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Saltwater reduces CO_2 and CH_4 production in organic soils from a coastal freshwater forested wetland

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Abstract A major concern for coastal freshwater wetland function and health is saltwater intrusion and the potential impacts on greenhouse gas production. Coastal freshwater wetlands are likely to experience increased hydroperiod with rising sea level, as well as saltwater intrusion. These potential changes to wetland hydrology may also alter forest structure and lead to a transition from forest to shrub/marsh wetland ecosystems. Loss of forested wetlands is already evident by dying trees and dead standing trees ("ghost" forests) along the Atlantic Coast of the US, which will result in significant alterations to plant carbon (C) inputs, particularly that of coarse woody debris, to soils. We investigated the effects of salinity and wood C inputs on soils collected from a coastal freshwater forested wetland in North Carolina, USA, and incubated in the laboratory with either freshwater or saltwater (2.5 or 5.0 ppt) and with or without the additions of wood. Saltwater additions at 2.5 ppt and 5.0 ppt reduced CO_2 production by 41 and 37 %, respectively, compared to freshwater. Methane production was reduced by 98 % (wood-free incubations) and by 75-87 % (wood-amended incubations) in saltwater treatments compared to the freshwater treatment. Additions of wood resulted in lower CH_4 production from the freshwater treatment and higher CH_4 production from saltwater treatments compared to wood-free incubations. The $\delta^{13}CH_4$-C isotopic signature indicated that in wood-free incubations, CH_4 produced from the freshwater treatment was from the acetoclastic pathway, while CH_4 produced from the saltwater treatments was more likely from the hydrogenotrophic pathway. These results suggest that saltwater intrusion into subtropical coastal freshwater forested wetlands will reduce CH_4 fluxes, but long-term changes in C dynamics will likely depend on how changes in wetland vegetation and microbial function influences C inputs to the soil.

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1 Introduction

Sea-level rise (SLR) threatens coastal regions around the world. Significantly, the rate of SLR is not uniform around the globe, with the highest rate occurring along the Atlantic coast of North America between Cape Hatteras and Cape Cod, due to factors including local currents, tides and glacial isostatic rebound (Karegar et al., 2017; Sallenger et al., 2012). Along with economic and cultural impacts, health of coastal forested ecosystems are expected to be impacted by sea-level rise (Langston et al., 2017). For instance, salinization of coastal freshwater wetlands will likely impact vegetation community dynamics and regeneration in low lying (< 1m) wetlands (Langston et al., 2017). Understanding how coastal wetland ecosystems respond to extreme events, long-term climate change and a rapidly rising sea is essential to developing the tools needed for sustainable management of natural resources, and the building of resilient communities and strong economies. Because it has more than 5,180 km^2 of coastal ecosystems and urban areas below 1 m elevation, the state of North Carolina is highly vulnerable to climate change and SLR and therefore saltwater intrusion (Riggs and Ames, 2008, Titus and Richman, 2001).

As sea level changes, coastal plant communities move accordingly up and down the continental shelf. In recent geologic time, sea level has risen about 3 m over the past ~2,500 years from sea level reconstructions adjacent to our study site (Kemp et al., 2011). The rate of SLR has varied greatly over that time, with periods of stability and change, and a geologically unprecedented acceleration in recent decades. The current distribution of coastal forested wetlands reflects the hydrologic equilibrium of the recent past climate, but the widespread mortality of such forests suggests that the rate of SLR is in a time of rapid change at a rate

potentially faster than the forest's capacity to move upslope. Furthermore, dying coastal forests will alter the quantity and quality of organic matter inputs to the soil as vegetation shifts occur, as well as introduce a large pulse of woody debris into soils. This has the potential to alter C cycling processes responsible for storage of C in the soil or loss of C as CO_2 and CH_4 (Winfrey and Zeikus, 1977).

Wetlands store more than 25% of global terrestrial soil C in deep soil organic matter deposits due to their unique hydrology and biogeochemistry (Batjes, 1996; Bridgham et al., 2006). Carbon storage capacity is especially high in forested wetlands characterized by abundant woody biomass, forest floors of *Spaghnum* spp., and deep organic soils. Across the US Southeast, soil organic C (SOC) in soils increases with proximity to the coast and is greatest in coastal wetlands (Johnson and Kern, 2003). Carbon densities are even higher in the formations of organic soils (Histosols) that occur across the region, typically ranging from 687 to 940 t ha^{-1}, but can be as high as 1,447 t ha^{-1} (Johnson and Kern, 2003). As noted, forested wetlands, which historically have contributed to terrestrial C sequestration, are in serious decline and processes leading to destabilization of accumulated soil C are not represented in broad-scale ecosystem and land-surface models. The extent of changes in soil C cycling processes attributable to altered hydroperiod, saltwater intrusion and structural changes in vegetation in these ecosystems remains unclear.

Saltwater intrusion, a direct result of SLR, into freshwater wetlands alters soil C cycling processes (Ardón et al., 2016; Ardón et al., 2018), particularly that of methanogenesis (Baldwin et al., 2006; Chambers et al., 2011; Dang et al., 2018; Marton et al., 2012), and microbial activity (e.g., extracellular enzyme activity, Morrissey et al., 2014; Neubauer et al., 2013). Saltwater contains high concentrations of ions, particularly SO_4^{2-}, which support high rates of sulfate

reduction compared to freshwater wetlands (Weston et al., 2011). Sulfate acts as a terminal electron acceptor in anaerobic respiration of soil organic C, and sulfate reducers will typically increase in abundance in response to saltwater intrusion and out-compete other anaerobic microorganisms particularly methanogens for C (Bridgham et al. 2013; Dang et al., 2019; Winfrey and Zeikus, 1977). The effect of SO_4^{2-} on soil C cycling and competitive interactions with other anaerobic microorganisms processes also appears dependent on the concentration of the ion (Chambers et al., 2011). Even within freshwater forested wetlands, hydrology and microtopography can interact to influence the amount of SO_4^{2-} within soils experiencing different levels of saturation and therefore rates of SO_4^{2-} reduction (Minick et al., 2019a). A majority of saltwater intrusion studies on soil C dynamics though have focused on tidal freshwater wetlands, whereas non-tidal freshwater wetlands have received relatively little attention, partially due to there being less dispersed geographically across the landscape. Nonetheless, they occupy critical zones within the coastal wetland ecosystem distribution and will be influenced by SLR differently than that of tidal wetlands. Tidal wetlands are likely to experience short-term pulses of saltwater with tidal movement of water, while sea level rise effects on saltwater intrusion into non-tidal wetlands may result in more long-term saltwater inundation. This difference in saltwater inundation period may influence rates of soil CO_2, CH_4 production, and microbial activity (Neubauer et al., 2013) and therefore should be considered in light of the hydrologic properties of specific wetlands.

Saltwater intrusion into freshwater systems may also influence the CH_4 producing pathways (Dang et al., 2019; Weston et al., 2011), as a result of saltwater-induced shifts in methanogenic microbial communities (Baldwin et al., 2006; Chambers et al., 2011; Dang et al., 2019). Stable isotope analysis of CO_2 and CH_4 indicate that acetoclastic methanogenesis is the

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major CH_4 producing pathway in these freshwater wetlands (Angle et al., 2016, Minick et al., 2019b), but the influence of saltwater on the pathway of CH_4 formation in non-tidal freshwater forested wetlands has rarely been studied, particularly through the lens of CO_2 and CH_4 stable C isotope analysis. As ^{13}C isotopic analysis of CH_4 is non-destructive and is long-proven as a reliable indicator of the CH_4 production pathway (Whiticar et al., 1986), utilization of this analysis provides easily attainable information on the effects of freshwater compared to saltwater on CH_4 production dynamics in coastal wetland ecosystems experiencing SLR-induced changes in hydrology and vegetation..

