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

Article

Accepted Version

Contribution type: Regular paper

Number of text pages: 20

Number of tables: 3

Number of figures: 2

Title: Elevated CO_2 and high salinity enhance the abundance of sulfate reducers in a salt marsh ecosystem

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Running head: Elevated CO_2 and high salinity on saltmarsh microbes

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Abstract

Salt marshes are anticipated to be exposed to elevated atmospheric CO_2 and high salinity due to sea-level rise in the future. This study aims to investigate the effects of elevated atmospheric CO_2 and high salinity on microbial communities using intact cores collected from a salt marsh in North Wales, UK. The cores were exposed to two levels of CO_2 (ambient vs. ambient + 200 ppm) and two levels of salinity (control vs. control + 10 ppt) over a growing season in the Free-Air Carbon Dioxide Enrichment (FACE) facility. We focused on the abundances of bacteria, sulfate reducers (SRB), methanogens and denitrifiers as they play a central role in greenhouse gas emissions. In addition, the activities of extracellular enzymes were determined to assess the effects on microbial activity, followed by Structural Equation Modelling (SEM) to elucidate possible mechanism for the changes we observed. Elevated CO_2 significantly increased DOC in pore water for the control salinity treatment during a vigorous growing season (i.e., May - Aug) but not the high salinity treatment. Microbial diversity presented by Shannon's diversity derived from T-RFLP analysis showed no significant changes except for *nirS* genes, suggesting potential influence of elevated CO_2 on denitrification. Microbial abundances changed substantially for certain functional groups; For example, the abundance of SRB increased both by elevated CO_2 and high salinity. In contrast, total bacterial abundance declined under the treatment of high salinity. SEM suggests that elevated CO_2 increases DOC in pore-water, which increased sulfate reducers. Salinity plays an additional role in this process to selectively increasing SRB without affecting methanogens. Overall, the results of this study suggest that SRB will play a key role in organic matter decomposition in salt marshes as atmospheric CO_2 and salinity increase. This is most likely to be mediated by changes in the quantity and the quality of organic carbon derived from salt marsh vegetation.

Key words: Elevated CO_2; Sulfate reducers; Salinity; Salt marsh; DOC

Introduction

Salt marshes are one of the most productive ecosystems on earth and play an important role in the global biogeochemical cycle, water quality amelioration, and habitat provisioning for wildlife. Those processes are mainly mediated by microorganisms in coastal soils, which decompose organic matter by releasing enzymes and metabolize organic carbon completely to CO_2 or CH_4 depending on the types of microbes and environmental conditions (Kang et al., 1998).

It is expected that these ecosystems will experience substantial environmental change under future climate scenarios, which may act to undermine these key functions. The first impact relates to elevated atmospheric CO_2 for which concentration is expected to increase up to 1,000 ppm by the end of this century (Kiehl, 2011). Previous studies have suggested that elevated CO_2 can stimulate above- and belowground productivity, resulting in organic matter accumulation or stimulation of heterotrophic respiration as a result of increased plant photosynthate in salt marshes (Cherry et al. 2009; Langley et al. 2009; Erickson et al. 2013). In addition, various factors (e.g., increasing oxygen release from roots, high root turnover and rhizodeposition, temperature rise, etc.) may give rise to a negative feedback with the fertilizing effect of elevated CO_2, promoting the release of soil organic carbon into the atmosphere as greenhouse gases (Wolf et al. 2007). Vegetation may also release more carbon compounds into belowground and adjacent ecosystems in the form of dissolved organic carbon (DOC), phenolics in particular, under elevated CO_2 (Freeman et al. 2004; Kim and Kang, 2008), leading to more rapid carbon mineralization in salt marshes due to a mechanism called the ‘priming effect’ (Bardgett et al. 2008).

