

# *Elevated CO<sub>2</sub> and high salinity enhance the abundance of sulfate reducers in a salt marsh ecosystem*

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8

9 **Authors:** Seon-Young Kim<sup>1,3</sup>, Chris Freeman<sup>2</sup>, Martin Lukac<sup>2</sup>, Seung-Hoon Lee<sup>1,4</sup>, Sean D.  
10 Kim<sup>5</sup>, Hojeong Kang<sup>1\*</sup>

11

12 **Running head:** Elevated CO<sub>2</sub> and high salinity on saltmarsh microbes

13

14 **Affiliations:**

15 <sup>1</sup> School of Civil and Environmental Engineering, Yonsei University, Seoul, 120-749, South  
16 Korea

17 <sup>2</sup> School of Natural Sciences, Bangor University, Bangor, LL57 2UW, UK

18 <sup>3</sup> Water Environment Research Department, National Institute of Environmental Research,  
19 Incheon, Korea

20 <sup>4</sup> Shine Biopharm Inc., Seoul, Korea

21 <sup>5</sup> Claremont High School, Claremont, CA91711, USA

22

23 **\*Address for Correspondence**

24 Prof. Hojeong Kang

25 School of Civil and Environmental Engineering

26 School of Engineering

27 Yonsei University

28 Seoul, 120-749, Korea

29 E-mail: hj\_kang@yonsei.ac.kr

30 Telephone: +82-2-2123-5803

31 Fax: +82-2-364-5300

32

33 **Abstract**

34 Salt marshes are anticipated to be exposed to elevated atmospheric CO<sub>2</sub> and high salinity due  
35 to sea-level rise in the future. This study aims to investigate the effects of elevated  
36 atmospheric CO<sub>2</sub> and high salinity on microbial communities using intact cores collected  
37 from a salt marsh in North Wales, UK. The cores were exposed to two levels of CO<sub>2</sub> (ambient  
38 vs. ambient + 200 ppm) and two levels of salinity (control vs. control + 10 ppt) over a  
39 growing season in the Free-Air Carbon Dioxide Enrichment (FACE) facility. We focused on  
40 the abundances of bacteria, sulfate reducers (SRB), methanogens and denitrifiers as they play  
41 a central role in greenhouse gas emissions. In addition, the activities of extracellular enzymes  
42 were determined to assess the effects on microbial activity, followed by Structural Equation  
43 Modelling (SEM) to elucidate possible mechanism for the changes we observed. Elevated  
44 CO<sub>2</sub> significantly increased DOC in pore water for the control salinity treatment during a  
45 vigorous growing season (i.e., May - Aug) but not the high salinity treatment. Microbial  
46 diversity presented by Shannon's diversity derived from T-RFLP analysis showed no  
47 significant changes except for *nirS* genes, suggesting potential influence of elevated CO<sub>2</sub> on  
48 denitrification. Microbial abundances changed substantially for certain functional groups; For  
49 example, the abundance of SRB increased both by elevated CO<sub>2</sub> and high salinity. In contrast,  
50 total bacterial abundance declined under the treatment of high salinity. SEM suggests that  
51 elevated CO<sub>2</sub> increases DOC in pore-water, which increased sulfate reducers. Salinity plays  
52 an additional role in this process to selectively increasing SRB without affecting methanogens.  
53 Overall, the results of this study suggest that SRB will play a key role in organic matter  
54 decomposition in salt marshes as atmospheric CO<sub>2</sub> and salinity increase. This is most likely to  
55 be mediated by changes in the quantity and the quality of organic carbon derived from salt  
56 marsh vegetation.

57 **Key words:** Elevated CO<sub>2</sub>; Sulfate reducers; Salinity; Salt marsh; DOC

58 **Introduction**

59

60 Salt marshes are one of the most productive ecosystems on earth and play an important role in  
61 the global biogeochemical cycle, water quality amelioration, and habitat provisioning for  
62 wildlife. Those processes are mainly mediated by microorganisms in coastal soils, which  
63 decompose organic matter by releasing enzymes and metabolize organic carbon completely to  
64 CO<sub>2</sub> or CH<sub>4</sub> depending on the types of microbes and environmental conditions (Kang et al.,  
65 1998).

66 It is expected that these ecosystems will experience substantial environmental change  
67 under future climate scenarios, which may act to undermine these key functions. The first  
68 impact relates to elevated atmospheric CO<sub>2</sub> for which concentration is expected to increase up  
69 to 1,000 ppm by the end of this century (Kiehl, 2011). Previous studies have suggested that  
70 elevated CO<sub>2</sub> can stimulate above- and belowground productivity, resulting in organic matter  
71 accumulation or stimulation of heterotrophic respiration as a result of increased plant  
72 photosynthate in salt marshes (Cherry et al. 2009; Langley et al. 2009; Erickson et al. 2013).  
73 In addition, various factors (e.g., increasing oxygen release from roots, high root turnover and  
74 rhizodeposition, temperature rise, etc.) may give rise to a negative feedback with the  
75 fertilizing effect of elevated CO<sub>2</sub>, promoting the release of soil organic carbon into the  
76 atmosphere as greenhouse gases (Wolf et al. 2007). Vegetation may also release more carbon  
77 compounds into belowground and adjacent ecosystems in the form of dissolved organic  
78 carbon (DOC), phenolics in particular, under elevated CO<sub>2</sub> (Freeman et al. 2004; Kim and  
79 Kang, 2008), leading to more rapid carbon mineralization in salt marshes due to a mechanism  
80 called the 'priming effect' (Bardgett et al. 2008).

