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Citation

Drigo, B. (2009, January 21). *The impact of increased atmospheric carbon dioxide on microbial community dynamics in the rhizosphere*. Netherlands Institute of Ecology, Faculty of Science, Leiden University. Retrieved from https://hdl.handle.net/1887/13419

Chapter 3

Impact of elevated CO_2 on the rhizosphere communities of *Carex arenaria* and *Festuca rubra*

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Global Change Biology, **13**, 2396-2410 (2007)

Abstract

The increase in atmospheric CO_2 levels is predicted to stimulate plant carbon fixation, potentially influencing the size, structure and function of micro and mesofaunal communities inhabiting the rhizosphere. To assess the effects of increased atmospheric CO_2 on bacterial, fungal and nematode communities in the rhizosphere, *Carex arenaria* (a non-mycorrhizal plant species) and *Festuca rubra* (a mycorrhizal plant species) were grown in three dune soils under controlled soil temperature and moisture conditions, while subjecting the above-ground compartment to defined atmospheric conditions differing in CO_2 concentrations (350 and 700 µl l^{-1}). Real-time PCR and PCR-denaturing gel electrophoresis methods were used to examine effects on the size and structure of rhizosphere communities. Multivariate analysis of community profiles showed that bacteria were most affected by elevated CO_2, and fungi and nematodes to a lesser extent. The influence of elevated CO_2 was plant dependent, with the mycorrhizal plant (*F. rubra*) exerting a greater influence on bacterial and fungal communities. Biomarker data indicated that arbuscular mycorrhizal fungi (AMF) may play an important role in the observed soil community responses. 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. These results indicate that responses of soil-borne communities to elevated CO_2 are different for bacteria, fungi and nematodes and dependent on the plant type and soil nutrient availability.

Introduction

Although a growing body of research has been devoted to the potential effects of increased CO_2 concentrations on vegetation dynamics, less attention has been paid to either the potential associated changes in soil-borne microbial communities or the key ecosystem functions they perform. Elevated atmospheric CO_2-levels have generally been observed to result in higher rates of photosynthesis (Ainsworth & Long 2005), particularly in C_3 plants (Long *et al.* 2004). Net photosynthesis under elevated CO_2 is likely to stimulate production of the above-ground vegetation and have a direct effect on carbon fluxes from the above-ground compartment into the soil. Under current ambient CO_2-levels, it is estimated that up to 50 % of the total assimilated carbon may be released into the soil. Half of this amount is subsequently found in the roots and about one-third as CO_2 evolved from the soil by root respiration and microbial utilization of root-borne organic substances. The remaining part of below-ground translocated C is incorporated into the soil organisms and soil organic matter (Kuzyakov & Domanski 2000). Under elevated atmospheric CO_2-levels, carbon input to soil is generally expected to increase due to improved plant carbohydrate status (Barron-Gafford *et al.* 2005), even if there is no significant CO_2 stimulation of above-ground growth (Korner & Arnone 1992). The quantity and quality of carbon substances actively released into the rhizosphere are known to influence the structure and function of soil-borne microbial communities (Darrah 1995; Hodge *et al.* 1998). It is therefore likely that changes in rhizodeposition in response to increased atmospheric CO_2 concentrations will also have an impact on soil microbial communities (Jones *et al.* 1998).

It is hypothesized that soil community responses to increased atmospheric CO_2 concentrations occur mostly via indirect mechanisms, especially via interactions with changing vegetation patterns and physiology. However, little is known about the nature or consequences of these responses. This lack of knowledge stems primarily from two sources. Firstly, belowground microbial communities have received less research attention than above-ground macrophytes in studies examining effects of global atmospheric changes. Secondly, the linkages between vegetation dynamics, physiology and associated microbial communities are not yet fully understood (Torsvik & Ovreas 2002). Of the few studies that have addressed structural changes within the microbial communities, the focus has primarily been on specific microbial populations such as *Rhizobium* species (Schortemeyer *et al*. 1996; Montealegre *et al*. 2000), *Pseudomonas* species (Marilley *et al.* 1999; Tarnawski *et al.* 2006; Tarnawski & Aragno, 2006), β/α-Proteobacteria or Acidobacteria (Lipson *et al.* 2006) and mycorrhizal fungi (Gamper *et al.* 2005).

To gain further insight into the effects of elevated atmospheric CO_2 on soil-borne communities, we assessed the plant-driven impact of elevated CO_2 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 nematode 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 $\mu l\ l^{-1}$) or elevated (700 $\mu l\ l^{-1}$) CO_2 concentrations. Bacterial, fungal and nematode community sizes were determined by Q-PCR and biomarker analyses, and molecular community profiles were generated for these communities by PCR-DGGE. Subsequently, multivariate statistical analyses were used to compare the relative impact of elevated CO_2 treatment versus plant and soil effects on these communities (Ter Braak & Verdonschot 1995; Borcard *et al.* 1992; Filion *et al*. 2000).

Material and methods

Plant species

Sand fescue, *Festuca rubra* ssp. *arenaria* (Caespitose), is a clonal perennial grass commonly occurring on coastal and inland sand dunes worldwide. *F. rubra* benefits from burial by windblown sand. The vigor of *F. rubra* declines if fore-dunes become stabilized, and the species is typically replaced by *Carex arenaria* (Van der Putten 1997). *F. rubra* is recognized as a mycorrhizal species (Greipsson & El-Mayas 1999; Orlowska *et al.* 2005). Sand sedge, *Carex arenaria* (Cyperaceae) is an established clonal perennial plant with long-lived tiller connections (rhizomes) (Tietema 1981), commonly occurring on costal and inland sand dunes in Europe, and on the Atlantic coast of North and South America (Tietema 1981; Olff *et al*. 2000; De Ridder-Duine *et al.* 2005). The position of *C. arenaria* within the plant community can vary widely from dominant to subdominant and forest under-story, and this species is thought to be non-mycorrhizal (Rickel *et al.* 1994).

