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REGULAR ARTICLE

Nitrogen fertilization affects bacteria utilizing plant-derived carbon in the rhizosphere of beech seedlings

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Abstract
Background and aims Forest ecosystems may act as sinks for or source of atmospheric CO_2. While inorganic nitrogen (N) fertilization increases aboveground tree biomass, the effects on soil and rhizosphere microorganisms are less clear, indicating potentially unpredictable changes in nutrient cycling processes maintaining ecosystem functioning. Although plant-derived carbon (C) is the main C source in soils during the vegetation period, information on the response of rhizosphere bacteria assimilating rhizodeposits to increased soil N availability mainly for trees is missing.
Methods We performed a greenhouse experiment with ^{13}C-CO_2 labelled young beech seedlings grown under different N fertilization levels. DNA Stable Isotope Probing (DNA-SIP) in combination with TRFLP and pyrosequencing enabled us to identify bacteria assimilating plant-derived C and to assess the main responders phylogenetically.
Results Although above- and belowground allocation of recently fixed photosynthates remained unchanged, microbial rhizosphere community composition was clearly affected by fertilization. Besides, we found lower ^{13}C incorporation into microbial biomass in fertilized soil. Moreover, it could be shown that only a small subset of the rhizosphere microbiome incorporated recently fixed C into its DNA, dominated by Proteobacteria (Alpha- and Betaproteobacteria) and Actinobacteria (Actinomycetales). and.
Conclusions Our results suggest that N fertilization may change both the diversity of bacterial communities using rhizodeposits and assimilation rates of recently fixed photosynthates. Given the close interaction of beneficial and/or deleterious microbes and plants in the rhizosphere, this could potentially have positive or negative implications for plant performance on long-term.

Keywords Fertilization · Plant-derived carbon · Rhizosphere microbiome · DNA-SIP · Pyrosequencing

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Introduction

The rapid increase in atmospheric CO_2 concentration due to fossil fuel burning and changes in land-use pattern (Ball and Drake 1997) has led to a considerable interest in the potential of biological systems to mitigate the effect of this greenhouse gas by enhanced carbon (C) sequestration. In particular forests accounting for a large proportion of the global net primary productivity (King et al. 2005) have been the focus of research. As forest ecosystems in boreal and temperate regions are often nitrogen (N) limited (Tamm 1991) fertilization is widely practiced in the frame of forest management strategies. In addition to active fertilization, anthropogenic N deposition attenuates nitrogen limitation of ecosystems, and consequently forests in the northern hemisphere presently act as C sinks (Erisman and de Vries 2000; Stinson et al. 2011). Besides the positive effect on aboveground biomass, N fertilization may decrease tree root biomass (Majdi and Kangas 1997; Majdi and Ohrvik 2004) and allocation of C to the roots (Giardina et al. 2004; Phillips and Fahey 2007), suggesting a significant impact on soil microorganisms.

During the vegetation period, photosynthetically assimilated CO_2 is released by the plants via rhizodeposition (root exudates, mucilage, enzymes and sloughed root cells) into the soil. Mainly microbes in the rhizosphere benefit from the surplus of easily degradable C provided by plants and in turn enhance plant growth and health via nutrient mobilization, production of plant growth hormones, induction of systemic resistance and controlling pathogens (Raaijmakers et al. 2009). Due to this close plant-microbe interaction plants are able to select for a certain rhizosphere microbiome by governing the quality and quantity of root exudates. However, recent studies could show that despite the high diversity of microorganisms in the rhizosphere of various plants (Bulgarelli et al. 2012; Donn et al. 2015; Mendes et al. 2014) only a small subset assimilates rhizodeposits effectively (Ai et al. 2015; Hernández et al. 2015).

Increased soil fertility due to N fertilization reduces belowground C allocation (Giardina et al. 2004; Phillips and Fahey 2007), resulting in enhanced competition and a reduction of microbial biomass and respiration in the rhizosphere (Phillips and Fahey 2007; Treseder 2008). Simultaneously, microbes able to grow fast at high soil nutrient levels and in the presence of labile C sources like Actinobacteria and Firmicutes are favored in the rhizosphere of fertilized soils whereas oligotrophs like Acidobacteria and Verrucomicrobia decrease (Ramirez et al. 2012). In addition, enzymes responsible for the degradation of recalcitrant C pools are reduced, probably resulting in increased soil C sequestration (Ramirez et al. 2012). However, N fertilization was also found to increase nitrification and denitrification (Forge and Simard 2001; Prescott et al. 1992; Wallenstein et al. 2006; Wertz et al. 2012), carrying the risk of nitrate leaching and emission of the potent greenhouse gas N_2O. However, effects of soil N amendment were site specific and not generalizable (Jandl et al. 2007), indicating potentially unpredictable alterations in nutrient cycling processes maintaining ecosystem functioning.

Most studies investigating the impact of N fertilization on the microbiome of the rhizosphere have been performed using annual plants, mainly crops used in agriculture. We predict that seedlings or young trees might react differently as primarily they have to establish a strong rooting system, harboring a highly active microbiome, that ensures the best possible allocation of nutrients (mainly phosphorous) during growth to be able to compete with older trees. Thus, C allocation patterns of young trees or seedlings into the rhizosphere might be unaffected by N fertilization. However, a direct effect of N fertilizers on the rhizosphere microbiome is likely and, consequently, changes in the diversity of bacteria, fungi and archaea associated to the belowground part of the plants independent from stable exudation rates may be predicted. Therefore, the aims of the present study were: (1) to quantify short-term N fertilization effects on growth and C fluxes of young beech seedlings and (2) to evaluate the short-term effect of N fertilization on total bacterial rhizosphere communities as well as on bacteria involved in the incorporation of plant-derived C. We conducted a greenhouse experiment with 3 weeks old beech seedlings grown under a ^{13}C-CO_2 enriched atmosphere in unfertilized and fertilized (10 kg N ha^{-1}) soil. Those microbes incorporating recently fixed C into their DNA were identified via DNA Stable Isotope Probing (DNA-SIP) in combination with molecular fingerprinting and barcoding of the rhizosphere microbial community.

