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SOIL MICROBIOLOGY

Effects of Elevated Atmospheric CO_2 on Microbial Community Structure at the Plant-Soil Interface of Young Beech Trees (*Fagus sylvatica* L.) Grown at Two Sites with Contrasting Climatic Conditions

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Abstract Soil microbial community responses to elevated atmospheric CO_2 concentrations (eCO_2) occur mainly indirectly via CO_2-induced plant growth stimulation leading to quantitative as well as qualitative changes in rhizodeposition and plant litter. In order to gain insight into short-term, site-specific effects of eCO_2 on the microbial community structure at the plant-soil interface, young beech trees (*Fagus sylvatica* L.) from two opposing mountainous slopes with contrasting climatic conditions were incubated under ambient (360 ppm) CO_2 concentrations in a greenhouse. One week before harvest, half of the trees were incubated for 2 days under eCO_2 (1,100 ppm) conditions. Shifts in the microbial community structure in the adhering soil as well as in the root rhizosphere complex (RRC) were investigated via TRFLP and 454 pyrosequencing based on 16S ribosomal RNA (rRNA) genes. Multivariate analysis of the community profiles showed clear changes of microbial community structure between plants grown under ambient and elevated CO_2 mainly in RRC. Both TRFLP and 454 pyrosequencing showed a significant decrease in the microbial diversity and evenness as a response of CO_2 enrichment. While *Alphaproteobacteria* dominated by *Rhizobiales* decreased at eCO_2, *Betaproteobacteria*, mainly *Burkholderiales*, remained unaffected. In contrast, *Gammaproteobacteria* and *Deltaproteobacteria,* predominated by *Pseudomonadales* and *Myxococcales*, respectively, increased at eCO_2. Members of the order *Actinomycetales* increased, whereas within the phylum *Acidobacteria* subgroup Gp1 decreased, and the subgroups Gp4 and Gp6 increased under atmospheric CO_2 enrichment. Moreover, *Planctomycetes* and *Firmicutes*, mainly members of *Bacilli*, increased under eCO_2. Overall, the effect intensity of eCO_2 on soil microbial communities was dependent on the distance to the roots. This effect was consistent for all trees under investigation; a site-specific effect of eCO_2 in response to the origin of the trees was not observed.

Keywords Rhizosphere · Beech · Bacterial communities · CO_2

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Introduction

The global atmospheric CO_2 concentration has increased by >30 % since the industrial revolution to approximately 395 ppm currently (www.esrl.noaa.gov) and is expected to reach 1,000 ppm by 2100 [1], due to fossil fuel burning and changes in land use patterns [2]. This rapid increase in the atmospheric CO_2 concentration led to a considerable interest in the potential of biological systems to mitigate the effect of this greenhouse gas by enhanced carbon (C) sequestration.

Especially, forests accounting for a large proportion of global net primary productivity [3] are in the focus of research [4–7]. It could be demonstrated that elevated CO_2 (eCO_2) increased the carboxylation efficiency of Rubisco [8], resulting in an increased C fixation [9], a higher C allocation and thus an altered plant chemistry (C/N ratio, concentrations of sugars, starch, and total nonstructural carbohydrates) [10], and higher aboveground and belowground plant biomass [11]. While the stimulating effects of eCO_2 on plants have been extensively studied since more than 20 years, the impact of atmospheric CO_2 enrichment on soil microbial communities and their activities has received scarce attention. However, microorganisms are key drivers of the nutrient turnover, and consequently, getting more insight into soil microbial responses to eCO_2 is critical for the understanding of the global nutrient cycling and of the potential of soils acting as C sink or source under future climatic conditions [12, 13].

As the CO_2 concentration in soil is 10 to 15 times higher than in the atmosphere [14], it is unlikely that atmospheric CO_2 enrichment has direct effects on soil communities. However, eCO_2 may influence soil ecosystems indirectly through CO_2-induced plant responses, mainly through changes in the rhizodeposition (including plant secretions like enzymes and mucilage, sloughing off of dead plant cells, root turnover, and root exudation) [15] and litter quality [10]. Several studies showed an increase in organic C input to soils due to higher plant root biomass [16, 17], increased root exudation [12], and enhanced fine root turnover [17, 18]. This additional C input results on one hand in increased microbial biomass and activity in soils under plants grown at eCO_2 concentrations [17, 19, 20]. CO_2-induced biochemical changes in plant leaves, e.g., increased C/N ratio due to enhanced C allocation, on the other hand, could slow down the decomposition of leaf litter [2, 10], indicating that altered litter quality might have severe effects on the soil nutrient cycling. Moreover, enhanced plant growth under eCO_2 results in an increased uptake of mineral nutrients such as nitrogen (N) from soil [18], leading to decreased soil N concentrations mainly in soils that have not been fertilized [5, 21]. Consequently, N becomes the main growth limiting factor in most terrestrial ecosystems [22], and the competition between plants and soil microbes for N will increase under future climatic conditions. Particularly, microorganisms using N compounds as energy source (ammonia oxidizers and nitrite oxidizers) or alternative electron acceptor (denitrifiers) may be negatively affected by stimulated plant growth under eCO_2. As these functional groups are important drivers of the terrestrial N cycle, their decrease would have a negative feedback to the availability of N for plant growth, which, in turn, would limit the primary productivity and thus the potential for soil C sequestration.

Previous studies on the effects of eCO_2 on microbial communities have examined either total microbial community patterns [4, 7, 14, 23–26], metabolic patterns [27, 28], or specific taxonomic or functional groups like rhizobia [29, 30], pseudomonads [31, 32], ammonium oxidizers, and denitrifiers [6]. However, due to the complex interactions with biotic and abiotic factors and the limitation of conventional molecular methods used for the characterization of soil microbial communities, the responses of microbial communities to eCO_2 are still poorly understood and contradictory. It has been assessed that the diversity and activity of microbial communities increase [4, 14, 25], decrease [6, 33], or remain unchanged [7, 23]. The apparent disparity could on one hand be due to different methods used targeting the microbial phenotypic (lipid analysis) or genotypic (analysis of nucleic acids) and the related differences in the degree of resolution. On the other hand, the contrasting results might be due to real differences among various ecosystems as eCO_2 effects have been considered both ecosystem and plant specific [24, 26] or even depending on the plant age and plant development stage. In this respect, still studies are rare where questions related to the resilience of microbial communities at the plant soil interface from genetically similar plants of comparable ontogenetic age grown at sites with differing climatic conditions have been addressed. Therefore, in the present study, potential effects of eCO_2 on the microbial rhizosphere community of young beech trees (*Fagus sylvatica* L.) originating from opposing mountainous slopes (northeast (NE) and southwest (SW)), resulting in different natural microclimates due to higher radiation at SW, were investigated. Although soil type and nutrient availability are similar at both slopes, previous studies investigating fungal communities showed significant differences in ectomycorrhizal assemblies still present when growing the plants under similar greenhouse conditions [34], indicating site-specific adaptations of the respective plant-soil systems due to different climatic conditions.

