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 (CH4) emissions to the atmosphere, which 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 CH4 budget. Our study examined CH4 production along a natural salinity gradient in fully inundated coastal Alaska wetlands. In the laboratory, we incubated natural sediments to compare CH4 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 seawater intrusion and enhanced organic matter availability, which we predicted would have contrasting effects on coastal wetland CH4 production. Tidal brackish wetlands produced less CH4 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. Seawater addition experiments with freshwater sediments, however, did not reduce CH4 production, perhaps because the 14-day incubation period was too short to elicit a shift in microbial communities. In contrast, increased organic matter enhanced CH4 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 CH4 production in coastal wetlands, and therefore their overall contribution to the global CH4 cycle, will be sensitive to increased organic matter availability and potentially seawater intrusion. To better predict future wetland contributions to the global CH4 budget, future studies and modeling efforts should investigate how multiple global change mechanisms will interact to impact CH4 dynamics.

1 Introduction

Wetlands contribute about 60% of all natural methane (CH4) emissions to the atmosphere (Kirschke et al., 2013). As global temperatures continue to increase, some models predict that wetland CH4 emissions will double by 2100 (Gedney et al., 2004). Because CH4 is a potent greenhouse gas, whose radiative forcing continues even after its oxidation to CO2 (Neubauer and Megonigal, 2015), higher wetland emissions could trigger a positive feedback loop that further increases temperatures and CH4 release. Higher future CO2 levels could result in further warming, an extended growing season (Walther et al., 2002), and CO2 fertilization of photosynthetic plants (Matthews, 2007; Ringeval et al., 2011). If the resulting increases in plant productivity provide additional organic matter to fuel additional CH4 production, this effect could shift the wetland greenhouse gas emission baseline. Predicting the response of these ecosystems to global change is challenging because we do not fully understand the sensitivity of the CH4 cycle to enhanced productivity of wetland plants (McGuire et al., 2009; Ringeval et al., 2011).

Any global change element that directly alters the availability of electron donors or electron acceptors could change CH4 production rates and baseline emissions, thereby exacerbating or mitigating the radiative forcing of climate. Methanogens generally use substrates provided by the fermentation of organic matter as electron donors, producing
CH$_4$ via two pathways: (1) acetoclastic methanogenesis, where acetate is electron donor and acceptor, 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, assuming competition for the same electron donor, methanogens can be outcompeted for these substrates because carbon as CH$_3$ or CO$_2$ is not an energetically favorable electron acceptor in comparison to those used by other microbes (e.g., NO$_3^-$, SO$_4^{2-}$). The presence of alternative electron acceptors can indicate 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 allow for depletion of alternative electron acceptors (Achtnich et al., 1995). Both the availability of electron donors and acceptors will therefore play an important role in determining the effects of global change on CH$_4$ production.

Accurately forecasting the effects of sea-level rise and increased organic matter on coastal wetland greenhouse gas budgets requires a process-level understanding of responses to potential changes in electron acceptors and donors (Fig. 1). 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 (Memonigal and Schlesinger, 1997; Vann and Memonigal, 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 seawater intrusion 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 (US 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 Rahmsdorf, 2009) will be exacerbated along the south central Alaskan coast where tectonic subsidence is prominent (Freymueller et al., 2008). For example, the Copper River delta, which is subsid-T -OM availability

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.

2 Materials and methods

2.1 Study area

The Copper River in south central Alaska is the eighth largest river in the United States (US 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 relatively 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 el-

<table>
<thead>
<tr>
<th>CH$_4$ production</th>
<th>Warming</th>
<th>Sea-level rise</th>
<th>OM availability</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

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we observed only sites dominated by Carex spp.). Because we observed temporal fluctuations in salinity with a YSI Pro Plus multiparameter water quality meter indicative of tidal influence, we collected 1 L of water and one sediment sample at five different sites along a salinity gradient. Although sediment and water from tidal brackish wetland sites were collected in one continuous wetland complex, they were considered separately in analyses due to large differences in salinity.