Material and methods

Experimental setup

Soil was collected from a low mountainous beech forest site in Tuttlingen, Southern Germany (8°45'E/47°59'N), classified as Rendzic Leptosoil (Sceletic),

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consisting of 68 % clay, 28 % silt and 4 % sand. The soil was characterized by a pH ($CaCl_2$) of 6.1, a water holding capacity (WHC) of 49 % and a total C and N content of 2.5 % and 0.2 %, respectively. To remove plant residues and gravel the soil was sieved (< 4 mm), filled into 500 mL plastic pots (400 g soil per pot) and adjusted to 60 % of WHC (Linn and Doran 1984). In total, 100 pots were set up and after 1 week pre-germinated beech nuts (certificate no. D090002446209, Samenklenge Laufen, Germany) were planted into 60 of them (1 seedling per pot) in June 2011. The half of the planted and unplanted pots was fertilized with NH_4NO_3 (10 kg N ha^{-1}) directly after planting while the other half remained unfertilized but received a similar volume of water (volume was calculated to obtain 60 % WHC). The plants were grown in the greenhouse at ambient daylight, 22/15 °C day/night temperature and relative humidity 50 %. Irrigation was performed by hand with 10–20 mL deionized water every 72 h to keep the water content of the soil between 50 and 60 % of the WHC. When the plants reached the young leaf developmental stage after 3 weeks, 15 fertilized and unfertilized planted pots, respectively, were placed into a tent built of transparent plastic foil (ethylene-tetrafluorethylene ETFE, film thickness 80 μm, Koch Membranen GmbH, Germany) to separate the plants from the outer atmosphere. To estimate the amount of soil autotrophic CO_2 fixation, 10 fertilized and 10 unfertilized unplanted pots were also placed within the tent. The tent atmosphere was enriched with ^{13}C-CO_2 (99 atom% ^{13}C, Air Liquide, Düsseldorf, Germany) as described previously (Gschwendtner et al. 2011), resulting in a $^{13}CO_2$ concentration of 45–55 % of the total CO_2 concentration. This labelling occurred for 8 h per day for a period of 2 weeks. During night, the tent CO_2 concentration was kept stable at approximately 350 μmol mol^{-1} by CO_2 depletion using a membrane pump combined with vials containing soda lime (Gschwendtner et al. 2011). Sampling occurred after 2, 4, 7, 10 and 14 days in triplicates for the labelled and unlabelled pots. Rhizosphere soil (defined as soil still attached to the roots after vigorous shaking (Yanai et al. 2003)) was stored at 4 °C for analysis of extractable soil C and N pools and microbial biomass carbon (C_{mic}) and at −80 °C for DNA extraction. The soil of the unplanted pots was treated similarly. Plants were cleaned with deionized water and roots, stems and leaves were dried at 65 °C for 48 h for determination of C and N content.

Total C and N content

The dried plant material was ball-milled, weighted into tin capsules and analyzed using an Elemental Analyzer coupled with an Isotopic Ratio Mass Spectrometer (EA-IRMS; Eurovector, Milan, Italy coupled with a MAT 253, Thermo Electron, Bremen, Germany). The $\delta^{13}C$ values were related to the international Vienna-Pee Dee Bee Belemnite (V-PDB) standard and were calculated as described by Werner and Brand (2001).

Extractable soil carbon and nitrogen pools

Within 4 days of sampling, 5 g soil was extracted in 0.01 M $CaCl_2$ solution (1:4, *w*/*v*) and filtered through 0.45 μm pore-sized polycarbonate filters (Whatman Nucleopore Track-Etch Membrane filters). Water extractable organic carbon (WEOC) was determined on a Total Carbon Analyzer (Shimadzu TOC 5050, Tokyo, Japan) by catalytic high temperature oxidation. Measurement of $\delta^{13}C$ was done via online coupling of liquid chromatography and stable isotope ratio mass spectrometry (LC-IRMS, Thermo Electron, Bremen, Germany). Total nitrogen, ammonium and nitrate in the extract was quantified using Skalar Analyzer (Thermo Fisher Scientific, Waltham, USA), while water extractable organic nitrogen (WEON) was calculated as difference between total nitrogen and inorganic N (ammonium plus nitrate).

Microbial biomass carbon (C_{mic})

C_{mic} was determined by the chloroform-fumigation method according to Vance et al. (1987) using 5 g soil extracted in 0.01 M $CaCl_2$ solution (1:4 *w*/*v*) (Joergensen 1995). Measurement of $\delta^{13}C$ in the extracts was done via online coupling of liquid chromatography and stable isotope ratio mass spectrometry (LC-IRMS, Thermo Electron, Bremen, Germany). The $\delta^{13}C$ values were related to the international Vienna-Pee Dee Bee Belemnite (V-PDB) standard and computed as described by Marx et al. (2007).

Nucleic acid extraction, isopycnic centrifugation and fractionation

DNA was extracted from 0.4 g of rhizosphere soil of each of the 60 pots using a FastDNA Spin kit for Soil (MP Biomedicals, Santa Ana, USA) and the Precellys24

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Instrument (PeqLab, Erlangen, Germany). Quality and quantity of the nucleic acids were checked spectrophotometrically (Nanodrop, PeqLab, Erlangen, Germany) and by gel electrophoresis. Isopycnic centrifugation of selected samples for molecular analyses (see below) was performed in CsCl gradients containing 5 ml CsCl stock solution (1.84 g ml^{-1}) and 1 ml gradient buffer (0.1 M Tris-HCl, pH 8; 0.1 M KCl; 1 mM EDTA) including 4 μg DNA at 20 °C at 45,500 rpm (180,000 g_{av}) for 48 h in a VTI 65.2 vertical rotor (Beckman Coulter, Krefeld, Germany). Prior to centrifugation, density of the gradients was checked via AR200 digital refractometer (Reichert Technologies, Munich, Germany) and adjusted to 1.72 g ml^{-1} (Lueders 2004b). Centrifuged gradients were fractionated from bottom to top into 12 equal fractions using Perfusor compact S (Braun AG, Melsungen, Germany). The density of each fraction was measured with a refractometer. Afterwards, the fractions were purified as described by Lueders et al. (2004a) and nucleic acids were quantified using a PicoGreen assay.

