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Elevated CO_2 alters the structure of the bacterial community assimilating plant-derived carbon in the rhizosphere of soya bean

Y. H. WANG[a, b†], Z. H. YU[a†], Y. S. LI[a], G. H. WANG[a], C. TANG[c], X. B. LIU[a], J. J. LIU[a], Z. H. XIE[a] & J. JIN[a,c]

[a] *Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China*

[b] *Guiyang University of Chinese Medicine, Guiyang 550025, China*

[c] *Centre for AgriBioscience, La Trobe University, Melbourne Campus, Bundoora, Vic 3086, Australia*

Correspondence: J. JIN. Email: jinjian29@hotmail.com

[†] These authors contributed equally to the work.

Summary

Elevated CO_2 (eCO_2) increases rhizodeposits, which in turn alters the soil microbial community. However, it is relatively unknown how the microbial community metabolizes plant-derived carbon (C) in the rhizosphere under eCO_2, especially in agricultural soils. This study used a $^{13}CO_2$ labelling technique combined with DNA-stable isotope probing (SIP) to fractionate the ^{13}C-DNA and ^{12}C-DNA from the rhizosphere of soya bean plants (*Glycine max* (L.) Merr. cv. Suinong 14) grown for 54 days under ambient CO_2 (aCO_2) (390 μmol mol^{-1}) or eCO_2 (550 μmol mol^{-1}). The DNA fractions were then subjected to Illumina Miseq sequencing. The results showed that eCO_2 decreased the richness and diversity of the ^{13}C-assimilating bacterial community compared to aCO_2 ($p < 0.05$). Elevated CO_2 decreased the abundances of genera including *Pseudarthrobacter*, *Gaiellales_uncultured*, *Microlunatus*, *Gemmatimonas*, *Gemmatimonadaceae_uncultured*, *Ramlibacter*, *Massilia*, *Luteimonas*, *Acidobacteriaceae_uncultured*, *Bryobacter* and *Candidatus_Solibacter*. These genera were probably fast-growing bacteria and sensitive to labile C. In contrast, eCO_2 stimulated the growth of genera *Novosphingobium*, *Acidimicrobiales_uncultured*, *Bacillus*, *Flavisolibacter* and *Schlesneria* which were able to assimilate complex C compounds. Moreover, the increased population of *Novosphingobium* under eCO_2 might have accelerated electron flow from the oxidation of organic C. Correspondingly, eCO_2 did not affect the concentration of the dissolved organic C but increased the plant-derived ^{13}C in the rhizosphere. These results indicated that an eCO_2-induced increase in non-labile C in rhizodeposits contributed to the increase in the population size of a number of the plant-C metabolizing genera that might become the mechanism for the turnover of fresh C in the rhizosphere, modifying the soil C cycle under eCO_2 environments.

Keywords: Mollisols; Stable Isotope Probing; ^{13}C labelling; Illumina Sequencing; Microbial Community

Introduction

The efflux of plant-derived carbon (C) into the rhizosphere (root–soil interface) stimulates microbial activity (Kuzyakov & Blagodatskaya, 2015). This C efflux contributes to 4 to 20-fold increases in microbial biomass and up to 3-fold increases in the rates of decomposition of organic substrates in the rhizosphere compared to the bulk soil (Blagodatskaya *et al*., 2009). Plant roots release up to 20–30% of total photosynthetically-fixed C into the rhizosphere (Calvo *et al*., 2017), which is metabolized by microorganisms and incorporated into soil C pools. However, elevated atmospheric CO_2 (eCO_2) concentration not only increases the quantity of rhizodeposits but also alters their quality in many plant species (Jin *et al*., 2014). Elevated CO_2 generally decreases the proportion of nitrogen (N)-rich metabolites but increases the proportion of C-rich metabolites in rhizodeposits, increasing the C/N ratios of rhizodeposits (Grayston *et al*., 1998). The eCO_2-induced changes in the quantity and quality of rhizodeposits are likely to affect the structure and functions of the soil microbial community and hence the soil C cycle.

A number of studies have indicated that the response of the soil microbial community to eCO_2 is associated with altered soil C cycling (Drigo *et al*., 2008). Using an experiment where 16 herbaceous plant species had been grown under FACE (Free Air CO_2 Enrichment) for 9 years, Xu *et al*. (2013) showed that eCO_2 significantly increased the abundance of C-degrading genes that are involved in the breakdown of starch, cellulose and hemicellulose in soil compared to ambient CO_2 (aCO_2). He *et al.* (2014) further revealed that eCO_2 stimulated the expression of key functional genes involved in C decomposition in a fine-silty Typic Endoaquoll, thereby highlighting the importance of soil microorganisms in regulating the turnover of rhizodeposits under eCO_2.

The DNA stable isotope probing (DNA-SIP) techniques were recently used to track plant-C flow into the microbial community in the rhizosphere of sugarcane grown under eCO_2, and showed an eCO_2-induced increase in the populations of Bacilli and Betaproteobacteria genera in comparison to aCO_2 (Da Costa *et al*., 2018). However, because of the complex nature of soil properties and their interactions with different plant species, the response of soil microbial communities associated with C cycling in response to eCO_2 may differ between ecosystems. Therefore, further research is required in agricultural systems where the effects of eCO_2 on soil microbial community structure are largely unknown.

Mollisols are a major soil type used for growing soya bean in northeast China (Sui *et al*., 2017). However, soil organic C (SOC) in Mollisols has markedly decreased (up to 50%) with intensive farming practices during the past five decades (Tong *et al*., 2017). The increase of atmospheric CO_2 concentrations associated with global climate change poses great uncertainty on predicted SOC stocks. Therefore, it is essential to understand how eCO_2 affects plant-derived C fluxes into the microbial community in the rhizosphere of widely-grown crops like soya bean. Although Yu *et al* (2016) reported that eCO_2 (550 μmol mol^{-1}) increased the abundances of bacterial genera such as *Arthrobacter*, *Catelliglo-bosispora*, *Bryobacter*, *Bradyrhizobium* and

Pedomicrobium in the rhizosphere of soya bean, knowledge is still limited with respect to the eCO_2-induced changes of specific members of the bacterial community that actively metabolize plant-C. This study aimed to investigate the influence of eCO_2 on the plant-C metabolizing bacterial community in the rhizosphere of soya bean growing in a Mollisol, and to assess the potential contribution of these bacteria to plant-C turnover under eCO_2.

Materials and methods

Experimental set-up

Using a soil sampler, soil was collected from the tillage layer (0–10 cm) at a farmer's paddock in Hailun (47º26´N, 126º38´E), Heilongjiang Province, northeast China, where soya bean is a major crop. The soil is classified as a Mollisol or Phaeozem (FAO-UNESCO, 1974). The soil was air-dried at room temperature and sieved (< 2 mm) prior to mixing with siliceous sand (1:1, w:w) to aid collection of the rhizosphere soil at harvest (Jin *et al*., 2014). Each pot (height 15 cm, diameter 12 cm) contained 1.5 kg of the soil and sand mixture, which was supplied with basal nutrients at the following rates (mg kg^{-1} soil): 217 urea, 219 KH_2PO_4, 167 $CaCl_2 \cdot 2H_2O$, 43 $MgSO_4 \cdot 7H_2O$, 6.7 $MnSO_4 \cdot H_2O$, 10 $ZnSO_4 \cdot 7H_2O$, 2 $CuSO_4 \cdot 5H_2O$, 0.3 $CoSO_4 \cdot 7H_2O$, 0.7 H_3BO_3, 0.2 $Na_2MoO_4 \cdot 2H_2O$ and 9 Fe-EDTA (Jin *et al*., 2009). Soil water content was maintained at 80 ± 5% of field water capacity by daily additions of deionized water to maintain the target weight.

