Dear Helge and reviewers,

Thank you for considering our manuscript for revision and resubmission following minor revisions. We have carefully considered your and the reviewers' comments and have revised the manuscript accordingly. Our responses to comments are provided in italicized black font below each comment. We also did a careful revision of the manuscript, as well, for consistency in writing style, wording, and correct grammar. The authors have uploaded a revised manuscript changes incorporated, a revised manuscript with changes still visible (as a supplement), as well as this response to reviewer's comments. We appreciate the timely feedback and consideration of this manuscript for publication in Biogeosciences.

Thank you,

Kevan

Associate Editor Decision: Publish subject to minor revisions (review by editor) (15 Sep 2019) by Helge Niemann

Comments to the Author:

Dear Kevan Minick,

Your revised version has been checked by the reviewers and although both agree that your MS has improved, there are still some issues open, and I agree here fully with the reviewers.

I am happy to receive a second revision with considerations of the remaining reviewers concerns. Please provide a clear statement with arguments if you chose not to consider a certain concern.

Best wishes, Helge Niemann

<u>Reviewer 1:</u>

Comments on the revised manuscript.

I acknowledge that the authors considered my comments. From my perspective the manuscript did improve very much – methods are better understandable and the discussion is well structured.

> *We appreciate the constructive feedback and detailed comments provided in both rounds of revisions. These comments have greatly improved the quality of the manuscript.*

Nevertheless, I would appreciate, if the authors would state that the in situ measurements of redox potential are unpublished data (add unpublished data in brackets; L467) to avoid confusion between data presented in this study and data that are not shown in the results. Additionally, please, check the manuscript for spelling mistakes and comma placement.

> *We have added "unpublished data" to the end of that sentence. We have also checked the manuscript for spelling, comma placement and other grammatical errors pointed out by both reviewers.*

Only a few things that caught my eye:

L2011 (approximately2 g fresh weight) -> (approximately 2 g fresh weight)

This has been fixed.

L558, 563, 820, 841 salt water -> saltwater

We have made these corrections

I am not a native speaker, so I only guess that in a term like "Changes in fresh- and salt-water" (e.g. L182, 186) 'salt-water' should be written as 'saltwater' as it was done throughout the manuscript, but I can be wrong. To me, it would make more sense to be consistent. Same with fresh-water in L183.
I state that version 4 of the manuscript with the title "Saltwater reduces potential CO2 and CH4 production in peat soils from a coastal freshwater forested wetland"

We agree with the reviewer. We have written out freshwater and saltwater in each of the identified instances (as well as a few other places) to be consistent throughout the manuscript.

<u>Reviewer 2:</u>

Thanks to the authors for answering questions and addressing some of the concerns raised by the reviewers. It is regrettable that several concerns were dismissed in their response. My biggest concern at this point is that both reviewers noted that we were not familiar with the methods for isotopic analysis or microbial enzymatic processes, so this – in essence – has not been reviewed at this time. These comments may be shared with the authors.

We acknowledge the reviewers concern about dismissal of previous comments. We tried to provide detailed responses and explanations to both reviewer's comments, where appropriate, and did not purposefully dismiss comments by either reviewer. We apologize if we did overlook specific comments.

The authors have recently (Minick et al. 2019a, b: in reference list of manuscript) published manuscripts with the same enzyme methods and isotopic analysis. The methods are well established, particularly the enzyme assays. For the isotopic analysis, we have added the equation and reference for the calculation of 13C derived CO2 (Figure 4c), using a two pool isotopic mixing model, as this may not be as well known. We have also added information on solid and gas sample calibrations used for the Picarro instrument and in determining unknown sample C concentration and 13C signature. Hopefully this will help alleviate concerns over this methodology.

<u>Comments on response to Reviewer 1</u>

Reviewer 1, point 13. Apparently, at least one reader wasn't aware of the formula initially, so "not necessary" is arguable

We previously provided equations for MBC and 13C calculations of MBC, which are less common. Upon rereading the previous comment, it appears unclear what particular equations the reviewer wanted to see. The other equations to potentially include are those for calculations of enzyme activity and wood derived CO2 using the 13CO2 data. We have 13CO2 calculations as readers may not be as familiar with these. The calculation of enzyme activity was described in writing. This calculation is common and straight forward and therefore is not typically represented as an equation in a manuscript.

Comments responding to request for physicochemical discussion fall off mid-sentence

We apologize for the incomplete answer to this comment. We measured ions in water and have some previous measurements on ion exchangeable elements from a previous study and have also measured soil C pools and 13C signature in different organic and mineral soil horizons at this site. Some of this is referenced in the manuscript, but not directly discussed in relation to this current study. We have added some properties of the soils in the methods that may be of use (C concentration, 13C of SOC, and pH), from Minick et al. 2019b. We also briefly discuss the ion exchangeable data in the introduction (Minick et al. 2019a). Other than those two studies, we do not have any further soil physicochemical data at his date.

Salinity levels addressed in comments but not the manuscript

We have added information about reasoning for the salinity levels chosen to the "sample collection" section of the methods and with reference to Figure 1

Comments on Figure 2. This reviewer was not suggesting different levels of CWD would or should have been tested. It was an observation that, as the authors note, might be interesting & worthy of further study.

In the last revised manuscript, we added a couple of sentences in the discussion pointing out this observation that there may be some interesting interactions between CWD and salt water at different levels.

LATEST VERSION

Substantive

L163: I still contend that a mean over such a long period is not very useful without some indicator of variability. I had suggested presenting the range, but std dev or other metrics would be useful.

We have added average annual temps and cumulative precip, with standard error for each, from the years 2008 to 2018.

L241: (no soil) incubations: does that mean it was just water (and maybe CWD) in the jars? This isn't immediately obvious to me (so then likely other readers).

These blank incubations had no soil, water, or wood in them. We have added information in the text to clarify what the blank samples represent.

Why were the masses of samples and dates of measurements changed between versions? This makes me question methodology and data integrity… (L286, L316, L327)

When reviewing some of the data, we realized that the initial statement of 0.5 g of dry weight, for enzyme analysis, should be stated as 0.8. The samples range from about 0.7 to 0.9 in dry weight depending on the total amount removed for each measurement period. For MBC, the value should have remained at 0.5, which was initially stated. We have changed this back. We are sorry for the confusion. All of the correct data was used in the calculations of enzyme activity and microbial biomass.

Minor

Authors use Oxford comma in some instances and not others. It should be used or not used consistently throughout the manuscript.

> *We have tried to be consistent with our use of commas, including making the suggested changes in the comments below*

The paragraph beginning L89: I find the switch between "sulfate" and SO43- inconsistent and think most readers would prefer the use of one or the other rather than bouncing back and forth.

> *We have changed all occurrences of the word sulfate to SO_4^{2-}, except with it was initially used (in same paragraph) and when used at the beginning of a sentence*

Sentence beginning L102: needs a grammatical revision for clarity. If "there" is referring to the forests, it should be "their" – and the entire sentence is difficult to follow.