Quantification of 16S rRNA genes

To minimize cross-feeding the shortest possible labelling time was determined by screening the gradient fractions for bacterial abundance shifts towards fractions with higher buoyant density (BD). Therefore, the sample with the highest ^{13}C incorporation into C_{mic} for each fertilized/unfertilized and labelled/unlabeled sample after 2, 4, 7, 10 and 14 days was chosen and fractionated as described above. Bacterial 16S rRNA genes in 240 gradient fractions (12 fractions each sample) were determined by quantitative real-time PCR (qPCR) on an ABI 7300 Cycler (Life Technologies, Darmstadt, Germany) using the primers FP16S/RP16S (Bach et al. 2002). Each 25 μl PCR reaction contained 1× Power SYBR Green PCR master mix (Life Technologies, Darmstadt, Germany), 0.2 μM of each primer (Metabion, Martinsried, Germany), 15 μg BSA (Sigma Aldrich, Taufkirchen, Germany) and 2 μl of template DNA. Thermal cycling started with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of amplification (95 °C for 45 s, 58 °C for 45 s, 72 °C for 45 s) and a melting curve analysis to confirm the specificity of the SYBR Green-quantified amplicons. Serial dilutions (10^1–10^6 copies μl^{-1}) of plasmid DNA containing the PCR product of the 16 rRNA gene of *Pseudomonas putida* were used to calculate standard curves. The amplification efficiency, calculated with the formula Eff = $10^{(-1/slope)}$ -1, was 93 %.

TRFLP analysis

As qPCR results revealed bacterial abundance shifts towards fractions with higher BD in labelled compared to unlabeled samples for the first time after 4 days in unfertilized pots and after 7 days in fertilized pots (detailed information is given in the results section), these time points were selected for further molecular analysis. Therefore, 144 gradient fractions (12 fractions for each triplicate replicate of labelled/unlabeled pots after 4 days of labelling (unfertilized pots) and 7 days (fertilized pots)) were selected for TRFLP fingerprinting. For the amplification of 1.4 kb fragments of the 16S rRNA gene the primers 27f (FAM-labelled) and 1401r (Gschwendtner et al. 2015) were used with the following PCR conditions: 94 °C for 10 min for initial denaturation, followed by 30 cycles of amplification (94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.5 min) and a final extension step at 72 °C for 10 min. PCR reactions (total volume 50 μl) contained 1× Taq buffer (Life Technologies, Darmstadt, Germany), 2.5 mM $MgCl_2$ (Fermentas, St. Leon Rot, Germany), 0.2 mM dNTPs (Fermentas, St. Leon Rot, Germany), 0.2 μM of each primer (Metabion, Martinsried, Germany), 150 μg BSA (Sigma Aldrich, Taufkirchen, Germany), 2.5 U Taq Polymerase (Life Technologies, Darmstadt, Germany) and 20 ng of template DNA. Amplicons were purified using Nucleospin Gel and PCR Cleanup kit (Macherey Nagel, Düren, Germany), digested with MspI (Fermentas, St. Leon Rot, Germany) and separated on an ABI 3730 sequencer using MapMarker 1000 (Eurogentec, Köln, Germany) as internal standard (Gschwendtner et al. 2015). Size and relative abundances of terminal restriction fragments (TRFs) were analyzed using PeakScanner v1.0 software (Life Technologies) and T-REX (http://trex.biohpc.org/) with a binning range of 2 bp.

Sequencing analysis

One sample for each fertilized/unfertilized and labelled/unlabeled treatment was selected randomly from the TRFLP triplicates. Based on previous TRFLP analysis, for each sample, one fraction with medium (1.73 g ml^{-1}) and high (1.77 g ml^{-1}) BD, respectively, was sequenced

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(in total 8 gradient fractions). Sequencing was performed on a 454 GS FLX Titanium system (Roche, Penzberg, Germany) as described previously (Gschwendtner et al. 2015), using the universal eubacterial primers 27f and 519r extended with unique Multiplex Identifiers, a four base library key and the respective A or B adapters for sample identification. 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. PCR conditions were: initial denaturation (94 °C, 5 min), followed by 25 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). All samples were purified using a Gel and PCR Cleanup Kit (Macherey Nagel, Düren, Germany), quantified using PicoGreen and quality checked via Bioanalyzer 2100 on a DNA 7500 chip (Agilent, Böblingen, Germany). Afterwards, the samples were pooled in an equimolar ratio of 10^9 molecules μl^{-1} followed by an emulsion PCR (Gschwendtner et al. 2015) and a two-region sequencing run (including amplicons generated using the forward and the reverse primer). The nucleotide sequence data obtained in this study have been submitted to the GenBank database under accession numbers SRR2470191 – SRR2470198.

Sequence data were processed in MOTHUR v.1.33.3 (Schloss 2009) as described previously (Gschwendtner et al. 2015). Briefly, quality trimming was done setting a minimum length of 250 bp per sequence and one mismatch for barcode and primers, respectively. For analyzing the sequencing data with the reverse primer, also the reverse complement was considered. After removing chimeras by alignment to the SILVA database provided by MOTHUR, phylogenetic classification was performed using the Ribosomal Database Project dataset and mitochondrial sequences were excluded. The calculated distance matrix resulted in operational taxonomical units (OTUs) obtained by the nearest neighbor clustering algorithm at 97 % sequence similarity and was used for calculating rarefaction curves. For analysis of Proteobacteria and Actinobacteria (which dominated the bacteria assimilating rhizodeposits), representative sequences of each OTU were aligned to the SILVA database for construction of a phylogenetic tree in ARB (Ludwig et al. 2004). OTUs represented by less than 0.5 % of reads were omitted from the phylogenetic dendrograms.

