Soil communities and functions as affected by multiple global change drivers

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MSc Sustainable Development - Global Change and Ecosystems

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April 8, 2016

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Summary

Global change driven by human activities and its impact on the world ecosystems' properties and functioning has been the object of many scientific studies done to date. However, soils ecosystems' response to global change has not received sufficient attention, despite the big portion of the global biodiversity that they host and the essential ecosystem functions and services that derive from them. Soil food webs carry out the decomposition and the regulation of nutrient cycling in belowground systems, which are determinant functions for soil quality. The present study focuses on soil food webs structure and functioning as affected by four global change agents: elevated CO_2 concentrations, nitrogen addition, warming and reduced precipitation; in a long-term grassland experiment.

Soil structure and functioning was determined by analyzing the decomposition and activity mediated by the microbial community and the structure and functional diversity of the nematode community as a representative of higher trophic levels. Microbial biomass and nematode densities were measured in consecutive years to assess the consistency of soil biota responses to the treatments. They varied through time on their response to global change agents. However, microbial biomass always tended to decrease, whereas nematode density tended to increase under global change. An extracellular enzyme analysis of enzymes involved in the carbon, nitrogen and phosphorous cycles in 2015 was performed. The activity of all enzymes relative to the microbial biomass increased under global change, and all of them were affected by the significant interaction between nitrogen, temperature and precipitation. Nematode community from 2014 was studied by its biodiversity and by calculating nematode functional indices. Nematode richness and diversity increased whereas evenness decreased. All the nematode functional indices were affected by the significant interaction between precipitation and temperature, indicating a degraded food web with a higher resource availability to primary consumers, and a bacterial-dominated decomposition under elevated temperature and reduced precipitation. Opportunistic nematode became more dominant whereas K-strategist presented a loss in density.

These results indicate that, despite the context-dependency of soil ecosystems response to global change, soil quality decreased. Soil food webs under global change in this experiment were representatives of a disturbed and stressed system with less trophic links, and where carbon, nitrogen and phosphorous cycles' stoichiometry was altered.

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Cover Figure: Photograph showing the edge of one FACE ring and some of the plots with the lamps used for the warming treatment of the TeRaCON experiment in Minnesota, USA. Photo credit: Prof. Dr. Nico Eisenhauer.

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Abbreviations list

AAP: Alanine aminopeptidase

aCO$_2$: Ambient CO_2

AMF: Arbuscular mycorrhizal fungi

aN: Ambient nitrogen

aP: Ambient precipitation

APh: Acid phosphatase

aT: Ambient temperature

βG: β-D-1,4-glucosidase

CI: Channel index

EC: Enzyme commission number

eCO$_2$: Elevated CO_2

EEA: Extracellular enzyme analysis

EI: Enrichment Index

eN: Elevated nitrogen

eT: Elevated temperature

FACE: Free-air CO_2 enrichment

MI2-5: Maturity index

MSEA: Mass-specific enzyme activity

NAG: β-1.4-N-acetyl-glucosaminidase

rP: Reduced precipitation

SI: Structure index

vwc: Volumetric water content

Introduction

Background

Human activities have an impact on the climate and biochemistry of the globe with significant consequences for ecosystem functions (IPCC, 2007). One of the main causes of climate change are anthropogenic greenhouse gas emissions (CO_2, CH_4) released into the atmosphere driven by population and economic growth (IPCC, 2014). As a consequence, we are experiencing increased atmospheric CO_2 concentrations and subsequent global warming and changes in precipitation regimes (IPCC, 2007; IPCC, 2014). Also, due to burning fossil fuels and fertilization, humankind is increasing the turnover rates of the nitrogen cycle (Gruber & Galloway, 2008). Those changes have tremendous impacts on Earth's ecosystems, such as biodiversity loss, changes in species distribution, and eutrophication of fresh waters (Millennium Ecosystem Assessment, 2005). Here, the object of study are soil ecosystems and the impact of global change on them. Soil ecosystems are essential given that a big portion of the global biodiversity belongs to them (Decaëns, 2010) and that they maintain crucial ecosystem functions and services from which humans benefit directly or indirectly (De Groot et al., 2002; Millennium Ecosystem Assessment, 2005; Nielsen et al., 2015). Decomposition of organic matter, cycling of minerals and nutrients, and sequestration of carbon are just some of the essential ecosystem functions and services driven by soil food webs decisive for resource retention, environmental maintenance, and for buffering the effects on global change (Ferris et al, 2001).

The capability of soil biota to sustain their proper functioning and regulate nutrient cycling is an indicator of soil health and, therefore, soil quality (Anderson, 2003). The main contribution of microbial communities to nutrient cycling is their role in decomposition and mineralization, which is mediated by extracellular enzymes (Burns et al., 2013). Higher trophic levels in the soil also play a role in nutrient cycling (Thakur et al., 2015). Global change-induced alterations in soil food web complexity can lead to serious changes in soil ecosystems (Blankinship et al., 2011): changes in soil structure, fertility, nutrient availability, and responsiveness to disturbances (Ferris et al., 2001).

State of the art

Regardless of the importance of soil ecosystems, research on the impact of global change agents has focused mainly on aboveground and not on belowground ecosystems, although they are tightly connected (Hu et al., 1999; West et al., 2006). Therefore, given their functional significance, it is necessary to enlarge our understanding on the effects of global change on soil ecosystems and how the soil system react to those impacts in the future.

It is important to approach this topic through long-term research experiments where global change agents' effects are studied in concert rather than separated, given that they interactively affect soil biota and ecosystems (Blankinship et al., 2011; Cesarz et al., 2015; Eisenhauer et al., 2012; Reich et al., 2012). The global change agents that are studied here are elevated atmospheric CO_2 concentration, nitrogen deposition, warming, and decreased precipitation.

The impact of elevated CO_2 concentrations on plant physiological processes cascades to soil biota. For instance, reduced plant stomata conductance as a response to increased atmospheric CO_2 leads to a consecutive increase in soil moisture content (Field et al., 1995), which is beneficial for most soil biota (Coleman et al., 2004, in: Eisenhauer et al. 2012). Further, elevated CO_2 concentrations enhance carbon acquisition by plants, increasing plant biomass production (Ainsworth & Long, 2005) which leads to elevated carbon inputs into the soil (Adair et al., 2011) and increases soil microbial biomass (Eisenhauer et al., 2012). However, this is accompanied by an increment in C-to-N ratio of plant tissues which means a reduced source quality for consumers (Körner, 2000).

The increase of nitrogen concentrations in the soil due to fertilization and increase of atmospheric nitrogen deposition from farming, traffic and industry (Erisman et al., 2011; Vitousek et al., 1997) brings changes in soil ecosystem processes, such as decomposition, mineralization, and nitrification (Swift et al., 1979). For example, nitrogen addition to the soil increases the rate of degradation of labile organic matter but it hampers the degradation of more complex substrates like lignine (Fog, 1988 in: Sinsabaugh et al., 2002). Nitrogen fertilization often benefits plant productivity but may decrease rizhodeposition and soil animal biodiversity (Dijkstra et al., 2005; Eisenhauer et al., 2012).

Increased temperatures and changes in precipitation regimes also have significant impacts on soil ecosystems. Warming has been shown to increase nitrogen mineralization in some soils (Rustad et al., 2001) and to affect the composition of soil biota communities (Zogg et al., 1997). Warming can also decrease water availability (Wan et al., 2002), which may have detrimental effects on soil life, which is highly

dependent on water. For that reason, precipitation is a key factor determining soil biota functioning as well, mainly through soil moisture content (Nielsen et al., 2015). Soil fauna abundance mostly decreases with drought, which may lead to the disappearance of some taxa and altered community composition (Lindberg et al., 2002).

There is limited research done on soil ecosystem responses to multiple, interacting global change drivers. Only few experiments have been performed with more than two global change agents in order to study the outcomes of their interactions. They show that many global change agents interact with each other in affecting the soil. For instance, ambient CO_2 concentrations, elevated temperature and drought were shown to have deleterious effects on some nematode trophic groups, but elevated CO_2 concentrations can negate those effects (Kardol et al., 2010). Soil moisture, CO_2 concentrations, and N availability may also interact with each other, since elevated CO_2 concentrations increase soil moisture (Field et al., 1995), and high moisture increases N availability (Zhang & Wienhold, 2002). Warming also enhances mineralization and soil respiration, but when drought takes place, mineralization declines with a subsequent decrease in N availability (Felzer et al., 2011; Melillo et al., 2011). Furthermore, given that most of terrestrial ecosystems are N-limited, unexpected responses may happen when elevated CO_2 concentrations and increased N deposition are taking place at the same time (Reich et al., 2006).

Research aim

This experiment embraces the complexity of ongoing global change by manipulating four global change agents and studying the responses of soil communities and functions. Thus, this project helps to advance the knowledge on the consequences of global change for soil ecosystems and their functioning. This research focuses on the question:

How does global change affect soil food web structure and functioning?

Theory and applied methodology

Most of the research up to now has been focusing only on biodiversity, assuming that this is an accurate proxy for soil functioning, but the soil biodiversity-ecosystem functioning relation is not yet well determined (Anderson, 2003). Diversity indices are useful for descriptive food webs assessments, but they lack information about the magnitude or nature of their functions (Ferris, 2010). Sometimes, a loss in biodiversity can lead to an even bigger loss in the functioning of the ecosystem (Barnes et al., 2014). Therefore it is important not only to assess biodiversity but also the soil food web structure and its functioning through different indicators to directly study the impact of global change on soil ecosystems.

To determine the status of the soil food web structure and its functioning, I analyzed (a) the decomposition and activity mediated by the microbial community; and (b) the structure and functional diversity of higher trophic levels through analysis of the nematode community. Analysis of the microbial community provides information about the functioning of the basis of the soil food web, while nematode community analysis provides functional and structural information of higher trophic levels (Ferris et al., 2001). Together, this information helps to determine the status of the soil food web as affected by multiple global change drivers.

Microbial community

Soil microorganisms exert essential functions and processes in soil ecosystems. Soil microbial biomass provides the basal resource for soil biota, influences nutrient uptake of plants, and is correlated with the quantity of organic matter in soils (Pankhust et al. 1995, in: Yao et al., 2000). Thus, microbial biomass is considered a suitable indicator of soil quality (Yao et al., 2000).

Decomposition of organic matter is a crucial function driven by soil bacteria, archaea and fungi with great importance for nutrient cycling (Burns et al., 2013; Van Der Heijden et al., 2008). Decomposition is driven by extracellular enzymes that break down complex organic compounds from microorganism, plant and animal debris into smaller assimilable subunits (Caldwell, 2005; Sinsabaugh et al. 1993). Different microorganisms have different target substrates rich in carbon, nitrogen and/or phosphorus. Depending on the substrate, they synthesize different substrate-specific extracellular enzymes (Sinsabaugh et al., 2008; Sinsabaugh et al., 2009). Enzyme production imply high energy and nutrient costs for soil microorganisms. Microorganisms seek for obtaining the greatest benefits consuming the minimum amount of resources possible. Consequently, enzymes generally follow the so called

cellular economics. This is that to make sure that the resources are not being wasted, the synthesis of extracellular enzymes is induced by the presence of substrates in the soil environment. Once the demand is satisfied and the assimilable products reached a certain concentration, the production of enzymes ceases (Allison et al., 2010; Burns et al., 2013).

Extracellular enzymes catalyze the rate limiting steps in the processes of decomposition (Sinsabaugh, 1994), hence they are good indicators of microbial activity and soil fertility (Ajwa et al., 1999). Also, since extracellular enzymes are not always present in the soil environment but only when there are certain organisms and substrates, their presence helps to understand the current composition and functioning of the soil.

Expected effects of global change agents on soil microbial community

Global change affects microbial community composition directly or indirectly through changes in plant biomass, exudates and rhizodeposition (Finzi et al., 2006; Phillips et al., 2011). This can also lead to changes in extracellular enzyme activities indirectly following changes of microbial biomass or composition, or directly changes of denaturalization, turnover, and sorption rates (Burns et al., 2013). Here I focus on enzymes involved in the carbon, nitrogen and phosphorus cycles to study how global change affect the three different cycles.

Temperature and moisture are key factors influencing the composition and functioning of microbial communities (Baldrian et al., 2010; Rustad et al., 2001). Temperature directly increases soil microbial activity and biomass by enhancing metabolic rates (Zogg et al., 1997), but it can also indirectly be detrimental by intensifying increasing drought effects through increased evapotranspiration (Norby & Luo, 2004) and thus, decreasing moisture in soil which is a main determinant of microbial biomass (Nielsen et al., 2015). Due to the very sandy soil at the field site, decreased precipitation and elevated temperature were expected to have detrimental effects, decreasing microbial biomass.

Temperature and precipitation influence enzyme production, enzyme efficiency, and substrate availability. It is very difficult to predict the effects of elevated temperature on soil enzyme activity (Burns et al., 2013). Warming increases enzyme activity but at the same time it increases their denaturalization rates (Wallenstein et al., 2012; Wallenstein & Weintraub, 2008). Also some experiments experienced increased enzyme production to level up the increased cellular maintenance cost (Schimel & Weintraub, 2003; Wang et al., 2013; Wang & Post, 2012) but in others, synthesis and secretion of enzymes by microbes decreased (Allison, 2005). In the present

experiment, elevated temperature was expected to be rather beneficial for extracellular enzyme activity (EEA) despite of the predicted decreased microbial biomass. Other studies already showed results where microbial biomass and enzyme activity went different directions under global change agents. Therefore, EEA and microbial biomass, although related with each other, are two variables that should be considered separately (Steinweg et al., 2013).

Reduced soil moisture is expected to decrease EEA. Soil water content determines the substrates, products, and enzyme diffusion rates through soil as well as soil microbial biomass, which is why reduced precipitation may cause a consecutive decrease of EEA (Allison, 2005) and lower turnover (Steinweg et al., 2012). Therefore, precipitation was expected to decrease EEA.

Although temperature and soil moisture play a major role in soil functioning, they interact with other factors such as nutrient availability. Elevated nitrogen availability in soil has a negative effect on soil microbial biomass as a result of changes in rhizodeposition and decreased root exudation (Dijkstra et al., 2005; Eisenhauer et al., 2012). On the other hand, it increases decomposition rates, since in most of terrestrial systems decomposition is a nitrogen limited process (Sinsabaugh et al., 1993). Hence, most extracellular enzymes normally have elevated activity with nitrogen amendments (Ajwa et al., 1999; Saiya-Cork et al., 2002), except for those involved in nitrogen mineralization (Saiya-Cork et al., 2002). Nitrogen amendments redirect the energy and resources away from nitrogen mineralization and towards phosphorous acquisition (Sinsabaugh et al., 2002). Given that the study area is limited in nitrogen due to glacial outwash during episodic glaciations (Reich et al., 2001; Sinsabaugh et al., 2008) and previous studies in the same experimental area resulted in increased decomposition under elevated nitrogen scenarios (Cesarz et al., 2015), I expected elevated decomposition but reduced nitrogen mineralization rates and decreased microbial biomass.

Elevated CO_2 usually does not directly affect soil microbial activities and decomposition because soil normally already have high CO_2 concentrations (Burns et al., 2013). However, indirect effects through the impact on plants are likely. CO_2 increases soil microbial activity and biomass (Blagodatskaya et al., 2010; Eisenhauer et al., 2012), by increasing soil water content and rhizodeposition (Eisenhauer et al., 2012; Field et al., 1995; Jones et al., 2009). Also, changes in the nitrogen cycle may happen, since nitrogen and carbon are bounded in soil organic matter (McGill & Cole, 1981). In this experiment, elevated CO_2 was expected to have a positive effect on soil microbial biomass and minor positive effects on decomposition and EEA.

Nematode community

The soil nematode fauna has been increasingly used to study the composition and complexity of soil food webs (Yeates, 2003;Ferris et al., 2001). Nematode communities have many characteristics that make them unique bioindicators, as they are a very well known group -the most abundant taxa of metazoa- with different morphological and behavioral attributes and occupying key positions at different trophic levels. Further, they are a useful tool for assessing the functional composition of soil food webs (Ferris et al., 2001).

Here I focus on (1) the food resource status of the soil ecosystem, (2) the main decomposition channel, (3) the structure of the trophic web, and (4) nematode diversity to determine the effect of global change agents on soil biodiversity.

The food resource status indicates how enriched is the system and the resource availability to primary consumers. It is determined by the abundance of a functional nematode group called enrichment opportunists, which are bacterial-feeding opportunistic nematodes. This nematode group responds positively when the system is enriched, irrespective of the current condition of the trophic soil food web and environmental quality (Bongers & Ferris, 1999; Bongers, 1990). A disturbance may increase the resource availability through higher mortality, turnover or favorable changes in the system (Odum, 1985). This generates higher microbial activity from which bacterial-feeding nematodes benefit (Ferris et al., 2001).

The quality of entering resources determines whether decomposition is more bacterial -or fungal- driven. This is the basal energy source for the soil food web and will determine if bacterial -or fungal- feeding nematodes are more abundant (Ferris et al., 2001; Ruess & Ferris, 2004). Bacteria prefer moist and N-rich soils and readily decomposable substrates, while fungal decomposition is favored by a high C:N ratio and by more complex sources like cellulose and lignin (Ruess & Ferris, 2004). Therefore the C:N ratio of the plant material is essential for the balance of the different decomposition pathways. When the C:N ratio of the substrates is low, bacterial-feeding nematodes are more abundant than when the C:N is high, which in this case fungal-feeding nematodes are more dominant (Ferris et al., 2001; Ruess & Ferris, 2004).

