Response to referee comments
"Saltwater intrusion into tidal freshwater marshes alters the biogeochemical processing of organic carbon"
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We thank each of the referees for their critical and thorough analysis of our manuscript. The supplemental pdf document contains responses to each of their comments and documents changes to the manuscript that were made as a result of their comments.

Anonymous referee #1

Comment 1: *"This project is timely and interesting."* Thank you.

Comment 2: *"The biggest questions I have with this paper…"* This comment has two main parts: First, a request for additional detail about the plant community, and second, the proposal of an additional hypothesis to explain the observed long-term effects of saltwater intrusion on soil biogeochemistry. We will address these separately below.

All three reviewers requested additional detail about the changes in plant community composition and/or the responses of plant C:N ratios to saltwater intrusion. We appreciate their interest in this aspect of the larger project and request patience as their questions will be more fully answered in the Neubauer and Sutter manuscript that is in preparation [as an aside, note that Biogeoscience's style requires that "in prep" manuscripts are cited with the current year; thus Neubauer and Sutter 2013 refers to this "in preparation" manuscript]. To address the referees' comments, we have added to the 'Study site' section some details about the effects of the saltwater additions on the plant community. We do not have information on C:N ratios of all the plants that were found in each plot, so we cannot specifically answer Referee #1's question about whether the plants in the +salt plots have a higher C:N ratio than the plants in other plots. However, we do know that the field treatments did not affect the C:N ratio of individual species – for both *Zizaniopsis miliacea* and *Phyla lanceolata*, which were found in most or all of the experimental plots, the C:N ratio did not differ as a function of experimental treatment. We also know that plants with low C:N ratios (e.g., *Peltandra virginica*) were generally absent from the +salt plots, suggesting that changes in plant species composition affected the C:N ratio of the entire plant community. However, given that we do not have sufficient C:N data to determine if this is, in fact, true, we have chosen to keep the text in its current form. **(text added at end of first paragraph of section 2.1)**

The referee's second major point here is that the initial increase in soil C mineralization following saltwater intrusion could also be contributing to both the reduction in soil C content in the +salt soils and a change in their quality. We agree. We have done some back-of-the-envelope calculations (envelope available upon request!) that suggest that the saltwater-induced stimulation of soil C mineralization would take at least 1.7 years (and most likely considerably longer) to explain the observed reduction in soil C content. However, previous work on the effects of saltwater "pulses" has shown that the effects on soil C mineralization typically last less than ~6 months (e.g., Weston et al. 2011; Chambers et al. 2011). Still, it is apparent that this mechanism is plausible on both a conceptual and an order-of-magnitude basis. Thus we have added sentences in sections 4.2.1 and 4.2.3 to mention that the depletion of soil C and a change in its quality/composition could also be partially due to the initial acceleration of soil C mineralization that follows saltwater intrusion. **(text added sections 4.2.1 and 4.2.3)**

Comment 3: *"I personally found the first 2 paragraphs…"* The referee is correct that the focus of our study was not to use the slurry-based gas production measurements to generate an ecosystem-scale C balance. We also agree with the referee that there are multiple reasons why slurry and field measurements would not be equivalent; we mentioned several of those reasons at the start of the second paragraph in section 4.2.4. The point of examining the slurry:field ratio was not to see how close to 1.0 the ratio was. Instead, we were more interested in seeing if the ratio (whatever it was) was similar between treatments. Because the slurry:field ratio for CH_4 (but not CO_2) was significantly lower for the +salt plots relative to the other treatments, we were able to speculate on the importance of plants as sources of low molecular weight organic compounds that are converted to CH_4 and note that the lower slurry:field ratio in the +salt plots suggests that the pool of labile substrates must be smaller in these plots relative to those of the other treatments. We have added several lines of text to this paragraph to better clarify possible interpretations and limitations of examining slurry:field ratios **(text added in second paragraph of section 4.2.4)**

