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OIKOS

Research article

Early spring snowmelt and summer droughts strongly impair the resilience of bacterial community and N cycling functions in a subalpine grassland ecosystem

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Subalpine grasslands support biodiversity, agriculture, and tourism but their resilience to extreme climatic events is challenged accelerating their vulnerability to tipping points. Microbial communities, central in ecosystem functioning, are usually considered more resistant and highly resilient to extreme events albeit their functional redundancy and strong selection by local harsh climatic conditions. This study explored the soil microbial responses upon recurrent spring-summer droughts associated with early snowmelt in subalpine grasslands mesocosms set-up at the Lautaret Pass (French Alps). Potential soil microbial respiration, nitrification and denitrification activities were monitored over a period of two growing seasons along with quantification of related gene abundances. Impacts of simulated spring-summer drought and early snowmelt were quantified to assess their resistance and recovery. Results revealed that droughts had a low and short-term adverse impact on bacterial total respiration supporting their hypothesized high resilience, i.e. resistance and ability to recover. Nitrification and abundances of the corresponding functional guilds showed relatively strong resistance to summer droughts but declined in response to early snowmelt. This resistance of nitrification was paralleled by the recovery of denitrification and abundances of denitrifying communities from all climatic extremes, except from the summer droughts where nitrifiers were collapsed. Denitrification and respective functional groups faced high impact of applied stresses with strong reduction in abundance and activity. Although, consequently lower denitrifiers' competition for nitrate may be positive for plant biomass production, warnings exist when considering the potential nitrate leaching as well as risks of greenhouses gases emission such as N_2O from these ecosystems.

Keywords: climate change, (de)nitrification, grasslands, N_2O, snowmelt, weather extremes

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Introduction

Subalpine grassland ecosystems are important for biodiversity, agriculture, and a wide range of key ecosystem services (Tappeiner et al. 2008, Grêt-Regamey et al. 2012, Zoderer et al. 2016, Pecher et al. 2017). They are also under extreme pressure of climatic changes drastically affecting their functioning, e.g. primary productivity, nutrient cycling etc. (Berauer et al. 2019, Bernard et al. 2019). Considering global changes (Engler et al. 2011, Schirpke et al. 2013, Berauer et al. 2019), and importance of conserving ecosystems functions (Donohue et al. 2016), it is crucial to investigate whether subalpine grasslands are highly vulnerable, have attained over time an altered recovery state (Seabloom et al. 2020), or have developed resilience which comprises resistance: i.e. ability to maintain the existing state (Wipf and Rixen 2010, Bernard et al. 2019), and recovery: i.e. to recover from disturbances (Hodgson et al. 2015, Ingrisch et al. 2018, Karlowsky et al. 2018).

The effects of recurring climatic extremes on microbial functioning in these grasslands need to be assessed as they may shift the fate of particular functions especially when resilience limits are crossed and tipping points are reached, and where a potentially irreversible change may occur or is already occurring sensu Schnellnhuber et al. (2006). These estimations are crucial and possibly protect the stability of ecosystems and inhabiting species (de Vries and Shade 2013, Griffiths and Philippot 2013). Where weather extremes such as droughts are predicted to be severe (Karlowsky et al. 2018, Fuchslueger et al. 2019), alpine and subalpine grasslands are also expected to face less snow falls which ultimately lead to subsequent droughts (Gobiet et al. 2014, Klein et al. 2016). However, their vulnerability to collapse or support a particular function towards the combined (cumulative) impact of weather extremes is poorly documented.

As key players in ecosystem functioning (Bardgett and van der Putten 2014, Pommier et al. 2018, Chen et al. 2020), soil microbial communities respond strongly to environmental variability (Piton et al. 2020). They can thus be used as early indicators of disruption of ecosystem functional stability (Griffiths et al. 2000, de Vries and Shade 2013). However, because they are highly diverse and functionally redundant across environments, soil microbes are usually expected to be highly resilient to climatic fluctuations albeit the strong selection under local harsh climatic conditions (Griffiths and Philippot 2013, Benot et al. 2014, Capdeville et al. 2019). Despite these yet known inherent capabilities, whether these microbial communities can withstand correlated acceleration of weather extremes is largely unknown. Especially, assessment of the functional boundaries of the subalpine grasslands is critical since they are susceptible to extreme events (Bernard et al. 2019).

Here we determined the short-term effect of drought, snow removal, and their additive (recurrent) effect on N-cycling activities and abundances of involved functional guilds and following responses, to measure the resistance and recovery, respectively. Since microbes, driving broad (e.g. respiration) and specialized soil processes (e.g. (de)nitrification), may respond differentially to various perturbations (Wertz et al. 2007, Chaer et al. 2009, Jusselme et al. 2016, Dai et al. 2020), we conducted our work at these distinct functional scales. Such distinction allows evaluating the impact of stress (i.e. resistance) and recovery of microbial community abundances and their activity.

We tested how total bacterial community (16S *rRNA*) and related substrate-induced respiration (SIR) were influenced under droughts, snow removal and their cumulative impact and how much they recovered. In parallel, we investigated nitrogen (N) cycling that is critical in ecosystem sustainability (Kuypers et al. 2018, Chen et al. 2020), by 1) determining the relative abundances of nitrifiers (*nxrA*, *NS*; involved in nitrite oxidation step), and of denitrifiers (*nirK*, *nirS*, *nosZ*; involved in nitric and nitrous oxide reduction to dinitrogen); and 2) measuring potential enzyme activities (nitrification and denitrification enzyme activities (DEA) – NEA and DEA, respectively) since they carry implications for both primary productivity (loss of N – an important plant nutrient) and environment (N_2O emissions-global warming) (Galloway et al. 2004, Dai et al. 2020).

The current work was carried out on functionally contrasted and assembled grasslands set up in a common garden at Lautaret Pass (see full description in Bernard et al. 2019) with several sampling campaigns during two growing seasons. Based on an assumed functional redundancy (Griffiths and Philippot 2013, Jia and Whalen 2020), we hypothesized that broad scale function (i.e. SIR) and gene abundances of total bacterial community (i.e. *16S rRNA*) should resist and/or recover once the stresses/extreme events were over. SIR, being representative of broad scale function, was expected to be influenced by soil moisture and abundance of *16S rRNA* (Hallin et al. 2009). However, more specific functions of N-cycling such as (de)nitrification (DEA and NEA) should be more sensitive because we expected comparatively less functional redundancy and, despite being able to resist to single stress, the combined effects of earlier snow removal and droughts would trigger a tipping point, disturbing various steps of these processes. Moreover, any shift in soil moisture, substrate availability and abundance of denitrifiers may strongly impact DEA (Philippot et al. 2007), both under drought and no drought, while NEA might be sensitive to ammonium availability (Jia and Conrad 2009) and snow removal (Jusselme et al. 2016). To address these questions, we quantified the immediate responses of studied microbial communities' abundances and processes to droughts and early snowmelt extreme events to explore the resistance and their following recovery across functionally contrasted and assembled subalpine grasslands.