81 Another change in the salt marsh environment is sea-level rise, for which these  
82 ecosystems will experience more frequent influxes and prolonged inundation with sea water

83 (Church et al. 2013). Numerous studies have focused on the responses of the salt marsh  
84 vegetation to high salinity in terms of growth, species composition / transition, and  
85 community structure (Munns and Tester, 2008). However, less is known about how organic  
86 matter degradation will respond to increasing salinity in these ecosystems. Salt marshes  
87 exhibit extremely high productivity and low decomposition rates, resulting in the  
88 sequestration of significant amounts of carbon (Bridgham et al. 2006). Any changes in  
89 edaphic environments (e.g., redox potential, terminal electron acceptors and donors, etc.) may  
90 accelerate or decelerate organic carbon mineralization through changes in microbial function  
91 and activity (Sutton-Grier et al. 2011). Therefore, understanding the effects of changing  
92 conditions on microbial processes and communities is crucial for determining the fate of  
93 organic matter decomposition under high salinity in salt marshes. This is of great importance  
94 not only locally but also globally, as effects of elevated CO<sub>2</sub> and water chemistry change on  
95 marsh biogeochemistry through microbial processes have been noted both in Asia and the  
96 north America (Lee et al., 2015; Lee et al., 2017b)

97         Despite potential consequences of increasing atmospheric CO<sub>2</sub> and high salinity in  
98 salt marshes, few studies have investigated the integrated effects of those environmental  
99 changes. Furthermore, most studies have focused on vegetation and organic matter content  
100 (Drake, 2014; Morrissey et al. 2014), while even less is known about the effects on microbial  
101 processes and microbial communities, with only limited data available on the effects of  
102 elevated CO<sub>2</sub> on microbial communities (Dunbar et al. 2012; Lee and Kang, 2016). Studies  
103 investigating salinity-induced changes in microbial communities have mostly been conducted  
104 along a naturally occurring salinity gradient in coastal areas where other variables present a  
105 challenge in establishing the consequence of high salinity (Bernhard et al. 2005; Henriques et  
106 al. 2006). To address this shortfall in our understanding of the fate of organic matter in salt  
107 marshes, it is essential that the integrative effects of elevated CO<sub>2</sub> and high salinity on

108 microbial processes and communities are now elucidated.

109           Among diverse microbial communities, sulfate reducing bacteria (SRB) play a crucial  
110 role in organic matter decomposition in saline environments such as salt marshes (Koretsky et  
111 al. 2005). Denitrifiers are also involved in buffering coastal N loading from terrestrial  
112 ecosystems through denitrification in salt marshes, which removes  $\text{NO}_3^-$  from the ecosystem  
113 (Davis et al. 2004; Wigand et al. 2004). Methanogenesis is another important pathway of  
114 carbon mineralization under anaerobic conditions. Although methanogenesis has been  
115 considered less important in salt marshes due to competitive advantage of SRB over  
116 methanogens (Dowrick et al. 2006), recent studies have suggested that methane emissions  
117 from coastal wetlands could be substantial (Vizza et al. 2017). The ability of those microbial  
118 communities to cope with increasing atmospheric  $\text{CO}_2$  and salinity is critical to determining  
119 the extent to which organic matter decomposition will respond to future climate changes.

120           In this study, we investigated the effects of elevated  $\text{CO}_2$  and high salinity on  
121 microbial communities and activities. In addition, we attempted to identify mechanisms  
122 underlying changes in microbial community by considering changes in carbon supply from  
123 vegetation exposed to higher  $\text{CO}_2$  concentrations. To achieve this, we incubated intact soil  
124 cores from a salt marsh under 2 levels of  $\text{CO}_2$  and salinity over a growing season, followed by  
125 microbial analysis.

126

127

## 128 **Materials and methods**

129

### 130 **Sampling site**

131 A total of 16 intact vegetation-soil cores were collected from Traeth Lafan nature reserve at  
132 Abergwyngregyn, which is located on the banks of the Menai Straits between mainland North

133 Wales and the island of Anglesey (53°14'N / 04°03'W; UK Grid ref. SH629728) in the UK.  
134 The reserve contains extensive salt marshes covered with halophytes including *Spartina* spp.  
135 and *Distichlis* spp. The soils are classified as sandy clay loam (alluvial gley) and detailed  
136 chemical properties are presented in S-Table 1.

137

138 Experimental design and incubation

139 Cylindrical plastic cores (12 cm diameter × 25 cm depth) were used to collect intact soil and  
140 vegetation (*Spartina* spp. and *Distichlis* spp.) from the salt marsh. The core was incubated in a  
141 plastic container (25 cm diameter × 40 cm depth) containing sea water diluted to either  
142 salinity at 20 ppt (control salinity) or 30 ppt (high salinity) with distilled water. The lateral  
143 side of the inner core had several holes for water exchange with the seawater in the outer  
144 container. The sea water collected in Conwy Bay, located near the sampling site, was filtered  
145 and sterilized using UV irradiation. The vegetation-soil cores were allowed to acclimate to the  
146 salinity of the diluted seawater (20 ppt, control salinity) for 4 weeks in water reservoir tanks.  
147 Each vegetation-soil system selected from control and high salinity treatments was randomly  
148 assigned to two different levels of atmospheric CO<sub>2</sub> including ambient air and ambient air +  
149 200 ppm. We prepared four replicate samples for each treatment.

150 During the experimental period, the 2 atmospheric CO<sub>2</sub> concentrations were  
151 automatically simulated in eight FACE (Free-Air Carbon Dioxide Enrichment, 8.5 m  
152 diameter) rings at the Bangor-FACE facility in North Wales. Four ambient air rings were left  
153 under natural conditions and the others were exposed to elevated CO<sub>2</sub> concentrations provided  
154 by the FACE technique. The vegetation-soil systems were maintained permanently flooded to  
155 the surface of soils during the incubation period using salinity-modified sea water. The  
156 salinity in the plastic containers was regularly monitored. The upper margins on the surface of  
157 the outer container except the area of vegetation-soil core were screened with plastic sheets to



158 prevent any disturbances due to precipitation or contaminants. The incubation was conducted  
159 over the growing season (April - November).

