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Plant and soil microbe responses to light, warming and nitrogen addition in a temperate forest

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Abstract

1. Temperate forests across Europe and eastern North America have become denser since the 1950s due to less intensive forest management and global environmental changes such as nitrogen deposition and climate warming. Denser tree canopies result in lower

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light availability at the forest floor. This shade may buffer the effects of nitrogen deposition and climate warming on understorey plant communities.

2. We conducted an innovative in-situ field experiment to study the responses of co-occurring soil microbial and understorey plant communities to nitrogen addition, enhanced light availability, and experimental warming in a full-factorial design.
3. We determined the effects of multiple environmental drivers and their interactions on the soil microbial and understorey plant communities, and assessed to what extent the soil microbial and understorey plant communities co-vary.
4. High light led to lower biomass of the soil microbes (analysed by phospholipid fatty acids), but the soil microbial structure, i.e., the ratio of fungal biomass to bacterial biomass, was not affected by light availability. The composition of the soil bacterial community (analysed by high-throughput sequencing) was affected by both light availability and warming (and their interaction), but not by nitrogen addition. Yet, the number of unique operational taxonomic units was higher in plots with nitrogen addition, and there were significant interactive effects of light and nitrogen addition. Light availability also determined the composition of the plant community; no effects of nitrogen addition and warming were observed. The soil bacterial and plant communities were co-structured, and light availability explained a large part of the variance of this co-structure.
5. We provide robust evidence for the key role of light in affecting both the soil microbial and plant communities in forest understoreys. Our results advocate for more multifactor global-change experiments that investigate the mechanism underlying the (in)direct effects of light on the plant–soil continuum in forests.

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Keywords: Climate change; Community responses; Bacteria community; Co-inertia analysis; Fungi; Herb layer; Light availability; Nitrogen addition

Introduction

Global change is affecting ecosystems through multiple environmental drivers (Vitousek 1994; Schroter *et al.* 2005). Increased atmospheric nitrogen (N) deposition and climate warming are amongst the most important ones, and they interact in complex ways (Sala *et al.* 2000). In temperate forests across Europe and eastern North America, the canopy cover has been increasing since the 1950s, mainly caused by changes in forest management, atmospheric N deposition, and climate (Schroter *et al.* 2005; Gold, Korotkov & Sasse 2006; McMahon, Parker & Miller 2010; Rautiainen, Wernick, Waggoner, Ausubel & Kauppi 2011; Kauppi *et al.* 2015). Denser forest canopies cause more shading and lower ground-level temperatures and may buffer the effects of both N deposition and climate warming on the forest understorey. Denser canopies can thus buffer the temperature experienced by understorey plants (De Frenne *et al.* 2013; Melin *et al.* 2014; Scheffers, Edwards, Diesmos, Williams & Evans 2014; Valladares, Laanisto, Niinemets & Zavala 2016), which can explain the lagged response of understorey plants to climate warming (Bertrand *et al.* 2011; De Frenne *et al.* 2013). In addition, shade potentially limits plant responses to increased N deposition (Hautier, Niklaus & Hector 2009; Verheyen *et al.* 2012; Borer *et al.* 2014; Farrer & Suding 2016; Walter *et al.* 2016).

To date, studies on the effect of global environmental change have involved various drivers, such as N deposition, climate warming, precipitation, and CO_2 concentration (Ciais *et al.* 2005; Hyvonen *et al.* 2007; Ramirez, Craine & Fierer 2012; von Rein *et al.* 2016). However, the role of light has rarely been considered, in spite of its importance in light-limited habitats such as the

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forest understorey. The few light-related studies showed that enhanced light availability can increase the cover of *Rubus* spp. (Walter *et al.* 2016), and enhance plant community responses to climate warming (De Frenne *et al.*, 2015). A direct experimental test of the effects of light, integrated with other global environmental change drivers, is still lacking, for both the soil microbial and understorey plant communities in temperate forests.

The soil microbial community can be severely influenced by environmental change drivers such as enhanced atmospheric N deposition and climate warming (Yergeau *et al.* 2012; De Vries, Dobbertin, Solberg, Van Dobben & Schaub 2014; Carey 2016; Farrer & Suding 2016). For example, in plots with N addition, N-demanding taxa may become more abundant than oligotrophic taxa, which eventually affects the metabolic capabilities of the soil microbial community (Ramirez, Craine & Fierer 2012). Experiments showed that climate warming may cause shifts in soil microbial composition or function that affect the global carbon and N cycles via changes in, e.g., the biomass of microbial decomposers (Allison, Wallenstein & Bradford 2010), the rate of heterotrophic respiration (Suseela, Conant, Wallenstein & Dukes 2012), and the efficiency of recalcitrant substrate utilization (Frey, Lee, Melillo & Six 2013). In natural conditions, multiple global environmental change drivers co-occur (Leuzinger *et al.* 2011), and the influence of multiple, combined global environmental change drivers on soil microbial communities may differ from the influences of the drivers applied in isolation (e.g., effects may be additive or one driver may dominate the overall effect).

