Soil Biology and Biochemistry 135 (2019) 184–193

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Soil microbial, nematode, and enzymatic responses to elevated CO_2, N fertilization, warming, and reduced precipitation

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ARTICLE INFO

Keywords:
Microbial biomass C
Soil enzymes
Nematodes
Carbon cycle
Nitrogen cycle
Phosphorus cycle

ABSTRACT

Ecological communities are increasingly confronted with multiple global change factors, which can have wide-ranging consequences for ecosystem structure and functions. Yet, we lack studies on the interacting effects of multiple global change factors on ecological communities – particularly long-term studies in field settings. Here, using a grassland field experiment in temperate North America, we report the interactive effects of four of the most common and pressing global change factors of the Anthropocene (elevated CO_2, elevated nitrogen, warming, and summer drought) on soil microbial and free-living soil nematode communities, which together form an extensive share of terrestrial biodiversity. In addition, we measured microbial mass-specific soil enzyme activities related to carbon, nitrogen, and phosphorus cycles. Our results showed that mass-specific soil enzyme activities and their stoichiometry were strongly affected by higher-order interactions among the global change factors. In particular, the three-way interaction among elevated CO_2, reduced precipitation, and warming decreased the ratio of carbon-to phosphorus-acquiring enzymes as well as nitrogen-to phosphorus-acquiring enzymes in the soil, indicating a relative increase in the breakdown of organic phosphorus in the soil. We also found that the three-way interaction among elevated CO_2, reduced precipitation, and warming altered the predominant decomposition pathway in the soil (towards a bacterial-dominated energy channel in future environments), indicated by the Channel Index of nematode communities. Further, the three-way interaction among nitrogen fertilization, reduced precipitation, and warming enhanced acid phosphatase (related to the P cycle). Nematode density increased at elevated nitrogen and ambient CO_2 as well as at ambient nitrogen and elevated CO_2, whereas it did not differ from controls at elevated nitrogen and elevated CO_2. Changes in microbial biomass were mainly driven by the additive effects of elevated CO_2 and temperature. Our results reveal various ways in which global change factors affect (both additively and interactively) soil biotic responses mainly via altering nutrient demands of soil microorganisms and changing soil community structure and energy channels.

1. Introduction

Since the industrial revolution, Earth's ecosystems have been experiencing increasing concentrations of atmospheric CO_2, subsequent global warming, and alterations in precipitation patterns (IPCC, 2014). Further, the intensive use of chemical fertilizers in contemporary agriculture has dramatically modified the cycling of reactive nitrogen across ecosystems (Erisman et al., 2008). The consequences of these global change factors for ecosystems are widely observed, such as in the form of biodiversity decline and deterioration of ecosystem services (Bellard et al., 2012; Hooper et al., 2012). However, most of the previous empirical studies have rarely considered several global change

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https://doi.org/10.1016/j.soilbio.2019.04.020
Received 29 November 2018; Received in revised form 25 April 2019; Accepted 28 April 2019
Available online 02 May 2019
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factors simultaneously (García-Palacios et al., 2014; Tylianakis et al., 2008), thereby leaving a crucial gap in our current understanding of the consequences of higher-order interactions among multiple global change factors on ecosystems. Moreover, long-term community and ecosystem responses to global change factors can change in unexpected ways over time, e.g, due to complex plant-soil interactions in terrestrial ecosystems (Reich et al., 2018; Reich and Hobbie, 2013). In this study, we aim to address these gaps by investigating the effects of higher-order interactions among four global change drivers (elevated CO_2, warming, elevated nitrogen, and summer drought) from a long-term grassland experiment on soil organisms, which form a major portion of terrestrial biodiversity and contribute to several ecosystem functions (Orgiazzi et al., 2016, Wall et al., 2015; Bardgett and Van Der Putten, 2014).

As soil organisms mainly depend on local soil abiotic conditions, plant inputs in the soil, and microclimate (Decaëns, 2010; Ettema and Wardle, 2002), we may expect global change factors that alter these factors the most to be the most important ones to have consequences for soil organisms (Blankinship et al., 2011; Nielsen et al., 2015). Accordingly, most of the previous global change studies investigating interactive global change effects on soil organisms have explained the variation in soil organism responses either via soil water and soil temperature or resources related to plant inputs. For instance, warming and reduced precipitation interactively reduced the feeding activity of soil invertebrates in situations when soil water content was reduced (Thakur et al., 2018). Further, such amplified deleterious effects of warming and drought on soil organisms have been shown to disappear at elevated CO_2 (Kardol et al., 2010). The positive effect of elevated CO_2 on soil organisms could be related to greater carbon inputs to soils via higher root production by plants and/or higher soil moisture at elevated CO_2 (Eisenhauer et al., 2012; Nie et al., 2013). Studies have also consistently shown that warming-induced greater nitrogen mineralization and soil respiration occur at ambient precipitation, and decrease in drier soil environments (Carey et al., 2016; Thakur et al., 2018), which subsequently increase soil N limitations (Felzer et al., 2011; Melillo et al., 2011). Thus, elevated N can subsidize the N deficit in soil in warmer and drier environments, whereas elevated N in warm and moist soil conditions may further enhance the negative effects on soil microbial growth. Moreover, plant inputs into the soil at elevated CO_2 may also multiply at elevated N, when soil is sufficiently wet relative to lower N and drier soil conditions (Reich et al., 2014).

Here, we studied the effects of higher-order interactions among four global change factors on two important groups of soil organisms: soil microorganisms and nematodes. These two groups are the most abundant and highly diverse organisms and contribute to several ecosystem functions (Ferris et al., 2001; Van Der Heijden et al., 2008). We explored the response of soil microorganisms by studying active soil microbial biomass and their extracellular enzymatic activities (EEA). Microbial biomass is a crucial biological property of soils, which when altered can influence numerous soil-related processes and functions, such as carbon sequestration and nutrient dynamics (Alvarez et al., 1998; Miltner et al., 2012). Exploring the responses of extracellular enzymatic activities enhances the understanding of how microbial-mediated processes in the soil, such as nutrient mineralization, may change with global change factors (Henry, 2013). For instance, elemental imbalances in the soil owing to global change factors can alter the nutritional demands of soil microorganisms (Steinweg et al., 2013), which could directly impede or even enhance microbial-mediated processes in the soil. In this vein, stoichiometric alterations of extracellular enzymatic activities in response to global change factors can provide insights into how those factors alter soil microbial communities and their functions (Sinsabaugh et al. 2008, 2009).