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 (Tables 1 and 2). Because non-tidal freshwater wetland ponds had distinct boundaries and extensive habitat heterogeneity within each wetland (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 rate from each system could be accurately assessed. In contrast, the tidal brackish wetland complex was continuous, lacking distinct boundaries, and generally exhibited less habitat heterogeneity than the non-tidal freshwater wetlands (i.e., we observed only sites dominated by Carex spp.). Because we observed temporal fluctuations in salinity with a YSI Pro Plus multiparameter water quality meter indicative of tidal influence, we collected 1 L of water and one sediment sample at five different sites along a salinity gradient. Although sediment and water from tidal brackish wetland sites were collected in one continuous wetland complex, they were considered separately in analyses due to large differences in salinity.

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). To capture the greater habitat heterogeneity of the non-tidal freshwater wetlands, we conducted five CH$_4$ production assays for each wetland (5 sediment samples × 9 wetlands × 2 time periods = 90 total incubations). We therefore characterized the non-tidal freshwater wetlands to a greater spatial extent than the brackish tidal wetlands where we conducted 10 total incubations (5 sites along a salinity gradient within the continuous tidal brackish wetlands complex × 2 time periods). To account for this difference in spatial sampling, we then 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 Seawater addition experiment

To assess the effects of seawater addition 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 × 4 treatments). This paired design acted as a control for “within wetland” sediment heterogeneity to better capture the response of the methanogens to adding organic matter, or ΔCH$_4$ production (treatment minus control). Our four organic matter treatments were based upon the dominant aquatic macrophyte species on the CRD –
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. For the freshwater wetlands, physicochemical parameters are from spot measurements of the hypolimnion conducted throughout summer 2014 (n = 4 per freshwater wetland), whereas DOC and sulfate were measured in the surface layer in June and August 2014 (5 sites × 2 time periods, n = 10 per wetland). For the brackish wetlands, physicochemical parameters are from one spot measurement of the surface layer. Tidal brackish wetlands A–E were sampled in June and F–J were sampled in August.

<table>
<thead>
<tr>
<th>Wetland</th>
<th>Elevation (m)</th>
<th>Depth (m)</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>SpC (µS cm⁻¹)</th>
<th>Salinity (ppt)</th>
<th>DOC (mg L⁻¹)</th>
<th>Sulfate (µM)</th>
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<tr>
<td>Eyak N</td>
<td>5.2</td>
<td>0.60±0.09</td>
<td>15.3±0.9</td>
<td>5.5±0.4</td>
<td>13±3</td>
<td>0.01±0.01</td>
<td>6.5±1.9</td>
<td>1.6±0.3</td>
</tr>
<tr>
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<td>5.5</td>
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<td>16.1±1.3</td>
<td>7.0±1.1</td>
<td>11±2</td>
<td>0.00±0.01</td>
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<td>2.0±0.2</td>
</tr>
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<td>Lily</td>
<td>8.2</td>
<td>0.65±0.04</td>
<td>13.1±0.8</td>
<td>5.9±0.2</td>
<td>60±19</td>
<td>0.03±0.01</td>
<td>3.5±1.0</td>
<td>6.0±2.2</td>
</tr>
<tr>
<td>Rich Hate Me</td>
<td>18.3</td>
<td>0.57±0.15</td>
<td>11.6±2.9</td>
<td>6.1±0.4</td>
<td>56±7</td>
<td>0.03±0.01</td>
<td>2.1±0.5</td>
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<td>14.2±0.9</td>
<td>6.3±0.3</td>
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<td>74±11</td>
<td>0.04±0.01</td>
<td>11±0.6</td>
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<tr>
<td>Storey S</td>
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<td>0.60±0.04</td>
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<td>7.3±0.7</td>
<td>70±6</td>
<td>0.03±0.00</td>
<td>4.2±0.3</td>
<td>7.9±0.5</td>
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<td>0.66±0.03</td>
<td>16.6±1.1</td>
<td>6.0±0.5</td>
<td>13±3</td>
<td>0.01±0.01</td>
<td>6.7±0.7</td>
<td>1.8±0.2</td>
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<td>Tiedeman S</td>
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<td>0.73±0.03</td>
<td>15.4±1.4</td>
<td>6.7±0.6</td>
<td>8.8±1.7</td>
<td>0.00±0.00</td>
<td>5.2±0.6</td>
<td>1.7±0.3</td>
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<td>Brackish A</td>
<td>2.4</td>
<td>0.22</td>
<td>14.9</td>
<td>6.9</td>
<td>190</td>
<td>0.09</td>
<td>0.79</td>
<td>130</td>
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<td>Brackish B</td>
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<td>0.30</td>
<td>17.6</td>
<td>7.6</td>
<td>19000</td>
<td>11</td>
<td>0.97</td>
<td>8100</td>
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<tr>
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<td>0.38</td>
<td>17.4</td>
<td>7.8</td>
<td>20000</td>
<td>12</td>
<td>1.2</td>
<td>8900</td>
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<td>Brackish D</td>
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<td>0.49</td>
<td>16.0</td>
<td>7.6</td>
<td>570</td>
<td>0.28</td>
<td>0.68</td>
<td>37</td>
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<td>Brackish E</td>
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<td>0.35</td>
<td>15.3</td>
<td>7.6</td>
<td>2100</td>
<td>1.1</td>
<td>0.67</td>
<td>1100</td>
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<td>Brackish F</td>
<td>0.6</td>
<td>0.73</td>
<td>11.3</td>
<td>6.4</td>
<td>1500</td>
<td>0.78</td>
<td>0.88</td>
<td>250</td>
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<td>Brackish G</td>
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<td>0.78</td>
<td>12.2</td>
<td>6.8</td>
<td>160</td>
<td>0.07</td>
<td>0.78</td>
<td>11</td>
</tr>
<tr>
<td>Brackish H</td>
<td>2.7</td>
<td>0.65</td>
<td>12.9</td>
<td>7.5</td>
<td>13000</td>
<td>7.7</td>
<td>1.7</td>
<td>6000</td>
</tr>
<tr>
<td>Brackish I</td>
<td>0.3</td>
<td>0.80</td>
<td>12.2</td>
<td>7.5</td>
<td>24000</td>
<td>15</td>
<td>2.2</td>
<td>8300</td>
</tr>
<tr>
<td>Brackish J</td>
<td>2.7</td>
<td>0.89</td>
<td>13.1</td>
<td>7.6</td>
<td>3800</td>
<td>2.0</td>
<td>21</td>
<td>930</td>
</tr>
</tbody>
</table>

