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Global impacts of fertilization and herbivore removal on soil net nitrogen mineralization are modulated by local climate and soil properties

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Abstract:

Soil nitrogen (N) availability is critical for grassland functioning. However, human activities have increased the supply of biologically-limiting nutrients, and changed the density and identity of mammalian herbivores. These anthropogenic changes may alter net soil N mineralization (soil net N_{min}), i.e., the net balance between N mineralization and immobilization, which could severely impact grassland structure and functioning. Yet, to date, little is known about how fertilization and herbivore removal individually, or jointly, affect soil net N_{min} across a wide range of grasslands that vary in soil and climatic properties. Here, we collected data from 22 grasslands on five continents, all part of a globally replicated experiment, to assess how fertilization and herbivore removal affected potential (laboratory-based) and realized (field-based) soil net N_{min}. Herbivore removal in the absence of fertilization did not alter potential and realized soil net N_{min}. However, fertilization alone and in combination with herbivore removal consistently increased potential soil net $N_{min.}$ Realized soil net N_{min}, in contrast, significantly decreased in fertilized plots where herbivores were removed. Treatment effects on potential and realized soil net N_{min} were contingent on site-specific soil and climatic properties. Fertilization effects on potential soil net N_{min} were larger at sites with higher mean annual precipitation (MAP) and temperature of the wettest quarter (T.q.wet). Reciprocally, realized soil net N_{min} declined most strongly with fertilization and herbivore removal at sites with lower MAP and higher T.q.wet. In summary, our findings show that anthropogenic nutrient enrichment, herbivore exclusion, and alterations in future climatic conditions can negatively impact soil net N_{min} across global grasslands under realistic field conditions. This is important context-dependent knowledge for grassland management worldwide.

Keywords: anthropogenic change, grazers, nitrogen, phosphorus, potassium, potential and realized soil net nitrogen mineralization, precipitation, temperature, global grasslands, NutNet

Introduction

The availability of biologically limiting nutrients, such as nitrogen (N), phosphorus (P), and potassium (K), drives the productivity (Elser *et al.*, 2007; Harpole *et al.*, 2011; Fay *et al.*, 2015) and functioning of grassland ecosystems worldwide (Neff *et al.*, 2000). Soil N availability is largely determined by the breakdown and depolymerization of organic material to monomers and inorganic N for which plants and microbes compete (Schimel & Bennett, 2004; Butterbach-Bahl & Gundersen, 2011; Kuzyakov & Xu, 2013; Mooshammer *et al.*, 2014). The net balance between

N mineralization and immobilization, further referred to as soil net N mineralization (soil net N_{min}), is largely controlled by soil physical and chemical properties (e.g., clay content, bulk density, pH, carbon (C) and N content), the type and amount of above- and belowground organic matter inputs (e.g., plant production), plant and soil microbial composition and activity (release of enzymes, nutrient uptake), and climatic factors (Giardina *et al.*, 2001; Schimel & Bennett, 2004; Booth *et al.*, 2005; Craine *et al.*, 2010; Dessureault-Rompré *et al.*, 2010; Conant *et al.*, 2011; Giese *et al.*, 2011; Risch *et al.*, 2019).

Human activities that alter the biodiversity and structure of grassland plant communities worldwide also can have important direct and indirect consequences for soil functioning, including soil net N_{min} (Rockström *et al.*, 2009; Steffen *et al.*, 2015). Two of the most pervasive human impacts threatening grassland ecosystems are increases in the supply of biologically limiting nutrients, e.g., through burning of fossil fuels or fertilization (Fowler *et al.*, 2013; Peñuelas *et al.*, 2013; Steffen *et al.*, 2015; Sardans *et al.*, 2017), and alterations in the density of native mammalian herbivores by over-exploitation or their replacement by livestock (Estes et al. 2011, Dirzo et al. 2014, Ripple et al. 2015, WWF 2018). However, we still do not know the relative contribution of climatic and edaphic factors versus anthropogenic drivers, such as increases in soil nutrient content and altered grazing regimes, in regulating soil net N_{min} in global grasslands (e.g., Thébault *et al.*, 2014).

Grassland soil net N_{min} can be impacted by the presence and activity of herbivores through plant biomass consumption, trampling, burrowing, and deposition of urine and dung (e.g., Bakker *et al.*, 2004; Olofsson, 2009; Schrama *et al.*, 2013; Risch *et al.*, 2015; Zhou *et al.*, 2017). However, the response of a system to a change in grazing conditions depends on soil texture and water availability (Schrama *et al.*, 2013), grazing intensity (Zhou *et al.*, 2017), herbivore species identity (Risch *et al.*, 2015; Zhou *et al.*, 2017), and herbivore body-size (Bakker *et al.*, 2004; Risch *et al.*, 2015, 2018). Similarly, the impacts of anthropogenic nutrient inputs on grassland soil net N_{min} depend on plant and soil characteristics, as well as local climatic conditions (Mueller *et al.*, 2013; Changhui *et al.*, 2014; Ochoa-Hueso *et al.*, 2014; Wei *et al.*, 2017; Chen *et al.*, 2019a; Hicks *et al.*, 2019). Both herbivore removal and nutrient additions can have positive, negative, or neutral effects on soil net N_{min}, largely depending on site conditions (e.g., Bakker *et al.*, 2004; Changhui *et al.*, 2014; Risch *et al.*, 2015; Wei *et al.*, 2017). This strong context-dependency makes it difficult to estimate how increases in soil nutrient availability and shifts in the presence of mammalian herbivores, individually and in combination, influence the ability of grassland soil communities around the world to mineralize N from soil organic matter.

