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# High resistance of soils to short-term re-grazing in a long-term abandoned alpine pasture

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## ABSTRACT

Grazed alpine pastures have shaped landscapes of the European Alps for millennia, but have partially been abandoned since the 1950s. Re-grazing of abandoned pastures could preserve this cultural landscape with its high species diversity, but also alter soil carbon and nitrogen cycles, as well as microbial communities, potentially affecting ecosystem services (e.g., water purification, carbon and nitrogen storage). However, there is a lack of information on the resistance of soil characteristics to re-grazing effects. After characterising the distribution of vegetation types of an abandoned pasture in the German Alps, we investigated short-term effects of re-grazing on soil organic carbon and nitrogen biochemistry, soil microbial communities, and water quality along a gradient of grazing intensity. The abandoned grassland studied presented a remarkably high diversity of species and habitats even 60 years after abandonment. It was also found to be particularly rich in terms of microbial biomass, as well as in soil carbon and nitrogen. A few months after re-grazing started, extractable organic carbon, gross nitrogen mineralisation rates and inorganic nitrogen concentrations were increased only in intensively grazing-affected areas with bare soil (i.e. concentration of cows with excreta inputs), which insignificantly contributed to the overall area. Re-grazing did not affect the microbial abundance, whatever the grazing intensity, but induced a community shift towards a smaller proportion of fungi compared to bacteria and an increase of ammonia oxidizers (archaea/bacteria) under bare soil conditions. Concentrations of dissolved organic carbon and nitrate in the draining creek remained very low. Overall, re-grazing of pastures in the first season had very limited effects on microbial communities and associated carbon and nitrogen turnover and concentrations, highlighting the resistance of the studied alpine soils to extensive re-grazing. Our results indicate how to develop sustainable management strategies that preserve alpine pastures from degradation.

## 1. Introduction

Traditionally, grazed pastures (called “Alm” or “Alp” in the Alpine region of central Europe) have been shaping big parts of the landscapes of the European Alps for millennia (Ringler, 2010; Bätzing, 2015). The European Alps are recognized as one of the six key biogeographical zones for plant and animal species, as well as habitats in the European Habitats and Species Directive (92/43/EEC) (MacDonald et al., 2000). Grasslands of the Alps also play an essential role in providing

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ecosystem services such as carbon (C) and nitrogen (N) storage, water purification, as well as aesthetic and recreational values (Lamarque et al., 2011; Schirpke et al., 2016; Vigl et al., 2016; Kohler et al., 2017; Kühnel et al., 2019). However, alpine pastures, especially those on steep slopes, have largely been abandoned since the 19th century (abandonment rate >60%) (Zimmermann et al., 2010) mainly due to the intensification of agricultural practices in the valleys and for economic reasons (Gellrich et al., 2007). Without grazing, the highly diverse plant communities of pastures are replaced by tall grass swards and ultimately by secondary forest. These changes in plant communities often lead to a decline in biodiversity and conservation value (Tasser and Tappeiner, 2002; Maurer et al., 2006; Spiegelberger et al., 2006; Kampmann et al., 2008; Peco et al., 2012), although the magnitude of decline is highly variable depending on the landscape context (Zimmermann et al., 2010).

The impacts of abandonment and re-grazing on soil functions, and especially soil organic matter (SOM) quality, storage, and turnover are still uncertain and vary according to grassland type, time since land use change and management intensity (Meyer et al., 2012; Rumpel et al., 2015). Grazing cessation in grasslands of the Alps tends to increase aboveground plant biomass (Gamper et al., 2007; Tappeiner et al., 2008; Puissant et al., 2017). The resulting litter accumulates due to its low decomposability (Zeller et al., 2001; Gamper et al., 2007) and less labile C is transferred to the rhizosphere (Karlowsky et al., 2018). The low quality of the litter (e.g., high C:N ratio and lignin content) in abandoned grasslands generally leads to a decrease of microbial biomass (Bardgett et al., 1997) and an increase of the fungal-to-bacterial ratio (Donnison et al., 2000; Zeller et al., 2001; Sjögersten et al., 2011). Furthermore, abandonment favoured spontaneous reforestation of high elevation pastures in recent decades, a trend that is accelerated by climate change (Tasser et al., 2007; Schirpke et al., 2017). Reforestation of mountain pastures would lead to a decrease of the forage quantity, the overall plant and animal biodiversity including habitat loss of endangered animal species such as grouse, as well as potential decrease of touristic values (Sitzia et al., 2010; Schirpke et al., 2017). Although spontaneous reforestation could possibly help to mitigate climate change via C storage mainly in the above-ground biomass, it can induce a critical loss of biodiversity, as highlighted in the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) report (Scholes et al., 2018).

The above-mentioned studies mostly reported on changes occurring after abandonment. However, very few papers addressed the resistance of ecosystems, i.e. the capacity of "staying essentially unchanged despite the presence of disturbances" (Grimm and Wissel, 1997), under future land use change scenarios (Pykälä, 2003; Schirpke et al., 2017; Tasser et al., 2017). Kohler et al. (2017) highlighted the development of extensive pastures by re-grazing as a plausible land-use option to preserve the high species diversity of the Stubai Valley in Austria. In addition, grazing with robust and slow growing cattle can also induce an increase of plant species richness (Pauler et al., 2019). While current knowledge of effects of extensive grazing is largely derived from abandonment studies, there is a lack of information on the effects of extensive re-grazing of abandoned mountain pastures on vegetation and soil characteristics, biogeochemical cycles, and microbial communities. Grazing of formerly abandoned grasslands can alter the physical properties of soil via trampling, generally leading to a compaction of soil (Steffens et al., 2008; Wen et al., 2016). Further changes in nutrient cycling and thus soil fertility are expected due to grazing. The effects of grazing on soil fertility are either direct via dung- and urine-borne nutrient inputs and nutrient redistribution (Peco et al., 2006, 2017), which increase the amounts of rather labile organic matter and N in soil (Bardgett and Wardle, 2003; Abdalla et al., 2018), or indirect via the modification of plant species composition, richness, and functional structure (Bardgett and Wardle, 2003; Valencia et al., 2015). Soil fertility also affects soil microbial communities whereby animal excreta inputs tend to increase microbial biomass and activity (e.g., Bardgett et al., 1997). Consequently gross N mineralisation and nitrification, and thus mineral N availability as well as N losses along hydrological and gaseous pathways, can increase (Bardgett et al., 1997; Mikola et al., 2009; Cai et al., 2017; Peco et al., 2017). However, depending on management, soil and climate, the grazing-induced increase of labile organic matter and nutrients does not necessarily lead to nutrient leaching at the expense of the water quality (Tamminga, 2003; O'reagain et al., 2005; Wu et al., 2011) or to increased soil nitrous oxide emissions (Wolf et al., 2010). While spatio-temporal variations (e.g. soil temperature and moisture, soil parameters) tend to have an overall greater effect on soil microbial biomass compared to grazing (Bardgett et al., 1997, 1999; Zeller et al., 2001), grazing or aboveground biomass harvest can significantly alter the microbial community structure and decrease the fungal-to-bacterial ratio (Bardgett et al., 1996; Zeller et al., 2001; Fuchslueger et al., 2019). The aforementioned studies exposed the impact of grazed vs abandoned pastures on soil and water characteristics but did not address how re-grazing of abandoned alpine grassland would affect soils.

Our study investigated short-term effects of extensive re-grazing of a long-term (> 60 years) abandoned mountain pasture in the German Alps (Brunnenkopfalm: 1500–1700 m.a.s.l.) on multiple soil characteristics. After characterising the distribution of vegetation types and the spatial heterogeneity of grazing, we examined re-grazing effects on soil microbial communities, C and N turnover and contents, as well as water quality. We hypothesised that re-grazing will (1) enhance the amount of extractable organic matter due to animal excreta inputs and trampling, (2) increase the abundance and alter the structure of microbial communities, resulting in (3) functional changes of the N cycle with increased gross N mineralisation and nitrification, leading to an increased mineral N availability in the soil. This will eventually result in increasing dissolved organic C (DOC) and nitrate ($NO_3^-$) loads in the creek water draining the grazed area. In order to gain a functional understanding of soil responses to re-grazing, we used a wide array of techniques to characterize soil biogeochemical properties (salt-extractable and total organic C (OC), gross N turnover rates, soil mineral N availability), as well as the abundance and characteristics of microbial communities (microbial biomass, phospholipid-derived fatty acids (PLFA) analysis, abundance of N-related microbial communities).

