were significantly lower than soils amended with high-quality C amendments (-0.13) and the control mesocosm sets (-0.12).

Initial mean soil pH was higher in soils receiving an organic C amendment, and soil pH decreased as clay content increased (**Table 4**). Similar trends were also observed for days 14, 34, and 62 (**Table 5**). For example, mean soil pH for days 14, 34, and 62 were significantly lower than in control soils compared to soils treated with high (-0.25) and low (-0.5) C substrates; mean soil pH did not differ statistically between C amendments. Mean soil pH for the different soil types varied for each destructive sampling date. On day 14, Natural and Restored soils were both subtantially higher than Restored +17% soil types. By day 32, Restored soil types had the highest mean soil pH compared to all other soil types. Soil pH remained high in Restored soils until day 62, and Natural soil type remained significantly lower compared to Restored (+0.33) and Restored +7% (+0.26); mean soil pH did not differ significantly between the Natural and Restored +17% soil type.

Redox values were higher and more variable at the beginning of the incubation and stabilized by the end of the incubation (**Table 5**). For day 14 and 34, mean redox values were significantly higher in control mesocosms compared to high (+154 mV) and low (+187 mV) C treatments, but did not differ between soils C treatments. On day 32, overall redox values were lower compared to days 14 and 34. While overall soil redox values were lower for day 32, potential redox for Restored + 17% clay was significantly higher than Natural (- 62 mV), Restored (- 69 mV), and Restored +7% (- 55 mV) soil types (**Table 5**). By day 62, soil redox values were higher and similar across all

treatments. When plotting these data on an Eh-pH diagram (**Supplemental Figure 6**), Natural soil types fell right above the technical standard for hydric soils and all C treatments fell below the technical and iron oxide ($Fe(OH)_3/Fe_2^+$) standards. By day 34, redox and soil pH values shifted for all treatments. As a result, all points fell below the technical standard and plotted alongside the $Fe(OH)_3/Fe_2^+$ standard. By day 62, all treatment pH and redox values shifted in the same direction.

Initial bacterial gene copy numbers (16S rRNA genes g^{-1} wet soil) were variable between the different soil and C treatments (**Table 4**). This trend held true for each incubation day and overall gene abundance decreased as time increased (**Figure 4**). On day 14, bacterial 16S rRNA gene abundance was similar among the different treatments except gene abundance was lower in Restored +17% clay. Mean gene abundance for day 34 did not differ significantly among the four soil types but was significantly higher in C amendments compared to control mesocosms. For day 62, mean gene abundance was similar among the four soil treatments except mean gene abundance was significantly lower in control Restored and Restored +7% mesocosms.

Overall 16S rRNA gene abundance for *Archaea* was lower than the *Eubacteria* (**Figure 4**). Natural soils had a lower archaea gene abundance compared to all other soil types on day 14. Natural soils gene abundance was significantly higher than Restored, Restored +7%, and Restored +17% treatments. Additional, gene abundance was significantly similar between Restored 7% and Restored 17%, but both were lower than Restored. By day 62, control mesocosm gene abundance was lower in all Restored soils compared to the Natural soil type. Archaeal gene abundance was similar between C

amendments. Additionally, Natural soils amended with low-quality C had a significantly higher mean gene abundance compared to Restored +17% clay.

Total soil respiration (mL g-1 soil) measured on days 14, 35, and 63 were correlated with total microbial gene abundance (16S Eub + 16S Arc per g soil) measured on days 14, 34, and 62. Because total soil respiration for control mesocosms produced less than two mL of gas for the entire duration of the experiment, these values were excluded from the correlation assessment. A significant positive correlation was detected between total soil respiration and total microbial gene abundance (proxy for biomass) for days 14 (Pearson's $r = +0.58$, $p < 0.01$) and days 34 (Pearson's $r = 0.68$, $p < 0.01$). The correlation between soil respiration and microbial gene abundance for day 63 was negative and not significant (Pearson's $r = -0.11$, $p = 0.59$).

Discussion

Unlike previous studies that reported decreasing C mineralization with increasing clay concentration (43, 46, 67), we did not observe differences among the Restored clay treatments (**Figures 1 and 2**). This was surprising because we expected the addition of fine clay to primarily sandy soil systems would enhance C sorption and decrease mineralization. In a similar study by Royland and Marschner (48), the effect of clay on soil respiration was affected by residue loading. Given that Restored soils have a low total soil C pool (< 2%), it is likely that some organic matter was sorbed to clay surfaces, but the organic matter addition overwhelmed the system. The addition of novel C substrate to the low C system may have also influenced microbial enzyme activity and selected for more opportunistic organisms, like copiotrophs, resulting in greater soil respiration.

Basal soil respiration observed in control mesocosms was similar among the four soil types for the entire duration of the experiment (< 2 mL; **Figure 1**). The addition of substrates to Natural and Restored soils increased total soil respiration and DOC leaching (**Figures 1 and Table 5**). Substrate-induced respiration was not unexpected because previous studies have demonstrated organic and inorganic additions enhances heterotrophic microbial activity (68). However, these results did not support our original hypothesis, that soil respiration would be lower in soils treated with high-quality substrates due to greater carbon use efficiency (**Figures 1 and 2**). Instead, we observed C mineralization and greenhouse gas emissions was greater in Restored soils treated with high-quality organic amendments (+ P. virginica) compared to low-quality organic amendments (+ P. australis). Litter chemical composition may be primarily response for these differences. High-quality litter has been shown to decompose faster because it has less complex litter C-chemistry (i.e., relative concentrations of lignin, cellulose, lipids) and higher N content (69–71). It is likely lower extractable TN concentrations in *P. australis* (15 ± 8 and 25 ± 8 mg N L^{-1}, respectively) reduced litter decomposition.

Unlike Restored soils, total soil respiration was similar between the two C treatments in the Natural soil system (**Figures 1 and 2**). Diminished C mineralization in higher organic matter soils was also observed in Yavitt and Lang (72). In this study, organic-rich soils did not respond to organic and inorganic substrate additions and produced a significantly lower amount of CH_4 compared to other soil types. In our study, soil C levels in the Natural soil system are high (~45%), which may be responsible for the reduced response. These results may also suggest that the soil microbial community in the Natural system is resilient to changes in litter quality. The high marsh zone

supports a diverse plant community (15+ plant species), which changes over space and time. Soil microorganisms occupying this habitat may be dominated by slow-growing oligotrophs (39) and have the extracellular enzymes to decompose multiple resources.

The composition of the gas changed over time. (**Table 5 and Figure 3**). For the first 14 days of incubation, CO_2 was the dominant respiration byproduct. Iron reduction was probably a dominant anaerobic respiration pathway in these systems. High concentrations of reduced Fe^{+II} in the soil profile of both parks were discovered during an initial inspection of these two sites (α-α-dipyridyl strip data not shown). During excavation and preparation, some Fe^{+II} was probably re-oxidized to Fe^{+III} and available for heterotrophs. Additionally, clay, organic matter, and glauconite pellets are all potential sources of Fe. Denitrification may also have been a source of CO_2 and other unaccounted trace gases. For the first several weeks of incubation, total respired gas exceeded total mL of CO_2 + CH_4. We monitored H_2S production for the first 28 d and confirmed sulfate reduction was not contributing to soil respiration. Therefore, it is likely denitrification was active in these systems. As water column DO concentrations, and soil redox values decreased (**Tables 4 and 5**), CH_4 production became the dominant respiratory product.

We observed a higher total C turnover (total mg of C respired as CO_2 + CH_4 ÷ total soil C + total C amendment (72)) in Restored soils (14 to 22%) compared to Natural soils (2 to 3%). However, the ratio of total respired CO_2: CH_4 was relatively similar across leaf and soil treatments (**Table 6**). High yields of CH_4 emission from these systems are unrealistic because anaerobic incubations suppress methane oxidation and uncouple methane cycling. It is estimated that 20 to 40% of the CH_4 produced in soils is

autoxidized or biological oxidized (33). If we correct for potential oxidation in this system, mean total CH_4 emissions from Natural soils is negligible (+High Quality: 19 ± 1 to 25 ± 1mL g^{-1} SOC; +Low Quality: 13 ± 1 to 17 ± 2 mL g^{-1} SOC) compared to Restored soils (+High Quality: 135 ± 12 to 180 ± 16 mL g^{-1} SOC; +Low Quality: 93 ± 12 to 125 ± 16 mL g^{-1} SOC). Therefore, even after correcting for oxidation, substrate induced respiration in Restored soils are a potential source of the potent greenhouse gas CH_4.

The microbial response to organic additions and incubation over time were also unexpected. Overall, we saw microbial 16S rRNA gene abundance, especially archaeal gene abundance, decrease over time (**Fig 4**). McLatchey and Reddy (73) found that microbial biomass C, N, P fell as soils became more reduced. It is likely that changes in redox may account for decreasing 16S rRNA gene abundance, but it may also be the result of a shift in community composition. Copiotrophs tend to utilize readily available resources and have fluctuating populations (39). As new resources become less available, copiotroph population decline and soils are dominated by oligotrophs. We are still waiting for sequencing results, but I expect we will detect changes in the relative abundance of copiotrophs (e.g., β-Proteobacteira and Bacteroidetes) to oligotrophs (e.g., Acidobacteria) to evolve over time. Furthermore, I would expect a higher relative abundance of Acidobacteria in low C systems (i.e., Restored soils) compared to high organic matter systems (i.e., Natural site). We found a weak but significant correlation between 16S rRNA archaea genes and CH_4 production for days 34/35 ($r = + 0.45$, $p = 0.03$), but all other correlations were insignificant. Once the sequence and methyl coenzyme reductase A (*mcrA*) gene abundance data are available, it will also be

interesting to evaluate the diversity and abundance of methanogen with methane production. Because we see a decrease in archaeal gene abundance over time, I suspect that we shift from a diverse group of organisms to a few dominant species of methanogens.

