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

Drigo, B.

Chapter 7

Three-year exposure to CO_2 enrichment modifies microbially-mediated carbon flow

Barbara Drigo, George A. Kowalchuk, Agata S. Pijl, Brigitte M Knapp, Henricus T.S. Boschker and Johannes A. van Veen

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Abstract

The aim of this study was to identify the microbial communities that are involved in the assimilation of rhizosphere-C and are most responsive to elevated CO_2 over the course of 3 years of CO_2 enrichment. To assess the effects of increased atmospheric CO_2 on bacterial, general fungal and arbuscular mycorrhizal fungal (AMF) communities in the rhizosphere, *Carex arenaria* (a non-mycorrhizal plant species) and *Festuca rubra* (a mycorrhizal plant species) were grown in dune soil under controlled soil temperature and moisture conditions, while subjecting the above-ground compartment to defined atmospheric conditions differing in CO_2 concentration (350 and 700 µl l^{-1}). ^{13}C signatures through microbial communities by fatty acid biomarker analyses (NLFA and PLFA), RNA-Stable Isotope Probing (SIP), Real-time PCR and PCR-denaturing gel electrophoresis (DGGE) after *in-situ* $^{13}CO_2$ pulse-labeling experiments were used to examine effects on the size and structure of rhizosphere communities. ^{13}C-N/PLFAs and ^{13}C-RNA-SIP measurements at 6 months, 1, 2 and 3 years of elevated atmospheric CO_2 incubations revealed that the influence of elevated CO_2 was plant dependent, with the mycorrhizal plant (*F. rubra*) exerting greater influence on bacterial and fungal communities. Biomarker data indicated that rhizodeposited C was first processed by AMF and only later translocated to bacterial and fungal communities in the rhizosphere soil. Over the course of three years, elevated CO_2 caused an increase in the proportional of ^{13}C enrichment retained in the AMF specific biomarker 16:1ω5 and delayed the translocation of C to the bacterial community.

Introduction

In the last 150 years, the atmospheric CO_2 concentration has increased by approximately 33% due to human activity, and is predicted to continue to rise by 0.4% per year (Alley *et al.* 2007). A continued rise in CO_2 may result in higher rate of photosynthesis (Ainsworth and Long 2005), especially in the C_3 plants (Long *et al.* 2004), which is likely to stimulate plant biomass production as well as root growth when sufficient mineral nutrients are available (Curtis and Wang 1998). This could have a direct effect on carbon fluxes from the above-ground compartment into the soil. Carbon input to soil generally increases in response to elevated CO_2 concentrations owing to improved plant carbohydrate status (Barron-Gafford *et al.* 2005), even when there is no significant CO_2 stimulation of above-ground growth (Körner and Arnone 1992). Quantitative and qualitative changes in C inputs to the soil include higher rates of plant litter-fall, root turnover, enhanced rhizodeposition, increased C supply to symbionts (mycorrhizae and rhizobium), as well as alteration in the chemical composition of plant tissue (e.g. higher C/N ratio) and root exudates (Hu *et al.* 1999). Any changes in the amount and/or composition of plant material input into the soil in response to elevated CO_2 may significantly affect the composition of soil-borne communities, which rely heavily on organic C supply for their own growth. Hence, these alterations in C inputs can significantly affect microbial processes, particularly decomposition and nutrient cycling, thereby feeding back to the atmospheric CO_2 concentration (Field *et al.* 1992; Zak *et al.* 2003).

Table 1: Average of root and shoot biomass of F. rubra and C. arenaria harvest after 6 months, 1, 2 and 3 years of incubation under ambient and elevated CO_2 conditions.

Plant species		CO_2 treatments	6 months	1 year	2 years	3 years
			g (dry weight)	g (dry weight)	g (dry weight)	g (dry weight)
F. rubra	shoots	AMB	20.40^{a}	45.96^{c}	82.22^{e}	154.81^{g}
		ELEV	38.78^{b}	63.89^{d}	134.49^{f}	254.70^{h}
C. arenaria	shoots	AMB	22.00^{a}	42.00^{c}	85.00^{e}	162.97^{g}
		ELEV	35.76^{b}	60.33^{d}	120.53^{f}	240.78^{h}
F. rubra	roots	AMB	12.81^{a}	35.60^{c}	73.33^{e}	144.74^{g}
		ELEV	18.65^{b}	53.77^{d}	104.94^{f}	204.24^{h}
C. arenaria	roots	AMB	10.00^{a}	22.56^{c}	45.35^{e}	97.07^{g}
		ELEV	15.76^{b}	37.53^{d}	109.26^{f}	203.38^{h}

Different letters within a column refer to significantly (P<0.05) different averages based upon an unequal N Tukey-HSD test.

It is difficult to speak of a typical response of soil-borne microbial communities to elevated CO_2 since experiments conducted to date have examined highly disparate ecosystems and experimental condition (Gamper *et al.* 2005; Denef *et al.* 2007; Marilley *et al.* 1999; Lipson *et al.* 2006; Tarnawski *et al.* 2006; Rillig *et al*. 1997; Grayston *et al.* 1998; Mayr *et al.* 1999; Montealegre *et al.* 2000; Janus *et al.* 2005; Jossi *et al*. 2006; Schortemeyer *et al*. 1996; Griffiths *et al.* 1998; Insam *et al.* 1999; Bruce *et al.* 2000; Zak *et al.* 2000; Klamer *et al.* 2002; Ebersberger *et al.* 2004). Despite the variation in experimental designs and obtained results, a number of generalizations can be drawn from previous studies. For instance, when microbes are C-limited, enhanced C inputs appear to stimulate microbial activities and CO_2 production. However, as plants increase N uptake in response to elevated CO_2 or when N is translocated to less-available pools, such as standing plant biomass and soil organic matter, N availability becomes the major limiting factor (Hu *et al.* 1999). Under these conditions enhanced C input under increased atmospheric CO_2 levels could alter microbial community composition in favor of fungi. Fungi are capable of colonizing nutrient–poor, recalcitrant substrates due to their greater and more variable C:N ratio, their wide-ranging enzymatic capabilities and their ability to translocated essential nutrient through their hyphae over considerable distances (Hu *et al.* 2001). Thus, alterations in soil microbial composition could have significant consequences for C and N transformations. For instance, it has been postulated that bacteria-dominated food webs lead to higher short-term mineralization rates of organic C and N (Wardle *et al.* 2004), while fungal stimulation, in particular of arbuscular mycorrhizal fungi, may enhance C sequestration (Tresender and Allen 2000) and N immobilization through hyphal translocation (Frey *et al.* 2000).