In our study, we hypothesize (1) that eCO_2 will alter the microbial community composition due to changed quantity and quality of C input into soil in the rhizosphere of beech trees, even after short incubation periods (2 days). We postulate further (2) that beech ecotypes from sites with contrasting environmental conditions will respond differently to eCO_2, resulting in site-specific changes in the associated soil microbial communities. To test these hypotheses, young beech trees from two contrasting mountainous sites in Germany were transferred to the greenhouse and grown under ambient (360 ppm) CO_2 conditions. A subset of the trees was incubated for 2 days under elevated (1,100 ppm) CO_2 conditions. For characterizing changes in the microbial community structure in the root rhizosphere complex as well as in the adhering soil, TRFLP and 454 pyrosequencing were used. Furthermore, alterations in the microbial community composition were linked to plant biomass and soil N pools.

Materials and Methods

Experimental Design

In July 2010, young beech trees (*F. sylvatica* L.) with a stem height of 50 cm were collected from two sites (32 trees per site) with opposing exposure (NE and SW) from a beech-dominated forest in the Swabian Jura, a low mountain range in Southern Germany (8° 45′ E/47° 59′ N). The sites were within a distance <1 km of each other and showed similar forest population genetics [35]. The soil on both sites was classified as shallow Rendzic Leptosol (sceletic) with a pH of 5.7 in the organic layer and 7.5 at 0.6 m depth [36, 37]. The higher radiation at SW resulted in increased daily maximum of air and soil temperatures and thus reduced water availability [38].

After removing the root-adhering soil using running water, the trees were planted separately in 5 l pots (17×17×17 cm) containing a homogenous mixture of coarse sand (diameter 0.7–1.2 mm, Melo Schwimmbadtechnik, Göttingen, Germany), fine sand (diameter 0.4–0.8 mm, Melo Schwimmbadtechnik), and peat (Torfwerk Zubrägel, Vechta, Germany) in a ratio of 4.5:4.5:1. To match stand light conditions, the trees were shaded with a 65 % shading net (Herrmann Meyer KG, Rellingen, Germany) and placed outside (Forest Botanical Garden, University of Göttingen, 9° 57′ E / 51° 33′ N). Each plant was watered daily with 50 ml tap water. After 1 month, the trees were transferred into a greenhouse with 20 °C temperature, 50 % relative humidity, a photoperiod of 16 h daylight (additional light by MT 400 DL/BH lamps, Eye Iwasaki Electric Co. Ltd., Tokyo, Japan), and ambient CO_2 atmosphere (360 ppm CO_2, UNOR 610 CO_2 analyzer, Maihak, Hamburg, Germany). Each tree was watered daily with 50 ml Hoagland nutrient solution [39]. Nitrogen was applied as ammonium-nitrate (concentration 0.4 mM), resulting in reduction of 60–80 % of bioavailable N sources (ammonium, nitrate, DON) compared to the original soil and consequently N limiting growth conditions for plants (data not shown). After the equilibration phase in the greenhouse, the trees were still well developed and did not show any signs of senescence.

On Oct. 4, 2010, eight trees per site were sampled and treated as control plants; immediately afterwards, CO_2 concentrations were increased to 1,100 ppm CO_2 (UNOR 610 CO_2 analyzer, Maihak, Hamburg, Germany) using ^{13}C-labeled CO_2 gas (1.5 % ^{13}C–CO_2, Cambridge Isotope Laboratories, Andover, USA) for 2 days. Eight plants per site were sampled 1, 3, and 8 days after the start of the ^{13}C–CO_2 pulse. For analysis of microbial community, responses to eCO_2 samples taken 8 days after the start of the labeling were chosen as this represented the time point with the highest ^{13}C enrichment in tree root biomass. A scheme of the experiment is shown in the supplement (Fig. S2).

For nucleic acid analyses, subsamples of rhizosphere soil (RS, defined as soil attached to roots after slight shaking) and root rhizosphere complex (RRC, defined as fine roots with tightly adhering soil that cannot be removed by vigorous shaking) [40] were immediately frozen on dry ice and stored at −80 °C for DNA extraction. A subsample was dried at 105 °C for determination of the water content. Another soil subsample was directly extracted 1:2 (soil/solution) with 0.5 M K_2SO_4 on a rotary shaker for 1 h. Afterwards, the extracts were vacuum filtered using pumps and glass fiber filters [41] and stored at −20 °C for the analysis of extractable soil C and N pools. Plant material was separated into leaves, stem, and roots and dried at 60 °C for 1 week to determine dry biomass and subsequently ^{13}C enrichment.

Extractable Soil Carbon and Nitrogen Pools

Extractable soil C and N pools were analyzed as described by Dannenmann et al. [41]. For the determination of ammonium and nitrate concentrations, a subsample of the filtered extracts was analyzed colorimetrically by a commercial laboratory (Dr. Janssen, Gillersheim, Germany). Total organic carbon (TOC) and total nitrogen (TN) in the extracts were quantified on an Infrared TOC analyzer with a coupled chemoluminescence-based total N module (DIMATEC GmbH, Germany). TOC concentrations were referred to as extractable dissolved organic carbon (DOC), while extractable dissolved organic nitrogen (DON) was calculated as the difference between TN and dissolved inorganic N (ammonium plus nitrate).

Nucleic Acid Extraction

DNA was extracted from 0.4 g RS and 0.1–0.2 g RRC, respectively, using the NucleoSpin Soil Kit (Macherey Nagel, Düren, Germany) with SL2 lysis buffer and enhancing solution SX according to manufacturer's instructions and the Precellys 24 Instrument (Bertin Technologies, Montigny-le-Bretonneux, France). Quality and quantity of the extracted nucleic acids were checked with a spectrophotometer (Nanodrop, PeqLab, Erlangen, Germany) and standard gel electrophoresis. The extracts were stored until use at −20 °C.