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

(Church et al. 2013). Numerous studies have focused on the responses of the salt marsh vegetation to high salinity in terms of growth, species composition / transition, and community structure (Munns and Tester, 2008). However, less is known about how organic matter degradation will respond to increasing salinity in these ecosystems. Salt marshes exhibit extremely high productivity and low decomposition rates, resulting in the sequestration of significant amounts of carbon (Bridgham et al. 2006). Any changes in edaphic environments (e.g., redox potential, terminal electron acceptors and donors, etc.) may accelerate or decelerate organic carbon mineralization through changes in microbial function and activity (Sutton-Grier et al. 2011). Therefore, understanding the effects of changing conditions on microbial processes and communities is crucial for determining the fate of organic matter decomposition under high salinity in salt marshes. This is of great importance not only locally but also globally, as effects of elevated CO_2 and water chemistry change on marsh biogeochemistry through microbial processes have been noted both in Asia and the north America (Lee et al., 2015; Lee et al., 2017b)

Despite potential consequences of increasing atmospheric CO_2 and high salinity in salt marshes, few studies have investigated the integrated effects of those environmental changes. Furthermore, most studies have focused on vegetation and organic matter content (Drake, 2014; Morrissey et al. 2014), while even less is known about the effects on microbial processes and microbial communities, with only limited data available on the effects of elevated CO_2 on microbial communities (Dunbar et al. 2012; Lee and Kang, 2016). Studies investigating salinity-induced changes in microbial communities have mostly been conducted along a naturally occurring salinity gradient in coastal areas where other variables present a challenge in establishing the consequence of high salinity (Bernhard et al. 2005; Henriques et al. 2006). To address this shortfall in our understanding of the fate of organic matter in salt marshes, it is essential that the integrative effects of elevated CO_2 and high salinity on

microbial processes and communities are now elucidated.

Among diverse microbial communities, sulfate reducing bacteria (SRB) play a crucial role in organic matter decomposition in saline environments such as salt marshes (Koretsky et al. 2005). Denitrifiers are also involved in buffering coastal N loading from terrestrial ecosystems through denitrification in salt marshes, which removes NO_3^- from the ecosystem (Davis et al. 2004; Wigand et al. 2004). Methanogenesis is another important pathway of carbon mineralization under anaerobic conditions. Although methanogenesis has been considered less important in salt marshes due to competitive advantage of SRB over methanogens (Dowrick et al. 2006), recent studies have suggested that methane emissions from coastal wetlands could be substantial (Vizza et al. 2017). The ability of those microbial communities to cope with increasing atmospheric CO_2 and salinity is critical to determining the extent to which organic matter decomposition will respond to future climate changes.

In this study, we investigated the effects of elevated CO_2 and high salinity on microbial communities and activities. In addition, we attempted to identify mechanisms underlying changes in microbial community by considering changes in carbon supply from vegetation exposed to higher CO_2 concentrations. To achieve this, we incubated intact soil cores from a salt marsh under 2 levels of CO_2 and salinity over a growing season, followed by microbial analysis.

Materials and methods

Sampling site

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

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

Experimental design and incubation

Cylindrical plastic cores (12 cm diameter × 25 cm depth) were used to collect intact soil and vegetation (*Spartina* spp. and *Distichlis* spp.) from the salt marsh. The core was incubated in a plastic container (25 cm diameter × 40 cm depth) containing sea water diluted to either salinity at 20 ppt (control salinity) or 30 ppt (high salinity) with distilled water. The lateral side of the inner core had several holes for water exchange with the seawater in the outer container. The sea water collected in Conwy Bay, located near the sampling site, was filtered and sterilized using UV irradiation. The vegetation-soil cores were allowed to acclimate to the salinity of the diluted seawater (20 ppt, control salinity) for 4 weeks in water reservoir tanks. Each vegetation-soil system selected from control and high salinity treatments was randomly assigned to two different levels of atmospheric CO_2 including ambient air and ambient air + 200 ppm. We prepared four replicate samples for each treatment.

During the experimental period, the 2 atmospheric CO_2 concentrations were automatically simulated in eight FACE (Free-Air Carbon Dioxide Enrichment, 8.5 m diameter) rings at the Bangor-FACE facility in North Wales. Four ambient air rings were left under natural conditions and the others were exposed to elevated CO_2 concentrations provided by the FACE technique. The vegetation-soil systems were maintained permanently flooded to the surface of soils during the incubation period using salinity-modified sea water. The salinity in the plastic containers was regularly monitored. The upper margins on the surface of the outer container except the area of vegetation-soil core were screened with plastic sheets to