The relative abundances of bacterial and fungal-feeding nematodes also shift with the succession in the decomposition progress. In early successional stages, readily decomposable sources are more abundant than in more mature systems, where complex substrates prevail. This translates into a shift during succession from bacterial- to fungal- dominated decomposition (Ruess & Ferris, 2004).

The structure and length of the trophic links are also related to the succession status of the soil ecosystem. When in a system the relative abundance of r-strategists

(opportunistic species) is high, the food web is rather simple. On the contrary, a food web with more trophic links reflects that the system is more mature and undisturbed (Ferris & Bongers, 2006; Ferris et al., 2004). In food webs with a high structure level, there are more links at higher trophic levels, and the proportion of K-strategist species is high. K-strategist have lower metabolic rates than r-strategists, which means that they are more energy-efficient. A food web with a highly developed structure must preserve the energy and carbon through many trophic levels (Ferris et al., 2001; Ferris & Bongers, 2006).

Resource enrichment may be related to the structure succession. When the enrichment status is sustained the succession may stop and the structure may be constant, but the structure and enrichment of the trophic food web are independent, since the structure is also affected by life history factors (Sudhaus et al. 1988 in: Ferris & Bongers, 2006). Therefore, they should be analyzed independently (Ferris et al., 2001).

Expected effects of global change agents on nematode community

Temperature was expected to have positive effects on nematode density and diversity (Blankinship et al., 2011). Precipitation was expected not to cause significant changes in the nematode community (Cesarz et al., 2015; Porazinska et al., 1998). Nematodes can stand dry conditions through anhydrobiosis (Treonis, Wall, & Virginia, 2000) and specifically in this experiment where the soil is very sandy, nematodes may be adapted to dry conditions. Only mild negative effects of reduced precipitation on diversity, density of K-strategist species and subsequently simplified community structure were expected (Cesarz et al., 2015). Mild positive effects on fungal-feeding nematodes were expected, since bacteria perform better in moist environments and fungi tolerate better dry conditions (Kardol et al., 2010; Griffiths et al. 1995 in: Ruess & Ferris, 2004). Fungi may take advantage of it due to less competence increasing their biomass (Wardle & Yeates, 1993). Reduced precipitation effects were expected to be intensified with elevated temperature and diminished by elevated CO_2.

A study in the same experimental area found nitrogen addition to decrease nematode richness (Eisenhauer et al., 2012). In Cesarz et al. (2015) - also in the same experimental area- as well as in Eisenhauer et al.(2012), nitrogen addition led to higher densities of opportunistic nematodes, reduced densities of K strategists, and a food web with simplified soil food webs. Given that elevated nitrogen is supposed to benefit the bacterial community, I expected a change in the proportion of bacterial and fungal-feeding nematodes towards a higher proportion of bacterial-feeding nematodes (Ruess

& Ferris, 2004) and a higher enrichment of the soil (Cesarz et al., 2015). Nevertheless, responses to nitrogen are complex and difficult to predict (Dijkstra et al., 2005).

Elevated CO_2 was expected to benefit opportunistic nematodes (Eisenhauer et al., 2012) as a response to a higher enrichment of the soil ecosystem (Ferris et al., 2001) and also to increase nematode density (Runion et al., 1994). But it was expected to find detrimental effects on K-strategist nematodes, with a subsequent negative effect on soil food web complexity (Cesarz et al., 2015).

Table 1: Expected main effects of the different global change agents on soil microbial and nematode communities (see text for more details and hypotheses for interactions).

		Elevated Temperature	Reduced Precipitation	Elevated Nitrogen	Elevated CO_2
Microbial community	Biomass	Decreased microbial biomass	Decreased microbial biomass	Decreased microbial biomass	Increased microbial biomass
	Decomposition	Increased decomposition	Decreased decomposition	Increased decomposition but lower nitrogen mineralization	Minor positive changes on decomposition and mineralization rates
Nematode community	r- Str			Higher density	Higher density
	K-Str		Lower density	Lower density	Lower density
	Structure		Simplified structure	Simplified structure	Simplified structure
	Enrichment			More enriched	More enriched
	Bacterial vs Fungal-		Benefit fungal-feeding nematodes	Benefit bacteria-feeding nematodes	
	Diveristy	Increased diversity and density	Decreased diversity	Decreased richness	Increased density

Research questions and hypotheses

My research questions are about the effects of elevated CO_2, nitrogen addition, elevated temperature, and reduced precipitation acting together that potentially have on soil food web structure and functioning. My main question is:

How does global change affect soil food web structure and functioning?

The following sub-questions regarding the microbial biomass, decomposition and nematode community were defined in order to answer the main research question.

1) How does global change affect the soil microbial biomass?

Hypothesis 1.1*: Global change factors interactively affect the soil microbial community.*

Hypothesis 1.2*: The ultimate expected outcome of a scenario with elevated CO_2, elevated nitrogen, elevated temperature, and reduced precipitation is a decreased microbial biomass compared to ambient conditions, although the biomass reduction will be mainly driven by warming and reduced precipitation.*

2) How does global change affect the soil microbial enzyme activities involved in the carbon, nitrogen, and phosphorous cycles?

Hypothesis 2.1*: Global change factors interactively affect the soil microbial enzyme activities involved in C, N, and P cycles. The final result of these interaction will be that C, N and P mineralization rates will be uncoupled, meaning this that the stoichiometry of the mineralization rates will be different than under ambient conditions.*

> ***Hypothesis 2.1.1****: Elevated nitrogen will cause a shift from nitrogen to phosphorous acquisition, increasing the activity of the enzymes involved in the phosphorous cycle and reducing the activity of those in the nitrogen cycle.*
>
> ***Hypothesis 2.1.2****: Although generally warming increases the activity, together with reduced precipitation is expected to intensify the effects of water deficiency and decrease the extracellular enzyme activity: however, these effects will be lower in elevated CO_2 scenarios.*

3) How does global change affects the structure and functional composition of the nematode community?

Hypothesis 3.1*: Global change factors interactively affect the structure and functional composition of soil food webs.*

Hypothesis 3.1.1*: Diversity is expected to decrease in response to the four global change agents interaction.*

Hypothesis 3.1.2*: Opportunistic nematodes will increase in abundance while K-strategists will decrease in response to elevated CO_2 and nitrogen. As a consequence the structure of the food web is simplified.*

Hypothesis 3.1.3*: Soil enrichment is expected to be higher due to the disturbance that these global change agents have on the system.*

Hypothesis 3.1.4*: Bacterial-feeding nematodes are expected to be increased relative to the fungal-feeding, proper of less mature and unstructured systems.*

Methods

Experimental design

This project was embedded in the framework of the TeRaCON experiment. It is a long-term global change experiment un grassland nested in the BioCON experiment at Cedar Creek, Minnesota, USA, established in 1997 (Reich et al., 2001). The region has a continental climate with warm summers (July average temperature 22ºC) and cold winters (January average temperature -11ºC) with a mean annual precipitation of 660mm (Reich et al., 2001) The experiment is set up on a sandy outwash (94.4% sand, 2.5% clay) soil in a secondary successional grassland (Eisenhauer et al., 2012; Reich et al., 2001). The area is divided in six FACE (free-air CO_2 enrichment) (Hendrey et al., 1993) rings, and those are again divided in plots (2x2 m) separated by metal barriers 30 cm deep and a 20 cm walkway buffer (Eisenhauer et al., 2012). Each plot receives a different set of treatments. TeRaCON manipulates **Te**mperature, **Ra**in, **CO_2**, and **N**itrogen in a full factorial design (2 temperature levels x 2 rain levels x 2 CO_2 levels x 2 nitrogen levels x 3 replicates; makes a total of 48 plots).

- Temperature: Ongoing since 2012. Ambient temperature and elevated temperature (ambient + 2ºC, 24h/d for ≈ 8 months per year) of both soil and plants. It is done with soil cable-based and lamp-based warming (Fig. 1, left). The magnitude of the increased 2ºC was established since it is the minimum expected change to occur in the next century in North America central regions (IPCC, 2007; TeRaCON NSF proposal). Plots are warmed in months when ambient temperatures are greater than -3 °C on average (TeRaCON NSF proposal).
- Rain: Ongoing since 2007. Summer drought is simulated with the help of rainout shelters that removed the rain (Fig. 1, right). It is used to reduce the number of precipitation events from May to August, to remove ~45% of the total rainfall (Eisenhauer et al., 2012). These quantity was chosen after some studies predicting a 10-40% lower moisture in this area in 2100 (Wuebbles & Hayhoe, 2004; IPCC, 2007).
- CO_2: Ongoing since 1997. Ambient CO_2 and elevated CO_2 (ambient +180ppm, 24h day^{-1} , ~ 560µmol mol^{-1} using FACE technology (Hendrey et al., 1993) at the ring level (Reich et al., 2001) (Fig. 1, right and cover figure).
- Nitrogen: Ongoing since 1997. 4gN $m^{-2}a^{-1}$ of slow-release ammonium nitrate (NH_4NO_3) has been added to eN plots in early May, June and July. This

quantity doubles the natural N availability in the system (Eisenhauer et al., 2012)

For my project, only plots that initially had a richness of 9 plant species were chosen. In each plot there were representatives from the four plant functional groups C_3 grasses, C_4 grasses, forbs, and N-fixing legumes (Eisenhauer et al., 2012). Those treatments and their combinations are applied to study mainly potential outcomes from the interactions rather than focusing on the effects of a particular future scenario (Eisenhauer et al., 2012).

Figure 1: (Left) Photograph showing the technology for the warming treatment in a plot in the TeRaCON experiment. Photo credit: Dr. Roy Rich. (Right) Photograph showing one FACE ring and four of the portable rainout shelters used for the reduced precipitation treatment in the TeRaCON experiment. Photo credit: Kary Worm (Eisenhauer et al., 2012).

Soil sampling

Samples for soil microbial measurements were taken in June 2012, August 2013, June 2014, and June 2015 when soil was neither too dry nor wet. Similarly, samples for nematode measurements were taken in June 2012, July 2013 and June 2014. Three soil samples (2cm diameter, 6 cm depth) were taken at each plot using steel corers for each measurement (if both microbial and nematode measures were sampled at the same time, six samples were taken). At least 10 cm would separate the sample points from each other. Soil samples were stored at 4ºC until further processing.

Soil microbial measurements

Soil microbial biomass were done by Eisenhauer lab group and provided for the present thesis. Microbial biomass (C_{mic}, µg Cg^{-1} soil dry mass) and microbial basal respiration (µL $O_2 \cdot h^{-1} \cdot g$ soil dry mass $^{-1}$) were determined using an O_2-microcompensation apparatus (Scheu, 1992). Soil microbial biomass was calculated adding D-glucose using the substrate induced method (SIR; Anderson & Domsch, 1978) by 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). Basal respiration was measured without substrate additions and by calculating the mean of the O_2 consumption rates after 14 and 24 hours from the start of the measurements (Eisenhauer et al., 2010).

The specific respiratory quotient (qO_2;µL O_2·mg C_{mic}^{-1}·h^{-1}) was also calculated by dividing the basal respiration by the microbial biomass. It is an indicator of the efficiency of carbon use by the microbial community (Eisenhauer et al., 2010) and it can reflect the microbial community succession status. Communities that recently suffered a disturbance use more energy per biomass unit than communities in equilibrium (Odum, 1985).

Extracellular enzyme activity analysis

I carried out an extracellular enzyme activity (EEA) analysis with samples obtained in June 2015 from the TeRaCON experiment. The selected enzymes were β-D-1,4-glucosidase, β-1.4-N-acetyl-glucosaminidase, alanine aminopeptidase, and acid phosphatase. Those 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 (βG): involved in the degradation of short chain cellulose oligomers (cellobiose) by catalyzing the hydrolysis of β-D-glucopiranosides (Eivazi & Tabatabai, 1988; Nannipieri et al., 2012).
- β-1.4-N-acetyl-glucosaminidase (NAG): catalyzes the hydrolysis of N-acetyl-β-D-glucosamine, an oligomer from chitin (German et al., 2011; Parham & Deng, 2000). Chitin is present in arthropods exoskeletons and fungal cell walls, and this enzyme is in mainly synthesized by fungi in soil ecosystems (Gooday, 1994). Although it forms part of both C and N cycles, its role in the N cycle is more important since chitin is one of the main components through which organic N is entering the soil (Olander & Vitousek, 2000).
- Alanine aminopeptidase (AAP): catalyzes the mineralization of peptides from alanine (Nannipieri et al., 2012).
- Acid phosphatase (APh): Hydrolyzes phosphoric mono-esters bonds into P. It is the principal enzyme involved in P mineralization in acidic soils (Olander & Vitousek, 2000).

They were measured using a fluorimetric enzyme assay, adapted from Saiya-Cork et al. (2002). The enzymes react with their respective MU-labeled substrates (methyl-umbelliferone) except for AAP. For the latter the substrate is dyed with another

fluorescent compound named methilcoumarin. The activity of these reactions is measured through their fluorescence.

For the essay, first the pH of the soil was determined (pH 5.5) and used to prepare the sample suspensions (1g soil of each sample and 125mL of 50mM sodium acetate buffer). Samples were homogenized for 1 min in an ultrasonic bath. Substrate solutions were prepared after testing for each enzyme the substrate concentration that better fitted for the activity assessment. I prepared four replicates 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 sample (50 µL distilled water and 200 µL respective sample suspension). Also, a quench for the calibration curve was prepared per essay with standard substrate concentrations (50 µL of the substrate solution with the concentrations 0 µmol/L, 2.5 µmol/L, 5 µmol/L, 10 µmol/L and 20 µmol/L) and 200 µL of the sample suspension, and the five respective negatives (200µL buffer). After incubating the samples for specific times for each enzyme in the dark (βG: 5.5h; NAG: 4h; AAP: 5.25h; APh: 2.5 h), I measured the activity with a fluorescence microplate reader at 365 nm excitation and 450 nm emission.

Table 2 :Extracellular enzymes analyzed, the corresponding substrate used for the assay, the enzyme commission number (EC) and the nutrient cycle they are involved in (German et al., 2011; Olander & Vitousek, 2000; Saiya-Cork et al., 2002).

Enzyme	Substrate	Code	Nutrient cycle
βG	4-MUB-β-D-glucoside	3.2.1.21	**C**
NAG	4-MUB-N-acetyl-β-D-glucosaminide	3.1.6.1	**C and N**
AAP	L-alanine-7-amido-4-methylcoumarin	3.4.11.1	**N**
APh	4-MUB-phosphate	3.1.3.2	**P**

Once the EEA had been measured, also the mass-specific enzyme activity (MSEA) was calculated, which is the EEA per unit of microbial biomass (Steinweg et al., 2013).

The stoichiometry of the EEA regarding the different cycle were estimated to assess relative changes of the cycles and to assess if the nutrient cycles are coupled/uncoupled. It was calculated as βG:(NAG+AAP) for C:N; C:P as βG:APh and N:P as (NAG+AAP):APh (Sinsabaugh et al., 2009; Steinweg et al., 2013).

Nematode community calculations and indices

Nematode extraction was done with approximately 10 g soil (fresh weight) with a modified Baermann method (Ruess, 1995). After the extraction (30 hours), nematodes were preserved in 4% formaldehyde. For 2012 and 2013, only density was calculated because identification could not be done to the genus level. In 2014 a more extensive

identification of the nematodes was done, so they were counted and identified to the family level. When an identified family presented more than one feeding type, it was identified to genus, so each nematode would be assigned a functional guild. Each group is assigned a colonizer-persister value (cp), which indicates their life strategy, ranging from 1 (r- strategist species) to 5 (K-strategist species) (Bongers, 1990), and the respective feeding habits (bacterivores, fungivores, omnivores or carnivores) to form functional guilds (Ba_x, Fu_x, Om_x, Ca_x, where x=cp) (Ferris et al., 2001). The following, different nematode indices were calculated based on the functional grouping of nematodes.

- Maturity index (MI2-5): It is the weighted mean of the cp values excluding cp1 nematodes. Cp1 nematodes are enrichment opportunist and respond positively to any enrichment in the environment. Therefore it is a powerful indicator of (pollution-induced) stress (Bongers & Bongers, 1998; Ferris et al., 2001).

$$\mathrm{MI2-5} = \sum \frac{v_i \times f_i}{n}$$

with v_i being the c-p value assigned to a family; f_i being the frequency of the family i; and n being the total number of individuals in the sample (Neher & Darby, 2006).

- Structure index (SI): represents the stability and structure of the ecosystem and the stability of trophic links. An ecosystem with a high SI means that it has many trophic links and that it is highly structured.

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

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) the weighted frequencies of Ba_2 and Fu_2 (Ferris et al., 2001).

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

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

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

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

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

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

Additionally, some biodiversity indices were calculated:

- Richness: number of different taxa.
- Shannon H': calculates the diversity (Pielou, 1966).

$$H' = -\sum(p_i \ln p_i)$$

- Shannon J': calculates the eveness (Shannon and Weaver, 1949).

$$J = \frac{H}{H_{max}}$$

Nematodes were extracted and identified by the Eisenhauer lab group.

Environmental data

Monthly mean temperature and monthly mean soil moisture were determined at the plot level. Soil temperature and volumetric water content (vwc) data were calculated for the months June 2012, August 2013, and June 2014. Soil temperature was determined by sensors at a soil depth of 13 cm. The vwc was determined hourly by hard-wired probes at a soil depth of 20 cm below ground (TeRaCON NSF proposal). Environmental data was measured by the Peter Reich's lab group.