Terminal Restriction Fragment Length Polymorphism (TRFLP)

The bacterial community structure of RS and RRC was analyzed for samples maintained at ambient CO_2 atmosphere (aCO_2) and 6 days after the ^{13}C–CO_2 pulse (eCO_2), respectively. For the amplification of 1.4 kb fragments of the 16S ribosomal RNA (rRNA) gene, the universal eubacterial primers 27f (5′-aga gtt tga tcm tgg ctc ag-3′, 6-Fam-labelled) and 1401r (5′-cgg tgt gta caa gac cc-3′) were used [42]. PCR

reactions were performed in 50 µl, containing 60 ng template DNA, 0.3 % bovine serum albumin (BSA), (Sigma-Aldrich, Taufkirchen, Germany), 5 % dimethyl sulfoxid (DMSO) (Sigma Aldrich), 0.2 mM deoxynucleotide (dNTP) (Fermentas, St. Leon Rot, 287 Germany), 2.5 mM $MgCl_2$, 2.5 U Taq Polymerase (Life Technologies, Darmstadt, Germany), 1× Taq buffer (Life Technologies), and 10 pmol of each primer. PCR was performed in a T3 thermocycler (Biometra, Göttingen, Germany) using the following conditions: 10 min at 94 °C for initial denaturation, followed by 30 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1.5 min at 72 °C, and a final extension step for 10 min at 72 °C. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The enzymatic restriction was conducted using 200 ng of labeled PCR product, 5 U of MspI (Fermentas), and 1× reaction buffer in a final volume of 25 µl. After incubation at 37 °C for 6 h and subsequently 20 min at 65 °C, the samples were purified using the Gel and PCR Cleanup Kit (Macherey Nagel, Düren, Germany). The fragments were separated by an ABI 3730 sequencer using MapMarker 1000 (Eurogentec, Köln, Germany) as internal standard. Size and relative abundances of terminal restriction fragments (TRFs) were analyzed using GeneMapper v4.0 software (Life Technologies) and T-REX (http://trex.biohpc.org/) with a binning range of 2 bp. TRFs smaller 1 % of the total peak height were excluded from the analysis. The data analysis of the TRFLP profiles was performed in R v3.0.1 (http://www.R-project.org/) by calculating the relative abundance of TRFs normalized by the total peak height of the respective TRF patterns. The TRFLP profiles were analyzed via Yue Clayton dissimilarity, Euclidean distances of Hellinger transformed data, and principal component analysis of Hellinger transformed data [43], using the R packages shown in the supplement (Table S1).

Barcoded Pyrosequencing and Sequence Data Analysis

As TRFLP and 454 pyrosequencing are capable of recovering highly comparable community structures [44], samples for sequencing were chosen based on previous TRFLP analysis. We selected RS and RRC at ambient and elevated CO_2 concentrations. 454 pyrosequencing was performed using the manufacturer's instructions of the Genome Sequencer FLX System (Roche). For the sequencing analysis of RS, all 32 samples were pooled equimolar to form one composite RS sample. As the TRFLP profiles of RRC showed different diversity patterns between ambient and elevated CO_2, the respective 16 samples were pooled equimolar to form one composite RRC sample for ambient and elevated CO_2 treatment, respectively.

Amplicon pyrosequencing was performed on a 454 GS FLX Titanium system (Roche, Penzberg, Germany) as previously described [44]. Briefly, the universal eubacterial primers 27f (5′-aga gtt tga tcm tgg ctc ag-3′) and 519r (5′-tat tac cgc ggc kgc tg-3′) were extended with unique Multiplex Identifiers (MID), a four-base library key, and the respective A or B adapters for sample identification. Sequencing PCR was performed in a T3 thermocycler (Biometra GmbH, Göttingen, Germany) with the following conditions: initial denaturation (94 °C, 5 min), followed by 28 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and elongation (72 °C, 60 s), ending with a final extension (72 °C, 10 min). Each 50 µl PCR reaction contained 1× PCR buffer with 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U High-Fidelity polymerase (Roche), 0.5 mM of each primer, and 50 ng of template DNA. All samples were purified using Gel and PCR Cleanup Kit (Macherey Nagel, Düren, Germany) and quantified via Quant-iT Pico Green dsDNA Assay Kit (Invitrogen, Karlsruhe, Germany). After measuring fragment size and concentration via Bioanalyzer 2100 device on a DNA 7500 chip (Agilent, Böblingen, Germany), samples were pooled in an equimolar ratio of 10^9 molecules μl^{-1}. Subsequently, an emulsion PCR was performed with the following conditions: 94 °C for 4 min, followed by 50 cycles of 94 °C for 30 s and 60 °C for 10 min each. The sequencing run was performed on the 16^{th} part of a picotiter plate. The nucleotide sequence data obtained in this study were uploaded and made available in the NCBI database under the accession numbers SRR1439456, SRR1439619, and SRR1439848.

To reduce sequencing errors, the raw sequences were processed with MOTHUR v.1.14.0 [45] as described in detail by Mrkonjic-Fuka et al. [46]. Briefly, flowgrams were separated according to barcode and primers, with a minimum length of 200 bp per sequence and one respective two mismatches for barcode and primers. After removing the primers, barcode, and homopolymers, the sequences were checked for chimeras by alignment to the SILVA database provided by MOTHUR, which is derived from a sequence collection of the Genomes online database [47]. Mitochondrial sequences were subsequently excluded. A distance matrix was calculated from the high-quality aligned sequences, resulting in operational taxonomical units (OTUs) obtained by the furthest neighbor clustering algorithm. For phylogenetic analysis of *Acidobacteria* and *Alphaproteobacteria*, which were the phyla with the highest responsiveness to eCO_2, representative sequences of each OTU were clustered in MOTHUR at 90 % similarity level and were aligned to the SILVA database using ARB [48]. OTUs represented by less than two reads were omitted from the phylogenetic dendrograms.

Statistical Analysis

TRFLP profiles were analyzed in R v3.0.1 (http://www.R-project.org/) by calculating the relative abundance of TRFs normalized by the total signal height of the respective TRF patterns. Fragments smaller than 50 bases and TRFs

contributing <1 % to the total peak height were excluded from the analysis. Significant differences between compartment, site origin, and CO_2 treatment were evaluated using multivariate analysis of variance (Adonis function) based on Yue Clayton dissimilarity and Euclidean distances of Hellinger transformed data. Furthermore, Shannon diversity and Pielou's evenness indices were calculated for the respective TRFLP profiles. The R packages were listed in the supplement (Table S1).

