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Short communication

Elevated CO_2 causes a change in microbial communities of rhizosphere and bulk soil of salt marsh system

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ABSTRACT

Using TRFLP and real-time qPCR, this study aimed to investigate the way elevated CO_2 (eCO_2) affects bacteria, fungi, archaea, and sulfate-reducing bacteria in salt marsh systems containing halophyte *Suaeda japonica*. Moreover, it also aimed to evaluate the effects of eCO_2 in terms of plant interaction by analyzing the rhizosphere and bulk soil separately. We observed that the gene abundance and community structures were affected by eCO_2, and the rhizosphere and bulk soil communities showed a different response. The rhizospheric microbes responded to eCO_2 more strongly than the bulk soil microbes. The results also showed that the sulfate-reducing bacteria (SRB) community was more sensitive to eCO_2 than the general bacterial community. In addition, the findings suggested that bacteria and archaea competed severely when exposed to eCO_2, which caused a dominance of bacteria over archaea or the co-presence of bacteria and archaea with a different micro-niche. Overall, eCO_2 caused a strong change in the microbial community in salt marsh with halophytes, but the overall functional activity of the microbial community was unchanged and balanced by the different response patterns of the bacterial, fungal, and archaeal communities in our system.

1. Introduction

Since the concentration of atmospheric CO_2 has been anticipated to increase dramatically, many studies have been conducted to examine various aspects of the effects of elevated CO_2 (eCO_2) on ecosystems (Bachman et al., 2010; Mayr et al., 1999; Stock and Midgley 1995). Although studies initially mainly concentrated on the response of the plant and soil chemistry to eCO_2 (AbdElgawada et al., 2015; Langley and Megonigal, 2010; Lindroth, 2010; Perry et al., 2012), the effects of eCO_2 on microorganisms in ecosystems have received increased attention lately (Freeman et al., 2004; Hayden et al., 2012; Lipson et al., 2014; Zhou et al., 2011), as microbes play a key role in the biogeochemical cycle of ecosystems.

The salt marsh system is a very rich ecosystem in which a dynamic nutrient cycle takes place. Accordingly, many researchers have shown an interest in the way eCO_2 affects salt marshes (Drake, 2014; Erickson et al., 2013; Pastore et al., 2016). However, the number of reports about microbes in salt marshes remains small in comparison with other ecosystems. Accordingly, the response of the microbial community to eCO_2 in salt marshes has rarely been studied (Weber et al., 2011). In particular, there is little information about the fungal and archaeal communities in salt marshes (Nelson et al., 2009; Torzilli et al., 2006). Fungal community is a central contributor to decomposition of recalcitrant organic matter, and the archaeal community has also been recently suggested to play a comparable role in the biogeochemical cycle of various ecosystems, including salt marshes (Seyler et al., 2014). As such, to clearly understand the dynamics of the nutrient cycle in ecosystem under eCO_2, it is necessary to investigate the responses of microbes, including the archaeal and fungal communities.

In terms of microbial composition, it has been reported that the proportion of sulfate-reducing bacteria (SRB) was higher in salt marshes than in other ecosystems (Klepac-Ceraj et al., 2004). With respect to the activity of microorganisms, sulfate reduction is considered to be one of the key processes for organic matter decomposition in salt marshes (Holmer and Storkholm, 2001), and it can contribute to up to 50% of the anaerobic carbon mineralization of wetlands. However, the way eCO_2 affects the SRB community has not been well-addressed. Therefore, it is useful to investigate the way eCO_2 may affect the abundance and distribution of SRB in salt marsh systems. In turn, this can help to understand the overall response of microbes to eCO_2.

As fixed carbon from plants is a primary source of microbial activity, the presence and type of plants may influence the

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magnitude of the effect of the eCO_2 on microbes (Lee et al., 2015). In salt marsh, halophytes tolerant to salt stress are typical plant biome. *Suaeda japonica* is one of representative halophytes distributing in coastal areas of East Asia region. As they play a central role in protecting coastal area from erosion by climate change such as sea-level rise as well as maintaining function of ecosystem, their ecology and distribution are regarded as one of the indicator for normality of salt marsh. The salt-tolerance of halophytes is known to be related with rhizospheric microbes (Rodriguez et al., 2008). The rhizosphere is known as a hot spot for interactions between plants and microbes (Marschner et al., 2011) and it has been reported that the microbial communities of the rhizosphere and bulk soil differed (Grover et al., 2015) and that eCO_2 could change the functional groups of the rhizosphere microbial communities, such as nitrogen-fixing bacteria (Xu et al., 2013). As such, it is also important to investigate the effects of eCO_2 on microbes in the rhizosphere and bulk soil separately.

In this study, we investigated the effects of elevated CO_2 on the microbial communities – including bacteria, fungi, archaea, and sulfate-reducing bacteria – in a salt marsh system with halophyte *Suaeda japonica* using TRFLP (terminal restriction fragment length polymorphism) and real-time qPCR (quantitative polymerase chain reaction).