Reliably estimating soil net N_{min} is not straightforward, and methodological constraints can limit, or even misguide, our understanding of this key process under real-world, field conditions (Arnold *et al.*, 2008; Makarov *et al.*, 2017; Risch *et al.*, 2019; Pinto *et al.*, 2020). For example, measures of potential soil net N_{min} assessed in the laboratory may allow us to better understand the processes by which global change affect the overall magnitude of soil N availability across grasslands worldwide; i.e., they reflect the potential of grasslands to respond to global change (Risch *et al.*, 2019). Measures of realized soil net N_{min} obtained directly in the field, in contrast, may provide a more realistic indication of how grasslands will respond to fertilization and herbivore removal under heterogeneous environmental and climatic field conditions (Risch *et al.*, 2019). Moreover, these two distinct measures, potential and realized soil net N_{min}, only weakly correlate across grasslands worldwide (Risch *et al.*, 2019) and it is unclear whether knowledge about global change effects gained from laboratory assessments of soil net N_{min} will allow us to estimate field rates. Thus, to understand and generalize how fertilization and herbivore removal affect soil net N_{min}, standardized, globally replicated experiments are required. These experiments should span a wide range of environmental and climatic contexts, and measure both potential and realized soil net N_{min} simultaneously.

To address this knowledge gap, we assessed how the removal of mammalian herbivores (Fence) and fertilization with growth-limiting nutrients (N, P, K, plus nine essential macro- and micronutrients; NPK) individually, and in combination (NPK+Fence), affected potential and realized soil net N_{min} across 22 natural and semi-natural grasslands on five continents (Fig. 1, Supplementary Fig. S1). Our sites spanned a comprehensive range of climatic and edaphic conditions found across the grassland biome (Fig. 1; Supplementary Table S1 & S2). We focused on grasslands, because they cover 40-50% of the ice-free land surface and provide vital ecosystem functions and services. They are particularly important for forage production and C sequestration. Worldwide, grasslands store approximately 20-30% of the Earth's terrestrial C, most of it in the soil (Schimel, 1995; White *et al.*, 2000). We assessed how treatment differed in potential and realized soil net N_{min} compared to control plots using linear mixed-effects models (LMMs). We also analyzed how the treatments affected potential and realized soil net ammonification and net nitrification, which are the two main steps of soil net N_{min}. To gain a mechanistic system-level understanding of how fertilization and herbivore removal affect soil net N_{min}, we used structural equation modelling (SEM) to test a conceptual model that also considered the role of potentially modulating environmental and climate variables (Grace, 2006; Eisenhauer *et al.*, 2015).

Overall, we expected that our grazing exclusion and nutrient addition treatments, individually and in combination, should lead to higher soil nutrient availability and higher quality of plant litter returned to the soil (Coley *et al.*, 1985; Anderson *et al.*, 2018), which, in turn, should have a positive effect on both potential and realized soil net N_{min} across our globally-distributed grassland study sites (Frank & Groffmann, 1998; Bakker *et al.*, 2004; Hobbie, 2015; Risch *et al.*, 2015; Ouyang *et al.*, 2018). However, we predicted that the magnitude of response of potential soil net N_{min} to our treatments would be larger than that for realized soil net N_{min} due to the optimal and standardized conditions associated with laboratory incubations. Finally, we expected that treatment responses in both potential and realized soil net N_{min} would be modulated by soil properties and long-term climatic conditions, such as precipitation and temperature, because the structure and abundance of soil communities, and the processes they drive, are conditional on long-term water availability (Ochoa-Hueso *et al.*, 2018).

Methods

Study sites and experimental design

The 22 sites contributing to this project are part of the Nutrient Network Global Research Cooperative (NutNet, https://nutnet.umn.edu/). Mean annual temperature across our 22 sites ranged from -4 to 22°C, mean annual precipitation from 252 to 1,592 mm, and elevations from 6 to 4,261 m above sea level (Fig. 1, Supplementary Table S1). Soil organic C varied from 0.8 to 7.8%, soil total N from 0.1 to 0.6%, and the soil C:N ratio from 9.1 to 21.5. Soil clay content spanned from 3.0 to 35%, and soil pH from 3.4 to 7.6 (Supplementary Table S2). Thus, the sites covered a wide range of environments in which grasslands occur (Fig. 1, Supplementary Table S1 & S2).

At each site, the effects of nutrient addition and herbivore removal were tested via a randomized-block design (Borer *et al.*, 2014; Supplementary Fig. S1a). Three replicate blocks with 10 treatment plots each were established at each site, with the exception of the site at bldr.us, where only two blocks were established (Supplementary Fig. S1a). The 10 plots were randomly assigned to a nutrient or fencing treatment, but only a subset of four plots was used in the current study, each with a different treatment (see below; Supplementary Fig. S1a). All plots were 5 x 5 m and divided into four 2.5 x 2.5 m subplots (Supplementary Fig. S1b). Each subplot was further divided into four 1 x 1 m square sampling plots, one of which was set aside for soil sampling (Borer *et al.*, 2014; Supplementary Fig. S1b). Plots were separated by at least 1 m wide walkways.

In this study, we collected data from the following four treatments: (i) untreated control plots (Control), (ii) herbivore removal plots (Fence), (iii) plots fertilized with N, P, K, plus nine essential macro and micronutrients (NPK), and (iv) plots with simultaneous fertilizer addition and herbivore removal (NPK+Fence; Supplementary Fig. S1a). The number of years of treatment differed among sites (2 – 9 years since start of treatment; Supplementary Table S1). For the nutrient additions, all sites applied 10 g N m^{-2} yr^{-1} as time-release urea; 10 g P m^{-2} yr^{-1} as triple-super phosphate; 10 g K m^{-2} yr^{-1} as potassium sulfate. A micro-nutrient mix (Fe, S, Mg, Mn, Cu, Zn, B, Mo, Ca) was applied at 100 g m^{-2} together with K in the first year of treatments but not thereafter.

The vertebrate herbivore removal treatment (Fence) was established by fencing two plots, one control and one NPK plot, within each of the blocks (Supplementary Fig. S1a). We designed the fences so that they would effectively exclude aboveground mammalian herbivores with a body mass of over 50 g (Borer *et al.*, 2014). At the majority of sites, the height of the fences was 180 cm, and the fence design included wire mesh (1 cm holes) on the first 90 cm along with a 30 cm outward-facing flange stapled to the ground to exclude burrowing animals; climbing and subterranean animals may potentially still access these plots (Borer *et al.*, 2014). For slight modifications in fence design at a few sites see Supplementary Table S3. While most sites only had native herbivores, a few sites (4) were also grazed by domestic animals (Supplementary Table S1).