160

161 Water chemistry

162 Pore-water samples were collected from each core on Julian days 90, 130 180, 210, 230, 270  
163 and 290 using a syringe and silicone tubing inserted into the soil at 15 cm below the soil  
164 surface. Pore-water samples were filtered (0.25  $\mu\text{m}$  pore size) and analyzed to determine  
165 DOC (TOC analyzer; Shimadzu, Model TOC-5000, Japan) and phenolic content (Folin-  
166 Ciocalteu phenol reagent). Salinity, pH and temperature were also measured in the plastic  
167 containers at the time of sampling.

168

169 Microbial abundance and community structure

170 To measure microbial community structure and quantity, 1  $\text{cm}^3$  of soil was collected from a  
171 depth of 5 cm below the soil surface at the end of the incubation period. DNA was isolated  
172 using the UltraClean Soil DNA Isolation Kit as specified by the manufacturer (MoBio  
173 Laboratories, CA. USA).

174 Microbial abundances were determined by quantitative real-time PCR (q-PCR)  
175 targeting bacterial 16S rRNA, *nirS*, *dsr* and *mcrA* genes. The primers used in the analysis are  
176 presented in S-Table 2. The q-PCR was performed on the I-Cycler<sup>TM</sup> (Version 3.0a, Bio-Rad,  
177 Hercules, CA) using SYBR Green as a detection system in a reaction mixture of each primer  
178 and SYBR Green Supermix including iTaq DNA, SYBR Green I and fluorescein mix,  $\text{MgCl}_2$ ,  
179 dNTP mixture, stabilizers (Bio-Rad, Hercules, CA), DNA template, bovine serum albumin  
180 (Sigma) and RNase-free water. The amplification followed a three step PCR: for bacterial  
181 16S rRNA, 35 cycles with denaturation at 94 °C for 25 s, primer annealing at 50 °C for 25 s,  
182 and extension at 72 °C for 25 s; for *nirS* genes, 40 cycles with denaturation at 95 °C for 30 s,

183 primer annealing at 65 °C for 45 s, and extension at 72 °C for 30 s; for *dsr* genes, 40 cycles  
184 with denaturation at 95 °C for 25 s, primer annealing at 60 °C for 30 s, and extension at 72 °C  
185 for 30 s; for *mcrA* genes, 60 cycles with denaturation at 95 °C for 30 s, primer annealing at  
186 50 °C for 60 s, and extension at 72 °C for 60 s. Two independent real-time PCR assay were  
187 performed on each of the two replicate soil DNA extracts. A standard curve was created using  
188 10-fold dilution series of plasmids containing the bacterial 16S rRNA, *nirS*, *dsr* and *mcrA*  
189 genes from environmental samples.

190 Community structures of bacteria, denitrifiers and SRB were determined by T-RFLP  
191 analysis. Fragments of bacterial 16S rRNA (approximately 900 bp), *nirS* (approximately 800  
192 bp) and *dsr* (1900 bp) genes were amplified for bacteria, denitrifiers, and SRB, respectively,  
193 using FAM (5-carboxyfluorescein)-labeled primer pairs as described in previous studies (S-  
194 Table 2). PCR was performed in a model PTC-100 thermal cycler (MJ Research, Waltham,  
195 MA) following methods outlined by Lane (1991). PCR products were purified using the Ultra  
196 PCR clean up kit (MoBio Laboratories, Inc., Carlsbad, CA) and digested with 8U *HhaI*  
197 (Promega, Madison, WI) at 37°C for 4 hours. After inactivation of the restriction enzyme by  
198 heating, the lengths of fluorescently labeled fragments were determined with a 3730 ABI  
199 electrophoretic capillary sequencer (Applied Biosystems, Foster City, CA) in conjunction  
200 with the Genemapper Software (Foster City, CA). Terminal reaction fragments (T-RFs) were  
201 quantified by peak area integration using a minimum peak height threshold of 50 relative  
202 fluorescent units. We excluded T-RFs below size 35 and calculated the proportion of T-RFs  
203 in each sample. Shannon diversity index was measured based on T-RFLP profiles of 16S  
204 rRNA, *nirS*, and *dsr* genes. PC-ORD version 4.01 software was used for multivariate  
205 statistical analysis of the T-RFLP profiles (McCune and Mefford, 1999).

206

207 Statistical analysis

208 All statistical analyses were performed using SPSS statistical software (version 12.0, SPSS  
209 Inc., Chicago, IL). To test effects of atmospheric CO<sub>2</sub>, salinity, time, and their interaction on  
210 the measurements collected on Julian days 130, 180, 210, 230, and 270, we applied repeated  
211 measures analysis in the general linear model (GLM). Comparisons of treatments were  
212 performed by a student t-test or a two-way analysis of variance (ANOVA) at each sampling  
213 time. We ran SEM (Structural Equation Modelling) using the levels of CO<sub>2</sub> and salinity, DOC,  
214 phenolics, pH, conductivity and microbial information that we collected in this study. The  
215 model was built using *sem* function in the *lavaan* R package with maximum likelihood. We  
216 further estimated standardized covariance value on each connection.

217

218

## 219 **Results**

220

### 221 Pore-water analysis

222 Elevated CO<sub>2</sub> was significantly associated with high DOC during certain periods. For  
223 example, elevated CO<sub>2</sub> significantly increased DOC for the control salinity treatment on the  
224 Julian days 130, 180 and 230 (i.e., May - August), but not for the high salinity treatment  
225 except for day 130 (Fig. 1). However, the results of repeated measures ANOVAs indicate no  
226 significant effects of elevated CO<sub>2</sub> or high salinity on DOC in pore-water across the  
227 incubation period (Table 1). As for phenolics, no significant differences were found between  
228 ambient and elevated CO<sub>2</sub> levels, but the high salinity treatment showed significantly higher  
229 phenolics than the control salinity treatment (Table 1).