Conclusions

In conclusion, we rejected both our hypotheses. In Restored soils, we found that clay additions did not decrease total soil C respiration. We also found the addition of high-quality organic amendments to Restored soils induced more C respiration in Restored soils. Organic amendments to Natural soils produced half the volume of gas compared to Restored soils, suggesting mechanisms controlling C mineralization are quite different between the two habitats. Because Restored soils are C limited, the addition of different organic amendments may be fueling opportunistic copiotrophs resulting in a significant loss of C from the soil system to the atmosphere as CH_4. It would be interesting to replicate this study in the greenhouse and field to examine how clay and C amendments influence C cycling at larger scales with fluctuating oxic conditions. Until then, the quality and quantity of C amendments applied to the topsoil of Wootons Landing Wetland Park should be carefully considered.

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Table 1: Soil characteristics of the high marsh zone at two tidal freshwater wetlands (n = 3, mean ± SEM). Soil texture was determined via particle size analysis (PSA: Bouyoucos G.J. (74)). Procedures to quantify soil organic matter (%) and pH were outlined in the *Methods: Physicochemical Properties* section.

Site	**Patuxent Wetlands Park** "Natural"				**Wootons Landing Wetland Park** "Restored"			
Soil Profile Description	Depth (cm)	Horizon	Color	Field Texture	Depth (cm)	Horizon	Color	Field Texture
	0-17	Oe	2.5Y 3/2	Muck	0-10	Oa	10Y 2.5/1	Muck
	17-81	Oa1	2.5Y 4/1	Muck	10-24	A	N 2.5	Mucky sand
	81-200	Oa2	2.5Y 3/1	Muck	24-37	C1	5Y 6/1	Sand
					37-91	C2	5Y 4/2	Sandy
Characteristics of Homogenized Soils (no amendments)								
Texture Class	Silty Clay				Sandy Loam			
% Clay	47.9 ± 1.2				13.0 ± 0.8 (-34.9)			
% Sand	5.7 ± 0.2				75.6 ± 1.3 (+69.9)			
Soil Organic Matter (%)	22.9 ± 0.18				3.3 ± 0.13 (-19.6)			
Soil pH_{CaCl2}	5.1 ± 0.05				6.5 ± 0.01 (+1.4)			

Table 2. Primers, reaction conditions, and efficiencies for quantitative PCR.

Target Gene		**Pure Culture**	**Primers & References** (Forward / Reverse)	**Thermocycler Conditions** (Acquisition Step Bolded)	**Number of Cycles**	**Plasmid standard and soil correction efficiency (%) (r^2 values)**
EUB	Bacteria 16S rRNA	*Escherichia coli*	EUB338 (75) EUB518 (75)	95°C for 5 min 95°C for 5 s / 55°C for 15 s / **72°C for 10 s**	1 40	Plasmid = 97% - 105% Soil = 97% All $r^2 \geq 99\%$
ARC	Archaeal 16S rRNA	*Sulfolobus solfataricus*	A915-for (76) Arc1059r (76)	95°C for 5 min 95°C for 5 s / 55°C for 15 s / **72°C for 10 s**	1 40	Plasmid = 94% - 100% Soil = 94% r^2 = 96 - 99%

Table 3. Initial mesocosm soil characteristics. One additional soil sample was prepared the day of mesocosm setup and immediately destructively sampled to assess initial soil characteristics for each of the twelve treatments. The mean ± SEM is presented for soil pH and median ± SD is presented for Eubacteria and Archaea.

	Carbon Amendment (n=4)	**Soil Type**			
		Natural	Restored	Restored + 7% Clay	Restored + 17% Clay
Soil pH_{CaCl2}					
Site (n=3)	**---**	***5.8 ± 0.35***	***6.4 ± 0.00***	***6.3 ± 0.03***	***6.1 ± 0.03***
Control	**6.0 ± 0.30**	5.1	6.4	6.3	6.2
High Quality	**6.2 ± 0.06**	6.2	6.4	6.2	6.1
Low Quality	**6.2 ± 0.08**	6.1	6.4	6.3	6.1
Eubacteria (16S rRNA genes g^{-1} wet soil)					
Site (n=3)	**---**	***2.14 x 10^{10} ± 3.61 x 10^{8}***	***5.02 x 10^{10} ± 4.07 x 10^{10}***	***2.77 x 10^{10} ± 2.17 x 10^{10}***	***2.57 x 10^{10} ± 1.65 x 10^{10}***
Control	**7.44 x 10^{9} ± 8.25 x 10^{9} ^{b}**	2.14 x 10^{10}	8.29 x 10^{9}	6.58 x 10^{9}	2.25 x 10^{9}
High Quality	**3.79 x 10^{10} ± 3.13 x 10^{10} ^{a}**	2.12 x 10^{10}	8.96 x 10^{10}	5.00 x 10^{10}	2.57 x 10^{10}
Low Quality	**3.10 x 10^{10} ± 1.22 x 10^{9} ^{a}**	2.19 x 10^{10}	5.02 x 10^{10}	2.77 x 10^{10}	3.42 x 10^{10}
Archaea (16S rRNA genes g^{-1} wet soil)					
Site (n=3)	**---**	***1.39 x 10^{9} ± 9.67 x 10^{8} ^{A}***	***7.94 x 10^{8} ± 1.03 x 10^{8} ^{A}***	***6.21 x 10^{8} ± 8.47 x 10^{7} ^{AB}***	***2.79 x 10^{8} ± 1.04 x 10^{8} ^{B}***
Control	**6.56 x 10^{8} ± 1.16 x 10^{8}**	2.74 x 10^{9}	6.90 x 10^{8}	6.21 x 10^{8}	1.35 x 10^{8}
High Quality	**6.77 x 10^{8} ± 2.99 x 10^{8}**	8.65 x 10^{8}	8.96 x 10^{8}	4.89 x 10^{8}	2.79 x 10^{8}
Low Quality	**7.21 x 10^{8} ± 4.42 x 10^{8}**	1.39 x 10^{9}	7.94 x 10^{8}	6.47 x 10^{8}	3.36 x 10^{8}

Table 4. Two-way analysis of variance (ANOVA) summary table. Variables that were log_{10} transformed prior to statistical analysis are marked with a hash tag (#).

Variable	Full Model ($F_{(11, 24)}$)	Variance Explained	Soil Type ($F_{(3,24)}$)	Carbon Amendment ($F_{(2,24)}$)	Soil × Carbon ($F_{(6,24)}$)
Total Soil Respiration (mL of gas g^{-1} SOC) (microcosm experiment)					
Day 14	95.4, $p < 0.01$*	98%	38.7, $p < 0.01$*	423, $p < 0.01$*	14.4, $p < 0.01$*
Day 35	188, $p < 0.01$*	99%	150, $p < 0.01$*	681, $p < 0.01$*	42.9, $p < 0.01$*
Day 63	206, $p < 0.01$*	99%	189, $p < 0.01$*	699, $p < 0.01$*	50.9, $p < 0.01$*
Dissolved Oxygen (mg of O_2 L^{-1})					
Day 14	19.9, $p < 0.01$*	90%	1.46, $p = 0.25$	103, $p < 0.01$*	1.48, $p = 0.23$
#Day 34	60.3, $p < 0.01$*	97%	0.76, $p = 0.53$	322, $p < 0.01$*	2.77, $p = 0.03$*
#Day 62	19.6, $p < 0.01$*	90%	1.78, $p = 0.18$	100, $p < 0.01$*	1.60, $p = 0.19$
*Dissolved Organic Carbon (mg C L^{-1}) **					
#Day 14	7.53, $p < 0.01$*	78%	0.87, $p = 0.47$	28.4, $p < 0.01$	3.93, $p < 0.01$*
#Day 34	5.21, $p < 0.01$*	71%	8.45, $p < 0.01$*	13.7, $p < 0.01$*	0.76, $p = 0.61$
Day 62	9.46, $p < 0.01$*	81%	6.23, $p < 0.01$*	28.0, $p < 0.01$*	5.23, $p < 0.01$*
Water pH					
Day 14	9.44, $p < 0.01$*	81%	4.67, $p < 0.01$*	40.8, $p < 0.01$*	1.37, $p = 0.27$
Day 34	10.8, $p < 0.01$*	83%	14.7, $p < 0.01$*	17.6, $p < 0.01$*	6.44, $p < 0.01$*
Day 62	3.75, $p < 0.01$*	63%	8.09, $p < 0.01$*	4.91, $p = 0.02$*	1.19, $p = 0.35$
Soil pH					
Day 14	4.75, $p < 0.01$*	69%	7.23, $p < 0.01$*	9.74, $p < 0.01$*	1.84, $p = 0.13$
Day 34	6.67, $p < 0.01$*	75%	6.91, $p < 0.01$*	24.7, $p < 0.01$*	0.53, $p = 0.78$
Day 62	7.96, $p < 0.01$*	78%	10.2, $p < 0.01$*	26.0, $p < 0.01$*	0.83, $p = 0.56$
Redox					
#Day 14	3.73, $p < 0.01$*	63%	1.17, $p = 0.34$	17.3, $p < 0.01$*	0.49, $p = 0.81$
#Day 34	6.06, $p < 0.01$*	74%	7.00, $p < 0.01$*	16.5, $p < 0.01$*	2.10, $p = 0.09$
Day 62	2.16, $p = 0.06$	50%	--	--	--
Bacterial 16S rRNA genes (EUB)					
#Day 14	17.2, $p < 0.01$*	89%	45.6, $p < 0.01$*	18.2, $p < 0.01$*	2.63, $p = 0.42$*
#Day 34	3.11, $p < 0.01$*	59%	1.99, $p = 0.14$	9.40, $p < 0.01$*	1.57, $p = 0.20$
#Day 62	9.20, $p < 0.01$*	81%	3.55, $p = 0.03$*	31.6, $p < 0.01$*	4.58, $p < 0.01$*
Archaeal 16S rRNA genes (ARC)					
#Day 14	8.83, $p < 0.01$*	80%	29.9, $p < 0.01$*	1.41, $p = 0.26$	0.77, $p = 0.60$
#Day 34	35.6, $p < 0.01$	94%	126, $p < 0.01$*	6.79, $p < 0.01$	0.31, $p = 0.92$
#Day 62	24.0, $p < 0.01$*	92%	36.4, $p < 0.01$*	63.9, $p < 0.01$*	4.40, $p < 0.01$*