Statistical analysis

Four-way ANOVA (RStudio Team 2015: RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA) was used to test the effects of CO_2 (ambient and elevated), nitrogen (ambient and elevated), temperature (ambient and elevated) and precipitation (ambient and reduced) and all possible combinations on soil microbial biomass (2012, 2013, 2014, 2015), qO_2 (2014, 2015), basal respiration (2014, 2015), nematode density (2012, 2013, 2014), nematode indices (MI2-5, SI, EI and CI, 2014), nematode functional guilds (cp1, cp2, cp3, cp4, and cp5, 2014), nematode diversity (Richness, Shannon H' and Shannon J', 2014), and MSEA (βG, NAG, AAP, APh 2015). Afterwards, a post-hock Tukey HSD test was performed to test significant differences among means. The effect of CO_2 was tested against the random effect of ring nested within CO_2, since the CO_2 treatment was applied at the ring level (Reich et al., 2001). Additionally, repeated measures ANOVA was used to test treatment effect and time effects on microbial biomass, nematode density, soil temperature and moisture.

Results

Soil microbial community analysis

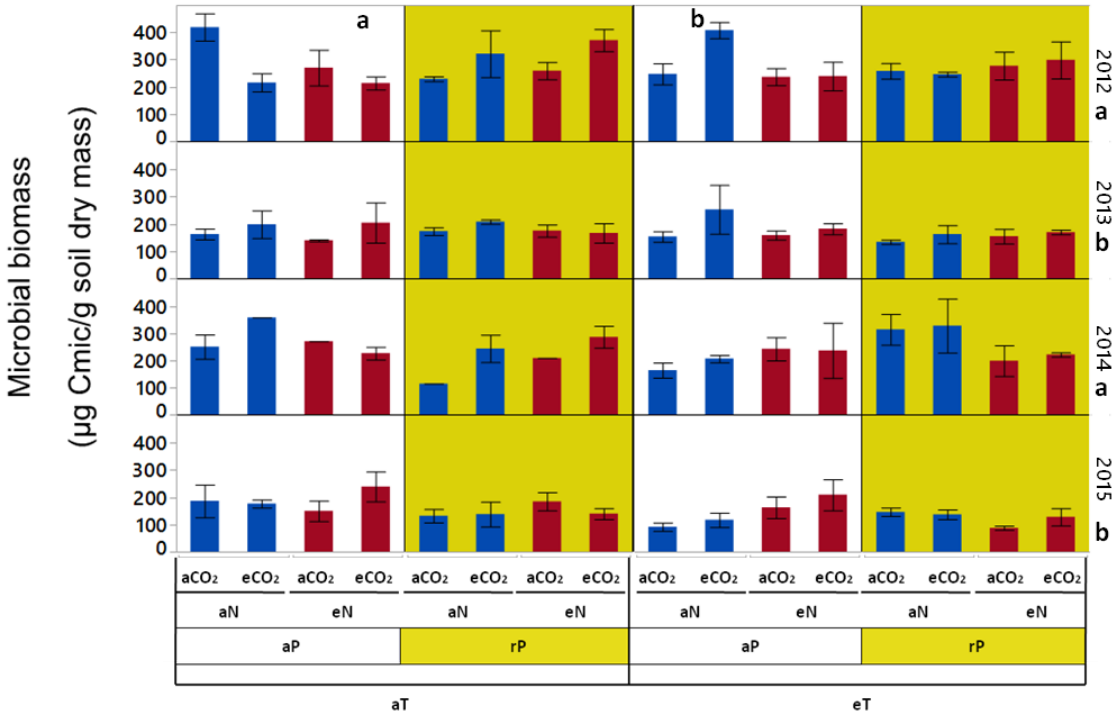

Figure 2: Microbial biomass (µg C_{mic} g^{-1}soil dry mass) as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature), and all the possible combinations in 2012, 2013, 2014 and 2015; and the comparison of microbial biomass between years (right). Mean ± SE. Bars with letters indicate what treatments combinations differed significantly, and letters on the right indicate what years differed significantly regarding microbial biomass (Tukey's HSD test, P<0.05).

Soil microbial properties (i.e. soil microbial biomass, qO_2 and basal respiration) and their dependence on the treatments differed among years, as they showed significant differences under different treatments interactions (Tab. 3, 4 and 5). To show significant differences under a certain treatments interaction means that the results are better explained by the effect that had those treatments and the interaction between them on the object of study. In this case, soil microbial biomass in 2014 showed significant differences under the interaction between the nitrogen, temperature and precipitation treatments (3-way interaction) (Tab.3). This means that the differences presented by soil microbial biomass in 2014 are better explained by the effect that nitrogen (ambient and elevated), temperature (ambient and elevated) and precipitation (ambient and reduced), and their combinations had on it. Soil microbial biomass in 2012 was affected by a significant 4-way interaction (i.e. four treatments interaction: CO_2, nitrogen, temperature and precipitation) while in 2013 and 2015 soil microbial biomass was not significantly affected by the experimental treatments. The general

microbial biomass also varied significantly among years (p-value: <0.000) being 2012 and 2014 significantly different from 2013 and 2015 (Fig 2) Monthly mean temperature and moisture (June 2012, August 2013 and June 2014) also differed significantly between years (soil temperature p-value: <0.0000; soil moisture p-value: <0.0000).

In 2012, soil microbial biomass was significantly higher at eCO_2 x aN x eT x aP than at eCO_2 x eN x aT x aP (significant 4-way interaction: Tab. 3, Fig. 2). In 2014, soil microbial biomass was affected by a significant interaction between nitrogen, temperature and precipitation (Tab. 3) , with the highest value on aN x eT x rP and the lowest on aN x eT x aP (Fig. 3). No significant treatment effects were found for qO_2 in 2014 (Tab. 4). Basal respiration in 2014 was affected by a significant interaction between CO_2 and temperature with the highest value at eCO_2 x aT and the lowest at aCO_2 x aT (Tab. 5, Fig. 4), and also by significant interaction between temperature and precipitation with the highest value at aT x aP and the lowest at eT x aP (Fig. 5). In 2015, there were no significant treatment effects on microbial biomass nor basal respiration (Tab. 3 and 5). qO_2 in 2015 was affected by a significant interaction between CO_2 and precipitation (Tab.4) with the highest value at eCO2 x rP and the lowest value was at eCO2 x aP (Fig. 6). This increase in qO_2 was due to a slight decrease in basal respiration and a substantial decrease in microbial biomass.

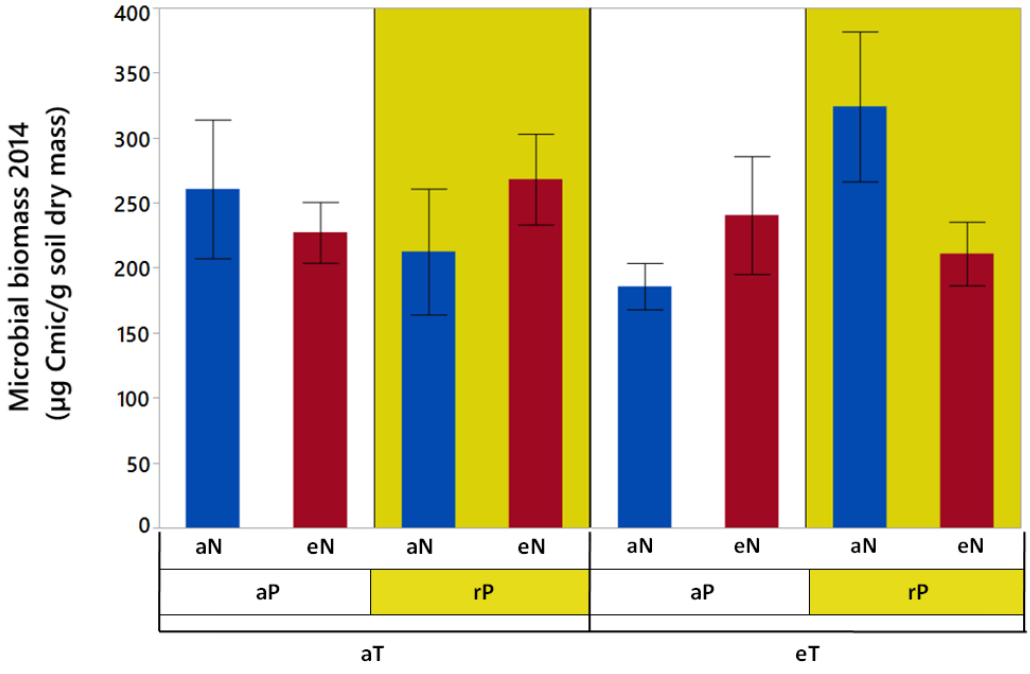

Figure 3: Microbial biomass (µg C_{mic} g^{-1}soil dry mass) as affected by nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all possible combinations in 2014. Mean ± SE.

Table 3: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated) , nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on microbial biomass in 2012, 2013, 2014 and 2015. Significant effects (P<0.05) are given in bold.

Treatment	2012			2013			2014			2015		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.1602	1	0.6890	3.2151	1	0.0730	0.6752	1	0.4112	0.3385	1	0.5607
Nitrogen	1.2581	1	0.2620	0.1318	1	0.7166	0.1395	1	0.7087	2.0955	1	0.1477
Temperature	0.3217	1	0.5706	0.3618	1	0.5475	0.0766	1	0.7819	3.2524	1	0.0713
Precipitation	0.0056	1	0.9402	0.7618	1	0.3828	0.0291	1	0.8645	2.7754	1	0.0957
CO_2 x Nitrogen	0.0664	1	0.7967	0.1628	1	0.6866	1.7494	1	0.1860	0.6155	1	0.4327
CO_2 x Temperature	2.0604	1	0.1512	0.0136	1	0.9072	0.3144	1	0.5750	0.1667	1	0.6831
Nitrogen x Temperature	0.0491	1	0.8246	0.0006	1	0.9801	0.3491	1	0.5546	0.1566	1	0.6923
CO_2 x Precipitation	3.7697	1	0.0522	1.1409	1	0.2855	0.0022	1	0.9627	1.7117	1	0.1908
Nitrogen x Precipitation	9.0798	1	**0.0026**	0.0585	1	0.8089	0.1557	1	0.6931	2.2865	1	0.1305
Temperature x Precipitation	0.5012	1	0.4790	0.1525	1	0.6961	7.9358	1	**0.0048**	0.7525	1	0.3857
CO_2 x Nitrogen x Temperature	3.3497	1	0.0672	0.2012	1	0.6537	2.7198	1	0.0991	0.0263	1	0.8712
CO_2 x Nitrogen x Precipitation	0.1585	1	0.6905	0.0164	1	0.8981	2.4553	1	0.1171	1.1909	1	0.2751
CO_2 x Temperature x Precipitation	14.8790	1	**0.0001**	0.0779	1	0.7801	2.5971	1	0.1071	0.3349	1	0.5628
Nitrogen x Temperature x Precipitation	0.0162	1	0.8989	0.4299	1	0.5120	8.9685	1	**0.0027**	3.1549	1	0.0757
CO_2 x Nitrogen x Temperature x Precipitation	3.9721	1	**0.0463**	0.5227	1	0.4697	0.3997	1	0.5272	2.0217	1	0.1551

Table 4: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated) , nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on the specific respiratory quotient (qO_2) in 2014 and 2015. Significant effects (P<0.05) are given in bold.

Treatment	2014			2015		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.0020	1	0.9641	0.0072	1	0.9325
Nitrogen	2.1797	1	0.1398	1.4622	1	0.2266
Temperature	0.2161	1	0.6421	0.0619	1	0.8035
Precipitation	2.7782	1	0.0956	0.1426	1	0.7057
CO_2 x Nitrogen	0.0776	1	0.7805	2.2868	1	0.1305
CO_2 x Temperature	3.6478	1	0.0561	0.0055	1	0.9411
Nitrogen x Temperature	0.4719	1	0.4921	0.4517	1	0.5015
CO_2 x Precipitation	1.3629	1	0.2430	5.0110	1	**0.0252**
Nitrogen x Precipitation	0.3411	1	0.5592	2.4817	1	0.1152
Temperature x Precipitation	0.4795	1	0.4887	3.0404	1	0.0812
CO_2 x Nitrogen x Temperature	0.1957	1	0.6582	0.1449	1	0.7034
CO_2 x Nitrogen x Precipitation	1.2875	1	0.2565	0.0911	1	0.7627
CO_2 x Temperature x Precipitation	1.8689	1	0.1716	0.0116	1	0.9141
Nitrogen x Temperature x Precipitation	0.2679	1	0.6047	3.4404	1	0.0636
CO_2 x Nitrogen x Temperature x Precipitation	0.8186	1	0.3656	0.8245	1	0.3639

Table 5: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated), nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on basal respiration 2014 and 2015. Significant effects (P<0.05) are given in bold.

Treatment	2014			2015		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.8033	1	0.3701	0.0090	1	0.9243
Nitrogen	3.1613	1	0.0754	0.0414	1	0.8387
Temperature	0.6710	1	0.4127	3.1613	1	0.0754
Precipitation	1.4719	1	0.2251	0.7311	1	0.3925
CO_2 x Nitrogen	1.5110	1	0.2190	2.3962	1	0.1216
CO_2 x Temperature	4.2117	1	**0.0401**	1.6285	1	0.2019
Nitrogen x Temperature	0.5093	1	0.4754	1.4054	1	0.2358
CO_2 x Precipitation	0.7821	1	0.3765	0.8909	1	0.3452
Nitrogen x Precipitation	0.1103	1	0.7398	0.0584	1	0.8091
Temperature x Precipitation	4.6800	1	**0.0305**	0.6049	1	0.4367
CO_2 x Nitrogen x Temperature	1.7637	1	0.1842	1.1072	1	0.2927
CO_2 x Nitrogen x Precipitation	0.0009	1	0.9765	0.0164	1	0.8981
CO_2 x Temperature x Precipitation	0.4993	1	0.4798	0.1537	1	0.6950
Nitrogen x Temperature x Precipitation	2.1055	1	0.1468	1.1241	1	0.2890
CO_2 x Nitrogen x Temperature x Precipitation	2.1214	1	0.1453	1.4902	1	0.2222

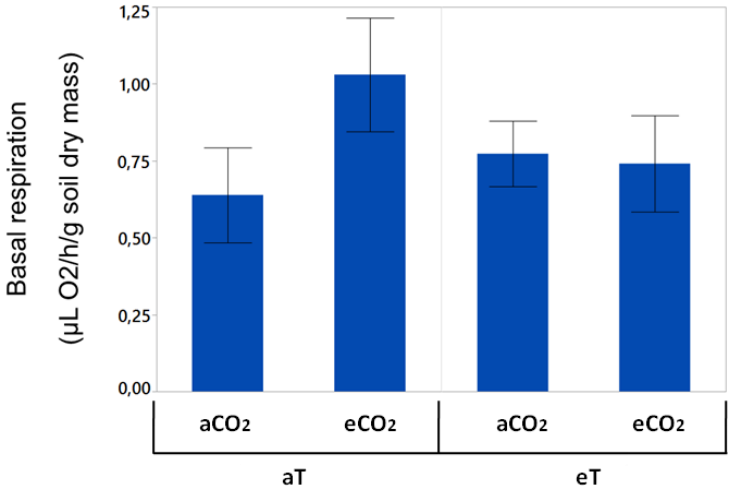

Figure 4: Basal respiration (µL $O_2 \cdot h^{-1} \cdot g$ soil dry mass $^{-1}$) as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all possible combinations in 2014. Mean ± SE.

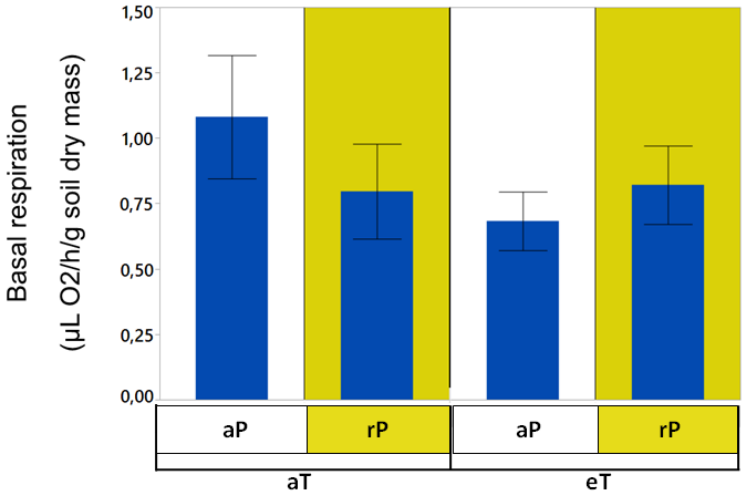

Figure 5: Basal respiration (µL $O_2 \cdot h^{-1} \cdot g$ soil dry mass $^{-1}$) as affected by precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature), and all possible combinations in 2014. Mean ± SE.

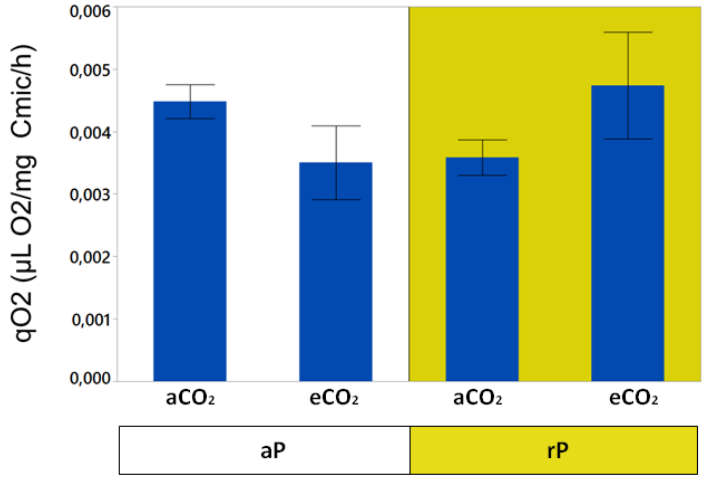

Figure 6: Specific respiratory quotient (qO_2;µL $O_2 \cdot$mg $C_{mic}^{-1} \cdot h^{-1}$) as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), and precipitation treatments (aP, ambient precipitation; rP, reduced precipitation), and all possible combinations in 2015. Mean ± SE.