We used a laboratory experiment to investigate the effects of saltwater and wood additions on CO_2 production, CH_4 production, and microbial activity in a non-tidal temperate freshwater forested wetland in coastal North Carolina, US. Although many studies have focused on salinity pulses in tidal freshwater wetlands, less attention has been given to the effects of sustained saltwater intrusion on soil C dynamics. Therefore, we tested the effects of sustained saltwater intrusion over the course of a 98 day laboratory incubation on soil C cycling and microbial activity (e.g., microbial biomass C and extracellular enzyme activity). Furthermore, we added wood to a subset of incubations in order to tease out effects of hydrology and wood inputs on C cycling.

2 Methods

2.1 Field Site Description

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138 The field site was located in the Alligator River National Wildlife Refuge (ARNWR) in
139 Dare County, North Carolina (35°47'N, 75°54'W). The ARNWR was established in 1984 and is
140 characterized by a diverse assemblage of non-tidal pocosin wetland types (Allen et al., 2011).
141 ARNWR has a network of roads and canals, but in general contains vast expanses of minimally
142 disturbed forested- and shrub-wetlands. Thirteen plots were established in a 4 km^2 area in the
143 middle of a bottomland hardwood forest surrounding a 35-meter eddy covariance flux tower
144 (US-NC4 in the AmeriFlux database; Minick et al., 2019a). Of the 13 plots (7 m radius), four
145 central plots were utilized for this study. Over-story plant species composition was
146 predominantly composed of black gum (*Nyssa sylvatica*), swamp tupelo (*Nyssa biflora*), bald
147 cypress (*Taxodium distichum*), with occasional red maple (*Acer rubrum*), sweet gum
148 (*Liquidambar styraciflua*), white cedar (*Chamaecyparis thyoides*), and loblolly pine (*Pinus*
149 *taeda*). The understory was predominantly fetterbush (*Lyonia lucida*), bitter gallberry (*Ilex*
150 *albra*), red bay (*Persea borbonia*), and sweet bay (*Magnolia virginiana*). The mean annual
151 temperature and precipitation from climate records of an adjacent meteorological station
152 (Manteo AP, NC, 35°55′N, 75°42′W, National Climatic Data Center) for the period 1981-2010
153 were 16.9 °C and 1270 mm, respectively. These wetlands are characterized by a hydroperiod
154 that operates over short time scales and is driven primarily by variable precipitation patterns.
155 Soils are classified as a Pungo series (very poorly managed dystic thermic typic Haplosaprist)
156 with a deep, highly decomposed muck layer overlain by a shallow, less decomposed peat layer
157 and underlain by highly reduced mineral sediments of Pleistocene origin (Riggs, 1996). Ground
158 elevation is below < 1 m above sea level. Sea-level rise models of coastal NC show that
159 ARNWR will experience almost complete inundation by 2100, with attendant shifts in
160 ecosystem composition (DOD, 2010).

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162 **2.2 Sample Collection**

163

164 Soil samples were collected on February 6, 2018, from surface organic soils by removing
165 seven 10x10 cm^{-2} monoliths from hummocks to the depth of the root mat (approximately 6.3 cm)
166 using a saw and a 10x10 cm^{-2} PVC square. The seven soil samples were composited by plot and
167 stored on ice for transport back to the laboratory. In the laboratory, roots and large organic
168 matter were removed by hand and gently homogenized. Soils samples were stored at 4°C for
169 seven weeks before initiating the laboratory incubation.

170 Freshwater and saltwater for the experiment was collected from water bodies surrounding
171 the ARNWR on March 7, 2018. Freshwater was collected from Milltail Creek, which runs
172 Northwest from the center of ARNWR to Alligator River and is drainage for our forested
173 wetland study site. Freshwater salt concentration was 0 ppt. Saltwater was collected from
174 Roanoke Sound to the east of ARNWR and had a salt concentration of 19 ppt. Fresh- and salt-
175 water were mixed together to get the desired salt concentration for the saltwater treatments (2.5
176 and 5.0 ppt). Four water samples of each fresh- and salt-water mixture were sent to the NCSU
177 Environmental and Agricultural Testing Service laboratory for analysis of total organic C (TOC),
178 ammonium (NH_4^+), nitrate (NO_3^-), phosphate (PO_4^-), sulfate (SO_4^-), calcium (Ca^{2+}), magnesium
179 (Mg^{2+}), sodium (Na^+), potassium (K^+), and chlorine (Cl^-).

180

181 **2.3 Incubation Setup**

182

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Incubation water treatments included: 1) soils incubated at 65 % water holding capacity (WHC) (Dry); 2) soils incubated at 100% WHC with freshwater (0 ppt); 3) soils incubated at 100% WHC with 2.5 ppt saltwater (2.5 ppt); and 4) soils incubated at 100% WHC with 5.0 ppt (5.0 ppt). A subsample of each soil was dried at 105°C to constant mass to determine gravimetric soil water content. Water holding capacity (WHC) was calculated by placing a subsample of fresh soil (~2 g fresh weight) in a funnel with a Whatman #1 filter and saturating with deionized H_2O (dH_2O). The saturated sample was allowed to drain into a conical flask for 2 h. After 2 h, the saturated soil was weighed, dried at 105°C to constant mass, and then weighed again to determine WHC.

Two sets of incubations were set up with the above mentioned water treatments. We added ^{13}C-depleted American sweetgum (*Liquidamber styraciflua*) wood to half the incubation vessels (0.22 g wood per g soil) (wood-amended), while the other half were incubated without wood (wood-free). Trees were grown at the Duke FACE site under elevated CO_2 concentrations (200 ppm CO_2 above ambient) using natural gas derived CO_2 with a depleted ^{13}C signature compared to that of the atmosphere (Feng et al., 2010; Schlesinger et al., 2006). The site was established in 1983 after clear cut and burn (Kim et al., 2015). Trees were grown under elevated CO_2 from 1994 to 2010 (Kim et al., 2015). Cookies were removed from harvested trees, dried at to a constant moisture level and stored at -20 °C until use. For the current incubation study, wood from control (non-fertilized) trees grown in the elevated CO_2 were used. The bark layer was removed and the outer five to seven tree rings of multiple cookies was removed with a chisel. Wood was then finely ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) and analyzed for C content and ^{13}C signature. Wood removed from the outer six tree rings

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had a C content of 45.6 ± 0.21 % and $\delta^{13}C$ value of -40.7 ± 0.06 ‰, which was within the range of -42 to -39 ‰ measured on fresh pine needles and fine roots (Schlesinger et al., 2006).

2.4 CO_2 and CH_4 Analysis

Headspace gas samples were collected from incubation vessels 15 times over the course of the 98 d incubation (days 1, 4, 8, 11, 15, 19, 25, 29, 29, 47, 56, 63, 70, 84, 98). Incubation lids were loosened between measurements to allow for gas exchange with the ambient atmosphere. Prior to each measurement, incubation vessels were removed from incubators, sealed tightly, and flushed at 20 psi for three minutes with CO_2/CH_4 free zero air (Airgas, Radnor, PA, USA). Following flushing, incubation vessels were immediately placed in the dark (2-6 h over the first 39 days and 12-18 h over the remainder of the incubation) before taking a gas sample for analysis. Approximately 300 mL of headspace gas was removed using a 50 mL gas-tight syringe and transferred to an evacuated 0.5 L Tedlar gas sampling bag (Restek, Bellefonte, PA, USA). Simultaneous analysis of CO_2 and CH_4 concentrations and $\delta^{13}C$ isotopic signature were conducted on a Picarro G2201-i Isotopic CO_2/CH_4 Analyzer (Picarro Inc., Sunnyvale, CA USA). Flux rates of CO_2-C and CH_4-C were calculated as well as daily cumulative CO_2-C and CH_4-C production summed over the course of the 98 d incubation. Small subsamples of soil were removed periodically from each incubation vessel for extracellular enzyme analysis (see below). Incubation vessel water levels (mass basis) were checked and adjusted three times per week using either freshwater or saltwater.