> *We have clarified the ending of this sentence. Including correcting the grammatical error.*

L111: run-on sentence

> *We have added a semicolon after the citation.*

L131: needs a comma after "dynamics" (run-on sentence)

> *We have added a comma*

L912 surrounding states water bodies – should that be "states' "?

> *This sentence was awkwardly worded with that, so we have removed the word "states"*

L190: SLR rather than "sea level rise"

> *We have changed this here and in a couple other places.*

L215: What is "fresh" soil?

> *These are samples stored at 4C after pre processing and until the start of the incubation. We have added a note stating what this term refers to.*

L258: grammar- use "were" not "was"

> *We have corrected this.*

L307: assuming the numerator intends to be the difference between delC X cond of fumigated and non-fumigated? If so, additional parentheses are needed, else the order of operations will change the results

> *The reviewer is correct. Thank you for catching that. We have added more parenthesis in the numerator*

L319+: I greatly appreciate the authors adding rationale for the chosen enzymes. Will all your readers know what the “EC a.b.c.d” references are for? A brief indication of Enzyme Commission numbers might be helpful?

We have added a sentence describing what EC stands for and what the EC number is used for

L479-480: keep one “is” that was stricken

We have added one back

L526: subject-verb agreement needs correction (e.g. appear, not appears)

We have made this change

L566: comma splice – remove the comma after “incubations”

We have made this change

L605: please provide data supporting this statement comparing productivity, as salt marshes generally considered one of the most productive systems on the planet

Our initial thought was that forested wetlands likely contain a much larger amount of aboveground biomass compared to marshes but this appears to not necessarily be supported by the literature. Therefore, we agree with the reviewer and have removed this part of the sentence.

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

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Keywords: *extracellular enzyme activity, sea-level rise, methanogenesis, microbial biomass carbon, carbon isotopes, ghost forest*

Abstract A major concern for coastal freshwater wetland function and health are the effects of saltwater intrusion on greenhouse gas production from peat soils. Coastal freshwater forested 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 forested wetland 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 plus wood treatment. Additions of wood also 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~~ suggested that in wood-free incubations, CH_4 produced from the freshwater treatment ~~was~~ originated primarily from the acetoclastic pathway, while CH_4 produced from the saltwater treatments ~~was more likely~~ originated primarily from the hydrogenotrophic pathway. These results suggest that saltwater intrusion into ~~subtropical~~ coastal freshwater forested wetlands will reduce CH_4 ~~fluxes~~production, but long-term changes in C dynamics will likely depend on how changes in wetland vegetation and microbial function influence~~s~~ C ~~inputs~~ cycling ~~to the~~in peat soils.

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 ri~~SLR~~se~~ (Langston et al., 2017; Kirwan and Gedan 2019). 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, resulting in widespread death of coastal freshwater forested wetlands (Kirwan and Gedan 2019). 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~~ peat 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 sulfate (SO_4^{2-}), which support high rates of SO_4^{2-} ~~sulfate~~ reduction compared to freshwater wetlands (Weston et al., 2011). Sulfate acts as a terminal electron acceptor in anaerobic respiration of ~~soil organic~~SO C, and SO_4^{2-} ~~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~~ microbial 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~~, partially due to their more confined distribution 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~~may experience short-term pulses of saltwater with tidal movement of water, while SLR effects on saltwater intrusion into non-tidal freshwater 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 non-tidal wetlands.

Saltwater intrusion into freshwater systems may also influence the CH_4 production 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 major CH_4 producing pathway in ~~these~~ freshwater wetlands (Angle et al., 2016), 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.

Our goal in this study was to test whether saltwater additions alter the production of CO_2, CH_4~~,~~, and microbial activity from organic soils of a non-tidal temperate freshwater forested wetland in coastal North Carolina, US, and whether effects differ in response to additions of wood. 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. ~~and~~ ~~w~~We expect saltwater intrusion due to SLR will be more persistent in ~~these~~ non-tidal wetlands. Therefore, we investigated the effects of sustained saltwater inundation, using a laboratory microcosm experiment, on greenhouse gas production and microbial activity (e.g., microbial biomass C and extracellular enzyme activity). Wood additions to microcosms were utilized to mimic the potential large ~~amount~~ pulses of wood to peat soils ~~inputs that will occur~~ as forest~~s~~ dieback occurs along the aquatic-terrestrial fringes of the Atlantic Coast and these wetlands transition to shrub/marsh ecosystems (Kirwan and Gedan 2019); ~~,~~ thereby providing a large and widespread pulse of coarse woody debris to wetland soils and potentially altering soil C cycling.

2 Methods

2.1 Field Site Description

The field site was located in the Alligator River National Wildlife Refuge (ARNWR) in Dare County, North Carolina (35°47'N, 75°54'W) (Figure 1). The ARNWR was established in 1984 and is characterized by a diverse assemblage of non-tidal pocosin wetland types (Allen et al., 2011). ARNWR has a network of roads and canals, but in general contains vast expanses of minimally disturbed forested- and shrub-wetlands. Thirteen plots were established in a 4 km^2 area in the middle of a bottomland hardwood forest surrounding a 35-meter eddy covariance flux tower (US-NC4 in the AmeriFlux database; Minick et al., 2019a). Of the 13 plots (7 m radius), four central plots were utilized for this study which have been more intensively measured for plant and soil properties and processes (Miao et al. 2013, Miao et al., 2017, Minick et al 2019a, 2019b, Mitra et al. 2019). Over-story plant species composition was predominantly composed of black gum (*Nyssa sylvatica*), swamp tupelo (*Nyssa biflora*), bald cypress (*Taxodium distichum*), with occasional red maple (*Acer rubrum*), sweet gum (*Liquidambar styraciflua*), white cedar (*Chamaecyparis thyoides*), and loblolly pine (*Pinus taeda*). The understory was predominantly fetterbush (*Lyonia lucida*), bitter gallberry (*Ilex albra*), red bay (*Persea borbonia*), and sweet bay (*Magnolia virginiana*). Mean air temperature and precipitation from climate records of an adjacent meteorological station (Manteo AP, NC, 35°55′N, 75°42′W, National Climatic Data Center) for the period of 2008 – 2018 was 17.0 ± 0.30 °C and 932 ± 38 mm, respectively. ~~The mean annual temperature and precipitation from climate records of an adjacent meteorological~~

~~station (Manteo AP, NC, 35°55′N, 75°42′W, National Climatic Data Center) for the period 1981-2010 were 16.9 °C and 1270 mm, respectively.~~ These wetlands are characterized by a hydroperiod that responds over short time scales and is driven primarily by variable precipitation patterns. Soils are classified as a Pungo series (very poorly ~~managed~~ drained dystic thermic typic Haplosaprist) with a deep, highly decomposed muck layer overlain by a shallow, less decomposed peat layer and underlain by highly reduced mineral sediments of Pleistocene origin (Riggs, 1996). Soils from the surface of hummocks have a pH of 4.2 ± 0.1, C concentration of 49 ± 1.3 %, and a $\delta^{13}C$ value of -29.1 ± 0.29 ‰ (Minick et al. 2019b). Ground elevation is below < 1 m above sea level. Sea-level rise models of coastal NC show that ARNWR will experience almost complete inundation by 2100, with attendant shifts in ecosystem composition (DOD, 2010).