Changes in the soil microbial community composition and structure can affect the plant community through, for instance, plant-soil feedbacks associated with the processes of soil organic matter decomposition and mineralization, or pathogenic and beneficial interactions (De Deyn & Van Der Putten 2005; Bardgett & Van Der Putten 2014; Larios & Suding 2015; Van

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Der Putten, Bradford, Brinkman, Van De Voorde & Veen 2016). Likewise, changes in plant community composition and diversity can also modulate soil microbial activities (Lange *et al.* 2015). To better understand the mechanisms behind the combined effects of multiple environmental drivers on light-limited ecosystems such as forest understoreys, it is important to not only look at the responses of the understorey plants or the responses of the soil microbes and the understorey plants separately, but to also quantify co-occurring responses (von Rein *et al.* 2016). Hence, studies should investigate the plant-soil continuum together to make realistic predictions on the effects of global environmental changes (von Rein *et al.* 2016). Plants and soil microbes might respond differently to different global environmental change drivers (Farrar & Suding, 2016), and the interaction between the multiple drivers may attenuate or increase the magnitude of the responses of both plants and soil microbes. A recent meta-analysis of a large database of studies on multiple environmental drivers showed that global change drivers indeed affect terrestrial ecosystems and that the effects may be less dramatic than expected if several drivers occur concurrently (Leuzinger *et al.* 2011).

Here we report findings from an innovative, *in-situ* experiment under a scenario of multiple environmental changes. We assessed the individual and interactive effects of N deposition, light availability, and climate warming on the biomass of the soil microbes, as well as the composition and diversity of the soil bacterial and understorey plant communities in a full-factorial experiment in an ancient temperate deciduous forest. We hypothesized that: (i) illumination and warming under the dense canopy of the studied ancient forest will shift the composition of the soil microbial and understorey plant communities. Meanwhile, we expect weak or no effects of N addition because of N saturation in the study area; (ii) the interactive effects of two or three simultaneously applied drivers on the composition of soil microbial and plant communities will

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differ from the effects of the drivers applied in isolation. We specifically expected illumination to enhance community responses to N and warming; (iii) the soil microbial and plant community will co-vary, as both communities are expected to respond concurrently to the applied global environmental change drivers.

Materials and Methods

Study area

The experiment was performed in the Aelmoeseneie forest, a temperate deciduous forest in Belgium (50.97°N, 3.81°E) owned by Ghent University, which has been forested since at least the 1770s. Ash (*Fraxinus excelsior* L.) and pedunculate oak (*Quercus robur* L.) were the dominant tree species. The soil developed from a quaternary layer of sandy loam on a shallow impermeable clay and sand complex of tertiary origin (Vanhellemont, Baeten & Verheyen 2014). The recorded mean annual temperature and mean annual precipitation (1981-2010) were 10.6 ℃ and 786 mm, with the precipitation evenly distributed over the year and the mean temperature of the warmest and coldest month 16.8 ℃ and 2.4 ℃. The N deposition in 2009 was 25.3 kg ha^{-1} (Verstraeten, Sioen, Neirynck, Roskams & Hens 2012).

Experimental design

Forty 0.5 m × 0.5 m plots with a similar canopy cover and without any disturbance were randomly established in 2011. The distance between two plots ranged from 5 m to 200 m (the closest two plots – the farthest two plots). The similar canopy cover ensured homogenized natural light availability for all plots. There were no statistically significant differences in plant species richness, evenness, and composition between the plots at the beginning of the experiment (De Frenne *et al.* 2015). The three manipulated environmental drivers were N addition (N), illumination (L), and

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warming (W). A full-factorial design resulted in eight possible combinations: all drivers applied in isolation, the two-factor and three-factor interactions (with five replicates each, i.e., 40 plots in total).

Transplantation. In addition to the naturally occurring species, we introduced three characteristic, relatively tall, competitive native plant species (*Urtica dioica* L., *Rubus fruticosus* agg. and *Aegopodium podagraria* L.) at the start of the experiment in September 2011 in all plots. Three rhizome fragments of c. 7 cm length per species were transplanted along the two diagonal axes of each plot (min. 10 cm away from the corners of the plot). The rhizomes of each of these species were collected within the same forest at < 500 m distance. This was key for the experiment because these species are expected to strongly respond to resource alterations and affect the understorey dynamics, but did not occur in any of the plots due to closed-canopy conditions before the experiment started (except for *A. podagraria* 3% ground cover in one single plot and *R. fruticosus* 0.5% and 2% ground cover in two of the 40 plots, repectively). Transplantation was thus needed to overcome dispersal limitation because the selected plots are surrounded by dense forest and these species would most likely not naturally colonize the plots within the timeframe of this study. By transplanting these light-demanding plants to the naturally light-limited study area, we can assess the effects of resource manipulation on plant community change unequivocally. Interestingly, the transplanted species disappeared almost completely in the plots without resource manipulation. More description of the full experiment design can be found in De Frenne *et al.* (2015).

N addition. In half of the plots, we added 10 g N m^{-2} $year^{-1}$. Ammonium nitrate was dissolved in 400 ml distilled water and applied four times per year (Jan., Apr., Jun., and Sep.). After the solution was sprayed onto the plots, 200 ml distilled water was immediately added to avoid salt deposition on the plants. Plots without N addition received 600 ml distilled water. Each spraying amounted to

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0.33 % of the total mean annual precipitation. This resulted in a mean top soil concentration increase of 4.39 g inorganic-N kg^{-1} dry soil in N added plots.

