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

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Chapter 8

General discussion

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This thesis has focused on the effects of increased atmospheric carbon dioxide on the structure and function of soil microbial communities.

Rising atmospheric CO_2 levels are predicted to have major consequences on carbon cycle feedbacks and the overall functioning of terrestrial ecosystems (Zhou *et al.* 2006). Photosynthetic activity and the structure of terrestrial macrophytes, especially C_3 plants, is expected to change, but it remains uncertain how this will affect soil-borne communities, which are strongly dependent on plant-derived carbon and exert feedbacks effects on ecosystem function (Hu *et al.* 2001; Carney *et al.* 2007; Staddon 2005; Rillig *et al.* 1999; Phillips *et al.* 2007).

Most of the conceptual models of plant-microbial interactions under elevated CO_2 have focused on broad-scale soil processes. Although such models are appropriate for understanding some ecosystem responses in the longer term, they do not consider how spatial and temporal dynamics in the rhizosphere can influence ecosystem responses to elevated CO_2. Elevated CO_2 can increase rhizosphere C flux through increases in fine root biomass (Norby *et al.*1994; Uselman *et al.* 2000) and/or in mass-specific exudation (Phillips *et al.* 2006). Moreover, elevated CO_2 may also induce changes in the chemical composition of exudates (Phillips *et al.* 2006). Exudates are the preferred substrates for the rhizosphere microflora (Cheng 1999), and the rapid assimilation of exudates creates a ‘rhizosphere effect’ around roots, where the tight coupling between substrate availability and soil microbial activity is likely to influence the C cycle (Paterson 2003). An emerging view in elevated CO_2 research is that microbial interactions are likely to play an increasingly important role in controlling ecosystem-scale responses to global change, highlighting the need for a more rhizo-centric view in this field (Phillips 2007).

In the context of global environmental change, mycorrhizal fungi may play a pivotal role in the terrestrial C cycle. There is currently little information on the input of C to the soil via mycorrhizal extraradical mycelia. Nevertheless, two key aspects of mycorrhizal functioning, namely the rapid acquisition of recent photosynthetate (Johnson *et al.* 2002) and the rapid turnover of the hyphal network in soil (Staddon *et al.* 2003), suggest that mycorrhizal fungi may represent a significant pathway for C sequestration into the soil. Furthermore, AMF biomass, alive or dead, in the soil may account for a very large proportion of the total soil microbial biomass (Olsson *et al.* 1999), and the mycelial network can also facilitate the movement of carbon into the bulk soil.

The use of DNA and RNA-based techniques has facilitated microbial ecology studies, allowing the study of organisms without the need for cultivation. Recent developments have also provided new means of coupling phylogenetic identification of organisms with their activity (Neufeld *et al.* 2007). Moreover, the development of RNA-based stable isotope probing (SIP) has made it possible to link the consumption of given compounds carrying a ^{13}C signature with the small subunit ribosomal RNA molecules of the microbes that consume them (Manefield *et al.* 2002) This important advance has enhanced our understanding of C flow in terrestrial ecosystems.

There have been two major aims of this thesis. The first was to study the plant-driven impacts of elevated CO_2 concentrations on shifts within both broad microbial groups as well as the dynamics of specific groups in mycorrhizal and non-mycorrhizal plants. The second aim was to track the fate of plant-assimilated C in mycorrhizal and non-mycorrhizal plants to the belowground microbial community, and to examine the impact of elevated atmospheric CO_2 levels on these processes in the short- (6 months) and long-term (3 years).