Table 5. Soil physiochemical properties measured on incubation days 14, 35, and 63. The mean ± SEM is presented for each soil type (n = 9), carbon amendment (n = 12), and soil × carbon (n = 3). Tukey's HSD post hoc test was used to detect mean differences for significant models. Variables that were log10-transformed prior to statistical analysis are marked with a hash tag (#).

DAY 14	Carbon Amendment	Soil Type			
		Natural	Restored	Restored +7%	Restored +17%
Dissolved Oxygen (mg of O_2 L^{-1})					
Soil Type	**---**	***1.43 ± 0.50***	***1.72 ± 0.45***	***1.60 ± 0.55***	***1.94 ± 0.43***
Control	**3.48 ± 0.17** ^{a}	3.30 ± 0.62	3.43 ± 0.17	3.77 ± 0.19	3.40 ± 0.35
High Quality	**0.63 ± 0.11** ^{b}	0.40 ± 0.00	0.87 ± 0.27	0.57 ± 0.32	0.67 ± 0.23
Low Quality	**0.93 ± 0.20** ^{b}	0.60 ± 0.20	0.87 ± 0.33	0.47 ± 0.03	1.77 ± 0.43
#Dissolved Organic Carbon (mg C L^{-1})					
Soil Type	**---**	***11.4 ± 2.11***	***20.1 ± 8.66***	***22.0 ± 6.70***	***10.7 ± 2.66***
Control	**5.19 ± 1.23**	10.3 ± 3.39 ^{ABCDE}	2.03 ± 0.07 ^{E}	3.23 ± 0.90 ^{DE}	5.23 ± 0.97 ^{CDE}
High Quality	**30.2 ± 6.31**	16.4 ±4.50 ^{ABCD}	46.3 ± 18.0 ^{AB}	43.4 ± 7.89 ^{A}	14.8 ± 6.31 ^{ABCD}
Low Quality	**12.7 ± 2.69**	7.43 ± 0.75 ^{BCDE}	11.9 ± 5.97 ^{ABCDE}	19.4 ± 8.15 ^{ABC}	12.0 ± 4.45 ^{ABCDE}
Water pH					
Soil Type	**---**	***6.5 ± 0.04*** ^{A}	***6.5 ± 0.06*** ^{A}	***6.5 ± 0.05*** ^{AB}	***6.4 ± 0.07*** ^{B}
Control	**6.6 ± 0.02** ^{a}	6.6 ± 0.06	6.7 ± 0.06	6.6 ± 0.03	6.6 ± 0.03
High Quality	**6.3 ± 0.03** ^{c}	6.4 ± 0.06	6.3 ± 0.03	6.3 ± 0.03	6.2 ± 0.07
Low Quality	**6.4 ± 0.04** ^{b}	6.5 ± 0.06	6.5 ± 0.06	6.4 ± 0.09	6.3 ± 0.00
Soil pH					
Soil Type	**---**	***6.2 ± 0.16*** ^{A}	***6.2 ± 0.09*** ^{A}	***6.0 ± 0.11*** ^{AB}	***5.7 ± 0.08*** ^{B}
Control	**5.8 ± 0.09** ^{b}	5.7 ± 0.23	6.0 ± 0.06	5.8 ± 0.26	5.6 ± 0.07
High Quality	**6.0 ± 0.12** ^{a}	6.5 ± 0.12	6.1 ± 0.12	6.0 ± 0.15	5.5 ± 0.12
Low Quality	**6.3 ± 0.08** ^{a}	6.3 ± 0.22	6.5 ± 0.10	6.2 ± 0.10	6.0 ± 0.09
#Redox (mV)					
Soil Type	**---**	***148 ± 31***	***190 ± 51***	***212 ± 43***	***200 ± 40***
Control	**308 ± 35** ^{a}	260 ± 45	352 ± 96	323 ± 109	296 ± 39
High Quality	**147 ± 23** ^{b}	96 ± 23	122 ± 26	169 ± 4	200 ± 82
Low Quality	**108 ± 15** ^{b}	90 ± 3	95 ± 30	143 ± 4	105 ± 46

Table 5 (cont.)

DAY 34	Carbon Amendment	Soil Type			
		Natural	Restored	Restored +7%	Restored +17%
[#]Dissolved Oxygen (mg of O_2 L^{-1})					
Soil Type	**---**	***1.47 ± 0.59***	***1.80 ± 0.67***	***1.70 ± 0.68***	***1.63 ± 0.62***
Control	**4.17 ± 0.17**	3.70 ± 0.68 ^{A}	4.43 ± 0.09 ^{A}	4.43 ± 0.09 ^{A}	4.10 ± 0.10 ^{A}
High Quality	**0.36 ± 0.04**	0.40 ± 0.06 ^{BC}	0.27 ± 0.03 ^{C}	0.33 ± 0.03 ^{BC}	0.43 ± 0.13 ^{BC}
Low Quality	**0.43 ± 0.07**	0.30 ± 0.00 ^{BC}	0.70 ± 0.25 ^{B}	0.33 ± 0.03 ^{BC}	0.37 ± 0.03 ^{BC}
[#]Dissolved Organic Carbon (mg C L^{-1})					
Soil Type	**---**	***10.1 ± 1.54*** ^{B}	***37.1 ± 9.01*** ^{A}	***26.4 ± 5.65*** ^{A}	***22.3 ± 3.86*** ^{A}
Control	**11.9 ± 2.26** ^{b}	8.20 ± 4.05	13.8 ± 6.74	11.3 ± 3.40	14.2 ± 5.25
High Quality	**33.6 ± 7.13** ^{a}	10.3 ± 1.83	53.0 ± 21.6	43.0 ± 10.8	28.1 ± 3.65
Low Quality	**26.4 ± 3.86** ^{a}	11.8 ± 2.29	44.4 ± 6.30	25.0 ± 1.21	24.5 ± 3.20
Water pH					
Soil Type	**---**	***6.4 ± 0.04***	***6.6 ± 0.05***	***6.7 ± 0.04***	***6.7 ± 0.06***
Control	**6.7 ± 0.07**	6.3 ± 0.07 ^{C}	6.8 ± 0.06 ^{A}	6.8 ± 0.06 ^{A}	6.8 ± 0.06 ^{A}
High Quality	**6.6 ± 0.03**	6.5 ± 0.03 ^{BC}	6.6 ± 0.06 ^{AB}	6.7 ± 0.03 ^{AB}	6.5 ± 0.00 ^{BC}
Low Quality	**6.5 ± 0.02**	6.5 ± 0.07 ^{BC}	6.5 ± 0.03 ^{BC}	6.5 ± 0.03 ^{BC}	6.5 ± 0.03 ^{BC}
Soil pH					
Soil Type	**---**	***6.34 ± 0.06*** ^{B}	***6.54 ± 0.09*** ^{A}	***6.34 ± 0.08*** ^{B}	***6.26 ± 0.05*** ^{B}
Control	**6.1 ± 0.05** ^{b}	6.13 ± 0.07	6.27 ± 0.17	6.07 ± 0.07	6.10 ± 0.06
High Quality	**6.5 ± 0.05** ^{a}	6.47 ± 0.03	6.63 ± 0.07	6.47 ± 0.13	6.33 ± 0.03
Low Quality	**6.5 ± 0.05** ^{a}	6.43 ± 0.03	6.73 ± 0.03	6.50 ± 0.06	6.33 ± 0.05
[#]Redox (mV)					
Soil Type	**---**	***75 ± 11*** ^{B}	***73 ± 17*** ^{B}	***84 ± 15*** ^{B}	***141 ± 22*** ^{A}
Control	**133 ± 13** ^{a}	107 ± 5	124 ± 30	131 ± 23	170 ± 36
High Quality	**69 ± 20** ^{b}	46 ± 13	30 ± 4	38 ± 1	161 ± 52
Low Quality	**78 ± 7** ^{b}	71 ± 19	65 ± 19	83 ± 12	92 ± 4

Table 5 (cont.)