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

Water chemistry

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

Microbial abundance and community structure

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

Microbial abundances were determined by quantitative real-time PCR (q-PCR) targeting bacterial 16S rRNA, *nirS, dsr* and *mcrA* genes. The primers used in the analysis are presented in S-Table 2. The q-PCR was performed on the I-Cycler™ (Version 3.0a, Bio-Rad, Hercules, CA) using SYBR Green as a detection system in a reaction mixture of each primer and SYBR Green Supermix including iTaq DNA, SYBR Green I and fluorescein mix, $MgCl_2$, dNTP mixture, stabilizers (Bio-Rad, Hercules, CA), DNA template, bovine serum albumin (Sigma) and RNAse-free water. The amplification followed a three step PCR: for bacterial 16S rRNA, 35 cycles with denaturation at 94 °C for 25 s, primer annealing at 50 °C for 25 s, and extension at 72 °C for 25 s; for *nirS* genes, 40 cycles with denaturation at 95 °C for 30 s,

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

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

Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 12.0, SPSS Inc., Chicago, IL). To test effects of atmospheric CO_2, salinity, time, and their interaction on the measurements collected on Julian days 130, 180, 210, 230, and 270, we applied repeated measures analysis in the general linear model (GLM). Comparisons of treatments were performed by a student t-test or a two-way analysis of variance (ANOVA) at each sampling time. We ran SEM (Structural Equation Modelling) using the levels of CO_2 and salinity, DOC, phenolics, pH, conductivity and microbial information that we collected in this study. The model was built using *sem* function in the *lavaan* R package with maximum likelihood. We further estimated standardized covariance value on each connection.

Results

Pore-water analysis

Elevated CO_2 was significantly associated with high DOC during certain periods. For example, elevated CO_2 significantly increased DOC for the control salinity treatment on the Julian days 130, 180 and 230 (i.e., May - August), but not for the high salinity treatment except for day 130 (Fig. 1). However, the results of repeated measures ANOVAs indicate no significant effects of elevated CO_2 or high salinity on DOC in pore-water across the incubation period (Table 1). As for phenolics, no significant differences were found between ambient and elevated CO_2 levels, but the high salinity treatment showed significantly higher phenolics than the control salinity treatment (Table 1).

Activity of extracellular enzymes

Correlation analysis between DOC concentrations and enzyme activities revealed significant

correlations for β-glucosidase (r = 0.749, $P<0.001$), N-acetylglucosaminidase (r = 0.619, $P<0.01$), Aminopeptidase (r = 0.535, $P<0.05$) and phosphatase (r = 0.641, $P<0.01$) (S-Table 3). The mean values for the enzyme activities were generally higher under elevated CO_2 than ambient CO_2 for both salinity treatments, although the differences were not statistically significant due to high variability between replicates. Thus, we were unable to detect any significant differences due to the main effects or possible interactions.

Microbial abundance and diversity

We noted that for SRB, there was a significant effect of elevated CO_2 and high salinity on the quantity ($P < 0.01$) (Table 2). The abundance of SRB was 25% higher in the elevated CO_2 and high salinity treatment than that of elevated CO_2 and control salinity, while the lowest abundance was noted for ambient CO_2 and control salinity. Consequently, the abundance of SRB was highest for the high salinity under elevated CO_2, indicating an additive effect of these two factors. The quantity of denitrifiers and methanogens did not differ in terms of the level of atmospheric CO_2 and salinity, and there was no interaction between these factors (Table 2). High salinity caused a significant decline in bacterial gene copy numbers which ranged from 1.0×10^9 to 2.5×10^8 target numbers g^{-1} dry soil (Table 2). Meanwhile, bacterial abundance was higher by 115% in the elevated CO_2 treatment compared to the ambient air treatment though this difference was not statistically significant (P = 0.13).

Microbial communities varied among treatments in terms of Shannon diversity index using T-RFLP profiles of bacterial 16S rRNA, *nirS* and *dsr* genes amplified for bacteria, denitrifiers, and SRB, respectively. Only the diversity of *nirS* genes increased under the high salinity treatment, and no other functional groups showed changes in diversity under elevated CO_2 or high salinity (Table 3). For example, NMS ordination of *dsr* gene profiles were not clearly separated even between high and control salinities (S-Fig. 1).

Discussion

Elevated CO_2 led to a significant increase in pore-water DOC during the vigorous growth period of May – August under control salinity (Fig. 1-(A)), suggesting a stimulation of the release of recently photosynthesized carbon compounds from the roots into rhizosphere under elevated CO_2, consistent with previous studies conducted in northern peatlands and brackish marsh systems demonstrating increases in labile DOC and phenolics under elevated CO_2 (Freeman et al. 2004; Keller et al., 2009). However, overall, repeated-measures ANOVA found no significant effect of elevated CO_2, potentially due to the confounding effects of high salinity and temporal variations (Table 1).