To achieve the first aim, I assessed the plant-driven impact of elevated CO_2 on changes in rhizosphere communities of two dominant coastal sand dune plant species, *F. rubra* (mycorrhizal) and *C. arenaria* (non-mycorrhizal). Coastal dune systems were chosen as the model due to their relative simplicity and particular relevance to issues of global climate change. Plants were grown under controlled temperature and moisture conditions, while subjecting the aboveground compartment to defined atmospheric conditions with either ambient (350 ppm) or elevated (700 ppm) CO_2 conditions in three different costal dune soils. Using PCR-Denaturing Gel Electrophoresis (DGGE), real time PCR and lipid analysis (P(N)LFA), I examined the structure and abundance of the broad microbial and meso-faunal groups, such as bacteria, actinomycetes, fungi and nematodes, as well as the dynamics of specific microbial groups, such as *Pseudomonas* spp., *Burkholderia* spp, *Bacillus, Trichoderma, Fusarium*, arbuscular mycorrhizal fungi (AMF) and phloroglucinol, phenazine and pyrrolnitrin producers in *C. arenaria* and *F. rubra* rhizospheres (**chapters 3 and 4**). A high performance liquid chromatography (HPLC) procedure was also used to analyze the composition and quantity of substrates exuded from the roots of both plants under elevated versus ambient CO_2 (**chapter 4**).

To achieve the second aim, I conducted $^{13}CO_2$ pulse-chase labelling experiments on short- and long-term plant incubations (**chapters 5, 6** and **7**). The $^{13}CO_2$ pulse-chase labelling methodology used overcame the common SIP problem of insufficient label incorporation. ^{13}C Stable Isotope Probing (SIP) applications for tracking plant-derived C fluxes into microbial nucleic acids (RNA-SIP; **chapters 6** and **7**) or biomarkers (^{13}C-N-PLFA; **chapter 5** and **7**) were applied in order to identify the active community using plant-derived ^{13}C labelled exudates in the *F. rubra* and *C. arenaria* root zone and to improve our understanding in the microbial community dynamics associated with rhizosphere carbon flow under ambient and increased CO_2 levels. To gain insight into the flow of carbon to different soil-borne microbial groups, specific fatty-acid biomarkers for AMF, total bacteria, *Pseudomonas* spp., *Burkholderi*a spp., *Bacillus*, actinomycetes and protozoa were used to track the ^{13}C allocation from the atmosphere into rhizosphere communities (**chapter 5**). I selected *Pseudomonas* spp. and *Burkholderia* ssp. as typical rhizosphere bacteria and actinomycetes and bacilli as representative bulk soil groups (**chapters 4** and **5**). AMF, bacterial and general fungal adaptations to increased levels of atmospheric CO_2 were studied in **chapter 7**, using a 3-year elevated CO_2 greenhouse experiment. I monitored the activity, size and structure of the rhizosphere microbial communities of *C. arenaria* and *F. rubra* at regular intervals. Throughout the three years of incubation, I labeled the plants at four separate times. I then tracked the C flow and the subsequent shifts in community composition by using RNA-SIP, biomarkers analysis, real-time PCR and PCR-DGGE approaches.

In the following section, I will address the research questions posed in the general introduction (**chapter 1**) in order to highlight the main findings achieved in this thesis.

1. What is the plant-driven effect of enhanced atmospheric CO_2 concentrations on the composition of the bacterial, fungal and nematode communities in the rhizosphere?

The molecular PCR-Denaturing Gel Electrophoresis (DGGE) community fingerprinting approach on 16S rRNA and 18S rRNA genes proved useful in assessing the influence of elevated CO_2 on the rhizosphere of the mycorrhizal and non-mycorrhizal plants (**chapters 3 and 4**). Analyses of variation partitioning for bacterial, fungal and nematode community profiles revealed that the bacterial community structure was the most affected by elevated

CO_2, with fungal and nematode communities being influenced to a somewhat lesser extent. The factors most influencing fungal community structure were soil origin and plant species, whereas, the structure of nematode communities was best explained by differences in soil origin. Moreover, the response of the rhizosphere communities to elevated CO_2 depended on the plant species. The mycorrhizal plant, *F. rubra,* showed a strong increase in AMF infection under elevated CO_2 and a more pronounced effect of elevated CO_2 on the structure of the rhizosphere microbial community. This suggests that mycelial products, potentially coupled with altered root exudation patterns, may have an important impact on the size and structure of the soil microbial community in response to elevated CO_2.