Soil microbial biomass has been shown to be more responsive to single global change factors than to interactive effects of multiple global change factors. For instance, Gutknecht et al. (2012) showed that elevated N was the only global change factor that reduced microbial biomass in a six-year study at the Jasper Ridge Global Change Experiment, which simultaneously manipulated CO_2, N, warming, and increased precipitation. Similarly, in a grassland global change experiment, Eisenhauer et al. (2012) reported an increase in soil microbial biomass in soils with elevated CO_2, while no interactive effects among any combination of global change factors (CO_2, N, and drought) were found. Moreover, previous global change studies have also indicated a lack of higher-order interactions in driving microbial EEA in the soil. In a grassland study, Henry et al. (2005) noted that only precipitation treatments consistently affected microbial EEA (both increase and decrease depending on the enzyme type) among other global change drivers (CO_2, N, and warming). The dominant role of precipitation manipulation on microbial EEA was also confirmed by Kardol et al. (2010), although also revealing the importance of plant species and elevated CO_2 via a significant three-way interaction. Further, microbial EEA in soils have also been shown to increase at lower temperature, but only when precipitation levels were higher (Gutknecht et al., 2010). The patterns of higher-order interaction effects on microbial biomass and microbial EEA could thus depend on a number of factors, such as nullifying effects among global change factors, and resources made available by plants (Steinauer et al., 2015; Steinweg et al., 2013).

The effects of higher-order interaction among four global change drivers on free-living soil nematodes are less well explored compared to soil microbial responses. Global change studies on soil nematodes have mainly shown that soil water availability is the main factor driving nematode density (Kardol et al., 2010; Thakur et al., 2017). In a higher-order interaction context, the total number of nematodes was shown to be much lower in drier soils, but elevated CO_2 buffered the negative effects of the drought treatment on nematode densities in soils exposed to ambient temperature (Kardol et al., 2010). Elevated CO_2 seems to buffer nematode communities against drought by increasing resource availability in the soil potentially via enhanced plant inputs and/or higher soil moisture (Cesarz et al., 2015). In contrast, Eisenhauer et al. (2012) found no evidence of higher-order interactions (CO_2, N, and drought) on nematode densities in a grassland experiment, but only a strong adverse effect of N enrichment on nematode diversity.

The wide-range of soil microbial and soil nematode responses to multiple global change factors thus need to be studied with repeated measurements in long-term experiments to develop a better understanding of how soil organisms respond and potentially adapt to ongoing anthropogenic global change. Accordingly, in a long-running multi-factor global change experiment, we quantified active biomass of the microbial community over four years and nematode densities over three years. We expected that elevated CO_2 and elevated N can buffer the negative effects of reduced precipitation on most of the soil microbial and nematode response variables, but mainly so in soils with ambient temperature.

2. Methods

2.1. Experimental design

This study was conducted in the framework of the TeRaCON (Temperature, Rain, CO_2, and N) experiment, a long-term global change experiment in a temperate grassland at Cedar Creek, Minnesota, USA (Reich et al., 2001). The region has a continental climate with warm summers (July average temperature 22 °C) and cold winters (January average temperature −11 °C) with a mean annual precipitation of 660 mm (Reich et al., 2001). TeRaCON was established within the BioCON experiment (Reich et al., 2001), which was set up on a sandy outwash soil (94.4% sand, 2.5% clay) in a secondary successional grassland (Reich et al., 2001). Within six FACE (free-air CO_2 enrichment; Hendrey et al., 1993) rings, the BioCON plots (2 × 2 m) were established in 1997 with a distance of 20 cm between each other and separated by metal barriers belowground (30 cm deep) (Eisenhauer et al., 2012). The experiment has a full factorial design with two temperature levels (ambient and +2 °C), two precipitation levels (ambient

and −45%), two CO_2 levels (ambient and +180 ppm), two nitrogen levels (ambient and +100%), and 3 replicates (48 plots in total). TeRaCON was established in 2007 (rainfall reduction, see below) and 2012 (temperature, see below) in plots that were planted with nine randomly selected plant species from a pool of 16 total species in four plant functional groups (C_3 grasses, C_4 grasses, forbs, and N-fixing legumes) (Eisenhauer et al., 2012).

In 1997, both the CO_2 and nitrogen treatments were established. The CO_2 treatments consisted of ambient CO_2 and elevated CO_2 (ambient +180 ppm, 24 h day^{-1}, ~560 μmol mol^{-1}) achieved using FACE technology (Hendrey et al., 1993) at the ring level (Reich et al., 2001) (3 rings at ambient, 3 rings at elevated CO_2, 8 nine-species plots per ring). The nitrogen (N) treatment comprised ambient or elevated N (4 g N m^{-2}y^{-1} of slow-release ammonium nitrate, NH_4NO_3) added to half of the nine-species plots in each ring in early May, June, and July. This quantity roughly doubled the natural N availability in the system (Eisenhauer et al., 2012).

Beginning in 2007, summer precipitation from May to August was reduced by ~45% of the total rainfall using rainout shelters in four plots per ring, crossed in a fully factorial manner with the other global change treatments (Eisenhauer et al., 2012). Four additional plots per ring with ambient precipitation were used as a control. Rainout shelters were manually deployed according to the weather forecast to minimize the time the shelters could influence microclimatic conditions on the plots.