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 nmol C g⁻¹ 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). All vegetation for each species was collected from the same plant individual to ensure minimal difference in quality within each treatment. 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 Tiesg et al. (2013); (1) horsetail had the lowest carbon content at 38%, while the other three species contain approximately 44–47% C; (2) lily tissue had the highest % N (2.5) and % P (0.24) followed by marestail with 1.7% N and 0.17% P; and (3) buckbean had 0.94% N and 0.15% P and horsetail had 1.1% N and 0.11% P.

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. To remove oxygen introduced to the inundated sediments during sample collection and slurry making, each bottle was made anaerobic by purging it with N₂ gas for 5 min. 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 CH₄ production potential were considered conservative. However, we do acknowledge that CH₄ production potentials generated by bottle incubations may not exactly reproduce CH₄ 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 3 months until the samples could be analyzed using gas chromatography. To maintain atmospheric pressure in the slurry incubations, 10 mL of N₂ gas was added after each sampling point. CH₄ 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, CH₄ production rates were inferred from the slope of the linear regressions of CH₄ concentrations over time and are reported as nmol CH₄ per gram of dry sediment per day (nmol g⁻¹ day⁻¹).
### Table 2. Sediment chemical characteristics (mean ± SD) of the wetlands 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. For the freshwater wetlands, measurements were conducted in June and August 2014 (5 sites × 2 time periods, n = 10 per wetland). For the brackish wetlands, sediment chemistry is from one measurement only. Tidal brackish wetlands A–E were sampled in June and F–J were sampled in August. 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. Porewater nitrate concentrations were extremely low with many below what we considered our detection limit (i.e., <2 µM).

