Soil Biology and Biochemistry 165 (2022) 108515

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Seasonal drought in Mediterranean soils mainly changes microbial C and N contents whereas chronic drought mainly impairs the capacity of microbes to retain P

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ARTICLE INFO

Keywords:
Drought stress
Microbial nutrients
Microbial osmolytes
Soil enzymes
Soil nutrients
Water limitation

ABSTRACT

Intensification of droughts may aggravate the generally low capacity of Mediterranean soils to store C and nutrients and induce soil C:N:P stoichiometric imbalances through its impact on soil microbial biomass and activity. Soil microbes may nonetheless have different responses to seasonal and chronic drought, but very few studies investigate long-term drought periods under field conditions. This study compares the effects of seasonal drought *versus* the impacts of 16 years of chronic experimental drought on microbial biomass and nutrients and assess the implications for soil nutrient availability and biogeochemical functioning in a Mediterranean forest. The chronic drought treatment reduced substantially and persistently microbial biomass C, N and particularly P, probably due to P-sparing community shifts or microbial adaptations. The smaller microbial N pool and lower mineralization activity contributed to the accumulation of C- and N-rich organic compounds in the soil and to a lower availability of mineralized forms of N during the vegetation growing season. As a result, chronic drought conditions may increase the risks of N losses from the plant-soil system in Mediterranean ecosystems. Microbial C:N ratios remained unaltered under chronic drought compared to control, likely associated with the equivalent accumulation of C- and N-rich osmolytes by microbial communities. In contrast, microbial biomass increased its C content relative to N content in response to seasonal drought, but also reduced considerably its N and P pool. Therefore, while microbial P was more sensitive to chronic water stress, microbial N and C were more closely coupled to the seasonal fluctuations of water availability.

1. Introduction

The frequency and intensity of droughts in the Mediterranean region have increased since 1950, accompanied by more irregular and torrential rain (Hartmann et al., 2013; Vicente-Serrano et al., 2014). Intensification of droughts and changed patterns of precipitation associated with climate change can have large impacts on the frequently nutrient-poor Mediterranean soils (Yaalon, 1997). Summer droughts decrease soil enzymatic activity (Sardans and Peñuelas, 2005; 2010), litter decomposition (Santonja et al., 2017) and soil organic matter (SOM) mineralization rates (Marañón Jiménez et al., 2011), with the consequent lower nutrient release in assimilable forms for plants. Drought impacts generally take longer to appear in Mediterranean vegetation (Vicente-Serrano et al., 2013). In the long term, the direct effect of water limitation together with the consequent lower nutrient accesibility imposed by drought also reduce plant growth, nutrient uptake and foliar nutrient concentrations (Kreuzwieser and Gessler 2010; Sardans and Peñuelas 2007; Sardans et al. 2008a, 2020).

Abbreviations: **acp**, acid phosphatase activity; **akp**, alkaline phosphatase activity; **bgl**, β-glucosidase activity; $\mathbf{C_{micro}}$, microbial C; **CUE**, carbon use efficiency; **EOC**, extractable organic carbon; **ETN**, extractable total nitrogen; **Pt**, extractable total phosphorus; $\mathbf{K_{micro}}$, microbial K; $\mathbf{Mn_{micro}}$, microbial Mn; $\mathbf{Mo_{micro}}$, microbial Mo; $\mathbf{N_{micro}}$, microbial N; **Pi**, bicarbonate-extractable inorganic P; $\mathbf{P_{micro}}$, microbial P; **Po**, bicarbonate-extractable organic P; **prot**, protease activity; **SOM**, soil organic matter; **sresp**, soil CO_2 efflux; **stemp**, soil temperature; **swclab**, gravimetric soil water content measured in the laboratory; **ure**, urease activity.