To gain further insight into the long term effects of elevated atmospheric CO_2 on soil-borne communities, we assessed the plant-driven impact of elevated atmospheric CO_2 over the course of three years on changes in rhizosphere communities of two dominant coastal sand dune plant species, *Festuca rubra* ssp. *arenaria* (sand fescue) and *Carex arenaria* (sand sedge). Analyses focused on bacterial, fungal and AMF abundance and community structure and the ratios between these groups. Coastal dune systems were chosen as the model due to their relative simplicity and particular relevance to issues of global climate change. We determined the effects of increased atmospheric CO_2-levels on microbial communities in the rhizosphere of *Carex arenaria* (a non-mycorrhizal plant species) and *Festuca rubra* (a mycorrhizal plant species) by growing these plants in different dune soils under controlled soil temperature and moisture conditions, while subjecting the above-

ground compartment to either ambient (350 µl l^{-1}) or elevated (700 µl l^{-1}) CO_2 concentrations. Bacterial, fungal and AMF community sizes were determined via an *in situ* ^{13}C-CO_2 pulse-labeling approach, utilizing determinations of ^{13}C-N/PLFAs, ^{13}C-RNA-SIP, q-PCR and molecular community profiling by PCR-DGGE. Subsequently, multivariate statistical analyses were used to compare the relative impact over time of elevated CO_2 treatment versus plant and soil effects on these communities (Ter Braak and Verdonschot 1995; Borcard *et al.* 1992; Filion *et al*. 2000).

Table 2: Abundance of specific PLFAs for bacterial biomass in the rhizosphere of *F. rubra* and *C. arenaria*. The plants were grown at elevated (ELEV) or ambient (AMB) CO_2 in Bergharen soil for 6 months, 1, 2 and 3 years. Different letters within a column refer to significantly ($P<0.05$) different averages based upon an unequal N Tukey-HSD test.

Plant species	Bacterial species	CO_2 treatments	6 months nmol g^{-1}	1 year nmol g^{-1}	2 years nmol g^{-1}	3 years nmol g^{-1}
F. rubra	*Burkholderia*	AMB	40.40^{l}	30.96^{j}	2.22^{bc}	0.81^{ab}
		ELEV	5.78^{d}	3.89^{c}	1.49^{b}	0.70^{ab}
	Pseudomonas	AMB	250.10^{q}	146.27^{op}	4.41^{cd}	1.41^{b}
		ELEV	3.60^{c}	0.95^{ab}	0.23^{ab}	0.10^{ab}
	Actinomycetes	AMB	75.78^{mn}	62.65^{m}	1.21^{b}	0.62^{ab}
		ELEV	1.65^{b}	0.06^{a}	0.76^{ab}	1.21^{b}
	Bacillus	AMB	170.56^{p}	134.35^{op}	0.25^{ab}	0.10^{ab}
		ELEV	1.15^{b}	0.06^{a}	0.16^{ab}	0.36^{ab}
	Protozoa	AMB	0.34^{ab}	0.02^{a}	0.01^{a}	0.00^{a}
		ELEV	0.36^{ab}	0.00^{a}	0.05^{a}	0.08^{a}
	AMF (NLFA)	AMB	15.16^{f}	18.34^{fg}	19.56^{g}	20.23^{h}
		ELEV	21.23^{h}	23.42^{h}	24.43^{h}	25.64^{h}
	AMF (PLFA)	AMB	7.15^{de}	4.47^{cd}	3.34^{c}	2.45^{bc}
		ELEV	9.90^{e}	7.71^{de}	5.23^{d}	4.12^{cd}
	Bacteria	AMB	556.08^{s}	454.07^{r}	38.07^{i}	27.36^{h}
		ELEV	40.34^{l}	34.68^{j}	83.61^{n}	120.01^{o}
C. arenaria	*Burkholderia*	AMB	97.32^{o}	94.00^{o}	77.09^{m}	20.12^{f}
		ELEV	35.76^{g}	32.06^{g}	52.41^{i}	60.12^{l}
	Pseudomonas	AMB	4.43^{d}	3.87^{c}	2.93^{c}	1.00^{ab}
		ELEV	3.46^{c}	2.12^{b}	2.20^{b}	2.39^{b}
	Actinomycetes	AMB	26.47^{f}	33.24^{g}	64.17^{l}	73.11^{m}
		ELEV	7.75^{e}	9.22^{e}	65.07^{l}	102.09^{q}
	Bacillus	AMB	1.21^{ab}	0.51^{ab}	0.22^{ab}	0.02^{a}
		ELEV	0.45^{ab}	0.60^{ab}	0.70^{ab}	2.26^{b}
	Protozoa	AMB	0.37^{ab}	0.26^{ab}	0.00^{a}	0.02^{a}
		ELEV	0.00^{a}	0.00^{a}	0.03^{a}	1.99^{b}
	AMF (NLFA)	AMB	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
		ELEV	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	AMF (PLFA)	AMB	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
		ELEV	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	Bacteria	AMB	123.45^{q}	110.33^{p}	101.48^{op}	60.12^{l}
		ELEV	34.73^{g}	42.34^{h}	85.48^{n}	115.02^{p}

