Early Spring Snowmelt and Summer Droughts Strongly Impair the Resilience of Key Microbial Communities in Subalpine Grassland Ecosystems

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ABSTRACT

Subalpine grassland ecosystems are important from biodiversity, agriculture, and touristic perspectives but their resilience to seasonally occurring climatic extremes is increasingly challenged with climate change, accelerating their vulnerability to tipping points. Microbial communities, which are central in ecosystem functioning, are usually considered as more resistant and highly resilient to such extreme events due to their functional redundancy and strong selection in residing habitats. To investigate this, we explored the soil microbial responses upon recurrent summer droughts associated with early snowmelt in grasslands mesocosms set-up at the Lautaret Pass (French Alps). Potential respiration, nitrification and denitrification were monitored over a period of two growing seasons along with quantification of community gene abundances of total bacteria as well as (de)nitrifiers. Results revealed that droughts had a low and short-term impact on bacterial total respiration supporting their hypothesized high 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 triggered a cascading effect on denitrification but also on the abundances of denitrifying communities which could recover from all climatic extremes except from the summer droughts where nitrifiers were collapsed. Denitrification and the respective functional groups faced high impact of applied stresses with strong reduction in the abundance and activity of this specialized community. Although, the consequently lower microbial competition for nitrate may be positive for plant biomass production, warnings exist when considering the potential nitrogen leaching from these ecosystems as well as risks of greenhouses gases emission such as N_2O.

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

1. Introduction

Subalpine grassland ecosystems are important for biodiversity, agriculture, and a wide range of key services (Pecher et al., 2017; Zoderer et al., 2016; Tappeiner et al., 2008; Grêt-Regamey et al., 2012). These grasslands are under extreme pressure of climatic changes drastically affecting their functioning and disrupting the linked ecosystem services, *e.g.*, plants productivity, nutrient cycling etc. (Berauer et al., 2019; Bernard et al., 2019). Considering the continuous human perturbations and global changes (Berauer et al., 2019), it is direly required to investigate whether these grasslands are highly vulnerable, have developed resilience (resistance: ability to maintain the existing state, and recovery characteristics: to recover from disturbances) (Ingrisch and Bahn, 2018; Hodgson et al., 2015) or have attained an altered recovery state (Seabloom et al., 2020) over the time which is crucial in conserving the ecosystems functions (Donohue et al., 2016).

The multiple effects of the global climatic changes not only impact the biodiversity and ecosystem services (Berauer et al., 2019; Schirpke et al., 2013, Engler et al., 2011) but also their associated resistance (Bernard et al., 2019; Wipf and Rixen, 2010) and recovery in Alpine grasslands (Ingrisch et al., 2020; Karlowsky et al., 2018). The effect of the recurrent of climatic extremes on microbial functioning in these grasslands needs to be assessed as they may shift the fate of particular biogeochemical functions especially when resilience limits are crossed and tipping points (where a potentially irreversible change is about to occur or is already occurring *sensu* (Schnellnhuber et al. (2006)) are reached. Such estimations are crucial to understand and possibly protect the stability of ecosystems and inhabiting species (de Vries and Shade, 2013; Griffiths and Philippot, 2013), but also to forecast the unforeseen consequences of adverse climatic extremes. Where weather extremes including droughts are predicted to be severe (Fuchslueger et al., 2019; Karlowsky et al., 2018), alpine grassland ecosystems are expected to face less snow falls that ultimately lead to subsequent droughts

(Klein et al., 2016; Gobiet et al., 2014). However, their vulnerability to collapse or support a particular function towards the combined (cumulative) impact of different weather extremes is poorly documented.

Microbial communities are crucial actors in ecosystem functioning (Chen et al., 2020; Bardgett and van der Putten, 2014) and respond strongly to environmental variability (Piton et al., 2020), thus are critical in estimating the functional stability perspectives (de Vries and Shade 2013; Griffiths et al., 2000). They are highly diverse, functionally redundant, and can be more resilient to climatic fluctuations as compared to many other environmental actors albeit the strong selection resulting from the local harsh climatic conditions (Capdeville et al., 2019; Griffiths and Philippot, 2013, Benot et al., 2014). Despite these yet known inherent capabilities, whether these microbial communities can withstand to correlated acceleration of weather extremes is largely unknown. Especially, the assessment of the functional boundaries of the subalpine grasslands and the linked ecosystems services is critical since they are susceptible to extreme events (Bernard et al., 2019).

The characteristics of existing plant species and nutrient availability as well as their interaction with microorganisms to ultimately respond to climatic changes are important in assessing the soil functioning resilience (Bernard et al., 2020). For example, any shift in the native plant community can influence the soil nutrient usage leading to the selection in the pool of residing microbes hence the corresponding functions (Legay et al., 2016; de Vries et al., 2012). Similarly, the weather extremes directly influencing the plant community may also impact the microbes (Bardgett et al., 2013; de Vries and Shade, 2013, Saccone et al., 2013). Earlier, summer droughts in subalpine grasslands are reported to increase leaf senescence while reducing plant productivity (Benot et al., 2014), as well as affecting N cycling-related microbial community and functions (Dai et al., 2020; Cantarel et al., 2012).

Here we determined the short-term effect of drought, snow removal, and their additive (recurrent) effect on microbial activities and size of the involved functional guilds and the following responses, to measure the resistance and recovery, respectively. Since the microbes, driving broad (*e.g.*, respiration, decomposition) and specialized soil processes (*e.g.* (de)nitrification), may respond differentially to various perturbations (Dai et al., 2020; Jusselme et al., 2016; Chaer et al., 2009; Wertz et al., 2007), we conducted our work both at broad and functional scales. Investigating the response of these grasslands is crucial since the variable impact of natural and manmade perturbations might have considerably influenced the nutrient cycling including that of N (Galloway et al., 2004), but also the involved microbial functional guilds. First, 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 they recovered from these stresses. Then we investigated the nitrogen (N) cycling (that is critical in ecosystem sustainability (Chen et al., 2020; Kuypers et al., 2018)) processes by determining the abundances (nitrifiers (*nxrA*, *NS*), denitrifiers (*nirK*, *nirS*, *nosZ*)) and activities (nitrification and denitrification enzyme activities -NEA and DEA, respectively) of the involved microbial communities, since they carry implications for plant productivity (loss of N - an important plant nutrient) and environment (N_2O emissions-global warming) (Dai et al., 2020; Galloway et al., 2004).

The current work was carried out on functionally contrasted and assembled grasslands set up in a common garden at Lautaret Pass with various sampling campaigns during two growing seasons. Based on an assumed functional redundancy (Jia and Whalen 2020; Griffiths and Philippot 2013), we hypothesized that the broad SIR and the relevant microbial community should resist and/or recover once the stresses/extreme events are over. SIR being representative of the total microbial communities is expected to be influenced by the soil moisture and abundance of *16S rRNA* (Hallin et al., 2009). However, the more specific

functions of N-cycling should be more sensitive due to comparatively less functional redundancy and, despite being able to resist to single stress, the combined effects (SR+D) may trigger a tipping point, disturbing the various steps of these processes. Moreover, any shift in soil moisture, substrate availability and abundance of denitrifiers can strongly impact DEA through affecting the micro-environment conditions (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. To address these questions, we quantified the immediate responses of the microbial communities' abundances and processes to droughts and early snowmelt extreme events to explore the resistance and the following recovery across subalpine grassland ecosystems.