For the sequence data analysis, distance matrices based on OTUs defined by 97 and 90 % similarity were generated for calculating rarefaction curves, richness, and diversity indices, using MOTHUR v.1.14.0. Moreover, population diversity was calculated on finite mixture models and coverage-based estimates using Catch-All v.2.0 [49]. Phylogenetic analysis of *Acidobacteria* and *Alphaproteobacteria* was done on 90 % similarity level with exclusion of OTUs representing less than two reads for phylogenetic trees constructed in ARB.

Results

Plant Biomass and ^{13}C Enrichment

Root, stem, and leaf biomass were not affected by eCO_2. Moreover, no difference between beech trees originating from two opposing mountainous slopes with contrasting climatic conditions (NE, cool-moist and SW, warm-dry) was observed, resulting in mean values of 5.28 g (root), 4.30 g (stem), and 1.66 g (leaf). The plant roots showed highest $\delta^{13}C$ values 8 days after the start of ^{13}C–CO_2 pulse (data not shown).

Soil Biochemical Parameters

Mean values of soil biochemical parameters are summarized in Table 1. Ammonium concentrations were neither impacted by site nor eCO_2 and ranged from 0.69 μg g^{-1} dry weight (dw) to 0.36 μg g^{-1} dw. In contrast, the nitrate contents decreased significantly at eCO_2 on both sites to 0.19 μg g^{-1} dw in average. While DON did not differ between NE and SW and remained constant at eCO_2 with 1.73 μg g^{-1} dw, overall, DOC concentrations decreased significantly at eCO_2 on both sites from 18.95 to 13.50 μg g^{-1} dw at NE and 17.39 to 12.05 μg g^{-1} dw at SW. Gravimetric soil moisture was neither influenced by site nor eCO_2 and was 8.1 % dw, overall.

TRFLP Analysis of Bacterial Communities Based on 16S rRNA Gene Amplicons

The TRFLP analysis was carried out to perform fingerprinting of bacterial communities in different soil compartments (RS and RRC) of beech trees originating from NE and SW slopes under ambient and elevated CO_2 concentrations and to screen for replicate variability (Table 2, Fig. S1). The TRF profiles of RS and RRC differed significantly, but while in samples from RS, no effect of slope origin and CO_2 treatment was detected; bacterial community pattern in samples from RRC showed a clear shift after eCO_2 (Table S2). The observed TRFs ranged from 51 to 620 bp, with 14 to 18 TRFs larger than 1 % of the total peak height in the respective samples. For RS, TRF 138 bp was the most dominant fragment at both sites and at both CO_2 concentrations, with a relative abundance of 21.5 % in average, followed by TRF 264 bp (12.4 %), TRF 148 bp (10.8 %), and TRF 437 bp (5.5 %). The diversity and evenness of the bacterial community in RS were neither influenced by slope origin nor eCO_2 treatment (Table 2). In contrast, the bacterial community structure of RRC was highly affected by the eCO_2 concentration. For RRC at ambient CO_2, TRF 403 bp was the most dominant fragment, accounting for 29.1 % of the total peak height in average, followed by 138 bp (19.2 %), 148 bp (6.8 %), 264 bp, and 132 bp (each 4.6 %). At eCO_2, TRF 491 bp was most abundant (29.2 %), followed by TRFs with the length 138 bp (14.7 %), 403 bp (10.5 %), 51 bp (6.2 %), and 132 bp (6.0 %). Both diversity and evenness of bacterial community structure of RRC

Table 1 Extractable soil N and C pools and gravimetric soil moisture of soil with beech trees originating from NE-exposed and SW-exposed mountainous slopes grown under ambient and elevated CO_2 concentrations (aCO_2 and eCO_2, respectively) (n=8, standard deviation in parentheses)

		NH_4^+ μg g^{-1} dw	NO_3^- μg g^{-1} dw	DON μg g^{-1} dw	DOC μg g^{-1} dw	Soil moisture % dw
NE	aCO_2	0.46 (0.23)	0.39 (0.16)	1.73 (0.20)	18.95 (1.87)	7.55 (1.19)
	eCO_2	0.44 (0.22)	0.18 (0.10)	1,76 (0.32)	13.50 (2.46)	8.30 (1.49)
SW	aCO_2	0.69 (0.56)	0.31 (0.27)	1.70 (0.40)	17.39 (3.03)	7.62 (1.18)
	eCO_2	0.36 (0.11)	0.20 (0.18)	1.71 (0.26)	12.05 (1.39)	8.79 (1.88)
p values	p slope	0.790	0.698	0.623	0.073	0.100
	p CO_2	0.206	**0.027**	0.765	**0.000**	0.175

Significant differences between slope and CO_2 treatment were calculated by multivariate ANOVA and are indicated by p values <0.05 (*bold letters*)

Table 2 Diversity indices based on TRFLP profiles for microbial communities in rhizosphere soil (RS) and root rhizosphere complex (RRC) associated with beech trees originating from NE-exposed and SW-exposed mountainous slopes grown under ambient and elevated CO_2 concentrations (aCO_2 and eCO_2, respectively) (n=8, standard deviation in parentheses)

		RS		RRC	
		Shannon	Evenness	Shannon	Evenness
NE	aCO_2	2.74 (0.19)	0.74 (0.05)	2.35 (0.26)	0.66 (0.07)
	eCO_2	2.79 (0.22)	0.76 (0.05)	1.93 (0.50)	0.58 (0.08)
SW	aCO_2	2.94 (0.15)	0.80 (0.04)	2.42 (0.19)	0.69 (0.07)
	eCO_2	2.95 (0.08)	0.80 (0.02)	2.04 (0.58)	0.62 (0.12)
p values	p slope	0.165	0.096	0.547	0.236
	p CO_2	0.635	0.263	**0.011**	**0.035**

Significant differences between slope and CO_2 treatment were calculated by multivariate ANOVA and are indicated by p values <0.05 (*bold letters*)