DAY 62	Carbon Amendment	Soil Type			
		Natural	Restored	Restored +7%	Restored +17%
[#]Dissolved Oxygen (mg of O_2 L^{-1})					
Soil Type	**---**	***1.41 ± 0.45***	***1.89 ± 0.77***	***1.30 ± 0.63***	***1.73 ± 0.73***
Control	**3.99 ± 0.40** [a]	3.13 ± 0.47	4.97 ± 0.23	3.23 ± 1.38	4.63 ± 0.30
High Quality	**0.39 ± 0.05** [b]	0.63 ± 0.03	0.30 ± 0.00	0.33 ± 0.09	0.30 ± 0.06
Low Quality	**0.37 ± 0.04** [b]	0.47 ± 0.09	0.40 ± 0.00	0.33 ± 0.07	0.27 ± 0.07
Dissolved Organic Carbon (mg C L^{-1})					
Soil Type	**---**	***4.14 ± 0.37***	***6.11 ± 0.88***	***7.21 ± 1.46***	***7.53 ± 1.44***
Control	**3.08 ± 0.35**	3.87 ± 0.43 [CD]	2.97 ± 0.79 [CD]	3.37 ± 0.89 [CD]	2.13 ± 0.59 [D]
High Quality	**7.54 ± 0.80**	4.77 ± 1.01 [BCD]	7.80 ± 0.72 [ABC]	6.53 ± 0.57 [ABCD]	11.1 ± 1.26 [A]
Low Quality	**8.13 ± 1.07**	3.80 ± 0.38 [CD]	7.57 ± 0.87 [ABC]	11.7 ± 2.60 [A]	9.40 ± 0.70 [AB]
Water pH					
Soil Type	**---**	***6.5 ± 0.04*** [C]	***6.7 ± 0.05*** [A]	***6.7 ± 0.03*** [AB]	***6.5 ± 0.04*** [C]
Control	**6.6 ± 0.05** [a]	6.5 ± 0.10	6.8 ± 0.07	6.7 ± 0.06	6.5 ± 0.07
High Quality	**6.7 ± 0.03** [a]	6.5 ± 0.09	6.7 ± 0.03	6.7 ± 0.06	6.6 ± 0.03
Low Quality	**6.5 ± 0.04** [b]	6.5 ± 0.00	6.6 ± 0.09	6.6 ± 0.03	6.4 ± 0.06
Soil pH					
Soil Type	**---**	***6.4 ± 0.07*** [B]	***6.8 ± 0.08*** [A]	***6.7 ± 0.07*** [AC]	***6.5 ± 0.07*** [BC]
Control	**6.4 ± 0.05** [b]	6.3 ± 0.09	6.5 ± 0.06	6.5 ± 0.15	6.3 ± 0.03
High Quality	**6.8 ± 0.06** [a]	6.5 ± 0.12	7.0 ± 0.00	6.9 ± 0.00	6.7 ± 0.07
Low Quality	**6.7 ± 0.05** [a]	6.6 ± 0.13	6.8 ± 0.07	6.7 ± 0.06	6.6 ± 0.03
Redox (mV)					
Soil Type	**---**	***173 ± 14***	***182 ± 17***	***181 ± 8***	***191 ± 10***
Control	**206 ± 14**	161 ± 41	237 ± 30	205 ± 14	221 ± 15
High Quality	**169 ± 7**	192 ± 8	154 ± 19	168 ± 5	162 ± 9
Low Quality	**171 ± 6**	166 ± 18	156 ± 7	170 ± 9	191 ± 11

Table 6. Ratio of total cumulative mg of $C\text{-}CO_2$ to $C\text{-}CH_4$ respired in soils treated with contrasting C amendments.

Soil Type	C Amendment	
	Low Quality	High Quality
Natural	0.52 ± 0.02	0.61 ± 0.00
Restored	0.49 ± 0.00	0.59 ± 0.01
Restored +7% Clay	0.54 ± 0.02	0.59 ± 0.00
Restored +17% Clay	0.60 ± 0.00	0.61 ± 0.02

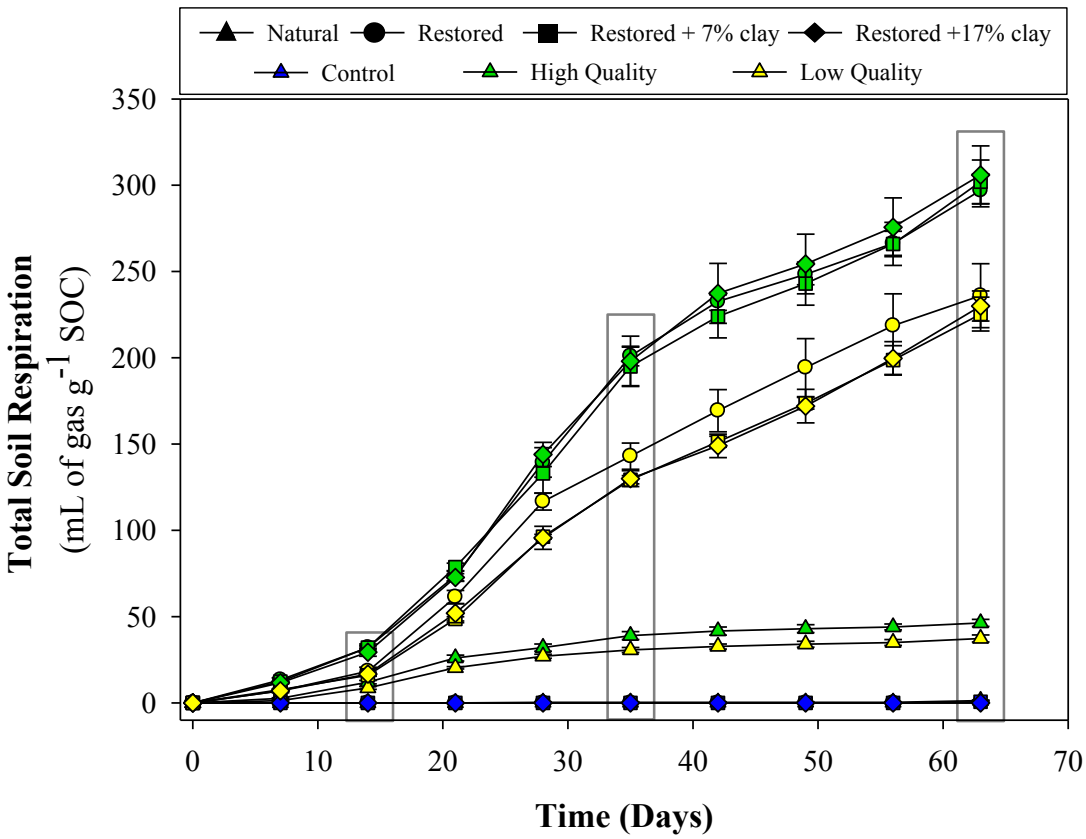


Figure 1. Total soil respiration over time (mean ± SEM). Mesocosms were incubated under anaerobic conditions at 20.6°C for 9 wk and gas measurements were collected every 2-3 d. Gray boxes correspond to the three destructive sampling days.

