The impact of increased atmospheric carbon dioxide on microbial community dynamics in the rhizosphere

Drigo, B.

Intermezzo

Climate change modulates carbon flow through soil food webs

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Abstract

Rising atmospheric CO_2 levels are predicted to have major consequences on carbon cycling and the functioning of terrestrial ecosystems[1]. Increased photosynthetic activity is expected, especially for C-3 plants, thereby influencing vegetation dynamics, yet little is known about the path of fixed C into soil-borne communities, and resulting feedbacks on ecosystem function[2,3,4,5]. Here, we demonstrate that arbuscular mycorrhizal fungi (AMF) act as the main conduit in the transfer of carbon between plants and soil and that elevated atmospheric CO_2 modulates the belowground translocation pathway of plant-fixed carbon. Shifts in active AMF species at under elevated atmospheric CO_2 conditions trigger downstream changes within the active rhizosphere bacterial and fungal communities. Thus, as opposed to increasing the activity of soil-borne microbes via enhanced rhizodeposition, elevated atmospheric CO_2 clearly evokes the emergence of distinct opportunistic plant-associated microbial communities. Analyses involving RNA-based stable isotope probing (SIP)[6,7], neutral/phosphate lipid fatty acids (N/PLFA-SIP)[8], community fingerprinting and real-time PCR allowed us to trace plant-fixed carbon to the affected soil-borne microorganisms. Based upon our data, we present a conceptual model in which plant-assimilated carbon is rapidly transferred to AMF, followed by a slower release from AMF to the bacterial and fungal populations well adapted to the prevailing (myco-)rhizosphere conditions. This model provides a general framework for reappraising carbon flow paths in soils, facilitating predictions of future interactions between rising atmospheric CO_2 concentrations and terrestrial ecosystems.

Anthropogenic CO_2 emissions are clearly contributing to rising atmospheric CO_2 levels, but the rate of atmospheric CO_2 elevation remains uncertain[9]. A major contribution to this uncertainty is our lack of knowledge concerning the path of carbon flow via plants into the soil, and the potential for climate-carbon cycle feedbacks involving vegetated terrestrial ecosystems[9]. Elevated atmospheric CO_2 leads to higher C assimilation by plants[10], and root-soil interactions facilitate movement of C to the soil[11], which represents the largest and most stable C pool in the terrestrial biosphere[12]. In addition to potential long-term changes in litter quantity and quality, C fixed by plants can enter the soil through increased root turnover, greater sloughing off of cells, enhanced plant tissue breakdown or increased root exudation[1,11,13]. Plant-derived exudates provide energy for rhizosphere microbial communities, thereby influencing their structure and function[4,10,13]. AMF, which form symbioses with the majority of land plants[3,14], have been recognized as a potentially important functional group involved in the sequestration of plant-derived C[3]. Although recent progress has been made in our understanding of C fluxes from the plant, to AMF, rhizosphere microorganisms and the soil food-web, knowledge is still scarce with respect to the relative flow of C to different biological groups in plant-soil systems[3,11,15]. Such knowledge is critical to our understanding of carbon cycling in terrestrial ecosystems and to assessing soil carbon storage potential in mitigating rising atmospheric CO_2 conditions.