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https://doi.org/10.1016/j.soilbio.2021.108515
Received 2 May 2021; Received in revised form 3 December 2021; Accepted 4 December 2021
Available online 6 December 2021

Seasonal and chronic drought, however, may have divergent effects on soil biogeochemical functioning (Rousk et al., 2013; Hoover and Rogers, 2016; Zuccarini et al., 2020). Short term seasonal drought can increase the accumulation of SOM and nutrients in forms unavailable to plants (Sardans et al., 2008b; Peñuelas et al., 2018) by increasing the total amount of above- and belowground litter (Ogaya and Peñuelas, 2007; Martí-Roure et al., 2011; Padilla and Pugnaire 2007) together with reductions in potential soil enzymatic activities and microbial mineralization rates at low soil water contents (Sardans and Peñuelas, 2010; Manzoni et al., 2012). Long term chronic drought reduces plant nutritional status, growth and cover, decreasing the quantity and quality of plant litter inputs to the soil (Sardans et al., 2008a, 2020, Lloret and Granzow-de la Cerda, 2013) and soil nutrient availability (Peñuelas et al., 2018; Sardans et al., 2020). Lower soil protection and permeability increase soil erosion (Li et al., 2011; Navarro-García et al., 2012) and nutrient loss (Zaimes et al., 2012, Bloor and Bardgett, 2012), especially if drought is accompanied by more frequent and torrential rain. Chronic drought may therefore aggravate the generally low capacity of Mediterranean soils to store C and nutrients (Beier et al., 2009) and induce soil C:N:P stoichiometric imbalances (Sardans and Peñuelas, 2012; García-Angulo et al., 2020).

Soil microbial biomass plays a fundamental role in the stabilization of soil C (Liang et al., 2017; Miltner et al., 2012) and in the regulation of nutrient availability in climates with marked seasonality (Bardgett et al., 2013), such as in Mediterranean ecosystems (Qiu et al., 2009). A large fraction of the available nutrients in Mediterranean soils is stored in microbial biomass (Díaz-Raviña et al., 1995; Aponte et al., 2010). This microbial nutrient pool oscillates throughout the year with seasonal changes in environmental factors, and these temporal dynamics may determine the amounts of nutrients released or immobilized in the soil and therefore their availability to plants (Díaz-Raviña et al., 1995). Soil microbial communities adapt during dry periods by synthesizing extracellular polysaccharides (Marchus et al., 2018) and storing simple C- and N-rich osmolytes, such as amino compounds in bacteria and polyols in fungi, in high concentrations to combat osmotic stress (Schimel et al., 2007). These adaptations allow microbes to survive and preserve part of their cellular and nutrient contents during drought (Gutiérrez-Girón et al., 2014). The capacity of the soil microbial biomass pool to store nutrients becomes particularly important during periods of drought, because the decrease in vegetation nutrient uptake and increase in foliar senescence may lead to the accumulation of nutrients in partially decomposed organic matter on the soil surface and to increase the risk of nutrient loss (Schaeffer et al., 2017). Microbes release stored osmolytes during rewetting periods and reactivate the mineralization of these and other organic substrates accumulated in soil during dry periods, therefore releasing assimilable nutrients for plant growth (Fierer and Schimel, 2002; Schimel, 2018). Microbial biomass may therefore buffer the seasonal oscillations in soil nutrients and act as a nutrient reservoir for plant nutrition during favorable periods (Aponte et al., 2010; Bardgett et al., 2005).

Chronic drought conditions, however, may impair the role of microbial biomass in regulating the availability of soil nutrients. Low levels of soil water content reduce the connectivity among nutrient-rich soil micropores and limit the mobility of soluble substrates and extracellular enzymes within the soil matrix (Borken and Matzner 2009; Moyano et al., 2013). The lower substrate accessibility for soil microbes increases the resources allocation of soil microbes to enzyme production and increases their C costs for resources acquisition (Schimel 2018; Asensio et al., 2021). Drought also forces microbes to synthesize structures to resist the effects of drought, to accumulate C- and N-rich compounds for osmotic regulation (Schimel 2018) and to increase the allocation of resources to sustain basal metabolic rates rather than to growth, thus decreasing microbial C use efficiency (CUE) (Tiemann and Billings 2011; Manzoni et al., 2012). These elevated C costs for maintenance when substrate mobility and accessibility are low can potentially reduce microbial biomass and consequently soil C stabilization and the capacity of this compartment to store nutrients, although the net effects of drought on microbial biomass C are largely inconsistent in the literature (Deng et al., 2021; Abbasi et al., 2020), and largely depend on the length and intensity of the drought period.