## 2. Methods

### 2.1. Experimental site

In May 2018, we set up a grazing experiment at Brunnenkopfalm (1500–1700 m.a.s.l.) in the Northern Limestone Alps (Ammergauer Alpen, Germany, 10.9188°W, 47.5818°N). The Brunnenkopfalm is located on south-exposed slopes of the crest between the Klammspitze and the Brunnenkopf summits in the lower subalpine zone. The slopes are relatively steep with inclinations up to 40°. The dominating parent material is dolomite with minor areas of sandstone and conglomerates that form the crest and the eastern part of the pasture (not part of the re-grazed area and this study). A heterogeneous distribution of soils of varying soil depth was found that were characterized as Leptosols and Cambisols (WRB, 2014) (general characteristics presented in Table 1). The development of soils is affected by planar erosion ("Blaiken") and deposition of eroded material from the sandstones and conglomerates forming the crest above the pasture.

In general, the climate of Northern Limestone Alps can be classified as a transition from Köppen–Geiger climate type *Cfb* to *Dfb* (oceanic to hemiboreal climate) (Kottek et al., 2006). In 2018, the cumulative precipitation during the period April – October at the nearby agro-meteorological station Ettal-Linderhof (940 m.a.s.l.) was 851 mm (Bavar-

**Table 1**
General climatic and soil characteristics (0-5 cm, ungrazed plots) on the pilot experiment. The monthly mean climate data were obtained from the meteorological station of Brunnenkopfhütte, with the exception of the precipitation data taken from the Ettal-Linderhof station, during the month of July and October 2018.

| | July 2018 | October 2018 |
|---|---|---|
| **Climatic characteristics** | | |
| Air temperature (°C) | 13.8 | 8.5 |
| Precipitation (mm) | 95 | 78 |
| Air Humidity (%) | 73.4 | 69.1 |
| Global radiation ($W\ m^{-2}$) | 219.7 | 141.6 |
| **Soil characteristics** ($n = 8$) | | |
| pH | 6.6 ± 0.6 | |
| Organic carbon ($mg\ g^{-1}$) | 142.4 ± 25.9 | |
| Inorganic carbon ($mg\ g^{-1}$) | 31.8 ± 37.7 | |
| Nitrogen ($mg\ g^{-1}$) | 14.0 ± 3.1 | |
| OC:N ratio | 10.3 ± 0.9a | |

ian-State-Agency-for-Environmental-Protection, 2019), and the mean temperature obtained from station Brunnenkopfhütte was 10.8 °C. More specific climate data for the sampling months (July and October) are available in Table 1.

The Brunnenkopfalm was first mentioned in a document in 1454 and used for grazing of around 50–60 young cattle until the 1920s. During the Second World War, the greater Alm area (roughly 44 ha in total) was intensively grazed by up to 141 young cattle before it was abandoned in 1955 (Heigl, 2016). In 2018, 4.2 ha were fenced and re-grazing was induced between May 30 and August 31 by a herd of 5 cattle of rustic, local and endangered breeds (Murnau-Werdenfelser and Tiroler Grauvieh breeds, approx. 1.2 LU/ha. Before the start of the re-grazing experiment five fenced grazing exclosures (control plots) with a size of 100 $m^2$ each were created (Fig. 1). For our experiment on the short-term effects we considered the western part of the 2018 grazing area (3.4 ha) and excluded the fifth replicate (the westernmost fenced area and its surrounding), as the eastern part and fifth replicate area were not grazed at the time of the first sampling in June.

### 2.2. Vegetation releves and biotope mapping

Plant species composition, vegetation and biotope types on the experimental site were characterized by Fütterer and Ewald (2019). Vegetation releves were conducted on 3 × 3m plots (in total 57 plots distributed over the fenced area). The mapping of biotopes and vegetation types was based on the methodology described by the Bayerisches-Landesamt-für-Umwelt (2018a),(Bayerisches-Landesamt-für-Umwelt, 2018b).

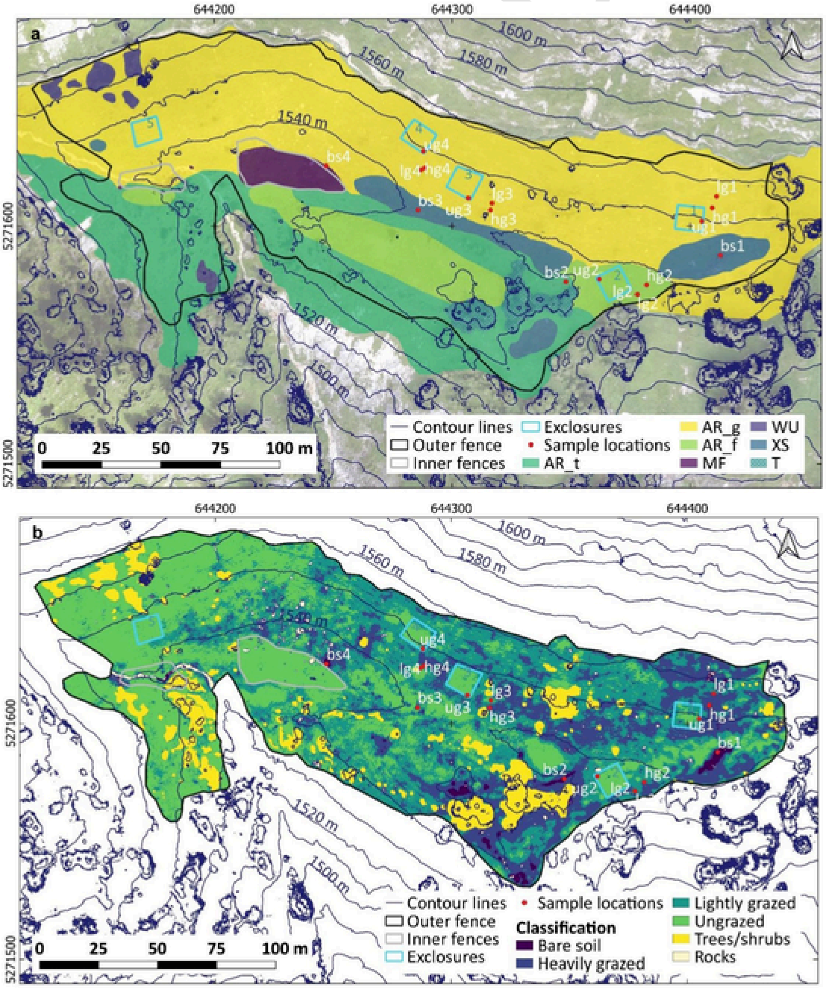

**Fig. 1.** Biotope types (**a**) and grazing intensity classes (**b**) on the pilot experiment of the Brunnenkopfalm. The different biotope types correspond to: alpine grassland (AR), with typical (AR_t), grass dominated (AR_g), and forb dominated (AR_f) variation; a complex of fen and spring fen (MF); mountain pine shrub (WU); megaforb (XS). T represents closed tree canopy. Four replicates were sampled per grazing intensity: ungrazed (ug); lightly grazed (lg); heavily grazed (hg); bare soil (bs). Used coordinate reference system: ETRS89/UTM zone 32 N (EPSG: 25832).

### 2.3. Sampling and sample preparation

Considering this grazing-induced heterogeneity, we collected soil samples along a gradient from ungrazed (i.e. control) to bare soil areas. The locations of the sampling spots were chosen in a way to represent four quantitative and visually distinguishable grazing intensities at the site: ungrazed, lightly grazed, heavily grazed and bare soils.

- Ungrazed zones were defined based on the fenced control areas where the cows did not have any access, i.e. the vegetation was at full height.
- Lightly grazed zones were defined as only partly grazed vegetation with mean standing height comprised between 10 and 20 cm.
- Heavily grazed corresponded to almost entirely grazed vegetation with only few cm height left so that the organic layer of greyish colour was visible.
- Bare soil is characterized by complete absence of vegetation due to trampling in the cow´s preferential dwelling spots.