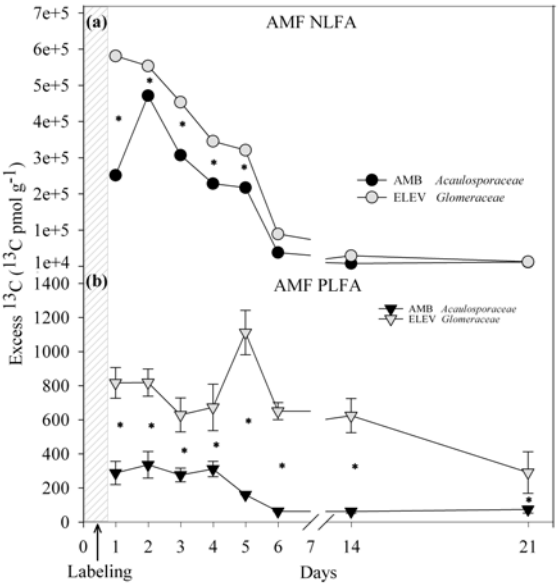

Figure 1: ^{13}C enrichment in the AMF signatures 16:1ω5 was determined in *F. rubra* rhizosphere soil for NFLA (circles; a) and PLFA (triangles; b) at ambient CO_2 (black) and elevated CO_2 (grey). The given AMF families, Acaulosporaceae and Glomerales, denote the affiliations of the 18S rRNA gene fragments recovered from the ^{13}C-labelled RNA fractions by RNA-SIP at ambient CO_2 and elevated CO_2, respectively. Asterisks designate significant differences ($P < 0.001$) between CO_2 concentrations.

The application of ^{13}C Stable Isotope Probing (SIP)[6,7,8] to track plant-derived C fluxes into microbial nucleic acids[6,7] or other biomarkers[8] has opened up a window toward understanding the flux of C through plant-associated microbial communities. In order to track the fate of plant-assimilated C to belowground microbial communities in response to elevated atmospheric CO_2, we conducted a $^{13}CO_2$ pulse-chase labelling experiment with *Festuca rubra* (mycorrhizal C-3 grass species) and *Carex arenaria* (non-mycorrhizal C-3 sedge) plants grown for six-months under ambient (350 ppm) or elevated (700 ppm) CO_2 conditions. Using RNA and neutral/phosphate lipid fatty acids (N/PLFA) stable isotope probing (SIP), community fingerprinting and taxon-specific real-time PCR, we tracked active microbial populations *in situ,* focusing on total bacterial, total fungal, *Pseudomonas* spp., *Burkholderia* spp., *Bacillus*, actinomycete and protozoan communities.
For *F. rubra*, AMF signature biomarkers[15] (N/PLFA 16:1ω5) showed strong ^{13}C-labelling within one day, followed by a significant decrease ($P < 0.001$), reaching a plateau 14 days after labelling (Fig. 1). NLFA 16:1ω5 enrichment was significantly increased at elevated CO_2 from day 1 to day 5 (Fig.1 a; days × CO_2: $F_{7,14} = 920.14$; $P < 0.001$), and PLFA 16:1ω5 enrichment at elevated CO_2 was significantly higher during the entire incubation

period (Fig. 1b; days × CO_2: $F_{7,4} = 1682.53$, $P < 0.001$). Increased ^{13}C-labelling of NLFA suggested enhanced production of AMF storage organs, and increased ^{13}C-labelling of PLFA implied AMF growth stimulation[15].