Table 6: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated), nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on microbial extracellular enzyme activity (EEA) of alanine aminopeptidase, β-1,4-N-acetyl-glucosaminidase, β-D-1,4-glucosidase and acid phosphatase in 2015. Significant effects (P<0.05) are given in bold.

Treatment	Alanine aminopeptidase			β-1,4-N-acetyl-glucosaminidase			β-D-1,4-glucosidase			Acid phosphatase		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.2168	1	0.6415	0.2480	1	0.6185	0.6485	1	0.4207	0.1752	1	0.6756
Nitrogen	8.1427	1	**0.0043**	0.6983	1	0.4034	0.8329	1	0.3614	17.5439	1	**0.0000**
Temperature	0.0598	1	0.8069	1.1157	1	0.2908	6.6075	1	**0.0102**	0.3165	1	0.5737
Precipitation	1.7165	1	0.1902	4.8066	1	**0.0284**	0.8152	1	0.3666	1.7900	1	0.1809
CO_2 x Nitrogen	2.0606	1	0.1512	0.2728	1	0.6015	0.6956	1	0.4043	0.0887	1	0.7659
CO_2 x Temperature	1.5601	1	0.2117	0.5300	1	0.4666	2.8159	1	0.0933	0.1014	1	0.7501
Nitrogen x Temperature	0.0522	1	0.8192	0.0078	1	0.9298	1.3144	1	0.2516	3.0747	1	0.0795
CO_2 x Precipitation	0.1893	1	0.6635	1.4359	1	0.2308	0.0169	1	0.8965	0.0113	1	0.9155
Nitrogen x Precipitation	0.8518	1	0.3560	0.8779	1	0.3488	0.0811	1	0.7758	0.0016	1	0.9680
Temperature x Precipitation	0.7822	1	0.3765	0.9007	1	0.3426	1.2694	1	0.2599	5.7163	1	0.0168
CO_2 x Nitrogen x Temperature	1.7545	1	0.1853	0.2723	1	0.6018	2.5198	1	0.1124	1.4721	1	0.2250
CO_2 x Nitrogen x Precipitation	0.6688	1	0.4135	0.6168	1	0.4322	0.1269	1	0.7217	0.0003	1	0.9858
CO_2 x Temperature x Precipitation	2.1601	1	0.1416	1.9131	1	0.1666	0.8309	1	0.3620	2.3623	1	0.1243
Nitrogen x Temperature x Precipitation	0.6844	1	0.4081	0.0026	1	0.9594	0.7468	1	0.3875	3.6417	1	0.0564
CO_2 x Nitrogen x Temperature x Precipitation	0.0496	1	0.8238	0.7633	1	0.3823	2.8972	1	0.0887	0.0118	1	0.9136

Table 7: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated), nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on microbial mass specific enzyme activity (MSEA) of alanine aminopeptidase, β-1,4-N-acetyl-glucosaminidase, β-D-1,4-glucosidase and acid phosphatase in 2015. Significant effects (P<0.05) are given in bold.

Treatment	Alanine aminopeptidase			β-1,4-N-acetyl-glucosaminidase			β-D-1,4-glucosidase			Acid phosphatase		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.2175	1	0.6410	0.0040	1	0.9497	0.0022	1	0.9627	0.4676	1	0.4941
Nitrogen	0.0161	1	0.8991	1.2233	1	0.2687	0.5807	1	0.4460	1.5757	1	0.2094
Temperature	3.3507	1	0.0672	0.0442	1	0.8335	0.1810	1	0.6705	3.1042	1	0.0781
Precipitation	0.2943	1	0.5875	7.3692	1	**0.0066**	1.3698	1	0.2418	0.0051	1	0.9432
CO_2 x Nitrogen	1.0135	1	0.3141	2.3287	1	0.1270	1.1560	1	0.2823	0.1308	1	0.7176
CO_2 x Temperature	1.4748	1	0.2246	3.1310	1	0.0768	1.2535	1	0.2629	0.0010	1	0.9751
Nitrogen x Temperature	0.1634	1	0.6861	0.6456	1	0.4217	0.7600	1	0.3833	1.2094	1	0.2714
CO_2 x Precipitation	3.2207	1	0.0727	2.0138	1	0.1559	0.6201	1	0.4310	2.5287	1	0.1118
Nitrogen x Precipitation	6.8760	1	**0.0087**	2.4599	1	0.1168	4.0507	1	**0.0442**	4.2118	1	**0.0401**
Temperature x Precipitation	0.6733	1	0.4119	1.5258	1	0.2167	3.2136	1	0.0730	0.4613	1	0.4970
CO_2 x Nitrogen x Temperature	0.7680	1	0.3808	0.0284	1	0.8663	1.5231	1	0.2171	0.2736	1	0.6009
CO_2 x Nitrogen x Precipitation	0.3062	1	0.5800	0.2581	1	0.6114	0.0321	1	0.8578	0.1170	1	0.7323
CO_2 x Temperature x Precipitation	0.2350	1	0.6278	4.5186	1	**0.0335**	2.9492	1	0.0859	0.0688	1	0.7931
Nitrogen x Temperature x Precipitation	5.6639	1	**0.0173**	3.8586	1	**0.0495**	6.3397	1	**0.0118**	9.2515	1	**0.0024**
CO_2 x Nitrogen x Temperature x Precipitation	0.4757	1	0.4904	0.0056	1	0.9405	0.0153	1	0.9015	1.3412	1	0.2468

Table 8: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated), nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on C:N [βG:(AAP+NAG)], C:P [βG:APh] and N:P [(AAP+NAG):APh] ratios in 2015. Significant effects (P<0.05) are given in bold.

Treatment	C:N			C:P			N:P		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.0919	1	0.7617	0.5026	1	0.4783	0.5857	1	0.4441
Nitrogen	0.2405	1	0.6239	8.6325	1	**0.0033**	4.2112	1	**0.0402**
Temperature	0.0283	1	0.8665	5.0962	1	**0.0240**	1.1989	1	0.2735
Precipitation	0.5030	1	0.4782	6.5479	1	**0.0105**	3.9173	1	**0.0478**
CO_2 x Nitrogen	0.0477	1	0.8272	0.8997	1	0.3429	0.4900	1	0.4839
CO_2 x Temperature	0.1914	1	0.6618	2.2052	1	0.1375	1.7452	1	0.1865
Nitrogen x Temperature	0.0663	1	0.7968	1.6001	1	0.2059	1.2541	1	0.2628
CO_2 x Precipitation	0.1576	1	0.6913	0.1497	1	0.6989	0.8538	1	0.3555
Nitrogen x Precipitation	0.2012	1	0.6537	0.0836	1	0.7725	0.0636	1	0.8008
Temperature x Precipitation	0.2357	1	0.6273	8.1154	1	**0.0044**	2.4832	1	0.1151
CO_2 x Nitrogen x Temperature	0.8495	1	0.3567	0.1694	1	0.6807	1.2937	1	0.2554
CO_2 x Nitrogen x Precipitation	0.1191	1	0.7300	0.1034	1	0.7478	0.1525	1	0.6962
CO_2 x Temperature x Precipitation	0.0001	1	0.9936	5.5893	1	**0.0181**	2.5865	1	0.1078
Nitrogen x Temperature x Precipitation	0.1548	1	0.6940	0.3416	1	0.5589	0.2146	1	0.6432
CO_2 x Nitrogen x Temperature x Precipitation	0.6640	1	0.4151	8.1539	1	**0.0043**	1.5932	1	0.2069

EEA results

The overall result from the EEA analysis shows that the activity of all the enzymes analyzed were affected by global change. The EEA analysis had significant differences under the effect of different single global change agents for each enzyme (Tab. 6). The four enzymes MSEA analysis were also affected by global change, being the four of them affected by the significant 3-way interaction between nitrogen, temperature and precipitation (Tab. 7), proving that those are important factors concerning the mineralization of SOM in the C, N and P cycles. This is in concordance to what it was expected according elevated CO_2 not to be an important agent in determining soil enzymatic activity.

The EEA analysis of the enzyme βG showed a significantly higher activity at ambient temperature (Fig. 7, left). The MSEA analysis showed a significantly higher activity at aN x eT x aP than at eN x eT x aP (significant 3-way interaction between nitrogen, temperature and precipitation: Fig. 8).

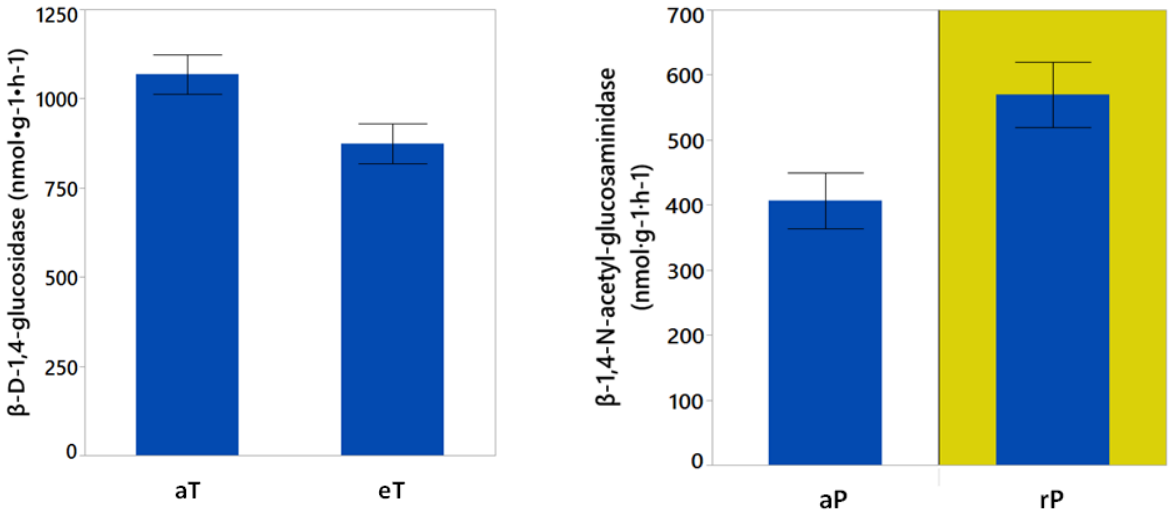

Figure 7: (Left) β-D-1,4-glucosidase activity (nmol·g^{-1}·h^{-1}) as affected by the temperature treatment (aT, ambient temperature; eT elevated temperature) in 2015; (Right) β-1,4-N-acetyl-glucosaminidase activity (nmol·g^{-1}·h^{-1}) as affected by the precipitation treatment (aP, ambient precipitation; rP, reduced precipitation) in 2015. Mean ± SE.

NAG results on the EEA analysis revealed that precipitation significantly affected it showing a higher activity under reduced precipitation (Fig. 7, right).The MSEA analysis presented a significantly higher value at aN x aT x rP than eN x aT x aP (significant 3-way interaction between nitrogen, temperature and precipitation: Fig. 8). Also the interaction CO_2, temperature and precipitation was significant, with a significantly higher activity at eCO_2 x aT x rP than at eCO_2 x aT x aP and eCO_2 x eT x aP (Fig. 9). This was the only case where CO_2 resulted to be an explanatory factor in the MSEA

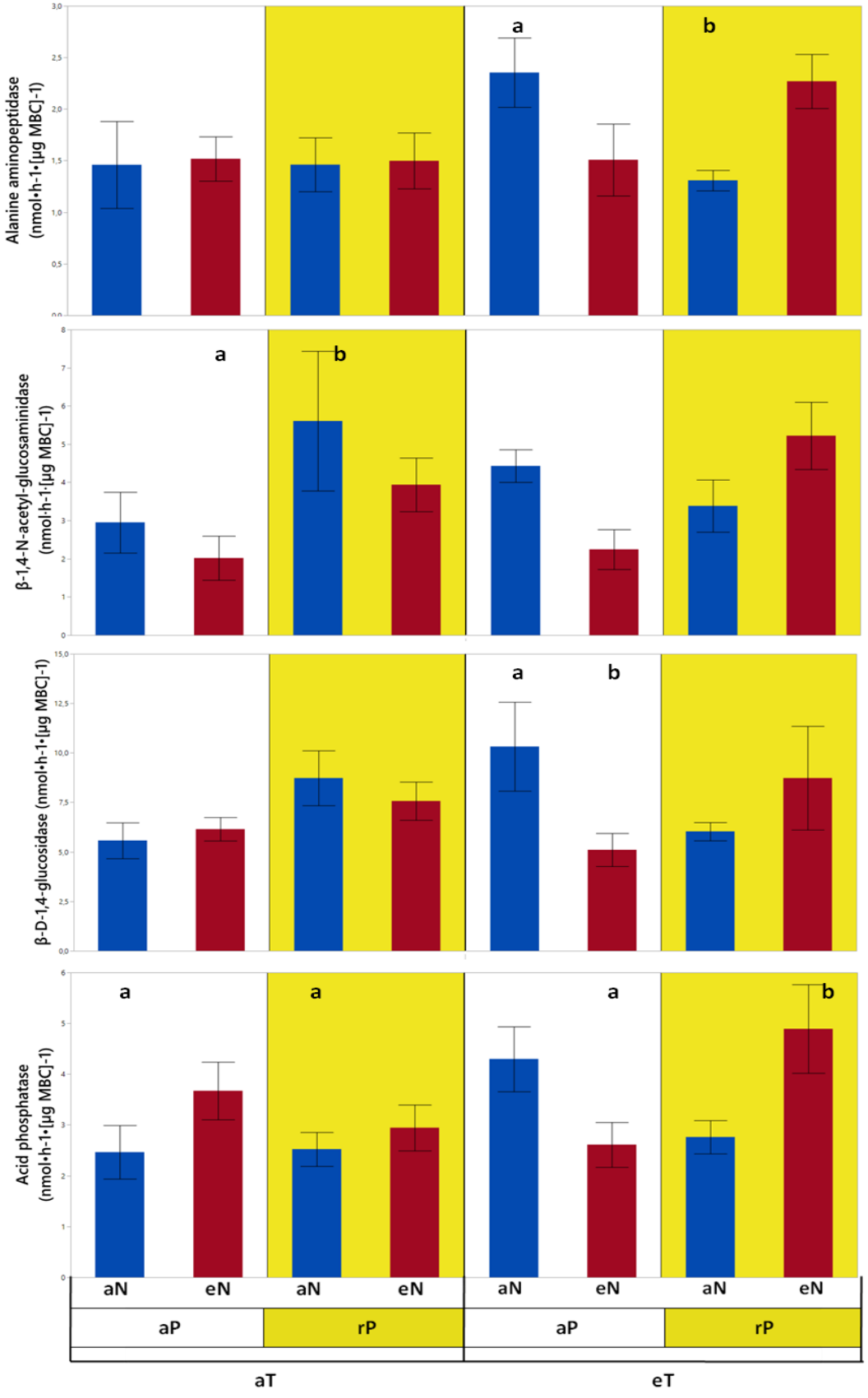

Figure 8: Microbial mass specific enzyme activity (MSEA; nmol·h^{-1} [µg MBC]$^{-1}$) of alanine aminopeptidase, β-1,4-N-acetyl-glucosaminidase, β-D-1,4-glucosidase and acid phosphatase as affected by nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature), and all possible combinations in 2015. Mean ± SE. Bars with letters indicate what treatments combinations differ significantly (Tukey's HSD test, P<0.05).

analysis results. This accords with Dorodnikov et al., (2009) findings, where chitinases were the only enzymes measured in their experiment affected by elevated CO_2 concentrations. This is may be due to rhizosphere microbes allocating C towards the production of enzymes in charge of N mineralization (Burns et al., 2013), although AAP, also involved in N mineralization, did not give the same result.

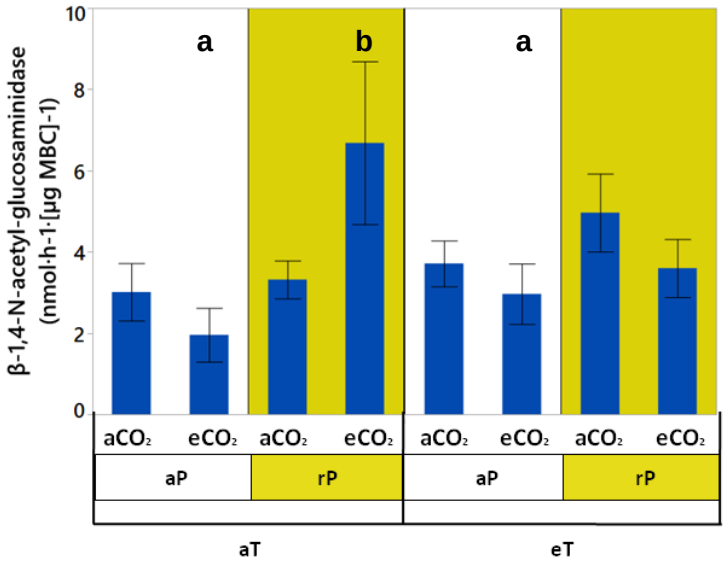

Figure 9: Microbial mass specific enzyme activity (MSEA; nmol·h^{-1} [µg MBC]$^{-1}$) of β-1,4-N-acetyl-glucosaminidase, as affected by CO_2 (aCO_2, ambient CO_2 ; eCO_2 , elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature (aT, ambient temperature; eT elevated temperature), and all possible combinations in 2015. Mean ± SE. Bars with letters indicate what treatments combinations differ significantly (Tukey's HSD test, P<0.05).