2. Materials and methods

2.1. Experimental site, Mesocosms and Treatments' implementation

The study was set up at Lautaret pass proximity in southeast France (2,100 m a.s.l, 45°02'13''N, 6°24'01''E) with 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. 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 8 blocks (Fig. 1). Within each of the 8 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 8 mesocosms were installed; and 4 were submitted to plant cuttings and fertilization (CF) while the other half remained untreated (NCF) (n=16+16). During spring 2014, slow-release fertilizer was applied as pellets in the CF mesocosms (150 kg N/ha). 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.

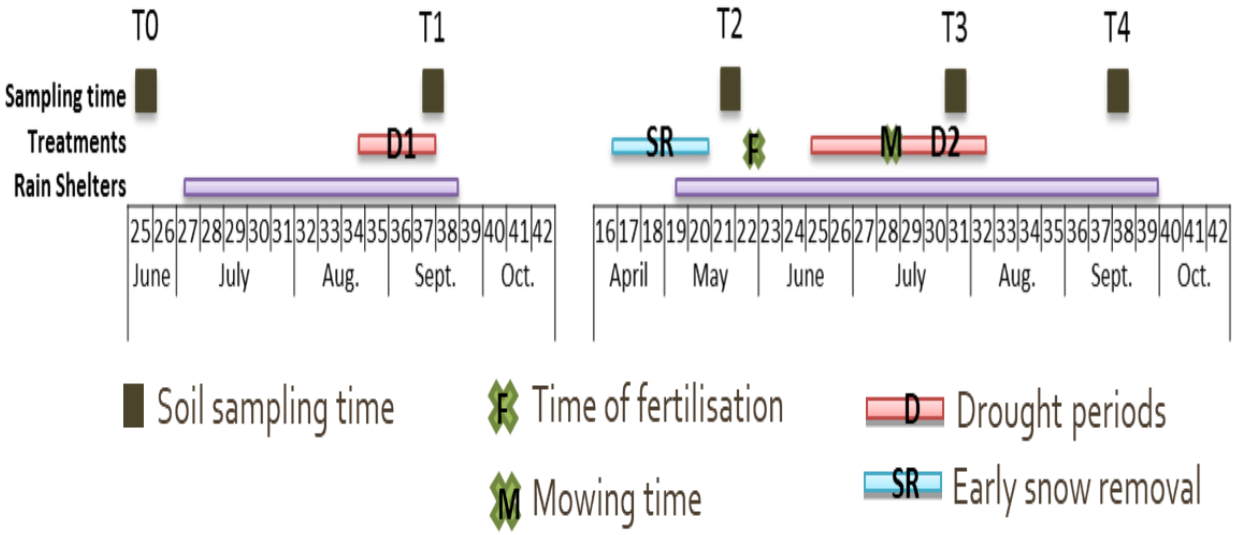

Fig. 1. Experimental timeline showing the implementation of various treatments as well as the sampling dates.

2.2. Soil sampling campaigns

Five sampling campaigns were carried out in 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. 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 sampling campaign (Fig. 1). During all sampling campaigns composite soil samples (5 cores mixed together, 0-10 cm depth) were collected from all 128 mesocosms (128x5: 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 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 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). 10 mg soil subsample were air dried, ground to powder and analyzed for soil total C and N concentrations using a C/N elemental analyzer (FlashEA 1112, ThermoFisher Scientific, MA, USA). 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, College Station, TX, USA) (Jones and Willett, 2006).

2.3. Soil DNA extractions and quantifications

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

2.4. Microbial community abundances

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; 1 X LightCycler DNA Master Mix SYBR® Green I (Roche Diagnostics); 1-ng of sample DNA or 10^8 –10^2 copies of the standard DNA pQuantAlb16S plasmid (Zouacheet al., 2012). The abundances of the *NS, nxrA, nirS, nirK and nosZ* genes were estimated using the primers developed previously (Attard et al., 2011; Henry et al., 2004; Henry et al., 2006; Kandeler et al., 2006). The total volume of the reaction was made 20 µl for *nxrA* and *nirK*, 500 nM for *nxrA* while 1µM for *nirK* for both the forward as well as reverse primers; 10 µL of the SYBR® Green I kit was used for the *nxrA* but also for the *nirK*, 0.4 µg of T4 protein (Qbiogene, Carlsbad, CA USA) for *nirK*. The total volume of the reaction was made 25 µl for *NS*, *nirS* but also for the *nosZ* while 400 nM with *NS* while 1µM for *nirS* and *nosZ* for both the forward as well as reverse primers; 12.5 µL of the SYBR® Green I kit was used for the *NS*, *nirS* but also with *nosZ*; for *nirS* the T4 protein was 0.5 µg while it was 0.4 µg for *nosZ*. The curves for the standards for *NS, nxrA, nirS, nosZ and also for the nirK* were acquired after the quantitative PCR assays with regular dilutions with already known concentrations of the plasmid DNA with corresponding gene (10^7 - 10^1 number of gene copies). Quantification of all gene community abundances was conducted out on a lightcycler 480 (Roche Diagnostics). The amplification conditions used for qPCR assays were: 10 min at 95°C for each 40 cycles for *16S rRNA*, 45 for *nxrA*, *NS*, *nirK* and *nirS* and 40 for *nosZ* (after 6 cycles of touchdown); 95°C for 15 s for *16S rRNA*, *nirK*, *nirS*, *nosZ* and for 30 s for *nxrA* and NS; 63°C for 1 min for *16S rRNA*, 55°C for 45 s for *nxrA* and 66°C for 30 s for *NS*, 30 s for *nirK* and *nirS*, 60°C for 15 s for *nosZ*. 72°C for 30 s for 16S *rRNA*, *nirK*, *nirS* and *nosZ*, 45 s for *nxrA*, 1 min for NS; and additional step of 80°C for 15 s *nirS* and *nosZ*. Then 95°C for 5 s for 16S

rRNA and *nxrA*, 1 s for *NS*, *nirK* and *nirS* and 15 s for *nosZ*; 65°C for 1 m for *16S rRNA* and *nxrA*, 68°C for 20 s for *NS* and *nirK*, 60°C for 20 s for *nirS* and 15 s for *nosZ*. And a continuous increase to 98°C for *16S rRNA*, *nxrA*, *NS*, *nirK* and *nirS* and 95°C for *nosZ* to determine the melting point; and finally, 10 s at 40°C for *16S rRNA*, *nxrA*, *NS*, *nirK* and *nirS* while 30 s for *nosZ* for cooling. 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.

2.5. 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) then incubated at 28°C. CO_2 concentrations were measured using a TECAN spectrophotometer after 6-hours 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-hours at 180-rpm and 28°C. The flask was sealed with parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA) 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-hours 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, Salt Lake City, UT, USA).