Sites and soils

To test the effect of elevated CO_2 on *F. rubra* and *C. arenaria* planted on different grassland sandy soils, we sampled in a coastal dune site (Middelduinen), a former beach plain (Kwade Hoek) and a river dune site (Bergharen) in the Netherlands. *F. rubra* and *C.*

arenaria belonged to the dominant species at all three sites. Although the soils from all study sites had a sandy texture, the physical, chemical and biochemical properties of the soils were quite disparate (Table 1). The dune grassland of Middelduinen and the forest of Bergharen were acidic, with low calcium carbonate contents. The Kwade Hoek dune grassland soil contained about 3% calcium carbonate, resulting in a high pH (8.61). In addition to these differences, Kwade Hoek soil contained less organic matter and ergosterol (an indicator of fungal biomass) than the other soils. Each sampling site was divided into 4×4 m subplots. For each subplot, ten cores were taken within tussocks of *F. rubra* and *C. arenaria* and the 5-15 cm layer was collected. Soil samples were put in plastic bags and transported in a cooling box to the lab. Per sampling site, the material (250 kg) was sieved (4 mm mesh), homogenized and stored at 4 °C until use (within one week after sampling).

Establishment of plants

F. rubra and *C. arenaria* seeds (Cruydt Hoek, Groningen, The Netherlands) were sterilized with 70% alcohol and 6% sodium hypochlorite and germinated on sterilized glass beads (3 mm diameter) in a growth chamber set at 25 ºC light (16 h) and 15 ºC dark (8 h). Plastic containers (750 cm^3) were filled with 1 kg of soil and wetted to 10% volumetric water content (based on dry weight). Uniform four-week-old seedlings (plumule length 3-5 cm) were selected and transferred to containers (three seedlings per container), which were put into one of four controlled CO_2 flow cabinets (see below). For each soil origin and CO_2 flow cabinet, six replicates of unplanted soil were incubated to test if the bulk soil communities were related to the rhizosphere communities under the different treatments.

CO_2 flow cabinets

Pots were incubated in four controlled CO_2 flow cabinets (1.9 m x 2.4 m x 0.9 m; Vötsch, Industrietechnik GmbH, Germany). Each cabinet provided an airtight system (1500 L airtight units) enabling the maintenance of a constant atmospheric CO_2-level of 350 $\mu l\ l^{-1}$ or 700 $\mu l\ l^{-1}$. CO_2 concentrations were maintained automatically using mass flow controllers (Brooks Smart, DMFC, Emmerson process Management, USA). A board infrared gas analyser (IRGA, CARBOCAP, GMT222, Dual Wavelength NDIRsensor, Vaisala, Oyj, Finland) was fitted in each of the flow cabinets to control the correct delivery of pure CO_2.

Table 1: Mean soil characteristics for bulk soil cores (0-15cm depth) collected at the three different soil origins

Soil origins	Org. C %	NH_4 mg kg^{-1}	NO_3 mg kg^{-1}	Total N %	C:N -	pH -	Total P mg kg^{-1}	$CaCO_3$ %	Particle > 150 µ	Ergosterol mg kg^{-1}
Bergharen	1.97^a	1.03^a	7.46^a	0.0605^a	19.15^b	4.32^a	34.13^a	0^a	87	1.7^a
Kwade Hoek	0.21^b	0^b	1.28^b	0.0058^b	44.62^c	8.61^b	71.11^b	3.27^b	92	0.2^c
Middelduinen	3.38^c	1.1^a	6.94^a	1.1165^c	17.89^a	4.34^a	95.52^c	0.04^a	77	1.6^a

Different letters within columns refer to significantly ($P < 0.05$) different averages based.

The elevated CO_2 concentration (700 µl l^{-1}) was obtained by injection of CO_2 from a pressurized cylinder and the ambient (350 µl l^{-1}) by removal of CO_2 from the air by a solid carbon soda filter (Sofnoline, SIGMA). In each CO_2 flow cabinet, maximum daily temperatures ranged from 21 – 22 ºC; daily minimum temperatures ranged from 16 – 18 ºC. Light intensity averaged 250 µE, with a 16 h photoperiod and a relative humidity of 70%. Climate data for the cabinets were stored digitally during the entire incubation period.

Experimental design

The experiment was set-up according to a split-plot design. In this design we considered as whole plot the CO_2 flow cabinets, while the two plant species (*F. rubra* and *C. arenaria*), and three soil origins (Bergharen, Kwade Hoek and Middelduinen) were considered as sub-treatments. Within each CO_2 flow cabinet there were 30 pots with *F. rubra* (10 per each soil origin) and 30 with *C. arenaria* and 18 pots of unplanted soil (6 for each soil origin), yielding a total number of 312 pots distributed in the four cabinets.
Germinating weeds were removed upon detection, and the soil moisture content was maintained at 10% using demineralized water. Within each chamber, all 78 pots were shuffled after each watering period to reduce potential position effects within the growth chambers.

Harvesting procedure

F. rubra and *C. arenaria* were harvested respectively at 73 and at 62 days after germination, when plants were dominated by shoot growth. In *C. arenaria*, the root/soil systems were first separated from rhizomes. Upon harvest, roots were shaken gently to remove loosely adhering soil. Approximately 80% of the initial soil remained in the pots, and this portion was considered 'bulk soil'. Pieces of roots with remaining adhering soil were cut and taken as a combined rhizosphere sample. Sub-samples of rhizosphere and bulk soil for DNA and ergosterol analyses were stored at -80 ºC until use. Rhizosphere and bulk soil for phospholipid (PLFA) and neutral (NLFA) lipid fatty acids was freeze-dried immediately after sampling. Sub-samples of bulk soil for physical and chemical analyses were stored at 4 ºC for no more than one week prior to analysis.

Soil analysis and microbial biomass measurement

Soil analyses were carried out using standard protocols (Carter 1993). Microbial biomass assessments were performed by lipid fatty acid analyses and ergosterol detection (fungal biomarkers). PLFA and NLFA lipid fatty acids were extracted and analyzed by chromatography (Boschker 2004). Peak areas were calculated relative to the internal standard methyl nonadecanoate fatty acid (19:0), which was added before methylation. The following fatty acids were summed up to estimate bacterial biomass: i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, i17:0, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0 (Frostegard & Baath 1996). The NLFA 16:1ω5 was employed as a signature for arbuscular mycorrhizal fungal (AMF) biomass estimation (Olsson 1999; Van Aarle & Olsson 2003; Olsson & Johnson 2005). The PLFA 16:1ω5 was used for the same purpose but to a lesser extent, because PLFA 16:1ω5 has a background originated from bacteria. We analyzed NLFA and PLFA 16:1ω5 separately since they represent different structures and developmental stages of AMF (Olsson & Johnson 2005).