Illumination. Additional light was provided by two 14 W fluorescent tubes (coolwhite 4000 K, spectral peaks at 546 nm and 611 nm), which were suspended at 65 cm above the forest floor with a wooden frame and protected by a plastic cover in 20 plots (one plot in treatment L was destroyed, leaving 19 plots for data analyses). The lamps switched on at sunrise and off at sunset to simulate the natural photoperiod of the study area. The timing was adjusted every two or three weeks throughout the whole year to match the seasonal variation in day length. Unilluminated plots were equipped with the same frame and cover but with dummy lamps. Photosynthetically active radiation (PAR) was measured after canopy flush on 14 June 2012 under cloudless conditions at five height intervals (20 cm, 30 cm, 40 cm, 50 cm, and 60 cm above the soil surface) in three plots per treatment using PAR Quantum sensors connected to Spectrosense 2+ meters (Skye Instruments, UK). The illuminated and unilluminated plots differed significantly in PAR influx (76.4 ± 10.8 μmol m^{-2} s^{-1} vs. 6.7 ± 0.4 μmol m^{-2} s^{-1}), and there was no effect of illumination on the measured temperatures (De Frenne *et al.* 2015). The applied increase in light availability is similar to the difference between denser canopy conditions in deciduous forest during the growing season and the typical light intensity in small clearings or more open forest types. During the growing season, the PAR levels in the understorey of temperate deciduous forests with a high leaf area index typically vary between 5 – 77 μmol m^{-2} s^{-1}, whereas the PAR levels in small clearings or in forests with a lower leaf area index vary between 11 – 121 μmol m^{-2} s^{-1} (Hutchison & Matt 1977; Clinton 1995; Nilsen *et al.* 2001; Fladeland, Ashton & Lee 2003; Augspurger, Cheeseman & Salk 2005).

Warming. Hexagonal open-top chambers (OTCs) passively warmed the air and soil temperatures. The OTCs were 60 cm high, consisted of six inclined plexiglas walls and an open top, and covered

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a ground surface area of 1.15 m^2. We set our 0.5 m × 0.5 m plots right beneath the open top, in the core area of the open-top chamber, to avoid the effects of the inclined OTC walls on precipitation, soil moisture, and air humidity. We installed three Type T miniature thermocouples (TC Direct, Nederweert, NL) in one unwarmed-unilluminated plot; one unwarmed-illuminated plot; one warmed-unilluminated plot and one warmed-illuminated plot (12 sensors in total). The temperatures were logged at 5 min intervals at 20 cm above the soil surface, at the soil surface and at 5 cm below the soil surface. Overall, the mean temperature (14 Sep. 2011 – 01 Apr. 2015) was raised by 1.43 ℃ (20 cm), 1.26 ℃ (soil surface), and 3.21 ℃ (-5 cm) in the OTCs compared to unwarmed plots, i.e., plots without OTC. The mean daily minimum and maximum temperatures were raised by 1.63 ℃ and 1.46 ℃ (20 cm), 0.85 ℃ and 1.79 ℃ (soil surface), 3.72 ℃ and 2.59 ℃ (-5 cm).

Data collection

Soil sampling. Soil cores for the analyses of microbial biomass and bacterial community composition were taken with a 2.7 cm diameter auger from 0-10 cm soil depth on 2 April 2015. In each plot, two opposite sample sites were selected at 5 cm distance from the plot centre. The two soil cores from one plot were pooled and homogenized. Soil samples were immediately sieved through a 1-mm mesh and stored at -18 ℃ until the start of the extraction. To avoid cross contamination, the auger was sterilized with 75 % ethanol between the plots.

Soil microbial biomass. Phospholipid fatty acids (PLFAs) were extracted and derived according to Huygens *et al.* (2011). In total, 42 PLFA biomarkers were detected. We selected 19 PLFAs as useful biomarkers, which accounted for 91 % of the detected biomass. Those selected biomarkers were assigned to different functional microbial groups. Soil microbial structure was calculated as

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the ratio of total fungal biomass to total bacterial biomass. See Supplementary Materials and Methods and Table S1 in Supporting Information for the details of extraction and assignment.

Soil bacterial community. The bacteria dominated the microbial community in the experimental plots; they made up 90.7 % to 91.9 % of the total microbial biomass in the different treatments. Hence, we only quantified the composition of the soil bacterial community, using high-throughput sequencing. The total DNA extraction from the soil samples was carried out with the PowerSoil®DNA Isolation kit and purified by means of the Wizard®DNA Clean-Up System, following the manufacturer's instructions. The 16 S rRNA gene v3-v4 region was amplified by PCR using the barcoded versions of the primers described by Klindworth *et al.* (2013). Sequencing was done on an Illumina MiSeq platform. The operational taxonomic units (OTUs) table was created using *MOTHUR* (v.1.38) (Schloss *et al.* 2009). See Supplementary Materials and Methods for details of the extraction, sequencing and OTUs table obtainment.

Plant community. The cover of all plants below 1 m height in each plot was assessed in 2015, in April for *Anemone nemorosa* L. and *Ranunculus ficaria* L. and in June for all other species.

Data analysis

We investigated how the three drivers - N addition, illumination, and warming - (interactively) affected soil microbial biomass, soil bacterial community composition, and plant community composition. Next, we investigated whether and how the aboveground (plants) and belowground (soil microbes) communities were linked. All data analyses were performed in R 3.3.2 (R Development Core Team 2016).

Soil microbial biomass. We used one-way analysis of variance (ANOVA) to test for the effects of the treatments on the biomass of each functional microbial group and the microbial structure (PLFA data), and Tukey's post-hoc test to investigate the differences among the treatments. We

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then applied three-way ANOVA to test for the main and interactive effects of the three drivers on each functional microbial group (the three-way interaction was never significant and therefore omitted).