The role of microbial biomass in regulating nutrient availability in Mediterranean soils has nonetheless been poorly addressed. The most studies mainly focus on the short-term droughts, whereas there is a lack of comprehensive understanding on the long-term drought effects (Deng et al., 2021). Very few studies have identified the transient effects of seasonal drought and the permanent impacts of chronic drought on microbial stoichiometry and on the size of nutrient storage in the microbial biomass pool. We compared the effects of seasonal drought *versus* the impacts of 16 years of chronic experimental drought on the size of the nutrient pool stored in the microbial biomass compartment and assessed the implications for soil C, nutrient availability and soil biogeochemical functioning in a Mediterranean forest. We call hereafter microbial storage the size of the nutrient pool stored in the microbial biomass compartment. We hypothesized that 1) both seasonal and chronic drought would lead to reductions in microbial biomass and in the nutrients stored in this compartment; 2) this reduction in microbial biomass and nutrients caused by chronic drought is persistent across seasons; 3) the diminished capacity of the microbial biomass community (as a whole) to store nutrients under chronic drought conditions is followed by a lower nutrient availability for vegetation during its growing period.

2. Material and methods

2.1. Study site

The study was carried out in a natural holm oak (*Quercus ilex*) forest in the Prades Mountains in Catalonia, northeastern Iberian Peninsula (41°21′N, 1°2′E). The vegetation is characterized by a dense forest, with *Q. ilex*, *Phillyrea latifolia* and *Arbutus unedo* the main arboreal species. The soil is a Dystric Cambisol with a depth of 35–90 cm (Sardans and Peñuelas 2010). The climate is typically Mediterranean, with hot and dry summers (mean temperature of 20.5 ± 0.3 °C, total precipitation of 64.8 ± 7.0 mm, and mean soil moisture of 10.9 ± 0.7% vol.) and warm winters (mean temperature of 4.8 ± 0.2 °C, total precipitation of 110.6 ± 16.0 mm, and mean soil moisture of 20.8 ± 1.1% vol.). The springs (mean temperature of 10.8 ± 0.4 °C, total precipitation of 237.4 ± 24.0 mm and mean soil moisture of 20.7 ± 1.6% vol.) and autumns (mean temperature of 12.7 ± 0.6 °C, total precipitation of 198.9 ± 23.9 mm and mean soil moisture of 17.6 ± 2.1% vol.) are usually rainy (mean values from 1998 to 2014, Figs. S1 and S2).

2.2. Experimental design

The drought experiment began in 1999 and consisted of partial rainfall exclusion that simulated a 30% reduction in precipitation for the forest (hereafter chronic drought treatment). Eight plots (10 × 15 m) were established at the same altitude (930 m a.s.l.) along the southern face of the mountain (25% slope). Half of the plots received the chronic drought treatment, and the other four plots served as controls (natural conditions). At the start of the experiment, just before the rainfall exclusion, soil water content measured during the previous autumn and winter was similar in the plots that received the chronic drought treatment and the control plots (24.7% and 24.8%, respectively). Soil total N concentration in 1999 was 0.37 ± 0.07%. Precipitation was reduced a 30% in the chronic drought treatment by the installation of plastic strips 0.5–0.8 m above the ground and covering 30% of the ground surface of the plots, and by the interception of water runoff by ditches. The precipitation intercepted by the plastic strips and ditches was conducted outside the plots. As a result, soil moisture was reduced a 15% in the chronic drought treatment throughout the entire duration of the experiment (Fig. S2). Further details of the chronic drought treatment

are described by Ogaya et al. (2020).

2.3. Soil sampling

We sampled the soil in 2014 on three consecutive days in the middle of each season: winter (25–27 January), spring (16–18 May), summer (31 July to 2 August) and autumn (24–26 October). We sampled five soil cores per plot and season (5 cm diameter and 10 cm deep). The cores were collected in all seasons around 15:00, after soil respiration was measured (see below). The samples were sieved in the laboratory (2-mm sieve) and stored at 4 °C until analysis. Subsamples were used for the determination of gravimetrical soil water content (swclab). Additional subsamples were used for the analysis of microbial biomass C, N, P, K, Mn and Mo concentrations, soil extractable C, N, P, K, Mn and Mo concentrations and potential activities of soil extracellular enzymes.