The proportion and rate of wood-derived CO_2 at each sampling date was calculated using $^{13}CO_2$ data and using the ^{13}C of depleted wood (-40.07) in a two pool flux model, with the

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depleted wood signature as the one end-point and the $^{13}CO_2$ of wood-free incubations as the other endpoint. Total wood-derived CO_2 was calculated using cumulative CO_2 produced over the 98 d incubation and the average $^{13}CO_2$ across the whole incubation.

2.5 Soil Characteristics

Soil organic C concentration and $\delta^{13}C$ was analyzed on initial soil samples and on soils from each of the thirty incubations following the 98 d incubation period. Initial SOC properties were measured on the four plot replicates prior to incubation. Soils were finely ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) prior to analysis on a Picarro G2201-i Isotopic CO_2/CH_4 Analyzer outfitted with a Costech combustion module for solid sample analysis (Picarro Inc., Sunnyvale, CA USA).

Soil pH and redox potential (Eh = mV) were measured in each incubation within one hour following sampling of headspace gas. Soil pH was measured on fresh soil samples with a glass electrode in a 1:2 mixture (by mass) of soil and distilled water (dH_2O). Soil redox potential (Eh = mV) was measured using a Martini ORP 57 ORP/ºC/ºF meter (Milwaukee Instruments, Inc., Rocky Mount, NC, USA) .

2.6 Microbial Biomass Carbon and $\delta^{13}C$ Isotopic Signature

Microbial biomass C was estimated on soils collected from incubations on day 1 (after 24 hour post-treatment incubation) and day 98 (following the end of the incubation). The chloroform fumigation extraction (CFE) method was adapted from Vance et al. (1987) in order

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to estimate MBC and $\delta^{13}C$. Briefly, one subsample of fresh soil (approximately 0.5 g dry weight each) was placed in a 50 mL beaker in a vacuum desiccator to be fumigated. Another subsample was placed into an extraction bottle for immediate extraction in 0.5 M K_2SO_4 by shaking for 1 hr and subsequently filtering through Whatman #2 filter paper to remove soil particles. The samples in the desiccator were fumigated with ethanol-free chloroform ($CHCl_3$) and incubated under vacuum for 3 d. After the 3 d fumigation, samples were extracted similar to that of unfumigated samples. Filtered 0.5 M K_2SO_4 extracts were dried at 60 °C in a ventilated drying oven and then ground to a fine powder with mortar and pestle before analysis of C concentration and $\delta^{13}C$ on a Picarro G2201-i Isotopic CO_2/CH_4 Analyzer outfitted with a Costech combustion module for solid sample analysis (Picarro Inc., Sunnyvale, CA USA). Microbial C biomass was determined using the following equation:

$$MBC = EC / k_{EC}$$

where the chloroform-labile pool (EC) is the difference between C in the fumigated and non-fumigated extracts, and k_{EC} (extractable portion of MBC after fumigation) is soil-specific and estimated as 0.45 (Joergensen, 1996).

The $\delta^{13}C$ of MBC was estimated as the $\delta^{13}C$ of the C extracted from the fumigated soil sample in excess of that extracted from the non-fumigated soil sample using the following equation:

$$\delta^{13}C_{MBC}\ (‰) = (\delta^{13}C_f \times C_f - \delta^{13}C_{nf} \times C_{nf})/(C_f - C_{nf})$$

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where C_f and C_{nf} is the concentration (mg kg^{-1} soil) of C extracted from the fumigated and non-fumigated soil samples, respectively, and $\delta^{13}C_f$ and $\delta^{13}C_{nf}$ is the ^{13}C natural abundance (‰) of the fumigated and non-fumigated soil samples, respectively.

2.5 Extracellular Enzyme Analysis

The potential activity of five extracellular enzymes were quantified on initial soil samples (day 0) and on days 1, 8, 35, and 98 of the soil incubation. The specific enzymes measured were: β-glucosidase (BG; EC: 3.2.1.21), peroxidase (PER; EC: 1.11.1.7), β-glucosaminidase (NAGase; EC: 3.2.1.30), alkaline phosphatase (AP; EC: 3.1.3.1), and arylsulfatase (AS; EC: 3.1.6.1). Substrates for all enzyme assays were dissolved in 50 mM, pH 5.0 acetate buffer solution for a final concentration of 5 mM substrate.

Hydrolytic enzymes (BG and XYL) were measured using techniques outlined in Sinsabaugh et al. (1993). Approximately 0.5 g dry weight of soil sample was suspended in 50 mL of a 50 mM, pH 5.0 acetate buffer solution and homogenized in a blender for 1 min. In a 2 mL centrifuge tube, 0.9 mL aliquot of the soil-buffer suspension was combined with 0.9 mL of the appropriate 5 mM p-nitrophenyl substrate solution for a total of three analytical replicates. Additionally, duplicate background controls consisted of 0.9 mL aliquot of soil-buffer suspension plus 0.9 mL of acetate buffer and four substrate controls were analyzed consisting of 0.9 mL substrate solution plus 0.9 mL buffer. The samples were agitated for 2-5 hr. Samples were then centrifuged at 8,160 g for 3 min. Supernatant (1.5 mL) was transferred to a 15 mL centrifuge tube containing 150 µL 1.0 M NaOH and 8.35 mL dH_2O. The resulting mixture was vortexed and a subsample transferred to a cuvette and the optical density at 410 nm was

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measured on a spectrophotometer (Beckman Coulter DU 800 Spectrophotometer, Brea, CA, USA).

The oxidative enzyme (PER) were measured using techniques outlined in Sinsabaugh et al. (1992). PER is primarily involved in oxidation of phenol compounds and depolymerization of lignin. The same general procedure for hydrolytic enzymes was followed utilizing a 5 mM L-3,4-Dihydroxyphenylalanine (L-DOPA) (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) solution plus 0.2 mL of 0.3% H_2O_2 to all sample replicates and controls as the substrate. After set up of analytical replicates and substrate and background controls, the samples were agitated for 2-3 hr. Samples were then centrifuged at 8,160 g for 3 min. The resulting supernatant turns an intense indigo color. Supernatant (1.4 mL) was transferred directly to a cuvette and the optical density at 460 nm was measured on a spectrophotometer.

For all enzymes, the mean absorbance of two background controls and four substrate controls was subtracted from that of three analytical replicates and divided by the molar efficiency (1.66/µmol), length of incubation (h), and soil dry weight. Enzyme activity was expressed as µmol substrate converted per g dry soil mass per hour (µmol g^{-1} h^{-1}).

2.6 Statistical Analysis

Water chemistry, cumulative CO_2 production, cumulative CH_4 production, cumulative enzyme activity, post-incubation SOC concentration and $\delta^{13}C$ SOC, and wood-derived and wood-associated SOC, CO_2, and MBC were analyzed using one-way ANOVA (PROC GLM package). Microbial biomass C, MBC ^{13}C, pH, Eh, $\delta^{13}CO_2$, and $\delta^{13}CH_4$ were analyzed using repeated-measures ANOVA (PROC MIXED package) with time (Time) as the repeated measure

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and the incubation treatments as fixed effects. All data for wood-free and wood-amended soils were analyzed separately. Raw data were natural log-transformed where necessary to establish homogeneity of variance. If significant main effects or interactions were identified in the one-way ANOVA or repeated-measures ($P < 0.05$), then post-hoc comparison of least-squares means was performed. All statistical analyses were performed using SAS 9.4 software (SAS Institute, Cary, NC, USA).

3 Results

3.1 Water and Soil Properties

Freshwater had higher concentrations of TOC compared to the saltwater treatments (Table 1). Concentration of SO_4^{2-}, Cl^-, Na^+, Ca^{2+}, Mg^{2+}, and K^+ were higher in saltwater treatments compared to freshwater and were approximately twice as high in the 5.0 ppt saltwater treatment compared to 2.5 ppt saltwater (Table 1).