230

### 231 Activity of extracellular enzymes

232 Correlation analysis between DOC concentrations and enzyme activities revealed significant

233 correlations for  $\beta$ -glucosidase ( $r = 0.749$ ,  $P < 0.001$ ), N-acetylglucosaminidase ( $r = 0.619$ ,  
234  $P < 0.01$ ), Aminopeptidase ( $r = 0.535$ ,  $P < 0.05$ ) and phosphatase ( $r = 0.641$ ,  $P < 0.01$ ) (S-Table  
235 3). The mean values for the enzyme activities were generally higher under elevated CO<sub>2</sub> than  
236 ambient CO<sub>2</sub> for both salinity treatments, although the differences were not statistically  
237 significant due to high variability between replicates. Thus, we were unable to detect any  
238 significant differences due to the main effects or possible interactions.

239

#### 240 Microbial abundance and diversity

241 We noted that for SRB, there was a significant effect of elevated CO<sub>2</sub> and high salinity on the  
242 quantity ( $P < 0.01$ ) (Table 2). The abundance of SRB was 25% higher in the elevated CO<sub>2</sub>  
243 and high salinity treatment than that of elevated CO<sub>2</sub> and control salinity, while the lowest  
244 abundance was noted for ambient CO<sub>2</sub> and control salinity. Consequently, the abundance of  
245 SRB was highest for the high salinity under elevated CO<sub>2</sub>, indicating an additive effect of  
246 these two factors. The quantity of denitrifiers and methanogens did not differ in terms of the  
247 level of atmospheric CO<sub>2</sub> and salinity, and there was no interaction between these factors  
248 (Table 2). High salinity caused a significant decline in bacterial gene copy numbers which  
249 ranged from  $1.0 \times 10^9$  to  $2.5 \times 10^8$  target numbers g<sup>-1</sup> dry soil (Table 2). Meanwhile, bacterial  
250 abundance was higher by 115% in the elevated CO<sub>2</sub> treatment compared to the ambient air  
251 treatment though this difference was not statistically significant ( $P = 0.13$ ).

252 Microbial communities varied among treatments in terms of Shannon diversity index  
253 using T-RFLP profiles of bacterial 16S rRNA, *nirS* and *dsr* genes amplified for bacteria,  
254 denitrifiers, and SRB, respectively. Only the diversity of *nirS* genes increased under the high  
255 salinity treatment, and no other functional groups showed changes in diversity under elevated  
256 CO<sub>2</sub> or high salinity (Table 3). For example, NMS ordination of *dsr* gene profiles were not  
257 clearly separated even between high and control salinities (S-Fig. 1).

258 **Discussion**

259

260 Elevated CO<sub>2</sub> led to a significant increase in pore-water DOC during the vigorous growth  
261 period of May – August under control salinity (Fig. 1-(A)), suggesting a stimulation of the  
262 release of recently photosynthesized carbon compounds from the roots into rhizosphere under  
263 elevated CO<sub>2</sub>, consistent with previous studies conducted in northern peatlands and brackish  
264 marsh systems demonstrating increases in labile DOC and phenolics under elevated CO<sub>2</sub>  
265 (Freeman et al. 2004; Keller et al., 2009). However, overall, repeated-measures ANOVA  
266 found no significant effect of elevated CO<sub>2</sub>, potentially due to the confounding effects of high  
267 salinity and temporal variations (Table 1).

268 Labile DOC acts as an important substrate for microorganisms and can cause a  
269 “priming effect” often reported in soil ecosystems (Kuzyakov et al. 2000, 2019). It has been  
270 reported that the leakage of DOC from *Spartina* roots to the rhizosphere enhances microbial  
271 activity in the soil (Hines et al. 1999). We expected that microbial enzyme activities would  
272 increase under elevated CO<sub>2</sub> conditions as a result of stimulated microbial decomposition  
273 linked to enhanced DOC availability through increased plant productivity. Although not  
274 statistically significant, in most cases, enzyme activities were higher for the elevated CO<sub>2</sub>  
275 treatment than the ambient air. Furthermore, all enzyme activities showed positive  
276 correlations with DOC concentrations when data from both treatments were analyzed  
277 simultaneously (S-Table 3). These results indicate that increased DOC production through  
278 elevated CO<sub>2</sub> could enhance overall mineralization of organic matter in salt marsh ecosystems,  
279 as was noted in forest soil (Kim and Kang, 2011) and freshwater wetlands (Kang et al., 2005).

280 Interestingly, increases in DOC under elevated CO<sub>2</sub> were found only in the control  
281 salinity treatment while those in high salinity were only significant on one sampling occasion  
282 (Fig. 1-(B)). Elevated CO<sub>2</sub> may have increased DOC concentrations in both control and high

283 salinity conditions by enhanced photosynthesis, but decomposition of DOC may be  
284 accelerated under higher salinity conditions (Weston et al., 2011), offsetting the increase  
285 effects of elevated CO<sub>2</sub>. This speculation is supported by the fact that SRB abundance  
286 increased by elevated CO<sub>2</sub> and high salinity (Table 2). Despite that significant reduction in  
287 bacterial abundance under high salinity was observed in this study as well as reported by  
288 other studies (Yang et al. 2016; De León-Lorenzana et al. 2018), SRB abundance selectively  
289 increased under elevated CO<sub>2</sub> and salinity. It has been widely known that elevated CO<sub>2</sub>  
290 generally increases DOC input in wetland ecosystems (Freeman et al. 2004; Kim and Kang,  
291 2008), which can stimulate both methanogens and SRB (Alewell et al. 2008; Sela-Adler et al.  
292 2017). In general, SRB out-compete methanogens for carbon substrates in saline  
293 environments with high availability of sulfate (Lovley and Klug, 1983), dominating the  
294 process of carbon mineralization. This may also be associated with changes in carbon quality  
295 in the soil following the exposure to high salinity and elevated CO<sub>2</sub> for a growing season.  
296 This proposal is supported by high phenolics in pore-water for the high salinity treatment, and  
297 a positive relationship ( $r = 0.50$ ,  $P < 0.05$ ) between the abundance of SRB and the ratio of  
298 phenolics to DOC in our study. This warrants further investigation where detailed chemical  
299 analysis for the composition of pore-water phenolics should be conducted. Overall results of  
300 our study suggest that a specific functional group of SRB in salt marsh would selectively  
301 increase in the future environment of elevated CO<sub>2</sub> and sea water intrusion, and may dominate  
302 carbon mineralization.