To gain further insight into the effects of CO_2 enrichment on the sizes of bacterial, fungal and nematode community, I evaluated the bacterial, fungal and nematode community size in the rhizosphere of plants grown under elevated CO_2 by a real time PCR approaches and lipid analyses (**chapter 3**). CO_2 enrichment led to an increase in the sizes of the fungal and nematode communities and to a lesser extent the bacterial community size. Similar to the community composition, rhizosphere community size strongly depended on the plant species. Effects of elevated CO_2 were also soil dependent, with greater influence observed in the more organic-rich soils, which also supported higher levels of AMF colonization.

Overall, these results allowed me to conclude that responses of soil-borne communities to elevated CO_2 are different for bacteria, fungi and nematodes and depend on plant species and soil type.

2. Do enhanced CO_2 concentrations result in shifts in the composition of specific bacterial and fungal rhizosphere groups?

To gain more detailed insight into the specific impacts of elevated CO_2 on soil-borne communities, I examined the dynamics of specific bacterial groups, such as phloroglucinol, phenazine, pyrrolnitrin producers, *Pseudomonas*, *Burkholderia*, *Bacillus,* and actinomycetes in the mycorrhizal and non-mycorrhizal plants (**chapter 4**). Among the bacterial groups, I selected *Pseudomonas* spp. and *Burkholderia* ssp. as typical rhizosphere bacteria and actinomycetes and the genus *Bacillus* (Smalla *et al.* 2001) as representative groups for bulk soil bacterial communities. Distance-based redundancy analysis (db-RDA) of resulting PCR-DGGE profiles revealed that elevated atmospheric CO_2 influenced *Pseudomonas* and *Burkholderia* more strongly than actinomycetes and *Bacillus* spp. The compositions of actinomycetes and *Bacillus* spp. were mostly influenced by the soil properties and nutrient availability. These results were consistent with the hypothesis that rhizosphere bacteria, such as *Pseudomonas* spp. and *Burkholderia* spp., would be more influenced than bulk soil bacteria in response to increased plant-derived C input in the soil. Moreover, the response of *Pseudomonas* and *Burkholderia* rhizosphere community structures to elevated CO_2 depended on the plant species. The mycorrhizal plant, *F. rubra,* had the stronger influence on these rhizo-competent bacterial groups, in accordance with the findings reported in **chapter 3**.

Using an HPLC approach, a quantitative and qualitative analysis of root exudation patterns was performed, comparing the model mycorrhizal and non-mycorrhizal plants under ambient and elevated CO_2 conditions (**chapter 4**). Two-fold greater levels of sugar exudation were observed in the *F. rubra* rhizosphere, compared to *C. arenaria*. Moreover, I observed that *F. rubra* at elevated CO_2 produced four times more trehalose than at ambient CO_2, whereas *C. arenaria* showed no detectable trehalose production under either of the

CO_2 levels. AMF, which are known to exude trehalose, may be involved in this increased trehalose release, which may in turn affect microbial populations in the mycorhizosphere.

Novel real-time PCR assays were used to detect genes involved in antagonistic activities, targeting pathways for the production of DAPG, PCA and PRN (**chapter 4**). CO_2 enrichment influenced the sizes of the PRN and DAPG producers in the rhizosphere and to a lesser extent the PCA producers. The density of antibiotic producers also strongly depended on the plant species studied and on the soil origin.

The colonization densities of two important fungal genera, *Fusarium* and *Trichoderma* were also examined via real-time PCR assays (**chapter 4**). The genus *Fusarium* is known to contain numerous important plant pathogens, while many *Trichoderma* species are known for their antagonistic capabilities against plant pathogens. The density of *Fusarium* spp. was significantly reduced in the rhizosphere of the mycorrhizal plant exposed to elevated atmospheric CO_2. In contrast, *Trichoderma* spp. increased in density under elevated CO_2 in the *F. rubra* rhizosphere, following the same trend as the general fugal community size**.** Although still preliminary in nature, these results suggest that the balance of pathogenic and antagonistic pressures may be affected by elevated CO_2 conditions.