Comment 4: *"You mention that fluxes were 'typically linear'…"* As noted in the manuscript, the median correlation coefficients (r2) for the CO_2 and CH_4 vs. time relationships were 0.99 (for CO_2) and 0.97 (for CH_4). We visually examined all plots that had a r2 < 0.85 and, when appropriate, removed an outlying data point from the regression. After removing outliers, only 16 of 180 CH_4 curves had r2 values less than 0.85 (and all were above 0.65). Every CO_2 curve had r2 > 0.90. We have added a sentence to the Methods section to indicate that a relatively small number of samples had relatively poor correlation coefficients but were still used in subsequent analyses. **(text added to section 2.2.3)**

Comment 5: *"As it reads now…"* After careful thought, we have decided to leave the organization of the manuscript in its original state. Although we recognize the appeal of presenting the short-term measurements apart from the long-term ones, we find that grouping the Methods and Results sections by measurement, rather than by time scale, allows for a smoother flow of the text. We have added text to sections 3.2 and 3.3 to remind the reader that these sections deal with samples from the long-term field manipulations. We note that the Discussion *is* organized by time scale, as we feel that this approach is perfectly suitable for that portion of the manuscript. **(text modified in sections 3.2 and 3.3)**

Comment 6: *"It seems to me*…" The referee is correct. We measured potential rates of CO_2 and CH_4 production. The text and the headings for sections 2.2 and 3.2 have been updated to reflect this. **(text modified throughout Methods and Results sections)**

Comment 7: *"Section 2.2.2. I would consider removing…*" We have revised the beginning of this section as suggested by the referee. Some text was deleted, and now the section begins "To determine the short-term effects of elevated salinity on potential rates of anaerobic CO_2 and CH_4 production, …" **(text modified at beginning of section 2.2.2)**

Anonymous referee #2

Comment 1: "*This is an interesting paper…*" Thank you.

Comment 2: "*The MS would benefit from more discussion…*" This comment has been addressed as part of our response to Comment #2 by Referee #1. **(text changed as noted in that response)**

Comment 3: *"The contrast between long-term and chronic effects is valuable…"* We appreciate this comment and entirely agree with the reviewer. We think that combining measurements across multiple time scales of disturbance adds insights that are unavailable when only one time scale is considered. **(no text change necessary or requested)**

Comment 4: *"Could the watering regime affect oxygen delivery…"* Yes, it is likely that the watering regime increased O_2 delivery to subsurface layers in the +fresh and +salt plots. However, we suggest that this potential artifact is not significant for the following two reasons. 1) For most of the measurements that we made (as reported in this paper and in our other published and unpublished work), the control and +fresh plots were generally similar to each other, and both were generally distinct from the +salt plots. If there was a large "flushing effect," we would expect that the +fresh and +salt plots would be similar (since both receive extra water relative to the control) and distinct from the controls (no water additions). Since that typically was not observed, we interpret our data to mean that the "flushing effect" is generally much less than any "saltwater effect." 2) One possible reason why there was a minimal "flushing effect" is that the soils at this site are well-flushed due to natural hydrology. In Neubauer (2013), it was reported that the porewater in the upper 10 cm at this site turned over at a rate of 0.52 day-1 (i.e., 52% per day). Indeed, this high turnover of porewater is why we needed to make experimental water additions to the +fresh and +salt plots twice per week. Thus, even in the absence of experimental water additions, there is still a possibility of delivering oxygenated water to subsurface soils. **(no text changed)**

Comment 5: "*Throughout the authors use 'parameters' …*" We respectfully disagree that our use of 'parameters' is incorrect. One of the definitions of 'parameter' is: "any of a set of physical properties whose values determine the characteristics or behavior of a system" (Merriam Webster's 3rd Unabridged Dictionary); it is in this sense that we used the word. However, to avoid any potential confusion, we have replaced 'parameters' with 'properties.' **(text changed in sections 2.4, 2.5, 3.3, and Fig. 4 legend)**

Anonymous referee #3

Comment 1: *"The plots and their manipulations are not sufficiently described…"* The first portion of this comment has been addressed as part of our response to Comment #2 by Referee #1. As for the second portion, we do not have sulfate concentrations for the freshwater and seawater that were used in making the soil slurries. It is commonly observed that sulfate concentrations are higher in marine waters vs. freshwaters (e.g., Fig 2C in Poffenbarger et al. 2011, as cited in the manuscript). Because we do not actually have sulfate data but are aware of these broad-scale patterns in sulfate availability, our discussion of the reasons for the lower CH_4 production in the saltwater-amended treatments is written to note that we are speculating on the importance of sulfate availability and not relying on our own (unavailable) concentration data. **(text changed as noted in our response to Referee #1's second comment)**