Statistical analysis

Statistical analysis was done in R v3.1.2 (http://www.R-project.org/) using multivariate analysis of variance (Adonis function). For plant and soil parameters, significant differences between sampling time and fertilization treatments were analyzed. TRFLP profiles were evaluated 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 fertilization treatments and gradient fractions were calculated with multivariate analysis of variance (Adonis function) based on Yue Clayton dissimilarity and Euclidean distances of Hellinger transformed data. Furthermore, replicate similarity was checked by clustering all TRFs according to a dissimilarity matrix based on the Yue Clayton coefficient, resulting in the Unweighted Pair Group Method (UPGMA) dendrogram (Yue and Clayton 2005). For sequence data analysis, distance matrices based on OTUs defined by 97 % sequence similarity were generated for calculating rarefaction curves.

Results

Soil nitrogen pools

As expected, for all extractable soil N pools a fertilization effect was observed, resulting in 17 % (WEON, 7d) to 67 % (nitrate, 2d) higher concentrations in the rhizosphere samples of the fertilized compared to the unfertilized pots (Table 1).

Plant biomass and ^{13}C enrichment

While stem and leaf biomass increased during the experiment for both fertilized and unfertilized pots, ranging from 0.05 to 0.10 g (stem) and 0.21 to 0.28 g (leaf), root biomass remained constant (Table 2). A fertilization effect was observed only for leaves, resulting in 10–20 % higher leaf biomass for fertilized compared to unfertilized pots. Nevertheless, ^{13}C incorporation was not affected by fertilization (Table 2) but increased with incubation time to 15,064 $^0/_{00}$ V-PDB (root) and 14,843 $^0/_{00}$ V-PDB (leaf). For both fertilized and unfertilized

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t1.1 Q3 **Table 1** Extractable soil N and C pools and ^{13}C incorporation into WEOC and Cmic of unfertilized (f0) and fertilized (f10) soils after 2, 4, 7, 10 and 14 days of incubation ($n = 6$ for nutrient pools; $n = 3$ for $\delta^{13}C$ values; sd means standard deviation)

			Extractable nutrient pools [mg kg^{-1}]										$\delta^{13}C$ [‰ V-PDB]			
			Ammonium		Nitrate		WEON		WEOC		C_{mic}		WEOC		C_{mic}	
			Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
t1.5	f0	2d	0.07	0.01	23.50	1.57	13.12	1.25	1.34	0.22	42.30	6.12	−24.53	1.06	14.37	2.99
t1.6		4d	0.09	0.01	28.80	3.81	10.99	1.39	1.36	0.21	45.26	3.01	−24.32	1.98	70.36	6.42
t1.7		7d	0.10	0.00	21.68	5.35	9.93	0.65	1.55	0.14	46.17	3.64	−22.30	0.85	266.59	34.31
t1.8		10d	0.14	0.01	20.87	4.47	9.93	0.65	1.72	0.33	45.00	3.74	−20.96	0.34	335.54	27.16
t1.9		14d	0.14	0.01	16.68	1.40	9.60	0.98	1.82	0.23	47.14	1.72	−19.44	0.63	391.22	32.96
t1.10	f10	2d	0.10	0.00	39.31	2.32	17.14	0.44	1.83	0.09	45.30	7.49	−22.75	1.22	2.34	1.08
t1.11		4d	0.12	0.01	41.23	3.12	13.45	1.23	1.82	0.15	43.92	6.01	−22.36	0.45	24.06	6.44
t1.12		7d	0.12	0.01	33.79	3.80	11.64	0.98	1.83	0.01	46.89	3.75	−20.80	0.92	115.64	19.69
t1.13		10d	0.17	0.01	28.49	3.37	9.87	0.28	1.99	0.15	48.25	2.68	−18.69	0.76	246.10	22.15
t1.14		14d	0.17	0.01	27.71	6.17	9.68	0.22	1.94	0.08	50.37	4.44	−17.71	1.45	284.64	69.63
t1.15	p_{fertil}		**<0.001**		**<0.001**		**<0.001**		**<0.001**		0.202		**<0.001**		**<0.001**	
t1.16	p_{time}		**<0.001**		**<0.001**		**<0.001**		0.062		0.189		**<0.001**		**<0.001**	
t1.17	$p_{fertil*time}$		0.898		0.320		**0.001**		0.384		0.773		0.979		**0.006**	

Significant differences between fertilization treatment and incubation time were calculated by multivariate ANOVA and are indicated by p values <0.05 (bold letters)

t2.1 **Table 2** Plant biomass and ^{13}C incorporation into roots and leaves of unfertilized (f0) and fertilized (f10) soils after 2, 4, 7, 10 and 14 days of incubation ($n = 6$ for plant biomass; $n = 3$ for $\delta^{13}C$ values; sd means standard deviation)

			Biomass [g]						$d^{13}C$ [‰ V-PDB]			
			Root		Stem		Leaf		Root		Leaf	
			Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
t2.5	f0	2d	1.14	0.45	0.05	0.02	0.21	0.05	969	483	3435	467
t2.6		4d	1.03	0.17	0.07	0.02	0.21	0.03	2880	1057	6147	1912
t2.7		7d	0.83	0.31	0.06	0.01	0.23	0.04	5445	2881	8777	1518
t2.8		10d	0.94	0.48	0.06	0.02	0.23	0.05	11,082	3697	9548	2895
t2.9		14d	0.97	0.30	0.08	0.02	0.25	0.04	13,206	3837	14,843	3539
t2.10	f10	2d	1.24	0.31	0.05	0.02	0.23	0.04	417	162	2892	637
t2.11		4d	0.95	0.31	0.06	0.01	0.25	0.02	2751	799	6238	842
t2.12		7d	0.97	0.27	0.08	0.02	0.26	0.04	4544	2576	6745	1292
t2.13		10d	1.18	0.17	0.08	0.03	0.26	0.06	7743	2748	8612	1161
t2.14		14d	1.01	0.27	0.10	0.03	0.28	0.04	15,064	1757	12,408	3154
t2.15	p_{fertil}		0.220		0.059		**0.001**		0.369		0.053	
t2.16	p_{time}		0.131		**<0.001**		**0.046**		**<0.001**		**<0.001**	
t2.17	$p_{fertil*time}$		0.700		0.870		0.970		0.203		0.620	

Significant differences between fertilization treatment and incubation time were calculated by multivariate ANOVA and are indicated by p values <0.05 (bold letters)

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pots, ^{13}C enrichment was higher in leaves compared to roots up to 7 days of incubation. C and N content of the plants was not influenced by fertilization (data not shown).