2.2 Sample Collection

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

Freshwater and saltwater for the experiment was collected from water bodies surrounding the ARNWR on March 7, 2018 (Figure 1). Freshwater was collected from Milltail Creek, which runs Northwest from the center of ARNWR to Alligator River and ~~is drainage for~~drains our forested wetland study site. Freshwater salt concentration was 0 ppt. Saltwater was collected from Roanoke Sound to the east of ARNWR and had a salt concentration of 19 ppt (Figure 1). Freshwater~~-~~ and salt~~-~~water were mixed together to get the desired salt concentration for the saltwater treatments (2.5 and 5.0 ppt). These concentrations of saltwater were chosen due to the salinity levels in the Croatan and Pamlico Sounds which are adjacent to ARNWR (Figure 1). Salinity in these waters range from approximately 1 to 5 ppt (unpublished data). Prior to mixing, freshwater~~-~~ and salt~~-~~water was filtered through a Whatman #2 filter (8 µm). Neither saltwater~~-~~ nor fresh~~-~~water were sterile filtered, therefore microbial communities from each water source were mixed together and added to the incubations. This could influence the response of soil microbes to the various treatments, but also represents what would occur under future projections of ~~sea level rise~~SLR in this region and the resulting mixing of freshwater~~-~~ and salt~~-~~water ~~sources~~ within the wetland. Four water samples of each freshwater~~-~~ and salt~~-~~water mixture were sent to the NCSU Environmental and Agricultural Testing Service laboratory for analysis of total organic C (TOC), ammonium (NH_4^+), nitrate (NO_3^-), phosphate (PO_4^-),~~ sulfate (~~SO_4^-~~)~~, calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^+), potassium (K^+), and chlorine (Cl^-). Analysis of TOC was made using a TOC analyzer (Schimadzu Scientific Instruments, Durham, NC). Analysis of NH_4^+, NO_3^-, and PO_4^-, was made using Latchat Quikchem 8500 flow injection analysis system (Lachat Insturments, Milwaukee, WI). ~~For~~ ~~SO_4^{2-}~~Sulfate and Cl^- were measured on~~,~~ a Dionex ion chromatograph ~~was used to measure concentration~~ (Thermo Fisher Scientific, Waltham, MA). Finally, a Perkin Elmer 8000 inductively-coupled plasma-optical emission spectrometer (Perkin Elmer, Waltham, MA) was used to analyze water samples for Ca^{2+}, Mg^{2+}, Na^+, K^+, and Cl^-.

2.3 Incubation Setup

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 a saltwater concentration of 2.5 ppt ~~saltwater~~ (2.5 ppt); and 4) soils incubated at 100% WHC with a saltwater concentration of ~~with~~ 5.0 ppt ~~saltwater~~ (5.0 ppt). A subsample of each fresh soil (soils stored at 4 ºC) was dried at 105°C to constant mass to determine gravimetric soil water content. Approximately 150 – 200 g fresh soil (20 – 25 g dry weight) collected from each plot was weighed into 1 L canning jars. For water addition estimates, WHC was calculated by placing a subsample of fresh soil (approximately 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 weighed again to determine WHC. It is important to note that the 100% WHC moisture level resulted in soils being completely flooded (with either freshwater~~-~~ or salt~~-~~water) with water covering the surface of the incubated soils, thereby allowing for the development of ~~methane~~ CH_4 producing conditions similar to that observed in the field for surface soils. ~~A subsample of each soil was dried at 105°C to constant mass to determine gravimetric soil water content. Soils were incubated in the dark in the laboratory for 98 d at 20 – 23 ºC in 1 L canning jars.~~ After soil and water additions, the remaining headspace was estimated for each individual incubation vessel (approximately 750 mL) and used in the calculation of gas ~~flux~~ production rates. ~~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 (approximately2 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.~~ Following wood-additions (see below), incubation vessels from each of the eight treatments were incubated in the dark in the laboratory for 98 d at 20 – 23 ºC.

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., 2016). Trees were grown under elevated CO_2 from 1994 to 2010 at which point they were harvested (Kim et al., 2016). Cookies were removed from harvested trees, dried to a constant moisture level and stored at -20 °C until use. The bark layer was removed and the outer six tree rings of multiple cookies were 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 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). For $\delta^{13}C$ analysis of solids (e.g., wood, microbial biomass extracts, soils), certified solid standards were used to develop a standard curve from the expected and measured $\delta^{13}C$ values ($R^2 > 0.999$). These standards included USGS 40 (L-glutamic acid) ($\delta^{13}C$ = -26.39 ‰; USGS Reston Stable Isotope Laboratory, Reston, VA, USA), protein ($\delta^{13}C$ = -26.98 ‰; Elemental Microanalysis Ltd, Okehampton, UK), urea ($\delta^{13}C$ = -48.63 ‰; Elemental Microanalysis Ltd, Okehampton, UK), atropine ($\delta^{13}C$ = -18.96 ‰; Costech Analytical

Technologies, Inc, Valencia, CA, USA), and acetanilide ($\delta^{13}C$ = -28.10 ‰; Costech Analytical Technologies, Inc, Valencia, CA, USA). For C concentration, atropine standards were weighed out over a range of C concentrations that encompassed the expected C concentrations of the unknown samples and within the measurement range of the instrument. A standard curve for C concentration was also developed from the expected and measured C concentration of the atropine standards ($R^2 > 0.99$). All unknown sample's C concentration and $\delta^{13}C$ value were adjusted using the linear equations derived from the appropriate standard curve. The $\delta^{13}C$ values were reported in parts per thousand (‰) relative to the Vienna Pee Dee Belemnite (VPDB) standard. Wood 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), and more depleted in $1^{3}C$ compared to that measured in hummock surface soils from our site (-29.1 ± 0.29 ‰; Minick et al. 2019b).