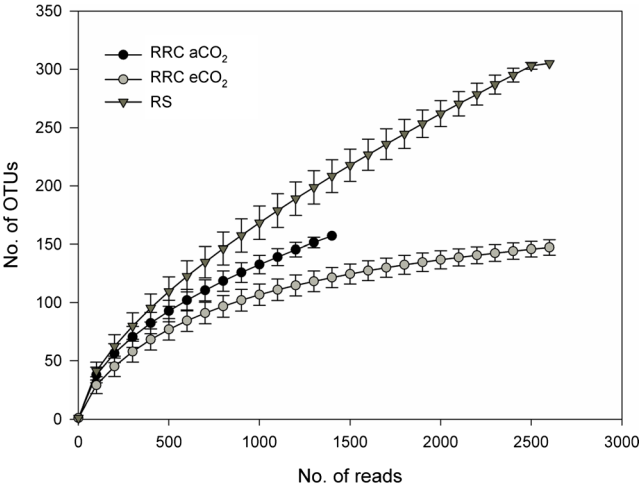

Fig. 1 Rarefaction curves of partial 16S rRNA gene sequences after DNA extraction and PCR amplification from rhizosphere soil (RS) and root rhizosphere complex (RRC) under ambient and elevated CO_2 concentrations (aCO_2 and eCO_2, respectively) at 90 % similarity level normalized with respect to sample size. *Error bars* represent 95 % CI values

decreased at eCO_2 treatment to 1.99 and 0.60 (mean of NE and SW), respectively (Table 2). When comparing bacterial communities of RS and RRC, RS showed higher diversity and evenness with 2.86 and 0.78, in average (Table 2).

Sequencing Analysis of Bacterial Communities Based on 16S rRNA Gene Amplicons

In total, 17,456 bacterial raw sequence reads were generated from the PCR amplicons by 454 pyrosequencing. After filtering, chimera check, and removing erroneous reads, 8006 high-quality partial 16S rRNA gene sequences with a minimum length of 200 bp remained, which were represented by 1957 OTUs (97 % similarity) and 569 OTUs (90 % similarity), respectively. Rarefaction curves suggested that for RRC, major OTUs were covered, while RS showed still no plateau phase at 90 % similarity level (Fig. 1). Coinciding with TRFLP patterns, the highest bacterial diversity and evenness was observed in RS (5.96 and 0.93), followed by RRC aCO_2 (5.59 and 0.89) and RRC eCO_2 (3.42 and 0.65). This ranking was confirmed by the CatchAll analysis providing diversity estimates based on modeling. The best fitted model describing the population diversity at RS was a mixture of three geometric distributions, while for both RRC samples, the diversity was best estimated with two-mixed geometric models (Table S3). In total, 10 phyla were detected: *Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria*, TM7, and *Verrucomicrobia*. More than 80 % of the annotated reads were grouped into three major phyla, which were ranked in abundance *Proteobacteria*>*Actinobacteria*>*Acidobacteria* (Fig. 2). *Proteobacteria* were dominated by members of the class *Alphaproteobacteria* belonging to the orders *Rhizobiales* and *Rhodospirillales* (Fig. 3 and Table S4). *Rhizobiales* were the most abundant *Alphaproteobacteria* in all samples, but while at RRC aCO_2, this order accounted for 14.0 % of all reads; eCO_2 treatment caused a decrease to 7.4 %. In RS, *Rhizobiales* made

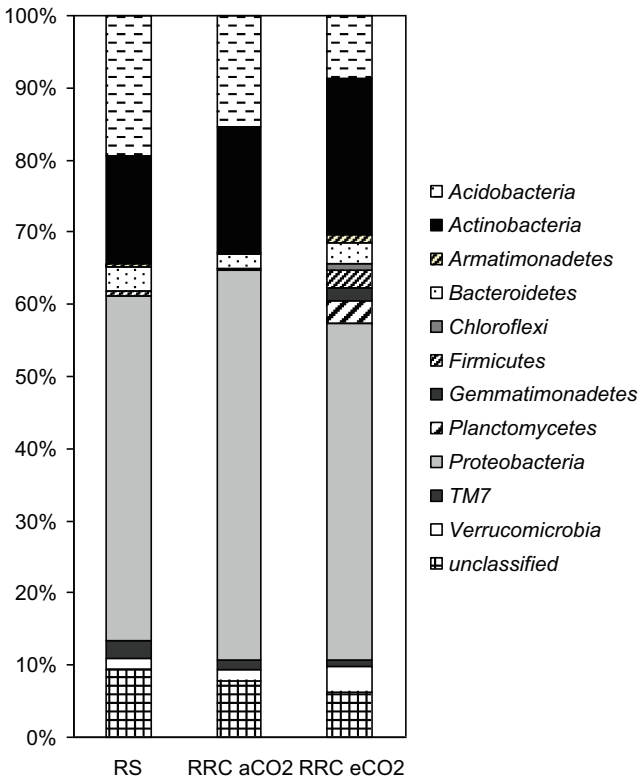

Fig. 2 Relative abundance (%) of sequences assigned to phylum level (8,006 reads in total) from rhizosphere soil (RS) and root rhizosphere complex (RRC) under ambient and elevated CO_2 concentrations (aCO_2 and eCO_2, respectively) based on partial 16S rRNA gene sequences after DNA extraction and PCR amplification