Initial hummock SOC concentration was 490 ± 27 g kg^{-1} with a $\delta^{13}C$ value of -28.5 ± 0.32 ‰. After 98 d of incubation, SOC concentration in wood-free incubations was lower in the 5.0 ppt saltwater treatment, although no difference in soil $\delta^{13}C$ was found between treatments (Table 2). For wood-amended incubations, post-incubation SOC concentration was lower in the 5.0 ppt saltwater treatment compared to the dry and freshwater treatment (Table 2). The $\delta^{13}C$ of wood-amended soils after 98 days of incubation was not different between treatments, but was depleted in ^{13}C compared to wood-free soils.

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342 Soil pH was significantly lower in the saltwater treatments in both wood-free and wood-
343 amended soils compared to the dry and freshwater treatments (Table 3; Figure 1a-b). After an
344 initial drop of pH in saltwater treatments to between 3.2 and 3.4 pH, pH steadily climbed back up
345 to between 4.0 and 4.2 p/H (Figure 1a-b). In wood-free soils, differences in soil Eh between
346 treatments was variable over time, with both the 5.0 ppt saltwater treatment and the freshwater
347 treatment having the lowest redox potential at different time points throughout the incubation
348 (Table 3; Figure 1c), but never got below -124 mV on average. In wood-amended soils, Eh
349 dropped quickly to between -200 and -400 mV over the first 30 days for saltwater incubated soils
350 (Table 3; Figure 1d), before rising to between -100 to 0 mV for the rest of the incubation period.
351 In freshwater incubated soils, Eh rose quickly back to between -50 to 0 mV by day 15 and
352 remained at this level for the rest of the incubation period, while saltwater treatments had
353 significantly lower Eh between days 8 and 25.
354
355 **3.2 CO_2, CH_4, $\delta^{13}CO_2$-C, and $\delta^{13}CH_4$-C**
356
357 In wood-free incubations, cumulative CO_2 production was not different between the dry
358 and freshwater treatments, but were higher than that produced from saltwater treatments (Table
359 4; Figure 2a). Cumulative CO_2 produced from wood-amended soils was highest in the dry
360 treatment compared to all other treatments (Table 4; Figure 2b). Wood-derived CO_2 (calculated
361 as the difference between cumulative CO_2 produced from wood-amended and wood-free
362 incubations) was highest in the dry treatment (Table 4; Figure 2c). This finding was also
363 confirmed by calculating cumulative wood-derived C using the ^{13}C two-pool mixing model, with

the highest proportion found in the dry treatment (54 ± 4.6 %) compared to soils incubated with freshwater (42 ± 1.7 %), 2.5 ppt saltwater (37 ± 1.0 %), and 5.0 ppt saltwater (38 ± 1.5 %).

Cumulative CH_4 production was highest in the freshwater treatment compared to the saltwater treatments in both wood-free and wood-amended incubations (Table 4; Figure 2d-e). The difference between cumulative CH_4 produced from wood-amended and wood-free incubations was lower (and exhibited a negative response to wood additions) in the freshwater treatment compared to both saltwater treatments (Table 3; Figure 2f), which both had a slight positive response to wood additions.

The CO_2:CH_4 ratio, in wood-free incubations, was calculated only for soils incubated under saturated conditions with freshwater or saltwater. The CO_2:CH_4 ratio, in wood-free incubations, was highest in freshwater (6 ± 3.4), compared to the 2.5 ppt saltwater (136 ± 33.9) and 5.0 ppt saltwater (102 ± 30.3) ($F = 24.8$; $P = 0.0002$). The CO_2:CH_4 ratio, in wood-amended incubations, was highest in freshwater (9 ± 0.8), compared to the 2.5 ppt saltwater (53 ± 20.3) and 5.0 ppt saltwater (107 ± 37.7) ($F = 9.2$; $P = 0.007$).

The $\delta^{13}CO_2$-C and wood-derived CO_2 (estimated by ^{13}C two-pool mixing model) exhibited a time by treatment interaction for both wood-free and wood-amended incubations (Table 3; Figure 3a-b). In general, $\delta^{13}CO_2$-C in wood-free and wood-amended incubations was depleted in the dry treatment (and remained steady throughout the incubation period) compared to all other treatments, especially after day 15. The proportion of wood-derived CO_2 was initially higher in saltwater treatments but gradually dropped over the course of the incubation, while the proportion of wood-derived CO_2 remained steady (approximately 50 %) for a good portion of the incubation but increased in the final couple measurements periods to a maximum of 75 % (Figure 3c).

The $\delta^{13}CH_4$-C (Table 3; Figure 4) exhibited a treatment and time effect (Table 3; Figure 4a-b), but only for wood-free incubations. For wood-free incubations, average $^{13}CH_4$-C across the course of the incubation was most enriched in the freshwater treatment (-67.8 ± 2.4 ‰) compared to the 2.5 ppt (-80.1 ± 2.4 ‰) and 5.0 ppt (-82.3 ± 2.0 ‰) saltwater treatments (Figure 4C). No difference in the $\delta^{13}CH_4$-C was found in wood-amended incubations (Figure 4b, d), ranging from between -78 to -75 ‰ for all treatments.

3.3 Microbial Biomass Carbon and Extracellular Enzyme Activity

Initially, in wood-free incubations, MBC was highest in the 2.5 ppt saltwater treatment compared to the dry treatment (Table 3; Table 5). Following the 98 day incubation, MBC in wood-free incubations was highest in the dry treatment, with no differences between the other treatments. In wood-amended soils, no difference in MBC was found initially, but following the 98 day incubation MBC was highest in the dry treatment followed by the freshwater treatment with the MBC of the saltwater treatments being the lowest. Initial $\delta^{13}C$ of MBC did not differ between treatments in either the wood-free or wood amended soils (Table 3; Table 5). After the 98 day incubation, ^{13}C of MBC in the wood-free treatments was most depleted in the freshwater treatment and most enriched in the 5.0 ppt saltwater treatment. In wood-amended incubations, ^{13}C of MBC was most depleted in the dry treatment and most enriched in the freshwater and 5.0 ppt saltwater treatments. Furthermore, the proportion of wood-derived MBC (as estimated by ^{13}C mixing model calculations) was highest in the dry treatment (31 %) and the 2.5 ppt saltwater treatment (21%) compared to the freshwater treatment (4%) (Table 5).

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409 In wood-free incubations, activity of BG, PER, and NAGase were higher in the dry
410 treatment compared to the saltwater treatments (Table 4; Table 5). Activity of AS was higher in
411 the dry and freshwater treatments compared to saltwater treatments, in both wood-free and
412 wood-amended incubations. In wood-amended incubations, BG and NAGase were highest in the
413 dry treatment compared to the saltwater treatments. In the freshwater treatment, wood addition
414 reduced activity of BG and NAGase compared to wood-free incubations (Figure 5a-b), but
415 enhanced PER activity (Figure 5c). Wood addition also reduced AS and P activity across all
416 treatments compared to wood-free incubations (Figure 5d-e).
417
418 **4 Discussion**
419
420 As forests within the lower coastal plain physiographic region of the southeastern US
421 continue to experience increasing stresses from sea level rise on hydrology, changes in microbial
422 C cycling processes should be expected. Our results, combined with other field and lab
423 experiments, confirm that saltwater intrusion into coastal freshwater wetlands can result in
424 reductions in CO_2 and CH_4 fluxes (Ardón et al., 2016; Ardón et al.,2018), but this will be
425 balanced by long- and short-term effects of saltwater intrusion on C cycling processes (Weston et
426 al., 2011) as well as changes in C inputs due to forest-marsh transition. Further, increased coarse
427 woody debris inputs to soils may reduce CH_4 emissions under freshwater conditions, but enhance
428 CH_4 emissions under saltwater conditions. Our results also clearly demonstrate that substantial
429 quantities of CH_4 can be produced from soils with redox potential between -100 to 100 mV,
430 which may be related to the specific pathway of CH_4 production (acetoclastic versus
431 hydrogenotrophic), and challenges the widespread assumption that methanogenesis only occurs