The temperature treatment was applied for 8 months per year (growing season) crossed in full-factorial combination with the N and rainfall treatments, beginning in 2012, using lamp-based infrared heating to warm the vegetation and soil surface, and vertical rod-based heating to warm below the soil surface, using techniques developed by Rich et al. (2015). For above- and belowground warming, power to each plot was controlled with and integrated microprocessor-based feedback control, to create a fixed temperature difference for both above and belowground. Aboveground, four 1000-W heaters (FTE-1000, 240 V, 245 mm × 60 mm; Mor Electric Heating Assoc., Inc. Comstock Park, MI) warmed each plot (Supplementary figure 1). This configuration provided a minimum power capacity of 400 W m^{-1}. Belowground soil warming to 1 m depth was accomplished through a hybridization of buried heating cable systems (Bergh and Linder, 1999; Peterjohn et al., 1993) and deep soil heating techniques (Hanson et al., 2011). This technique used vertical warming 'pins' made from resistance cable and inserted at 4 per m^2 to achieve a temperature increase of the entire soil profile with minimal site disturbance (only 15-mm entry holes), and low energy requirements. Soil warming was controlled via in-ground thermocouples at 10-cm depth positioned immediately adjacent to warming pins.

2.2. Soil sampling

Soil samples for soil microbial measurements were taken in June 2012, June 2013, June 2014, and June 2015. Samples taken in June 2012, July 2013, and June 2014 were also used for soil nematode extraction. During these sampling years, the elevated temperature treatments were higher by ~2 °C compared to ambient plots (Supplementary figure 2). Further, we were able to reduce summer precipitation by ~34%, when averaging over the four years in the May–August period (Supplementary figure 3).

Three soil samples (2 cm diameter, 6 cm depth, at least 10 cm apart) were taken per plot and pooled (when both microbial and nematode samples were taken at the same time, six samples were taken and pooled). Soil samples were stored at 4 °C until further processing. The soil samples were sieved with a 2 mm mesh, and measurements/extractions were performed < 7 days after soil sampling.

2.3. Soil microbial and enzyme measurements

Soil microbial respiration and biomass were determined using an O_2-microcompensation apparatus (Scheu, 1992). Soil microbial biomass (C_{mic}, μg C g^{-1} soil dry mass) was calculated using the substrate-induced method (SIR; Anderson and Domsch, 1978) by adding D-glucose and determining the maximum initial respiratory response (MIRR) as the mean of the three lowest readings within the first 10 h of the measurement. Microbial biomass was calculated as 38 x MIRR (Beck et al., 1997).

The activities of four soil enzymes were measured to gain insights into soil microbial community functioning using samples obtained in June 2015. The selected enzymes were β-D-1,4-glucosidase, β-1,4-N-acetyl-glucosaminidase, alanine aminopeptidase, and acid phosphatase. These are hydrolytic enzymes commonly used to assess changes in activities involved in the carbon, nitrogen, and phosphorous cycles (German et al., 2011). β-D-1,4-glucosidase is involved in the degradation of short chain cellulose oligomers (cellobiose) by catalyzing the hydrolysis of β-D-glucopyranosides (Eivazi and Tabatabai, 1988; Nannipieri et al., 2012). β-1,4-N-acetyl-glucosaminidase catalyzes the hydrolysis of N-acetyl-β-D-glucosamine, an oligomer of chitin (German et al., 2011; Parham and Deng, 2000). Chitin is one of the main forms of organic N that enters the soil and is present in arthropods exoskeletons and fungal cell walls (Olander and Vitousek, 2000), and is mainly synthesized by soil fungi (Gooday, 1994). Alanine aminopeptidase catalyzes the mineralization of peptides from alanine (Nannipieri et al., 2012). Acid phosphatase hydrolyzes phosphoric mono-ester bonds to P. It is the principal enzyme involved in P mineralization in acidic soils (Olander and Vitousek, 2000).

The enzyme activities were measured using a fluorimetric method based on the release of methylumbelliferone (MU) from MU-labeled substrates adapted from Saiya-Cork et al. (2002). However, to measure the activity of alanine aminopeptidase, methylcoumarin was used instead of MU. We determined the soil pH (pH 5.5) with a glass electrode and prepared a sodium acetate buffer solution with the same pH. The sample solution was prepared using 1 g of fresh soil and 125 mL of 50 mM sodium acetate buffer. Samples were shaken for 15 min and homogenized for 1 min in an ultrasonic bath. Four replicates were prepared per sample well (50 μL from the substrate solution, plus 200 μL from the respective sample suspensions), four negative wells (50 μL substrate solution and 200 μL buffer), and four blanks per well (50 μL buffer and 200 μL of the respective sample suspension). Also, a quench for the calibration curve was prepared per assay with standard substrate concentrations (50 μL of the substrate solution with the concentrations 0, 2.5, 5, 10, and 20 μmol/L, and 200 μL of the sample suspension) and the five respective negatives (200 μL buffer and 50 μL substrate solution). After incubating the samples for specific times for each enzyme in the dark (β-D-1,4-glucosidase: 5.5 h; β-1,4-N-acetyl-glucosaminidase: 4 h; alanine aminopeptidase: 5.25 h; acid phosphatase: 2.5 h), the activity was measured with a microplate reader using an endpoint measurement (BMG Labtech, Ortenberg, Germany) at 365 nm excitation and 450 nm emission. The endpoint for each enzyme was determined by saturation curves (when enzyme activity levels off) based on the optimal substrate concentration, buffer solution and its pH. We calculated mass-specific enzyme activity (MSEA), which is the extracellular enzyme activity per unit of microbial biomass, to determine if the changes in the extracellular enzyme activity were caused by changes of soil microbial biomass (Steinweg et al., 2013).

We also estimated the stoichiometry of the extracellular enzyme activities related to the different elemental cycles. These enzymatic ratios provide insights into the nutrient demand of soil microorganisms (Steinweg et al., 2013). It was calculated as β-D-1,4-glucosidase:(β-1,4-N-acetyl-glucosaminidase + alanine aminopeptidase) for C:N; C:P as β-D-1,4-glucosidase:acid phosphatase; and N:P as (β-1,4-N-acetyl-

glucosaminidase + alanine aminopeptidase):acid phosphatase (Sinsabaugh et al., 2009; Steinweg et al., 2013). All these ratios were based on mass-specific enzyme activities.

