Dear Drs. Scranton and Neubauer,

We very much appreciate the time and effort you put into reviewing this manuscript. We have synthesized your comments and questions into a single response in the form of an author's comment on the discussion site, but we kept different reviews separate. We have also attached the revised manuscript as a supplement, where the changes inspired by Dr. Scranton's comments are marked with blue text and those inspired by Dr. Neubauer's are highlighted in yellow. Again, we appreciate the feedback and recognize that your efforts have resulted in a greatly improved manuscript.

Respectfully,

Carmella Vizza, Will West, Stuart Jones, Julia Hart, and Gary Lamberti

RESPONSE TO REVIEWER 1, DR. MARY SCRANTON:

Response to 2nd set of comments (SC2):

Dear Dr. Scranton,

Thank you again for taking the time to review our response to your comments. We appreciate the friendly tone of the conversation, and we acknowledge that many of the misunderstandings have resulted from differences between marine and freshwater fields. Having your perspective as a marine geochemist has helped us to hone our language and hopefully the manuscript will now be more easily understood across fields. Although we know that you disagree with our specific sea-level rise simulation, we appreciate your comment that the study "does contribute to an understanding of how increasing sulfate concentrations might change methane production." In the hope of keeping the second response succinct, we will briefly address some of the major points in your second and first set of comments in that sequence. We hope that our actions will speak louder than our words in the supplement that we have attached, which is a revised version of the manuscript with blue text for the changes that have been made in light of your comments and suggestions.

First of all, we agree that there may be differences in terminology between scientists studying marine and freshwater ecosystems, so we have tried to make our terminology clearer throughout the manuscript by describing our wetlands as fully inundated (e.g., **please see pg. 4, lines 6-12**), and we also separated water column parameters from sediment parameters (**please see Tables 1 and 2**) and added data on porewater acetate, nitrate, and sulfate concentrations. Regarding the pooling of samples, if we had wanted to assess the "electron tower" paradigm in freshwater wetlands, we agree that it would be not be appropriate to make composite sediments for microbial samples or even make homogenous 20-cm sediment slurries for methane production without doing more explicit surveys of how porewater chemistry and microbial communities vary by depth of the sediment. However, we believe that assessing the validity of this paradigm is beyond the scope of this paper. While we call on it to inform our results, the main purpose of the first portion of the paper was to assess how average methane production varies across freshwater and brackish wetlands and how microbial communities and normative chemical conditions may

influence this process. Our goal was thus to characterize methane production over a large spatial extent, which inevitably involves some homogenizing and compositing of sediments for study feasibility.

Additionally, we explicitly present how much carbon we added in the organic matter simulation (**please see pg. 5, lines 14-17**) and appreciate you detecting this oversight. We also formalized our analyses of litter quality, discussed litter quality more in depth, and tried to be explicit about what Tiegs et al. 2013 previously measured in the Methods, Results, and Discussion (**please see pg. 5, lines 17-18, pg. 8, lines 28-29, p. 10, lines 20-21, and pg. 15, lines 2-9**). We also added more details in the Methods about our chemistry and statistical analyses (**see Sections 2.3.2 and 2.3.3 and pg. 8, lines 1-8**). Regarding your concern with the sea-level rise simulation, we now refer to it throughout the manuscript as a "biogeochemical sea-level rise simulation" (e.g., **please see pg. 1, line 14, pg. 3, lines 19-23, pg. 13, lines 20-25**). Lastly, we agree with you that hydrogenotrophic methanogenesis is potentially important and that we cannot rule it out in our ecosystems (**please see pg. 12, lines 14-18, and pg. 12, last paragraph**).

Again, we appreciate the time and effort you have taken to make this manuscript a better one. Below you will find your set of comments point-by-point to which we have already responded and posted online, but we have added in the exact changes that we have made in the **revised manuscript in bold** for your reference.

Respectfully,

Carmella Vizza, Will West, Stuart Jones, Julia Hart, and Gary Lamberti

Response to 1st set of comments (SC1):

Dear Dr. Scranton,

Thank you for taking the time to read and comment on our manuscript. We appreciate your observations and questions. Below is our response to your comments.

Comment: "The basic premise on which the authors base their study is that methane production depends on substrates produced during fermentation of organic matter and that methane production and sulfate reduction usually do not occur in the same sediment, presumably because of competition for these substrates." **Response:** In this comment, you seem to imply that methane production and sulfate reduction do not occur in the same sediment. Methanogens and sulfate-reducing bacteria do directly compete for organic substrates such that when sulfate or other more energetically profitable alternative electron acceptors are available, some methane is still produced, but it is at lower rates (please see manuscript citations: Achtnich et al. 1995; Lovley and Klug 1983, 1986).

Comment: "The authors mimic sea level rise by adding brackish water to their samples (which ignores the possible importance of increased water logging and decreased oxygen in the sediments)." **Response:** These sediments came from freshwater wetland ponds (0.4 – 1.2 m total water depth) that were already waterlogged and therefore had decreased oxygen in the

sediments. We have no reason to suspect that the physical act of sea-level rise would change the saturation status of the sediments. **Please see pg. 4, lines 2-8.**

Comment: "All incubations are done after vials are flushed with nitrogen although the natural sediments apparently all had some level of oxygen present in situ." **Response:** In this comment and throughout your review, you seem to imply that purging vials with nitrogen gas is problematic. It is widespread practice in methane production bottle assays to purge with nitrogen gas in order to remove the oxygen that was introduced by removing sediment from below the water's surface and exposing it to the air in the field and in the lab (Lofton et al. 2014; Sinke et al. 1992; West et al. 2012, 2015). Of course, it is possible that there are microsites where oxygen is present in the freshwater wetland sediments, but we believe that the same may be true in the bottle experiments. **Please see pg. 5, lines 23-27.**

Comment: "Values for nitrate (another potential substrate for carbon remineralizing organisms) and for acetate (the putative important substrate for methanogens) were measured but data were apparently quite variable and are not reported." **Response:** Summary porewater acetate levels are reported in Results section 3.1.1. You are correct in that we did not report actual values for nitrate, and we will add a short line in the results to indicate that nitrate was usually 2+ orders of magnitude lower than total sulfate levels. We measured acetate, nitrate, and sulfate fairly extensively in both the water column and the porewater as well as other parameters. We did not present all the data we collected because we did not want to overwhelm the reader, but these data are all available in the archived data. If reviewers and editors feel that these data would enhance the manuscript, we will add them in tabular or figure form to the manuscript or as supplemental information. **Please see Table 2.**

Comment: "No measurements were made of H_2, another potential substrate for methanogens and methanogens were not examined to see whether they were actually acetoclastic or hydrogenoclastic." **Response:** We did not measure H_2 production as we do not currently have an instrument available to measure this nor did we classify the type of methanogens. However, acetate was the predominant factor explaining methane production patterns in almost all experiments, which suggests that a large proportion of the methanogenesis is acetoclastic (e.g., pg. 8 lines 29-30). However, we do not deny the potential of hydrogenotrophic methanogenesis occurring in our ecosystems (please see last paragraph on page 10). **Please see revised manuscript, pg. 12, lines 14-18, and pg. 12, last paragraph.**

Comment: "The data for the dsrA and mcrA genes are again not presented and in the results section appear to contract the conclusions made about their abundance. The authors also ignore the fact that numbers of genes do not directly relate either to number of cells (a cell can have more than one copy of a gene) or to gene activity." **Response:** The *dsrA* and *mcrA* summary data are presented in the Results section 3.1.3, and individual gene data per sample are also archived in the data set (see microbial tab). Although we acknowledge that the number of genes does not equate with number of cells or gene activity, qPCR of functional genes for particular guilds is a commonly used approach to estimate the abundance of a functional group. We also acknowledge in the manuscript that it is possible that some of the genes we detect are from dormant microbial

communities (please see pg. 11, lines 8-12). The number of genes corresponding to abundance is always going to be a bit of an assumption, but in a broader survey of our wetlands, we have unpublished data from our system that *mcrA* abundance relative to *dsrA* abundance is correlated with methane production rates. Also, in *Letters in Applied Microbiology* (Volume 62, Issue 2, Pages 111-118), Morris et al. 2016 found that hydrogenotrophic methane production rates corresponded to *mcrA* abundance. Although observation of functional gene transcripts or even the actual enzymes catalyzing the reactions would clearly be preferred, logistics associated with our remote field sites precluded work with these more labile macromolecules. Despite challenges associated with interpreting DNA copy number of functional genes, we believe that our use of qPCR substantially adds to the literature as there are few studies that simultaneously measure methane production and microbial functional group abundances. For example, the studies listed on pg. 9, lines 22-26, have hypothesized that microbial community processes are behind methane patterns along a salinity gradient, but none of these studies actually tested this hypothesis by measuring microbial communities. **Please see revised manuscript, pg. 7, lines 7-9 and pg. 11, lines 12-25.**

Comment: "Finally the authors use natural organic matter to enrich their incubations but do not indicate the amount of carbon added or the relative lability of that carbon. (Clearly a gram of sucrose and a gram of twigs would not be expected to stimulate microbial activity to the same extent)." **Response:** We added 3 g of live tissue per macrophyte species, which translated to 0.10-0.12 mol of C added to each incubation, and we will add these values to the methods of the manuscript. Dr. Scott Tiegs of Oakland University has measured the % C, % N, and % P of the four litter species we added: Marestail (45% C, 1.7% N, 0.17% P), Buckbean (44% C, 0.94% N, 0.15% P), Lily (45% C, 1.7% N, 0.17% P), and Horsetail (47% C, 2.5% N, 0.24% P), but the patterns we observed did not follow %C, %N, %P, C:N or C:P, which are considered standard indices of litter quality. We discuss the phosphorus in detail because Tiegs et al. 2013 found that litter decomposed more rapidly when P was higher (see pg. 11, lines 14-24). You are correct in that we did not specifically measure the lability of the carbon from each macrophyte species, but we are leveraging information from Tiegs et al. 2013, whose study had already assessed this by measuring decomposition rates of these litter types in Copper River Delta ponds. We will clarify this in the discussion. **Please see revised manuscript pg. 5, lines 14-18, pg. 8, lines 28-29, pg. 10, lines 21-22, and pg. 15, lines 2-9.**

Comment: "P2 Line 6: I do not like the term green house compensation point. It is not widely used and does not directly relate to what you are measuring (as you say nothing about carbon sequestration in this system)." **Response:** We believe that the term "greenhouse compensation point" accurately describes wetlands' dual roles in carbon sequestration and emissions, and we believe that because northern wetlands are on the edge of this compensation point that it is so important to study how global change may alter the methane cycle. Greenhouse compensation point is a good way to set up the broader context of this study. **Please see pg. 2, lines 6-8.**

Comment: "You do not mention carbon fertilization again, and there is no indication in the text of whether any increases in carbon production in the CRD system would be due to warming or to carbon fertilization. I think you hurt yourself by trying

to draw connections to too many issues. I would recommend drastic simplification of this section and sticking to the facts." **Response:** Increased CO_2 levels lead to warming as well as CO_2 fertilization, both of which could affect the amount of substrate available. We are not precisely sure whether increased organic matter will result from warming, from longer growing seasons directly, or from CO_2 fertilization; therefore, it seems prudent to mention both potential mechanisms. Regardless, we will reevaluate our discussion of these points based on your recommendation and consider ways to focus our comments. **Please see pg. 2, lines 9-12 and pg. 3, lines 4-5.**

Comment: "P2 Line 16: redox conditions are only indirectly related to climate change. You seem to imply the mere presence of nitrate or sulfate changes redox conditions but this is not true, especially if oxygen is present. In fact there can be a lot of nitrate and sulfate in oxic surface sediments. Did you ever have sulfide in your samples? Does oxygen penetration vary with ecosystem? How far below the surface were the samples collected?" **Response:** True, redox conditions are a potential indirect effect of climate change. Sea-level rise could increase sulfate levels just as higher decomposition rates due to warming could deplete oxygen levels, both of which affect redox conditions. The sediment came from the top 20 cm, and although some oxygen is likely present in small amounts, it is also likely depleted quickly. For example, the layer of water directly above the sediments often has low DO levels of around 1 mg/L, particularly in the evening, and it is not uncommon to have anoxic groundwater upwelling in these systems. We also observe that water column DO levels drop throughout the season as vegetation begins to senesce. As for hydrogen sulfide, we did not directly measure that, but sediment characteristics (black coloration and pungent odor) suggest the presence of sulfide. Usually sediments become anaerobic within the first few cm of freshwater ponds, which is another reason that we make the incubations anaerobic by purging with nitrogen gas. Of course oxygen matters, but the amount of nitrate in particular is orders of magnitude lower than agricultural and other human impacted systems. Lastly, if significant levels of oxygen were present in these sediments, it would kill the methanogens because they are extremely sensitive to O_2 (please see manuscript citation: Whalen 2005) and we would therefore see very little methane production in our experiments. **Please see pg. 2, line 18.**

Comment: "P2 Line 23: I don't think an "abundant supply of organic matter can reduce competition for methanogens by increasing substrate availability". Try instead "abundant supply of organic matter can increase substrate availability"" **Response:** We appreciate the wording suggestion and will make the change. **Please see pg. 2, lines 28-29.**

Comment: "P2 Line 28: replace "are likely results of" with "may be influenced by" " **Response:** We appreciate the wording suggestion and will make the change. **Please see pg. 3, line 2.**

Comment: "P3 lines 7 and 10. Use same unit for sealevel rise (100 and 170 cm)" **Response:** We appreciate the wording suggestion and will make the change. **Please see pg. 3, lines 14-16.**

Comment: "P3 line 15: Numbers of methanogens not as important as whether or not the methanogens are active." **Response:** Please see our response to your major comment above about qPCR and the feasibility of RNA work. **Please see revised manuscript, pg. 7, lines 7-9.**

Comment; "P4 line4: The range of physicochemical parameters in table 1 are actually pretty small for most measurements. Perhaps more important is whether the intertidal sediments are exposed to the atmosphere at low tide (tidal range?). How long are they submerged? What is the water content? Again it matters how far below the surface these sediments were collected. From the table it must be shallow since there was more O2 in these sediments than the freshwater wetlands." **Response:** The intertidal sediments were collected from the top 20 cm just as in the freshwater wetlands, and they are covered with freshwater during low tide and increasingly brackish water during high tide. So again these sediments are waterlogged with depleted oxygen levels. In Table 1, the DO data are actually from the water column, as we do not have oxygen data for the sediment. In the freshwater ponds where limnological profiles could be conducted, we reported DO levels from the bottom of the water column, but in intertidal marsh, DO levels came from the surface layer. This is detailed in the legend for Table 1. We will remove the water column DO data altogether since they seem more misleading than helpful. **Please see Table 1.**

Comment: "P4 line 26: Were no replicates run for sediments from a single site? How can you tell if observed variability is just typical of replicate samples? I would also think you MUST indicate how much macrophyte tissue you added (probably in terms of gC/g sediment or something like that) to even know if these treatments were similar since the lability of the carbon is likely not the same." **Response**: We ran five control replicates without added substrate at each freshwater wetland (n = 5), which we then averaged to form the basis of the delta CH4 production metric. Each macrophyte treatment was replicated in 5 different ponds. In the increased organic matter simulation, we used pond as the replicate because we were more interested in capturing how wetlands differing in biogeochemistry along a glacial to oceanic gradient would respond to organic matter addition rather than how much variability there exists within a single wetland's response. **Please see pg. 5, lines 7-12.**

Comment: "P5 Line 6: You mean incubation temperature not ambient temperature?" **Response"** Yes, we do. We appreciate this good suggestion for a wording change. **Please see pg. 5, line 24.**

Comment: "P5 line 8: Purging with nitrogen will likely have a bigger effect than incubating at a few degrees cooler than the actual sediment. I would expect stimulation by this as you allow more anaerobic processes to occur. Were any blanks or controls run?" **Response:** Please see our response to your major comment above. **Please see pg. 5, lines 23-27.**

Comment: "P5 line 12: "flame ionization" not "flame ionizing" detector" **Response:** We will make this change. **Please see pg. 5, lines 31.**

Comment: "P5 line 20: You report that only sulfate was detectable in the water column. Did you present any water column data? Do they mean anything? What are detection limits for pore waters? Present nitrate and acetate data for sediments as well as sulfate." **Response:** We do have extensive water column data for these wetlands, some of which are in the archived data.