at very low redox potentials. The ARNWR is characterized by a hydroperiod that operates over short time scales and is driven primarily by variable precipitation patterns (Miao et al., 2013), which results in the influx of oxygenated waters. Periodic *in situ* measurements of redox potential indicate that standing water is relatively aerated (Eh = 175 - 260 mV), while surface soils of hummocks when not submerged are more aerated (Eh = 320 mV) than submerged hollow surface soils (Eh = 100 - 150 mV) and deeper organic soils (20-40 cm depth; Eh = 50 - 90 mV). Furthermore, our results indicate that additions of new C to soils as wood may result in short-term reductions in redox potential as anaerobic processes are enhanced due to the added C substrate and terminal electron acceptors are quickly reduced. As SLR continues to rise over the next century, more persistent saltwater intrusion may occur as rising brackish waters mix with non-tidal freshwater systems having important implications for both above- and below-ground C cycling dynamics. Although our study only looked at these effects in a controlled laboratory experiment, these data provide a baseline understanding of potential changes in C cycling dynamics due to SLR.

Saltwater additions decreased CO_2 production compared to freshwater in the wood-free soils, although MBC and extracellular enzyme activity were not different between these treatments. This has been found in other pocosin wetland soils on the coast of North Carolina (Ardón et al. 2018). Variable effects of salinity (and or sulfate additions) have been found on soil respiration, with some studies showing an increase (Marton et al., 2012; Weston et al., 2011), a decrease (Lozanovska et al. 2016; Servais et al. 2019), or no change (Baldwin et al., 2006). Krauss et al. (2012) found that permanently flooded saltwater treatments (expected in non-tidal wetlands) in a simulated coastal swamp mesocosm reduced soil respiration, whereas saltwater pulses (expected in tidal wetlands) had a variable effect on soil respiration. Alternatively, CO_2

production was not reduced in the saltwater compared to freshwater treatments in wood-amended soils, while MBC was lower in the saltwater compared to freshwater, which suggests a shift in microbial carbon use efficiency.

Methane production was higher in the freshwater compared to saltwater treatments in both wood-amended and wood-free incubations. Numerous others studies have found that saltwater reduces CH_4 fluxes compared to freswater, both within the field and laboratory. Reduced CH_4 production from saltwater treated soils primarily results from the availability of more energetically favorable terminal electron acceptors (primarily SO_4^{2-}), which leads to the competitive suppression of methanogenic microbial communities by sulfate reducing communities (Bridgham et al., 2013; Chambers et al., 2011; Winfrey and Zeikus, 1977), as methanogens and sulfate reducers compete for acetate and electrons (Le Mer and Roger, 2001). Dang et al. (2019) did find partial recovery overtime of the methanogenic community following saltwater inundation to freshwater soil cores, but interestingly this community resembled that of microbes performing hydrogenotrophic methanogenesis and not acetoclastic methanogenesis. Activity of arylsulfatase was also lower in saltwater amended soils. This also indicates a functional change in the microbial community, as microbes in the saltwater treatment are utilizing the readily available SO_4^{2-} pool, while microbes in the freshwater and dry treatments are still actively producing SO_4^{2-}-liberating enzymes to support their metabolic activities. Findings by Baldwin et al. (2006) support the effects of saltwater on changing the microbial community structure as well, in which reductions in CH_4 production in NaCl treated freshwater sediments were accompanied by a reduction in archaeal (methanogens) microbial population, establishing a link between shifting microbial populations and changing CH_4 flux rates due to saltwater intrusion.

Changes in fresh- and salt-water hydrology due to rising seas is leading to dramatic shifts in the dominant plant communities within the ARNWR and across the southeastern US (Connor et al., 1997; DOD, 2010; Langston et al., 2017). This has the potential to alter the soil C balance due to introduction of large amounts of coarse woody debris as trees die. In our laboratory experiment, additions of wood resulted in changes in both CO_2 and CH_4 production, but the direction of change depended on if soils were incubated with freshwater or saltwater. Wood additions increased CO_2 production except in the freshwater treatment. This was particularly evident in the dry treatment where wood additions increased CO_2 production by approximately 32 %. For the dry treatment, wood-amended soils had the highest MBC and NAGase activity as microbes were likely immobilizing more N to support metabolic activities in the presence of added C (Fisk et al., 2015; Minick et al., 2017). Higher respiration with wood additions in the saltwater treatments likely resulted from enhanced metabolic activity of sulfate reducing microbes in the presence of an added C source. On the other hand, wood additions resulted in a decline in CH_4 production from the freshwater treatment, while slightly enhancing CH_4 production from the saltwater treatments. Wood additions also resulted in much lower redox potential, particularly in the saltwater treatments, and coupled with $^{13}CH_4$ stable isotope composition may have driven the higher levels of CH_4 production (via hydrogenotrophic methanogenesis) in the wood plus saltwater treatments. The suppression of CH_4 production by wood additions in the freshwater treatment was somewhat surprising given the positive effects of C additions on CH_4 production recently found in freshwater sediments (West et al. 2012), but likely resulted from enhancement of other, more energetically favorable redox reactions with the addition of a C source (e.g., wood). Furthermore, wood additions to freshwater incubations resulted in a decrease in MBC and activity of BG and NAGase enzymes compared to wood-free

incubations, but an increase in PER activity. This suggests that the microbial communities have altered their functional capacity in response to wood-addition when exposed to freshwater. The CO_2:CH_4 ratio further indicated that, in freshwater, CH_4 production was quite high in relation to CO_2 production. This ratio was significantly higher though for saltwater treatments as CH_4 production dropped drastically compared to freshwater.

Changes in the CH_4 production due to saltwater additions appears to be related to the dominant CH_4 producing pathway. The $^{13}CH_4$ isotopic signature in wood-free freshwater incubated soils indicated that acetoclastic methanogenesis was the dominant CH_4 producing pathway, while hydrogenotrophic methanogenesis dominated in the saltwater treatment. Acetoclastic methanogenesis produces isotopically enriched CH_4 compared to that of the hydrogenotrophic methanogenesis (Chasar et al., 2000; Conrad et al. 2010; Krohn et al. 2017; Sugimoto and Wada, 1993; Whiticar et al., 1986; Whiticar 1999), given that methanogens discriminate against heavier $^{13}CO_2$ during the hydrogenotrophic methanogenesis. The differences in C discrimination between the two pathways is greater for the hydrogenotrophic compared to the acetoclastic pathway which results in more depleted (-110 to -60 ‰) and more enriched (-60 ‰ to -50 ‰) $^{13}CH_4$, respectively. This has been confirmed in field and laboratory experiments (Conrad et al. 2010; Krohn et al. 2017; Krzycki et al., 1987; Sugimoto and Wada, 1993; Whiticar et al., 1986; Whiticar, 1999). Baldwin et al. (2006) also found that saltwater additions promoted the hydrogenotrophic methanogenic pathway. Further, Dang et al (2019) showed that saltwater additions to soil cores resulted in a shift in the relative abundance of hydrogenotrophic methanogens, supporting the idea that saltwater may alter not only the flux of CH_4 but also the production pathway. Chambers et al. (2011) found a shift in the methanogenic microbial community under saltwater treatments as well, which could have implications for the dominant

pathway of methane production. Previous work at our site showed that freshwater saturated soils from different microsites (hummocks, hollows, and subsurface Oa horizon soil) also had $\delta^{13}CH_4$ values more like that found from CH_4 produced via acetoclastic methanogenesis (Minick et al., 2019b).