Potential and realized soil net N mineralization, ammonification, nitrification and other soil properties

Each site participating in the study received a package containing identical material from the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL) to be used for sampling and on-site N incubations. For the field incubation, we followed the protocol by Risch *et al.* (2015, 2019). Briefly, we drove a 5 x 15 cm (diameter x depth) steel cylinder 13.5 cm deep into the soil after clipping the vegetation at randomized locations in each plot. The top 1.5 cm of the cylinder remained empty to capture incoming N from run-off or deposition with a polyester mesh bag (mesh-size 250 μm) filled with 13.2 ± 0.9 g of acidic and alkaline exchanger resin (1:1 mixture;

ion-exchanger I KA/ion-exchanger III AA, Merck AG, Darmstadt). The bag was fixed in place with a metal Seeger ring (Bruetsch-Rüegger Holding, Urdorf, Switzerland). We then removed 1.5 cm soil at the bottom of the cylinder and placed another resin bag to capture N leached from the soil column. The exchange resin was saturated with H^+ and Cl^- prior to filling the bags by stirring the mixture in 1.2M HCl for 1 h and then rinsing it with demineralized water until the electrical conductivity of the water reached 5 μS/cm. The cylinders were then re-inserted into the cored hole, level with the soil surface, and incubated for an average of 42 days (range 40 to 57days). The individual site coordinators chose the timing of incubation to start approximately six weeks prior to peak plant biomass production. All incubations were completed between February 2015 and January 2016 accounting for differences in growing season between northern and southern hemispheres. At the end of the incubation, the cylinders were re-collected and immediately shipped to WSL in an insulated box together with cold packs to halt further mineralization. Gloves were worn at all times to avoid contamination of the samples. Upon arrival at WSL, we extracted the resin bags and a 20 g subsample of sieved soil (4 mm) separately in a 100 ml PE-bottle with 80 ml 1 M KCl for 1.5 h on an end-over-end shaker and filtered through ashless folded filter paper (DF 5895 150, ALBET LabScience). We measured NO_3^- (colorimetrically; Norman & Stucki, 1981) and NH_4^+concentrations (flow injection analysis; FIAS 300, Perkin Elmer) on these filtrates.

At the start of the field incubation, we additionally collected two soil cores of 5 x 12 cm (diameter x depth) in each sampling plot and composited them to measure potential soil net N_{min}, soil chemical and biological properties (see below). We also collected an additional sample (5 x 12 cm) to assess soil physical properties, which remained within the steel cylinder. Both ends were tightly closed with plastic caps. Cores were carefully packed to avoid further disturbance, and together with the composited soil samples, were shipped to the laboratory at WSL within a few days after collection.

From the composited samples, we extracted an equivalent of 20 g dry soil with KCl, as described above, and measured NO_3^- and NH_4^+concentrations. Realized soil net N_{min} was then calculated as the difference between the inorganic N content of samples collected at the end of the incubation (plus N extracted from the bottom resin bag) and the N content at the beginning of the incubation; values were scaled to represent daily mineralization rates (mg N kg^{-1}soil d^{-1}; Risch *et al.*, 2015). Realized soil net N_{min} values represent an average period of 42 days prior to peak biomass, typically the highest period of biological activity, and not the entire year (Risch *et al.*, 2019).

A second subsample of the composited sample was used to determine potential soil net N_{min} in the laboratory (Risch *et al.*, 2019). Briefly, we weighed duplicate samples (8 g dry soil) into 50-ml Falcon tubes. Soil moisture was brought to 60% of the field capacity of each plot, the Falcon tubes tightly closed and then incubated at 20°C for 42 days in a dark room. Every week the Falcon tubes were opened and ventilated. At the end of the incubation, the soil samples were extracted the same way as described above and NO_3^- and NH_4^+ was determined. Potential soil net N_{min} was calculated as the difference between the N content before and after the incubation and scaled to represent daily values (mg N kg^{-1}soil d^{-1}). Using our NO_3^- and NH_4^+ measures we also calculated potential and realized soil net nitrification and soil net ammonification to be able to better understand the drivers of fertilization and herbivore removal effects on potential and realized soil net N_{min}.

A third subsample of the composite soil sample was sieved (2 mm mesh) and microbial biomass (µg C_{mic} g^{-1} soil dry weight) was estimated by measuring the maximal respiratory response to the addition of glucose solution (4 mg glucose per g soil dry weight dissolved in distilled water; substrate-induced respiration method) on approximately 5.5 g of soil (Anderson & Domsch, 1978). The rest of the composited sample was dried at 65°C for 48 h, ground and sieved (2 mm mesh) to assess a series of soil chemical properties (Risch *et al.*, 2019). We measured the percentage of clay as an indicator of soil texture (Gee & Bauder, 1986; Risch *et al.*, 2019).

Statistical analyses

Potential and realized soil net N_{min} were square root transformed to account for a highly skewed data distribution ($y_{t\,=}$ sign(y)*sqrt|y|; negative values in the data set impeded log transformation). To assess treatment effects on potential and realized soil net N_{min}, we used linear mixed effects models (LMMs) fitted by maximum likelihood using the lme function from the nlme package (version 3.131.1; Pinheiro *et al.*, 2016), R version 3.6.1; R Foundation for Statistical Computing. Treatment (Control, Fence, NPK, NPK+Fence) was a fixed factor, with site and block as random factors, where block was nested within site. We also tested for effects of time since start of treatments in preliminary analyses by adding total treatment years as an additional fixed factor. We did not find a significant effect of years of treatment, and thus dropped this variable from the models. The LMMs were corrected using varIdent if the homogeneity of variance criterion was not met (Pinheiro *et al.*, 2016). To visualize our results, we calculated treatment effects using Cohens' d statistic (Cohen, 1977; Koricheva *et al.*, 2013). Note that calculating response ratios (or log response ratios) was not possible with our data, as we have both negative and positive values.