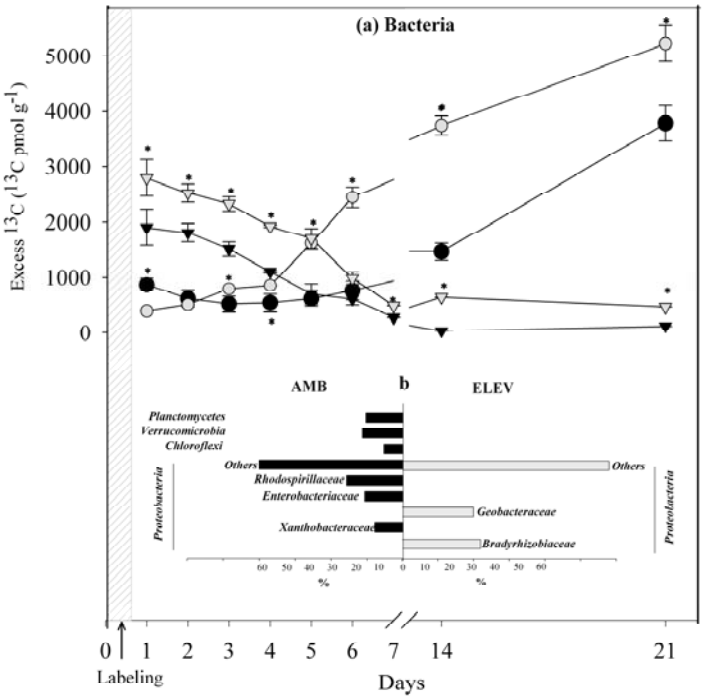

Figure 2: ^{13}C enrichment (a) in the bacterial PLFAs at ambient CO_2 (black) and at elevated CO_2 (grey) in the rhizosphere soil of *F. rubra* (circles) and *C. arenaria* (triangles). (b) Significantly different groups in clones libraries derived from *F. rubra* ^{13}C-labelled 16S rRNA at ambient (AMB) and elevated (ELEV) CO_2, harvesting on day 21. Asterisks ($P < 0.001$) designate significant differences between CO_2 concentrations.

RNA-SIP revealed a complete shift in AMF species receiving plant-derived C under ambient versus elevated CO_2 conditions (Fig. 1, S2b). In the communities under ambient atmospheric CO_2, an AMF taxon affiliated to the *Acaulosporaceae* (*A. lacunosa*) received most of the fixed C, while, under elevated CO_2, the dominant AMF incorporating ^{13}C label was affiliated to the *Glomeraceae* (*G. claroideum*). These two AMF families are known to exploit different spatial niches and exhibit disparate life history strategies[16]. Fungal-specific 18S rRNA gene clone libraries were also in accordance with a greater C allocation to AMF at elevated CO_2, with ^{13}C-labeled fractions taken one day after pulse-labeling yielding approximately 50% AMF sequences at elevated CO_2 compared to 36% AMF sequences at ambient atmospheric CO_2 concentrations ($P < 0.001$).

Elevated CO_2 conditions also significantly affected the spectrum of non-AMF fungi incorporating plant-derived C ($P > 0.001$), mirroring observed changes in fungal

community structure in response to elevated CO_2[17]. Differences included the disappearance of *Trichosporm porosum, Capnobotrytella, Heteroconicum chaetospira*-like sequences from the 'heavy' fraction associated with plants grown at elevated CO_2 as compared to sequences recovered from ambient CO_2-grown plants and the presence of *Tricoderma harzianum, Eimeriidae*-like sequences and an unidentified fungal species at ambient CO_2, 24 hours after labeling.

The gradual decrease of the ^{13}C incorporation in AMF-specific biomarkers was accompanied by a significant increase in ^{13}C incorporation in the bacterial community, commencing 4-5 days after labeling in the mycorrhizal plant (*F. rubra*) (Fig. 2a). The non-mycorrhizal plant, *C. arenaria*, showed an opposite pattern, with a rapid incorporation of ^{13}C in the bacterial community only at the beginning of the experiment, followed by a gradual decrease. PCR-cloning analyses based on ^{13}C-labeled 16S rRNA fractions showed a significant effect of CO_2 treatment ($P < 0.001$), in agreement with observed shifts in total bacterial community structure[17]. Shifts in dominant C-incorporating bacteria were observed during the course of the experiment (Fig. 2b). For instance, at ambient CO_2 for *F. rubra*, Proteobacteria represented 57%, 90% and 73% of bacterial sequences 1, 6, and 14-21 days post-labeling, respectively (not shown). The remaining sequences were mostly affiliated with Chloroflexi, Planctomycetes and Verrucomicrobia, with the latter only detected in the ^{13}C fraction at day 21. At elevated CO_2, all clones derived from ^{13}C fractions were affiliated with Proteobacteria. Within the Proteobacteria, significant differences ($P < 0.001$) were observed between the 'heavy' bacterial clone libraries derived from the ambient versus elevated CO_2 treatments at all sampling times. Differences included presence of the Xanthobacteraceae, Rhodospirillaceae and Enterobacteriaceae-like sequences only in ambient CO_2 libraries, and Geobacteraceae and Bradyrhizobiaceae-like sequences only in the elevated CO_2 libraries. Interestingly, within this last family, *Bradyrhizobium japonicum*-like sequences were detected. This species has been identified as a mycorrhizal helper bacteria (MHB) of the genus *Glomus*[14]. For both plant species, the peak of ^{13}C incorporation into protozoan biomass, as judged by PLFA 20:4ω6[18] (Fig. S1a, S2c), coincided with the highest ^{13}C incorporation into the bacterial community, 5-6 and 1 day(s) post-labeling for *F. rubra* and *C. arenaria*, respectively, suggesting a coupling of bacterial and protozoan growth.