A soya bean variety [*Glycine max* (L.) Merr. cultivar Suinong 14] which is widely cropped in northeast China was used in this study. Seven uniform seeds were sown in each pot and thinned to two plants seven days after emergence. Then, a total of 12 pots were randomly allocated into six open-top chambers (OTC) with two pots per OTC. Three OTCs were for aCO_2 (390 ± 30 μmol mol^{-1}) and the other three for eCO_2 (550 ± 30 μmol mol^{-1}). The OTC was an octagonal steel frame with a 3.5-m diameter, 2.0-m high and canopy at a 45° angle and 0.5-m high (Zhang *et al*., 2014). Polyethylene sheet with more than 95% of transparency was used to construct the OTC. The atmospheric CO_2 level in each OTC was regulated by a digital CO_2-regulating system (VK2010, Victecher, Beijing, China). An electronic pump was installed at the ground level in each OTC to facilitate the circulation of inner air. More information about the OTC design can be obtained in Li *et al.* (2017).

The $^{13}CO_2$ labelling

Soya bean plants were labelled with $^{13}CO_2$ for 25 days commencing at Day 29 (the third node stage, V_3). The labelling was carried out in air-tight clear polymethyl methacrylate chambers (area 50 cm ×50 cm, height 40 cm). Three pots from each treatment were placed in the labelling chambers and labelled with $^{13}CO_2$ of the same concentration as the respective CO_2 treatment. Before labelling, the $^{12}CO_2$ in the chambers was depleted by filtering air through 3 M NaOH solution for 30 minutes using a membrane pump (MOD.LS30L, Guangli Inc., Wuhan, China). Then, six beakers containing the calculated amount of $Na_2{}^{13}CO_3$ (≥ 99.8 atom%, Sigma-Aldrich, St Louis, MO, USA) were placed in each of the six chambers, and 9 M H_2SO_4 was injected with a syringe needle through a rubber tube into each beaker every 90 minutes for 8 hours daily. The amount of $Na_2{}^{13}CO_3$ for the labelling procedure was

calculated based on the designated CO_2 concentrations and the CO_2 assimilation by plants in the chamber (Lian *et al*., 2017). To determine the assimilation rate of CO_2 in the chambers, non-labelled controls (three replicates in each treatment) were set up in an additional two chambers under the same conditions using $Na_2{}^{12}CO_3$ to produce non-labelled CO_2 (Yu *et al*., 2017). The $^{12}CO_2$ concentration inside labelling chambers was monitored with a CO_2 analyser (CI-301PS; CID Bio-Science Inc., Gamas, Washington, USA). The labelling chambers were equipped with electric fans to homogenize the atmosphere inside the chambers. The other environmental conditions in chambers were a photosynthetically-active photon flux density of approximately 1000 μE m^{-2} s^{-1}, the temperature at 27−29ºC and relative humidity of 60 ± 5%.

After labelling for 25 days the soya bean plants were harvested (Day 54). Shoots were cut at the ground level. Roots were carefully removed from pots, and the rhizosphere soil was sampled by gently shaking the roots (Jin *et al*., 2014). Plant samples were oven-dried at 60ºC for 72 hours, weighed and ground in a ball mill (Retsol MM2000, Retsch, Haan, Germany). Approximately 2 g of the rhizosphere soil was immediately frozen in liquid N and then kept at –80ºC until DNA extraction. The remaining soil in each sample was separated into two parts. Approximately 20 g of fresh soil was used for measurements of microbial biomass C (MBC), dissolved organic C (DOC), ammonium (NH_4^+) and nitrate (NO_3^-), and approximately 10 g of soil was air-dried for measurements of Olsen phosphorus (P), available K, total C, total N and $\delta^{13}C$.

Microbial biomass C was determined using the fumigation method (Vance, 1987). Total organic C (TOC) in soil extracts (1:4 in 0.5 M K_2SO_4) was determined using a TOC analyser (Multi N/C 2100, Analytik Jena, Germany). The value of MBC was calculated as the difference in TOC concentration between fumigated and non-fumigated soils. Total organic C in the extracts of non-fumigated soil was considered as DOC (Chen *et al*., 2016). The NH_4^+-N and NO_3^--N were extracted in 2 M KCl solution (1:10), and their concentrations were measured using a Continuous Flow Analyzer (San^{++}, Skalar, Holland). Olsen P in soil was extracted in 0.5 M $NaHCO_3$ (1:20) and determined with the Mo-Sb colorimetric method (Olsen *et al*., 1954). The soil available K was extracted in 1 M NH_4Ac (1:10) and determined with flame photometry (FP640, INASA, China). Total C and N concentrations of soil samples were analysed using an EL III Elemental Analyzer (Hanau, Germany), and the $\delta^{13}C$ was determined using a MAT 253 isotope ratio mass spectrometer (Thermo Fisher, Germany). Using a Wettler Toledo 320 pH meter, pH was measured after shaking with 0.1 M CaCl solution (1:5 = w:v) for 30 min.

Soil DNA extraction and isopycnic centrifugation

Total DNA in soil was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals; Solon, OH, USA). In the process of DNA extraction, water samples were included as the negative control to test potential contamination. Three technical replicates of each soil sample were performed to provide a sufficient amount of DNA for isopycnic centrifugation. The soil DNA quality was assessed by gel electrophoresis, and DNA

was quantified by Nanodrop 2000 spectrophotometer (Bio-Rad Laboratories Inc., Hercules, USA). Density gradient isopycnic centrifugation was performed using 7000 ng soil total DNA, together with caesium trifluoroacetate (CsTFA, Sigma, USA) and gradient buffer (0.1 M Tris HCl, 0.1 M KCl, 1 mM EDTA, pH = 8.0) to reach a density of 1.60 g mL^{-1}. Subsequently, the centrifugation was performed in a Beckman Coulter Optima L-XP ultracentrifuge on a VTi 65 rotor (Beckman Coulter, Brea, California, USA) at 179 000 *g* for 40 hours at 20ºC. Then, the centrifuged solution was fractionated into 14 fractions. The buoyant density of each fraction was determined with a digital refractometer (AR200 Digital Handheld Refractometer, Depew, New York, USA). DNA in each fraction was precipitated with ice-ethanol and sodium acetate and then washed with 70% ethanol after being centrifuged at 15 000 *g* for 1 hour. The DNA was finally re-suspended in autoclaved Milli-Q water for further use.

The DNA purification and Illumina MiSeq sequencing

The fractions from 2 to 4 (^{13}C-DNA) and those from 8 to 10 (^{12}C-DNA) were pooled separately. The DNA templates were amplified with primers of 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Biddle *et al*., 2008). The forward primer was modified by a unique 6 nt barcode at the 5′ end. The PCR was performed under the following conditions: initial denaturation at 95ºC for 10 minutes, followed by 40 cycles of denaturation at 95ºC for 15 s, annealing at 60ºC for 10 s and extension at 72ºC for 10 s, with a final extension at 72ºC for 10 minutes. The amplification was performed in duplicate, then PCR products were pooled together and purified with an Agarose Gel DNA purification kit (TaKaRa, Dalian, China). The purified PCR products were submitted to Biozeron (Shanghai, China) for paired-end sequencing on an Illumina MiSeq platform (Caporaso *et al*., 2012).