We also fitted LMMs for potential and realized soil net ammonification and nitrification to gain more insight into how global change affects the processes underpinning potential and realized soil net N_{min}. We also sqrt-transformed (see above) these dependent variables. Treatment was included as a fixed factor with random factors as described above. In addition, we assessed how potential and realized soil net N_{min} were related to potential and realized soil net ammonification and nitrification, respectively. For this, we calculated site averages for each treatment separately. We then ran LMMs, with potential and realized soil net N_{min} as the dependent variable, potential and realized soil net ammonification/nitrification as the independent ones. Site was included as a random factor.

Based on our previous work (Risch *et al.*, 2019) and the existing literature (Schimel & Bennett, 2004; Liu *et al.*, 2017), we developed *a priori* causal conceptual models of relationships among treatments, environmental drivers, and potential and realized soil net N_{min} (Supplementary Fig. S2) to test with structural equation modelling (SEM) using a *d-sep* approach (Shipley, 2009; Lefcheck, 2016). The variables included in the model were long-term climatic conditions, specifically, site-level mean annual precipitation (MAP) and temperature of the wettest quarter (T.q.wet), plot-level soil texture (clay content) and soil microbial biomass. Mean annual precipitation and T.q.wet were obtained from WorldClim (Hijmans *et al.*, 2005) (http://www.worldclim.org/) and together with the experimental treatments were predicted to directly affect soil properties and soil net N_{min} (Supplementary Fig. S2). Soil clay content was, in turn, predicted to affect microbial biomass and soil net N_{min}. Because we determined microbial biomass prior to incubating the samples in the laboratory or field, we assumed that the abundance of these microbes would be responsible for N process rates and not *vice versa* (Supplementary Fig. S2). We tested our conceptual model (Supplementary Fig. S2) using the piecewiseSEM package (version 2.0.2; Lefcheck, 2016) in R 3.4.0, in which a structured set of linear models are fitted individually. This approach allowed us to account for the nested experimental design, and overcome some of the limitations of standard structural equation models, such as small sample sizes (Shipley, 2009; Lefcheck, 2016). We used the lme function of the nlme package to model response variables, including site as a random factor. Good fit of the SEM was assumed when Fisher's C values were non-significant ($p > 0.05$). For all significant interactions between covariates and experimental treatments detected in the SEMs, we calculated treatment effect sizes, i.e. the differences in potential or realized soil net N_{min} between Control and treatments (Fence, NPK, NPK+Fence) and plotted these values against the climate or soil covariates. Finally, we fitted LMMs for the soil variables included in our SEMs, with treatment as the fixed factor, and with site and block as random factors, where block was nested within site.

Results

As predicted, our treatments increased potential soil net N_{min}. Mineralization was higher in fertilized plots, both with mammalian herbivores present (NPK: +34% on average across all sites) and with herbivores excluded (NPK+Fence: +66%), but there was no response to herbivore removal alone (Fence; Fig. 2a,b, Supplementary Table S4). In contrast and counter to our hypothesis, realized soil net N_{min} was significantly lower in fertilized plots where herbivores were excluded compared to control plots (NPK+Fence: -42%, Fig. 2a, Supplementary Table S4). However, herbivore removal (Fence) and fertilization (NPK) on their own did not lead to any discernable difference in realized soil net N_{min} compared to the control plots (Fig. 2a,c, Supplementary Table S4). The fertilization and fencing treatments led to greater variability (standard deviation [SD]), compared to control plots, in both potential (Control: 0.33 (SD); Fence: 0.49; NPK: 0.63; NPK+Fence: 0.56) and realized (Control: 0.28; Fence: 0.41; NPK: 0.75; NPK+Fence: 0.77) soil net N_{min} across the 22 global grassland sites (see also Fig. 2b,c, Supplementary Fig. S3).

Potential soil net ammonification had a negative but non-significant response to the NPK+Fence treatment (-29%), whereas realized soil net ammonification was significantly lower in fertilized treatments (NPK: -44%, NPK+Fence: -61%) compared to the control plots (Fig. 2d-f, Supplementary Table S4). In contrast, potential soil net nitrification was significantly higher in both fertilized plots compared to the control plots (NPK: +62%; NPK+Fence: +71%), whereas realized soil net nitrification remained unaffected by our treatments (Fig. 2g-j, Supplementary Table S4). Potential soil net N_{min} was higher at sites with higher potential nitrification (Supplementary Fig. S4a,b), while realized soil net N_{min} was higher at sites where both realized soil net ammonification and nitrification were high (Supplementary Fig. S4c,d). Soil clay content and microbial biomass did not differ among our treatments (Supplementary Fig. S5).

Our SEM explained 19% (marginal R^2) of the variability in potential soil net N_{min} across our grasslands and showed that potential soil net N_{min} increased directly in response to fertilization, independent of herbivore presence (Fig. 3a), in line with the results of our LMM (Fig. 2a). However, fertilization in combination with herbivore removal (NPK+Fence) had a larger positive effect on potential soil net N_{min} at sites with higher MAP (Fig. 3a, Fig. 4a). Similarly, we found larger increases in potential soil net N_{min} when nutrients were added at sites with higher T.q.wet, and this was not modified by presence of herbivores (NPK, NPK+Fence; Fig. 3a, Fig. 4b). Sites with higher MAP also had higher microbial biomass, which directly and positively affected potential soil net N_{min} (Fig. 3a).

Our SEM for realized soil net N_{min} explained 33% (marginal R^2) of the variability in this measure across our grasslands (Fig. 3b). Fertilization with herbivore removal directly and negatively affected realized soil net N_{min}, also in line with our LMM results (Fig. 2b). However, we found that the negative joint effect of fertilization with herbivore removal (NPK+Fence) on realized soil net N_{min} was larger at sites with lower MAP and higher T.q.wet (Fig. 4d,e). Moreover, the decline of realized soil net N_{min} with nutrient additions in the presence of herbivores (NPK) was conditional to sites with higher T.q.wet and more clay in the soil (Fig. 3b, Fig. 4d,e). Site-dependent decreases in realized soil net N_{min} in response to herbivore removal regardless of nutrient additions (Fence, NPK+Fence) were only evident at sites with greater microbial biomass (Fig. 3b, Fig. 4f). Sites with higher clay content and higher MAP had higher soil microbial biomass (Fig. 3b).