AAP activity showed significant higher activity under elevated nitrogen (Fig. 10, left). The MSEA analysis showed the scenario aN x eT x aP had a significantly higher activity than aN x eT x rP (significant 3-way interaction between nitrogen, temperature and precipitation: Fig. 8).

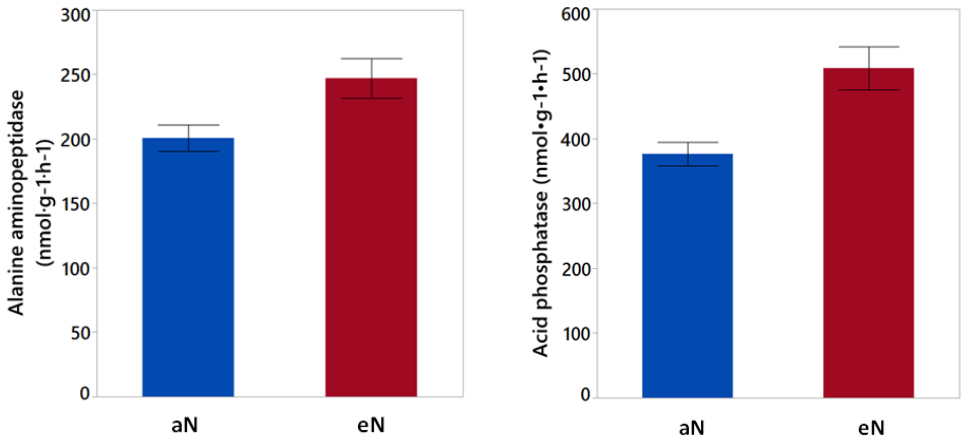

Figure 10:(Left) Alanine aminopeptidase activity (nmol·g^{-1}·h^{-1}) as affected by the nitrogen treatment (aN, ambient nitrogen; eN, elevated nitrogen) in 2015; (Right) Acid phosphatase activity (nmol·g^{-1}·h^{-1}) as affected by the nitrogen treatment (aN, ambient nitrogen; eN, elevated nitrogen) in 2015. Mean ± SE.

EEA analysis for APh presented a significant higher activity with elevated nitrogen (Fig. 10, right). This response to elevated nitrogen agrees to what it was predicted, showing higher activity towards P acquisition under elevated N. The MSEA analysis showed a significantly higher values at eN x eT x rP than at aN x aT x aP, aN x aT x rP and eN x eT x aP (significant 3-way interaction between nitrogen, temperature and precipitation: Fig. 8).

All enzymes presented high activity with eN x eT x rP (Fig. 8). It is unexpected that low precipitation show higher rates of activity, specially acting together with temperature, since the water stress for the microbial biomass is higher.

AAP results do not coincide either with what it was expected, which was a decrease in the activity of the enzymes involved in the N cycle under elevated nitrogen. However, that NAG and AAP activities were not significantly affected by the same factors -even though they are both importantly involved in the N cycle- is not unexpected, given that both enzymes work on different substrates (Saiya-Cork et al., 2002).

The ratio C:N did not give any significant difference (Table 8). The ratios C:P was significantly affected by the 4-way interaction. The ratio N:P was significantly affected by the single effects of nitrogen and precipitation. The figure 12 (B) shows that the relative activity of the enzymes also changes. All the enzymes present higher MSEA under global change (Fig 12, C and D), and the EEA of all enzymes except for βG increased with global change (Fig. 12, A and B). These results suggest that the carbon, nitrogen and phosphorous cycles have been affected by global change.

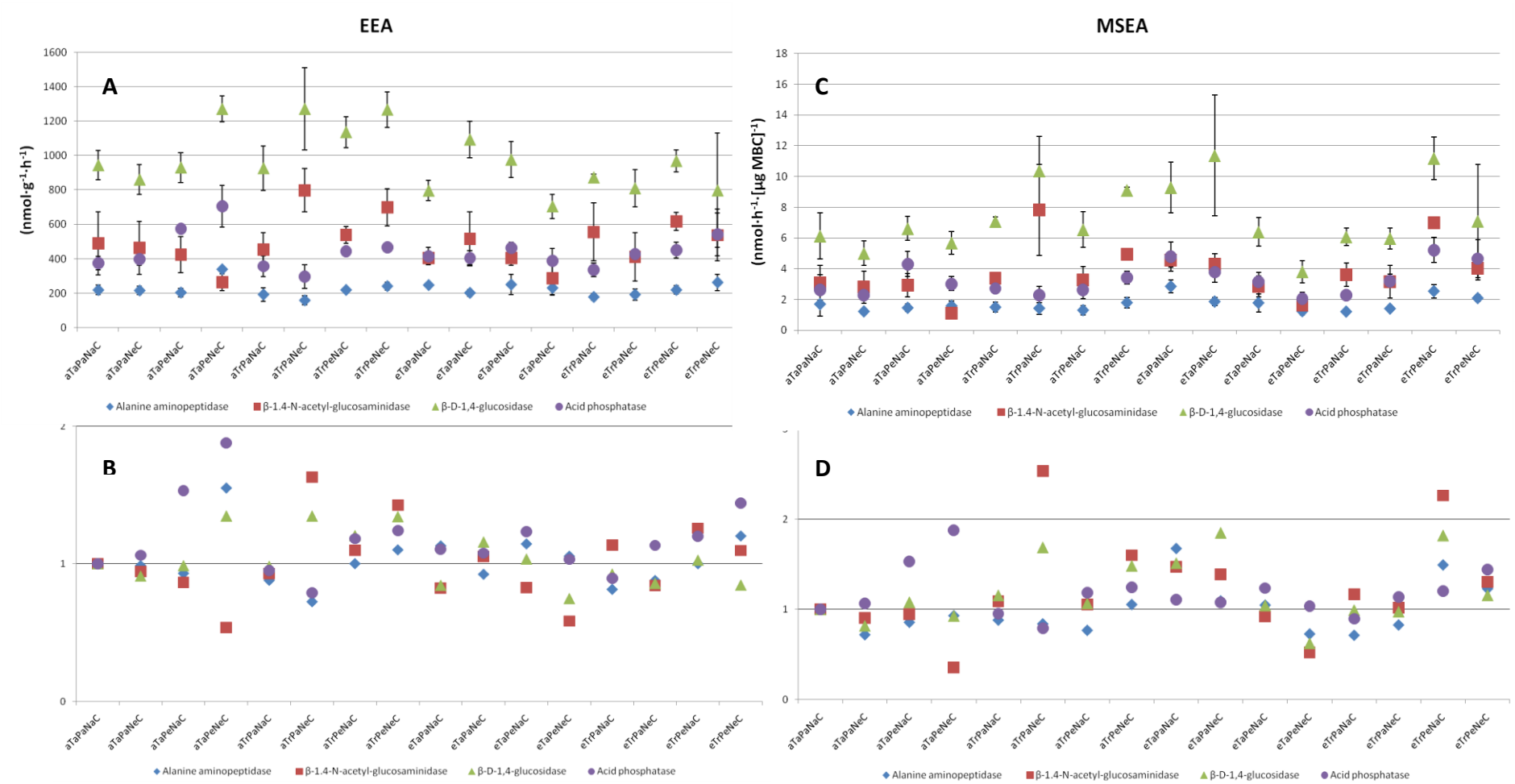

Figure 12: (A) Activity (nmol·g^{-1}·h^{-1}), (B) relative activity to the ambient scenario, (C) mass specific enzyme activity (MSEA; nmol·h^{-1} [µg MBC]$^{-1}$) and (D) relative MSEA to the ambient scenario of alanine aminopeptidase, β-1,4-N-acetyl-glucosaminidase, β-D-1,4-glucosidase and acid phosphatase as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature), and all the different combinations. Mean ± SE.

Table 9: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated) , nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on nematode density in 2012, 2013, and 2014. Significant effects (P<0.05) are given in bold.

Treatment	2012			2013			2014		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	2.1730	1	0.1405	0.0045	1	0.9466	0.4711	1	0.4925
Nitrogen	0.0241	1	0.8765	0.0001	1	0.9924	1.6300	1	0.2017
Temperature	0.0793	1	0.7782	0.6306	1	0.4271	4.5278	1	**0.0333**
Precipitation	0.0008	1	0.9768	0.9712	1	0.3244	0.0591	1	0.8079
CO_2 x Nitrogen	4.9890	1	**0.0255**	0.8613	1	0.3534	0.5323	1	0.4657
CO_2 x Temperature	0.4234	1	0.5153	0.6306	1	0.4271	2.0412	1	0.1531
Nitrogen x Temperature	0.8874	1	0.3462	0.0572	1	0.8110	0.2670	1	0.6053
CO_2 x Precipitation	0.0416	1	0.8384	0.0770	1	0.7814	1.0682	1	0.3014
Nitrogen x Precipitation	0.2649	1	0.6068	0.0770	1	0.7814	3.9041	1	**0.0482**
Temperature x Precipitation	0.1826	1	0.6692	0.2022	1	0.6529	0.2670	1	0.6053
CO_2 x Nitrogen x Temperature	0.0793	1	0.7782	2.6768	1	0.1018	0.0333	1	0.8553
CO_2 x Nitrogen x Precipitation	0.0001	1	0.9923	2.7397	1	0.0979	0.0748	1	0.7844
CO_2 x Temperature x Precipitation	0.0966	1	0.7560	2.5530	1	0.1101	0.4472	1	0.5036
Nitrogen x Temperature x Precipitation	0.5164	1	0.4724	2.4922	1	0.1144	0.6736	1	0.4118
CO_2 x Nitrogen x Temperature x Precipitation	0.1509	1	0.6977	1.1279	1	0.2882	1.9553	1	0.1620

Table 10: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated) , nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on the nematode maturity index (MI2-5), enrichment index (EI), structure index (SI) and channel index (CI) in 2014. Significant effects (P<0.05) are given in bold.

Treatment	MI 2-5			EI			SI			CI		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.0986	1	0.7535	0.7344	1	0.3914	0.0073	1	0.9321	0.1493	1	0.6992
Nitrogen	0.4712	1	0.4924	0.5163	1	0.4724	0.1512	1	0.6974	0.2382	1	0.6255
Temperature	3.2837	1	0.0700	1.1292	1	0.2879	0.8613	1	0.3534	2.4722	1	0.1159
Precipitation	1.4325	1	0.2314	5.2110	1	**0.0224**	1.2191	1	0.2695	1.0650	1	0.3021
CO_2 x Nitrogen	0.3970	1	0.5286	0.7748	1	0.3787	0.3490	1	0.5547	1.0773	1	0.2993
CO_2 x Temperature	1.5674	1	0.2106	0.0594	1	0.8074	0.8958	1	0.3439	1.1483	1	0.2839
Nitrogen x Temperature	0.7594	1	0.3835	0.5301	1	0.4666	0.7734	1	0.3792	0.1937	1	0.6599
CO_2 x Precipitation	1.1835	1	0.2766	0.0009	1	0.9759	1.9417	1	0.1635	0.8473	1	0.3573
Nitrogen x Precipitation	1.5375	1	0.2150	0.8871	1	0.3463	2.0029	1	0.1570	0.3143	1	0.5750
Temperature x Precipitation	7.6235	1	**0.0058**	6.8461	1	**0.0089**	7.0902	1	**0.0078**	9.9427	1	**0.0016**
CO_2 x Nitrogen x Temperature	0.2449	1	0.6207	4.2667	1	**0.0389**	0.0491	1	0.8247	1.7203	1	0.1897
CO_2 x Nitrogen x Precipitation	0.0448	1	0.8324	2.8541	1	0.0911	0.0798	1	0.7776	0.1797	1	0.6717
CO_2 x Temperature x Precipitation	0.9528	1	0.3290	0.5291	1	0.4670	1.3255	1	0.2496	0.9701	1	0.3247
Nitrogen x Temperature x Precipitation	0.0899	1	0.7643	0.0261	1	0.8717	0.0385	1	0.8445	0.1741	1	0.6765
CO_2 x Nitrogen x Temperature x Precipitation	5.1506	1	**0.0232**	0.1765	1	0.6744	6.7857	1	**0.0092**	0.3121	1	0.5764

Table 11: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated) , nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on the density of nematode groups according to their colonizer-persister value (cp) in 2014. Significant effects (P<0.05) are given in bold.

Treatment	cp1			cp2			cp3			cp4			cp5		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	0.1100	1	0.7401	0.5392	1	0.4628	0.0000	1	0.9988	0.4910	1	0.4835	0.1488	1	0.6997
Nitrogen	0.0158	1	0.9000	0.0173	1	0.8954	1.6467	1	0.1994	0.5800	1	0.4463	0.1117	1	0.7382
Temperature	0.1617	1	0.6876	1.1709	1	0.2792	0.4731	1	0.4916	2.8829	1	0.0895	0.0106	1	0.9180
Precipitation	0.5339	1	0.4650	1.8056	1	0.1790	5.1808	1	**0.0228**	1.2345	1	0.2665	0.2440	1	0.6213
CO_2 x Nitrogen	0.0043	1	0.9476	0.1418	1	0.7065	0.3334	1	0.5637	0.3193	1	0.5720	1.5725	1	0.2098
CO_2 x Temperature	0.0232	1	0.8789	1.3775	1	0.2405	0.4742	1	0.4911	1.4144	1	0.2343	0.2719	1	0.6021
Nitrogen x Temperature	0.5642	1	0.4526	2.1643	1	0.1413	1.0301	1	0.3101	0.0000	1	0.9998	1.1672	1	0.2800
CO_2 x Precipitation	0.0494	1	0.8241	1.0850	1	0.2976	0.5038	1	0.4779	0.5891	1	0.4427	0.0109	1	0.9168
Nitrogen x Precipitation	0.4068	1	0.5236	0.9021	1	0.3422	1.9592	1	0.1616	0.4335	1	0.5103	0.1892	1	0.6635
Temperature x Precipitation	2.8026	1	0.0941	0.4966	1	0.4810	0.7809	1	0.3769	1.5411	1	0.2145	2.4831	1	0.1151
CO_2 x Nitrogen x Temperature	1.4726	1	0.2249	0.0335	1	0.8547	3.0403	1	0.0812	0.6007	1	0.4383	0.0003	1	0.9868
CO_2 x Nitrogen x Precipitation	5.1564	1	**0.0232**	2.4309	1	0.1190	0.0399	1	0.8418	0.0003	1	0.9858	0.0034	1	0.9536
CO_2 x Temperature x Precipitation	0.0123	1	0.9116	0.0234	1	0.8785	0.6026	1	0.4376	1.4865	1	0.2228	1.6949	1	0.1930
Nitrogen x Temperature x Precipitation	0.3283	1	0.5667	0.3896	1	0.5325	0.8220	1	0.3646	0.1596	1	0.6895	0.4166	1	0.5187
CO_2 x Nitrogen x Temperature x Precipitation	0.2741	1	0.6006	4.6039	1	**0.0319**	2.2543	1	0.1332	1.7397	1	0.1872	0.2048	1	0.6509

Table 12: ANOVA table of X^2 and P values on the effects of CO_2 (ambient and elevated) , nitrogen (ambient and elevated), precipitation (ambient and reduced), and temperature (ambient and elevated), and all possible interactions on nematode richness, diversity (Shannon H') and eveness (Shannon J') in 2014. Significant effects (P<0.05) are given in bold.

Treatment	Richness			Shannon H'			Shannon J'		
	X2	**df**	**pValue**	**X2**	**df**	**pValue**	**X2**	**df**	**pValue**
CO_2	1.9246	1	0.1654	4.6562	1	**0.0309**	0.7947	1	0.3727
Nitrogen	0.3859	1	0.5344	0.5362	1	0.4640	5.2598	1	**0.0218**
Temperature	8.5772	1	**0.0034**	4.3751	1	**0.0365**	2.7223	1	0.0990
Precipitation	1.7722	1	0.1831	1.5054	1	0.2198	0.1912	1	0.6619
CO_2 x Nitrogen	2.0163	1	0.1556	2.3912	1	0.1220	0.0409	1	0.8398
CO_2 x Temperature	1.1342	1	0.2869	1.1557	1	0.2824	0.6078	1	0.4356
Nitrogen x Temperature	0.0079	1	0.9293	0.3124	1	0.5762	1.5724	1	0.2099
CO_2 x Precipitation	0.0000	1	1.0000	0.1229	1	0.7259	2.7359	1	0.0981
Nitrogen x Precipitation	4.1665	1	**0.0412**	2.5442	1	0.1107	0.0011	1	0.9737
Temperature x Precipitation	0.0709	1	0.7901	0.0107	1	0.9178	0.0787	1	0.7791
CO_2 x Nitrogen x Temperature	0.0315	1	0.8591	0.1786	1	0.6726	0.3287	1	0.5664
CO_2 x Nitrogen x Precipitation	0.0315	1	0.8591	0.0041	1	0.9488	0.4521	1	0.5014
CO_2 x Temperature x Precipitation	0.7876	1	0.3748	0.0041	1	0.9488	1.9323	1	0.1645
Nitrogen x Temperature x Precipitation	0.3859	1	0.5344	0.7681	1	0.3808	0.3727	1	0.5415
CO_2 x Nitrogen x Temperature x Precipitation	0.1260	1	0.7226	0.6691	1	0.4134	3.5535	1	0.0594

Nematode community

The four nematode indices in 2014 were affected by a significant interaction between temperature with precipitation (Tab. 10). Interestingly, in all of them the scenario eT x rP had a lower effect than elevated temperature or reduced precipitation acting alone (Fig. 13), showing non-additive effects of both global change agents.