2.4 CO_2 and CH_4 Sample Collection and 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. Four blank ~~(no soil)~~ incubations (empty jars; no soil, water, or wood) were set up and treated in the exact same manner as incubations containing soil~~s~~, water, and wood. Blanks were used to measure soil-free CO_2 and CH_4 concentrations in incubations, which were always well below the detection limit of the gas analyzer (described below). Prior to each measurement, incubation vessels were removed from ~~incubators~~the dark, 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 back 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). For $\delta^{13}C$ analysis of gases (e.g., CO_2 and CH_4), certified gas standards were used to develop a standard curve from the expected and measured $\delta^{13}C$ values ($R^2 > 0.99$). The gas standards for $^{13}CO_2$ analysis included gas tanks containing: 1) 372 ppm CO_2 with a $\delta^{13}C$ value of -11.0 ± 0.25 ‰ (Airgas, Inc., Radnor, PA); 2) 420 ppm CO_2 with a $\delta^{13}C$ value of -10.3 ± 0.18 ‰ (Airgas, Inc., Radnor, PA); 3) 768 ppm CO_2 with a $\delta^{13}C$ value of -29.5 ± 0.14 ‰ (Airgas, Inc., Radnor, PA); and 4) 3000 ppm CO_2 with a $\delta^{13}C$ value of -34.4 ± 0.3 ‰ (Airgas, Inc., Radnor, PA). The gas standards for $^{13}CH_4$ analysis included gas tanks containing: 1) 1.75 ppm CH_4 with a $\delta^{13}C$ value of -43.2 ± 0.07 ‰ (Airgas, Inc., Radnor, PA); 2) 2.00 ppm CH_4 with a $\delta^{13}C$ value of -42.7 ± 0.20 ‰ (Airgas, Inc., Radnor, PA); 3) 10.00 ppm CH_4 with a $\delta^{13}C$ value of -29.5 ± 0.14 ‰ (Airgas, Inc., Radnor, PA); and 4) 15.08 ppm CH_4 with a $\delta^{13}C$ value of -68.6 ± 1.00 ‰ (Airgas, Inc., Radnor, PA). For CO_2 and CH_4 concentration, a concentrated gas standard (gas mix containing 4043 ppm CO_2 and CH_4) (Airgas, Inc., Radnor, PA) was diluted with zero air gas, providing a range of CO_2 and CH_4 concentrations that encompassed the expected gas concentrations of the unknown samples. A standard curve for gas concentration was developed from the expected and measured gas concentration of the diluted gas standards ($R^2 > 0.99$). All unknown gas sample CO_2 and CH_4 concentrations and $\delta^{13}C$ values were adjusted using the linear equations derived from the appropriate standard curve. The $\delta^{13}C$ values were reported in parts per thousand (‰) relative to the Vienna Pee Dee Belemnite (VPDB) standard. ~~Flux~~ Production 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 (approximately 1.0 g dry weight) of soil were removed periodically from each incubation vessel for extracellular enzyme analysis (see below). Removal of soil was accounted for in subsequent calculations of gas production rates. 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 (Fry 2006), with the depleted wood signature as ~~the~~ one end-point and the $^{13}CO_2$ of wood-free incubations as the other endpoint.

$$\% C = ((\delta^{13}CO_{2\text{wood + soil}} - \delta^{13}CO_{2\text{wood-free soil}}) / (\delta^{13}C_{\text{wood}} - \delta^{13}CO_{2\text{wood-free soil}})) *100$$

Where $\delta^{13}CO_{2\text{wood+ soil}}$ is the $\delta^{13}C$ value of CO_2 produced from soils incubated with the addition of ^{13}C-depleted wood, $\delta^{13}C_{\text{wood-free soil}}$ is the $\delta^{13}C$ value of CO_2 produced from soils incubated without the addition of ^{13}C-depleted wood, and $\delta^{13}C_{\text{wood}}$ is the average $\delta^{13}C$ value of the ^{13}C-depleted wood. 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~~ were analyzed on the four replicate soil samples prior to the start of the incubation (initial soil samples) and on soils from each of the thirty incubations following the 98 d incubation period. The initial C analysis was performed on samples removed 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). Carbon concentration and ^{13}C calibration standards were the same as those described for the analysis of the ^{13}C-depleted wood.

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 the four replicate soil samples immediately prior to the start of the incubation 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 (MBC) 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 to estimate MBC and $\delta^{13}C$. Briefly, one subsample of soil (approximately ~~1.0~~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~~ non-fumigated 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). Carbon concentration and ^{13}C calibration standards were the same as those described for the analysis of the ^{13}C-depleted wood. 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:

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

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 was quantified on soil samples and on days 1, 8, 35, and 98 of the soil incubation. The enzymes chosen for this experiment represent a range of compounds ~~they target~~ in which they degrade individually, including fast and slow cycling C compounds, as well as ones that target nitrogen (N), phosphorus (P), and sulfate (S) containing compounds. The Enzyme Commission number (EC) is stated in parenthesis after each enzyme, which classifies them by the chemical reaction catalyzed by each enzyme. The specific enzymes measured were: β-glucosidase (BG; EC: 3.2.1.21), xylosidase (XYL; EC 3.2.1.37), 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). Carbon-degrading enzymes BG, XYL, and PER degrade sugar, hemicellulose, and lignin, respectively, while the N-degrading enzyme, NAGase, degrades chitin. Enzymes AP and AS degrade phosphorus and SO_4^{2-} ~~sulfate~~ containing compounds, respectively. 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, XYL, NAGase, AP, and AS) were measured using techniques outlined in Sinsabaugh et al. (1993). Approximately 0.8 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, a 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~~ consisting of 0.9 mL aliquot of soil-buffer suspension plus 0.9 mL of acetate buffer were analyzed, as well as ~~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, followed by the addition of ~~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 measured on a spectrophotometer (Beckman Coulter DU 800 Spectrophotometer, Brea, CA, USA).

The oxidative enzyme (PER) was measured using techniques outlined in Sinsabaugh et al. (1992). PER is primarily involved in oxidation of phenolic 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 as the substrate plus the addition of 0.2 mL of 0.3% H_2O_2 to all sample replicates and substrate 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}). Daily cumulative enzyme activity was calculated and summed over the course of the 98 d incubation.

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 a 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 and the incubation treatment~~s~~ as a fixed effect~~s~~. 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 or repeated-measures 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 (pre-incubation) 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). Overall, ~~T~~the $\delta^{13}C$ of wood-free (-29.5 ± 0.08 ‰) and wood-amended soils (-30.5 ± 0.12 ‰) after 98 days of incubation ~~was~~ were significantly different (F = 49.6; *P* < 0.0001)~~between treatments (Table 2)~~.

Soil pH was significantly lower in the saltwater treatments in both wood-free and wood-amended soils compared to the dry and freshwater treatments (Table 3; Figure 2A-B). After an initial drop of pH in saltwater treatments (wood-free and wood-amended) to between 3.2 and 3.4 pH, pH steadily climbed back up to between ~~4.0~~3.8 and 4.2 pH (Figure 2A-B). In wood-free soils, differences in soil Eh between treatments was variable over time, with both the 5.0 ppt saltwater treatment and the freshwater treatment having the lowest redox potential at different time points throughout the incubation (Table 3; Figure 2C), but ~~never got~~fell below -124 mV on average. In wood-amended soils, Eh dropped quickly to between -200 and -400 mV over the first 30 days for saltwater incubated soils (Table 3; Figure 2D), before rising to between -100 to 0 mV for the rest of the incubation period. In freshwater incubated soils, Eh rose quickly back to between -50 to ~~0~~ +50 mV by day 15 and remained at this level for the rest of the incubation period, while saltwater treatments had significantly lower Eh between days 8 and 25.

