



Elevated CO₂ and high salinity enhance the abundance of sulfate reducers in a salt marsh ecosystem

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11

12 **Running head:** Elevated CO₂ and high salinity on saltmarsh microbes

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33 **Abstract**

34 Salt marshes are anticipated to be exposed to elevated atmospheric CO₂ and high salinity due
35 to sea-level rise in the future. This study aims to investigate the effects of elevated
36 atmospheric CO₂ 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 CO₂ (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 CO₂ 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 CO₂ on
48 denitrification. Microbial abundances changed substantially for certain functional groups; For
49 example, the abundance of SRB increased both by elevated CO₂ and high salinity. In contrast,
50 total bacterial abundance declined under the treatment of high salinity. SEM suggests that
51 elevated CO₂ 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 CO₂ 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 CO₂; 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 CO₂ or CH₄ 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 CO₂ 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 CO₂ 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 CO₂, 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 CO₂ (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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 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 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 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 CO_2 concentrations. To achieve this, we incubated intact soil
124 cores from a salt marsh under 2 levels of 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 CO₂ 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 CO₂ 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 CO₂ 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 µm pore size) and analyzed to determine
165 DOC (TOC analyzer; Shimadzu, Model TOC-5000, Japan) and phenolic content (Folin-
166 Ciocalteau 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 cm³ 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-CyclerTM (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, MgCl₂,
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 CO₂, 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 CO₂ 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 CO₂ was significantly associated with high DOC during certain periods. For
223 example, elevated CO₂ 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 CO₂ 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 CO₂ 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 β -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 CO₂ than
236 ambient CO₂ 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 CO₂ and high salinity on the
242 quantity ($P < 0.01$) (Table 2). The abundance of SRB was 25% higher in the elevated CO₂
243 and high salinity treatment than that of elevated CO₂ and control salinity, while the lowest
244 abundance was noted for ambient CO₂ and control salinity. Consequently, the abundance of
245 SRB was highest for the high salinity under elevated CO₂, 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 CO₂ 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 1.0×10^9 to 2.5×10^8 target numbers g⁻¹ dry soil (Table 2). Meanwhile, bacterial
250 abundance was higher by 115% in the elevated CO₂ 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 CO₂ 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 CO₂ 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 CO₂, consistent with previous studies conducted in northern peatlands and brackish
264 marsh systems demonstrating increases in labile DOC and phenolics under elevated CO₂
265 (Freeman et al. 2004; Keller et al., 2009). However, overall, repeated-measures ANOVA
266 found no significant effect of elevated CO₂, 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 CO₂ 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 CO₂
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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂. This speculation is supported by the fact that SRB abundance
286 increased by elevated CO₂ 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 CO₂ and salinity. It has been widely known that elevated CO₂
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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 N₂O.
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 CO₂ is known to increase CH₄ 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 CO₂ treatments (Table 2). It
324 is noteworthy that there is little consensus on the responses of methanogens to elevated CO₂ 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 CO₂
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 CO₂ and
336 high salinity in salt mash ecosystems, therefore, the soil emission of CO₂ may be further
337 accelerated rather than that of CH₄, which was proposed by other studies (Kuzyakov et al.
338 2019).

339

340

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348

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485 community structure in surface sediments of the Qinghai-Tibetan Lakes. Scientific
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487

488 **Tables**

489

490 Table 1. The F statistic, degrees of freedom, and *p*-value for the main effects (CO₂, 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

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

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498 Table 2. Mean gene copy numbers (target numbers g⁻¹ dry soil) (\pm SEM) of bacteria,
 499 denitrifiers, SRB and methanogens exposed to two levels of atmospheric CO₂ and salinity.
 500 Statistical comparisons are based on two-way ANOVA.