Findings from this study indicate that substantial changes in the greenhouse gas flux and microbial activity are possible due to saltwater intrusion into freshwater wetland ecosystems but that the availability of C in the form of dead wood (as forests transition to marsh) may alter the magnitude of this effect. Sea level rise will likely lead to dramatic changes in vegetation, particularly transitioning forested wetlands into shrub or marsh wetlands. As forested wetlands are lost, dead trees could provide a significant source of C to already C-rich peat soils. The long-term effect of forest to marsh transition on ecosystem C storage will likely depend on the balance between dead wood inputs and effects of sea level rise and vegetation change on future C inputs and soil microbial C cycling processes. Future work should include investigation of these C cycling and microbial processes at the field-scale and expand to a wider range of non-tidal wetlands within the southeastern US region.

Author contribution

All authors contributed to the conception and design of the study. KM wrote the first draft of the manuscript. KM collected the samples from the field and performed laboratory analysis. All authors contributed to manuscript revision and approved the submitted version.

Competing Interest

The authors declare that they have no conflict of interest.

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Tables and Figures

Table 1. Total organic C (TOC) and ion concentrations in freshwater (0 ppt), 2.5 ppt saltwater, and 5.0 ppt saltwater. Standard errors of the mean are in parenthesis (n=4). Values with different superscript lowercase letters are significantly different ($P < 0.05$).

Treatment	TOC	SO_4^{2-}	Cl^-	Na^+	NH_4^+	NO_3^-	PO_4^{3-}	Ca^{2+}	Mg^{2+}	K^+
0 ppt	44 (0.3)^a	1 (0.1)^a	17 (0.2)^a	8 (0.1)^a	0.00 (0.000)^a	0.00 (0.000)^a	0.00 (0.000)^a	1 (0.0)^a	1 (0.0)^a	0.2 (0.0)^a
2.5 ppt	40 (0.7)^b	162 (1.3)^b	1391 (42.8)^b	538 (19.2)^b	0.06 (0.004)^b	0.06 (0.000)^a	0.01 (0.000)^a	23 (0.3)^b	64 (2.6)^b	19 (0.3)^b
5.0 ppt	38 (0.1)^b	319 (6.5)^c	2695 (22.6)^c	1039 (15.9)^c	0.07 (0.004)^b	0.07 (0.004)^a	0.01 (0.000)^b	44 (1.0)^c	125 (2.1)^c	36 (0.4)^c

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Table 2. Post-incubation soil organic C (SOC) concentration (g kg^{-1}), SOC $\delta^{13}C$ (‰), wood-derived SOC (%) (estimated from ^{13}C two pool mixing model) for soil samples collected from the field and incubated for 98 d in the laboratory under dry conditions (Dry) or fully saturated with freshwater (0 ppt) or saltwater (2.5 and 5.0 ppt) and with (+ Wood) or without addition of ^{13}C-depleted wood. Pre-incubation data was measured from the four replicates prior to incubation and therefore have the same for each treatment. Standard errors of the mean are in parenthesis (n=4). Data from wood-free and wood-amended soils were analyzed separately. Values followed by different superscript lowercase letters are significantly different between the four treatments of the non-wood or wood amended soils ($P < 0.05$).

Treatment	Post-SOC Concentration (g kg^{-1})	Post-SOC $\delta^{13}C$ (‰)	Wood-derived SOC (%)
Dry	495 (1.5)^{b}	-29.5 (0.20)^{a}	.
0 ppt	493 (3.3)^{b}	-29.5 (0.18)^{a}	.
2.5 ppt	488 (4.9)^{b}	-29.5 (0.20)^{a}	.
5.0 ppt	460 (8.6)^{a}	-29.5 (0.16)^{a}	.
Dry + Wood	491 (4.7)^{ab}	-30.4 (0.30)^{a}	8 (2.5)
0 ppt + Wood	502 (4.6)^{a}	-30.7 (0.22)^{a}	12 (0.4)
2.5 ppt + Wood	477 (4.9)^{bc}	-30.6 (0.35)^{a}	10 (1.4)
5.0 ppt + Wood	470 (4.6)^{c}	-30.4 (0.14)^{a}	10 (2.0)

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Table 3. Results (F-values and significance) from the repeated measures ANOVA of pH, Eh, microbial biomass C (MBC), $\delta^{13}C$ isotopic signature of MBC, $\delta^{13}CO_2$ and $\delta^{13}CH_4$ measured in soils collected from a coastal freshwater forested wetland and incubated in the laboratory for 98 d under fully saturated with either freshwater or salt water (2.5 ppt and 5.0 ppt). Data from wood-free and wood-amended soils were analyzed separately.

Source	pH	Eh	MBC	MBC ^{13}C	$\delta^{13}CO_2$	$\delta^{13}CH_4$
Wood-Free						
Treatment	26.6***	4.5*	3.7*	3.2*	351.7***	60.5***
Time	4.4***	40.7***	40.9***	15.8**	24.2***	8.3***
Treatment x Treatment	1.22	3.7***	27.3***	3.3*	6.4***	1.1
Wood-Amended						
Treatment	29.0***	13.6***	39.9***	2.6	129.8***	0.3
Time	18.3***	30.1***	111.0***	3.7	34.8***	1.4
Treatment x Treatment	1.4	3.4***	24.2***	5.5**	8.3***	1.0

*P < 0.05, **P < 0.01, ***P < 0.0001

Table 4. Results (F-values and significance) from the one-way ANOVA of cumulative gas production and extracellular enzyme activity (BG: β-glucosidase; PER: peroxidase; NAGase: glucosaminidase; AP: alkaline phosphatase; and AS: arylsulfatase) from soils collected from a coastal freshwater forested wetland and incubated in the laboratory for 98 d under dry conditions or fully saturated with either freshwater or salt water (2.5 ppt and 5.0 ppt). Data from wood-free and wood-amended soils were analyzed separately.

Source	CO_2	CH_4	BG	PER	NAGase	AP	AS
Wood-Free							
Treatment	20.4***	15.6***	7.2**	11.9**	9.5**	0.9	15.8**
Wood-Amended							
Treatment	13.3**	36.7***	16.6**	2.5	32.0***	2.3	31.2***

*P < 0.05, **P < 0.01, ***P < 0.0001

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Table 5. Initial (1 d) and final (98 d) microbial biomass C (MBC) concentration (mg kg^{-1}), MBC $\delta^{13}C$ (‰), wood-derived MBC (%) (estimated using ^{13}C two pool mixing model) and cumulative extracellular enzyme activity (µmol g^{-1}) (BG: β-glucosidase; PER: peroxidase; NAGase: glucosaminidase; AP: alkaline phosphatase; and AS: arylsulfatase) for soils incubated under dry conditions (Dry) or saturated with freshwater (0 ppt) or saltwater (2.5 and 5.0 ppt) and with (+ Wood) or without addition of ^{13}C-depleted wood. Standard errors of the mean are in parenthesis (n=4). Values followed by different superscript lowercase letters are significantly different between the four treatments for the wood-free or wood-amended soils ($P < 0.05$).