Material and methods

Soil, classified as river dune, was collected in the spring of 2004 from sites dominated by *F. rubra* and *C. arenaria* at Bergharen (51° 51'31.37"N; 5°40'9.86"E; The Netherlands). The soil characteristics and sampling strategy are previously described in chapter 3.
Seeds of *C. arenaria* and *F. rubra* were collected from the same foredune sites and stored dry until used. In order to obtain seedlings, the seeds were germinated for 4 weeks on moist glass beads in a climate room at a 16/8 h light/dark regime at a temperature of 25/15°C respectively. When the first leaf was 2–3 cm long, the seedlings were transplanted to 6.0 l plastic pots filled with 6,000 g of Bergharen soil. In each pot, four seedlings of *C. arenaria* and *F. rubra* were planted. Soil moisture content was adjusted to 10% w/w and maintained at this level throughout the experiment by weighing the pots twice a week and resetting their initial weight using demineralized water. Once every three months, 75 ml full-strength Hoagland nutrient solution was added per pot (Brinkman *et al.* 2004). This nutrient supply was to compensate for effects of nutrient release as a result of soil nutrient depletion in soil (Troelstra *et al.* 2001; van der Putten *et al.* 1988). The experiment was carried out over a period of 3 years (September 2004 - September 2007) in which 200 *F. rubra* and 200 *C. arenaria* plants were grown, half at an ambient atmospheric CO_2 concentration (350 µl/l) and half at double this concentration (700 µl/l). For each CO_2–treatments 10 pots with unplanted soil were also incubated. Within each chamber, all pots were shuffled after each watering (twice a week) to reduce potential position effects.
The $^{13}CO_2$ pulse-labeling was carried out 6 months (March 2005), 1 year (September 2005), 2 year (September 2006) and 3 years after germination (September 2007). In each pulse labeling event a total of 32 *F. rubra* and 32 *C. arenaria* plants, plus 6 unplanted pots were subjected to $^{13}CO_2$ pulse-labeling, half from the 350 µl/l CO_2 treatment and the other half from the 700 µl/l CO_2 treatment. The remaining pots were incubated in two separate CO_2 flow cabinets to ensure that there was no contamination with respired ^{13}C enriched CO_2. Pulse labeling used 99 at. % ^{13}C enriched CO_2 (Cambridge Isotope Laboratories, Andover, MA, USA). The three pulse labeling events and the harvesting procedure were conducted as previously described in chapter 5, except that samples were collected at 24h, 72h, 6, 14 and 21 days after labeling.
The lipid biomarker analysis was conducted as described in chapter 5. Extraction and analysis of DNA and RNA, isopycnic centrifugation, synthesis of cDNA and domain-specific PCR quantification of density-resolved 16S and 18S rRNA and community analyses by 16S and 18S rRNA-based PCR-DGGE were performed as described in chapter 6. The statistical analysis was performed as described in chapters 3 and 5, with the exception that changes in community composition of bacteria, fungi and AMF to the treatments were analyzed by Principal Coordinate Analysis (PCoA).

Results and Discussion

^{13}C enrichment and distributions of nucleic acid in centrifugation gradients

Real-time PCR, based upon SSU rRNA across the CsCl gradient profile, revealed pronounced bacterial and fungal peaks in fractions detected at densities >1.80 g/ml for each of the four pulse labelling times (March 2005 and September 2005, 2006 and 2007) for both plant species and CO_2 treatments (Fig 1). Total fungal SSU rRNA template numbers were approximately 70% of those detected for bacteria, similar to patterns observed in

chapter 6. Rhizosphere soil rRNA was still highly enriched in ^{13}C relative to the non-labelled soil samples at 21 days post-labelling for all plants, CO_2 treatments and sampling times. Bacterial and fungal ^{13}C-rRNA quantities were comparable across the different years for both CO_2 conditions and plant species, reflecting comparable levels of ^{13}C-CO_2 incorporation for the four pulse-labelling events. As controls, the q-PCR for bacterial and fungal SSU rRNA was performed along the CsCl gradient profiles of unlabelled rhizosphere materials. No low buoyancy ('heavy') RNA peaks were detected for any such unlabeled samples (data not shown).

Rhizosphere-C uptake and translocation within microbial communities

Fungal communities have been shown to be highly responsive to increases in rhizosphere-C supply (Griffiths *et al.* 1999; Denef *et al.* 2007). The *F. rubra* rhizosphere soil samples taken after each of the pulse-labelling events showed a large incorporation of ^{13}C in the AMF NLFA 16:1ω5 and to a lesser extent in the corresponding PLFA, yet ^{13}C incorporation in bacterial PLFAs remained low until 6 days after pulse-labelling (Fig 1 and 2). This suggests that AMF are closely associated with the root system (Butler *et al.* 2003; Olsson and Johnson 2005). AMF appear to be actively utilizing and incorporating newly produced plant-derived C into their biomass to a far greater extent than bacteria. Recent *in situ* studies using PLFA-based on SIP by ^{13}C-CO_2 pulse labelling have also reported a much faster incorporation of rhizosphere-C into AMF biomass (Johnson *et al.* 2002; Olsson and Johnson 2005; Denef *et al.* 2007) as compared to incorporation into bacterial biomass (Treonis *et al.* 2004). The $\delta^{13}C$ enrichment in the AMF NLFA and PLFA was still prominent at 21 days post-labelling and increased over the course of the 3-year experiment (Fig. 2). This increase in AMF N-PLFA-^{13}C enrichment over time was probably due to the increasing size of the plants (Table 1). Interestingly, the timing of maximum ^{13}C incorporation into bacterial PLFAs subsequent to pulse-labelling events shifted over the course of the experiment. Peaks of bacterial ^{13}C incorporation occurred at 6 days post labelling for the first two labelling events (after 6 and 12 months of plant growth), yet shifted to 14 and 21 days post labelling after 2 and 3 years of plant growth, respectively. These results are consistent with those of Olsson and Johnson (2005) who, over a time period of 32 days, found a decrease in the ^{13}C enrichment of AMF PLFA, 16:1ω5, extracted from roots, and a concomitant increase in ^{13}C enrichment of bacterial PLFAs extracted from soil. These data confirmed our hypothesis described previously that the major pathway of C flux from the roots into the soil microbial community was through mycorrhizal fungi and only subsequently into the non-symbiotic microbial community (see chapter 5 and 6). The increase of ^{13}C enrichment of AMF N-PLFAs over the different years after the pulse labelling suggests an increasing retention time of rhizodeposited-C in AMF biomass, which could be of great importance to soil organic C sequestration in grasslands ecosystems. However, as lipids (N/PLFAs) are major components of the cells membranes of living organisms and, crucially, only remain intact in viable cells (White *et al.* 1979), this may also suggest very little activity and extremely slow turnover of AMF biomass, assuming no continued active ^{13}C-assimilation in the period following the pulse-labelling. Such slow microbial cell turnover would be in contrast to earlier findings of Treonis *et al.* (2004), who found a decrease in ^{13}C enrichment for all PLFAs between 4 and 8 days post-labelling and root-derived C turnover was even greater through fungal (16: 1ω5, 18: 1ω9, 18: 2ω6,9) and gram-negative (16: 1ω7, 18:1ω7, cy19:0) bacterial biomarker PLFAs

compared to gram-positive bacterial biomarker lipids (15:0a, 15:0i, 16:0i).