If reviewers feel these data add to the manuscript, we can add them in tabular form or in supplemental information. Some (i.e., DOC and sulfate) are also already presented in Table 1, but acetate and nitrate were not detectible in the water column (we will add concentration detection limits to the manuscript which were about 10 and 5 µM, respectively). **Please see Table 1 for water column data and Table 2 for sediment porewater data.**

Comment: "P5 line 27: the method you describe is typically called "loss on ignition" and represents a loss of organic matter. Did you convert to organic C? Is this data reported anywhere in the paper? Was DOC measured the same way? Blanks? Detection limit?" **Response:** Yes, we took the sediment organic matter data (in % of OM) from loss on ignition and converted it to organic C (pg. 5, lines 25-29). We will add in the following citation about conversion to organic C: Thomas et al. 2005; Aquat. Sci. 67:424–433. The data were not reported in the paper as to not overwhelm the reader, especially since the amount of carbon did not affect the results, but the data are archived and available online. DOC was measured using a Shimadzu TOC-V (pg. 5, lines 18-19), and we did run blanks. All samples had detectible DOC, but our lowest standard was 1 mg/L and all samples registered above this level with the exception of five of the intertidal samples, which fell in between the blank and the lowest standard. If reviewers would like more detail on these analyses in the methods, we will add them to the manuscript. **Please see sections 2.3.2 and 2.3.3.**

Comment: "P6 line 2: I would use the word "converted" rather than "scaled" " **Response:** Thank you for the wording suggestion. **Please see pg. 6, lines 18 and 23.**

Comment: "P6 line 5: I couldn't tell what this composite sediment was used for. Were incubations for each site or was all sediment made into a composite? Why do this? It seems that then the averages for the genes refer to different samples than the physiochemical properties. Can you properly do statistics comparing the combined samples in one parameter to the individual samples in one parameter?" **Response:** The composite sediment was a combination of the five sediment samples from different sites for each freshwater wetland, but this was only done for the microbial analyses. Making a composite is highly practiced in soil microbial ecology for the purpose of controlling analytical costs while still capturing significant spatial heterogeneity. We made a composite for each freshwater wetland which were then compared against ten sediment samples from intertidal marsh. So yes, it is true that we could not directly link methane production of a single sample or its physicochemical properties to the functional group abundances, but overall we were able to characterize methanogen and sulfate-reducer abundances across ecosystems and relate these to average methane production rates. **Please see pg. 6, lines 30-31 and pg. 7, line 1.**

Comment: "P7 line 2: move "log transformed" to immediately follow "four factors"" **Response"** Methane production rates were log-transformed, not the factors. We cannot make this change because it would misrepresent our statistics. **Please see pg. 7, line 27-29 where we rearranged the wording for clarity.**

Comment: "P7 line 26ff: You use a lot of significant figures for something that is so variable. Maybe you are justified in two significant figures but not 3 or more. Again blanks and detection limits need to be mentioned as your averages are pretty close

to zero (or at least almost include zero). I would like to see these data associated with specific samples" **Response:** We will reduce the number of significant figures and make the needed detection statements in the methods. We only reported parameters if they were detectible. The reason that these averages are close to zero in this particular line is that we converted actual concentrations to the total amount in the incubation bottle (i.e., µM * PW volume in incubation * (PW volume per ml of sediment) * sediment volume in bottle). **Please see sections 2.3.2, 2.3.3, 3.1.1., 3.2 and Tables 1 and 2.**

Comment: "P8 line 14: This paragraph seemed very odd. You seem to contradict yourself a lot. For intertidal samples, three had no dsrA but this was found in all freshwater AND the dsrA was independent of ecosystem type. This seems to contradict your hypothesis which you nevertheless cling to. Remember the presence of a gene doesn't mean the organisms are doing anything at the moment and the number of genes does not necessarily indicate the number of cells of a particular organisms." **Response:** Indeed, we did not detect *dsrA* in 3 of the 10 intertidal samples. Nevertheless, *dsrA* abundance tended to be higher in intertidal ecosystems, although not significantly so. In contrast, the *mcrA* presence and abundance did vary significantly by ecosystem, and methanogens are the group that we tend to use to explain our methane production results directly, while we use sulfate and sulfate-reducer abundance as supporting evidence (please see pg. 9, lines 14-16). **Please see revised manuscript pg. 9, line 22, and pg. 11, lines 18-25.**

Comment: "P8 line 27ff: I really don't like your equating sealevel rise purely with sulfate concentration. It is ok to say that sea level rise will flood current intertidal areas, but the vegetation will change and the water will be permanently water logged rather than periodically exposed to air. You have no data on estuarine wetlands to compare to the freshwater wetlands (although others have done this comparison in other systems). I would guess the reason you see no effect here is that you are mimicking the process the wrong way. You are really looking at increased sulfate levels, not sealevel rise." **Response:** In these systems, which are waterlogged throughout the year, we expect sulfate concentration to be a major biogeochemical change. Our simulation not only adds sulfate, but it also adds other nutrients and the microorganisms brought in with the saltwater. You are correct that over the long term vegetation and other characteristics of these ecosystems will also change. We will add comments at pg. 3., line 12, and pg. 4 line 20 to reflect our biogeochemical focus on the effects of sea-level rise. Our data on intertidal ecosystems for comparison with freshwater ecosystems can be found in Results section 3.1.2 and Figure 3. **Please see revised manuscript, pg. 1, line 14, pg. 4, lines 6-8, pg. 13, lines 20-25.**

Comment: "P10,line2: The sentence beginning "our study also demonstrates..."seems to directly contradict your own data as you said before that dsrA did not correlate with ecosystem type" **Response:** Just because *dsrA* presence and abundance did not significantly correlate with ecosystem type does not mean the presence or abundance of sulfate-reducing bacteria does not affect methane production in intertidal ecosystems. Even though these bacteria are also present in freshwater ecosystems, they could be limited by sulfate availability. Also *dsrA* tended to be higher on average relative to freshwater ecosystems, just not

significantly so. We will change the wording to "tended to have generally higher sulfate-reducer abundances when present" to address this concern. **Please see revised manuscript pg. 9, line 22, and pg. 11, lines 18-25.**

Comment: "P10 line 13ff and next paragraph: These two sections are wild speculation with absolutely no data behind them. There are a lot of other factors that might be important that you haven't included." **Response:** We provide potential hypotheses and explanations for our data that were grounded in the literature, which is not an uncommon practice in the discussion. Specifically, we discuss the acetoclastic pathway of methane production and how we might expect that to change in the future as well as possible reasons for why methane production rates varied by an order of magnitude throughout the season. We could omit these sections, but felt that it was important to say something about the results we observed. Regardless, we will reevaluate the nature and extent of our explanations. **Please see pg. 12, line 13, where we have extensively re-worked this paragraph. Also, we acknowledge that the last paragraph on pg. 12 involves hypothesizing about seasonal differences in CH_4 production because the data we collected fall short of explaining them, but the discussion is backed up by literature and we put forward a more formal hypothesis stating that future study is needed.**

Comment: "P11line14: This whole section is hurt by the fact that you don't characterize the macrophytes at all in terms of potential lability. Did you add a constant amount of C or organic N? Or just dry mass? Or just a "chunk" of leaves? Again much of this section is speculation without more facts" **Response:** Please see our response above to your major comment about lability of macrophytes. We evaluated many different possibilities for explaining the results of the different macrophyte treatments including % C, % N and % P, but the quality measurement that seemed to matter the most was antimicrobial properties. None of these potential indicators of lability appeared to have an effect on methane production, which we will clarify in the Discussion pg 11, lines 14-30, and pg 12, lines 1-9. **Please see revised manuscript pg. 5, lines 14-18, pg. 8, lines 28-29, pg. 10, lines 21-22, and pg. 15, lines 2-9.**

Comment: "P12 line19: You have no idea whether there are hydrogen utilizing methanogens around or how much hydrogen there might be. You can reiterate what other people have said in other papers but I don't see you connecting these to your system with any facts." **Response:** We will try to improve the clarity of the manuscript, by acknowledging that methanogens in general utilize acetate, directly (acetoclastic) or indirectly (hydrogenotrophic). We will also add that CO_2 and H_2 are compounds that also come from fermentation of organic matter such as acetate. Hence, this is why acetate is important and was measured in this study. **Please see pg. 12, lines 14-18.**

Comment: "Modeling: I didn't really understand the models. If you are making linear equations including various parameters and then constant factors for each, I would like to see more detail on how this worked. If you have a lot of variables, you can fit most data but figure 6 is completely mysterious and doesn't convince me of the value of your model. I also didn't understand the columns in tables 2 and 3. I have never heard of an AIC before, for example. Explain the statistics a bit to an audience that may include non-biologists." **Response:** Akaike Information Criterion (AIC) is an increasingly utilized form of model

selection that generates estimates of the model being the best representation given the data and the set of models being explored. It also penalizes for the number of parameters in the model. We will add more description of this in the methods. Basically, general linearized models are created, each of which is associated with an AIC value, which is then used to rank the models. We also corrected for small sample sizes (AIC_c). The model with the lowest AIC_c value is considered the best, and all remaining models are compared relative to the best approximating model using delta AIC_c (Δ_i). Models with a Δ_i less than or equal to 2 are considered to have substantial support, while models having a Δ_i greater than 7 have little support (Burnham and Anderson 2002). The relative strength of the models was then evaluated with Akaike weights (ω_i), which indicate the probability of a model being the best model, given the data and the set of candidate models (Burnham and Anderson 2002). **Please see revised manuscript pg. 8, lines 1-13.**

Respectfully,

Carmella Vizza, Will West, Stuart Jones, Julia Hart, and Gary Lamberti

RESPONSE TO REVIEWER 2, DR. SCOTT NEUBAUER

Response to 1st set of reviewer comments (R1):

Dr. Neubauer,

We greatly appreciate the extensive time and effort you put into reviewing our manuscript. Your comments were insightful and extremely thorough, and we believe that your suggestions have greatly improved the manuscript. Below please find our point-by-point responses to your suggestions and comments with the location of changes we made to the revised manuscript in bold. The attached supplement is the revised manuscript, which also has the changes your comments inspired highlighted in yellow.

Comment: "I was very interested in the topics covered by this manuscript and think that the measurements and experiments can provide some insight into questions about controls of CH4 production and how global changes will affect CH4 dynamics. However, I was a bit disappointed in the analysis and interpretation of the data, especially in how the authors tied their findings to existing knowledge. Similarly, I felt that the authors could have done a better job exploring the literature on how plants and organic matter inputs affect CH4 production." **Response:** We are pleased that you found our study interesting, and we thank you for pointing us towards several studies we had not found in our original literature search. We hope you find our discussion greatly expanded in light of the literature you recommended as well as a few other papers we came across in the process. **Please see pg., 11 lines 25-30, and pgs. 12-14.** Also, we hope that the expanded information on the AIC model selection as well as the fleshing out of some of our hypotheses in the discussion will aid the reader in understanding our data analysis and interpretation.

Specific comment 1: p. 1, line 13; p. 3, line 12; and throughout manuscript, "freshwater and intertidal wetlands". As someone who studies tidal freshwater wetlands, I take issue with the way you are classifying wetlands as either freshwater *or* intertidal.

Wetlands can be both! Indeed, there are over 19,000 ha of tidal *and* freshwater wetlands within the Copper River Delta (see Hall's chapter in the 2009 "Tidal Freshwater Wetlands" book; Barendregt, Whigham, Baldwin (eds). Backhuys Publishers). A better way of characterizing your two groups of sites would be "brackish intertidal" and "non-tidal freshwater" (unless, of course, your freshwater sites were also intertidal). **Response:** Certainly, wetlands are difficult to classify and the terminology is something that we should clarify because it appears to be a source of confusion. In light of Dr. Scranton's comment, we have added that the 'freshwater wetlands' are constantly inundated with fresh water so as to distinguish them from our 'intertidal wetlands', which are covered in freshwater at low tide and covered in increasingly brackish water at higher tides (rarely exposed to air at low tide). Although our freshwater wetlands currently do not receive any tidal influence, their surrounding sloughs sometimes do, which is why they are at risk of seawater intrusion. We have concluded that the best terms to call our wetlands in light of yours and Dr. Scranton's comments are "tidal brackish wetlands" and "non-tidal freshwater wetlands." **Please see pg. 1, line 13 in the Abstract and the terms were changed throughout the rest of the manuscript (we only highlighted the first instance in the revised manuscript so as not to distract from other changes) as well as pg. 4, lines 9-12.**

Specific comment 2: p. 1, lines 15-16: Your data clearly show that rates of CH4 production and porewater sulfate were each higher in the brackish sites. But, how did you determine that the high sulfate was the cause of the lower CH4 production? You also reported differences in porewater nitrate and acetate (top of p. 8) between ecosystem types, and presumably there were differences in salinity as well. How did you conclude that sulfate was the driving factor? Sulfate wasn't even the most important factor from your modeling exercise (p. 8, lines 11-12). **Response:** Table 3 shows that the top model (lowest AIC_c score) included all factors (ecosystem type, acetate, sulfate, and time period) and the second best model included ecosystem type, acetate and sulfate. Therefore, for explaining methane production rates in the non-tidal freshwater wetlands and tidal brackish wetlands, the most important factors are ecosystem type, acetate, and sulfate. First, nitrate availability was negligible in comparison to the total sulfate availability so we decided to remove that from the analysis (also based on your comment #16). Second, we concluded that acetate was not responsible for lower methane production rates in the brackish sites because acetate was actually higher than in the freshwater sites, and higher acetate availability should theoretically increase methane production. We believe that the variable 'ecosystem type' captures factors other than sulfate such as microbial communities and perhaps even salinity. It is difficult to disentangle the effects of sulfate from salinity since those two variables are highly correlated **(please see our expanded discussion of this on pg. 11, lines 18-30 and pg. 12, lines 1-12)**. However, mechanistically from a redox perspective, we believe that it is most plausible that lower methane production rates in brackish sites resulted from sulfate-reducing bacteria (which tended to be higher when present in brackish versus freshwater sites) that outcompete methanogens (which were significantly lower in brackish versus freshwater sites). One could also argue that stress from salinity might also lower methanogenic activity, but if this were the case, this should be a more immediate effect that

would have decreased methane production in the sea-level rise simulation. Conversely, the turnover of microbial communities in response to redox conditions may be a less immediate effect. You are correct in pointing out that our hypothesis, although supported by our data, does require some assumptions and deduction on our part. We have changed the wording to "probably due to higher sulfate availability" on **pg. 1, lines 15-16.**

Specific comment 3: p. 1, line 19, ". . .increased organic matter generally enhanced CH4 production rates." This statement is too strong for your data set. You used four different organic matter amendments (= 4 plant species). The CH4 production rates increased for only two of the species you tested (Fig. 5); the other two species had no effect. So, based on your data, I don't see how you can justify saying that the amendments "generally enhanced CH4 production." If something happening 50% of the time means that it is a "general" occurrence, you could also say that the amendments generally had no effect on CH4 production. **Response:** In calculating the difference in methane production between the paired treatment and control, this metric was positive in 15 out of 20 cases and so we believe that 75% of the cases is enough to say "generally." However, we agree with your comment in light of the particular species and will change the wording to say "enhanced CH_4 production in 75% of the incubations, but this response depended on the macrophyte species added with half of the species treatments having no significant effect," **please see pg. 1, lines 20-21.**

Specific comment 4: p. 2, line 4, ". . .21 times more effectively. . ." This value of the global warming potential for CH4 over a 100-year time period is quite old and has been updated in each of the two IPCC reports that have been published after the Whalen paper you cited. The bigger point of this comment is that the global warming potential may not be the best way to compare CO2 and CH4 when one is talking about ecosystem processes, where C3 gases are emitted or sequestered year after year. I discussed this in a paper that was published last year (Neubauer and Megonigal. 2015. Moving beyond global warming potentials to quantify the climatic role of ecosystems. Ecosystems. 18:1000-1013). **Response:** We appreciate your comment and acknowledge that using GWP may not be the best or most updated way of addressing why methane is an important greenhouse gas. We changed the wording to better reflect this, **please see pg. 2, lines 4-5**.

Specific comment 5: p. 2, lines 5-7, "Currently, wetlands at northern latitudes. . ." Other studies that have taken a longer temporal perspective have concluded that many northern wetlands have had a net cooling effect for the last 8,000-11,000 years (Frolking and Roulet 2007. Global Change Biology. 13:1079-1088). Because CH4 is broken down in the atmosphere – and therefore the warming due to CH4 emitted in any given year is transient – but the cooling due to C sequestration lasts "forever," a wetland that is old enough can have a lifetime net cooling effect, even if its radiative balance over a shorter period implies net warming. So, a single wetland could have a warming or cooling effect, depending on what time scale you consider. **Response:** Thank you for your insightful comment. We agree with you that a wetland's role in carbon sequestration and emissions is highly dependent on temporal perspective. **Please see pg. 2, lines 6-12** where we attempt to better reflect this point.

Specific comment 6: p. 3, line 28: Why is the word "wetlands" in quotes? Are you suggesting that your sites aren't actually wetlands? Also, why are you comparing brackish intertidal marshes with (unvegetated?) freshwater ponds? My understanding is that you are trying to compare sites that differ in salinity and sulfate due to their effects on CH4 production. Why not compare vegetated brackish marsh with vegetated freshwater marsh? Or, brackish ponds with freshwater ponds? **Response:** We were attempting to name a term that we would use to refer to them throughout the manuscript and therefore should have put both CRD and wetlands in quotes. For clarification, we are comparing vegetated freshwater non-tidal wetlands or "ponds" or with vegetated brackish tidal wetlands. We consider the freshwater wetlands to be "pond-like" because they have more clearly delineated boundaries, whereas the brackish tidal wetlands are continuous. Unfortunately, there are no brackish water ponds that we know of in the Copper River Delta to which to compare our freshwater systems. We also wanted our sites to be comparable in depth. The freshwater sites with depths greater than 0.4 m all tend to be "pond-like" due to the clay-like sediments in the area that allow very little drainage, whereas the brackish sites tend to have a depth of up to 2 m during high tide and 1 m during low tide. In general, the sites we sampled in the brackish tidal wetlands tended to be a bit shallower than the average depth for logistical reasons (i.e., using a handheld bucket auger from a jet boat). We have attempted to make this clearer in this paragraph, **please see pg. 4, lines 5-12**.