DNA isolation and PCR-DGGE analyses

Soil genomic DNA was isolated from a 500 mg (wet weight) sub-sample using the PowerSoil™ DNA Isolation kit according to the manufacturer's specifications (MoBio Laboratories, Solana Beach, CA, USA). DNA was eluted in 50 µl of deionized water and stored at -20°C until use.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) strategies were applied to assess the composition of bacterial, fungal and nematode communities (supplementary data, figure S1). Table 2 summarizes the primers, thermocycling regimes, and electrophoresis conditions used to analyze the different target communities. All PCRs were carried out in 25 µL volumes containing 2.5 µL of 10×PCR buffer, 2.5 µL of bovine serum albumine (BSA; 4 mg mL^{-1}), 0.75 µL of each primer (30 pM), 2.5 µL of dNTPs mix (8 mM), and 0.056 U of Expand High Fidelity polymerase (Roche, Mannheim, Germany). All primers were synthesized by Alpha DNA (Montreal, Quebec). To reduce variation caused by pipetting errors, PCR mixtures were prepared using a Corbett Robotics CAS-1200 precision liquid handling system (Sydney, Australia). All amplifications were carried out on a PTC-200 thermal cycler (MJ-Research, Waltham, MA). All thermocycling programs were preceded by an initial denaturation step (95°C for 5 min) and followed by a final elongation step phase (72°C for 10 min). For each cycle of PCR, denaturation was at 95 °C for 1 min, annealing at the specified temperature (Table 2) for 1 min and elongation at 72°C for 1 min. Touchdown protocols started with the highest annealing temperature, which was subsequently lowered by 2°C for each 2 cycles until the target annealing temperature was reached. In addition to the target soil DNA, a 'negative' (without DNA) control sample was included with every PCR run. PCR products were examined by standard 1.5% (w/v) agarose 0.5×TBE gel electrophoresis with ethidium bromide staining, to confirm product integrity and estimate yield. Approximately 0.5 µg of PCR product was used for DGGE analysis, using the method of Muyzer *et al.* (1993) as modified by Kowalchuk *et al.* (2002), except that the linear gradients indicated were as in Table 2. DGGE were carried using a D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA).

Table 2: Primers, PCR, DGGE and real time PCR conditions used in this paper.

Community	Primers	Amplification protocol[1]	Types of analyses	Reference
Bacteria	968-gc/1378	Touchdown 65°C to 55°C; 35 cycles	DGGE gradient[2] (45-65% denaturant)	(Heuer *et al.*, 1997)
	Ba519f/Ba907r	52°C; 40 cycles	real-time PCR	(Lueders *et al.* 2004a)
Fungi	FR1-gc/FF390	Touchdown 55°C to 47°C; 37 cycles	DGGE gradient[2] (40-55% denaturant)	(Vainio and Hantula 2000)
	Fung5f/FF390r	48°C; 40 cycles	real-time PCR	(Lueders *et al.* 2004b)
Nematodes	NEMF1-gc/ NEM896r	53°C; 40 cycles	DGGE gradient[2] (25-50% denaturant)	(Waite *et al.,* 2003)
	NEMF1/ NEM896r	53°C; 40 cycles	real-time PCR	

[1] **PCR protocols are given as: annealing temperature; number of cycles. The remaining of the procedure is given in the text.**

[2] **100% denaturant is defined as 40% (v/v) formamide and 7 M urea.**

All gradient gels were topped with 10 ml of acrylamide containing no denaturant and electrophoresis was carried out at 60°C and 200V for 10 min followed by an additional 16h at 70V. Gels were stained in ethidium bromide and digital images captured using an Imago apparatus (Gentaur, Brussels, Belgium) upon UV transillumination.
We randomly selected 216 DNA extracts obtained from rhizosphere soil samples and 36 from unplanted soil at different treatments and analysed those for the composition of the bacterial, fungal and nematode communities. Due to the number of samples, multiple DGGE gels were run for the three different soil origins (Bergharen, Middelduinen, Kwade Hoek) and the different plants species (*F. rubra* and *C. arenaria*). Each sample was assessed in at least two different runs to confirm the reproducibility of the DGGE fingerprint across gels. In each, to facilitate comparable statistical analysis, all gels of the same community were combined into a composite image using Corel PHOTO-PAINT 12 prior to further analysis (Corel Corporation, 2003). The gel images were normalized with respect to migration pattern of the standard lanes of each gel, and sample patterns were compared using the Image Master 1D program (Amersham Biosciences, Roosendaal, the Netherlands) with rolling circle (r = 10) background subtraction and automated band detection. Matching of bands was performed in reference to a hypothetical composite lane containing bands at all positions found across the entire dataset.

Real-time PCR

Real-time PCR was performed using the ABsolute QPCR SYBR green mix (AbGene, Epsom, UK) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) to quantify fungal, bacterial, and nematode DNA. 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 soil was carried as described in Table 2. Standards were made from full-length PCR-amplified 18S or 16S genes from pure fungal and bacterial isolates, respectively. For nematodes, the same amplification protocol was used as for PCR-DGGE analyses except that the ABsolute QPCR SYBR green mix was substituted for the normal PCR mix and the forward primers was without GC clamp. To make the nematode standard, extracted rhizosphere DNA was PCR-amplified, and cloned. One resulting clone that contained a proper insert of nematode origin was randomly chosen and used in a colony PCR procedure using plasmid enclosed primer, and the resulting PCR product was purified and diluted to known concentrations. Using 10-fold increments, the standard concentrations were adjusted from 10^8 to 10^1 SSU rRNA gene copies μl^{-1} for bacteria, from 10^5 to 10^1 SSU rRNA gene copies μl^{-1} for nematodes and from 10^7 to 10^1 SSU rRNA gene copies μl^{-1} for fungi. Most of the samples, and all standards, were assessed in at least two different runs to confirm the reproducibility of the quantification.