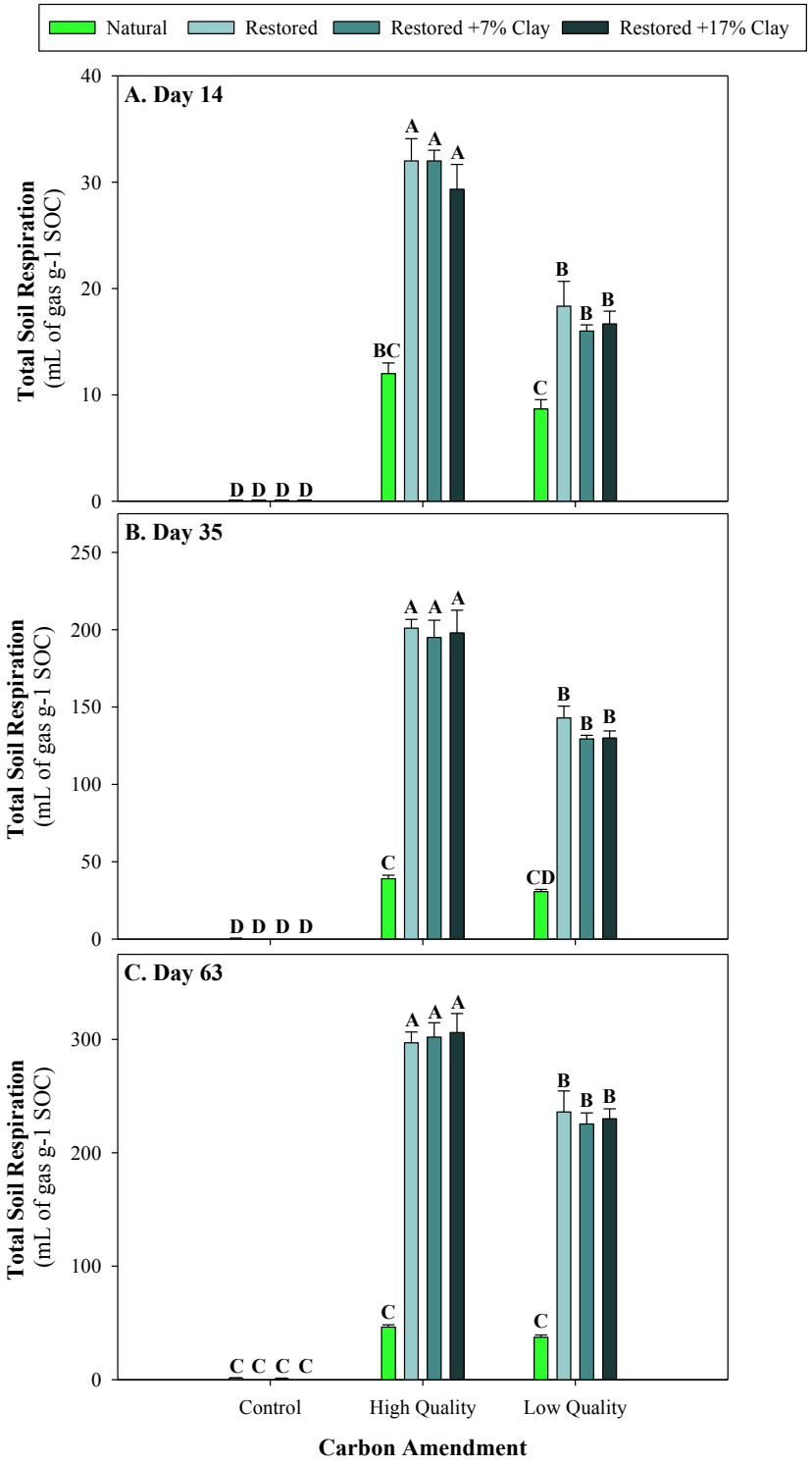


Figure 2. Total soil respiration (mL g^{-1} SOC) for days 14, 35, and 63 (mean ± SEM).

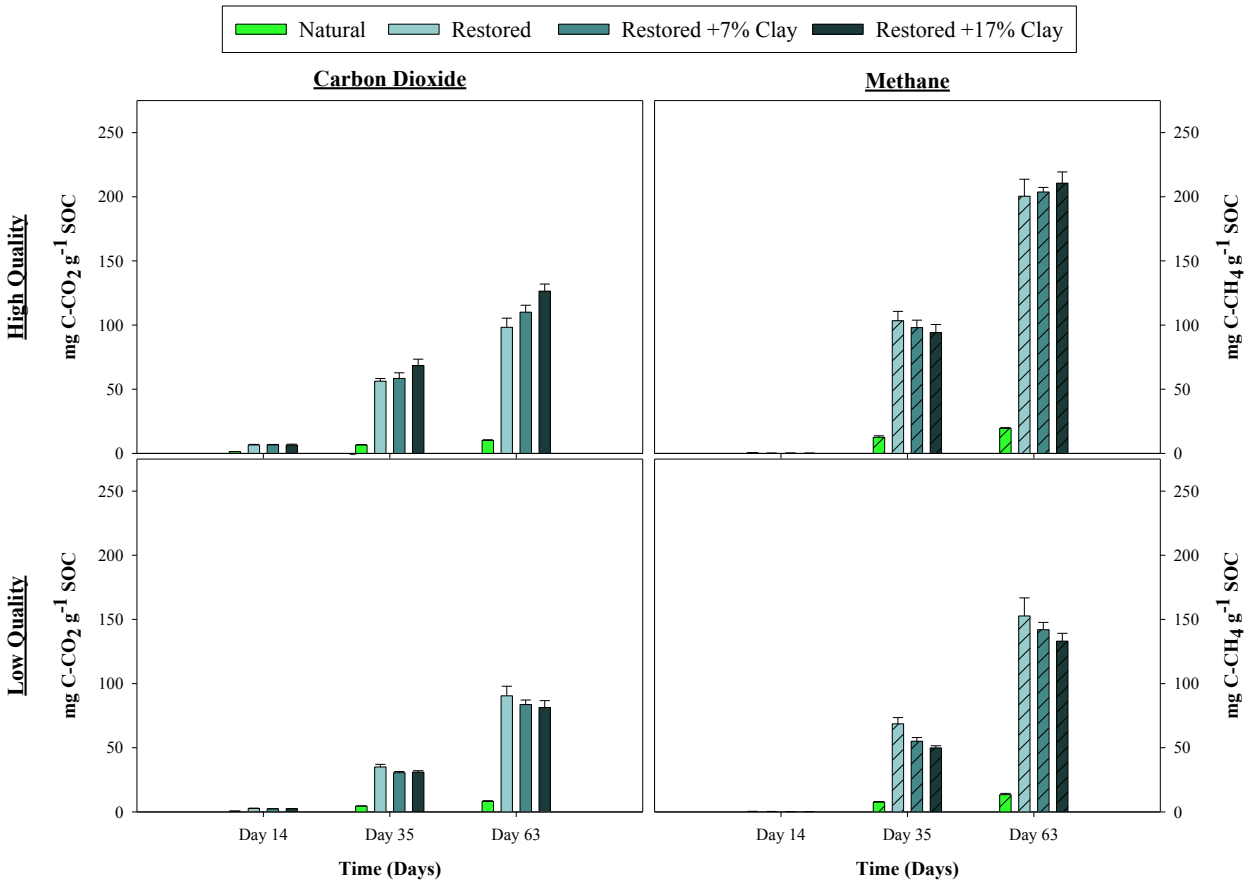


Figure 3. Total production of $C\text{-}CO_2$ and $C\text{-}CH_4$ g^{-1} soil organic carbon (SOC) for days 14, 35, and 63 (mean ± SEM). Control mesocosms respired less than 2 mL of gas for the entire duration of the experiment and thus were excluded from gas composition analysis.

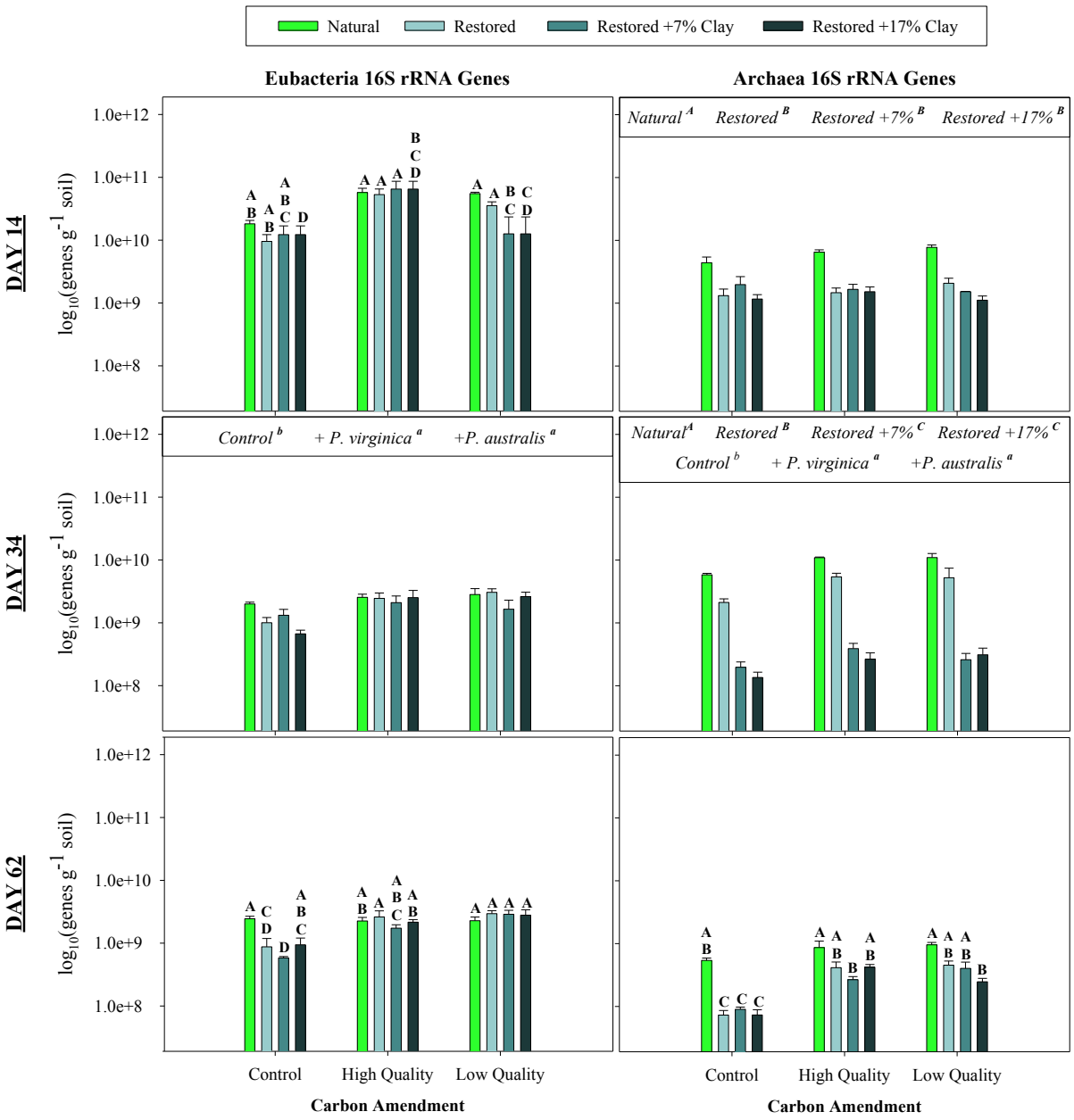
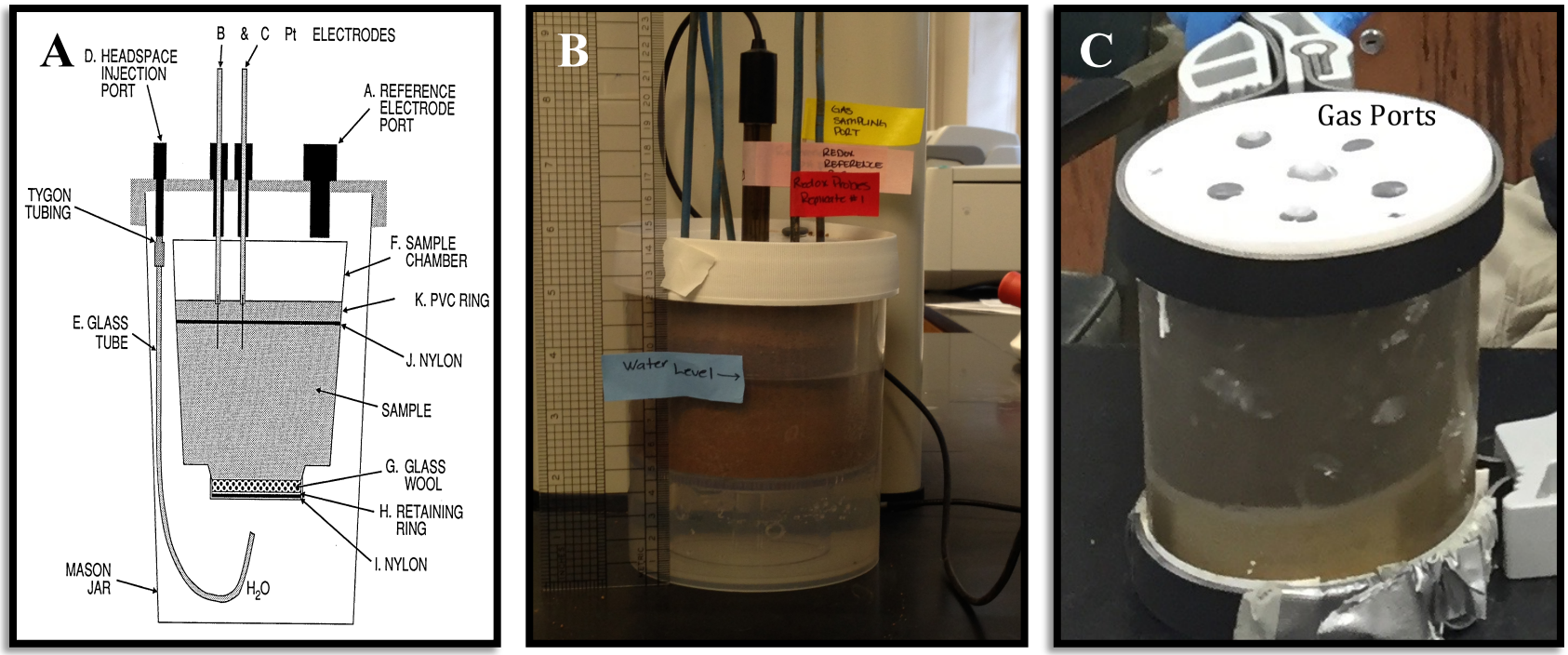