Typical rhizosphere bacteria[19] also showed a pronounced response to the CO_2 treatment throughout the labeling experiment. At ambient CO_2, *P. fluorescens* was the main *Pseudomonas* species incorporating plant-derived C (Fig. 3). This species is also known to function as a MHB, enhancing AMF development[14]. Under elevated CO_2, the biodiversity of the active *Pseudomonas* community increased, with active populations of *P. fluorescens*, *P. aeruginosa, P. trivialis,* and *P. putida* being detected (Fig. 3). Isolates of several of these species have been shown to have MHB, biocontrol or pathogenic activities[14]. *Large shifts were also observed within* dominant C-incorporating Burkholderiaceae species, with an opposite trend with respect to diversity. *B. fungorum*, *B. cepacia, B. glathei, B. phenazinum, B xenoforans* and a "2,4–degrading bacterial" species were detected at ambient CO_2, yet only the first three of these species were detected at elevated CO_2. To quantify C uptake by *Pseudomonas* ssp. and *Burkholderia* ssp., cyclopropyl PLFAs, cy17:0 and cy19:0 were used as biomarkers for *Pseudomonas* spp. and *Burkholderia* spp. respectively[20]. As judged by these biomarkers, these genera became highly enriched in ^{13}C for *F. rubra* at elevated CO_2 (Fig. 2, Fig. 3, Fig S2 a1-a2). This coincided with a lower biomass, suggesting a more rapid turnover at elevated CO_2.