^{13}C incorporation into WEOC and C_{mic}

Both WEOC and C_{mic} remained constant over time, ranging from 1.3 to 1.8 mg kg^{-1} and 42.3 to 47.2 mg kg^{-1}, respectively, for unfertilized pots (Table 1). While C_{mic} was unaffected by fertilization, WEOC concentration was 10–36 % higher in fertilized compared to unfertilized pots. The ^{13}C enrichment of both WEOC and C_{mic} increased with incubation time up to −19.4 $^{0}/_{00}$ V-PDB (WEOC) and 391.2 $^{0}/_{00}$ V-PDB (C_{mic}) for unfertilized pots (Table 1). While $\delta^{13}C$ of WEOC was higher in fertilized compared to unfertilized pots, ^{13}C incorporation into C_{mic} was increased in unfertilized pots. Microbial autotrophic CO_2 fixation could be neglected, as microbial biomass of labelled and unlabeled unplanted control pots showed no difference in ^{13}C enrichment (data not shown).

Quantification of 16S rRNA genes of density-resolved bacterial communities

For the identification of bacteria assimilating ^{13}C-labelled plant-derived carbon, microbial communities were analyzed with DNA-SIP. To resolve ^{13}C-enriched DNA from the background of unlabeled DNA, DNA extracts were isopycnically centrifuged and fractionated according to the buoyant density (12 fractions per CsCl gradient). Quantification of 16S rRNA genes in gradient fractions by real-time PCR revealed similar abundance pattern among the gradient fractions of fertilized and unfertilized samples, with highest 16S rRNA gene copies in “medium” fractions (BD of 1.70 g ml^{-1}) and lowest in “heavy” fractions (1.77 g ml^{-1}), ranging from 10^8 and 10^3 gene copy numbers, respectively (ESM 1). However, fertilization-dependent abundance shifts between labelled and unlabeled pots were observed: while in unfertilized pots bacterial abundance was shifted towards fractions with higher BD already after 4 days, a shift in abundance in fertilized pots was observed after 7 days for the first time. Therefore, the respective time points were selected for further analyses of the microbial community.

TRFLP fingerprinting of density-resolved bacterial communities

To compare bacterial diversity between the different fertilization levels and sampling time points, we reconstructed the total microbial soil communities by summarizing the abundances of each TRF individually within the 12 fractions for each density gradient. Although aware that this must not necessarily 100 % reflect the original soil community due to possible losses during fractionation and purification, we assume that this approach allows a better comparison of total bacterial community structure (reconstructed from gradients) and bacteria incorporating ^{13}C than a direct fingerprinting of bacterial communities from the extracted DNA without density gradient centrifugation. TRFLP profiles for fertilized and unfertilized soils did not change significantly between 4 and 7 days of incubation for the respective fertilization treatment (data not shown). In total, 95 and 96 TRFs were detected for fertilized (7d) and unfertilized (4d) soils, respectively, ranging from 52 to 1121 bp, with 51 TRFs > = 1 % of relative abundance (Fig. 1). Both fertilization levels were dominated by TRFs 85, 139, 403 and 487 bp, which accounted in sum for 62 % (unfertilized) and 60 % (fertilized) of all TRFs. Although TRFLP profiles contained similar TRFs for fertilized and unfertilized soils, the pattern differed significantly due to altered relative abundances of 16 TRFs: While TRFs 80, 139, 441 and 497 bp were significantly enriched in unfertilized soils, TRFs 60, 128, 158, 201, 276, 279, 398, 434, 469, 487, 510 and 540 bp were higher in fertilized compared to unfertilized soils. A table showing the distribution of all TRFs among the gradient fractions is given in Online Resource 2 (ESM 2).

For the identification of TRFs representing bacteria assimilating ^{13}C derived from rhizodeposits, the microbial diversity within the gradient fractions was screened for abundance shifts towards fractions with higher BD in ^{13}C-labelled pots compared to unlabeled pots. While four TRFs were observed in “heavier” fractions in the ^{13}C gradient compared to the unlabeled gradient in both fertilized and unfertilized samples (TRFs 60, 72, 85 and 491 bp), TRF 55 bp was shifted only in fertilized samples (Fig. 2). For all gradients, labelling shifts within the bacterial community structure occurred gradually over at least two gradient fractions and were most pronounced between fractions with a density of 1.71–1.73 g ml^{-1}

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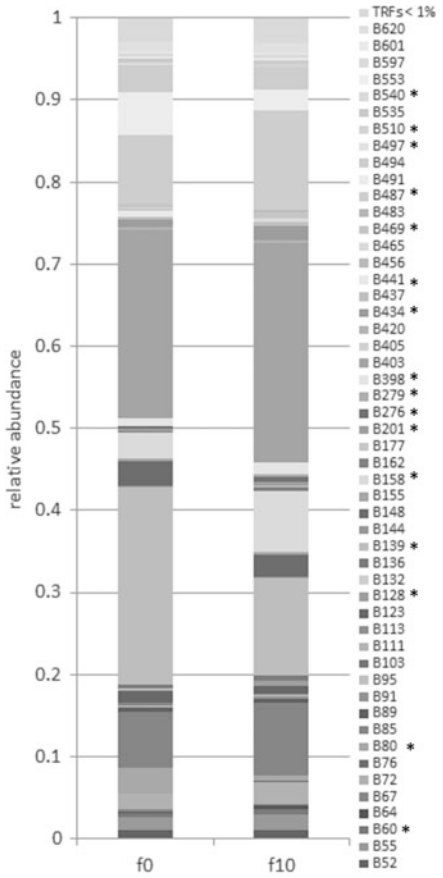