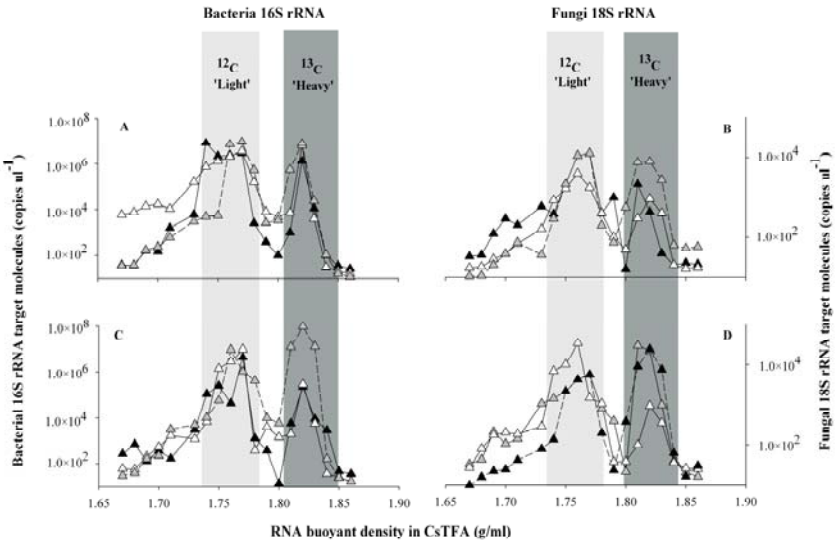

Figure 1: CsTFA density gradient centrifugation of rRNA extracted from *F. rubra* rhizosphere soil at 1 year (white triangles), 2 year (black triangles) and 3 years (grey triangles) of growth at ambient (A, B), and at elevated CO_2 (C, D) after 6 days of incubation with ^{13}C-CO_2. Bacterial and fungal SSU rRNA template distribution within gradient fractions was quantified with real-time reverse transcriptase-PCR. The *C. arenaria* rhizosphere soil had a similar ^{13}C-rRNA trend (not displayed). The density-range characteristic for the 'light' ^{12}C-rRNA is shaded in light grey and for the 'heavy' ^{13}C-rRNA in dark grey. All the density separated fractions were used for the PCR-DGGE fingerprinting analysis. The fractions from which the clone libraries of selected templates were generated are included in the light grey and dark grey shaded areas.

Effect of elevated CO_2 on AMF, bacterial and fungal community structure

Fungal communities have been shown to be highly responsive to increases in rhizosphere-C supply (Griffiths *et al.* 1999; Denef *et al.* 2007). In contrast with our previous findings, the central role of mycorrhizae in the belowground C-pathway changed at ambient and at elevated CO_2. The capacity of AMF to act as a sink for C increased over the three years of the experiment at elevated CO_2 conditions (Fig 2). At ambient CO_2 the major incorporation of plant derived C was observed 24 hours after pulse-labelling, remaining stable in the sizes and fluxes over all samplings during the three years (Fig 1A). Several studies have observed enhanced AMF activities in response to elevated atmospheric CO_2 concentrations (Rillig *et al.* 1999; Klironomos *et al*. 1996; Zak *et al.* 2000; Treseder 2004), generally attributed to the greater substrate use efficiency of fungi and AMF. Although in nutrient limited ecosystems, most aboveground biomass responses to elevated CO_2 are weak or neutral (Schäppi and Körner 1996; Stöcklin *et al*. 1998; Körner 2000), an increase in

aboveground biomass with elevated CO_2 was observed from along the different years (Table 1).

In addition to the increased capacity of AMF to act as a carbon sink, there was also a shift in the active AMF taxa over the course of the experiment at both ambient and elevated CO_2 concentrations. AMF-specific PCR-DGGE banding patterns derived from the 'heavy' RNA fractions under elevated CO_2 were significantly different ($P < 0.001$) from those observed at ambient CO_2 (Fig. 3). The first ordination axis, which explained 55.1% of the variability in taxon composition, revealed a clear separation of the ^{13}C-incorporating AMF community structure at ambient and elevated CO_2 throughout the 3 years of CO_2 enrichment. As hypothesized in chapter 6, the shift in dominant populations of AMF under elevated CO_2 over the three years of incubation may induce major shifts in rhizobacterial communities by modifying the quality, quantity and timing of rhizodeposition (see chapter 3 and 4). Indeed, the taxonomic composition of ^{13}C-RNA incorporating bacterial (Fig. 4 A) and fungal communities (Fig. 5 A) associated with *F. rubra* over the three years were significantly different under elevated CO_2 ($P < 0.001$) from the ambient CO_2. A clear separation in time was also observed for the ^{13}C-incorporating bacterial (Fig. 4 B) and fungal (Fig. 5 B) communities associated with *C. arenaria* at elevated CO_2. However, variation partitioning analyses on datasets separated by plant species revealed that sampling time explained a greater amount of variation for ^{13}C-bacterial and fungal community composition at elevated CO_2 in *F. rubra* than in *C. arenaria* (Fig. 6).