2. Materials and methods

2.1. Microcosm of salt marsh system and sampling

Sampling was conducted in a salt marsh located at Hwangsan-do, where *Suaeda japonica* predominates. Intact soil samples were collected using PVC pots (diameter: 6 cm). Samples were transported to the lab and distributed into pots for incubation in growth chambers. Growth chambers were operated under two CO_2 concentration conditions – 380 ppm for the ambient condition and 760 ppm for the elevated condition. Forty pots were placed in each chamber. Artificial seawater (30‰; Sigma, St. Louis, MO, USA) was supplied to each pot to prevent desiccation of sediment every 3 days. Humidity was maintained 65%, and temperature was maintained 12–20 °C during incubation. Incubations lasted for 48 days and all measurements were investigated for both surface bulk soil (0–2 cm) and rhizosphere soil (4–6 cm) at approximately 2-week intervals (0 day, 8 day, 22 day, 36 day, 48 days after incubation). Surface bulk soil samples were collected by sterile spatula and plant roots were vigorously shaken by hand to remove the adhering rhizosphere soil.

2.2. Measurements of physicochemical factors and microbial activity

Soil pH was measured in slurries (soil-to distilled water ratio of 1:10) using a pH meter (Orion 3Star, ThermoScientific). Soil water

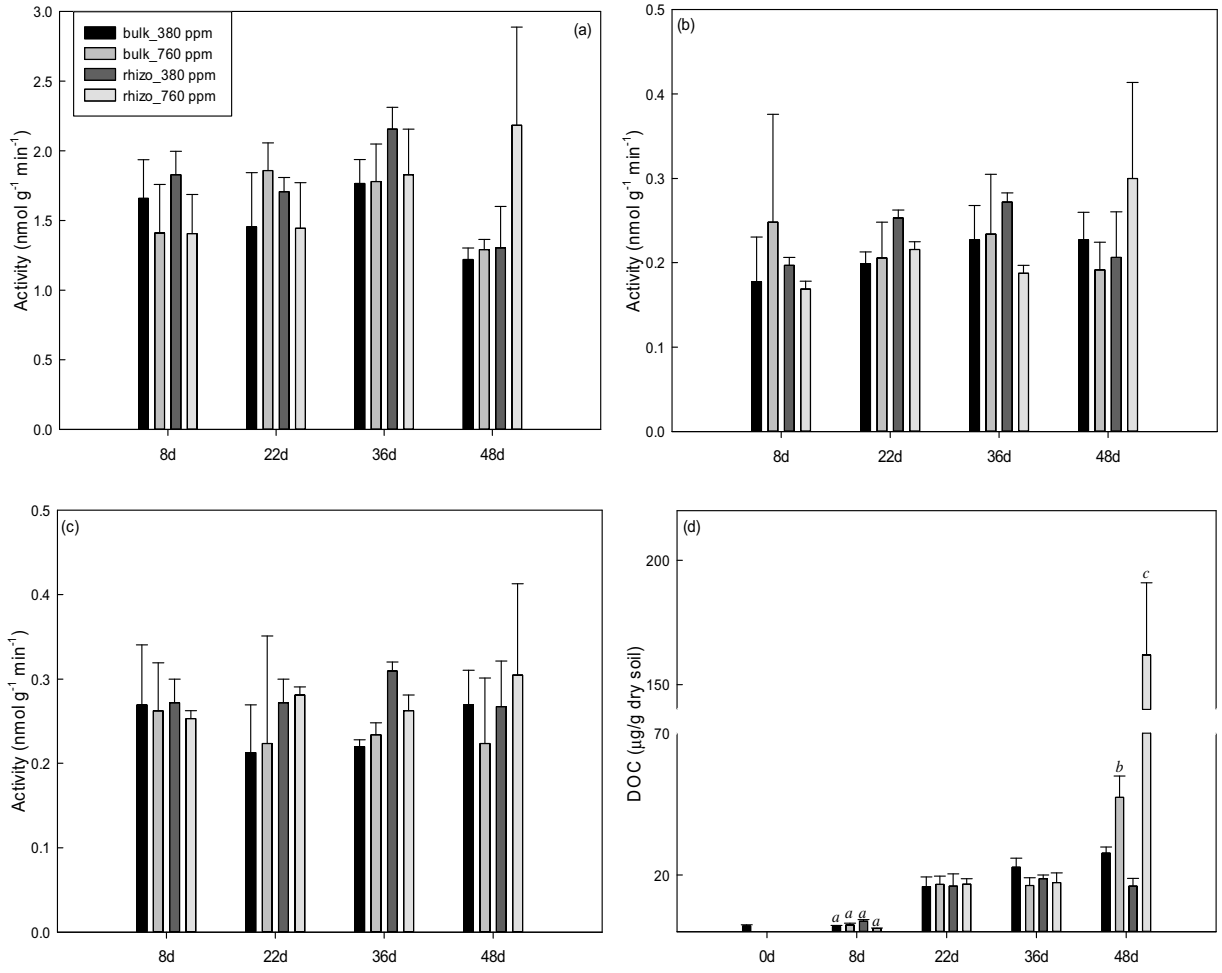

Fig. 1. Physicochemical factors of soil. (a) aminopeptidase (b) β-glucosidase (c) *N*-acetyl glucosaminidase (d) DOC. The values labeled with different letters were found by the ANOVA to be significantly different ($p < 0.05$).

content was determined as weight loss after drying overnight at 105 °C. Dissolved organic carbon (DOC) was extracted by filtering the soil slurries through a 0.2-μm filter, followed by quantification using a TOC analyzer (TOC-VCPH, Shimadzu). Extracellular enzyme activities of β-glucosidase, *N*-acetylglucosaminidase, and amino-peptidase were determined to assess general microbial activities in samples. We used methylumbelliferyl or methlycoumarin compounds as a model substrate (Hoppe, 1993; Kang and Freeman, 1999).