Fig. 1 Relative abundance of TRFLP fragments obtained from the reconstructed TRFLP profile by summarizing the abundance among the 12 gradient fractions from unfertilized (f0) and fertilized (f10) soils based on partial 16S rRNA gene sequences after DNA extraction and PCR amplification ($n = 6$). TRF names refer to bp lengths. Significant differences ($p < 0.05$) between fertilization treatments for individual TRFs are indicated by *asterisks*

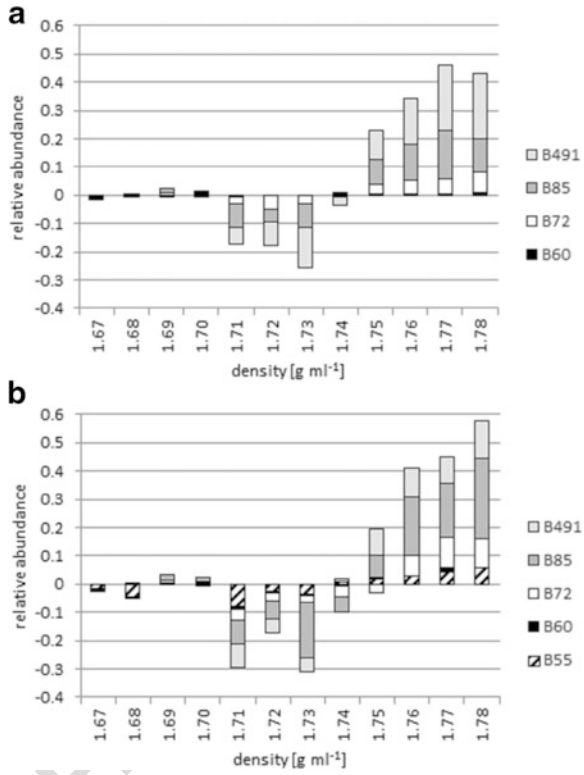

Fig. 2 TRFLP fragments showing significant shifts ($p < 0.05$) towards fractions with higher buoyant density in ^{13}C-labelled microcosms compared to unlabeled microcosms presented as mean difference between the relative abundance in ^{13}C-labelled and unlabeled DNA extracts from **a** unfertilized (f0, 4 days) and **b** fertilized (f10, 7 days) soils among 12 gradient fractions ($n = 3$)

(medium) and 1.77–1.78 g ml^{-1} (heavy) (Fig. 2). Along with the high replicate similarity (ESM 3), for both fertilized and unfertilized pots one gradient fraction with medium and high density, respectively, was selected for phylogenetic analyses of bacterial community.

Sequencing analysis of density-resolved bacterial communities

Based on TRFLP results showing high reproducibility (ESM 3) and most pronounced shifts between medium and heavy fractions, eight gradient fractions were selected for pyrosequencing analysis: one fraction with medium and high density for both fertilized/unfertilized soils and labelled/unlabeled pots. In total, 38,066 bacterial raw sequence reads were generated from the 16S rRNA gene PCR amplicons. After filtering, chimera check and removing erroneous reads, 14,616 high quality sequence reads with a minimum of 250 bp remained, which were represented by 862 OTUs revealed from the forward primer dataset and 701 OTUs from the dataset generated by the reverse primer at 97 % sequence similarity. Rarefaction curves indicated diversity coverage of 80–95 % for both forward and reverse datasets (ESM 4).

Using the forward primer dataset, the annotated reads obtained for the heavy fractions of the ^{13}C-labelled samples of both unfertilized (f0) and fertilized (f10) soils could be grouped into three major phyla: Actinobacteria, Bacteroidetes and Proteobacteria, accounting in sum for 78 % (f0) and 74 % (f10) of all reads (ESM 5) at day 4 and day 7, respectively. Proteobacteria were the most abundant and were dominated by members of Betaproteobacteria belonging to the order

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Burkholderiales including the families Oxalobacteraceae (11.5 % (f0) and 15.5 % (f10) of all reads) and Comamonadaceae (*Acidovorax*, 3.6 % (f0) and 2.6 % (f10) of all reads). Alphaproteobacteria were the second most abundant Proteobacteria with Rhizobiales (6.8 % (f0) and 7.4 % (f10) of all reads) as predominant order. Actinobacteria were dominated by members of the order Actinomycetales (14.1 % (f0) and 15.5 % (f10) of all reads) which remained mainly unclassified on family level. Members of the families Flavobacteriaceae (*Flavobacterium*) and Sphingobacteriaceae (*Pedobacter*) were the predominant Bacteroidetes in the heavy fractions, accounting in sum for 6.8 % (f0) and 1.2 % (f10) of all reads. In the heavy fraction of the unlabeled samples similar taxa were observed but with altered abundances. While Proteobacteria and Actinomycetales were lower, Bacteroidetes were higher compared to the heavy fraction of the labelled samples for both fertilized and unfertilized soils. Similar results were obtained when analyzing the dataset generated by the reverse primer (data not shown).

Screening the diversity pattern for abundance shifts (threshold =50 % difference) towards the heavy fraction in ^{13}C-labelled gradients compared to unlabeled gradients resulted in the identification of 4 (fertilized) and 7 (unfertilized) OTUs representing bacteria assimilating ^{13}C-labelled plant-derived C, mainly belonging to Proteobacteria and Actinobacteria (Fig. 3, Table 3). While OTU005 (Actinomycetales) and OTU009 (Rhizobiales) were shifted in both fertilized and unfertilized samples, OTU004 (Oxalobacteraceae) and OTU015 (*Arthrobacter*) were shifted only in fertilized samples. By contrast, OTU008 (*Flavobacterium*), OTU002 (Proteobacteria), OTU018 (Rhodobacteraceae), OTU027 (*Massilia*) and OTU017 (*Haloferula*) showed abundance shifts only in unfertilized soils. However, the shifts were not statistically supported due to the lack of replication. The distribution of OTUs related to Proteobacteria and Actinobacteria is shown in phylogenetic trees (ESM 6)).