Discussion

In this study, we did not detect any differences in potential or realized soil net N_{min} when herbivores were removed from global grasslands in the absence of fertilization. However, fertilization led to consistently higher potential soil net N_{min}, either individually (NPK), or when combined with herbivore removal (NPK+Fence). In contrast, and counter to our expectations, realized soil net N_{min}, was significantly lower compared to the control plots when we simultaneously added fertilizer and removed herbivores (NPK+Fence). This was surprising, as we expected increases in both potential and realized soil net N_{min} with our treatments, although with lower values for realized soil net N_{min}. We discuss potential reasons for these findings below. Moreover, despite the overall patterns in treatment response in potential and realized soil net N_{min}, we found that site-specific differences in soil and climatic properties strongly influenced how fertilization and herbivore removal affected both potential and realized soil net N_{min}. This context-dependence, together with the increased variability in both potential and realized soil net N_{min}

caused by our treatments, could explain the previous lack of consensus over the direction of grassland N mineralization responses to fertilizer additions and herbivore removal.

Herbivores alone do not affect potential and realized soil net N_{min} across global grasslands

The lack of a clear-cut response in soil net N_{min} to herbivore removal, in the absence of fertilization, is consistent with a study conducted in the Swiss Alps, where the removal of large ungulates did not affect soil net N_{min} (Risch *et al.*, 2015). However, it contrasts with other findings where higher (Frank & Groffmann, 1998; Bakker *et al.*, 2004; Zhou *et al.*, 2017) or lower (Wang *et al.*, 2020) soil net N_{min} were detected in grazed compared to ungrazed grasslands. In our study, we only found a reduction in realized soil net N_{min} with herbivore removal at sites with higher microbial biomass; however, this context-dependent response was not strong enough to allow for a clear overall pattern to emerge across sites. Apart from microbial biomass, differences in population densities, stocking rates, or composition of herbivore communities that result in different grazing intensities may also contribute towards explaining our variable responses, but we lacked this information in our study. A recent meta-analysis, which included mixed data for both potential and realized soil net N_{min}, showed, however, that moderate grazing led to significantly higher soil net N_{min} compared to ungrazed plots across global grasslands, whereas light and heavy grazing may had no effect (Zhou *et al.*, 2017). Similarly, the removal of large ungulates alone did not affect realized soil net N_{min} in the Swiss Alps, but when all mammalian herbivores were excluded and only invertebrates were present, realized soil net N_{min} increased (Risch *et al.*, 2015). In a Dutch grassland, only the removal of cattle led to increases in realized soil net N_{min}, while the additional removal of rabbits and voles did not lead to further changes (Bakker *et al.*, 2004). These studies highlight the importance of considering the functional diversity of the excluded herbivores (Wang *et al.*, 2019). Finally, time since treatment implementation, i.e., establishing the fences, may potentially explain some variability in the response of grassland mineralization to herbivore removal (Frank & Groffmann, 1998; Bakker *et al.*, 2004; Risch *et al.*, 2015; Wang *et al.*, 2020), although we did not find any statistical evidence for this in our study.

Nutrient addition alone and with herbivore removal affects potential and realized soil net N_{min}

We found higher potential soil net N_{min} in both fertilized treatments (NPK, NPK+Fence) compared to the control plots. We are not aware of other studies that have assessed how potential soil net N_{min} responded to NPK fertilizer additions, but N additions have been shown to increase potential soil net N_{min} in a semi-arid grassland with loamy-sand soils (Chen *et al.*, 2019a). Our findings suggest that in our fertilized treatments, soil microbes, either generally or certain microbial groups specifically, consistently increased their activity under the standardized and optimized conditions in the laboratory, particularly for samples collected from sites with higher MAP and higher T.q.wet. This context-dependence on long-term climatic conditions may, in turn, be mediated by greater plant productivity and soil microbial biomass at wetter and warmer sites leading to a greater pool of readily mineralizable soil organic N. This finding is similar to what has been shown in the Mongolian steppe, where fertilization increased potential soil net N_{min} under both moderate and heavy grazing, but with additional irrigation, potential soil net N_{min} was only higher at the moderately grazed sites (Chen *et al.*, 2018).

In contrast to findings for potential soil net N_{min}, fertilization alone (NPK) did not alter realized soil net N_{min} across our global grasslands, which is similar to results reported from several site-specific fertilization experiments (Mueller *et al.*, 2013; Wei *et al.*, 2017). Fertilization in combination with herbivore removal (NPK+Fence) did, however, lead to a decrease in soil net N_{min}, which was unexpected, yet similar to findings from a Californian serpentine grassland study (Esch *et al.*, 2013). This decrease might be due to the strong context-dependence in the response of realized soil net N_{min} to our nutrient additions, with or without herbivores. We found that fertilization led to lower realized mineralization rates at sites with higher soil clay content (NPK) and higher T.q.wet (NPK, NPK+Fence). The treatment interaction with soil clay content could potentially be attributed to the fact that our treatments disrupted organo-mineral interactions within the soil matrix (Zhao *et al.*, 2020). Moreover, given that higher soil clay content was generally associated with higher microbial biomass, our findings may also suggest that microbial communities were downregulating the ‘mining’ for nutrients from soil organic matter and released less mineral nutrients to the soil system when limiting nutrients were added (Dijkstra *et al.*, 2013). Consistent with these findings, a recent meta-analysis including field-based studies from different terrestrial biomes found that microbial extracellular enzyme activities were downregulated by application rates of 100 kg N ha^{-1} y^{-1} (Jia *et al.*, 2020). Yet, four years of adding N and P fertilizer had no effect on enzyme activities related to N cycling in three different Chinese grasslands (Chen *et al.*, 2019b). Similarly, fertilization and herbivore removal individually or in combination did not affect microbial extracellular enzyme activities in a Californian grassland (Esch *et al.*, 2013), further emphasizing the variability of results found across studies.