Shared and unique OTUs of the bacterial community. The analyses of the bacterial community (high-throughput sequencing data) were conducted after proportional normalization. The abundance of each OTU was rescaled by taking the proportion of the OTU's read in the total reads, multiplying the result with the minimum sample size (1803 reads) and then rounding to the nearest integer to account for sample size differences (McMurdie & Holmes 2014). This resulted in a scaled taxon-abundance matrix comprised of 4110 OTUs. We calculated the number of shared and unique OTUs between the pairs of treatments: 'N added' vs. 'No N added', 'Illuminated' vs. 'Unilluminated', and 'Warmed' vs. 'Unwarmed'. We used chi-square tests to assess differences in the number of unique OTUs in each pair of treatments (Schmidt, White & Denef 2016).

Alpha diversity of the soil bacterial and plant community. We calculated two indices of alpha diversity: (i) the species richness, i.e., the number of species or OTUs in a plot, and (ii) the inverse Simpson diversity index, taking into account the number of plant species or OTUs present and the relative abundance of each plant species or OTU, using the package *vegan* (Oksanen *et al.* 2016). Three-way ANOVA was used to characterize the individual and interacting effects of the three drivers (the three-way interaction was never significant and therefore omitted).

Beta diversity of the soil bacterial and plant community. We used model-based multivariate abundance analysis to test beta diversity of the soil bacterial and the plant community (Wang, Naumann, Wright & Warton 2012). The model fits individual generalized linear models (GLMs) to each plant or bacterial species (OTU) and then uses these models to make community-level

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inferences about the importance of the model predictors based on resampling. The multivariate abundance models were constructed using a forward selection procedure, i.e., by systematically adding variables to the null model and testing for significant model improvement between nested models by likelihood-ratio tests (*anova.manyglm* function, 500 for the bacteria community, 5,000 PIT-trap resampling runs for the plant community). Model improvements were considered significant at the 0.05 significance level. Mean-variance relationships were modelled by a negative binomial model, and Dunn-Smythe residuals of each model were evaluated for normality and homoscedasticity. The significance of the final model parameters was used to assess community-level effects of the model predictors.

The linkage of the soil bacterial and plant community. We conducted co-inertia analysis (hereafter referred to as COIA), a general and flexible eigenvector framework with no constraint regarding the number of variables that allows to measure the concordance (i.e. co-structure) between two multivariate datasets that share the same objects (plots in our case) (Dolédec and Chessel, 1994; Dray et al., 2003). The method finds a common space into which the plots and species of the datasets can be projected and compared (the distance between plots reflects their similarity). We used the *ade4* package (Dray et al., 2007) to apply COIA to two pairs of datasets: i.e., plant data vs. PLFA biomarkers and plant data vs. bacterial OTU data (at phylum level). We also applied COIA to two subsets of the PLFA data and looked at plant data vs. fungal biomarkers and plant data vs. bacterial biomarkers. Prior to the COIA, we performed Principal Component Analysis (PCA) on the Hellinger-transformed community datasets. We evaluated the strength of the coupling between each pair of datasets with the RV coefficient, which is a multivariate generalization of the Pearson correlation coefficient. The RV coefficient gives a measure of the global similarity of the two datasets between 0 and 1: the closer the coefficient is to 1, the stronger

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the correlation between the datasets. We then used the Monte-Carlo test (with 999 random permutations) to assess the significance of the co-structure between the datasets.

Results

Soil microbial biomass

The biomass of the functional microbial groups differed significantly between the treatments (Fig.1, Table S2).The highest total microbial biomass occurred in the treatment of warming and N addition combined (WN), while the lowest total microbial biomass occurred in the treatment of N addition, illumination, and warming combined (WLN). The WN treatment significantly differed from the WLN treatment for all functional microbial groups. Three other significant differences in the biomass of functional microbial groups between treatments were found. First, the WN treatment differed from the LN treatment (combined illumination and N addition), with significantly higher biomasses of Gram-negative bacteria, total biomass of bacteria and total biomass of the microbial community in the WN treatment (i.e., the treatment without illumination). Second, the WN treatment differed from the L treatment, with a significantly higher biomass of Gram-negative bacteria in the WN treatment (i.e., the treatment without illumination). Third, the W treatment differed from the WLN treatment, with a significantly higher biomass of Gram-positive bacteria and *Actinobacteria* in the W treatment (i.e., the treatment without illumination).

Illumination was the only driver that showed a strong negative effect on all functional microbial groups ($P < 0.001$); neither N addition, warming, nor the two-way interactions had an effect (Fig.1, Table S3). The three drivers did not affect the soil microbial structure (Table S3).

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Composition and diversity of the soil bacterial and plant communities

Seventeen phyla were identified in the soil bacterial community. *Acidobacteria, Actinobacteria,* and *Proteobacteria* accounted for 94 % of all reads and were the main phyla in all treatments (unclassified phyla were not taken into account) (Fig. S1). Concerning alpha diversity, none of the three drivers had an effect on phylum richness, but the interactive effect of N addition and warming was significant for the inverse Simpson index ($P = 0.031$). The beta diversity of the soil bacterial community was significantly affected by illumination ($P = 0.004$), warming ($P = 0.012$) (Table 1 and Table S4), the interaction between illumination and warming ($P = 0.006$), and the interaction between illumination and N addition ($P = 0.002$) (Table 1). N addition did not significantly affect the soil bacterial community composition ($P = 0.09$). The three drivers also affected the number of unique OTUs found in the plots. The number of unique OTUs was significantly ($P < 0.001$) higher in plots in which the driver was manipulated, i.e., in the plots with N addition, in illuminated plots, and in warmed plots (Fig. 2a), and the highest number of unique OTUs was found in the plots with N addition (Fig. 2a). The shared OTUs made up between 92.6 and 95.6 % of the overall OTU abundance (Fig. 2b), and there was no significant effect of the three drivers on the number of shared OTUs (Fig. 2a).