Material and methods

Experimental site, mesocosms and treatments' implementation

The study was set up at Lautaret pass proximity in southeast France (2100 m a.s.l., 45°02′13″N, 6°24′01″E) with

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a multifactorial experimental design where 128 mesocosms (n = 128), 50 cm width and 40 cm depth, were installed in a common garden beside subalpine grasslands. Mesocosms were filled with previously sieved (5 mm) in situ soil and planted with varying relative abundances of four species ranging along a plant economics spectrum (for further detail on plant treatments see Bernard et al. 2019). The assumption was that the varying plant functional diversity may increase the resilience of grassland functioning to climate manipulation. Species richness and functional composition remained stable throughout the whole experimentation due to low plant mortality.

No snow removal (NSR) and snow removal (SR) treatments (n = 64 + 64) were performed and, for each of the NSR and SR, there was an additional (sub) climate change treatment, i.e. maintained average soil moisture (ND) or drought (D) (n = 32 + 32). Treatments receiving no snow removal with maintained average soil moisture were named as control (CTRL). All the 128 mesocosms were installed inside eight blocks to build a multifactorial experimental design. Within each of the eight blocks with or without snow removal, there were two sub-blocks either maintained at long-term average soil moisture or subjected to summer drought. Within each sub-block, eight mesocosms were installed, and four were submitted to plant cuttings and fertilization (CF) (following farmers' practices) before the 2nd drought, while the other half remained untreated (NCF) (n = 16 + 16). Slow-release fertilizer was applied after the snow removal treatment and before the 2nd drought as pellets in the CF mesocosms (150 kg N ha^{-1}, it was slow NPK release fertilizer (13–13-13 + 2 MgO + oligoelements). Mowing was mimicked by clipping the vegetation 10 cm above the soil surface once during the 2nd drought (Fig. 1).

To simulate early snowmelt, snow cover was removed one month earlier than the average snowmelt period which ultimately simulated a shorter snow-covered period followed by an early spring drought. The drought treatment was implemented using rainout shelters during spring, summer and fall. These shelters were thus in use during the whole vegetation season and all mesocosms were watered manually to maintain long-term average soil moisture. This manual watering was discontinued to achieve drought that reduced soil moisture for more than one month in August–September 2013 and June–July 2014. The drought was for 4–8 weeks during the growing season without water addition.

Soil sampling campaigns

Five sampling campaigns in total were carried out at different time intervals including June 2013 (T0), September 2013 (T1), May 2014 (T2), July 2014 (T3) and September 2014 (T4). 1st and 2nd D were applied in August–September 2013 and June–July 2014 respectively (Fig. 1). The SR treatment was applied before T2 sampling campaign. Resistance to 1st D of the microbial communities was measured at T1, resistance to 1st SR and recovery from 1st D at T2, resistance to 2nd D and recovery from 1st SR at T3; and recovery from overall D and SR treatments was evaluated at T4 (Fig. 1). At T0, no difference in the microbial parameters investigated here was observed, and control was already placed at each date – T1–T4, so we chose to compare only these. During all sampling campaigns composite soil samples (five cores mixed together, 0–10 cm depth) were collected from all 128 mesocosms at five time points (128 × 5: n = 640). Fresh soil samples were sieved at 2 mm, homogenized, and brought to the laboratory to measure the enzyme activities in fresh soil while a sub-sample was stored at −20°C for subsequent DNA extraction. Soil properties including soil moisture content, SOM% (soil organic matter), soil pH, soil %C, soil %N, total dissolved nitrogen-TDN, and the dissolved organic nitrogen-DON, soil NH_4^+, and soil NO_3^- were also measured in an

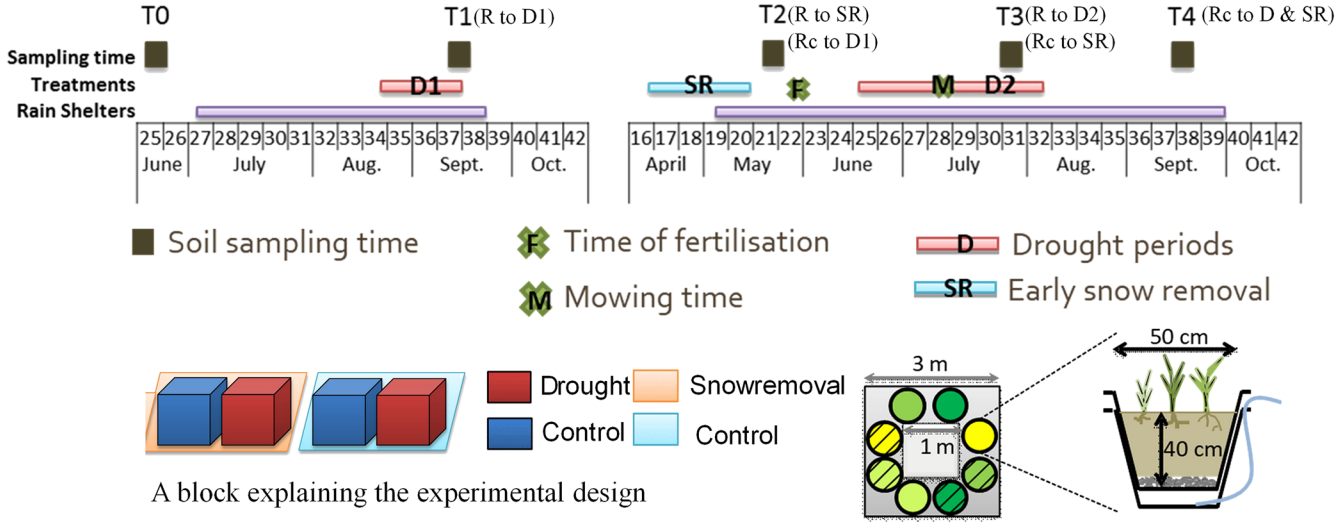

Figure 1. Experimental timeline showing the implementation of various treatments as well as the sampling dates. R and Rc represent resistance and recovery respectively. F and M in green represent the fertilization and mowing, respectively. D in red and SR in sky color indicate drought and snow removal, respectively.

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associated work with focus on the effects of local environmental changes on subalpine grasslands functioning (Bernard 2017). Specifically, subsamples of 5 g fresh soil were dried at 70°C for 1 week to determine soil moisture content (in g g^{-1} dw calculated as the 70°C dry soil weight relative to the fresh mass), followed by 4 h at 550°C to determine SOM% (calculated as the 550°C soil weight relative to the 70°C dry mass). Ten mg soil subsample were air dried, ground to powder and analyzed for soil total C and N concentrations using a C/N elemental analyzer. Soil pH was measured in a 1 : 4 (air dry soil/distilled water) solution. Soil N-NH_4^+ and N-NO_3^-, N-TDN and N-DON were determined in separated 10 mg fresh soil subsamples extracts (K_2SO_4, 0.5 M) using an FS-IV colorimetric chain (OI-Analytical) (Jones and Willett 2006).