2.4. Nematode community calculations and indices

Nematodes were extracted from approximately 10 g of fresh soil with a modified Baermann method (Ruess, 1995). Extracting rather low amounts of mixed soil from multiple subsamples was shown to result in high nematode extraction efficiency for the Baermann funnel method (Schulz et al., 2018). After the extraction (30 h) at room temperature, nematodes were preserved in 4% formaldehyde. For 2012, 2013, only total nematode densities were counted. In 2014, nematodes were counted and identified to family level. When an identified family contained more than one feeding type (i.e., plant feeder, bacterial feeder, fungal feeder, predator, omnivore), it was identified to genus level, so that each nematode could be assigned distinctly to a given feeding type. Each nematode family was also assigned to a colonizer-persister value (c-p), which indicates their life strategy, ranging from 1 (*r*-strategist species, "colonizers") to 5 (*K*-strategist species, "persisters") (Bongers, 1990). C-p 1 nematodes (only bacterial feeders) have a high fecundity and are relatively resistant to pollutants due to their non-permeable cuticle. Only c-p 1 nematodes are able to form dauer larvae to overcome unfavorable conditions, such as resource depletion. Combining the c-p class of the nematode family with the respective feeding type allowed sorting nematodes according to functional guilds (Bacterial feeder (Ba_x), Fungal feeder (Fu_x), Omnivore (Om_x), Predators (Pr_x), where x = c-p class) (Ferris et al., 2001). We excluded plant root feeding nematodes for the calculation of the nematode indices because of their inverse relationship following Bongers (1990).

Different nematode indices were calculated based on c-p values and functional guilds. The Maturity Index (MI) is the weighted mean of the c-p (colonizer-persister) values excluding c-p 1 nematodes to evaluate stress reaction independent of strong enrichment reactions caused by c-p 1 nematodes (Bongers and Bongers, 1998). C-p 1 nematodes are enrichment opportunists and respond positively to any enrichment in the environment (Ferris et al., 2001). The Maturity Index is a powerful indicator of stress (Bongers, 1990; Ferris et al., 2001).

$$MI = \frac{1}{N}\sum_{i=1}^{N} v_i \times f_i$$

with v_i being the c-p value assigned to family i; f_i being the total number of individuals of the family i, and N being the total number of individuals in the sample for all families.

The Structure Index (SI) reflects the stability and structure of the soil food web and the stability of trophic links. An ecosystem with a high Structure Index means that it has many trophic links and that it is highly structured.

$$SI = 100 \times \left[\frac{s}{s+b}\right]$$

with s (structure food web component) calculated as the weighted frequencies of Ba_3–Ba_5, Fu_3-Fu_5, Pr_3–Pr_5, and Om_3-Om_5, and b (basal food web component) as the weighted frequencies of Ba_2 and Fu_2 (Ferris et al., 2001).

The Enrichment Index (EI) represents the status of primary enrichment of the soil food web.

$$EI = 100 \times \left[\frac{e}{e+b}\right]$$

with e (enrichment component) calculated as the weighted frequencies of Ba_1 and Fu_2 (Ferris et al., 2001).

The Channel Index (CI) indicates the main decomposition channel (bacterial- or fungal-dominated). High Channel Index values indicate a more fungal-dominated system, while low values indicate a more bacterial-dominated system.

$$CI = 100 \times \left[\frac{Fu_2}{3.2 \times Ba_1 + 0.8 \times Fu_2}\right]$$

where 0.8 and 3.2 are coefficients of enrichment weightings for Fu_2 and Ba_1, respectively (Ferris et al., 2001).

Additionally, nematode diversity (Shannon diversity) and evenness (Pielou evenness) were calculated as:

$$\textit{Shannon diversity } (H') = -\sum_{i=1}^{S} (p_i \ln p_i)$$

$$\textit{Pielou evenness } (J) = \frac{H'}{\log S}$$

where p_i is the proportion of taxon i, and S is the number of nematode taxa (identified at the family level).

2.5. Statistical analysis

The effects of higher-order interactions (four-way interactions) among global change factors on soil microbial and soil nematode responses were assessed using mixed-effects models with CO_2 rings as the random effect accounting for the nested design (Eisenhauer et al., 2012). All other global change treatments were nested in CO_2 rings in the TeRaCON experiment. For microbial biomass (C_{mic}) and nematode density, we used sampling year as an additional random intercept to account for the interannual variability. We adopted a model averaging approach to find the best explanatory model for explaining the responses of both soil microorganisms and nematodes. For this, we selected the best model using the delta AIC value. Whenever the best model (delta AIC = 0) was an intercept only model, we opted for the model with at least a single interaction term among the global change drivers nearest to delta AIC = 0. Delta AIC for a model is the difference between its AIC value and the minimum AIC value from the best model. Moreover, when delta AIC equalled or exceeded the value of 4 and no model with any interaction term was found, we opted for the models with at least a single global change factor. Although such a threshold is arbitrary, it provided us the flexibility to explore the best model with as many interaction terms as possible among global change factors as also previously done (e.g. Ding et al., 2011; Halfwerk et al., 2011). After the best model was identified, we ran that very model to obtain the coefficients and respective p-values. Further, the p-value for a given fixed effect was obtained running (semi-) parametric bootstraps on the mixed-effects model parameters. We also performed post-hoc Tukey's HSD tests on the linear models with significant interaction terms. For nematode density (count data), we used negative binomial error terms due to overdispersion in the models with Poisson error terms (Zuur et al., 2009). All other linear models were run with Gaussian error terms. All statistical analyses were performed in R statistical software (R Core Team, 2017). Model selections based on delta AIC were carried out with the MuMIN package (Barton, 2018). The same package was used to estimate conditional R^2-values (Nakagawa and Schielzeth, 2013) (combined R^2 of fixed and random terms) from the best linear mixed-effects model, except for the model with negative binomial error. The conditional R^2 from the model with negative binomial error was calculated as explained in Nakagawa et al. (2017). All mixed-effects models were analyzed with the lme4 package (Bates et al., 2015), and bootstrapped p-values were obtained using the boot (Canty and Ripley, 2017) and the lme4 package. The post-hoc multiple comparisons were carried out using the multcomp package (Hothorn et al., 2008). The linear model assumptions (e.g. homogeneity of variance) and overdispersion in the data were tested using the DHARMa package (Hartig, 2017). We performed log-transformations on certain response variables (indicated in Table 1) to improve the model fit and meet the linear model assumptions.