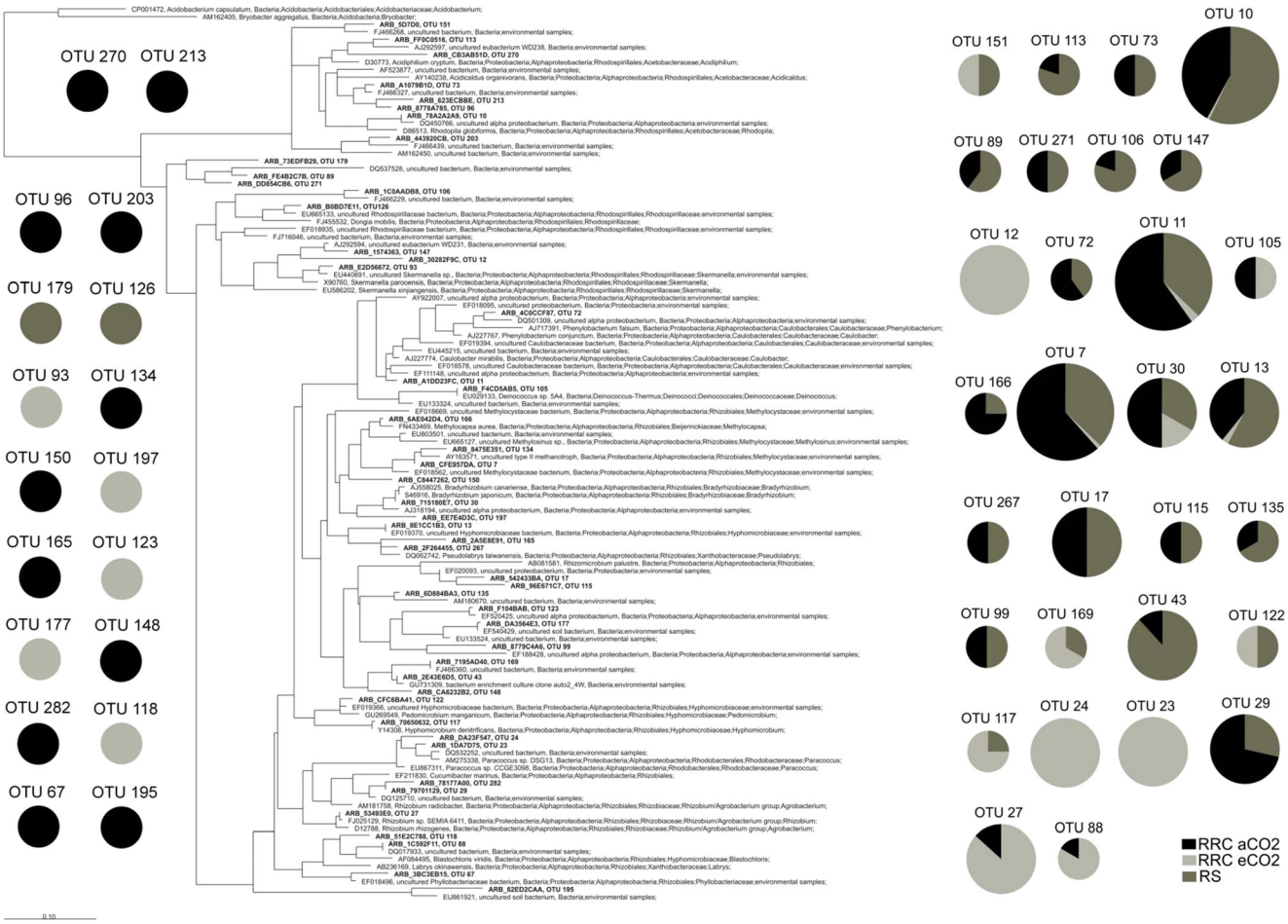

Fig. 3 Phylogenetic dendrogram (maximum likelihood consensus tree) showing the distribution of sequences related to *Alphaproteobacteria* derived from rhizosphere soil (RS) and root rhizosphere complex (RRC) under ambient and elevated CO_2 concentrations (aCO_2 and eCO_2, respectively). The pie charts represent the relative abundance of the corresponding OTUs among the samples. The pie chart size indicates the number of reads obtained for the representative OTUs: >100 reads (large), 10 to 100 reads (medium), and <10 (small)

up 11.3 % of total reads. The abundance of *Rhodospirillales* was 6.3 and 6.8 % in RS and RRC aCO_2, respectively. At RRC eCO_2, *Rhodospirillales* accounted only for 4.2 % of all reads, but instead *Rhodobacterales*, which were not detected at aCO_2, increased to 3.4 %. *Betaproteobacteria* were the second most abundant *Proteobacteria*, with *Burkholderiales* as predominant order, ranging from 10.2 % at RRC aCO_2 to 6.3 % in RS. *Gammaproteobacteria* increased with eCO_2 from 2.7 to 8.2 % of total reads in RRC and were dominated by *Pseudomonadales*. Accordingly, the abundance of *Deltaproteobacteria* increased at eCO_2 in RRC up to 2.5 %, while in RRC aCO_2 and RS, it was <1 % of all reads. Members of the order *Myxococcales* accounted for 60–72 % within this class and consequently were the predominant *Deltaproteobacteria* in all samples. *Actinobacteria* was the second most abundant phylum in RRC, with 17.4 and 21.5 % of total reads at aCO_2 and eCO_2, respectively. In RS, members of *Actinobacteria* accounted for 15 % of all reads. *Actinomycetales* were predominant in all samples, accounting for 55 to 76 % of all *Actinobacteria*, followed by *Acidimicrobiales* with 5 to 20 % of all *Actinobacteria*. While *Actinobacteria* were highest in RRC eCO_2, members of the phylum *Acidobacteria* were lowest with only 8.7 % of all reads and thus only half as abundant as in RRC aCO_2. The highest number of *Acidobacteria* was found in RS, accounting for 19.5 % of total reads. Members of *Acidobacteria* Gp1 subdivision were predominant in all samples, ranging from 52 to 90 % of all *Acidobacteria* (Fig. 4 and Table S4). In RS and RRC aCO_2, Gp3 subdivision was the second most abundant, accounting for 9 to 13 % of the *Acidobacteria.* In contrast, in RRC eCO_2, subgroups Gp4 and Gp6 were more abundant (each 16 % of all *Acidobacteria*) compared to Gp3, which was <1 % of all reads under eCO_2 conditions. *Bacteroidetes* were observed in all samples and were dominated by the order *Sphingobacteriales*, accounting for 2.0 to 2.7 % of total reads. Members of the phyla *Planctomycetes*, *Gemmatimonadetes*, and *Firmicute*s were only found in RRC eCO_2 with >1 % of all reads. All *Planctomycetes* belonged to the order *Planctomycetale*s, accounting for 3.0 % of all reads. Similarly, all detected *Gemmatimonadetes* were members of the order *Gemmatimonadales*, accounting for 2.0 % of total reads. *Firmicutes* (2.3 % of all reads) were dominated by