Soil DNA extractions and quantifications

DNA was extracted from soil stored at −20°C using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc.) and DNA concentration was determined by using the Quant-iTTM PicoGreen dsDNA Assay Kit (Invitrogen).

Abundances of total bacteria and N-cycling related bacteria

The abundance of the total bacterial communities was estimated in all DNA extracts using a 16S rRNA primer-based quantitative PCR (qPCR) assays as described by Lane (1991). Final reaction volume was 20 µl, with 300 nM of forward and reverse primers; 1X LightCycler DNA Master Mix SYBR Green I (Roche Diagnostics); 1 ng of sample DNA or 10^8–10^2 copies of the standard DNA pQuantA-lb16S plasmid (Zouache et al. 2012). The abundances of the genes *NS, nxr*A, *nir*S, *nir*K *and nos*Z were estimated using the primers developed previously (Henry et al. 2004, 2006, Kandeler et al. 2006, Attard et al. 2011). The total volume of the reaction was made 20 µl for *nxr*A and *nir*K each, with concentration of 500 nM for *nxr*A while 1 µM for *nir*K for both forward as well as reverse primers; 10 µl of the SYBR Green I kit was used for the *nxr*A and *nir*K, and 0.4 µg of T4 protein (Qbiogene) was used for *nir*K. The total volume of the reaction was made 25 µl for *NS*, *nir*S and *nos*Z, with concentration of 400 nM for *NS* while 1 µM for *nir*S and *nos*Z both for forward as well as reverse primers; 12.5 µl of the SYBR Green I kit was used for the *NS*, *nir*S and *nos*Z; for *nir*S the T4 protein was 0.5 µg while it was 0.4 µg for *nos*Z. The curves for the standards for *NS*, *nxr*A, *nir*S, *nos*Z and *nir*K were acquired after the quantitative PCR assays with regular dilutions of already known concentrations of the plasmid DNA with corresponding genes (10^7–10^1 number of gene copies). The detail of thermocycler conditions used for quantification of respective genes are provided in the Supporting information. At least two independent quantitative PCRs were done for every sample while the average was used. Co-amplification of the standards and the dilution of samples were used to control any inhibition in the quantification.

Microbial activities

Susbtrate induced respiration microResp. (SIR) was measured as CO_2 production using MicroResp plates (Chapman et al. 2007). Soil was added to the wells of 96-well plates. 0.5 ml of a nutritive solution including glucose (1.2 mg of C-glucose g of dried soil) was added then incubated at 28°C. CO_2 concentrations were measured using a TECAN spectrophotometer after 6 h of incubation and the slope of the linear regression was used to estimate aerobic respiration as the CO_2 produced (ppmV CO_2 h^{-1} g^{-1}).

Nitrifying enzyme activity (NEA) was measured on fresh soil samples as described by Patra et al. (2005). In brief, the fresh soil sample (equal to 3 g dry weight) was incubated with 3 ml solution of N-NH_4^+ (200 µg N-$(NH_4)_2SO_4$ g^{-1}) in the flask for 72 h at 180 rpm and 28°C. The flask was sealed with parafilm to produce the aerobic conditions. With distilled water, the total volume of the suspension was made up to 15 ml. During the incubation, at regular intervals of 5, 24, 48 and 72 h soil slurry was filtered with a pore size of 0.2 µm. The rates of production of NO_2^-/NO_3^- were measured by ionic chromatography (DX120. Dionex).

DEA was measured by incubating the fresh soil sample in a 150 ml airtight plasma-flask sealed by a rubber stopper for 4 h at 28°C followed by the N_2O measured each 30 min for 3 h a gas chromatograph coupled to a micro-catharometer detector (IGC-R3000; SRA instruments) using the method adapted from Dassonville et al. (2011). Briefly, a substrate solution (2 ml) containing glucose (0.5 mg of C-glucose g^{-1} of dried soil), glutamic acid (0.5 mg of C-glutamic acid g^{-1} of dried soil) and potassium nitrate (50 µg of N-KNO_3 g^{-1} of dried soil) were added to the soil. A 90 : 10 He–C_2H_2 atmosphere provided anaerobic conditions and inhibited N_2O-reductase activity using the acetylene inhibition technique that blocks the last step in the denitrification pathway resulting in the accumulation of N_2O (Yoshinari et al. 1977). The soil was incubated for 4 h at 28°C and after incubation the amount of N_2O was measured each 30 min for 3 h. The amount of N_2O produced was measured using a gas chromatograph coupled to a micro-catharometer detector (IGC-R3000; SRA instruments).

Statistics and structural equation model

The data obtained were analyzed using JMP8(SAS Inst. Inc.). First, we run a descriptive test in which normality of data was checked through Kolmogorov–Smirnov and Shapiro–Willk tests. Both of these showed that the data was not normally distributed as none of these had significant values above 0.05. Since the data was not normally distributed, Kruskal–Wallis pairwise test (suited for non-normally distributed data) were applied to know which treatment is different from the other for studied parameters at various sampling times. The KMO and Bartlett's test was also run through factor analyses. The KMO value of 0.812 revealed that the factor analyses showed promising results whereas Bartlett's test significant value was less than 0.05 depicting homoscedasticity in the data. In addition to

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linear modeling, for block effect, and repeated measures considerations (to consider time component-sampling dates or sampling from same place at different times), we used cluster analyses approach using R (www.r-project.org) (the package of R for hierarchical cluster analysis is 'cluster' and the function is *hclust* and to make dendogram we used function *dendogram*) to determine the effect of treatments within each block (to investigate the block effect) at different sampling times (to investigate the repeated measures) on each other. Structural equation model (SEM) was constructed using AMOS (Amos Development Corporation) to link the environmental and microbiological variables and investigate their relationship under different climatic extremes. The fitness of the model was assessed by using the χ^2 test and the model was considered as correct if the χ^2 test produced a non-significant p-value – $p > 0.05$. Since the factors controlling the microbial functioning under drought and/or snow removal treatments were changing upon these treatments, the hypothesized models were changed to increase the level of significance and to fit the model. SEM were constructed to understand the relationship and path coefficients among the various causal environmental and microbial factors under D, SR, SR+D treatments. The choices for SEMs were made considering the parameters showing strong correlations obtained through linear regression modeling. Initially, the SEMs were constructed for each sampling interval separately for each treatment to observe how the various environmental and microbial factors influenced the SIR, NEA, and DEA over each period of sampling time ($n = 128$ for each sampling interval). Then the strongly correlated parameters across different treatments were linked through SEMs to understand the variable impact of treatments for different parameters hence variability was also considered.