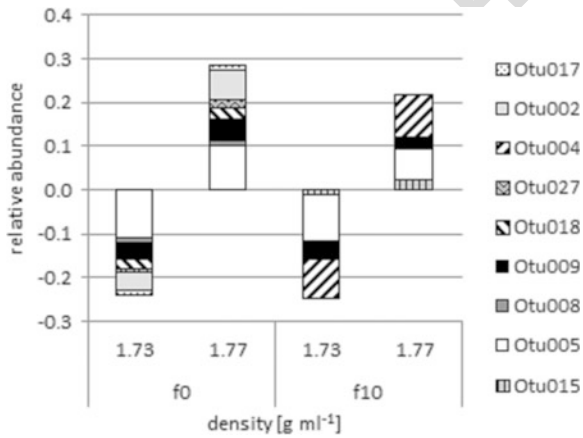

Fig. 3 Operational taxonomic units (OTUs) showing shifts towards the fraction with high buoyant density in ^{13}C-labelled pots compared to unlabeled pots presented as difference between the relative abundance in ^{13}C-labelled and unlabeled DNA extracts from **a** unfertilized (f0, 4 days) and **b** fertilized (f10, 7 days) soils (n = 1). Data are obtained from the forward primer dataset. Phylogenetic classification of OTUs is presented in Table 3

Discussion

When plants start growing in soil, they immediately get in contact with soil microorganisms, resulting in the establishment of a microbial rhizosphere community closely interacting with the plants. Although it is acknowledged that additional N supply affects soil microbial community structure (Ai et al. 2015; Wertz et al. 2012; Zhu et al. 2015), limited information is available on how N fertilization influences the plant associated microbiome in the rhizosphere and its utilization of plant-derived carbon. In this study, we investigated the impact of low N fertilization levels (10 kg N ha^{-1}) on the rhizosphere microbial community of 3 weeks old beech seedlings and identified bacteria assimilating root-derived carbon using DNA-SIP. This method has found widespread application in microbial ecology as it allows the identification of ^{13}C-labelled microbes by sequencing of DNA directly isolated from soil. However, it has to be taken into account that isotope incorporation occurs only during DNA replication, resulting often in the need for long incubation periods to generate sufficiently high isotopically enriched DNA, harboring the risk of labelled by-products which may become substrates for non-target organisms (Lueders et al. 2004b). This cross-feeding can be minimized when selecting the shortest possible incubation time by sampling along a time series as was done in the present study. Although RNA-SIP would be a more sensitive approach because RNA synthesis in active cells occurs at high rates and thus ^{13}C is incorporated independent of cell replication, DNA was chosen as target molecule because (1) the density-resolved separation of isotopically labelled DNA via gradient centrifugation is better compared to RNA (Lueders et al. 2004a) and (2) complete genomes are labelled allowing follow-up analyses of different

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t3.1 **Table 3** List of ^{13}C-labelled OTUs (forward primer dataset) obtained from unfertilized and fertilized soils and its phylogenetic classification

t3.2 OTU	Phylum	Class	Order	Family	Genus
t3.3 Otu002	Proteobacteria	unclassified	unclassified	unclassified	unclassified
t3.4 Otu004	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	unclassified
t3.5 Otu005	Actinobacteria	Actinobacteria	Actinomycetales	unclassified	unclassified
t3.6 Otu008	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*
t3.7 Otu009	Proteobacteria	Alphaproteobacteria	Rhizobiales	unclassified	unclassified
t3.8 Otu015	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Arthrobacter*
t3.9 Otu017	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Haloferula*
t3.10 Otu018	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	unclassified
t3.11 Otu027	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Massilia*

functional genes or high-resolution metagenomics of target groups (Kalyuzhnaya et al. 2008).

With ongoing plant age, aboveground plant biomass increased independent from the fertilizer treatment, with a significant fertilization effect on leaf biomass, while root biomass was unaffected by fertilization and remained constant during the experiment. The positive fertilization effect on leaves is in line with previous studies observing higher aboveground plant biomass with additional N supply (Giardina et al. 2004; Zhu et al. 2015). Root growth has been found to be negatively (Majdi and Kangas 1997; Majdi and Ohrvik 2004) or positively (Majdi and Andersson 2005; Zhu et al. 2015) influenced by fertilization, indicating that effects might be plant species- and site-specific. The lack of fertilization impact on root growth in the present study may, on the one hand, be due to the early plant development stage resulting in the need for high investment into root biomass to get established in soil independent of fertilization. On the other hand, the short duration of the present experiment (14 days) may also preclude more distinct fertilization effects on plant growth. Simultaneously, photosynthesis rates seemed to be unaffected by fertilization, despite increased leaf growth, as ^{13}C contents of plant biomass were similar between fertilized and unfertilized soils.

Moreover, fertilization did not impact C_{mic} content, which is in contrast to various studies that observed a significant reduction of microbial biomass, respiration and activity in the rhizosphere of fertilized plants (Bowden et al. 2004; Phillips and Fahey 2007; Treseder 2008). Our results are in line with Zhu et al. (2015) who also reported also no effect on microbial biomass when investigating different grass species for a short duration (80 days) under different fertilization regimes. Interestingly, we found higher ^{13}C incorporation into C_{mic} in unfertilized soils, indicating that utilization of recently fixed plant-derived C was decreased in fertilized soils. Coinciding, soil WEOC content and ^{13}C enrichment of WEOC were higher in fertilized compared to unfertilized soils. As microbial biomass remained constant, rhizosphere microorganisms in fertilized soils must have used alternative carbon sources for growth. This decrease in the dependence of root-derived C with increasing soil nutrients was observed previously: Ai et al. (2015) showed that inorganic N fertilizers suppressed the utilization of wheat rhizodeposits by certain taxa but increased their preference for SOM as nutrient source, leading to a reduced bacterial diversity in the ^{13}C-labelled microbiome and thus simpler plant-microbe interactions. The changed utilization pattern probably resulted from altered quantity and quality of plant-derived C (Giardina et al. 2004; Kuzyakov 2002) which may influence the efficiency of microbial carbon assimilation (Fisk and Fahey 2001). We also observed faster ^{13}C incorporation into bacterial DNA extracted from unfertilized soils, reaching sufficient enrichment for gradient separation after 4 days of incubation, whereas in fertilized soils an abundance shift was first detected after 7 days of incubation.