3.2 CO_2, CH_4, $\delta^{13}CO_2$-C, and $\delta^{13}CH_4$-C

In wood-free incubations, cumulative CO_2 production was not different between the dry and freshwater treatments, but ~~were~~ was higher than that produced from saltwater treatments (Table 4; Figure 3A). Cumulative CO_2 produced from wood-amended soils was highest in the dry treatment compared to all other treatments (Table 4; Figure 3B). Wood-derived CO_2 (calculated as the difference between cumulative CO_2 produced from wood-amended and wood-free incubations) was highest in the dry treatment (Table 4; Figure 3C). This finding was also 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 %) (F = 10.1; $P = 0.001$).

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 3D-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 3F), 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 4A-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 freshwater and saltwater treatments (after day 1) but gradually dropped over the course of the incubation, while the proportion of wood-derived CO_2 from the dry treatment dropped quickly after the first sampling date (day 1) and remained steady (approximately ~~40~~50-60 %) for the remainder of the incubation period (Figure 4C).

The $\delta^{13}CH_4$-C (Table 3; Figure 5) exhibited a treatment and time effect (Table 3; Figure 5A-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 5C). No difference in the $\delta^{13}CH_4$-C was found in wood-amended incubations (Figure 4b, d), ~~ranging~~ which ranged from between -78 to -75 ‰ for all treatments.

3.3 Microbial Biomass Carbon and Extracellular Enzyme Activity

Initially, MBC was lowest in the dry treatment of wood-free incubations and ~~lowest~~ in the 5 ppt treatment of wood-amended incubations (Table 3; Table 5). Following the 98 day incubation, MBC was highest in the dry treatment of wood-free incubations, with no differences between the other treatments. In wood-amended incubations, final MBC was also highest in the dry treatment ~~and lowest in~~compared to both saltwater treatments. 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).

In wood-free incubations, activity of BG~~, PER, and~~ and NAGase ~~were~~ was higher, while PER was lower, in the dry treatment compared to the saltwater treatments (Table 4; Table 5). Activity of AS was higher in the dry and freshwater treatments compared to saltwater treatments, in both wood-free and wood-amended incubations. In wood-amended incubations, BG and NAGase were highest in the dry treatment compared to the saltwater treatments. In the freshwater treatment, wood addition reduced activity of BG and NAGase compared to wood-free incubations (Figure 6A-B), but enhanced PER activity (Figure 6C). Wood addition also reduced AS and P activity across all treatments compared to wood-free incubations (Figure 6D-E).

4 Discussion

As forests within the lower coastal plain physiographic region of the southeastern US continue to experience increasing stresses from SLR~~ on hydrology~~, changes in microbial C cycling processes should be expected. Our results, combined with other field and lab experiments, confirm that saltwater intrusion into coastal freshwater forested wetlands can result in reductions in CO_2 and CH_4 production (Ardón et al., 2016; Ardón et al.,2018~~) in the presence or absence of wood~~), but this may be balanced by long- and short-term effects of saltwater intrusion on these C cycling processes (Weston et al., 2011), as well as changes in C inputs due to forest-to-marsh transition. Further, wood additions to these wetland soils may reduce CH_4 production under freshwater conditions compared to the absence wood additions (Figure 3C and 3F), but slightly enhance CH_4 production under saltwater conditions. Our results also ~~clearly~~ demonstrate that substantial quantities of CH_4 can be produced from freshwater wetland soils with redox potential between -100 to 100 mV, which may be related to the specific pathway of CH_4 production (acetoclastic versus hydrogenotrophic) (Angle et al., 2016), and challenges the widespread assumption that methanogenesis only occurs at very low redox potentials. Changes in the water table depth at the ARNWR is driven primarily by precipitation patterns (Minick et al., 2019a), resulting in the influx of oxygenated waters. Periodic *in situ* measurements of redox potential at the ARNWR 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 ~~–~~to 150 mV) and deeper organic soils (20 — 40 cm depth; Eh = 50 ~~–~~to 90 mV) (unpublished data). 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 in these wetlands due to SLR.

Saltwater additions decreased CO_2 production compared to freshwater in the wood-free soils, although post-incubation MBC and extracellular enzyme activity (e.g., BG, NAGase, and AP) 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 SO_4^{2-} ~~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 post-incubation 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 freshwater, 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 SO_4^{2-} ~~sulfate~~ reducing communities (Bridgham et al., 2013; Chambers et al., 2011; Winfrey and Zeikus, 1977), as methanogens and SO_4^{2-} ~~sulfate~~ reducers compete for acetate and electrons (Le Mer and Roger, 2001). Dang et al. (2019) did find partial recovery over time 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 the CH_4 production due to saltwater additions appear~~s~~ 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 treatments. 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, resulting ~~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, recent studies have found that saltwater additions to soils result in a shift in the relative abundance of hydrogenotrophic methanogens (Chambers et al. 2011; Dang et al 2019), supporting the idea that saltwater may alter not only the ~~flux~~ production of CH_4 but also the ~~dominant~~ pathway of methane production.

Changes in freshwater- 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; Kirwan and Gedan 2019). 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 compared to wood-free soils, 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 SO_4^{2-} ~~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~~,~~ and an increase in PER activity. This suggests that the microbial communities have altered their functional capacity in response to wood~~-~~ additions 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. In wood-free incubations, the CO_2:CH_4 trend between freshwater and saltwater treatments was parabolic, but was linear upward in wood-amended soils. This suggests that interactions between saltwater concentration and coarse woody debris (in the form of dead and dying trees; Kirwan and Gedan 2019) may be important to understand ~~in~~ when determining effects of salt~~-~~water intrusion on greenhouse gas production in freshwater forested wetlands.

