

RESEARCH ARTICLE

# Climate Change Impairs Nitrogen Cycling in European Beech Forests

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## Abstract

European beech forests growing on marginal calcareous soils have been proposed to be vulnerable to decreased soil water availability. This could result in a large-scale loss of ecological services and economical value in a changing climate. In order to evaluate the potential consequences of this drought-sensitivity, we investigated potential species range shifts for European beech forests on calcareous soil in the 21<sup>st</sup> century by statistical species range distribution modelling for present day and projected future climate conditions. We found a dramatic decline by 78% until 2080. Still the physiological or biogeochemical mechanisms underlying the drought sensitivity of European beech are largely unknown. Drought sensitivity of beech is commonly attributed to plant physiological constraints. Furthermore, it has also been proposed that reduced soil water availability could promote nitrogen (N) limitation of European beech due to impaired microbial N cycling in soil, but this hypothesis has not yet been tested. Hence we investigated the influence of simulated climate change (increased temperatures, reduced soil water availability) on soil gross microbial N turnover and plant N uptake in the beech-soil interface of a typical mountainous beech forest stocking on calcareous soil in SW Germany. For this purpose, triple <sup>15</sup>N isotope labelling of intact beech seedling-soil-microbe systems was combined with a space-for-time climate change experiment. We found that nitrate was the dominant N source for beech natural regeneration. Reduced soil water content caused a persistent decline of ammonia oxidizing bacteria

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and therefore, a massive attenuation of gross nitrification rates and nitrate availability in the soil. Consequently, nitrate and total N uptake of beech seedlings were strongly reduced so that impaired growth of beech seedlings was observed already after one year of exposure to simulated climatic change. We conclude that the N cycle in this ecosystem and here specifically nitrification is vulnerable to reduced water availability, which can directly lead to nutritional limitations of beech seedlings. This tight link between reduced water availability, drought stress for nitrifiers, decreased gross nitrification rates and nitrate availability and finally nitrate uptake by beech seedlings could represent the Achilles' heel for beech under climate change stresses.

## Introduction

European beech (*Fagus sylvatica* L.) dominates the natural forest vegetation in moist to moderately dry areas of the sub-mountainous altitude range in Central Europe [1]. About one third of the potential beech forest area in Central Europe is located on calcareous soil that is highly susceptible to water deprivation. For future forestry in Central Europe, it has even been suggested to replace spruce by beech [2,3]. However, beech responds to lower water availability with reduced growth and competitive power, and this apparent drought sensitivity of beech is a matter of current concern and debate [4–10] due to observations of increased heat waves and drought periods in wide regions of Central Europe [11]. This trend is expected to continue and intensify in the coming decades [12,13]. Two different mechanisms have been proposed to explain the sensitivity of beech to increased temperature and drought [4–6]: (1) tree physiological limitations; and (2) nutritional limitations due to decelerated microbial N turnover in soil, specifically due to impaired liberation and provision of bioavailable N by soil microbes. The latter mechanism may be of particular importance in calcareous soils and apply especially for beech seedlings with their restricted root system, because Rendzic Leptosols are poor in bioavailable N [14,15]. Furthermore, these soils are characterized by a shallow profile, high gravel content, clayey texture, and low water and nutrient retention capacity. Hence, bioavailable N released by microorganisms is frequently limiting growth of this forest type [5,15–17]. However, due to the lack of experimental evidence, and a range of severe methodological problems in the quantification of gross N turnover in the plant-soil-microbe system, this mechanism remained a hypothesis.

Nitrogen mineralization and nitrification as well as the subsequent competitive partitioning of bioavailable organic N monomers and inorganic N compounds between the soil microbes and vegetation regulate tree N uptake in N-limited forests. There is now ample evidence that trees—including European Beech—successfully compete for organic N as well as for mineral N with free living microorganisms in a wide range of forest ecosystems with or without the help of mycorrhizal fungal symbionts [5,15,16,18]. Hence, trees can actively influence soil microbial N turnover processes by competing for the same substrates at several stages of the N cycle: (1) Trees may take up monomeric organic compounds such as amino acids thus limiting this substrate for microbial uptake of dissolved organic nitrogen (DON) and microbial ammonification. (2) Trees may take up ammonium ( $\text{NH}_4^+$ ) thus reducing substrate availability for autotrophic nitrification and microbial immobilisation. (3) Trees may take up nitrate ( $\text{NO}_3^-$ ) thus limiting microbial  $\text{NO}_3^-$  utilization [5,18,19–22]. Furthermore, trees influence microbial N turnover in soil by the determination of organic matter composition via plant residues, thus

influencing substrate quality for N mineralization [20] and by direct carbon (C) allocation to microorganisms via root exudation [5,16,20].

Surprisingly, the recognition of the importance of plant-microbe interactions for gross N turnover has only rarely been reflected in experimental designs for quantification of soil or plant N turnover. In contrast, soil scientists usually determine gross N turnover rates in plant-free disturbed soil, and plant physiologists frequently deploy N uptake measurements with washed roots in absence of soil and soil microorganisms [23]. Such experimental approaches neglect plant-soil-microbe C/N interactions such as plant-microbe competition for N, microbial resupply of plant nutrients and plant rhizodeposition effects on microbial soil N turnover [5]. Hence it may be questioned if N flux rates determined based on isolated views of the plant or soil compartments are realistic. Studies interlinking both sides are less common and have been performed aiming at elucidating plant-microbial competition for N in different ecosystems like tundra [24], alpine meadow [25], grassland [26–30] and temperate forests [31–36]. However, these studies almost exclusively performed simple tracing experiments aiming at the determination of  $^{15}\text{N}$  recovery rates in plant and microbial N pools rather than providing actual N turnover rates. Studies characterizing the competition for N between plants and microorganisms in a process-oriented way, i.e., by determining simultaneously occurring rates of microbial N turnover in soil and N uptake by plants are still very rare [5,22,37].

Thus, our goals were (1) to explore the extent of potential species range shifts of European Beech on marginal calcareous soils in the 21<sup>st</sup> century and (2) to test whether there is a soil microbial mechanism limiting beech seedling N nutrition and establishment and thus competitive strength under reduced water availability in a changing climate, potentially contributing to expected species range shifts on top of plant physiological constraints. Therefore, we conducted statistical species range modelling for European beech forests on calcareous soil for present day conditions and for the year 2080. In view of the importance of seedling establishment for species range shifts we assessed beech seedling performance under exposure to climate change conditions in a field experiment, thereby considering soil microbial community composition, gross rates of microbial N turnover, soil N availability and N nutrition of beech seedlings. For this purpose, we deployed an enriched stable isotope-based experimental approach to simultaneously quantify all major N turnover processes in intact beech seedling-soil-microbe systems, thereby maintaining plant-soil microbe interactions and competition for N. By translocation of soil-mesocosms containing natural beech regeneration across a narrow valley from the northwest (NW, cool moist microclimate) to the southwest (SW, warm-dry microclimate) aspect, we combined this approach with a space-for-time climate change experiment. In addition to exposure-induced reduction of soil water availability, a roof system accelerated drought during a 39 days period at the SW aspect. Supporting measurements included abundance of genes related to ammonia oxidation, mycorrhizal colonization and N metabolite levels in fine roots as well as plant biomass and isotope recovery in beech seedlings three months after  $^{15}\text{N}$  isotope labelling.

## Materials and Methods

### Statistical species distribution model

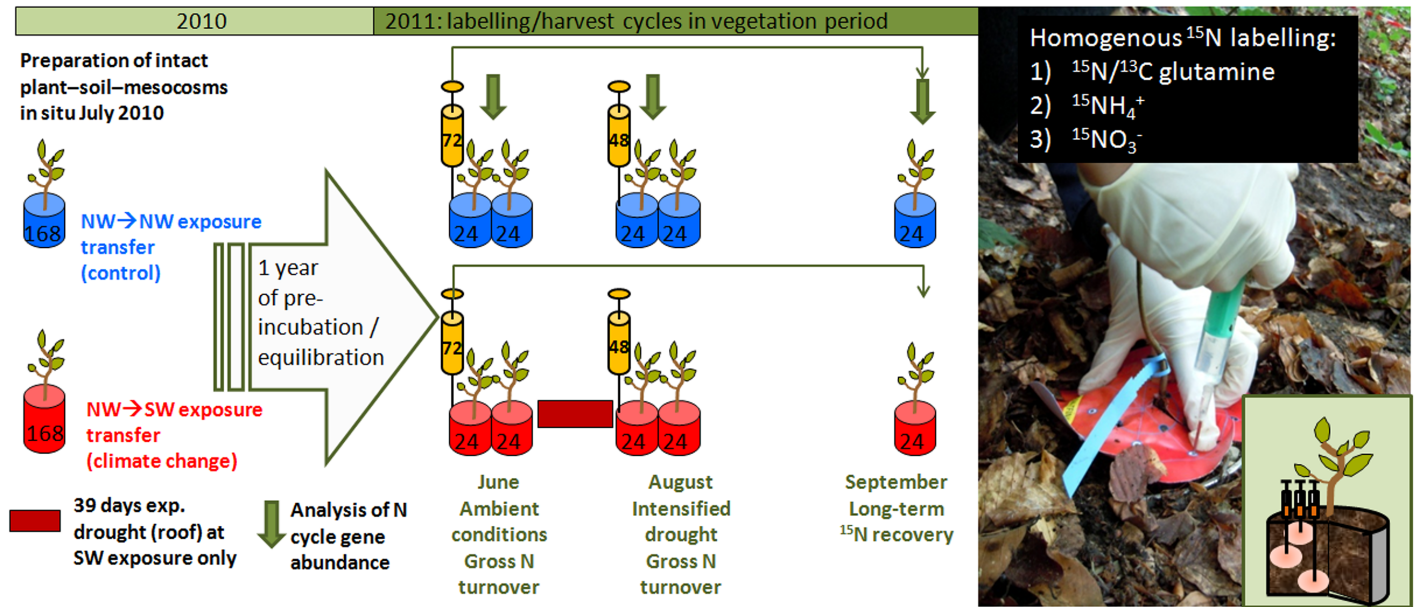
For the species distribution model we used presence/absence information for European beech derived from an international monitoring network (ICP level I—more than 8000 plots with 1097 presence observations of European beech) as response variable that we combined with high-resolution derivations of precipitation and temperature from the WorldClim- database, which implies modelling the realized niche of the species. For the projection under future conditions, we used output from the global circulation model HADCM3 driven by the SRES

scenario A2 [38] until the year 2080, which was calibrated and statistically downscaled to 30-arc-second tiles. We fitted a Generalized Linear Model (GLM) with logit link function and calibrated the model by a stepwise selection using the Bayesian Information Criterion (BIC) with five bioclimatic variables entering the model including: yearly sum of degree days above 5°C, iso-thermality, a yearly drought index, sum of precipitation in the warmest quarter of the year and the precipitation of the most humid month. As the threshold value for transforming predicted probabilities into binary presence information we used Cohen's Kappa. We used the area under curve (AUC) of the receiver operating characteristic (ROC) and Cohen's Kappa to evaluate the performance of the model. Both, AUC with a value of 0.86 and Kappa with a maximum value of 0.43, indicate good performance of the model (S1 Fig). To validate the model we carried out a 10-fold cross-validation, for which we randomly split the data into a training and an evaluation dataset. The model appears to be robust with the cross validation means being very close to the model values and the variation within cross validation results being low (S1 Table). The species distribution maps for current and projected climate were intersected with a map of the Geology of Europe depicting calcareous and limestone substrates. More detailed information on the statistical species distribution modelling is provided in the Supplementary Materials and Methods.