Labile DOC acts as an important substrate for microorganisms and can cause a "priming effect" often reported in soil ecosystems (Kuzyakov et al. 2000, 2019). It has been reported that the leakage of DOC from *Spartina* roots to the rhizosphere enhances microbial activity in the soil (Hines et al. 1999). We expected that microbial enzyme activities would increase under elevated CO_2 conditions as a result of stimulated microbial decomposition linked to enhanced DOC availability through increased plant productivity. Although not statistically significant, in most cases, enzyme activities were higher for the elevated CO_2 treatment than the ambient air. Furthermore, all enzyme activities showed positive correlations with DOC concentrations when data from both treatments were analyzed simultaneously (S-Table 3). These results indicate that increased DOC production through elevated CO_2 could enhance overall mineralization of organic matter in salt marsh ecosystems, as was noted in forest soil (Kim and Kang, 2011) and freshwater wetlands (Kang et al., 2005).

Interestingly, increases in DOC under elevated CO_2 were found only in the control salinity treatment while those in high salinity were only significant on one sampling occasion (Fig. 1-(B)). Elevated CO_2 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_2. This speculation is supported by the fact that SRB abundance
286 increased by elevated CO_2 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_2 and salinity. It has been widely known that elevataed CO_2
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_2 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_2 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_2 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_2 and nitrogen additions. It has also
307 been reported that diversity of plant community confines the intensity of microbial responses

to environmental changes (Lange et al. 2015). We expected substantial changes in microbial community by high salinity as distinctive differences in community structure was reported along an estuarine salinity gradient (Campbell and Kirchman, 2013). However, the difference in salinity of our study (20 vs. 30 ppt) was much smaller than the other study (0.09 to 30 ppt), for which the impact of salinity change was not huge enough to induce microbial shift. Future study should explore more details about the diversity increase in *nirS* genes under high salinity, which is responsible for the key step in denitrification as well as release of N_2O. Previous studies have shown that *nirS* community differs along the salinity range of 8.7 to 33.6 even within 40 m distance, indicating the sensitive response of denitrifiers along the salinity gradient (Santoro et al. 2006).

In general, elevated CO_2 is known to increase CH_4 emissions and methanogen activity in wetlands (Hutchin et al. 1995; Megonigal and Schlesinger, 1997), which is mediated by the size and the lability of recently photosynthesized DOC, a key substrate for methanogens (Chasar et al. 2000). However, the aforementioned mechanism of competition from SRB may hinder the proliferation of methanogens in our system. This is well reflected in the fact that methanogen abundance was unchanged by elevated CO_2 treatments (Table 2). It is noteworthy that there is little consensus on the responses of methanogens to elevated CO_2 if other factors are involved (Lee et al. 2012; Kao-Kniffin and Zhu, 2013; Lee et al. 2017b). In particular, it has been reported that acetoclastic methanogens are strongly inhibited by the presence of SRB due to the competition for substrates (Lovley and Klug, 1983) and that acetoclastic pathway is more dominant than hydrogenotrophic pathway of methanogenesis (Conrad, 1999; Chasar et al. 2000).

Summing up the findings in this study, our SEM results indicate that elevated CO_2 would increase DOC concentrations, which in turn increase SRB abundances. Here, salinity plays a central role in the increment of SRB abundances (Fig. 2). Overall variations in

microbial abundance and SEM results suggest that the main pathway of carbon decomposition in salt marshes in the future environments would be sulfate reduction, while roles of methanogensis would be comparably minor. In the future environment of elevated CO_2 and high salinity in salt mash ecosystems, therefore, the soil emission of CO_2 may be further accelerated rather than that of CH_4, which was proposed by other studies (Kuzyakov et al. 2019).

Acknowledgements

This work was supported by the National Research Foundation of Korea Grant [NRF-2009-352-C00145], NIER Research Fund [NIER-2019-01-02-058] and Fellowship Program for Overseas Studies [2017-ES-0002] funded by the Korean Government. C. Freeman acknowledges support from the Royal Society, Leverhumle Trust and NERC. H. Kang is grateful to NRF (20110030040, 2016R1D1A1A02937049) and Forestry Service (2017096A00179BB01).