Fourteen plant species were observed across all plots. The two most abundant species were *Anemone nemorosa* L. and *Oxalis acetosella* L. (Fig. S2). The alpha diversity of the plant community was not significantly affected by the three drivers, but the effect of the two-way interaction of N addition and warming on plant species richness was marginal significant ($P = 0.063$). The beta diversity of the plant community was significantly affected by illumination only ($P = 0.026$, Table S4).

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Co-structure between the soil microbial and the plant community

There was no significant co-structure between the soil microbial (measured as PLFA biomarkers) and plant communities or the fungal biomarkers (Table 2) in all plots. Yet, we saw significant co-structure between the soil bacterial and plant communities, for both the bacterial biomarkers (PLFA data, Table 2) and the bacterial phyla (OTU data, Table 2, Fig. 3). When we split the dataset into illuminated plots and unilluminated plots, we only saw co-structure in the illuminated plots, between the plant community on the one hand and the overall soil microbial community (19 PLFA biomarkers) and the soil bacterial community (15 PLFA biomarkers, 17 phyla) on the other hand (Table 2).

Discussion

Light availability emerged as a critical driver for both soil microbes and understorey plants in our experiment. Denser canopies in forests do not merely reduce light availability in the understorey but may also affect, for instance, light quality, temperature, humidity, soil mineralisation, and decomposition (Neufeld & Young 2003; Valladares, Laanisto, Niinemets & Zavala 2016). In our full-factorial experiment, we were able to separate the effects of nitrogen availability, light availability, and temperature in the understorey unequivocally, one of the strengths of our study.

In illuminated plots, the soil microbial biomass was significantly lower and the composition of the soil bacterial community and the plant community differed when compared to unilluminated plots. The response to light of the soil microbial and plant communities may be explained in two (non-exclusive) ways. (i) Light drives the species composition of the plant community through asymmetric resource competition (Valladares, Laanisto, Niinemets & Zavala 2016), and differences in plant community composition result in a different quality and quantity of litter,

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which in turn affects the nutrient supply to the soil microbial community (Strickland, McCulley, Nelson & Bradford 2015). (ii) Light availability affects the allocation of the photosynthetic products in plants, resulting in, e.g., more investment in aboveground plant biomass and less belowground carbon allocation (to root exudates and rhizodeposition), which eventually results in reduced soil microbial biomass (Drake *et al.* 2013; Balasooriya, Denef, Huygens & Boeckx 2014). It is likely that the plant community in our plots changed first, as a result of illumination, and that this change in the plant community then affected the soil microbial community composition. We speculate that the time scale in which soil microbial community starts to change is after 2.5-year manipulation as we have documented increases of plant heights under illuminated plots than in unilluminated plots in 2014 (De Frenne *et al.* 2015). Light availability is a key environmental driver in shaping plant community composition and diversity, and in linking the aboveground and belowground communities, in various ecosystems. In grasslands, for instance, taller plants are more competitive and reduce the light availability for smaller plants, which results in changes in the plant community composition and diversity loss with fertilization (Hautier, Niklaus & Hector 2009). An altered plant community can affect soil heterotrophic activities via, for instance, litter decomposition (Spehn, Joshi, Schmid, Alphei & Korner 2000). In an agro-ecosystem for example, Lau *et al.* (2012) found that the aboveground biomass of soybean significantly increased with light availability (PAR 279.12 ± 44.65 μmol m^{-2} s^{-1} vs. 51.25 ± 8.45 μmol m^{-2} s^{-1}) most likely through *Rhizobia*. Because the process of N-fixation by *Rhizobia* is highly correlated with the products of photosynthesis. Hence, light availability can affect both aboveground and belowground communities in direct and indirect ways in different ecosystems. The co-structure we found between the soil bacterial and plant communities in illuminated plots supports the idea that the abiotic factor light availability affects the soil bacteria through its effect

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on the understorey plants. We found no co-structure between the soil fungal and plant communities. Cassman *et al.* (2016), on the contrary, found co-structures between the plant and soil fungal communities but not between the plant and soil bacterial communities in grassland ecosystems with long-term N addition. The difference between our study and Cassman *et al.* (2016) may be caused by the differences in resource limitation and the composition of the soil microbial and plant communities in different ecosystem types, e.g., in forests vs. grasslands (Nacke *et al.* 2011; Kaiser *et al.* 2016).

