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DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„The impacts of single and multiple global change drivers on soil microbial communities and functions“

verfasst von / submitted by

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Abstract

Atmospheric CO_2 concentrations and global temperatures have been steadily increasing, as a consequence of industrialization, intensive agriculture, land use change, and other anthropogenic activities. In addition, future climate predictions forecast an increasing frequency of climatic extreme events such as droughts and heat waves. Microorganisms play a major role in terrestrial ecosystem functioning and there is growing evidence that ecosystem level responses to climate change can be tightly linked to changes in microbial physiology, community structure and composition.

In this thesis I assess the effect of single and combined climate change drivers on soil microbial communities, with a special focus on the terrestrial nitrogen cycle. Results indicated that drought is a major driver of general and functional microbial community structure in grassland soils. More specifically, a summer drought caused a decrease in *amoA* transcription levels from ammonia-oxidizing archaea and complete nitrifiers, while ammonia-oxidizing bacteria remained relatively unaffected. In addition, one year after the end of the summer drought treatment, significant differences in bacterial/archaeal and fungal community structure remained observable in soils previously exposed to drought, in comparison to controls. The effects of future climate conditions (i.e., elevated temperature and CO_2) on community structure were more pronounced during spring and autumn, than in summer.

Furthermore, microorganisms exposed to long-term warming showed increased transcription levels of genes encoding secreted extracellular enzymes involved in the depolymerization of high molecular weight organic nitrogen such as proteins and peptidoglycan. This provides evidence of the importance of microbial necromass as a C and N source in response to sustained warming, where substrate depletion is a common phenomenon.

Taken together, the results from this thesis highlight gene expression changes as a mechanism by which soil microorganisms adjust their physiology in order to cope with direct and indirect climate change drivers. More specifically, drought emerged as a major factor driving changes in general and functional community structure, with potential consequences for terrestrial N biogeochemistry.

Zusammenfassung

Die CO_2-Konzentration in der Atmosphäre und die globalen Temperaturen steigen infolge der Industrialisierung, der Intensivierung der Landwirtschaft, der veränderten Landnutzung und anderer anthropogener Aktivitäten stetig an. Darüber hinaus prognostizieren zukünftige Klimavorhersagen eine zunehmende Häufigkeit von klimatischen Extremereignissen wie Dürren und Hitzewellen. Mikroorganismen spielen eine wichtige Rolle für das Funktionieren terrestrischer Ökosysteme, und es gibt immer mehr Belege dafür, dass die Reaktionen der Ökosysteme auf den Klimawandel eng mit Veränderungen der mikrobiellen Physiologie, Gemeinschaftsstruktur und -zusammensetzung verknüpft sind.

In dieser Arbeit untersuche ich die Auswirkungen einzelner und kombinierter Faktoren des Klimawandels auf mikrobielle Gemeinschaften im Boden, mit besonderem Augenmerk auf den terrestrischen Stickstoffkreislauf. Die Ergebnisse zeigen dabei, dass Trockenheit ein wichtiger Faktor für die allgemeine und funktionelle Struktur der mikrobiellen Gemeinschaften in Graslandböden ist. Insbesondere verursachte Sommertrockenheit einen Rückgang der *amoA*-Transkripte von Ammonia-oxidierenden Archaeen und von vollständigen Nitrifikanten (complete nitrifiers), während Ammonia-oxidierende Bakterien relativ unbeeinflusst blieben. Darüber hinaus waren ein Jahr nach dem Ende einer Sommertrockenheit signifikante Unterschiede in der Struktur der Bakterien-/Archaeen- und Pilzgemeinschaften in Böden, die zuvor der Trockenheit ausgesetzt waren, im Vergleich zu Kontrollen zu beobachten. Die Auswirkungen künftiger Klimabedingungen (erhöhte Temperatur und CO_2 Werte) auf die Struktur der Gemeinschaften waren im Frühjahr und Herbst stärker ausgeprägt als im Sommer.

Darüber hinaus zeigten Mikroorganismen, die einer langfristigen Erwärmung ausgesetzt waren, eine erhöhte Transkription von Genen, die für sekretierte extrazelluläre Enzyme kodieren, die an der Depolymerisierung von hochmolekularen organischen Stickstoffverbindungen, wie Proteinen und Peptidoglykan, beteiligt sind. Dies belegt die Bedeutung der mikrobiellen Nekromasse als C- und N-Quelle in Reaktion auf eine anhaltende Erwärmung, bei der die Substratverarmung ein häufiges Phänomen ist.

Insgesamt unterstreichen die Ergebnisse meiner Arbeit die Bedeutung von Veränderungen in der mikrobiellen Genexpression als physiologische Adaptation, um mit direkten und indirekten Einflüssen des Klimawandels zurechtzukommen.

Insbesondere Trockenheit erwies sich als eine der Hauptursachen für Veränderungen in der allgemeinen und funktionellen mikrobiellen Gemeinschaft, was potenzielle Folgen für die terrestrische N-Biogeochemie mit sich bringt.

Overview of manuscripts

Chapter II

Manuscript title: Composition and activity of nitrifier communities in soil are unresponsive to elevated temperature and CO_2, but strongly affected by drought

Author names: Joana Séneca, Petra Pjevac, Alberto Canarini, Craig W. Herbold, Christos Zioutis, Marlies Dietrich, Eva Simon, Judith Prommer, Michael Bahn, Erich Pötsch, Wolfgang Wanek, Andreas Richter.

Reference: *The ISME Journal*, 14:3038-3053 (2020). https://doi.org/10.1038/s41396-020-00735-7

Author contributions: AR, MB and WW conceived and supervised the study. EP and MB established and maintained the experimental sites. JS, AC, MD, ES, AR, MB collected the samples. JS, JP, and AC processed and analyzed soil process rates and physicochemical parameters supervised by AR and WW. JS quantified, sequenced, and analyzed all functional genes' data with the help of CZ, PP and CWH. JS and AC wrote the manuscript with input of all authors.

Chapter III

Manuscript title: Drought legacy effects on microbial community structure in a managed grassland

Author names: Joana Séneca, Alberto Canarini, Eva Simon, Marlies Dietrich, Judith Prommer, Ivana Bogdanovic, Victoria Martin, Moritz Mohrlok, Bela Hausmann, Erich M. Pötsch, Andreas Schaumberger, Wolfgang Wanek, Michael Bahn, Hannes Schmidt, Andreas Richter

Reference: Draft manuscript

Author contributions: AR, MB and WW conceived the experiment. AR, HS and AC supervised the study. EP and AS was responsible for the site management. JS, AC, ES, VM, and MD did field work. JS, AC, ES, JP, IB and MM processed samples and produced data. BH assisted with data analysis. JS wrote the manuscript with input of all authors.

Chapter IV

Manuscript title: Increased microbial expression of organic nitrogen cycling genes in long-term warmed grassland soils

Author names: Joana Séneca, Andrea Söllinger, Craig W. Herbold, Petra Pjevac, Judith Prommer, Erik Verbruggen, Bjarni D. Sigurdsson, Josep Peńuelas, Ivan A. Janssens, Tim Urich, Alexander T. Tveit, Andreas Richter.

Reference: *ISME Communications*. 2021;1(1):69. doi:10.1038/s43705-021-00073-5

Author contributions: AR and TU conceived and supervised the study. BDS, EV, AR, IJ, JPe established the experimental sites. JP and AR collected the samples. JS, JP, and ATT did laboratory work. JS analyzed the data, with assistance from ATT. AS provided data and assisted with data analysis. CWH and PP assembled the metagenomes, assisted with data analysis, and provided valuable input. JS wrote the manuscript with input from all authors

Chapter I

Introduction, thesis outline and main goals

Introduction

1. Soil microorganisms and climate change

It is now universally accepted that soil microorganisms play a major role in nutrient transformations and all biogeochemical cycles. Soil microorganisms produce and consume greenhouse gases, influence soil acidity, and mediate the cycling of nutrients within soil profiles (Falkowski et al., 2008; Fierer, 2017). In addition, they link aboveground and belowground processes, with consequences for soil health and plant productivity. Consequently, microorganisms are central to our understanding of future ecosystem-level responses to climate change (Jansson and Hofmockel, 2020).

Anthropogenic activity has a profound effect on global ecosystem functioning. In particular, the extensive use of nitrogen-rich fertilizers that currently sustain about 50% of the human population has nearly doubled the reactive nitrogen inputs to both terrestrial and aquatic ecosystems (Galloway et al., 2004). In parallel, the burning of fossil fuels, land use change and intensive agriculture practices have substantially contributed to a more than 40% increase in atmospheric CO_2 concentrations in comparison to pre-industrial times (Hu et al., 1999; Kuzyakov et al., 2019). As a consequence, global average air temperatures have been steadily rising and are already more than 1.1 ^{o}C above pre-industrial times (Allen et al., 2018). Current climate change projections predict that the upper limit of +1.5-2 ^{o}C in global temperature increase by the end of the 21st century will be exceeded (Hoegh-Guldberg et al., 2018). On top of it, the frequency of extreme weather events such as heat waves, droughts, torrential rainfall events are also predicted to increase, placing an even greater strain on natural ecosystems (Hoegh-Guldberg et al., 2018).

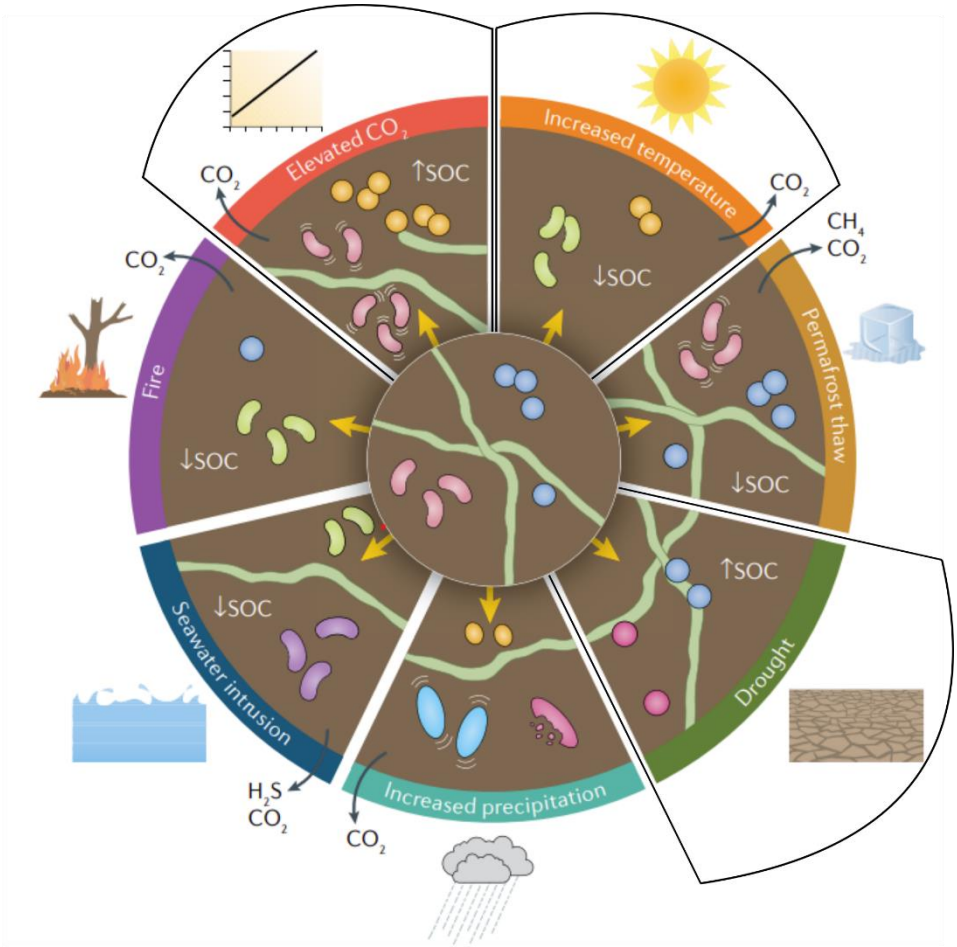

Figure 1. Schematic representation of soil microbial responses to climate change. An undisturbed microbial community (represented at the center) can respond differently to distinct environmental stressors. Changes in cell color represent changes in community structure. White lines represent increases in microbial activity. The main factors investigated in this dissertation are highlighted. Figure adapted from (Jansson and Hofmockel, 2020)

1.1. Elevated atmospheric CO_2

Net CO_2 emissions to the atmosphere are originally the result of a tight interplay between CO_2 fixation by plants and autotrophic and heterotrophic respiration. Industrialization and anthropogenic activities have contributed to an environmental imbalance characterized by substantial increases in atmospheric CO_2 emissions (IPCC, 2021). Elevated atmospheric CO_2 concentrations (eCO_2) can have direct effects on primary productivity and studies have reported a 20-30% increase in plant biomass under eCO_2 in comparison to ambient conditions

(Hu et al., 1999; de Graaff et al., 2006). However, since the natural CO_2 concentration in soils is about one to two orders of magnitude higher than in the atmosphere, increases in atmospheric CO_2 in the range of 100-400ppm are unlikely to have strong direct effects on belowground processes (Hu et al., 1999; Kuzyakov et al., 2019). Therefore, eCO_2 effects on soil processes and microorganisms are most often indirect, via changed plant activity, and can be substantially variable. These indirect effects of elevated atmospheric CO_2 are expected to accelerate belowground biogeochemical nutrient and carbon cycling (van Groenigen et al., 2014). Indeed, an increase in fine and secondary root production under elevated eCO_2 may lead to an increase in rhizodeposition and exudation (Paterson et al., 1997; Phillips et al., 2011). As soil microorganisms are intrinsically C limited, higher plant-derived labile organic C input under eCO_2 will positively affect or even alleviate microbial C limitation, resulting in higher microbial activity, biomass levels, NH_4^+ mineralization and nitrification rates in the rhizosphere (Singh et al., 2010). Indeed, a recent meta-analysis has suggested a shift in in soil microbial communities from slow-growing k-strategists to fast-growing r-strategists as a potential result of higher belowground C inputs in response to eCO_2 (Sun et al., 2021). However, long term eCO_2 could also lead to a progressive nitrogen (N) limitation, as N gets sequestered in plant and microbial biomass and is no longer available for uptake (Luo et al., 2004). In this case, plant growth under eCO_2 becomes constrained by the lack of mineral nutrients such as N or P while competition between plants and microbes for nutrients increases, potentially resulting in lower microbial activity levels and process rates.

Another indirect effect of eCO_2 is its influence on soil moisture content (and consequently on the surrounding microbial communities) through its positive effect on plant water use efficiency (Hu et al., 1999; Kuzyakov et al., 2019). This becomes particularly relevant in combination with other climate change factors, since increased plant water use efficiency is one of the mechanisms by which eCO_2 may offset the effects of reduced water content caused by warming and/or drought.

1.2. Increasing global temperatures

As all chemical reactions are inherently temperature sensitive, soil warming is predicted to have direct effects on microbial physiology and belowground processes in contrast to eCO_2. Soil warming often starts a phase of accelerated microbial respiration, which increases atmospheric CO_2 releases. Then, respiration rates often deaccelerate and, in most cases, return to pre-warmed states of soil CO_2 emissions. There are two mechanisms which can explain these phenomena: i) physiological acclimation of microorganisms, whereby microbial

activity returns to pre-warmed rates after a short-term, initial acceleration (Crowther and Bradford, 2013) and ii) substrate depletion, under which temperature-induced increases in microbial activity lead to a decline in labile nutrient pools (Hartley et al., 2008). This constrains further microbial activity, resulting in decreased microbial biomass levels and process rates per unit of soil under sustained long-term warming (Walker et al., 2018). The acceleration on microbial activity and processes due to warming may lead to a redistribution of the organic C stored belowground towards the atmosphere, initiating a positive feedback on which higher temperatures lead to elevated respiration rates and increasing atmospheric CO_2 concentrations, which in turn causes further temperature increases (Singh et al., 2010; Bradford, 2013; Niboyet et al., 2011; Greaver et al., 2016).

The storage capacity of terrestrial ecosystems and the sensitivity of C pools is thought to vary substantially. High-latitude ecosystems are especially vulnerable to global warming, since temperature constraints microbial decomposition processes, which may lead to SOC accumulation (Davidson and Janssens, 2006). Moreover, high latitude ecosystems are disproportionally more affected by climate warming than other ecosystems (García-Palacios et al., 2021). Indeed, a study on subarctic grasslands has shown a linear reduction of 1.28 ± 0.16-ton SOC ha^{-1} per ^{o}C of warming from the upper 10 cm soil layer (Marañón-Jiménez et al., 2019). Another meta-analysis has estimated ~5-15% of the terrestrial permafrost C pool to be vulnerable to be released under the form of greenhouse gases within this century, under the current global warming trajectory (Schuur et al., 2015)

There have been a wide range of studies on the effect of elevated temperature on the structure and size of soil microbial communities, that have yielded contrasting results which are likely dependent on the ecosystem type and duration of warming (Sheik et al., 2011; DeAngelis et al., 2015). A study using cocultures revealed that soil warming is predicted to favor slow growing microorganisms, which goes in line with the substrate-depletion mechanism in response to long-term warming and consequent community structure shifts towards recalcitrant-OM degrading microorganisms (Pold et al., 2015; Lax et al., 2020).

1.3. Drought

In addition to eCO_2 and soil warming, future climate change scenarios also include an increase on the frequency of extreme events such as droughts. The definition of ‘drought’ varies across studies, and for the purpose of this thesis, I will refer to a drought event as “a period of time with an abnormal precipitation deficit”, according to its IPCC definition (IPCC 2018: Annex I:

Glossary, 2018). Drought can have indirect effects on belowground microbial communities through an overall decrease in plant biomass, coupled with a reduction on belowground carbon allocation (Fuchslueger et al., 2014; Meeran et al., 2021). In addition, drought can directly alter the physical soil environment. As soils dry, there is decrease in connectivity due to a receding water film which decreases the accessibility of substrates and microbial mobility and is coupled with higher oxygen availability belowground (Schimel, 2018) (Fig. 2). These changes result in the formation of disconnected resource islands, lower decomposition rates and a decline in overall microbial growth.

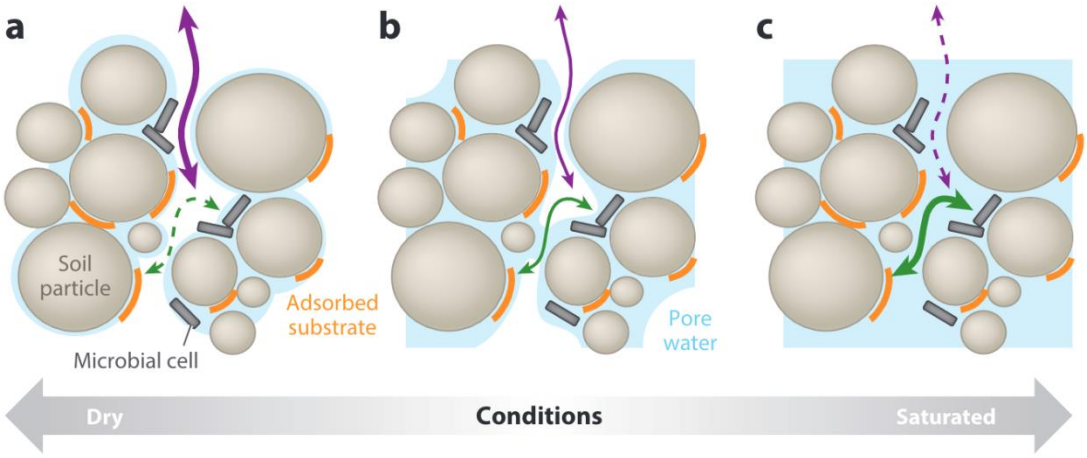

Figure 2. Effects of moisture on soil architecture. Moisture effects on soil architecture are depicted under conditions ranging from dry (a) to water saturated (c). The arrow thickness represents relative changes between the three conditions. Purple arrows represent gas transport, and green arrows represent solute transport. Figure adapted from (Schimel, 2018)

Soil microorganisms possess numerous physiological strategies to cope with drought, which include the accumulation of osmolytes to deal with differences in osmotic potential, switching to dormant states, and production of extracellular polymeric substances (EPS) to retain higher levels of moisture. In general, bacterial biomass has been shown to decrease under drought as a consequence of resource limitation and lower microbial growth rates (Schimel et al., 2007). However, some studies have reported either no changes or increases in biomass under drought (Fuchslueger et al. 2014; Hartmann et al., 2013). The exact biological mechanisms explaining biomass changes are not entirely understood but may be linked to variations in microbial growth vs death rates (Schimel, 2018). In addition, responses may vary according to the ecosystem and according to the plant response to drought. Finally, the use of different methods (e.g. total DNA quantification vs PLFA quantifications vs chloroform fumigation

methods) to quantify microbial biomass may introduce additional confounding factors and hamper comparisons across studies (Naylor and Coleman-Derr, 2018).

Microbial community composition has been shown to be significantly impacted by drought (Bouskill et al., 2013; Fuchslueger et al., 2014). Community composition changes following a severe drought can entail changes on the relative abundance of particular groups and/or can result from the death of drought-sensitive taxa and consequent proliferation of more resistant community members (Cordero et al., 2021; Griffiths & Philippot, 2013). Most of the studies that tackled the responses of belowground community composition to drought have reported changes at broad-level phylogenetic resolution (i.e., phyla) often coupled with information on cell-wall type, as a result of the use of Gram-staining specific PLFA biomarkers. A number of potential biological mechanisms have been put forward to attempt to explain these broad shifts in soil community composition in response to drought. The first mechanism concerns differences in substrate preferences and growth strategies between Gram-positive and Gram-negative bacteria, whose growth and life strategies often align with the definitions of 'oligotrophs' and 'copiotrophs' respectively (Naylor and Coleman-Derr, 2018; Schimel, 2018). Drought environments are typically characterized by lower plant-derived soil carbon inputs, high soil oxygen levels and a high level of spatial heterogeneity. Reduced connectivity and changes in plant-derived inputs force soil microorganisms to change their resource acquisition traits, potentially causing shifts in community composition (Malik et al., 2020; Malik and Bouskill, 2022). Another mechanism pertains to different microbial strategies in tolerating loss of soil moisture, whereby thicker cell walls, EPS production and spore forming potential may render particular groups more resistant to drought at the cost of reduced growth rates (Malik & Bouskill, 2022; Schimel, 2018).

The relief in water limitation induced by a rainfall event after a period of drought often causes pulses in microbial activity and soil organic matter turnover, as a result of the sudden availability of previously inaccessible substrates (Schimel, 2018). However, depending on the intensity of drought, soils might have changed their capacity to retain water. This could potentially lead to losses of carbon and nutrients by runoff, as well as to the development of anaerobic conditions, which could fuel denitrification processes. Indeed, N_2O emissions have been shown to substantially increase following rewetting events (Leitner et al., 2017; Harris et al., 2021) . A study in a semi-arid grassland revealed a 5-fold increase in N_2O emissions within 1h after rewetting and a higher contribution of inorganic N to the rewetting N flush(Leitner et al., 2017) Together with classic denitrifying microorganisms, nitrifiers can also contribute to N_2O emissions via nitrifier-denitrification (Kool et al., 2011). Consequently, drought-induced changes in general community structure – including the nitrifiying community structure - could result in altered levels of N_2O emissions (Hink et al., 2017, 2018; Kits et al., 2019).

1.4. Interactive effects of climate change drivers

Predicting the extent by which microbially mediated ecosystem-level processes will be affected by the nature and intensity of multiple climate change drivers is challenging, as the effects may not be always simply additive (Castro et al., 2010; Dieleman et al., 2012; Carrillo et al., 2018). While some studies have addressed the effects of multiple climate change drivers on soil nutrient pools and processes, there are fewer studies focused on changes in microbial community structure and on their potential consequences for ecosystem functioning (Dieleman et al., 2012; Meyer-Grünefeldt et al., 2015; Wild et al., 2018; Fuchslueger et al., 2019). In addition, it is also likely that the relative importance of multiple climate change drivers changes across ecosystems (Naylor et al., 2020).

As mentioned in the previous sub-sections, soil warming generally contributes to higher microbial activity levels and an overall acceleration in process rates. Drought – on the other hand – can lead to limited microbial activity levels and decreased process rates (Fuchslueger et al., 2014). A study that compared the effects of warming and drought on grassland microbial communities demonstrated that the lower moisture contents resulting from the interaction treatment combination resulted in a 50-80% decrease in microbial population size in comparison to drought conditions alone (Sheik et al., 2011), demonstrating that interactive effects of more than one climate change drivers may lead to non-linear system behaviors. In addition, the effects of multiple climate change drivers on microbial physiology metrics such as microbial growth and respiration have been shown to vary seasonally (Simon et al., 2020). More specifically, during peak growing season, eCO_2 was shown to offset some negative effects of soil warming on microbial growth, resulting in higher biomass specific growth rates in comparison to ambient CO_2 conditions. On the other hand, at the end of the growing season, temperature alone played a more relevant role explaining the observed higher growth and respiration rates (Simon et al., 2020).

2. Microbial nitrogen cycling

Nitrogen is a key element of life and its biogeochemical cycling is closely linked to the carbon cycle. Nitrogen availability often limits net primary production in agricultural, as well as natural and semi-natural ecosystems (Robertson and Vitousek, 2009). In addition, all living organisms use N to assemble a wide range of complex organic compounds such as amino and nucleic acids, chitin and proteins. In contrast to the global C cycling, where the largest fluxes are associated with the net primary production of terrestrial and marine systems, the biogeochemical cycling of N is dominated by microbially mediated processes in soils, sediments and water bodies (Butterbach-Bahl, 2011).

2.1. Organic nitrogen cycling

While the processes and microbial responses that underly C dynamics have been extensively studied, somewhat less attention has been given to the overall microbial contributions to soil N cycling despite its close link to the C cycle and common vulnerability to climate change (Knicker, 2011). The terrestrial N cycle is governed by the bioavailability of assimilable N forms to soil microorganisms and plants. Atmospheric N_2 can be fixed by specific microbial groups, but it is estimated that more than 90% of soil N occurs in the form of high molecular weight (HMW) compounds, amino sugar polymers, and aromatic N compounds (Simpson et al., 2007; Geisseler et al., 2010; Warren, 2014). Therefore, the microbially mediated depolymerization of these complex organic N forms is often considered the rate limiting step that drives the remaining processes of the terrestrial N cycle (Wanek et al., 2010). In addition, there is growing evidence that heterotrophic microorganisms themselves play a major role in SOM formation and decomposition through death and biomass recycling (Fig. 3) (Liang et al., 2019, 2020; Sokol et al., 2022). The depolymerization of SOM is a complex process that involves the interaction of different microbial functional groups, such as decomposer microorganisms that produce a variety of extracellular enzymes, and cheaters, that exploit the catalytic activity of decomposers (Kaiser et al., 2015). It is often the interplay between these two groups, coupled with the quality and quantity of organic matter, that regulates turnover rates in soils.

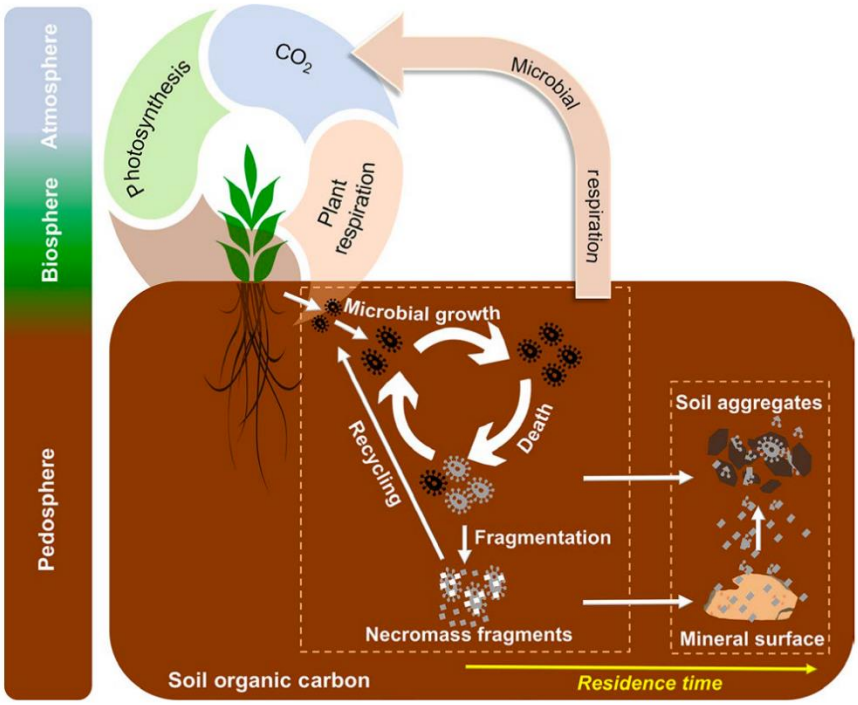

Figure 3. The role of soil microorganisms in SOM formation and decomposition. In order to sustain growth, heterotrophic soil microorganisms convert a portion of plant exudates and existing SOM into CO_2 via microbial respiration (mineralization). The other portion of assimilated C contributes to SOM formation via microbial assimilation into biomass and/or becomes attached to mineral surfaces. Upon death, microbial remains (necromass) contribute to the SOM pool. Figure from (Liang et al., 2019)

Extracellular enzymes depolymerize HMW organic N forms into smaller oligomers and monomers which can be easily taken up by heterotrophic microorganisms and eventually further mineralized to ammonium (NH_4^+), fueling inorganic N transformation processes. In addition, as organic N compounds also contribute to the organic C fraction, soil microorganisms may decompose organic N compounds to meet their energy and C demands (Knicker, 2011; Soong et al., 2020). Depolymerizing bacteria and fungi secrete a wide array of enzymes to their extracellular space with the goal of mining surrounding soil organic matter for C and nutrients. Secreted enzymes include glycosyl hydrolases, numerous proteases and peptidases, and exo-nucleases (Nguyen et al., 2019). The genomic potential to produce such extracellular enzymes is phylogenetically broadly distributed (Berlemont & Martiny, 2016; Nguyen et al., 2019). This means that there is a high level of functional redundancy with regard to the production of depolymerizing enzymes.