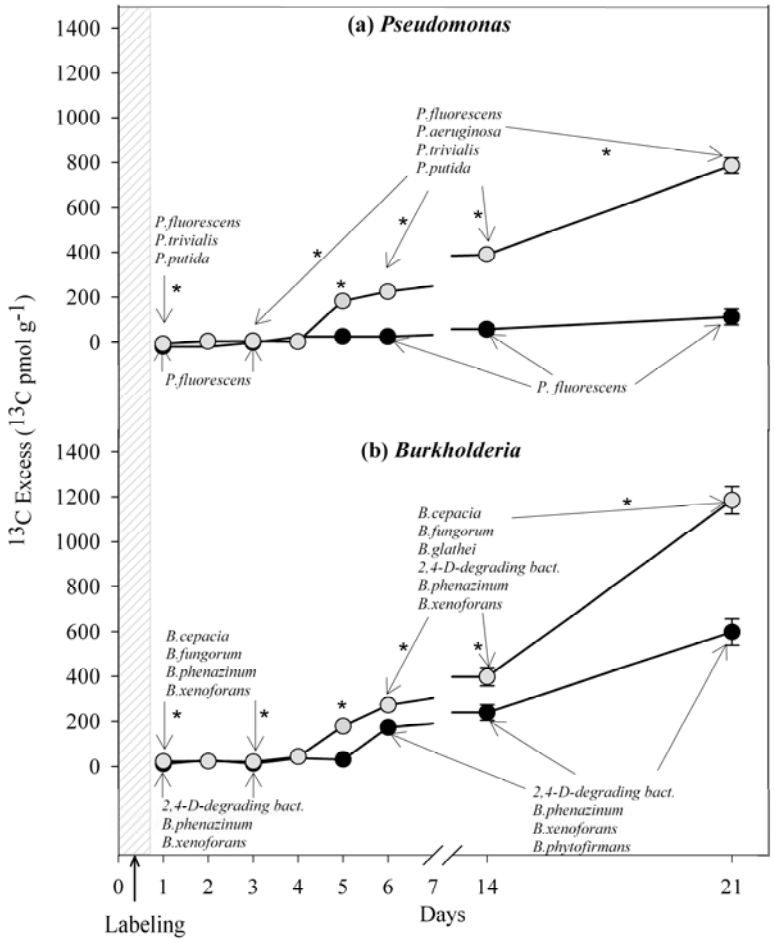

Figure 3: ^{13}C enrichment in rhizosphere of *F. rubra* at ambient (black) and elevated CO_2 (grey) in the PLFAs of *Pseudomonas* (a) and *Burkholderia* (b) specific signatures determined at ambient (black) and elevated CO_2 (grey). *C. arenaria Burkholderia* and *Pseudomonas* showed the highest ^{13}C enrichment on day 1 at elevated CO_2 (not shown). The active *Pseudomonas* and *Burkholderia* species identified by RNA-SIP, DGGE and cloning are indicated along the different time course at ambient and elevated CO_2. Asterisks ($P < 0.001$) designate significant differences between CO_2 concentrations.

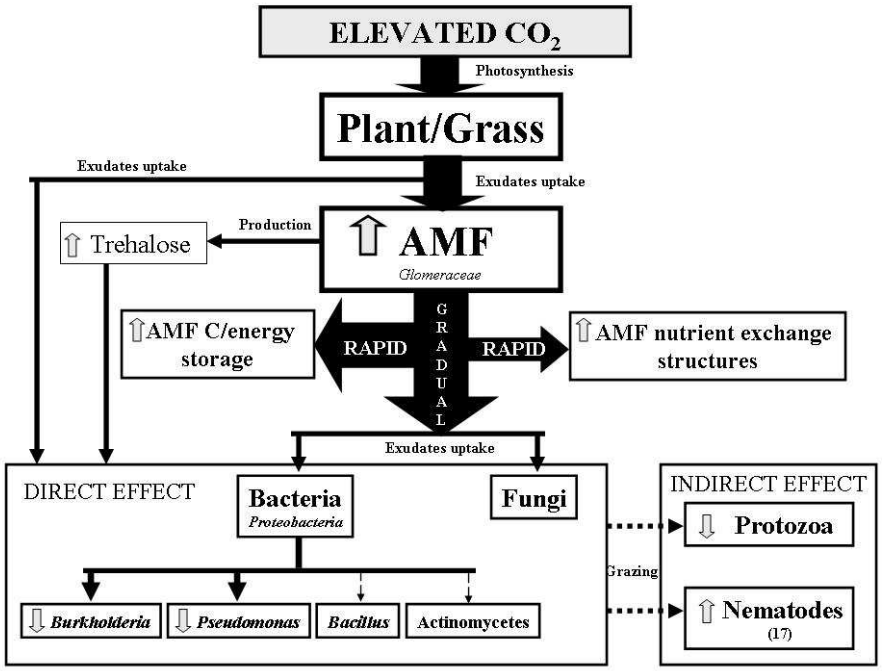

Figure 4: Conceptual model of C flow in mycorrhizal plant-soil systems summarizing the effect of elevated CO_2 atmospheric concentrations on soil communities. Grey arrows indicate increases and decreases in the respective community sizes, as determined by real-time PCR and lipid analysis from this study and (17). Absence of an arrow indicates that no significant changes in the communities size where detected. The mechanism and magnitude of the C-flow along the soil food-web are indicated by the black arrows.