303 Unlike microbial abundance, microbial community structure we measured in this  
304 study appeared to be unresponsive to elevated CO<sub>2</sub> or high salinity except for *nirS* genes.  
305 Similarly, Lee et al. (2017a) reported changes in microbial abundances but not the community  
306 structure when salt marsh was exposed to elevated CO<sub>2</sub> and nitrogen additions. It has also  
307 been reported that diversity of plant community confines the intensity of microbial responses

308 to environmental changes (Lange et al. 2015). We expected substantial changes in microbial  
309 community by high salinity as distinctive differences in community structure was reported  
310 along an estuarine salinity gradient (Campbell and Kirchman, 2013). However, the difference  
311 in salinity of our study (20 vs. 30 ppt) was much smaller than the other study (0.09 to 30 ppt),  
312 for which the impact of salinity change was not huge enough to induce microbial shift. Future  
313 study should explore more details about the diversity increase in *nirS* genes under high  
314 salinity, which is responsible for the key step in denitrification as well as release of N<sub>2</sub>O.  
315 Previous studies have shown that *nirS* community differs along the salinity range of 8.7 to  
316 33.6 even within 40 m distance, indicating the sensitive response of denitrifiers along the  
317 salinity gradient (Santoro et al. 2006).

318 In general, elevated CO<sub>2</sub> is known to increase CH<sub>4</sub> emissions and methanogen  
319 activity in wetlands (Hutchin et al. 1995; Megonigal and Schlesinger, 1997), which is  
320 mediated by the size and the lability of recently photosynthesized DOC, a key substrate for  
321 methanogens (Chasar et al. 2000). However, the aforementioned mechanism of competition  
322 from SRB may hinder the proliferation of methanogens in our system. This is well reflected in  
323 the fact that methanogen abundance was unchanged by elevated CO<sub>2</sub> treatments (Table 2). It  
324 is noteworthy that there is little consensus on the responses of methanogens to elevated CO<sub>2</sub> if  
325 other factors are involved (Lee et al. 2012; Kao-Kniffin and Zhu, 2013; Lee et al. 2017b). In  
326 particular, it has been reported that acetoclastic methanogens are strongly inhibited by the  
327 presence of SRB due to the competition for substrates (Lovley and Klug, 1983) and that  
328 acetoclastic pathway is more dominant than hydrogenotrophic pathway of methanogenesis  
329 (Conrad, 1999; Chasar et al. 2000).

330 Summing up the findings in this study, our SEM results indicate that elevated CO<sub>2</sub>  
331 would increase DOC concentrations, which in turn increase SRB abundances. Here, salinity  
332 plays a central role in the increment of SRB abundances (Fig. 2). Overall variations in

333 microbial abundance and SEM results suggest that the main pathway of carbon decomposition  
334 in salt marshes in the future environments would be sulfate reduction, while roles of  
335 methanogenesis would be comparably minor. In the future environment of elevated CO<sub>2</sub> and  
336 high salinity in salt mash ecosystems, therefore, the soil emission of CO<sub>2</sub> may be further  
337 accelerated rather than that of CH<sub>4</sub>, which was proposed by other studies (Kuzyakov et al.  
338 2019).

339

340

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348



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487

488 **Tables**

489

490 Table 1. The F statistic, degrees of freedom, and *p*-value for the main effects (CO<sub>2</sub>, salinity  
 491 and time) and all possible interactions on DOC and phenolics in pore-water on days 90, 130  
 492 180, 210 and 230 based on repeated measures ANOVAs. Asterisks indicate statistically  
 493 significant differences at (\*) *p* < 0.1, \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001

494

| Water chemistry | Effects                           | F     | Df | <i>p</i> value |
|-----------------|-----------------------------------|-------|----|----------------|
| DOC             | CO <sub>2</sub>                   | 0.109 | 1  | 0.749          |
|                 | Salinity                          | 1.401 | 1  | 0.267          |
|                 | CO <sub>2</sub> × salinity        | 0.469 | 1  | 0.511          |
|                 | Time                              | 3.758 | 4  | 0.012*         |
|                 | Time × CO <sub>2</sub>            | 1.124 | 4  | 0.360          |
|                 | Time × salinity                   | 0.568 | 4  | 0.688          |
|                 | Time × CO <sub>2</sub> × salinity | 0.407 | 4  | 0.802          |
| Phenolics       | CO <sub>2</sub>                   | 1.863 | 1  | 0.200          |
|                 | Salinity                          | 6.803 | 1  | 0.024*         |
|                 | CO <sub>2</sub> × salinity        | 0.867 | 1  | 0.372          |
|                 | Time                              | 7.996 | 4  | 0.000***       |
|                 | Time × CO <sub>2</sub>            | 1.182 | 4  | 0.332          |
|                 | Time × salinity                   | 5.022 | 4  | 0.002**        |
|                 | Time × CO <sub>2</sub> × salinity | 0.165 | 4  | 0.692          |

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498 Table 2. Mean gene copy numbers (target numbers g<sup>-1</sup> dry soil) ( $\pm$  SEM) of bacteria,  
 499 denitrifiers, SRB and methanogens exposed to two levels of atmospheric CO<sub>2</sub> and salinity.  
 500 Statistical comparisons are based on two-way ANOVA.