Enzyme-mediated depolymerization processes in soils can be controlled either by enzyme amount (enzyme limitation) or by resource availability (substrate limitation) (Wanek et al., 2010; Noll et al., 2019a). Both are strongly dependent on environmental conditions, input rates, and/or soil physicochemical factors which influence protein availability and microbial community structure (Noll et al., 2019b).

2.2. Inorganic nitrogen transformations

The mineralization of organic N molecules in microorganisms and subsequent release of NH_4^+ constitutes the link between the organic and inorganic parts of the N cycle. Nitrification is a key process of the inorganic terrestrial N cycle that includes the microbial oxidation of NH_4^+ to NO_2^- and NO_3^- and has been far better studied than the upstream processes of organic N transformations. In agricultural systems, nitrification contributes to fertilizer loss as NO_3^- easily leaches out of the soil and is further converted to volatile N compounds. This phenomenon can contribute to an increase on the eutrophication of downstream aquatic ecosystems and to the production of large amounts of the potent greenhouse gas nitrous oxide (N_2O) (Kuypers et al., 2018). Nitrification was for a long time considered to be a product of the division of labor between two distinct groups of autotrophic ammonia-oxidizing microorganisms (AOM) and nitrite oxidizing bacteria (NOB) (Costa et al. 2006). First, ammonia-oxidizing bacteria (AOB) and archaea (AOA) oxidize ammonia to nitrite (NO_2^-), which is subsequently oxidized to NO_3^- by NOB. The recent discovery that some species closely related to *Nitrospira* NOB – the comammox *Nitrospira* CMX - can catalyze both reactions called for a reassessment of the relative contribution of each microbial group to nitrification (Daims et al., 2015, 2016; van Kessel et al., 2015) (Fig.4).

Canonical AOB are restricted to four known genera: *Nitrosococcus*, *Candidatus* Nitrosoglobus (Gammaproteobacteria), *Nitrosomonas*, and *Nitrosospira* (Betaproteobacteria), out of which the latter is thought to be the dominant AOB in soils according to 16S rRNA and functional marker gene surveys (Di et al., 2009; Aigle et al., 2019). Regarding AOA, five clusters have been proposed within the phylum Thaumarchaeota: *Nitrososphaera*, *Nitrosocosmicus*, *Nitrosocaldus*, *Nitrosotalea* and *Nitrosopumilus* (Stahl and de La Torre, 2012; Lehtovirta-Morley, 2018).

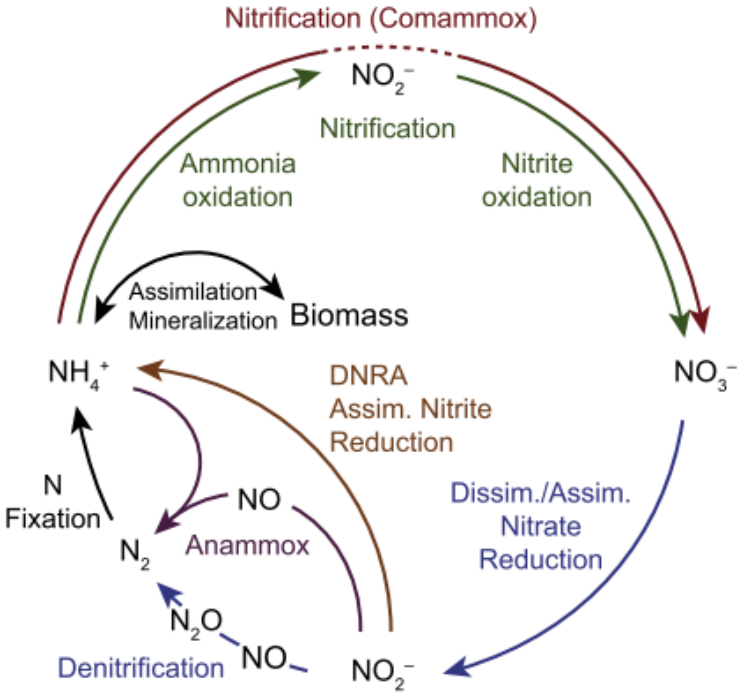

Figure 4. Schematic illustration of the main steps of the N cycle. Nitrification, consisting of the oxidation of ammonium and nitrite was thought to only be mediated by two distinct groups of microorganisms. Comammox microorganisms can perform the full conversion of ammonium to nitrate via nitrite. Abreviations: Anammox, anaerobic ammonium oxidation; DNRA, dissimilatory nitrite reduction to ammonia; assim., dissimilatory. Figure from Daims et al. 2016.

AOA often thrive in extreme habitats such as strongly acidic and high temperature ecosystems, but they are also known to co-occur with AOB in mesophilic soils (Tourna et al. 2011, PNAS). So far, less is known about the ecology of comammox *Nitrospira* (phylum Nitrospirae) other than that they are composed of two main clusters A and B with ubiquitous distribution patterns. While there are some studies on the physiology of the few pure culture CMX representatives, less is known about their role in the environment. (Pjevac et al., 2017; Koch et al., 2019; Wang et al., 2019; Li et al., 2020, 2022). Canonical NOB have traditionally received less attention than AOM due to the idea that ammonia-oxidation was the rate-limiting step of nitrification and that the NO_2^- in soils was short-lived and usually does not accumulate. In addition, progress in NOB research was further hampered by the long withstanding difficulties of growing these organisms in pure cultures (Daims et al., 2016). The emergence of metagenomics and other 'omics approaches has aided the culture-independent characterization of this highly diverse group and provided valuable insights into its

ecophysiology (Daims et al., 2016). Currently, NOB are found across four bacterial phyla, spanning seven genera: *Nitrobacter*, *Nitrotoga*, *Nitrococcus*, *Nitrospira*, *Nitrospina*, *Nitrolanceae* and *Candidatus* Nitromaritima. In terrestrial ecosystems, *Nitrobacter* and *Nitrospira*-like NOB are believed to be the main contributors to the oxidation of NO_2^- to NO_3^- and their relative abundance is thought to be strongly influenced by their different substrate and oxygen affinity levels (Daims et al., 2001, 2016; Attard et al., 2010; Pester et al., 2014; Han et al., 2018).

Environmental factors determine the structure and activity of the different nitrifying groups, but the mechanisms behind the selective dominance of one group over others are not entirely understood. AOA, AOB and CMX display fundamental differences in their physiology and cellular architecture which likely influence their distribution, their ability to adapt to environmental change and their capacity for N turnover (Prosser and Nicol, 2012; Lehtovirta-Morley, 2018).

Two key differences that attempt to explain the selective dominance of certain AOM groups over others concern ammonia oxidation kinetics and substrate affinities. In comparison to AOA and CMX, AOB have lower substrate affinities but higher maximum activity (V_{max}) thus providing AOB with a potential selective advantage over other AOM in nutrient-rich environments (Arp et al., 2007; Kits et al., 2017; Koch et al., 2019; Martens-Habbena et al., 2009). This idea is supported by results from ecological surveys, which show a dominance of AOA over AOB in the open ocean and low N soils, while AOB dominate in wastewater treatment plants and fertilized agricultural soils (Jia and Conrad, 2009; Rütting et al., 2021). Another factor influencing the abundance and distribution of nitrifying organisms is pH since the interconversion between NH_3 and NH_4^+ strongly depends on pH. The lower the pH, the lower the concentration of bioavailable substrate – NH_3 – for nitrification. The traditional observation that *Nitrosomonas europaea* was only able oxidize NH_3 (and not NH_4^+) was pointed as the main reason behind the lack of AOB-derived ammonia-oxidation in acidic environments but did not explain the mechanisms behind observed high nitrification rates at low pH (Suzuki et al., 1974). It was not until the discovery of the first acidophilic AOA *Nitrosotalea devanaterra* that this view changed (Lehtovirta-Morley et al., 2011; Herbold et al., 2017). In addition, the isolation of an AOB from soil (*Candidatus* Nitrosoglobus) capable of ammonia-oxidation at pH levels around 3 came to further complicate the niche differentiation dynamics between AOM under acidic conditions (Hayatsu et al., 2017). Further studies have additionally revealed the ability of AOB to use urea as substrate instead of NH_3 in acidic environments, as well as to grow in biofilms and aggregates (Burton and Prosser, 2001; Hayatsu et al., 2017). Moreover, the discovery of *Nitrosocosmicus*, a group of AOA isolated from soil capable of NH_3 oxidation at high nutrient concentrations further reduced support for

niche differentiation between AOM based solely on substrate concentration (Lehtovirta-Morley et al., 2016; Nicol et al., 2019).

Even though nitrifying microorganisms are found across five phyla spanning two domains of life, their phylogenetic distribution is still considered narrow in comparison to microorganisms involved in HMW SOM decomposition and organic matter assimilation (Nelson et al., 2016). Consequently, nitrification could be potentially more sensitive to environmental stress and climate change via changes in community composition (Schimel and Schaeffer, 2012).

3. Thesis outline and main goals

The central topic of this dissertation revolves around gaining a better understanding of the response of microbial communities to single and multiple climate change drivers, with a particular focus on microbial community composition and on microorganisms involved in organic and inorganic N transformations. To this purpose, I investigated differences in community structure at the DNA (presence) and RNA (potential activity) levels using standard phylogenetic markers (e.g. 16S rRNA gene, ITS1 region) and analyzed the expression of functional genes involved in inorganic N transformations (*amoA*) in a multifactorial climate change experiment (warming x eCO_2 x drought) in a managed grassland in central Austria. In addition, I evaluated the response of microorganisms involved in organic N decomposition processes through the analysis of soil metagenomes and metatranscriptomes in a geothermal warming experiment in Iceland.

The **second chapter** of this thesis focuses on inorganic N transformations in a managed sub-montane grassland. Here I investigated the responses of different ammonia-oxidizing microorganisms via *amoA* gene and transcript sequencing and quantification under elevated temperature, eCO_2 and drought conditions. The goal of the study was to understand how these climate change drivers alone or in combination affected the relative abundance of nitrifier communities and their activities (i.e gross nitrification rates). Results highlighted drought as a main driver causing group-specific changes in nitrifier community abundance, irrespective of temperature and eCO_2 manipulations.

In the **third chapter**, I analyzed the effect of seasonality in modulating single and combined effects of elevated temperature, CO_2 and drought on belowground bacterial/archaeal and fungal community structure and composition in a sub-montane grassland. Community structure was assessed by sequencing and quantifying phylogenetic marker genes such the 16S rRNA gene and the ITS1 fungal region. In addition, I looked into the structure of the

potentially active bacterial/archaeal communities via 16S rRNA sequencing in RNA extracts. Results indicated that climate change drivers on community structure were season specific. As drought emerged as a strong factor affecting microbial community structure, I attempted to identify potential drought legacy effects on the active fraction of the bacterial/archaeal community structure by analyzing the soil microbiome one year after the end of the drought treatment.

Finally, in the **fourth chapter** I performed a comprehensive analysis of microbially-mediated organic N transformations in response to soil warming. The successful implementation of comprehensive functional gene assays on genes that encode HMW-degrading enzymes is hampered by their high diversity and phylogenetic distribution. In order to overcome this, I sequenced and analyzed whole soil metagenomes and metatranscriptomes along natural soil temperature gradients. Here, the goal was to conduct an in-depth investigation of expression patterns of genes encoding enzymes involved in the extracellular degradation of HMW organic N polymers in response to soil warming. Results demonstrated that sustained soil warming leads to higher microbial investment into enzymes that involved in the breakdown and recycling of microbial remains such as peptidoglycan and proteins.

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Chapter II

Composition and activity of nitrifier communities in soil are unresponsive to elevated temperature and CO_2, but strongly affected by drought

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ARTICLE

Composition and activity of nitrifier communities in soil are unresponsive to elevated temperature and CO_2, but strongly affected by drought

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Abstract

Nitrification is a fundamental process in terrestrial nitrogen cycling. However, detailed information on how climate change affects the structure of nitrifier communities is lacking, specifically from experiments in which multiple climate change factors are manipulated simultaneously. Consequently, our ability to predict how soil nitrogen (N) cycling will change in a future climate is limited. We conducted a field experiment in a managed grassland and simultaneously tested the effects of elevated atmospheric CO_2, temperature, and drought on the abundance of active ammonia-oxidizing bacteria (AOB) and archaea (AOA), comammox (CMX) *Nitrospira*, and nitrite-oxidizing bacteria (NOB), and on gross mineralization and nitrification rates. We found that N transformation processes, as well as gene and transcript abundances, and nitrifier community composition were remarkably resistant to individual and interactive effects of elevated CO_2 and temperature. During drought however, process rates were increased or at least maintained. At the same time, the abundance of active AOB increased probably due to higher NH_4^+ availability. Both, AOA and comammox *Nitrospira* decreased in response to drought and the active community composition of AOA and NOB was also significantly affected. In summary, our findings suggest that warming and elevated CO_2 have only minor effects on nitrifier communities and soil biogeochemical variables in managed grasslands, whereas drought favors AOB and increases nitrification rates. This highlights the overriding importance of drought as a global change driver impacting on soil microbial community structure and its consequences for N cycling.

Introduction

Nitrogen (N) cycling is a fundamental process in terrestrial ecosystems, critical for the functioning of all living organisms. Soil ecosystems are currently undergoing substantial changes, as a consequence of anthropogenic activities [1, 2]. Microorganisms catalyze most soil N transformation processes, and thus play a central role in mediating N exchange between the atmosphere, plants, and soils [3]. Thus, understanding the nature and the intensity of the responses of soil microorganisms to climate change is of utmost importance to predict the future terrestrial N cycle,

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including its repercussion on plant productivity and climate-carbon feedbacks [3, 4]. Yet, our understanding of the relative role of specific microbial taxa in mediating N transformations in terrestrial environments in response to climate change is still incomplete. In spite of current climate projections [5] the interactive effects of elevated temperature, elevated atmospheric CO_2, and changes in precipitation patterns have rarely been assessed together with regards to microbially mediated soil N transformations [6, 7].

Nitrification represents a key process in the terrestrial N cycle. It has long been considered to be a two-step process initiated with the oxidation of ammonia to NO_2^- by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), followed by NO_2^- oxidation to NO_3^- by nitrite-oxidizing bacteria (NOB) [8]. Based on metagenomic surveys, marker gene assays and functional gene studies targeting the *amoA gene*, (gene that codes for the alpha subunit of ammonia monooxygenase) several studies demonstrated the influence of different environmental factors on the abundance and composition of nitrifying communities, most notably substrate concentration, soil water content and soil pH [9–11]. Furthermore, all known ammonia-oxidizers contribute to nitric (NO) and nitrous (N_2O) oxide emissions, but the yield by which they do differs strongly between groups, with AOB showing the highest N_2O yield per mol of oxidized ammonia when compared with other groups [12]. Several studies have reported a numeric dominance of AOB over AOA in agricultural and grassland soils to which fertilizers are applied on a regular basis, leading to the idea that AOB are responsible for the ammonia-oxidation step in these environments [13, 14]. In contrast, AOA were thought to be the main ammonia-oxidizing taxa in N poor environments, as well as in acidic soils [15]. Notably, all known AOB have a relatively low substrate affinity, whereas AOA encompass members with a broad range of substrate affinities [12]. NOB span six genera, out of which *Nitrospira* is the most phylogenetically diverse and widespread across different habitat types including terrestrial ecosystems [16]. Recently, it was discovered that some members of this genus (Comammox *Nitrospira*; CMX) can catalyze both steps of nitrification [17, 18]. Although our knowledge on the distribution and diversity of comammox *Nitrospira* is still comparatively limited, several studies have reported their presence and activity in terrestrial environments [19, 20]. Furthermore, it has been demonstrated that the only cultured comammox species *N. inopinata*, has a high substrate affinity [12]. Thus, a joint assessment of the relative contribution of the aforementioned microbial groups to nitrification is needed, as well as of their specific responses to climate change.

Current global change in temperate ecosystems is characterized, amongst other changes, by rising air and soil temperature, elevated CO_2 concentrations, and changes in water availability, such as increased frequencies of drought events. Elevated atmospheric CO_2 concentrations affect soil microorganisms by promoting plant growth and photosynthetic activity [21]. This can result in increased plant belowground C allocation and rhizodeposition [22, 23], which stimulates heterotrophic microbial activity and mobilizes N. However, higher plant growth can indirectly affect soil microorganisms by gradually depleting the soil N pool available for microbial uptake [24, 25], potentially causing microorganisms to become N limited. Previous studies revealed a decrease in the relative abundance of AOA and AOB in response to elevated CO_2 [26, 27]. In contrast to elevated CO_2, elevated temperature directly accelerates microbial processes and turnover rates [28], alters water and nutrient availability, extends the plant growing season [29] and may favor the growth of slow growing bacteria [30]. Concomitantly, studies have reported significant changes in *amoA* gene abundance and expression of AOA and AOB in response to temperature [27, 31]. The growth, activity, and community structure of AOA, but not of AOB have been shown to change in response to elevated temperature in soil mesocosms [31]. On the other hand, a comprehensive study across 23 different soil types has highlighted temperature as the most important factor affecting AOB community structure [32]. Finally, extreme events such as droughts can cause soil microorganisms to decrease their activity levels, to accumulate osmoprotectants/compatible solutes, and often to switch to dormancy [33]. A study in mountain grasslands showed that drought had distinct effects on the relative abundance of ammonia-oxidizing microorganisms, with reduced AOA *amoA* abundance with drought and no observed changes in AOB *amoA* abundance [11].

Multifactorial studies that particularly target nitrifier communities are scarce, and most studies are rather focused on the response of broad ecosystem N processes to global change [34–36].

Therefore, the goal of this study was to understand how elevated atmospheric CO_2 (eCO_2), elevated temperature (eT) and drought (D), alone or in combination, affect the relative abundance and diversity of nitrifier communities and their activity (i.e., gross nitrification rates) in a managed submontane grassland. We expected to observe: (i) a shift in active nitrifier community structure towards high affinity nitrifiers (AOA and CMX) as a consequence of lower NH_4^+ availability at eCO_2; (ii) an overall increase in nitrification rates and *amoA* expression levels of AOA, AOB, and potentially CMX in response to eT; and (iii) a reduction of the abundance and activity of all nitrifying groups by drought. Our study also intended to explore whether the combined effect of eCO_2 and eT—with or without drought—would have additive or nonadditive effects on the diversity and abundance of nitrifiers.

Methods

Site description and experimental design

The experimental site is located at the agricultural research institute HBLFA Raumberg-Gumpenstein in Styria, Austria (47°29′38″N, 14°06′03″E) and is part of a long-term climate change experiment (ClimGrass). With a mean annual temperature of 8.2 °C and a mean annual precipitation of 1056 mm, this area is representative of a large part of alpine grasslands. The grassland under study was established in 2007 and is dominated by the grasses *Arrhenatherum elatius* and *Festuca pratensis* together with legume species such as *Lotus corniculatus* and *Trifolium pratense*. It also includes a few subdominant non-leguminous forbs such as *Taraxacum officinale and Plantago lanceolata*. The soil type is a Cambisol (WRB classification) with loamy sand texture and a pH of 5.

The experimental design was originally based on a response surface regression approach that aimed to study the nature and intensity of the grassland's responses to manipulations of elevated atmospheric temperature and CO_2, and drought [37]. The experimental site comprised a total of 54 plots showing individual and combined effects of three levels of temperature (+1.5 °C, + 3 °C), atmospheric CO_2 concentrations (ambient, +150 ppm, +300 ppm) and drought (Fig. S1). A detailed explanation regarding the reasons behind the choice of treatments and corresponding number of replicates can be found in [37]. Each treatment had a different number of replicates, which reflected a balance between statistical power and budget constraints [37].

For this specific experiment, we selected only a set of soil samples from 26 plots (4 × 4 m each) during peak growing season in July 2017 (Table 1; Fig. S1). Treatments included soil plots on which temperature (eT; +3 °C) and CO_2 (eCO_2; +300 ppm CO_2) were manipulated individually or in combination, as well as plots on which drought (D) was simulated through the use of automatic rainout shelters installed 8 weeks before sampling (Table 1; Fig. S1). Manipulations in atmospheric CO_2 concentrations were done through a miniFACE—free air CO_2 enrichment system, whereas atmospheric temperature was manipulated through the use of infrared heaters. Since 2014, infrared heaters were continuously on except when snow cover exceeded a depth of 10 cm. The soil temperatures measured by soil probes in selected plots between June and July 2017 showed an average of +1.86 °C for plots affected by elevated temperature, compared with the controls (data not shown). CO_2 fumigation was only applied during the growing season (April to November). Site management included mowing (three times a year) and mineral fertilization (90 kg N ha^{-1} a^{-1} as NH_4NO_3, 65 kg P ha^{-1} a^{-1} as $Ca(H_2PO_4)^2$ and 170 kg K ha^{-1} a^{-1} as K_2O), which corresponds approximately to the amount of NPK removed by biomass harvest, in order to replicate local farming practices.

Table 1 Soil plots sampled in this study.

Treatment	Treatments used for statistical analyses		
	Description	Individual and combined effects of temperature and CO_2	Individual and combined effects of future climate and drought
[Amb]	Ambient conditions ($n = 8$)	×	×
[eT]	+3 °C above ambient ($n = 3$)	×	
[eCO_2]	+300 ppm CO_2 above ambient ($n = 3$)	×	
[eT] × [eCO_2]	+3 °C and +300 ppm CO_2 above ambient ($n = 4$)	×	×
[Amb] × [D]	Ambient conditions + rainout shelter ($n = 4$)		×
[$eTeCO_2$] × [D]	+3 °C and +300 ppm CO_2 above ambient + rainout shelter ($n = 4$)		×

Biological replicates are shown in parenthesis. Specific soil plots were selected in order to test for either the individual and combined effects of elevated temperature and CO_2 or the individual and combined effects of future climate and drought. This separation was done in order to have a full factorial design for each set. The spatial arrangement of all the plots is shown in Fig. S1.

Sample collection and soil physicochemical analyses

Immediately after removal of aboveground biomass several (4–11) soil cores were taken per plot to a depth of 10 cm using a stainless-steel soil corer with 2 cm inner diameter. Stones and roots were picked, washed, dried and weighed, and soils were sieved through a 2 mm sieve directly after collection. Fresh soil subsamples for molecular analyses were immediately frozen in liquid N. The remaining fresh soil was transported to the laboratory and incubated shortly (2–3 days) at the respective in situ temperatures measured at the time of collection (17 °C for ambient temperature plots and 20 °C for elevated temperature plots) until analysis.

Gravimetric soil water content was determined by oven drying (105 °C for 24 h) 5 g of fresh, sieved soil. Dissolved soil organic C (DOC), nitrogen (DON) and total dissolved N (TDN) were measured in 1 M KCl extracts (using a soil to solution ratio of 1:7.5 w–v) after filtering through ash-free cellulose filters (Whatman, GE Healthcare Life Sciences) on a TOC/TN analyzer (TOC-VCPH/TNM-1, Shimadzu, Austria). In parallel, soils were chloroform fumigated for 48 h in order to estimate microbial biomass nitrogen (MBN) [38]. After fumigation, soils were also extracted with 1 M KCl, and MBN was calculated as the difference in TDN between fumigated and non-fumigated

soils. Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were determined photometrically in the 1 M KCl extracts of non-fumigated soils [39]. Soil pH was determined in a 1:5 mix of dry soil and 0.01 M $CaCl_2$ solution with a pH meter (Sentron Europe BV).

Isotope pool dilutions

Gross rates of N mineralization, and nitrification were determined and calculated from fresh, sieved soil samples using ^{15}N pool dilution assays [40].

Two to three grams of duplicate soil samples were incubated with $^{15}NH_4Cl$ and $K^{15}NO_3$ (98 at%) tracer solutions for 4 and 24 h at the corresponding in situ field temperatures. We aimed to increase the ^{15}N enrichment of the target pool to ~20 at% ^{15}N and added 100 µl of tracer solution per gram of fresh soil, after a previous photometrical determination of background NH_4^+ and NO_3^-pool sizes. The addition of liquid the tracer increased the soil water in ambient and drought plots by 1.79 and 4.56-fold respectively. Incubations were stopped after 4 and 24 h by the addition of 1 M KCl (1:7.5 w–v), extraction by horizontal shaking on an orbital shaker for 30 min (150 rpm) and filtration through ash-free cellulose filters. Mineralization extracts were prepared using a microdiffusion method [41] followed by the measurement of $^{15}/^{14}N$ isotope ratios and concentrations of NH_4^+ in the acid traps by elemental analyzer-isotope ratio mass spectrometry (EA-IRMS; EA1110 coupled via ConFlo III interface to a Delta^{PLUS} IRMS, Thermo Fisher, Bremen, DE [42]. Concentrations and $^{15}/^{14}N$ isotope ratios of NO_3^- in 1 M KCl extracts were determined by converting NO_3^- to NO_2^- with vanadium (III) chloride (VCl_3) and further reduction of NO_2^- to N_2O by sodium azide (NaN_3) [42]. Concentrations and $^{14}/^{15}N$ isotope ratios of the resulting N_2O were determined by purge-and-trap isotope ratio mass spectrometry (PT-IRMS), using a Gasbench II headspace analyzer (Thermo Fisher, Bremen, DE) with a cryo-focusing unit, coupled to a Finnigan Delta V Advantage IRMS (Thermo Fisher, Bremen, DE).

TNA extraction, RNA purification, and cDNA synthesis

Total nucleic acids (TNA) were extracted from 0.4 g frozen soil by bead-beating in the presence of a cetyl trimethylammonium bromide buffer and phenol, according to a previously published extraction protocol [43] and eluted in 250 µl of Low-Tris-EDTA buffer. Following extraction, samples were purified using the OneStep™ PCR Inhibitor Removal Kit (Zymo, Irvine, CA, USA) and TNA was quantified using the Quant-iT™ PicoGreen® Kit (Thermo Fisher Scientific), according to the manufacturers' instructions. DNA was digested in 1–3 µg of TNA extract with Turbo DNase (Thermo Fisher Scientific), and RNA was purified using the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Fisher Scientific) and eluted in 25 µl of RNase Storage Solution (Thermo Fisher Scientific). The RNA yield was quantified using the Quant-iT™ RiboGreen® assay (Thermo Fisher Scientific). Successful DNA digestion was confirmed by negative results on a gel electrophoresis after a DNA-targeted SSU rRNA gene PCR assay on the purified RNA extracts using the primer pair 515F-mod/806R-mod [44, 45]. Afterwards, cDNA was synthesized from 400 ng of RNA extract using random hexamer primers and SuperScript™ IV reverse transcriptase (Thermo Fisher Scientific) following the manufacturer´s instructions and eluted in 54 µl of nuclease-free water.

amoA/nxrB gene and transcript amplification and sequencing

The diversity of the ammonia-oxidizing microbial community, as well as the NOB community was assessed via amplification and sequencing of the ammonia monooxygenase enzyme subunit A (*amoA*) gene, and the nitrite oxidoreductase subunit beta (*nxrB*) gene respectively, with functional group specific primers modified to include a linker sequence ('head') for barcoding PCRs at the 5′ end, as described previously [46]. Detailed amplification conditions can be found in the Supplementary Material and Methods file. Library preparation and sequencing services were provided by Microsynth (Balgach, Switzerland). The library was prepared by adapter ligation and PCR using the TruSeq Nano DNA Library Prep Kit according to the TruSeq nano protocol (Illumina, FC-121–4003), but excluding the fragmentation step. Sequencing was performed on a MiSeq platform, v3, 2 × 300 bp (Illumina). Paired MiSeq reads were processed according to [46]. AOB, and AOA *amoA* gene OTUs were clustered at 95% and 96% sequence identity, respectively [47, 48]. A sequence identity cutoff of 95% was used for NOB *nxrB* OTUs and for CMX clade B *amoA* OTUs. Taxonomic classification of AOA, AOB-related (*Nitrosomonas*), and NOB OTUs was performed using the evolutionary placement algorithm implemented in RAxML to place OTUs into a reference tree using a set of classified full-length nucleotide sequences [49, 50]. Uncultured *Nitrosospira* (AOB) *amoA* OTUs were classified by local nucleotide BLAST (version 2.8.1+) searches against a *Nitrosospira* reference database [51] since phylogenetic trees among reference *Nitrosospira* showed no stable tree topology for mapping reads. Represented sequences for all CMX clade B *amoA* were aligned against a reference *pmoA* and *amoA* database [52] using the SINA aligner [53] and a maximum likelihood phylogenetic tree was calculated based on this alignment using W-IQ-Tree [54] with 1000 bootstrap iterations and ModelFinder to determine the best fitting base

substitution model [55]. The resulting tree was visualized with iTOL [56].