The distinction of grazing intensity classes was made in the field, considering the RGB image of the area, and was later confirmed by the analysis of the RGB image (see Unmanned Aircraft Systems (UAS)-based remote sensing. Two (July 24th, 2018) and five (October 9th, 2018) months after the beginning of grazing, topsoil samples (0–5 cm) were taken from the four grazing intensity classes, with four replicated sampling spots for each class. The sampling spots were approximatively the same for both sampling dates. Sampling spots for the grazing classes ungrazed, lightly grazed and heavily were all situated in alpine grassland biotopes on slope with the same exposure. However, bare soil was not present in alpine grassland biotopes so that these sampling spots were located in other biotopes (Fig. 1).

At each sampling spot, five undisturbed soil samples were sampled (around 10 cm from each other) using stainless steel cylinders ($d = 5.6$ cm, $h = 4$ cm, $V = 100$ cm$^3$) at a sampling depth of 0–5 cm. Undisturbed samples were directly processed in the field for gross N mineralisation and nitrification analyses (four cores), while the fifth core was used for determination of soil inorganic N background concentrations and gravimetric water content.

Two further disturbed samples (~ 1.5 kg each) for SEOC, PLFA, C and N analyses, as well as for DNA extraction and qPCR analysis (~ 200 g), were collected adjacent to the intact cores. Samples for DNA extraction and qPCR analysis were stored in dry ice directly in the field. Other samples were kept at 20 °C overnight, sieved at 5 mm and sorted for coarse roots the next day. Fresh soil subsamples were used for microbial biomass and SEOC analyses, while freeze-dried subsamples were used for PLFA analyses, as well as for C and N analyses.

### 2.4. Unmanned Aircraft Systems (UAS)-based remote sensing

In July 18th, 2018, the grazing experiment was surveyed by an UAS in order to obtain high resolution aerial images of the study area. We used a fixed wing drone (eBee, Sensefly) equipped with an RGB camera (Sony DSC-WX220, 18 M P resolution). Processing of single images to generate an RGB image and a digital surface model (DSM) with a spatial resolution of 0.05 m was performed with Pix4Dmapper software.

The RGB image was used to estimate grazing intensity classes in the study area on the date of image acquisition. We aimed at classifying the image using the following land cover/land use classes: trees/shrubs, rocks, and the four qualitative grazing intensity classes (ungrazed, lightly grazed, heavily grazed, bare soil) used in soil sampling (see sub-chapter Sampling and sample preparation).

First, we manually digitized the visually identifiable trees and shrubs using the RGB image and the DSM. Then, we calculated the Red Green Blue Vegetation Index (RGBVI) as introduced by Bendig et al. (2015) and used it together with the blue band to define the remaining land cover/land use classes based on certain thresholds (**Table A**). Different threshold values were tested to identify those that best represent the visual differences between the classes in the RGB image. For example, RGBVI values ≤ 0.12 represented well areas covered with rocks and bare soil. To further differentiate between rocks and bare soil the blue band was used (**Table A**). In a last step the different land cover/land use classes were transformed to vector layers and the area of the class polygons was calculated. It has to be noted that the used classification approach aimed at providing a rough estimation of the land cover/land use based on simple thresholds of RGB data. While this classification is acceptable for distinguishing rocks, bare soil and heavily grazed areas, the distinction between lightly grazed and ungrazed areas is limited. One reason is that the RGBVI was originally designed for aboveground biomass estimation (Bendig et al., 2015). For the estimation of grazing intensity classes we assumed that a higher grazing intensity is related to lower biomass. However, variations in biomass can also be caused by other factors than differences in grazing intensity, e.g. by different species composition. Therefore, e.g. some areas in the un-grazed reference plots could be classified as lightly grazed due to naturally induced lower biomass values. Nonetheless, general spatial patterns were detected well, e.g., the fenced areas were largely detected as ungrazed as well as the entire western part of the study area, which had not been grazed by the cattle at the time of UAS flight as reported by the permanent shepherds. The QGIS software (version 3.0.0-Girona) was utilized for the further processing and analysis of the RGB image.

### 2.5. Bulk C and N analyses and salt-extractable organic C (SEOC)

Freeze dried soil samples were analysed for organic carbon and total N concentrations by dry combustion in duplicate using an elemental analyser ("vario MAX cube", Elementar, Langenselbold, Germany). To evaluate the amount of a OC pool comparable to dissolved organic matter and thus presumably more bioavailable, we extracted the soils using 0.05 M $K_2SO_4$, following a similar procedure as described in Mueller et al. (2012), yielding a salt extractable OC (SEOC). The SEOC extracted with $K_2SO_4$ was reported as more similar to the natural dissolved organic matter compared to deionized water and $CaCl_2$ extraction (Rennert et al., 2007). In brief, 100 *g* of fresh soil were shaken overhead with 400 mL of $K_2SO_4$ solution for 10 min at constant room temperature (20 °C), centrifuged and filtered through polypropylene membrane filters (GH Polypro, Pall; 0.45-µm mesh size). The extracted total OC was determined using a TOC-5050A (Shimadzu, Kyoto, Japan) which measures non-purgeable organic carbon using a non-dispersive infrared detector after high temperature combustion.

### 2.6. Gross nitrogen turnover rates and soil inorganic N concentrations

Gross N mineralisation and nitrification rates were determined using the $^{15}N$ pool dilution technique with intact soil cores in the field as described for similar soil by Wang et al. (2016a). In brief, two adjacent intact soil cores (100 cm$^3$) were labelled with $^{15-}NH_4^+$ solution and another two with $^{15}NO_3$- solution using a multi-injector (30 injections of 0.1 mL each per core in 3 depths) directly in the field. The injected solutions were isotopically enriched to 30 atom % $^{15}N$. Total N addition amounted to 3 mg N $kg^{-1}_{SDW}$. A first extraction ($t_1$ cores) was conducted approximately two hours after $^{15}N$ labelling, while a second extraction ($t_2$ cores) followed approximately 22 h after $^{15}N$ labelling. The $t_2$ cores were reburied after $^{15}N$ labelling at the sampling spots in the field for *in situ* incubation until extraction the following day. The $^{15}N$ labelled soil was extracted by submerging the entire content of the soil cores in 120 mL of 0.5 M $K_2SO_4$ and shaking in a plastic bottle. Extracts were further transported to the laboratory of KIT Campus Alpin in Garmisch-Partenkirchen within 3 h and were shaken there at 150 rpm for 60 min, followed by vacuum filtration and syringe filtration to 0.45 µm (Wang et al., 2016a). Filtered extracts were frozen with one subsample of >30 mL being used for analysis of $^{15}$N enrichment in $NH_4^+$-N (for determination of gross N mineralisation) or $^{15}NO_3^-$—N (for determination of gross nitrification) using diffusion on acid traps for subsequent analysis of $^{15}$N enrichment with coupled elemental analysis (Flash EA1112; Thermo, Bremen, Germany) - isotope ratio mass spectrometry (Delta Plus XP; Thermo) at the Center of Stable Isotope Analysis of KIT-IMK-IFU (Wang et al., 2016b). A further subsample of the filtered extracts was analysed for soil $NH_4^+$-N, $NO_3$--N and $NO_2$--N concentrations by continuous flow analysis using a photometric autoanalyzer (CFA-SAN Plus; Skalar Analytik, Germany). The fifth unlabelled soil core was used for determination of soil inorganic background concentrations using the same extraction procedure. Gross rates for N mineralisation and nitrification were calculated using isotope pool dilution approaches according to Dannenmann et al. (2016).

### 2.7. Microbial biomass

The microbial biomass was measured using the fumigation-extraction method adapted from Vance et al. (1987). In brief, 5 g of fresh soil were submitted to chloroform vapour for 16 h and subsequently extracted with 20 mL of 0.05 M $K_2SO_4$ solution, together with the non-fumigated samples. The extracted OC was determined for the fumigated and non-fumigated samples by the C analyser TOC-5050A (Shimadzu, Kyoto, Japan). The extracted microbial C ($E_c$) expressed in mg C $kg^{-1}{}_{SDW}$ was calculated by applying Eq. (1).

$$\text{Microbial biomass} = (\text{CF} \times \frac{V_{K2SO4} + V_{sw}}{m_s} - \text{CT} \times \frac{V_{K2SO4} + V_{sw}}{m_s}) \times \text{kEc} \quad (1)$$

Where $C_F$ is the C extracted from the fumigated soil, $C_T$ is the C extracted from the control/non-fumigated soil, is the volume of $K_2SO_4$ in ml, is the volume of water in the soil in ml, is the dry soil mass in g and kEc is the conversion factor (0.45).