501

| Microbial community | Atmospheric CO <sub>2</sub> | Salinity | Gene copy numbers (target numbers g <sup>-1</sup> dry soil) |                                    | Between-Subjects Effects               |                                      |                                   |
|---------------------|-----------------------------|----------|---|------------------------------------|--|--------------------------------------|-----------------------------------|
|                     |                             |          | Mean $\pm$ SEM  |                                    | CO <sub>2</sub>                        | Salinity                             | CO <sub>2</sub> $\times$ salinity |
| Bacteria            | Ambient                     | Control  | 5.8 $\times$ 10 <sup>8</sup>                                | $\pm$ 3.1 $\times$ 10 <sup>8</sup> | F = 2.61<br><i>p</i> = 0.13            | F = 7.781<br><i>p</i> = <b>0.02*</b> | F = 2.48<br><i>p</i> = 0.14       |
|                     |                             | High     | 2.4 $\times$ 10 <sup>8</sup>                                | $\pm$ 4.6 $\times$ 10 <sup>7</sup> |  |                                      |                                   |
|                     | Elevated                    | Control  | 1.5 $\times$ 10 <sup>9</sup>                                | $\pm$ 5.7 $\times$ 10 <sup>8</sup> |  |                                      |                                   |
|                     |                             | High     | 2.5 $\times$ 10 <sup>8</sup>                                | $\pm$ 5.2 $\times$ 10 <sup>7</sup> |  |                                      |                                   |
| Denitrifiers        | Ambient                     | Control  | 9.1 $\times$ 10 <sup>6</sup>                                | $\pm$ 7.8 $\times$ 10 <sup>5</sup> | F = 1.24<br><i>p</i> = 0.29            | F = 1.67<br><i>p</i> = 0.222         | F = 0.26<br><i>p</i> = 0.62       |
|                     |                             | High     | 7.9 $\times$ 10 <sup>6</sup>                                | $\pm$ 1.6 $\times$ 10 <sup>6</sup> |  |                                      |                                   |
|                     | Elevated                    | Control  | 1.2 $\times$ 10 <sup>7</sup>                                | $\pm$ 2.5 $\times$ 10 <sup>6</sup> |  |                                      |                                   |
|                     |                             | High     | 8.9 $\times$ 10 <sup>6</sup>                                | $\pm$ 1.4 $\times$ 10 <sup>6</sup> |  |                                      |                                   |
| SRB                 | Ambient                     | Control  | 8.7 $\times$ 10 <sup>5</sup>                                | $\pm$ 5.1 $\times$ 10 <sup>4</sup> | F = 10.25<br><i>p</i> = <b>0.008**</b> | F = 8.59<br><i>p</i> = <b>0.01*</b>  | F = 0.03<br><i>p</i> = 0.88       |
|                     |                             | High     | 1.2 $\times$ 10 <sup>6</sup>                                | $\pm$ 1.0 $\times$ 10 <sup>5</sup> |  |                                      |                                   |
|                     | Elevated                    | Control  | 1.2 $\times$ 10 <sup>6</sup>                                | $\pm$ 4.0 $\times$ 10 <sup>4</sup> |  |                                      |                                   |
|                     |                             | High     | 1.6 $\times$ 10 <sup>6</sup>                                | $\pm$ 1.7 $\times$ 10 <sup>5</sup> |  |                                      |                                   |
| Methanogens         | Ambient                     | Control  | 5.4 $\times$ 10 <sup>6</sup>                                | $\pm$ 1.0 $\times$ 10 <sup>6</sup> | F = 1.06<br><i>p</i> = 0.33            | F = 0.01<br><i>p</i> = 0.92          | F = 1.03<br><i>p</i> = 0.33       |
|                     |                             | High     | 4.8 $\times$ 10 <sup>6</sup>                                | $\pm$ 1.7 $\times$ 10 <sup>6</sup> |  |                                      |                                   |
|                     | Elevated                    | Control  | 3.9 $\times$ 10 <sup>6</sup>                                | $\pm$ 1.1 $\times$ 10 <sup>6</sup> |  |                                      |                                   |
|                     |                             | High     | 4.7 $\times$ 10 <sup>6</sup>                                | $\pm$ 2.0 $\times$ 10 <sup>6</sup> |  |                                      |                                   |

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506 Table 3. Shannon's diversity ( $\pm$  SEM) at different atmospheric CO<sub>2</sub> and salinity based on T-  
 507 RFLP fingerprints of amplified for 16S rDNA, *nirS* and *dsr* genes. Statistical comparisons are  
 508 based on two-way ANOVA. Significant differences are labelled with different letters ( $p <$   
 509 0.05).

| Target genes | Atmospheric CO <sub>2</sub> concentrations |                          | Salinity                     |                              |
|--------------|--|--------------------------|------------------------------|------------------------------|
|              | Ambient CO <sub>2</sub>                    | Elevated CO <sub>2</sub> | Control salinity             | High salinity                |
| 16S rDNA     | 2.43 $\pm$ 0.73                            | 2.27 $\pm$ 1.14          | 2.48 $\pm$ 0.83              | 2.25 $\pm$ 0.92              |
| <i>nirS</i>  | 1.52 $\pm$ 0.41                            | 1.56 $\pm$ 0.40          | 1.49 $\pm$ 0.38 <sup>a</sup> | 1.60 $\pm$ 0.43 <sup>b</sup> |
| <i>dsr</i>   | 2.45 $\pm$ 0.68                            | 2.42 $\pm$ 0.70          | 2.42 $\pm$ 0.65              | 2.45 $\pm$ 0.74              |

510

511 **Figure captions**

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513 Figure 1. Effects of elevated CO<sub>2</sub> on DOC in pore water for the control salinity (A) and high  
514 salinity (B) treatments. Statistical comparisons between ambient and elevated CO<sub>2</sub> treatments  
515 are based on Student t-test. Significant differences are shown with \* at  $p < 0.05$ .