All sequence alignments were performed with Mafft [57] and manually inspected. OTUs with no similarity to the functional group of interest were considered unspecific co-amplification and excluded from further analysis (Table S1).

AmoA gene and transcript quantification by qPCR

Gene and transcript copy numbers of *amoA* genes of beta-proteobacterial AOB, AOA, and CMX clade B were determined by qPCR. Gene and transcript copy numbers of *Nitrospira nxrB* were not assessed in this study due to the highly variable gene copy number per genome, which would hamper precise quantifications. In addition, the primer pair used for amplicon sequencing co-amplifies several nonspecific amplicons, which would also make quantifications inaccurate [58].

Assays with different sample dilutions (5× to 10,000×), resulting in template amounts ranging from 0.004 to 24 ng, were performed to minimize possible PCR inhibition caused by excess template or co-extracted inhibitory substances. For all samples, the highest dilutions that yielded consistent results were used to calculate gene and transcript copy numbers. All assays were performed in triplicate for each dilution. Detailed amplification conditions can be found in the Supplementary Material and Methods file. For each assay, standard series were generated by tenfold serial dilutions (10^8–10^1 gene copies μl^{-1}) from purified M13-PCR products obtained from environmental samples (CMX clade B *amoA*) or pure cultures of *Nitrosomonas europaea* and *Nitrososcosmicus hydrocola*, as described previously [52, 59]. The average amplification efficiency for the comammox clade B *amoA*, archaeal *amoA*, and betaproteobacterial *amoA* assays was 90.6%, 93.6% and 89,2% respectively.

Statistical analyses

All statistical analyses were performed in R (version 3.5.1). Statistical analyses were performed with two separate linear models using the *Anova()* function of the package "car" following a full factorial design that allowed to test for interactions between all factors under analysis. This approach was chosen because the drought treatment was imposed on a reduced set of treatments (Table 1; Fig. S1) that did not include soil plots where eT and eCO_2 were manipulated individually. Therefore, the first model (eT vs. eCO_2 dataset) included [eCO_2], [eT] and their interactions. The second model (drought dataset) included drought, [eT × eCO_2] and their interactions. Both models were used to assess if individual global change effects were modified when variables were combined (interactive effects) in order to reveal potential additive, synergistic or antagonistic effects (Tables 2 and 3, Fig. 1, Table S2). Model assumptions of normality and homoscedasticity were checked on the model residuals [60] and variables were transformed when needed to meet model assumptions. Nonparametric tests were conducted in case assumptions were not met after data transformation. Multiple comparisons were performed on the interactive effects using the Least Square Means within the R package "lsmeans" (Table S3). In order to account for the potential effect of different treatment replicates, we used a type II ANOVA, except whenever an interaction effect was observed and a type III ANOVA was used instead [61]. Sequencing data were analyzed with the "phyloseq" package [62]. The Shannon diversity index was calculated after removing OTUs not observed more than three times in at least 10% of the samples. Specific sequencing barcodes per sample, and the number of reads that passed the filtering criteria can be found in the Supplementary Datafile 1. We used a permutational analysis of variance (PERMANOVA) with 9999 permutations on a dissimilarity matrix (Morisita–Horn on top of a Hellinger transformed OTU table) to assess the effect of the variables under study on functional gene diversity using the function *adonis()* in the "vegan" package with a two-factorial design [63] (Table S4). Similarly to the ANOVA analyses, we developed two models, the first model (eT vs. eCO_2 dataset) included [eCO_2], [eT], and their interactions, the second model (drought dataset) included drought, [eT × eCO_2] and their interactions. This test is sensitive to dispersions among groups, which might be more pronounced in situations where there is a different number of replicates per treatment and thus confound significant PERMANOVA results. Therefore, we confirmed *a priori* that groups did not differ significantly in their dispersion by performing an analysis of multivariate homogeneity (PERMDIST) using the function *betadisper()* of the package 'vegan', with the bias.adjust=T argument, to account for differences in sample size [64, 65]. All raw sequencing data were deposited into the NCBI SRA under BioProject ID PRJNA612521. All the remaining data are available as Supplementary Material.

Results

Soil biogeochemical variables and processes are sensitive to drought but not to other global change factors

Manipulation of CO_2 concentration and temperature had little or no effect on soil N and C pools, and N processes in the studied grassland (Table 3). No significant statistical effect was found for [eCO_2], neither alone nor in combination with [eT], for soil N, SWC, pH and gross N process

Table 2 Average values for soil parameters, organic and inorganic pools and process rates (means ± SE).

	Mean values per treatment											
	[Ambient]		[eT]		[eCO_2]		[eT] × [eCO_2] [eT × eCO_2]		[Ambient] × [D]		[eT × eCO_2] × [D]	
	Mean ± SE		Mean ± SE		Mean ± SE		Mean ± SE		Mean ± SE		Mean ± SE	
	($n = 8$)		($n = 3$)		($n = 3$)		($n = 4$)		($n = 4$)		($n = 4$)	
Soil parameters												
SWC	0.34	0.01	0.32	0.01	0.34	0.00	0.33	0.01	0.08	0.01	0.06	0.01
pH	4.95	0.03	5.02	0.05	4.99	0.03	4.97	0.03	4.96	0.04	5.01	0.02
Soil N	0.31	0.01	0.27	0.02	0.26	0.02	0.29	0.02	0.32	0.01	0.33	0.03
MBN	79.31	5.89	93.39	14.16	63.65	9.89	93.34	6.59	99.44	7.09	120.26	6.03
Extractable organic and inorganic pools												
NO_3^-	2.97	0.64	5.18	2.30	4.19	1.47	5.95	2.46	5.98	1.72	5.26	1.09
NH_4^+	1.11	0.20	0.87	0.12	0.95	0.13	0.89	0.11	12.23	4.54	13.13	2.99
DIN	4.09	0.76	6.04	2.25	5.13	1.45	6.84	2.51	18.21	6.15	18.39	3.56
DON	14.54	2.21	8.51	1.90	12.37	0.67	12.65	1.83	19.34	1.18	23.78	3.45
TDN	18.63	2.78	8.51	1.91	12.37	0.68	19.49	1.07	37.55	7.08	42.16	4.48
DOC	87.28	8.65	93.79	3.26	79.99	0.88	94.07	7.93	165.68	45.37	215.09	59.17
Gross process rates												
NH_4^+ mineralization[a]	2.72	0.38	1.75	0.76	2.62	0.35	2.97	0.49	4.03	0.30	6.97	0.81
NH_4^+ immobilization[ab]	2.08	0.49	1.14	0.51	1.82	0.23	3.44	1.05	5.18	1.73	10.59	3.89
Nitrification	1.77	0.42	2.24	0.80	2.73	0.20	2.38	0.73	3.89	0.17	4.56	0.62

The number of biological replicates is shown in parenthesis. Soil water content (SWC) is expressed in % of fresh soil; *MBN* microbial biomass N and *TDN* total dissolved nitrogen are expressed in μg N g^{-1} DW; *DIN* dissolved inorganic nitrogen and *DON* dissolved organic nitrogen are expressed in μg N g^{-1} DW. Ammonium (NH_4^+) and nitrate (NO_3^-) are expressed in μg N g^{-1} DW. *DOC* dissolved organic carbon is expressed in μg C g^{-1} DW. All process rates are expressed in μg N g^{-1} DW d^{-1}.

[a] $n = 3$ for treatment [Ambient] × [D].

[b] $n = 3$ for treatment [eT × eCO2].

rates. In contrast, only microbial MBN and DOC increased significantly in response to [eT] alone (Table 3).

Drought had a significant effect on most of the measured variables and processes. SWC decreased from 33% in the ambient plots to around 6% in the drought plots (Tables 2 and 3, $F = 1164.6$, $p = 2.25 \times 10^{-16}$). In addition, drought caused a loss of 24.6 % (in ambient conditions) and 50% (in future climate scenarios) of aboveground plant biomass, which corresponded to a reduction in plant N uptake of 15.8% and 46.6% respectively (data not shown). Furthermore, MBN was significantly higher in both the drought plots ($F = 11.31$, $p = 0.003$) and in [eT × eCO_2] plots ($F = 6.11$, $p = 0.025$), with no significant interaction term (Table 3).

There was a significant increase of DIN ($F = 15.24$, $p = 0.001$), DON ($F = 10.96$, $p = 0.004$), TDN ($F = 26.35$, $p = 0.0001$), and DOC ($F = 24.44$, $p = 0.0001$) in the drought plots relative to ambient plots, but no significant effect of [eT × eCO_2] conditions. The response of inorganic N forms to drought was mainly driven by increases in NH_4^+ contents under drought (Table 3; $F = 93.35$, $p = 4.23 \times 10^{-8}$), since NO_3^- levels remained unchanged. Moreover, N mineralization rates increased significantly in response to individual and combined effects of drought and [eT × eCO_2] (Table 3). Concomitantly, nitrification rates were significantly higher in the drought plots (Tables 2 and 3). These results should however be interpreted with caution due to the potential short-term rewetting effect introduced by the application of a liquid tracer during the isotope pool dilution experiments [66].

AOB are the most abundant ammonia-oxidizer group and did not decrease with drought

Betaproteobacterial AOB *amoA* gene copy numbers ranged between 1.43×10^9 and 3.79×10^9 copies g^{-1} DW,

Table 3 Individual and combined effects of eT, eCO_2, and drought in soil parameters, organic and inorganic pools and process rates.

	Treatment effects											
	eT vs. eCO_2 dataset						Drought dataset					
	[eT]		[eCO_2]		[eT] × [eCO_2]		[$eTeCO_2$]		[D]		[eT × eCO_2] × [D]	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Soil parameters												
SWC	3.140	0.09	0.72	0.40	1.180	0.29	1.740	0.21	1164.59	$\mathbf{2.24 \times 10^{-16}}$	0.621	0.42
pH	0.557	0.47	0.01	0.93	1.280	0.27	0.890	0.36	0.28	0.60	0.320	0.57
Soil N	0.128	0.73	1.39	0.26	3.140	0.09	0.262	0.62	1.08	0.31	0.422	0.52
MBN	5.620	**0.03**	0.99	0.33	0.769	0.40	6.110	**0.02**	11.30	$\mathbf{3.00 \times 10^{-3}}$	0.240	0.63
Extractable organic and inorganic pools												
NO_3^-	1.570	0.23	0.40	0.53	0.010	0.89	0.990	0.33	1.03	0.32	1.720	0.20
NH_4^+	0.510	0.48	0.05	0.82	0.080	0.77	0.030	0.86	93.35	$\mathbf{4.23 \times 10^{-8}}$	0.670	0.42
DIN	1.230	0.28	0.31	0.58	0.005	0.94	0.700	0.41	15.24	$\mathbf{1.00 \times 10^{-3}}$	0.100	0.75
DON	3.120	0.09	0.54	0.47	2.260	0.15	0.007	0.93	10.96	$\mathbf{4.00 \times 10^{-3}}$	1.070	0.31
TDN	0.140	0.71	1.40	0.25	1.250	0.28	0.770	0.39	26.35	$\mathbf{1.00 \times 10^{-4}}$	0.037	0.84
DOC	5.330	**0.02**	0.01	0.89	NA	NA	1.680	0.21	24.44	$\mathbf{1.00 \times 10^{-4}}$	0.000	0.99
Gross process rates												
NH_4^+ mineralization	0.560	0.46	0.84	0.37	1.550	0.23	8.180	**0.01**	22.81	$\mathbf{2.00 \times 10^{-4}}$	5.830	**0.03**
NH_4^+ immobilization	0.020	0.88	1.19	0.29	3.110	0.10	2.210	0.16	5.60	**0.03**	0.068	0.79
Nitrification	0.030	0.85	0.95	0.34	0.430	0.51	1.320	0.26	15.26	$\mathbf{1.40 \times 10^{-3}}$	0.002	0.96

Statistical effects were assessed by a 2-way ANOVA type II or a 2-way ANOVA type III whenever a significant interaction term was found. Least square means (lsmeans) multiple comparison tests were ran on interactive effects. Significant effects from the ANOVA are shown in bold. NA depict variables for which nonparametric tests were used and therefore an interactive effect could not be calculated.

SWC soil water content, *MBN* microbial biomass nitrogen, *DIN* dissolved inorganic nitrogen, *DON* dissolved organic nitrogen, *TDN* total dissolved nitrogen, *DOC* dissolved organic nitrogen.

indicating that they were the most abundant of all ammonia-oxidizing microorganisms in these montane-grassland plots (Fig. 1). Archaeal *amoA* gene copy numbers ranged between 1.21×10^8 and 2.70×10^8 copies g^{-1} DW, and CMX clade B *amoA* gene copy numbers ranged from 9.10×10^7 to 2.50×10^8 copies g^{-1} DW. We observed no significant effect of individual and combined [eT] and [eCO_2] effects on *amoA* gene abundance (Fig. 1a). We found an interactive effect of [$eTeCO_2$] and [D] in both CMX and AOB (Fig. 1b), although post-hoc analyses revealed significant differences between individual treatments for AOB only (Table S3).

Betaproteobacterial AOB *amoA* transcript copy numbers ranged between 1.14×10^7 and 3.62×10^7 copies g^{-1} DW, whereas AOA and CMX clade B had similar *amoA* transcript copies g^{-1} DW ($0.27–0.99 \times 10^7$ and $0.23–1.46 \times 10^7$, respectively) (Fig. 1). We observed no significant individual [eT] and [eCO_2] effects on *amoA* transcription (Fig. 1a; Table S2). There was an interactive effect in CMX *amoA* transcription (Fig. 1a), although not supported by the post-hoc analysis (Table S3). On the other hand, both AOA and CMX clade B *amoA* transcript copy numbers decreased significantly in response to drought (Fig. 1b). Notably, AOB *amoA* transcription was not affected by drought, but a significant decrease in AOB *amoA* transcript copy numbers was observed in plots affected by [eT × eCO_2].

AOA and NOB community structure is significantly affected by drought

The richness of nitrifying communities in all treatments was relatively low. We recovered 12 CMX *amoA* OTUs, 23 AOA *amoA* OTUs, and 15 AOB *amoA* OTUs which were robustly assigned to the corresponding phylogenetic clades after excluding unspecific OTUs (Figs. S2, S3). Unspecific OTUs comprised 0.01%, 0.05%, and 0.3% of all reads for AOA, CMX, and AOB, respectively, (Table S1) and are therefore unlikely to interfere with the qPCR quantifications. In addition to excluding nonspecific AOA OTUs, the coverage of the qPCR primers was checked in silico due to the use of different AOA primer pairs for archaeal *amoA* quantification and sequencing. This resulted on the exclusion of three more AOA OTUs, which comprised 32% of all reads (Table S1). Notably, CMX clade A *amoA* amplification was not detectable in all samples. Furthermore, 40 *nxrB* OTUs also displayed a robust taxonomic classification

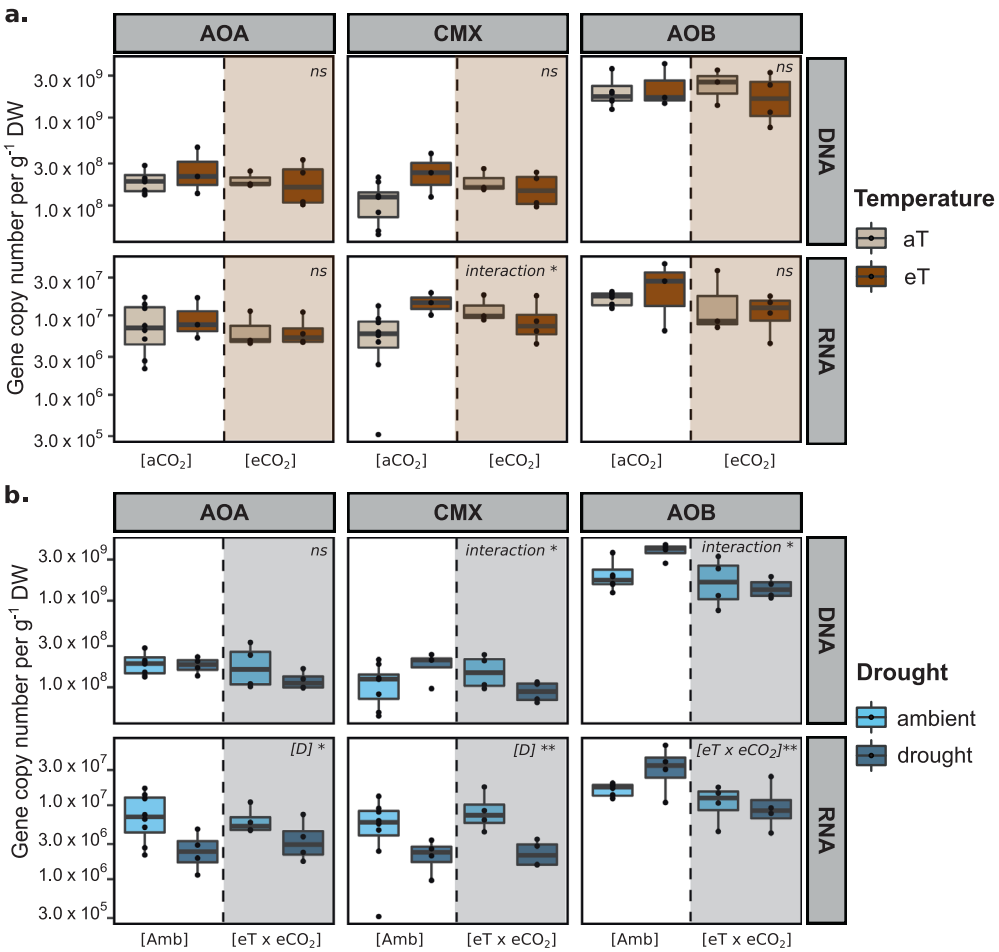

Fig. 1 qPCR quantifications of amoA gene and transcript copy numbers per gram of dry soil from the ammonia-oxidizing microbial groups. a Quantifications from plots affected by elevated temperature (eT) and atmospheric CO_2 concentrations (eCO_2). The dashed lines represent plots affected by elevated atmospheric CO_2 conditions (eCO_2). The colors represent plots affected by either ambient temperature (aT) or elevated temperature (eT). **b** Quantifications from plots affected by future climate (eT x eCO_2) and drought (D). The dashed lines separate plots affected by drought (D). The colors represent the drought treatment. The caption on the upper right corner of each subplot represents the ANOVA results. *p value <0.05; **p value <0.01. Whenever the ANOVA results showed a significant interaction term, lsmeans multiple comparison tests were ran. For details on the ANOVA and post-hoc tests, see Tables S2 and S3.

within the reference tree after excluding nonspecific OTUs (Table S1, Fig. S4).

The most abundant AOA *amoA* gene and transcript OTU was affiliated with genus *Nitrosotalea* and comprised 12–65% of the reads per treatment. The remaining OTUs were affiliated with members of the family *Nitrososphaeraceae*, which comprises the genera *Nitrososcosmicus* and *Nitrososphaera*. Within the *Nitrososphaeraceae*, we observed a sister group to the genus *Nitrososphaera*, comprising ten OTUs and 13–37% of the reads per treatment (Fig. S2a). Statistical analyses showed that the AOA *amoA* gene community structure was significantly affected by [eT] alone (PERMANOVA, $F = 8.50$; $p = 0.02$; Table S4; Fig. S5a), and plots on which temperature was manipulated individually showed a higher Shannon index (ANOVA, $F = 5.18$; $p = 0.03$; Fig. 2a). In addition, variance partitioning revealed a significant difference in AOA *amoA* gene and transcript community structure in response to drought (PERMANOVA, $F = 36.48$, $p \le 0.001$; and $F = 3.91$, $p = 0.04$, respectively) (Fig. 3b; Table S4; Fig. S5b). Changes in community structure resulted in a significantly higher Shannon diversity index of AOA *amoA* gene (ANOVA, $F = 10.908$, $p = 0.004$) in the drought plots (Fig. 2b). The changes in AOA community composition at both transcript and gene levels reflected a clear decrease in the relative abundance of *Nitrosotalea*-affiliated OTUs in response to individual manipulations of drought (Fig. 3a; Fig. S5b) and [eT] (Fig. 3a; Fig. S5a).

Regarding CMX clade B *amoA* gene and transcript diversity, a clear dominance of one single OTU, which comprised an average of 87.3% and 87.5% of gene and transcript reads per treatment, respectively, (Fig. 3; Fig. S5),

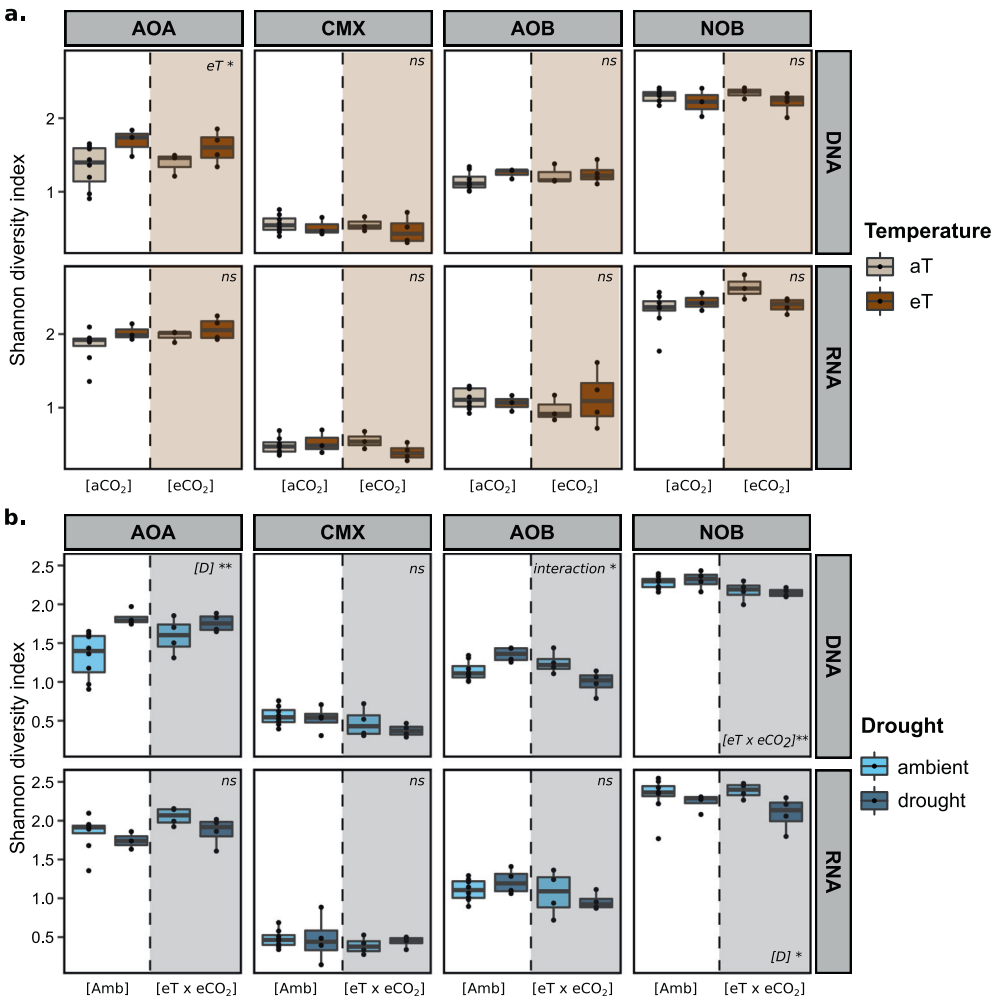

Fig. 2 Shannon diversity index based on amoA/nxrB gene and transcript sequences from all microbial groups. a Quantifications from plots affected by elevated temperature (eT) and atmospheric CO_2 concentrations (eCO_2). The dashed lines represent plots affected by elevated atmospheric CO_2 conditions (eCO_2). The colors represent plots affected by either ambient temperature (aT) or elevated temperature (eT). **b** Quantifications from plots affected by future climate (eT x eCO_2) and drought (D). The dashed lines separate plots affected by drought (D). The colors represent the drought treatment. The caption on the upper right corner of each subplot represents the ANOVA results. *p value <0.05; **p value <0.01. Whenever the ANOVA results showed a significant interaction term, lsmeans multiple comparison tests were ran. For details on the ANOVA and post-hoc tests, see Tables S2 and S3.

was observed. There were no significant effects of any tested variable on CMX clade B community composition and diversity indexes.

The betaproteobacterial AOB *amoA* diversity was dominated by OTUs affiliated with the genus *Nitrosospira*, and the abundance of *Nitrosomonas*-related *amoA* OTUs was very low (average of 0.24% and 0.73% of *amoA* gene and transcript reads per treatment, respectively). At the transcript level, the most abundant OTU was most similar to an uncultured *Nitrosospira* clade D19 OTU (100% identity), which comprised an average of 53% of the reads per treatment (Fig. 3), dropping to 24.4% at the gene level (Fig. S4). On the other hand, the most abundant OTU at the gene level was most similar to an uncultured *Nitrosospira* clade D12 OTU (100% identity), with an average of 56.7% of the reads per treatment (Fig. S4). The most abundant transcript OTU was 87.6% identical to the most abundant gene OTU. Variance partitioning showed a significant interaction effect of $[eT \times eCO_2] \times [D]$ on the diversity and relative abundance of AOB *amoA* transcripts (PERMANOVA, $F = 5.34$, $p = 0.02$; Table S4). Notably, this treatment showed the lowest Shannon diversity index across all treatments (Shannon $= 0.95 \pm 0.05$; Fig. 2b). At the AOB *amoA* gene level, a significant increase in the Shannon index was observed in ambient plots versus $[eT \times eCO_2]$ plots, only under drought conditions (Fig. 2b; Tables S2 and S4).