### *In situ* climate change experiment: site description and experimental design

**The Tuttlingen experimental beech forest.** The *in situ* climate change experiment with its biogeochemical, microbiological and plant physiological field studies was based on intact plant-soil-mesocosms with beech seedlings and was conducted in a typical N-limited mountainous beech forest growing on a Rendzic Leptosol in Southern Germany (Tuttlingen experimental beech forest, 8°45'E; 47°59'N, ca. 800 m a.s.l.). Mean annual air temperature is approximately 6.5°C and the average annual rainfall amounts to 854 mm (1961–1990). The clay-rich soil is classified as Rendzic Leptosol (Skeletal) according to the International Union of Soil Sciences Working Group WRB (2007) derived from horizontally bedded limestone and marls. Soil profiles are shallow followed by weathered parent rock containing > 45% gravel and stones. Due to nutrient poor soils and low atmospheric N input, soil N cycling is characterized by competitive partitioning of N between beech and associated mycorrhiza vs. free living soil microorganisms [14–16]. Permits for the experiment were issued by the Landratsamt Tuttlingen, Germany.

**Experimental design.** The climate change treatment was established using a space-for-time approach and combined with isotope-based process studies (Fig 1). We used topographic variability as a model for climate variability, thus identifying model ecosystems for present day and future climate conditions located on opposing slopes of a narrow valley. The control site with its NW aspect and cool-moist microclimate (representing current climate conditions) is located at a distance of less than 1 km to the climate change site with its SW aspect and warm-dry microclimate, representing “future climate conditions”. Microclimate at SW exposure is characterized by increased daily maximum of air and topsoil temperatures and thus reduced water availability [14]. Therefore, the SW aspect is considered to constitute a model ecosystem with local climatic patterns equalling the climatic conditions predicted for coming decades [39]. Climate change was simulated by transferring stainless steel cylinders (15 cm height, 16.4 cm inner diameter) containing intact beech seedling-soil-microbe mesocosms of uniform size from NW exposure to SW exposure, with control transfers at the NW exposure sampling site [40].



**Fig 1. Experimental design.** The figure illustrates coring of beech-soil-mesocosms by use of stainless steel cylinders with subsequent pre-incubation for one year either under cool-moist microclimate at the coring site (NW exposure, control) or warm-dry microclimate (SW-exposure, climate change). After pre-incubation and equilibration, homogeneous labelling of the intact beech seedling-soil-microbe systems with either  $^{15}\text{N}/^{13}\text{C}$ -enriched glutamine,  $^{15}\text{N}$ -ammonium ( $\text{NH}_4^+$ ) or  $^{15}\text{N}$ -nitrate ( $\text{NO}_3^-$ ) and subsequent double harvests (6 and 48 hours after labelling) were conducted for determination of gross N turnover rates in the beech seedling-soil-microbe system in June (comparison of ambient NW vs. SW climatic conditions) and August (ambient NW conditions vs. roof-intensified drought at SW). A final sampling of mesocosms labelled in June allowed investigating long-term isotope recovery in September (three months after labelling). All three sampling dates were accompanied by determination of supporting soil and plant parameters such as abundance of microbial genes related to ammonia oxidation, mycorrhization, and plant metabolites.

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The incubation areas were in fenced zones of  $400\text{ m}^2$  either in immediate vicinity to the sampling area (NW) or at the SW slope in 1 km distance characterized by the same inclination. Both NW and SW incubation areas showed closed canopy and only beech trees were present at a distance of 50 m. After full development of leaves, photosynthetic active radiation levels at the forest floor of the research site are mainly regulated by the canopy rather than by slope exposure [3]. The simulated climate change conditions at the SW slope were intensified through a temporal (June 27, 2011–August 05, 2011) rain-out sheltering roof approximately 1 m above ground level (Fig 1). All mesocosms incubated at SW exposure were under the roof in this period.

After an equilibration period of one year, short-term-isotope-tracing-based process studies were conducted via soil  $^{15}\text{N}$  labelling followed by destructive sampling to simultaneously quantify all relevant gross N turnover processes in the plant-soil-microbe system. Labelling of beech-soil-microbe-mesocosms took place on June 22, 2011 (72 beech-soil-mesocosms at NW and SW each), to (1) compare gross N turnover between ambient conditions at SW and NW exposure based on full destructive sampling six hours and 48 hours after labelling, and (2) to investigate  $^{15}\text{N}$  recovery at the end of growing season through a final sampling in September. A second labelling campaign took place after the roof period on August 2 (48 beech-soil-mesocosms at NW and SW each), followed again by sampling six and 48 hours after labelling. The August labelling/sampling cycle allowed to compare gross N turnover between ambient conditions at NW and roof-intensified drought (39 days) conditions at SW. Isotope recovery in the plant-soil-N pools three months after labelling was investigated in a third, final sampling in September 2011 (Fig 1). At all three sampling dates, supporting soil, microbial, mycorrhizal

and plant parameters as well as abundance genes encoding for ammonia monooxygenase in soil were determined as described in the following sections.

**Coring and transfer of intact beech seedling-soil-mesocosms.** Overall, 256  $^{15}\text{N}$ -labelled and 32 unlabelled beech seedling-soil-mesocosms were analysed in this study. Coring and Transfer of beech seedling-soil-mesocosms was conducted within one week in absence of precipitation in July 2010. For coring, a suitable sampling area of 50 \* 50 m with identical slope ( $18^\circ$ ) and similar soil profile was identified. In this representative area, finer grained soil (Ah horizon) was only found in the uppermost 15–20 cm (i.e., the main rooting zone of beech natural regeneration). There was a sharp transition between this almost gravel-free clay-rich finer grained soil and the deeper gravel-dominated periglacial layers which were not suitable for coring. In this sampling area, beech natural regeneration trees of 2.2–2.5 mm stem diameter and 30–40 cm height were selected. Custom-made stainless steel cylinders (height 150 mm, outer diameter 168 mm, sharp edge at bottom, wall thickness 2 mm, open at bottom and top) were manually driven into the soil with the selected beech seedling growing in the centre. A custom-made extension tool fitting to the stainless steel cylinders facilitated coring under avoidance of damage to the aboveground part of the plant. After coring, the cylinders, containing an intact beech-soil-mesocosm, were carefully excavated under preservation of an even ending at the bottom. Excavated beech-soil-mesocosms were immediately reburied vertically either within NW or after transfer to SW. The litter layer was preserved and contained in the cylinders during coring. Pre-tests showed that the entire root system of beech seedlings of this size fitted well to the embraced volume of the used steel cylinders. Only mesocosms without visible damage or cut beech roots were used.

While reburying beech-soil-mesocosms, extreme care was taken to create a realistic transition between the bottom of the soil cores and the ambient soil. For this purpose, beech-soil-mesocosms were placed vertically in holes with a 5 cm thick horizontal bed made of homogenized NW soil and subsequently gently hammered down 2 cm at the new position. Finally, continuous contact between the litter layer on the transferred beech-soil-mesocosms and the surrounding litter layer at the new position was established. After transfer, all reburied intact beech-soil-mesocosms at both sites were irrigated with 500 ml of water over a period of two hours to avoid drying or death of enclosed beech seedlings following transfer. The amount of water corresponded to a precipitation event of  $23.7 \text{ l m}^{-2}$ , i.e., a typical summer convective rainfall event. The survival rate of transferred beech seedlings was 97%.

Ten further beech-soil-mesocosms were transferred (5 within the NW slope, 5 to the SW slope) to monitor soil temperature and soil moisture in 5 cm depth in the mesocosms ( $n = 5$  each slope) using horizontally installed combined soil moisture/temperature probes (DECA-GON EC-5, Decagon Devices, Inc., Pullman, USA) with an hourly temporal resolution.

### Isotope labelling of beech seedling-soil-mesocosms

In order to facilitate the simultaneous quantification of gross rates of all major N turnover processes in the beech-soil-microbe system, the intact soil-root-mesocosms were labelled with either  $^{15}\text{N}/^{13}\text{C}$ -enriched glutamine,  $^{15}\text{NH}_4^+$  or  $^{15}\text{NO}_3^-$ . To enable homogeneous distribution of isotopes in the root-soil system, we applied an isotope injection method developed for large soil cores [41] and optimized and adapted it for the Tuttligen soil. For this purpose, we conducted pre-experiments in March 2011 using Brilliant Blue FCF colour dye instead of  $^{15}\text{N}$  enriched label solution. Various injection patterns and depths, numbers of injections per mesocosm and solution volumes per single injection were tested to achieve homogeneous 3-dimensional distribution of label solution in the soil-root-system at minimized amounts of injected liquid to minimize label artefacts such as stimulation of N turnover and leakage of labeled

solution at the bottom of the beech-soil-mesocosms. In this pre-experiment, mesocosms were harvested one day after dye application by removing soil stepwise from bottom to top in layers of 2 cm. The distribution of the colour dye in the soil was monitored visually. Outflow at the bottom of the labelled beech-soil-mesocosms was examined by storing the beech-soil-mesocosms on white paper sheets. To inject dye and later  $^{15}\text{N}$  solution we used custom-made stainless steel side port cannulas with lasered depth check marks. In this labelling optimization experiment the following setup facilitated homogeneous colour dye distribution with only minimal leakage: 16 single amounts of 3 ml solution each were injected into the beech seedling-soil-mesocosms to a depth of 1 and 3 cm each, and another 8 injections of 3 ml each to a depth of 6 cm. Additionally, 10 ml of  $^{15}\text{N}$ -labelled solution were homogeneously spread on top of the soil surface. Hence, the total amount of added label solution was 130 ml, applied to 2.3 kg dry soil contained on average in the beech-soil-mesocosms. Paper calibres indicating injection patterns and depths were constructed to fit onto the stainless steel cylinders in order to ensure reproducible labelling. Before  $^{15}\text{N}$ -labelling, the litter layer was removed from the top of the soil surface and later replaced. An earlier study showed that the litter layer is of very minor significance for N nutrition of beech natural regeneration [42].