No significant effects of plant functional composition and fertilization/mowing on the investigated microbial parameters were found ($p > 0.10$). The findings were independent of the sampling locations and time and no block effect was found. The observations were also supported by the Cluster analyses which are shown in the dendrogram (Supporting information). Cluster analyses used to determine the effect of treatments within each block at different sampling times revealed five different clusters. In each cluster, specific treatments grouped separately and showed that effect of one specific treatment does not influence any block. The further effect of outliers was analyzed to know the difference in descriptive values after non-consideration of outliers.

Results

Changes in broad scale function (i.e. SIR) and total bacterial community (i.e. 16S rRNA)

Soil moisture in CTRL showed the significant effect of drought (D), snow removal (SR) and SR+D treatments as shown at T1, T2 and T3 sampling times (Fig. 2a, Supporting information). The resistance and recovery of the N-cycling activities both for nitrification and denitrification, and the abundances of corresponding genes, were calculated as the relative change in D, SR, and SR+D treatments in comparision to CTRL. SIR was found to be significantly ($p = 0.001$) lowered upon both droughts and was recorded up to 89% of CTRL (43.83 ppmV CO_2 h^{-1} g^{-1} ± 0.58) but recovered ultimately ($p = 0.639$) (Fig. 2b). Although the interaction of SR+D treatments marginally ($p = 0.0123$) affected the SIR, it was not affected by SR alone ($p = 0.625$). It was noteworthy that the abundance of the total bacterial community (as expressed by 16S rRNA gene copies) followed a similar trend as SIR and was significantly ($p = 0.0006$) reduced up to 56.3% of CTRL (4.87×10^8 copies per g of dry soil) with a complete recovery observed at T4 (Fig. 2, Supporting information).

Response of N cycling processes to droughts and snowmelt

Nitrification (NEA) showed resistance to both droughts at T1 and T3 and thus remained independent of the change in moisture content. Whereas, there was a significant reduction in NEA (up to 76% of CTRL) upon SR treatment at T2 (Fig. 3, Supporting information). To assess linkages, multiple regression analyses were carried out for the parameters in addition to moisture contents and the findings showed that the NEA was positively correlated to NH_4^+, %N, TDN and *nxr*A ($r = 0.56, 0.20, 0.31, 0.20$; $p < 0.005$). On the contrary, DEA was significantly lowered up to 85 and 68% of CTRL upon first and second drought, respectively (Fig. 4, Supporting information). Recovery from the first drought was observed for DEA at T2 except for the interaction of SR+D treatment. However, the second drought (at T3, Fig. 4) strongly ($p = 0.0001$) impacted DEA which did not recover from SR. Hence, at T4 there was an incomplete recovery in denitrification acitivity as the DEA for SR+D mesocosms could not return to the initial levels (0.362 µg N-N_2O g^{-1} dry soil h^{-1} ± 0.022).

Impact on N cycling functional guilds

Our results revealed that the abundances of N cycling functional guilds including nitrifiers and denitrifiers were strongly impacted by both D and SR climatic extremes ($p = 0.001$). We observed that the *Nitrospira* and *Nitrobacter* (*nxr*A and *NS*, respectively) abundances were significantly ($p = 0.001$) reduced upon applied stresses and could recover only for *nxr*A (Fig. 3). The denitrifiers were particularly impacted by the second drought, that strongly lowered their gene abundances. At T4, the community gene abundances of *nir*S and *nos*Z denitrifiers were completely recovered. However, nirK denitrifiers could not recover in agreement to the observations as recorded for DEA (Fig. 4, Supporting information). The multiple correlation analyses showed the strong positive correlations of DEA with the %C, %N, DON, SOM, soil moisture, NEA as well as with the abundances of denitrifying genes. However, these correlations were found more prominent in the climate change treatments than the CTRL

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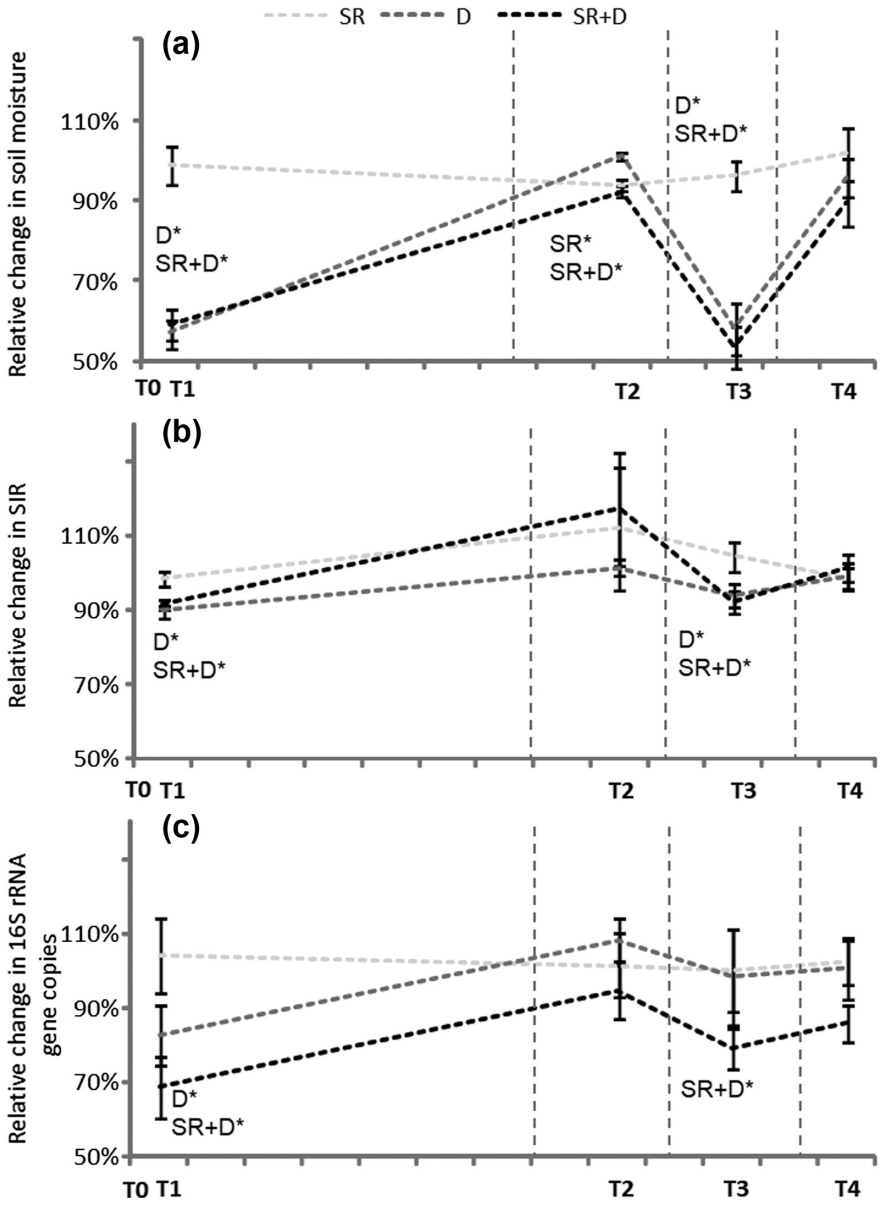