Nitrospira nxrB OTUs were affiliated with lineage 1, 2, 5, and 6, with a clear dominance of lineage 2, which comprised an average of 88.9% and 79.9% of gene and

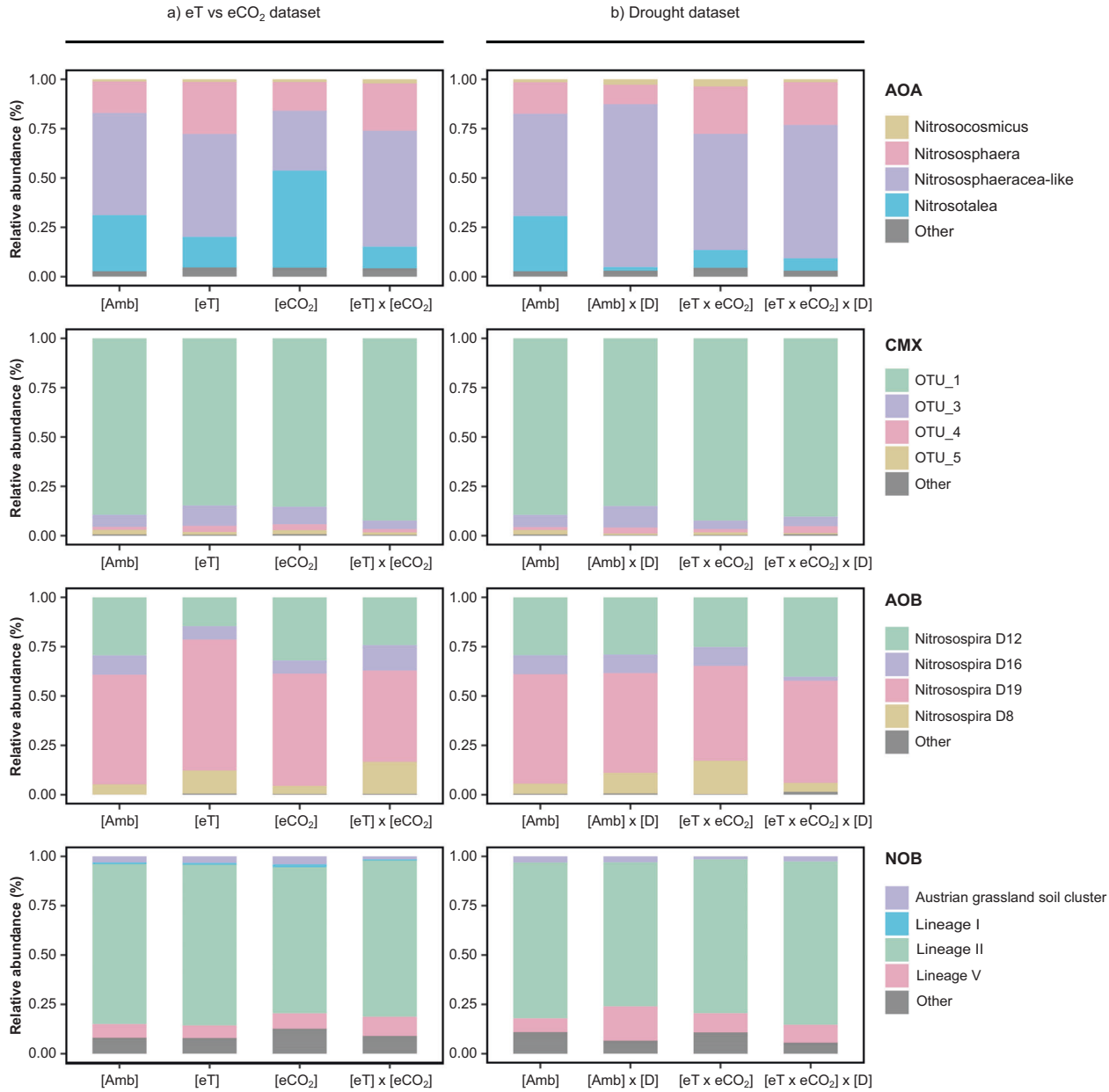

Fig. 3 Relative abundance (%) of all nitrifying groups obtained by amoA and nxrB transcript sequences. a Data for plots affected by single and interactive effects of elevated temperature (eT) and atmospheric CO_2 (eCO_2) concentrations. **b** Plots affected by single and interactive effects of future climate conditions (eT x eCO_2) and drought (D). The color code represents different genera/OTU/clades/lineages. "Amb" stands for "ambient conditions". Multiple horizontal lines within the same color represent individual OTUs within a group. Taxa that comprised <0.1% of all reads per treatment are grouped as "Other".

transcripts, respectively (Fig. 3; Fig. S5). The closest culturable representative of the most abundant OTU was *Nitrospira japonica* (99.92% identity), and most of the OTUs clustered together with environmental sequences from Austrian and Namibian soil clusters (Fig. S4). Amplicon sequencing showed that NOB were the most diverse group of nitrifiers, showing the highest Shannon index values of all microbial groups. (Fig. 2). Variance partitioning showed that [eT] alone caused significant differences in *nxrB* gene community structure (PERMANOVA, $F = 4.06$, $p = 0.02$; Table S4; Fig. S5a), although there were no significant differences in alpha diversity. Drought caused significant differences in both *nxrB* gene and transcript community composition (PERMANOVA, $F = 5.34$, $p = 0.02$, PERMANOVA, $F = 6.53$, $p < 0.01$; Table S4). These changes were coupled with significantly lower levels of alpha diversity under drought at the transcript level (ANOVA, $F = 5.75$, $p = 0.03$; Fig. 2b), although the same trend was not observed at the gene level. Contrastingly, *nxrB* gene community structure was

significantly affected by [eT × eCO_2] (PERMANOVA, $F = 5.52$, $p = 0.02$; Table S4), which was reflected in significantly lower Shannon index values (ANOVA, $F = 9.12$, $p = 0.008$; Fig. 2b).

Discussion

Ammonia-oxidation, usually regarded as the rate-limiting step of nitrification [67], is mediated by specific groups of bacteria and archaea. Therefore, climate change induced alterations in nitrifier community structure could affect soil nitrification to a higher degree than other N cycling processes, such as protein decomposition [11, 68] which are carried out by broader sets of microorganisms. In addition, since the discovery of complete nitrifiers, there is a need for the reassessment of the relative role of each microbial group in ammonia oxidation under climate change. Here we report an in-depth assessment on the effect of multiple climate change drivers on soil nitrification, including (i) the quantification of functional genes and transcripts by quantitative PCR, (ii) a census of the nitrifying microorganisms by sequencing at the functional gene and transcript levels, and (iii) an estimation of gross ammonification and nitrification rates.

In contrast to our first hypothesis we found that the nitrification process was relatively resistant to increases in atmospheric CO_2 with no significant changes in gross nitrification and in nitrifier functional gene abundance and expression. Effects of increased atmospheric CO_2 on soil microbial processes are primarily determined by changes in plant belowground carbon inputs and in plant nutrient uptake [69], where rhizodeposition can promote heterotrophic microbial activity, but greater plant N uptake may cause substrate (NH_4^+) limitation for nitrifiers. At our site, no increase in above and belowground net primary productivity was found in response to elevated CO_2 or temperature (Canarini et al., in preparation). Likewise, eCO_2 did not affect gross N mineralization and NH_4^+ levels, highlighting that substrate availability for nitrifiers did not change in response to eCO_2. In addition, other studies also reported very few effects of elevated atmospheric CO_2 concentration on belowground N processes in heathlands and temperate grasslands [70–72] showing that soil parameters such as pH and inorganic N concentrations likely play a more significant role than increased atmospheric CO_2 on overall nitrification.

Elevated temperature alone also did not significantly affect most of the soil processes and variables studied. More importantly, in contrast to our second hypothesis, elevated temperature did not affect the abundance, composition, and activity of soil nitrifier communities, indicating a high tolerance of the nitrification process to temperature in this ecosystem. Also, our results are in line with meta-analyses that observed that the effect of temperature on N cycling processes is less pronounced in grasslands, in comparison with other ecosystem types [36, 73]. In addition, other studies in grassland systems also reported no significant effects of elevated temperature on nitrification [72, 74]. Our results indicate that increased temperature might have altered abiotic and biotic soil parameters that may be masking individual temperature effects. Specifically, the soil water content at our field site was found to decrease throughout summer (Simon et al., under review) which can constrain substrate availability and/or accessibility to nitrifiers and mask potential stimulatory effect of warming on the nitrification process. Also, plant production of biological nitrification inhibitors (BNIs) could explain these results. However, BNIs were not assessed in this study, and their importance has mostly been reported in N-limited ecosystems [75, 76].

We further hypothesized that drought would reduce the abundance and activity of all nitrifying groups but found that responses to drought were group specific. The *amoA* expression levels of AOA and CMX clade B significantly decreased, whereas AOB either maintained (eT × eCO_2 plots) or increased (under ambient temperature and CO_2) their *amoA* transcription levels under drought. Drought can directly affect microbial growth by reducing the soil water potential and forcing microorganisms to invest into osmoregulation rather than growth and replication [33]. It can also have indirect effects on microbial communities by reducing plant belowground C allocation [77, 78], and by changing the availability and mobility of nutrients through a reduced soil pore connectivity and reduced plant uptake [79]. Studies have shown that plants can alter their root exudate abundance and composition during drought periods [80] as an attempt to better cope with the osmotic stress and/or to recruit particular fungi to the vicinity of the roots [81, 82]. Within root exudates, plants can also actively secrete BNIs, but given that fertilized grasslands are often not N limited, and our results point to higher NH_4^+ levels under drought, there is little evidence to support a relevant role for BNIs in these systems [76, 83]. Also, at the same study site, a decrease in plant biomass has been observed under drought, as well as a reduction in total plant N uptake. As a consequence of the plant response to drought, organic and inorganic dissolved N forms accumulated in the soil (Table 2) as reported in other studies [33]. The same pattern was observed with regards to microbial biomass N. Previous studies reported an increase in microbial biomass with drought, and have indicated that major pools of C- and N-based microbial metabolites are dynamic in response to drought [84, 85]. The accumulation of organic and inorganic N forms might have created optimal conditions for the proliferation of AOM with low ammonia (NH_3) affinities and high V_{max}, such as AOB, as opposed to AOA and CMX

[12]. AOA have been reported to be more sensitive to perturbations and variations in NH_4^+ concentrations when compared with AOB [11, 86]. Also, the nitrification activity of both AOA and CMX saturates faster due to lower maximum rates of NH_3 oxidation, compared with AOB [12]. However, no pure culture representative exists for CMX clade B yet, and the kinetic parameters of *Nitrospira inopinata*—the only CMX *Nitrospira* clade A isolate so far—might not be representative of all CMX members [12]. AOB, and particularly members of the genus *Nitrosospira*, are ubiquitous in grasslands and tolerate high concentrations of NH_4^+ [87]. In addition, genes involved in the protection of bacterial cells from hypoosmotic stress have been found in *N. multiformis*, showing that these organisms are well adapted to changes in soil water potential. However, osmoprotectants have also been detected in genomes of cultivated AOA [88–91], indicating that these organisms can also adapt to osmotic stress. Nevertheless, a dominance of AOB in managed grasslands has been reported due to frequent fertilization events [13, 14], and AOB are known to outcompete AOA in substrate-rich environments [9].

Along with an overall decrease in archaeal *amoA* gene and transcript copy number in response to drought, we found a reduction in the relative abundance of AOA OTUs affiliated to *Nitrosotalea*. This could be explained by the accumulation of organic compounds under drought, some of which were shown to inhibit *Nitrosotalea* activity and growth in pure cultures [92] compared with other AOA such as *N. viennensis* and *N. gargensis* [88, 93]. Furthermore, inhibition by organic compounds has been shown to be stronger in AOA than in AOB, although it varies between AOA strains [94, 95].

Even though we lack quantitative data on *nxrB* gene and transcript abundance due to still unresolved methodological challenges, we observed that the existing *Nitrospira-like* NOB community structure differed significantly in the drought plots compared with the non-drought plots, which was reflected on a decrease in the Shannon diversity index at the transcript level. *Nitrospira* were shown to have a high affinity for nitrite, and often dominate NOB communities in N-limited soils [96]. Given that NO_2^- often does not accumulate in soils, *Nitrospira* are considered to be the main nitrite-oxidizing genus in these ecosystems [97, 98]. Regardless of the decrease in diversity under drought, *Nitrospira* sublineage II remained the most abundant sublineage. Pure culture studies have reported a high metabolic flexibility of members of this sublineage, and it is possible that some microorganisms affiliated with sublineage II are better able to cope with the higher osmotic stress and levels of inorganic N imposed by drought [99, 100]. In addition, another study has reported higher rates of autotrophic growth of a *Nitrospira* sublineage II phylotype upon the addition of NH_4^+ [101].

Emission of the greenhouse gas N_2O has been shown to increase up to eightfold following the end of a drought period in grassland ecosystems, and a single short rain event can increase the annual net N_2O flux between 2 and 50% [102]. Therefore, our findings may also have broader implications since a drought-induced alteration in nitrifier community structure in favor of AOB over AOA and CMX might lead to higher N_2O emissions upon rewetting. In soil, N_2O is produced by denitrifying and ammonia-oxidizing microorganisms [103]. Within the latter group, AOB produce relatively high yields of N_2O via hydroxylamine oxidation under oxic conditions [104], while AOA and CMX produce much lower yields of N_2O during aerobic ammonium oxidation [105, 106]. Changes in redox potential could affect nitrification processes, specifically at the interface between aerobic and (partially) anaerobic soil portions. However, the soils in this study did not reach anaerobic conditions (maximum 34% soil water content), therefore making it unlikely that redox changes would have played a significant role.

Finally, we found few to no significant interactive effects between the different climate change drivers. Global reviews have shown that the interaction of different climate change factors can lead to nonadditive effects [6], although a more recent meta-analysis showed that antagonistic and synergistic effects are quite rare [7]. A recent study in a grassland system assessed the effect of elevated temperature and elevated CO_2 and found that while combinatory effects were antagonistic, they were mostly nonsignificant [72]. Regarding nitrifier community composition, only active AOB were significantly affected by the interactive effect of future climate conditions and drought [$eTeCO_2 \times [D]$). Under these conditions, the AOB diversity and abundance were lowest, as assessed by *amoA* amplicon sequencing and gene and transcript copy number quantification, which agrees with our last hypothesis.

In conclusion, in our study we demonstrated that nitrifying communities in grassland soils were remarkably unresponsive to elevated CO_2 and elevated temperature, alone or in combination, resulting in unaltered nitrification rates. We also showed that drought significantly changed the structure of the existing nitrifying communities, possibly through a strong reduction of plant biomass and N uptake. We hypothesize that the specific conditions created by drought (such as the accumulation of NH_4^+ and organic N, and, reduced pore connectivity) resulted in favorable niches for AOB, that showed higher or unaltered levels of *amoA* transcription and drove the observed peak in nitrification rates at the expense of ammonium-oxidizing archaea and comammox *Nitrospira*. Nevertheless, caution should be taken when interpreting nitrification rates due to the potential short-term rewetting effect introduced by the application of the liquid tracer (see Methods section). Future

climate conditions further interacted with drought and caused an antagonistic effect on the diversity and abundance of this group, without reducing gross nitrification rates. Given that a shift in nitrifier community structure towards AOB could potentially result in higher N_2O emission rates at the end of the drought period, special emphasis should be put into understanding the sensitivity of different nitrifiers to individual and combined global change variables in terrestrial ecosystems.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter III

Drought legacy effects on microbial community structure in a managed grassland

Tentative title: Drought legacy effects on microbial community structure in a managed grassland

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Author contributions

AR, MB and WW conceived the experiment. AR, HS and AC supervised the study. EP and AS was responsible for the site management. JS, AC, ES, VM, and MD did field work. JS, AC, ES, JP, IB and MM processed samples and produced data. BH assisted with data analysis. JS wrote the manuscript with input of all authors.

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Data availability: Raw amplicon sequencing data are available at the NCBI Short Read Archive (SRA) under BioProject ID PRJNA862760. The R code used for analyzing the data and generating figures is available upon request. All supplementary tables can be found at: https://figshare.com/s/698f9929535b20ceaa82

Abstract

The last decades were characterized by rising atmospheric CO_2 concentrations and global temperatures, and an increasing frequency of extreme events, such as drought. Microorganisms are the major drivers of soil biogeochemical processes, yet the response of community structure to climate change across temporal scales and the effects of climate history in shaping microbial community assemblages remains understudied.

Here we report on a study that aims to understand how future climate conditions (+3 ^{o}C; +300 ppm CO_2) and drought, alone and in combination, affect the microbial community composition throughout the vegetation period in a sub-montane grassland. Towards this aim, we analyzed the bacterial, archaeal, and fungal community structure in different seasons. We further sampled fourteen months after the end of an 8-week summer drought to investigate potential drought legacy effects on community structure.

We found a strong drought effect shaping the DNA-based bacterial/archaeal and fungal community structure during peak drought conditions. In addition, drought effects on community structure could still be detected two months after the end of the drought treatment. Fungal communities were more sensitive than bacteria and archaea, and the community structure between droughted and non-droughted soils did not fully recover even fourteen months after the end of the drought treatment. Future climate conditions also impacted the RNA-based bacterial/archaeal community structure at in May and October, while drought caused significant differences in community structure two and fourteen months after the end of the drought treatment.

In conclusion, we report a minor influence of future climate conditions shaping the microbial community structure. Furthermore, our findings suggest that drought history can have lasting effects on the microbial community structure, potentially contributing to the establishment of new guilds and communities, with consequences for plant-health and plant-soil feedbacks.

1. Introduction

The importance of soil microorganisms in maintaining global biogeochemical cycles, soil health and soil carbon sequestration has long been recognized (Falkowski et al., 2008). Grasslands store an estimated amount of 20% of the total soil carbon stock due to the existence of deep roots and abundant rhizosphere systems that deliver carbon (C) to the soil (Jansson and Hofmockel, 2020). Consequently, the predicted vulnerability of grasslands to climate change is tightly linked to plant-microbe interactions and bulk soil microbial processes that recycle C and other nutrients.

Among the most prominent and frequent climate change factors affecting microbial communities are increased atmospheric CO_2 concentrations, rising soil and air temperatures and extreme weather events such as droughts (Hoegh-Guldberg et al., 2018). Increased atmospheric CO_2 concentrations (eCO_2) have indirect effects on soil microbial communities by promoting higher plant photosynthetic activity, higher fine root production and belowground C allocation via rhizodeposition (Hu et al., 1999). In the short term, eCO_2 results in higher microbial activities resulting in higher mobilization of soil organic matter (van Groenigen et al., 2014). Additionally, it has been suggested that eCO2 affects the recruitment of fast growing microbial and fungal groups in the vicinity of roots (Hu et al., 1999; Naylor et al., 2020; Sun et al., 2021). As for specific changes in the composition of the overall microbial community, studies do not show consistent results, which is not surprising given the different length of the studies, different climatic and edaphic conditions that include soil and ecosystem types, and site histories. A meta-analysis across several soil ecosystems reported that the only common response to eCO_2 was a depletion of two Acidobacteria groups (Dunbar et al., 2012). An additional microarray study reported higher abundance levels of copiotroph bacteria (*Bacilli*, Firmicutes) under elevated CO_2 in a native grassland ecosystem (Hayden et al., 2012). On the other hand, long-term exposure to eCO_2 can cause a depletion in labile substrates in soil, as plants compete with microorganisms for available substrates. Under such conditions, shifts in

microbial community structure towards slow-growing microorganisms have been reported (Yang et al., 2018; Naylor et al., 2020).

Elevated temperatures (eT) can impact microorganisms both directly via a general acceleration of process rates and faster growth and turnover rates, and indirectly by expanding the plant growing season, altering plant community composition, and affecting plant growth and rhizodeposition (Cleland et al., 2006; Bradford et al., 2008; Piao et al., 2019). Elevated temperatures can additionally alter soil physicochemical properties such as water content and nutrient availability. Microbial community structure has been observed to indirectly change in response to warming via adaptations to the quantity and quality the available substrates (Melillo et al., 2017; Jansson and Hofmockel, 2020). Particularly, members of Alphaproteobacteria and Acidobacteria have been reported to be relatively enriched in 20-year-old warmed forest soils in comparison to ambient conditions, whereas some members of the Actinobacteria showed an opposite trend (Sheik et al., 2011; DeAngelis et al., 2015). Other studies did not report significant changes in the bacterial and fungal community composition in short and long-term (i.e, more than 50 years) warming of up to +6 °C (Radujković et al., 2018; Walker et al., 2018).

The frequency of drought events - defined as time periods with abnormal precipitation deficits – is also predicted to increase (IPCC 2018: Annex I: Glossary, 2018). Drought can alter the physical soil environment by reducing soil connectivity through receding water films, which in turns decreases the accessibility of substrates for microorganisms and plants. In addition, drought can have indirect effects on microbial communities via a decrease in belowground plant-derived C. As soils dry and soil water potential decreases, microorganisms are forced to invest into osmoregulation processes rather than in growth, which often culminates in reduced microbial activity levels under drought (Schimel, 2018). Microbial physiology adaptations include changes in active state (e.g., induction of dormancy), the production of osmolytes to cope with the osmotic stress and the production of extracellular polymeric substances (EPS) to reduce water loss (Naylor and Coleman-Derr, 2018; Kakumanu et al., 2019).

Drought events can have long-lasting legacy effects on soil microbial community structure through changes in soil-plant feedbacks, plant growth and belowground nutrient allocation (Hasibeder et al., 2015; Fuchslueger et al., 2016; de Vries et al., 2018; Canarini et al., 2021; Meeran et al., 2021). Moreover, reduced plant-derived C as a consequence of drought affects the availability of nutrients and consequently shapes microbial community structure in the vicinity of roots (Williams and de Vries, 2020). Using phospholipid fatty acids (PLFA) markers, several studies have reported increases in the abundance of Gram-positive bacteria, such as Firmicutes and Actinobacteria in response to drought, and decreases in Gram-negative bacteria such as Proteobacteria, Verrucomicrobia and Bacteroidetes (Barnard et al., 2013; Acosta-Martínez et al., 2014; Naylor and Coleman-Derr, 2018). Different life strategies of soil microorganisms have been suggested as the main reason behind the differential abundance of microbial groups under drought conditions (Malik et al., 2020). Particularly, the spore-forming ability of Actinobacteria and Bacilli may allow them to enter a stable state during periods of environmental stress, leading them to persist under drought conditions.

Knowing how ecosystems will respond to the increase in frequency and severity of drought events is crucial. However, most studies have focused on immediate short-term effects of drought while neglecting possible legacy effects on microbial community structure, which are key for ecological memory formation (Fuchslueger et al., 2016; Kaisermann et al., 2017; Meisner et al., 2018). Supporting this view, a recent study in a montane grassland showed that the dissimilarities in microbial community structure increased with the increasing number of drought events (Canarini et al. 2021). In particular, after ten years of recurrent droughts, soils exhibited significantly higher abundance levels of members of Actinobacteria, Ascomycota and Glomerales, and lower abundance levels of Acidobacteria, Bacteroidetes and Basidiomycota, in comparison to control plots (Canarini et al. 2021). This shows that soil can pre-adapt to further drought periods, but legacy effects on the microbial communities in between drought periods remain less understood.

A realistic understanding of the role of climate change on soil microbial community structure cannot be achieved in single-factor studies since multiple climate change effects may not be additive (Dieleman et al., 2012). Therefore, assessing the nature and magnitude of interactive effects in multifactorial experiments is of particular importance. More recently, many recent studies have incorporated multifactorial designs (reviewed in (Naylor et al. 2020; Jansson and Hofmockel 2020) and references therein). Their contrasting findings in comparison to single factor studies emphasize the importance of this approach. In addition, including amplicon sequencing data extends traditional microbial metrics such as growth, respiration and enzymatic activities and enables a more in-depth assessment of how specific microbial communities are shaped by single and combined climate change drivers.

Microbial community composition changes across seasons, but to date there are only a few studies that address the effect of climate change drivers over seasons and across different stages of plant growth (Voříškova et al. 2014; Campisano et al. 2017; Shigyo, et al. 2019). Indeed, a ^{13}C-PLFA profiling study on a seasonally dry California oak-grassland soils indicated that microbial community structure showed repeated seasonal cycles, and that soil microclimate and plant composition were stronger drivers of microbial community composition than available C (Waldrop and Firestone, 2006). In addition, bacterial community structure was found to be positively correlated with physical and chemical soil properties on an unfertilized grassland in spring and sutumn, while in summer the density and diversity of plants played an additional role (Regan et al., 2014). Another study has shown that the effects of temperature and increased CO_2 on microbial physiology were interactive and season specific (Simon et al., 2020). More specifically, the interactive effect of temperature and eCO_2 in spring pointed towards a potentially stronger dependence of microbial growth on plant-derived inputs at this time of the year in comparison to autumn, where temperature alone played a more relevant role than eCO_2 explaining the observed higher growth and respiration rates (Simon et al., 2020). During this time, a combination of plant senescence, lower basal temperatures,

and reduced substrate availability likely represented a stronger limiting factor to microbial activity in comparison to other seasons (Simon et al., 2020; Maxwell et al., 2022).

The overall goal of this study was to understand how future climate conditions (i.e, soil warming and increased atmospheric CO_2) and drought, alone and in combination, affected the microbial community composition in a managed permanent sub-montane grassland soil. We hypothesized that i) changes in community structure would be season specific, and the effect of warming and eCO_2 would be stronger in spring and autumn than in summer; ii) there would be significant drought effects on microbial community structure at peak drought, which would remain observable one year later.

Towards this goal, we made use of an ongoing multifactorial field experiment in central Austria where soil warming, increased atmospheric CO_2 and drought are manipulated at different levels. We conducted three sampling campaigns during the growing season in 2017 to identify the role of seasonality in modifying single and combined effects of soil warming, increased atmospheric CO_2 and drought on soil microbial community structure. We additionally sampled fourteen months after the end of the drought treatment to investigate potential drought legacy effects on community structure. We analyzed differences on the DNA- and RNA-based bacterial/archaeal microbial community structure over seasons by 16S rRNA gene and transcript sequencing, respectively. In addition, we assessed the fungal community response to the climate change treatments by sequencing the ITS1 region. Finally, we quantified total bacterial and fungal biomass via digital droplet PCR (ddPCR) using the same marker genes which, in combination with sequencing data, allowed for a quantitative microbiome profiling.

2. Methods

2.1. Site description, experimental design, and sample collection

The experimental site is located at the Agricultural Research and Education Centre (AREC) in Raumberg-Gumpenstein in Styria, Austria (47°29´38´´N, 14°06´03´´E) and is part of a multifactorial climate change experiment (ClimGrass). The experimental design was based on a response surface regression approach that aimed to study the nature and intensity of the grassland's responses to manipulations of elevated atmospheric temperature and CO_2, and drought (Piepho et al., 2017). The experimental site comprised a total of 54 plots (4 x 4 m each) showing individual and combined effects of three levels of temperature (ambient, +1.5 °C, +3°C), atmospheric CO_2 concentrations (ambient, +150 ppm, + 300 ppm) and drought (Séneca et al., 2020; Simon et al., 2020)**.**

We collected a selected set of soil samples from 20 plots in spring (May 30-31st 2017), summer (July 25-26th 17), autumn (October 3-4th 2017) and autumn the following year (October 5-6th 2018) **(Fig. 1)**. These 20 soil plots included plots in which temperature (eT; +3°C) and CO_2 (eCO_2; +300 ppm CO_2) were manipulated simultaneously (hereafter called "Future Climate" treatments), and plots on which drought (D) was simulated via automatic rainout shelters. We sampled all corresponding control plots, in a fully factorial experimental design **(Fig.1a)**. Rainout shelters were installed five days before the spring sampling (May 17) and remained in place for 8 weeks, being removed shortly after the summer sampling, at peak drought (July 17). Atmospheric CO_2 concentrations were manipulated through a miniFACE – free air CO_2 enrichment system, whereas atmospheric temperature was manipulated through infrared heaters (Simon et al., 2020; Meeran et al., 2021) **(Fig1b)**. Since 2014, infrared heaters have been running continuously except when snow cover exceeded a depth of ten centimetres. CO_2 fumigation was only applied during the

A.

Treatment	Replicates	eCO_2	eT	Rainout shelter
Amb	8	Ambient	Ambient	No
Amb + D	4	Ambient	Ambient	Yes
$eTeCO_2$	4	+ 300 ppm	+ 3 °C	No
$eTeCO_2$ + D	4	+ 300 ppm	+ 3 °C	Yes

B.

C.

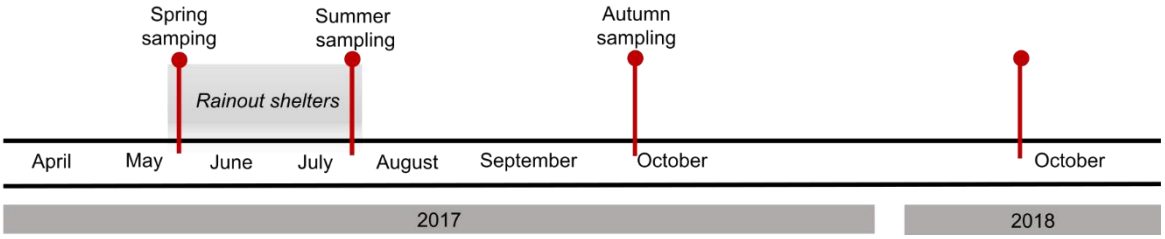

Fig. 1. Sampling site and strategy

Schematic representation of the experimental design (A), site (B) and sampling strategy (C). eCO_2 represents elevated CO_2 manipulations; eT represents elevated temperature manipulations.

growing season (April to November). Site management resembled local farming practices and included mowing three times a year and mineral fertilization (90 kg N ha^{-1} a^{-1} as NH_4NO_3, 65 kg P ha^{-1} a^{-1} as $Ca(H_2PO_4)^2$ and 170 kg K ha^{-1} a^{-1} as K_2O).