Specific comment 7: p. 4, lines 10-11 and 17: I'm a bit confused by your sampling design. You collected a single sample from five sites along the salinity gradient. Elsewhere (e.g., p. 3, lines 16-17), you explained that you expect that the availability of sulfate will be an important driver of rates of CH4 production. Given that, why would you combine all the sites along the salinity gradient into a single "brackish intertidal" value? **Response**: We combined all the tidal brackish sites into one value for the tables in the manuscript because this ecosystem was rather continuous and lacked clearly distinct boundaries like the non-tidal freshwater wetlands, **please see pg. 4, lines 15-18**. They are also combined in the figures as to contrast two distinctive ecosystem types. However, when it comes to data analysis through AIC model selection, each tidal brackish site is considered on its own such that widely varying sulfate levels are appropriately taken into account, **please see pg. 7, lines 27-29.**

Specific comment 8: You have quite a wide range of sulfate values (Table 1); was there a significant relationship between porewater sulfate and CH4 production? This would be another way of getting at your hypothesis about the effects of sulfate on methanogenesis. **Response**: Yes, if we log-transform methane production rates from the brackish and freshwater wetland comparison and run correlation tests on both porewater sulfate and total sulfate (porewater + water column), the results are $r = -0.67$, $P = 0.0001$, and $r = -0.71$, $P = 0.00003$, respectively. Although we believe that doing separate correlations by factor are redundant with AIC model selection (although different philosophically), we are happy to include these correlations in the manuscript if reviewers and editors feel they would improve its clarity.

Specific comment 9: p. 4, line 20: Does this sulfate concentration indicate the sulfate concentration in the water that was added when making the slurry or does it indicate the sulfate concentration in the slurry itself? Can you also report the salinity

of the water added for the slurries (or the final salinity of the slurry, whatever is consistent with the sulfate concentration)? **Response:** We have added another table to distinguish between water and sediment chemistry data, with the water column concentrations presented in Table 1 and the porewater concentrations presented in Table 2. The salinity concentrations of the water column are reported in Table 1, but unfortunately our salinity probe is only designed for measurements of the water column in the field so we were not able to also measure the salinity of the porewater (i.e., we could only extract a few mL of porewater per sediment sample). **Please see Table 2**, where we have presented both the porewater sulfate data as well as the total amount of sulfate available in the slurry (PW + WC).

Specific comment 10: p. 4, "Increased organic matter simulation" section: How much organic matter did you add to each bottle? Did you characterize the organic matter (e.g., C, N, P contents? lignin content?)? Did you use aboveground or belowground tissues? Were the tissues first cut to a standard size (e.g., passed through a grinder) before added to the bottles? Details such as those should be added to this section. **Response:** This is a good suggestion. **Please see pg. 5, lines 14-18** where we have updated this information in the manuscript.

Specific comment 11: p. 4, line 30: The genus is Menyanthes, not Menanythes. The same genus name is misspelled in some of the figure legends. **Response:** We greatly appreciate you catching this error. It has been changed throughout the manuscript. **Please see pg. 5, line 13, and Figure Captions 5 and 6**.

Specific comment 12: p. 5, lines 5 and 9, and elsewhere in the manuscript. Generally, you include a space between a number and its units (e.g., "60 mL" on line 5) but other times you don't (e.g., "250mL" on line 5). I also remember seeing some places where you said that your experiment lasted for "14d" (instead of "14 d"). When editing, check throughout the manuscript to see that you include a space between a number and its units. **Response:** We agree with you on consistency and will check all instances. In general, we did not include a space when a unit was used an adjective, as in describing a 250-mL serum bottle, which according to the style of *Biogeosciences* should be listed as a "250mL serum bottle." Nouns such as "250 mL of sediment" should indeed contain a space as you suggest. In light of the confusion, we have gone back and hyphenated all the adjectives and highlighted these instances for reference. We hope that the editor will clarify whether he prefers the lack of space or the hyphenated adjectives in light of these instances.

Specific comment 13: p. 5, line 12: It is a flame ionization detector, not a flame ionizing detector. **Response:** Thank you. We have made this change, **please see pg. 5, line 31.**

Specific comment 14: In the context of comparing between treatments within your study, it is fine to report your CH4 production rates as µmol per bottle per time. However, this really limits your ability to compare your results with those of others. Note, for example, that you would have gotten different rates (on a per bottle basis) if you had used a different volume of sediment, even if everything else was identical. At a minimum, you should tell the reader the weight of sediment in each bottle (e.g., "The 60 ml of added sediment was equivalent to 80-90 g of dry sediment."). It would be even better if you reported

your rates as µmol CH_4 per gram sediment per time. **Response: Please see pg. 5, line 22,** where we have added in the average amount of wet sediment. We agree with you that we would have gotten different methane production rates if we had added widely variable amounts of sediments, but the coefficient of variation in the wet sediment masses was approximately 3% and therefore did not affect the major trends when we reran all the analyses with CH_4 production rates as nmol CH_4 per gram of dry sediment per day. Originally, we reported values on a per bottle basis because we were treating each bottle as its own "wetland microcosm." This is also the reason we ran analyses with the total amount of each anion per incubation converted from porewater concentrations. Nonetheless, we agree with your argument that it is important to be able to make this comparable to other studies. Therefore, we have added the line in the methods clarifying how much wet sediment was added (dry sediment mass is available in the archived data) and have completely re-done all figures, tables, and analyses with CH_4 production rates being reported and analyzed as nmol CH_4 per gram of dry sediment per day. **Please see pg. 6, line 3, Tables 3-4 and Figures 3-6.**

Specific comment 15: p. 6, lines 1-2, "Porewater concentrations were scaled. . ." I don't understand what this means. Are you saying that you multiplied the anion concentrations by the volume of porewater in order to determine the total amount of each anion in the bottle? Based on your nutrient/anion results (bottom of p. 7 to top of p. 8), I think this is what you did. But, why did you do this? As with the CH4 production rates (previous comment), reporting things on a per-bottle basis makes your numbers completely dependent on the amount of sediment (and its water content) that you ran through the centrifuge. It seems more straightforward to report your nutrients using molar units (e.g., mM) because those numbers are independent of the volume of sediment that was processed and can be easily compared with other studies. **Response:** We have clarified how and why we converted porewater concentrations to the total amount per g of dry sediment (we removed bottle rates based on your comment 14), **please see pg. 6, lines 21-25**. Essentially, we converted the porewater concentrations to the total amount of each anion per bottle because the volume of porewater extracted from the sediments varied widely among samples (~47% CV). In light of your suggestion of being able to make comparisons between studies, we have now reported both the amount of each anion in the porewater per g of dry sediment as well as the traditional porewater concentrations **in Table 2**.

Specific comment 16: p. 7, line 4, "total sulfate and nitrate" Is this fourth factor the sum of sulfate and nitrate? If so, why did you add them together? I recognize that both sulfate and nitrate are electron acceptors. Adding them together makes the implicit assumption that one mole of nitrate is "the same" as one mole of sulfate. However, the thermodynamics (i.e., energy yield) and stoichiometry (i.e., moles of sulfate or nitrate reduced per mol of carbon oxidized) differ for sulfate reduction and nitrate reduction. So, in terms of competing with methanogens for electron donors, one mole of nitrate is not equivalent to one mole of sulfate. **Response:** Thank you for this insightful comment. Originally, we combined sulfate and nitrate as an overall representation of the presence of alternative electron acceptors and in order to minimize the number of parameters in model selection. Although we agree with you that the thermodynamics and stoichiometry varies per reaction, we thought that trying

to account for this would be negligible in light of the fact that nitrate was such a small component of this factor (~ 4.5%) in comparison to total sulfate (~ 95.5 %). In light of how negligible nitrate is and because of the stoichiometric concerns you voice here, we have decided to remove nitrate from analyses and instead just focus on total sulfate availability and therefore redid all analyses to account for this. **Please see pg. 7, line 31 and pg. 8, line 1.**

Specific comment 17: p. 7, line 7: What is delta i? **Response: Please see pg. 8, lines 1-13** where we have updated the manuscript to reflect more details about AIC model selection.

Specific comment 18: GLMs: I am approaching this manuscript as someone who is interested in the questions you are addressing but is getting lost when trying to understand and interpret the GLMs. Admittedly, this is because I have not ever received formal (or informal) training in GLMs. I am not expecting the revised manuscript or your "Response to reviewers" document to provide a tutorial in how GLMs work, but I hope that you will be able to make some modifications to the manuscript text that will make it easier for someone who isn't familiar with GLMs (such as myself) to follow the analyses that you did. **Response: Please see pg. 8, lines 1-13,** where we have updated the manuscript to reflect more details about AIC model selection. **Specific comment 18a**: In a GLM, what is a parameter estimate (e.g., as mentioned on p. 7, line 7)? I am familiar with multiple linear regression analyses, where each explanatory variable has its own parameter (or, "slope"). But, multiple linear regressions use continuous explanatory variables. In contrast, you have some nominal variables (e.g., ecosystem type, time period, macrophyte species). I can't begin to translate from my experience with multiple linear regressions to guess how you would come up with a parameter estimate for a nominal variable, or what such a parameter would even mean. **Response:** For categorical variables, a GLM essentially adjusts the intercept. For example, in considering ecosystem type, both freshwater and brackish wetlands would have their own intercept. **Specific comment 18b:** How do you estimate the importance of different variables? And, what does "relative importance" mean? I first thought that relative importance would be where everything is expressed as a fraction of the importance of the most important variable (so, the relative importance of the most important variable would be 1 and everything else would have a lower relative importance). But, that must not be the case since none of the variables for the GLM from the organic matter addition experiment have a relative importance of 1 (p. 9, lines 10-11). **Response:** Great question, and we have included more details about this in the manuscript. **Please see pg. 8, lines 11-13.** Also, we have included the updated Akaike weights after model averaging in **Tables 3 and 4** so that the reader can mentally calculate the relative importance in their head. For example, in Table 3, there were three models included in model averaging with a $\Delta_i > 4$, and sulfate is a factor included in 2 of the 3 models whose $\omega_{i\,(MA)}$ were 0.61 and 0.26, so its relative importance is 0.87. **Specific comment 18c**: Given Figure 6, it is apparent that you can use GLMs to generate predictive equations. Would it be worthwhile to include your best predictive equations in the manuscript for the reader to see? **Response:** We will be glad to report this should the editor feel this would be helpful. Although since these models are fairly specific to this study and these ecosystems, we would not necessarily recommend that others try to extrapolate methane production rates

from them. **Specific comment 18d**: I have no idea how to interpret the "Akaike weights" numbers in Tables 2 and 3. **Response: Please see pg. 8, lines 3-8. Specific comment 18e:** What is a null model? **Response:** A null model includes an intercept only and we have updated this in the methods for clarification, **please see pg. 8, line 9, and Tables 3 and 4.**

Specific comment 19: p. 7, Statistical Analyses: What are you using as your level of statistical significance – 0.01? 0.05? 0.10? **Response:** Great question, and we have updated our methods to reflect this. **Please pg. 8, lines 16-17.**

Specific comment 20: p. 7-8, Water column and porewater chemistry results: A student of mine did an experiment where he measured porewater concentrations of 130 µM sulfate, 5 µM nitrate, and 4 µM acetate. How do those numbers compare with yours? I don't actually want you to make that comparison but I want to make the point (again) that it is impossible for the reader to make that kind of comparison. I only know that you processed "~50 ml" of sediment but I have no idea of the water content of that sediment. Therefore, I cannot convert your values of the stock of sulfate or nitrate or acetate in a ~50 ml chunk of soil to a concentration. **Response:** Again, you are absolutely right that it is important for others to be able to compare their ecosystems to ours in terms of chemistry, and therefore porewater concentrations have been added to **Table 2**.

Specific comment 21: p. 8, line 9 and Tables 2 & 3, "total sulfate/nitrate" Earlier, I thought that you calculated sulfate + nitrate. Did you actually use the ratio of these anions in your analyses? What is the rationale for doing that? **Response:** No, we did not use a ratio, but rather we originally added sulfate and nitrate together. However, we have removed nitrate from analyses based on your comment 16.

Specific comment 22: p. 9, lines 18-20 and p. 13, lines 4-5: I am unclear how you concluded that it is important to consider the interactions of multiple global change mechanisms. I totally agree with that idea, but do not see how your results demonstrate the importance of studying interactions. After all, you only looked at individual factors, not at interactions. If you had done an experiment where you manipulated salinity and organic matter availability individually and together and had found that the interacting factors gave results that were unexpected based on single factor experiments, then you would have support for the idea that it is important to consider the interactions of multiple global change mechanisms. **Response:** Good point, as our study did not directly assess the interactions of simulated sea-level rise and increased organic matter availability. However, in assessing the differences in methane production between freshwater and brackish wetlands as well as in our sea-level rise simulation, we included both redox conditions (i.e., total sulfate availability) and a component of organic matter (i.e., acetate availability) as factors in our models. Both redox conditions and acetate availability were important factors for both modeling efforts, but interestingly the influence of each factor for the freshwater/brackish comparison was in the opposite direction for the organic matter simulation. This suggests that environmental context matters, i.e., higher sulfate levels generally lead to lower methane production, but methanogens in areas with more alternative electron acceptors might be more likely to respond to increased organic matter availability. This is why we believe that other studies should not only look into these factors in different ecosystems, but also examine how they interact. **Please see pg. 11, 3-6.**

Specific comment 23: p. 9, line 22-26, "Many studies. . ." Weston et al. (2014; Biogeochemistry. 120:163- 189) is an example of a study that measured CH4 fluxes along a salinity gradient *and* measured rates of methanogenesis. Neubauer et al. (2013; Biogeosciences. 10:8171- 8183) reported CH4 fluxes and CH4 production for a wetland that experienced >3 years of experimental saltwater intrusion. **Response:** We thank you for drawing our attention to these sources. **Please see pg. 11, lines 12-16.**

Specific comment 24: p. 9, line 27, "directly linked lower CH4 production to higher sulfate and nitrate concentrations" This is odd phrasing since you saw higher CH4 production where you had higher nitrate; your sentence suggests the opposite pattern. I think the confusion here is related to the way you did the GLMs with sulfate+nitrate (or perhaps sulfate/nitrate) as a model factor. You chemically analyzed these anions separately, but then combined them in some way for the statistical analyses. As noted in earlier comments, I do not understand how/why you combined these anions for statistical analysis. **Response:** Our results did show lower methane production when sulfate availability was high (**please see Table 3**), and we removed nitrate based on your comment 15. Methane production and sulfate availability were inversely related (please see our response to your comment 8). We attempted to clarify the wording, **please see pg. 11, lines 14-16.**

Specific comment 25: In your Statistical Analysis section (p. 7), you say that you used "general linear models." In the legends for Tables 2 and 3, you say that you used "general linearized models." A quick Google search for the exact phrase "general linearized models" did not reveal any statistics-related results in the first two pages of search results (I didn't look any deeper than that). A search for the same term (without the quotes) suggested Wikipedia pages for "general linear model" and "generalized linear model" as the top two search results. These are not the same thing. So, this comment is a long-winded request that you clarify whether you used "general linear models," "generalized linear models," or "general linearized models" (whatever they are) and to make sure that you use the correct terminology throughout your manuscript. **Response:** We appreciate you catching this, it is most common to just refer to them as GLMs, but the analyses used were actually named "generalized linear models" and **pg. 7, line 28,** has been changed accordingly as well as in **Tables 3 and 4**.

Specific comment 26: p. 10, lines 11-15: You cannot directly compare your CH4 production rates with those reported by Hines et al. (2008). Most importantly, Hines used 50 ml of slurry with 1 part soil to 3 parts total slurry volume; in each of your bottles, you used 120 ml of slurry that was 1 part wet sediment to 1 part water. All else being equal, we would expect higher CH4 production in your study simply because you had more sediment in your bottles. Expressing the rates per gram of soil/sediment, as I suggested earlier, would go a long way toward making your results comparable with those from other studies. [Helpfully, Hines et al. reported the typical weight of dry soil per milliliter of their slurry so you can get a rough idea of what their rates would be if expressed per gram of soil. Your data repository file lists sediment weights (wet or dry?) for each bottle.] **Response:** Originally, we had wanted to present Hines' data in bottle rates so that they could be comparable to

the rates we originally report in the manuscript, but we agree with your point that rates should be compared based on g of sediment. **Please see pg. 12, lines 18-25**, where we have updated these calculations and extensively re-worked this paragraph.