<table>
<thead>
<tr>
<th>Wetland</th>
<th>SOM (%)</th>
<th>Total sediment C (nmol g⁻¹)</th>
<th>PW acetate (µM)</th>
<th>PW nitrate (nmol g⁻¹)</th>
<th>PW sulfate (µM)</th>
<th>Total sulfate (nmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyak N</td>
<td>2.0 ± 0.5</td>
<td>0.81 ± 0.22</td>
<td>57 ± 57</td>
<td>360 ± 350</td>
<td>1.2 ± 0.7</td>
<td>9.6 ± 9.9</td>
</tr>
<tr>
<td>Eyak S</td>
<td>1.8 ± 0.5</td>
<td>0.71 ± 0.21</td>
<td>18 ± 13</td>
<td>120 ± 82</td>
<td>1.4 ± 1.0</td>
<td>4.3 ± 3.1</td>
</tr>
<tr>
<td>Lily</td>
<td>2.1 ± 0.5</td>
<td>0.86 ± 0.21</td>
<td>58 ± 43</td>
<td>620 ± 560</td>
<td>0.86 ± 0.26</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Rich Hate Me</td>
<td>3.1 ± 3.9</td>
<td>1.3 ± 1.6</td>
<td>29 ± 44</td>
<td>110 ± 140</td>
<td>3.7 ± 4.9</td>
<td>2.2 ± 4.2</td>
</tr>
<tr>
<td>Scott S</td>
<td>1.5 ± 2.2</td>
<td>0.60 ± 0.89</td>
<td>31 ± 34</td>
<td>300 ± 340</td>
<td>2.2 ± 2.4</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>Storey N</td>
<td>1.8 ± 0.2</td>
<td>0.73 ± 0.09</td>
<td>10 ± 5</td>
<td>120 ± 64</td>
<td>0.92 ± 0.39</td>
<td>1.2 ± 0.8</td>
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<tr>
<td>Storey S</td>
<td>1.9 ± 3.1</td>
<td>0.76 ± 1.2</td>
<td>15 ± 16</td>
<td>160 ± 120</td>
<td>0.58 ± 0.39</td>
<td>2.8 ± 3.1</td>
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<td>Tiedeman N</td>
<td>2.8 ± 2.9</td>
<td>1.1 ± 1.2</td>
<td>25 ± 14</td>
<td>190 ± 95</td>
<td>1.7 ± 1.2</td>
<td>3.7 ± 3.8</td>
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<tr>
<td>Tiedeman S</td>
<td>2.3 ± 0.8</td>
<td>0.93 ± 0.32</td>
<td>17 ± 7</td>
<td>120 ± 45</td>
<td>2.1 ± 1.9</td>
<td>4.3 ± 3.4</td>
</tr>
</tbody>
</table>

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⁻¹); 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 CH₄ 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 h at 60°C, and the dry weight was recorded. Subsequently, the organic matter in the sediment was combusted at 500°C for 4 h, and the sediment was re-wetted and then dried at 60°C for at least 48 h before re-weighing (Steinman et al., 2007). 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 gram of dry sediment (Table 2). To extract porewater from the sediment, another portion (~50 mL) was centrifuged for 45 min at 4°C at ~4000 RCF (relative centrifugal force). 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 extracted from sediment (0.17 ± 0.09 mL porewater per milliliter of sediment), porewater concentrations were converted to the total amount of each anion (nmol) per gram of dry sediment (i.e., µM X porewater volume in incubation X porewater volume per milliliter of sediment X sediment volume in bottle/mass of dry sediment X 1000; Table 2).