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519 Figure 2. Conceptual diagram of the pathway showing how elevated CO<sub>2</sub> and salinity affect  
520 sulfate reducing bacteria (SRB). Numbers denote standardized parameter values for the  
521 relationship of covariance, with the sign indicating positive or negative effect. Solid lines  
522 indicate significant pathway ( $n=4$ ,  $p < 0.01$ ). Dashed lines indicate non-significant pathway.

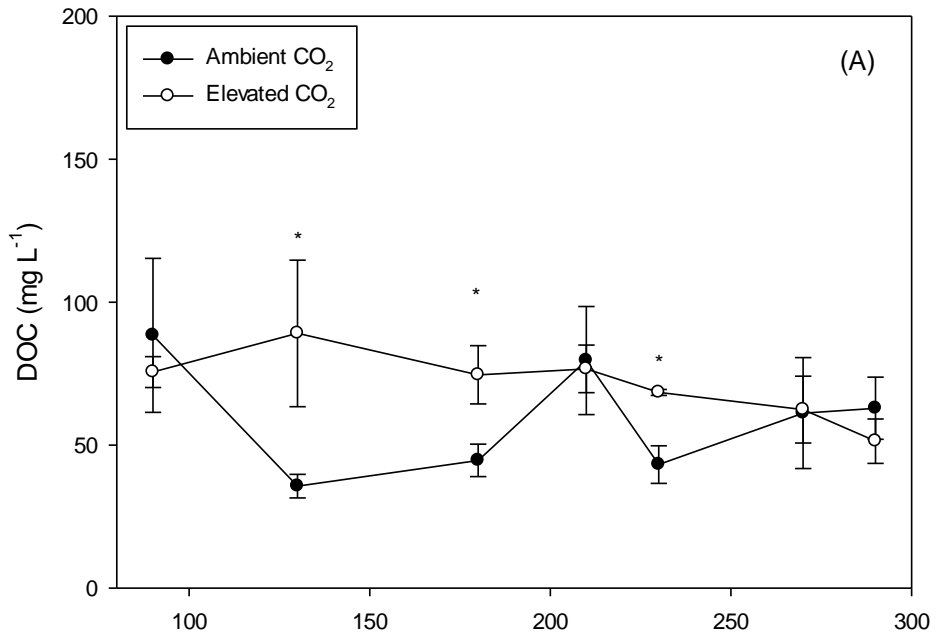
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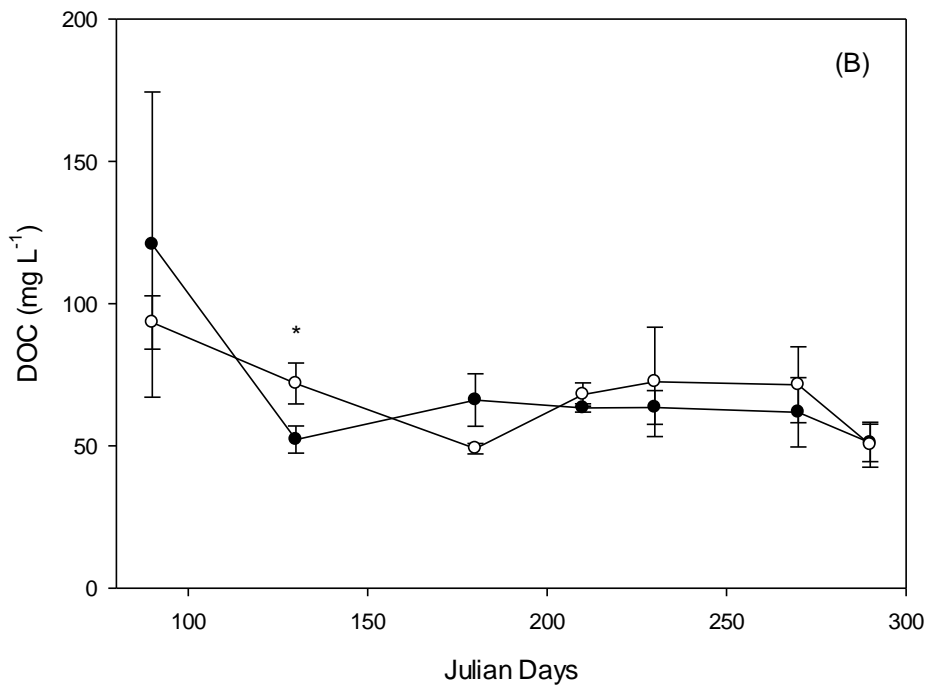
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526 **Figures**

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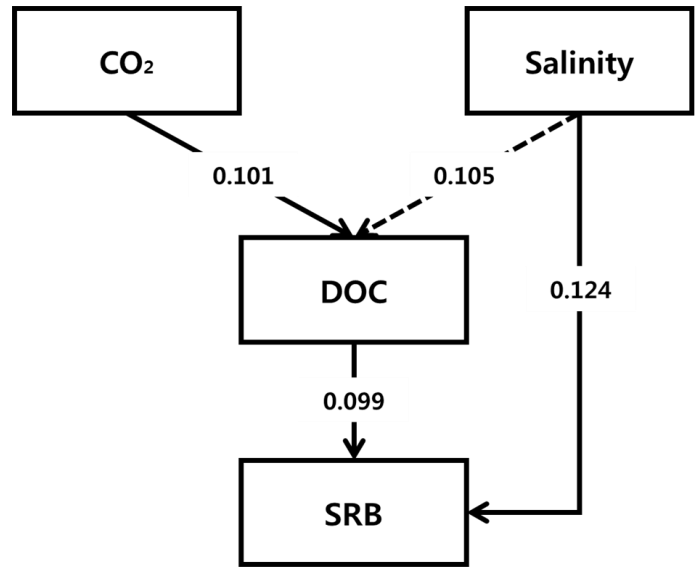
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531 **Figure 1**

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537 Figure 2.