Nematode community structure (MI2-5 and SI indices) was affected by a significant interaction between temperature and precipitation. Both SI and MI2-5 showed very similar results, with a significant higher value at aT x aP than at aT x rP (and eT x aP in the case of MI2-5) (Fig.13). SI and MI2-5 were also significantly affected by the 4-way interaction. Here the highest value is at aCO_2 x aN x aT x aP, and the lowest under aCO_2 x aN x aT x rP (Fig. 3 Appendix). Reduced precipitation showed to have the expected detrimental effect in the community structure. It was expected that CO_2 and nitrogen were also expected to present detrimental effects in SI and MI2-5, but the results showed different responses to those global change agents, not always being detrimental. This suggests that the effect of the four global change agents acting in concert has an important effect on the composition of the nematode community. This relates to the results of the different nematode functional groups (Tab. 11). Nematode functional groups showed complex results and different from each other, reflecting that each functional nematode groups were affected in different manners by global change. Cp1 was significantly affected by the interaction between CO_2, nitrogen and precipitation (Fig. 6 Appendix), while cp2 by the 4-way interaction. Both nitrogen and CO_2 were variables expected to benefit those two r-Strategists groups, specially cp2, but it does not seem to be the general pattern (Fig. 5 Appendix). The nematode functional group cp3 was significantly affected by precipitation, and presented lower densities at reduced precipitation (Fig. 7 Appendix); cp4 and cp5 did not show any significant differences.

The EI index presented at the scenario aT x rP a significant higher value than aT x aP (significant 2-way interaction between temperature and precipitation: Fig. 13). The interaction elevated temperature and reduced precipitation (eT x rP) did not show the highest value, showing again a non-additive effect of both agents. EI was also significantly affected by the 3-way interaction between CO_2, nitrogen and temperature, with the highest value was under eCO_2 x aN x aT and the lowest value under eCO_2 x eN x aT (Fig. 4 Appendix).

The CI index was only affected by the significant interaction between temperature and precipitation. At aT x aP the index was significantly higher than at aT x rP and eT x aP (Fig. 13). This means that decomposition channel is fungal-dominated under

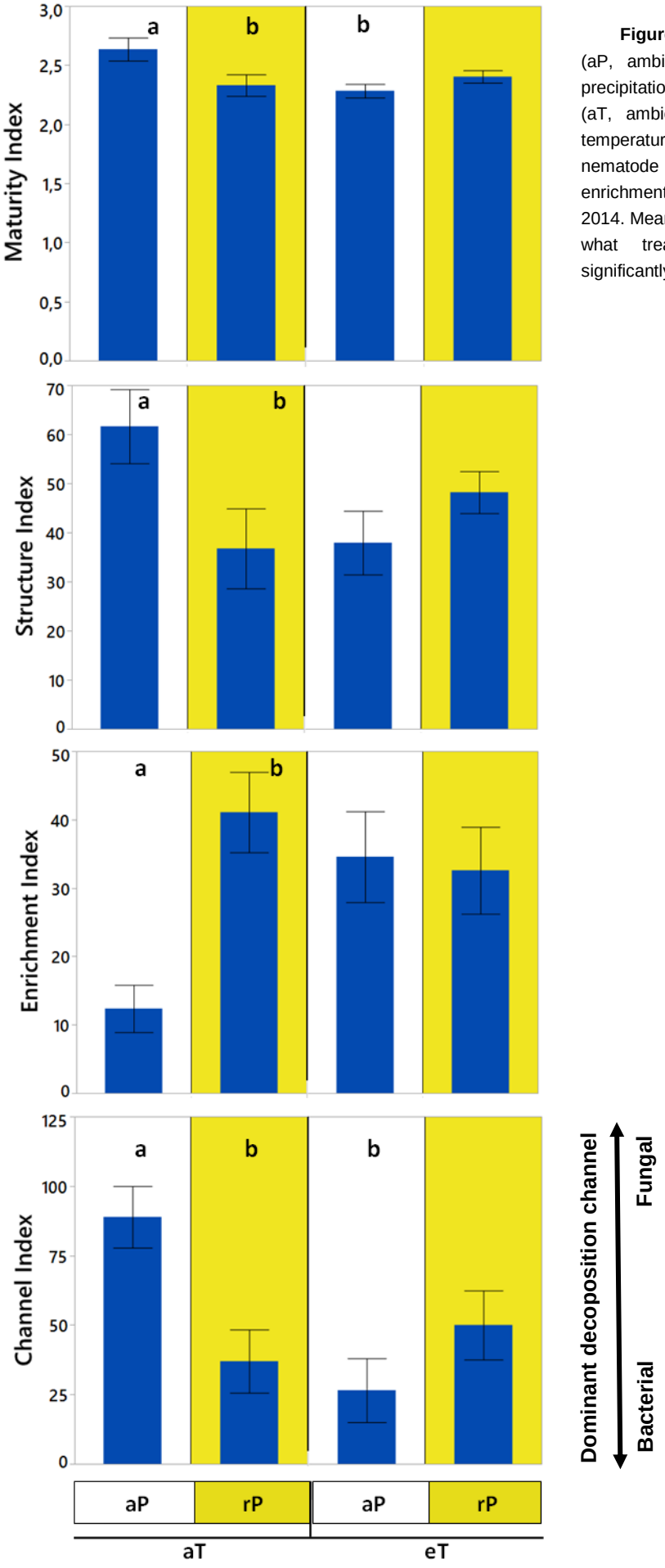

Figure 13: The effect of precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature) and their combinations on the nematode maturity index, structure index, enrichment index, and channel index in 2014. Mean ± SE. Bars with letters indicate what treatments combinations differ significantly (Tukey's HSD test, P<0.05).

ambient conditions, while at aT x rP and eT x aP it is more bacterial-dominated. In contrast to what it was expected, nitrogen did not play a significant role in the nature of the decomposition channel and lower moisture did seem to lower the fungal feeders.

The nematode profile was done for only the interaction between temperature and precipitation, since it was the only common significant interaction for both SI and EI (Fig. 14). It manifests that disturbed scenarios falls under the D quadrat, and undisturbed (aT x aP) under the C quadrat. This suggests that under undisturbed scenarios the enrichment of the environment is moderated, the main decomposition channel is fungal, the C:N ratio is rather moderated to high and the food web is structured. Disturbed scenarios are more stressed, the C:N ratio is higher and food web is degraded, corroborating the results of the previous nematode indices (Ferris et al., 2001).

These results are in contrast with Cesarz et al.(2015), from the same experiment in the August 2010, where nematode indices were mainly affected by nitrogen, moderately by CO_2 and little by precipitation. However, my results showed to be precipitation the main agent together with temperature.

Nematode density - as microbial biomass- showed very different responses to global change among the three years (2012, 2013 and 2014) (Tab. 9, Fig. 13). In 2012, nematode density was significant for the interaction between CO_2 and nitrogen, with a significantly higher density at eCO_2 x aN than at aCO_2 x aN (Fig. 13). No significant differences were found for nematode density in 2013. In 2014, nematode density was significantly higher at elevated temperature (Fig. 16, right). The interaction nitrogen with precipitation also had significant effects on nematode density, with the highest nematode density at eN x rP and the lowest at aN x rP (Fig. 16, left).

Nematode richness in 2014 was also significantly higher at elevated temperature (Tab. 12, Fig. 19, right). It was -as well as nematode density- affected by the significant interaction between nitrogen and precipitation with the highest nematode richness at eN x rP and the lowest at aN x rP (Fig. 19, left).

Nematode diversity (Shannon H') was significantly higher at elevated CO_2 as it was predicted (Fig. 20 left). Nematode diversity was also significantly higher at elevated temperatures (Fig. 20 right). Evenness (Shannon J') was significantly higher at ambient nitrogen concentrations (Figure 18). Nevertheless, temperature was expected to be the main agent influencing the evenness.

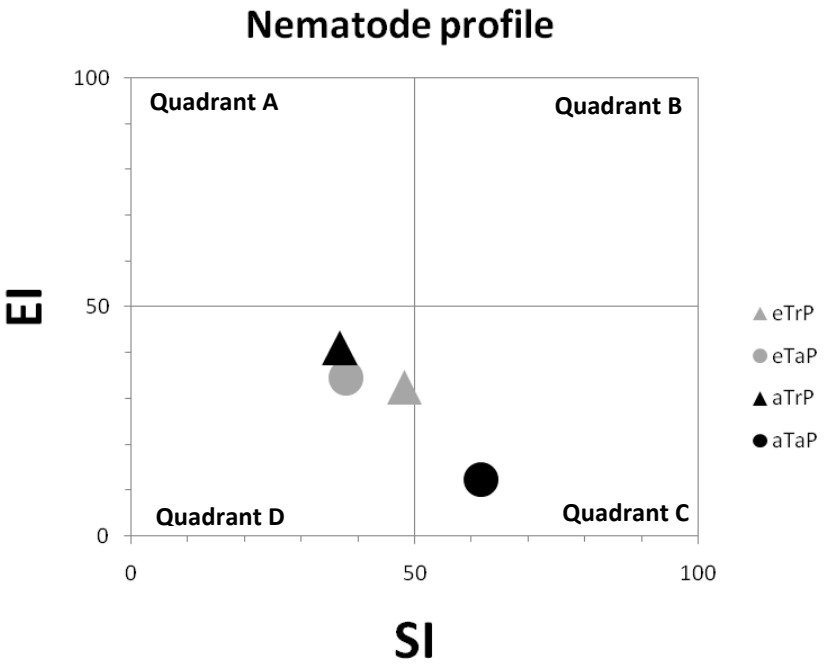

Figure 14:Nematode index, where the nematode enrichment index (EI) is plotted against the structural index (SI), as affected by the precipitation (ambient = circles; reduced = triangles) and temperature treatments (ambient = black; elevated = grey).

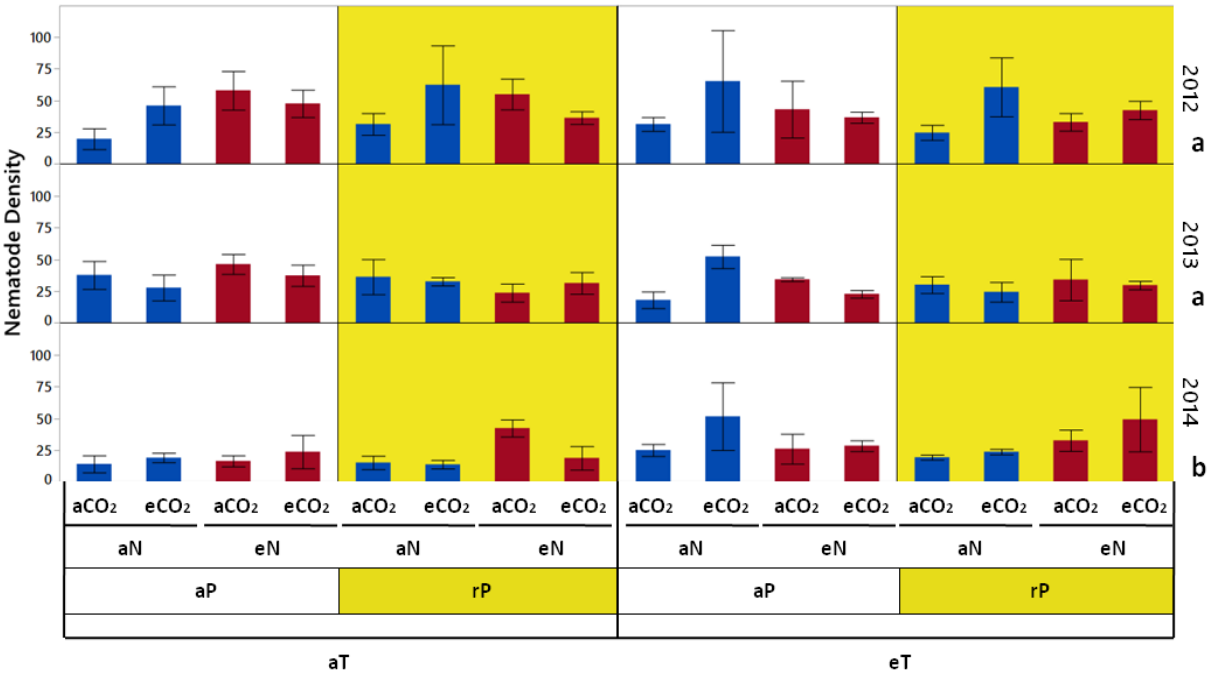

Figure 15: Nematode density as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature (aT, ambient temperature; eT elevated temperature) and all the possible combinations in 2012, 2013, and 2014; and the comparison of nematode density between years (right). Mean ± SE. Letters on the right indicate what years differed significantly regarding nematode density (Tukey's HSD test, $P<0.05$).

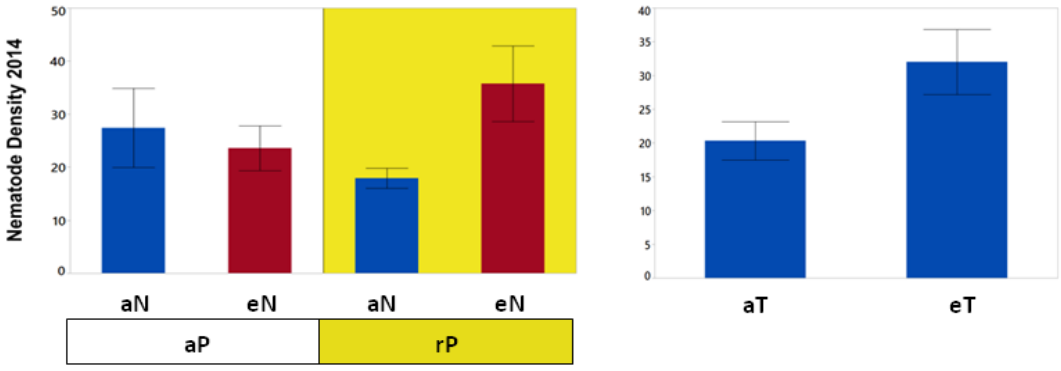

Figure 16: Nematode density as affected by (left) nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), and precipitation (aP, ambient precipitation; rP, reduced precipitation) and all possible combinations, and (right) by and temperature (aT, ambient temperature; eT elevated temperature) in 2014. Mean ± SE.

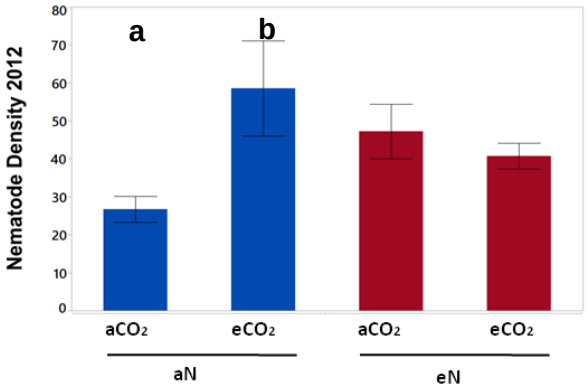

Figure 17: Nematode density as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), and all possible combinations in 2012. Mean ± SE.

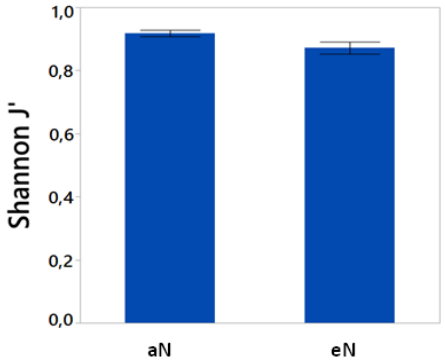

Figure 18: Nematode evenness (Shannon J') as affected by nitrogen (aN, ambient nitrogen; eN, elevated nitrogen) in 2014. Mean ± SE.

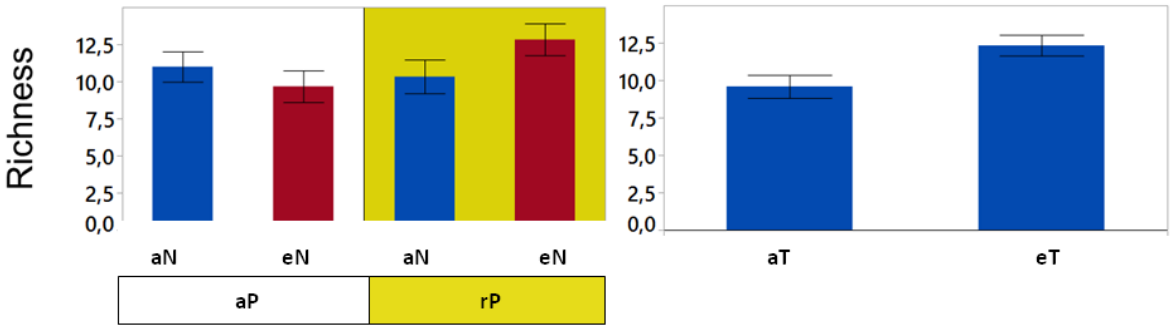

Figure 19: Nematode richness as affected by (left) nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), and precipitation (aP, ambient precipitation; rP, reduced precipitation) and all possible combinations, and (right) by and temperature (aT, ambient temperature; eT elevated temperature) in 2014; Mean ± SE.

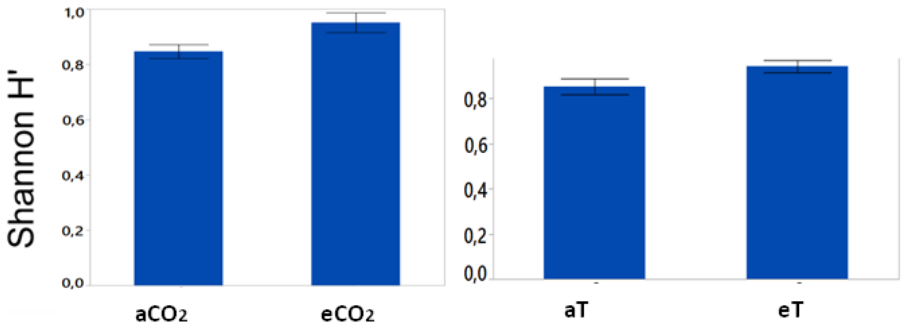

Figure 20: Nematode diversity (Shannin H') as affected by (left) CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), and (right) by and temperature (aT, ambient temperature; eT elevated temperature) in 2014; Mean ± SE.