Findings from this study indicate that substantial changes in the greenhouse gas ~~flux~~ production 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. At ARNWR and similar coastal freshwater forested wetlands, salt~~-~~water intrusion may reduce both CO_2 and CH_4 emissions from soils to the atmosphere. Sea~~-~~-level rise will likely lead to dramatic and visually striking changes in vegetation, particularly transitioning forested wetlands into shrub or marsh wetlands (Kirwan and Gedan 2019), which has resulted in the widespread occurrence of “ghost” forests along the Atlantic coast (Kirwan and Gedan 2019). ~~which will reduce the primary productivity and the C uptake potential of these ecosystems as more productive forests transition to less productive marsh systems.~~ As forested wetlands are lost, dead trees could provide a significant source of C to already C-rich peat soils, with the potential to ~~also increase~~alter CO_2 ~~emissions~~ and ~~slight increases in~~ CH_4 production. 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~~SLR 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 (mg L^{-1}) 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}

Table 2. Post-incubation soil organic C (SOC) concentration (g kg^{-1}), SOC $\delta^{13}C$ (‰), and 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~~wood-free 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)

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 conditions 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 conditions 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

Table 5. Initial (1 d) and final (98 d) microbial biomass C (MBC) ~~concentration~~ (mg kg^{-1}), MBC δ^{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 conditions 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 δ^{13}C (‰)	Final MBC δ^{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}

Figure 1. Location of the Alligator River National Wildlife Refuge (ARNWR) in eastern North Carolina (NC) and ~~the~~ surrounding ~~states~~ water bodies. The enlarged map shows surrounding freshwater (Alligator River and Albermarle Sound) and saltwater (Pamlico Sound, Croatan Sound, and Roanoke Sound) bodies. The star represents the approximate location of soil and freshwater (from Milltail Creek) sampling locations within the freshwater forested wetlands of ARNWR. The black circle represents the approximate location of saltwater sampling (at the Melvin Daniels Bridge, Roanoke Sound) from the Roanoke Sound. The saltwater was sampled approximately 20 miles east of the soil and freshwater samples.

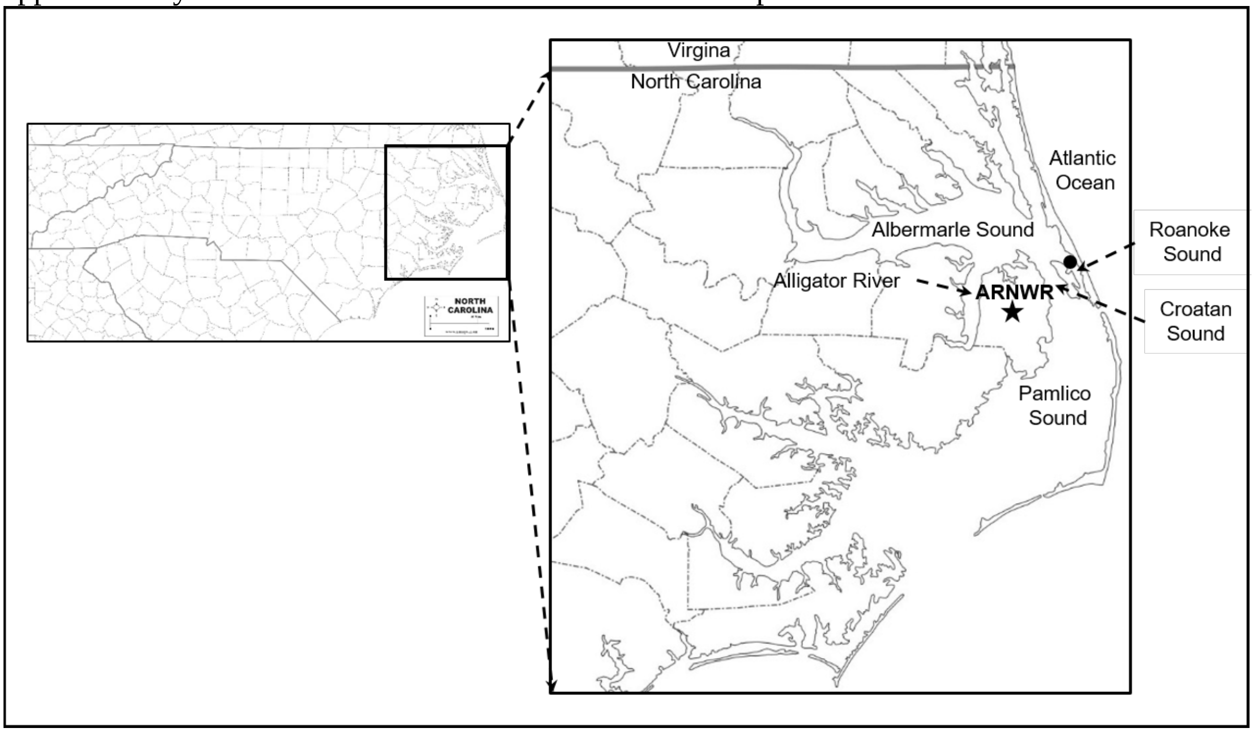

Figure 2. pH for wood-free soils (A) and wood-amended soils (B) and redox potential for wood-free soils (C) and wood-amended soils (D) measured over the course of the 98 d laboratory incubation. Symbols represent mean with standard error (n=4). Treatment means with different lowercase letters are significantly different within a sampling time point ($P < 0.05$).