Effect of elevated CO_2 on specific bacterial PLFAs

Several studies have reported an enrichment of gram-negative bacteria under elevated atmospheric CO_2 conditions (Sonnemann and Wolters 2005; Montealegre *et al*. 2002; Drissner *et al.* 2007) in contrast to fungal biomass which was not affected. However, some other studies were unable to detect changes in microbial biomass (Allen *et al*. 2000; Kandeler *et al.* 2006), nor shifts bacterial and fungal communities under elevated CO_2 (Zak *et al.* 1996; Rønn *et al.* 2002; Niklaus *et al.* 2003; Ebersberger *et al.* 2004). To date, *in situ* studies of microbial community responses to elevated atmospheric CO_2 have been limited to examination of total PLFA-C distribution, which does not distinguish between the metabolically-active and inactive microbial communities. The advantage of a pulse-labelling approach in combination with ^{13}C-PLFA analysis is the additional information provided regarding the response of those microbial communities that are actively assimilating newly fixed C. The response to elevated CO_2 of metabolically-active microbial communities is not detectable through conventional total PLFA-C analysis and RNA/DNA analysis due to the large background concentration of the mostly inactive total soil microbial community, but this information is of great importance to better understanding C cycling in terrestrial ecosystems under increasing atmospheric CO_2 concentrations. ^{13}C-PLFA results indicated a greater ^{13}C enrichment bacterial PLFAs associated with *C. arenaria* (Fig. 8) as compared to *F. rubra* (Fig. 7) in both CO_2 treatments. Interestingly, in *F. rubra*, the incorporation of ^{13}C in general bacterial biomarkers decreased significantly over time ($P < 0.001$) at elevated CO_2 compared to ambient CO_2 treatment (Fig. 7). In contrast, for *C. arenaria,* the incorporation of ^{13}C labelled exudates into the general bacterial biomarkers showed an increasing trend over time both at ambient and elevated CO_2 (Fig. 8).

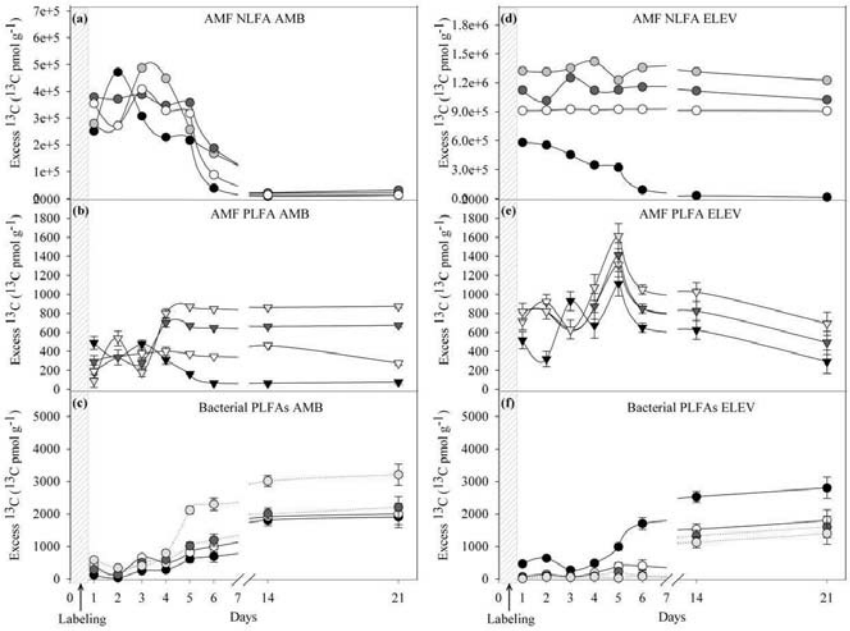

Figure 2: ^{13}C enrichment in the arbuscular mycorrhizal fungal signature 16:1ω5 was determined in *F. rubra* rhizosphere soil for NFLA (circles) at ambient CO_2 (2 a) and elevated CO_2 (2 d) after 6 months incubation (black), 1 year (white), 2 years (dark grey) and 3 years (light grey). PLFA 16:1ω5 (triangles) fractions were determined) at ambient CO_2 (2 b) and elevated CO_2 (2 e) after 6 months incubation (black), 1 year (white), 2 years (dark grey) and 3 years (light grey). The ^{13}C enrichment in the bacterial PLFAs was determined as the sum of 15 bacteria-specific PLFAs at ambient CO_2 (2 a) and elevated CO_2 (2 d) after 6 months incubation (black), 1 year (white), 2 years (dark grey) and 3 years (light grey) in the rhizosphere soil of *F. rubra* (circles). ^{13}C enrichment denotes the excess ^{13}C after subtraction of natural background as determined for non-labeled systems. Shaded area indicates period of ^{13}C-CO_2 incubation.

The cyclopropyl PLFAs cy17:0 and cy19:0 have been demonstrated to be useful biomarkers for *Pseudomonas* spp. and *Burkholderia* spp. respectively, due to their high presence in these typically rhizo-competent genera (Berg *et al.* 2005; Lugtenber *et al.* 2001; Vancanneyt *et al.* 1996; Treonis *et al.* 2004). Based on the behaviour of these biomarkers, both genera decreased in the rhizosphere of *F. rubra* and increased in the rhizosphere of *C. arenaria* at elevated CO_2 (Fig. 7 and 8).
Interestingly, this coincides with lower biomass for these two genera in both plants at elevated CO_2 concentrations, suggesting a more rapid turnover at elevated CO_2 associated with *C. arenaria* and the opposite for *F. rubra*. The patterns of ^{13}C incorporation for these bacterial genera were similar to that of the total bacterial communities for both plants, but it did not account for the full level of bacterial labeling, indicating that bacterial labeling is not limited to these genera.