Analysis of Illumina MiSeq sequencing data

Raw data were processed by the QIIME software package (version 1.8.0, Caporaso *et al*., 2010). The reads were quality-trimmed by eliminating sequence length shorter than 380 bp and quality scores lower than 20. In total, 445 928 high quality and chimera-free reads with an average length of 392 bp were obtained. The number of sequences ranged from 30 357 to 43 254 across samples. We randomly selected 30 357 sequences based on the minimum reads among all samples before further analysis. The operational taxonomic units (OTUs) were defined at the 97% similarity level by Usearch (vsesion 7.1 http://drive5.com/uparse/) with the remaining and unique sequences. In total, 1172 OTUs were finally obtained. At 70% of the confidence threshold, the taxonomic identity of phylotypes was classified using the Ribosomal Database Project RDP Classifier (Release 11.1 http://rdp.cme.msu.edu/). All sequences were deposited in the GenBank Sequence Read Archive SRP116808.

Statistical analysis

Statistical comparisons between two CO_2 treatments were performed for soya bean shoot and root dry weights, root-to-shoot biomass ratio, soil biochemical properties, number of bacterial OTUs and alpha diversity in the ^{13}C-DNA fraction using Student's *t*-test (two-tailed) at the 0.05 significance level (SPSS 20.0 for Windows).

Changes in microbial community structure in the ^{13}C-DNA and ^{12}C-DNA fractions in response to eCO_2 were analysed with principal component analysis (PCA) using the vegan package in R version 3.3.1 for Windows (R Development Core Team, 2010). The PCA analysis was done on the sums-of-squares-and-products matrix (S). To assess the significance of the difference in the microbial community composition between aCO_2 and eCO_2, permutational multivariate ANOVA (PERMANOVA) was conducted using the ADONIS function in the R package of vegan (ADONIS; Oksanen *et al*., 2014). Genera and OTUs with more than 0.3% of the relative abundance in the ^{13}C-DNA fraction were selected and Student's *t*-test (two-tailed) was conducted to test the difference between two CO_2 treatments for each genus or OUT, using GenStat (version 13.0, VSN International, Hemel Hemspstead, UK). Genera affiliated to 'unclassified' and 'norank' were excluded in results of this study.

Results

Biochemical properties of the rhizosphere soil

In this study, the soil had a total C of 17.2 g kg^{-1}, total N of 0.98 g kg^{-1}, Olsen P of 25.3 mg kg^{-1}, NH_4Ac-extractable potassium (K) of 157 mg kg^{-1} and a pH of 5.1. The soil ^{13}C abundance in the rhizosphere under eCO_2 was 2.7-fold greater than that under aCO_2. Elevated CO_2 increased MBC by 32% ($p < 0.05$), but decreased available K in the rhizosphere ($p < 0.05$) (Table 1). There was no significant CO_2 effect on concentrations of total C, total N, DOC, NH_4^+-N, NO_3^--N and Olsen P, and C/N ratio in the rhizosphere soil. In terms of plant growth, eCO_2 increased the dry weights of shoots and roots by 19 and 46%, respectively ($p < 0.05$), but not root-to-shoot ratios (Figure S1, Supporting Information).

Separation of ^{13}C-DNA

There were no bands in DNA fractions 2–4 for the non-labelled control, whereas bands in these fractions were observed in the ^{13}C-labelled treatment (Figure S2, Supporting Information). This indicated that ^{13}C-DNA (high buoyant-density fractions) was successfully separated. The DNA bands in fractions 8–10 (low buoyant-density fractions) were ^{12}C-DNA.

Bacterial community composition in the ^{13}C-DNA fraction

Elevated CO_2 decreased the number of OTUs, Ace estimator, Chao1 estimator and Shannon index in the ^{13}C-DNA fraction by 10, 7, 7 and 18%, respectively (Table 2) ($p < 0.01$). In the PCA, the first and second principal components explained 59.3% and 23.0% of the variance, respectively. The scores from the first two principal component analysis were plotted in their plane. They show that the bacterial community in the ^{13}C-DNA fraction ($p < 0.05$, PERMANOVA) but not the ^{12}C-DNA fraction ($p > 0.05$, PERMANOVA) significantly differed between the CO_2 treatments (Figure 1).

Genera in the ^{13}C-DNA fraction in response to eCO_2

With the exclusion of the 'unclassified' or 'norank' genera, 35 genera affiliated to ten phyla in the ^{13}C-DNA fraction showed a significant response to eCO_2 (Figure 2). Actinobacteria (8.0–13.6%), Gemmatimonadetes (5.6–11.1%), Proteobacteria (10.8–25.7%) and Acidobacteria (2.5–7.2%) were the predominant phyla (Figure 2), and also the main phyla that had responded to eCO_2. Within Actinobacteria and

Proteobacteria, the relative abundances of most genera such as *Pseudarthrobacter*, *Gaiellales_uncultured*, *Microlunatus*, *Ramlibacter*, *Massilia* and *Luteimonas* in the ^{13}C-DNA fraction decreased under eCO_2 ($p < 0.05$) (Figure 2, Table S1, Supporting Information), while the relative abundances of *Novosphingobium* and *Acidimicrobiales_uncultured* increased from 0.2 to 21.8% and from 0.2 to 0.4% in response to eCO_2, respectively. In Firmicutes and Bacteroidetes, eCO_2 increased the relative abundances of genera from 0.7 to 2.1% for *Bacillus*, and from 0.1 to 0.2% for *Flavisolibacter*. Elevated CO_2 decreased the relative abundances of genera in other phyla, such as Gemmatimonadetes, Acidobacteria, Armatimonadetes, Chloroflexi, Planctomycetes and Nitrospirae ($p < 0.05$).

Discussion

This study has shown for the first time that eCO_2 significantly reduced the richness and diversity of the bacterial community that metabolized plant-derived C in the rhizosphere of soya bean. It was evident that eCO_2 decreased the number of OTUs, Chao1 estimator and Shannon index in the ^{13}C-DNA fraction (Table 2). This result was consistent with a previous study that reported that eCO_2 significantly decreased the soil bacterial diversity in a rice paddy Fluvisol, which was considered to be partially due to an increase in the abundance of Burkholderiaceae (14.9–46.5%) under eCO_2 (Okubo *et al*., 2015). In this present study, the competitive growth of *Novosphingobium* might have constrained the responses of other genera in response to eCO_2 (Table 2, Figure 2) and decreased the evenness of the species distribution. This pattern of competitive diversity has been shown to decrease diversity of the microbial community (Torsvik *et al*., 2002). Furthermore, as eCO_2 probably increases the C/N ratio of rhizodeposits (Grayston *et al*., 1998) and increased the C/N ratio in soya bean roots (Li *et al*., 2017), the activity of fast-growing *r*-strategists which are the dominant microbial population in the rhizosphere might be relatively constrained by N deficiency (Chen *et al*., 2014) leading to the observed decrease in the richness and diversity of plant-C-metabolizing bacterial community under eCO_2.