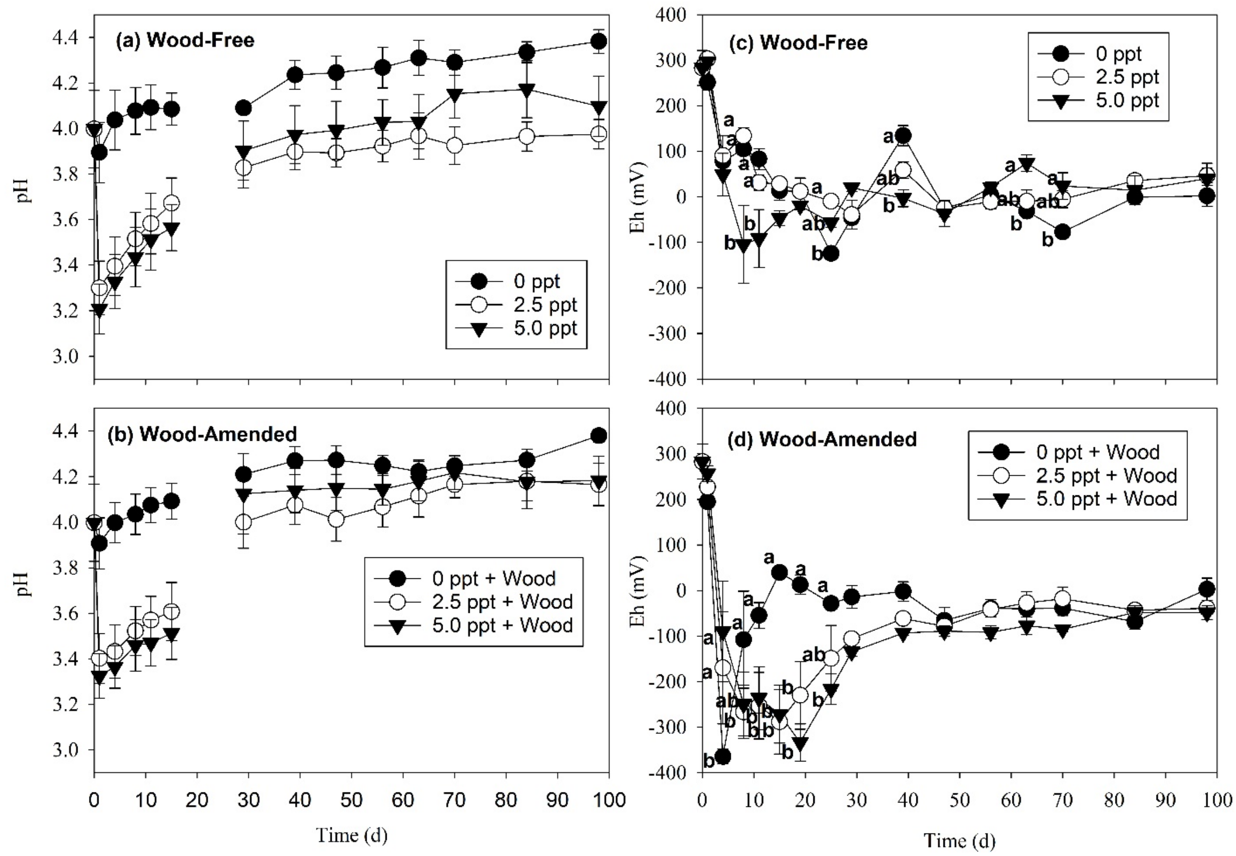

Figure 3. Cumulative CO_2 production from wood-free soils (A), wood-amended soils (B), and the wood-associated CO_2 production (C); and cumulative CH_4 production for wood-free soils (D), wood amended soils (E), and the wood-associated CH_4 production (F). Panels C and F refer to the difference between wood-amended and wood-free soils. Bars represent mean with standard error (n=4). Bars with different uppercase letters are significantly different ($P < 0.05$).

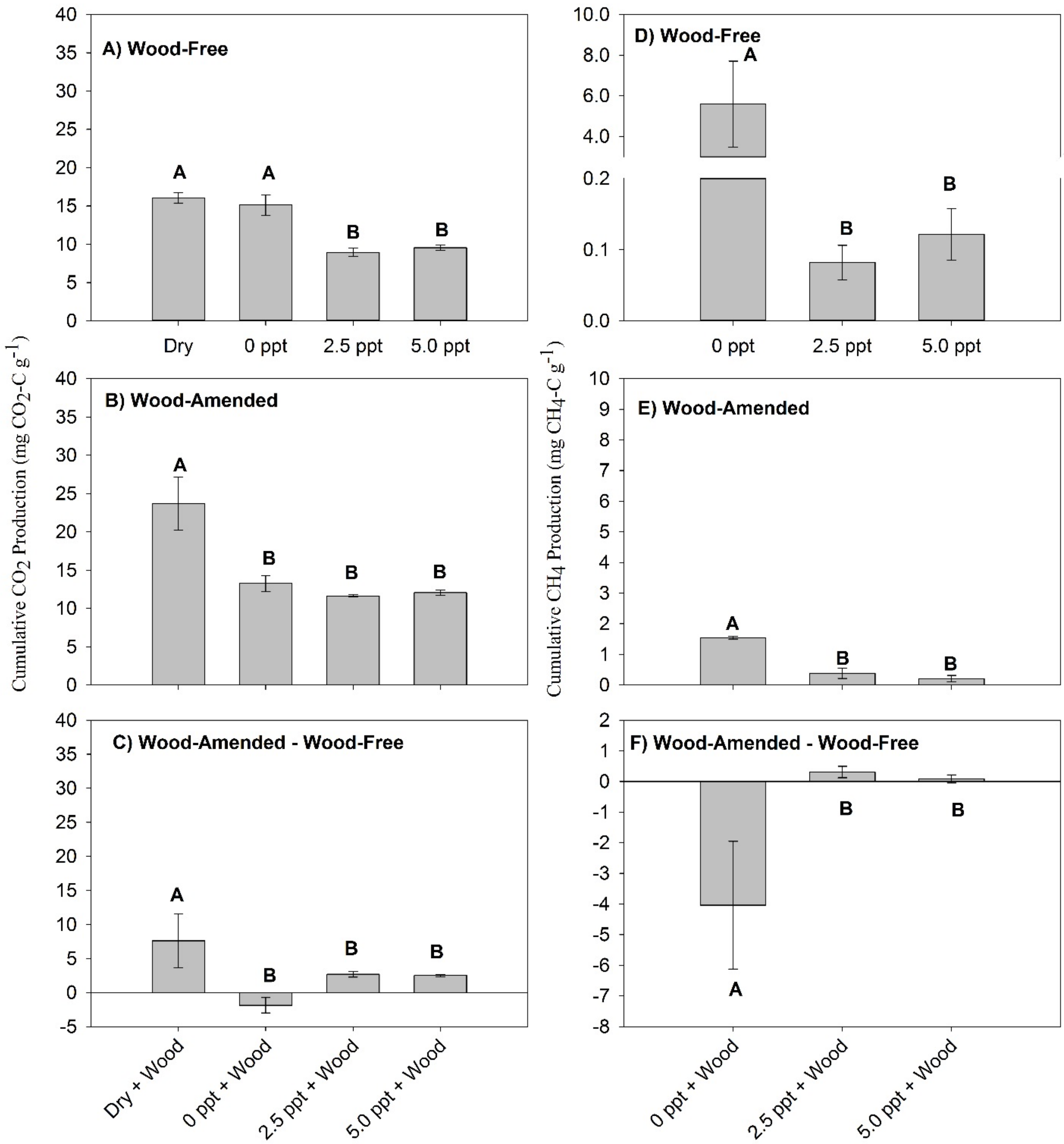

Figure 4. The $\delta^{13}CO_2$ values measured over the course of the 98 d laboratory incubation for wood-free soils (A), wood-amended soils (B), and the proportion of wood-derived CO_2 (C). Bars represent mean with standard error (n=4). Treatment means with different lowercase letters are significantly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