Comment 2: *"The description of processing, storage and incubation…"* The referee asks several important questions about the processing, storage, and incubation of our samples. <u>Processing and storage:</u> We respectfully disagree with the referee that we failed to describe storage and processing conditions. At the end of section 2.1, we note that all samples were stored at 4 °C. In section 2.2.1, we note that sample processing (preparation of soil slurries) was done in a N_2-filled glove bag and that deoxygenated water was used when preparing the soil slurries. For clarification, we have revised the text to note that the removal of large roots and woody debris was also conducted in the glove bag. In section 2.2.2, we note that the samples in that experiment were processed anaerobically as described in section 2.2.1. <u>Incubation temperature:</u> Samples were incubated at 25 °C because our focus was on determining potential rates of CO_2 and CH_4 production. We were not trying to generate an ecosystem carbon balance using the slurry incubation data. See also, our reply to Comment #3 by Referee #1. Effect of storage time: The referee's description of the storage time is correct. Ideally, the storage time would have been shorter, but logistical and scheduling constraints prevented that. Regardless, the storage conditions were identical for samples from each treatment plot, so we are uncertain if the referee is suggesting that long-term storage and/or the storage conditions would affect one treatment differently than the others. To us, that seems somewhat unlikely. **(text modified, section 2.2.1)**

Comment 3: *"From the Abstract…"* The reviewer is correct that our experimental interpretation depends on the assumption that "all plots were similar prior to the field manipulations." We explicitly stated that in the manuscript (the text that the referee quoted comes from section 4.2.1) and in the Abstract ("Working with tidal freshwater marsh soils that had experienced roughly 3.5 years of in situ saltwater additions..."). Also, we have re-read the Methods section and think that it also indicates clearly that we did not do any before-and-after analyses (e.g., end of section 2.1, "the day after the field manipulations ceased, soil cores…were collected from all fifteen plots"). We are therefore at a loss as to why the reviewer expected that we had a before-and-after sampling design. As a related note, one major reason that we did not do a before-and-after type of analysis is because our field plots were small and collecting soil cores from each plot at the beginning of the experiment would have compromised the integrity of the plots (i.e., each plot would have had a large deep hole, relative to the size of the plot, throughout the entire 3.5 year experiment). The referee's comments about soil parameters and salinity measurements are discussed in more detail below (see Comments 9 and 10 below). **(no text changed)**

Comment 4: *"The comparisons of incubation-based CO_2…"* In this study, we were interested in knowing how soil biogeochemical processes responded to saltwater intrusion. We do have field measurements of ecosystem CO_2 and CH_4 emissions, but that was not sufficient because field measurements capture the integrated effects of both plant and soil microbial processes. When asking questions about the ecosystem-scale effects of a disturbance, a metric that combines both plant and microbial measurements may be the most appropriate approach. However, we were interested only in soil processes. In order to exclude direct plant effects (e.g., plant respiration), we decided to remove the soils from the field. The reasons that the field emission measurements do not completely agree with the slurry measurements were discussed throughout section 4.2.4, especially in the first sentence of the second paragraph of that section. A large part of the reason is that field measurements include plant and soil emissions, whereas the slurries reflect soil-only processes. (**no text changed)**

Comment 5: *"How was the salinity…"* In both sets of slurry experiments, salinity was manipulated by mixing river water with water from the flow-through seawater system at the Baruch Marine Field Laboratory. We have revised the text in question to indicate that the salinity was "adjusted using seawater." **(text changed, section 2.2.2)**

Comment 6: *"What are the detection limits…"* We did not quantify the minimum detection limits for CO_2 and CH_4 using these instruments. However, based on the lowest measured gas concentrations, the detection limits were 0.5 ppm CH_4 and 26 ppm CO_2, or better. This information has been added to the Methods section. **(text added, section 2.2.3)**

Comment 7: *"Were any efforts made…"* Substrate limitation was not tested as part of this particular experiment. However, the values we used for substrate addition were based on our prior published work, which included a full analysis of Michaelis-Menten kinetics for soil samples from several comparable wetlands (Prasse 2009 and Morrissey et al. 2013).