Figure 2. Relative change compared to CTRL for different treatments at various sampling dates (a) in soil moisture; (b) in SIR-MicroResp; (c) in abundance of total bacterial community (number of 16S rRNA gene copies g^{-1} dry soil). Light grey dotted lines show variations in time of the variables in the snow removal (SR) treatment relative to CTRL; dark grey dotted lines show variations of the drought (D) treatment relative to CTRL; and the black dotted lines show the variations of cumulative SR+D treatment relative to CTRL. The steric '*' denotes the significant differences for the respective treatment.

especially for soil moisture. For example, the Spearman correlation coefficient showed a non-significant correlation between soil moisture and *nirK* denitrifiers ($r = 0.04$; $p > 0.652$) only for the CTRL whereas it was positive in SR+D treatment ($r = 0.33$; $p < 0.005$).

Microbial variables and climatic treatments, a structural equation modeling

The results obtained from structural equation modeling approach showed how the various environmental and microbial parameters influenced the broad scale and N-cycling activities when droughts, snow removal, or their interaction, occurred. For instance, SEM revealed that the SIR was linked to soil moisture and 16S rRNA for various treatments at different sampling times except at T1 and T2 ($\chi^2 = 14.35$, $p = 0.35$; Fig. 5). In contrast, NEA was found to be mainly linked with soil NH_4^+ concentration and community gene abundances of the *NS* nitrifiers, except for the SR treatment. Interestingly, under droughts, major part of the DEA variance (up to 55%) was explained by soil moisture with *nir*S as dominant denitrifiers except at T3 when it was *nos*Z (Fig. 5b). It was also observed that the overall impact of snow removal and drought was variable for different denitrifiers with severe

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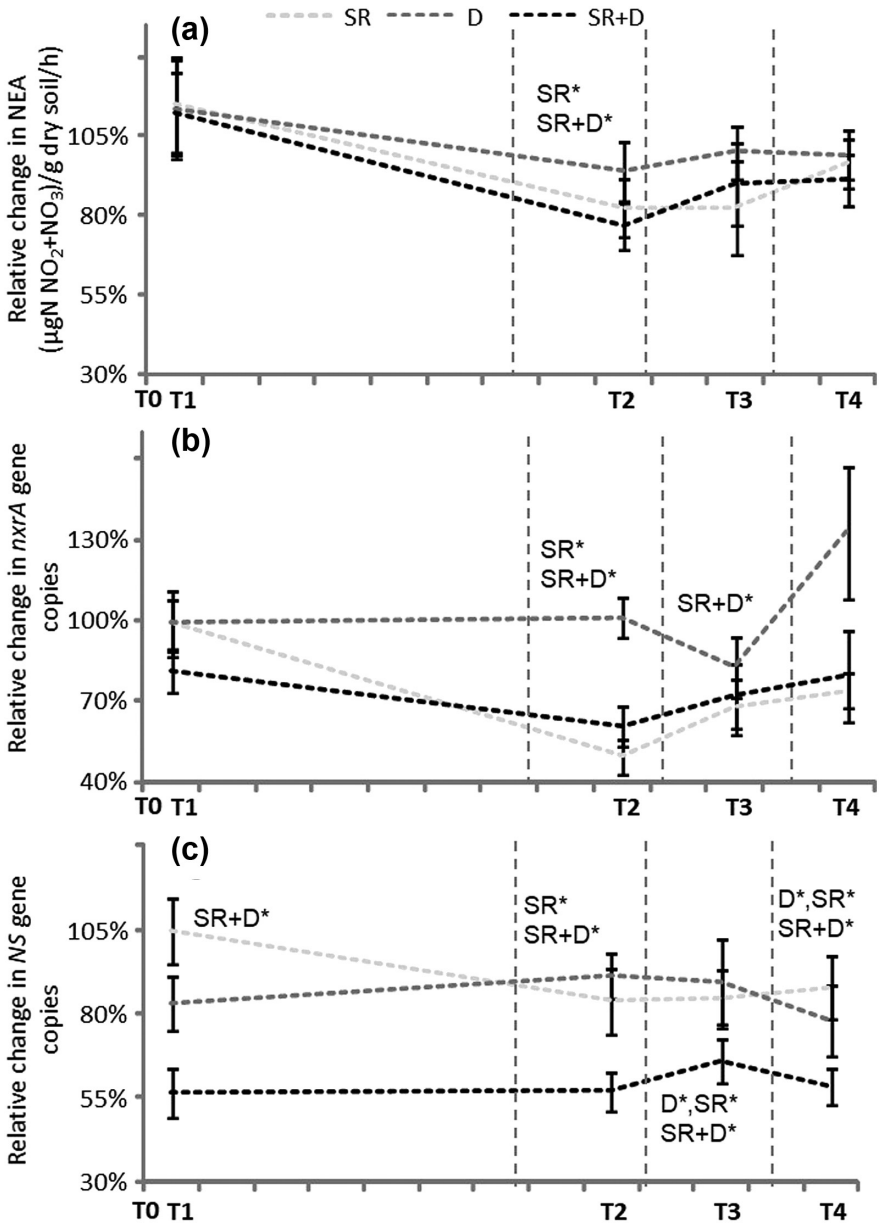

Figure 3. Relative change compared to CTRL in (a) nitrification enzyme activities (µg N-(NO^{2-} + NO^{3-}) g^{-1} dry soil h^{-1}); (b) in abundance of nxrA nitrite reductases (number of nxrA gene copies g^{-1} dry soil); (c) in abundance of NS nitrite reductases (number of NS gene copies g^{-1} dry soil). Light grey dotted lines show variations in time of the variables in the snow removal (SR) treatment relative to CTRL; dark grey dotted lines show variations of the drought (D) treatment relative to CTRL; and the black dotted lines show the variations of cumulative SR+D treatment relative to CTRL. The steric '*' denotes the significant differences for the respective treatment.

impact on *nir*K which could not recover by the end of the experiment (Fig. 4b).