Denitrification enzyme activities (DEA) was measured by incubating the fresh soil sample in a 150-ml airtight plasma-flask sealed by a rubber stopper for 4-hours at 28°C followed by the N_2O measured each 30 minutes for 3-hours a gas chromatograph coupled to a

micro-catharometer detector (lGC-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-hours at 28°C and after incubation the amount of N_2O was measured each 30 minutes for 3-hours. The amount of N_2O produced was measured using a gas chromatograph coupled to a micro-catharometer detector (lGC-R3000; SRA instruments).

2.6. Statistics and Structural Equation Model

The data obtained were statically analyzed using JMP8® (SAS Institute Inc., SAS Campus Drive, NC, USA). Normally distributed data was analyzed using the Student's test and Tukey pairwise comparison. Where the data was not normally distributed after a log transformation further analysis was carried using the Kruskal-Wallis and Kruskal-Wallis pairwise comparison. Structural Equation Model (SEM) was constructed using the AMOS (Amos Development Corporation, Crawfordville, Florida, USA) to link the environmental and microbiological variables to investigate the relationship between these parameters under different climatic extremes. The fitness of the model was assessed by using the $\chi 2$ test and the model was considered as correct if the $\chi 2$ test produced a non-significant p-value - $p>0.05$. Since the factors controlling the microbial functioning under the 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. We also carried out linear regression modeling for the

studied microbial parameters and associated soil properties and the SEM were fitted considering the parameters showing strong correlation.

3. Results

3.1. Changes in Broad Scale Function and Community

The trajectory followed for the soil moisture at all sampling campaigns compared to CTRL showed the significant effect of the D, SR and SR+D treatments (Fig. 2a; Fig. S1). The resistance and recovery of the N-related microbial 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. It was observed that the 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 ($p=0.639$). 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 the 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; Fig. S1).

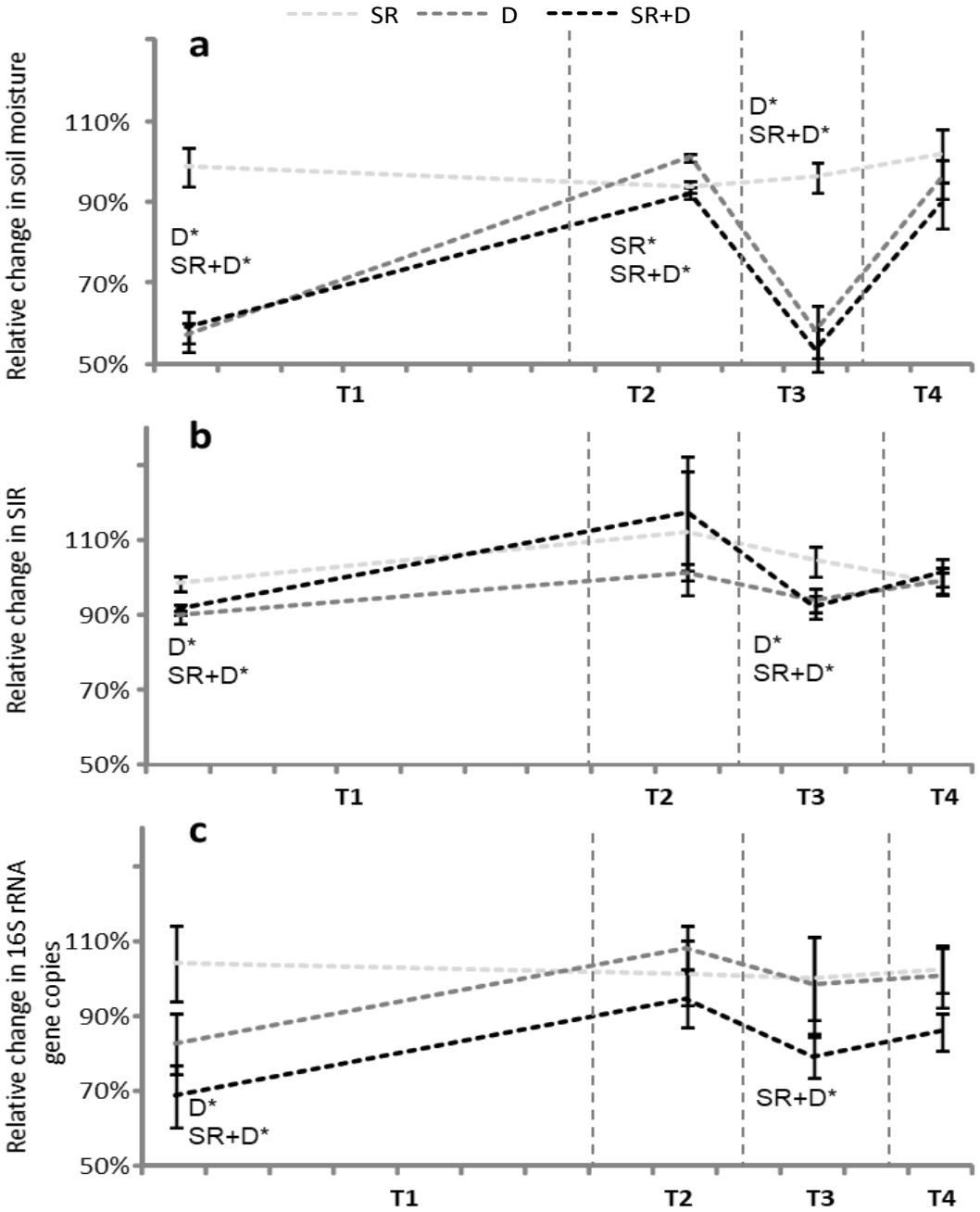

Fig. 2. Relative change compared to CTRL for different treatments at various sampling dates **a)** in soil moisture; **b)** in SIR-MicroResp™; **c)** in abundance of total bacterial community (number of 16S *rRNA* gene copies g^{-1} dry soil); Light grey dotted lines show variations in time of the variables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

3.2. 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) as observed upon SR treatment at T2 ($p=0.01$, Fig. 3; Fig. S2). To assess the linkages, the 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 *nxrA* ($r = 0.56, 0.20, 0.31, 0.20$; $p < 0.005$). On the contrary, DEA was significantly affected by both droughts and was found to be lowered up to 85% and 68% of CTRL upon first and second drought, respectively (Fig. 4; Fig. S3). 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 the SR+D mesocosms could not return to the initial levels (0.362 µg $N\text{-}N_2O$/g dry soil/h ± 0.022).