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539 **Supplementary Information**

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542 S-Table 1. Soil characteristics collected from sampling sites at Traeth Lafan nature reserve.

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| Soil chemistry                        |                 |
|---------------------------------------|-----------------|
| SOM (%)                               | 6.7             |
| T-N (%)                               | 0.202           |
| T-P (%)                               | 0.067           |
| K <sup>+</sup> (mg/kg)                | 792.1           |
| Ca <sup>2+</sup> (mg/kg)              | 2690.0          |
| Mg <sup>2+</sup> (mg/kg)              | 643.0           |
| SO <sub>4</sub> <sup>2-</sup> (mg/kg) | 45.0            |
| pH                                    | 8.19            |
| Soil texture (%)                      | Sandy clay loam |
| Sand                                  | 55.2            |
| Silt                                  | 18.6            |
| Clay                                  | 26.3            |

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547 S-Table 2. Primers used for Q-PCR and T-RFLP analyses

| Assays | Primers  | Sequence (5'→3')                  | Target gene  | Target group | Ref.  |
|--------|----------|-----------------------------------|--|--------------|---|
| Q-PCR  | 341F     | 5'-CCT ACG GGA GGC AGC AG-3'      | Bacterial 16S rDNAs                                  | Bacteria     | Lane (1991)                                   |
|        | 515R     | 5'-ATT CCG CGC CTG GCA-3'         |  |              |   |
|        | NirS832F | 5'-TAC CAC CCC GAG CCG CGC GT-3'  | <i>nirS</i> (NO <sub>2</sub> <sup>-</sup> reductase) | Denitrifiers | Liu et al. (2003)<br>Braker et al. (1998)     |
|        | NirS3R   | 5'-GCC GCC GTC RTG VAG GAA-3'     |  |              |   |
|        | DSR1F+   | 5'-ACS CAC TGG AAG CAC GGC GG-3'  | <i>dsr</i> (dissimilatory sulfite reductase)         | SRB          | Kondo et al. (2004)                           |
|        | DSR-R    | 5'-GGT TRK ACG TGC CRM GGT G-3'   |  |              |   |
|        | ME 1     | 5'-GCM ATG CAR ATH GGW ATG TC -3' | <i>mcrA</i> (methyl-coenzyme reductase A)            | Methanogens  | Hales et al. (1996)<br>Springer et al. (1995) |
|        | MCR1R    | 5'-ARC CAD ATY TGR TCR TA -3'     |  |              |   |
| T-RFLP | 27F      | 5'-AGA GTT TGA TCM TGG CTC AG-3'  | Bacterial 16S rDNAs                                  | Bacteria     | Lane (1991)                                   |
|        | 907R     | 5'-CCG TCA ATT CCT TTR AGT TT-3'  |  |              |   |
|        | cd3F     | 5'-GTN AAY GTN AAR GAR CAN GG-3'  | <i>nirS</i> (NO <sub>2</sub> <sup>-</sup> reductase) | Denitrifiers | Liu et al. (2003)                             |
|        | cd4R     | 5'-ACR TTR AAY TTN CCN GTN GG-3'  |  |              |   |
|        | DSR1F    | 5'-ACS CAC TGG AAG CAG CAC G-3'   | <i>dsr</i> (dissimilatory sulfite reductase)         | SRB          | Wagner et al. (1998)                          |
|        | DSR4R    | 5'-GTG TAG CAG TTA CCG CA-3'      |  |              |   |

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549

550 S-Table 3. Correlation coefficients between DOC (Dissolved Organic Carbon) and enzyme activities in cores at the end of the incubation (N=4).

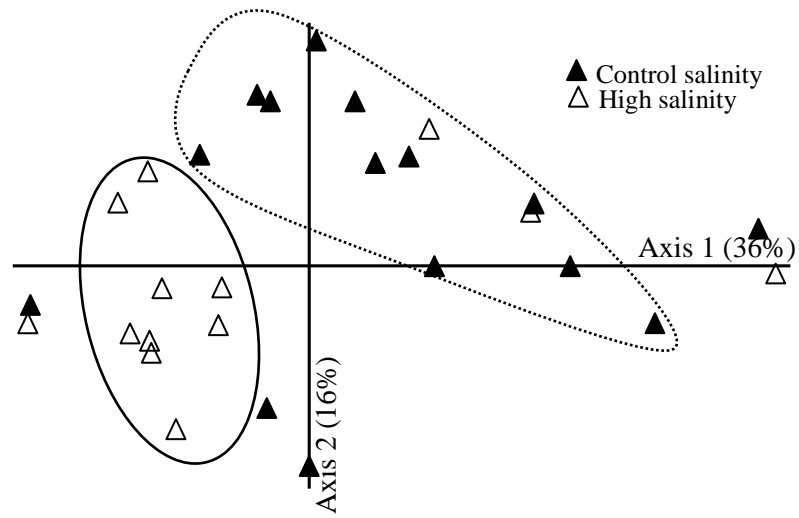
| Enzymes                 | r     | P values |
|-------------------------|-------|----------|
| $\beta$ -glucosidase    | 0.749 | < 0.001  |
| N-acetylglucosaminidase | 0.619 | < 0.01   |
| Aminopeptidase          | 0.535 | < 0.05   |
| Phosphatase             | 0.641 | < 0.01   |
| Arylsulfatase           | 0.166 | 0.54     |

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553 S-Figure 1. NMS ordination of *dsr* gene profiles obtained from control and high salinity treatments. The percent variation explained by each axis  
554 is shown in parentheses.

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