Moreover, the values are consistent with other literature on enzyme activity in wetlands. Because our plots of activity versus incubation time were highly linear (all $R^2 > 0.95$, as reported in the Methods), we are confident that substrate limitation was not a concern. **(no text change necessary or requested)**

Comment 8: *"'the'? 'then'?"* This mistake has been corrected. **(text changed, section 2.3)**

Comment 9: *"Salinity is estimated…"* We are not sure what the reviewer is asking us to do here. The YSI3200 measured conductivity values and converted those measurements to salinity values using the Practical Salinity Scale 1978. This is how all conductivity-based instruments measure salinity. Before and after drying our samples, we weighed them so we could determine soil water content. Subsequently, we were able to use the measured salinity values and the measured water contents to calculate how much salt was in each sample. It sounds like the reviewer might be asking us if we separated salts from the soil matrix in order to confirm that our measurements were accurate. We did not do that and, in fact, are not even sure how one would go about separating salts from the organic and mineral soil particles. We also did not dry down samples of water to determine the mass of salts and other dried substances. **(no text changed)**

Comment 10: *"Why were these parameters…"* The measurements were performed on the soils from the slurry incubations because we were interested in determining if there were any relationships between soil properties (e.g., organic matter content, %C, %N) and rates of CO_2 and CH_4 production. We reasoned that we would be more likely to find such relationships if we measured soil properties on the *exact same samples* that were incubated for the CO_2 and CH_4 production measurements, as opposed to incubating one set of samples for gas production and analyzing a separate set of samples for soil properties. We have also measured many of the same soil properties on samples from a parallel core (data not in this manuscript) that was not used in the slurry experiments and the data are generally comparable with those from the slurried soils. **(no text changed)**

Comment 11: *"How did the authors account for…"* None of these factors was accounted for as we deemed their effects to be minor. <u>Evaporation:</u> Soil samples were sealed in plastic bags from the time of collection until their use in the gas production assays. It took only a few minutes to transfer soil from the sealed bags into the serum bottles used for the gas production assays. We expect that evaporation during this time was trivial. <u>C degradation:</u> The maximum average rate of CO_2 production was ~2 µmol g-1 h^{-1} (from Fig. 1). Over the roughly 72 h duration of the slurry measurements, ~1.7 µg of C would be mineralized to CO_2…if we use this maximum rate. For most samples, the rate will considerably lower. Even the maximum rate of C degradation would result in the loss of only ~0.5% of the soil C pool during the incubation (using soil C contents from Fig. 4). <u>Error of water addition</u>*:* Water was added to the slurries using a pipette with a reported accuracy of 3%. However, this does not matter in our calculations of water content (or organic matter, or carbon, or nitrogen) because soil samples were weighed at field moisture before being added to the serum bottles (i.e., before any water was added to them) and were then weighed again after they were dried completely. **(no text changed)**

Comment 12: *"If this is the data…"* This change has been made as requested. (**text changed, beginning of section 3.1.1)**

Comment 13: *"Referring to the treatment effects…"* For clarification, we have added "for total anaerobic C mineralization" to this sentence. **(text changed, section 3.1.1)**

Comment 14: *"The authors should elaborate…"* The referee raises an important question that we are happy to address. Within a field treatment (control, +fresh, +salt) and laboratory salinity level (freshwater, low salinity, moderate salinity), there was a very high degree of variability in absolute rates of both CO_2 and CH_4 production. The situation was most extreme for CH_4 production where, in soils from the control and +fresh plots, the error bars spanned one to two orders of magnitude. Thus, even if the absolute rate of CH_4 production for a particular field treatment increased by a factor of two due to a change in laboratory salinity, we would be unable to statistically detect such a change because of the high sample-to-sample variability. As explained in both the Methods section and the legend to Figure 2, we examined relative changes in gas production rates as a way of accounting for the high sample-to-sample variability in absolute gas production rates. The text at the beginning of section 3.1.2 has been modified and expanded to better explain why it was necessary to examine relative rates of CO_2 and CH_4 production. **(text modified, section 3.1.2)**