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488 **Tables**

489

490 Table 1. The F statistic, degrees of freedom, and *p*-value for the main effects (CO_2, 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	*p* 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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Table 2. Mean gene copy numbers (target numbers g^{-1} dry soil) (± SEM) of bacteria, denitrifiers, SRB and methanogens exposed to two levels of atmospheric CO_2 and salinity. Statistical comparisons are based on two-way ANOVA.

Microbial community	Atmospheric CO_2	Salinity	Gene copy numbers (target numbers g^{-1} dry soil) Mean ± SEM	Between-Subjects Effects		
				CO_2	Salinity	CO_2× salinity
Bacteria	Ambient	Control	$5.8 \times 10^8 \pm 3.1 \times 10^8$	F = 2.61 p = 0.13	F = 7.781 ***p* = 0.02***	F = 2.48 p = 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 p = 0.29	F = 1.67 p = 0.222	F = 0.26 p = 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 ***p* = 0.008****	F = 8.59 ***p* = 0.01***	F = 0.03 p = 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 ***p* = 0.33**	F = 0.01 p = 0.92	F = 1.03 p = 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	$4.7 \times 10^6 \pm 2.0 \times 10^6$			

506 Table 3. Shannon's diversity (± SEM) at different atmospheric CO_2 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 ± 0.73	2.27 ± 1.14	2.48 ± 0.83	2.25 ± 0.92
nirS	1.52 ± 0.41	1.56 ± 0.40	1.49 ± 0.38[a]	1.60 ± 0.43[b]
dsr	2.45 ± 0.68	2.42 ± 0.70	2.42 ± 0.65	2.45 ± 0.74

510

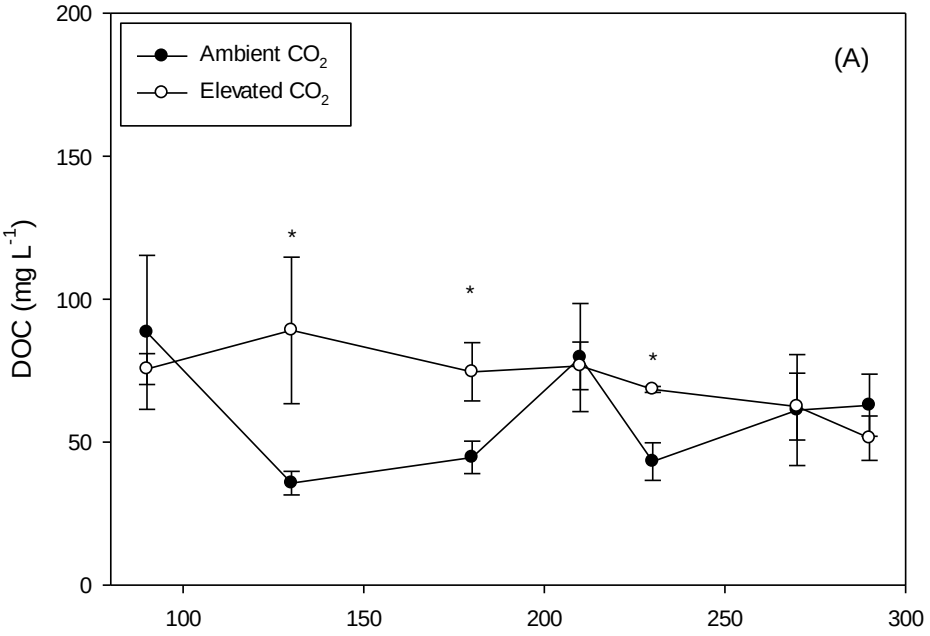
Figure captions

Figure 1. Effects of elevated CO_2 on DOC in pore water for the control salinity (A) and high salinity (B) treatments. Statistical comparisons between ambient and elevated CO_2 treatments are based on Student t-test. Significant differences are shown with * at $p < 0.05$.