Soil samples were collected as described previously (Séneca et al., 2020). Physicochemical parameters, soil processes and pools for the 2017 sampling points are described elsewhere (Maxwell et al., 2022). For the October 18 sampling, we quantified dissolved organic carbon (DOC), nitrogen (DON), total dissolved nitrogen (TDN), NH_4^+, NO_3^-, soil C, N and P pools as described in (Séneca et al. 2020; Simon et al. 2020). We additionally determined microbial biomass carbon (MBC) and nitrogen (MBN) via chloroform-fumigation. Gravimetric soil water content of the collected samples was determined by oven drying (105 °C for 24 h) 5 g of fresh, sieved soil (Simon et al., 2020). Soil moisture sensors measured water content *in situ* in the top soil layers at 1-minute intervals and recorded data at 15-minute averages throughout the

entire growing season (Simon et al. 2020). All these data can be found as in **Table S1** and **Fig. S1.**

2.2. Nucleic acid extraction and sequencing

DNA and RNA were co-extracted from approximately 0.4 g frozen soil by bead-beating in the presence of a cetyl trimethylammonium bromide (CTAB) buffer and phenol and eluted in 250 µl of Low-Tris-EDTA buffer (Angel et al. 2012). Following extraction, samples were purified using the OneStep™PCR InhibitorRemoval Kit (Zymo, Irvine, CA, USA). RNA was purified from the total nucleic acid extract, and cDNA was synthetized as described previously (Séneca et al., 2020)**.** The V3-V4 hypervariable region of the bacterial and archaeal 16S rRNA gene was amplified and sequenced in DNA and cDNA extracts using the primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015; Parada et al., 2016). The fungal intergenic spacer region (ITS1) was PCR-amplified and sequenced only in DNA extracts using the primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) (White et al., 1990; Smith and Peay, 2014). Extracts were prepared and sequenced at the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna (JMF, project ID JMF-22006-6; JMF-2201-13) following a 2-step PCR, as described previously (Pjevac et al., 2021). First and second step PCR protocols are presented in **Table S2**. Sequencing libraries were prepared with the Illumina TruSeq Nano Kit excluding the fragmentation step, and sequenced in paired-end mode (600 cycles; V3 chemistry) on and Illumina MiSeq sequencer following the manufacturer's instructions. Paired reads were demultiplexed, chimera checked and processed into ASVs using DADA2 v.1.14.1 (Callahan et al., 2017). Fungal and bacterial/archaeal ASVs were classified using the UNITE database v.8.2 and the SILVA SSU database v.138 respectively.

2.3. ddPCR 16S rRNA gene and ITS1 region quantification

Both the 16S rRNA genes and ITS1 regions were quantified by digital droplet PCR (ddPCR) on TNA extracts using the same primer sets used for sequencing. Each ddPCR reaction (22 µl) contained 1x QX200 ddPCR EvaGreen Supermix (BioRad), 0.1 µmol l^{-1} of each primer and either 1 ng or 0.1 ng of template for the quantification of fungi or bacteria/archaea, respectively. Droplets were generated on a QX200™ Droplet Generator (BioRad) and non-template controls were included in each assay. Amplification conditions are presented in **Table S2**. The fluorescence intensity was measured on a QX200 Droplet Reader (BioRad). Gene copy numbers were calculated using the QuantaSoft (BioRad) software. Here, thresholds between positive and negative droplet populations were set manually for each sample using the fluorescence of the negative control as a guide. Final ddPCR results were expressed as gene copy number per gram of dry weight soil.

2.4. Data analysis

All data analysis steps were performed using R v3.6.1 (R Core Team, 2019). Sequencing data were analyzed primarily with the "ampvis2", "phyloseq" and "rstatix" R packages (McMurdie and Holmes, 2013; Albertsen et al., 2015; Kassambara, 2021). Raw sequencing datasets were pre-filtered to remove unspecific reads such as non-bacterial and chloroplast sequences. Samples were then split by nucleic acid type and marker gene and subsequently filtered by removing ASVs with less than 5 total counts in at least two samples. Filtering at this stage was chosen instead filtering at a later stage to avoid introducing a 'sampling point' effect. Richness and diversity indexes were calculated using the R package "microbiome" (Lahti and Shetty, 2017). The effect of sample timepoint on richness and diversity indexes was analyzed by either a one-way ANOVA or a Kruskal-Wallis test according to whether the variables met parametric assumptions or not. Following a significant result, either a Tukey HSD multiple comparison test or a Dunn's post-hoc test was conducted in order to determine which pairs of sampling

timepoints were significantly different. We considered a variable suitable for parametric tests if the ANOVA residuals were normally distributed (Shapiro's test) and the variable variance was homogeneous (Levene's Test). Within each sampling point, the single and interactive effect of drought and future climate were analyzed by a two-way ANOVA whenever parametric conditions were met. Following a significant interaction term, a Tukey HSD multiple comparisons test was conducted. Whenever parametric assumptions were not met, we performed a Kruskal-Wallis test using the variable "Treatment" as a factor, to circumvent the limitation of non-parametric tests to determine interactive effects. To assess the effect of the environmental variables under study on the community composition and structure, we performed a permutational analysis of variance (PERMANOVA) using the function *adonis()* after checking the homogeneity of group dispersions, using the *betadisper()* function from the vegan R package (Oksanen, 2020). The PERMANOVA analysis was run using 999 permutations on a dissimilarity matrix generated using the Euclidian distance on top of a center log ratio (CLR) transformed ASV table. This approach is equivalent to using the Aitchison distance and is recommended for compositional data (Gloor et al., 2017). Principal component analyses (PCAs) were additionally performed using the function *ordinate()* from the phyloseq R package. We further used the DESeq2 package to assess which ASVs were significantly more or less abundant either in drought vs ambient conditions, in future climate vs ambient conditions or between sampling points (Love et al., 2014). We also assessed which ASVs were differentially abundant in ambient plots in the growing season of 2017. To this purpose, the DESeq2 model was ran using "Sampling timepoint" as factor. ASV log2fold changes between July 17 vs May 17 (reference="May17"), October 17 vs July 17 (reference="July17") and October 17 vs May 17 (reference="May17") were obtained. For heatmap visualization, counts corresponding to significantly abundant ASVs were extracted from a *rlog()* transformed count matrix and subsequently normalized by row (z-score).

ddPCR gene/region copy numbers were log10 transformed and the single and interactive effects of drought and future climate at each sampling point were assessed using parametric or non-parametric statistical methods, as mentioned above.

For quantitative microbiome profiling, the unfiltered 16S rRNA gene and ITS1 region ASV tables were normalized using ddPCR gene copy numbers. Briefly, relative abundance tables were multiplied by the total gene copy number per gram dry weight soil of each sample. The quantitative ASV tables were subsequently filtered by removing ASVs with less than 20000000 and 700000 copies per gram dry weight soil for bacteria and fungi, respectively, in at least two samples to mimic the proportion of data kept on the relative abundance dataset and to enable future comparisons. Matrices used for ordination analyses were transformed by first adding a pseudo count (+1) and *log()* transformed. PERMANOVAs and PCAs were performed using the Euclidian distance as described above.

We assessed the effect of drought or future climate conditions on the ddPCR normalized abundance of bacterial and fungal families, genera and ASVs via a non-paired Mann-Whitney test using the basic Rstats function *wilcox.test()*. Differences between Drought and Ambient and Future Climate and Ambient were expressed in log2fold changes. All multiple comparison p-values were adjusted for the false-discovery rate using the Benjamin-Hochberg correction method.

3. Results

3.1. ddPCR quantification of fungal and bacterial/archaeal abundances

Bacterial/archaeal 16S rRNA gene copy numbers per gram of soil (dry weight) ranged between 7.0 x 10^9 and 2.1 x 10^{11} and did not differ significantly between sampling points (Kruskal-Wallis, Q=6.02, p=0.11). Bacteria and archaea did not show a strong seasonal response to single and combined effects of drought and future climate conditions **(Fig. 2; Table S3).**

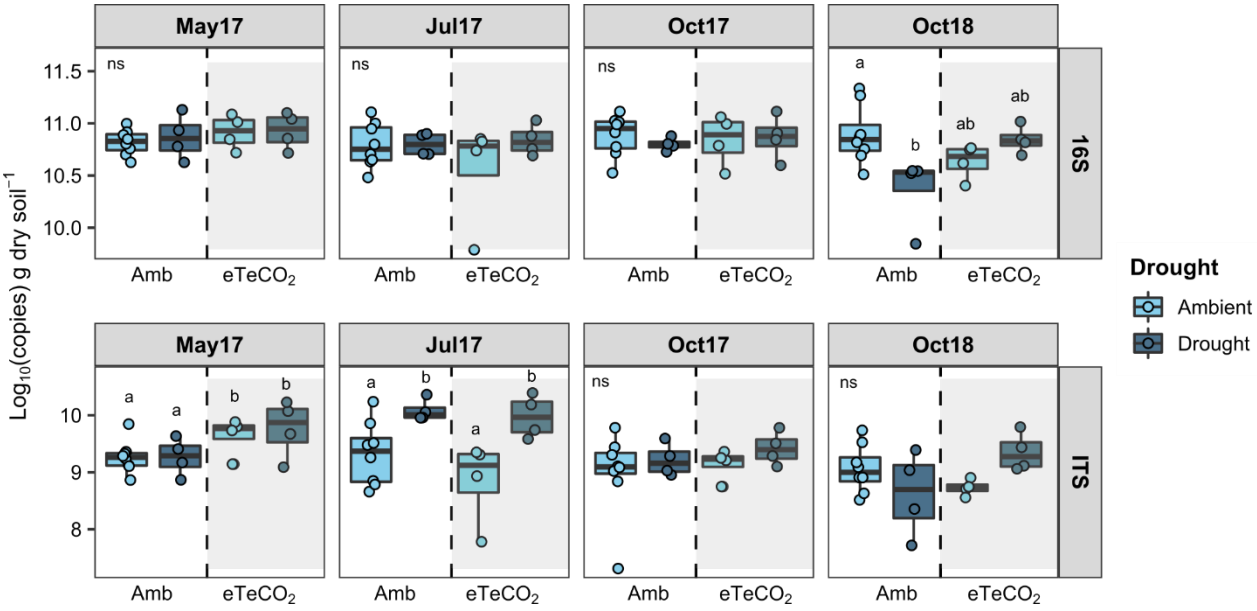

Fig. 2. Quantification of the 16S gene and ITS1 region by ddPCR in TNA extracts.

The boxplots depict gene/region copy numbers per gram dry weight soil across biological replicates of the same treatment. Inside the boxplot, the median value is shown. The dashed line separates plots subjected to either ambient or future climate conditions. The colors represent the drought treatment. The letters inside each subplot indicate the statistical significance, as assessed by parametric or non-parametric tests (see Methods). Ns indicates that no significant single or interactive effect of drought and future climate was found. Detailed statistical results can be found in Table S3.

Amb: ambient temperature and atmospheric CO_2; $eTeCO_2$, future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

In October 2018 we observed a significant decrease in 16S rRNA gene copy numbers in response to drought, only in ambient plots with no manipulations of temperature and atmospheric CO_2 (Tukey HSD post-hoc test, Q=-0.52, p.adj=0.01; **Table S3A**). The abundance of the fungal ITS1 region ranged between 2.0 x 10^7 and 2.4 x 10^{10} and varied significantly between sampling points, with copy numbers in May 17 and July 17 being significantly higher than those in October 18 (Dunn's post-hoc test, Q=-1.58, p.adj=0.01; Q=-3.35, p.adj=0.004). In addition, higher ITS1 copy numbers were detected under future climate conditions compared to ambient conditions in May 17 (ANOVA, F=6.74, p=0.01). In July 17, there was a clear significant increase in copy numbers in response to drought (ANOVA, F=14.6, p=0.002). In October 17 and October 18, no significant effect of drought or future climate was observed, even though the pattern of ITS abundances across treatments in October 2018 were similar to the ones observed for 16S rRNA genes (**Fig. 2**).

3.2. Seasonal responses of DNA-based bacterial/archaeal community structure to future climate and drought

A total of 1,008,130 reads and 8,273 16S rRNA gene ASVs were obtained across the four sampling points after removing non-bacterial and chloroplast sequences. After transforming relative abundances into absolute abundance data using the ddPCR quantifications, low abundant ASVs (0.1 % of total reads) were removed resulting in 7,858 ASVs.

ASV richness did not change between sampling points in 2017 but was significantly lower in October 18 in comparison to the 2017 sampling points **(Table S4; Table S4A).** In July 17, we observed a strong effect of drought causing lower ASV richness and diversity. (**Fig. S2**; **Tables S5-S6)**. No differences on richness and diversity levels in response to future climate and drought were observed in May and October 17. In October 18, ambient plots subjected to drought showed significantly higher diversity levels than ambient plots (Dunn's post-hoc test, Q=2.86, *p.adj*=0.03; **Table S6A)**.

Concomitantly, we did not observe significant effects of drought and future climate on bacterial/archaeal community structure in May 17. In contrast, we observed a strong drought effect in July 17 which was also visible in October 17. In October 18, we found no evidence of a drought legacy effect on community structure, but a marginally significant interactive effect of drought and future climate **(Fig. 3d; Table S7).**

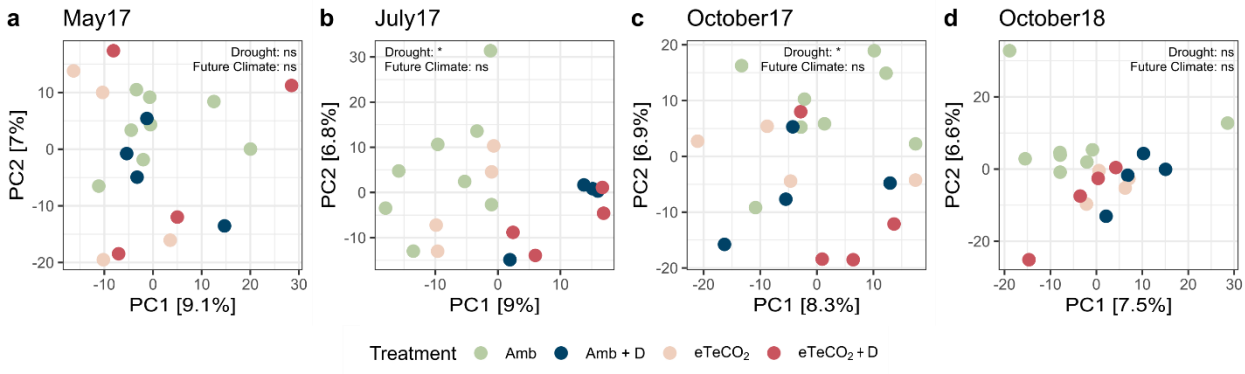

Fig. 3. DNA-based bacterial and archaeal community structure across sampling points

PCA depicting changes in microbial community structure (i.e. 16S rRNA gene sequencing) in response to single and combined effects of drought and future climate. The input data was normalized using ddPCR 16S rRNA gene copy numbers per sample. The statistical significance was assessed by a two-way PERMANOVA using drought and future climate as factors and results are shown inside each sub-plot. Detailed statistical results can be found in Table S7.

Amb: ambient temperature and atmospheric CO_2; $eTeCO_2$, future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

** p-value < 0.01; * p-value < 0.05; ns = not significant

The core bacterial/archaeal community composition remained relatively stable across all sampling points, and dominated by taxa from phyla Acidobacteria, Verrucomicrobia, Actinobacteria, Bacteroidetes and Alphaproteobacteria **(Fig. S3)**. In July 17, drought plots were characterized by significant increases in the abundances of taxa affiliated with proteobacterial families *Erwiniacea* and *Oxalobacteraceae*, Actinobacteria (particularly family *Nocardioidaceae*) and Bacteroidota orders Flavobacteriales and Cytophagales. On the other hand, we detected a significant decrease of members of family *Omnitrophaceae*, particularly Candidatus *Omnitrophus* in response to drought **(Table S8).** In October 17 and October 18, we detected no clear increases or decreases of particular groups in plots previously exposed to drought.

3.3. Seasonal responses of DNA-based fungal community structure to future climate and drought

A total of 587,957 reads and 1,442 ASVs were obtained across the four sampling points through sequencing of the fungal ITS1 region in TNA extracts. After transforming relative abundances into absolute abundance data using the ddPCR, low abundant ASVs (0.4 % of total reads) were removed resulting in 1,365 ASVs.

We observed few to no differences in fungal ASV richness across sampling points. Diversity levels increased significantly across the seasons in 2017 but were lowest in October 18 **(Fig. S4; Table S5 and S9)**. Within each sampling point, we observed no responses in fungal richness and diversity to single and combined effects of future climate and drought **(Fig. 4)**. On the other hand, the fungal community structure responded strongly to drought on all sampling points (**Fig. 4**, **Table S7).** Additionally, in October 17, future climate conditions significantly affected fungal community structure but in the following year this effect was only marginally significant (PERMANOVA, F=1.18, p=0.07, **Table S7**).

The fungal communities in May 17 were dominated by Ascomycota, Basidiomycota and Mortiellomycota and future climate did not cause any significant taxonomic shifts **(Fig. S5).** In July 17, taxa affiliated with families *Cladosporiaceae*, *Ceratobasidiaceae*, *Sporidiobolaceae*, *Phaeosphaeriacea* and *Nectriaceae* were among the groups that showed the highest increase in response to drought conditions **(Table 10).** In October 17, the phyla-level phylogenetic dominance remained similar to July and May, but the drought effects were less pronounced and reflected on an increase in abundance of members of family *Aspergillaceae* and *Didymosphaeriaceae* **(Table S11).** Future climate conditions caused an increase in abundance of taxa affiliated with orders Pleosporales and Xylariales, particularly families *Microdochiaceae* and *Hyponectriaceae* **(Table S11A)**. In October 18, there were no clear taxonomic shifts in plots previously exposed to drought.

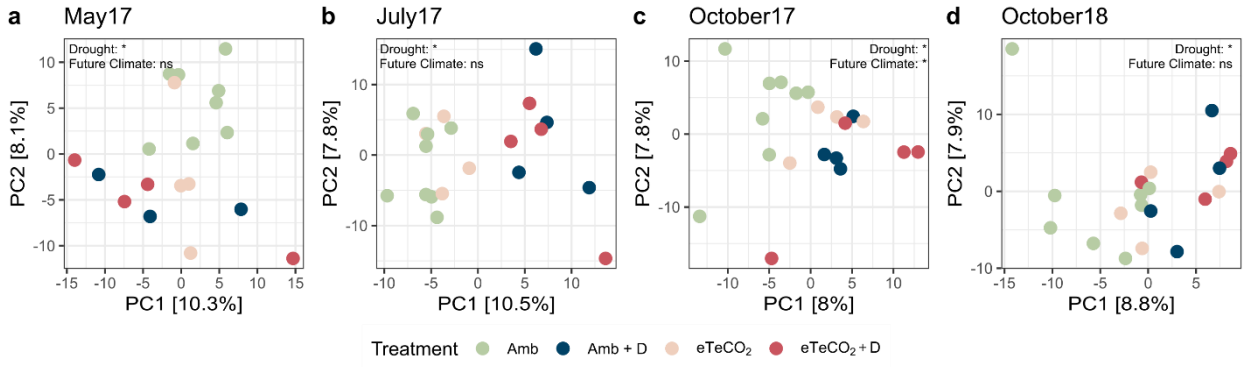

Fig. 4. DNA-based fungal community structure across sampling points

PCA depicting changes in active microbial community structure (i.e. ITS region sequencing) in response to single and combined effects of drought and future climate. The input data was normalized using ddPCR ITS region copy numbers per sample. The statistical significance was assessed by a two-way PERMANOVA using drought and future climate as factors and results are shown inside each sub-plot. Detailed statistical results can be found in Table S7.

Amb: ambient temperature and atmospheric CO_2; $eTeCO_2$, future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

** p-value < 0.01; * p-value < 0.05; ns = not significant

3.4. Seasonal changes of RNA-based bacterial/archaeal community structure in ambient plots

A total of 587,680 reads and 7,427 16S rRNA ASVs were obtained across the four sampling points after removing non-bacterial and chloroplast sequences. The removal of low abundant ASVs (0.29 % of total reads) resulted in 6,749 ASVs.

We observed a significant seasonal effect on the active microbial community structure in ambient plots (i.e not subjected to any climate change manipulation) across the three seasons in 2017 (PERMANOVA, F=1.41, p=0.001) **(Fig. S6).** Post-hoc analysis indicated significant differences between all season pairs **(Tables S12)** indicating the existence of distinct seasonal microbial communities in ambient soil plots. No statistically significant seasonal effects were observed on the remaining soil treatments according to post-hoc tests **(Table S12A)**. The seasonal differences in ambient plots were not caused by significant differences on the number of unique ASVs (ANOVA, F=0.78, *p*=0.47) nor differences in the Shannon diversity index (Kruskal-Wallis, Q=0.18, *p*=0.9) but due to changes in the abundance of specific ASVs **(Fig. S7; Table S12B)**. There was a total of 42 differentially abundant ASVs between the ambient plots of May and July 17, as inferred by the DeSeq2 model **(Fig. S7a).** Among the differentially abundant taxa were members of classes Actinobacteria and Gammaproteobacteria, particularly families *Oxalobacteriaceae*, *Erwiniaceae* and *Streptomycetaceae*. On the other hand, several ASVs affiliated with Bacteroidota and Verrucomicrobia, particularly families *Cytophagaceae*, *Microscilaceae* and *Pedosphaeraceae*, significantly decreased their abundance levels in July in comparison to May 17. Between July and October 2017, the number of differentially abundant ASVs was 72 **(Fig. S7b)**. We observed a reverse abundance pattern compared to the one observed between May and July, whereby taxa that significantly increased their levels of abundance in July 17 in comparison to May significantly decreased their abundance levels in October 17 in comparison to July and vice-versa, to a lesser extent. The closer similarity between the ambient community structure of May and October 2017 was supported by the ordination analysis and further backed up by

the low number of differentially abundant ASVs between the two sampling points (n=20) **(Fig.S6; Fig. S8).**

3.5. Seasonal responses of RNA-based bacterial/archaeal community structure to future climate and drought

We observed a significant effect of future climate affecting the RNA-based microbial community structure in May 17 (PERMANOVA, F=1.13, *p*= 0.03) **(Fig. S9a).** In July 17, we observed no effects of drought and/or future climate on microbial community structure. In October 17, we observed an effect of both drought and future climate conditions on active microbial community composition with no significant interaction term (PERMANOVA, F=1.35, p=0.002; F=1.19, p=0.01) suggesting that future climate did not modify any of the observed drought effects **(Fig. 9c)**. One year later, in October 18, we observed a significant drought legacy affecting community structure (PERMANOVA, F=1.18, p=0.02) **(Fig. 9d)**.

We additionally observed a significant effect of sampling timepoint on ASV richness (Kruskal-Wallis, Q=10.2, p=0.01) **(Table S4).** ASV richness was significantly lower in July 17 and October 18 in comparison to May 17 (Dunn's post-hoc test, Q=-2.54, *p.adj*=0.03; Q=-2.96, *p.adj*=0.02) **(Table S4A, S5).** Within each sampling point, there were no significant climate change effects on ASV richness and diversity **(Fig. S10)**. The only observed significant climate change effect was observed on the number of unique ASVs in October 18, whereby ASV richness was significantly lower in plots previously exposed to drought, in comparison with the corresponding controls (ANOVA, F=6.25, *p*=0.02). **(Fig. S10b).**

The drought legacy effect on the RNA-based bacterial/archaeal community structure two and fourteen months after the end of the drought treatment was translated on the differential abundance of specific ASVs between control plots and plots exposed to drought **(Fig. 5)**. In October 17, two distinct microbial response types were observed: a cluster of taxa affiliated with Bacteroidota (order Sphingomonadales) and Gammaproteobacteria (order

Burkholderiales) significantly increased their abundance levels in plots previously subjected to drought whereas taxa affiliated with Polyangiales, *Nostocaceae* and *Nitrosotaleacea* showed the opposite abundance pattern **(Fig. 5a)**. In October 18, all differentially abundant ASVs showed decreased abundance levels in plots previously subjected to drought in comparison to control plots **(Fig. 5b)**.

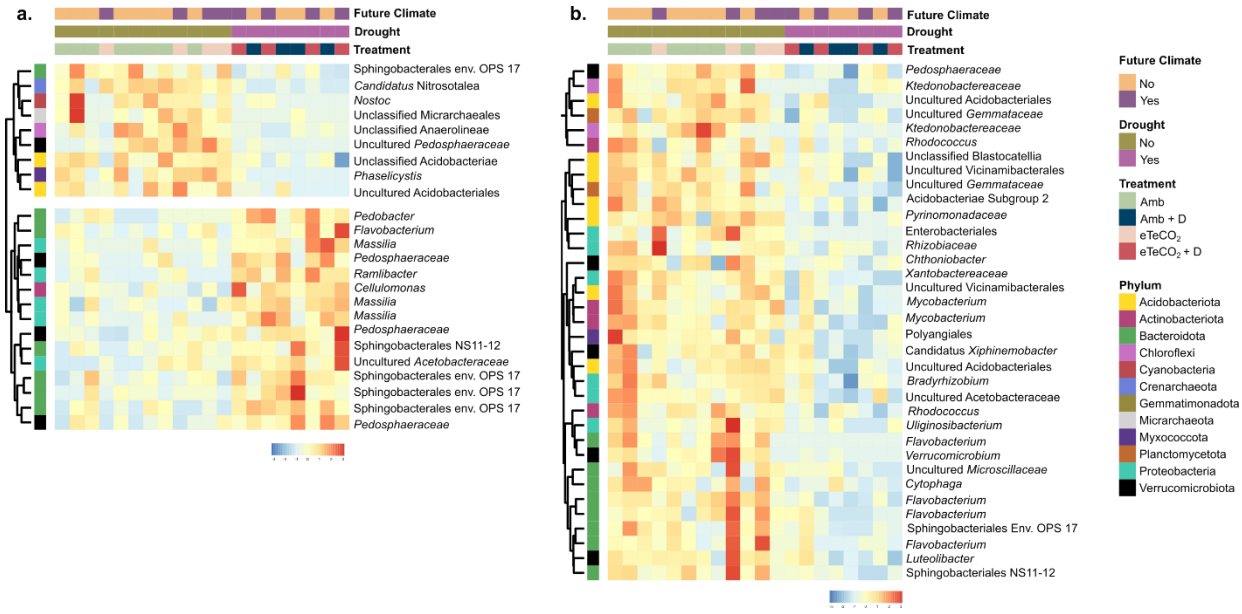

Fig. 5. Differential 16S rRNA transcript abundance between ambient and droughted plots two and fourteen months after the end of the drought treatment

The heatmap shows log2 transformed read counts of significantly abundant ASVs between ambient and drought plots in (a) October 2017 and (b) October 2018, as inferred by the DESeq2 model. The data was normalized by row (z-score) and clustered using complete linkage according to similar taxonomic abundance patterns (see Methods). Colored row annotations depict phyla, whereas colored column annotations depict Future Climate (+3ºC, + 300 ppm), Drought, and specific treatments.

4. Discussion

4.1. Fungal responses to future climate and drought across seasons

In our study, we observed distinct responses of bacterial/archaeal and fungal communities to single and combined effects of future climate and drought. One of the major findings was that fungal abundance was significantly higher during peak drought (July 2017) and that the community structure showed significant differences in response to drought on all sampling points. Due to their extended hyphal structures, soil fungi are thought to be either less affected by drought and/or better adapted to it than bacteria and archaea (de Vries et al., 2018). However, the nature of the fungal response to drought has been shown to vary across studies, with some reporting community structure changes between dry and wet conditions (Hawkes et al., 2011) and others reporting increases, decreases or no changes in biomass in response to drought or irrigation (Scheu and Parkinson, 1994; Gordon et al., 2008; Haugwitz et al., 2014; Fuchslueger et al., 2016; Hartmann et al., 2017). Thus, the tolerance, sensitivity, or opportunistic response of specific fungal species to drought might be strongly dependent on the ecosystem type and intensity of disturbance. Other studies at this field site reported a significant decrease in aboveground plant biomass during peak drought conditions and a consequent accumulation in dissolved organic matter, as a result of a reduced relief on the competitive pressure between plants and soil microorganisms (Séneca et al., 2020; Meeran et al., 2021). In line with these results, we detected higher absolute abundance levels of known plant pathogens such as *Microdochium*, *Fusarium* and family *Phaeosphaeriaceae*. These taxa could have proliferated under drought conditions as a result of reduced plant fitness, as it has been reported in other studies (Preece et al., 2019). In contrast, we also observed higher abundance levels of known saprotrophs such as *Cystofilobasidium* and *Cladosporium* at peak drought in July 17. While *Cystofilobasidium* members have been described to thrive in nutrient-rich environments (Jeewani et al., 2020), members of the xerophilic genus *Cladosporium* have

been reported to feed on decaying plant material and tolerate low water content levels (Araújo et al., 2020).