Comment 15: *"'upon the addition of seawater and…"* These sentences have been rephrased to specify that the enzyme activities were higher or lower in certain experimental plots, without attributing those changes to a specific mechanism (e.g., the addition of saltwater or freshwater). Later in the manuscript, we talk about why the different treatments could have affected enzyme activities. **(text changed, section 3.2)**

Comment 16: *"Lower compared to?"* The second half of this sentence provides a comparison to the data mentioned in the first half of the sentence. Thus, the water content in the 23-28 cm interval is lower than the water content in the top three depths that we sampled. **(no text changed)**

Comment 17: *"Reference?"* This sentence is intended as a topic sentence that outlines the major points that will be developed in the rest of the paragraph. The references that support it are found throughout the paragraph as the details of this recent work are explored. (**no text changed)**

Comment 18: *"Do you mean microbial activity…"* Yes, that phrasing was a mistake on our part. The sentence has been changed to indicate that changes in rates of C mineralization are generally observed as changes in CO_2 production, and not driven by those changes. **(text changed, beginning of section 4.1)**

Comment 19: *"I don't understand this..."* At present, it is unknown why the Weston et al. (2011) study found a large increase in CH_4 emissions following saltwater intrusion. Those authors noted that saltwater intrusion must have made a pool of previously-inaccessible low molecular weight DOC available to methanogens, and/or increased the production of low molecular weight DOC. However, the literature has not yet provided any indications why Weston et al. found an increase in CH_4 production and emissions whereas other studies have shown that saltwater intrusion decreases CH_4 production and/or emissions. **(text modified, section 4.1)**

Comment 20: *"Please make clear…"* We do not understand the referee's comment. There is only one citation in this sentence (Weston et al. 2006) and only one Weston et al. (2006) reference in the References section. (**no text changed)**

Comment 21: *"Please explain why…"* The point of the highlighted sentence was to note that *if* saltwater was going to affect soil properties and/or microbial communities, any effects would have already happened in soils that had already been exposed to saltwater for the previous 3.5 years. The reviewer is correct that previous research (though somewhat limited in scope) has not indicated that organic carbon desorption is an important mechanism in tidal freshwater marshes. Thus, we have modified the text to more broadly refer to soil physico-chemical properties, which could include desorbable organic matter.

(text modified, last paragraph of section 4.1)

Comment 22: *"Please elaborate…"* This comment has been addressed as part of our response to Comment #2 by Referee #1. We have added some text to talk about how the plant community changed in response to the field manipulations, although we acknowledge that connecting changes in our plant community to soil properties is still speculative. **(text added, section 2.1)**

Comment 23: *"Why should the concentrations…"* The production of extracellular enzymes can be increased when appropriate substrates are present in the environment. Since we observed lower activities of glucosidase and phenol oxidase in our saltwater-treated soils, one possible explanation is that the substrates that those enzymes attack (namely, cellulose and lignin) were present at lower concentrations in those soils. As discussed throughout section 4.2.2 of the Discussion, changing substrate availability is not the only mechanism that can explain the observed changes in extracellular enzyme activity. We are simply noting that it is one possibility. **(no text changed)**

Comment 24: *"Why should saltwater intrusion…"* Saltwater intrusion can lead to desorption of NH_4^+ from soil particles due to cation exchange, potentially leading to the loss of inorganic N from the ecosystem. This is one possible explanation of the reduced soil N concentrations in our +salt plots and we have added a reference that supports this mechanism. Regardless of the mechanism, we did observe that our saltwater-exposed plots had lower concentrations of soil N and a higher C:N ratio. Thus, if any processes in the marsh were initially N-limited (i.e., before the experiment began), the degree of N-limitation would presumably be more severe following saltwater intrusion. We have further modified the text to specify that we are talking about nitrogen limitation and not the more general "nutrient" limitation. **(text modified, second paragraph of section 4.2.2)**