Specific comment 27: p. 10, last paragraph: Ultimately, this paragraph is unsatisfying. After seeing the huge June vs. August difference in rates of CH4 production, I was really hoping that you would be able to provide some strong insight into the cause(s) of that difference. I guess you are limited by data availability. Still, I wonder if others working in similar systems have reported order-of-magnitude changes in rates over such a short time period. I don't know anything about your system except what is in the manuscript but I'm wondering if the pattern could be related to the timing of soil thaw in the early growing season or perhaps the phenology of plant growth. Finally, I'll note that the measured concentration of acetate reflects the balance between rates of acetate production and acetate consumption. So, if higher acetate production in August was balanced by higher acetoclastic methanogenesis, you would see high rates of CH4 production without correspondingly high acetate concentrations. **Response:** Yes, you are correct that we are limited by data availability, but wish we had been able to explain these intriguing seasonal differences. However, in the hope of making this paragraph more satisfying, we have fleshed out our hypothesis about why these seasonal differences might occur and it indeed pertains to plant phenology! Also, you are correct that porewater acetate availability is a balance between acetate production and consumption. **Please see pg. 13, lines 8-18.**

Specific comment 28: p. 5, line 20 and p. 11, line 8: Change detectible to detectable. **Response:** Done, **please see pg. 6, line 10 and pg. 14, line 17.**

Specific comment 29: p. 12, lines 5-8: The possible role of antimicrobial compounds is an interesting hypothesis and you presented some information to support it. However, I did not see where you tested for the effects of litter quality (e.g., C:N:P, percent lignin, lipid content) on CH4 production rates. Without having run those analyses (either the chemical analyses or the statistical analyses), why are you discounting the possible influence of those factors that have previously been shown to be important? **Response:** We have formalized our analyses of the Tiegs et al. (2013) stoichiometry for the macrophytes used in this study and can therefore rule out C:N:P. **Please see revised manuscript pg. 5, lines 14-18, pg. 8, lines 28-29, pg. 10, lines 21-22, and pg. 15, lines 2-9.** Although we cannot rule out percent lignin or lipid content, it is our hypothesis that antimicrobial compounds were responsible for why methanogens responded differently to macrophyte species based on the literature we found about their antimicrobial properties. Even if we had measured percent lignin or lipid content, it would be difficult to conclusively rule out that some other quality measure contributed to the differences we observed without extensively characterized the quality of these macrophyte species. Again, we acknowledge that antimicrobial compounds hypothesis is untested, but this study does provide some anecdotal evidence suggesting that it might be worthy of further examination. **Please see pg. 15, line 24.**

Specific comment 30: p. 12, line 13, "Fewer studies have examined. . ." There have been studies looking at CH4 emissions when wetland plants are grown in an elevated CO2 environment. Although there are important differences between CH4

emissions and CH4 production, the elevated CO2 studies generally find that CH4 emissions increase with elevated CO2, with this increase often being attributed to higher plant production (see, for example, Vann and Megonigal 2003. Biogeochemistry. 63:117-134). **Response:** We appreciate you pointing us toward some interesting studies. **Please find our discussion updated on pg. 15, lines 29-31, and pg. 16, lines 1-6.**

Specific comment 31: Table 1: The legend says that you made 4-10 spot measurements per site. Given that, why aren't there any standard deviations or other estimates of error/variability for pond depth, temperature, pH, and salinity? **Response:** In light of the fact that these variables did not vary all that much, we originally did not report the error in order to avoid the table from becoming too cluttered. However, we are happy to report them, and please find standard deviations for all these parameters **in Table 1.**

Specific comments 32 and 33: Data repository: I took a look at the data that you made available at the knd.informatics.org site and had a question about your CH4 production rates on the "All CH4 data" Excel worksheet. In column R, you reported "areal CH4 production (umol/m2/d)." How did you determine areal rates? What does an areal rate even mean in the context of a soil slurry in a bottle? Is the "area" the same as the cross-sectional area of the bottle? If so, that seems meaningless to me since a cylindrical bottle is going to have the same cross-sectional area whether the bottle is 1 4 full, 1 2 full, or 3 4 full but, C10 presumably, would have different rates of CH4 production due to the different amounts of soil in the bottle. Given that you didn't report the areal rates in your manuscript, this whole things would be a question of curiosity. . .except that you used these areal rates to calculate the per-bottle rates (column S of the Excel file) that *are* reported in your manuscript. So, I need to know more about these areal rates before I can judge the validity of the per-bottle rates. I do not understand the formula that you used to go from areal rates (umol/m2/d) to per-bottle rates (umol/d): per-bottle rate = areal rate * 0.2 * sediment volume in liters. In order for the units to work out, the 0.2 factor must have units of m2/L. Those are odd units. Where does 0.2 m2/L come from and what does that conversion factor represent? **Response:** We apologize for any confusion caused by not removing the areal rates from the data repository, and we have updated those data included in the repository accordingly, **please see https://knb.ecoinformatics.org/#view/doi:10.5063/F1028PF8**. We have plans to use areal CH_4 production rates as a parameter for another manuscript, one that involves a process-based model with a methane budget for each pond. In order to use the methane production data as an input we needed to come up with a conversion factor of sediment volume to area, which is what the 0.2 m^2 per L represents. That conversion factor has nothing to do with this study though; it is just that we simply back-calculated the bottle rates in this particular spreadsheet from the areal rates. Just to be clear about our process: To get bottle rates, the GC concentrations are in ppm that are regressed against incubation duration in days (since we have measurements from multiple time points), which we then convert to µmol L^{-1} d^{-1} which we then multiply by headspace volume. To determine areal rates, we take the GC concentrations in ppm that are regressed against incubation duration in days, which we then convert to µmol L^{-1} d^{-1} which we then multiply by headspace volume and divide by sediment volume, and lastly we

multiply by the areal conversion factor (1 L/0.2 m^2). This is why in the spreadsheet online we took the areal rates, divided by that areal conversion factor (or multiplied the values by 0.2 m^2/L), and then multiplied that value by sediment volume in mL and divided that by 1000 to convert to L. Again, this was only a back calculation since we did not have the raw µmol L^{-1} d^{-1} data in this particular spreadsheet.

Respectfully,

Carmella Vizza, Will West, Stuart Jones, Julia Hart, and Gary Lamberti

Regulators of coastal wetland methane production and responses to simulated global change

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Abstract. Wetlands are the largest natural source of methane (CH_4) to the atmosphere, but their emissions vary along salinity and productivity gradients. Global change has the potential to reshape these gradients and therefore alter future contributions of wetlands to the global CH_4 budget. Our study examined CH_4 production along a natural salinity gradient in fully inundated coastal Alaska wetlands. In the laboratory, we incubated natural sediments to compare CH_4 production rates between non-tidal freshwater and tidal brackish wetlands, and quantified the abundances of methanogens and sulfate-reducing bacteria in these ecosystems. We also simulated the short-term biogeochemical effects of sea-level rise and enhanced organic matter availability, which we predicted would have contrasting effects on coastal wetland CH_4 production. Tidal brackish wetlands produced less CH_4 than non-tidal freshwater wetlands probably due to high sulfate availability and generally higher abundances of sulfate-reducing bacteria, whereas non-tidal freshwater wetlands had significantly greater methanogen abundances. Simulating the biogeochemical effects of sea-level rise in freshwater sediments, however, did not reduce CH_4 production, perhaps because the 14-day incubation period was too short to elicit a shift in microbial communities. In contrast, increased organic matter enhanced CH_4 production in 75% of the incubations, but this response depended on the macrophyte species, added with half of the species treatments having no significant effect. Our study suggests that CH_4 production in coastal wetlands, and therefore their overall contribution to the global CH_4 cycle, will be sensitive to increased organic matter availability and potentially sea-level rise. To better predict future wetland contributions to the global CH_4 budget, future studies and modeling efforts should investigate how multiple global change mechanisms will interact to impact CH_4 dynamics.

Keywords: Methanogenesis, seawater intrusion, saltwater incursion, redox conditions, microbial communities

1 Introduction

Wetlands contribute about 60% of all natural methane (CH_4) emissions to the atmosphere (Kirschke et al., 2013). As global temperatures continue to increase, some models predict that wetland CH_4 emissions will double by 2100 (Gedney et al., 2004). Because CH_4 is a potent greenhouse gas whose radiative forcing continues even after its oxidation to CO_2 (Neubauer and Megonigal 2015), higher wetland emissions could trigger a positive feedback loop that further increases temperatures and CH_4 release. Currently, wetlands at northern latitudes are thought to be on the brink of the "greenhouse compensation point," wherein carbon sequestration is offset by greenhouse gas emissions (Whiting and Chanton, 2001), but this balance between sequestration and emissions is highly dependent on the temporal perspective being considered (e.g., Frolking and Roulet 2007). Nevertheless, higher future CO_2 levels, which could result in further warming, an extended growing season, and CO_2 fertilization, could upset this present balance by converting northern wetlands to net sources of carbon to the atmosphere in the short-term, especially if the resulting increases in plant productivity provide additional organic matter to fuel additional CH_4 production (Ringeval et al., 2011). Predicting the response of these ecosystems to global change is challenging because we do not fully understand the sensitivity of the CH_4 cycle to enhanced productivity of wetland plants (McGuire et al., 2009; Ringeval et al., 2011).

Warming associated with increasing CO_2 levels will also lead to sea-level rise in coastal areas and longer growing seasons at northern latitudes (Walther et al., 2002), thus further enhancing the CO_2 fertilization effect (Matthews, 2007; Ringeval et al., 2011), all of which could affect where wetlands stand in relation to their current greenhouse compensation point. These elements of global change could indirectly alter two of the main factors that influence CH_4 production – substrate availability and redox conditions (Whalen, 2005). Methanogens generally use substrates provided by the fermentation of organic matter, producing CH_4 via two pathways: (1) acetoclastic methanogenesis, where acetate is the substrate of choice, and (2) hydrogenotrophic methanogenesis, where H_2 and CO_2 are the substrates utilized (Conrad, 1999). Acetate is therefore an important substrate that methanogens either directly use (acetoclastic pathway) or indirectly use via the H_2 and CO_2 resulting from its fermentation and that of other organic matter (hydrogenotrophic pathway). However, methanogens can be outcompeted for these substrates because carbon is not an energetically favorable electron acceptor in comparison to those used by other microbes. High redox potential and the presence of alternative electron acceptors (e.g., NO_3^-, SO_4^{2-}) can signal intense microbial competition for the fermentative substrates that methanogens utilize (Lovley and Klug, 1983; 1986; Lovley and Phillips, 1987). For example, Winfrey and Ward (1983) observed much greater rates of sulfate reduction than CH_4 production in intertidal sediments until sulfate became depleted. However, an abundant supply of organic matter can increase substrate availability, act as an electron donor, and lower redox potential as alternate electron acceptors are consumed (Achtnich et al., 1995). Both redox conditions and substrate availability will therefore play an important role in determining the effects of global change on CH_4 production.

To accurately forecast the future global CH_4 budget, it is critical that we understand the effects of sea-level rise and increased organic matter availability on CH_4 production in wetlands (Fig. 1), which may be influenced by rising global CO_2 concentrations and temperatures. Laboratory studies and field surveys report increased CH_4 production and emissions with warming (Moore and Dalva, 1993; Klinger et al., 1994; Lofton et al., 2014). Additionally, elevated CO_2 levels can also lead to higher photosynthesis and CH_4 emission rates (Megonigal and Schlesinger 1997; Vann and Megonigal 2003). However, despite their potential importance in regulating CH_4 emissions from wetlands, especially those at northern latitudes, few studies have attempted to simulate the effects of sea-level rise or increased substrate availability on CH_4 production. Both of these global change mechanisms are likely to disrupt coastal wetland biogeochemical cycles, especially at northern latitudes where their effects are likely to be stronger and more abrupt.

We studied wetland ecosystems in the Copper River Delta of Alaska, an area vulnerable to global change because of its northern location and proximity to the ocean. Over the past 50 years, average annual temperatures in Alaska have increased 1.9 °C, with winter temperatures rising 3.6 °C (U.S. Global Climate Change Program, 2009), which is extending the growing season. In addition, the projected global sea-level rise of 100 cm by 2100 (Vermeer and Rahmstorf, 2009) will be exacerbated along the southcentral Alaskan coast where tectonic subsidence is prominent (Freymueller et al., 2008). For example, the Copper River Delta, which is subsiding at about 0.85 cm per year (Freymueller et al., 2008), is at risk of a relative sea-level rise of about 170 cm by 2100.

Our study objectives were to (1) compare CH_4 production rates and microbial community abundances in sediments from constantly inundated non-tidal freshwater and tidal brackish wetlands in the Copper River Delta, (2) simulate the short-term biogeochemical effects of sea-level rise for freshwater wetlands, and (3) simulate increased organic matter availability in freshwater wetlands. We hypothesized that (1) tidal brackish wetlands sediments will have lower CH_4 production rates than those from the non-tidal freshwater wetlands, (2) tidal brackish wetland sediments will have higher abundance of sulfate-reducing bacteria, but lower numbers of methanogens than non-tidal freshwater wetlands, (3) simulating the biogeochemical effects of sea-level rise in freshwater sediments will decrease CH_4 production rates, with sulfate availability largely being responsible for this effect, and (4) increasing the amount of organic matter available will enhance CH_4 production, but substrate quality will moderate this effect. Our conceptual model for these interactions is depicted in Fig. 1.

2 Materials and Methods

2.1 Study area

The Copper River in southcentral Alaska is the eighth largest river in the United States (U.S. Geological Survey, 1990). Draining a large region of the Chugach Mountains and the Wrangell Mountains into the Gulf of Alaska, the Copper River and

its sediment deposits have shaped the largest contiguous wetland on the Pacific Coast of North America. The Copper River Delta (CRD) encompasses about 283,000 hectares of wetland habitat and supports extraordinary biodiversity (Bryant, 1991) in a largely pristine landscape. Wetlands and shallow ponds (0.2 to 2 m in depth) were created and modified by the Great Alaska earthquake in 1964 that elevated the CRD by 1−4 m depending on location (Thilenius, 1995). A natural succession of wetlands thereby emerges from the ocean to the uplands (Fig. 2). Our study focused on the brackish tidal wetlands and non-tidal freshwater wetland/pond habitats. The brackish tidal wetlands we chose to study were increasingly brackish during rising high tide but were fully inundated with freshwater during low tide so as to be comparable to the fully inundated non-tidal freshwater wetlands. The freshwater wetland habitats currently receive little to no tidal influence, but their surrounding sloughs and rivers are tidally influenced, which could result in future seawater intrusion with sea-level rise. We consider the freshwater wetlands to be "pond-like" because they have clearly delineated boundaries, whereas the brackish wetlands are more continuous in nature. We chose these two ecosystem types because they are the most prevalent yet distinctive habitats on the CRD with which to contrast CH_4 production.

2.2 Experimental design

2.2.1 Sample collection

Using a handheld bucket auger, sediment samples (~ 250 mL) were collected from nine non-tidal freshwater wetlands and five tidal brackish wetland sites varying in physicochemical parameters (Table 1); tidal brackish wetland sites were combined when presenting summary data due to their continuous nature in contrast to the freshwater wetlands, which had distinct boundaries. Due to extensive habitat heterogeneity within the non-tidal freshwater wetlands (i.e., open water and several different macrophyte zones), we collected at least five sediment samples representative of the different habitats at each wetland (n = 9) along with at least 1 L of hypolimnetic water during each sampling period, so that the average CH_4 production rates from each system could be accurately assessed. In contrast, the tidal brackish wetland sites generally exhibited less habitat heterogeneity than the non-tidal freshwater wetlands (i.e., we observed only sites dominated by *Carex* spp.), but we observed temporal fluctuations in salinity with a YSI Pro Plus multiparameter water quality meter indicative of tidal influence. We, therefore, collected 1 L of water and one sediment sample at five different sites along a salinity gradient.

2.2.2 Non-tidal freshwater and tidal brackish wetland comparison

To assess CH_4 production, laboratory incubations were conducted using sediment and water samples collected during two sampling periods (June and August 2014). Specifically, we conducted five incubations for each non-tidal freshwater wetland (n = 9) and five incubations for tidal brackish wetlands (n = 5 separate locations in the continuous tidal zone). We then we used the average CH_4 production rates from each non-tidal freshwater wetland as a replicate in comparing CH_4 production rates between non-tidal freshwater (n = 9) and tidal brackish (n = 5) systems at each sampling period.

2.2.3 Sea-level rise simulation

To assess the effects of sea-level rise on CH_4 production, additional sediments were collected in June from a single site in five of the freshwater wetlands (n = 5) and then incubated with tidal brackish water (6.3 mM sulfate). We then compared them to the average CH_4 production rates of the five sediment samples incubated with freshwater from that same subset of non-tidal freshwater wetlands (n = 5) during June 2014.

2.2.4 Increased organic matter simulation

To assess the effects of increased organic matter on CH_4 production, four sediment samples from different sites were used from five of the non-tidal freshwater wetlands (n = 20). An aliquot of each sediment sample from each wetland was incubated with fresh macrophyte tissue from one of four species (treatment) and then compared to an aliquot that served as a paired control sediment sample (total pairs = 20; 5 wetlands x 4 treatments). This paired design controlled for "within wetland" sediment heterogeneity to better capture the response of the methanogens to adding organic matter, or ΔCH_4 production (treatment–control). Our four organic matter treatments were based upon the four dominant aquatic macrophyte species on the CRD – buckbean (*Menyanthes trifoliata*), horsetail (*Equisetum variegatum*), lily (*Nuphar polysepalum*), and marestail (*Hippuris vulgaris*). Specifically, we cut aboveground tissue to a standard size per species such that 3.0 g of live biomass could be added to each incubation resulting in approximately 0.23 ± 0.02 mmol C per gram of dry sediment (mean ± sd). In most incubations this addition of organic matter increased the total amount of carbon already available in the sediment by 45 ± 15 % (Table 2). Differences in substrate quality between these treatments, as described by % C, % N, and % P as well as C:N and C:P, are available from Tiegs et al. (2013). All vegetation for each species was collected from the same plant individual to ensure minimal difference in quality within each treatment.