### 2.8. Phospholipid-derived fatty acids (PLFA) analysis

We quantified and classified microorganisms using analyses of phospholipid-derived fatty acids (PLFA) as developed by Frostegård et al. (1991) and modified by Baumert et al. (2018). Briefly, lipids were extracted from 0.5 g of soil with Bligh & Dyer solution (i.e. a methanol:chloroform:citrate buffer solution (2:1:0.8; v/v/v)) and subsequently separated using a silica column (0.5 g SiOH, Chromabond ®, Macherey-Nagel, Düren, Germany). The PLFA fraction was collected and subjected to alkaline methanolysis. The resulting fatty acid methyl esters (FAMEs) were analysed using a gas chromatograph with flame ionization detection (GC-FID) and equipped with a ZB-5HT fused silica capillary column (60 m, 0.25 I.D., 0.25 µm film thickness; Phenomenex LTD, Aschaffenburg, Germany). We used nonadecanoic acid methyl ester (19:0) as an internal standard to calculate the PLFA concentrations and normalized them to the mean long-term results of a standard soil extracted in parallel (2 g of soil). PLFAs were classified as bacterial biomarkers (i15:0, a15:0, i16:0, i17:0, cy17:0, cy19:0, 15:0, 16:1n7 and 17:0) and fungal biomarkers (18:2n6 and 18:1n9). The bacterial PLFAs were further separated into Gram-positive (GP: i15:0, a15:0, i16:0, i17:0) and Gram-negative (GN: cy17:0, cy19:0) bacteria to calculate the GP-to-GN ratio. The fungal and bacterial PLFA contents were used to express the PLFA-based fungal-to-bacterial ratio. Bacterial and fungal biomarkers were summed together with the unspecified microbial PLFAs 14:0, 16:0, 18:1n9, 18:1n9t, 18:0 and 20:0 to obtain total microbial PLFA content.

### 2.9. DNA extraction

DNA was extracted from 0.5 g fresh weight of soil samples following a modified phenol-chloroform extraction procedure (Lueders et al., 2004; Töwe et al., 2011) and using the Precellys24 Instrument (Bertin Technologies, France). DNA concentrations were determined by absorbance measurements at 260 nm using a spectrophotometer Nanodrop (PeqLab, Germany) and the extraction quality of all the samples was estimated by absorbance ratio calculations ($A_{260}/A_{280}$ and $A_{260}/A_{230}$). DNA concentrations were in the range of 859–3933 ng $g^{-1}$ SDW. DNA samples were stored at −20 °C until further processing.

### 2.10. Quantitative real-time PCR assay

Nitrogen related genes of microbial mineralizers (*apr* and *chi*A genes) and ammonia oxidizers (*amoA* of ammonia-oxidizing archaea (AOA) and bacteria (AOB)) in soil were quantified by quantitative Real-Time PCR (qPCR). Four separated qPCRs were carried out in 96-well plates (Applied Biosystems, Germany) using SYBR green as fluorescent dye and performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany). A pre-experiment was first conducted to avoid PCR inhibition, this resulted in an optimal sample dilution of 1:128 for all the samples (data not shown). Details on the marker genes and qPCR conditions are described in Table B. Serial plasmid dilutions ($10^1$ – $10^7$ gene copies $ml^{-1}$) were used for standard curve calculations. The PCR efficiencies of the amplifications were calculated as described in Töwe et al. (2010) and resulted respectively for each marker gene in the following values: for alkaline protease (*apr*) 82%, chitinase (*chi*A) 94%, AOA 88% and AOB 95%. The coefficient of determination ($R^2$) of the standard curves was determined to be above 0.99 for each qPCR. The specificity of the amplified products was checked by melting curves of the amplicons and 2% agarose gels.

### 2.11. Water temperature, nitrate and DOC concentrations in drainage water

The grazed area is drained by a perennial spring with a mean discharge of 0.65 L $s^{-1}$. An open spring capture (30 × 20 cm) was built containing a *UV/Vis spectrometer* probe (spectro::lyser, s::can Messtechnik GmbH, Vienna, Austria) for continuous measurement of $NO_3$- and DOC concentrations as well as water temperature (Jacobs et al., 2018). Nitrate concentrations were determined as described above. Manual discharge measurements were conducted by measuring the time needed to fill a pot of defined volume.

### 2.12. Statistical analyses

Statistical analyses were performed with the R statistical software (Team, 2013) with the package `agricolae´. As most data were not normally distributed (Shapiro-Wilk test), we used the non-parametric Kruskal-Wallis test followed by a pairwise comparison of groups to compare treatment (ungrazed, lightly grazed, heavily grazed, bare soil) and the Wilcoxon test to analyze the time effect (July or October). For all analyses, statistical significance was set as α = 0.05. The Spearman coefficient of correlation, calculated from not normally distributed ranked data, was computed between all the variables investigated and displayed by using heat maps with the, `readxl´ and `corrplot´ packages.

## 3. Results

### 3.1. Spatial heterogeneity of vegetation types and grazing intensities

Before the onset of re-grazing, four vegetation type were distinguished and mapped on the 3.4 ha experimental area (Fig. 1a). According to the mapping manual of the Bavarian State Agency for Environmental Protection (Bavarian-State-Agency-for-Environmental-Protection, 2018) vegetation type included alpine grassland vegetation (AR, Alpine and subalpine calcareous grassland), a complex of fen and spring fen (MF, Alkaline fens), mountain pine shrub land (WU, Bushes with *Pinus mugo* and *Rhododendron hirsutum*) and megaforb (XS, *Rumicion alpini*, tall herb anthropogenic vegetation). Alpine grassland covered most of the study area and occurred in three main variations (typical, tall grass dominated, forb dominated). All vegetation types together created a high structural heterogeneity. A floristic survey by the Bavarian Botanical Society detected 228 vascular plant species on the same experimental site (Ewald et al., 2018).

The result of the classification derived from the UAS-based remote sensing approach shows that, in July, most of the area was ungrazed (34.1%) or lightly grazed (35.6%). Bare soil comprised only ~1% of the site and heavily grazed soil accounted for 18.0% of the total area (Fig. 1b). Grazed patches were predominantly situated in the eastern part of the site. Lightly and heavily grazed areas were distributed homogeneously across the biotope types, bare soil areas were mainly located in the southern part of the site, on or close to megaforb, forb vegetation or fen biotopes. Trees and shrubs covered 10.0% of the total area, while rocks were present at 1.3% of the total area.

### 3.2. Carbon and nitrogen contents under different grazing intensities

The experimental site presented a significant OM contribution in the top soil, and mean OC and N contents of 155 ± 44 mg $g^{-1}{}_{SDW}$ and 15 ± 4 mg $g^{1}{}_{SDW}$, respectively (Fig. 2). Overall, OC (Fig. 2a) and N (Fig. 2b) contents followed identical patterns and did not vary significantly along the grazing gradient and between the two sampling dates. Although not always statistically significant, OC and N contents in bare soil tended to be higher (203 ± 51 and 19 ± 4 mg $g^{-1}{}_{SDW}$, respectively) compared to the other grazing intensities (mean of 139 ± 26 mg $g^{-1}{}_{SDW}$ and 13 ± 2 mg $g^{-1}{}_{SDW}$, respectively). In July, the amount of salt-extractable OC (SEOC) was significantly higher in bare soil compared to the other grazing intensities (1.5 versus mean of 0.9 mgC $gOC^{-1}{}_{SDW}$). However, this observation did not reappear in soils from the second sampling in October (Fig. 3a). The EC (Fig. 3b), ammonium ($NH_4^+$) concentrations (Fig. 3c) and gross N mineralisation (Fig. 3d) were around two fold higher in bare soil compared to ungrazed areas in July. This was however not always significant due to large spatial heterogeneity. The $NH_4^+$ concentrations significantly decreased in October under bare soil (31 versus 53 mg $NH_4^+$-N $kg^{-1}{}_{SDW}$) (Fig. 3c). Gross nitrification, as well as nitrate ($NO_3$-) concentrations in soil, were barely detectable (0.7 ± 1.4 mg $kg^{-1}{}_{SDW}$) and did not present specific trends between treatments (**Table C**). In July, EC, pH, N, OC and SEOC were significantly positively correlated with each other, as for example EC with SEOC ($r = 0.71$, $p$-value = 0.002) (Fig. 4a).