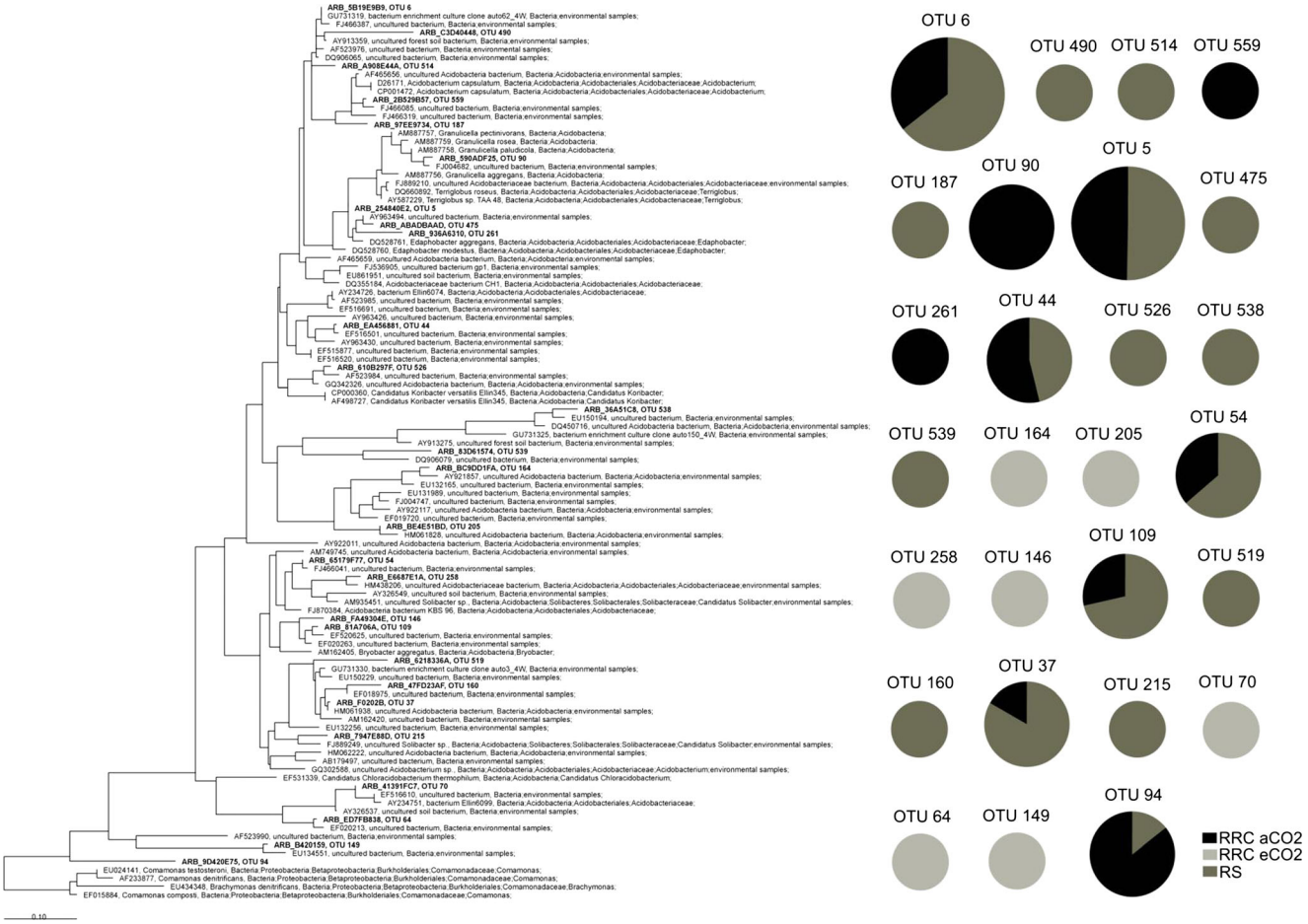

Fig. 4 Phylogenetic dendrogram (maximum likelihood consensus tree) showing the distribution of sequences related to *Acidobacteria* derived from rhizosphere soil (RS) and root rhizosphere complex (RRC) under ambient and elevated CO_2 concentrations (aCO_2 and eCO_2, respectively). The pie charts represent the relative abundance of the corresponding OTUs among the samples. The pie chart size indicates the number of reads obtained for the representative OTUs: >100 reads (large), 10 to 100 reads (medium), and <10 (small)

members of the class *Bacilli*, belonging to the orders *Bacillales, Lactobacillales*, and *Clostridiales*, which made up 74, 22, and 4 % of the *Bacilli*, respectively. The abundance of unclassified sequences ranged from 16.7 to 27.3 % on order level and 6.3 to 9.4 % on phylum level. All sequence data are presented in Figs. 2, 3, and 4 and Table S4.

Discussion

Effect of Elevated CO_2 on Beech Plant Biomass and Soil Biochemical Parameters

The short duration of eCO_2 precluded plant growth stimulation that had been observed in other studies under atmospheric CO_2 enrichment [11, 19, 50–53]. Regarding bioavailable soil N pools, a significant decrease in nitrate at eCO_2 was observed. This is in line with previous studies [5, 18, 21, 54] and might be due to decreased nitrification [21], increased denitrification [6], and/or higher plant uptake [18]. Responses of activity pattern of nitrifiers and denitrifiers may be related to an eCO_2-induced increase in the soil water content through reduced stomatal conductance [55] and evapotranspiration [56], although the observed increase in soil moisture was not significant in the present study due to the high standard deviations among the replicates. However, increased soil water content results in lower soil oxygen concentrations in soil and thus may have stimulated *in situ* denitrification rates. Surprisingly, besides nitrate. also DOC declined under eCO_2. This is in contrast to other studies reporting increased [18, 32] or unaffected [6] DOC concentrations in soils under atmospheric CO_2 enrichment. The decrease of DOC in the present study might be, on one hand, due to the short incubation time period with eCO_2, which did not allow the plant to fully adapt to the changed environmental conditions. Increased activity of denitrifiers, on the other hand, may also lead to fast consumption of DOC, as denitrifiers are

heterotrophic microorganisms depending on easily degradable C. Consequently, their growth is favored under eCO_2 due to enhanced plant root exudation [12].