2.3 Laboratory analyses

2.3.1 Sediment slurry incubations

For each incubation, approximately 60 mL (82 ± 2.5 g) of wet sediment and 60 mL of water were incubated in a 250-mL serum bottle in the dark at approximately 14.0 °C. In order to remove oxygen introduced to the inundated sediments during sample collection and slurry making, each bottle was made anoxic by purging it with N_2 gas for five minutes. Since incubation temperature was generally lower than average wetland temperature (June: 17.2 ± 0.9 °C, August: 18.4 ± 1.3 °C), estimated rates of CH_4 production potential were considered conservative. However, we do acknowledge that CH_4 production potentials generated by bottle incubations may not exactly reproduce CH_4 production rates in these ecosystems. Headspace samples (10 mL) were removed at 2, 5, 8, 11, and 14 days, injected into a 2-mL serum vial (pre-evacuated with a vacuum pump), sealed with silicone, and stored upside down in water for less than three months until the samples could be analyzed using gas chromatography. To maintain atmospheric pressure in the slurry incubations, 10 mL of N_2 gas was added after each sampling point. CH_4 concentrations were measured using an Agilent 6890 gas chromatograph equipped with a flame ionization detector

(Agilent Technologies, Santa Clara, CA, USA) as detailed by West et al. (2015). After accounting for headspace dilution due to sampling, CH_4 production rates were inferred from the slope of the linear regressions of CH_4 concentrations over time and are reported as nmol CH_4 per g of dry sediment per day (nmol g^{-1} day^{-1}).

2.3.2 Physicochemical measurements

Temperature, pH, dissolved oxygen, specific conductivity, and salinity were measured at each sampling location using a YSI Pro Plus multiparameter water quality meter (YSI, Yellow Springs, OH, USA). Dissolved organic carbon was analyzed using a Shimadzu TOC-VCSH (Shimadzu Scientific Instruments, Kyoto, Japan). All samples, with the exception of five of the tidal brackish samples, registered above the lowest standard (1 mg/L); the five exceptions registered between the blanks and the lowest standard. Acetate, nitrate, and sulfate concentrations were analyzed using a Dionex ICS-5000 (Thermo Fisher Scientific, Sunnyvale, CA, USA), but only sulfate was detectable in the water column. Detection limits for acetate, nitrate, and sulfate were approximately 10, 2, and 1 µM, respectively. Water chemistry analyses were performed using instrumentation at the University of Notre Dame Center for Environmental Science and Technology.

2.3.3 Sediment organic matter and porewater chemistry

To examine starting conditions for each CH_4 production assay, a subsample of sediment was frozen at the start of the incubation for later analysis. A portion of each subsample was dried for at least 48 hours at 60 °C, and the dry weight was recorded. Subsequently, the organic matter in the sediment was combusted at 500 °C for four hours, and the sediment was re-wetted and then dried at 60 °C for at least 48 hours before re-weighing (Steinman et al., 2011). Sediment organic matter was estimated as the percent of sediment material lost during combustion (SOM %) and converted to the total sediment organic carbon (Thomas et al., 2005) available per g of dry sediment (Table 2). To extract porewater from the sediment, another portion (~ 50 mL) was centrifuged for 45 minutes at 4 °C at ~ 4000 RCF. The total volume of supernatant per volume of sediment was recorded, and a subsample of the porewater was also analyzed on the Dionex ICS-5000 for acetate, nitrate, and sulfate. To account for the widely differing porewater volumes we were able to extract from sediment (0.17 ± 0.09 ml porewater per mL of sediment), porewater concentrations were converted to the total amount of each anion (nmol) per g of dry sediment (i.e., µM x porewater volume in incubation x porewater volume per mL of sediment x sediment volume in bottle / mass of dry sediment x 1000; Table 2).

2.3.4 Microbial analyses

DNA was extracted from frozen sediments used in other analyses, including multiple June tidal brackish sediments (n = 10), the freshwater sediments used in the sea-level rise simulation (n = 5), and a composite of the five sediment samples (1 g sediment per sample was added to make a 5-g composite) from the nine freshwater wetlands for the June time period (n = 9), according to the manufacturer's protocol with a PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA, USA). We chose to make composites for microbial analyses of the non-tidal freshwater wetlands for the purpose of controlling analytical costs

while controlling for the significant spatial heterogeneity in these ecosystems. Extracted DNA served as a template for quantitative PCR (qPCR) targeting of two genes – the alpha subunit of methyl coenzyme reductase (*mcrA*) and the alpha subunit of dissimilatory sulfite reductase (*dsrA*). The *mcrA* gene catalyzes the reduction of a methyl group to CH_4 (Thauer, 1998), and is possessed by all known methanogens thereby making it ideal for quantifying methanogen abundance (Luton et al., 2002; Earl et al., 2003; Castro et al., 2004). The *dsrA* gene catalyzes the final step in sulfate respiration, and its ubiquity in sulfate-reducing bacteria makes it powerful at assessing their abundance (Wagner et al., 1998; Klein et al., 2001; Zverlov et al., 2005). Although the number of genes does not necessarily equate with number of cells or gene activity, qPCR of functional genes for particular guilds is a commonly used approach to estimate the abundance of a functional group and these gene abundances have been correlated with functional processes such as CH_4 production (e.g., Morris et al., 2015).

The *mcrA* and *dsrA* genes were amplified using a 20-µL qPCR reaction in a Mastercycler ep realplex^2 gradient S (Eppendorf, Hamburg, Germany), using SYBR Green as the reporter dye. Each reaction contained 1 µL of brackish or freshwater wetland DNA template and was conducted using the PerfeCTa SYBR Green FastMix (Quanta BioSciences). For the *mcrA* qPCR, primer details and thermocycling conditions in West et al. (2012) were replicated except that we employed a fluorescent detection step at 78 ºC for 20 seconds. For the *dsrA* qPCR primer, details and thermocycling conditions in Kondo et al. (2008) were replicated. Melting curves for both *mcrA* and *dsrA* were run to ensure absence of non-specific amplification. Amplification, fluorescence data collection, and initial data analysis were all performed by the Eppendorf realplex^2 software.

Standard qPCR curves for *mcrA* and *dsrA* were generated by pooling gel-extracted amplicons containing our qPCR primer sites from a subset of our non-tidal freshwater and tidal brackish wetland samples. We amplified *mcrA* using primers detailed in Luton et al. (2002) and thermocycling conditions in West et al. (2012), and *dsrA* by replicating primer details and thermocycling conditions in Kondo et al. (2008). After amplification, we used gel electrophoresis and an Invitrogen PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA) to isolate the *mcrA* and *dsrA* amplicons. Following clean-up, we quantified the purified amplicons using Invitrogen's Qubit technology. We then used serial ten-fold dilutions of these genes to generate standard curves for qPCR. Our detection limit for each gene was approximately 1000 copies per g of wet sediment. Samples below detection were assigned a value of 999 copies per g for further analysis. We ran triplicate analyses of all samples for both the *mcrA* and *dsrA* qPCR, the averages of which were used in summary statistics and analyses.

2.4 Statistical analyses

For the non-tidal freshwater (n = 18, 9 sites x 2 time periods) and tidal brackish wetland comparison (n = 10, 5 sites x 2 time periods), we analyzed how four factors influenced log-transformed CH_4 production rates using generalized linear models (GLM) and Akaike Information Criterion (AIC) based model selection. The four factors were: (1) ecosystem type (non-tidal freshwater or tidal brackish), (2) time period (June or August), (3) porewater acetate availability (nmol g^{-1} dry sediment), and (4) total sulfate present (nmol g^{-1} dry sediment). As nitrate availability was extremely low in these ecosystems in comparison

to total sulfate availability (i.e., ~5%), we did not include nitrate as a factor in the GLMs. AIC-based model selection identifies the most likely model given the data while penalizing for model complexity (i.e., the number of parameters). In our analysis, we corrected for small sample sizes (AIC_c; Burnham and Anderson 2002). The model with the lowest AICc value is considered the most likely, and all remaining models are compared relative to the most likely model using delta AIC_c (Δ_i). Models with a Δ_i less than or equal to 2 are considered to have substantial support, while models having a Δ_i greater than 7 have little support (Burnham and Anderson 2002). The relative strength of our candidate models was then evaluated with Akaike weights (ω_i), which indicate the probability of a model being the most likely model, given the data and the set of candidate models (Burnham and Anderson 2002). We considered 16 candidate models (all possible additive combinations of the four factors including the null model) using the methods described above. A subset of those models, excluding the null model (i.e., intercept only) and those with relatively low support ($\Delta_i > 4$), were then used to determine model-averaged parameter estimates and to estimate the relative importance of variables (Burnham and Anderson, 2002). To estimate the relative importance of predictor variable *x*, we used the sum of Akaike weights for models including variable *x* (the closer the sum is to 1, the more important the variable *x*); we only considered models where $\Delta_i < 4$ for this analysis (Burnham and Anderson, 2002).

To compare the abundance of methanogens and sulfate-reducing bacteria, we first used a chi-squared test for each gene to determine whether the presence/absence of *mcrA* or *dsrA* was independent of ecosystem type. We then used a non-parametric Kruskal-Wallis tests to determine whether the number of copies of *mcrA* or *dsrA* varied by ecosystem type. For all statistical analyses excluding AIC model selection, α was set 0.05.

For the sea-level rise simulation, we conducted a paired *t*-test to determine whether CH_4 production rates in non-tidal freshwater wetland sediments were affected by being flooded with tidal brackish water instead of freshwater from their respective wetlands. Pearson correlations were computed (Zar, 2010) to determine whether porewater acetate or total sulfate levels were related to CH_4 production rates during this experiment.

To determine whether adding organic matter affected CH_4 production rates, we first used an analysis of variance (ANOVA) with treatment (i.e., macrophyte species) as the factor of interest and non-tidal freshwater wetland as a blocking variable. Then we analyzed how three factors influenced the response of each sediment, or ΔCH_4 production (treatment–control), using additive GLMs. The three factors were: (1) macrophyte species added, (2) total acetate available in the porewater (nmol g^{-1} dry sediment), and (3) total amount of sulfate present (nmol g^{-1} dry sediment). A total of eight candidate models (all possible additive combinations of the three factors including the null model) were compared as described above. To determine whether macrophyte species stoichiometry influenced the response of methanogens to increased organic matter, linear regressions were computed for % C, % N, % P, C:N, and C:P against ΔCH_4 production. All statistical analyses were conducted in the R software environment using the base and MuMIn packages (R Development Core Team, 2016).

3 Results

3.1 Non-tidal freshwater and tidal brackish wetland comparison

3.1.1 Water column and porewater chemistry

Water column and sediment porewater chemistry of the incubations varied more by ecosystem type than by time period (Tables 1 & 2). Total sulfate levels in non-tidal freshwater incubations (June: 84 ± 65; August: 48 ± 43 nmol gram^{-1} dry sediment; mean ± sd) were about two orders of magnitude lower than in tidal brackish incubations (June: 4300 ± 4300; August: 3500 ± 3700 nmol gram^{-1} dry sediment) and did not vary between time periods. In comparison to total sulfate levels, porewater nitrate availability was very low, with non-tidal freshwater wetlands (June: 1.5 ± 0.9; August: 1.8 ± 1.8 nmol gram^{-1} dry sediment) having relatively higher nitrate than the tidal brackish wetlands (June: 0.24 ± 0.51; August: 0.0092 ± 0.0025 nmol gram^{-1} dry sediment; Table 2). The total amount of acetate available in the non-tidal freshwater wetland incubations was similar in June (28 ± 22 nmol gram^{-1} dry sediment) and August (30 ± 17 nmol gram^{-1} dry sediment), while levels in the tidal brackish wetland incubations were generally higher and more variable especially in August (210 ± 260 nmol gram^{-1} dry sediment) than in June (130 ± 80 nmol gram^{-1} dry sediment).

3.1.2 CH_4 production

CH_4 production rates were higher in non-tidal freshwater wetlands than in tidal brackish wetlands and approximately an order of magnitude higher in both ecosystems in August compared to June (Fig. 3). Porewater acetate positively influenced CH_4 production rates, while total sulfate availability negatively influenced CH_4 production rates (Table 3). The most likely model contained all four factors – ecosystem type, time period, acetate, and total sulfate (Table 3). Based upon model averaging of the top three models (Table 3), all four factors appeared to influence CH_4 production with the relative importance of these variables being 1.00 for ecosystem, 1.00 for porewater acetate, 0.87 for total sulfate availability, and 0.74 for time period.

3.1.3 Functional group abundances

Tidal brackish sediments tended to have higher abundances of sulfate-reducing bacteria when present, while non-tidal freshwater sediments were characterized by higher numbers of methanogens. In the tidal brackish wetlands, three out of ten samples were below the detection limit for the *dsrA* gene, our proxy for sulfate-reducing bacteria abundance, but we detected this gene in all nine non-tidal freshwater wetland composite samples. The presence or absence of the *dsrA* gene was independent of ecosystem type ($\chi^2 = 3.21$, df = 1, $P = 0.07$). Tidal brackish sediments (n = 10) and non-tidal freshwater wetland sediments (n = 9) had 3.52 ± 5.39 x 10^5 and 5.20 ± 5.08 x 10^4 copies of *dsrA* per gram of wet sediment, respectively. Due to high variability, the number of copies of *dsrA* did not differ significantly by ecosystem (Kruskal-Wallis: $H = 1.31$, df = 1, $P = 0.25$). In contrast, we detected the *mcrA* gene, our proxy for methanogen abundance, in only two out of ten tidal brackish samples, but in all nine non-tidal freshwater wetland samples. The presence or absence of the *mcrA* gene was dependent on

ecosystem type (χ^2 = 12.44, df = 1, P = 0.0004). Tidal brackish samples had 2.14 ± 5.78 x 10^4 copies of the *mcrA* per gram of wet sediment, while non-tidal freshwater wetlands had 1.84 ± 1.25 x 10^5 copies of *mcrA* per gram of wet sediment. Methanogen abundance therefore differed significantly between ecosystem types (Kruskal-Wallis: H = 11.24, df = 1, P = 0.0008)

3.2 Sea-level rise simulation

Flooding of fully inundated non-tidal freshwater wetland sediments with brackish water did not affect CH_4 production rates (Fig. 4). Even though total sulfate levels increased from 63 ± 37 to 5400 ± 400 nmol $gram^{-1}$ dry sediment with the addition of tidal brackish water, CH_4 production rates did not differ between treatment and control incubations (paired t-test: t = 0.44, df = 4, P = 0.68). However, CH_4 production rates were significantly correlated with porewater acetate levels (r = 0.88, t = 5.18, df = 8, P = 0.0008), but not with total sulfate levels (r = 0.09, t = 0.24, df = 8, P = 0.81). The non-tidal freshwater wetland sediments used in this biogeochemical sea-level rise simulation (n = 5) had about an order of magnitude higher number of copies of *mcrA* (3.12 ± 4.40 x 10^5) than *dsrA* (5.32 ± 6.33 x 10^4) per gram of wet sediment.

3.3 Increased organic matter simulation

The organic matter treatments significantly influenced CH_4 production rates ($F_{4, 16}$ = 4.48, P = 0.01), but this effect varied with macrophyte species (Fig. 5). Adding buckbean and marestail had little effect on CH_4 production, while the lily and horsetail treatments generally increased methanogen activity (Fig. 5). The most likely model for predicting ΔCH_4 production (treatment – control) included acetate availability, which had a negative effect on the response (Table 4). The next best models included porewater acetate and species (Model 2) or porewater acetate and total sulfate availability (Model 3), which had a positive effect on the response (Table 4). Models 1–4 (Table 4) were averaged to determine parameter estimates with the relative importance of the variables being 0.88 for porewater acetate, 0.33 for macrophyte species, and 0.15 for total sulfate availability. Using the model-averaged parameters, our predictions of the response of CH_4 production rates to increased substrate availability closely followed the observed results (Fig. 6). Finally, macrophyte species stoichiometry (i.e., % C, % N, % P, C:N, and C:P) had no effect on ΔCH_4 production ($r^2 < 0.08$, $P > 0.24$ for all regressions).