Compared to aCO_2, eCO_2 fundamentally altered the structure of the plant-C metabolizing bacterial community in the rhizosphere of soya bean (Figure 1), indicating the potential shift of their eco-functions, especially C flow from the plant root to the soil. At the genus level, the populations of *Novosphingobium*, *Acidimicrobiales_uncultured*, *Bacillus*, *Flavisolibacter* and *Schlesneria* were larger under eCO_2 (Figure 2). Genera *Novosphingobium*, *Bacillus* and *Schlesneria* affiliated to orders of Sphingomonadales, Bacillales and Planctomycetales, respectively, can degrade complex C compounds (Eichorst *et al*., 2012; Wang *et al*., 2015). *Bacillus* is known as proteolytic bacteria, and is considered to be an important protease producer in soils (Sakurai *et al*., 2007), whereas *Novosphingobium* and *Schlesneria* were reported to be able to metabolize a wide range of xenobiotic aromatic compounds (Liu *et al*., 2005), and degrade various heteropolysaccharides (Dedysh, 2011). Moreover, *Novosphingobium* genera were found to be able to produce P450 enzymes that act as electron acceptors (Yang *et al*., 2010). Many members of the *Novosphingobium* genera are capable of competing for electrons to reduce nitrate to

nitrite (Nguyen *et al*., 2016), and the reduction of nitrate largely depends on the oxidation of organic C to supply electrons. Under eCO_2, increased amounts of C released from roots (Table 1) might act as an electron donor that accelerates the electron flow. *Novosphingobium*, therefore, may be stimulated under eCO_2 to reduce nitrate to nitrous oxide, and provide more energy for microbial metabolism during the process (Ribera-Guardia *et al*., 2014). The increased abundance of *Novosphingobium* DNA in the ^{13}C-enriched DNA fraction under eCO_2 indicated that this genus is important in the biodegradation of complex substrates and the acceleration of electron flow in the rhizosphere, consequently contributing to changes in soil C and N cycling in response to eCO_2.

The eCO_2-induced response of some genera might be temporal, as the positive response of genera *Acidimicrobiales_uncultured* (Acidimicrobiales) and *Flavisolibacter* (Bacteroidetes) to eCO_2 was inconsistent with the result reported by Yu *et al*. (2016). The different responses of the two genera to eCO_2 might be attributed to the different plant growth stages between the two studies (the initial podding stage in the study of Yu *et al*. compared to the third-node stage in this study) as conditions in the rhizosphere differ between growth stages because of changes in root exudation and functions (Li *et al*., 2017; Xu *et al*., 2017). Thus, the extent of change in bacterial communities of the rhizosphere in response to eCO_2 might depend on the changes of the plant-C efflux over time. The eCO_2-induced changes in plant-derived compounds in rhizodeposits might be the primary factor for stronger competition for plant-C by complex-substrate assimilators (Figure 2). However, this assumption needs further investigation by identifying the composition of rhizodeposits and tracing the bacteria that metabolize these compounds in soils in response to eCO_2.

A number of dominant plant-C metabolizing genera in the rhizosphere under aCO_2 were relatively constrained under eCO_2. This constraint might be attributed to the decreased degradability of rhizodeposits. These genera including *Pseudarthrobacter*, *Gaiellales_uncultured*, *Gemmatimonas* and *Acidobacteriaceae_uncultured* belong to the phyla Actinobacteria, Gemmatimonadetes, Proteobacteria and Acidobacteria (Figure 2) that are usually competitive and fast-growing bacteria in the rhizosphere of many plant species (Edwards *et al*., 2014; Mao *et al*., 2014; Naz *et al*., 2014) including rice (*Oryza sativa* L.) (Okubo *et al*., 2015), grasses (Hayden *et al*., 2012) and shrubs (Lesaulnier *et al*., 2008). Moreover, several studies reported that genera *Bradyrhizobium* and *Lysobacter* in the rhizosphere of switchgrass (*Panicum virgatum* L.) (Mao *et al*., 2014) and soya bean (Figure 2), and genera *Actinobacteria*, *Gemmatimonas* and *Gemmatimonadetes* in the rhizosphere of rice (Edwards *et al*., 2014) were the dominant users of rhizodeposits, and sensitive to the changes in quality of rhizodeposits (Grover *et al*., 2015). Although eCO_2 increased the plant-C efflux into the rhizosphere, it did not affect the concentration of DOC (Table 1). The result suggests that eCO_2 probably decreased the proportion of labile C in the rhizodeposits, thereby causing the relative suppression of fast-growing *r*-strategists.

Conclusions

Elevated CO_2 significantly decreased the richness and diversity of bacteria that utilized plant-C in the rhizosphere of soya bean grown in a Mollisol. Elevated CO_2 decreased the relative abundances of fast-growing bacteria including *Pseudarthrobacter*, *Gaiellales_uncultured*, *Gemmatimonas* and *Acidobacteriaceae_uncultured*, but increased the abundances of assimilators of complex C compounds including *Novosphingobium*, *Acidimicrobiales_uncultured*, *Bacillus*, *Flavisolibacter* and *Schlesneria*. Alteration of the bacterial community was most likely attributable to the eCO_2-induced changes in the quantity and quality of plant-derived C compounds. Thus, the turnover of eCO_2-derived plant-C in Mollisols might change as eCO_2 suppresses the growth of fast-growing bacteria that metabolize plant-derived C but increases the populations of bacteria that metabolize more complex-C compounds. The microbial contribution to the turnover of specific plant-C compounds produced under eCO_2 warrants further research.

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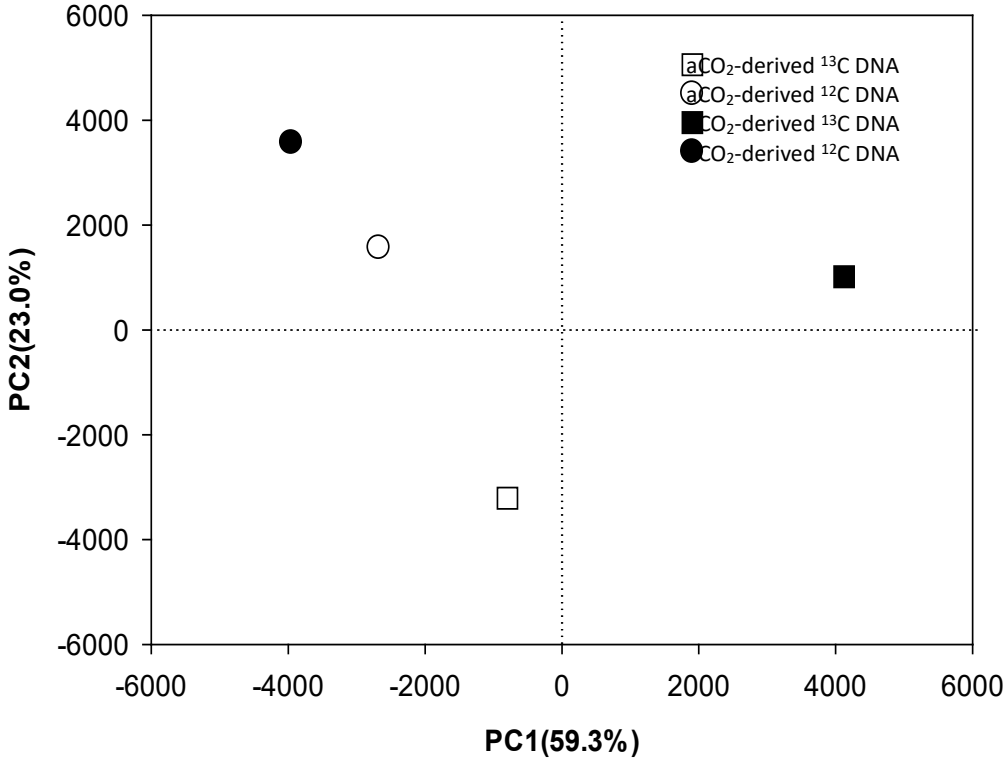

Figure 1 The scores of principal components 1 and 2 plotted in the plane of these two axes. The analysis was done on sequences of the ^{13}C- and ^{12}C-DNA fraction in the rhizosphere of soya bean exposed to aCO_2 (390 µmol mol^{-1}) or eCO_2 (550 µmol mol^{-1}) for 54 days and $^{13}CO_2$ labelling for 25 days. Each datum point represents the mean of 3 replicates.