Effect of Elevated CO_2 on the Bacterial Community Structure in the Rhizosphere of Young Beech Trees

Fingerprinting of bacterial rhizosphere communities performed by TRFLP showed clear differences between the observed compartments (RS and RRC) and their responsiveness to eCO_2. While the microbial population structure was unaffected by eCO_2 in RS, the diversity as well as the evenness decreased in RRC. This indicates that CO_2-induced effects on microbial communities differ depending on their distance to roots. Accordingly, Montealegre et al. [24] observed no influence of atmospheric CO_2 enrichment on microbial communities in RS of white clover (*Trifolium repens* L.) but a clear population shift toward gram-negative bacteria in bulk soil. This may be explained by increasing C supply to bulk soil via mycorrhizal hyphae, as mycorrhizal growth is stimulated under eCO_2 [57, 58]. A decreasing microbial diversity under eCO_2 was previously found [6, 30] and is likely due to changes in quality and quantity of rhizodeposition favoring specific microorganisms. Although TRFLP could not provide a detailed insight into the microbial community composition, it allowed a relative quantification of CO_2-induced changes and thus sample selection for subsequent sequencing analysis, as previous studies comparing TRFLP fingerprints and pyrosequencing data are capable of recovering the same amplicon pools from environmental samples and result in highly comparable overall microbial community pattern [44].

The 10 phyla observed in the present study are commonly found in soils, often with *Proteobacteria, Acidobacteria*, and *Actinobacteria* dominating [59]. Within all three phyla, clear differences between ambient and elevated CO_2 were found in RRC. Surprisingly, the abundance of *Rhizobiales* predominating *Alphaproteobacteria* decreased at eCO_2 in RRC. This order contains many diazotrophs, whose enhancement would be advantageous under eCO_2 because of increasing N limitation due to stimulated plant growth [11, 13] and enhanced microbial activity [17, 19, 20]. The observed decrease is in contrast with other studies reporting an increased N fixation in grassland ecosystems and plant-free marsh sediments [19]. However, Schortemeyer et al. [29] found an increase in *Rhizobium leguminosarium* associated with white clover at eCO_2, whereas *R. leguminosarium* in the rhizosphere of perennial ryegrass and in bulk soil was unaffected, indicating that the microbial response is plant-specific. While *Betaproteobacteria* remained unaffected by eCO_2 in the present study, *Gammaproteobacteria* dominated by the order *Pseudomonadales* increased in RRC. *Pseudomonas* sp. are known to be highly rhizo-competent heterotrophs depending on easily degradable C [60, 61] and are consequently stimulated by enhanced root exudation at eCO_2 [62]. Accordingly, Marilley et al. [31] observed higher dominance of *Pseudomonas* sp. in the rhizosphere of perennial ryegrass under atmospheric CO_2 enrichment. Moreover, the abundance of genes encoding for labile C degradation was found to increase at eCO_2 [19], indicating a fast consumption of root exudates. This is in line with the decreasing DOC concentrations at eCO_2 observed in the present study. Besides *Gammaproteobacteria*, also *Deltaproteobacteria* were stimulated under eCO_2 in RRC. The most abundant *Deltaproteobacteria* belonged to the order *Myxococcales*, which were previously found to be increased in the rhizosphere of two perennial grasses grown at eCO_2 [25]. *Myxococcales* are cellulolytic microorganisms [63] and thus likely to be stimulated by enhanced root growth due to eCO_2, leading to higher cellulose availability in soil [53].

Members of the order *Actinomycetales* dominating the phylum *Actinobacteria* also increased at eCO_2 in RRC. *Actinomycetales* are among the most important litter decomposers [64] and play an important role in the degradation of recalcitrant C including plant cell wall components, e.g., cellulose, hemicellulose, and chitin [65]. Consequently, they may be stimulated by enhanced plant growth due to eCO_2 [11]. However, actinomycetes are also known as slow growing, typical bulk soil inhabitants [66], which might explain the conflicting responses to eCO_2, ranging from positive (this study, [4]) to unaffected [32, 67] to negative [68].

In contrast to *Actinobacteria*, *Acidobacteria* decreased at eCO_2 in RRC, overall. *Acidobacteria* constitute on average 20 % of soil bacteria, with subgroups Gp1 to Gp4 and Gp6 being predominant [59]. Despite their abundance, the physiological properties of this phylum are still largely unknown. However, both pH and soil C content are negatively correlated with the abundance of subgroup Gp1 in a large number of soils [69, 70], indicating the adaptation to oligotrophic conditions [71]. This may explain the observed decrease under eCO_2 (this study, [4, 26]), as soil pH may increase via decomposition of organic acids [72] and organic matter with high C/N ratio [73], release of conjugate bases [74], or production of ammonia from organic matter decomposition [75]. Moreover, increased soil C availability can be expected by increased rhizodeposition due to eCO_2 [15, 62]. However, members of subgroups Gp5, Gp6, and Gp17 showed the highest relative abundance in nutrient-rich soils [70], indicating that certain *Acidobacteria* are more copiotrophic than previously suggested. This is in line with the CO_2-induced increase of subgroups Gp4 and Gp6 in RRC observed in the present study.

Members of the order *Planctomycetales* were enriched in RRC at eCO_2. *Planctomycetes* form a distinct phylum and possess phenotypic features which are highly unusual among bacteria, including the lack of peptidoglycan in cell walls and intracellular compartmentalization with the formation of a membrane-bound nucleoid [76]. Members of planctomycetes

are commonly found in environmental microbial communities from wastewater and soil [77], including some species with highly unusual physiology but mostly chemoheterotrophs [78, 79] contributing to global nitrogen turnover [76].

Besides *Planctomycetes*, also *Firmicutes* represented predominantly by the class *Bacilli* increased at eCO_2 in RRC. *Bacilli* are gram-positive bacteria that play an important role in the mineralization of plant-derived materials and humus in soils and consequently can be expected to increase in abundance under atmospheric CO_2 enrichment due to increased aboveground and belowground plant biomass [11]. This is in line with previous studies [68, 80] but in contrast to results from Drigo et al. [32], who observed no influence of eCO_2 on abundance and community composition of the genus *Bacillus* in the rhizosphere of *Carex arenaria* and *Festuca rubra*. However, Drigo et al. [32] observed that abundance and community structure differs regarding plant species and soil origin, which may explain the contradictory results.

Conclusion

The main aim of this study was to provide insight into microbial community responses in the rhizosphere of young beech trees from two different mountainous sites exposed to eCO_2 concentrations. The results demonstrated that atmospheric CO_2 enrichment clearly altered the microbial community composition already shortly after application, but the effect was dependent on the distance to the roots. Interestingly, an ecotype-related effect of different beech trees obtained from two contrasting sites was not detected, which may be due to the short time period of the study and/or the artificial soil used. This study nicely complements experiments where young beech trees have been incubated for longer time periods with increased CO_2 conditions, and the effects were less pronounced compared to the present study [81], indicating an adaption of trees and their associated microflora over time.

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