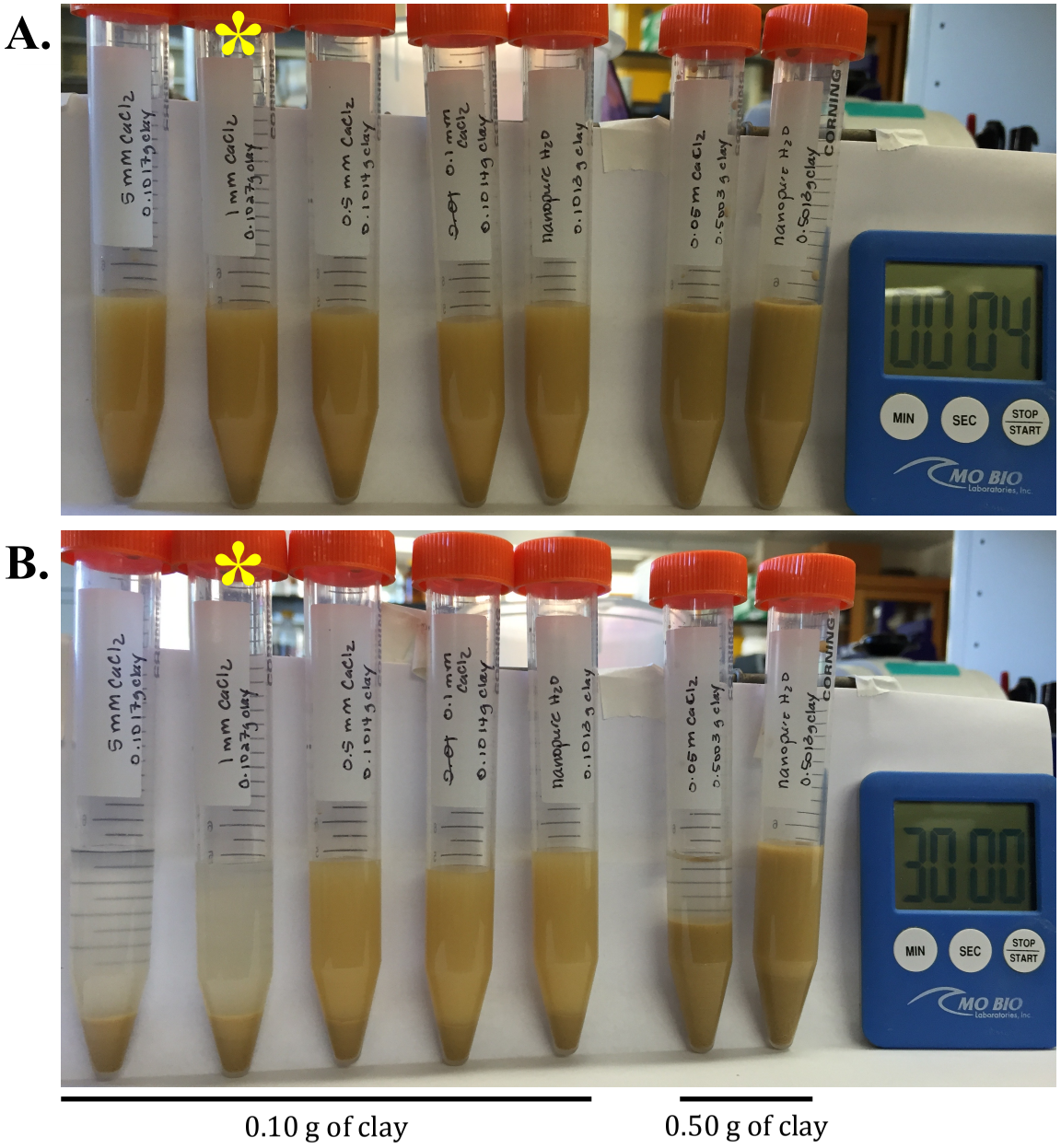
Figure 4. Estimated gene abundance of Eubacteria and Archaea 16S rRNA genes for incubation days 14, 34, and 62. A two-way ANOVA was used to detect mean differences by soil type, C amendment, and interaction of soil × carbon. Prior to statistical analysis, gene copy numbers were $\log_{10}$ transformed to meet normality assumptions. Tukey's HSD post hoc test was used to differentiate means for significant effects.

Supplemental Table 1. Experimental Design and Assembly

Soil Type	Clay Amendment				Carbon Amendment (g of dried leaf tissue)		
	Dry Soil Weight (g)	Starting Clay Content (%)	Clay Addition (g)	Final Clay Content (%)	Control (No Addition)	High Quality (+ *Peltandra virginica*)	Low Quality (+ *Phragmites australis*)
Natural	130	48	0.0	48	0	2.6	2.7
Restored	260	13	0.0	13	0	5.2	5.3
Restored + 7% Clay	241.8	13	18.2	20	0	5.2	5.3
Restored + 17% Clay	215.8	13	44.2	30	0	5.2	5.3