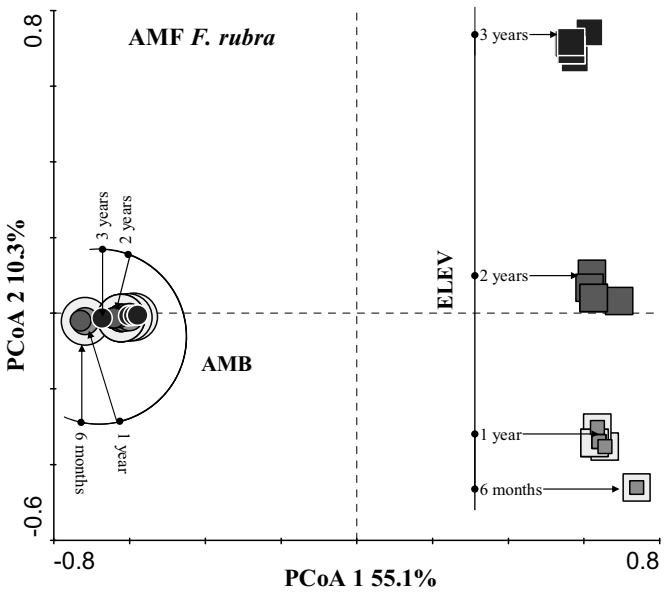

Figure 3: Representation for AMF DGGE pattern of the first two axes of PCoA with passively projected species centroid and position of the individual ^{13}C heavy labeled AMF in *F. rubra* rhizosphere plants grown at ambient (circles) and elevated CO_2 (squares). White is indicative of the 6 months incubation treatment, light grey stands for 1 year incubation, dark grey 2 years incubation and black 3 years incubation.

We considered the PLFA signature i17:0 as an indicator for the activity and biomass of *Bacillus* spp. (Kaneda 1991), and 10Me-PLFAs as indicative of actinomycetes (Bardgett *et al.* 1999; Billings & Ziegler 2005). These bacterial groups have previously been recognized as dominant bulk soil inhabitants (Smalla *et al.* 2001). For both plants, the dynamics of these bacterial groups, as judged by these biomarkers, were different from those observed in *Pseudomonas* and *Burkholderia*. Their ^{13}C incorporation was much lower that observed for *Pseudomonas* and *Burkholderia* and was not affected by elevated CO_2 (Fig. 7 and 8). These results are in accordance with the data presented in chapter 5, as well as information presorted by Zak *et al.* (1996) and Bardgett *et al.* (1999), who howed that slow-growing soil microorganisms, such as actinomycetes, were not affected by elevated CO_2.

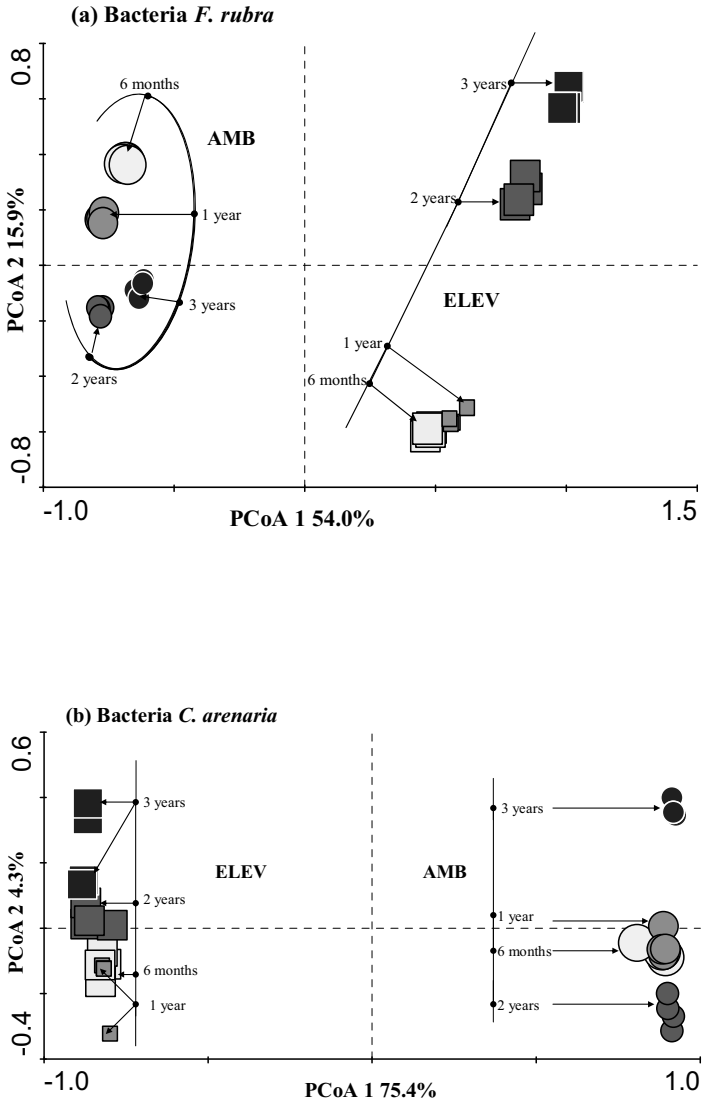

Figure 4: Representation for bacterial DGGE patterns of the first two axes of PCoA with passively projected species centroid and position of the individual ^{13}C heavy labeled bacteria in *F. rubra* (a) and *C. arenaria* (b) rhizosphere plants grown at ambient (circles) and elevated CO_2 (squares). White is indicative of the 6 months incubation treatment, light grey stands for 1 year incubation, dark grey 2 years incubation and black 3 years incubation.