3.3. Impact on N Cycling Functional Guilds

The results revealed that the abundances of the N cycling functional guilds including the nitrifiers and denitrifiers were strongly impacted by both D and SR climatic extremes ($p=0.001$). We observed that the *Nitrospira* and *Nitrobacter* (*nxrA* and *NS)* abundances were significantly ($p=0.001$) reduced upon applied stresses which could recover only for *nxrA* (Fig. 3). The denitrifiers' abundances were particularly impacted by the second drought, that strongly lowered the gene abundances of this functional group. At T4, the community gene abundances of the *nirS* and *nosZ* denitrifiers were completely recovered, but the *nirK* denitrifiers could not recover which was in agreement to the observations as recorded for DEA (Fig. 4; Fig. S3). The multiple correlation analyses as discussed in the next section

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

3.4. Microbial Variables and Climatic Treatments, a Structural Equation Modeling

Fig. 5 represents how the various environmental (soil moisture, NH_4, NO_3 etc.) and microbial parameters (gene abundances) influenced the global and functional activities of microbial communities at different levels of drought and snow removal as well as by their interaction. This initial SEM was constructed for each sampling interval to observe how the various environmental and microbial factors influenced the global and functional microbial activities over each period of time. Afterwards, we separately analyzed the most correlated parameters from the results of the linear regression modeling and these parameters were linked to fit the SEM for the drought, snow removal treatments but also for their interaction. The SEM revealed that the SIR was linked to the 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 never associated to the soil moisture and 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 the soil moisture with *nirS* as dominant denitrifiers except at T3 when it was *nosZ*. It was also observed that the overall impact of snow removal and drought was variable for the different denitrifiers with severe impact on *nirK* which could not recover even by the end of experiment. No significant effects of plant functional composition and fertilization on microbial parameters were recorded.

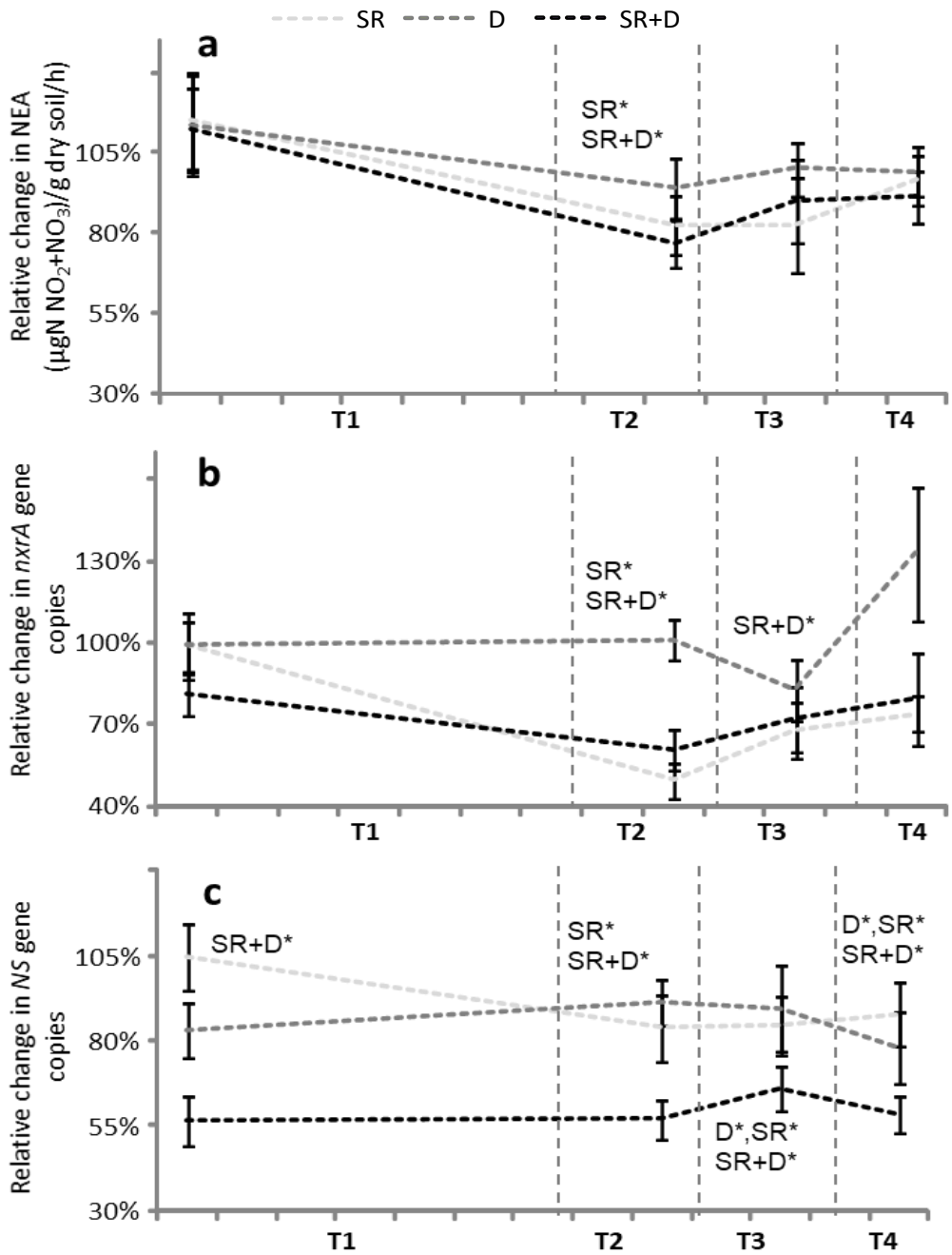

Fig. 3. a) Relative change compared to CTRL in **a)** nitrification enzyme activities (µgN-(NO_2+NO_3)/g dry soil/h); **b)** in abundance of *nxrA* nitrite reductases (number of *nxrA* gene copies g^{-1} dry soil); **c)** in abundance of *NS* nitrite reductases (number of *NS* gene copies g^{-1} dry 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; and the black dotted lines show the variations of cumulative SR+D treatment.