The composition of the bacterial community was more responsive to the manipulated drivers than the plant community and the soil microbial biomass. Both illumination and warming significantly affected the composition of the soil bacterial community. Light availability most likely affects the soil bacterial community in an indirect way, via root exudates for instance. Warming, on the other hand, can affect the soil bacterial community in a direct way, via metabolic carbon (Schindlbacher *et al.* 2011). We also observed pairwise interaction effects of the three drivers on the composition of the bacterial community, whereas we saw only a main effect (of illumination) for the plant community. There were interactive effects of illumination with N addition and with warming, while N addition alone did not affect the bacterial community. These results support our second hypothesis that when N addition and illumination are applied simultaneously, the interactive effects of the two drivers on the composition of the soil bacterial community differ from the effect of N addition alone. In N-saturated soils, the competition for N between the soil microbial community and plant roots is lower than that in N-limited soils (Kuzyakov & Xu 2013). In soils, however, all N transformation and uptake processes are correlated with soil carbon resources and regulated by soil microbes (Geisseler, Horwath, Joergensen & Ludwig 2010). Light availability can affect soil carbon sources via changes in the composition of aboveground plant community or their

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photosynthesis (Raven & Karley 2006; Strickland, McCulley, Nelson & Bradford 2015; Valladares, Laanisto, Niinemets & Zavala 2016). Similarly, increased temperatures can affect the decomposition rate of soil organic matter and thus potentially affect the composition of the soil bacterial community via the soil nutrient pool (Hopkins *et al.* 2014; Sierra, Trumbore, Davidson, Vicca & Janssens 2015). Warming and enhanced light also interactively affected the composition of the soil bacterial community in our study. Yet, to which degree the two drivers are synergetic or antagonistic in terms of affecting the soil bacterial community needs further research. von Rein *et al.* (2016), in a warming experiment in an incubator, showed interactive effects of the global change drivers warming and drought on the composition of the soil microbial community, with no effect of warming alone. Hence, when two or more global change drivers are considered simultaneously, the interaction of the drivers may result in additive or attenuated effects and thus cause different responses of the studied community when compared to drivers applied in isolation. The differences in the soil bacterial community composition between the treatments in our study (the unique OTUs, see Fig. 2) suggest a compositional trajectory of change in the bacterial community and a taxon-specific succession under different environmental drivers.

We observed no response of the soil microbial biomass and the plant community composition to N addition and warming. Many temperate forests in Europe and North America are N saturated, and this may cause the lacking response in the soil microbial biomass and plant community to extra N addition (De Schrijver *et al.* 2008; De Schrijver *et al.* 2011; Verstraeten *et al.* 2012). With regard to global warming, a lagged response of plant communities has been observed in lowland areas (Brohan, Kennedy, Harris, Tett & Jones 2006; Bertrand *et al.* 2011). The lagged response to warming is at least partly due to the large proportion of cosmopolitan and thermophilous species in these forests, which results in a higher tolerance to increased temperatures (Bertrand *et al.* 2011;

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De Frenne *et al.* 2015). The response of soil microbial biomass and plant community composition to N addition and warming may vary over time (Smith 2011; Contosta, Frey & Cooper 2015; Shi *et al.* 2015), which underpins the importance of the temporal scale in environmental change studies. At the global scale, warming is expected to change plant communities (Hooper *et al.* 2012). The lack in response of the plants to N addition and warming in our four-year study does not imply that these environmental changes will not affect the soil microbial biomass and understorey plants in the longer run. Chronical warming and accumulation of N (i.e., over more than ten years) can shift the composition and interaction of the soil microbial and plant communities in forests (Bradford *et al.* 2008), especially if the canopy is opened up (Verheyen et al. 2012).

Conclusion

In our four-year experiment in which we simultaneously manipulated three global change drivers in an ancient temperate deciduous forest, light availability emerged as a critical driver for both soil microbes and plants. Under additional illumination, the soil microbial biomass was lower, the composition of the soil bacterial and plant communities was different, and the composition of the soil bacterial and plant communities was co-structured. N addition and warming did not significantly affect the soil microbial biomass and plant community composition, but warming significantly altered the composition of the soil bacterial community. Our results underpin the need to concurrently investigate several communities of the plant-soil continuum and to integrate multiple environmental drivers when studying the effects of global environmental change on ecosystem functioning. In addition, the mechanisms underlying our results also merit further investigation.

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The authors declare no conflict of interest.

Authors' contributions

Shiyu Ma, Kris Verheyen, Pascal Boeckx, Nico Boon and Pieter De Frenne conceived the ideas and designed methodology; Shiyu Ma and Pieter De Frenne collected the data; Shiyu Ma, Ruben Props, Safaa Wasof and Margot Vanhellemont analysed the data; All authors led the writing of the manuscript.

Data accessibility

Data of soil microbial biomass, bacterial sequencing and plant cover deposited in the Dryad Digital Repository: https://doi:10.5061/dryad.q6789 (Ma *et al.* 2018). The sequencing data are also available from NCBI BioProject under accession ID: PRJNA429723 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA429723).

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Fig.1 Biomass (mean ± standard error) of the microbial functional groups based on PLFA concentrations. The treatments are control (C), N addition (N), illumination (L), warming (W), warming + N addition (WN), warming + illumination (WL), illumination + N addition (LN) and warming + illumination + N addition (WLN). Each symbol represents a functional microbial group: PLFAtot (total biomass of the microbial community), Btot (total biomass of bacteria), Ftot (total biomass of fungi), AB (*Actinobacteria*), NB (Non-specific bacteria), G+ (Gram-positive bacteria) and G- (Gram-negative bacteria).

Fig.2 (a) The number of unique and shared operational taxonomic units (OTUs) for the treatments in which a specific driver is manipulated or not manipulated. (b) The relative abundance of shared and unique OTUs within each group of treatments. *** Indicates significant differences in the number of unique OTUs between the pair of treatments based on a chi-square test ($P < 0.001$).