Differences in the response of potential and realized soil net N_{min} to nutrient additions and herbivore removal

In contrast to our expectations, our treatments only led to increases in soil net N_{min} in the laboratory under standardized, optimal conditions, but not in the field, where our treatments led to a reduction in realized soil net N_{min}. This shows that laboratory measures, although useful to explore and understand soil processes under standardized conditions, do not allow us to anticipate what happens under ambient conditions in the field, as already previously suggested by other studies (Arnold *et al.*, 2008; Risch *et al.*, 2019). Hence, to predict and estimate how global change drivers such as biodiversity loss, fertilization and climate change, alter the rates of N mineralization in grassland ecosystems, it is important to measure soil N processes in the field and not in the laboratory.

Mechanistically, the differences in the response of potential and realized soil net N_{min} to our treatments may be attributed to a combination of sample preparation (mixing, sieving, removing roots) and a shift in the composition or activity of different microbial groups under optimal versus ambient conditions. In the field, dead roots remained in the incubated soil cores while the roots were removed for the laboratory incubations. Hence, more labile C was available in the field, which is known to increase N immobilization and decrease soil net N_{min} (Hook & Burke, 1995; Knops *et al.*, 2002). Similarly, soil preparation (mixing, sieving) for determining potential soil net N_{min} makes formerly protected soil organic matter available. Fine aggregates have been shown to have much higher potential N mineralization than coarse aggregates (Bimüller *et al.*, 2016) or undisturbed soils (Hassink, 1992). In addition, soil microbial communities associated with different sized soil aggregates have been shown to interact differently with NPK fertilizer (Liao *et al.*, 2018).

Further, we found higher potential soil net nitrification with fertilization (regardless of presence or absence of herbivores), while there was only a weak effect of NPK+Fence on potential soil net ammonification. In contrast, realized soil net nitrification remained unaffected by our treatments. This indicates that nitrifiers were likely more active in the laboratory when limiting nutrients were added as indicated by much higher nitrification rates under fertilized conditions compared to the field. Hence, more NO_3-N was processed when fertilized, increasing potential soil net N_{min}. In line with these findings, potential soil net nitrification was higher in NPK fertilized agricultural soil under maize compared to the control sites (Li *et al.*, 2019). The authors explained this enhanced potential net nitrification with a higher abundance of ammonia oxidizing bacteria and archaea (nitrifying microbes). Similarly, the decrease of realized soil net N_{min} in response to fertilizer addition in our study could be due to the lower activity of ammonifiers, as we found lower realized soil net ammonification when fertilizer was added (regardless of herbivore presence/absence), but no change in realized soil net nitrification. Consequently, less NH_4-N was released, which ultimately led to lower realized soil net N_{min}. Unfortunately, we were not able to find any studies that assessed how fertilization or grazing affect the relationship between the activities of ammonifiers/nitrifiers and net ammonification/nitrification, and how this would feed back to potential and realized soil net N_{min} to compare with our result. Further studies are, therefore, needed to evaluate the role of different microbial taxa in regulating soil organic matter processing and nutrient cycling under different management regimes, which may allow for the identification of specific communities that function better under particular conditions.

Finally, across our grasslands, the response of both potential and realized soil net N_{min} to fertilization with herbivore removal (NPK+Fence), and potential soil net N_{min} to fertilization alone (NPK), depended on MAP and T.q.wet. Thus, expected future alterations in global precipitation regimes (Fischer & Knutti, 2014) due to global climate change will likely have a strong impact on grassland soil net N_{min} in combination with different grassland management regimes, as shown by Chen *et al.* (2018). Similarly, N additions in combination with increases in soil water availability can have more consistent positive effects on nitrogen-mineralizing enzyme activities than the two factors in isolation (Tian *et al.*, 2017).

Conclusions

Our study provides strong evidence that human activities impact the capacity of grassland ecosystems to provide key ecosystem functions such as soil net N_{min}. We show that a nutrient-enriched, herbivore-impoverished, and climatically more variable world, will have negative consequences for the ability of soil communities to mineralize N under realistic field conditions. In the long-term, this might lead to a reduced functional ability of grasslands to mineralize soil N, making them increasingly dependent on external nutrient inputs. Thus, our findings strongly support farmers and land managers advocating to move away from high input agriculture and promote a more sustainable management of grassland ecosystems and their soils. Moreover, our results show under which environmental conditions, fertilization and herbivore removal cause the strongest negative effects on soil nutrient cycling and which may thus require particular attention.