2.3. Molecular analysis of microbial communities in soil

2.3.1. DNA extraction and TRFLP

Soil samples collected for measurement of biogeochemical parameters were also used for DNA extraction. From about 0.5 g of each soil sample, DNA was isolated using an UltraClean Soil DNA Isolation Kit (MoBio, USA), as specified by the manufacturer. DNA samples were amplified by PCR using the fluorescently labeled forward primer 27F (5′-[6FAM]-AGAGTTTGATCCTGGCTCAG-3′) and the unlabeled reverse primer 927R (5′-CCGTCAATTCCTTTRAGTTT-3′), which target bacterial 16S rRNA genes (Lane, 1991). For the fungal community, PCR was performed using the fluorescently labeled forward primer ITS1F (5′-[HEX]-CTTGGTCATTTAGAGGAAGTAA-3′) and the unlabeled reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), which target the fungal internal transcribed spacer (ITS) region of the rRNA gene (White et al., 1990; Gardes and Bruns, 1993). For the archaeal community, PCR was performed using the unlabeled forward primer Arch109F (5′-AC(G/T)GCTCAGTAACACGT-3′) and the fluorescently labeled reverse primer Arch915R (5′-[NED]-GTGCTCCCCCGCCA ATTCCT-3′), which target the archaeal 16S rRNA gene (Großkopf et al., 1998; Stahl and Amann, 1991). For the sulfate reducing bacterial community, PCR was performed using the fluorescently labeled forward primer DSR1F (5′-AC(G/C)CACTGGAAGCACG-3′) and the unlabeled reverse primer DSR4R (5′-GTGTAGCAGTTACCGCA-3′), which target the dsrAB gene encoding for dissimilatory sulfite reductase (Wagner et al., 1998). Each PCR reaction was performed with a total volume of 50-μl reaction mixture containing approximately 100 ng of template DNA, PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris–HCl [pH 8.4], 0.1% Triton X-100), 200 μM of dNTPs, 2.0 U of *Taq* polymerase (Promega, USA), 4 μg of bovine serum albumin (Sigma, USA), and 25 pmol of each primer. All PCR was performed using a MJ Research thermal cycler PTC 100 (MJ Research, Waltham, MA) with the following program: 5 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 50 °C (for Bacteria and Fungi), 52 °C (for Archaea), or 54 °C (for SRB) and 72 °C for 1 min 30 s, and a final extension step at 72 °C for 10 min. To minimize the error due to unequivocal distribution of DNA and PCR bias, further divisions were made from each DNA sample and two independent PCR reactions were performed and pooled for each