Labelling was conducted simultaneously at NW and SW starting at 5:00 am and took 3 hours (June) and 2 hours (August). The label solution always contained all three N compounds used in this experiment, i.e.,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and glutamine, and depending on the labelling treatment, either  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  or glutamine was isotopically enriched (50 atom %  $^{15}\text{N}$ ). Glutamine was also enriched in  $^{13}\text{C}$  (50 atom%)  $^{13}\text{C}$  for glutamine). Glutamine was chosen as an organic N compound because earlier studies showed high glutamine uptake capacities of and abundance in beech seedlings and adult beech trees at this site [15,16]. The amount of N added via label solution equalled to 3.5 mg  $\text{NH}_4^+$ -N  $\text{kg}^{-1}$  soil dry weight (sdw), 3.5 mg  $\text{NO}_3^-$ -N  $\text{kg}^{-1}$  sdw, and 0.7 mg glutamine-N  $\text{kg}^{-1}$  sdw. Soil  $\text{NH}_4^+$  background concentrations in unlabeled soil were hardly different between NW and SW. However, background soil  $\text{NO}_3^-$  concentrations were generally much smaller at SW than at NW. Therefore, the ratio of added  $\text{NO}_3^-$ -N via label solution to background soil  $\text{NO}_3^-$  was larger for SW than for NW. In June, N added via label solution equalled to 76% and 80% of ambient  $\text{NH}_4^+$  concentrations and 35% and 78% of ambient  $\text{NO}_3^-$  concentrations at NW and SW, respectively. In August, this equalled 84% and 114% of ambient soil  $\text{NH}_4^+$  concentrations. However,  $\text{NO}_3^-$  levels were extremely low at SW, so that added label equalled to 211% and 762% of ambient  $\text{NO}_3^-$  concentrations at NW and SW, respectively. Hence, several-fold reduced soil  $\text{NO}_3^-$  levels were observed both in unlabelled mesocosms and in labelled mesocosms of the climate change treatment.

Mean total  $^{15}\text{N}$  isotope recovery (sum of extractable and non-extractable soil N pools plus plant N pools) six hours after isotope application was  $74\pm 4\%$  (standard error of the mean) after  $^{15}\text{N}$  glutamine labelling,  $86\pm 5\%$  after  $^{15}\text{NH}_4^+$  labelling, and  $61\pm 4\%$  after  $^{15}\text{NO}_3^-$  labelling.

## Beech-soil-mesocosm harvest and sample preparation

Eight intact beech-soil-mesocosms were harvested every sampling point for each of the three N-compound labelling treatments and each of the two slopes (NW exposure, control treatment and SW exposure, climate change treatment). Consequently, 48 beech-soil-mesocosms each were sampled on June 22, 2011 (6 hours after labelling), on June 24, 2011 (48 hours after the labelling), on August 2, 2011 (6 hours after labelling) on August 4 (48 hours after labelling) and on September 27, 2011 (3 months after labelling). The harvests on June 22–24, August 2–4 and September 27 were accompanied by additional sampling of unlabelled beech-soil-mesocosms to analyse ambient background inorganic N concentrations and isotopic natural abundance of investigated pools with 4–8 replicates per exposure.

For harvesting, beech-soil-mesocosms were excavated under maintenance of an even ending at the bottom and entirely harvested within two hours after excavation. The beech seedling was cut and further processed as described below. The remaining soil/root system was carefully separated by hand into soil, gravel, dead coarse organic material and living fine and coarse roots. Additionally, mycorrhizal root tips and a subsample of rhizosphere soil (defined as soil adhering to root after vigorous shaking) were sampled and further processed as described below.

The soil contained in each mesocosm was immediately homogenized by manual mixing for 10 minutes to assure full mixing to a homogeneous sample. All soil extraction steps for analysis of N compounds and their  $^{15}\text{N}$  enrichment in soil were immediately conducted during harvest in the field lab with field fresh soil (see below). A subsample of ca. 100g soil was air dried. For nucleic acid analysis, other subsamples of bulk soil as well as the rhizosphere soil were immediately frozen at  $-80^{\circ}\text{C}$ .

Fresh weight of the entire soil contained in the beech-soil-mesocosms and the weight of the stainless steel cylinders were recorded. Gravimetric soil water content was determined with a subsample of approximately 400-500g of soil by drying at  $105^{\circ}\text{C}$  until constant weight.

During each harvest, beech seedlings were carefully removed from mesocosms and separated into leaves, stems, coarse ( $>2$  mm diameter) and fine roots ( $<2$  mm diameter). Remaining adhering small soil was carefully washed from the roots and dried. The fresh weight of each plant tissue was determined. Samples were dried at  $60^{\circ}\text{C}$  until constant weight. After determination of the fresh weight, fine root samples were separated into two parts for subsequent analyses: one part for ectomycorrhizal analyses was wrapped in wet tissue and stored in plastic bags at  $4^{\circ}\text{C}$  until return to the laboratory, the second part was dried at  $60^{\circ}\text{C}$  for 2 days for later EA-IRMS analyses. For biochemical analyses of N metabolites in fine roots, additional samples were taken from unlabelled beech-soil-mesocosms on June 22 and shock-frozen in liquid N until sample analyses to avoid degradation of the metabolites.

## Soil analyses

**Total organic carbon (C), total C and N and  $\delta^{15}\text{N}$  in bulk soil.** Soil samples were immediately frozen and freeze-dried after sampling. After removing roots and gravel using tweezers, bulk soil samples were finely ground. Determination of total soil C, N and  $\delta^{15}\text{N}$  was performed in duplicate for every sample with an isotope ratio mass spectrometer (Delta V, Thermo Electron Corporation, Dreieich, Germany) coupled to an elemental analyser (Euro EA, Eurovector, Milan, Italy) at the Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Soil Ecology in Neuherberg. The  $^{15}\text{N}$  atom% excess enrichment was calculated by subtracting natural abundance values gained by the unlabelled mesocosms from the values obtained of the labelled soil-mesocosms.

**Analyses of extractable soil N pools.** A representative subsample of 100 g of homogenized soil out of every beech-soil-mesocosm was immediately extracted in the field with  $0.5\text{ M K}_2\text{SO}_4$  at a soil:solution ratio of 1:1.5. Addition of  $\text{K}_2\text{SO}_4$  solution was followed by 1 hour of shaking at 240 rotations per minute. After shaking, extracts were vacuum filtered using pumps and glass fibre filters [15]. Subsamples of the extract were transferred into different tubes and immediately frozen in the field for later analysis of (1) total organic C and total N concentrations, (2)  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations, (3)  $^{15}\text{N}$  enrichment in  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and dissolved organic N (DON), and (4)  $^{15}\text{N}$  enrichment in total dissolved (organic + mineral) N.

A second soil subsample was not immediately extracted, but first underwent a chloroform-fumigation over 24 hours as described in detail in an earlier publication [15] and was extracted afterwards as described above with subsequent freezing of the extract for later analysis of (1) total N (organic + organic) and (2)  $\delta^{15}\text{N}$  in total N. Ammonium and  $\text{NO}_3^-$  concentrations in



extracts were analysed colorimetrically by a commercial laboratory (Dr. Janssen, Gillersheim, Germany). Total organic C (TOC) and total N (TN) in extracts were quantified using an Infra-red TOC analyser with a coupled chemoluminescence-based total N module (DIMATEC GmbH, Germany) [15]. Dissolved organic N was calculated as the difference between total N and mineral N in extracts.

The  $^{15}\text{N}$  enrichment in soil  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and DON was quantified by sequential diffusion steps, based on conversion of the target compounds into  $\text{NH}_4^+$ , pH increase to induce volatilization as  $\text{NH}_3$ , and subsequent trapping of  $\text{NH}_3$  on acid traps prepared for isotope ratio mass spectrometry (IRMS) at the Center of Stable Isotopes of KIT-IMK-IFU as described in detail in earlier publications [41,42]. Microbial biomass N and  $^{15}\text{N}$  were quantified following the chloroform-fumigation extraction approach as described in detail in earlier publications [41,42]. No conversion factors ( $k_{\text{EN}}$ ) were used to render estimates of rather the active part of microbial biomass and newly immobilised N [42]. Total non-extractable soil N and the respective  $^{15}\text{N}$  recovery were calculated as the difference between total soil N and all extractable N compounds (i.e.,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , DON and microbial biomass N).

## Molecular analysis of ammonia monooxygenase gene abundance

**Nucleic acid extraction from bulk soil and rhizosphere soil.** DNA was extracted from 0.4 g bulk soil and 0.1 g rhizosphere soil, respectively, using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Heidelberg, Germany) and the Precellys 24 Instrument (Bertin Technologies, Montigny-le-Bretonneux, France). Quantity and quality of the extracted DNA were checked with a spectrophotometer (Nanodrop, PeqLab, Erlangen, Germany) and gel electrophoresis. The extracts were stored at  $-80^\circ\text{C}$  until real-time PCR analysis.

**Quantitative real-time PCR assay to quantify marker genes for ammonia oxidation.** Quantitative real-time PCR (qPCR) was performed using an ABI 7300 Cycloer (Life Technologies, Darmstadt, Germany) with the following assay reagents: dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) (Sigma Aldrich, Taufkirchen, Germany), primers listed in [S2 Table](#) (Metabion, Martinsried, Germany) and 2x Power SYBR Green master mix (Life Technologies, Darmstadt, Germany). The respective reaction mixtures (total volume 25  $\mu\text{l}$ ) for the quantification of archaeal (AOA) and bacterial (AOB) genes encoding for ammonia monooxygenase ([S2 Table](#)) consisted of: 12.5  $\mu\text{l}$  SYBR Green master mix, 5 pmol of each primer, 0.5  $\mu\text{l}$  3% BSA and 2  $\mu\text{l}$  DNA template.

For quantification, standard curves were calculated using serial dilutions ( $10^1$  to  $10^6$  gene copies  $\mu\text{l}^{-1}$ ) of plasmid DNA containing PCR products of the respective genes ([S2 Table](#)). According to manufacturer's instruction, the PCR detection limit was assessed to 10 gene copies. In advance, the optimal dilution for each amplification assay was determined by dilution series of randomly chosen DNA extracts, in order to prevent PCR inhibition. The qPCR assays were performed in 96-well plates (Life Technologies, Darmstadt, Germany) for all target genes ([S2 Table](#)). All PCR runs began with a hot start at  $95^\circ\text{C}$  for 10 minutes. After each run, the specificity of the SYBR Green-quantified amplicons was checked by melting curve analysis and gel electrophoresis. The amplification efficiency was calculated from the formula  $\text{Eff} = [10^{(-1/\text{slope})} - 1]$  and resulted in the following average efficiencies (standard deviation less than 5% of mean) for the different genes: AOA, 89%, AOB, 97%.

## Analysis of ectomycorrhizal colonization

During harvest in the field laboratory, precleaned fine root samples were placed in tap water filled petri dishes under a dissecting microscope (Stemi SV 11; Zeiss, Jena Germany) and were thoroughly cleaned from adhering soil particles using fine forceps.

Back in the laboratory randomly chosen, 2–3 cm long parts of the root system were used for examination under a dissecting microscope (205 FA; Leica, Wetzlar, Germany) and processed according to the method of Pena et al. [43]. In each sample, 300 vital root tips were counted recording simultaneously the number of dead root tips. Occasionally, samples contained less than 300 living root tips. The root tips were classified as mycorrhizal, non mycorrhizal and dry root tips.