Altogether, our data suggests a selective dominance of a reduced set of fungal groups under peak drought conditions, which either benefited from decaying plant fitness, higher organic matter accumulation, and/or were better equipped to deal with osmotic stress. In parallel, the death of some drought sensitive fungal groups coupled with potentially lower turnover rates under drought, could have contributed to an accumulation of fungal DNA in droughted plots. The abundance of fungi returned to pre-drought conditions two months after the end of the drought treatment, which could reflect an acceleration of turnover rates upon rewetting, as it has been observed for other groups and soil processes (Schimel, 2018). However, the community structure, remained significantly different between droughted and non-droughted plots up to fourteen months after the end of the drought treatment, which indicated that fungal communities at our sampling site did not fully recover.

4.2. Bacterial and archaeal responses to future climate and drought across seasons

Bacterial and archaeal abundances showed a remarkable resistance to future climate and drought throughout the growing season of 2017. Community structure, on the other hand, was strongly affected by drought in July 17 and was characterized by significantly lower levels of richness and diversity. Notably, the abundance of the known root colonizing genus *Massilia* (family *Oxalobactereaceae*) peaked under drought conditions in July 17, and remained at high abundance levels in the following sampling points. This strictly aerobic copiotrophic genus has been reported to exploit windows of opportunity at early successional stages in the vicinity of roots when substrates are not limiting, thus obtaining a competitive advantage over other microorganisms (Ofek et al., 2012). Concomitantly, actinobacterial genera *Curtobacterium*, *Marmoricola*, *Nocardioides* and *Streptomyces* increased their abundance levels under peak

drought, which goes in line with previous findings (Barnard et al., 2013). Higher abundance levels of members of these groups have been reported under drought and they are known alleviate drought stress in plants (Monohon et al., 2021; Wang et al. 2019; Warrad et al. 2020) and to secrete a wide range of carbohydrate degrading enzymes such as leucine aminopeptidase and chitinase (Zhang et al., 2021). In addition, the ability of some of these genera to produce biofilms could have acted as a buffer against water stress, facilitated access to substrates, and consequently enabled growth (Dees et al., 2016). Given the increased fungal abundance at peak drought coupled with increases in decaying plant-derived organic matter and fungal biomass it is possible that the higher levels of substrate created optimal conditions for the growth of members of these groups.

Two months after the end of the drought treatment, we observed a significant drought legacy effect causing a separation between the bacterial/archaeal communities of droughted and non-droughted soils. Fourteen months after the end of the drought treatment, significant drought effects on community structure were no longer observed which indicates a recovery of the microbial community structure to pre-drought conditions. In accordance, we reported no significantly abundant taxa between droughted and non-droughted plots. However, in ambient plots subjected to drought, marginally significant differences in RNA-based community structure remained in place fourteen months after the drought ended, in comparison to non-droughted ambient plots. These differences were coupled with significantly lower 16S rRNA transcript abundance levels in droughted ambient plots in comparison to non-drought plots. Drought under future climate conditions contributed to an increase on 16S rRNA transcript abundance in comparison to non-droughted plots under future climate. This indicated that future climate might alleviate the severity of drought effects on bacterial/archaeal abundance and community structure. Future climate conditions and in particular higher temperatures, could have led to a pre-adaptation of microbial communities to lower soil moisture, and consequently to a better tolerance of drought conditions, in comparison to communities in ambient plots.

4.3. Seasonal variations in RNA-based bacterial and archaeal community structure in ambient plots

Seasonality caused significant changes on the RNA-based bacteria/archaeal community structure in ambient plots not subjected to climate change manipulations. In particular, communities in May 17 and October 17 were more similar to each other than those of July 17. In addition, at our experimental site we did not observe strong variations in soil water content on the soil top 9 cm layer across the three 2017 sampling points, which led us to exclude soil water content as an explanatory variable. However, the temperature in the topsoil layers during October 17 was substantially lower than in either May or July 17 (data not shown). We hypothesize that the seasonal patterns of RNA-based bacterial/archaeal community structure in ambient plots are a result of a transition from a plant-controlled scenario in spring and summer, to a control based on edaphic soil properties such as temperature in autumn. More specifically, when plants are actively growing during, the competitive pressure between microorganisms and plants for nutrients is at its highest. Fresh plant-derived rhizodeposits often fuel microbial activity and enable growth, and microorganisms have been reported to outcompete plants for nutrients in the short-term (Xu et al., 2011; Kuzyakov and Xu, 2013; Philippot et al., 2013; Tian et al., 2020). Indeed, we found higher relative abundance levels of known root colonizers and plant-associated microorganisms of families Oxalobactereaceae, Streptomycetaceae and genus *Pantoea* during summer in comparison to spring. Supporting this view, ambient plots at our experimental sites showed the highest growth and respiration rates during summer (Simon et al., 2020). The beginning of plant senescence and continuous decrease on plant nutrient uptake in autumn can contribute to an increase on the availability of substrates for microbial consumption (Kuzyakov and Xu, 2013). However, during this time microbial growth can be constrained due to lower basal temperatures, as evidenced in other studies (Simon et al., 2020) and community structure may consequently be more similar to the one observed in spring.

4.4. RNA-based bacterial and archaeal responses to future climate and drought across seasons

Surprisingly, we observed no effects of the combined future climate and drought treatment on the structure of the RNA-based bacterial and archaeal communities at peak drought in July 2017, which partially contrasts with our second hypothesis. Our results rather point towards similar biological states between drought and ambient communities at peak drought, which goes against our findings at DNA levels and previous studies which reported significant drought effects on bacterial/archaeal community structure. We hypothesize that the different stabilities of RNA and DNA, in combination with other yet unknown factors related contributed to the discrepancies in results and further analyses are needed in order to understand their significance.

Differences in RNA-based bacterial/archaeal community structure between droughted and non-droughted plots were still found two and fourteen months after the end of the drought treatment. Nonetheless, only a small percentage of total ASVs were differentially abundant between droughted and non-droughted plots, which highlights the overall resilience of bacterial and archaeal communities to environmental stress. Rewetting typically causes an abrupt increase in microbial activity which is linked to the sudden increase in pore connectivity and subsequent availability of nutrients (Blazewicz et al., 2014; Placella et al., 2012). In parallel, the relief of osmotic stress causes previously dormant microorganisms to become active again (Schimel, 2018). Indeed, some studies suggest a higher importance of the intensity and duration of a rewetting event for nutrient cycling and recovery of the existing trophic food webs, than the drought period itself (Schimel 2018; Cordero et al., 2022.). Along these lines, previous studies have reported visible changes in community structure in on rewetted soils (Barnard et al., 2013). Our findings go in line with previous studies that suggest that drought history and rewetting can persist in soil microbial communities via specific response groups (Meisner et al. 2018; Cordero et al., 2022.). Indeed, we hypothesize that drought impacted the active bacterial/archaeal communities of droughted soils by causing the

death of some taxa and opening a window of opportunity for the potential proliferation of others (e.g. *Massilia*). This disturbance would be the driving force for the establishment of new members of the community at the cost of others, culminating in entirely different communities between soils with a drought history and soils without it.

We also showed that future climate conditions significantly affected the structure of the potentially active, RNA-based communities in May and October 2017. This goes in line with our first hypothesis and previous studies that suggested that the effect of future climate would be more important in spring and autumn than in summer, due to both a higher microbial dependency on fresh plant-derived inputs in spring (possibly enhanced by eCO_2) and to a potentially more significant effect of temperature on community structure and microbial activity in autumn, when basal temperatures and plant inputs are lower (Simon et al., 2020). Indeed, temperature data from the topsoil layers indicated that the magnitude of the differences between future climate and ambient plots was higher in October and May than in July (data not shown). In addition, other studies have also shown the higher importance of warming on soil processes and pools during colder seasons of the year (Maxwell et al. 2022).

4.5. Conclusion

In this study we used a multifactorial climate change experiment to assess the single and combined effects of future climate conditions and drought on belowground microbial communities in a sub-montane grassland. Our results highlight the role of drought history affecting present (DNA-based) and potentially active (RNA-based) microbial communities. We found soil bacteria and archaeal communities to be overall more resilient to drought than fungal communities. Altogether, we show that drought history has lasting effect on the structure of belowground microbial communities, with potential consequences for plant-health and plant-soil feedbacks. Given that the frequency of extreme events such as drought is predicted to increase, it becomes particularly timely to assess the extent by which the resistance and/or resilience of belowground communities can have permanent impacts on ecosystem functionality.

Supplementary Material

Supplementary Results – soil properties and nutrient pools

We detected no changes in soil properties and nutrient pools in response to drought October 18, one year after the removal of the rainout shelters. On the other hand, soil water content decreased under future climate conditions (Wilcoxon-test, U=75, p=0.03). In addition, the dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) pools significantly increased in response to future climate conditions (Wilcoxon-test, U=23, p=0.05, Wilcoxon-test, U=22, p=0.04) **(Table S1).**

Supplementary Figures

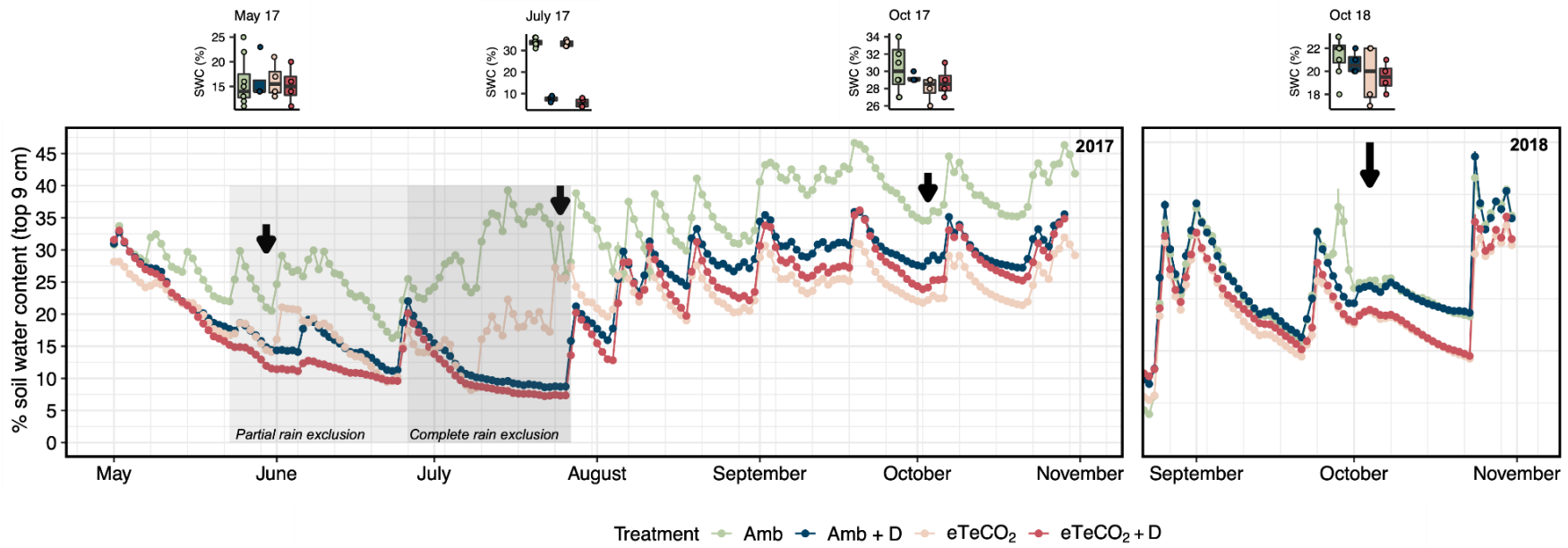

Fig. S1. Variation in soil water content across all sampling points

Daily averaged soil water content (%) across all treatments in the top 9 soil layer. The black arrows depict sampling timepoints. The grey boxes indicate the time period on which the rainout shelters were applied. The boxplots indicate the gravimetric soil water content at the time of sampling by oven drying (See Methods).

Figure S1.

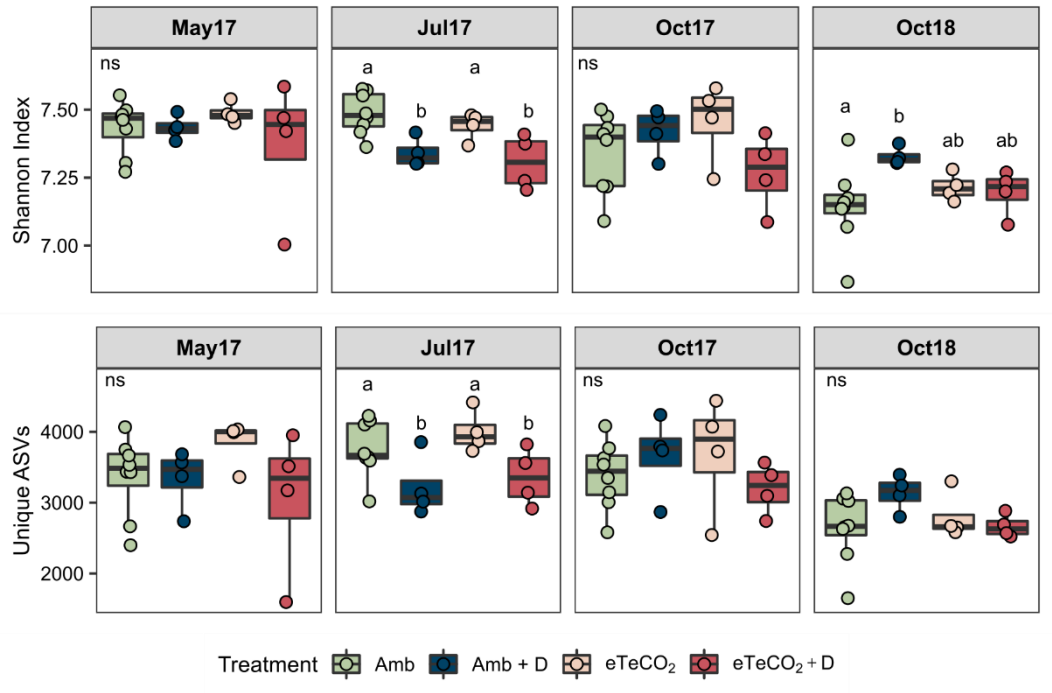

Fig. S2. 16S rRNA gene diversity and richness across sampling points

The Shannon diversity index (a) and the number of unique ASVs (b) are depicted for each of the sampling points. The input data was normalized using ddPCR 16S rRNA gene copy numbers per sample. The letters inside each subplot indicate the statistical significance, as assessed by parametric or non-parametric tests (see Methods). Ns indicates that no significant single or interactive effect of drought and future climate was found. Detailed statistical results can be found in Tables S4;S5.

Amb: ambient temperature and atmospheric CO_2; $eTeCO_2$, future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

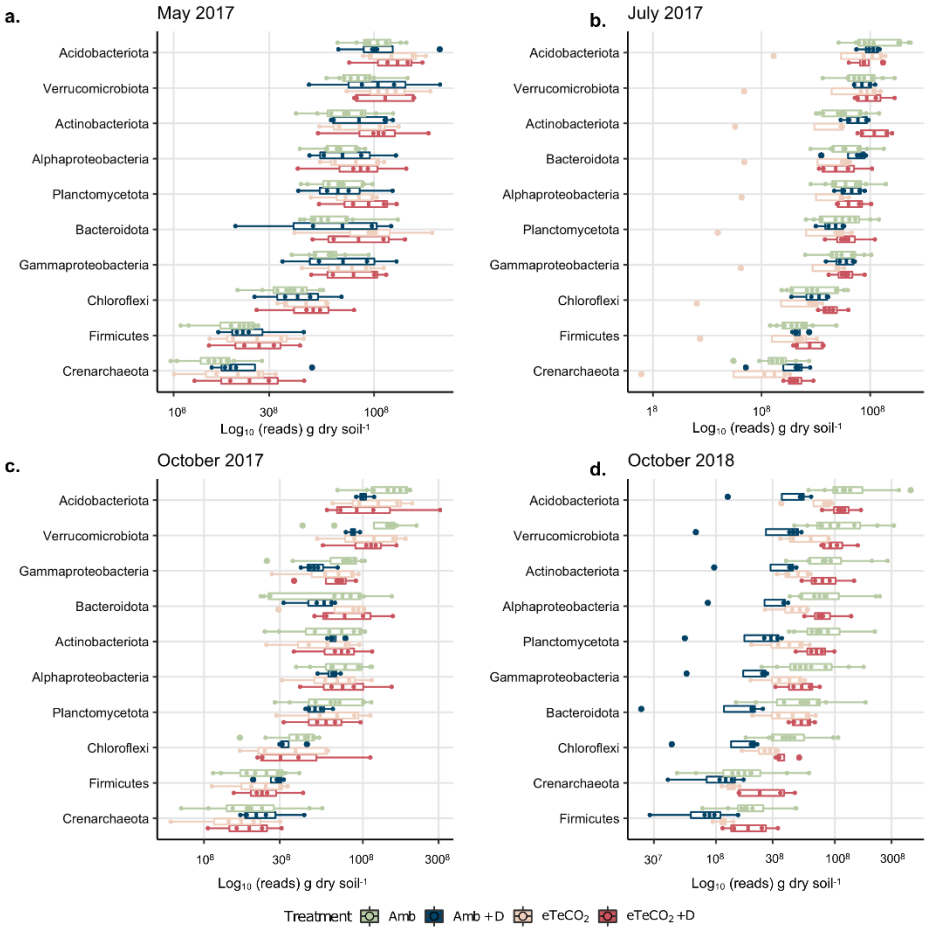

Fig. S3. Top 10 most abundant bacterial/archaeal phyla

The input data was normalized using ddPCR 16S rRNA gene copy numbers per sample.

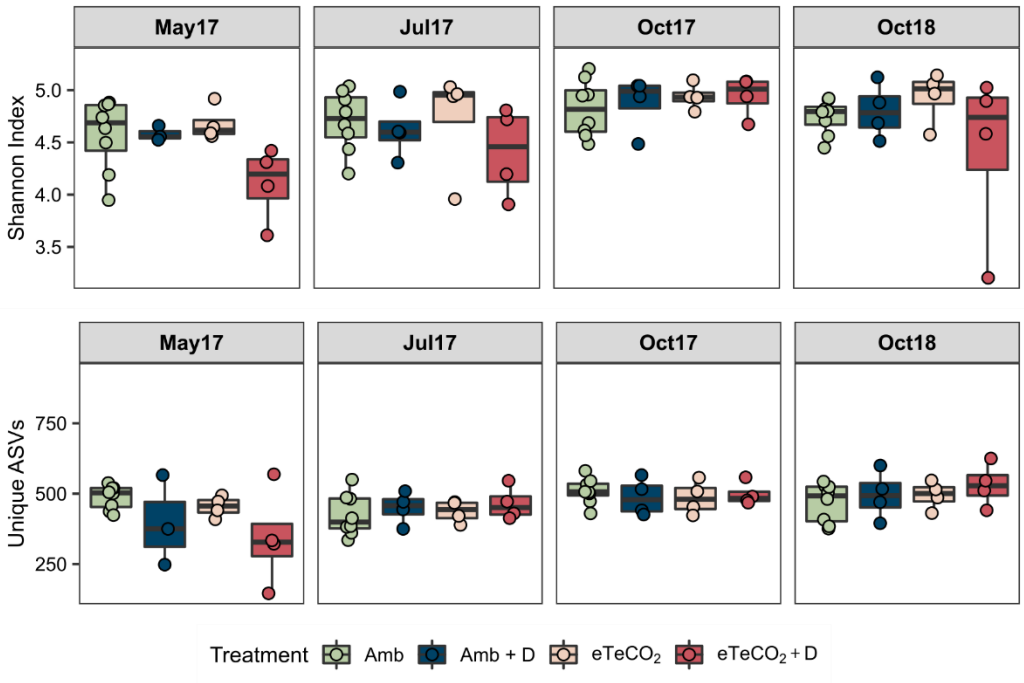

Fig. S4. ITS diversity and richness across sampling points

The Shannon diversity index (a) and the number of unique ASVs (b) are depicted for each of the sampling points. The input data was normalized using ddPCR ITS region copy numbers per sample. The letters inside each subplot indicate the statistical significance, as assessed by parametric or non-parametric tests (see Methods). Ns indicates that no significant single or interactive effect of drought and future climate was found. Detailed statistical results can be found in Tables S4;S5.

Amb: ambient temperature and atmospheric CO_2; $eTeCO_2$, future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

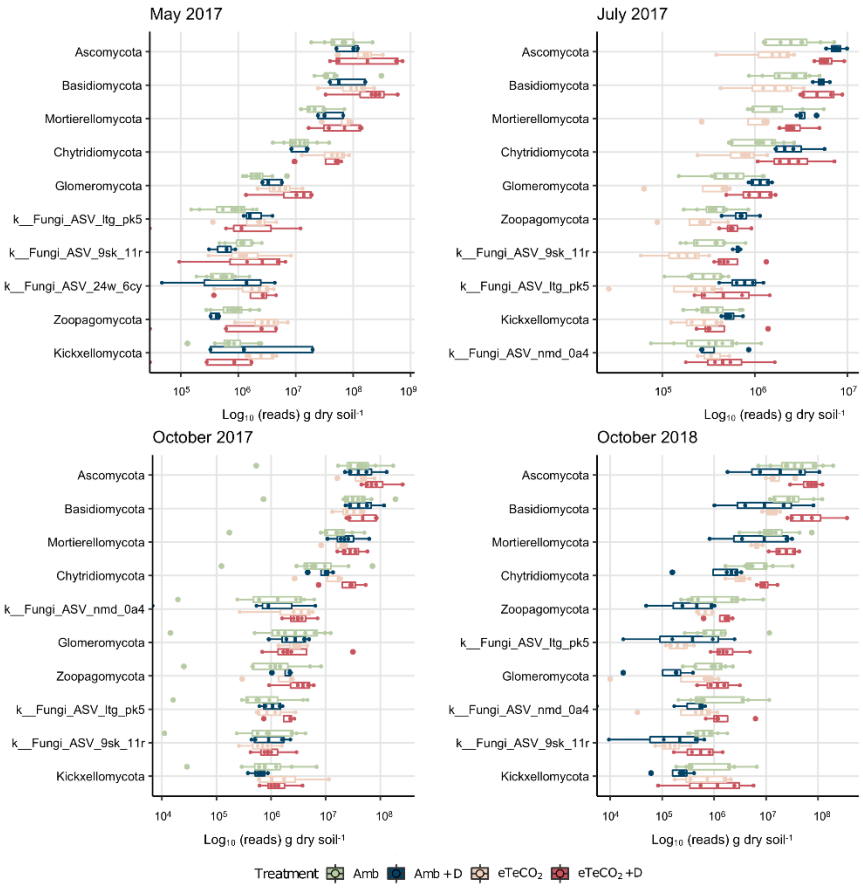

Fig. S5. Top 10 most abundant fungal phyla

The input data was normalized using ddPCR ITS region copy numbers per sample.

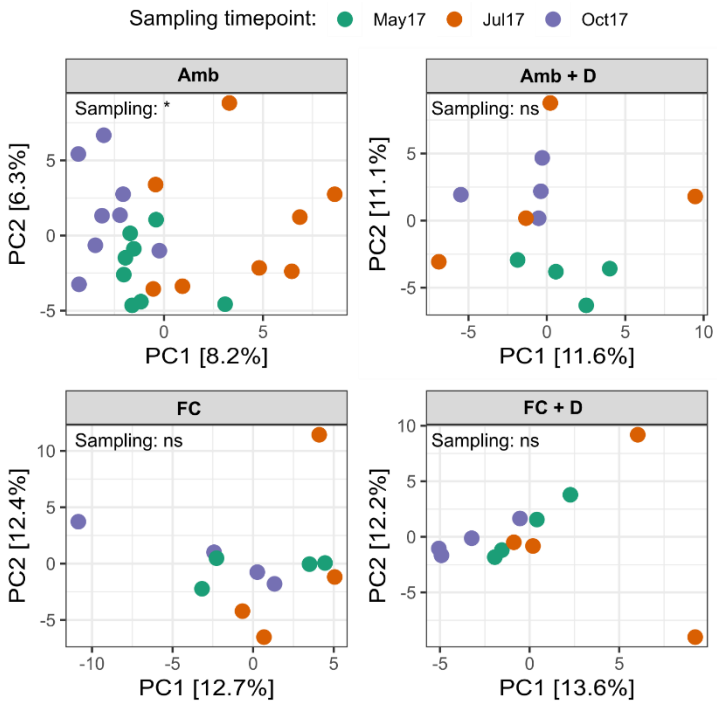

Fig. S6. Seasonal variation in RNA-based bacterial/archaeal microbial community structure per treatment

PCA depicting seasonal changes in active microbial community structure (i.e. 16S rRNA transcript sequencing) in 2017. Statistical significance was assessed by a pairwise PERMANOVA (see Methods) and results are summarized inside each subplot. Detailed statistical results can be found in Table S7.

Amb: ambient temperature and atmospheric CO_2; FC: future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

** p-value < 0.01; * p-value < 0.05; ns = not significant

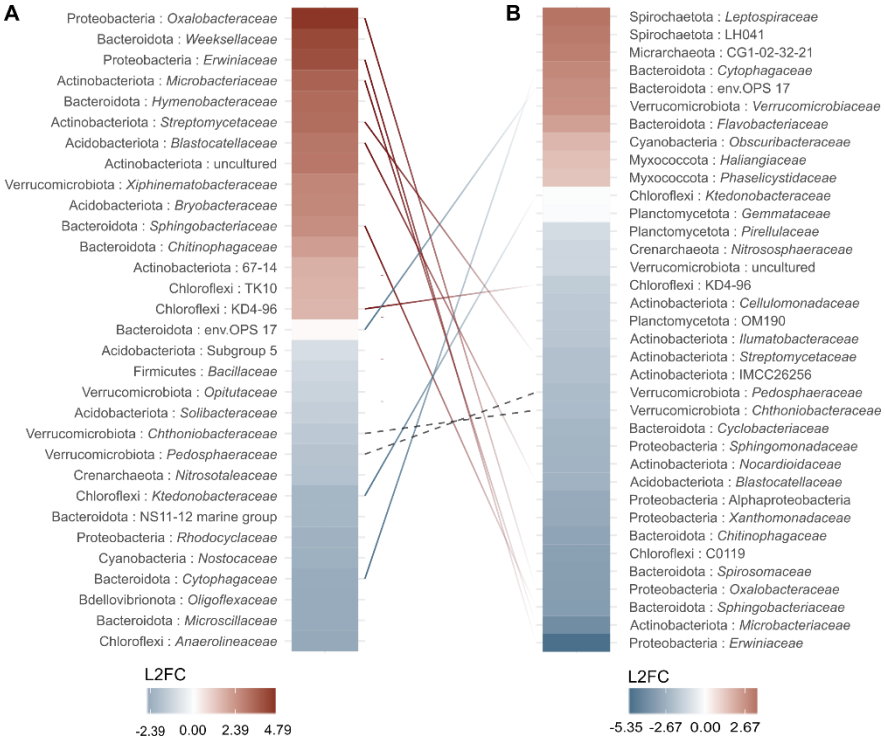

Fig. S7. Log2Fold changes of differentially abundant potentially active microbial taxa in ambient plots

The differential abundance of potentially active microbial taxa (i.e. 16S rRNA transcript) was inferred by the DESeq2 model in ambient plots (n=8) of July 17 vs May 17 (a) and October 17 vs July 17 (b). The heatmap shows average log2fold changes at the family level.

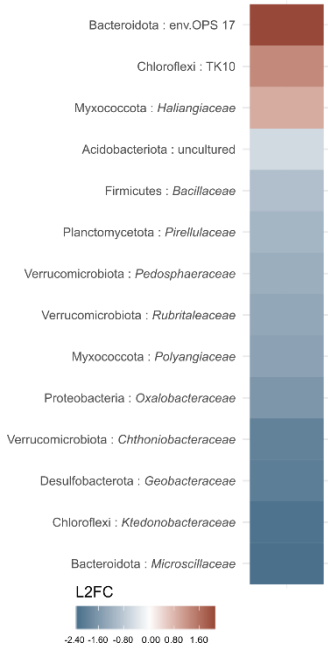

Fig. S8. Log2Fold changes of significantly abundant microbial taxa in ambient plots: October 17 vs May 17

The differential abundance of potentially active microbial taxa (i.e. 16S rRNA transcript) was inferred by the DESeq2 model in ambient plots (n=8) of October 17 vs May 17. The heatmap shows average log2fold changes at the family level.