2.3.4 Microbial analyses

According to the manufacturer’s protocol with a PowerSoil DNA (deoxyribonucleic acid) isolation kit (Mo Bio, Carlsbad, CA, USA), DNA was extracted from frozen sediments used in other analyses, including multiple June tidal brack-
ish sediments \((n = 10)\), the freshwater sediments used in the seawater intrusion simulation \((n = 5)\), and a composite of the five sediment samples \((1 \text{ g of sediment per sample was added to make a } 5 \text{ g composite})\) from the nine freshwater wetlands for the June time period \((n = 9)\). 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 polymerase chain reaction (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 in 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\(\mu\)L qPCR reaction in a Mastercycler ep realplex\(^2\) gradient S (Eppendorf, Hamburg, Germany), using SYBR Green as the reporter dye. Each reaction contained 1\(\mu\)L 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°C for 20 s. 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 the 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 10-fold dilutions of these genes to generate standard curves for qPCR. Our detection limit for each gene was approximately 1000 copies per gram of wet sediment. Samples below detection were assigned a value of 999 copies per gram 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 \((n = 18, 9 \text{ sites } \times 2 \text{ time periods})\) and tidal brackish wetland comparison \((n = 10, 5 \text{ sites } \times 2 \text{ time periods})\), we analyzed how four factors influenced log-transformed CH\(_4\) production rates using generalized linear models (GLMs) 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 AIC\(_c\) value is considered the most likely, and all remaining models are compared relative to the most likely model using delta AIC\(_c\) (\(\Delta_i\)). Models with a \(\Delta_i\) less than or equal to 2 are considered to have substantial support, while models having a \(\Delta_i\) greater than 7 have little support (Burnham and Anderson, 2002). The relative strength of our candidate models was then evaluated with Akaike weights \(\omega_i\), 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 \(\omega_i\), which indicate the probability of a model including the 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, \(\alpha\) was set at 0.05.
For the seawater addition experiment, we conducted a paired t test to determine whether CH$_4$ production rates in non-tidal freshwater wetland sediments were affected by being incubated anaerobically with brackish tidal 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 $\Delta$CH$_4$ production (treatment minus 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 (from Tiegs et al., 2013) against $\Delta$CH$_4$ 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 by ecosystem type (Tables 1 and 2), and variation by ecosystem type tended to be greater than temporal variation. Total sulfate levels in non-tidal freshwater incubations (June: 84 ± 65; August: 48 ± 43 nmol g$^{-1}$ dry sediment; mean ± SD) were about 2 orders of magnitude lower than in tidal brackish incubations (June: 4300 ± 4300; August: 3500 ± 3700 nmol g$^{-1}$ 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 g$^{-1}$ dry sediment) having relatively higher nitrate than the tidal brackish wetlands (June: 0.24 ± 0.51; August: 0.0092 ± 0.0025 nmol g$^{-1}$ dry sediment; Table 2). The total amount of acetate available in the non-tidal freshwater wetland incubations was similar in June (28 ± 22 nmol g$^{-1}$ dry sediment) and August (30 ± 17 nmol g$^{-1}$ dry sediment), while levels in the tidal brackish wetland incubations were generally higher and more variable especially in August (210 ± 260 nmol g$^{-1}$ dry sediment) than in June (130 ± 80 nmol g$^{-1}$ 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 was positively associated with higher CH$_4$ production rates, while higher total sulfate availability was associated with lower 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, whereas non-tidal freshwater sediments were characterized by higher numbers of methanogens. In the tidal brackish wetlands, 3 out of 10 samples were below the detection limit for the dsrA gene, our proxy for sulfate-reducing bacteria abundance, but we detected this gene in all 9 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 × 10$^3$ and 5.20 ± 5.08 × 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 2 out of 10 tidal brackish samples, but in all 9 non-tidal freshwater wetland samples. The presence or absence of the mcrA gene was dependent on ecosystem
Table 3. Generalized linear models (GLMs) wherein log-transformed CH$_4$ production rate is the response variable and ecosystem type (non-tidal 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$_C$ ($\Delta_i$) and Akaike weights (\(\omega_i\)) before and after model averaging (MA). Models with a $\Delta_i$ 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.

<table>
<thead>
<tr>
<th>Model no.</th>
<th>GLM</th>
<th>AIC$_C$</th>
<th>$\Delta_i$</th>
<th>$\omega_i$</th>
<th>$\omega_i$(MA)</th>
</tr>
</thead>
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<tr>
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<td>ecosystem + time period + acetate (↑) + sulfate (↓)</td>
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</tr>
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<td>4.4</td>
<td>0.062</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>ecosystem + time period + sulfate (↓)</td>
<td>137.7</td>
<td>12.4</td>
<td>0.001</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>ecosystem + sulfate (↓)</td>
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<td>13.9</td>
<td>0.001</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>time period + sulfate (↓)</td>
<td>140.2</td>
<td>14.9</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>sulfate (↓)</td>
<td>140.9</td>
<td>15.6</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>time period + acetate (↑) + sulfate (↓)</td>
<td>141.4</td>
<td>16.2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>acetate (↑) + sulfate (↓)</td>
<td>141.4</td>
<td>16.2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>ecosystem + time period</td>
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<td>17.6</td>
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<td>12</td>
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<td>19.0</td>
<td>0</td>
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<tr>
<td>13</td>
<td>null</td>
<td>158.2</td>
<td>33.9</td>
<td>0</td>
<td>-</td>
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</tbody>
</table>

type ($\chi^2 = 12.44$, df = 1, $P = 0.0004$). Tidal brackish samples had $2.14 \pm 5.78 \times 10^4$ copies of the mcrA per gram of wet sediment, whereas non-tidal freshwater wetlands had $1.84 \pm 1.25 \times 10^5$ 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 Seawater addition experiment