Our results revealed that fertilization not only altered incorporation of recently fixed C but also bacterial community composition in the rhizosphere as TRFLP profiles showed clear abundance shifts for several TRFs in fertilized soils, which was also found previously (Zhu et al. 2015). Using pyrosequencing, Ai et al. (2015) and Ramirez et al. (2012) could identify Actinobacteria, Firmicutes and Nitrospirae as highly responsive to

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inorganic fertilization of soils. Nitrospirae are capable of nitrification, which often increases in N fertilized soils (Forge and Simard 2001; Prescott et al. 1992; Wertz et al. 2012), while Actinobacteria and Firmicutes are known copiotrophs showing high growth rates with high soil nutrient levels and the availability of labile C sources like root exudates (Fierer et al. 2003).

Interestingly, only a small subset of the rhizosphere microbiome was found to be capable of assimilating plant-derived C, dominated by Proteobacteria and Actinobacteria in the rhizosphere of wheat (Ai et al. 2015) or Proteobacteria and Verrucomicrobia in the rhizosphere of rice (Hernández et al. 2015). Similarly, our results showed that <1 % of observed OTUs were shifted towards the heavy fraction in ^{13}C-labelled samples compared to unlabeled samples, thus representing bacteria incorporating ^{13}C derived from plant assimilates into their DNA. While in fertilized soils only Proteobacteria and Actinobacteria incorporated ^{13}C, in unfertilized soils also Verrucomicrobia (*Haloferula*) and Bacteroidetes (*Flavobacterium*) assimilated labelled plant-derived carbon. Moreover, Rhodobacteraceae belonging to Alphaproteobacteria incorporated recently fixed carbon into their DNA only in unfertilized soils. Proteobacteria have previously been described as highly rhizo-competent heterotrophs assimilating C derived from agricultural plants (Ai et al. 2015; Hernández et al. 2015); especially Betaproteobacteria were found to be enriched in the rhizosphere of various plant species (Donn et al. 2015; Mao et al. 2014; Peiffer et al. 2013), probably due to their high ability to utilize root exudates (Fierer et al. 2007). However, we observed also Proteobacteria in the medium gradient fraction, indicating that these bacteria relied on other C sources like SOM. Therefore, those Proteobacteria may be saprophytic bacteria that have been generally adapted to rhizosphere conditions among various plant species (Bulgarelli et al. 2012). In contrast, 80–92 % of Actinobacteria were shifted towards the heavy gradient fraction in the labelled samples (ESM 5), indicating that Actinobacteria may use plant-derived C almost entirely as their sole C source. This finding is consistent with a previous study identifying Actinobacteria as highly effective rhizodeposit consumers in the rhizosphere of wheat (Ai et al. 2015). However, Mao et al. (2014) found Actinobacteria only weakly labelled when investigating C fluxes in the rhizosphere of switchgrass, indicating that the utilization of plant-derived C by Actinobacteria is dependent on the quality of root exudates that may differ among plant species (Percival et al. 2001; Sauer et al. 2006). In unfertilized soils, also Bacteroidetes and Verrucomicrobia assimilated ^{13}C. While Verrucomicrobia have been reported previously as consumers of plant-derived C (Hernández et al. 2015), Bacteroidetes were mainly detected in the unlabeled rhizosphere fraction of wheat plants (Ai et al. 2015). As for Actinobacteria, the inconsistent results may be due to the different plant species investigated. However, Bacteroidetes are known as copiotrophic soil bacteria capable of high growth rates when labile C sources are available (Fierer et al. 2003).

Although our results indicate that fertilization may alter the diversity of bacteria incorporating plant-derived C, it has to be taken into account that no replication was done for pyrosequencing. Consequently, the question remains if the observed changes were due to treatment or inter-plant effects or a combination of both. However, sample selection for sequencing was based on TRFLP data as it could be shown that TRFLP fingerprints and pyrosequencing data are capable of recovering highly comparable community structures in environmental samples using the primer pairs also used in the present study (Pilloni et al. 2012). As TRFLP analysis showed (1) high replicate similarity and (2) significant abundance shifts occurring between medium and high density fractions, we assume that the observed diversity changes were more related to fertilization than to inter-plant effects.

In summary, our results suggest that already low inorganic N fertilization (10 kg N ha^{-1}) increased leaf biomass from 3 to 4 weeks old beech seedlings. Although photosynthesis rates and the quantity of rhizodeposition seemed to be unaffected, bacterial diversity and the assimilation of recently fixed C in the rhizosphere were clearly altered. This might be, on the one hand, due to the direct influence of the increased N availability in fertilized soils favoring subsets of microorganisms capable of certain functions (Forge and Simard 2001; Prescott et al. 1992; Wallenstein et al. 2006; Wertz et al. 2012) and, on the other hand, due to quality changes in plant-derived C, which were not investigated in this study. Given the close interaction of rhizosphere microbes and their host plant and the fragile balance of beneficial and deleterious microorganisms in the rhizosphere, fertilization could potentially affect plant growth and health positively or negatively on long-term. However, further studies including more replication and plants at later development stages are

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necessary to better understand how changes of plant-microbe interactions impact the ecological functions of the rhizosphere microbiome under different fertilizer regimes. Additionally, naturally occurring stressors, e.g. drought, should be included and, in that regard, also transcriptome-based studies should be performed.

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