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

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506 Table 3. Shannon's diversity (\pm SEM) at different atmospheric CO₂ 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 ₂ concentrations		Salinity	
	Ambient CO ₂	Elevated CO ₂	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 ^a	1.60 \pm 0.43 ^b
<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 CO₂ on DOC in pore water for the control salinity (A) and high
514 salinity (B) treatments. Statistical comparisons between ambient and elevated CO₂ 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 CO₂ 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.

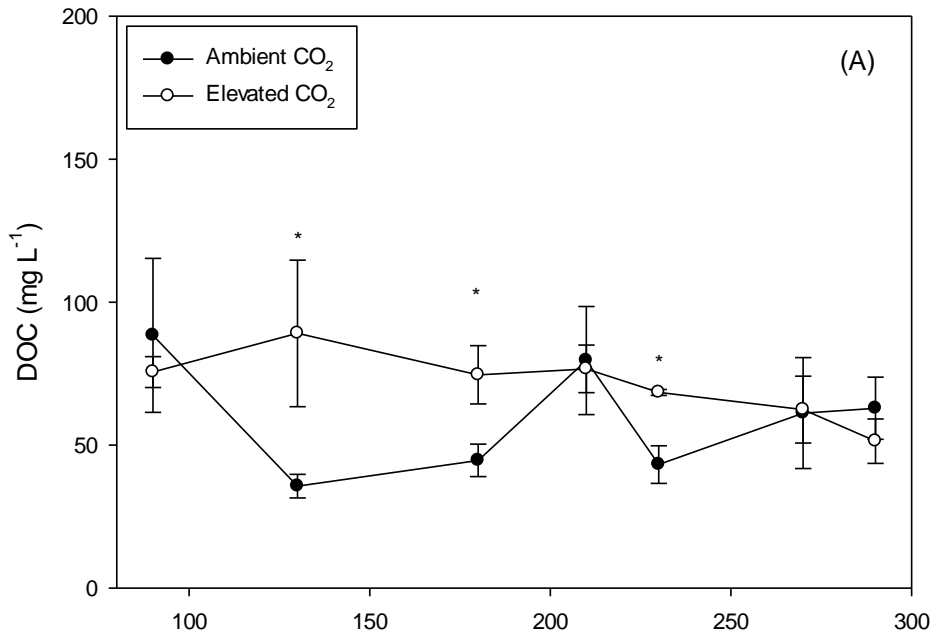
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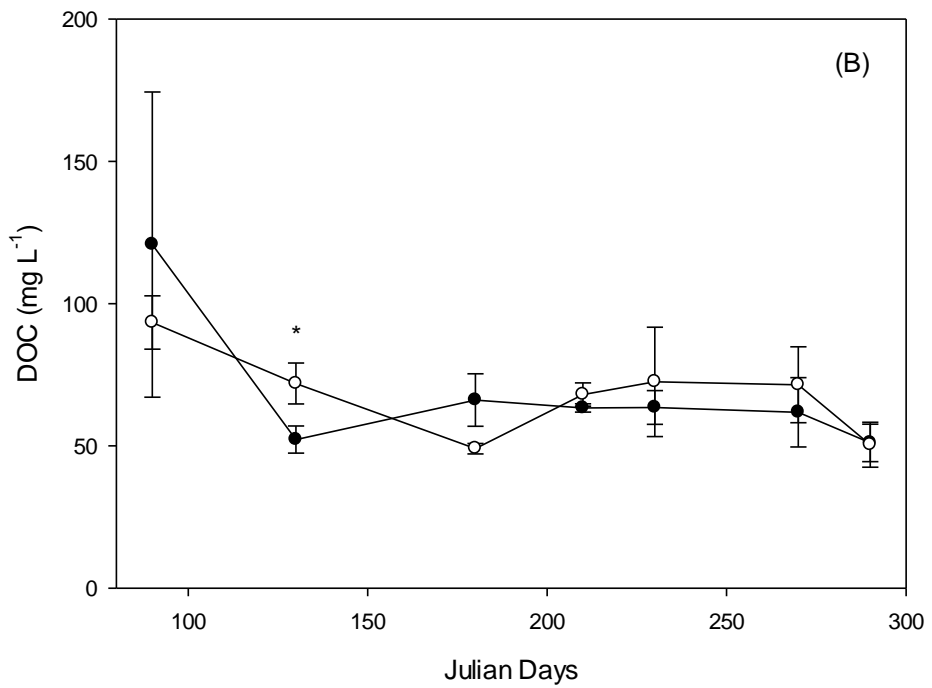
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526 **Figures**