### 3.3. Abundance and characteristics of microbial communities

Microbial abundance depicted by microbial biomass and total microbial PLFAs showed a mean (all grazing intensities included) of 31.6 ± 5.1 mgC $gOC^{-1}$and 4.6 ± 0.8 nmol $mgOC^{-1}$, respectively (Fig. 5a, **b**), with no specific trends between grazing intensities. This holds true for bacterial and fungal proxies (Fig. 5c, **d**) as well as Gram-positive:Gram-negative ratio (Fig. 5e). However, the fungal-to-bacterial ratio tended to be lower in the bare soil, with significant differences between bare soil and ungrazed areas in October (0.3 versus 0.5) (Fig. 5f). In October, a significant negative correlation was evidenced between the fungal-to-bacterial ratio and the N content (r = - 0.53, $p$-value = 0.03) (Fig. 4b). The abundances of N mineralizers (alkaline proteases and chitinases) were not significantly affected by the grazing but were 10–100 times higher than ammonia oxidizers (Fig. 6a, **b**). The abundance of ammonia oxidizing archaea (AOA) and bacteria (AOB) tended to be higher under bare soil (Fig. 6 **c, d**), and in July, AOA was significantly positively correlated with N ($r = 0.66$, $p$-value = 0.005), as well as gross nitrification ($r = 0.67$, $p$-value = 0.005) (Fig. 4a).

### 3.4. Nitrate and DOC concentrations in drainage water

Mean DOC and $NO_3^-$-N concentrations measured from May 29 to August 8 in the water of the creek draining the pasture were very low with 1.67 mg $L^{-1}$ and 0.18 mg N $L^{-1}$, respectively (Fig. 7). Regular peaks corresponding to precipitation events were measured, but did not exceed 14.5 mg C.$L^{-1}$ and 3.9 mg N $L^{-1}$ for DOC and $NO_3^-$-N, respectively.

## 4. Discussion

The mapped biotope types on the experimental site of Brunnenkopfalm (Fig. 1a) and the existing plant inventory (Ewald et al., 2018) documented remarkably high diversity of species and habitats and correspondingly high conservation value of the pasture even 60 years after abandonment. Patches of trees and shrubs covered around 10% of the area. Considering the long abandonment of the grassland (> 50 years),

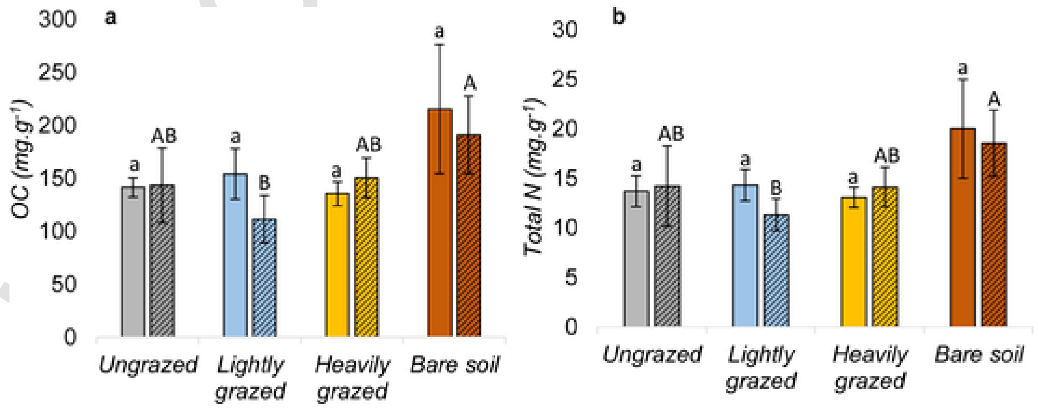

**Fig. 2.** Organic carbon (**a**) and total nitrogen (**b**) contents in the soil under different grazing intensities. The plain and crosshatched bars correspond to the samples collected in July and October, respectively. For all variables, $n = 4$. The lowercase letters indicate the results from the statistical tests applied to compare the effect of different grazing intensities in July. The uppercase letters highlight the statistical results for october.

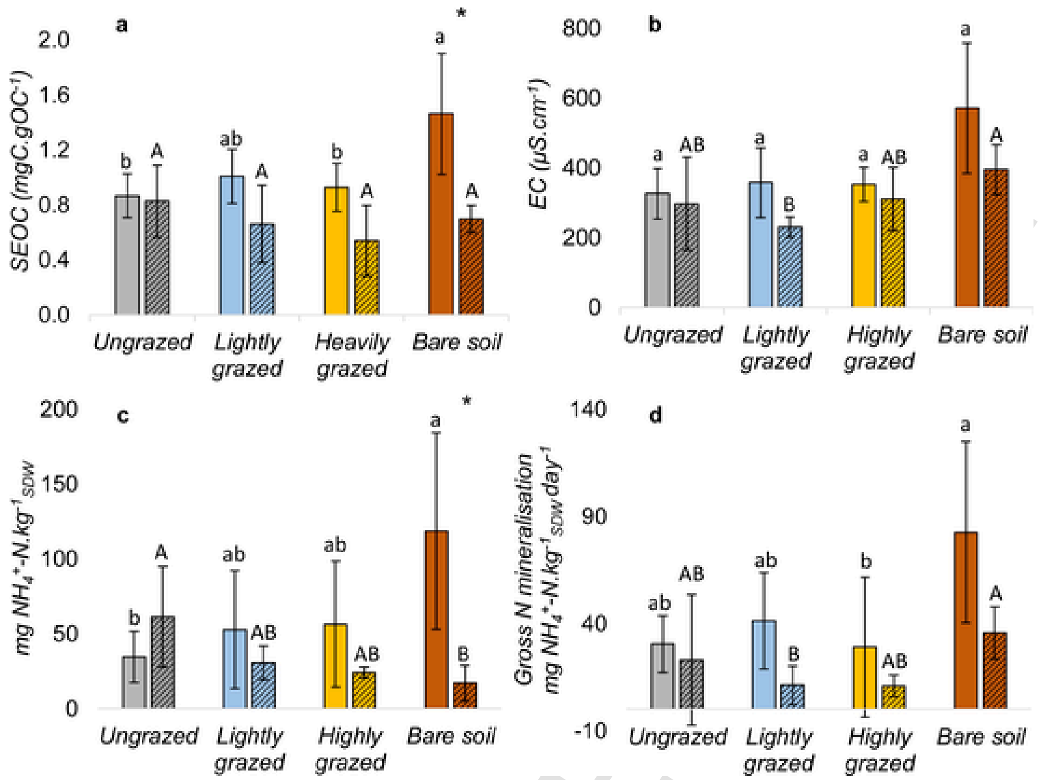

**Fig. 3.** Salt-extractable organic carbon (SEOC) and available nitrogen. (a) SEOCunder different grazing intensities, (**b**) electrical conductivity (EC), (**c**)ammonium ($NH_4^+$) contents and (**d**)gross N mineralisation rates. The plain and crosshatched bars correspond to the samples collected in July and October, The lowercase letters indicate the results from the statistical tests applied to compare the effect of different grazing intensities in July. The uppercase letters highlight the statistical results for october. The asterisk indicate a significant difference between July and October, for a given variable.

the expansion of trees might have been limited, but natural reforestation of the grassland is likely in the longer term, as it has been observed in the majority of abandoned alpine grasslands at comparable elevation (Tasser et al., 2007). Thus, the high plant diversity of the Brunnenkopfalm would depend on management that is adapted and compatible to nature conservation. Extensive re-grazing of abandoned alpine grasslands was highlighted as a plausible option to maintain the high plant diversity of the European Alps (Kohler et al., 2017).