Samples of ectomycorrhizal root tips were collected for isotope measurements. For determination of C and N content as well as  $^{15}\text{N}$  and  $^{13}\text{C}$  enrichment, root tips were dried for three days at 60°C. 1–5 mg of root tip material was weighted in 5 x 9 mm tin capsules (IVA Analyse-technik, Meerbusch, Germany) with a micro balance (Supermicro S4; Sartorius, Göttingen, Germany). Measurements were conducted at the service unit KOSI (Kompetenzzentrum für Stabile Isotope, University Göttingen, Germany) using an isotope ratio mass spectrometer (IRMS Delta Plus, Finnigan MAT, Bremen, Germany) coupled via an interface (Conflo III, Finnigan MAT, Bremen, Germany) to an elemental analyser (NA1110, CE-Instruments, Rodano, Milano, Italy). Acet anilide was used as standard, IAEA N1 ( $\delta^{15}\text{N}_{\text{air}}$  0.4 ‰) und  $\text{N}_2$  ( $\delta^{15}\text{N}_{\text{air}}$  20.3 ‰) for N calibrations and IAEA 600 ( $\delta^{13}\text{C}_{\text{VPDB}}$  -27.7 ‰) for C calibrations.

## Analysis of plant tissues

**Total N,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in beech tissues.** To determine total N, as well  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  notation in plant tissues, oven-dried (48h, 60°C) samples were ground to a fine powder using a ball mill (Retsch MM 100, Retsch GmbH, Haan, Germany) for leaves and fine roots, and liquid N for stems and coarse roots. Aliquots of 1.0–2.5 mg for each tissue were weighed into tin capsules (IVA Analysentechnik, Meerbusch, Germany). Samples were analysed using an elemental analyser (Vario EL, elementar Analysensysteme GmbH, Hanau, Germany) coupled via an interface (Conflow III, Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus, Thermo Finnigan MAT GmbH, Bremen, Germany). Working standards (glutamic acid), calibrated against primary standards USGS 40 (glutamic acid  $\delta^{13}\text{C}_{\text{CPDB}}$  = -26.39) and USGS 41 ( $\delta^{13}\text{C}_{\text{CPDB}}$  = 37.63) for  $\delta^{13}\text{C}$  and USGS 41 ( $\delta^{15}\text{N}_{\text{air}}$  = 47.600) for  $\delta^{15}\text{N}$ , were analysed after every twelfth sample to enable correction for the drift of isotopic analyses over time if required.

**Metabolites: quantification total soluble protein, total amino acids and  $\text{NO}_3^-$  in the fine roots of beech seedlings.** For biochemical analyses of N metabolites in the fine roots of beech seedlings, only fine roots from unlabelled samples were used from three time points (see above). Total amino acids in the fine roots were extracted [44] from frozen homogeneous fine root material (~ 50mg) in 1 mL methanol:chloroform (3.5:1.5, v:v) and 0.2 mL buffer (pH 7.0) containing 20 mM Hepes, 5 mM EGTA and 10 mM NaF. Total amino acid concentration was quantified photometrically (Beckman Coulter Inc., Fullerton, CA, USA) at 570 nm as described by Liu et al. [45] using the colour reaction with ninhydrin reagent. Glutamine was used as a standard (Sigma, Hamburg, Germany). Total soluble proteins were extracted from (~ 50mg) frozen and ground fine root material in 1 mL buffer (1 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 15% glycerol (v:v), 5 mM dithiothreitol (DTT) and 0.1% Triton-X 100) as previously described in detail [15]. Concentrations of total soluble protein were quantified photometrically at 595 nm using Bradford reagent (Ameresco Inc., Solon, Ohio, USA) in a UV-DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). Bovine serum albumine (BSA A-6918, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) was used as standard. Nitrate was extracted from c. 50 mg tissue in 0.1 g washed polyvinylpyrrolidone (PVP Sigma-Aldrich Inc., Steinheim, Germany) [15]. Nitrate concentrations were determined using an ion chromatograph (DX 120, Dionex, Idstein, Germany) combined

with an autosampler (AS 3500, Thermo Separation Products, Piscataway, USA) and equipped with the PeakNet software package (version 4.3, Dionex, Idstein, Germany). Nitrate, phosphate, and sulphate were used as standards.

### Calculation of N pools, isotope recovery and gross rates of N turnover

Measurements of N pool size in soil and plant as well as isotopic information of plant, soil organic, inorganic, microbial and mycorrhizal N pools was used to calculate (1) gross rates of ammonification and nitrification using isotope pool dilution approaches based on the data gained 6 hours and 48 hours after  $^{15}\text{N}$  labelling, (2) plant and microbial uptake of glutamine,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  N using  $^{15}\text{N}$  tracing approaches for the first 6 hours after  $^{15}\text{N}$  labelling, and (3)  $^{15}\text{N}$  recovery in the investigated N pools. Formulas and further details are provided in the Supplementary Materials and Methods.

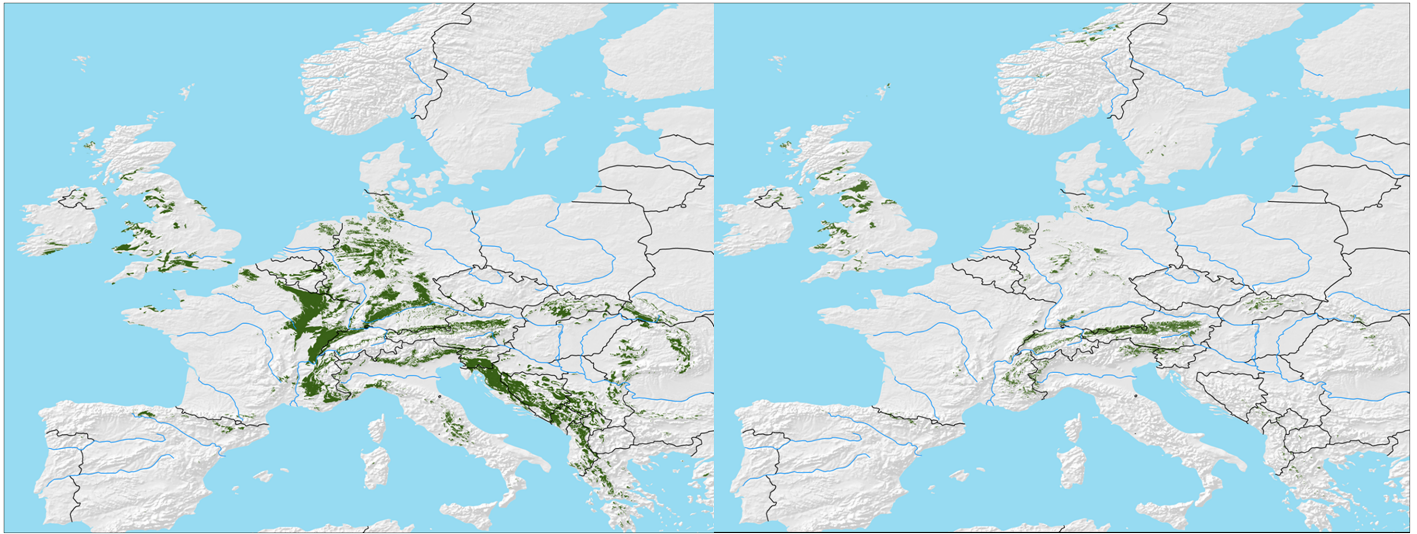
### Statistics

Experimental data are shown as mean values with standard errors (SE) of the mean, if not otherwise stated. Data were log-transformed if necessary to meet the requirement of normality and subsequently analysed using two-way ANOVA with the factors exposure (NW versus SW exposure, i.e., control and climate change treatments), time and their interaction. Durbin-Watson test statistics revealed absence of autocorrelation between time points. Depending on the parameters investigated, the factor time has different levels with independent observations, since samples were taken from different beech-seedling-soil-mesocosms. There were two levels for gross rates of N turnover (June, September), three levels for N pools and abundance of microbial ammonia monooxygenase genes (June, August, September) and five levels for isotopic composition of C and N pools (June\_6 hours after labelling; June\_48 hours after isotope labelling; August\_6 hours after isotope labelling; August\_48 hours after isotope labelling and September\_3 months after isotope labelling). Differences between the levels of the time factor within each treatment level were further tested by applying the Tukey post-hoc test. For plant metabolites in fine roots, single comparisons between SW and NW were performed with non-parametric U-tests due to smaller number of replicates.

## Results

### Potential species range distribution modelling for beech on calcareous soil

Based on statistical species distribution models driven by climatic predictors [46,47], we computed the distribution range in the form of the realized niche of beech forests on calcareous soil in Europe. For present day conditions, the modeled potential species range distribution of European beech (Fig 2, left panel) reflected well the distribution of limestone bedrock geology. East of the Rhine rift valley, distribution patterns followed the Jurassic limestone mountain ranges along the Swabian and Franconian Cuesta Mountains from SW to SE Germany. West of the Rhine valley, there was a continuation of distribution patterns following the similar Jurassic bedrock geology towards the edge of the Paris-Seine Basin. The Jurassic mountain ranges along the border between France and Switzerland were identified to be another important potential distribution area. The Southern distribution limit was ranging from the Cantabrian Mountains and Pyrenees in Spain to the Provence in Southern France, the calcareous Apennine Mountains in Central Italy to the Balkan Mountains and the mountainous regions of Greece.



**Fig 2. Modelled potential distribution of beech forests on calcareous soils in Europe (green colour) under current climatic conditions (left panel).** I.e., For the SRES A2 scenario, we computed a potential distribution of 7.2 million ha in the year 2080 (right panel), i.e., a reduction to 22% of the current distribution. Made with Natural Earth under CC0 license.