Statistical analysis

The DGGE fingerprints obtained were binary coded and used in statistical analysis as “species” presence-absence matrices. The influence of plant species (*C. arenaria* versus *F. rubra*), soil origin (Bergharen, Kwade Hoek and Middelduinen), CO_2 concentrations (ambient versus elevated) and the interactions between these three factors on the community structure, as examined by PCR-DGGE, was tested by distance-based redundancy analysis (db-RDA, Legendre & Anderson 1999). Jaccard‘s coefficients of similarity were first calculated between samples and used to compute principal coordinates

(PCoA) in the R-package (Casgrain & Legendre 2001). When necessary, eigenvectors were corrected for negative eigenvalues using the procedure of Lingoes (1971) and then all the PCoA axes were exported to Canoco version 4.5 (Ter Braak and Šmilauer, 2002) and treated as “species” data. To test the effects of the three groups of factors (plants, soil origin and CO_2), they were entered as dummy binary-variables. In Canoco one group of factors was entered as the explaining variables in the model while the other two groups of factors were entered as covariables. The significance of such models was tested with a Monte-Carlo test based on 999 permutations restricted for split-plot design, with whole-plots being the CO_2 flow cabinets. Further db-RDA analyses were also carried as above but on subsets of the whole dataset, by either analyzing plants species or soil origins separately.

The percentage of variation in the dataset, that was related to the different factors, was determined by variation partitioning analysis (Borcard *et al.* 1992) using the same strategy as for db-RDA analyses. The percentage of variation explained by a factor was the trace of the analysis constrained using this factor (RDA) divided by the trace of the unconstrained analysis (PCA). Unexplained variation was calculated by subtracting the trace of the overall amount of explained variation by the different factors in the model from the total inertia.

Relationships between microbial community structure, as assessed by PCR-DGGE, and soil environmental parameters were determined with Canonical Correspondence Analyses (CCA), excluding the CO_2 effect, (i.e. using CO_2 concentration levels as supplementary environmental variables). Variability caused by different soils and plant species was removed from the model by entering these factors as co-variables. Separate analyses were carried out for bacterial, fungal and nematode communities to distinguish key environmental parameters influencing the structure of the different microbial communities. Calculations were done with Canoco version 4.5 (Ter Braak and Šmilauer, 2002).

Soil parameters, PLFA/NLFA and SSU rRNA gene numbers were analyzed using analysis of variance (ANOVA). Analyses were carried out according to the split-plot design as described by Filion *et al*. (2000). The F statistic used for testing the significance of main effects of the CO_2 treatment applied to whole plots (CO_2 flow cabinets) was obtained by dividing the treatment mean-square by the mean-square for CO_2 flow cabinets nested within CO_2 treatments. The error term to test for interactions between CO_2 and soil origin or plant species was based on the mean square of the interaction between those treatments and cabinets nested in CO_2 (Filion *et al.* 2000). Analyses were carried in Statistica 7.0 (StatSoft Inc., Tulsa, OK). Normality was tested with a Shapiro-Wilks test and by inspection of residuals, and variance homogeneity by Levene’s test. When data failed to satisfy one of these tests, an appropriate transformation was applied (log or square-root transformation). Tukey’s honestly significant difference (HSD) method and the modified version for unequal sample size (Unequal N HSD in Statistica) were used for post-hoc comparisons with a 0.05 grouping baseline.

Results

Effect of elevated CO_2 on bacterial, fungal and nematode community profiles

Plant species, soil origin, CO_2 concentration and the interactions between these factors all explained a significant ($P < 0.001$) part of the variation in the bacterial, fungal and nematode communities (supplementary data, table S1). Variation partitioning of PCR-DGGE datasets for these three communities revealed that CO_2 treatment, soil origin, and plant species together explained about 50% of the variation in community profiles for bacteria and fungi, and about 30% for nematodes (Fig. 1a). The three groups of variables all explained a significant part of the variation, but their relative contribution differed between groups of organisms. For bacteria, the greatest degree of variation was explained by elevated CO_2 (18.9%), while soil origin and plant species both explained about 14-15% of the total variation. For fungi, the greatest degree of variation was explained by soil origin (18.5%) and plant species (17.6%), while elevated CO_2 explained only 10% of the total variation. For nematodes, soil origin was the most explanatory factor (13.3%), followed by elevated CO_2 (10.1%) and plant species (7.6%).

Variation partitioning analyses on datasets separated by plant species revealed different trends for *F. rubra* and *C. arenaria*. For *C. arenaria*, soil origin explained the greatest amount of variation for bacterial, fungal and nematode community composition (Fig 1b). In particular, fungal community composition in *C. arenaria* rhizosphere was strongly influenced by soil origin (63.1%). CO_2 treatment explained 9-24% of variation in *C. arenaria* ($P < 0.001$). In *F. rubra* rhizosphere, soil origin and elevated CO_2 explained roughly an equal amount of variation for all three groups of organisms (Fig. 1c). The amount of variation explained by elevated CO_2 was higher for *F. rubra* than for *C. arenaria*, especially for bacterial and fungal communities.

Variation partitioning for the three soil origins separately revealed that in Bergharen (Fig. 1d) and Middelduinen (Fig. 1f) elevated CO_2 (24.4% and 31.5%) and plant species (34.5% and 38.4%) both significantly ($P < 0.001$) explained variation in bacterial communities. In Kwade Hoek (Fig. 1e), variation in bacterial community composition could not be significantly explained by elevated CO_2 (0.32%; $P = 0.65$). For all three sites, elevated CO_2 and plant species both significantly ($P < 0.001$) explained a part of nematode and fungal community composition.

In the unplanted bulk soil, plant species, CO_2 treatment and the interactions between these factors did not explain a significant ($P = 0.76$) part of the variation in bacterial, fungal and nematode communities. As expected, only soil origin had a significant ($P<0.001$) effect on the structure of the unplanted soil borne communities (data not shown).

Canonical Correspondence Analysis (CCA; supplementary data, figure S2) showed that soil variables could significantly explain bacterial, fungal and nematode DGGE patterns (test of significance of all canonical axes: $P = 0.001$). The environmental parameters that were most strongly related to patterns of community profiles at elevated versus ambient CO_2 were total nitrogen, total carbon, total phosphate, extractable phosphate (Olsen-P), organic carbon and pH. Furthermore, variation in the bacterial rhizosphere community was related to calcium carbonate and C-to-N ratio.