Treatment	Initial MBC Concentration (mg kg^{-1})	Final MBC Concentration (mg kg^{-1})	Initial MBC $\delta^{13}C$ (‰)	Final MBC $\delta^{13}C$ (‰)	Wood-derived MBC (%)	BG	PER	NAGase	AP	AS
Dry	2238 (400)^{c}	4077 (387)^{a}	-27.0 (0.43)^{a}	-28.4 (0.28)^{ab}	.	547 (37)^{a}	176 (14)^{a}	240 (20)^{a}	7599 (1038)^{a}	47 (2)^{a}
0 ppt	3982 (196)^{ab}	2657 (344)^{b}	-27.3 (0.19)^{a}	-28.9 (0.16)^{a}	.	479 (18)^{ab}	197 (38)^{a}	194 (11)^{ab}	6308 (517)^{a}	47 (8)^{a}
2.5 ppt	7334 (1177)^{a}	2495 (195)^{b}	-27.8 (0.51)^{a}	-27.9 (0.03)^{ab}	.	389 (33)^{b}	412 (75)^{b}	159 (9)^{b}	6539 (183)^{a}	19 (3)^{b}
5.0 ppt	6483 (104)^{ab}	2114 (135)^{b}	-27.0 (0.30)^{a}	-27.4 (0.15)^{b}	.	379 (27)^{b}	490 (30)^{b}	154 (8)^{b}	6387 (529)^{a}	15 (2)^{b}
Dry + Wood	4444 (579)^{a}	5174 (249)^{a}	-29.3 (0.40)^{a}	-32.1 (0.44)^{a}	31 (4.9)^{a}	554 (37)^{a}	243 (22)^{a}	275 (17)^{a}	7247 (887)^{a}	40 (2)^{a}
0 ppt + Wood	5376 (330)^{a}	1832 (102)^{b}	-29.8 (0.37)^{a}	-29.4 (0.15^{b}	4 (1.1)^{b}	349 (24)^{b}	275 (44)^{a}	153 (11)^{b}	4965 (459)^{a}	36 (3)^{a}
2.5 ppt + Wood	5173 (405)^{a}	748 (124)^{c}	-30.1 (0.25)^{a}	-30.4 (0.95)^{ab}	21 (7.8)^{a}	368 (12)^{b}	365 (30)^{a}	150 (6)^{b}	5548 (653)^{a}	14 (3)^{b}
5.0 ppt + Wood	2123 (400)^{b}	790 (87)^{c}	-29.9 (0.43)^{a}	-29.7 (0.37)^{b}	18 (1.9)^{ab}	369 (13)^{b}	326 (38)^{a}	150 (6)^{b}	5893 (495)^{a}	13 (2)^{b}

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Figure 1. pH for wood-free soils (A) and wood-amended soils (B) and redox potential for wood-free soils (C) and wood-amended soils (D) measured over the course of the 98 d laboratory incubation. Symbols represent mean with standard error (n=4). Treatment means with different lowercase letters are significantly different within a sampling time point ($P < 0.05$).

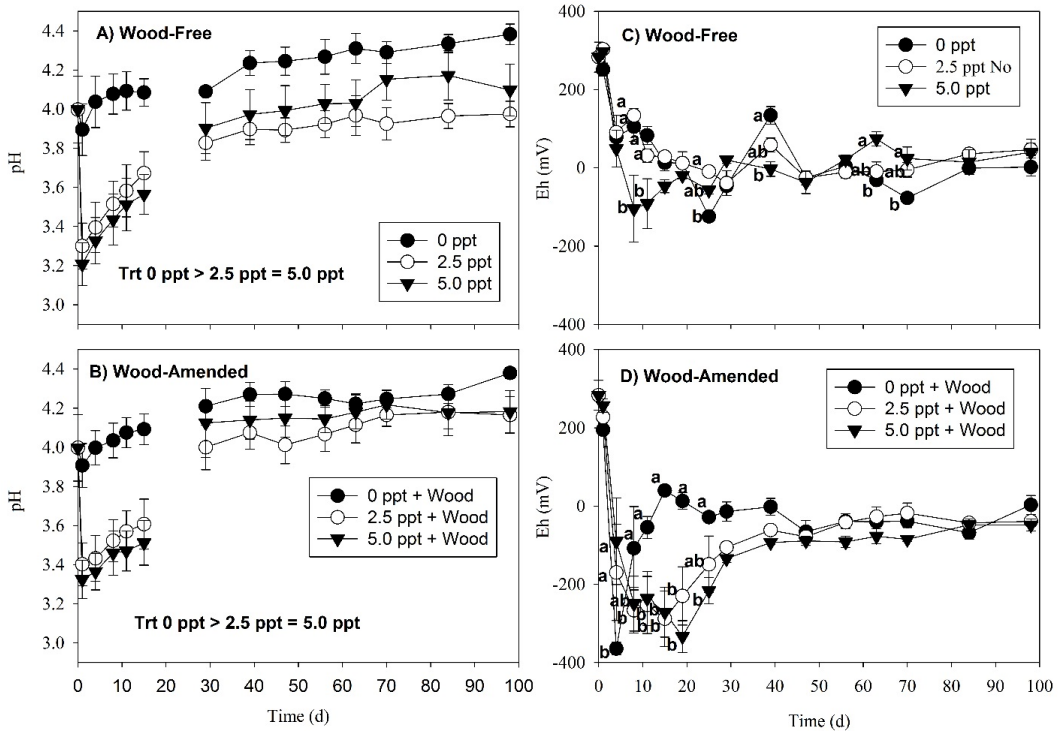

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Figure 2. Cumulative CO_2 production for wood-free soils (A), wood-amended soils (B), and the wood-associated CO_2 production (C); and cumulative CH_4 production for wood free soils (D), wood amended soils (E), and the wood-associated CH_4 production (F). Bars represent mean with standard error (n=4). Bars with different uppercase letters are significantly different ($P < 0.05$).