At current atmospheric conditions, mycorrhizal fungi receive up to the 20% of the plant photosynthetates. Trehalose synthesis is thought to contribute to the C drain to mycorrhizal fungi[14] and to play an important role in selecting specific bacterial communities in the mycorrhizosphere, specifically members of the genera *Bradyrhizobia*, *Pseudomonas* and *Burkholderia*[14]. We observed that trehalose concentrations in the mycorhizosphere increased by a factor 4 at elevated atmospheric CO_2 conditions (4.54 ppm, elevated CO_2 vs 1.21 ppm, ambient CO_2). This suggests that AMF-associated trehalose release may be involved in inducing the observed shifts in active bacterial populations in the rhizosphere of *F. rubra.*

Bacillus spp. and actinomycetes have been recognized as dominant bulk soil inhabitants[19]. Using the PLFA signatures i17:0 for *Bacillus* spp.[21] and 10Me-PLFAs for actinomycetes[22], we detected virtually no labeling for these groups throughout the experiment for both plant species studied (Fig. S1b). These results are consistent with previous findings that slow-growing soil microorganisms, such as actinomycetes, were unaffected by elevated CO_2[24] and are supported by the fact that we detected no Actinobacteria- or Firmicutes-like sequences in our ^{13}C-based bacterial clone libraries.

In the controlled growth systems used in our experiment, we were able to follow carbon incorporation patterns to different components of the soil-borne microbial community,

revealing major shifts in carbon flow routes and active diversity upon six-months of exposure to elevated atmospheric CO_2 conditions. It should be noted that these effects may have been enhanced by the quantum shift in CO_2 conditions in our experiment, as it has been suggested that long-term step-wise increases in CO_2 may have more limited effects on plant-soil systems[25]. We have, however, observed that similar shifts, including the importance of AMF as a C sink, are maintained in longer-term experiments of 3 ½ years (Drigo *et al*. submitted).

Figure 4 presents a conceptual model of C flow in mycorrhizal plant-soil systems, summarizing our findings. C allocation belowground proceeds principally via AMF, which rapidly receive plant-derived C. AMF subsequently release this C gradually to their associated microbes, highlighting the key position of AMF in the release of plant-derived C to the soil microbial community. Shifts in AMF populations in response to elevated CO_2 conditions therefore result in marked changes in bacterial diversity and activity, stimulating specific populations best capable of responding to the particular nutrient conditions of the (myco-)rhizosphere. These responses of soil-borne microbial communities are expected to impact biodiversity and soil food-web interactions, as well as the direction and magnitude of terrestrial ecosystem / atmosphere feedbacks that regulate global C cycling.

Methods

Plant and soil study systems

Soil was collected from a river dune at Bergharen (51°51'31.37"N; 5°40'9.86"E; the Netherlands), where *F. rubra* and *C. arenaria* were the dominant grass species. The soil had a sandy texture, pH 4.32, a low calcium carbonate content, 1.97% organic C, and showed 1.7 mg/kg fungal biomass (estimated from ergosterol data). Ten soil cores (5-15 cm depth) were collected from 4×4 m sampling plots, covered by *F. rubra* and *C. arenaria*. Pots were filled with sieved soil (1 kg), planted with three four-week-old seedlings of *F. rubra* and *C. arenaria*, and allocated to four CO_2 flow cabinets (for a detailed description see (17)). Two hundred pots per CO_2 (350 ppm, 700 ppm) and plant species (*F. rubra* and *C. arenaria*) treatment were grown for six months (211 total growing days) prior to the described pulse-chase ^{13}C labeling experiment. *F. rubra* plants was found to be heavily colonized by AMF.

$^{13}CO_2$ pulse-labeling

$^{13}CO_2$ pulse-labelling (99 at. % ^{13}C-CO_2, Cambridge Isotope Laboratories, Andover, MA, USA) was carried respectively at 350 ppm and 700 ppm. A total of 150 *F. rubra* and 150 *C. arenaria* plants, plus 16 unplanted pots, were subjected to $^{13}CO_2$ pulse-labeling, half from the 350 ppm CO_2 treatment and the other half from the 700 ppm CO_2. The remaining pots, used for natural abundance and background $^{13}C/^{12}C$ measurements, were incubated in two separate CO_2 flow cabinets (350 and 700 ppm) to ensure that there was no contamination with respired ^{13}C-enriched CO_2. The amount of $^{13}CO_2$ added during labeling was sufficient to label plants to 2545 ‰ $\delta^{13}C$ at ambient CO_2 levels and 2892 ‰ $\delta^{13}C$ at elevated CO_2. Actual ^{13}C-content (excess ^{13}C) in individual pools (shoots, roots and soil) was also calculated as described in (26). Total RNA was extracted using the method describe by (6).The integrity of the RNA preparations was visualized by LabChip® microfluidic technology and automated electrophoresis for RNA analysis using the Experion RNA