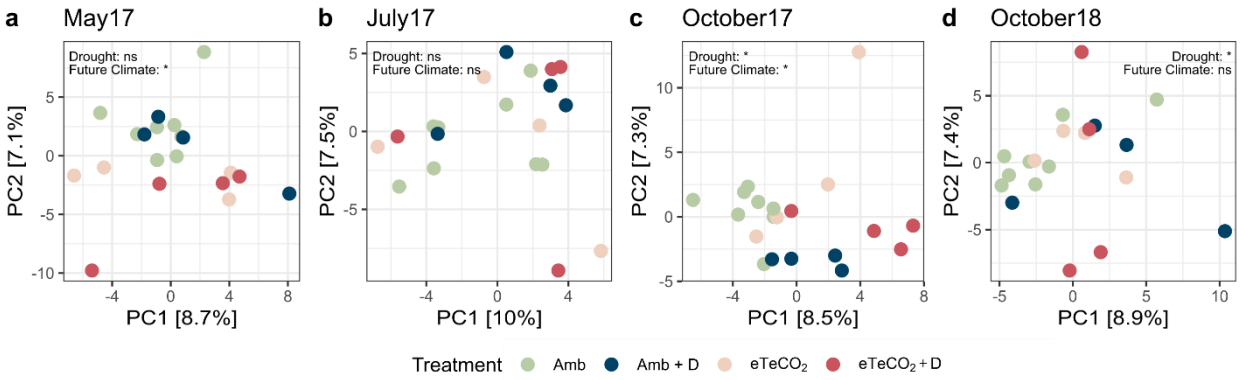

Fig. S9. RNA-based bacterial and archaeal community structure across sampling points

PCA depicting changes in the potentially active microbial community structure (i.e. 16S rRNA transcript based) in response to single and combined effects of drought and future climate. The statistical significance was assessed by a two-way PERMANOVA using drought and future climate as factors and results are shown inside each sub-plot. Detailed statistical results can be found in Table S7.

Amb: ambient temperature and atmospheric CO_2; $eTeCO_2$, future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

** p-value < 0.01; * p-value < 0. 05; ns = not significant

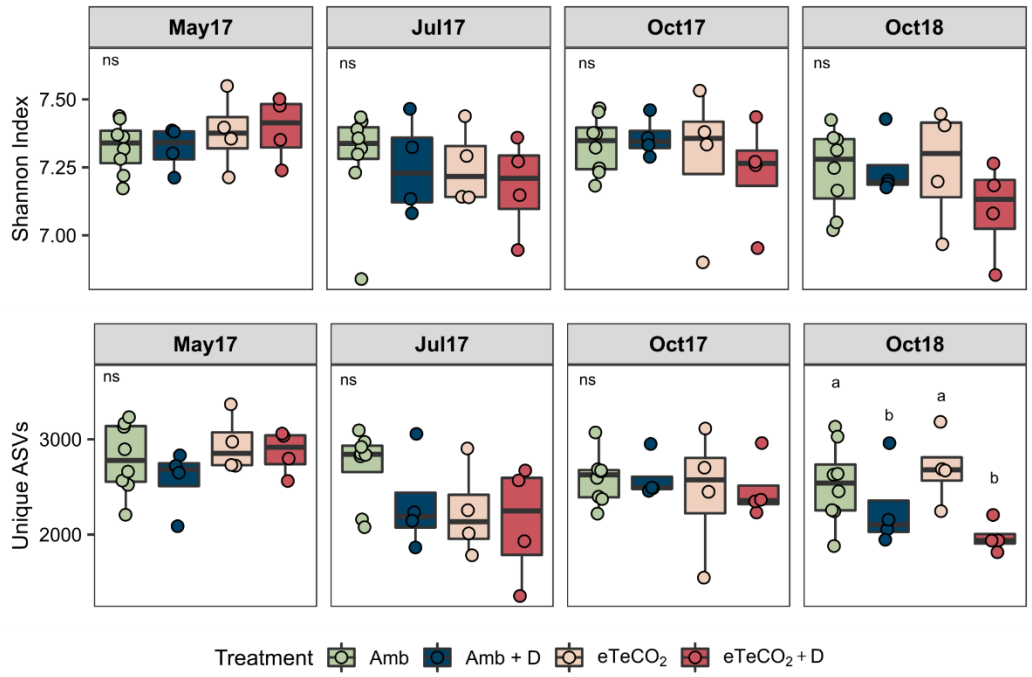

Fig. S10. 16S rRNA transcript diversity and richness across sampling points

The Shannon diversity index (a) and the number of unique ASVs (b) are depicted for each of the sampling timepoints. The letters inside each subplot indicate the statistical significance, as assessed by parametric or non-parametric tests (see Methods). Ns indicates that no significant single or interactive effect of drought and future climate was found. Detailed statistical results can be found in Tables S4-S5.

Amb: ambient temperature and atmospheric CO_2; $eTeCO_2$, future climate conditions: +3 ^oC, + 300 ppm CO_2; D: drought treatment

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Chapter IV

Increased microbial expression of organic nitrogen cycling genes in long-term warmed grassland soils

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Increased microbial expression of organic nitrogen cycling genes in long-term warmed grassland soils

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Global warming increases soil temperatures and promotes faster growth and turnover of soil microbial communities. As microbial cell walls contain a high proportion of organic nitrogen, a higher turnover rate of microbes should also be reflected in an accelerated organic nitrogen cycling in soil. We used a metatranscriptomics and metagenomics approach to demonstrate that the relative transcription level of genes encoding enzymes involved in the extracellular depolymerization of high-molecular-weight organic nitrogen was higher in medium-term (8 years) and long-term (>50 years) warmed soils than in ambient soils. This was mainly driven by increased levels of transcripts coding for enzymes involved in the degradation of microbial cell walls and proteins. Additionally, higher transcription levels for chitin, nucleic acid, and peptidoglycan degrading enzymes were found in long-term warmed soils. We conclude that an acceleration in microbial turnover under warming is coupled to higher investments in N acquisition enzymes, particularly those involved in the breakdown and recycling of microbial residues, in comparison with ambient conditions.

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INTRODUCTION

Soil organic matter (SOM) represents the largest global reservoir of organic carbon (C) and nitrogen (N), and its turnover plays a critical role in global element cycling and the climate system [1]. Rising global temperatures are expected to accelerate the rates of SOM decomposition by accelerating microbial activity and increased expression of enzymes that are responsible for the extracellular depolymerization of high-molecular-weight organic matter, potentially resulting in large releases of greenhouse gases such as CO_2 into the atmosphere and a positive feedback to climate change [2, 3]. While the processes underlying C dynamics and their repercussion on climate change have been extensively studied, soil N storage and the fate of organic N have received far less attention, despite their intrinsic link to organic C and common dependence on temperature.

Soil microorganisms play a major role in terrestrial N cycling [4, 5], and studies have shown that N accessibility for microorganisms may affect the degradation and turnover rates of the different SOM pools [6, 7]. Furthermore, organic N compounds also contribute substantially to the organic C fraction [7] and microorganisms may decompose organic N to meet their demand in C and energy, rather than their N demand [8]; additionally, the availability of N has been reported to stimulate C-mobilizing enzymes [9].

Organic N in soil consists of high-molecular-weight compounds, such as intracellular and cell-wall bound proteins and nucleic acids of microbial and plant origin, but the great majority of organic N is found in microbial residues (dead microbial cell walls) and its components, such as peptidoglycans, chitin, and cell-wall bound proteins, which may represent up to 80% of the organic N pool [10–12]. The first step in the depolymerization of soil organic N therefore requires the microbial secretion of extracellular enzymes that deconstruct organic N polymers into oligomers or monomers that are small enough to be taken up directly by soil microorganisms and plants and can be further mineralized and incorporated as ammonium (NH_4^+) or nitrate (NO_3^-). While microbially-mediated inorganic N transformations have been extensively studied [13] we know little of how organic nitrogen cycling is controlled in the environment.

All enzyme-mediated reactions are intrinsically temperature sensitive and warming often causes higher metabolic and turnover rates, as enzymes converge towards their temperature optimum. At the same time, sustained warming also depletes soils of easily accessible organic substrates, leading to reduced microbial biomass levels [14, 15]. In such nutrient-depleted environments, it is even more crucial for organisms to tightly regulate their resource acquisition strategies. The ability to degrade organic N forms is widespread among soil microorganisms [16–18], and substrate concentration and availability are important factors that regulate the production of extracellular enzymes [19, 20]. Under ammonia-limiting conditions, pure culture studies reported higher synthesis rates of proteins

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involved in the scavenging of alternative N sources as well as in the turnover of proteins [21, 22]. Similarly, increased transcription of genes encoding high-affinity ammonium transporters under N starvation conditions have also been observed [23, 24].

Physiological adaptations of microorganisms to environmental perturbations entail changes in gene expression patterns, and a better understanding on how these regulatory mechanisms respond to the lower organic matter availability caused by warming is urgently needed. Most of our knowledge of ecosystem function comes from cultivation-based studies, enzymatic assays, and/or functional gene amplification studies that target specific processes such as methanogenesis, nitrification and N fixation [25, 26]. However, none of these methods provide a holistic approach to study the functionally active potential of complex microbial communities in the environment. With the emergence of metagenomic and metatranscriptomic approaches we now have powerful tools at hand to study such processes in complex environmental settings in a global change context [27–30].

Here, we combined metatranscriptomics and metagenomics to determine if warming causes a potential acceleration in organic N cycling in soil. We hypothesized that long-term soil warming would lead to persistent transcriptional changes in microbial organic N cycling, specifically higher transcription levels of genes associated with the production of extracellular enzymes involved the depolymerization of organic N from microbial necromass (i.e., of proteins, nucleic acids, chitin, and other organic N-containing substances). Such enzymes would target microbial residues, which become relatively enriched in warmed soils after more labile sources are consumed, and thus constitute alternative sources of both N and C that are readily replenished [11, 31].

We used a geothermal warming experiment in a subarctic grassland in Iceland [32] that provides stable and continuous gradients of soil warming of different durations [33]. The experiment was thus ideal to evaluate possible transcriptional responses to soil warming in situ. Previous studies at these locations reported significant losses of about 40% of soil organic C and N with increased soil warming in the top soil, as well as a decrease in microbial biomass and RNA content [32, 34]. However, biomass-specific microbial growth and turnover rates were consistently higher, even after more than 50 years of warming [14]. Warming did not affect above- or belowground plant biomass or the soil C:N ratio, indicating proportional losses of C and N [14, 15, 35]. A parallel metatranscriptomic study on the potentially active microbial communities from the same set of samples assigned 93–95% of total putative mRNA reads to bacteria [34]. Although there are some known biases towards this group in public databases and extraction methods which require careful interpretation, these data suggest a potentially significant role of bacteria in maintaining the main metabolic pathways detected [34].

METHODS

Sampling sites and soil sampling

Soil samples were collected from two Icelandic grassland sites subjected to either long-term (>50 years) or medium-term (8 years) geothermal warming from the ForHot natural soil warming experimental site (64° 0′ N, 21° 11′ W). This study site was established in 2012 and comprises replicated transects (2 × 2 m soil plots) representative of different temperature regimes. For each temperature regime, physicochemical parameters such as mean daily soil temperature, mean annual precipitation and soil pH are available [33]. In mid-July 2016 we sampled 4 biological replicates of "Ambient" and "Warmed" (+6 °C above ambient) soil plots from both grasslands to a total of 16 samples (Fig. S1). At the time of sampling the mean ambient temperature of the medium-term warmed (MTW) and LTW soil plots were 13.2 °C and 12.8 °C respectively. The mean elevated temperature of the warmed plots was 22.5 °C and 21.8 °C. From the 16 collected samples, 4 samples representative of the 4 different conditions (i.e. LTW ambient, LTW warmed, MTW ambient, MTW warmed) were selected for metagenome sequencing (Fig. S1). Five subsamples of each soil plot were taken, mixed in a bag, sieved through a 2 mm filter sieve and immediately frozen in liquid nitrogen. Contextual soil data for this specific sampling as well as nutrient pools and potential extracellular leucine aminopeptidase and ß-1,4-N-acetylglucosaminidase activities were collected and measured shortly after sampling (Supplementary Material and Methods; [33, 34]).

Nucleic acid extraction and sequencing

Total nucleic acids were extracted from 0.3 g of soil from three technical replicates of each biological replicate (total = 48) by bead-beating in the presence of cetyl-trimethylammonium bromide (CTAB) buffer and phenol [36]. Extracts were split and treated with DNase (RQ1, Promega) or RNase A (Thermo Fisher Scientific) before metatranscriptome ($n = 16$) and metagenome ($n = 4$) sequencing, respectively. The quality and quantity of DNA and RNA were assessed with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the Quant-iT™ PicoGreen® and RiboGreen® kits (Thermo Fisher Scientific), respectively. The absence of DNA in the RNA extracts was verified by PCR assays targeting bacterial SSU rRNA genes. A total of 100 ng RNA was linearly amplified using the MessageAmp II-Bacteria Kit (Ambion Life Technologies) according to the manufacturer's instructions and an amplification step of 14 h. Metatranscriptomic sequencing libraries were generated using the NEBNext® Ultra RNA Library Prep Kit for Illumina® and sequenced an Illumina HiSeq2500 (v2 chemistry, 2 × 125 bp mode) at the Vienna Biocenter Core Facilities, Vienna, Austria. We used the same filtered metatranscriptomic reads as the ones described in [34]. Shortly, transcripts were quality filtered and trimmed as described in [34] and putative paired-end mRNA reads with at least one hit against the NCBI nr database as of September 2018 were kept for analysis (Table S1).

Metagenome sequencing libraries were generated using the NEBNext Ultra FS II DNA Library Prep Kit for Illumina (New England BioLabs) at the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna and sequenced on a HiSeq4000 with the HiSeq 3000/4000 SBS Kit (300 cycles) in 2 × 150 bp mode. Metagenomic paired-end reads were screened for residual adapters and PhiX, quality filtered, trimmed and merged using BBtools (BBmap package v. 33.57 http://sourceforge.net/projects/bbmap/) with parameters: ktrim = r k = 21 mink = 11 hdist = 2 minlen = 120 qtrim = r trimq = 15. Following quality filtering, reads were assembled using SPAdes (v3.11.1) with the following options: -m 2000 - only-assembler -k 21,31,121, –meta -t 32. Contigs with less than 1Kb were discarded. Metagenomic read processing and assembly statistics can be found in (Tables S2 and S3). Genes were predicted on assembled metagenomic contigs for each sample ($n = 4$) using Prodigal v2.6.3 [37] with the metagenome (-p) option. Metatranscriptomic read mapping was done using BBmap against the metagenomic genes using default settings (Table S1). The mapping was done against metagenomic instead of metatranscriptomic assemblies because most of the signal peptides that target a protein for secretion are present on N-terminus of the sequence, which is often fragmented and consequently underestimated during metatranscriptomic assemblies. Fragment counts for each gene were converted to fragment per kilobase (FPK) values by dividing the number of fragments mapping to each gene by the length of the gene (in kb). Outlier genes from each dataset with excessively high FPK values were determined by fitting FPK values into a continuous power law cumulative distribution function using the commands available in the powerRlaw R package [38]. Nonzero FPK values per dataset were used to estimate the Xmin parameter with the commands *conpl$new()* and *estimate_xmin()*. Regression between log-transformed FPK values above Xmin and log-transformed rank was performed using the command *lm()* from the base stats package in R. The influence of each FPK on the resulting regression was assessed with the command *influence.measures()* from the base stats package in R. Genes with FPK values that were flagged as "influential" by all six available diagnostics (dfbetas, dffits, covratio, cooks.distance, rstandard and rstudent) and positive residuals (excessively high) were flagged as outliers. The outlier genes that were identified considering each dataset independently were then removed from all datasets and the procedure was repeated until no further outlier genes could be identified. In total, 22 genes were removed using this procedure. A count matrix containing the number of remaining mapped transcripts per metagenomic gene was normalized into a transcript per million (TPM) matrix, taking into account the gene length and the metatranscriptomic library size.

All computations were performed using the CUBE computational resources, University of Vienna, Austria, or were run on the high-

performance computing resource STALLO at the University of Tromsø, Norway.

Identification and screening of genes encoding putatively secreted organic N degradation-related proteins

We first aimed to build a metagenomic backbone representative of all genes at the sampling sites, against which putative mRNA reads could be mapped. A collection of non-redundant metagenomic genes were screened for the presence of protein sequences of interest using Pfam hmm models ($n = 253$) and *hmmsearch* with the trusted cut-off of each individual model [39, 40]. We included known protein domains present in enzymes involved in peptidoglycan and chitin catabolism (e.g., glycosyl hydrolases from families 18, 19, 20, 22), as well as those involved in nucleic acid degradation (e.g., endo/exonucleases) and those involved in protein catabolism (e.g., metallo- and serine peptidases) (Table S4). We additionally included extracellular domains such as the LysM and LTD domains, which are found in a wide range of carbohydrate-targeting extracellular proteins. Only hits whose reverse search against the Pfam database (v.32) using *hmmscan* returned the target model as best hit were considered further. Hits with more than one attributed Pfam domain were individually checked, and only the one with the highest bitscore was kept. This list of candidate genes was further screened to identify conserved domains signaling putatively extracellular secretion: SignalP (v 4.1) was used to search for signal peptides, with the default settings for eukaryotes, Gram-negative bacteria and Gram-positive bacteria [41]. SecretomeP (v 2.0) was used to screen for alternatively secreted proteins in Gram-negative and Gram-positive bacteria with default settings [42]. Only proteins that had a SecP score above 0.5 and that simultaneously did not contain a signal peptide were considered further. Finally, predicted protein subcellular localization was inferred using Psort (v 3.0) with the default settings from archaea, Gram-negative, and Gram-positive bacteria [43]. Proteins whose location was set as "Extracellular", "Outer Membrane" and "Cell Wall" were considered further. We used these three approaches in order to circumvent limitations associated with the performance of each software in identifying different secretion pathways [41]. A total of 15409 metagenomic genes met the above-mentioned criteria and were subsequently annotated against an updated database containing predicted proteins from all protist, fungal, bacterial, and archaeal genomes and MAGs in the JGI and NCBI [44]. The database also included all fungal genomes from the NCBI RefSeq database. The total number of predicted proteins in this database was 73.4 million, as of March 2020. Additionally, genes were annotated against the CAZy V.9 database [45] as well as the NCBI nr protein database. Our criteria to identify homologs against these databases using BLASTP were an amino acid identity above 40%, bit scores above 50, a subject coverage of at least 30%, a minimum alignment length of 50 residues, and the –more-sensitive option on Diamond [46]. We excluded 1360 genes with no hits on the genomic database. In addition, we manually excluded 1910 genes that were not involved in organic N degradation, but still contained relevant protein family domains and had been flagged as encoding putatively secreted proteins. This was the case for chaperone and precursor proteins, as well as proteins involved in cell division, that might be secreted but not actively involved in the hydrolysis of organic N compounds. The final dataset consisted of 12012 genes encoding putatively secreted proteins involved in organic N cycling, which could be assigned to 144 Pfams (Table S5). We additionally used the functional annotation to better define the target substrate of proteins encoded by particular genes. This was particularly important for genes encoding for proteins containing extracellular domains such as LysM or LTD. For example, the LTD domain can be present in proteins that target either nucleic acids or proteinaceous compounds. Based on the annotation of genes containing this domain, we were able to either assign a preferred substrate, or exclude other target substrates. Finally, genes of interest were taxonomically classified using the LCA algorithm based on the NCBI taxonomy implemented in Diamond (--outfmt 102, top = 5, min_id = 40).

Data analysis

All statistical analyses were performed using R v3.6.1. Previously identified genes involved in extracellular organic N degradation were extracted from the main TPM matrix, in order to keep relative abundance expression levels in relation to the remaining genes. The table was filtered in order to keep only genes with mapped transcripts ($n = 9413$), which comprised 131 Pfams. We applied a function filter, by first aggregating the TPM matrix Pfams, and then keeping only the domains present in all the four biological replicates of at least one group (Table S6). We additionally performed the function filter on each grassland site individually (Table S6).

To filter the functions that showed similar responses under warming irrespective of warming duration, we aggregated the TPM matrix by their Pfam annotation and considered a Pfam to be more abundant under warmed conditions if the mean TPM abundance across the four biological replicates was higher under warming than under ambient conditions. The statistical significance of this trend was then assessed by parametric or nonparametric tests according to whether parametric conditions (normal distributions and homogeneity of variances) were met or not (Table S7). *P*-values were adjusted for the false-discovery rate using the Benjamin–Hochberg correction method (*p.adjust*(method = "BH")).

A nonmetric multidimensional scaling (NMDS) was performed with function *metaMDS()* from the R package vegan [47] to assess the effect of temperature and warming duration on the transcribed organic N-related functions. A principal component analysis (PCA) was performed with function *PCA()* from the FactoMineR [48] R package to investigate the effect of temperature and warming duration on the physicochemical soil properties and cumulative relative transcription levels of key genes encoding putatively secreted organic N-degrading enzymes. The cumulative sum of relative transcriptional abundances of key genes was calculated by adding up the TPM values of all genes associated with the degradation of a particular substrate, which yielded four values representative of the four organic N substrates under study.

The *adonis()* function was used to perform permutational analyses of variance (PERMANOVA) and assess the effect of temperature and warming duration on the observed functional and taxonomic abundance patterns. Heatmaps were generated using the *pheatmap()* function of the pheatmap R [49] package. Rows were clustered using complete linkage, according to similar distribution patterns. All dissimilarity matrices used were generated using the Canberra distance, and PERMANOVAs were ran with 9999 permutations. The number of unique genes and functions was calculated by iterative ($n = 10$) rarefaction with replacement at a fixed depth (75% of the reads of the sample with the lowest number of total genes), using the number of transcripts that mapped to each gene potentially involved in the degradation of organic N sources as a probability vector. The function *rmultinom()* from the R stats package was used, and a rounded mean of the multinomial probabilities was used to compute gene and function richness values using the function *richness()* from the microbiome R package. The same approach was used to calculate the number of unique taxonomic groups.

RESULTS

Common responses to soil warming

We obtained soil metatranscriptomes of four biological replicates of previously characterized locations representing ambient and warmed (+6 °C) conditions in two grasslands subjected to 8 and > 50 years of warming. We additionally obtained four metagenomes representative of each condition. Metatranscriptomic reads were mapped to predicted metagenomic genes in order to identify transcriptional differences in organic N degradation processes in response to warming.

In both grasslands, we obtained a total of 9074 unique metagenomic open reading frames (ORFs), representing predicted genes and contained 106 different protein family domains (Pfams) associated with organic N degradation processes. Most of the genes were affiliated with bacteria (85.1 and 86.7% in the MTW and long-term warmed (LTW) grasslands, respectively), whereas eukaryotic and archaeal genes represented approximately 0.01% of these genes. The remaining fraction of the identified genes could not be phylogenetically classified at the domain level. After excluding such genes and those assigned to "Unclassified Bacteria", 75% the genes had a phylum-level classification, 40% had a class-level classification and 19.3% had an order-level classification. Genes phylogenetically associated with phylum Proteobacteria were the most abundant in both grasslands (29–32%), followed by Acidobacteria (21–23%) and Actinobacteria (7.2–8.2%). We found no significant differences in the number of unique taxonomic groups between ambient and warmed soils regardless of the warming duration (Fig. S2).

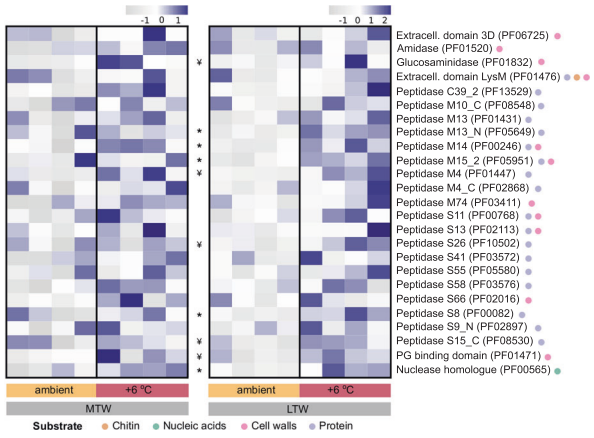

Fig. 1 Shared transcriptional responses to warming. Shared protein family domains (Pfams) associated with organic N degradation that had higher mean relative transcript abundances under warming than ambient conditions (see Methods for details). Each heatmap is normalized by row (z score). Biological replicates are ordered by their temperature profile, and the potential target substrates of each Pfam are indicated by the colored circles after the family domain name. Significant differences between ambient and warmed conditions for each Pfam were assessed by parametric or nonparametric *t*-tests (two-tailed), depending on whether or not the variable met parametric assumptions. All *p* values were adjusted for the false-discovery rate (Benjamin-Hochberg correction). Adjusted significant *p* values < 0.05 are indicated on the left of each heatmap by *, and *p* values that became nonsignificant after correction are indicated by ¥. The results of the full statistical analysis and exact *p*-values are presented in Table S7. PG peptidoglycan; Peptidase S_ serine peptidase; Peptidase M_ metallopeptidase; Peptidase C_ cysteine peptidase; LTW Long-term warmed grassland; MTW Medium-term warmed grassland.

Approximately 55% of the Pfams involved in organic N degradation identified in each grassland showed higher mean transcription levels in the warmed plots (LTW, 60 out of 104 Pfams; MTW, 50 out of 94 Pfams). Twenty-five Pfams were shared between both grasslands (i.e. showed the same response, irrespective of warming duration), and most were classified as metallopeptidases ($n = 8$) and serine peptidases ($n = 10$) or had domains involved in peptidoglycan catabolism ($n = 5$) (Fig. 1).

Warming influences physicochemical properties and transcription of genes encoding organic N-degrading enzymes

A PCA was used to examine the composition of soils with respect to physicochemical characteristics and cumulative sum of relative transcriptional abundances of key genes encoding putatively secreted organic N-degrading enzymes. Both axes captured between 66.2 and 73% of the variation in the MTW and LTW grassland respectively, and ambient and warmed soils showed a clear separation along the first dimension (Fig. 2; Fig. S3).

Warmed MTW grassland plots were characterized by non-significantly higher microbial CN ratios, leucine aminopeptidase potential activity, and higher relative transcription levels of genes encoding enzymes involved in the degradation of cell walls and proteins (Fig. S4; Table S7). Ambient plots were characterized by lower levels of organic C (DOC), total free amino acids (TFAA), and microbial biomass C and N [34]. In addition, genes encoding enzymes putatively involved in the degradation of chitin and nucleic acids also showed higher relative transcription levels under ambient conditions. In the LTW grassland, warmed plots were characterized by higher relative transcription of genes coding for enzymes involved in the degradation of all types of organic N sources, as well as higher potential enzymatic activities and microbial CN ratios (Fig. S4; Tables S7 and S8). Similarly to the MTW, the ambient plots of the LTW grasslands were characterized by higher microbial biomass, DOC and TFAA levels [34] (Fig. 2B; Fig. S4).

Warming leads to an upregulation of organic N-degrading genes and enzyme families

Warming resulted in significantly higher relative transcription levels of genes encoding putatively secreted proteins involved in organic N degradation in comparison with ambient plots regardless of warming duration (Fig. 3). In addition, a higher variability in the relative expression of genes encoding for secreted proteins putatively involved in organic N degradation was observed in the metatranscriptomes of the MTW grassland compared to the LTW grassland (3526 TPMs ± 227 and 3939 TPMs ± 77, in MTW and LTW grasslands respectively; Fig. S5B). The number of unique transcribed genes or functions also did not differ significantly between the ambient and warmed conditions (Fig. S6; Table S7).

Additional multivariate analyses (PERMANOVA) showed that the transcribed Pfams were significantly affected by temperature, warming duration and an interaction of the two (Fig. 3B). However, the dispersion between groups was not homogeneous (Fig. S7; Permdist, $p < 0.05$), an issue likely raised by the high group variance dispersion of ambient MTW plots. This meant that the PERMANOVA assumptions were not met, and results should be interpreted with care.

Individual grassland transcriptional responses to warming

We observed a significantly different community structure of the microbial taxa transcribing the genes of interest in response to warming in the LTW grassland (PERMANOVA, $F = 5.17$, $R^2 = 0.46$, $p = 0.03$) (Fig. S8B) in comparison with ambient soil plots. Warmed LTW grassland soils were characterized by higher transcription levels of genes affiliated with Planctomycetes, Beta- and Deltaproteobacteria, whereas in ambient soils, the transcription levels of Actinobacteria, Alpha- and Gammaproteobacteria were higher (Fig. S8B).