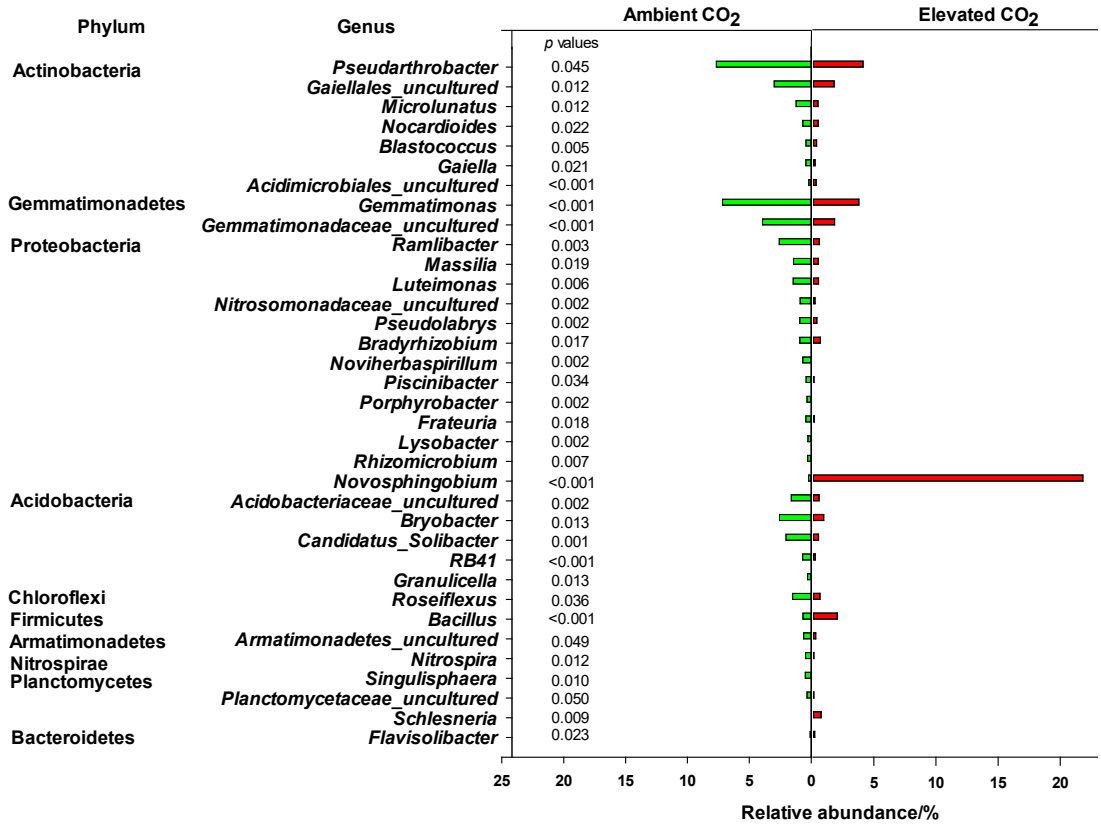

Figure 2 The relative abundances of bacteria at the genus level in the ^{13}C-DNA fraction in the rhizosphere of soya bean exposed to aCO_2 (390 μmol mol^{-1} or eCO_2 (550 μmol mol^{-1}) for 54 days and $^{13}CO_2$ labelling for 25 days. Only genera with significant ($p < 0.05$) responses to treatments are shown here.

Table 1 Soil total C and N, C/N ratio, microbial biomass C (MBC), dissolved organic C (DOC), available N, P and K and $\delta^{13}C$ in the rhizosphere of soya bean exposed to aCO_2 (390 µmol mol^{-1}) or eCO_2 (550 µmol mol^{-1}) for 54 days and $^{13}CO_2$ labelling for 25 days.

Treatments	Total C	Total N	C/N	MBC	DOC	NO_3^--N	NH_4^+-N	Olsen P	Available K	$\delta^{13}C$
	/g kg^{-1}			/mg kg^{-1}						/‰
aCO_2	17.2 ± 0.2	1.02 ± 0.0	16.9 ± 0.5	196 ± 3.6	49.8 ± 2.3	17.3 ± 4.1	40.9 ± 1.2	44.0 ± 4.4	119 ± 1.9	611 ± 97
eCO_2	17.5 ± 0.7	1.00 ± 0.1	17.6 ± 0.4	259 ± 6.5	52.7 ± 1.8	10.4 ± 2.6	40.5 ± 0.6	47.3 ± 2.7	106 ± 2.0	2252 ± 819
p value	0.71	0.75	0.22	0.002	0.33	0.31	0.64	0.61	0.007	0.009

The data are means of 3 replicates ± standard error. The *p* values indicate the significance of the CO_2 treatment effect.

Table 2 Summary of the number of operational taxonomic units (OTUs), Ace estimator, Chao1 estimator and Shannon's index of bacteria and rarefaction coverage in the ^{13}C-DNA fraction in the rhizosphere of soya bean exposed to aCO_2 (390 µmol mol^{-1}) or eCO_2 (550 µmol mol^{-1}) for 54 days and $^{13}CO_2$ labelling for 25 days.

Treatments	Number of OTUs	Ace estimator	Chao1 estimator	Shannon's index	Rarefaction coverage
aCO_2	891 ± 4	973 ± 7	986 ± 7	5.18 ± 0.07	0.99 ± 0
eCO_2	800 ± 2	908 ± 16	917 ± 11	4.23 ± 0.16	0.99 ± 0
p value	0.01	0.02	0.01	0.01	0.32

The data are means of 3 replicates ± standard error. The *p* values indicate the significance of the CO_2 treatment effect.