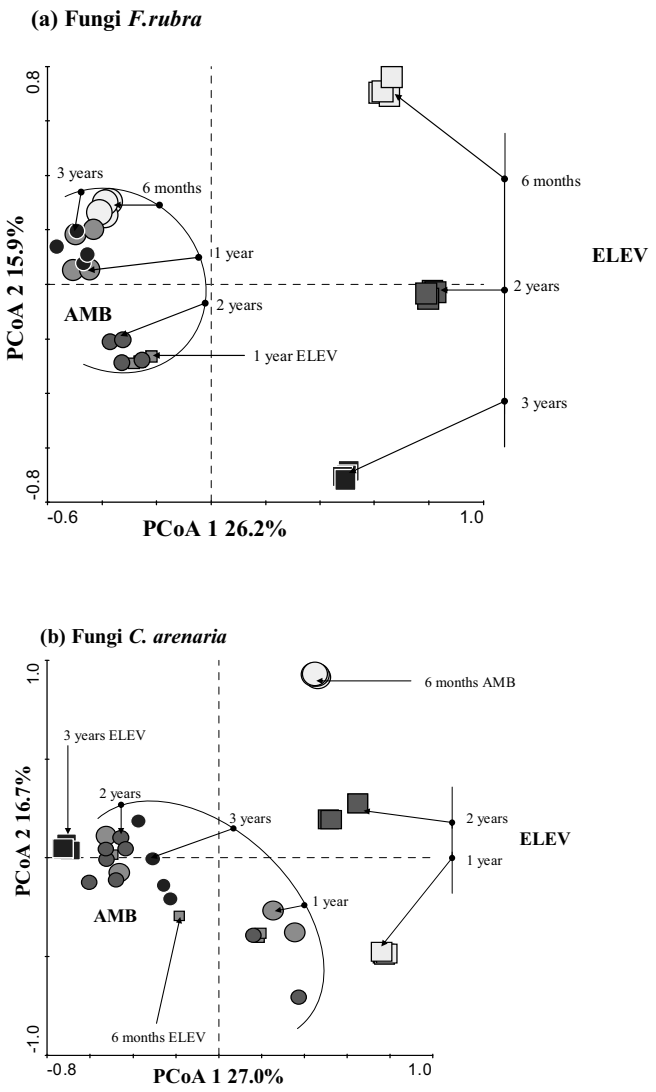

Figure 5: Representation for fungal DGGE patterns of the first two axes of PCoA with passively projected species centroid and position of the individual ^{13}C heavy labeled bacteria in *F. rubra* (a) and *C. arenaria* (b) rhizosphere plants grown at ambient (circles) and elevated CO_2 (squares). White is indicative of the 6 months incubation treatment, light grey stands for 1 year incubation, dark grey 2 years incubation and black 3 years incubation.

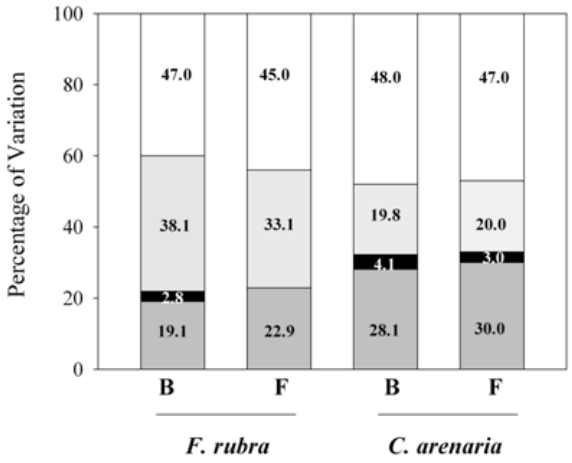

Figure 6: Variation partitioning representation for DGGE patterns of the bacterial (B) and fungal (F) rhizosphere communities under *C. arenaria* plants only and under *F. rubra* plants. All the different factors (CO_2 levels and sampling times) were having a significant effect ($P < 0.001$) on the microbial community structure as tested by db-RDA. Unexplained variance (white); CO_2 level (light gray); sampling times (dark gray); shared variation between sampling times + CO_2 (black).for *Pseudomonas* and *Burkholderia* and was not affected by elevated CO_2 (Fig. 7 and 8). These results are in accordance with the data presented in chapter 5, as well as information presorted by Zak *et al.* (1996) and Bardgett *et al.* (1999), who showed that slow-growing soil microorganisms, such as actinomycetes, were not affected by elevated CO_2.

The biomarker PLFA 20:4ω6 was used as biomass indicator for the protozoan community (Fig. 7 and 8). Unlike bacteria, the ^{13}C enrichment of the protozoan fraction was low throughput the entire experimental period. This suggests that the smaller size and higher activity of the bacterial community in *F. rubra* and *C. arenaria* under elevated CO_2 were independent of protozoan grazing.

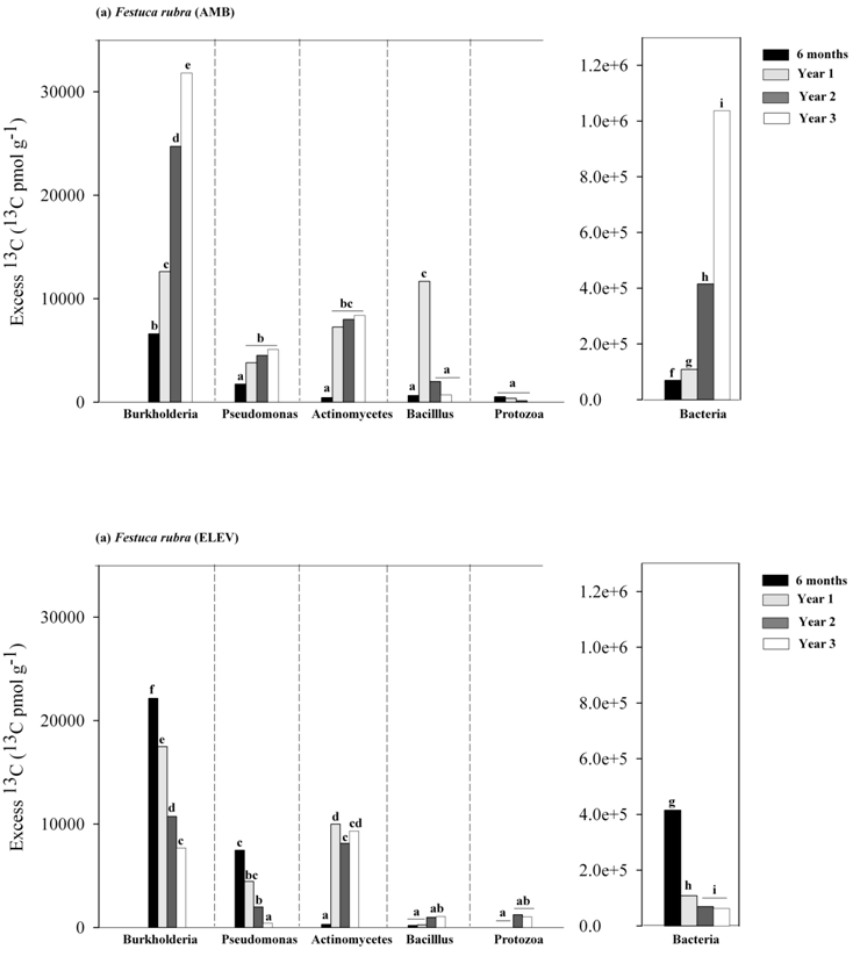

Figure 7: ^{13}C enrichment in the specific bacterial signatures was determined in rhizosphere soil of *F. rubra* at ambient (a) and elevated CO_2 (b) in the phospholipid fatty acids (PLFA) specific signatures for *Burkholderia* spp., *Pseudomonas* spp., actinomycetes, *Bacillus*, protozoa and in the total bacterial community. White is indicative of the 6 months incubation treatment, light grey stands for 1 year incubation, dark grey 2 years incubation and black 3 years incubation. ^{13}C enrichment denotes the excess ^{13}C after natural background subtraction as determined in non-labeled systems. Different letters designates within a graph refer to significantly different averages based upon Tukey HSD test.