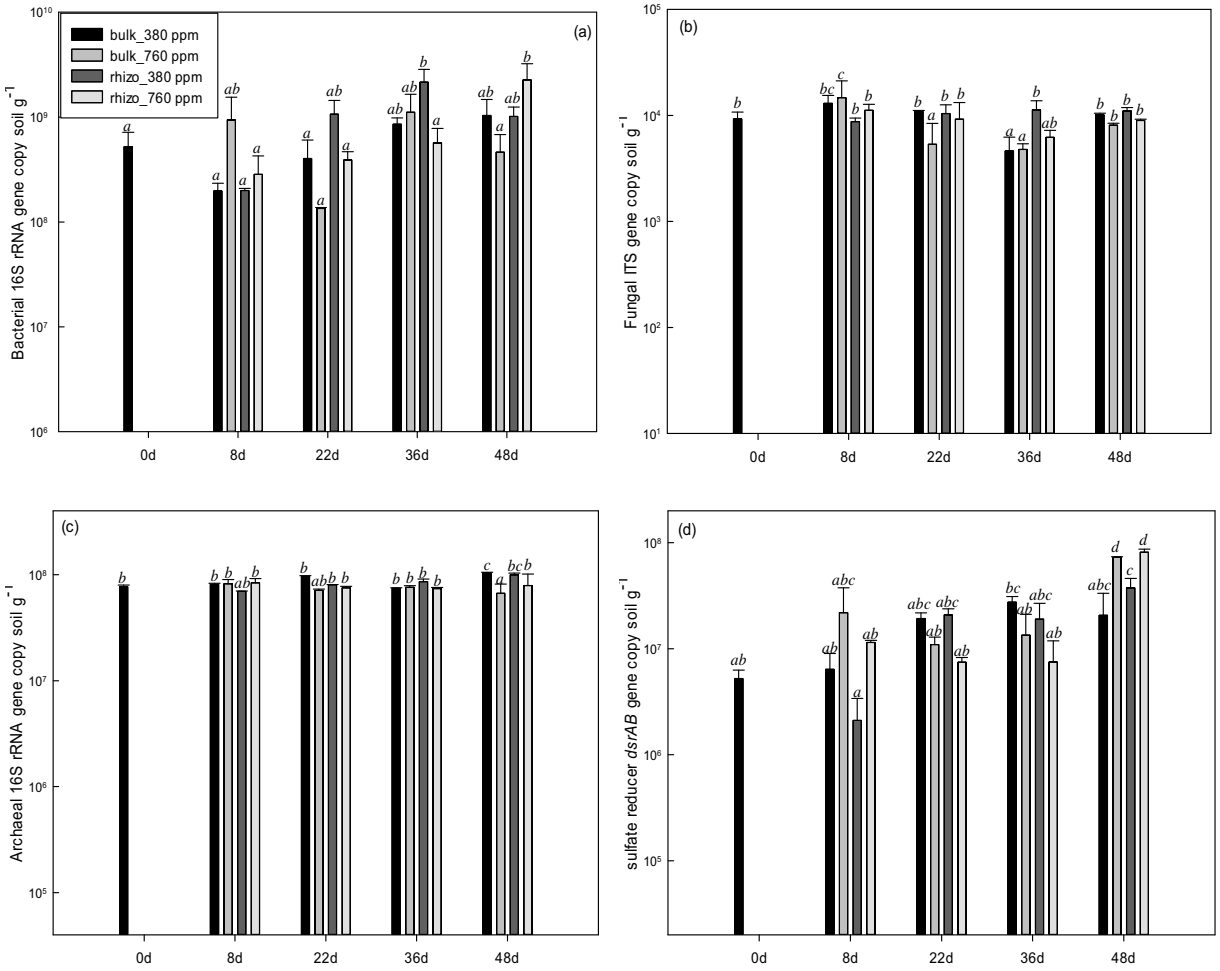

Fig. 2. Abundance of microbial community from real-time qPCR. (a) bacteria (b) fungi (c) archaea (d) sulfate-reducing bacteria. The values labeled with different letters were found by the ANOVA to be significantly different ($p < 0.05$). (bulk_380 ppm: bulk soil samples incubated at ambient CO_2 concentration (380 ppm), bulk_760 ppm: bulk soil samples incubated at elevated CO_2 concentration (760 ppm), rhizo_380 ppm: rhizosphere soil samples incubated at ambient CO_2 concentration (380 ppm), rhizo_760 ppm: rhizosphere soil samples incubated at elevated CO_2 concentration (760 ppm)).

sample. For TRFLP analysis, the pooled PCR products were purified using a NucleoSpin® Extract II PCR clean-up Gel extraction kit (MACHERY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Approximate 300 ng of purified PCR product were added to a reaction mixture (final volume 25 μl) containing 10 U of restriction endonuclease *Hha*I (Promega, Madison, WI) (for bacterial 16S rRNA and fungal ITS), *Taq*I (for archaeal 16S rRNA gene), or Sau3AI (for SRB) and incubated at 37 °C except archaea (65 °C) for 4 h. The digests were desalted using SigmaSpin Post-Reaction Purification Column (Sigma, USA), and aliquots (1 μl) were used for TRFLP analysis. The terminal fragment size analysis was performed using an ABI 3730 DNA Analyzer (Applied Biosystems) in conjunction with GeneScan software (Applied Biosystems). Terminal restriction fragments (TRFs) were quantified via peak area integration using a minimum peak height threshold of 50 relative fluorescent units. We excluded TRFs with a size less than 35 bases and calculated the proportion of each TRF in each sample. TRFs having a proportion less than 0.1% were excluded from subsequent analyses. The dominant TRFs in each microbial TRFLP profile were inferred through by *in silico* digestion of sequences of each gene present in database.

2.3.2. Real-time quantitative PCR

To estimate the bacterial, fungal, archaeal, SRB biomass, we performed qPCR using CFX96 (Bio-Rad, Hercules, CA) and SYBR Green as a detection system (Bio-Rad, USA). Each reaction in 20 μl contained the specific primer set for each group for Bacteria: 341 F (CCTACGGGAGGCAGCAG)-515R (ATTCCGCGGCTGGCA) (Lane, 1991); for Fungi: the ITS1F-ITS4 primer pair; for Archaea: Arch349F (5′-G(C/T)GCA(G/C)CAG(G/T)CG(A/C)GAA(A/T)-3′)-Arch806R (5′-GGACTAC(A/C/G)(G/C)GGGTATCTAAT-3′) (Takai and Horikoshi, 2000); for SRB: DSR1F (AC(G/C)CACTGGAAGCACG)-DSR-R(GTGG(A/C)(A/G)CCGTGCA(G/T)(A/G)TTGG) (Wagner et al., 1998; Kondo et al., 2004). The amplification followed a three-step PCR for all targeted genes: 40 cycles with denaturation at 94 °C for 25 s, primer annealing at 50 °C (for bacteria and fungi), 52 °C (for archaea), or 54 °C (for SRB) for 25 s, and extension at 72 °C for 25 s. Two independent real-time PCR assays were performed on each soil DNA extract. The standard curves were created using 10-fold dilution series of plasmids containing the bacterial 16S rRNA gene, fungal ITS region, archaeal 16S rRNA gene, and *dsrAB* gene from environmental samples for bacterial, fungal, archaeal, and SRB communities, respectively.