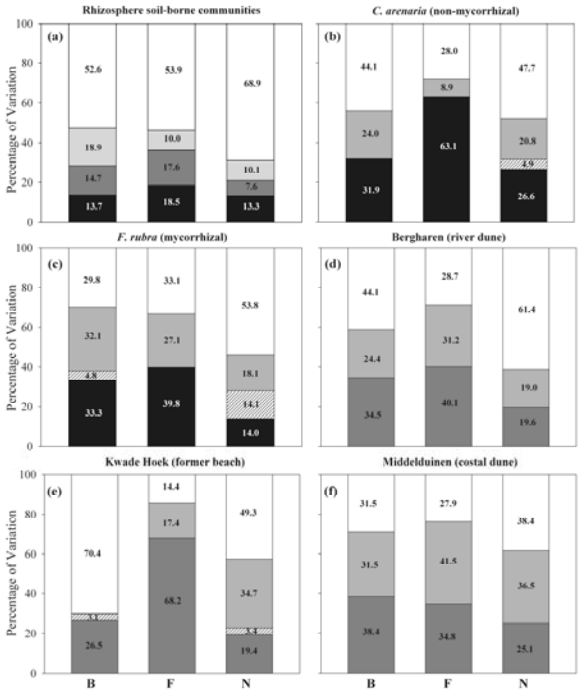

Figure 1: Variation partitioning representation for DGGE patterns of the (B) bacterial, (F) fungal and (N) nematode rhizosphere communities for: (a) complete dataset, (b) communities under *C. arenaria* (non-mycorrhizal) plants only, (c) communities under *F. rubra* (mycorrhizal) plants only and communities under Bergharen, river dune (d), Kwade Hoek, former beach (e) and Middelduinen, costal dune (f). All of the different factors (CO_2 levels, plant species and soil origin) had a significant effect ($P < 0.001$) on the microbial community structure as tested by db-RDA (see material and methods for details and table S1 for detailed results). Unexplained variance (white); CO_2 level (light gray); Plant species (dark gray); Soil origin (black); shared variation between Soil origins + CO_2, or Plant species + CO_2 (hash). Shared variations inferior at 3% were not represented.

Effect of elevated CO_2 on the rhizosphere community densities

SSU rRNA gene abundances for bacteria, fungi and nematodes were generally much higher for *F. rubra* than *C. arenaria* and differed with respect to soil origin (Table 3). In Kwade Hoek soil, *C. arenaria* generally yielded relatively low rRNA gene abundances for bacteria, fungi and nematodes, but this was not observed for *F. rubra*. For fungal ($F_{1,2} = 215.06$, $P < 0.01$) and nematode communities ($F_{1,2} = 1011.34$, $P < 0.001$), there was a significant $CO_2 \times$ Plant species interaction, indicating that CO_2 elevation had different effects on the two plants rhizospheres. More complex interactions between CO_2 concentration, plant species and soil origins were also observed. For *C. arenaria*, bacterial and fungal rRNA gene abundance decreased with elevated CO_2 in Bergharen and Middelduinen soils, whereas it

Table 3: Bacterial, fungal and nematode SSU rRNA genes abundance in the rhizosphere of *C. arenaria* and *F. rubra* grown in three different soils at elevated (ELEV) and ambient (AMB) CO_2 concentrations and associated ANOVA tests.

Plant species	Site location	CO_2 treatment	Bacterial 16S rRNA log copies	Fungal 18S rRNA log copies	Nematodes 18S rRNA log copies	Bacterial-Fungal ratio
C. arenaria	**Bergharen**	AMB	8.85^{bc}	7.56^{a}	5.56^{b}	1.17^{abc}
		ELEV	8.23^{dg}	7.41^{a}	6.34^{a}	1.10^{bcd}
	Kwade Hoek	AMB	7.59^{h}	5.46^{f}	5.26^{b}	1.26^{e}
		ELEV	8.06^{b}	6.41^{c}	6.51^{ac}	1.39^{d}
	Middelduinen	AMB	9.13^{de}	8.46^{b}	6.92^{cd}	1.08^{abc}
		ELEV	8.33^{bc}	7.45^{a}	4.37^{g}	1.12^{abc}
F. rubra	**Bergharen**	AMB	9.64^{af}	9.05^{de}	7.53^{ef}	1.06^{a}
		ELEV	9.96^{f}	9.36^{e}	8.87^{h}	1.06^{ab}
	Kwade Hoek	AMB	9.74^{af}	8.18^{c}	8.06^{ab}	1.48^{e}
		ELEV	9.45^{ae}	6.57^{b}	5.76^{f}	1.16^{abcd}
	Middelduinen	AMB	8.61^{cg}	7.29^{a}	6.50^{ac}	1.18^{bc}
		ELEV	9.29^{ade}	8.68^{bd}	7.35^{de}	1.07^{abc}
	Plants		***	***	***	n.s.
	Soil origin		***	***	***	***
	CO_2		n.s.	*	***	n.s.
	Soil origin × CO_2		n.s.	**	***	**
	Soil origin × Plants		***	***	***	**
	CO_2× Plants		n.s.	**	***	*
	Plants × Soil origin × CO_2		n.s.	**	**	n.s.

Different letters within a column refer to significantly (*P*<0.05) different averages based upon an unequal N Tukey-HSD test. Significant levels: * P<0.05, **P<0.01, ***P<0.001, n.s.: not significant.

increased in Kwade Hoek soil. For *F. rubra*, these patterns were exactly the opposite. Under elevated CO_2, bacterial/fungal ratios tended to increase in Kwade Hoek and Middelduinen soils for *C. arenaria*, but decreased for *F. rubra*. In Bergharen soil, bacterial/fungal ratios were unaffected by CO_2 level for both plant species. SSU rRNA gene abundances for bacteria, fungi and nematodes were also determined for unplanted bulk soil samples and were found to be significantly lower ($F_{1,2} = 214.03$; $P < 0.01$) than in the rhizosphere of both *C. arenaria* and *F. rubra* (data not shown).