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Figure captions

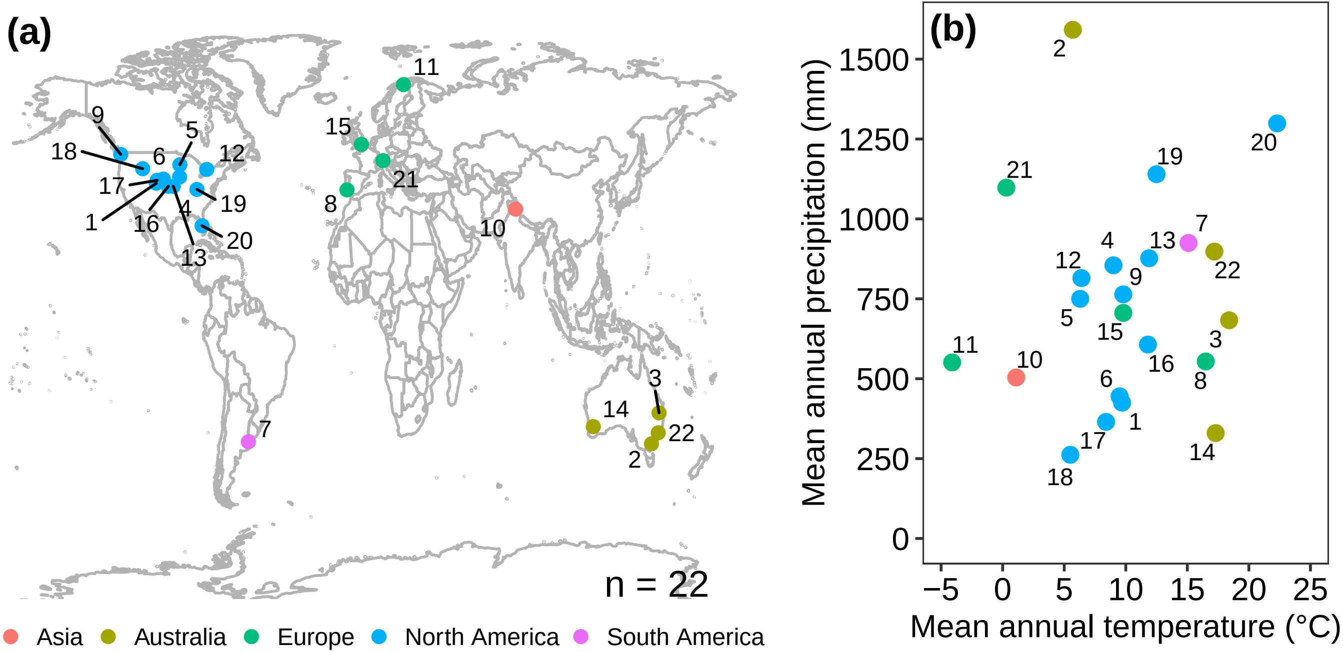
Fig. 1. Geographic and climatic distribution of experimental sites. (a) Location of the 22 NutNet sites where the field experiment was conducted and soil samples were collected for laboratory analyses. (b) The 22 study sites represent a wide range of mean annual temperature (MAT) and mean annual precipitation (MAP) conditions. Our sites also cover a wide range of soil edaphic conditions as described in the main text and shown in Supplementary Table S2. Numbers refer to # in Supplementary Table S1 & S2.

Fig. 2: Treatment effects on potential and realized soil net N mineralization across 22 grasslands globally. Potential and realized soil net N_{min} (a)-(c), soil net ammonification (d)-(f) and soil net nitrification (g)-(j). Treatment effect size presented as Cohens' d in (a), (d) and (g). Significant treatment effects are indicated with *. (b), (c), (e), (f), (h), (j) Boxplots of raw data. They show tge median (50th percentile), 25th and 75th percentile of the data across sites. Individual measures are shown in the background. Control = control plots, Fence = herbivores removed, NPK = fertilized with N, P, K and micronutrients, NPK+Fence = fertilized with N, P, K and micronutrients and herbivores removed. Potential and realized soil net N_{min}, net ammonification and nitrification values were square-root transformed.

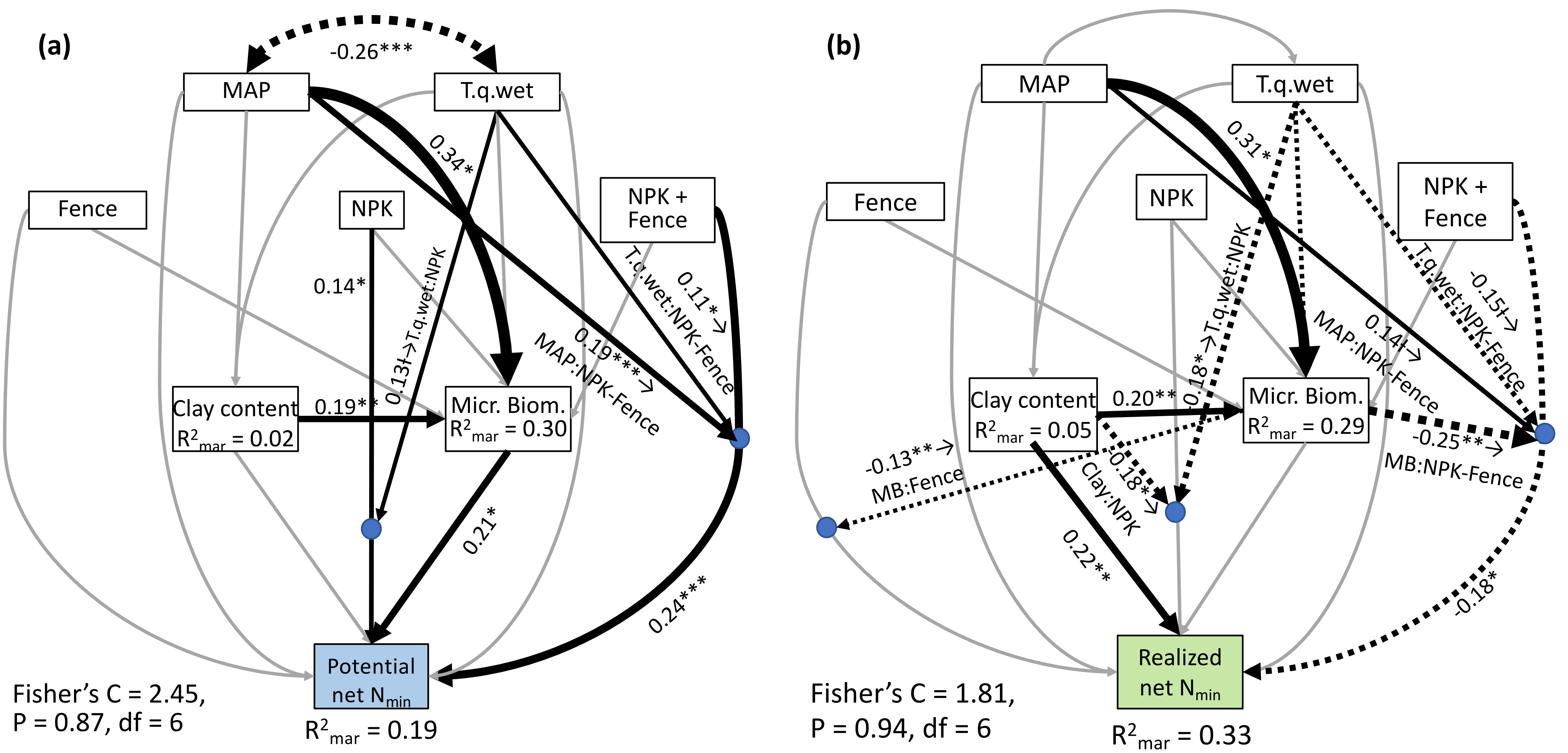
Fig. 3: Influence of local environmental conditions on the response of potential and realized soil net N_{min} to herbivore removal and fertilization. Structural equation model diagram representing connections between treatment, climatic conditions and soil properties found to influence (a) potential soil net N_{min} and (b) realized soil net N_{min}. The width of the connections represents estimates of the standardized path coefficients, with solid lines representing a positive relationship and dashed lines a negative relationship. Interaction effects are depicted with arrows pointing to solid blue dots. Significant connections and R^2 are shown in black, non-significant ones in light-grey. †$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. MAP = mean annual precipitation, T.q.wet = temperature of the wettest quarter, Treatments: Control = control plots, Fence = herbivores removed, NPK = fertilized with N, P, K and micronutrients, NPK+Fence = fertilized with N, P, K and micronutrients and herbivores removed, Clay content = soil clay content, Micr. Biom. = soil microbial biomass, total number of observations for potential soil net