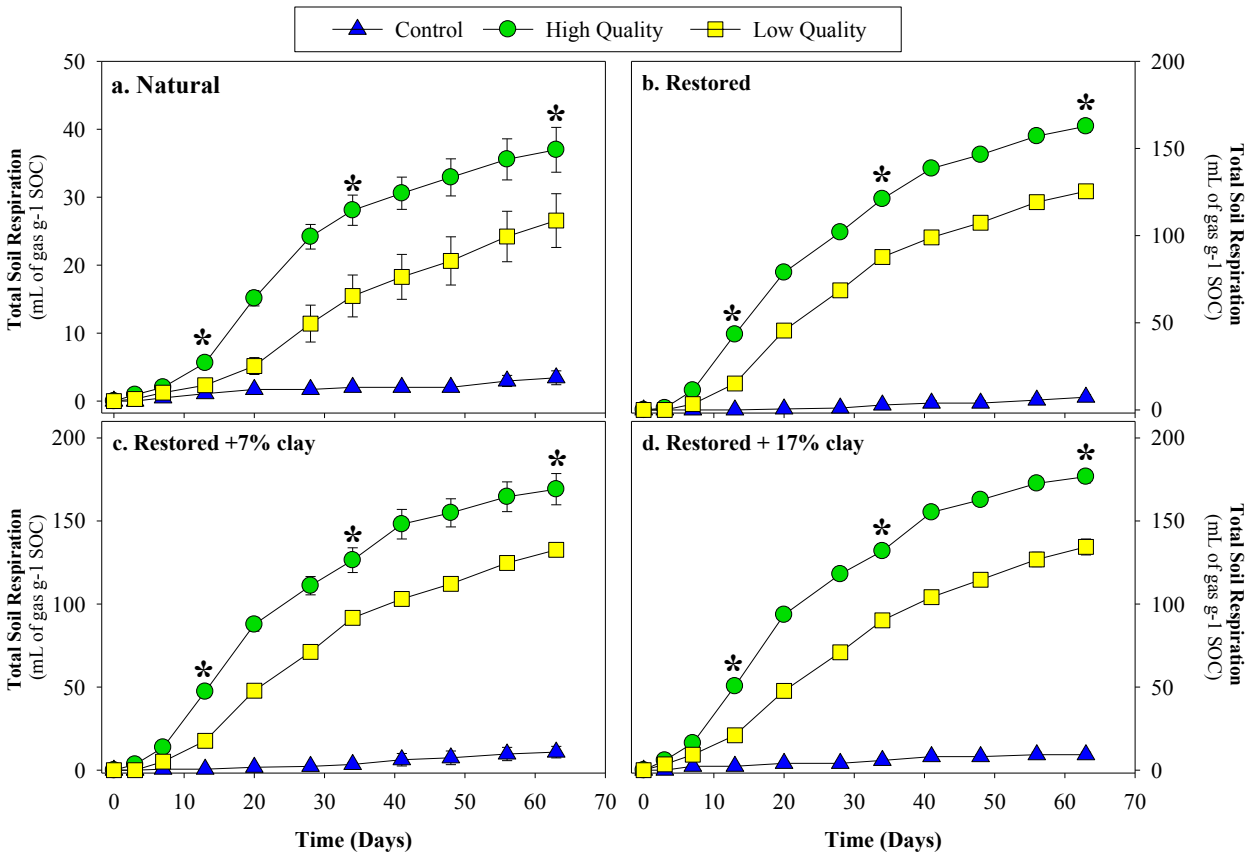


Supplemental Figure 1. (A) Schematic of the original mesocosm apparatus presented in Updegraff et al. (1995). (B) Original prototype of the laboratory mesocosm to monitor changes in soil characteristics over time. (C) A sealed mesocosm being open with two strap wrenches.

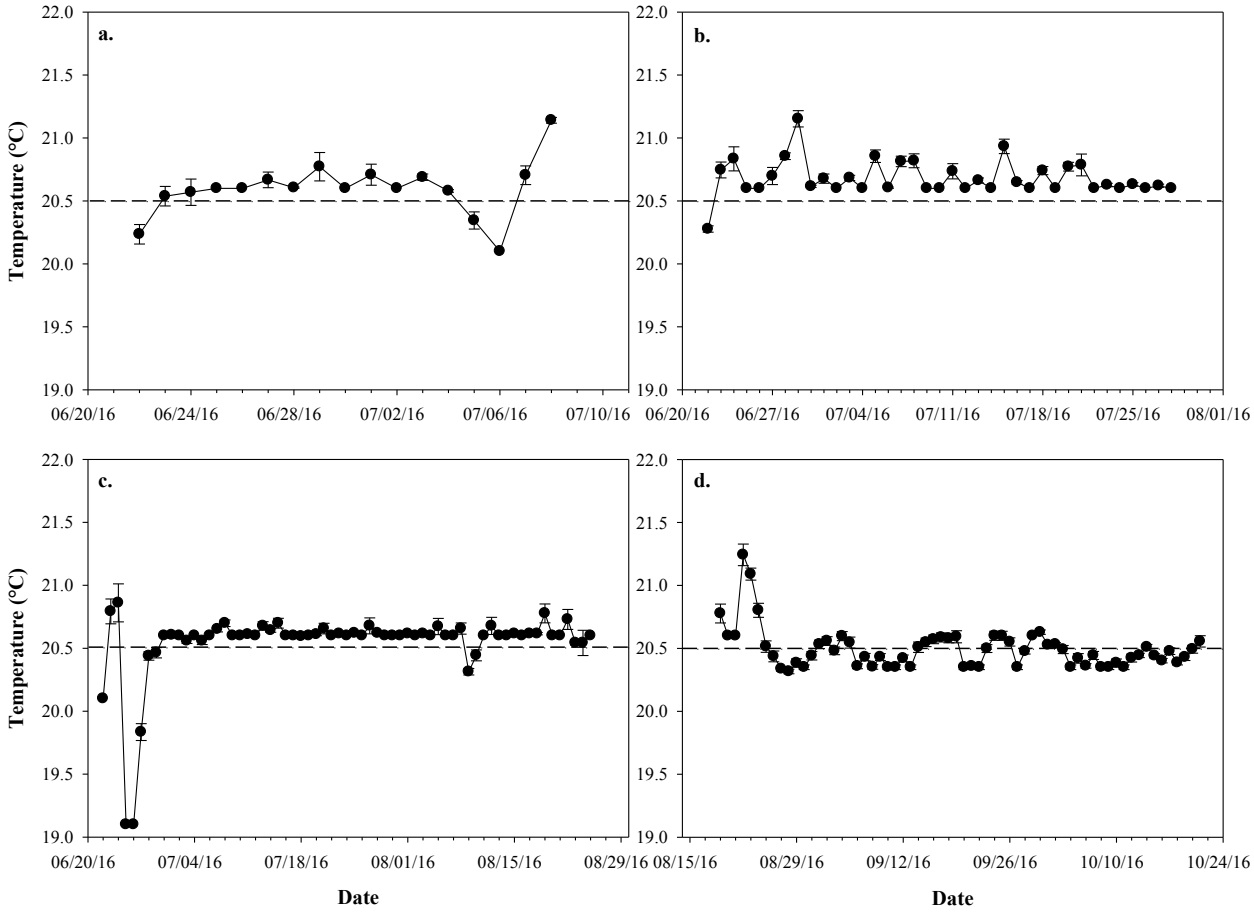


Supplemental Figure 2. Observing the behavior of 0.10 and 0.50 g of clay suspended in 5 mL of nanopure water and filter sterilized calcium chloride ($CaCl_2$) at 0.1, 0.5, 1.0, and 50 mM over time. After 30 min into the experiment, it was determined that a background ion concentration of 1 mM $CaCl_2$ would reduce clay from dispersing into the water column during initial preparation and throughout the experiment.

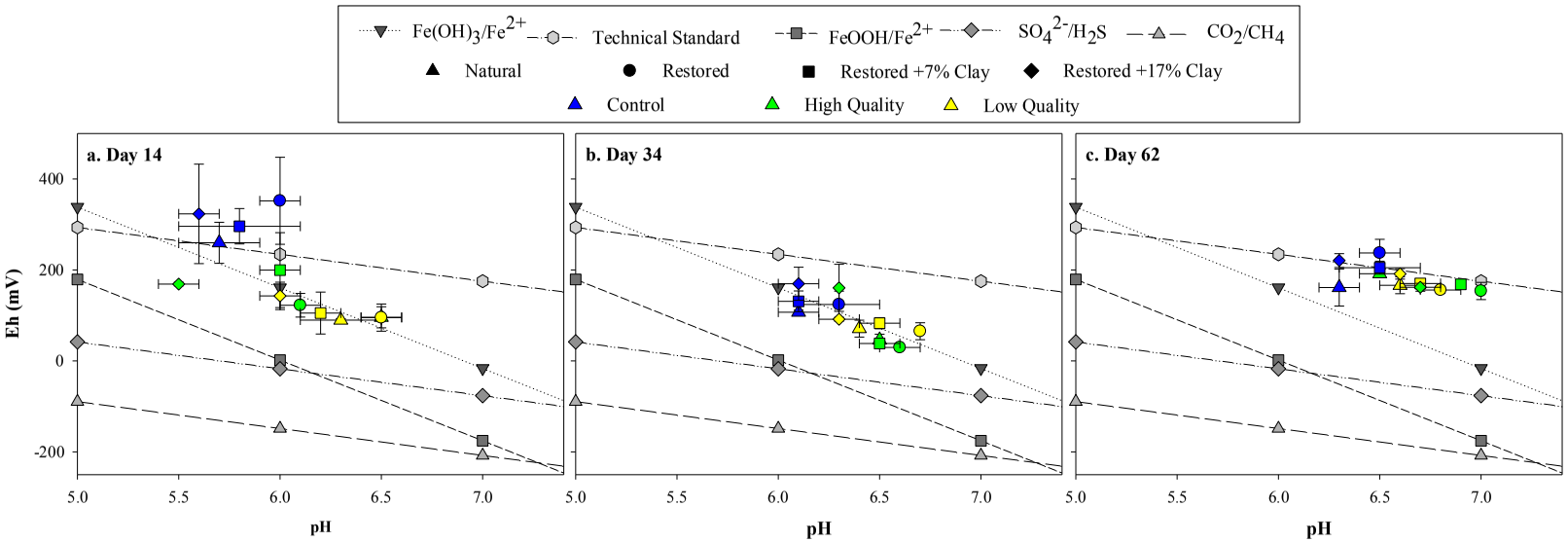
Supplemental Figure 3. Due to unexpected soil expansion in the full-scale mesocosm experiment, a separate, smaller mesocosm experiment was deployed to characterize total soil respiration and gas composition (CO_2 and CH_4) over time. The experiment was replicated following the experimental design outlined in the paper except the starting material for all 12 treatments were reduced to ¼ the size of the original setup (see Supplemental Table 1) to provide sufficient space for soil expansion.