4 Discussion

To begin to understand likely responses of wetlands to global change processes, we conducted a space-for-time substitution of how sea-level rise might affect CH_4 production in freshwater wetlands by comparing them to brackish systems. We found that CH_4 production was lower in tidal brackish than in non-tidal freshwater wetlands, likely due to differences in redox state (i.e., higher sulfate levels in the tidal brackish) and in microbial communities (i.e., lower methanogen abundances in the tidal

brackish). Short-term simulation of sea-level rise in non-tidal freshwater sediments (~14 d), however, did not influence CH_4 production rates. In contrast, higher organic matter availability generally enhanced CH_4 production rates in 75% of incubations, but this response varied by macrophyte species and the amount of substrate already available. Because acetate and sulfate availability had contrasting effects depending on the experiment (i.e., freshwater/brackish comparison vs. increased organic matter), these results demonstrate that the interaction of global change mechanisms must be considered when modeling the future contribution of coastal wetlands to the global CH_4 budget (Fig. 1).

4.1 Non-tidal freshwater and tidal brackish wetland comparison

CH_4 production rates in tidal brackish wetlands were substantially lower than those of non-tidal freshwater wetlands, as predicted. Many studies have attributed the decrease in wetland CH_4 emissions along increasing salinity and sulfate concentrations to sulfate-reducing bacteria outcompeting methanogens for substrates (DeLaune et al., 1983; Bartlett et al., 1987; Magenheimer et al., 1996; Poffenbarger et al., 2011), but none of these studies directly assessed whether lower CH_4 emissions resulted from reduced CH_4 production or higher CH_4 oxidation. Two recent studies documented lower CH_4 production with elevated salinity (Chambers et al. 2013; Neubauer et al. 2013), and attempted to link C mineralization rates to extracellular enzymes, but microbial communities were not quantified. In comparison, our study quantified CH_4 production along a similar spatial gradient and directly linked lower CH_4 production to higher sulfate availability and indirectly to relative abundance of functional microbial guilds. The presence of alternative electron acceptors such as sulfate likely signals that methanogens have to compete for organic substrates with sulfate-reducing bacteria (Oremland and Polcin, 1982; Lovley and Klug, 1986; Achtnich et al., 1995). Our study also demonstrates that tidal brackish sediments tended to have generally higher sulfate-reducing bacteria (*dsrA*) abundances when present, but significantly lower levels of methanogens (*mcrA*) than non-tidal freshwater sediments. Although we did not include microbial data in the model selection due to sample size limitations, we hypothesize that microbial community differences could help to explain why ecosystem type (freshwater vs. brackish) was an important factor during model selection. Collectively, these results along with higher sulfate availability in tidal brackish wetlands (and sulfate's importance in our model selection analysis) suggest that shifts in the relative abundance of functional microbial guilds between tidal brackish and non-tidal freshwater wetlands contribute to differences in CH_4 production between these ecosystems.

The difference between brackish and freshwater wetland CH_4 production could also be shaped by other ecosystem factors such as salinity and salinity-induced cation exchange. Because salinity and sulfate availability are often correlated, it can be difficult to disentangle these two factors; Chambers et al. (2011) isolated their effects in a laboratory manipulation and found that seawater (sulfate) had a more dramatic and longer lasting effect on CH_4 production than saltwater (NaCl). Nevertheless, salinity often places additional stress on organisms such that saltwater intrusion alters microbial and plant

communities (Herbert et al. 2015). Additionally, saltwater intrusion can influence cation exchange in the sediments such that calcium is mobilized, which can co-precipitate with phosphate, thereby releasing ammonium, all of which can shift a wetland towards P rather than N limitation (Herbert et al. 2015; van Dijk et al. 2015). Although we did not directly measure these effects of salinity and therefore cannot rule them out, we hypothesize that sulfate availability and differences in functional microbial guilds are primarily responsible for differences in CH_4 production rather than salinity and salinity-induced cation exchange. Our hypothesis relies on three observations: (1) N and P availability were extremely low in both freshwater and brackish ecosystems (DIN: < 25 µg N L^{-1}, SRP: < 15 µg P L^{-1}) and therefore different sediment cation exchange capacities were unlikely to change the N and P limitation of these wetlands, (2) salinity tended to be consistently low in freshwater wetlands, but CH_4 production was still negatively correlated with sulfate availability, and (3) sulfate availability was an important factor in ecosystem comparison model selection, and was the only factor where a direct mechanistic link can be made to the differences in CH_4 production between freshwater and brackish ecosystems (i.e., acetate availability was higher in brackish wetlands and therefore one might expect higher CH_4 production).

In addition to the influences of microbial communities and redox conditions on CH_4 production, acetate availability appeared to be an important factor. Substrate availability regulates CH_4 production (Whalen, 2005), and acetate is one of the major precursors for methanogenesis (Conrad, 1999) as it can be a direct (acetoclastic) or an indirect (hydrogentrophic) substrate for methanogens after further fermentation. Although the importance of acetate as a factor in our experiments suggests that acetoclastic methanogenesis may be prevalent in the CRD, we cannot rule out the potential of hydrogenotrophic methanogenesis, which is thought to be the primary pathway in other Alaskan wetlands (Hines et al., 2001). According to Hines et al. (2008), acetate tended to accumulate in Alaskan peat rather than be converted to CH_4 possibly due to homoacetogenic bacteria (i.e., those that make acetate) being able to outcompete methanogens for CO_2 and H_2 in colder temperatures and the general lack of acetoclastic methanogens. In contrast, CH_4 production in CRD wetlands was tightly coupled to acetate availability in the ecosystem comparison as well as in both simulations. Despite the differences between these Alaskan wetlands (CRD sediment is more similar to clay than to peat; see SOM % in Table 2), CRD freshwater wetlands exhibited similar CH_4 production rates to those conducted during August 2001 by Hines et al. (2008), which ranged from about 10 to 500 nmol g^{-1} dry peat day^{-1}. Because CH_4 production rates in Alaskan peat tended to increase with higher proportions of vascular plant cover (Hines et al. 2008) and the fermentation of this plant matter facilitates the production of acetate, it is possible that the role of the acetoclastic pathway may grow more important in northern wetlands in the future as vascular plant growth increases (Klady et al., 2011).

CH_4 production rates often vary seasonally as a function of temperature, but we observed August rates that were an order of magnitude higher than those conducted in June despite these incubations being conducted at the same temperature. Other factors affecting CH_4 production that could vary seasonally include (1) availability of organic matter such as acetate for

CH_4 production (Whiting and Chanton, 1993; Walter et al., 2001), (2) redox conditions including sulfate concentrations (Sinke et al., 1992), (3) microbial population densities (Yannarell and Triplett, 2005), or (4) the pathway by which CH_4 is produced (Avery et al., 1999). In our study, we did not observe large seasonal differences in porewater acetate or sulfate availability in CRD wetlands, but we did not assess seasonal variation in the abundances of methanogens and sulfate-reducing bacteria, their per-cell activity rates, or availability of H_2 or methanogenic substrates other than acetate. Therefore, it is possible that the observed seasonal differences in CH_4 production rates were the result of microbial community shifts, decreased per-cell activity of methanogens in June, greater CH_4 produced from the hydrogenotrophic pathway during August as acetate levels did not change, or some combination of these potential explanations. Additionally, we acknowledge that the porewater acetate level we measured is an indicator of the balance between acetogenesis and acetate consumption, so it is possible that acetogenesis rates increased during August and the acetoclastic pathway of methanogenesis correspondingly increased such that acetate availability appeared to be similar during these two months. Although we did not collect the data that satisfactorily explain these intriguing seasonal differences, we hypothesize that CH_4 production rates vary in accordance with macrophyte phenology in these ecosystems, which clearly affects both redox conditions and microbial processing rates. For example, in early growing season, CH_4 production is low, but steeply increases at peak growing season as more labile plant exudates are produced. The end of the growing season results in plant senescence, increased organic matter availability as plants decompose, and reduced oxygen levels, which then results in higher CH_4 production until colder temperatures start to decelerate microbial processing. All of these conditions could lead to seasonal succession in microbial communities and their activity rates. Future studies should seek to explain the mechanism behind seasonal differences in CH_4 production that are independent of temperature.

4.2 Sea-level rise simulation

Despite our finding that CH_4 production rates were significantly lower in tidal brackish wetlands sites, simulating the biogeochemical effects of sea-level rise in non-tidal freshwater sediments surprisingly did not affect CH_4 production rates. We acknowledge that our experiment simulated short-term consequences of sea-level rise such as increased sulfate availability and the addition of other marine nutrients and microbial communities, but we were not simulating longer term changes such as differences in plant communities and production that may result from increased salinity (Neubauer 2013; Hopfensperger et al. 2014; Herbert et al. 2015). Nevertheless, many other short-term studies conducting similar sea-level rise simulations have observed a decrease in CH_4 production rates with elevated salinity (DeLaune et al. 1983; Chambers et al. 2011; Marton et al. 2012; Chambers et al. 2013; Neubauer et al. 2013; van Dijk et al. 2015). In many of these studies, however, sulfate availability was much higher. For example, DeLaune et al. (1983) found that CH_4 production was inhibited with the addition of ~10 mM sulfate, which is higher than the sulfate concentration (~6 mM) used in this study. Chambers et al. (2011) observed a reduction in the treatments where sulfate concentrations were about 130 and 320 µmol per g^{-1} of dry sediment, which is about two orders

of magnitude larger than our sea-level rise simulation (5 μmol g^{-1} of dry sediment). Additionally, the majority of all these experiments were conducted at 25–30°C, or almost double the temperature used in this study (14°C), which could increase the rates at which microbial communities and their activities respond. It is therefore likely that the external environmental conditions imposed, such as the temperature, salinity, or sulfate availability used in a sea-level rise simulation, can influence the results.

In addition to environmental conditions, initial factors such as soil characteristics or site properties may mediate how methanogens respond to sea-level rise simulations (Neubauer et al. 2013). For example, van Dijk et al. (2015) found that elevated salinity decreases CH_4 production in peat but not in clay, and the sediment of the CRD wetlands is claylike in nature. Additionally, in some of these experiments, the sediments prior to incubation had been exposed to higher levels of sulfate (e.g., brackish sediments used by DeLaune et al. 1983) and were therefore more likely primed for sulfate reduction and the corresponding increase in competition for organic substrates. In contrast, the freshwater sediments used in this simulation had lower sulfate availability, and the sulfate-reducing bacteria abundances were an order of magnitude lower than methanogens. In some cases, however, sulfate reduction can increase without a corresponding decrease in CH_4 production (Hopfensperger et al. 2014), especially if saltwater intrusion increases both sulfate and organic matter availability (Weston et al. 2011).

Sea-level rise could therefore affect both redox conditions and organic matter availability, but their contrasting effects on CH_4 production are mediated by microbial communities and processes. Although the presence of sulfate-reducing bacteria was detectable in the sediments used in this simulation, we do not know whether these taxa were active or dormant. In fact, dormant taxa can account for almost 40% of taxon richness in nutrient-poor systems (Jones and Lennon, 2010), such as the CRD freshwater wetlands. Additionally, we conducted 14-day incubations, which may have been too short to allow for shifts in the relative abundance of sediment microbial populations (Hoehler and Jørgensen, 2013). For example, Edmonds et al. (2009) found no changes in microbial community composition of bacteria or archaea after sediment cores had been exposed to seawater for 35 days. We therefore hypothesize that the reason that CH_4 production in freshwater sediments did not respond to the sea-level rise simulation is a combination of environmental conditions, initial sediment factors, and a lag in response time from the microbial communities.

4.3 Increased organic matter simulation

Higher availability of organic matter generally increased CH_4 production rates, but this effect varied with the species of macrophyte added to the incubations. Differences in litter quality is known to influence methanogen communities and CH_4 production (Yavitt et al., 1990; 2000; Valentine et al., 1994). For example, West et al. (2012) found that adding algal carbon significantly enhanced CH_4 production relative to terrestrial carbon. Although aquatic macrophyte carbon may be of lower quality than that of algae, aquatic macrophytes are generally more labile than terrestrial plants (Schlickeisen et al., 2003). For

example, Tiegs et al. (2013) found that terrestrial plants decomposed more slowly than aquatic macrophytes in CRD wetlands. Additionally, Tiegs et al. (2013) conducted a decomposition assay of all the macrophyte species used in this study, as a way of assessing litter quality, and found that buckbean and lily leaves decomposed at about the same rate, but both were faster than marestail and horsetail. The rate of decomposition of different plant species was correlated with phosphorus content, and therefore indicative of litter quality differences (Tiegs et al., 2013). However, our CH_4 production response did not follow the decomposition pattern documented by Tiegs et al. (2013); we observed higher CH_4 production for the lily and horsetail treatment relative to the control, but not for buckbean and marestail. We also did not find that the CH_4 production response to organic matter treatment varied by % C, % N, % P, C:N, C:P, or any other measure of litter quality assessed by Tiegs et al. (2013).

Other measures of litter quality beyond elemental composition could explain differences in the methanogen response. West et al. (2015), for example, found that higher lipid content of phytoplankton enhanced CH_4 production rates. Alternatively, certain properties may influence the fermentative microbial communities associated with vegetation during decomposition (Boon et al., 1996), which are responsible for providing methanogenic substrates. For example, in a survey of 209 plants, Bishop and MacDonald (1951) reported that buckbean was one of the 10 most active species for antibacterial substances, while horsetail did not possess such properties. Specifically, buckbean extracts include aucubin, a defensive compound that can inhibit many strains of anaerobic bacteria (Weckesser et al., 2007). Marestail also contains aucubin as well as a verbascoside, another antimicrobial compound (Damtoft et al., 1994). In contrast, the only part of lily linked to potential antimicrobial properties is the rhizomes, which have been used in folk medicine (Padgett, 2007) and are more likely to require defensive compounds because of competition with the sediment microbial community than the floating leaves we used for this experiment. Therefore, we hypothesize that CH_4 production varied as a function of a different measure of litter quality than previously put forward (e.g., C:N:P, percent lignin, or lipid content), whereby the negative effects of the antimicrobial properties of buckbean and marestail on the fermentative bacteria superseded the positive effect of increasing the amount of organic matter. We suggest that this hypothesis is worthy of further examination.

Many other studies have documented that CH_4 production is enhanced by the addition of direct substrates such as acetate and H_2 (Williams and Crawford, 1984; Bachoon and Jones, 1992; Amaral and Knowles, 1994; Coles and Yavitt, 2002; Yavitt and Seidman-Zager, 2006), or the addition of indirect substrates such as dextrose and glucose (DeLaune et al., 1983; Williams and Crawford, 1984; Coles and Yavitt, 2002), which would need to be broken down by fermentative bacteria before methanogens could utilize them. Fewer studies have examined the effects of more biologically realistic, indirect substrates such as plant or algal matter on CH_4 production incubations (but see Valentine et al., 1994; West et al., 2012; 2015). However, two studies involving larger scale plots with elevated CO_2 levels exhibited greater photosynthetic rates and greater CH_4 emissions (Megonigal and Schlesinger 1997; Vann and Megonigal 2003). Although Vann and Megonigal (2003) observed

enhanced plant biomass that was strongly correlated with CH_4 emissions, Megonigal and Schlesinger (1997) did not see increased biomass and therefore hypothesized that lower transpiration rates, not increased substrate availability, led to higher CH_4 emissions by increasing flooding duration and stimulating anaerobic processes. In our study, increased substrate availability is likely the mechanism behind increased CH_4 production because our smaller scale simulation did not alter flooding duration, anaerobic conditions, or the physical structures by which plants can act as conduits for gas exchange (i.e., aerenchyma). Interestingly, the amount of acetate already available in the sediment appeared to moderate the methanogen response to enhanced substrate availability. The negative relationship between ΔCH_4 production and porewater acetate concentration suggests that methanogenic substrate concentrations can become saturated, which is expected from traditional Michaelis-Menten enzyme kinetics.

Another indication of substrate limitation is the positive relationship between the methanogenic response to added organic matter and the total amount of sulfate available in the incubation. This alternative electron acceptor provides more energy than either methanogenic pathway (acetoclastic or hydrogenotrophic) when coupled to the oxidation of organic matter (Stumm and Morgan, 1996; Schlesinger and Bernhardt, 2013). For example, Westermann and Ahring (1987) found that inhibiting sulfate reduction stimulated CH_4 production in an alder swamp, suggesting that methanogens and sulfate-reducing bacteria compete for common substrates. Sulfate availability, therefore, may signal strength of competition for electron donors (organic matter) that methanogens must overcome to produce CH_4. The higher the competition, the more likely that methanogens respond positively to the addition of organic matter. The response of methanogens to increased substrate availability, therefore, is likely regulated by the quality of the substrate (e.g., C:P, lipid content, or antimicrobial compounds), strength of competition for substrate (e.g., redox conditions, microbial community assemblages, or per-cell activity rates), and whether substrate availability is limiting or saturated in the environment. Although total sulfate availability played a less significant role than acetate and macrophyte species, the model using averaged estimates from all three parameters allowed us to accurately predict the response in CH_4 production for this experiment.