Effect of elevated CO_2 on the AM Fungal communities

As expected, 16:1ω5 NLFA and PLFA, which have been used as biomass estimators for intraradical (PLFA and NLFA 16:1ω5) and extraradical (NLFA 16:1ω5) AMF biomass, were significantly higher ($F_{2,4} = 88.44$; $P < 0.001$) in the mycorrhizal plant species (*F. rubra*) as compared to the non-mycorrhizal one (*C. arenaria,* $F_{1,2} = 2.91$, $P = 0.23$*;* data not shown). For *F. rubra*, NLFA 16:1ω5 (Figure 2a) increased significantly ($F_{2,4} = 571.19$, $P < 0.001$) at elevated CO_2 compared with ambient CO_2 at all three sites by a factor of 22 for Bergharen, 15 for Kwade Hoek and 8 for Middelduinen soil. PLFA 16:1ω5 (Figure 2b) increased significantly as well (Soil origin × CO_2: $F_{2,4} = 2171.82$, $P < 0.001$) in all three soils. Although PLFA 16:1ω5 occurs in AMF as well as bacteria, the increase due to the CO_2 enrichment followed the same patterns observed in NLFA 16:1ω5 (Figure 2a), supporting the notion that this biomarker was mostly from AMF origin in our study.

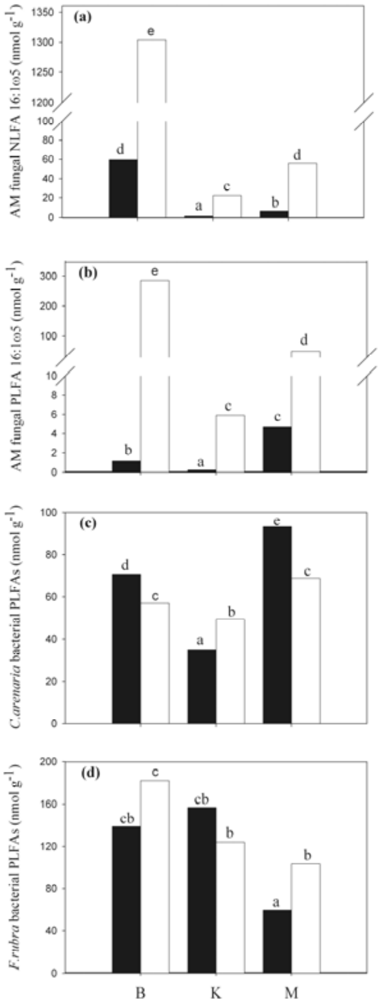

Figure 2: Abundance of 16:1ω5 NLFA (a), 16:1ω5 PLFA (b) in the rhizosphere of *F. rubra* and of specific PLFAs for bacterial biomass in the rhizosphere of *C. arenaria* (c) and *F. Rubra* (d). The plants have grown at elevated (white bars) and ambient (black bars) CO_2 in Bergharen, river dune (B), Kwade Hoek, former beach (K) or Middelduinen, costal dune (M) soils. Different letters within the graph refer to significantly ($P < 0.01$ for NLFA, $P < 0.001$ for PLFA, $P < 0.001$ for *C. arenaria* and *F. rubra*) averages based upon a Tukey-HSD test.

Bacterial specific PLFAs for *C. arenaria* (Figure 2c) and *F. rubra* (Figure 2d) confirmed the patterns described by SSU rRNA gene abundance, with much higher levels observed at elevated CO_2 for *F. rubra* as compared to *C. arenaria* and showing similar patterns with respect to soil origins.

As for the 16S rRNA density, the trend of the bacterial community in the two rhizospheres was significantly (Plants × CO_2: $F_{1,2} = 37.01$, $P = 0.03$) opposite under ambient and elevated CO_2 concentrations.

Discussion

Influence of elevated CO_2 on rhizosphere communities

The cultivation-independent approaches used revealed that increased atmospheric CO_2 exerted differential influences on bacterial, fungal and nematode communities in the rhizosphere of *C. arenaria* and *F. rubra*. These effects were observed within a background of other significant sources of variation, namely effects of different plant species and soil origins. It has become evident that general biomass and activity measures are inadequate to describe soil-borne microbial community responses to elevated atmospheric CO_2 concentrations (Janus *et al.* 2005). Those studies that have taken a more detailed approach have produced mixed results. The influence of changes in C allocation under elevated CO_2 on the composition of the microbial community have ranged from pronounced effects (Rillig *et al.* 1997; Grayston *et al.* 1998; Mayr *et al.* 1999; Montealegre *et al.* 2000; Janus *et al.* 2005; Jossi *et al.* 2006) to subtle or undetectable effects (Griffiths *et al.* 1998; Insam *et al.* 1999; Bruce *et al.* 2000; Montealegre *et al.* 2000; Zak *et al.* 2000; Klamer *et al.* 2002; Ebersberger *et al.* 2004). In our experiments, 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 could best be explained by differences in soil origin.

Our observed changes in bacterial and fungal community structure are in agreement with previous studies conducted under field conditions (Marilley *et al.* 1999; Janus *et al.* 2005; Sonnemann & Wolters 2005; Jossi *et al.* 2006) and studies addressing structural changes within bacterial communities (Marilley *et al.* 1999; Janus *et al.* 2005; Jossi *et al.* 2006). These latter studies focused mainly on specific bacterial communities and showed CO_2-related shifts in community composition of *Pseudomonas* spp. (Marilley *et al.* 1999), of *Rhizobium* species (Schortemeyer *et al.* 1996; Montealegre *et al.* 2000) or stimulation of *Proteobacteria* (Jossi *et al.* 2006).

The short term effects of CO_2 enrichment on the rhizosphere microbial community are likely to be mediated by the plant through quantitative and qualitative changes in root exudation (Paterson *et al.* 1996; Hodge *et al.* 1998). Our findings that bacteria are most influenced by CO_2 enrichment are consistent with this hypothesis, as bacteria would be expected to exhibit good competitive abilities for consumption of root exudates, which are typically composed mostly of simple organic substrates such as sugars, organic acids and amino acids (De Boer *et al.* 2005; De Boer *et al*. 2006). It would be expected that the effect of elevated CO_2 on the structure of fungal communities could become more evident only after longer exposure times, since fungi are important in the decomposition of more recalcitrant organic compounds, such as cellulose and lignin. In our study, *C. arenaria* and *F. rubra* were harvested during their vegetative development, which has been hypothesized to favor changes in the bacterial community. In contrast, fungal communities have been hypothesized to respond more to plants during reproductive stages (Mougel *et al.* 2006). Changes in bacterial community composition during the vegetative growth stages may correspond to changes in the composition of soluble root exudates (Duineveld *et al.* 2001).