Table 1
Results of linear mixed-effects models with four global change factors as fixed effects. In the model column, we provide the coefficients of the fixed effects corresponding to the given response variable. The delta AIC values equal to zero are the best fitted models according to model selection criterion. The reported R^2 are conditional R^2, which account for the variations explained by both fixed and random effects. The "-" sign in treatment columns indicate the absence of the given treatment from the model. C: CO_2, N: Nitrogen, P: Precipitation, T: Temperature. ·: p-value = 0.05, *: p-value < 0.05, **:p-value < 0.01, ***:p-value < 0.001. The statistically significant (p-value < 0.05) treatments are given in bold, whereas marginally significant ones (p-value = 0.05) are in italics under the model description.

Response variables	Model	Δ AIC	R^2 (%)	C	N	P	T	C.N	C.P	C.T	N.P	N.T	P.T	C.N.P	C.P.T	N.P.T	N.T.C	C.N.P.T
Microbial responses																		
Microbial biomass C (log(x))	5.31 + 0.1 × **C** −0.11 × **T**	3.23	41.76	*	-	-	*	-	-	-	-	-	-	-	-	-	-	-
β-D-1,4-glucosidase (log(x))	1.94–0.21 × N	0	15.97	-	ns	-	-	-	-	-	-	-	-	-	-	-	-	-
Alanine aminopeptidase	1.49 + 0.33 × T	0.77	31.98	-	-	-	ns	-	-	-	-	-	-	-	-	-	-	-
β-1,4-N-acetyl-glucosaminidase	3.02–0.14 × C −0.02 × N +0.50 × P +1.32 × T −1.81 × C.N +**4.29** × **C.P** +0.30 × C.T −0.40 × N.P −1.25 × N.T −1.13 × P.T −**5.13** × **C.P.T** +*4.61* × *N.P.T*	0	45.22	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	-	*	.	-	-
Acid phosphatase	2.46 + 1.20 × N +0.05 × P +**1.83** × **T** −0.78 × N.P −**2.89** × **N.T** −1.59 × P.T +**4.55** × **N.P.T**	0	39.93	-	ns	ns	**	-	-	-	ns	**	ns	-	-	***	-	-
C:N enzyme ratio	1.63–0.81 × P	2.93	2.5	-	-	ns		-	-	-	-	-	-	-	-	-	-	-
C:P enzyme ratio	2.34–0.02 × C −**0.47** × **N** +0.49 × P −0.05 × T +**1.26** × **C.P** +0.32 × C.T −0.09 × P.T −**2.08** × **C.P.T**	1.96	54.64	ns	*	ns	ns	-	*	ns	-	-	ns	-	*	-	-	-
N:P enzyme ratio	1.44–0.13 × C +0.31 × P +0.05 × T +**1.82** × **C.P** +0.17 × C.T +0.22 × P.T *-2.44* × *C.P.T*	3.31	29.39	ns	-	ns	ns	-	*	ns	-	-	ns	-	.	-	-	-
Nematode responses																		
Nematode density	3.20 + **0.43** × **C** +**0.40** × **N** −**0.55** × **C.N**	0	18.44	*	**	-	-	**	-	-	-	-	-	-	-	-	-	-
Nematode richness	8.97 + 2.47 × C −0.5 × N −1.57 × P +**2.44** × **T** −0.76 × C.N +1.07 × C.P +4.38 × N.P −2.11 × C.N.P	0	41.30	ns	ns	ns	*	ns	ns	-	ns	-	-	ns	-	-	-	-
Nematode diversity (Shannon)	2.4 + **0.27** × **C**	0	18.72	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nematode evenness	0.91–**0.04** × **N**	0	19.01	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-
Maturity index (2–5)	2.41–0.11 × N	3.78	4.22	-	ns	-	-	-	-	-	-	-	-	-	-	-	-	-
Enrichment index (log (x+1))	2.69 + **0.92** × **P** +**0.80** × **T** −**0.98** × **P.T**	0	22.43	-	-	***	**	-	-	-	-	-	*	-	-	-	-	-
Structural index (log (x+1))	3.56–0.69 × P −0.51 × T +1.49 × P.T	2.38	7.85	-	-	ns	ns	-	-	-	-	-	ns	-	-	-	-	-
Channel index (log (x+1))	4.61–0.53 × C −1.74 × P −**4.04** × **T** +0.57 × C.P +**3.27** × **C.T** +**4.78** × **P.T** −**3.49** × **C.P.T**	0	41.03	ns	-	ns	***	-	ns	**	-	-	***	-	*	-	-	-

3. Results

3.1. Soil microbial and enzyme responses

For the full models with four-way interaction terms, we did not find any significant four-way interactions among global change factors in driving microbial and enzymatic responses based on the model averaging procedure (Table 1). Microbial biomass C, which was measured every year once from 2012 to 2015, decreased at elevated temperature, whereas it increased at elevated CO_2 (Fig. 1). However, we did not find any significant interaction effect of these two global change factors on microbial biomass C, nor were there other statistically significant main effects or interactions (Table 1).

The activity of soil enzymes related to C cycling (e.g., β-D-1,4-glucosidase) and N cycling (e.g., Alanine aminopeptidase) showed no significant responses to any of the global change factors (Table 1). In contrast, N cycle-related soil enzyme (β-1.4-N-acetyl-glucosaminidase) activity was significantly affected by a three-way interaction among CO_2, precipitation, and temperature (Supplementary figure 4). Elevated CO_2 decreased this N cycle-related enzyme when soils were drier and warmer (Table 1), although we did not detect statistical differences in their mean based on post-hoc tests (Supplementary figure 4). The activity of the soil enzyme related to the phosphorus cycle (acid phosphatase) showed a similar pattern to that of β-1.4-N-acetyl-glucosaminidase but modulated via N treatments. That is, the activity of this enzyme also increased when soils were higher in nitrogen but drier and warmer (Table 1, Fig. 2). We further observed a three-way interaction among CO_2, precipitation, and temperature affecting the two enzymatic elemental ratios, namely C: P enzyme ratio (significantly, $p < 0.05$) and N: P enzyme ratio (marginally significantly, p-value = 0.05). The C:P enzyme ratio increased at elevated CO2 but only so at ambiently warmed and drier soils (Table 1, Fig. 3). The C: N enzyme ratio in soils did not significantly change in response to any of the global change factors (Table 1).