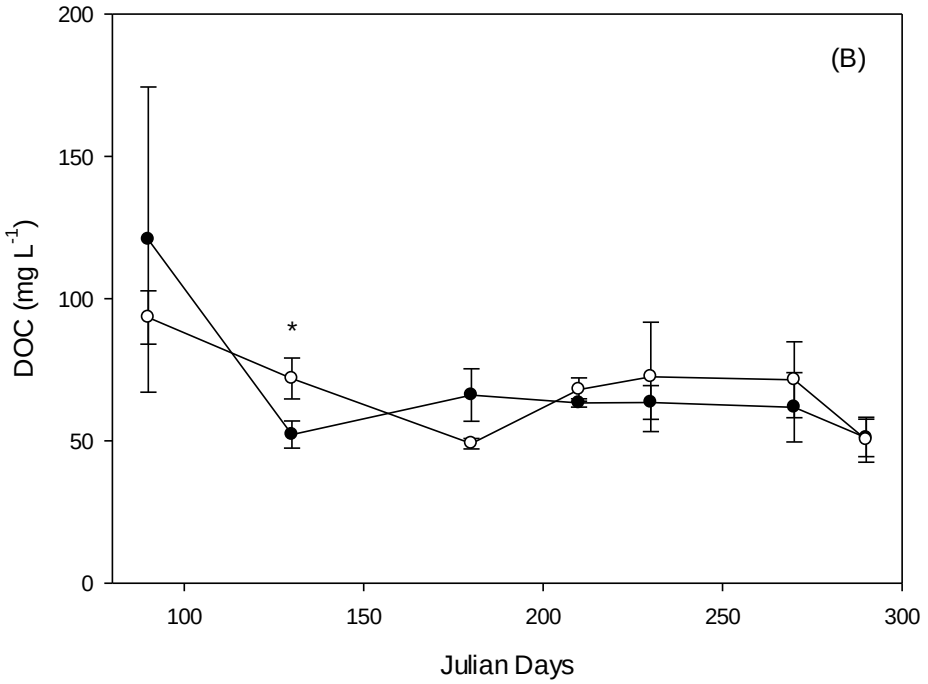
Figure 2. Conceptual diagram of the pathway showing how elevated CO_2 and salinity affect sulfate reducing bacteria (SRB). Numbers denote standardized parameter values for the relationship of covariance, with the sign indicating positive or negative effect. Solid lines indicate significant pathway (n=4, $p < 0.01$). Dashed lines indicate non-significant pathway.

526 **Figures**

527



528



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531 Figure 1

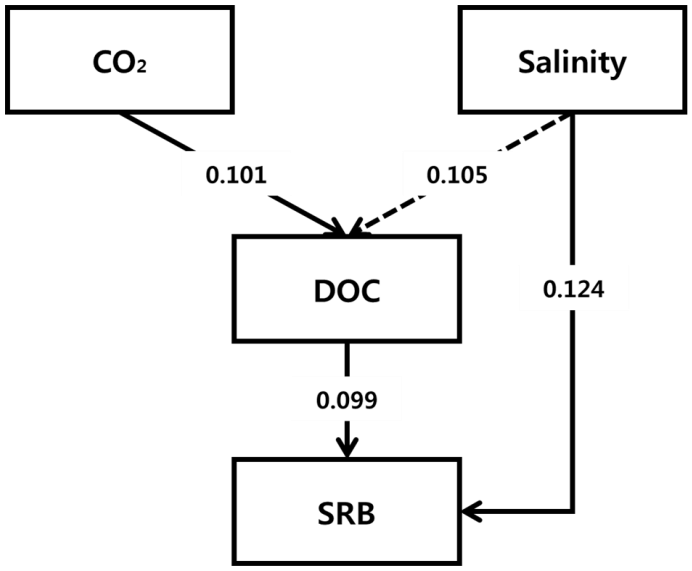

Figure 2.

Supplementary Information

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

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

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´	*nirS* (NO_2^- reductase)	Denitrifiers	Liu et al. (2003)
	NirS3R	5´-GCC GCC GTC RTG VAG GAA-3´			Braker et al. (1998)
	DSR1F+	5´-ACS CAC TGG AAG CAC GGC GG-3´	*dsr* (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´	*mcrA* (methyl-coenzyme reductase A)	Methanogens	Hales et al. (1996)
	MCR1R	5´-ARC CAD ATY TGR TCR TA -3´			Springer et al. (1995)
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´	*nirS* (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´	*dsr* (dissimilatory sulfite reductase)	SRB	Wagner et al. (1998)
	DSR4R	5´-GTG TAG CAG TTA CCG CA-3´			

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549

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

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