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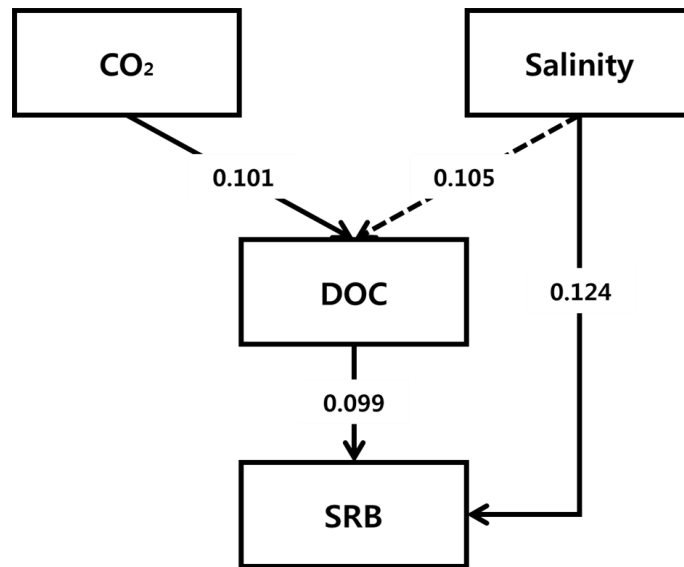
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531 **Figure 1**

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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 ⁺ (mg/kg)	792.1
Ca ²⁺ (mg/kg)	2690.0
Mg ²⁺ (mg/kg)	643.0
SO ₄ ²⁻ (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 ₂ ⁻ reductase)	Denitrifiers	Liu et al. (2003) 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) 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 ₂ ⁻ 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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550 S-Table 3. Correlation coefficients between DOC (Dissolved Organic Carbon) and enzyme activities in cores at the end of the incubation (N=4).

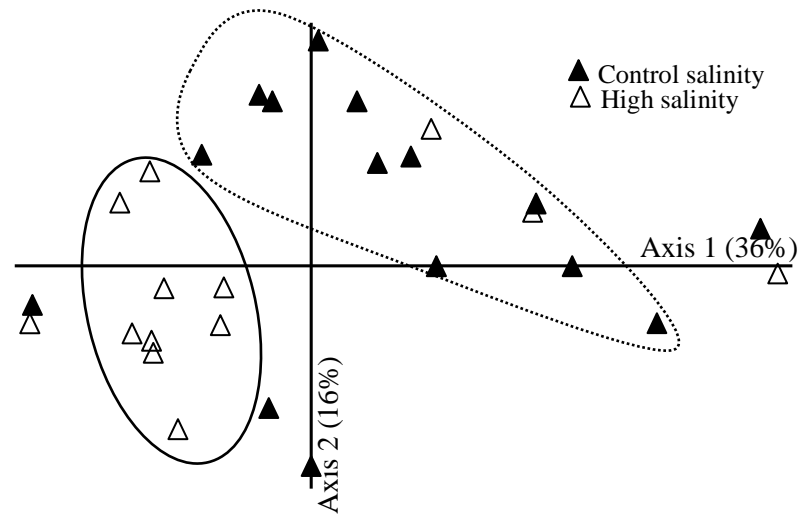
Enzymes	r	P values
β -glucosidase	0.749	< 0.001
N-acetylglucosaminidase	0.619	< 0.01
Aminopeptidase	0.535	< 0.05
Phosphatase	0.641	< 0.01
Arylsulfatase	0.166	0.54

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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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