Discussion

This work reported the resilience, comprising the resistance and recovery capabilities, of total bacterial activities and gene abundances in subalpine grassland ecosystems to simulated summer droughts and early spring snowmelt. Investigating the potential impact of these extreme events is essential since subalpine grasslands are under drastic influence of global warming that may ultimately disrupt their functioning and subsequent ecosystem services (Vittoz et al. 2009, Wipf and Rixen 2010, Schirpke et al. 2013, Pommier et al. 2018, Berauer et al. 2019, Bernard et al. 2019). Our results demonstrated the impact of successive droughts and early snowmelt events on assembled subalpine grassland ecosystem functioning, including nitrification and denitrification as model processes which are of valuable insights into resilience of overall N cycling (Chen et al. 2020, Dai et al. 2020).

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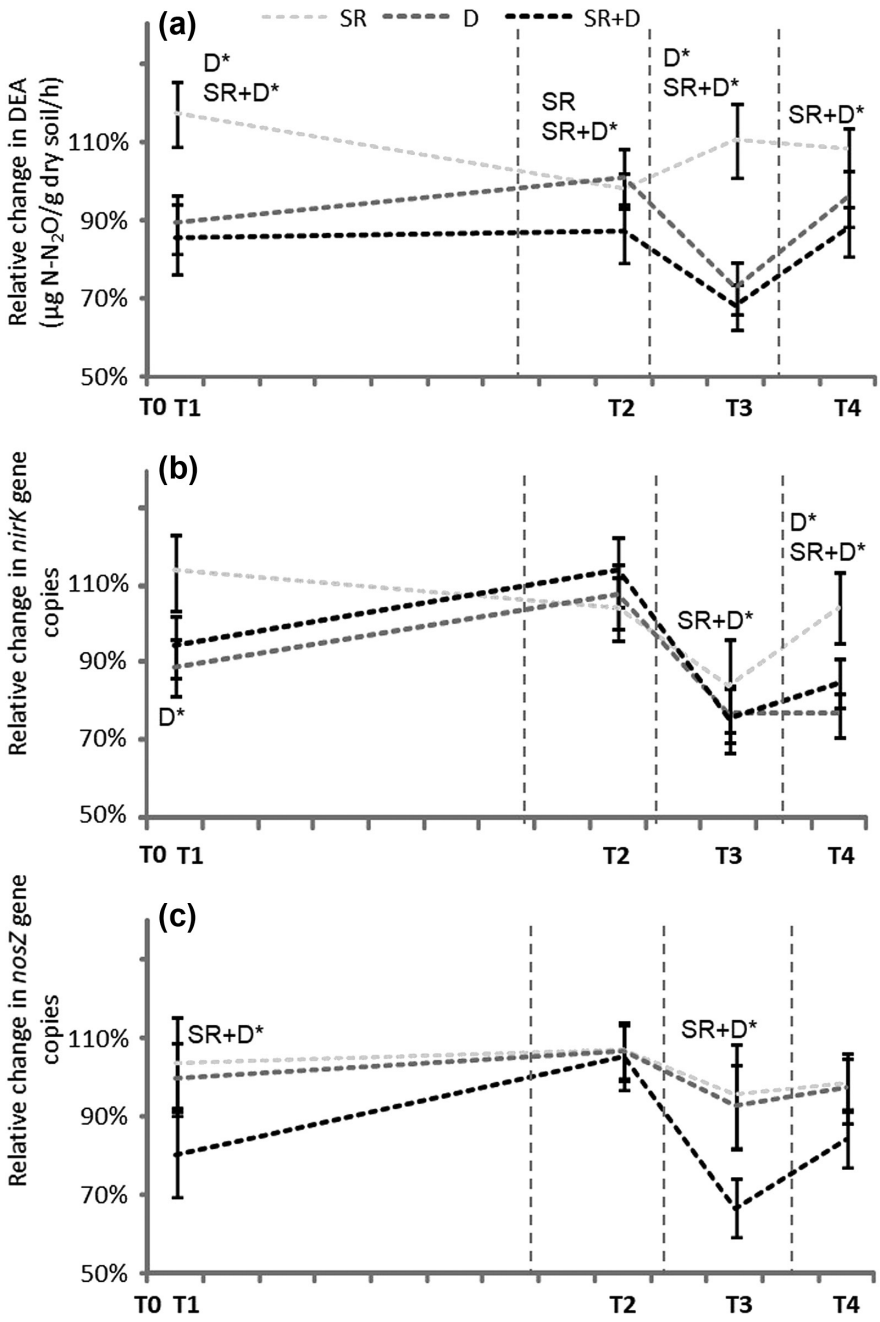

Figure 4. Relative change compared to CTRL in (a) DEA (µg N-N2O g^{-1} dry soil h^{-1}); (b) in abundance of nirK denitrifiers (number of nirK gene copies g^{-1} dry soil); (c) in abundance of nosZ denitrifiers (number of nosZ gene copies g^{-1} dry soil). Light grey dotted lines show variations in time of the variables in the snow removal (SR) treatment relative to CTRL; dark grey dotted lines show variations of the drought (D) treatment relative to CTRL; and the black dotted lines show the variations of cumulative SR+D treatment relative to CTRL. The steric '*' denotes the significant differences for the respective treatment.

The quantification of resistance and recovery by comparing the relative change in drought, snow removal, and cumulative stresses, over time showed the strong impact of these different treatments on studied microbial activities and the relative abundance of the corresponding functional groups. The substrate induced respiration (SIR) and total bacterial abundances were highly resistant to the applied stresses with high recovery patterns strengthening our assumption that this function is carried out by a wide range of bacterial communities and generally exhibit a fast recovery (Griffiths and Philippot 2013, Ingrisch et al. 2020, Jia and Whalen 2020). Conversally, short term reduction in SIR upon droughts have been reported to be associated to lower microbial activities under water deficit conditions (Bloor and Bardgett 2012, Bernard et al. 2019). Accordingly, broad scale bacterial functions are also reported to be less sensitive to various