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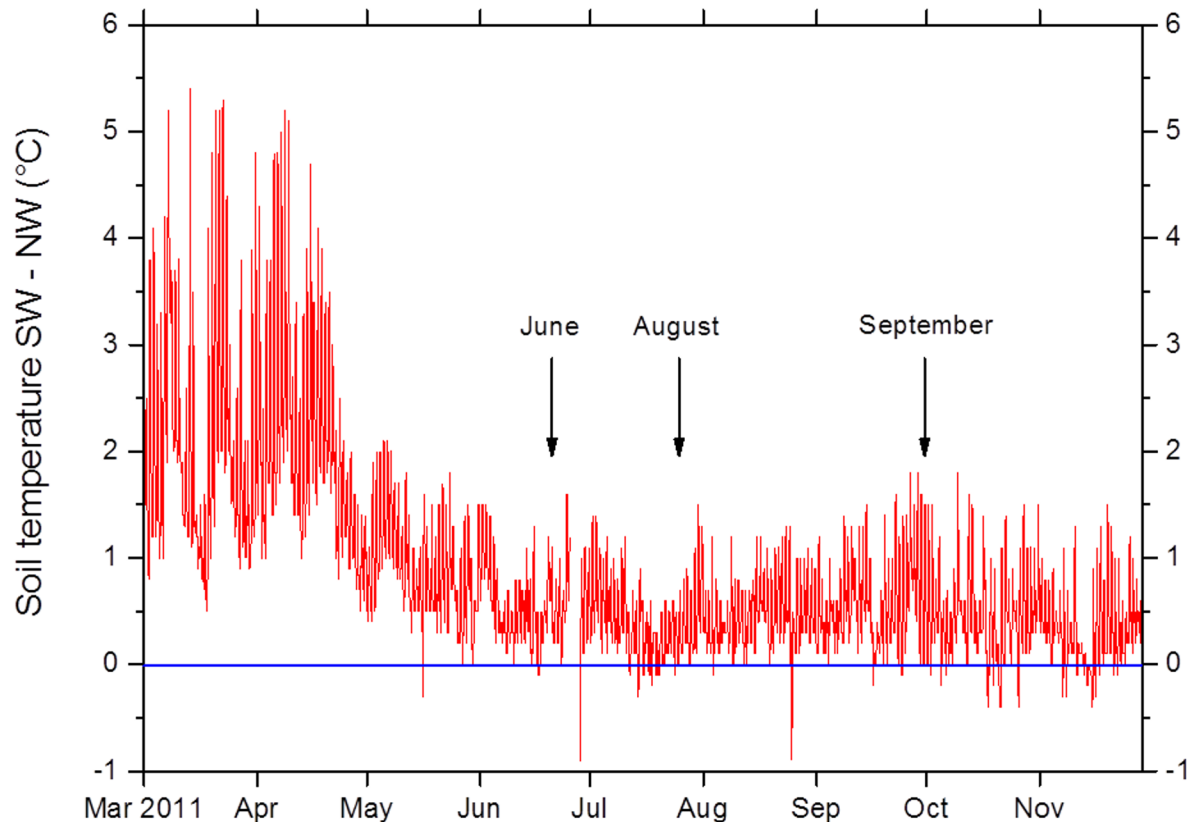
Overall these forests would cover an area of 31.4 million ha, i.e., approximately one third of the current potential beech forests in Europe (104.0 million ha), [47]. For the SRES A2 scenario [38] (Fig 2, right panel) we computed a potential distribution of 7.2 million ha in the year 2080, i.e., a drastic reduction to 22% of the current distribution. In this scenario, potential beech species range distribution is mainly reduced to Alpine regions and Great Britain with only residual spots in Northern and SW Germany (Fig 2, right panel).

### *In situ* climate change experiment

In order to evaluate whether N limitation could be a driver of the drought sensitivity of European beech, gross N turnover in intact beech seedling-soil-microbe-mesocosms was determined in the frame of a space-for-time translocation climate change experiment. Transfer from NW to SW increased soil temperature on average by 1°C (Fig 3) and—amplified by the roof—persistently decreased soil volumetric water content over the entire growing season (Fig 4). Also gravimetric water content was persistently lower in harvested mesocosms of the SW than of the NW treatments irrespective of addition of <sup>15</sup>N label solution (Fig 5).

Ammonification, nitrification and microbial immobilisation of inorganic N were approximately one order of magnitude larger than plant uptake (Fig 6). Microbial biomass N was several times larger than the plant N pool (Fig 6). Extractable soil NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> pools were in the magnitude of daily rates of gross inorganic N turnover. Nitrate was the dominant N source for beech seedlings in June, followed by NH<sub>4</sub><sup>+</sup>, whereas organic N supplied as glutamine was hardly recovered (Fig 6). Plant N uptake was generally larger in June than in August (Fig 6).

Considering data from all sampling dates in our statistical model showed that transfer to SW increased the soil DON concentrations but decreased NH<sub>4</sub><sup>+</sup> availability, indicating impaired mineralization-immobilisation turnover with also reduced plant NH<sub>4</sub><sup>+</sup> uptake (Table 1, Fig 6). While the N cycle in soil of the NW control treatment was characterized by high nitrification rates, transfer to SW exposure resulted in a five-fold decline of gross nitrification and soil NO<sub>3</sub><sup>-</sup> concentrations already in June (Fig 6). Consequently, plant NO<sub>3</sub><sup>-</sup> uptake



**Fig 3. Soil temperature differences (5 cm depth) between beech-soil-mesocosms incubated at SW exposure (warm-dry microclimate, climate change treatment) and at NW exposure (cool-moist microclimate, control treatment).** Data represent mean values of five temperature probes per treatment directly installed horizontally in soil of transferred beech-soil-mesocosms. Arrows indicate the three sampling campaigns. The period between the sampling in June and August equals the roof period of 39 days.

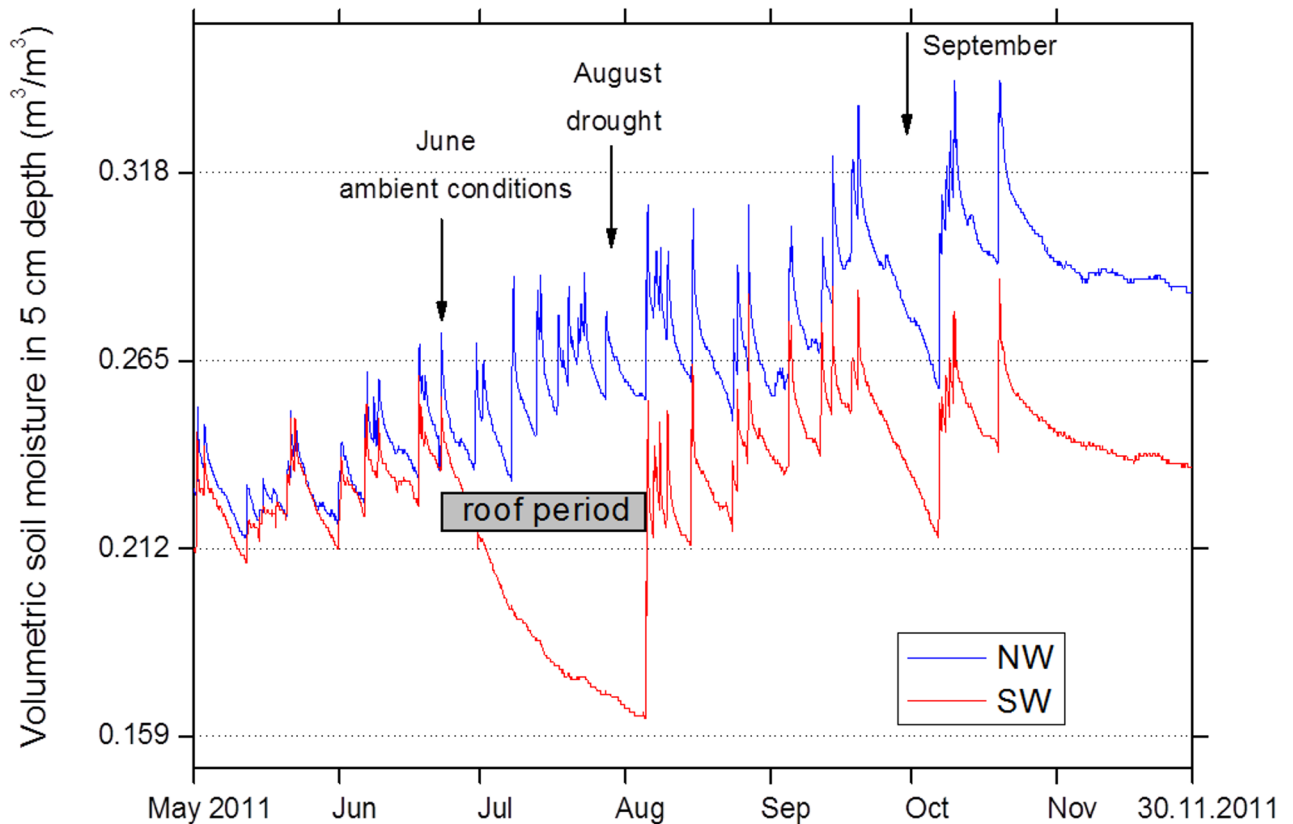
doi:10.1371/journal.pone.0158823.g003

was also reduced by a factor of five (Fig 6), although mycorrhizal colonization of vital roots remained unaltered with values close to 100% in both exposures (Table 2).

In conjunction with decreased  $\text{NH}_4^+$  uptake, this resulted in a significantly diminished plant N pool (Fig 6). From June to August, plant biomass N increased significantly at NW but only marginally at SW under drought conditions (Fig 6). Plant N uptake calculated from short-term  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$  tracing in June and August corresponded well with N uptake calculated from the plant N increment between June and August, with both approaches clearly showing a severely reduced uptake for SW (Table 3). In August, rates of N turnover and plant uptake were generally less than in June both at NW and SW with similar but less pronounced climate change treatment effects on N cycling as observed in August (Fig 6).

The observed effects of the climate change treatment on N processes (Table 1) in the plant-soil interface were confirmed by a range of supporting data. Concurrent analyses of microbial communities involved in selected processes of the N cycle revealed a pronounced reduction of ammonia oxidizing bacteria (AOB) in bulk soil in June and September and in the rhizosphere in August (Fig 7, S3 Table). Gross nitrification rates were strongly positively correlated with the abundance of AOB in soil (Fig 7).

Reduced N uptake by beech seedlings of the climate change treatment is also supported by persistently higher  $^{15}\text{N}$  enrichment in mycorrhizal root tips grown in NW exposure than in SW exposure, irrespective of  $^{15}\text{N}$  labelled compound (S4 Table). Furthermore,  $^{15}\text{N}$  recovery in



**Fig 4. Dynamics of volumetric soil moisture in 5 cm depth (mean values of  $n = 5$  measurements) in intact beech-soil-mesocosms of the control treatment (NW exposure, cool-moist microclimate) and climate change treatment (SW exposure, warm-dry microclimate) in the growing season 2011, i.e., 1 year after implementation of treatments by transferring beech seedling-soil-mesocosms within NW exposure or to SW exposure in summer 2010. Arrows indicate sampling campaigns (see Fig 1).**