2.4. Statistical analysis

Results for physicochemical analyses, real-time qPCR, and ribotype number were analyzed by ANOVA using SPSS 18.00 (SPSS Inc., USA). The significance level was set at $p < 0.05$. We analyzed the TRFLP profile using multivariate methods such as nonmetric

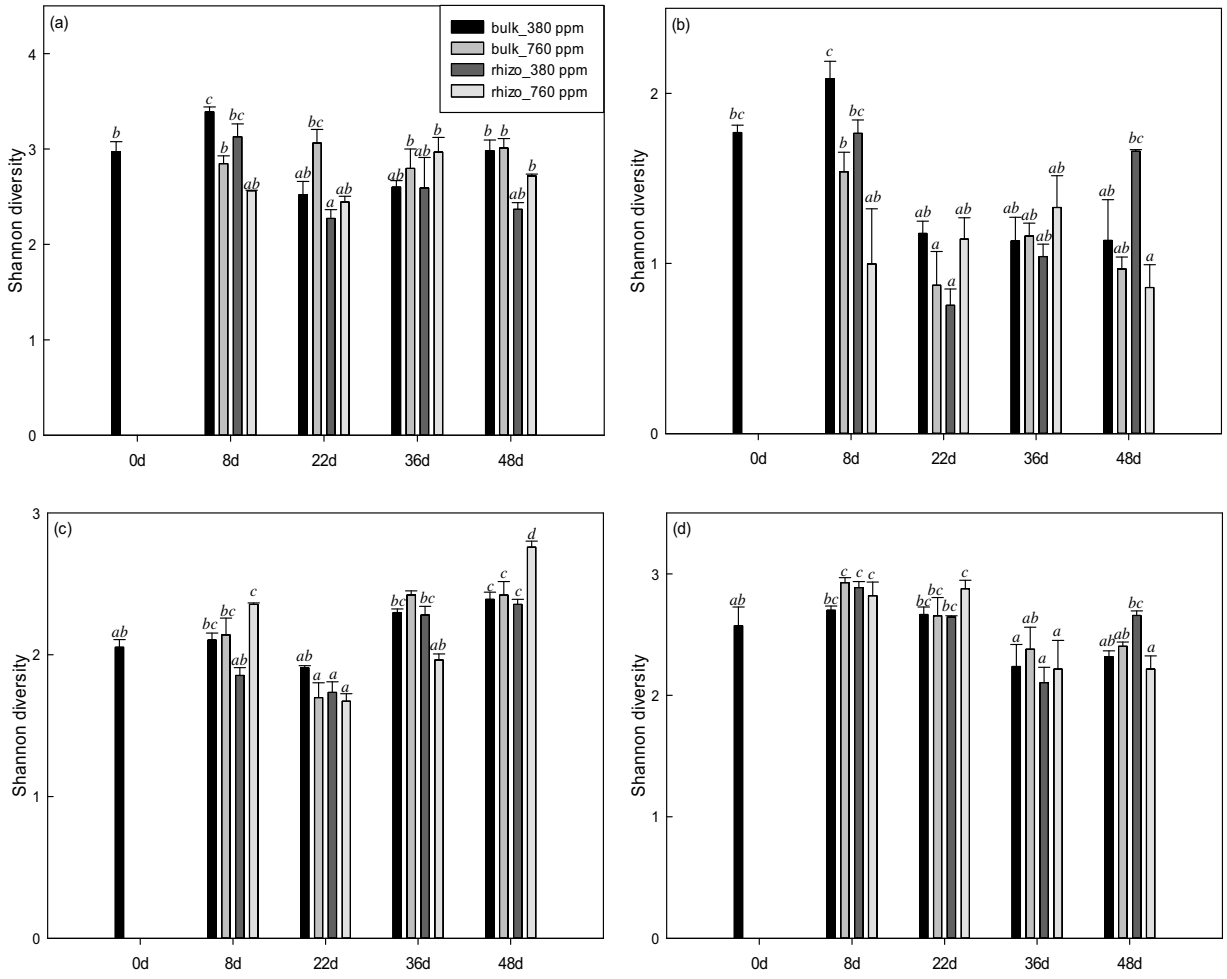

Fig. 3. Diversity of microbial community retrieved by TRFLP profile. (a) bacteria (b) fungi (c) archaea (d) sulfate-reducing bacteria. The values labeled with different letters were found by the ANOVA to be significantly different ($p < 0.05$). (bulk_380 ppm: bulk soil samples incubated at ambient CO_2 concentration (380 ppm), bulk_760 ppm: bulk soil samples incubated at elevated CO_2 concentration (760 ppm), rhizo_380 ppm: rhizosphere soil samples incubated at ambient CO_2 concentration (380 ppm), rhizo_760 ppm: rhizosphere soil samples incubated at elevated CO_2 concentration (760 ppm)).

multidimensional scaling (NMS). PC-ORD, v6.0 (MjM Software, Oregon, USA) was used for NMS and RDA, respectively.