N_{min} = 244, total number of observations for realized soil net N_{min}. = 256, total number of sites for potential soil net N_{min} = 21, total number of sites for realized soil net N_{min} = 22. Potential and realized soil net N_{min} values are square-root transformed.

Fig. 4: Treatment effects on soil net N_{min} in relation to site-specific differences in climate or soil properties for interactions in the SEMs. Relationships for potential soil net N_{min} (a), (b), and realized soil net N_{min} (c), (d), (e), (f). Control = control plots, Fence = herbivores removed, NPK = fertilized with N, P, K and micronutrients, NPK+Fence = fertilized with N, P, K and micronutrients and herbivores removed. Potential and realized soil net N_{min} values are square-root transformed. Treatment effects were calculated as the difference between values of control and treatment (Fence, NPK, NPK+Fence) plots. Note that only the relationships marked with blue dots in Fig. 3 are significant, but all relationships are presented here to facilitate comparisons.


(a)
9
18
6
5
12
17
1
16
4
19
20
13
11
15
21
8
10
3
14
22
2
7
n = 22
● Asia
● Australia
● Europe
● North America
● South America
(b)
Mean annual precipitation (mm)
1500
1250
1000
750
500
250
0
−5
0
5
10
15
20
25
Mean annual temperature (°C)
2
20
19
21
7
4
13
22
12
9
5
15
3
11
10
16
8
6
1
17
14
18


Soil net N mineralization
Potential: $F_{3,\,179} = 6.57$; $P < 0.001$
Realized: $F_{3,\,189} = 2.73$; $P = 0.045$
(a)
Effect size (Cohen's d)
1.0
0.5
0.0
-0.5
-1.0
*
*
*
Potential
Realized
Fence
NPK
NPK+Fence
Treatment
Soil net N mineralization
(b)
Net N min (sqrt mg N kg soil^{-1}day^{-1})
2
1
0
-1
-2
(c)
Net N min (sqrt mg N kg soil^{-1}day^{-1})
Control
Fence
NPK
NPK+Fence
Soil net ammonification
Potential: $F_{3,\,179} = 2.38$; $P = 0.071$
Realized: $F_{3,\,189} = 5.62$; $P < 0.001$
(d)
Effect size (Cohen's d)
*
*
*
Fence
NPK
NPK+Fence
Treatment
Soil net ammonification
(e)
Net ammon. (sqrt mg N kg soil^{-1}day^{-1})
2
0
-2
(f)
Net ammon. (sqrt mg N kg soil^{-1}day^{-1})
Control
Fence
NPK
NPK+Fence
Soil net nitrification
(g)
Effect size (Cohen's d)
*
*
Potential: $F_{3,\,179} = 14.63$; $P < 0.001$
Realized: $F_{3,\,189} = 0.23$; $P = 0.877$
Fence
NPK
NPK+Fence
Treatment
Soil net nitrification
(h)
Net nitr. (sqrt mg N kg soil^{-1}day^{-1})
3
2
1
0
(j)
Net nitr. (sqrt mg N kg soil^{-1}day^{-1})
3
2
1
0
-1
Control
Fence
NPK
NPK+Fence


(a)
-0.26***
MAP
T.q.wet
Fence
NPK
NPK + Fence
0.34*
0.14*
0.13†→T.q.wet:NPK
0.11*→ T.q.wet:NPK-Fence
0.19***→ MAP:NPK-Fence
Clay content
R²mar = 0.02
0.19**
Micr. Biom.
R²mar = 0.30
0.21*
0.24***
Potential net Nmin
R²mar = 0.19
Fisher's C = 2.45,
P = 0.87, df = 6
(b)
MAP
T.q.wet
Fence
NPK
NPK + Fence
0.31*
0.18*→T.q.wet:NPK
T.q.wet:NPK-Fence
-0.15†→
0.14†→ MAP:NPK-Fence
Clay content
R²mar = 0.05
0.20**
Micr. Biom.
R²mar = 0.29
-0.13**→ MB:Fence
-0.18*→ Clay:NPK
-0.25**→ MB:NPK-Fence
0.22**
-0.18*
Realized net Nmin
R²mar = 0.33
Fisher's C = 1.81,
P = 0.94, df = 6


Potential
Potential
Realized
Realized
Realized
Realized
Fence
NPK
NPK+Fence
(a)
(b)
(c)
(d)
(e)
(f)
Treatment effect on soil Net N mineralization (mg N kg soil^{-1}day^{-1})
Mean annual precipitation (mm)
Temperature of the wettest quarter (°C)
Soil clay content (%)
Microbial biomass (g^{-1} soil)
400
800
1200
1600
0
10
20
30
500
1000
-2
-1
0
1
2
4