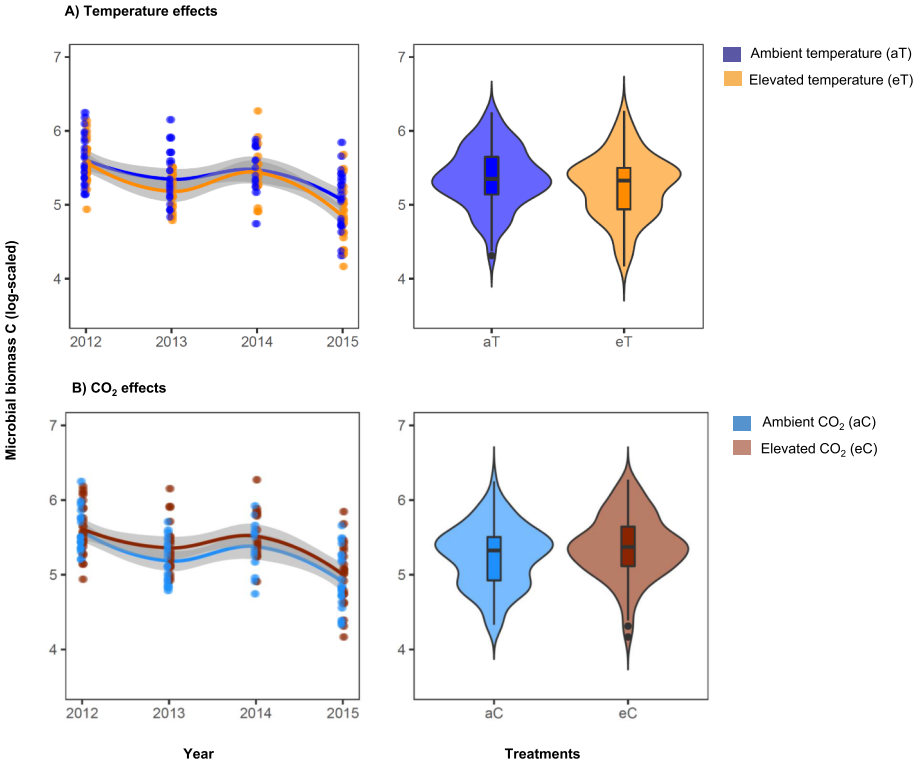

Fig. 1. A) (Left) Temperature effects on soil microbial biomass C (μg C g^{-1} soil dry mass) over the four years (2012–2015) of measurement. The shaded areas around the lines are standard errors. (Right) Median soil microbial biomass at ambient (aT) and elevated (eT) temperatures in boxplots. The shaded areas around the boxplots show the distribution of soil microbial biomass C at the two temperature treatments. B) (Left) CO_2 effects on soil microbial biomass C over the four years (2012–2015) of measurement. The shaded areas around the lines are standard errors. (Right) Median soil microbial biomass C at ambient (aC) and elevated (eC) CO_2 in boxplots. The shaded areas around the boxplots depict the distribution of microbial biomass C in the two CO_2 treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

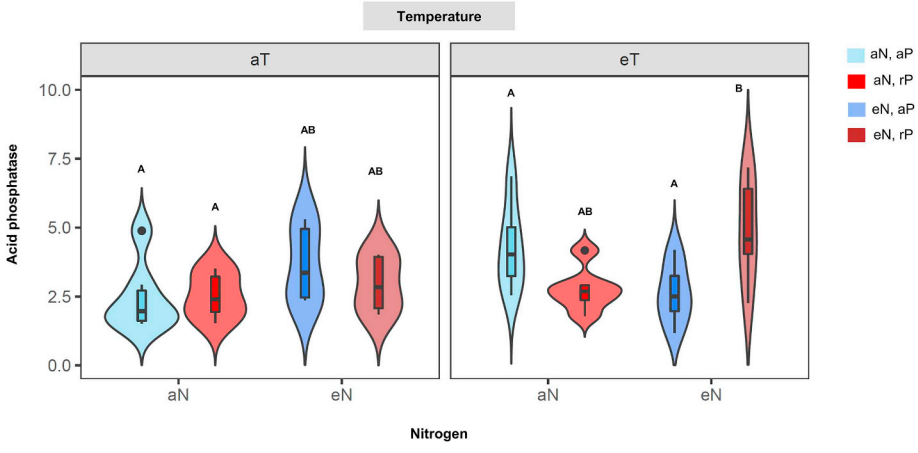

Fig. 2. Three-way interaction effects of nitrogen, precipitation, and temperature on mass-specific acid phosphatase (nmol g^{-1} h^{-1}), an enzyme related to phosphorus cycle in the soil. The shaded areas around the boxplots show the distribution of acid phosphatase in a given treatment. The letters above the shaded areas are from post-hoc Tukey's HSD tests. aN: ambient nitrogen, eN: elevated nitrogen, aP: ambient precipitation, rP: reduced precipitation, aT: ambient temperature, eT: elevated temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