3. Results and discussion

Our results showed that eCO_2 increased the dissolved organic carbon in both the rhizosphere and bulk soil (Fig. 1). This may have originated from the increase in plant exudate. This increase in exudates may cause the increase in bacterial abundance in the rhizosphere. The eCO_2 also increased the SRB abundance in the rhizosphere, while it decreased the abundance in the bulk soil. However, the fungal abundance was not affected by the eCO_2 and the archaeal abundance was reduced by the eCO_2 in both the rhizosphere and bulk soil (Fig. 2). For the diversity, the eCO_2 increased bacterial diversity but decreased the fungal and SRB diversity in the rhizosphere (Fig. 3).

Although the bacterial abundance was increased by eCO_2, the activities of the 3 types of extracellular enzymes were not significantly affected by the eCO_2 (Fig. 1). However, the result of correlation analysis showed that bacterial abundance was positively correlated with the DOC, β-glucosidase, aminopeptidase, and SRB, while the archaeal abundance was negatively correlated with the enzyme activity, and the fungal abundance was not correlated with any parameters (Supplementary data Table S1). This suggests that bacterial groups play a key role in the degradation of organic compounds.

In addition to the results of microbial abundance and diversity, the results of the NMS also showed differences in each microbial community's structure depending on the CO_2 concentration (ambient CO_2 vs. elevated CO_2) and soil type (rhizosphere vs. bulk soil) (Fig. 4). Overall, the eCO_2 affected the analyzed microbial communities in both the bulk and rhizosphere soil, and in general,