CO_2-plant interactions

The main sources of plant-dependent influence on soil-borne communities upon conditions of elevated CO_2 include changes in the release of carbon from roots into the rhizosphere, quantity and quality of plant litter and changes in the rate of root turnover (Korner, 2000). Although root exudation probably represent a relatively minor C transfer pathway compared to other fluxes (e.g. structural root components and litter), its impact on ecosystem functioning may be disproportionably large due to the relatively simple chemical nature of most root exudates (Cardon, 1996). Indeed, the C contained in root exudates is easily accessible and could potentially enhance the growth of soil microorganisms that are usually C limited. Under conditions of elevated CO_2, both the increase in root biomass that is frequently observed (Rogers *et al*., 1994; Allard *et al.*, 2005) and qualitative and quantitative changes in root exudation and other forms of rhizodeposition (Paterson *et al.* 1996; Hodge *et al.* 1998) are likely to create shifts in rhizosphere communities.

Because plant species differ in the quantity and quality of resources that they return to the soil, effects on soil-borne community composition may be plant species specific (Wardle *et al*., 2004; Smalla *et al.* 2001). For instance, grassland plant species differ in the composition of microbial communities around their roots (Bardgett *et al.* 1999), which helps explain why soils planted with different grassland species, support different rhizosphere communities (Griffiths *et al.* 1992).

In our study, the response of the rhizosphere communities to elevated CO_2 depended on the plant species. *F. rubra*, a species reported to depend on AMF infection (Greipsson & El-Mayas 1999; Orlowska *et al.* 2005), showed a strong increase in AMF infection at elevated CO_2, in accordance with previous studies (Staddon & Fitter 1998).

AMF abundance has been demonstrated to affect microbial community composition in the rhizosphere (Hodge *et al.*, 2000; Johansson *et al.*, 2004; Rillig *et al.*, 2006; Andrade *et al.*, 1997; Marschner & Baumann 2003), by modifying rhizodeposition (Linderman 1988) or by affecting the physical and chemical conditions of the soil adjacent to plant roots (Jones *et al.* 2004; Rillig & Mummey 2006). Following AMF colonization, root exudation patterns may be altered with the fungus acting as a C sink (Douds *et al*., 2000; Graham, 2000; Jones *et al*. 2004), retaining C photosyntate in mycorrhizal biomass and controlling its time of release to the soil microbial community (Olsson & Johnson 2005). Moreover AMF colonization could alter the carbohydrate metabolism of the roots (Shachar-Hill *et al.* 1995; Bago *et al.* 2003) and increase root respiration (Douds *et al.*, 2000; Shachar-Hill *et al.* 1995). Hyphal exudation of water, carbohydrates and glomulin (Gadkar *et al.*, 2006) from AMF may also occur (Jones *et al.* 2004). Changes in root exudation and rhizodeposition following AMF colonization typically include reductions in total sugar output (Azcon & Ocampo 1981; Bansal & Mukerji 1994) and alterations in amino acid ratios (Mathur *et al.* 2000). The altered pattern of exudation by the fungus may create an extended interface for physical and metabolic interactions between bacteria and other fungi with the cell surface structures or mucilage (Johansson *et al*., 2004: Toljander *et al*., 2006). Mycelial exudates could also function as chemo-attractants or –repellants, or display antagonistic or stimulatory activities toward certain microorganisms (Grayston *et al.* 1997; Filion *et al.*, 1999; Sood *et al.* 2003), thereby affecting bacterial community composition (Toljander *et al.* 2007). Our observations that CO_2 effects are most pronounced in the rhizosphere of the mycorrhizal plant species are consistent with these hypothesized influences of AMF. Mycelial products, coupled with altered root exudation patterns, may thus play important roles in direct and indirect interactions between AMF and other soil microorganisms, having a substantial quantitative and qualitative impact on the size and structure of the soil microbial community.

CO_2-soil interactions

Consistent with previous research (De Ridder-Duine *et al.* 2005; Marschner *et al*., 2001), we observed that soil origin greatly influenced soil community composition in our experiment. This effect has been attributed to the strong impact of soil properties and nutrient availability on selection of soil-borne organisms. Interestingly, the impact of elevated CO_2 conditions also differed between the soils. Bacterial rhizosphere communities in Bergharen and Middelduinen soils showed marked CO_2 treatment responses, whereas no significant effects were observed in Kwade Hoek soil for either of the plants tested. Although it is not clear exactly why this should be the case, the Kwade Hoek soil was most distinct, containing less organic matter and ergosterol and having a sandier texture and higher pH, than the other two soils.

For the mycorrhizal plant, *F. rubra*, AMF may also play a role in changing soil properties, such as aggregate formation or nutritional status, and such affects would be expected to vary between different soils (Rillig & Mummey 2006). Indeed, under elevated CO_2 in Bergharen soil planted with *F. rubra*, we observed changes in the structure of macroaggregates (data not shown). In addition to affecting bacterial and fungal communities, soil structure is known to influence nematode communities, for which a highly significant interaction between CO_2 level and soil type was observed for *F. rubra*. This link however is still speculative, as the mechanisms and the significance of changes in soil aggregation are still poorly understood (Rillig & Mummey 2006).

Effect of elevated CO_2 on rhizosphere community size

Previous studies on the effects of increased CO_2-levels on soil community biomass have yielded mixed results. Quantitative alterations in carbon supply have been shown to decrease (Diaz *et al.* 1993; Ebersberger *et al.* 2004), increase (Zak *et al.* 1993) or not affect (Randlett *et al*. 1996; Kandeler *et al*. 1998) the growth and activities (*e.g.* decomposition and nutrient cycling of soil-borne communities (Jones *et al.* 1998; Hu *et al.* 1999).