We also found higher transcription levels and a shift in the transcriptional structure of genes encoding enzymes potentially involved in the degradation of chitin in response to warming (Fig. 4E). Warmed LTW grassland plots were characterized by higher transcription of genes annotated as β-N-acetylglucosaminidases, chitosanases and transglycolases (Fig. 4E), but the chitin-degrading microbial community structure was not significantly affected by warming (Fig. S9A). Genes encoding proteins potentially involved in nucleic acid degradation also showed significantly higher transcription levels under warming in the LTW grassland in comparison with ambient conditions (Fig. 4F). Similarly, there were also significant changes in the relative transcription levels of genes encoding the different protein family domains associated with nucleic acid degradation. These differences also entailed taxonomic changes in the nucleic-acid-degrading microbial community between ambient and warmed plots (Fig. S9B). Warmed LTW grassland plots were characterized by higher transcription levels of genes affiliated with Betaproteobacteria, *Candidatus* phylum Rokubacteria, Deltaproteobacteria, and Verrucomicrobia (Fig. S9B), transcribing genes annotated as extracellular ribonucleases, endonucleases, and extracellular domains (Fig. 4F). The significantly higher relative transcription levels of genes potentially encoding proteins involved in cell wall degradation under warming were characterized by higher relative transcription levels of peptidoglycan hydrolases such as lysozymes, metallo- and serine peptidases, as well as several peptidoglycan-binding domains (Fig. 4G). Significant differences in the community structure of taxa transcribing genes encoding these enzymes were characterized by higher relative contributions of Alpha- and Deltaproteobacteria, Acidobacteria and *Candidatus* Rokubacteria under warming. (Fig. S9C). Notably, no significant

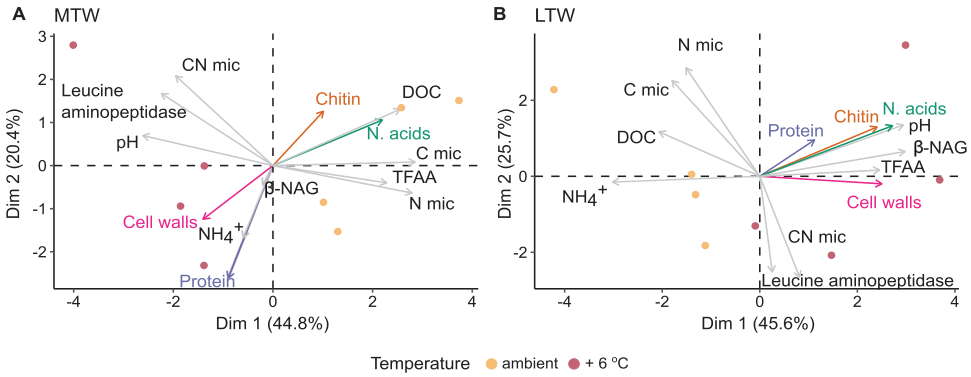

Fig. 2 Principal component analysis (PCA) depicting the effect of warming on soil physicochemical properties and relative transcription of genes involved in the degradation of organic N. Warming effect on soil physicochemical properties and the cumulative relative transcription level of genes encoding putative organic N-degrading enzymes are shown for the medium-term warmed (MTW) (**A**) and long-term warmed (LTW) (**B**) grasslands. DOC, dissolved organic (μg C g^{-1} dry weight soil); C mic, microbial biomass C (μg C g^{-1} dry weight soil); N mic, microbial biomass N (μg N g^{-1} dry weight soil); TFAA, total free amino acids (μg N g^{-1} dry weight soil); CN mic, microbial C to N ratio. DOC, pH, and microbial biomass C and N contents for these samples were retrieved from [34]. Enzyme data are expressed per unit of microbial biomass C (Table S8). The cumulative relative transcription of genes involved the degradation of the different organic N compounds is indicated by the colored arrows: chitin (orange); nucleic acids (green); cell walls (pink); protein (purple).

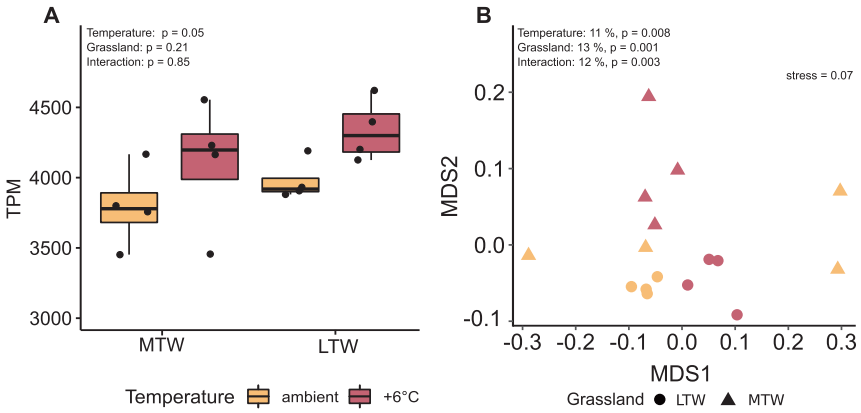

Fig. 3 Differences in relative transcription levels of genes involved in organic N degradation. The relative transcription levels of all genes encoding secreted proteins putatively involved in organic N degradation in relation to all protein-coding genes in each sample are shown as transcripts per million (TPM) between ambient and warmed soil plots of the medium-term warmed grassland (MTW) and the long-term warmed grassland (LTW) (**A**). Inside the boxplots, the median value is shown. A two-way ANOVA was used to assess statistical the statistical significance. Subplot (**B**) shows a nonmetric multidimensional scaling (NMDS) ordination of all samples, according to the transcribed functions under analysis. The percentage of variation explained by each factor in a PERMANOVA is shown in the NMDS plot. Unpaired *t*-tests (two-tailed) were used after checking the parametric assumptions, and *p*-values were adjusted for the false-discovery rate (Benjamin-Hochberg correction). Detailed statistical results can be found in Table S7.

effect of warming was found regarding the transcription levels of genes associated with protein degradation (Fig. 4H). Nevertheless, temperature significantly affected the structure of protein-degrading family domains, as indicated by higher transcription levels of genes annotated as aspartic, metallo-, and serine peptidases in the warmed plots. These functions were primarily associated with Planctomycetes, Acidobacteria and Betaproteobacteria (Fig. S5D).

The number of significant differences between warmed and ambient soils were fewer in the MTW grassland than in the LTW grassland (Fig. 4A; Figs. S8B and S10). However, functions associated with cell wall and protein degradation showed trends toward higher relative transcription levels under warming, similarly to what was observed in the LTW grassland (Fig. 4). Medium-term warming also resulted in significantly different functional changes of genes encoding enzymes involved in protein degradation, which were characterized by higher transcription of genes encoding serine peptidases and extracellular domains (Fig. 4D).

DISCUSSION

The depolymerization of complex organic N compounds to oligomers or monomers is considered to be the rate-limiting step in terrestrial N cycling [50–52]. Organic N compounds are also valuable sources of C and energy [8], and whenever they are scavenged for C or energy rather than N, microorganisms may excrete the excess N as ammonium, fueling inorganic N transformations. Studies have suggested that the main determinants of organic N depolymerization rates are substrate availability and accessibility, as well as the activity of depolymerizing enzymes of microbial origin [50, 53]. Warming directly accelerates decomposition processes by soil microorganisms, microbial activity as well as the growth and turnover of microbial communities, contributing to a gradual depletion of substrates [3, 32]. Lower microbial biomass in response to substrate limitation, coupled with higher mass-specific microbial activity, results in reduced microbial C and N immobilization and in consequent C and N losses from the system with potential positive feedbacks to climate change [14, 15, 54].

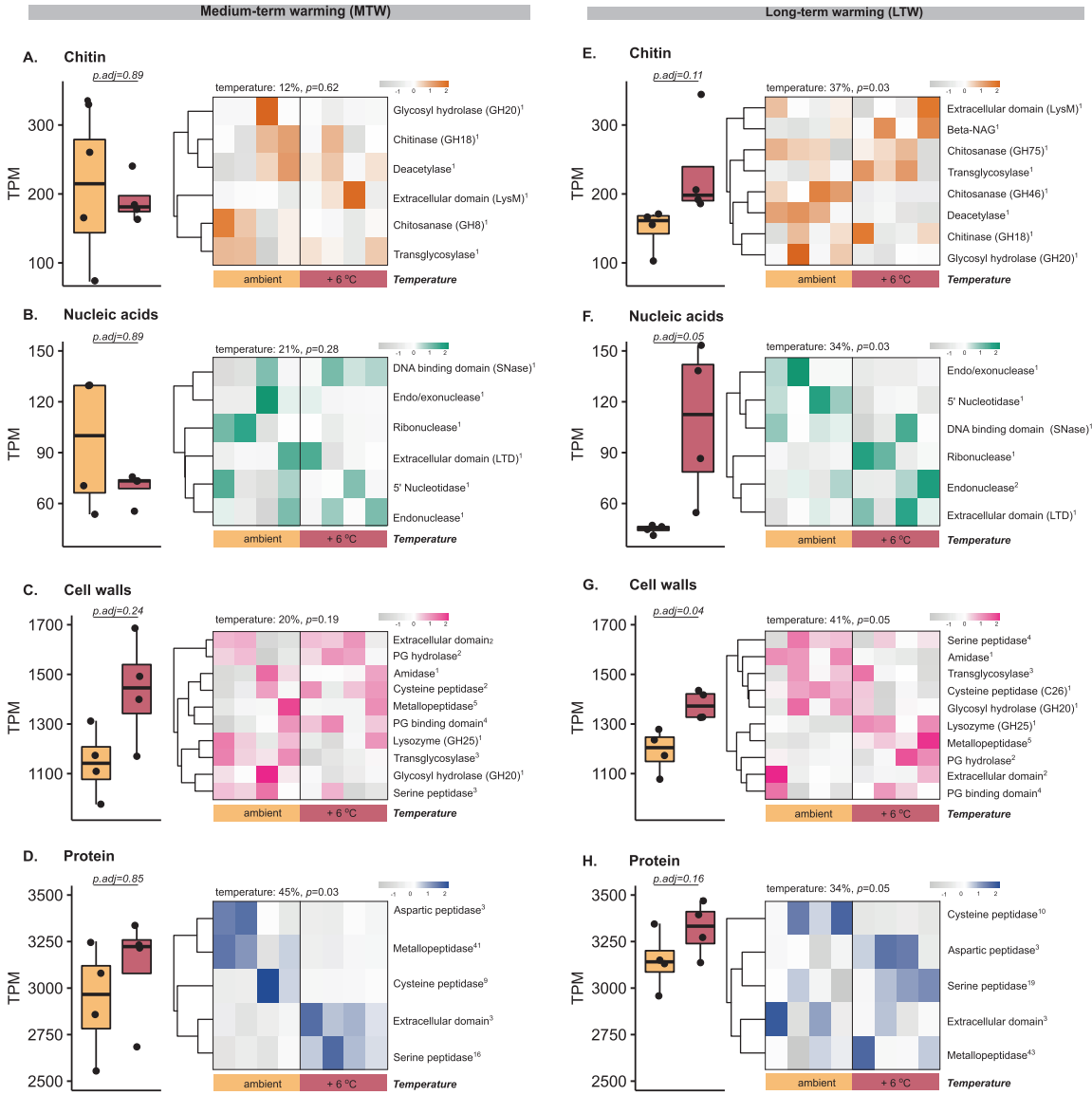

Fig. 4 Grassland-specific transcriptional responses to warming. Warming-induced differential transcription levels, in transcripts per million (TPM), of genes and enzyme families involved in the degradation of chitin (**A**, **E**), nucleic acids (**B**, **F**), cell walls (**C**, **G**) and proteins (**D**, **H**) in the MTW and LTW grasslands. The boxplots depict an aggregated sum of transcripts mapped to genes assigned to the degradation of a particular substrate relative to all transcripts, according to ambient or warmed (+6 °C) conditions. Significant differences between ambient and warmed conditions for each substrate were assessed by parametric or nonparametric *t*-tests, depending on whether or not the variable met the assumptions (Table S7). All *p* values were adjusted for the false-discovery rate (Benjamin–Hochberg correction). Heatmaps were built using TPM normalized count matrices for each substrate and depict the different functional domains (Pfams) assigned to a substrate. The data were normalized by row (z score) and clustered using complete linkage, according to similar abundance patterns. The superscripted numbers indicate the number of Pfams aggregated in each category. The percentage of variation explained by temperature is shown on top of the heatmaps (PERMANOVA analysis).

Increasing metabolic investment towards the production of depolymerizing enzymes that act on insoluble high-molecular-weight plant and SOM sources is therefore a likely key strategy to overcome the limitation of accessible nutrients [21, 22, 55, 56]. Indeed, we observed higher transcription levels of genes and enzyme families involved in organic N degradation in response to warming, particularly in the LTW grassland (Fig. 4), although we found no significant difference between the MTW and LTW grasslands (Fig. S5). This observation is consistent with a trend towards higher hydrolytic enzymatic activities per unit of microbial biomass that was also observed in response to long-term warming (Fig. S4). Even though measurements of potential enzymatic activities are general indicators, they reflect the activity of a limited set of enzymes under optimal conditions, thus providing an incomplete assessment of what happens under natural conditions.

In our study, most of the detected transcribed genes encoding putatively secreted proteins were associated with the degradation of microbial residues such as cell walls and proteins, consistent with these substrates representing major sources of organic N under warming [11] in line with our hypothesis. We detected significantly higher relative transcription levels of genes encoding extracellular peptidases from family M13, thermolysins from family M4 and serine peptidases from family S8 in LTW soils. These

peptidase families are described as having strong extracellular proteolytic activity and to act on proteins and small peptides to sustain bacterial nutrition [16, 57].

Additionally, genes encoding peptidases from families M14 and M15 also showed consistently higher relative transcription levels in the warmed plots of the LTW grassland. Extracellular zinc-containing carboxypeptidases from family M14 are synthesized without signal peptides and are involved in nutrition and in the metabolism of bacterial cell walls [58, 59]. Cell-wall hydrolases from family M15 include zinc-containing D-Ala-D-Ala carboxypeptidases and dipeptidases involved in cell-wall biosynthesis and metabolism [59].

The increased transcription levels of genes encoding these families of extracellular peptidases in the LTW grassland thus suggests that microorganisms exposed to long-term warming allocate more resources into cell wall and protein degradation processes, corroborating sustained higher microbial turnover rates reported previously for LTW soils [14]. Our results are further supported by previous observations that highlighted the importance of microbial residues as potential C and N sources under warming [11, 60].

We detected few to no changes in the structure of the microbial communities responsible for producing the enzyme families under study. This suggests that physiological changes rather than major changes in community composition were responsible for adaptations to soil warming in this subset of the microbial community of our experiment, especially since the ability to produce and secrete these types of enzymes is widespread among soil microorganisms. Nevertheless, the few observed phylogenetic changes between ambient and warmed conditions of the LTW grassland reflected a potentially relevant contribution of Deltaproteobacteria in degrading all organic N forms and of Planctomycetes in degrading proteinaceous compounds. On the other hand, Alpha- and Gammaproteobacteria and Actinobacteria showed higher transcription levels of genes involved in the degradation of all organic N forms under ambient conditions, suggesting the existence of group-specific responses to warming and/or substrate preferences.

Previous observations reported an absolute decrease in fungal phospholipid fatty acid markers under elevated temperatures [32] in comparison to ambient conditions. Supporting this view, we observed higher transcription levels of deacetylases and chitosanases (GH 46) in ambient conditions in the LTW grassland. Glycosyl hydrolases from family 46 known to degrade chitosan, a polymer obtained after the deacetylation of chitin. The higher relative transcription levels of genes encoding deacetylases and chitosanases under ambient conditions coupled to higher fungal PLFA levels suggests that fungal cell walls are contributing more to the microbial necromass under ambient than under warmed conditions and decreasing the importance of chitin as a source of organic matter under warming. While the observed higher relative transcription levels of chitin-degradation genes in the LTW grassland might contradict previous findings, high levels of functional redundancy are common to the degradation of microbial residues such as chitin and peptidoglycan present in microbial cell walls [18, 61], so chitinases may also be involved in the degradation of bacterial cell walls. Another possible explanation is that while fungi are less abundant in warmed soils, their turnover is increased, resulting in a potentially higher contribution of fungal cell-wall material for decomposition. Along these lines, we did not detect changes in the transcription levels of genes containing domains present in classic chitinases (i.e., glycosyl hydrolases from families 18 and 19) in response to warming.

Notably, we detected higher relative transcription levels of genes annotated as β-N-acetylglucosaminidases in the LTW soils, but the transcription of these genes was not even detected in MTW soils. Thus, regarding the LTW grassland, we cannot exclude the possibility that the transcription of genes associated with chitin degradation is also associated the degradation of other substrates present at higher levels or more easily accessible under natural conditions. A methodological limitation of this study is the impossibility of linking a protein family domain directly to the degradation of a particular substrate, given that there might be high levels of enzyme promiscuity (i.e., an enzyme acting on multiple substrates). We tried to account for this limitation by carefully evaluating the functional annotation of each gene encoding a putatively secreted enzyme and assigning it to the degradation of all the substrates that it had the potential to act on. Another limitation of this study is that only one sampling time point for metatranscriptomics was taken. This is particularly relevant, due to the short half-life of mRNA and its associated high temporal variability. However, our results demonstrate similar responses to warming in both grassland ecosystems. Therefore, we believe that the expression patterns we found are robust, even though we cannot draw conclusions about their variability in time.

We were surprised to find few to no transcribed fungal genes associated with organic N degradation in the metatranscriptomes in this study. Saprotrophic fungi are known to produce a wide range of extracellular enzymes that degrade organic N sources [62]. However, the underrepresentation of eukaryotes in metagenomic and metatranscriptomic datasets has been also reported in other studies [63]. We observed an increase in glycosyl hydrolases family 75 under warming in LTW which mostly includes chitosanases of fungal origin. However, all 12 genes that contained this functional domain were assigned to Bacteria and had no hits on the CAZy database, which suggests the presence of a still uncharacterized set of bacterial chitosanases.

In this study, we highlight the potential of metatranscriptomics as a tool that offers a comprehensive overview of the active functional potential for the degradation of organic N under warming. Our results also provide grounds for the formulation of hypotheses that enable further targeted research focused on specific sets of enzymes of interest.

Finally, our study demonstrates that sustained soil warming leads to higher transcription levels of genes and enzyme families involved in the degradation of N-rich polymers, especially those found in microbial necromass. This suggests that the recycling of microbial remains is a key process to support consistently higher growth and turnover rates of microbial communities when labile C and N substrates become limiting, already after 8 years of sustained warming. Considering that (sub)arctic ecosystems are especially vulnerable to climate change, our results provide a mechanistic insight into the micro-scale mechanisms that lead to ecosystem-scale responses to soil warming.

DATA AVAILABILITY

The metatranscriptomic reads were obtained from [34] and are available at the NCBI Short Read Archive (SRA) under BioProject ID PRJNA663238. The metagenomic raw reads and assemblies obtained in this study can be found under BioProject ID PRJNA746424. The corresponding metadata is available as Supplementary Material.

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AUTHOR CONTRIBUTIONS

AR and TU conceived and supervised the study. BDS, EV, AR, IJ, JPe established the experimental sites. JP and AR collected the samples. JS, JP, and ATT did laboratory work. JS analyzed the data, with assistance from ATT. AS provided data and assisted with data analysis. CWH and PP assembled the metagenomes, assisted with data analysis and provided valuable input. JS wrote the manuscript with input from all authors.

COMPETING INTERESTS

The authors declare no competing interests.

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Chapter V

Summary and concluding remarks

Summary

In the previous chapters, I have investigated the *in situ* effect of single and combined climate change drivers on the structure and composition of microbial communities, with a particular focus on those involved in organic and inorganic N transformations, across the vegetation period. Chapters II and III were dedicated to gaining a deeper understanding on how elevated temperatures alone or in combination with CO_2 and drought shaped the general and functional community structure in a sub-montane grassland. I additionally aimed to examine the effects of future climate conditions (+3 ^oC; +300 ppm CO_2) across different temporal scales as well as to assess short and mid-term drought legacy effects on community structure. Towards this goal, I collected samples from multiple timepoints during the plant growing season in 2017 and fourteen months after the end of an intense summer drought period (chapter III). Furthermore, I aimed at better understanding how an intense drought affected microbial communities involved in inorganic N transformations, so I specifically looked into the potential contributions of different groups of ammonia-oxidizing microorganisms (AOM) under peak summer drought conditions (chapter II).

The results from chapter II include, for the first time, a comprehensive assessment of the response of all known AOM to future climate change scenarios (elevated CO_2, temperature and drought) in light of the recent discovery of complete nitrifiers. Niche differentiation between ammonia-oxidizing microorganisms (AOM) is thought to revolve around three main interacting factors: i) substrate affinity and saturation rates, ii) mixotrophy, and ii) pH optimum (Prosser and Nicol, 2012). Climate change affects the quantity of available substrates for nitrification, which in turn show a strong dependence on pH (Lehtovirta-Morley, 2018). Together with environmental factors such as temperature and water content, these factors dictate the population dynamics of AOM and the selective proliferation of certain groups over others. Indeed, we observed an accumulation of organic and inorganic N forms under drought conditions, but no significant changes in pH.

In this chapter, I showed that both AOA and CMX displayed different sensitivity levels to drought in comparison to AOB. While both AOA and CMX have a high affinity for NH_3 and often thrive in nutrient poor environments, AOB possess an opposite

metabolic repertoire, having a low affinity for NH_3 and higher V_{max} optimum (Prosser and Nicol, 2012). Consequently, based solely in substrate availability AOB are expected to outcompete AOA and CMX in nutrient rich environments as the grassland under study (Di et al., 2009). However, some AOA members (e.g. *Nitrosocosmicus*) also thrive in nutrient rich environments, which reduces support for the decrease in AOA potential activity (as inferred by *amoA* transcript quantification) based solely on substrate availability (Lehtovirta-Morley et al., 2016; Nicol et al., 2019). On the other hand, the growth and activity levels of some AOA has been shown to be sensitive to the accumulation of organic compounds which was the case under drought conditions (Lehtovirta-Morley et al., 2013). Furthermore, microbial physiology studies on the only clade A CMX representative in pure culture indicated that this group possess the lowest substrate affinity levels of all AOM (Kits et al., 2017). Even though CMX in this study were all affiliated with clade B, the nature of their response mimicked that of AOA, suggesting similar ecological niches and sensitivities to drought. Similarly to the structure of AOM communities, nitrification rates did not respond to single and combined effects of temperature and eCO_2 but were significantly higher under drought in comparison to non-drought conditions. Even though the results from isotope pool dilution assays in dry soils must be interpreted with care, this study provides grounds for AOB-driven nitrification rates under drought at the expense of the remaining AOM.

In chapter III, in addition to investigating the how season modulates single and combined effects of eT, eCO_2 and drought on microbial communities, I assessed potential drought-legacy effects two and fourteen months after the end of an 8-week summer drought perturbation. As drought events become more frequent, the response of an ecosystem to each new disturbance will likely be dependent on perturbation history. While other studies have reported that changes in microbial community structure only started to be manifested after > 5 years of recurring drought events (Canarini et al., 2021), at our sampling site we observed differences on the DNA- and RNA-based bacterial/archaeal communities between droughted and non-droughted plots two months after an intense drought period, even if less extreme than at peak drought. This goes in line with other studies which report an acceleration in microbial activity and turnover following a dry period, as substrates become accessible again, communities are relieved from osmotic stress and some trophic interactions (e.g. predation) can resume (Schimel, 2018; Simon et al., 2020). Fourteen months after the

drought treatment, however, significant dissimilarities in bacterial/archaeal community structure between ambient and droughted plots were still observed, although only in RNA-based bacterial and archaeal community structure. This suggests that drought could have caused a restructuring of the existing microbial community members, potentially enabling the transition to a new state characterized by different guilds. Furthermore, at our experimental site, fungi displayed lower resilience levels to drought, which was reflected in drought-legacy effects on community structure two and fourteen months after the end of the drought treatment.

In addition to the drought legacy effects, our results also highlight the role of eT and eCO_2 in affecting the RNA-based bacterial/archaeal community structure at the beginning and at the end of the growing season. A parallel study focused on microbial physiology revealed that climate change effects on microbial biomass, growth and respiration rates were season-specific (Simon et al., 2020). Specifically, eT alone caused lower microbial growth during the peak growing season (May) but this effect was buffered by eCO_2 which, in combination with eT, led to higher growth rates in comparison to the controls. On the other hand, at the end of the growing season (October), eT alone caused higher microbial biomass-specific growth and respiration rates and the interactive effect with eCO_2 did not result in significant changes in microbial physiology metrics (Simon et al., 2020). Higher microbial growth rates in response to future climate ($eTeCO_2$) can be explained by higher plant-derived organic matter inputs, which can be further intensified by eCO_2. This would result in the priming of a specific portion of the existing microbial communities, causing increased respiration rates, and potentially explain differences in community structure. On the other hand, at the end of the growing season there is a general decrease in plant growth. Consequently, the direct influence of eCO_2 as a plant-growing promoting factor and its indirect effect on belowground communities could potentially decrease, leaving eT as a potential main driver of high microbial growth, respiration rates and belowground community structure.

Given the well documented role of soil warming on accelerating atmospheric CO_2 emissions through an acceleration on microbially mediated SOM processes, overall activity and turnover rates, chapter IV was dedicated to an in-depth assessment of the effects of soil warming on the expression of genes encoding for extracellular organic N degradation processes. Long-term soil warming has been shown to deplete soils

from easily assimilable organic matter, thus contributing to a decrease in microbial biomass but also to increased microbial activities per unit of microbial biomass (Walker et al., 2018). Based on these findings, we expected to find an up-regulation of genes encoding extracellular enzymes targeting SOM forms such as microbial necromass (composed of peptidoglycan and proteins), as these substrates would become a more viable source of C and N under sustained warming, due to the absence of more labile forms. Metagenomics and metatranscriptomics was chosen as a way to tackle the inherent difficulty that is to design and successfully apply marker gene assays to such a diverse set of gene families such as glycosyl hydrolases and peptidases. The chosen experimental site was a sub-arctic grassland in Iceland that is part of the longest *in situ* climate change experiment, whereby soils become naturally warmed due to geothermal activity, creating natural soil temperature gradients. High latitude ecosystems are characterized by low mean annual temperatures and store a disproportionally high percentage of soil C and N, and are known to be particularly vulnerable to rising global temperatures. Previous studies at this location reported that soil warming up to +6 ^{o}C above ambient conditions caused no significant changes in aboveground plant biomass, but significant losses of C and N from the top-soil layers as well as decreases in microbial biomass (Marañón-Jiménez et al., 2018, 2019; Walker et al., 2018; Verbrigghe et al., 2022). On the other hand, biomass-specific microbial growth and turnover rates were found to be higher in response to soil warming, suggesting a metabolic acceleration even after > 50 years of warming (Walker et al., 2018).

Results from chapter IV indicated that microorganisms exposed to long-term warming adjusted their transcription machinery and increased the expression levels genes encoding extracellularly secreted enzymes particularly those targeting microbial residues such as cell walls, proteins and other sources of recalcitrant organic matter. Many genes that showed higher transcription levels under warming encoded extracellular cell wall hydrolases as well as metallo- and serine peptidases, which have known proteolytic activity in soil and are encoded by a wide range of microorganisms. Indeed, we did not observe changes in the functional community composition expressing these genes between warmed and un-warmed soils, which reinforces i) how phylogenetically widespread these traits are and ii) the physiological plasticity levels of the existing microbial communities.

Concluding remarks

Soil research has come a long way and the combination of classic soil ecology methods with modern ‘omics approaches presents exciting opportunities for future research. No longer perceived as abiotic and static, soil has become a lively net of complex interacting networks and organisms. Overall, this thesis has contributed to a better understanding of the response of microbial communities to single and combined environmental press disturbances (elevated temperatures, CO_2 and drought) particularly the microbial responses in key steps of the terrestrial N cycling.

There are several general take-home messages to retrieve from this thesis:

(1) Microbial communities play a pivotal role in soil biogeochemistry, and display extraordinary levels of adaptability to environmental change through changes in gene expression pattern and/or changes in community composition.

(2) Analyzing the temporal dynamics of soil microbial communities to climate change is important to obtain a comprehensive overview as opposed to using data from a single snapshot to predict future climate change effects.

(3) Data collection from *in situ* multifactorial experiments (robustly replicated) is relevant in order to assess the nature and magnitude of the biological response, which most often than not is non-additive and non-linear.

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