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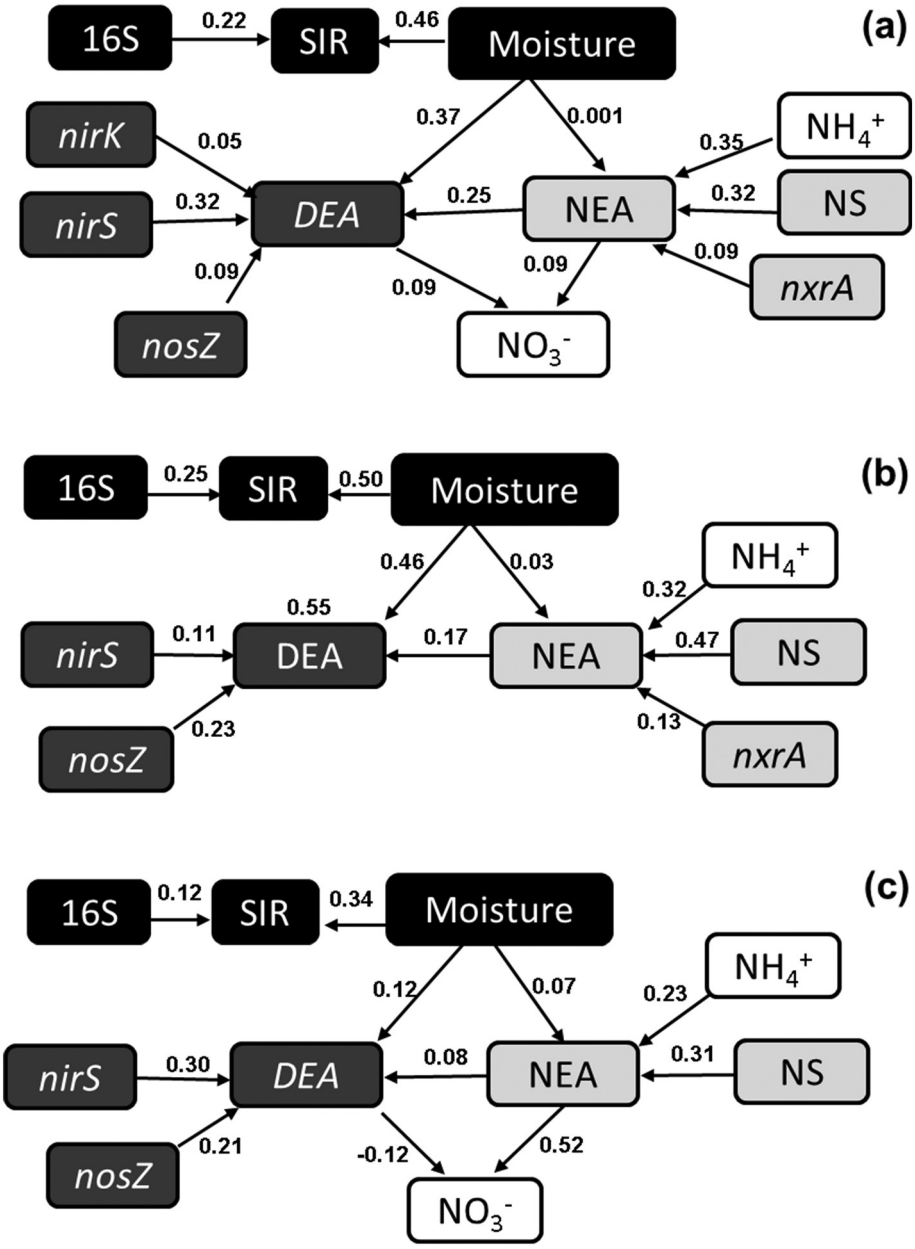

Figure 5. Structural equation models (SEM) bridging the effects of microbial and environmental factors. SEMs including the substrate induced respiration MicroResp (SIR), nitrification (NEA), denitrification (DEA), gene community abundances (16S rRNA, nxrA, NS, nirK, nirS, nosZ) and environmental factors (soil moisture, NH_4^-, NO_3^-) in (a), (b) and (c) are built using the data from treatments; SR, D, SR+D, respectively. The values shown beside the arrows are the path coefficients and correspond to the standardized coefficients calculated based on the analysis of correlation matrices. Values only for the significant relations (that fit the model, $p > 0.05$) are indicated in the SEMs.

perturbations (Chaer et al. 2009). Alike soil respiration and decomposition processes are generally driven by most heterotrohic microorganisms, and weak impact of climatic stresses on these processes has been reported in a joint experiment (Bernard et al. 2019), while high recovery to climatic stress of microbial biomass was observed in other mountainous grasslands (Piton et al. 2020).

The impacts of extreme climatic events including droughts on N cycling were more subtile (Fuchslueger et al. 2019). All applied treatments showed different impacts on nitrification and denitrification, where denitrification (community gene relative abundance and enzyme activity) was less resistance to drought (D) as compared to nitrification (both for community gene abundances and enzyme activities) (Fig. 3, 4, Supporting information). Furthermore, when snow removal and drought occurred in the same mesocosms (SR+D), DEA was first strongly reduced and followed an incomplete recovery (Fig. 4a). Contrastingly NEA was also affected by snow

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removal but completely recovered at T4 (Fig. 3a). Such findings implied a comparatively strong influence of droughts and associated soil moisture deficits, specifically for DEA (Philippot et al. 2009, Dai et al. 2020). This impact was pronounced when both climatic stresses (SR+D) occurred and may be associated to the additive and cascading effect of snow removal on nitrification that ultimately broke NO_3^- provision to denitrifiers. Interestingly, these observations were specific to both stress and microbial group, with variable response for microbial community size and functions. As found by Sorensen et al. (2020), a collapse in microbial pool size upon snowmelt may also be linked with the niche differentiation . In nearby subalpine grasslands, a strong turnover by nitrifying and denitrifying microbial communities and subsequent nitrogen transformations has been previously shown upon natural variations in snowpack depth (Jusselme et al. 2016). In wake of climatic extremes and considering the functional stability scenarios, such multiple cascading effects are important from microbial community perspectives and related ecosystem services. For example, these scenarios are crucial for N-cycling activities which can adversely impact greenhouse gas emissions (i.e. N_2O in particular), soil fertility and primary productivity, hence ask for adaptive management strategies to counter the unforeseen consequences (Bouwman et al. 2009, Di et al. 2014, Griffis et al. 2014, Pommier et al. 2018).

The reduction in gene abundances of nitrifiers and denitrifiers upon drought or snow removal further delineated the strong impact of climate change treatments in the studied subalpine grasslands. Denitrifiers are usually reported as more tolerant to droughts (Hammerl et al. 2019) than nitrifiers (Austin and Strauss 2011). Here, in line with DEA, denitrifiers' abundances were also negatively affected by droughts, especially after the second one. Like DEA, *nirK* denitrifiers' abundances were severely lowered and could not show a complete recovery at T4 (Fig. 4). DEA was apparently related to various denitrifying communities including *nirK*. In agreement, DEA and abundance of *nirK* denitrifiers have been found to be correlated upon variable snow depths under similar subalpine grasslands (Jusselme et al. 2016).

Soil abiotic properties are crucial in estimating N cycling processes, and resilience of microbial communities upon applied stresses may be influenced by inherent soil edaphic characteristics (Berard et al. 2015). Although we found overall strong positive correlations of DEA with %C, %N, DON, SOM, soil moisture, NEA, and denitrifiers' abundances, these observations shifted with treatments and during the study period. In mesocosms exposed to combined snow removal and drought (SR+D), soil moisture along with *nir*K, *nir*S and *nos*Z were correlated to DEA ($r = 0.33$, 0.15, 0.32, 0.38; $p < 0.005$). While soil moisture and *nir*K were not correlated to DEA ($r = 0.04$; $p > 0.64$) in the CTRL hence the variable dominance of different denitrifiers in D, SR and CTRL were observed. Under various environments, these parameters contrastingly affected the overall DEA and subsequent outcomes (Chroňáková et al. 2009, Dong et al. 2009, Petersen et al. 2012, Chen et al. 2019, 2020). At T4, DEA could not recover demonstrating a severe impact caused both by drought and the additive effect with snow removal. Moreover, the impact on nitrification during snow removal significantly passed on to DEA which collapsed upon the second drought showing the cascading effect for SR+D.