5 Conclusions

Our study demonstrates that potential interactions between elements of global change, specifically sea-level rise, longer grower seasons, and CO_2 fertilization, could have competing effects on CH_4 production from coastal wetlands (Fig. 1). Determining the timescale required for processes at the microbial scale to shift towards sulfate reduction is challenging, and the magnitude of seawater intrusion needed to induce this shift is currently unclear. Microbial community shifts can occur over longer timescales than several months, and CH_4 production can be more affected by long-term salinization (~ 3.5 years) than 2-day salinity pulses (Neubauer et al. 2013). As others have noted, the global carbon cycle is inextricably linked to other

elemental cycles (i.e., sulfur) by processes taking place at the microbial scale (Schimel, 2004; Burgin et al., 2011). In addition, the potential effects of sea-level rise are not limited to CH_4 production alone. Salinization also reduces aerobic and anaerobic methane oxidation, with aerobic organisms being particularly sensitive to salinity (Dalal et al. 2008; Herbert et al. 2015). Furthermore, the effects of sulfate availability on the CH_4 cycle extend beyond sea-level rise to other aspects of global change such as road salts and agricultural land use (Helton et al. 2014; Herbert et al. 2015).

In contrast to sea-level rise and increased sulfate availability, longer growing seasons and CO_2 fertilization will likely enhance carbon substrate supply and in turn CH_4 production. Our study demonstrates that the effect of increased organic matter depends on plant species, the availability of other methanogenic substrates, and the presence of alternative electron acceptors. It is possible that longer growing seasons and CO_2 fertilization could reduce competition between methanogens and other microbial communities by providing more substrates, as we saw in freshwater wetlands with higher sulfate concentrations, thereby superseding the effect of sea-level rise. Additionally, the CO_2 fertilization effect could increase organic matter accretion of marsh plants, which could physically counteract sea-level rise by raising marsh elevation (Langley et al., 2009). Future studies should consider how the interaction of sea-level rise, increased organic matter, and warming will affect both the microbial and ecosystem processes of the global methane cycle. This intersection of global change processes will be particularly important for projecting the future CH_4 budgets of coastal wetland ecosystems.

6 Data availability

The data will be freely accessible through the international repository, Knowledge Network for Biocomplexity (KNB) at: https://knb.ecoinformatics.org/#view/doi:10.5063/F1028PF8.

Author contributions. CV designed the study as sparked from discussions with SEJ. CV and JAH conducted the fieldwork and laboratory analyses. WEW played a key role in methodology and analyzing methane samples with the GC. SEJ and GAL played advisory roles in shaping this research. CV prepared the manuscript with contributions from all co-authors.

Competing interests. The authors declare that they have no conflict of interest.

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Table 1. Water column physical and chemical characteristics (mean ± sd) of the wetlands sampled in the Copper River Delta including elevation, depth, temperature, pH, specific conductivity (SpC), salinity, dissolved organic carbon (DOC), and sulfate concentrations. Freshwater physicochemical parameters, with the exception of elevation, DOC, and sulfate are from spot measurements of the hypolimnion conducted throughout summer 2014 (n = 4 per freshwater wetland). Tidal brackish wetlands parameters are from spot measurements of the surface layer (n = 10). DOC and sulfate are from June and August 2014 (n = 10 per wetland).

Wetland	Elevation (m)	Depth (m)	Temp (°C)	pH	SpC (µs cm^{-1})	Salinity (ppt)	DOC (mg L^{-1})	Sulfate (µM)
Eyak N	5.2	0.60 ± 0.09	15.3 ± 0.9	5.5 ± 0.4	13 ± 3	0.01 ± 0.01	6.5 ± 1.9	1.6 ± 0.3
Eyak S	5.5	0.61 ± 0.03	16.1 ± 1.3	7.0 ± 1.1	11 ± 2	0.00 ± 0.01	5.6 ± 0.5	2.0 ± 0.2
Lily	8.2	0.65 ± 0.04	13.1 ± 0.8	5.9 ± 0.2	60 ± 19	0.03 ± 0.01	3.5 ± 1.0	6.0 ± 2.2
Rich Hate Me	18.3	0.57 ± 0.15	11.6 ± 2.9	6.1 ± 0.4	56 ± 7	0.03 ± 0.01	2.1 ± 0.5	24 ± 5
Scott S	13.4	0.81 ± 0.07	14.2 ± 0.9	6.3 ± 0.3	61 ± 37	0.03 ± 0.02	2.1 ± 0.6	54 ± 17
Storey N	4.6	0.56 ± 0.04	16.8 ± 1.0	6.9 ± 0.4	74 ± 11	0.04 ± 0.01	11 ± 0.6	4.5 ± 0.7
Storey S	2.1	0.60 ± 0.04	16.6 ± 2.4	7.3 ± 0.7	70 ± 6	0.03 ± 0.00	4.2 ± 0.3	7.9 ± 0.5
Tiedeman N	5.5	0.66 ± 0.03	16.6 ± 1.1	6.0 ± 0.5	13 ± 3	0.01 ± 0.01	6.7 ± 0.7	1.8 ± 0.2
Tiedeman S	5.5	0.73 ± 0.03	15.4 ± 1.4	6.7 ± 0.6	8.8 ± 1.7	0.00 ± 0.00	5.2 ± 0.6	1.7 ± 0.3
Tidal brackish	1.4	0.56 ± 0.24	14.3 ± 2.3	7.3 ± 0.5	8500 ± 9600	5 ± 6	3.1 ± 6.3	3400 ± 3900

Table 2. Sediment chemical characteristics (mean ± sd) of the wetlands (n = 10 per wetland) sampled during June and August 2014 in the Copper River Delta including sediment organic matter (SOM %), total sediment carbon, and porewater (PW) concentrations of acetate, nitrate, and sulfate as well as total sulfate availability in the slurry incubations. All chemistry parameters were converted to the total amount of anion per gram of dry sediment (nmol g^{-1}) for analyses, but standard porewater concentrations (µM) are also reported for comparison with other studies.

5

Wetland	SOM (%)	Total Sediment C (mmol g^{-1})	PW Acetate (nmol g^{-1}) / (µM)		PW Nitrate (nmol g^{-1}) / (µM)		PW Sulfate (nmol g^{-1}) / (µM)		Total Sulfate (nmol g^{-1})
Eyak N	2.0 ± 0.5	0.81 ± 0.22	57 ± 57	360 ± 350	1.2 ± 0.7	9.6 ± 9.9	150 ± 160	970 ± 950	160 ± 160
Eyak S	1.8 ± 0.5	0.71 ± 0.21	18 ± 13	120 ± 82	1.4 ± 1.0	4.3 ± 3.1	65 ± 48	500 ± 350	67 ± 48
Lily	2.1 ± 0.5	0.86 ± 0.21	58 ± 43	620 ± 560	0.86 ± 0.26	0.4 ± 0.2	5.1 ± 3.2	49 ± 27	11 ± 3
Rich Hate Me	3.1 ± 3.9	1.3 ± 1.6	29 ± 44	110 ± 140	3.7 ± 4.9	2.2 ± 4.2	60 ± 130	96 ± 110	84 ± 140
Scott S	1.5 ± 2.2	0.60 ± 0.89	31 ± 34	300 ± 340	2.2 ± 2.4	0.8 ± 0.8	11 ± 11	120 ± 110	51 ± 14
Storey N	1.8 ± 0.2	0.73 ± 0.09	10 ± 5	120 ± 64	0.92 ± 0.39	1.2 ± 0.8	18 ± 11	210 ± 160	22 ± 12
Storey S	1.9 ± 3.1	0.76 ± 1.2	15 ± 16	160 ± 120	0.58 ± 0.39	2.8 ± 3.1	39 ± 44	450 ± 460	46 ± 45
Tiedeman N	2.8 ± 2.9	1.1 ± 1.2	25 ± 14	190 ± 95	1.7 ± 1.2	3.7 ± 2.8	56 ± 43	420 ± 320	93 ± 81
Tiedeman S	2.3 ± 0.8	0.93 ± 0.32	17 ± 7	120 ± 45	2.1 ± 1.9	4.3 ± 3.4	66 ± 53	440 ± 320	67 ± 53
Tidal brackish	6.4 ± 4.9	2.6 ± 2.0	170 ± 190	1800 ± 1800	0.13 ± 0.36	88 ± 69	1200 ± 860	11000 ± 4900	3900 ± 3800

Table 3. Generalized linear models (GLMs) wherein log-transformed CH_4 production rate is the response variable and ecosystem type (nontidal freshwater or tidal brackish), time period (June or August), porewater acetate level, and total sulfate availability are potential factors. Positive (↑) or negative effects (↓) of continuous factors are indicated. Models are ranked in order of the lowest Akaike information criterion corrected for low samples sizes (AIC_c) along with delta AIC_c (Δ_i) and Akaike weights (ω_i) before and after model averaging (MA). Models with a Δ_i larger than 4 were not included in the model averaging. The three models with a larger AIC_c than the null model (intercept only) are not presented.

Model #	GLM	AIC_c	Δ_i	ω_i	$\omega_{i\ (MA)}$
1	ecosystem + time period + acetate (↑) + sulfate (↓)	125.3	0.0	0.571	0.61
2	ecosystem + acetate (↑) + sulfate (↓)	127.0	1.7	0.244	0.26
3	ecosystem + time period + acetate (↑)	128.4	3.1	0.120	0.13
4	ecosystem + acetate (↑)	129.7	4.4	0.062	-
5	ecosystem + time period + sulfate (↓)	137.7	12.4	0.001	-
6	ecosystem + sulfate (↓)	139.2	13.9	0.001	-
7	time period + sulfate (↓)	140.2	14.9	0	-
8	sulfate (↓)	140.9	15.6	0	-
9	time period + acetate (↑) + sulfate (↓)	141.4	16.2	0	-
10	acetate (↑) + sulfate (↓)	141.4	16.2	0	-
11	ecosystem + time period	142.9	17.6	0	-
12	ecosystem	144.2	19.0	0	-
13	null	158.2	33.9	0	-

Table 4. Generalized linear models (GLMs) wherein ΔCH_4 production rate (treatment minus control) is the response variable and the macrophyte species added (buckbean, horsetail, lily, or marestail), porewater acetate availability, and total sulfate availability are potential factors. Positive (↑) or negative effects (↓) of continuous factors are indicated. Models are ranked in order of the lowest Akaike information criterion corrected for low samples sizes (AIC_c) along with delta AIC_c (Δ_i) and Akaike weights (ω_i) before and after model averaging (MA). The null model (intercept only) was not included in the model averaging, and the three models with a larger AIC_c than the null model are not presented.

Model #	GLM	AIC_c	Δ_i	ω_i	$\omega_{i\ (MA)}$
1	acetate (↓)	286.4	0.0	0.429	0.52
2	acetate (↓) + species	288.2	1.8	0.178	0.22
3	acetate (↓) + sulfate (↑)	288.9	2.5	0.121	0.15
4	species	289.4	3.0	0.096	0.12
5	null	290.0	3.6	0.072	-

Figure Captions

Figure 1. Conceptual diagram illustrating the potential effects of warming, sea-level rise, and increased organic matter (OM) availability on CH_4 production in coastal wetlands. These three global change mechanisms are all indirect consequences of rising CO_2 levels.

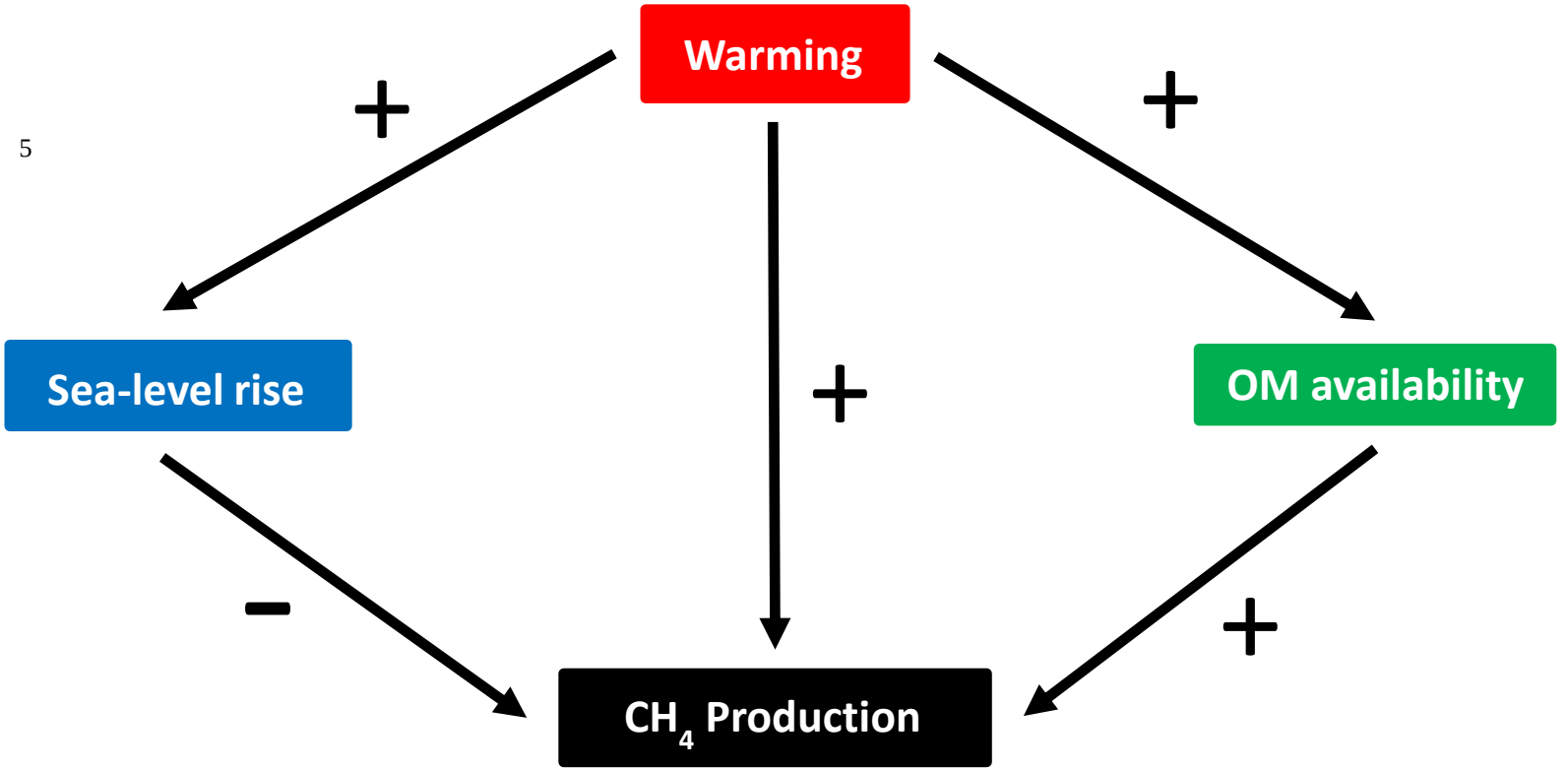
Figure 2. Aerial photo of the Copper River Delta taken by the USDA Forest Service depicting the major wetland ecosystem types extending from glaciers to ocean.

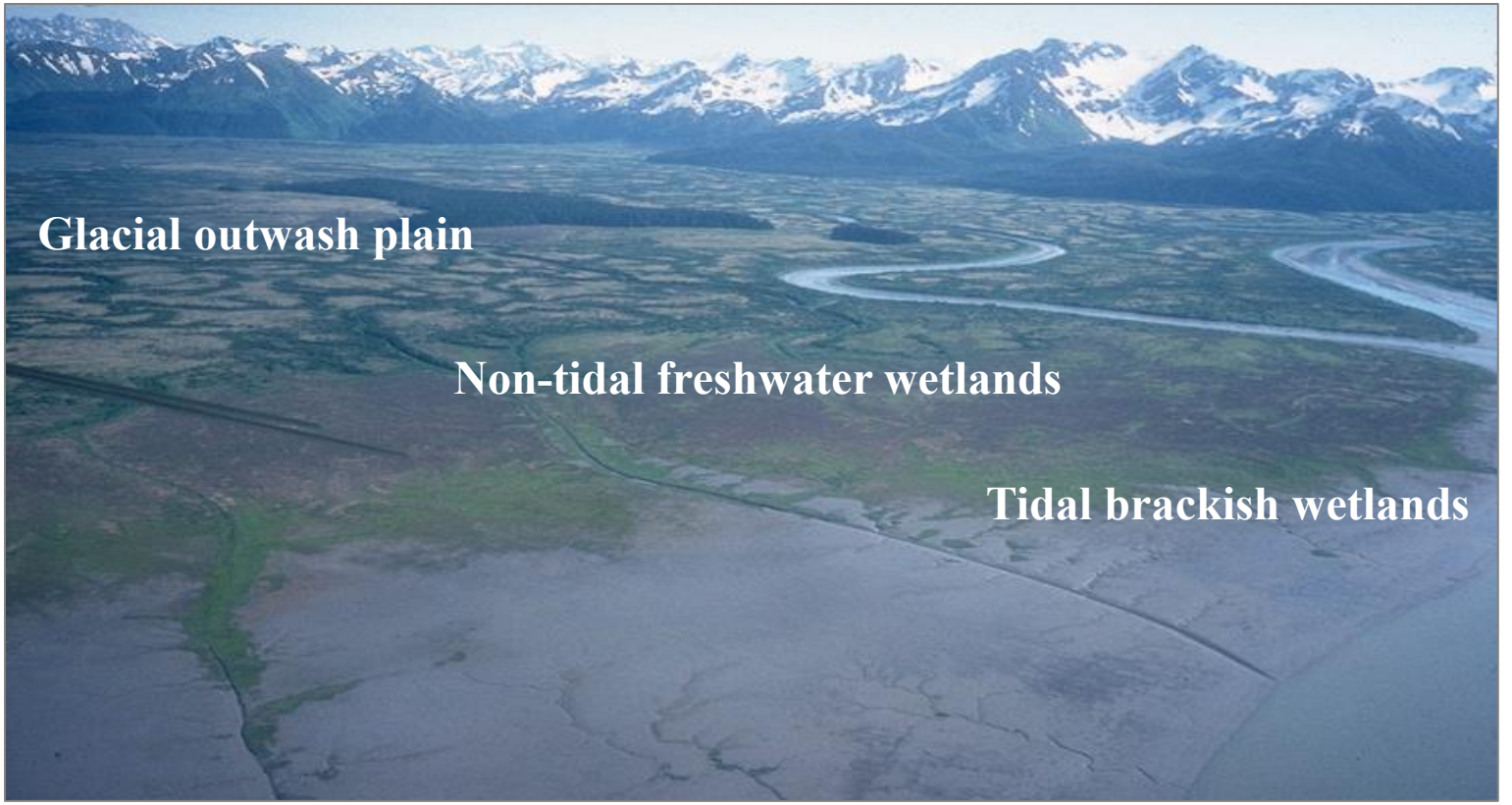
Figure 3. Mean CH_4 production rates (nmol g^{-1} of dry sediment day^{-1}) from Copper River Delta non-tidal freshwater (n = 9) and tidal brackish (n = 5) wetlands during A) June and B) August, 2014. Error bars represent standard errors.