Table 13: interactions where significant differences were found after the ANOVA test. (C=CO_2; N=Nitrogen; T=Temperature; P=Precipitation)

Year	2012	2013	2014	2015
Microbial biomass	CxNxTxP		NxTxP	
Specific respiration				CxP
Basal respiration			TxP, CxT	
AAP (EEA/MSEA)				N / NxTxP
NAG (EEA/MSEA)				P / NxTxP, CxTxP
βG (EEA/MSEA)				T / NxTxP
APh (EEA/MSEA)				N / NxTxP
MI			TxP, CxNxTxP	
SI			TxP, CxNxTxP	
EI			TxP, CxNxT	
CI			TxP	
Richness			T, NxP	
Shannon H'			C, T	
Shannon J'			N	
Cp 1			CxNxP	
Cp 2			CxNxTxP	
Cp 3			P	
Cp 4				
Cp 5				
Density	CxN		T, NxP	

Discussion

Time series analysis of global change effects on soil microbial biomass and nematode density revealed differences among the years. Nematode density and microbial biomass were affected by different significant interactions through the years. This suggests that global change effects on soil organisms are context dependent. This goes in line with the soil temperature and moisture results, that suggest that differences in abiotic environmental factors among years could be one of the causes contributing to this context dependency. This also implies that it is difficult to generalize these results and predict future responses of soil communities and transferring out present findings to other locations. However, the results from these experiments may provide first important insights to understanding the context-dependency of how and in what direction soil composition and functioning may change under global change

Microbial community

In line with the hypothesis 1.1: global change factors interactively affect soil microbial community biomass and functions, but treatment effect differed among years.

The results only partly confirmed the hypothesis 1.2. Under elevated CO_2, elevated nitrogen, elevated temperature and reduced precipitation microbial biomass decreased in comparison to the ambient scenario. However, this difference was not significant and neither can I confirm that temperature and precipitation were the main drivers in microbial biomass changes.

The results support the hypothesis 2.1: not only global factors did interactively affect the soil microbial enzyme activities involved in C, N and P cycles, but also the stoichiometry of the activities of the enzymes involved in the C, N and P cycles also changed with global change (Figure 14). Hence, it can be affirmed that the carbon, nitrogen and phosphorous cycles are uncoupled.

Activities of all measured enzymes were significantly affected by global change factors, with activity increasing with global change for all enzymes except for βG. βG activity decreased significantly with elevated temperature (Fig. 7, left) and tended to decrease under elevated CO_2, elevated nitrogen, elevated temperature and reduced precipitation (Fig.12, A and B). However, the MSEA for βG increased with global change (Fig. 12, C and D). This indicates that the decrease in activity under elevated temperature was due to a decrease in microbial biomass. A higher activity in βG per unit of biomass may indicate a higher availability of cellulose for microorganisms. Here, this might be due to changes in the plant community and/or that precipitation reduced

microbial biomass and as a consequence, the cellulose portion per unit of microbial biomass increased leading to a higher βG activity per unit of biomass.

NAG activity increased significantly with reduced precipitation (Fig. 7, right). Higher NAG activity indicates higher fungal biomass (Olander & Vitousek, 2000), which concords with the results, given that low moisture conditions are detrimental for bacteria (Ruess & Ferris, 2004) and this might benefit fungal communities, as explained before. Thus, changes in NAG activity and its MSEA point to a change in the microbial community composition under global change towards a higher fungal abundance.

I found no evidence to support the hypothesis 2.1.1. Elevated nitrogen significantly increased both AAP and APh activity significantly. Nevertheless, I expected an increase in APh and a reduction in APP activity as obtained in Saiya-Cork et al. (2002). Nitrogen amendments may cause a shift from nitrogen acquisition towards phosphorous acquisition (Sinsabaugh et al., 2002). Similar results were expected given the similar latitude and proximity of the two study sites and the fact that they share a history of episodic glaciations (Sinsabaugh et al., 2008), which may indicate similar geological properties. However, the observed differences between those experiments results may be due to different soil types. The Saiya-Cork et al. (2002) study was conducted in a forest, while this study was conducted in a grassland with very sandy soil. Our study site might be more nitrogen-limited and, thus the amelioration of energy employed towards nitrogen acquisition did not play a major role happen.

The overall analysis of the extracellular enzyme activity and MSEA suggests that changes in enzyme activity may have been due to (a) global change factors alter nutrient availability and subsequently, changes substrates concentration in the soil. This leads to changes in the microbial community composition, causing changes in the enzyme activity. It may be also due to (b) changes in soil climatic factors (i.e. elevated temperature and/or reduced soil moisture) that may have had a direct effect on the microbial community causing also a change in the composition with a following change in the enzyme activity, given that different microorganisms target different substrate and produce different enzymes; (c) changes in the enzymes turnover due to soil climatic factors (Steinweg et al., 2013); or (d) a combination of some of all of the previous.

Furthermore, all enzymes increased their MSEA under global change (Fig 12. C and D), which indicates an overall higher energy demand. This assumption is supported by the qO_2 results (Fig. 6), which reached the highest value at elevated CO_2 and reduced precipitation. This indicates that there was a higher energy demand per unit of biomass, which is typical for disturbed systems (Odum, 1985). This may indicate

compositional changes in the microbial community in response to global change, but it may also suggest that more resources were allocated to enzyme production to acquire nutrients to cope with environmental disturbances (Schimel & Weintraub, 2003; Wang et al., 2013).

Continuing with the MSEA results, they cannot confirm the hypothesis 2.1.2: warming did not intensified the effects of reduced precipitation. There is a repeating pattern in all the enzymes MSEA regarding nitrogen in scenarios with elevated temperature (eT) (Fig. 8). In scenarios with reduced precipitation (rP), scenarios with elevated nitrogen (eN x eT x rP) present higher activity than the scenarios with ambient nitrogen (aN x eT x rP). But in scenarios with ambient precipitation (aP), scenarios with elevated nitrogen (eN x eT x aP) present lower activity than scenarios with ambient nitrogen (aN x eT x aP). Also, the activity in the scenario with eN x eT x rP was always more or less as high as in the scenario with aN x eT x aP. This was not as predicted, since the hypothesized effect of elevated temperature and precipitation was an activity decrease regardless of nutrient availability. This may indicate that the interaction among nutrients and climate factors have an important role in the determining microbial community composition and functioning.

Nematode community

In line with the hypothesis 3.1: global change factors interactively affect the structure and functional composition of soil food webs. In the figure 13, it can be easily seen that the scenario with elevated temperature and reduced precipitation did not present the most drastic results, suggesting that global change agents do have interactive effects, some of them counteracting each other.

The results obtained support the hypotheses 3.1.2 and 3.1.3: MI2-5, SI show a general decrease under the presence of global change agents, while the EI increases (Fig. 3 Appendix). This is typical of disturbed systems that present simplified structures, with fewer trophic links which make them less stable, and the enrichment status is higher as a consequence of higher mortality (Ferris et al., 2001; Odum, 1985; Wardle & Yeates, 1993). Also hypothesis 3.1.4 can be confirmed, since results present lower CI, indicating a lower relative fungal-feeding nematode density compared to bacteria-feeding (Ferris et al., 2001). The indices are also consistent with each other. EI higher values indicates more bacterial activity as a result of enrichment, while CI higher values indicates a higher fungal density relative to bacteria (Ferris et al., 2001). As expected, EI and CI had opposite results (when EI increases CI decreases, and vice versa) indicating both coherently changes in the microbial communities and the bacteria and fungi relative proportion. In the figure 13, under ambient temperature and ambient

precipitation, both indices indicate that the microbial community is rather fungal-dominated. In the elevated temperature and reduced precipitation scenario CI decreased and EI increased, indicating a more enriched system with shift to a higher relative bacteria density. It is also consistent with MI2-5 and SI results, which also present higher values under ambient conditions and, as explained before, as the system is more mature and more structured, CI usually increases revealing a higher relative fungal density (Ruess & Ferris, 2004).

Nematode indices results under the interaction between temperature and precipitation (Fig. 13) showed that elevated temperature with reduced precipitation had higher values than ambient temperature with reduced precipitation, and elevated temperature with ambient precipitation for MI2-5, SI and CI, but lower for EI. These results were not expected. It was expected that the interaction between elevated temperature and reduced precipitation had higher detrimental effects than when the global change agents are acting alone. However, this might be a result of the different establishment time of the treatment. As said before, temperature was the latest treatment that was set up. This can be a sign of adaptation and later selection. Previous to the application of the temperature treatment, the soil communities under scenarios with reduced precipitation might already have suffered shifts in composition adapting to dryer conditions. After the implementation of elevated temperature, reduced precipitation scenarios may have presented even lower soil water content. Those populations better adapted to dry conditions might have been beneficed and selected. On the other hand, the communities under ambient precipitation after the implementation of the warming treatment may have been harmed since they did not adapt to low soil moisture. As a result, plots with reduced precipitation and elevated temperature present a more structured and stable community than those with elevated temperature and ambient precipitation.

All these results suggest changes in the community compositions and functioning of the trophic food web under global change. Additionally, the figure 5 (Appendix) shows changes in the different cp classes densities. They show different densities under the effect of global change. Under global change r- strategists (cp1-2) show a higher abundance that K-strategists (cp3-5) in comparison to the ambient scenario, partly confirming the hypothesis 3.1.2, although the expected effects of elevated CO_2 and elevated nitrogen were not shown in these results. Moreover, differences between r-strategists and K-strategists can be perceived. Cp1 show significant differences under the 4-way interaction (CO_2, nitrogen, temperature, and precipitation) and cp2 under the interaction between CO_2, nitrogen and precipitation. On the other hand, K-strategist are barely significantly affected by global change. Only Cp3 presents a significant

difference under the precipitation treatment. This may suggests that K-strategists are less directly affected by global change, and that their densities are mainly driven by bottom-up forces (Wardle & Yeates, 1993).

The results do not support the hypothesis 3.1.1 since diversity (Shannon H') increases with global change (Fig. 20, and 8 Appendix). Both density and richness present significant increase under temperature (Fig. 16 right and 19 right) and present significant differences under the interaction between nitrogen and precipitation increasing both under elevated nitrogen and reduced precipitation. However, evenness (Shannon J') tend to decrease with global change (Fig. 8 Appendix) and significantly decreases with elevated nitrogen (Fig. 18), indicating that nitrogen fertilization benefit dominant species, which is consistent with other results (Eisenhauer et al., 2012). In summary, although nematode diversity increases under global change, it has detrimental effects on the structure and functioning of the food web.

Common trends

There are some common trends in the microbial and nematode communities although those analyses were done in different years. The possible effect of the later implementation of the temperature treatment on the nematode community in 2014 was already mentioned above. A similar effect might have be shown in the enzymes MSEA in 2015. The different effect of nitrogen in combination with precipitation in scenarios with elevated temperature in the enzymes MSEA may be also a consequence of previous adaptation and community changes to reduced precipitation and, in this case, its interaction with nitrogen availability, and later selection when the temperature treatment was established.

On the other hand, nematode density and richness in 2014 show a similar response to elevated nitrogen than the enzymes MSEA in 2015. Elevated nitrogen has a positive effect in plots with reduced precipitation (Fig. 8), although this positive effect of nitrogen in enzyme MSEA is present only under elevated temperature. This response in the two years for both communities might be related. The cause may be due to changes parameters that have not been measured in this experiment, such as changes in plant diversity.

The CI results from 2014 and the NAG results from 2015 might seem contradictory at first sight. Lower CI values in scenarios under global change effects in 2014 indicated a lower fungal-feeding nematodes abundance relative to bacterial-feeding nematodes, while a higher NAG activity and MSEA in 2015 suggests a higher fungal abundance. This may mean that the two sampling dates differed in the soil fungal density, but it can also indicate that different fungi groups where differently affected by

global change agents. The CI index refers indirectly to the fugal biomass through fungal feeding nematodes, and it only takes into account the Fu_2 nematode functional guild. Cesarz et al. (2015) suggested that -in the same experimental area- Fu_2 nematodes relied more on saprothrophic fungi, whereas Fu_4 depended on arbuscular mycorrhizal fungi (AMF). Hence, this results may suggest changes within the fungi community under global change effects, where saprothrophic fungi decreases and AMF abundance increases. Thus, NAG activity may reflect rather the AMF biomass. Moreover, NAG was the only enzyme which MSEA was affected by CO_2, and AMF tend to increase under elevated CO_2 resulting from increased root carbon supply (Treseder, 2004).

Limitations and further research

The consideration of additional explanatory variables would have been helpful to better understand the causes of the changes in the soil structure and functioning obtained in this experiment. Some examples of additional explanatory variables could be plant biomass, quality of plant inputs and plant diversity, which would have helped principally to understand the changes in enzyme activity. Although at the initial setting of the experiment the studied plots had the same plant diversity, significant changes might have happen through all these years. The role of plant diversity and the above-belowground interaction in soil functioning and quality has been already widely recognized.

A PCA (Principal Component Analysis) would have helped to better understand changes in the nematode structure. It would be used to examine how nematode community composition changes with the different treatments and to determine whether the structural changes are due to distinct species presence in the plots. This would also confirm or contrast previous findings affirming that nematode functional guilds play a major role in soil structure and functioning than trophic groups (Cesarz et al., 2015).

To test my hypothesis on the effect of the later setting of the temperature treatment in the experiment on soil structure and functioning, I would carry out a PLFA (PhosphoLipid Fatty Acid) analysis. It would provide information on the microbial composition in the different plots. By taking a deeper look on the composition and observe if the microorganisms present in the plots differ in drought resistance I would confirm (or reject) my hypothesis.

Conclusion

The present study shows that is important to consider the context-dependency of global change effects on soil communities and functions. Therefore, long-term experiments and repeated measurements of key variables are needed to gain better knowledge of how global change will affect soil ecosystems.

Despite some inconsistent responses across years, our results indicate that global change significantly alters soil food web structure and functioning. Overall, global change pushes soil ecosystems towards more disturbed conditions. For instance, microbial biomass and microbial energy use efficiency decreased under global change. Furthermore, soil enzymes involved in the carbon, nitrogen, and phosphorous cycles as well as their stoichioimetry are altered by global change. Soil food web tend to get simplified by global change, showing characteristics of a less mature system. Less complex food web structure with a lower number of trophic links indicate that energy and nutrients are less efficiently retained in the system. Taken together, these results suggest that global change will decrease soil health and quality.

Acknowledgements

I want to thank firstly to Prof. Dr. Nico Eisenhauer for providing me the opportunity to carry out this research project in his lab, and for his supervision and guidance through these past months. I thank Katja Steinauer, who taught me how to do the extracellular enzyme activity analysis and helped me to solve the obstacles I found on the process. Also, Madhav Prakash Thakur for solving many doubts I had about statistics, and Dr. Simone Cesarz for helping me with the nematodes indices. I would also like to mention the Experimental Interaction Ecology research group at the iDiv, where I have been working these past months on my thesis and felt welcomed since the first day. And last but not least, I'm also thankful to Prof. Dr. Martin Wassen for supervising my master thesis, despite the difficulties that suppose to do it abroad, and for all his feedback and help provided.

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Appendix

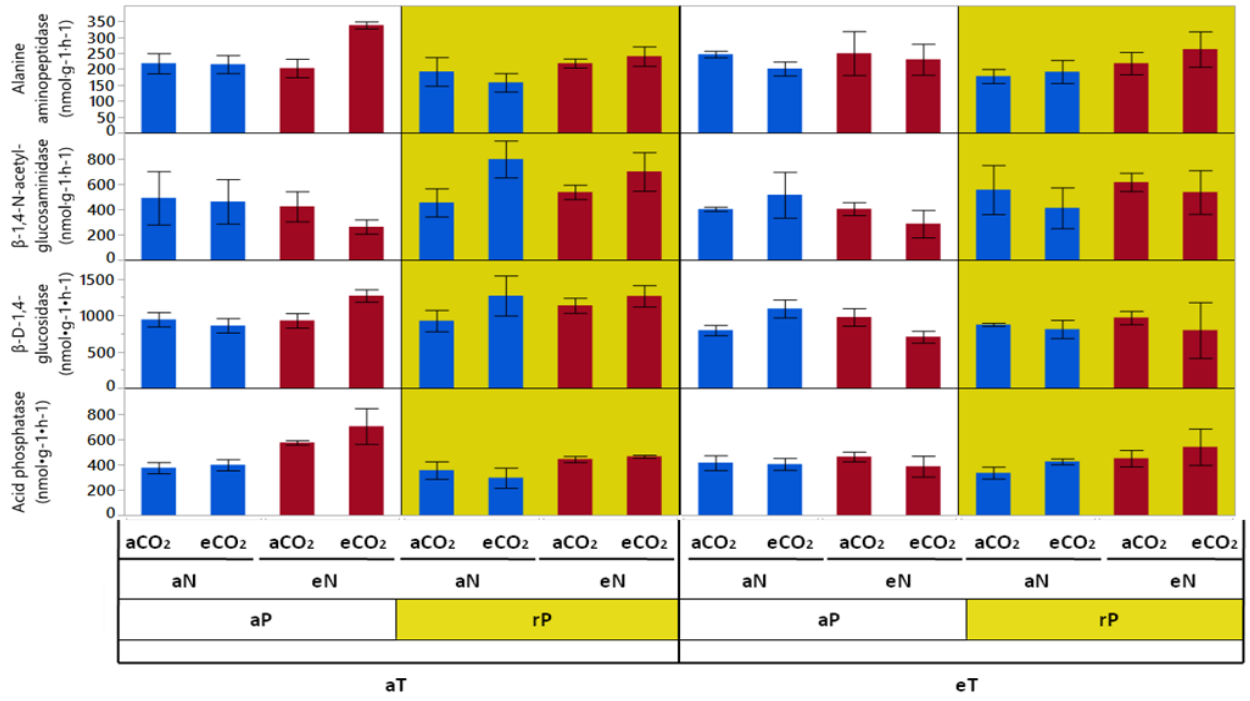

Figure 1: Microbial extracellular enzyme activity (nmol·g^{-1}·h^{-1}) of alanine aminopeptidase, β-1,4-N-acetyl-glucosaminidase, β-D-1,4-glucosidase and acid phosphatase as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all the different combinations in 2015. Mean ± SE.