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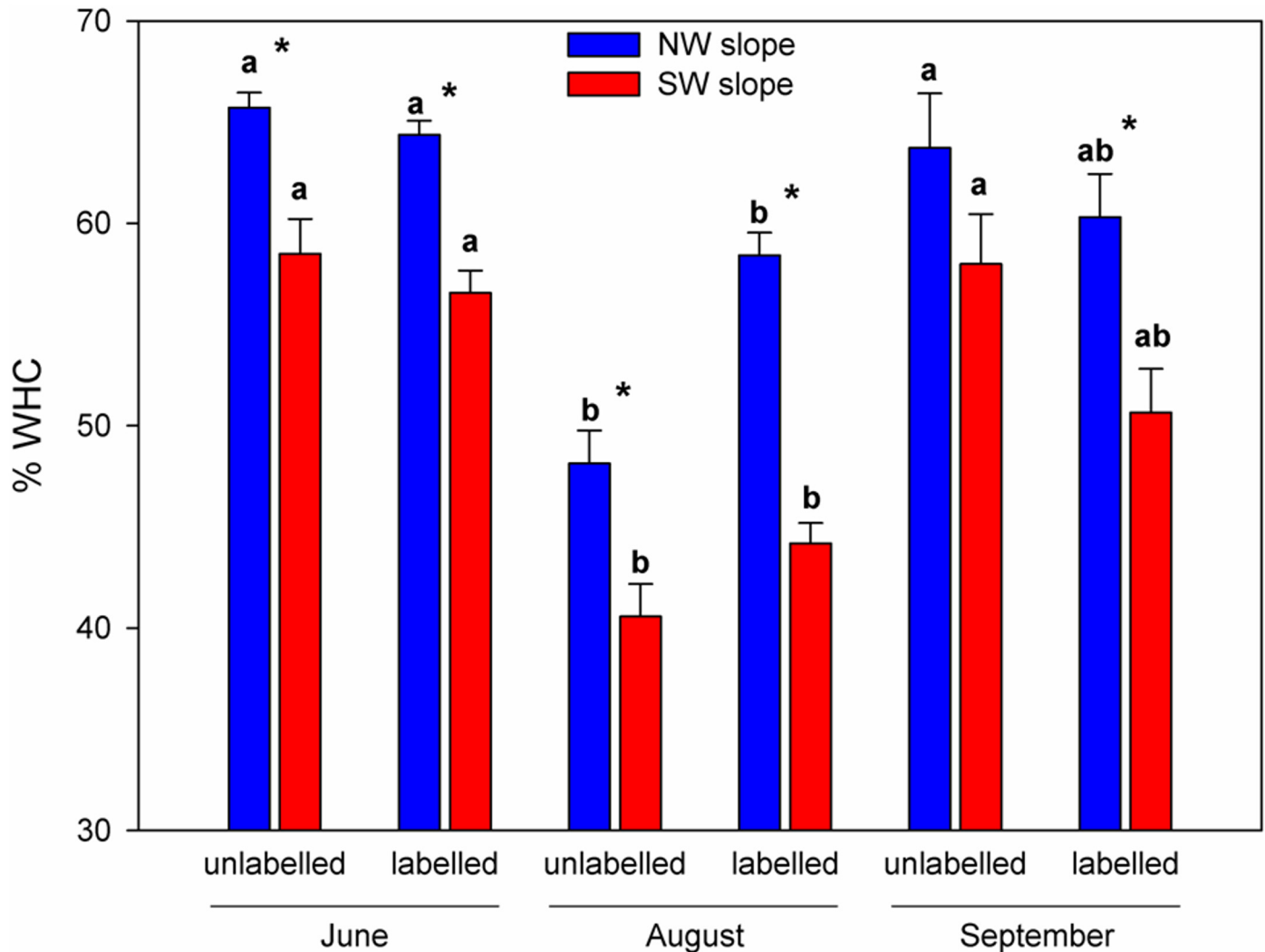
beech seedlings as determined in September, i.e., three months after <sup>15</sup>N labelling was persistently smaller at SW than at NW regardless of the <sup>15</sup>N source provided (Fig 8). Moreover, levels of N-bearing metabolites in fine roots of beech seedlings of the climate change treatment were reduced (Fig 9). Finally, above- and belowground biomass of beech seedlings were persistently smaller in SW than in NW beech-soil-mesocosms (Fig 6, S5 Table).

## Discussion

### Experimental and modelled climatic change

The statistical species range distribution modelling indicated a dramatic biome shift of European beech on calcareous soils, i.e., a reduction of almost 80% of the realized niche of beech in the year 2080. This approach is based on relationships to climate indicators and is—in contrast to process-based ecosystem models—neither based on physiological nor on biogeochemical processes. Furthermore, such predictions of the suitable growth area can underestimate the adaptive capacities of tree species [47]. Evidence for adaption of beech to drought conditions was observed close to their dry distribution limit [17].

Compared with the SRES A2 scenario used for the statistical modelling, the *in situ* space-for-time climate change experiment represents a smaller effect of climatic change. The NW exposure corresponds to a model climate for present day conditions of many beech forests in Central Europe, while the SW exposure is considered a model for climatic conditions expected

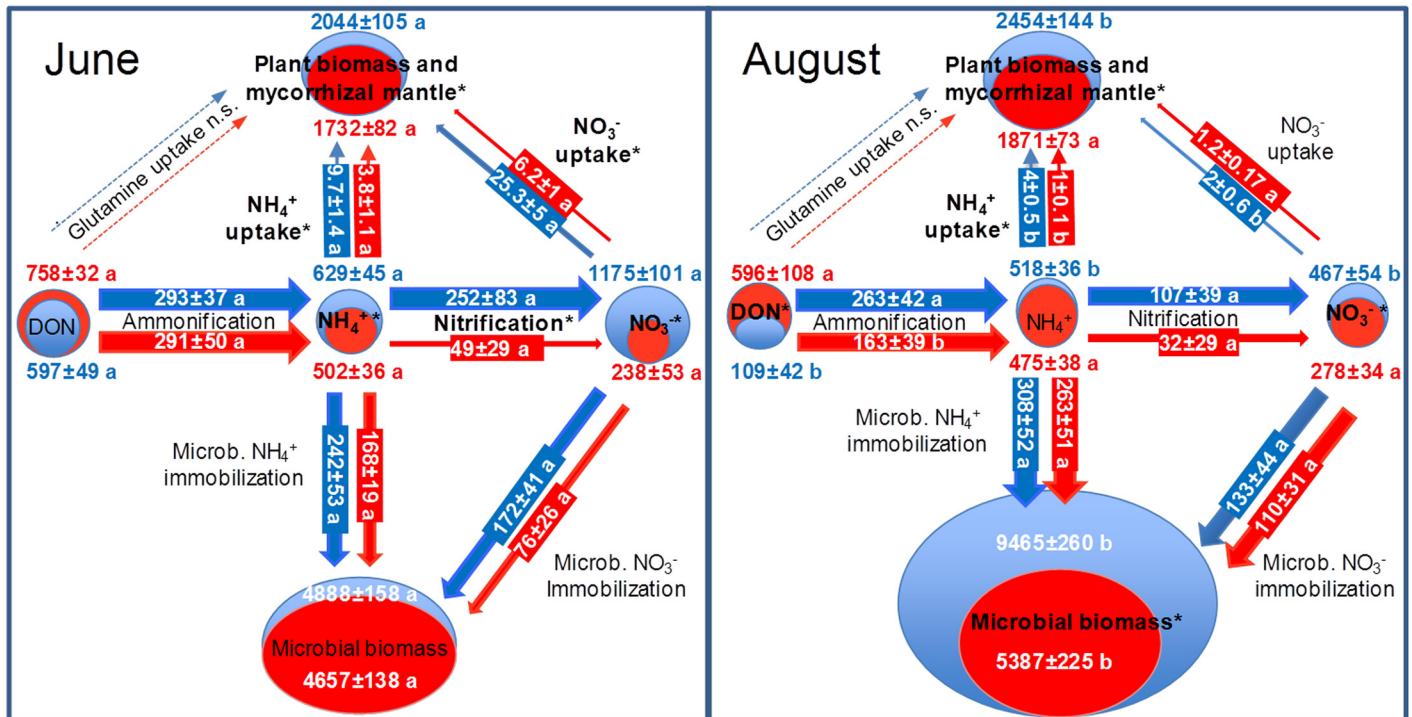


**Fig 5. Gravimetric soil moisture related to water holding capacity (WHC) as determined from labelled (n = 48) and unlabelled (n = 4 to 8) beech-soil-mesocosms in June (ambient conditions at both exposures), August (intensified drought at SW exposure due to roof) and September (final harvest).** Asterisks indicate significant differences (p<0.05) between NW and SW exposure at the respective harvest. Different indices indicate significant differences between different sampling dates and labelled and unlabelled beech-soil-mesocosms.

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in the target region for the following decades in the first half of the 21st century [48]. The differences in temperature across the valley of approx. 1°C correspond to expected climatic changes between the periods 1971–2000 and 2021–2050 [12,48]. Such a warming would correspond to an SRES B1 scenario, which has to be considered as very optimistic given the actual level of CO<sub>2</sub> emissions [49]. We therefore used the A2 scenario as a framework for a potential development of the area of beech under climate change and rather difficult site conditions.

The fast and clear changes in N biogeochemistry and N nutrition observed here due to translocation from NW to SW exposure raise questions regarding the extent the beech stands in the investigated valley were affected by aspect in the past decades. Indeed these two beech stands—planted at both sides of the valley 80 years ago—are characterized by marked differences with 35% reduced basal area, 5 m less tree height and a timber volume of 236 rather than 349 solid m<sup>3</sup> ha<sup>-1</sup> at SW compared to NW exposure [48]. Hence, already the past microclimatic



**Fig 6. Gross N turnover rates ( $\text{mg N m}^{-2} \text{ day}^{-1}$ ) ( $n = 8$ ) and N pool sizes ( $\text{mg N m}^{-2}$ ) ( $N = 48$ ) in intact beech seedling-soil-mesocosms.** Blue: NW exposure (control treatment); Red: SW exposure (climate change treatment). The June sampling represents the onset of the growing season after full development of leaves, while the August sampling was conducted in the middle of the growing season after 39 days of rainfall exclusion at SW. Gross rates of N turnover were calculated based on  $^{15}\text{N}$  tracing and pool dilution approaches following homogeneous labelling of the intact soil with double-labelled  $^{15}\text{N}/^{13}\text{C}$ -glutamine,  $^{15}\text{N}\text{NH}_4^+$ , or  $^{15}\text{N}\text{NO}_3^-$ . Thickness of process arrows and nitrogen pool signatures is representative for respective turnover rates and pool sizes. Processes and pools significantly affected by the climate change treatment are indicated by bold letters with asterisks. Different indices show significant differences between June and August for a given treatment/exposure.

doi:10.1371/journal.pone.0158823.g006

**Table 1. Results of two-way ANOVA analysis testing the factors exposure (NW vs. SW), time (June/August/September) and the interaction of exposure and time on gross rates of N turnover in the beech seedling-soil-microbe system and corresponding N pools.**

	Exposure	Time	Exposure x Time
Ammonification	0.223	0.064	0.24
Nitrification	<b>0.010</b>	0.119	0.213
Micr. $\text{NH}_4^+$ immob.	0.223	0.084	0.733
Micr. $\text{NO}_3^-$ immob.	0.113	0.946	0.318
Plant $\text{NH}_4^+$ uptake	<b>&lt;0.001</b>	0.002	0.633
Plant $\text{NO}_3^-$ uptake	<b>&lt;0.001</b>	<0.001	0.002
DON pool	<b>&lt;0.001</b>	<0.001	0.003
$\text{NH}_4^+$ pool	0.034	<0.001	0.506
$\text{NO}_3^-$ pool	<0.001	<0.001	<0.001
Plant N pool	<0.001	<0.001	0.517
Micr. biomass N	<b>0.0067</b>	<0.001	0.022

Numbers represent p values (significant at  $p < 0.05$ ). Significantly larger values in the control treatment are indicated by blue colour while red colour indicates larger values in the climate change treatment.