Incubating non-tidal freshwater wetland soils with brackish water did not affect CH$_4$ production rates (Fig. 4). Even though total sulfate levels increased from $63 \pm 37$ to $5400 \pm 400$ nmol g$^{-1}$ 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 seawater addition experiment ($n = 5$) had about an order of magnitude higher number of copies of mcrA ($3.12 \pm 4.40 \times 10^3$) than dsrA ($5.32 \pm 6.33 \times 10^3$) 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, whereas the lily and horsetail treatments generally increased methanogen activity (Fig. 5). The most likely model for predicting $\Delta$CH$_4$ production (treatment minus 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,
Table 4. Generalized linear models (GLMs) wherein ΔCH\textsubscript{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\textsubscript{c}) along with delta AIC\textsubscript{c} (Δ\textsubscript{i}) and Akaike weights (ω\textsubscript{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\textsubscript{c} than the null model are not presented.

<table>
<thead>
<tr>
<th>Model no.</th>
<th>GLM</th>
<th>AIC\textsubscript{c}</th>
<th>Δ\textsubscript{i}</th>
<th>ω\textsubscript{i} (MA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>acetate (↓)</td>
<td>286.4</td>
<td>0.0</td>
<td>0.429</td>
</tr>
<tr>
<td>2</td>
<td>acetate (↓) + species</td>
<td>288.2</td>
<td>1.8</td>
<td>0.178</td>
</tr>
<tr>
<td>3</td>
<td>acetate (↓) + sulfate (↑)</td>
<td>288.9</td>
<td>2.5</td>
<td>0.121</td>
</tr>
<tr>
<td>4</td>
<td>species</td>
<td>289.4</td>
<td>3.0</td>
<td>0.096</td>
</tr>
<tr>
<td>5</td>
<td>null</td>
<td>290.0</td>
<td>3.6</td>
<td>0.072</td>
</tr>
</tbody>
</table>

Figure 5. Mean CH\textsubscript{4} production rates (nmol g\textsuperscript{-1} dry sediment day\textsuperscript{-1}) from organic matter treatments (CTL is control, BB is buckbean Menyanthes trifoliata, HT is horsetail Equisetum variegatum, LI is lily Nuphar polysepalum, and MT is marestail Hippuris vulgaris) replicated in five non-tidal freshwater wetlands during August 2014. Error bars represent standard error.

4 Discussion

To begin to understand likely responses of wetlands to global change processes, we conducted a space-for-time substitution of how seawater intrusion might affect CH\textsubscript{4} production in freshwater wetlands by comparing them to brackish systems. We found that CH\textsubscript{4} production was lower in tidal brackish than in non-tidal freshwater wetlands, likely due to differences in availability of alternative electron acceptors (i.e., higher sulfate levels in the tidal brackish) and in microbial communities (i.e., lower methanogen abundances in the tidal brackish). Experimental addition of seawater in non-tidal freshwater sediments (∼14 days), however, did not influence CH\textsubscript{4} production rates. In contrast, higher organic matter availability enhanced CH\textsubscript{4} production rates in 75% of incubations, but this response depended on the amount of substrate already available and the macrophyte species added, with half of the species treatments having no significant effect. Because acetate and sulfate availability had contrasting effects depending on the experiment (i.e., freshwater/brackish comparison vs. increased organic matter), these results indicate that we do not have a sufficient mechanistic understanding of how changes in electron donors and electron acceptors will interact to ultimately influence CH\textsubscript{4} production. Future studies should consider the possible in-
teraction of global change mechanisms, such as sea-level rise and CO$_2$ fertilization/longer growing seasons, which will likely alter the availability of electron acceptors and electron donors, thereby influencing CH$_4$ production (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; Pofffenbarger 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 indicates that methanogens have to compete for organic substrates with sulfate-reducing bacteria (Oremoland and Polcin, 1982; Lovley and Klug, 1986; Achnich 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 coprecipitate with phosphate, and ammonium is released, 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 alternative electron acceptors 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 (hydrogenotrophic) 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 related 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) availability of alternative electron acceptors including sulfate (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 aceticlastic pathway of methanogenesis correspondingly increased such that acetate availability appeared to be similar during these 2 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 the availability of electron donors and microbial processing rates (e.g., Eviner and Chapin, 2003). We think the following seasonal trajectory is possible: (1) in early growing season, CH$_4$ production is low, but steeply increases at peak growing season as more labile plant exudates are produced, and (2) 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 Seawater addition experiment