Fig. 3 The co-inertia analysis of the soil bacterial (17 phyla) and plant community compositions across all plots. (a) The mutual ordination of the plots as a function of both the soil bacterial and plant community compositions, (b) projection of the plant species, and (c) projection of the bacterial phyla. Illuminated plots (with L) are shown in red; unilluminated plots (without L) are shown in black. The two first canonical axes account for 78.3 % of total co-inertia and the RV coefficient is 0.31 ($P = 0.002$). The arrow length in (a) is proportional to the difference between the ordinations of the plant and bacteria data; the position of the arrow tails is determined by the ordination of the plant community data, the arrowheads by the bacterial data. Only the plant species and bacterial phyla with the highest ordination scores are shown in figures (b) and (c). The abbreviations of the plant species in (b): Aep *Aegopodium podagraria* L., Hom *Holcus mollis* L., Lag *Lamium galeobdolon* (L.) Crantz, Oxa *Oxalis acetosella* L., Rir *Ribes rubrum* L., Rur *Rubus fruticosus* agg., Sth *Stellaria holostea* L. The abbreviations of the bacterial phyla in (c): Aci Acidobacteria, Bac Bacteroidetes, Chl Chlamydiae, Pro Proteobacteria, Ver Verrucomicrobia.

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Table 1 Final model testing the effects of nitrogen addition (N), illumination (L), and warming (W) on the soil bacterial community composition.

Driver	Residual df	Test statistic (LRTs)	*P* value
N	37	5571.543	0.088
L	36	5642.737	0.004**
W	35	5358.399	0.012*
L:W	34	1754.798	0.006**
N:W	33	1474.774	0.076
L:N	32	2080.481	0.002**

LRTs: Likelihood ratio tests.

* $P < 0.05$, ** $P < 0.01$

The full model selection is available in Table S4.

Table 2 The co-structures between the soil microbial (PLFA data, i.e., four fungal biomarkers and fifteen bacterial biomarkers; OTU data of the bacterial community, i.e., 17 phyla) and plant communities (14 species) in all plots and subset plots with and without illumination.

Soil microbial communities	RV value		
	All plots	Unilluminated plots	Illuminated plots
PLFA biomarkers (19)	0.25	0.31	0.44*
- Fungal biomarkers (4)	0.13	0.14	0.28
- Bacterial biomarkers (15)	0.24*	0.29	0.43*
Bacterial OTUs (17 phyla)	0.31**	0.38	0.43*

RV: Coefficient values.

* $P < 0.05$, ** $P < 0.01$ (Monte-Carlo tests).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Supplementary Materials and Methods: Soil microbial biomass and bacterial community

Figure S1 Relative abundance of the soil bacterial phyla in the different treatments: control (C), N addition (N), illumination (L), warming (W), warming + illumination (WL), warming + N addition (WN), illumination + N addition (LN), and warming + illumination + N addition (WLN).

Figure S2 Relative cover of the plant species in the different treatments: control (C), N addition (N), illumination (L), warming (W), warming + illumination (WL), warming + N addition (WN), illumination + N addition (LN), and warming + illumination + N addition (WLN).

Table S1 The functional microbial group assignment based on the biomarkers of phospholipid fatty acids (PLFAs).

Table S2 Mean biomass (μg/g) of each functional microbial group and the soil microbial structures for the different treatments.

Table S3 The effects of N addition (N), illumination (L), warming (W) and their interactions on the biomass of each functional microbial group and the soil microbial structure.

Table S4 Multivariate abundance testing of N addition (N), illumination (L), warming (W) and the two-way interactions for the beta diversity of the soil bacterial and the plant communities.

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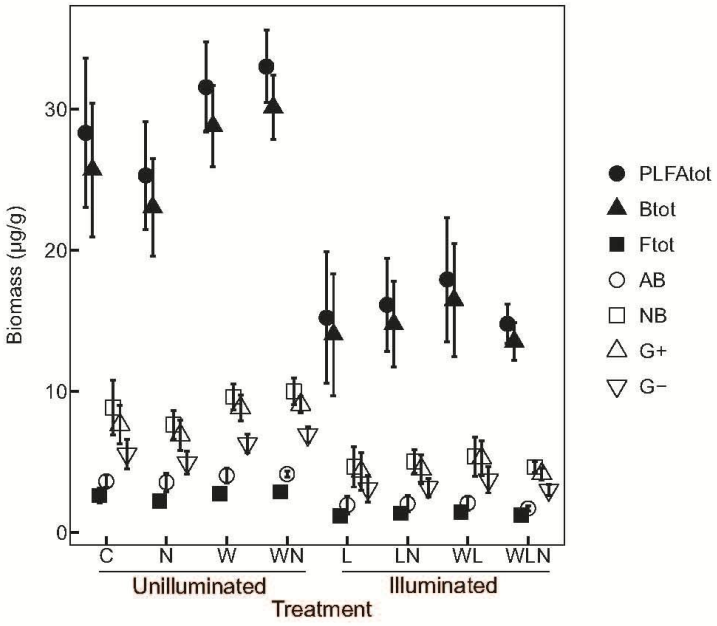