The present study is the first to evaluate the community size in the rhizosphere of plants grown under elevated CO_2 by a real time PCR approach. CO_2 enrichment influenced the sizes of the fungal and nematode communities and to a lesser extent the bacterial one. Similar to the community composition, rhizosphere community size also strongly depended on the plant species studied and on the soil origin. It is generally assumed that CO_2-induced increases in soil C availability will stimulate fungal densities more than those of bacteria (Klironomos *et al.* 1996; Rillig *et al.* 1999). Jones *et al.* (1998) reported similar results for the effects of elevated CO_2 on bacterial and fungal abundance. In their study, changes observed in soil fungi at elevated CO_2 were thought to be related to increased concentrations of dissolved organic carbon (DOC) in the rhizosphere and to increases in soil-water dissolved organic nitrogen (DON). It should be noted that in our study a large proportion of increases in fungal biomass in the *F. rubra* rhizosphere might be attributed to increases in AMF biomass. Given the important roles played by fungi in organic matter degradation, nutrient cycling, plant nutrition and soil aggregate formation, it would be expected that shifts in fungal communities might have important ramifications on soil functioning.

Elevated CO_2 had a variety of effects on soil nematode community size. *C. arenaria* and *F. rubra* planted in Kwade Hoek and Middelduinen appeared to influence the nematode community directly through changes in plant carbon allocation to the below-ground environment, and indirectly via changes in microbial communities that responded to plant carbon inputs. These findings are similar to those found in other studies involving elevated CO_2 (Hoeksema *et al*. 2000; Yeates *et al.* 2003). The size of the nematode communities in Bergharen soil increased in elevated CO_2, independent of plant species. This result is similar to those reported by Neher *et al*. (2004), who suggested that effects of elevated CO_2 on soil nematode communities would not necessarily have a simple functional relationship with rhizosphere carbon allocation. In confirmation of our nematode community profiling, preliminary nematode identification data for samples collected from *C. arenaria* soil (unpublished data), confirmed that elevated CO_2 had induced shifts in nematode community structure, increasing the bacterivores and consistently decreasing fungivores, as previously reported by Neher *et al.* (2004).

Concluding remarks

The main aim of this study was to provide insight into the plant-microbe interactions of the rhizosphere under elevated CO_2. The results demonstrated that elevated CO_2 influenced different parts of the soil microbial community, but that the effects depended on the plant species and soil type. It should be noted that short-term approaches as used in the present experiment, may not represent field conditions that have decades of equilibrium time (Klironomos & Hart 2001), and studies such as the present one should therefore be combined with long-term and incremental experiments on elevated CO_2 effects. These findings do suggest that plant-driven rhizosphere effects may be important in determining the impact of elevated CO_2 on terrestrial ecosystem functioning, including nutrient and carbon balances.

Acknowledgements

This study was supported by a Netherlands Research Council (NWO) grant of the Biodiversity and Global Change program awarded to J.A. van Veen and G.A. Kowalchuk (852.00.40). The authors thank Gregor Disveld for providing generous help in managing the CO_2 flow cabinets, Wiecher Smant for help with soil analyses and Wietse de Boer for helpful comments on the manuscript.

Supplementary data

Table S1: Distance-based redundancy analyses results for plant species (*C. arenaria* and *F. rubra*), soil origin (Middelduinen, Kwade Hoek and Bergharen) and ambient and elevated CO_2 concentrations on bacterial, nematodes and fugal communities PCR-DGGE patterns based on 999 Monte Carlo permutations test and split-plot analysis of variance (* P < 0.001).**

	Bacteria **16S rDNA priers**		**Nematodes** **18S rDNA primers**		**Fungi** **18S rDNA primers**	
Total PCR-DGGE bands observed	75		83		59	
Total PCR-DGGE samples analysed	204		204		204	
	P-value	Trace	P-value	Trace	P-value	Trace
Plants	***	0.138	***	0.253	***	0.176
Soil origin	***	0.129	***	0.141	***	0.185
CO_2	***	0.189	***	0.078	***	0.100
Soil origin × CO_2	***	0.059	***	0.183	***	0.130
Soil origin × Plants	***	0.139	***	0.231	***	0.207
CO_2 × Plants	***	0.064	***	0.038	***	0.064
Plants × Soil origin × CO_2	***	0.264	***	0.355	***	0.362

Figure S1: Example of 16S rRNA (Bacteria) and 18S rRNA (Fungi and Nematodes) gene-based denaturing gradient gel electrophoresis profiles obtained from rhizosphere soil of *F. rubra* cultivated on Bergharen soil under ambient and elevated CO_2 concentrations. R stands for reference pattern.

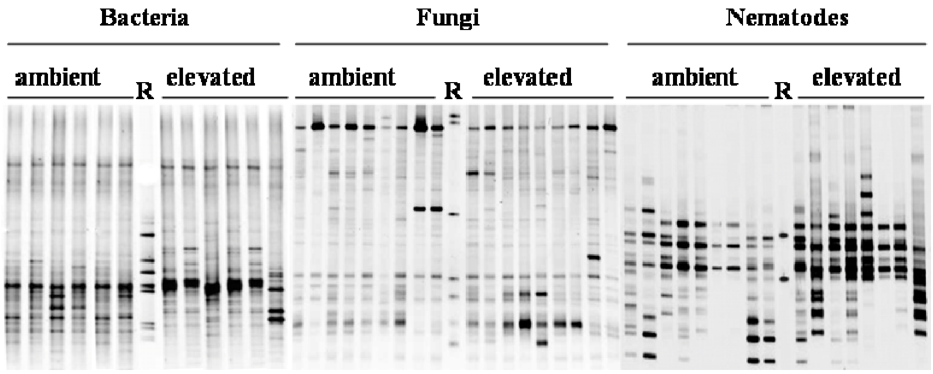

Figure S2: Relationship between the rhizosphere microbial community and soil chemical parameters assessed for *C. arenaria* and *F. rubra* cultivated in Bergharen, Kwade Hoek and Middelduinen soils at ambient (AMB) and elevated (ELEV) CO$_2$. Shown is a scatter plot of the soil chemical parameters based on Canonical Correspondence Analysis (CCA) of data obtained from small subunit rRNA genes (SSU rRNA) denaturing gradient gel electrophoresis. CO$_2$ levels were passively entered and did not influence the analyses. (A) Bacteria; (B) Fungi; (C) Nematodes.