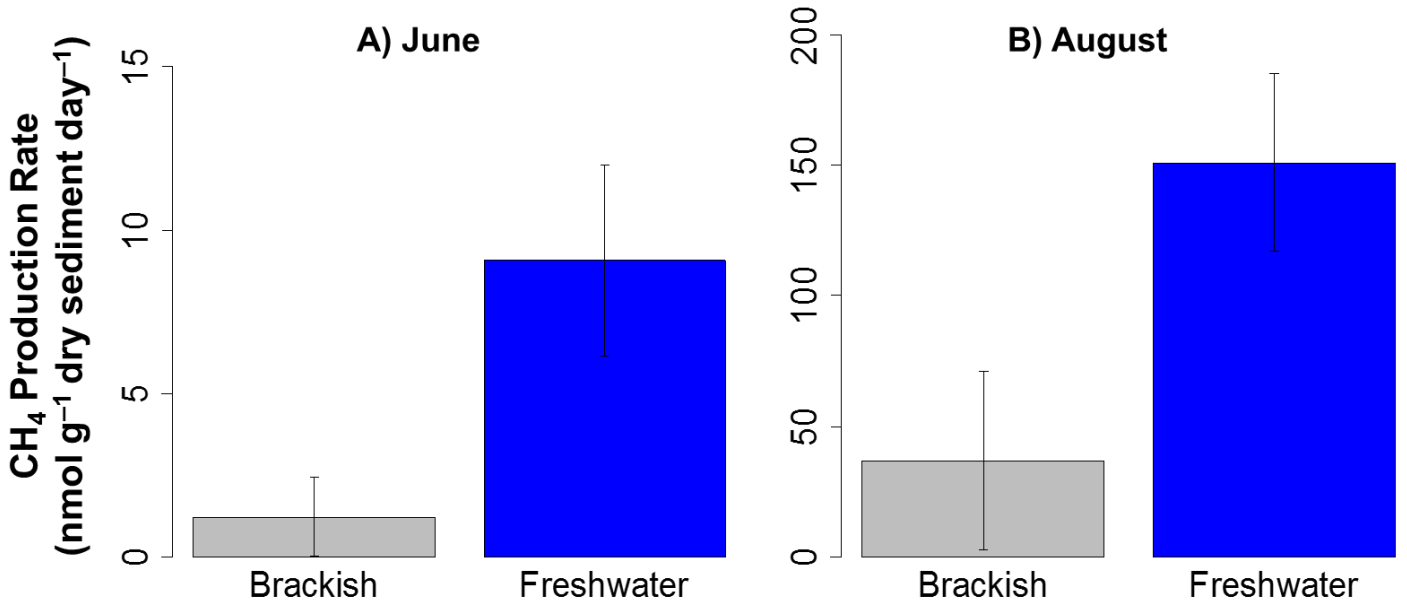
Figure 4. Mean CH_4 production rates (nmol g^{-1} of dry sediment day^{-1}) from non-tidal freshwater wetland sediments incubated with freshwater (FW/FW; n = 5) and other sediments from the same freshwater wetlands incubated with brackish water from tidal brackish wetlands (FW/BR; n = 5). Error bars represent standard errors. This sea-level rise simulation was conducted over a 14-day period in June 2014.

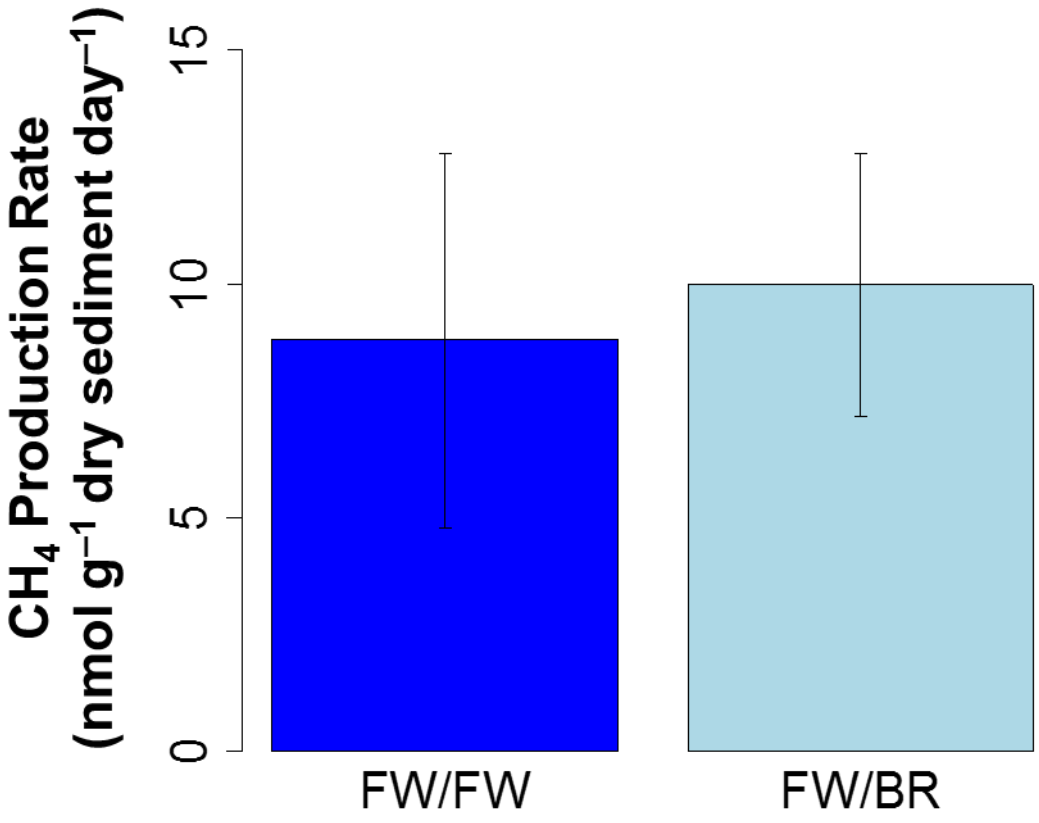
Figure 5. Mean CH_4 production rates (nmol g^{-1} of dry sediment day^{-1}) from organic matter treatments (CTL = control, BB = buckbean *Menyanthes trifoliata*, HT = horsetail *Equisetum variegatum*, LI = lily *Nuphar polysepalum*, and MT = marestail *Hippuris vulgaris*) replicated in five non-tidal freshwater wetlands during August 2014. Error bars represent standard error.

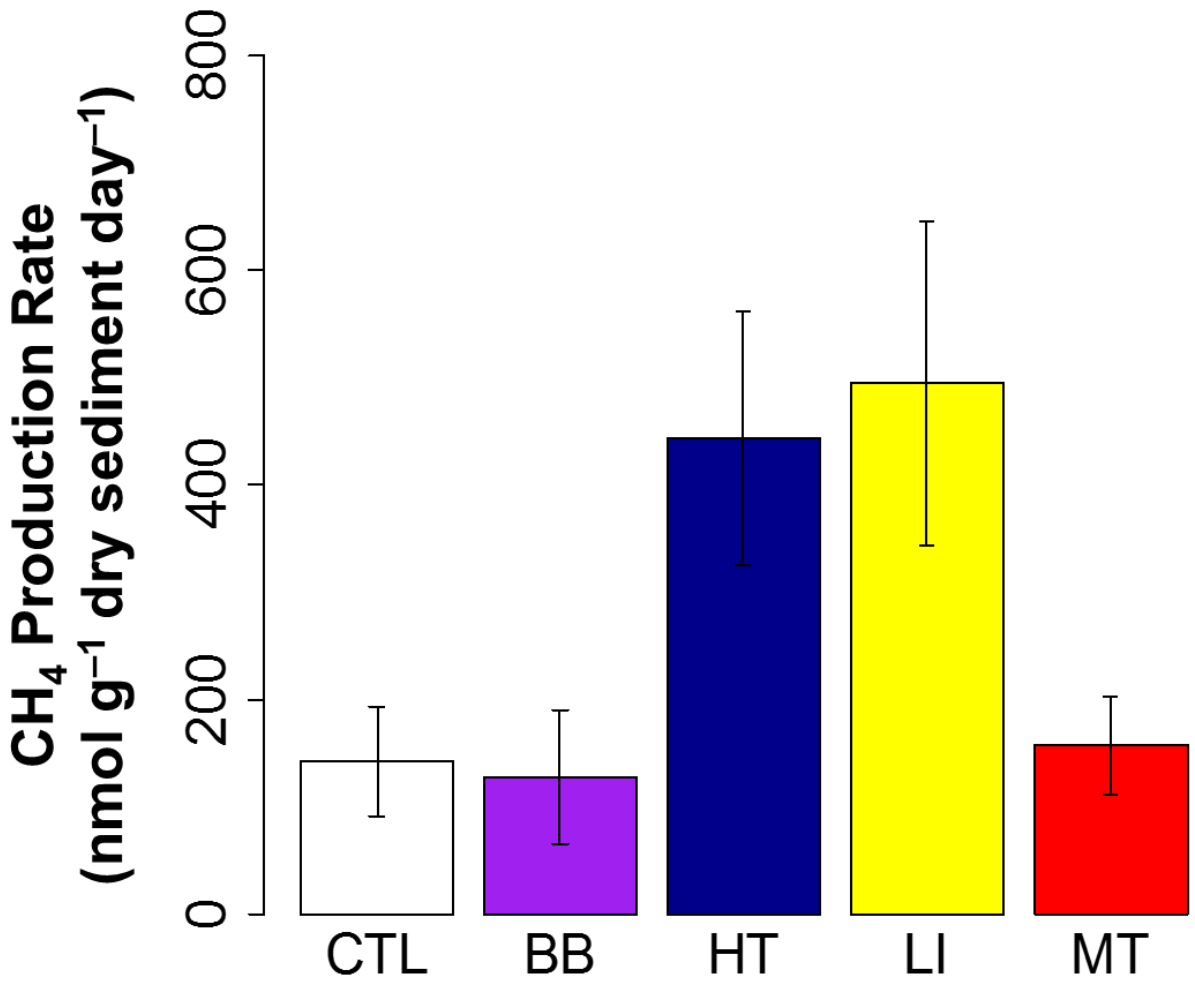
Figure 6. Actual response of ΔCH_4 production (treatment–control; nmol g^{-1} of dry sediment day^{-1}) plotted against the predicted response from model-averaged parameter estimates of the macrophyte species added (BB= buckbean *Menyanthes trifoliata*, HT = horsetail *Equisetum variegatum*, LI = lily *Nuphar polysepalum*, and MT = marestail *Hippuris vulgaris*), porewater acetate availability, and total sulfate availability. The dashed black line depicts the 1:1 line, and above the gray dotted line marks the point at which adding organic matter increased CH_4 production (or ΔCH_4 production > 0). The solid black line is the best-fit line between the actual and the predicted responses ($y = 0.95x - 86$; r^2 of 0.59), which demonstrates that although the model did a decent job of predicting relative changes in the response, it tended to underestimate ΔCH_4 production.

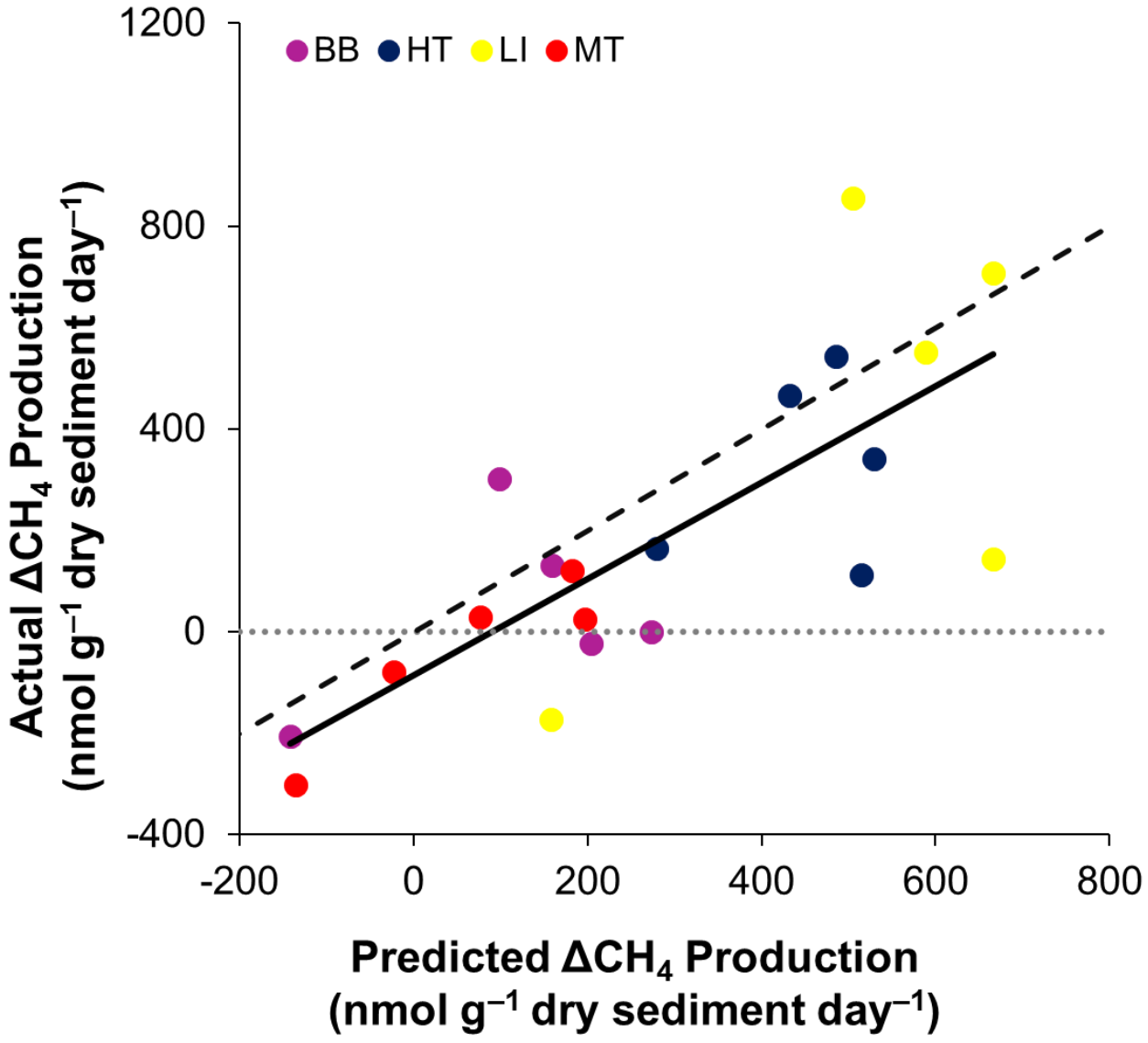

Warming
+
+
Sea-level rise
+
OM availability
−
+
CH4 Production


Glacial outwash plain
Non-tidal freshwater wetlands
Tidal brackish wetlands


A) June
B) August
CH_4 Production Rate
(nmol g^{-1} dry sediment day^{-1})
0
5
10
15
50
100
150
200
Brackish
Freshwater
Brackish
Freshwater


CH_4 Production Rate
(nmol g^{-1} dry sediment day^{-1})
0
5
10
15
FW/FW
FW/BR


CH_4 Production Rate
(nmol g^{-1} dry sediment day^{-1})
0
200
400
600
800
CTL
BB
HT
LI
MT


BB
HT
LI
MT
1200
800
400
0
-400
-200
0
200
400
600
800
Actual ΔCH4 Production
(nmol g−1 dry sediment day−1)
Predicted ΔCH4 Production
(nmol g−1 dry sediment day−1)