These pronounced impacts on DEA and corresponding denitrifiers' abundances were also explained through SEM (Fig. 5). Possibly the system, (as described by Schnellnhuber et al. 2006) upon disturbance was 'pulsed' (respond on alteration but return) and 'pressed' (respond on sustained alteration so shift) where 'press' brings the ecosystems to a tipping point (as observed for DEA in our study). This may shift the system to an irreversible new state (Wall 2007), or at least may lead to altered recovery patterns (Seabloom et al. 2020). Such changes in relative abundances of denitrifiers may also cause a selection in certain stable/unstable bacterial groups. We suggest that extreme climatic events may consequently impact (increase) N losses through NO_3^- leaching or N_2O emissions (de Vries and Shade 2013). In addition, studies in other ecosystems have suggested that these functional guilds may have contrasting resilience abilities against various disturbances (Capdeville et al. 2019).

Though SIR was found to be controlled by soil moisture and *16S* rRNA when SEM was fitted, NEA was highly linked to both soil NH_4^+ and *NS* nitrifiers' abundance but weakly to soil moisture. Therefore, *NS* communities seemed to play a dominant role in these grasslands. Higher abundance of *NS* nitrifiers than *nxr*A under drought as well as the dominant role of *NS* nitrifiers was earlier shown in other soils with low microbial activity (Attard et al. 2010). In contrast, DEA was linked both to soil moisture and abundances of *nir*S denitrifiers throughout the experiment except at T3 when the *nos*Z abundances responded to soil moisture, suggesting that the community selection and/or adaptation was an important phenomenon under changing climate or stress conditions. Yet, a more detailed description of community compositional change is required to provide such information. After the second drought at T3, a cascading effect of snow removal might have shifted the community dynamics from *nir*S to *nos*Z prevalent functional groups due to a better adaptation of the *nos*Z denitrifiers. The denitrifiers responded differentially, e.g. in contrast to *nir*K; *nir*S and *nos*Z abundances recovered, strengthening the assumption of a community specific impact of drought and snow removal (Karlowsky et al. 2018). This is consistent with *nir*K denitrifiers being reported as less abundant (Peterson et al. 2012), and relatively more sensitive to environmental change than *nir*S denitrifiers in subalpine and agricultural soils (Hallin et al. 2009, Szukics et al. 2019). Though the findings implied by Dai et al. (2020) contrasted these observations, seasonally driven drought effects have also been reported for *nir*K, *nir*S and *nos*Z abundances in other grassland ecosystems (Hammerl et al. 2019). Moreover, the denitrifiers harboring *nir*K genes might not contain *nos*Z genes, in agreement to the discovery that not all denitrifiers contain *nos*Z (Hallin et al. 2018), which may ultimately disturb the overall nitrous oxide budget from the soils.

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Plant functional composition and fertilization + mowing did not significantly affect the investigated soil microbial variables, suggesting they were not the main drivers of the studied processes. It can be inferred that specific plant trait-centered influence of abiotic variables on broad (i.e. SIR) and N-cycling (i.e. DEA, NEA) activities and abundances of involved functional guilds, in response to climatic stresses, was not observed (Griffin-Nolan et al. 2019). Previously, summer droughts in subalpine grasslands were reported to increase leaf senescence while reducing plant productivity (Benot et al. 2014), as well as affecting N cycling community and functions (Cantarel et al. 2012, Dai et al. 2020). Yet, leaf or root specific traits, rather than plant community composition, might have affected the nutrient availability hence the associated microbial processes. This could explain why we found that soil nutrient contents along with gene abundances were driving the N cycling processes. The characteristics of existing plant species, nutrient availability, their interaction with microorganisms and ultimate response to climatic changes are important in soil functioning resilience (Bardgett et al. 2013, Saccone et al. 2013, Kaisermann et al. 2017, de Vries et al. 2018, Bernard et al. 2020). For example, any shift in native plant community (in wake of changing climate) can influence the soil nutrient usage leading to selection in the pool of residing microbes hence the corresponding functions (de Vries et al. 2012, Legay et al. 2016).

Interestingly in similar grasslands, no direct effect of fertilization + mowing was observed either, but only specific plant traits were found to affect the same bacterial activities (Legay et al. 2016). This suggests a competition for nutrients between plants and microbes, as observed following snowmelt in nearby subalpine grasslands (Legay et al. 2013), which could result here in a non-significant fertilization + mowing effect on the investigated microbial parameters at least during the study period. However, such management strategies including fertilization practices may favor certain microbial communities ultimately causing faster resilience to stress conditions (Piton et al. 2020). It is noteworthy that due to the climatic extremes, the responses showed by the nitrifiers' and denitrifiers' groups were different to CTRL indicating an unanticipated internal competition for initially available nutrients. Importance of such competitions will be crucial to further investigate, especially in ecosystems facing climatic extremes, since these are suggested to be critical in controlling ecosystem N cycling (Grigulis et al. 2013, Legay et al. 2013, Le Roux et al. 2013).

Conclusions

This work demonstrated a strong and unprecedented impact of climate extremes such as early snowmelt and recurrent droughts, alone or in combination, on soil bacterial communities both at broad (i.e. soil respiration) and more specific N-related (i.e. nitrifiers' and denitrifiers' activities and abundances) scales. Low and short-term impacts of droughts on bacterial total respiration delineated its high resilience. Though nitrification activity and community gene abundances of corresponding functional guilds exhibited relatively strong resistance to summer droughts, these declined in response to early snowmelt strongly affecting the denitrification in a cascading effect. Our findings inferred that predicted climatic extremes may carry severe consequences for soil microbial community functioning in subalpine grasslands ultimately resulting in shifts in N availability, plant community diversity, and associated ecosystem services.

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Author contributions

Farhan Hafeez: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Software (equal); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal). **Jean-Christophe Clement**: Conceptualization (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Resources (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing – review and editing (equal). **Lionel Bernard**: Data curation (equal); Writing – review and editing-Supporting. **Franck Poly**: Conceptualization (equal); Data curation (equal); Methodology (equal); Resources-Supporting, Supervision-Supporting, Validation-Supporting, Visualization-Supporting. **Thomas Pommier**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Methodology (equal); Project administration (lead); Resources (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Zenodo Digital Repository: http://doi.org/10.5281/zenodo.5648316 (Hafeez et al. 2023).

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Supporting information

The Supporting information associated with this article is available with the online version.

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