### *4.1. Brunnenkopfalm, alpine grasslands with OM rich soils*

The topsoil of the experimental site shows pH values close to neutral (mean of 6.7) (Table C) and is rich in OM as reflected by the high OC and N concentrations (Fig. 2). This is in agreement with previous studies on Bavarian grasslands reporting the highest SOC stocks in alpine grasslands (Wiesmeier et al., 2013; Kühnel et al., 2019). In Kühnel et al. (2019), the high SOC stocks were correlated to high elevation and pH, reflecting a parent material rich in carbonates or dolomite. In the present study, the relatively low OC:N ratio (10.4 ± 0.7) suggest a high microbial contribution and relatively degraded OM (Janssen, 1996; Bengtsson et al., 2003). This is supported by the high microbial abundance depicted by microbial biomass (31.6 ± 5.1 mgC $gOC^{-1}{}_{SDW}$) and total microbial PLFAs (4.6 ± 0.8 nmol $mgOC^{-1}{}_{SDW}$) (Fig. 5a, **b**). Indeed, previous work investigating microbial community size in top soils of grasslands, including those in the European Alps (sampled from June to October), found mean total PLFA contents ranging from 0.2 to $<$ 4.0 nmol $mgOC^{-1}{}_{SDW}$(Frostegård and Bååth, 1996; Zeller et al., 2001; Djukic et al., 2010). Thus, the Brunnenkopfalm is found to be particularly rich in terms of microbial biomass, as well as in C and N.

### *4.2. The impact of short term re-grazing was not reflected in the total OC and N content*

Overall, grazing did not have a significant short-term effect (two and five months after the beginning of grazing) on the soil total OC and N contents. However, sampling spots affected by intense cow trampling and excreta (i.e. bare soil) tended to have slightly elevated OC and N contents as compared to the other grazing categories (Fig. 2).

Given the short re-grazing period in our experiment, elevated OC and N contents in the bare soil category are unlikely to be a direct result of re-grazing. Bare soil areas were mostly located either on moist areas, corresponding to the wet-adapted biotopes (e.g., megaforb) (Fig. 1) and thus soils sensitive to trampling, or areas on the lower slopes favoured by the cows due to the small scale topography inducing winds that reduce the presence of horseflies (De Miguel et al., 1997). Previous studies note that grazing-induced bare soil areas corresponded to moist zones where animals dwell preferentially (Carmona et al., 2013; Grigulis et al., 2013). Given that samples from bare soil were not only obtained in the vegetation type alpine grassland as those samples of the other grazing classes, elevated OC and total N concentrations in the bare soil areas might be also due to historical effects on SOM accumulation – be it due to topography, plant cover (Grigulis et al., 2013) and hence soil development processes. For instance, the vegetation types megaforb and fen where the bare soil areas were identified can be associated to moist soils where SOM tend to accumulate due to hampered OM decomposition. Consequently, trends towards higher soil total OC and N concentrations at the bare soil sampling spots might be either due to impaired mineralisation at high soil moisture, due to historical and/or new C and N inputs by grazing cattle, or most likely through a combination of all factors.

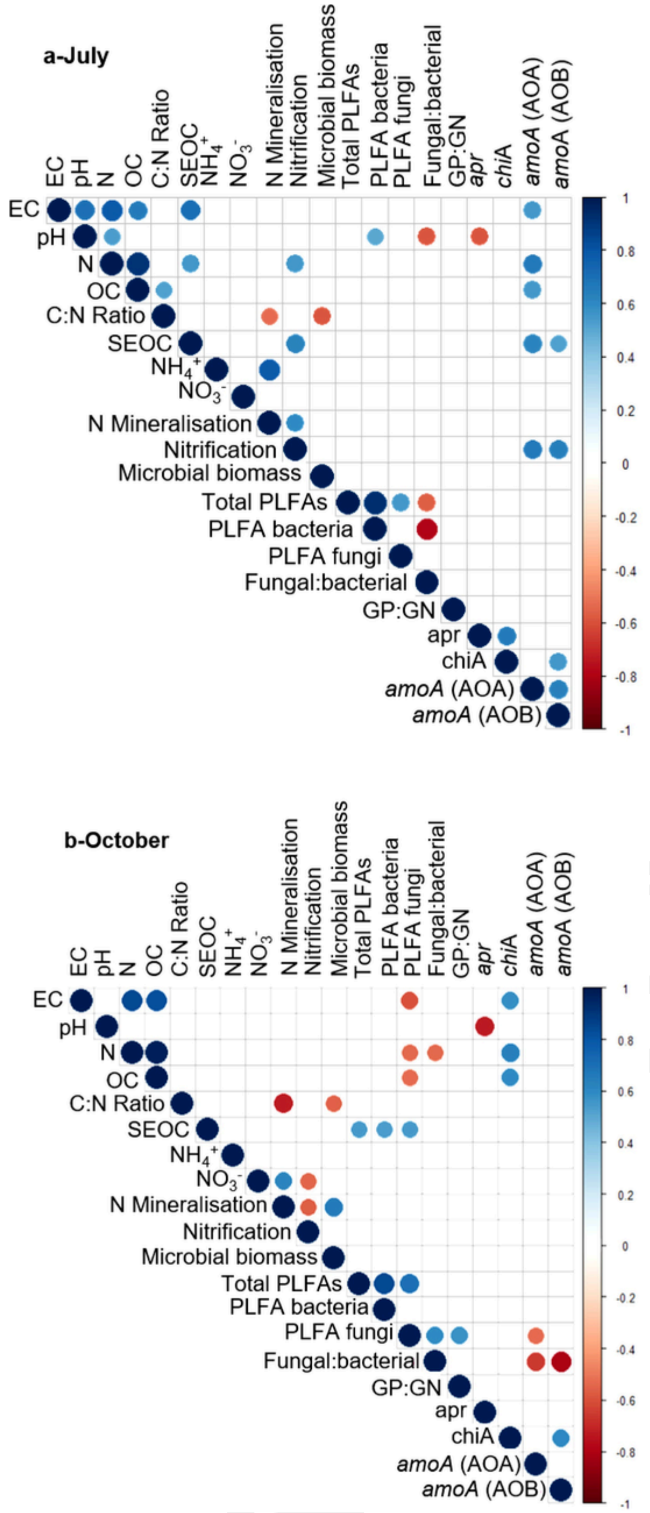

**Fig. 4.** Correlation matrix represented by using the Spearman coefficient between the variables studied in the present project for July (**a**) and October (**b**). The blue and red circles highlight positive and negative correlations, respectively. The circle diameter and the color gradient correspond to the degree of correlation, only the statistically significant correlations ($p$-value $< 0.05$) are represented. The values and units used for each variable are identical to those presented in previous figures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

### *4.3. Extractable OC and mineral N contents slightly affected by re-grazing*

In contrast to the total soil OC and N contents, the parameters with higher temporal variability, i.e. extractable OC and mineral N concentrations, can be assumed to be more sensitive to re-grazing on the short term and we hypothesised that grazing would for instance increase the amount of salt extractable OM and ammonium mainly due to the excreta production. In July, SEOC, EC and $NH_4^+$ concentrations, as well as gross N mineralisation were around two fold higher in bare soil compared to ungrazed areas (Fig. 3). However, as outlined above, changes in bare soil compared to the other grazing classes – besides of being spatially irrelevant – cannot be unequivocally related to recent grazing effects. In pastures, the concentration of animals on moist areas (Carmona et al., 2013) can lead to nutrient concentration through increase of available C and N due to excreta inputs (Peco et al., 2017) which are often more decomposable compared to plant residues (MacDiarmid and Watkin, 1972; Frank and Groffman, 1998). The differences in these measures (i.e. SEOC, EC and $NH_4^+$ concentrations, as well as gross N mineralisation) between the grazing intensities were levelled out in October, with a significant decrease of SEOC and $NH_4^+$ concentrations in the bare soil (Fig. 3). As cows were taken back from the experimental site to the farm on the 31st of August, additional cow excreta inputs were absent for more than one month before the sampling in October. This lead to a clear decrease in available OC and N, with no significant differences to the other grazing intensities. Thus, at the end of the season the availability of soluble OM forms levelled off between vegetated and bare soil areas. The temporal fluctuations are partly related to the seasonal changes of soil characteristics (e.g., soil temperature and moisture) (Bardgett et al., 1997, 1999; Zeller et al., 2001), which are key drivers for OC and N cycling independent of management and grazing intensities (Zeller et al., 2001). Indeed, the higher air temperature and global radiation in July compared to October (Table 1) can be assumed to have led to relatively high organic matter mineralisation in these relatively moist soils, and thus a release of extractable OC and available N (Burke et al., 1997). This is supported by high gross N mineralisation rates (Fig. 3d). The overall increase of available N in spring and early summer (July) in alpine soils might to some extent also be related to the rapid turnover of microorganisms which feed on senescent plant residues during autumn and winter (Lipson et al., 1999; Bardgett et al., 2005), as well as snow melt which is a considerably source of inorganic N in alpine grasslands (Hiltbrunner et al., 2005).