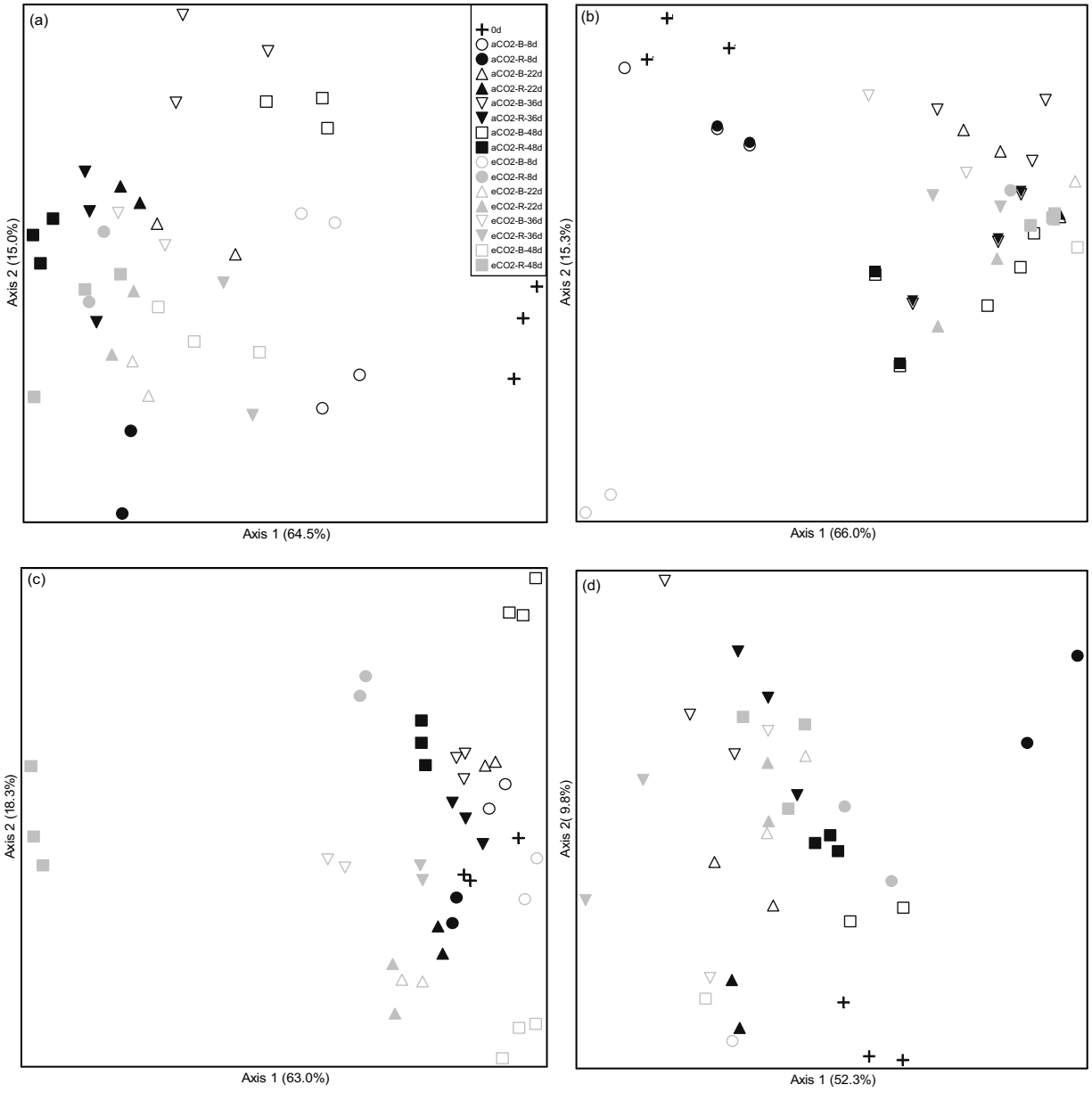

Fig. 4. Plots from NMS analysis of TRFLP profiles of microbial community. (a) bacteria (b) fungi (c) archaea (d) sulfate-reducing bacteria. Abbreviation indicates CO_2 concentration (ambient (a) or elevated (e)), soil type (bulk (B) or rhizosphere (R)), and incubation time. (e.g. aCO_2_B_8d indicates bulk soil samples incubated at ambient CO_2 concentration (380 ppm) for 8 days).

the effect of the eCO_2 was larger in the rhizosphere than in the bulk soil.

To verify the specific groups responding significantly to eCO_2 as well as playing a key role in the function of ecosystems, we also analyzed the top 10 dominant TRFs of each microbial community. For the bacterial community, the proportion of the predominant TRF (TRF 209) in the rhizosphere was not significantly changed by the eCO_2 (Fig. 5). The predominant TRF was inferred as the bacterial group affiliated with beta- or gamma-Proteobaceria (e.g. *Pseudomonas*, *Rhodobacter*, *Burkholeria*), which are known as the main group in the soil bacterial community.

For the fungal community, the proportion of the predominant TRF (TRF 129) was increased by the eCO_2 (Fig. 5). The predominant TRF was inferred as the fungal group affiliated with *Sordariales*, which have been identified as the dominant group in tidal marshes (Weber et al., 2011).

For the archaeal community, the predominant TRF (TRF 183) was inferred as the one affiliated with the Crenarchaeota group, and its proportion was decreased by the eCO_2, although the decrease was not significant (Fig. 5). The compositions of the overall dominant groups in the archaeal community of the rhizosphere were significantly modified by the eCO_2.

For sulfate-reducing bacteria, the dominant groups were increased by the eCO_2 in the rhizosphere (Fig. 5). The predominant TRF (TRF 212) was inferred as the SRB group with the highest activity (*Desulfotomaculum*), which is known to inhabit estuaries with low salinity (Leloup et al., 2006).

The abundance in specific SRB groups that can easily absorb nutrients would be increased by the eCO_2, while the overall diversity would be reduced in the rhizosphere. It has been reported that the SRB diversity and composition vary according to environmental conditions including the organic matter and sulfate concentration (Leloup et al., 2006). In our study, the TRFs (TRF 66 and TRF 170) that were seen to increase under eCO_2 conditions were inferred to be an SRB group with low activity (*Desulfobacterales*). The latter are known to prefer high nutrient conditions, and the increase in exudate caused by the eCO_2 may have driven the increase in this group.

Overall, the bacteria and SRB abundances showed similar patterns, while the archaeal abundance showed a contrasting pattern. In addition, the bacterial, fungal, and SRB diversities showed similar patterns throughout the experiment, while the archaeal diversity showed a contrasting pattern. This implies that there may be competition between the bacterial and archaeal communities, and that this competition may be more severe in eCO_2 conditions.

Due to the competition between bacteria and archaea, the archaeal abundance was reduced but the acclimation of the archaeal community to low nutrient conditions led to an increase in diversity. Actually, our results showed that the abundance in the predominant