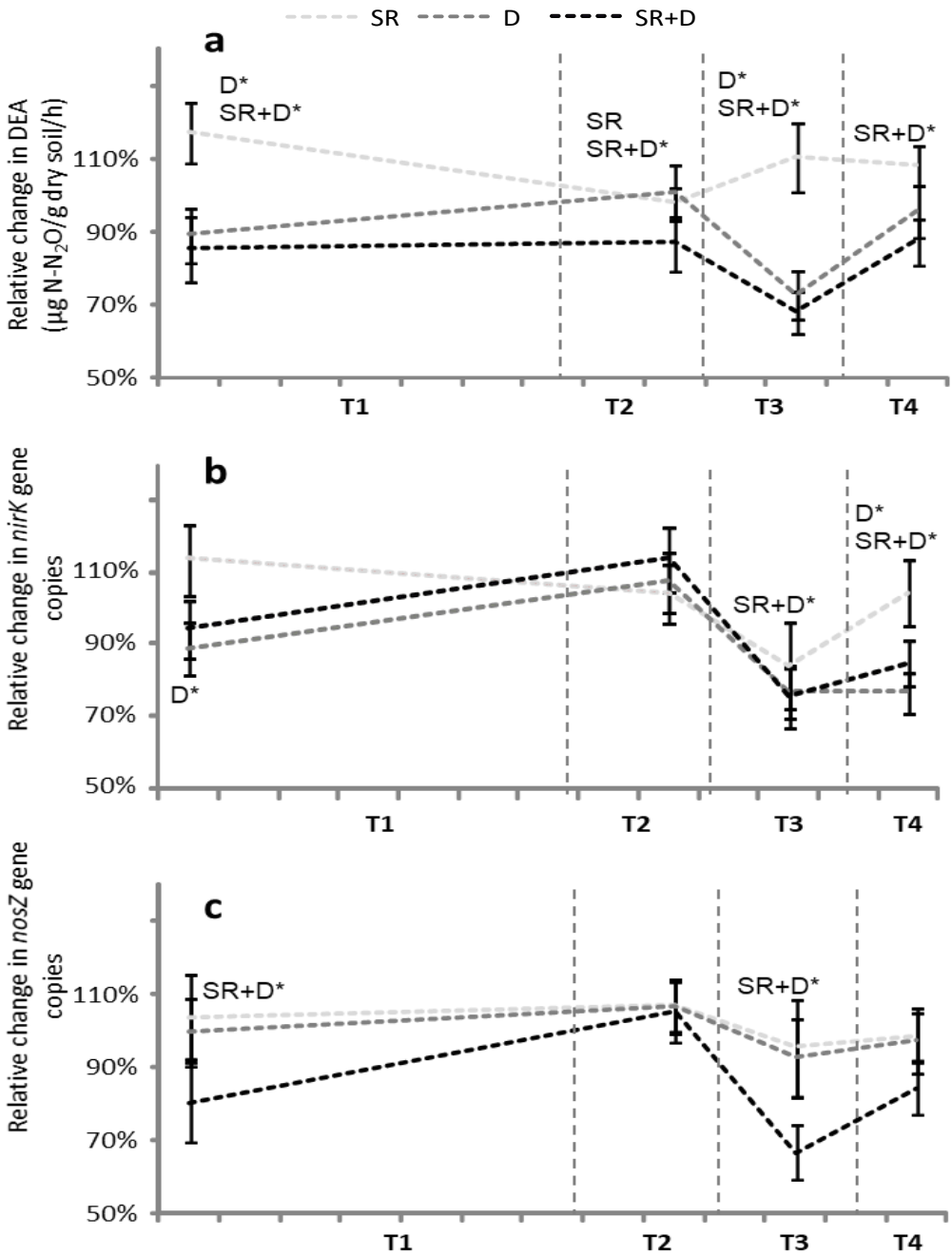

Fig. 4. a) Relative change compared to CTRL in **a)** denitrification enzyme activities (µg N-N_2O/g dry soil/h); **b)** in abundance of *nirK* denitrifiers (number of *nirK* gene copies g^{-1} dry soil); **c)** in abundance of *nosZ* denitrifiers (number of *nosZ* gene copies g^{-1} dry soil); Light 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; and the black dotted lines show the variations of cumulative SR+D treatment.

4. Discussion

Determining the resilience including the resistance and recovery capabilities at subalpine grassland ecosystems is crucial to understand since these ecosystems are under drastic influence of global climatic changes which ultimately may disrupt their functioning and the linked ecosystem services (Berauer et al., 2019; Bernard et al., 2019; Schirpke et al., 2013; Wipf and Rixen, 2010; Vittoz et al., 2009). This work demonstrates the impact of successive droughts and early snowmelt climatic extremes on subalpine grassland ecosystem functioning focusing on nitrification and denitrification as model processes since these give valuable insights into overall N cycling (Chen et al., 2020; Dai et al., 2020).

The quantification of resistance and recovery by comparing the relative change in D, SR, and SR+D treatments inferred how the microbial activities and the corresponding functional groups responded upon different climate change treatments. Broad scale function – SIR and the size of the total bacterial community - showed low resistance but high recovery for all treatments which strengthened the fact that the wide range of microbial communities are carrying this function generally exhibit a fast recovery (Ingrisch et al., 2020). SIR represents the broad scale microbial activity thus, in our study, disturbing a part of the global community could not affect the overall function greatly. Yet, the short term reduction in SIR upon droughts can be associated to the low microbial activity induced by the water deficit conditions (Bernard et al., 2019; Bloor and Bardgett, 2012). Earlier, Chaer et al. (2009) elaborated that the broad scale functions are less sensitive to disturbance. This is similar to the observations of weak impact of climatic stresses for decompostion processes in a joint experiment (Bernard et al., 2019), and high recovery of microbial biomass in other mountainous grasslands (Piton et al., 2020). These are in accordance to the functional redundancy concepts (Jia and Whalen, 2020; Griffiths and Philippot, 2013).