### *4.4. Short-term extensive grazing does not affect microbial biomass*

We expected that grazing would increase microbial abundance and alter microbial communities. This was only partly verified as grazing did not significantly affect the abundance of microorganisms (Fig. 5a**, b**), but led to a significant decrease of the fungal-to-bacterial ratio in the bare soil compared to the control in October (0.3 vs 0.5) (Fig. 5f). This support the fact that the impact of grazing on microbial community is strongly dependent on grazing intensity (Zhao et al., 2017). The lower fungal contribution under bare soil could result from the N fertilization via cow excreta which favoured the bacterial community over the fungal community (Bardgett and McAlister, 1999). This is supported by a negative correlation observed between the fungal to bacterial ratio and the N content (Fig. 4b).

### *4.5. High internal N cycling in soil*

The dominant N form in urine and dung is organic N (e.g., urea, peptides and amino acids), which are mineralized to become available to plants and microorganisms (Cai et al., 2017). This mineralisation is

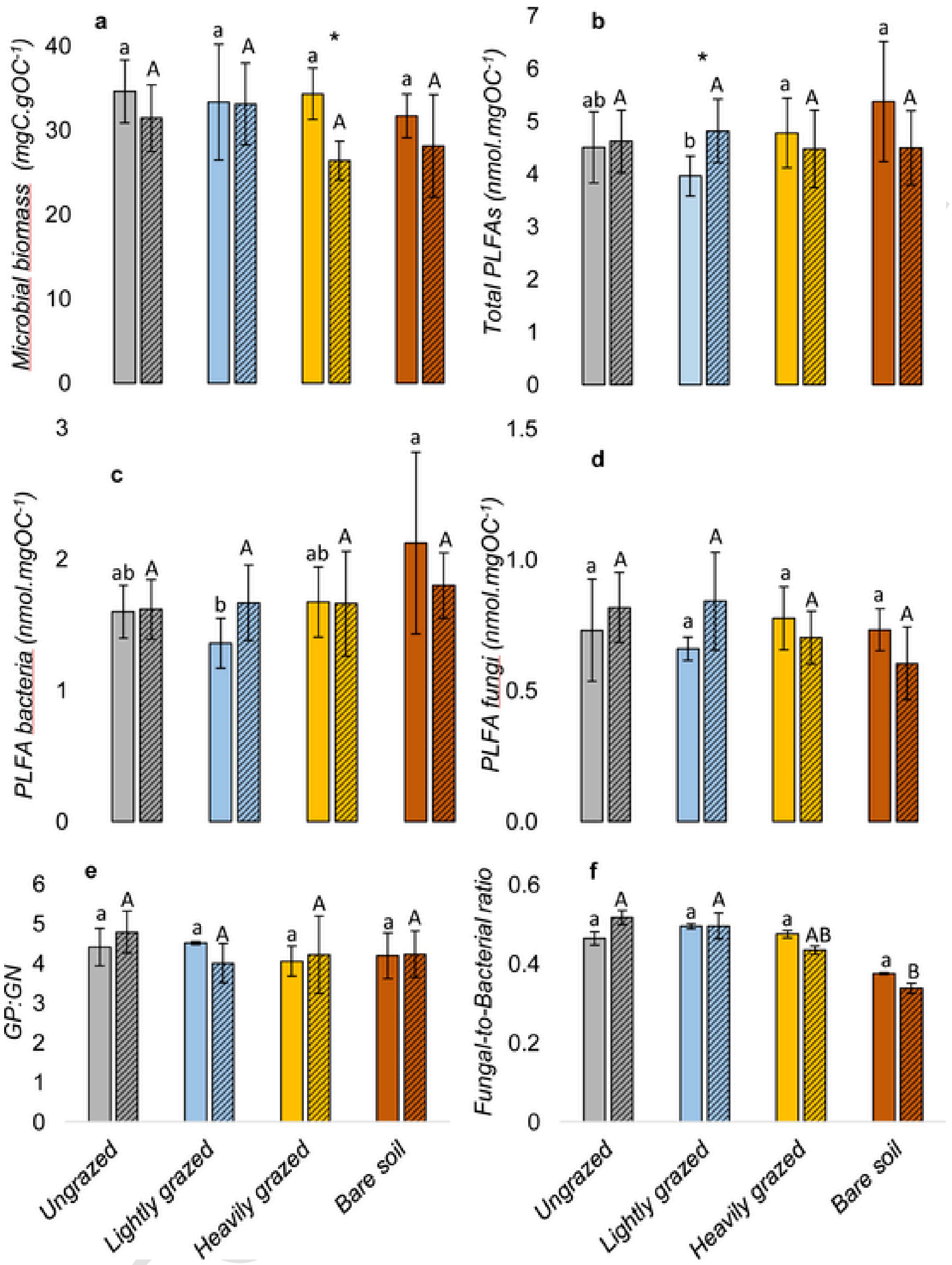

**Fig. 5.** Microbial community charateristics under different grazing intensities: microbial biomass (**a**), total PLFAs (**b**), bacterial (**c**) and fungal (**d**) PLFA biomarkers; Gram-positive (GP) to Gram-negative (GN) bacteria ratio (**e**) and PLFA-based fungal-to-bacterial ratio (**f**) Data are expressed relative to the soil organic carbon measured in the respective samples. The plain and crosshatched bars correspond to the samples collected in July and October, respectively. For all variables, $n = 4$. The lowercase letters indicate the results from the statistical tests applied to compare the effect of different grazing intensities in July. The uppercase letters highlight the statistical results for october. The asterisk indicate a significant difference between July and October, for a given variable.

realized by a series of extracellular enzymes produced by microorganisms (Geisseler et al., 2010). While $NH_4^+$ concentrations and gross N mineralisation were higher under bare soil (Fig. 3), N mineralizers did not significantly increase (Fig. 6a, **b**). Also, their abundance was not significantly correlated with $NH_4^+$ concentrations and gross N mineralisation (Fig. 4). However, it needs to be noted that protein-encoding genes do not necessarily highlight a significant correlation with the corresponding process (i.e. N mineralisation) and that for short-term processes, the activity (i.e. RNA abundance) might be a better proxy than the presence (i.e. DNA abundance as determined in this study) of microorganisms (Rocca et al., 2015).

In the N cycle, the produced $NH_4^+$ can be biologically oxidized to $NO_3^-$ by nitrifiers (Butterbach-Bahl et al., 2011). Due to the simultaneous presence of microbes involved in nitrification and low OC:N ratios in the studied soil, a high risk for grazing-induced increases in nitrification and associated N losses seems likely. Indeed, the low OC:N ratio suggest an advantage of autotrophic nitrification over heterotrophic $NH_4^+$ immobilization due to C shortage for soil microorganisms (Wu et al., 2011). The abundance of the ammonia monooxyge-