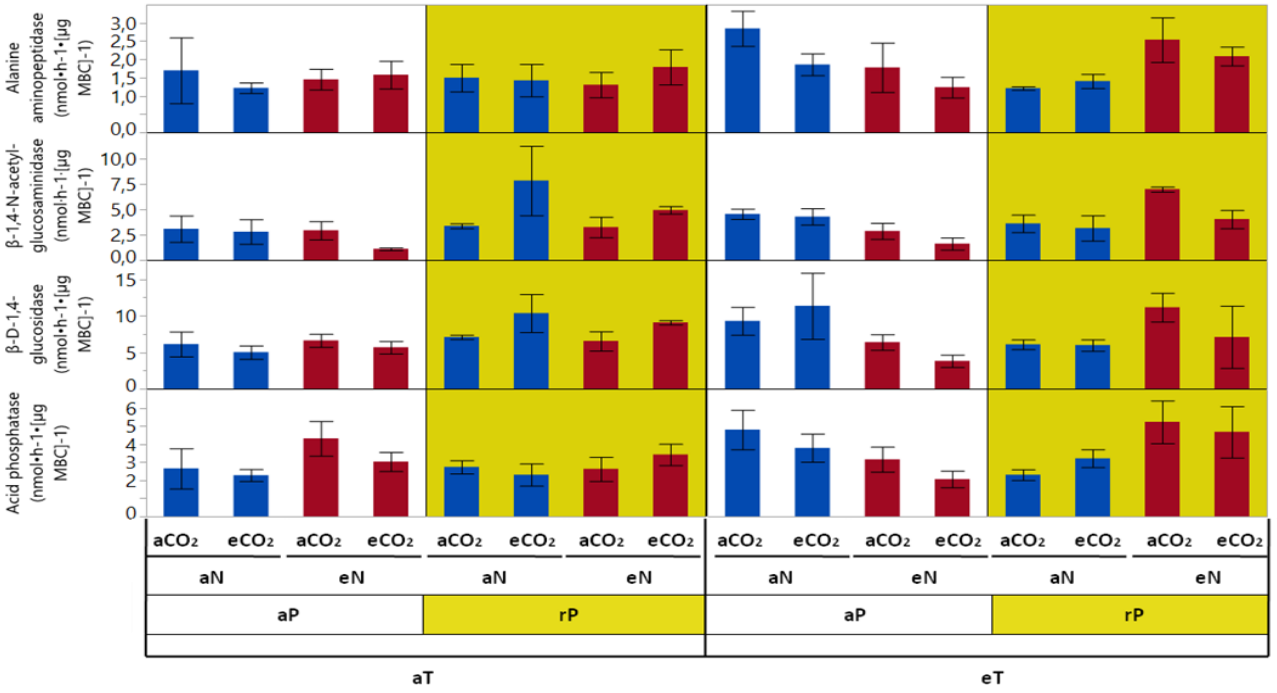

Figure 2: Mass specific enzyme activity (MSEA; nmol·h^{-1} [µg MBC]$^{-1}$) of alanine aminopeptidase, β-1,4-N-acetyl-glucosaminidase, β-D-1,4-glucosidase and acid phosphatase as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all the different combinations in 2015. Mean ± SE.

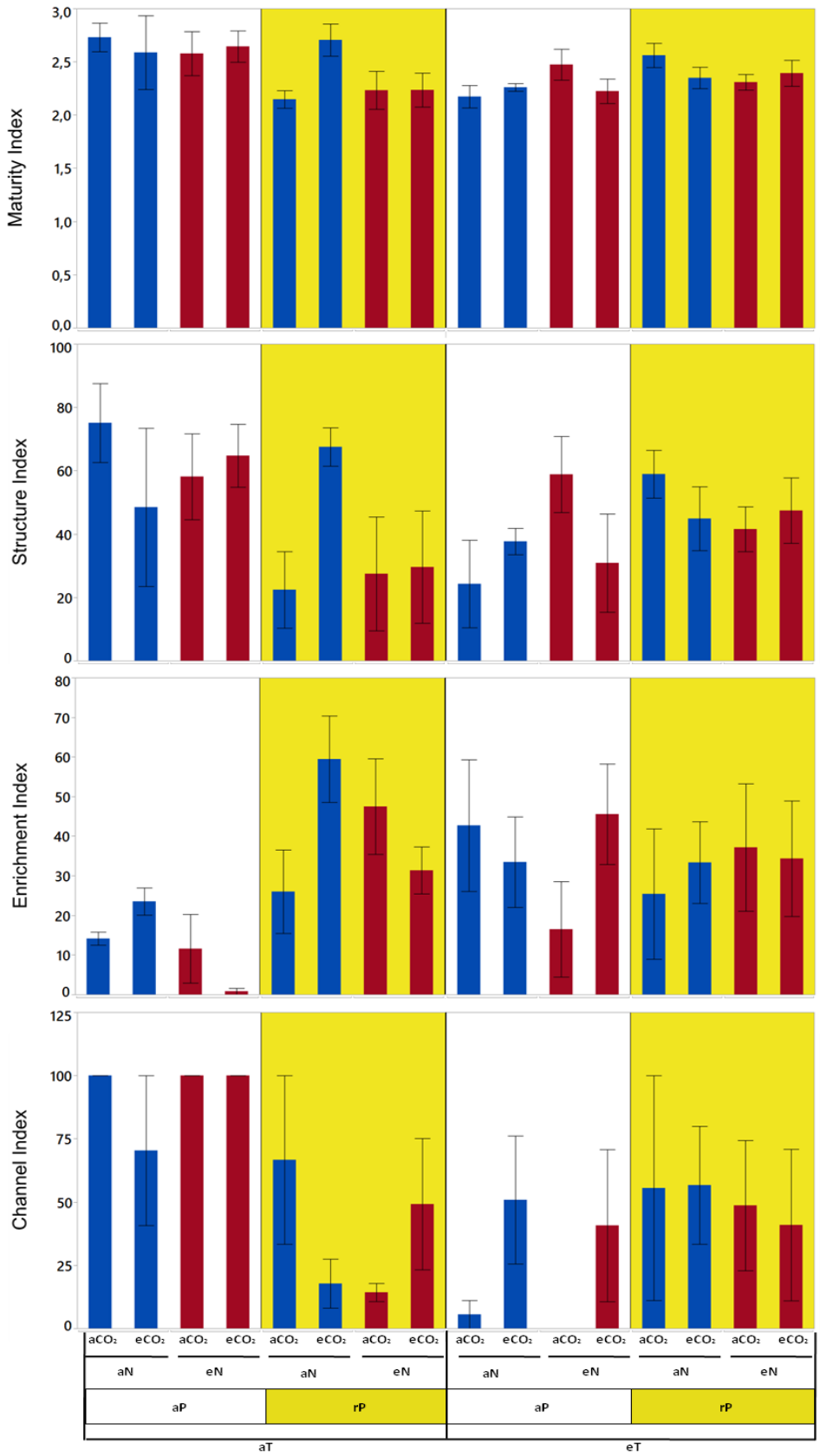

Figure 3: Nematode maturity index, structure index, enrichment index, and channel index as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all the different combinations in 2014. Mean ± SE.

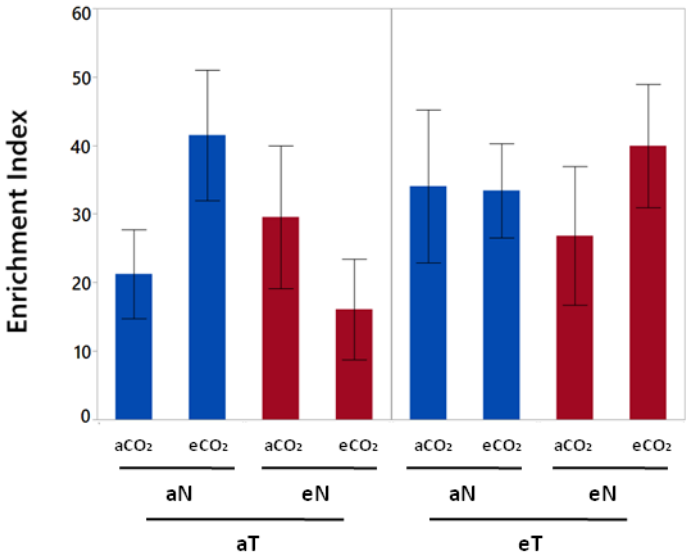

Figure 4: Nematode enrichment index as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all the different combinations in 2014. Mean ± SE.

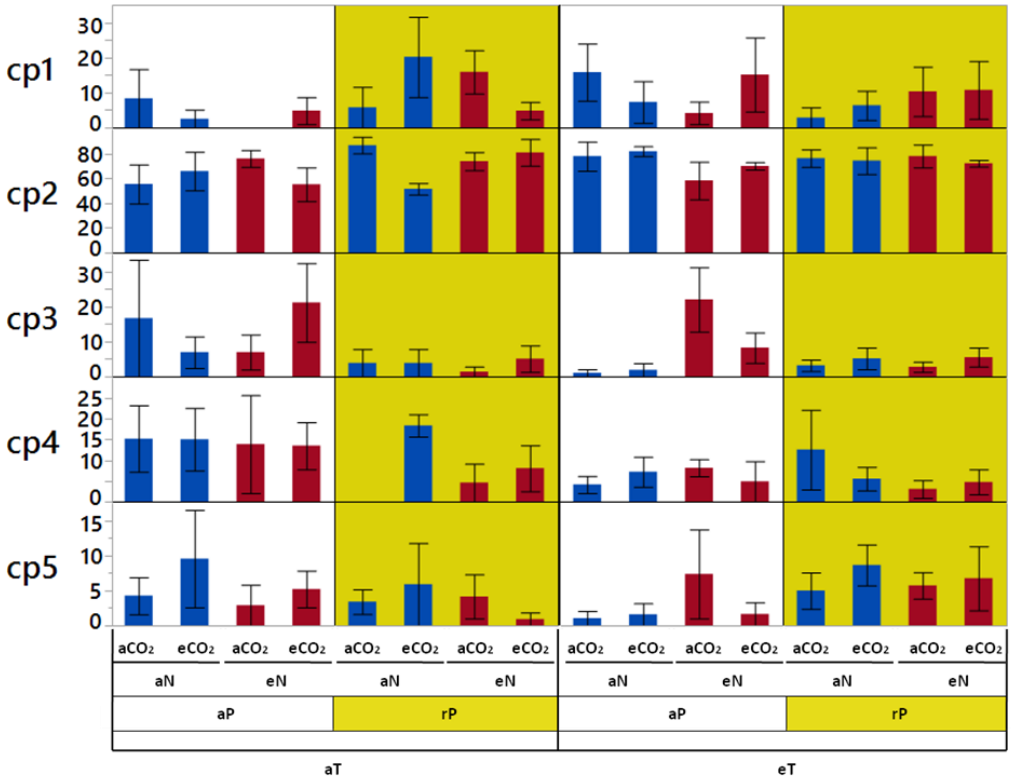

Figure 5: Nematode colonizer-persister groups as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all the different combinations in 2014. Mean ± SE.

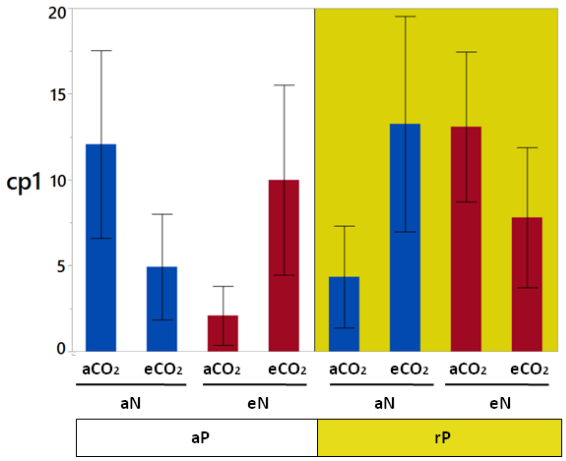

Figure 6: Nematode colonizer-persister group 1 as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), and precipitation (aP, ambient precipitation; rP, reduced precipitation), and all the different combinations in 2014. Mean ± SE.

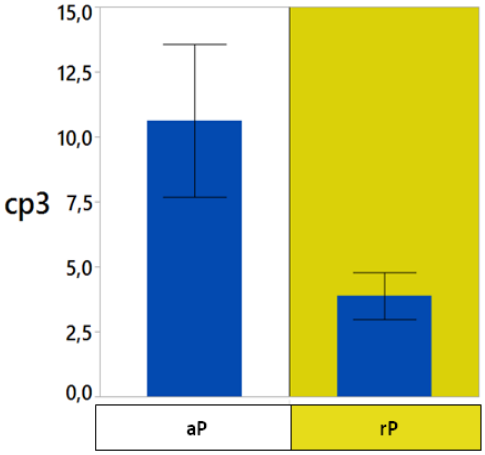

Figure 7: Nematode colonizer-persister group 3 as affected by precipitation (aP, ambient precipitation; rP, reduced precipitation) in 2014. Mean ± SE.

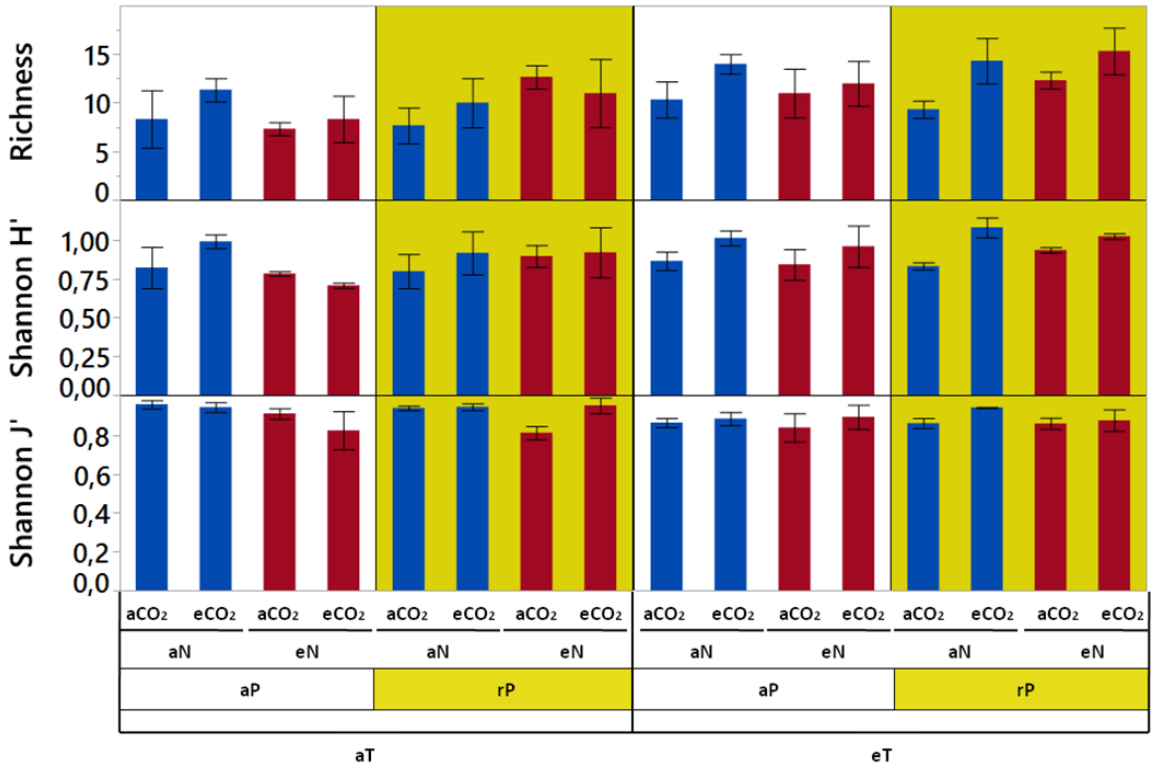

Figure 8: Nematode richness, diversity (shannon H'), and evenness (Shannon J') as affected by CO_2 (aCO_2, ambient CO_2; eCO_2, elevated CO_2), nitrogen (aN, ambient nitrogen; eN, elevated nitrogen), precipitation (aP, ambient precipitation; rP, reduced precipitation), and temperature treatments (aT, ambient temperature; eT elevated temperature) and all the different combinations in 2014. Mean ± SE.

Declaration of Authorship

I, Inés Martín del Real, hereby certify that this thesis has been composed by me and is based on my own work, unless stated otherwise. No other person's work has been used without due acknowledgment in this thesis. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

This paper was not previously presented to another examination board and has not been published.

Date and place: Signature:

Leipzig, April 8th 2016