Supporting Information

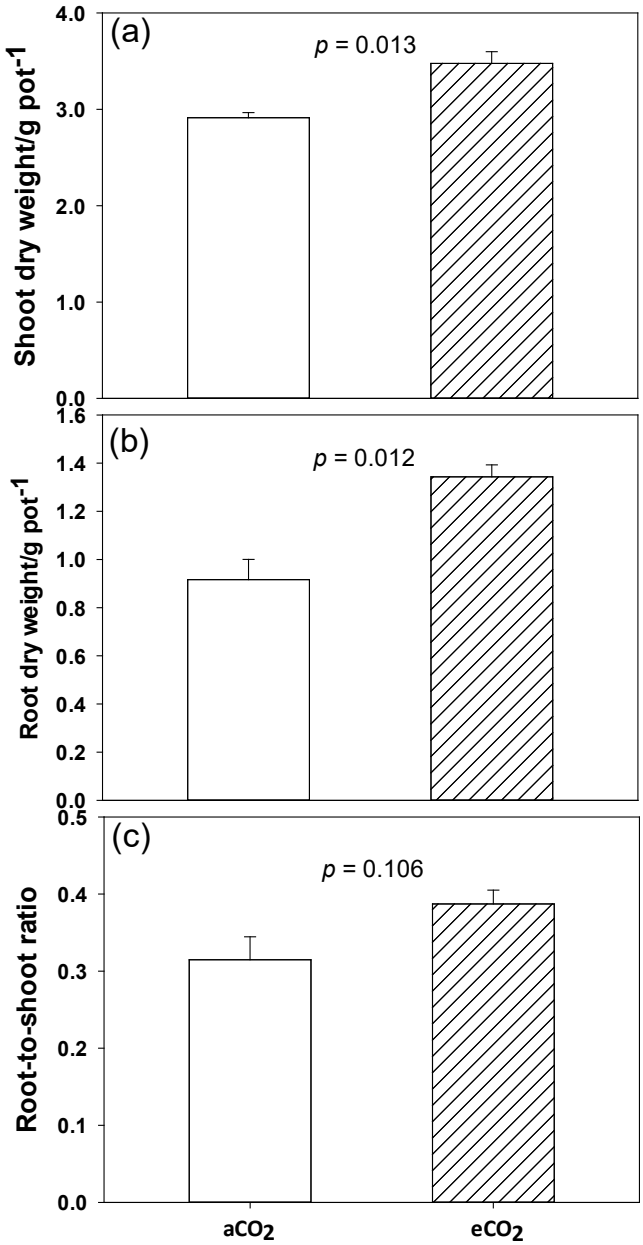

Figure S1 The effect of eCO_2 on dry weights of shoots (a) and roots (b) and root-to-shoot ratios (c) of soya bean exposed to aCO_2 (390 µmol mol^{-1} or eCO_2 (550 µmol mol^{-1}) for 54 days and $^{13}CO_2$ labelling for 25 days. The vertical error bars represent the standard error of the mean (n=3). The p values indicate the significance of the CO_2 treatment effect.

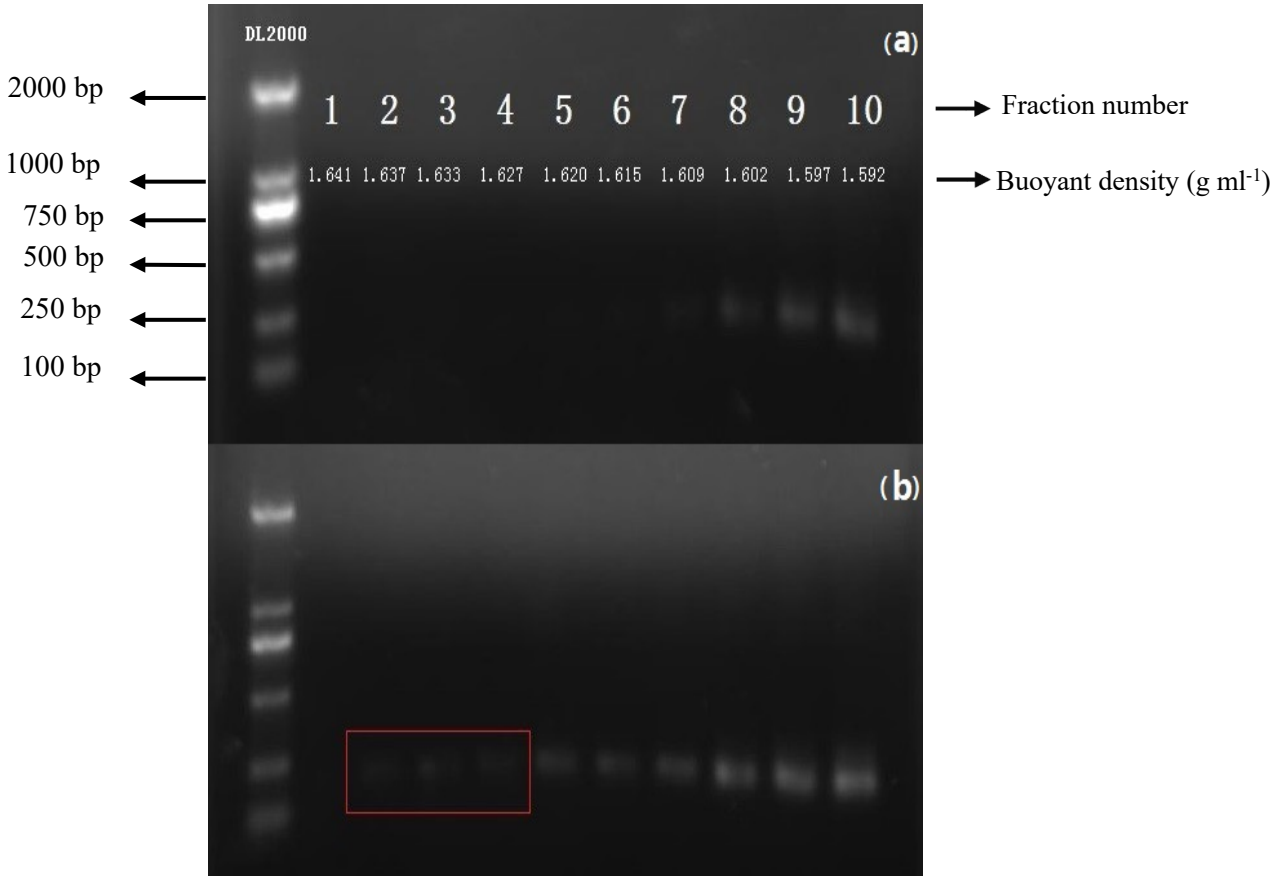

Figure S2 Aliquots of gradient fractions in non-labelled control (a) and $^{13}CO_2$-labelled treatment (b) after isopycnic centrifugation, the bands were PCR amplicons of DNA fractions and run on a 1% agarose gel. The marker on the left side was a DL 2000 ladder (Takara, Japan).

Table S1 Number of OTUs in accordance with relative abundances (≥ 0.3%) of genera that responded significantly to eCO_2. Genera were compared between ^{13}C-DNA fractions in the rhizosphere of soya bean exposed to aCO_2 (390 µmol mol^{-1}) or eCO_2 (550 µmol mol^{-1}).

Phylum	Genus	OTU ID	Number of OTUs	
			aCO_2	eCO_2
Acidobacteria	*Acidobacteriaceae_uncultured*	OTU985	128 ± 17	28 ± 4
		OTU374	10 ± 1	3 ± 2
		OTU852	20 ± 1	11 ± 0
		OTU331	51 ± 4	14 ± 6
		OTU672	100 ± 8	25 ± 3
		OTU414	12 ± 2	20 ± 10
		OTU1129	26 ± 6	15 ± 2
		OTU5	1 ± 0	1 ± 0
		OTU511	9 ± 0	4 ± 2
		OTU747	10 ± 1	2 ± 0
		OTU1185	2 ± 0	2 ± 1
		OTU295	1 ± 0	2 ± 1
		OTU990	54 ± 1	28 ± 1
		OTU818	9 ± 4	6 ± 0
	Bryobacter	OTU854	28 ± 5	9 ± 1
		OTU191	13 ± 2	3 ± 0
		OTU253	11 ± 3	2 ± 1
		OTU259	145 ± 11	51 ± 5
		OTU339	3 ± 1	0 ± 0
		OTU1086	75 ± 16	19 ± 1
		OTU251	6 ± 0	2 ± 1
		OTU1254	0 ± 0	6 ± 3
		OTU233	12 ± 0	6 ± 1
		OTU908	3 ± 0	2 ± 0
		OTU226	1 ± 1	61 ± 25
		OTU888	3 ± 0	1 ± 1
		OTU183	129 ± 25	30 ± 1
		OTU899	7 ± 1	4 ± 2
		OTU1195	12 ± 1	9 ± 0
		OTU873	106 ± 13	22 ± 9
		OTU1073	59 ± 8	12 ± 2
		OTU1077	17 ± 0	6 ± 0
		OTU224	25 ± 8	8 ± 1
		OTU235	32 ± 3	9 ± 1
	Candidatus_Solibacter	OTU211	66 ± 4	24 ± 5
		OTU202	23 ± 3	17 ± 4