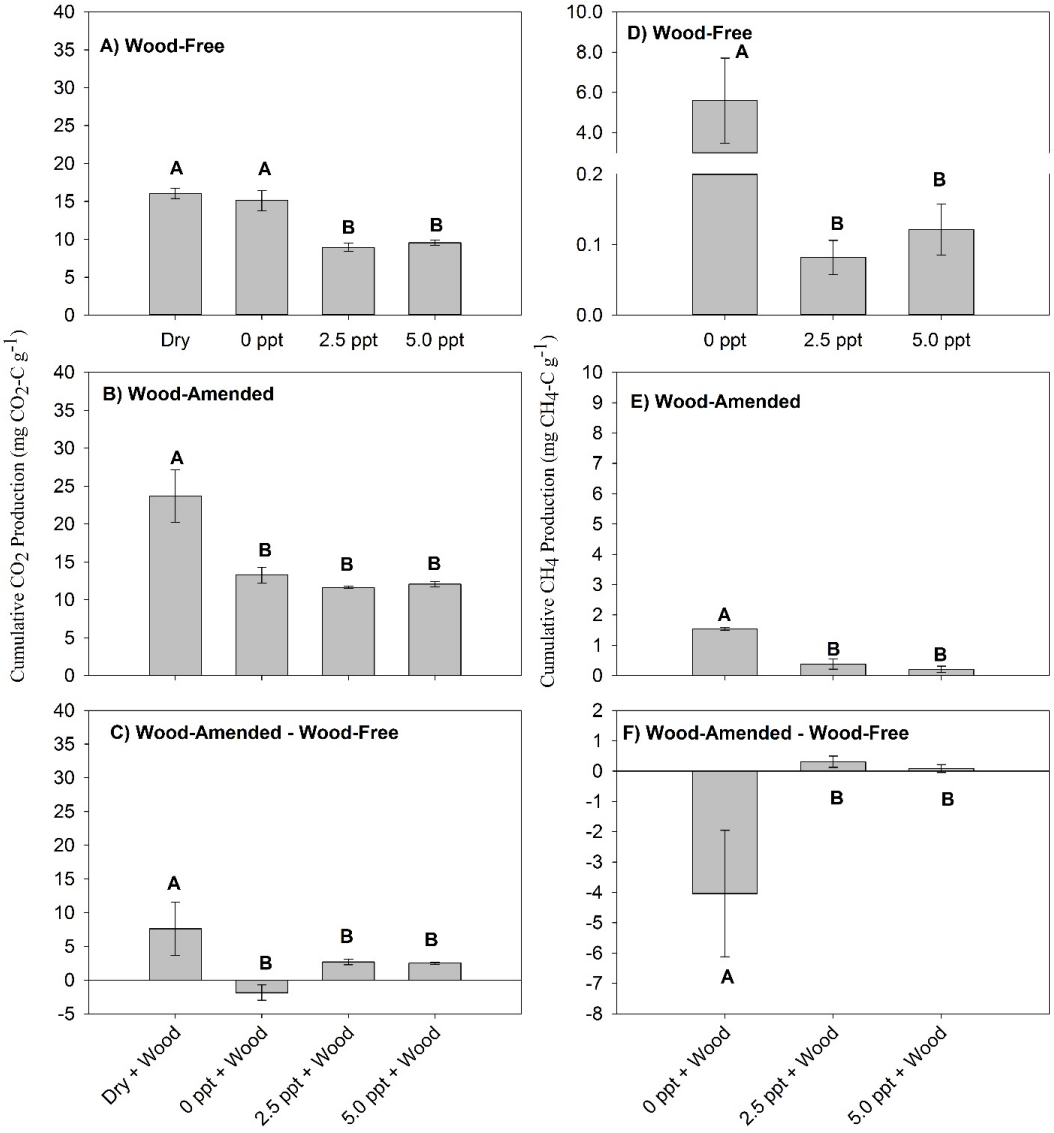

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Figure 3. The $\delta^{13}CO_2$ values measured over the course of the 98 d laboratory incubation for wood-free soils (A), wood-amended soils (B), and the proportion of wood-derived CO_2 (C). Bars represent mean with standard error (n=4). Treatment means with different lowercase letters are significantly different within a sampling time point ($P < 0.05$).

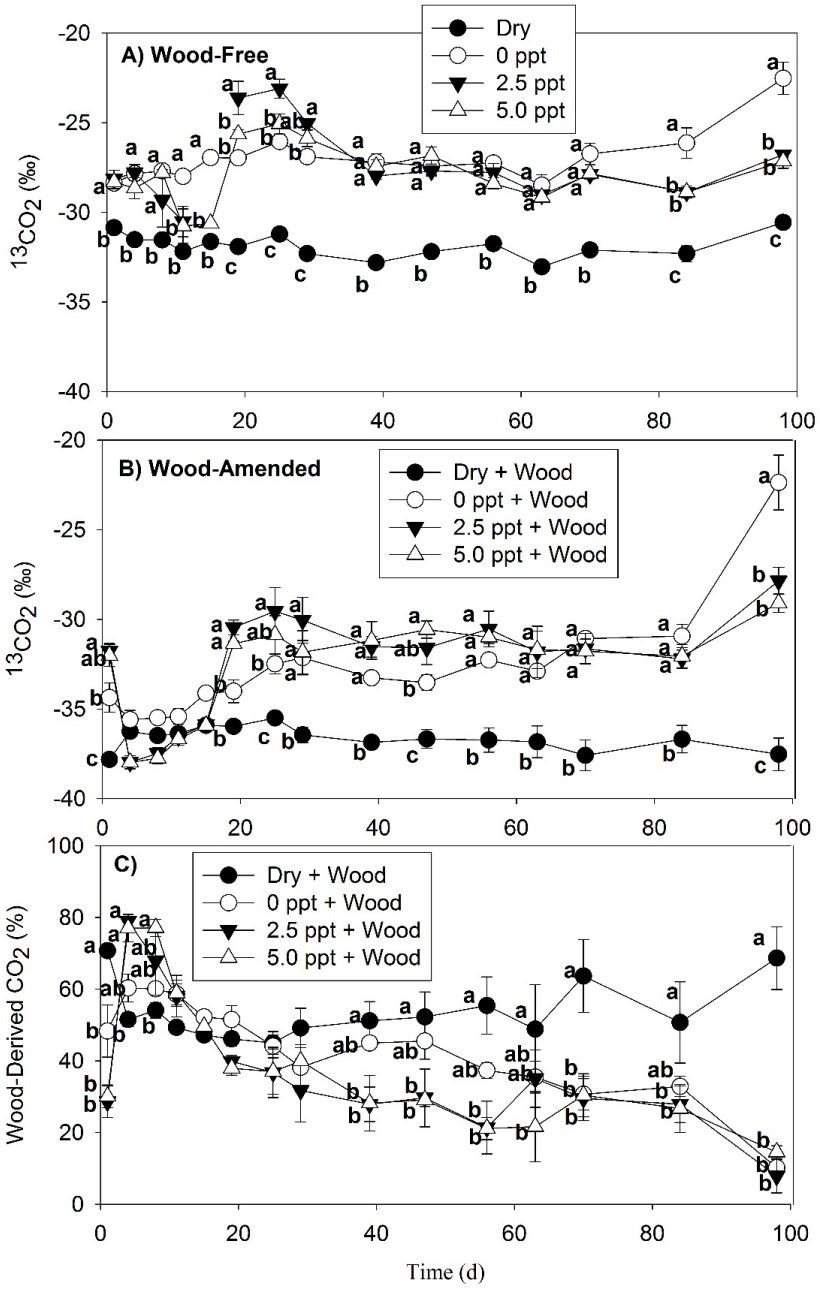

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Figure 4. The $\delta^{13}CH_4$ values measured over the course of the 98 d laboratory incubation for wood-free soils (A) and wood-amended soils (B) and the average $\delta^{13}CH_4$ across the entire incubation for wood-free soils (C) and wood-amended soils (D). Symbols or bars represent mean with standard error (n=4). Treatment means with different lowercase letters are significantly different within a sampling time point ($P < 0.05$).

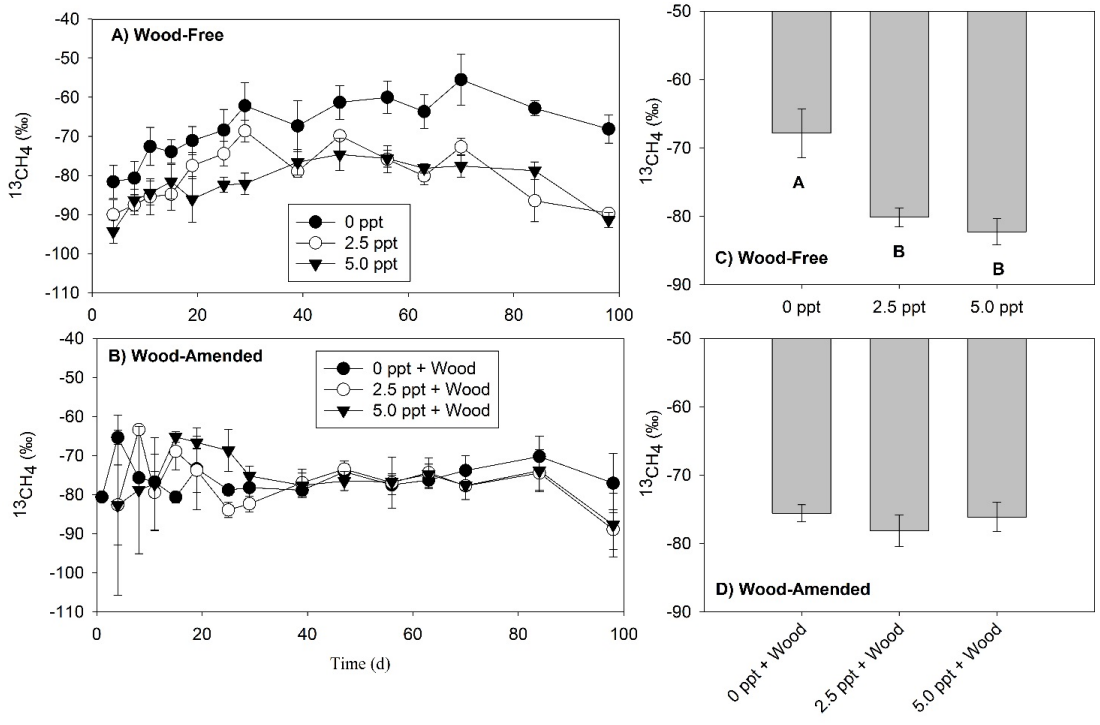

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Figure 5. Wood-associated (Wood-Amended – Wood Free) enzyme activity. Bars represent mean with standard error (n=4). Treatment means with different upper letters are significantly different ($P < 0.05$).