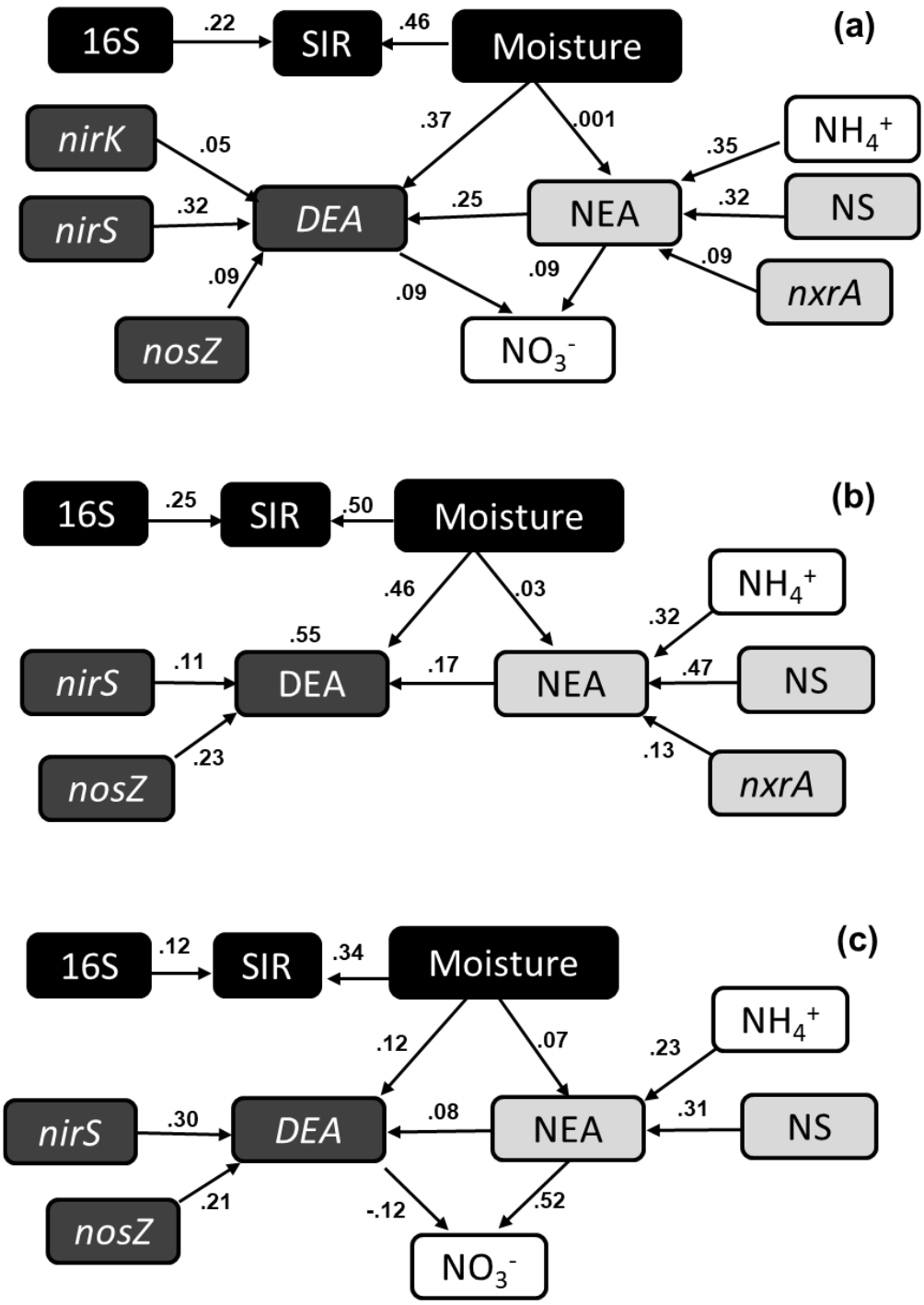

Fig. 5. Structural equation models (SEM) bridging the effects of different microbial and environmental factors. SEMs including the Substrate induced respiration MicroResp (SIR), nitrification (NEA), denitrification (DEA), gene community abundances (16S *rRNA*, *nxrA*, *NS*, *nirK*, *nirS*, *nosZ*) and environmental factors (soil moisture, NH_4, NO_3), in **a, b, c** are built using the samples 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.

The impact of extreme events including droughts on microbial N cycling are yet less clear (Fuchslueger et al., 2019), however the climate change treatments here showed contrasting impacts on N cycling processes with denitrification (community gene abundance and activity) showing less resistance to D as compared to nitrification (community gene abundance and activity). Moreover, at T3 when snow removal and droughts occurred in the same mesocosms (SR+D), they strongly reduced the DEA followed by an incomplete recovery, which was in contrast to the NEA that recovered compeletly at T4. This confirmed a strong influence of droughts and associated soil moisture deficits on DEA (Di et al., 2020; Philippot et al., 2009), which was especially pronounced for SR+D indicating the cascading effect of SR and declined nitrification upon snowmelt. The collapse in microbial pool size upon snowmelt has also been observed while studying the niche differentiation perspectives in soils (Sorensen et al., 2020). A strong turnover by these nitrifying and denitrifying microbial communities was previously shown for similar subalpine grasslands upon natural variations in snowpack depth (Jusselme et al., 2016). Such multiple cascading effects on N-related microbial activities are important since these can adversely impact the greenhouse gas emissions (i.e., N_2O in particular), soil fertility and plant productivity hence ask for adaptive management strategies (Griffis et al., 2014; Di et al., 2014; Bouwman et al., 2009).

The reduction in gene abundances of both nitrifiers and denitrifiers upon D and SR delineated the strong impact of studied climate change treatments on grasslands functioning. Although denitrifiers are reported as more tolerant to droughts (Hammerl et al., 2019) than nitrifiers (Austin and Strauss, 2010), here in agreement to DEA steadiness, the denitrifiers'

abundances were also negatively impacted by droughts, especially the second one. In line with DEA, the *nirK* denitrifiers' abundances were severely lowered and could not show a complete recovery at T4. Such correlations were previously recorded between DEA and the abundance of *nirK* denitrifiers upon variable snow depths under similar subalpine grasslands (Jusselme et al., 2016). Conversely, Wertz et al. (2007) found that denitrifiers were less affected by the heat stress in an agricultural soil and it was associated to the high functional redundancy. Besides, resilience of microbial communities is also influenced by the inherent soil edaphic characteristics (Berard et al., 2015). Although we found strong positive correlations between the DEA and %C, %N, DON, SOM, soil moisture, NEA, denitrifiers' abundances, interestingly these controlling factors shifted during the studied time. For example, in SR+D treatment, soil moisture along with *nirK*, *nirS* and *nosZ* were correlated to DEA ($r = 0.33, 0.15, 0.32, 0.38$; $p < 0.005$), while soil moisture and *nirK* had not shown any significant correlation with DEA ($r = 0.04$; $p > 0.64$) in the CTRL. This also shows the variable dominance of different denitrifiers upon D, SR and CTRL treatments. These different variables, across a range of environments, are shown to be differently correlated to DEA (Chen et al., 2020; Chen et al., 2019; Petersen et al., 2012; Chroňáková et al., 2009, Dong et al., 2009). At T4, DEA could not recover reflecting a severe impact of the subsequent warming caused both by D and the additive effect through SR+D. 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 case) and may shift to an irreversible new state (Wall, 2007), or at least may lead to altered recovery patterns (Seabloom et al., 2020). Furthermore, such changes in the relative abundances of the denitrifiers may also result in the selection of certain stable/unstable groups, which may consequently impact (increase) the N losses (NO_3 leaching or N_2O emissions) (de Vries and

Shade 2013). In addition, studies in other ecosystems have also suggested that these functional guilds may have contrasting resilience abilities against various disturbances (Capdeville et al., 2019).

Though SIR was related to soil moisture and *16S rRNA* when the SEM was fitted, NEA was highly linked to the soil NH_4^+ and the abundance of the *NS* nitrifiers hence showing a weak effect of soil moisture when droughts occurred. Higher abundance of *NS* nitrifiers than *nxrA* as well as the dominant role of *NS* nitrifiers under drought was earlier shown in low activity soils (Attard et al., 2010). However, DEA was related both to soil moisture and abundances of *nirS* denitrifiers throughout the experiment except at T3 when it was *nosZ*. After the second drought at T3, a cascading effect of SR might have shifted the community dynamics from *nirS* to *nosZ* with better adaptation of the *nosZ* harboring denitrifiers. The denitrifiers responded differentially, *e.g.*, in contrast to *nirK*, the *nirS* and *nosZ* were recovered, showing a community specific impact (Karlowsky et al., 2018) of drought and snow removal. The *nirK* were previously reported to be less abundant (Peterson et al., 2012), and relatively more sensitive to environmental change than *nirS* in agricultural soils (Szukics et al., 2019; Hallin et al., 2009). Though the findings implied by Dai et al. (2020) contrasted with these observations, seasonally driven drought effects have also been reported for *nirK*, *nirS* and *nosZ* dynamics in grassland ecosystems (Hammerl et al., 2019). Moreover, the denitrifiers harboring *nirK* genes here might not contain *nosZ* genes, in agreement to the discovery that not all denitrifiers contain *nosZ* (Hallin et al., 2018).