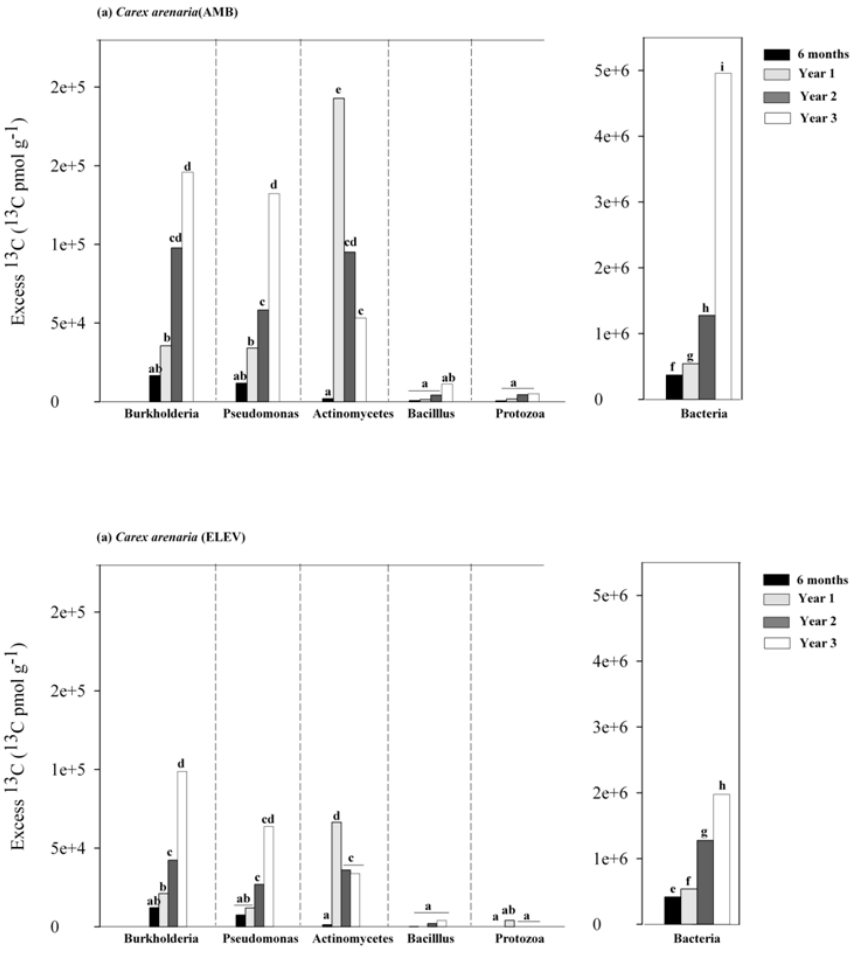

Figure 8: ^{13}C enrichment in the specific bacterial signatures was determined in rhizosphere soil of *C. arenaria* at ambient (a) and elevated CO_2 (b) in the phospholipid fatty acids (PLFA) specific signatures for *Burkholderia* spp., *Pseudomonas* spp., actinomycetes, *Bacillus*, protozoa and in the total bacterial community. White is indicative of the 6 months incubation treatment, light grey stands for 1 year incubation, dark grey 2 years incubation and black 3 years incubation. ^{13}C enrichment denotes the excess ^{13}C after natural background subtraction as determined in non-labeled systems. Different letters designates within a graph refer to significantly different averages based upon Tukey HSD test.

Unfortunately, PLFA analysis does not allow for specific detection of individual microbial species. Stable isotope labelling techniques in combination with molecular tools such as RNA-SIP combined with community profiling and sequencing methods (chapter 6; Griffiths *et al.* 2004; Rangel-Castro *et al.* 2005; Lu *et al.* 2006) provide a more detailed picture of active microbial communities and allowed for a better understanding of microbial community response to elevated CO_2.

Several studies have analysed the microbial community composition under elevated CO_2 using experimental approaches other than the NLFA/PLFA- and RNA- based SIP approaches used in this study. Examples include the study or extracellular enzyme activity (Moscatelli *et al*. 2005; Chung *et al.* 2006), PCR-DGGE analyses (Chung *et al.* 2006; Chapters 3 and 4), substrate-induced respiration measurements, and 16S rRNA clone libraries (Lipson *et al.* 2005).

Consistent with the findings presented here, most of these studies also suggested stimulated fungal pathways under increased atmospheric CO_2 concentrations.This fungal stimulation could be beneficial for the ecosystem functioning as AMF are believed to play a positive role in soil ecosystem functions, such as maintaining soil structure (Bossuyt *et al.* 2001; Rillig *et al*. 2002), C sequestration (Treseder and Allen 2000; Bailey *et al*. 2002) and N immobilization through hyphal translocation (Beare 1997; Frey *et al*. 2000). However, in a 6-year study subjecting a sandy scrub-oak ecosystem to double ambient CO_2 conditions, a decline in soil carbon was observed, despite higher plant growth and increased fungal abundance (Carney *et al.* 2007). Interestingly, Carney *et al.* (2007) also showed that an increased fungal abundance can reduce soil carbon storage, possibly by promoting lignolytic enzyme activity, potentially stimulating a priming effect with respect to the decomposition of recalcitrant organic materials. This could explain the small, or often undetectable, increases in soil carbon content in response to elevated CO_2 even when substantial increases in plant biomass are observed (e.g. Gill *et al.* 2002; Jastrow *et al.* 2005; van Groningen *et al.* 2006).

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