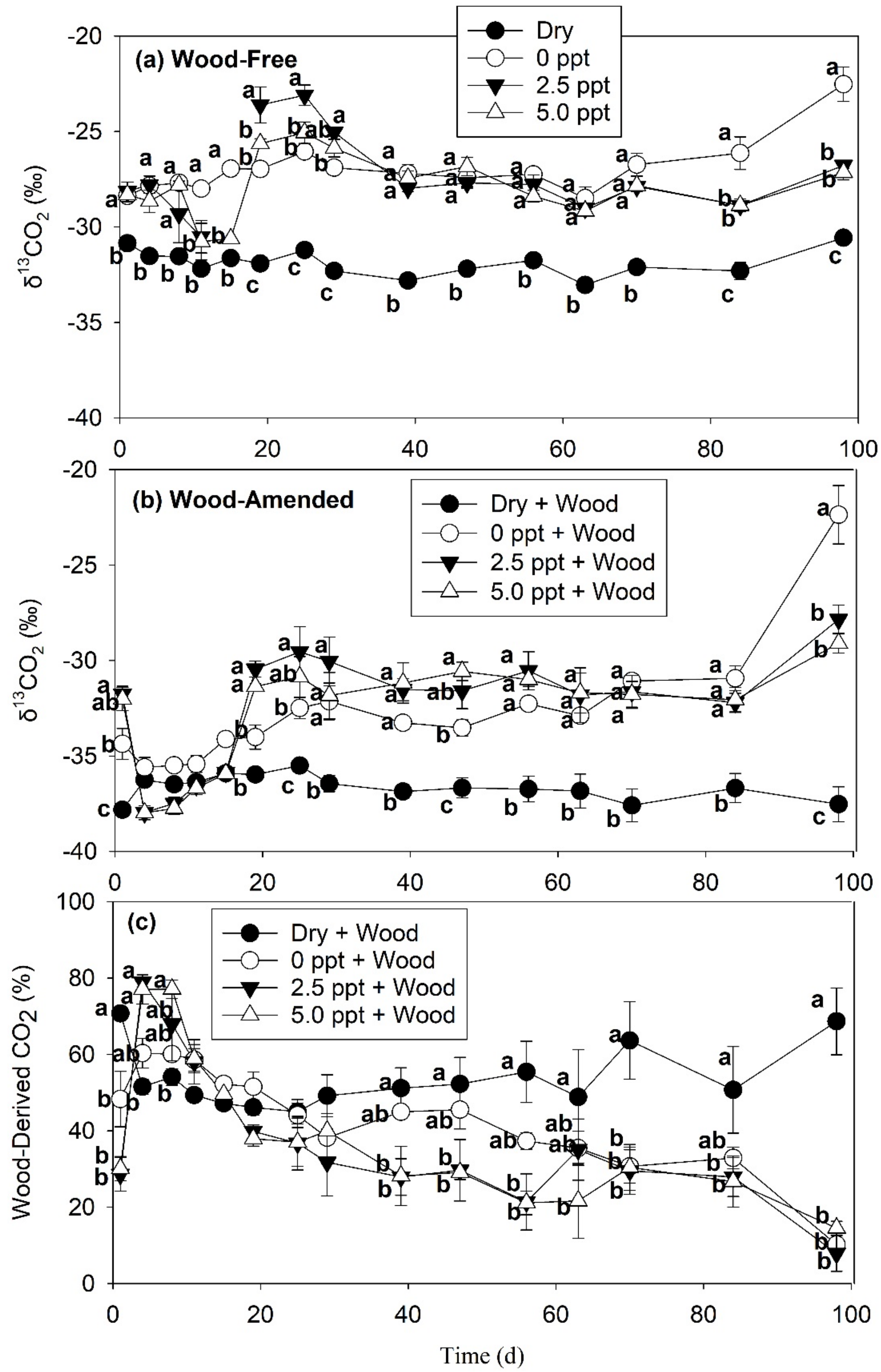

Figure 5. The $\delta^{13}CH_4$ values measured over the course of the 98 d laboratory incubation for wood-free soils (A) and wood-amended soils (B) and the average $\delta^{13}CH_4$ across the entire incubation for wood-free soils (C) and wood-amended soils (D). Symbols or bars represent mean with standard error (n=4). Treatment means with different lowercase letters are significantly different within a sampling time point (P < 0.05).

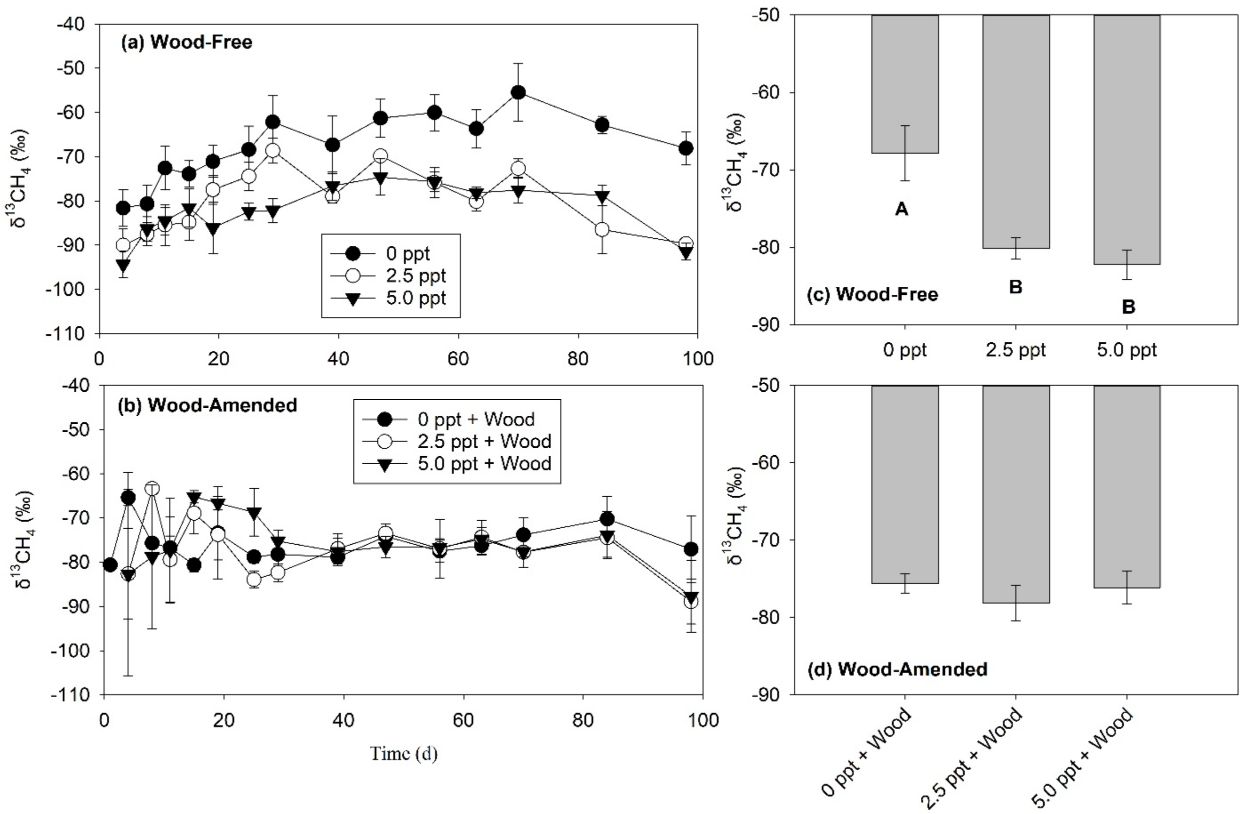

Figure 6. Wood-associated (~~Wood~~wood-~~Amended~~ amended – ~~Wood~~wood- ~~Free~~free) enzyme activity. Bars represent mean with standard error (n=4). Treatment means with different upper letters are significantly different (P < 0.05).