3. What is the impact of elevated CO_2 on the capability of the soil microbial community to incorporate plant-assimilated C?

The biomarker analysis of $^{13}CO_2$ pulse-chase labelling experiments allowed me to track plant-derived C fluxes into the root microbial community (**chapter 5**) in the model plant systems studied. To gain insight into the flow of carbon to different soil-borne microbial groups, specific fatty-acid biomarkers for AMF, total bacteria, *Pseudomonas* spp., *Burkholderia* spp., *Bacillus*, actinomycetes and protozoa were used to track the ^{13}C allocation from the atmosphere into rhizosphere communities. Results suggested that the major pathway of C flux from the roots into the soil microbial community is via mycorrhizal fungi. I observed a rapid transfer of photosyntates into mycorrhizal biomass, and a subsequent slow C release to bacterial genera known to colonize the rhizosphere. This pattern was particularly pronounced in the elevated CO_2 treatment.

The bacterial genera *Burkholderia* and *Pseudomonas* were strongly influenced by elevated CO_2, whereas the genus *Bacillus* and actinomycetes were not, confirming that effective accumulation of plant-derived carbon in the short term is restricted to efficient rhizosphere colonizers (**chapter 4**).

My results indicate that increased levels of plant-derived carbon enter soil-borne microbial communities principally via AMF, especially at elevated CO_2, with direct plant-bacterial transfer initially playing only a relatively minor role.

4. What are the functional consequences of changes in the community structure of some of the major microbial players brought about by elevated concentrations of atmospheric CO_2?

The combination of RNA-based stable isotope probing (SIP), community fingerprinting and real-time PCR analyses allowed me to trace plant-fixed carbon to distinct microbial populations incorporating plant-derived carbon under elevated atmospheric CO_2 (**chapter 6**). The analysis of labeled RNA confirmed the hypothesis proposed in **chapter 5** that AMF are the main conduit in the transfer of carbon between plants and soil. The ^{13}C-RNA-based clone libraries showed that the retention of C in AMF under elevated CO_2 atmospheric conditions coincides with pronounced shifts in plant-AMF interactions. At ambient atmospheric CO_2 I detected Acaulosporaceae, specifically *Acaulospora lacunosa*, playing as the dominant AMF incorporating ^{13}C-labelled exudates. In contrast, at elevated CO_2, I observed Glomeraceae, specifically *Glomus claroideum*, as the main AMF using ^{13}C exudates. The switch to elevated CO_2 levels thus resulted in an almost complete shift in the AMF populations receiving C from the plant. AMF species identity is known to affect (myco-) rhizosphere community structure and activity (Vandenkoornhuyse *et al.* 2007) , suggesting that this AMF shift may represent an important mechanisms driving the observed soil-borne community responses to elevated CO_2.

By analyzing the bacteria and fungal rhizosphere communities actively incorporating ^{13}C exudates, we observed that communities incorporating plant-derived carbon under different levels of atmospheric CO_2 were highly disparate. Elevated CO_2 conditions increased the diversity of fungi taking up plant-derived C. In contrast, the bacterial community consuming plant C at elevated CO_2 was represented only by Proteobacteria species, with no detection of the Chloroflexi, Planctomycetes and Verrucomicrobia as was the case at ambient CO_2. Within the Proteobacteria, the frequency and diversity of the sequences affiliated with the Pseudomonaceae and Burkholderiaceae showed higher species richness at elevated CO_2. At ambient CO_2, *P. fluorescens* was the main *Pseudomonas* species incorporating root exudates. Under elevated CO_2 conditions, the species richness of the active *Pseudomonas* community increased over time with *P. fluorescens*, *P. trivialis,* and *P. putida* also being detected. *The species richness of Burkholderiaceae under elevated CO_2 also increased by a factor of two.* Interestingly, the Pseudomonaceae and Burkholderiaceae species reported under elevated CO_2 atmospheric conditions in **chapter 6** have been shown to have the potential to act as mycorrhizal helper bacteria *in* Glomus *species.*