Supplemental Figure 4. Preliminary mesocosm experiment to monitor total soil respiration (mL gas g^{-1} SOC) and to select destructive sampling dates.



Supplemental Figure 5. Daily mean temperature ± standard error of the mean for incubators housing each mesocosm set (n = 36): (a) day 14, (b) day 35, (c) day 62, and (d) the smaller scale soil respiration experiment.



Supplemental Figure 6. Eh-pH diagram for destructive sampling days 14, 34, and 62.

Chapter 5: Conclusions

Tidal freshwater wetlands provide a range of ecosystem service, including abating floodwaters, intercepting pollution, and storing and transforming nutrients. While these habitats are prevalent along the east coast, these habitats are vulnerable to anthropogenic stressors, such as urbanization, salinization, and global climate change. Wetland restoration is helping to reestablish the presence of these wetlands in the watershed; however, restored wetlands frequently look physically, chemically, and biologically different than their natural counterparts. In this dissertation, field and laboratory studies were conducted to examine the plant, soil, and microbe interactions, as well as, how these relationships shape soil function, specifically carbon (C) cycling, in natural and restored tidal freshwater wetlands of the mid-Atlantic region.

Chapter 2 Summary

In chapter two, we investigated plant-microbe relationships in restored and natural tidal freshwater wetland soils from two tributaries of the Chesapeake Bay. We hypothesized that soil properties, such as SOM and mineral N concentration, would differ between natural and restored sites, and that these differences would correspond to differences in bacterial and archaeal composition and the abundance of functional genes. Furthermore, we tested if wetland microbial community composition and functional capacity would vary between plant species. For each marsh, soil samples were collected from the root zone of four plant species: *Typha latifolia*, *Phragmites australis*, *Peltandra virginica*, and *Lythrum salicaria*.

The results of this study did not identify a strong effect of plant species on microbial composition or functional potential in restored wetland sites. We did, however,

identify differences among microbial communities with different plant species in natural wetlands. There were many interesting patterns at the site level. For example, microbial community composition was more similar the urban and suburban natural wetland habitats. This was quite surprising because we expected urbanization to impact wetland soil function negatively. We are encouraged that the small remnant wetland appears to maintain a similar microbial community to the suburban natural reference wetland, demonstrating the importance of conserving small TFWs in other urban centers.

Similar patterns in microbial community composition and functional gene abundance were observed between the two urban marshes, Kenilworth and Kingman. Again, these results were unexpected because the two sites were restored eight years apart. Both restored sites were reestablished using dredged soil material from the adjacent Anacostia River, suggesting that restoration method may be driving soil microbial ecology and soil function in these sites. One of the most significant outliers was observed in the suburban restored marsh, Wootons Landing Wetland Park. Microbial community structure and soil function in this restored wetland were significantly different compared to all other marshes. Previous years of soil mining and a different restoration approach are likely the reasons for these significant differences.

The results from chapter two suggest the primary drivers of microbial composition and soil function appear to be related to substrate, surrounding land use, legacy, and restoration method. Based on these results, two studies were designed to examine the effects of land use and soil substrate on soil C storage and cycling.

Chapter 3 Summary

In chapter three, we examined the quantity and quality of C associated with soil aggregates, reactive mineral particles (i.e., silt and clay), and Fe-, Al-, Mn-oxides in two different tidal freshwater wetlands. Soils were collected from two soil horizons across three habitats (low marsh, high marsh, and an adjacent upland region) in Patuxent Wetlands Park (natural) and Wootons Landing Wetland Park (rest. 1992). A wet sieving procedure was used to fractionate bulk soils into five pools: floating particulate organic matter (fPOM), large macroaggregates (> 2000 μm), small macroaggregates (> 250 to < 2000 μm), microaggregates (> 53 to < 250 μm), and silt + clay minerals (< 53 μm). Total C was quantified for each of the four soil fractions, and the relative abundance of six C compounds was characterized for all five fractions. A sequential chemical extraction procedure was also used to evaluate the relationship of dissolved organic carbon (DOC) with Fe-, Al-, Mn-oxides.

Similar to other non-wetland studies, we found that most of the soil C was associated with large macroaggregates (> 2000 μm) in natural systems. In contrast, soil C in restored soils was associated with small macroaggregate (> 250 to < 2000 μm). Based on research in non-wetland habitats, loss of macroaggregates inhibits total C storage and reduces long-term stability of soil C in microaggregates (< 250). Regardless of these differences, approximately equal amounts of soil C was associated with the silt + clay mineral fraction, suggesting that a maximum amount of C is stored on these mineral surfaces. While Fe-, Al-, and Mn-oxide coatings and deposits can enhance soil C stabilization, a relatively small amount of total C was extracted using the sequential extraction procedure. These results suggest that the role of organo-metal complexes is not

the dominant factor stabilizing C in these soil systems. If anything, the large volume of DOC extracted with 1.0 M sodium pyrophosphate suggests most of the C is associated with weakly crystalline structures and thus is probably a transient pool.

Another particularly interesting result from this study was the quality of C associated with the five fractions. Even though we observed differences in C quantity across soil horizons, habitats, and sites, the relative abundance of the six chemical compounds classes were relatively similar. The most notable differences observed across the five fractions were differences in the relative abundance of lignin derivatives and N-containing compounds. Lignin derivatives tended to have a greater abundance in the fPOM pool and decreased with decreasing aggregate size. In contrast, a greater abundance of N-containing compounds was associated with the silt + clay mineral fraction (< 53 μm) and abundance decreased with increasing aggregate size.

The results of chapter three suggest that disturbance to the soil habitat negatively impacts aggregate formation and soil C storage. Because large macroaggregates (≥ 2000 μm) are important in the formation and stabilization of C with microaggregates, it is important to think about the soil characteristics and conditions that promote aggregate formation. Reactive particles, such as clay, silt, and OM, are of particular importance in aggregate formation. Clay is also an important substrate impacting microbial activity and C mineralization rates. While the results of this study need to be corroborated with additional research, these data suggest that practitioners should carefully select topsoil amendments that are representative of soil textures in natural systems of that region.

Chapter 4 Summary

In the last research chapter of this dissertation, we tested the influence of soil texture on soil C cycling and greenhouse gas emissions. Representative soils were collected from the high marsh zone of Wootons Landing Wetland Park, a primarily sandy-textured soil, and percent clay concentrations were increased from 13% to 20% and 30%. Additionally, soils were treated with either a high-quality leaf litter (i.e., low C: N ratios) or low-quality leaf litter (i.e., low C: N). We expected that increasing clay content would reduce total soil respiration and lead to an increased ratio of CO2: CH4. We also expected additions of high-quality organic substrates would increase microbial community biomass and decreases total soil respiration. Soils mesocosms were also constructed using soils collected from the high marsh zone of Patuxent Wetlands Park (clay content = 42%) to compare restored soil C responses to natural habitats.

The results of this study did not support our hypotheses. First, we found that increasing clay concentration in restored soils from 13% to 20% and 30% did not decrease soil respiration. Restored soils treated with high-quality leaf litter respired more C compared to low-quality leaf amendments. These results were unexpected particularly because soils from the natural habitat produced significantly less total C and were not affected by the quality of leaf litter. These results are supported by previous research, suggesting that organic additions to low C systems enhance heterotrophic decomposition and total soil respiration. Longer incubations or field studies are needed to evaluating the long-term effects of increasing clay content on soil C preservation and soil C respiration; until further analysis, practitioners should be cautious when applying organic matter amendments to low C systems.

Final Thoughts

The results of these three studies highlight the effects of disturbance to soil function in tidal freshwater wetlands. We saw that method of restoration drove similar microbial community structure and soil function between two wetlands that were restored eight years apart. Additionally, we observed major changes to the Wootons Landing Wetland Park landscape significantly altered soil microbial ecology and soil function. Further analysis of this restored site and comparison to its' natural counterpart, Patuxent Wetlands Park, highlighted soil C storage is significantly diminished. We also identified that soil C storage mechanisms (i.e., aggregates) and respiration were different between the two habitats. Given all the data we collected on Wootons Landing Wetland Park, we could hypothesize that this restored site is primarily serving as a net C source and not sink. While we cannot discount the other ecosystem services that this habitat provides to the region, if C sequestration and long-term storage of C is a primary objective, then it seems that this restored wetland is falling short. It has been ~25 years since restoration. Longer monitoring studies should be conducted to examine the trajectory of soil C cycling in this site.

In conclusion, we need to continue investigating restored tidal freshwater wetlands of the mid-Atlantic region because significant changes to the landscape and soil habitat have profound effects on soil function especially soil C dynamics. Additionally, practitioners should consider restoring wetlands with soils that are representative of the natural systems and use caution when applying organic matter amendments to low C systems.

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