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**Table 2. Percentage of vital root tips colonized with mycorrhizal fungi.**

	NW		SW		Two-Way-ANOVA		
	mean	SE	mean	SE		p	F
June	99.0	0.5	99.2	0.4	Slope	0.56	0.34
August	99.1	0.5	98.8	0.4	Time	0.08	2.51
September	99.7	0.1	99.9	0.1	Interactions	0.83	0.18

No significant differences in mycorrhizal colonization rates were observed between the control treatment (NW exposure) and the climate change treatment (SW exposure).

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differences across the valley appeared to have been sufficient to impair the development of the beech stand at the warm-dry stand with SW exposure.

### Experimentally simulated climate change decelerates beech N nutrition as a consequence of impaired N cycling

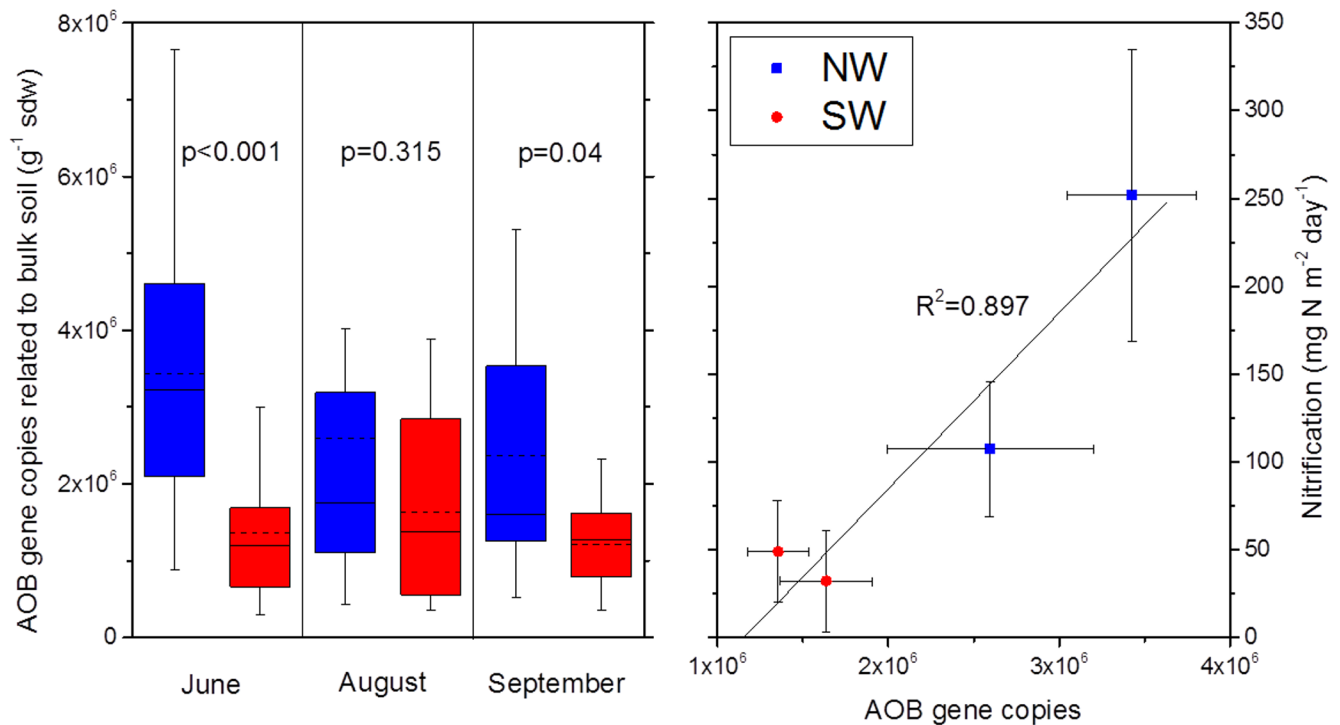
Homogeneous and reproducible triple <sup>15</sup>N isotope labelling allowed the simultaneous quantification of all major N turnover systems in the investigated intact beech seedling—soil-meso-cosms under persistence of plant-microbe interactions during the experimental incubation period. This is expected to have resulted in comparable realistic rates of gross N turnover. The N cycle revealed by this approach was characterized by high rates of microbial mineralization-immobilisation turnover. The comparably low soil inorganic N concentrations in the range of daily rates of gross inorganic N production and consumption rates show that microbial N turnover largely controlled soil inorganic N availability. The dominance of NO<sub>3</sub><sup>-</sup> over NH<sub>4</sub><sup>+</sup> as a source for beech seedling N nutrition is in line with earlier studies at the site conducted with adult trees [15,16]. Generally significantly larger N uptake in June than in August (Fig 6) confirmed an earlier study [16], showing highest N uptake capacity of beech seedlings at the onset of the growing season. Such studies on N uptake capacity also indicated that glutamine is of high importance for N nutrition of beech seedlings [16,50,51]. Here, the homogeneous application of double-labelled <sup>13</sup>C-carbon (<sup>13</sup>C)/<sup>15</sup>N glutamine to intact soil showed that <sup>15</sup>N but not <sup>13</sup>C was retrieved in mycorrhizal and plant tissues (Fig 6, S6 and S7 Tables). This suggests that either uptake of intact glutamine was not significant under such realistic field conditions or glutamine derived C was already subjected to respiration in the mycorrhizal mantle [18]. These findings show the limitation of uptake capacity studies to explain actual *in situ* uptake of organic N in the presence of microbial competition.

**Table 3. Total plant N uptake (mg N m<sup>-2</sup> day<sup>-1</sup>).**

	N increment June-August (42 days) (mg N m <sup>-2</sup> day <sup>-1</sup> )	Short-term <sup>15</sup> NH <sub>4</sub> <sup>+</sup> and <sup>15</sup> NO <sub>3</sub> <sup>-</sup> tracing June (mg N m <sup>-2</sup> day <sup>-1</sup> )	Short-term <sup>15</sup> NH <sub>4</sub> <sup>+</sup> and <sup>15</sup> NO <sub>3</sub> <sup>-</sup> tracing August (mg N m <sup>-2</sup> day <sup>-1</sup> )
NW	10	35 ± 7	6 ± 1.1
SW	3.4	9 ± 1.6	2.2 ± 0.31

Plant uptake was calculated (1) from the net increment of the mean plant N pool between June and August (n = 48 each), divided by the time span of 42 days between these sampling dates, (2) from the sum of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> uptake rates calculated from short-term (6h) <sup>15</sup>NH<sub>4</sub><sup>+</sup> and <sup>15</sup>NO<sub>3</sub><sup>-</sup> tracing into plant biomass in June and August. Nitrogen uptake was always significantly smaller at SW exposure than at NW exposure (p<0.05).

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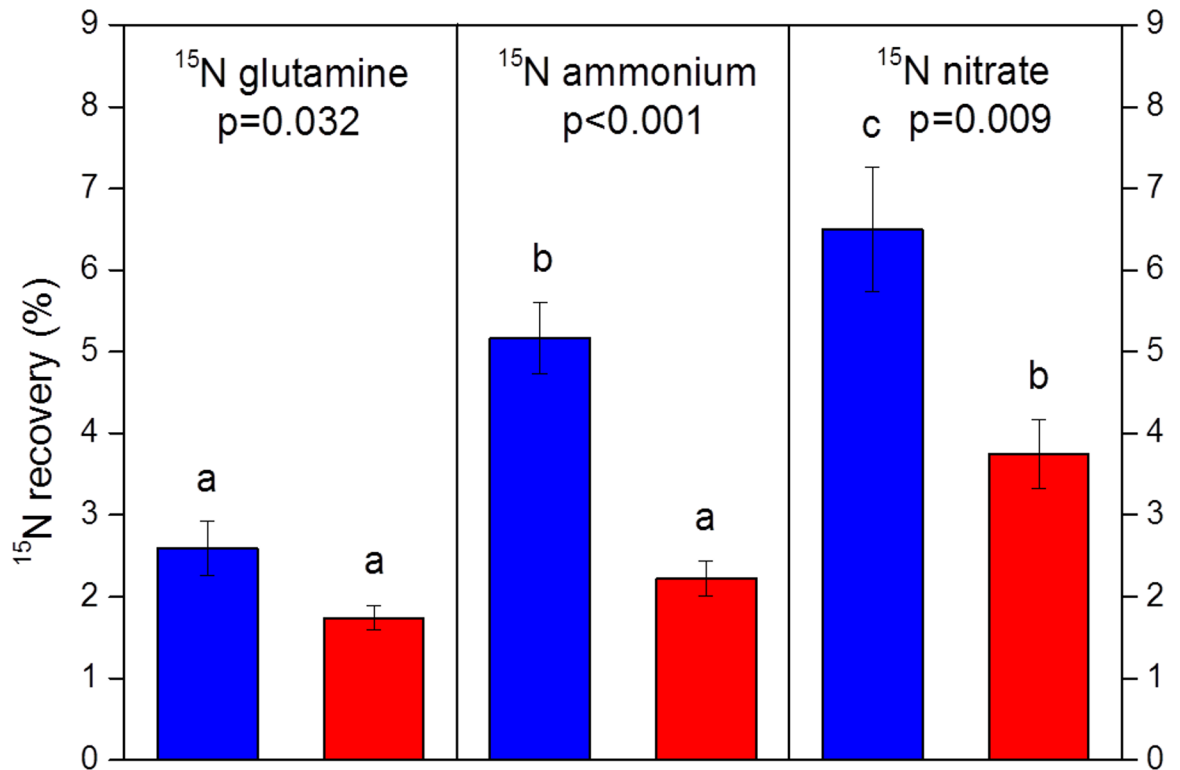
**Fig 7. Ammonia oxidizing bacteria and nitrification rates.** Abundance of genes encoding for ammonia monooxygenase of ammonia oxidizing bacteria in bulk soil (AOB, left panel) and relationships between AOB gene abundance and gross rates of nitrification (right panel). Blue colour: control treatment (NW exposure). Red colour: climate change treatment (SW exposure).

doi:10.1371/journal.pone.0158823.g007

The most striking finding of the simulation of climatic change via transfer from NW to SW exposure was a chain of effects related to  $\text{NO}_3^-$  production and consumption, i.e., the persistently reduced community of AOB and the associated five-fold decline of gross nitrification, soil  $\text{NO}_3^-$  concentrations and beech  $\text{NO}_3^-$  uptake. This suggests a tight link between soil water availability, nitrifier community, gross nitrification rates, soil  $\text{NO}_3^-$  availability and N nutrition of beech seedlings. Apparently this microbial mediated effect of climate change on beech seedling N nutrition was strong enough to reduce growth and competitive performance of beech seedlings already after one year, as indicated by plant biomass and N content (Fig 6). A parallel study using the same experimental facilities as our work but focusing on the quantification of transcripts of functional N cycle genes found a persistent reduction of the transcripts of ammonia oxidizing archaea in the climate change treatment [52]. This indicates that—though not visible in the gene abundance levels—also the nitrifying activity of archaea was negatively affected by the exposure to simulated climate change, which probably also contributed to the observed reduction of gross nitrification rates.