We did not find any significant effect of plant functional composition and fertilization on microbial parameters; hence these were not the main drivers of the studied processes but there might be a trait-centered influence of different variables (Griffin-Nolan et al., 2019). For example, leaf or root specific traits, rather than plant composition, might have affected the nutrient availability and ultimately associated microbial processes. Interestingly under similar

grasslands, no direct effect of fertilization was observed but only specific plant traits were affecting the same microbial activities (Legay et al., 2016). This may also suggest a competition for nutrients between plants and microbes resulting in a non-significant fertilization effect on the microbial parameters during the study period. However, such management strategies including fertilization practices may also favor certain microbial communities consequently causing their fast resilience to stress conditions (Piton et al., 2020). It is noteworthy that due to the climatic extremes, the responses showed by the various nitrifiers' and denitrifiers' groups were different to CTRL conditions 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 the ecosystem N cycling (Legay et al., 2013; Grigulis et al., 2013, Le Roux et al., 2013).

5. Conclusions

This work clearly demonstrated a strong and unprecedented impact of early snowmelt and recurrent droughts, alone or in combination, on soil microbial communities both at broad (i.e., soil respiration) and functional (nitrifiers' and denitrifiers' activities and abundances) scales. The droughts showed a low and short-term impact on bacterial total respiration supporting their assumed high resistance and ability to recover. Though the nitrification and community gene abundances of the corresponding functional guilds exhibited relatively strong resistance to summer droughts, it declined in response to early snowmelt leaving the strong impact on denitrification. The findings inferred that the predicted climatic extremes may carry severe consequences for the soil microbial community functioning in subalpine grasslands ultimately resulting in shifts in N availability, plant community diversity, and associated ecosystem services.

Conflict of Interest

The authors of this article declare that they have no financial conflict of interest with the content of this article.

Acknowledgements

This work was part of the ERA-Net BiodivERsA project REGARDS (ANR-12-EBID-004-01). Quantitative PCR were carried out at the platform DTAMB (IFR 41, Université Lyon 1) and N cycling activities at the platform AME (UMR5557-UMR1418). We want to thank all the people who helped us in the field or in the lab: C. Arnoldi, A. Foulquier, N. Guillaumaud. This research was conducted at 'Jardin du Lautaret'(UMS 3370 Univ. Grenoble Alpes – CNRS), a member of the Fédération FREE-Alpes and of the AnaEE-France (ANR-11-INBS-0001AnaEE-Services), and on the LTSER platform 'Zone Atelier Alpes', a member of the ILTER-Europe network.

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Supplementary Figures

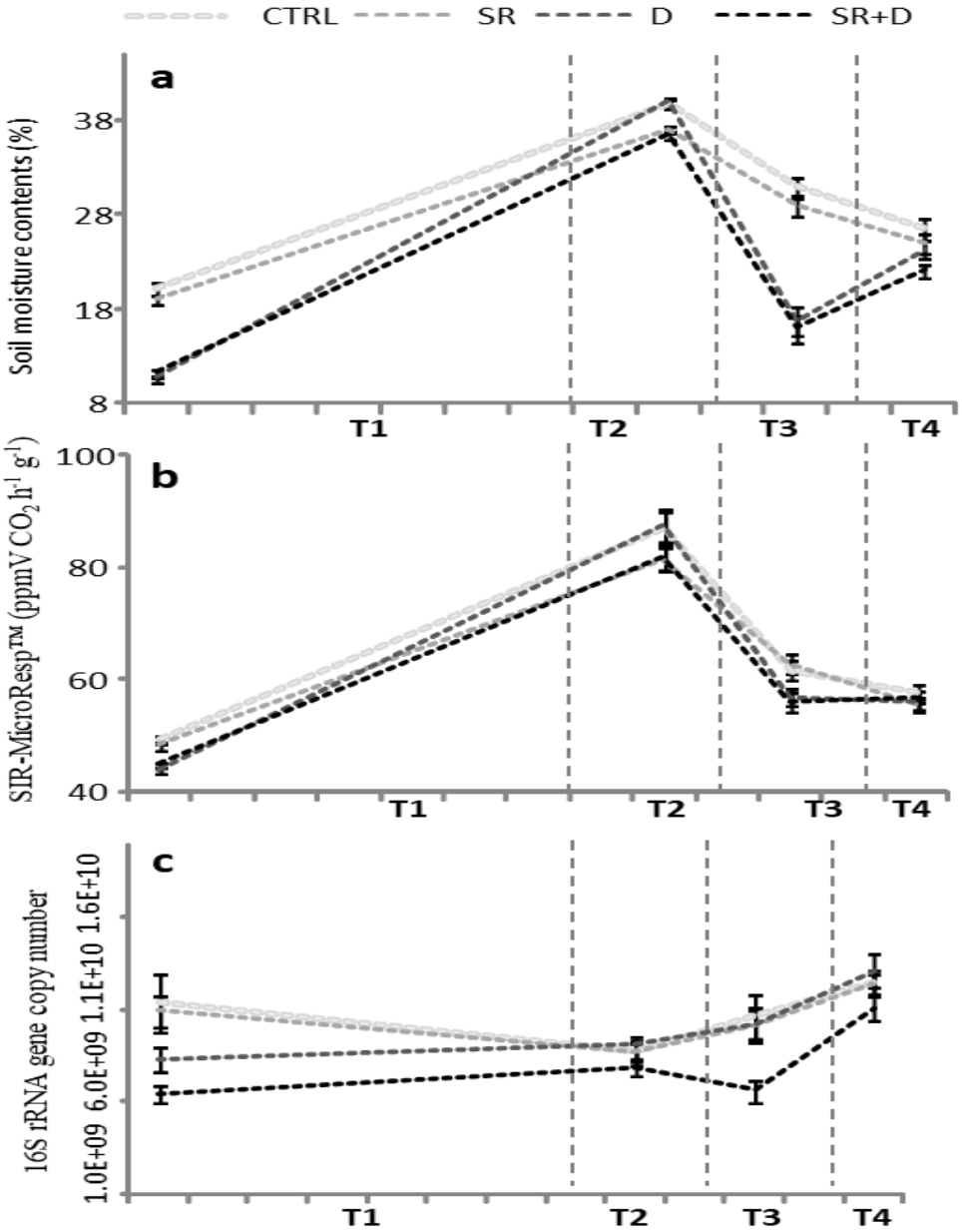

Fig. S1. a) Soil moisture in percentage of dry soil; **b)** SIR-MicroResp™ (ppmV CO_2 h^{-1} g^{-1}); **c)** Abundance of total bacterial community (number of 16S *rRNA* gene copies g^{-1} dry soil).