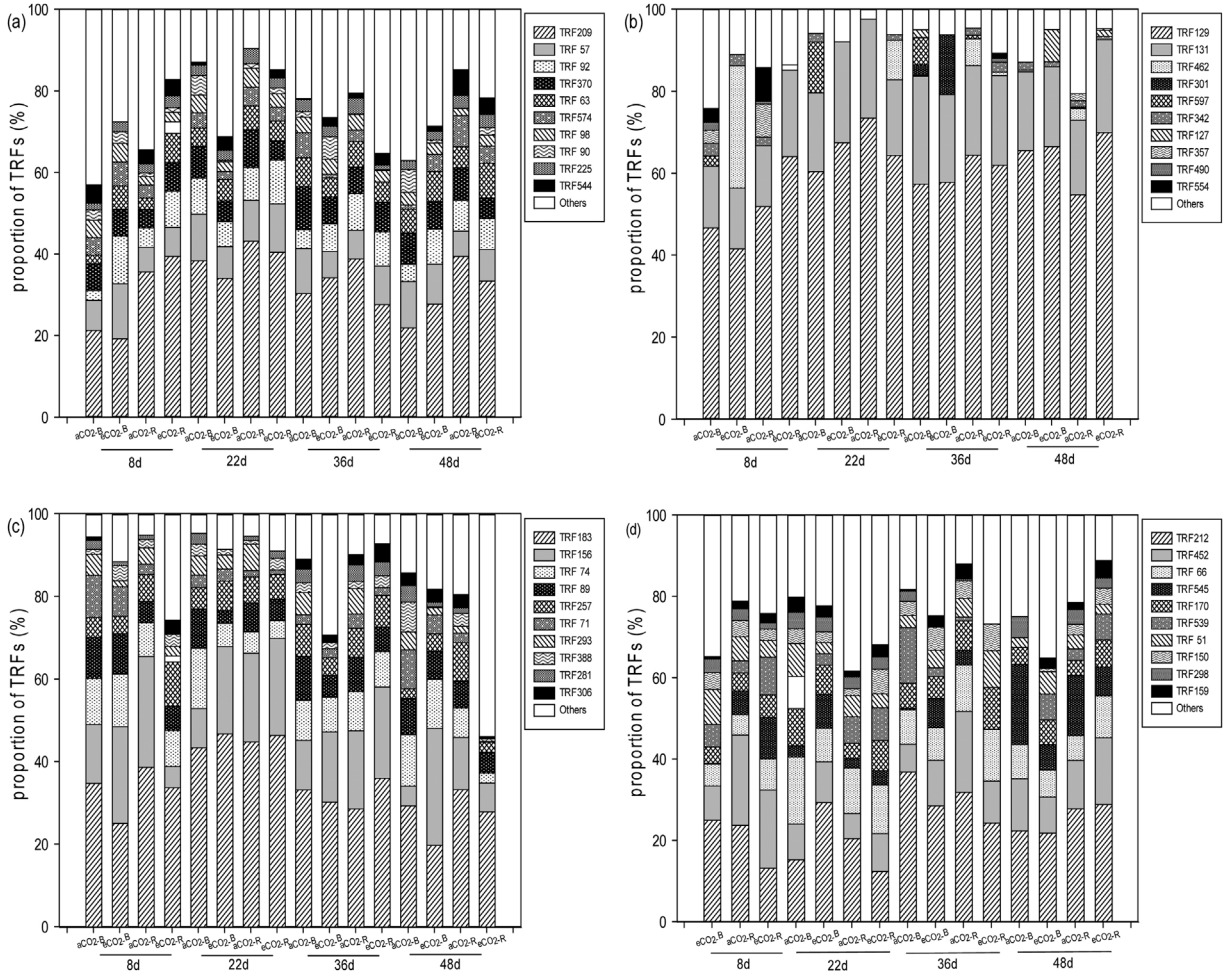

Fig. 5. Proportion of TRFs in microbial community. (a) bacteria (b) fungi (c) archaea (d) sulfate-reducing bacteria. Abbreviation indicates CO_2 concentration (ambient (a) or elevated (e)) and soil type (bulk (B) or rhizosphere (R)) (e.g. aCO_2-B indicates bulk soil samples incubated at ambient CO_2 concentration (380 ppm)).

TRF of the archaeal community was significantly reduced by the eCO_2, and that other members that could compete or co-exist with the bacterial groups became dominant. A recent study also reported that the bacterial and archaeal populations seemed to compete for the same resources (Seyler et al., 2014).

Overall, our results suggest that under eCO_2 conditions, the bacterial community may outcompete the archaeal community in the rhizosphere of halophyte. This implies that a dramatic change in environmental factors such as CO_2 concentration may temporarily disturb the balance of the microbial communities.

It has been reported that the sulfate reduction rate (SRR) in freshwater wetlands is often comparable to that of marine sediments (Leloup et al., 2006). Our results showed that the overall bacteria and SRB abundances were increased by the eCO_2, which suggested that SRB are important components of the salt marsh bacterial community. This implies that the SRB group plays a central role in organic carbon decomposition in salt marshes under eCO_2 conditions. Although we did not measure the CH_4 emissions and methanogen abundance, as the SRB and methanogen communities compete with one another, our results suggest that eCO_2 stimulates overall organic matter decomposition but does not drive an increase in CH_4 emissions in salt marshes.

Our results showed that the microbial community in salt marsh with halophytes was affected by the eCO_2 even within a very short space of time and, as expected, the rhizosphere's microbial community was sensitive to eCO_2. This is in line with the well-known observation that the effect of eCO_2 on the soil microbial community is indirect and is mediated by the vegetation (Kohler et al., 2010; Lagomarsino et al., 2007).

Although the microbial abundance and communities were modified by the eCO_2, the shift in the microbial abundance and communities did not directly or significantly lead to a change in the overall microbial activity. This may be due to the functional redundancy of the microbial community, or to the fact that the experimental durations were too short to observe a different metabolic activity.

In conclusion, in salt marsh systems with halophytes, eCO_2 mainly causes a strong change in the microbial community, but the overall functional activity of the microbial community is balanced by the different response patterns of the bacterial, fungal, and archaeal communities. To obtain more information about the effects of eCO_2 on microbial communities, further studies including the analysis of activity of functional microbial groups would be also desirable.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.09.009.

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