The still significantly reduced number of gene copies of AOB at the end of the growing season in September in conjunction with the positive correlation of the abundance of AOB in soil and nitrification (Fig 7) suggest that the climate-change triggered mechanism of impaired nitrification activity with associated consequences for  $\text{NO}_3^-$  availability and uptake by beech seedlings is persistent. This was also confirmed by the smaller long-term  $^{15}\text{N}$  recovery in beech seedlings in all labelling treatments (Fig 8) and the reduced N metabolite levels in the beech seedlings (Fig 9).

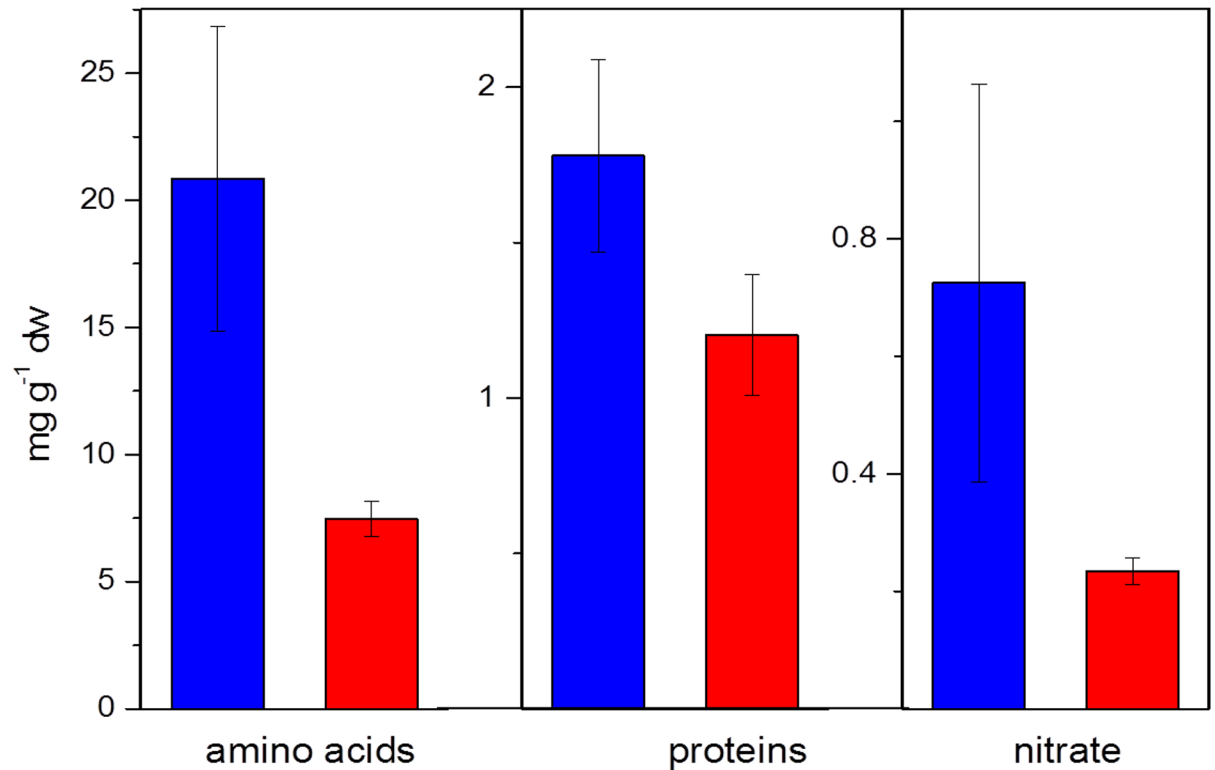
Ammonification and soil  $\text{NH}_4^+$  levels were only marginally reduced in the climate change treatment (Fig 6). This was confirmed by a parallel study [52], which did not detect a significant



**Fig 8. <sup>15</sup>N recovery (n = 8) in beech seedlings (sum of fine roots, coarse roots, stem and leaves).** Data were collected in September, i.e., three months after isotope labelling with glutamine, NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> and indicate recovered % of isotopic excess, i.e., after subtracting <sup>15</sup>N natural abundance. Blue: NW exposure (control treatment); red: SW exposure (climate change treatment). <sup>15</sup>N recovery was highest after nitrate labelling both for SW and NW as indicated by different indices. The climate change treatment always reduced <sup>15</sup>N recovery, as indicated by p<0.05.

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reduction of transcript levels of the mineralization related genes *chiA* due to translocation from NW to SW exposure. Unaltered mineralization rates in the climate change treatment indicate that lower soil moisture accounted for attenuated nitrification through detraction of AOB metabolism and limitation of N substrate diffusion [53]. Soil moisture is the major environmental driver of *in situ* nitrification in Rendzic Leptosols with maximum nitrification at 65% of maximal water holding capacity [14], i.e., the soil water levels frequently found at NW exposure (Fig 5). A strong reduction of gross nitrification rates when soil moisture falls below this optimum is in line with our general understanding of nitrification [53] as well as with earlier observations for Rendzic Leptosols in beech stands [14]. The strong sensitivity of the AOB community to climatic change conditions may be related to the fact that these microorganisms obligatorily depend on ammonia oxidation without metabolic alternatives. Thus, NO<sub>3</sub><sup>-</sup>-dominated N nutrition of beech seedlings may represent a major obstacle for beech performance under reduced soil water levels in marginal soil in a changing climate, leading to reduced growth and, thus, impaired competitive performance. The observed reduction of gross NO<sub>3</sub><sup>-</sup> supply at SW in June equals to 2 kg N ha<sup>-1</sup> day<sup>-1</sup> and thus has the potential to affect the N balance of the entire forest stand with its estimated N demand of roughly 100 kg N ha<sup>-1</sup> growing season<sup>-1</sup>. Adult beech growing on Rendzic Leptosol has been observed to exhibit decreased maximum NO<sub>3</sub><sup>-</sup> uptake capacity and basal stem area with declining soil water availability [39] and has no opportunities to access deeper soil N pools in this shallow soil type.



**Fig 9. Metabolites (total amino acids, total soluble proteins, NO<sub>3</sub><sup>-</sup>) extracted from fine roots of beech seedlings in June.** Blue colour represents the control treatment (NW exposure), red colour represents the climate change treatment (SW exposure). Error bars denote standard errors of the mean (n = 4 per time and treatment). Amino acid and NO<sub>3</sub><sup>-</sup> metabolite levels were significantly lower in beech seedlings of the climate change treatment.

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It needs to be noted that the experimental findings from the space-for-time climate change experiment on the significant contribution of nutritional limitations to the drought sensitivity of European beech on marginal calcareous soil cannot be directly linked to the results of potential species range distribution modelling (Fig 2), as this statistical modelling approach is not based on physiology or biogeochemistry. The statistical model used in this study compares presence or absence of beech with climatic parameters only so that regional differences in N availability and their impacts on beech seedling N nutrition are not considered. The present study suggests that this problem could be tackled by the use of indices of N limitation such as atmospheric N deposition and soil C:N ratios in statistical species range models, which should improve their performance with regard to predicting regional differences in vulnerability and resilience of beech stands in a changing climate. Dynamic vegetation models so far focus on light and water as determinants of stand development and species competition in a changing climate. Our work shows that N dynamics is a critical component to be included in such models. The presented data facilitate testing whether dynamic vegetation models coupled to process-oriented biogeochemical ecosystem models [54] can consider effects of climate change on beech performance more comprehensively, taking also into account N availability as mediated by soil microbes. For this purpose, such biogeochemical test data on plant-soil-microbe N dynamics is urgently needed also for other forest stands. The inclusion of microbial nutrient dynamics in such models is also important in the light of recent observational evidence that nutrient availability could dominate as control of net ecosystem productivity in forests at a global scale [55].

## Implications for future forest management and ecosystem services

Impaired microbial provision of bioavailable N may be a stressor for beech in addition to plant physiological limitations under reduced soil water availability, enhancing the drought sensitivity of beech under predicted climatic changes. The nutritional deficiency could be further exacerbated by enhanced N leaching after prolonged drought due to retarded stabilization of microbial N in organo-mineral associations [40].

The results of the present study were gained for beech seedlings, however also could have direct implications for adult beech stands, because the shallow soils and the shallow root system do not allow adult beech to tap much deeper water and nutrient resources than beech seedlings. Furthermore, the competitive performance and establishment of beech seedlings is of importance for the rejuvenation of the stands. By highlighting the relationships between drought, N cycling and beech N seedling nutrition, our work provides pointers to developing mitigation options to increase beech seedling resilience and adaptation potential in a changing climate. In intensively managed forests and plantations, nutritional limitations could be counteracted by fertilization to increase levels of bioavailable N in soil. However, trade-offs such as undesired rapid hydrological  $\text{NO}_3^-$  leaching in the joint aquifers of limestone karst systems would need to be quantified. The associated risk for nitrous oxide losses may remain small due to high nitrous oxide reductase activity in such soils, converting nitrous oxide into harmless dinitrogen [56]. Another option to promote beech seedling establishment and performance is silvicultural reduction of the stand density. This treatment increased soil water availability via increased throughfall and reduced competition for water in coniferous stands [57]. A further alternative is mixing beech stands with deep-rooting trees such as oak. Such mixing approaches appear to improve water balance of soil and beech via hydraulic lift of water from deeper aquifers followed by water efflux from oak roots [58], and thus may increase the resilience of beech seedlings to climate change in stands mixed with deep rooting tree species.

## Supporting Information

### **S1 Fig. Supporting materials and methods.**

(DOCX)

### **S1 File. Supporting materials and methods.**

(DOCX)

### **S1 Table. Model statistics and accuracy measures for the fitted model.**

(DOCX)

### **S2 Table. Primer sets and thermal profiles used for the absolute quantification of the respective genes.**

(DOCX)

### **S3 Table. Abundance of microbes involved in nitrification based on the quantification of marker genes.**

(DOCX)

### **S4 Table. $^{15}\text{N}$ enrichment (atom%) in excess of natural abundance in mycorrhizal root tips.**

(DOCX)

### **S5 Table. Aboveground and belowground dry plant biomass (mg) of beech seedlings for the three harvest dates.**

(DOCX)

**S6 Table.  $^{13}\text{C}$  recovery in plant.**  
(DOCX)

**S7 Table.  $^{13}\text{C}$  enrichment (atom%) in excess of natural abundance in mycorrhizal root tips harvested in June, August and September.**  
(DOCX)

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## Author Contributions

Conceived and designed the experiments: MD HR JS AP MS IKK. Performed the experiments: MD SB CB ML SG JT RG. Analyzed the data: MD HR JS AP MS IKK SB CB ML SG JT RG MH AB. Contributed reagents/materials/analysis tools: MH AB MD HR JS AP MS IKK RG. Wrote the paper: MD HR JS AP MS IKK JT MH.

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