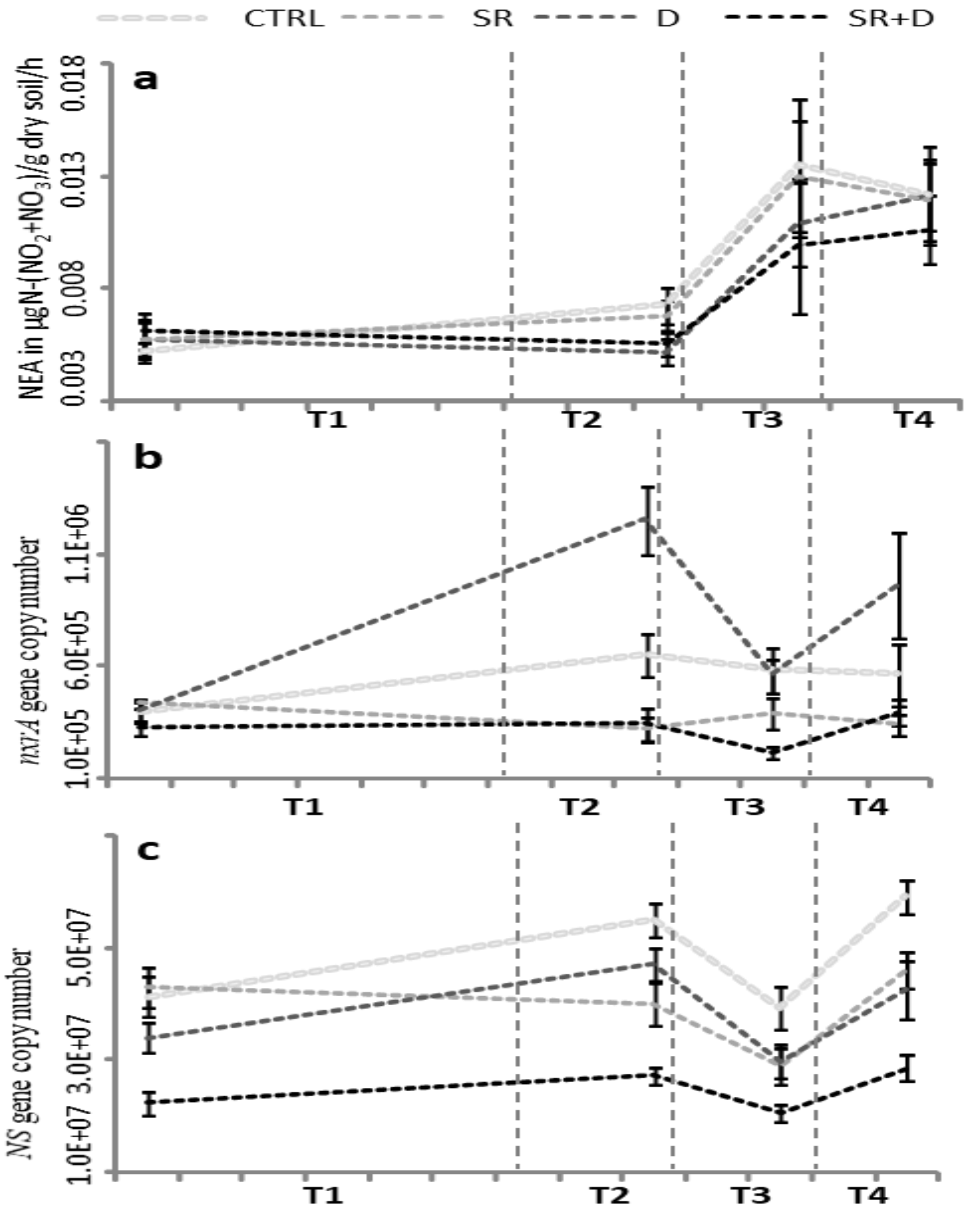

Fig. S2. a) Nitrification enzyme activities quantified in µgN-(NO_2+NO_3)/g dry soil/h for different treatments at various sampling dates; **b)** Abundance of *nxrA* nitrite reductase (number of *nxrA* gene copies g^{-1} dry soil) for different treatments at various sampling dates; **c)** Abundance of *NS* nitrite reductase (number of *NS* gene copies g^{-1} dry soil) for different treatments at various sampling dates.

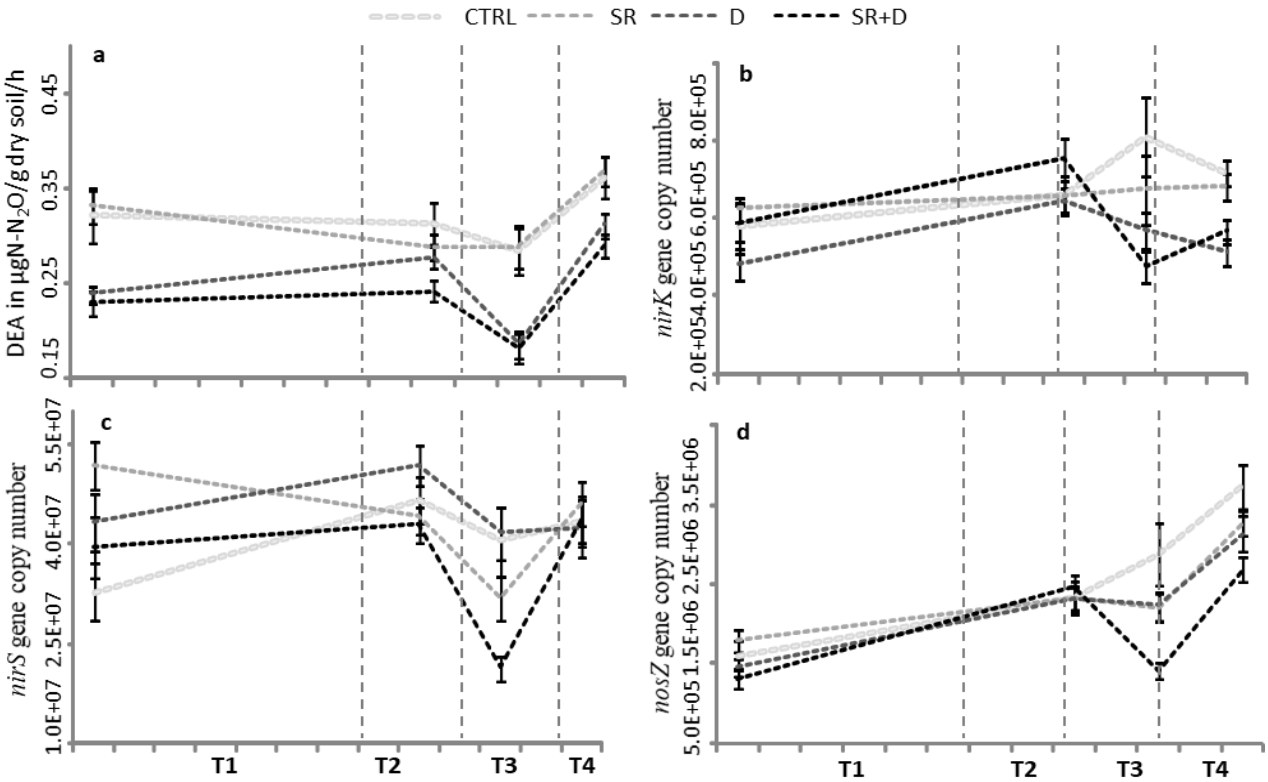

Fig. S3. a) Denitrification enzyme activities determined in µg N-N_2O/g dry soil/h for different treatments at various sampling dates; **b)** Abundance of *nirK* denitrifiers (number of *nirK* gene copies g^{-1} dry soil); **c)** Abundance of *nirS* denitrifiers (number of *nirS* gene copies g^{-1} dry soil); **d)** Abundance of *nosZ* denitrifiers (number of *nosZ* gene copies g^{-1} dry soil) for different treatments at various sampling dates.