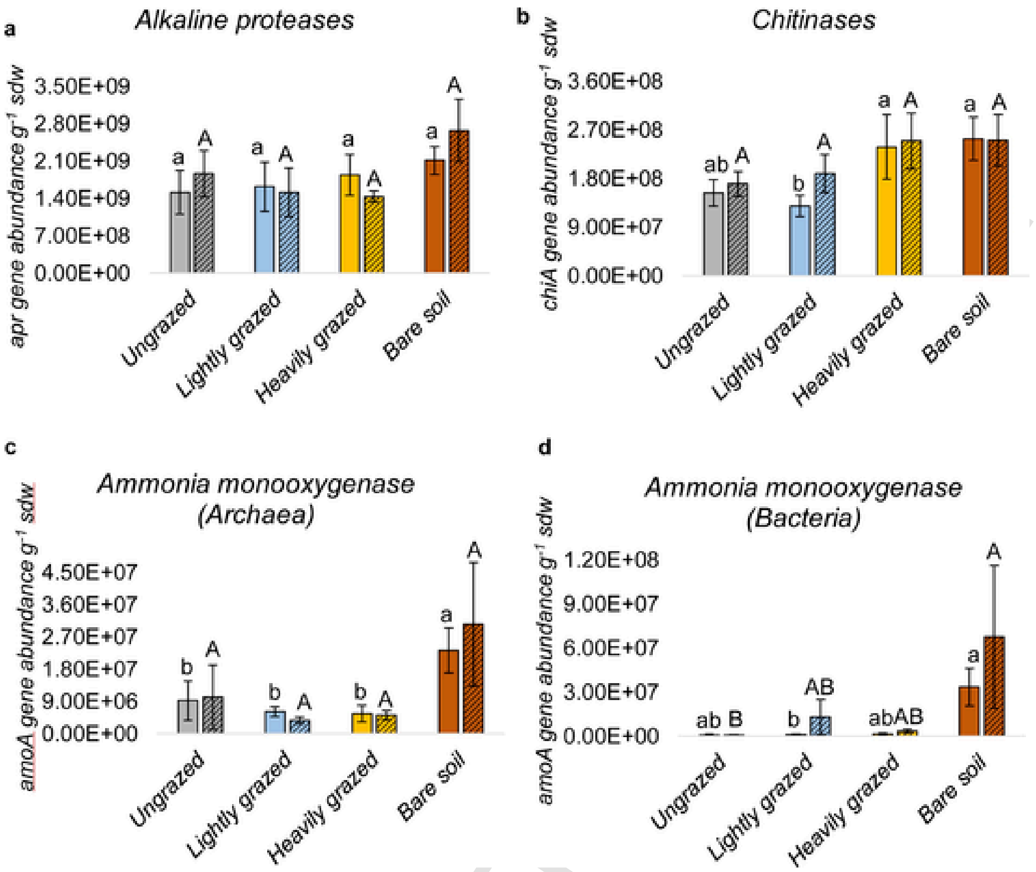

**Fig. 6.** Nitrogen related genes of microbial mineralizers (apr and chiA genes) and ammonia oxidizers (amoA of ammonia-oxidizing archaea (AOA) and bacteria (AOB)), $n = 4$. The lowercase letters indicate the results from the statistical tests applied to compare the effect of different grazing intensities in July. The uppercase letters highlight the statistical results for october. The asterisk indicate a significant difference between July and October, for a given variable.

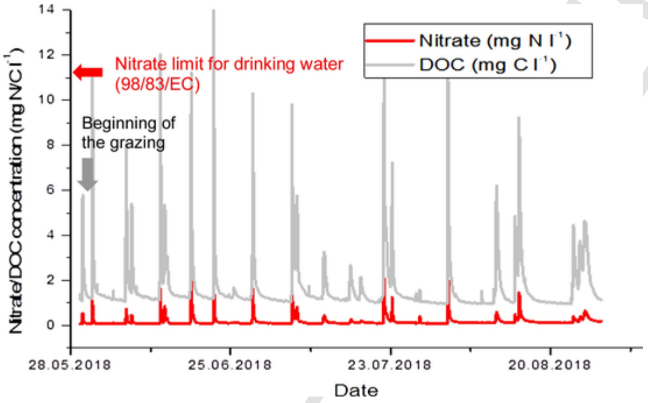

**Fig. 7.** Nitrate and dissolved organic carbon (DOC) concentration in the creek.

nase gene, *amoA*, for archaea (AOA) and bacteria (AOB) tended to be higher under bare soil conditions (Fig. 6c, **d**). In July, AOA were positively correlated with N, as well as gross nitrification (Fig. 4a). This is in agreement with previous findings showing an increase of AOA under dung patches containing labile OC (Wessén et al., 2010) and a strong positive response of AOB to fertilizer applications (Schauss et al., 2009; Szukics et al., 2019). However, we could not verify that AOA abundance was driven by soil properties while AOB abundance was related to $NO_3$- concentrations and N supply, as suggested in studies from mountain (Szukics et al., 2019) and lowland (Di et al., 2010; Shen et al., 2011) grassland. In the present study, the trend to higher AOA and AOB levels under bare soil did not translate into measurable higher rates of gross nitrification or $NO_3^-$ contents in soil. Both remained barely detectable and did not present specific trends between treatments (Table C). The mobile anion $NO_3$- producedas a result of nitrification, is the main agent regarding N loss along gaseous or hydrological pathways, if not immobilized into plant or microbial biomass (Lu et al., 2011; de Vries and Bardgett, 2012; Li et al., 2019). Thus, in our study, AOA and AOB gene abundances cannot serve as a proxy for gross $NO_3^-$ production as it was suggested by Dannenmann et al. (2016) due to limited metabolic alternatives of nitrifiers. These results indicate that the correlation between AOB and AOA abundances and nitrification is not fully understood, as was recently highlighted by Cai et al. (2017).

In sum, intense cow trampling together with additional nutrient inputs of animal excreta probably increased the extractable OC, as well as the production of $NH_4^+$ via gross N mineralisation. However, despite the low soil OC:N ratio, the more easily extractable OC might have favoured heterotrophic microbial $NH_4^+$ release over autotrophic nitrification, thus effectively preserving N in the soil for subsequent uptake and avoiding $NO_3^-$ accumulation.

### 4.6. Unaffected water quality after short-term re-grazing

Nitrification of $NH_4^+$ can lead to a loss of N via leaching (Butterbach-Bahl et al., 2011). Following the concept of Schimel and Bennett (2004) relating N availability in ecosystems to N cycling, N availability at Brunnenkopfalm is sufficient to meet microbial and plant needs, but still limiting for the $NH_4^+$ supply to nitrifiers. The resulting system is dominated by $NH_4^+$ of small mean residence time, with a limited production of $NO_3^-$. Thus, the mean $NO_3^-$-N concentrations measured in the water of the drainage creek were very low

(1.67 mg $L^{-1}$ and 0.18 mg N $L^{-1}$, respectively) (Fig. 7). For $NO_3^-$-N, this is 14 times lower than the limit of 11.3 mg N $L^{-1}$ set by the European Union in the Drinking Water Directive (98/83/EC) and the German drinking water ordinance (TrinkwV), and even lower than the threshold for water used in baby food production (< 2.2 mg N $L^{-1}$). Short-lived peaks corresponding to precipitation events were measured, but did not exceed 14.5 mg $L^{-1}$ and 3.9 mg N $L^{-1}$ for DOC and $NO^{3-}$ respectively. The DOC concentrations are within the typical range for streams of the Alpine region (Battin, 1999). Thus, the extensive grazing of our experiment did not affect water quality on the short term. These results confirm that extensive grazing of grasslands does not necessarily increase N loss (O'reagain et al., 2005). In the present study, the high microbial N retention capacity of the soils and plant N uptake appear to be instrumental to the mitigation of $NO_3^-$ leaching.

## 5. Conclusions

The soils at the Brunnenkopfalm are highly resistant to extensive re-grazing on the short-term. Grazing effects (cow trampling and excreta) were only observed at bare soil spots which represent a small fraction of the study area (~ 1%), and could at least partially also be driven by the initial site heterogeneity (soil and vegetation) rather than solely by recent grazing activities. Nonetheless, the outlined biogeochemical mechanisms indicate that strong trampling within re-grazing of abandoned pastures can induce a release of extractable OC and available N under bare soil conditions. At Brunnenkopfalm, this did not result into an alteration of water quality at the short-term because bare soil was spatially irrelevant. A slight increase in nitrifier abundances at bare soil spots, as well as the low OC:N ratios of soils suggest that a future increase in inorganic N accumulation is possible at least at bare soil areas. This could possibly endanger some biodiverse grassland biotopes via eutrophication and result in environmental N losses along hydrological or gaseous pathways. Thus, long-term studies are needed to verify whether soils are also resistant to re-grazing in the long-term. On the short-term, undesired re-grazing effects can be avoided by extensive, guided grazing with adapted cattle breeds targeted to avoid trampling-induced bare soil areas, especially close to open water (e.g., springs, rivers). Under these conditions, extensive re-grazing of abandoned alpine grasslands appears as a plausible option to preserve this cultural landscape without affecting the soil characteristics and water quality.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agee.2020.107008.

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