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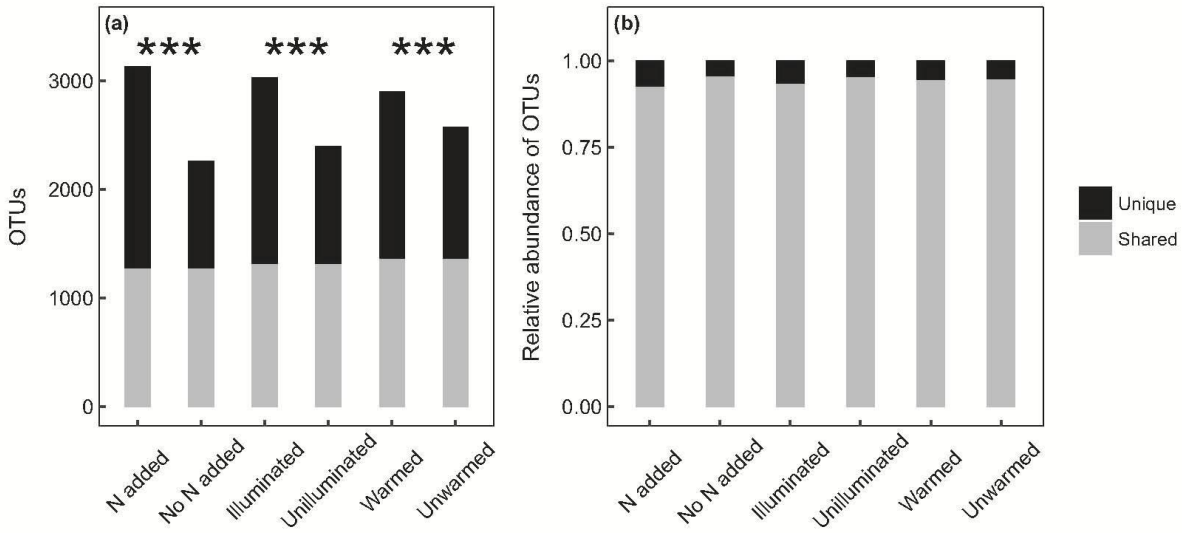

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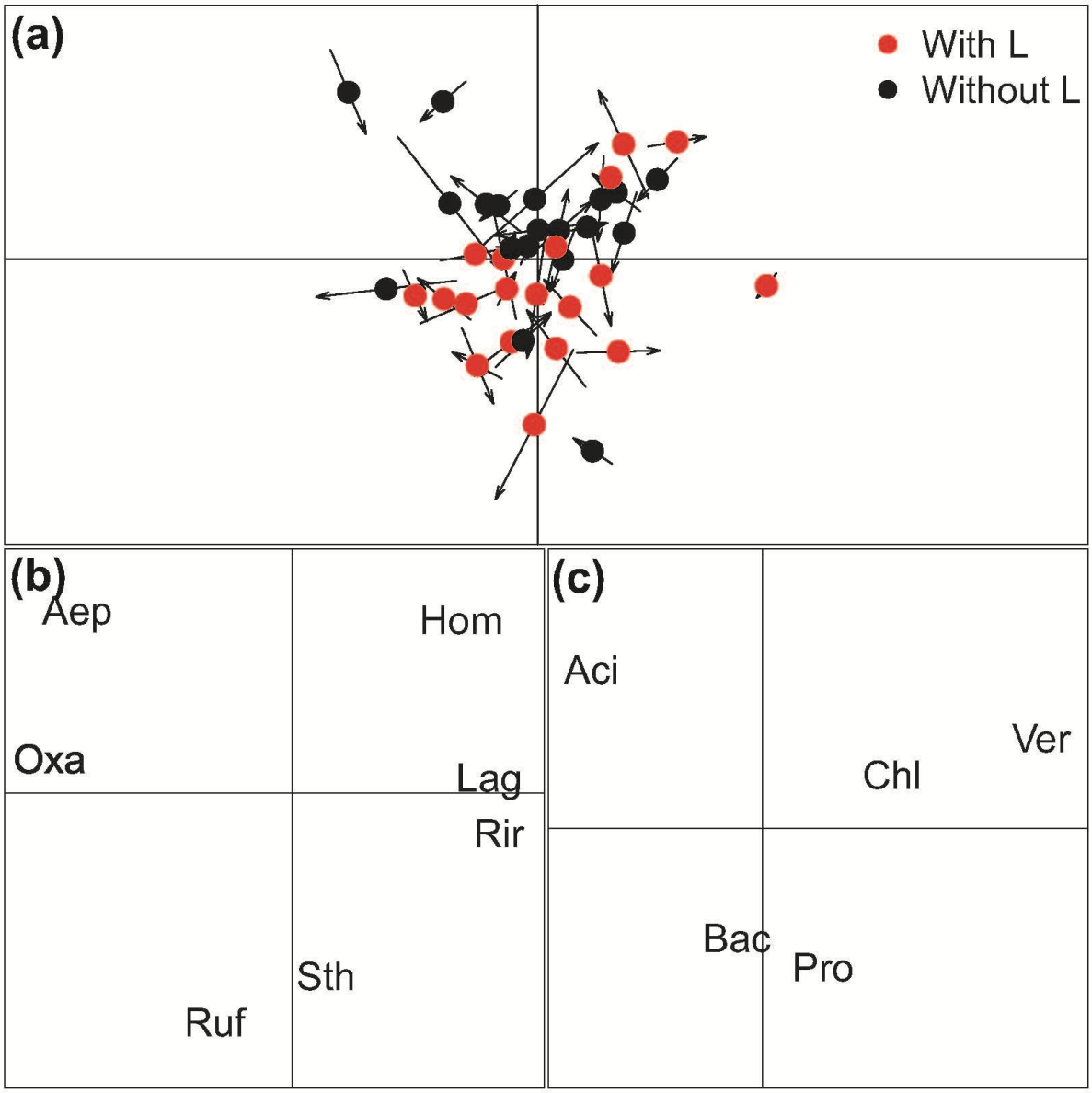

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