Despite our finding that CH$_4$ production rates were significantly lower in tidal brackish wetlands sites, adding seawater to non-tidal freshwater sediments surprisingly did not affect CH$_4$ production rates. We acknowledge that our experiment simulated short-term consequences of seawater intrusion, 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 seawater addition experiments have observed a decrease in CH$_4$ production rates with elevated salinity (DeLaune et al., 1983; Marton et al., 2012; Chambers et al., 2011, 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 $\sim 10$ mM sulfate, which is higher than the sulfate concentration ($\sim 6$ mM) used in this study. Chambers et al. (2011) observed a reduction in the treatments where sulfate concentrations were about 130 and 320 $\mu$mol g$^{-1}$ of dry sediment, which is over 1 order of magnitude larger than our seawater addition experiment (5 $\mu$mol g$^{-1}$ dry sediment). Additionally, the majority of all these experiments were conducted at 25–30 $^\circ$C, or almost double the temperature used in this study (14 $^\circ$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 seawater addition experiment, can influence the results.

In addition to environmental conditions, initial factors, such as soil characteristics or site properties, may mediate how methanogens respond to seawater addition experiments (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 microbial communities therefore could have been more likely to respond with higher rates of sulfate reduction, thereby increasing 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 seawater intrusion increases both sulfate and organic matter availability (Weston et al., 2011).

Seawater intrusion could therefore affect both availability of alternative electron acceptors and organic matter, 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 CH₄ production in freshwater sediments did not respond to the seawater addition experiment 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 CH₄ production rates, but this effect varied with the species of macrophyte added to the incubations. Differences in litter quality are known to influence methanogen communities and CH₄ production (Yavitt et al., 1990, 2000; Valentine et al., 1994). For example, West et al. (2012) found that adding algal carbon significantly enhanced CH₄ 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 (Schliekeisen et al., 2003). For example, Tiegts et al. (2013) found that terrestrial plants decomposed more slowly than aquatic macrophytes in CRD wetlands. Additionally, Tiegts 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 (Tiegts et al., 2013). However, our CH₄ production response did not follow the decomposition pattern documented by Tiegts et al. (2013); we observed higher CH₄ production for the lily and horsetail treatment relative to the control, but not for buckbean and marestail. We also did not find that the CH₄ production response to organic matter treatment varied by % C, % N, % P, C : N, C : P, or any other measure of litter quality assessed by Tiegts 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 CH₄ 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 CH₄ 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 CH₄ production is enhanced by the addition of direct substrates such as acetate and H₂ (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 CH₄ production incubations (but see Valentine et al., 1994; West et al., 2012, 2015). However, two studies involving larger-scale plots with elevated CO₂ levels exhibited greater photosynthetic rates and greater CH₄ emissions (Megenigal and Schlesinger, 1997; Vann and Megenigal, 2003). Although Vann and Megenigal (2003) observed enhanced plant biomass that was strongly correlated with CH₄ emissions, Megenigal and Schlesinger (1997) did not see increased biomass and therefore hypothesized that lower transpiration rates, not increased substrate availability, led to higher CH₄ emissions by increasing flooding duration and stimulating anaerobic processes. In our study, increased substrate availability is likely the mechanism behind increased CH₄ 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 ΔCH₄ 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 or-
ganic 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., availability of alternative electron acceptors, 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 seawater intrusion and increased organic matter from longer growing 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 seawater intrusion are not limited to CH\(_4\) production alone. Salinization also reduces aerobic and anaerobic methanogenesis, 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 seawater intrusion. 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 doi:10.5063/F1028PF8 (Vizza, 2016).

Author contributions. Carmella Vizza designed the study as it developed from discussions with Stuart E. Jones. Carmella Vizza and Julia A. Hart conducted the fieldwork and laboratory analyses. William E. West played a key role in methodology and analyzing methane samples with GC (gas chromatography). Stuart E. Jones and Gary A. Lamberti played advisory roles in shaping this research. Carmella Vizza 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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