		OTU1084	183 ± 14	51 ± 16
		OTU499	52 ± 3	4 ± 2
		OTU853	51 ± 7	5 ± 2
		OTU116	14 ± 1	8 ± 3
		OTU584	9 ± 1	7 ± 2
		OTU489	3 ± 1	1 ± 0
		OTU1176	2 ± 0	1 ± 0
		OTU212	37 ± 5	6 ± 1
		OTU128	3 ± 1	2 ± 1
		OTU316	77 ± 5	20 ± 1
		OTU745	27 ± 8	4 ± 2
	RB41	OTU821	110 ± 2	65 ± 7
		OTU168	2 ± 1	4 ± 2
		OTU317	7 ± 0	2 ± 0
		OTU930	28 ± 2	4 ± 1
		OTU1062	1 ± 1	0 ± 0
		OTU763	36 ± 5	2 ± 0
		OTU1015	3 ± 0	0 ± 0
	Granulicella	OTU427	59 ± 8	17 ± 3
		OTU375	21 ± 4	10 ± 2
Actinobacteria	*Gaiellales_uncultured*	OTU13	10 ± 1	5 ± 0
		OTU824	28 ± 2	11 ± 5
		OTU1063	30 ± 3	11 ± 2
		OTU68	3 ± 1	3 ± 1
		OTU636	1 ± 0	0 ± 0
		OTU673	128 ± 3	84 ± 15
		OTU9	12 ± 2	8 ± 2
		OTU425	39 ± 3	21 ± 4
		OTU1123	32 ± 2	19 ± 2
		OTU760	3 ± 0	1 ± 0
		OTU607	33 ± 2	27 ± 7
		OTU874	114 ± 9	63 ± 16
		OTU670	33 ± 1	27 ± 1
		OTU502	5 ± 0	4 ± 2
		OTU1004	11 ± 2	7 ± 0
		OTU898	176 ± 20	94 ± 17
		OTU1132	7 ± 2	5 ± 2
		OTU697	14 ± 1	18 ± 5
		OTU691	5 ± 1	3 ± 2
		OTU55	3 ± 1	2 ± 0
		OTU896	91 ± 11	61 ± 1
		OTU1121	17 ± 2	8 ± 0
		OTU1245	6 ± 1	3 ± 0

		OTU967	2 ± 0	1 ± 1
	Pseudarthrobacter	OTU581	2042 ± 268	1101 ± 187
	Microlunatus	OTU784	325 ± 32	134 ± 31
	Nocardioides	OTU447	40 ± 3	38 ± 1
		OTU1286	70 ± 7	57 ± 1
		OTU912	7 ± 2	5 ± 1
		OTU671	3 ± 1	1 ± 0
		OTU106	5 ± 0	5 ± 1
		OTU806	56 ± 1	36 ± 8
		OTU1006	1 ± 0	2 ± 0
	Blastococcus	OTU658	3 ± 1	4 ± 1
		OTU916	110 ± 2	101 ± 0
	Gaiella	OTU26	15 ± 0	12 ± 2
		OTU662	43 ± 1	23 ± 5
		OTU875	42 ± 2	31 ± 1
		OTU892	13 ± 3	7 ± 2
		OTU589	3 ± 0	2 ± 1
	Acidimicrobiales_uncultured	OTU856	3 ± 1	1 ± 1
		OTU242	3 ± 1	1 ± 0
		OTU653	9 ± 1	3 ± 0
		OTU1138	1 ± 0	2 ± 1
		OTU1180	2 ± 0	3 ± 1
		OTU724	3 ± 1	1 ± 0
		OTU17	0 ± 0	0 ± 0
		OTU884	3 ± 1	7 ± 1
		OTU438	7 ± 1	1 ± 1
		OTU739	5 ± 1	2 ± 0
		OTU1250	10 ± 1	9 ± 1
		OTU1224	2 ± 0	62 ± 0
		OTU740	1 ± 1	1 ± 0
		OTU453	3 ± 1	2 ± 0
		OTU531	3 ± 1	3 ± 2
Armatimonadetes	*Armatimonadetes_uncultured*	OTU683	4 ± 1	7 ± 2
		OTU682	11 ± 3	4 ± 0
		OTU1052	0 ± 0	0 ± 0
		OTU764	3 ± 1	1 ± 1
		OTU40	8 ± 0	2 ± 1
		OTU1029	6 ± 1	1 ± 0
		OTU385	12 ± 1	5 ± 1
		OTU1002	2 ± 1	3 ± 1
		OTU803	7 ± 3	0 ± 0
		OTU801	3 ± 0	2 ± 1
		OTU57	7 ± 1	2 ± 1

		OTU334	3 ± 1	0 ± 0
		OTU333	22 ± 2	10 ± 4
		OTU684	2 ± 1	2 ± 1
		OTU554	4 ± 0	2 ± 1
		OTU882	19 ± 4	3 ± 1
		OTU1145	8 ± 2	1 ± 1
		OTU1131	17 ± 1	22 ± 8
		OTU388	2 ± 1	1 ± 1
		OTU857	12 ± 4	8 ± 3
		OTU387	3 ± 0	4 ± 1
		OTU574	10 ± 1	2 ± 1
		OTU1199	0 ± 0	3 ± 2
		OTU354	4 ± 1	2 ± 1
		OTU1206	1 ± 0	3 ± 2
Bacteroidetes	*Flavisolibacter*	OTU153	5 ± 0	14 ± 1
		OTU462	2 ± 0	9 ± 1
		OTU571	0 ± 0	0 ± 0
		OTU437	6 ± 0	15 ± 6
		OTU1098	2 ± 0	4 ± 2
		OTU72	2 ± 0	0 ± 0
		OTU6	9 ± 1	24 ± 7
Chloroflexi	*Roseiflexus*	OTU27	23 ± 5	8 ± 4
		OTU600	1 ± 0	2 ± 1
		OTU601	22 ± 1	5 ± 0
		OTU604	2 ± 0	1 ± 0
		OTU599	15 ± 5	5 ± 2
		OTU1108	2 ± 1	3 ± 2
		OTU136	2 ± 1	5 ± 3
		OTU79	2 ± 0	0 ± 0
		OTU71	15 ± 1	4 ± 2
		OTU771	117 ± 24	40 ± 18
		OTU59	7 ± 1	1 ± 0
		OTU1055	33 ± 1	18 ± 9
		OTU174	42 ± 12	18 ± 4
		OTU114	1 ± 0	2 ± 1
		OTU595	55 ± 1	32 ± 4
		OTU1024	2 ± 0	1 ± 1
		OTU795	12 ± 0	5 ± 3
		OTU798	6 ± 0	1 ± 1
		OTU205	21 ± 4	5 ± 3
		OTU585	1 ± 0	0 ± 0
		OTU765	24 ± 3	18 ± 10
		OTU1252	2 ± 1	8 ± 4