StdSens analysis system (Experion™, Bio-Rad Laboratories Inc., the Netherlands) and subsequently stored at -80 ºC. Total RNA was quantified using both the Experion™ system and a NanoDrop, ND-1000 Spectrophotometer (Bio-Rad Laboratories Inc., the Netherlands). ^{13}C-enriched RNA was obtained by density-gradient centrifugation and analyzed as described in (6). RNA samples from equilibrium density gradient fractions were reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase with low RNAse H activity (200 u/µl, ReverseAid™ M-MuLVRT, Fermentas) using random hexaminer primers (0.2 µg/µl) according to the manufacture's protocol (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas). The cDNA produced was then used for bacterial 16S rRNA and fungal 18S rRNA quantification by real-time PCR using the ABsolute QPCR SYBR green mix (AbGene, Epsom, UK) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). All mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia). Quantification of fungal and bacterial SSU ribosomal RNA gene copies in rhizosphere soil was carried as described in (17). All the samples, and all standards, were assessed in at least two different runs to confirm the reproducibility of the quantification. PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis of bacterial, fungal, *Pseudomonas* sp., *Burkholderia* ssp. and AMF communities of reverse transcribed density-resolved RNA fractions followed the procedures described in [17, 27,28,29].

Cloning and sequencing of amplicons

PCR products using RNA or excised DGGE bands were obtained using several group-specific primer combinations as in (17), (26), (27) and (28). PCR products were purified with the High-Pure PCR product purification kit (Boehringer Mannheim, Almere, NL) and cloned into the pGEM-T Easy vector (Promega, Leiden, NL) according to the manufacturer's instructions. Plasmid extraction was performed using the Wizard Plus SV miniprep DNA purification kit (Promega, Benelux). 350 clones with confirmed inserts of the expected size, were selected randomly for sequencing, using the vector –encoded universal T7 primer, from the bacterial-, fungal-specific, *Pseudomonas*-, *Burkholderia*- and AMF specific libraries (Macrogen; South Korea). To confirm reliability of sequences derived from DGGE bands, three different colonies with the expected insert were sequenced per excised band. Sequences were aligned in the Bioedit Sequence Alignment Editor program (www.mbio.ncsu.edu/BioEdit/bioedit.html). To identify chimeric sequences in the clone libraries all recovered sequences were checked by using CHIMERA_CHECK 2.7 (Ribosomal Database Project II; http://rdp.cme.msu.edu). All cloned bacterial (~900 bp), *Pseudomonas* (~250bp*)* and *Burkholderia* (~ 500 bp) 16S rRNA gene sequences and fungal (~600 bp) and AMF (~400 bp) 18S rRNA gene sequences were compared at the species level with sequences in public databases by using NCBI Blast (http://www.ncbi.nlm.nih.gov/blast) and the Ribosomal Database Project II Classifier (http://rdp.cme.msu.edu). To estimate the probability of observing differences in the frequency between libraries recovered from ambient and elevated CO_2 treatments, the cloned sequences were compared by the classification and library compare algorithm published in naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences (Ribosomal Database Project II). Sequences were deposited in the GenBank database (Accession numbers: xxxxxxxx-yyyyyyyy).