The results described in **chapter 6** confirmed the findings of **chapters 3, 4** and **5**, supporting a model in which plant-assimilated carbon is first rapidly transferred to AMF, followed by a slower release from AMF to distinct bacterial and fungal communities (Fig 1). The model derived from our results provides a general framework for reappraising our view of carbon flow paths in soils and their effects on soil biodiversity under elevated atmospheric CO_2 concentrations.

5. What are the consequences of elevated CO_2 on C-incorporating soil communities in the longer term?

In **chapter 7,** I extended the findings of **chapters 3, 5,** and **6** to a longer-term study. The mycorrhizal plant (*F. rubra*) exerted a greater influence on bacterial and fungal communities over 3 years of incubation under elevated CO_2. Biomarker data indicated that arbuscular mycorrhizal fungi (AMF) incorporated plant-derived C, which was only later translocated to the bacterial communities. Elevated CO_2 over the course of three years caused an increase in the proportion of ^{13}C retained in the arbuscular mycorrhizal fungal biomarker 16:1ω5 and delayed the subsequent translocation of C to the bacterial community over the course of the experiment. Thus, the results found in the short-terms incubations experiments described in **chapters 3, 4, 5** and **6** were supported by the relatively long-term incubation experiment in which communities had a much longer time to adapt to elevated CO_2 conditions.

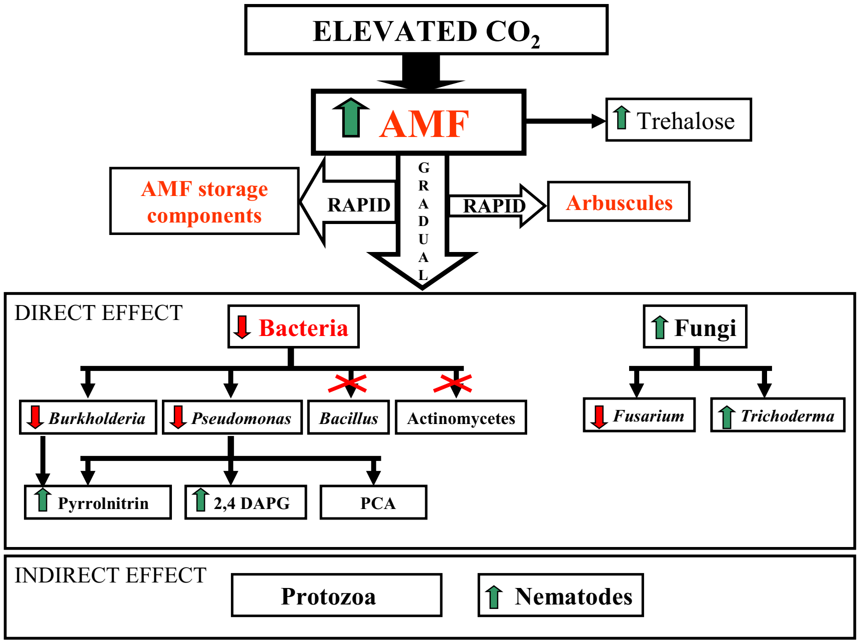

Fig 1: Conceptual model summarizing the mycorrhizal and micro- and macro-fauna affected by elevated CO_2 atmospheric concentration. Green arrows indicate increases and red arrows decreases in the respective community sizes, as determined by real-time PCR and lipid analysis. Absence of an arrow indicates that no significant changes in the communities size where detected. The mechanism and magnitude of the C-flow along the soil food-web is indicated by the black arrows.