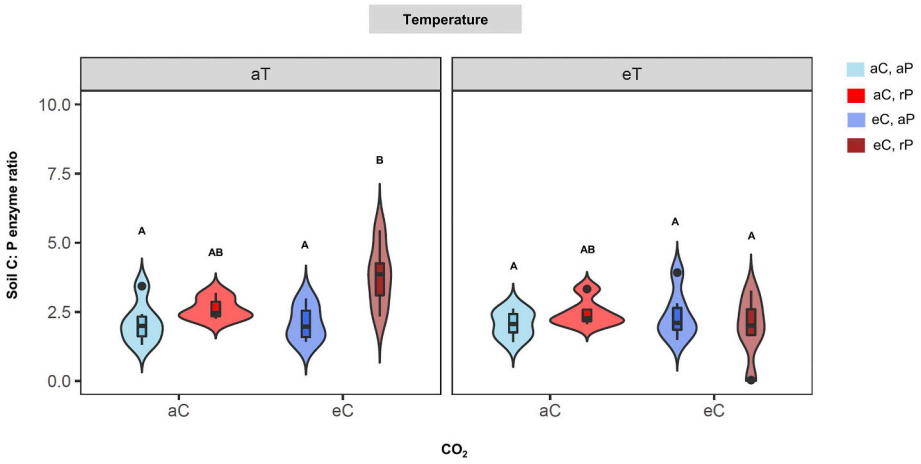

Fig. 3. Three-way interaction effects of CO_2, precipitation, and temperature on soil C: P enzyme ratio. The shaded areas around the boxplots show the distribution of soil C: P ratio in a given treatment. The letters above the shaded areas are from the post-hoc Tukey's HSD tests. aC: ambient CO_2, eC: elevated CO_2, aP: ambient precipitation, rP: reduced precipitation, aT: ambient temperature, eT: elevated temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

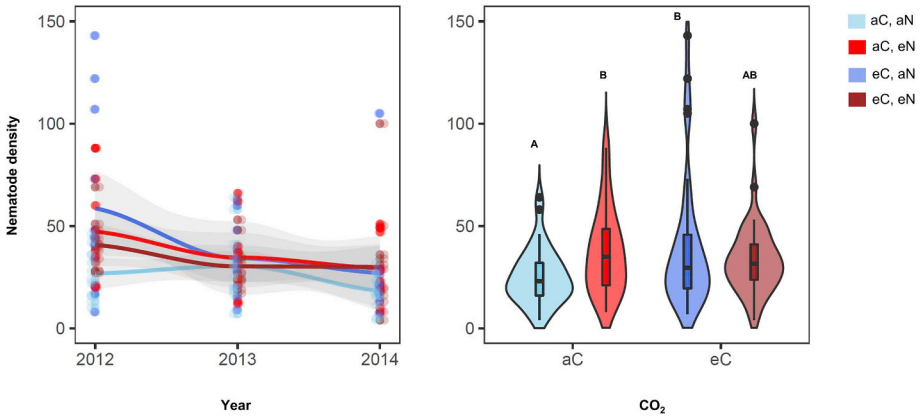

Fig. 4. Effects of elevated CO_2 and nitrogen on nematode density (measured three times each year from 2012 to 2014). In the right panel, the nematode density patterns are shown pooled from three years. The shaded areas around the boxplots (right panel) show the distribution of nematode density in a given treatment, whereas the shaded areas around the lines are standard errors (left panel). The letters on top of the shaded areas are from the post-hoc Tukey's HSD tests. aC: ambient CO_2, eC: elevated CO_2, aN: ambient nitrogen, eN: elevated nitrogen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Nematode responses

We found a significant interaction effect of CO_2 and N enrichment on nematode density, which was measured consecutively for three years (2012, 2013, and 2014). At ambient N, elevated CO_2 increased nematode density, whereas elevated CO_2 had no effect on nematode density at elevated N (Fig. 4). Although the model for nematode taxa richness contained several global change factors, only temperature significantly enhanced the taxa richness of nematodes. The Shannon diversity of nematodes increased at elevated CO_2, whereas nematode evenness decreased at elevated nitrogen (Table 1).

Among the several indices calculated for nematodes communities from the year 2014, we found only one significant three-way interaction among CO_2, precipitation, and temperature affecting the Channel Index of nematodes. This three-way interaction decreased the Channel Index values (Table 1). The Channel index significantly decreased at elevated temperature but only at ambient CO_2 and ambient precipitation (Supplementary figure 5). We further found that the Enrichment Index was reduced by the significant interaction between precipitation and temperature (Table 1). Both maturity and structural index of nematodes were unaffected by any of the global change drivers (Table 1). None of the nematode responses were significantly affected by a four-way interaction among the global change factors.

4. Discussion

The main finding of this study is that the nature of higher-order interactions among multiple global change drivers vary for different soil biotic responses. We did not find any four-way interaction among the tested global change drivers on any soil biotic variable. However, there were some consistent three-way interactions among CO_2, precipitation, and temperature affecting mass-specific soil enzymatic responses as well as the decomposition pathway in the soil (i.e. the Channel index) (Table 1). Notably, all significant three-way interactions among CO_2, precipitation, and temperature were consistently negative in direction (indicated by the sign of coefficients of fixed effects in model description in Table 1). This contradicts previous studies showing that elevated CO_2 can buffer the detrimental effects of warming and reduced precipitation (Cesarz et al., 2015; Kardol et al., 2010), except for P-limitation in the soil indicated by enzymatic ratios (Table 1). We also observed several significant two-way interactions between different global change factors driving soil enzymatic and nematode responses. These two-way interactions were mainly between CO_2 and precipitation in driving mass-specific soil enzyme stoichiometry, whereas nitrogen and CO_2 interactively affected variations in nematode density (Table 1, Fig. 4). Overall, we highlight that only certain combinations of global change drivers interactively influenced a given set of soil biotic responses in this study system agreeing with previous multi-global change studies (Eisenhauer et al., 2012; Gutknecht et al., 2012; Kardol et al., 2010). We elaborate on the implications of these findings on soil communities and ecosystem functions.

4.1. Soil microbial and mass-specific enzyme responses

Soil microbial biomass was greater at elevated CO_2 likely because CO_2 increases plant carbon acquisition and allocation belowground (Adair et al., 2011, 2009) as well as soil water content (Eisenhauer et al., 2012). Indeed, studies have shown that elevated CO_2 in the atmosphere stimulates microbial growth in the soil close to plant roots

(rhizosphere soils) rather than in bulk soils (Phillips et al., 2011). As our soil sampling consisted of both rhizosphere and bulk soils, positive effects of CO_2 on microbial biomass likely resulted from greater root growth and exudation in the soil (Adair et al., 2011, 2009). Past studies have shown that the stimulation of plant growth by CO_2 fertilization depended on supply of other resources, such as N and water (Reich et al., 2014). We thus expected that microbial biomass would mirror plant responses and that its response to elevated CO_2 would similarly depend on N and water availability. However, only the main effect of CO_2 significantly affected microbial biomass, and not its interactions with other resources. Temperature-induced decrease in soil microbial biomass could mainly be due to direct physiological stress in soil microorganisms and potentially drier soil conditions (Romero-Olivares et al., 2017), although reduced precipitation had negligible effects on microbial biomass (Table 1).