Lipid biomarker and stable isotope analysis

Neutral (NLFA) and phospholipid (PLFA) lipid fatty acids were extracted and analyzed according to the protocol described by (26). The N/PLFA 16:1ω5 was used as a signature for AMF biomass and ^{13}C incorporation[15]. The following fatty acids were used as biomarkers for bacterial biomass: i14:0, i15:0, a15:0, i16:0, 16:1ω7t, i17:1ω7, 10Me16:0, a17:1ω7, i17:0, a17:0, cy17:0, 10Me17:0, 18:1ω7c, 10Me18:0 and cy19:0[22]. The phospholipid fatty acids (PLFA) cy17:0 and cy19:0 were used as biomarkers for *Pseudomonas* spp. and *Burkholderia* spp. respectively[20]. 10Me16:0, 10Me17:0 and 10Me18:0 were used for actinomycetes[22] and i17:0 for *Bacillus*[21]. The signature 20:4ω6 was used to assess the ^{13}C incorporation and biomass of the protozoan community[18]. NLFA/PLFAs were analyzed using ANOVA (Statistica 7.0, StatSoft Inc., Tulsa, OK) according to a split-plot design, considering as whole plot the two different CO_2 treatments, while *F. rubra* and *C. arenaria* un/labeled, un/labeled unplanted soil, and the time courses as sub-treatments within each whole plot. The F statistic obtained by dividing the treatment mean-square by the mean-square for CO_2 flow cabinets nested within CO_2 treatments.

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Supplementary material

Figure S1: ^{13}C enrichment in rhizosphere soil of *F. rubra* and *C. arenaria* at ambient (black) and elevated CO_2 (grey) in the PLFA specific signatures for the (a) protozoa, (b) *Bacillus* (circles, *F. rubra*, ambient (black), elevated (light grey) ; triangles, *C. arenaria*, ambient (light grey), elevated (dark grey)) and actinomycetes (squares, *F. rubra*, ambient (grey), elevated (dark grey); diamonds, *C. arenaria*, ambient (grey), elevated (dark grey)). Asterisks ($P < 0.001$) designate significant differences between CO_2 concentrations.

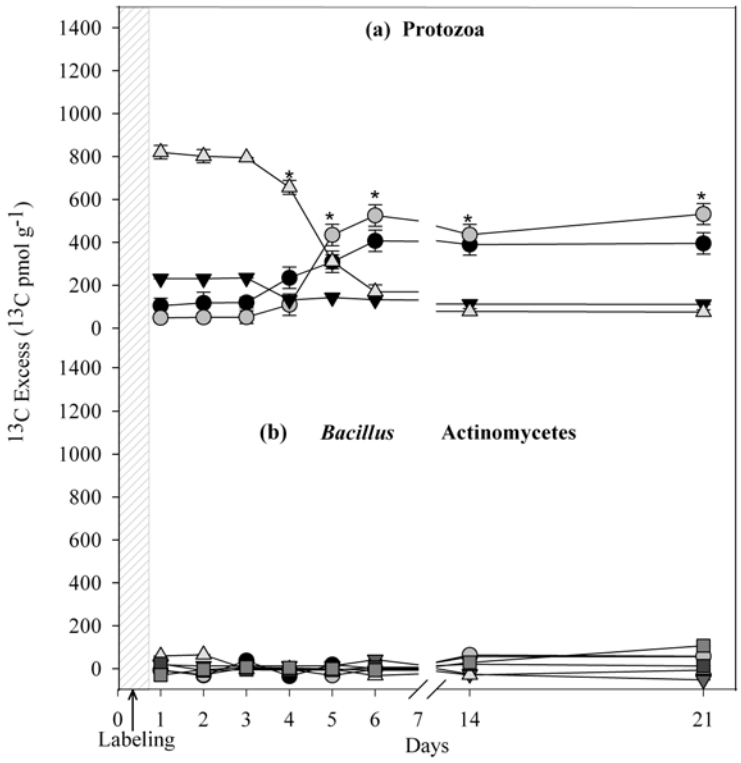

Figure S2: ^{13}C abundance in rhizosphere soil of *F. rubra* and *C. arenaria* at ambient (black) and elevated CO_2 (grey) in the PLFA specific signatures for the (a) bacteria (a1 *Pseudomonas*, a2 *Burkholderia*, a3 *Bacillus*, a4 Actinomycetes), (b) AMF (b1 NLFA; b2 PLFA) and (c) protozoa. Letters designate significant differences between CO_2 concentrations.