Firmicutes	*Bacillus*	OTU880	2 ± 1	5 ± 1
		OTU1214	6 ± 1	27 ± 3
		OTU960	1 ± 1	2 ± 0
		OTU1136	20 ± 1	46 ± 4
		OTU510	131 ± 11	409 ± 31
		OTU630	1 ± 0	3 ± 1
		OTU519	5 ± 1	5 ± 1
		OTU858	12 ± 1	45 ± 13
		OTU1221	2 ± 1	10 ± 4
Gemmatimonadetes	*Gemmatimonas*	OTU841	1 ± 0	0 ± 0
		OTU398	1 ± 1	1 ± 1
		OTU326	7 ± 0	2 ± 1
		OTU997	3 ± 0	2 ± 1
		OTU304	7 ± 1	1 ± 0
		OTU34	5 ± 1	3 ± 1
		OTU33	125 ± 7	89 ± 11
		OTU695	20 ± 3	10 ± 2
		OTU507	42 ± 1	24 ± 0
		OTU844	65 ± 7	30 ± 3
		OTU133	30 ± 1	21 ± 4
		OTU36	0 ± 0	1 ± 0
		OTU25	2 ± 0	1 ± 0
		OTU20	2 ± 0	2 ± 0
		OTU917	361 ± 5	211 ± 23
		OTU514	8 ± 2	2 ± 1
		OTU22	3 ± 0	1 ± 1
		OTU343	194 ± 5	136 ± 4
		OTU569	3 ± 0	1 ± 0
		OTU981	347 ± 12	166 ± 16
		OTU84	2 ± 1	3 ± 1
		OTU528	43 ± 4	15 ± 4
		OTU1044	145 ± 6	74 ± 1
		OTU199	7 ± 1	9 ± 3
		OTU943	93 ± 3	26 ± 1
		OTU980	33 ± 0	9 ± 1
		OTU605	14 ± 1	2 ± 0
		OTU1	4 ± 1	2 ± 1
		OTU987	1 ± 0	1 ± 0
		OTU409	36 ± 2	19 ± 5
		OTU1294	242 ± 8	126 ± 7
		OTU861	17 ± 0	5 ± 1
		OTU993	53 ± 5	26 ± 5
	Gemmatimonadaceae_uncultured	OTU63	3 ± 0	1 ± 0

		OTU509	148 ± 1	52 ± 10
		OTU508	468 ± 20	171 ± 18
		OTU430	5 ± 1	3 ± 0
		OTU46	8 ± 1	15 ± 8
		OTU974	81 ± 2	65 ± 14
		OTU866	72 ± 1	42 ± 7
		OTU591	240 ± 14	134 ± 5
		OTU12	2 ± 1	1 ± 0
		OTU657	16 ± 2	7 ± 3
		OTU524	1 ± 0	0 ± 0
		OTU1057	2 ± 0	0 ± 0
		OTU989	6 ± 1	4 ± 2
Nitrospirae	*Nitrospira*	OTU640	1 ± 0	0 ± 0
		OTU614	8 ± 1	5 ± 2
		OTU1031	29 ± 5	17 ± 5
		OTU277	1 ± 0	1 ± 0
		OTU335	5 ± 1	1 ± 0
		OTU713	7 ± 0	2 ± 0
		OTU1289	1 ± 1	4 ± 2
		OTU1101	1 ± 0	0 ± 0
		OTU959	11 ± 3	4 ± 1
		OTU19	59 ± 7	12 ± 2
		OTU1051	3 ± 2	1 ± 0
Planctomycetes	*Singulisphaera*	OTU400	42 ± 6	20 ± 1
		OTU797	12 ± 4	2 ± 1
		OTU288	3 ± 0	1 ± 0
		OTU669	1 ± 0	0 ± 0
		OTU475	13 ± 1	9 ± 1
		OTU703	0 ± 0	1 ± 0
		OTU937	4 ± 2	1 ± 1
		OTU1146	56 ± 7	5 ± 1
	Planctomycetaceae_uncultured	OTU750	3 ± 1	0 ± 0
		OTU1110	1 ± 0	0 ± 0
		OTU1118	1 ± 1	0 ± 0
		OTU556	3 ± 0	0 ± 0
		OTU1182	0 ± 0	1 ± 1
		OTU301	1 ± 0	0 ± 0
		OTU953	1 ± 0	0 ± 0
		OTU81	1 ± 1	1 ± 0
		OTU811	6 ± 2	1 ± 0
		OTU723	4 ± 1	0 ± 0
		OTU1026	2 ± 0	1 ± 0
		OTU1075	4 ± 0	1 ± 0

		OTU138	3 ± 1	1 ± 0
		OTU774	25 ± 8	4 ± 1
		OTU1035	1 ± 0	0 ± 0
		OTU515	8 ± 1	0 ± 0
		OTU213	2 ± 0	0 ± 0
		OTU1107	11 ± 1	8 ± 0
		OTU337	0 ± 0	1 ± 0
		OTU1027	2 ± 0	0 ± 0
		OTU14	1 ± 0	0 ± 0
		OTU1168	9 ± 1	3 ± 2
		OTU270	1 ± 0	2 ± 1
		OTU346	0 ± 0	1 ± 0
		OTU1305	0 ± 0	4 ± 2
		OTU1321	3 ± 0	18 ± 10
		OTU178	4 ± 2	0 ± 0
		OTU664	0 ± 0	1 ± 1
	Schlesneria	OTU187	0 ± 0	201 ± 42
Proteobacteria	*Ramlibacter*	OTU232	688 ± 50	166 ± 63
	Massilia	OTU1070	276 ± 18	111 ± 43
		OTU248	22 ± 1	12 ± 6
		OTU431	83 ± 8	20 ± 7
	Luteimonas	OTU423	389 ± 44	147 ± 12
	Nitrosomonadaceae_uncultured	OTU1313	1 ± 0	11 ± 5
		OTU848	52 ± 0	7 ± 3
		OTU711	9 ± 0	6 ± 1
		OTU602	5 ± 1	0 ± 0
		OTU311	172 ± 13	48 ± 12
	Pseudolabrys	OTU247	56 ± 5	18 ± 8
		OTU1080	154 ± 2	67 ± 12
		OTU513	34 ± 4	23 ± 2
		OTU1113	3 ± 1	2 ± 1
	Bradyrhizobium	OTU1071	243 ± 8	178 ± 8
		OTU1274	2 ± 0	9 ± 5
	Noviherbaspirillum	OTU260	180 ± 19	35 ± 8
	Piscinibacter	OTU1094	111 ± 5	48 ± 19
	Porphyrobacter	OTU156	90 ± 4	34 ± 7
	Frateuria	OTU700	115 ± 14	60 ± 1
	Lysobacter	OTU992	73 ± 6	25 ± 2
	Rhizomicrobium	OTU555	50 ± 7	9 ± 3
		OTU245	18 ± 1	8 ± 1
		OTU946	2 ± 1	0 ± 0
		OTU221	7 ± 2	8 ± 5
	Novosphingobium	OTU1152	12 ± 1	5771 ± 434

	OTU544	27 ± 2	27 ± 2
	OTU1184	18 ± 1	14 ± 3

Data are mean of 3 replicates ± standard error.