By contrast, mass-specific extracellular enzymes responses to interactive effects of multiple global change factors were more pronounced, indicating that microbial activities may have been more responsive to multiple global change drivers than microbial biomass (Romero-Olivares et al., 2017). In fact, we also observed that by reducing N limitations in the soil, the amount of acid phosphatase increased in the warmer and drier soil. In the Jasper Ridge Global Change Experiment, Menge and Field (2007) also showed that phosphatase activities increased in N-enriched soils; however, they did not detect any higher-order global change interactions as observed in our study. We suspect that differences in soil abiotic conditions as well as in plant communities between the annual grasslands in the Jasper Ridge Global Change Experiment and perennial grasslands in TeRaCON may have contributed to differences in P limitations in the soil. Moreover, an enzyme related to the N cycle (β-1,4-N-acetyl-glucosaminidase) was also increased by a higher-order interaction of N enrichment with warming and reduced precipitation (although only marginally significantly), indicating a potential decrease in soil N limitation. The same enzyme production was, however, constrained by a three-way interaction among elevated CO_2, reduced precipitation, and warming. This chitin-degrading enzyme is often synthesized by soil fungi and has been shown to respond to higher-order interactions among N enrichment, increased precipitation, and warming in a previous study (Gutknecht et al., 2010). It seems that elevated CO_2 relaxed presumably fungal N-demand in warmer and drier soils, while N enrichment tended to increase it in warmer and drier soils. We encourage future studies to understand how different resource enrichments can affect microbial nutrient demands in soils exposed to warming and drought. Overall, our results suggest that mass-specific enzyme production by soil microorganisms may alter nutrient dynamics in the soil, such as the phosphorus cycle, when exposed to multiple global change drivers. The mechanisms and consistency of such changes are unknown. The implications of these shifts on plant and soil communities therefore will be an important research area for global change studies, given that these enzymes play crucial roles in soil carbon sequestration (Henry, 2013).

Elevated CO_2, reduced precipitation, and elevated temperature also interacted to affect the ratio between carbon and phosphorus as well as the ratio between nitrogen and phosphorus-related mass-specific extracellular enzyme activities. As these two ratios have phosphatase in common, we assume that their overall reduction may indicate that the interactive effects of three global change drivers enhanced the breakdown of organic phosphorus in the soil. A recent study showed that enzymatic ratios in the soil had a minor response to warming and drought (Steinweg et al., 2013). Our results suggest that resource enrichment from elevated CO_2 can, in fact, increase microbial P-demand which may have resulted in higher EEA production in warmer and drier soils.

4.2. Nematode responses

The three-year nematode density patterns were primarily driven by resource-enriching global change drivers: elevated CO_2 and N enrichment. The positive effects of elevated CO_2 on soil nematode density seemed to hold true only in the ambient N environment. Similarly, the positive effects of enriched N on soil nematode density seemed to hold true only in the ambient CO_2 environment. These results contradict a previous study, which showed neutral effects of elevated CO_2 and N on the density of free-soil living nematodes (Eisenhauer et al., 2012). However, our study is based on a longer-term study and a greater number of sampling points across years, indicating that some of the interactive effects of global change drivers on soil organisms can only be realized after their application for a longer period of time.

Warming increased the taxon richness of nematodes (based on one year of measurement), agreeing with recent studies (Ma et al., 2018; Thakur et al., 2017). Positive effects of warming on taxon richness of nematodes have been shown to depend on greater resource availability in the soil (Ma et al., 2018; Thakur et al., 2017). Although we found no interaction effect of global change factors on nematode taxa richness, the best explanatory model contained several global change factors indicating the possibility that variations in nematode taxa richness could be a result of both additive and interactive effects among global change factors (Table 1).

Lower values of the Channel Index of nematodes in warmer soils suggest a more bacterial-dominated energy channel (Cesarz et al., 2015; Ferris et al., 2001). In fact, the same three-way interaction among elevated CO_2, reduced precipitation, and elevated temperature that explained variation in mass-specific enzymatic ratios seemed to also shift the energy channel from fungal to bacterial pathways. Although the measurement years varied for nematodes and enzymes, we suspect that complex interactions among these global change drivers can affect each other. For instance, the shifts toward the bacterial energy channel could potentially enhance the breakdown of organic matter (Wardle et al., 2004) that link well with the greater concentration of phosphorus-related extracellular enzymes in our study. Further, similar responses of microbial activity and nematode may indicate that these groups are linked through feeding relationships, as nematodes are a key regulator of soil microbial communities (Wardle et al., 1998).

We believe that a more holistic understanding of soil biotic responses to multiple global change factors can only be realized by employing a multi-biotic perspective to the soil. Toward this end, we have presented both soil biota as well as their activity responses to multiple global change factors. While the most complex form of interaction was not significant in our study, we observed several higher-order interactions (i.e. three-way interactions) among global change drivers. Importantly, our study indicates a greater importance of the interaction among elevated CO_2, reduced precipitation, and warming in structuring and functioning of soil communities.

Acknowledgement

We are grateful to three anonymous reviewers for their constructive suggestions on this manuscript. MPT acknowledges funding from the German Research Foundation (DFG, TH 2307/1-1). Further support came from the German Centre for Integrative Biodiversity Research Halle–Jena–Leipzig, funded by the German Research Foundation (FZT 118). The field experiment is supported by NSF Long-Term Ecological Research grants DEB-0620652 and DEB-1234162, Long-Term Research in Environmental Biology grant DEB-1242531, and Ecosystem Sciences grant DEB-1120064 and by U.S. Department of Energy Programs for Ecosystem Research grant DE-FG02-96ER62291.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.04.020.

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