2.4. Microbial elemental concentrations and extracellular enzymatic activities

Elemental C, N and P concentrations in the microbial biomass were measured using chloroform fumigation-extraction (Jenkinson and Powlson, 1976). Two subsamples (10 g fresh weight) of sieved soil were used for each sample. One subsample was fumigated for 24 h with ethanol-free chloroform and extracted using 0.5 M K_2SO_4 (4:1 v:w) for C and N and 0.5 M $NaHCO_3$ (10:1 v:w) for P, after shaking for 30 min. The other subsample was directly extracted following the same protocol. The extracts were then filtered through Whatman 42 equivalent paper. Total organic C and total N concentrations in the K_2SO_4 extracts were measured using a Multi N/C 3100 Total Organic Carbon/Total Nitrogen analyzer (Analytik Jena AG, Jena, Germany). Total P concentration in the $NaHCO_3$ extracts was determined after the digestion of 2.5 g of an aliquot with HNO_3; a correction for the adsorption of P during extraction was applied after measuring the recovery of samples spiked with P as described by Zuccarini et al. (2020). Elemental P concentrations in the digested samples and blanks were determined using an Optima 4300DV inductively coupled plasma/optical emission spectrometer (ICP-OES; PerkinElmer, Wellesley, USA). Microbial biomass C (C_{micro}), N (N_{micro}) and P (P_{micro}) concentrations were calculated as the difference between the fumigated and unfumigated samples. Due to their roles in microbial mineralization, the elemental concentrations of K, Mn and Mo in the microbial biomasses (K_{micro}, Mn_{micro} and Mo_{micro}, respectively) were also determined for the 0.5 M $NaHCO_3$ extracts using chloroform fumigation-extraction and ICP-OES analysis. All elemental concentrations in the microbial biomasses were expressed as μg of element per unit of soil dry mass.

The activities of acid and alkaline phosphatase (acp and akp), β-glucosidase (bgl), protease (prot) and urease (ure) were determined colorimetrically, based on original methods developed by Tabatabai and Bremner (1969) (acp and akp), Hoffmann and Dedeken (1965) (bgl), Ladd and Butler (1972) (prot) and Kandeler and Gerber (1988) (ure). The procedures were adapted from Schinner et al. (1996) and Sardans et al. (2008b,c) with one major modification: after the addition of substrate and incubation, the soil suspension was centrifuged at 11200 g for 5 min (instead of filtering), and the clear supernatant was photometrically compared to blank reagent (following the steps for color development if necessary). We then measured the optical density of the calibration standards, samples and controls using a Helios Alpha spectrophotometer (Thermo Spectronic, Cambridge, UK). Enzymatic activity was expressed as μmol of substrate released per gram of dry soil and incubation time (h).

2.5. Soil extractable elements

The concentrations of soil extractable total organic C (EOC) and extractable total N (ETN) were measured in the unfumigated K_2SO_4 extracts. The concentrations of extractable total P (Pt) and of potassium (K), manganese (Mn) and molybdenum (Mo) were measured in the unfumigated $NaHCO_3$ extracts following the procedures described above. One aliquot of the $NaHCO_3$ extracts was used for determining the concentration of inorganic extractable P (Pi) using the Olsen's method (Watanabe and Olsen, 1965). The correction for P adsorption was also applied to this fraction. The concentration of organic extractable P (Po) in the extracts was calculated as the difference between Pt and Pi. The elemental concentrations in the extracts were expressed as μg of extractable element per unit of soil dry mass.

Total organic C (TOC) and N (TN), ammonium and nitrate were determined from 0 to 15 cm depth soil samples taken in the same way as above in 2005, six years after the experiment started. For the determination of TOC in soil samples we used the Walkley-Black method (Walkley and Black 1934). TN concentrations in soils were determined by organic elemental analysis using combustion coupled to gas chromatography. For that, we used a Thermo Electron gas chromatograph model NA 2100 (C.E. Instruments-Thermo Electron, Milano, Italy). Ammonium and nitrate were extracted with a 2 M KCl solution and analyzed by colorimetrically using a spectrophotometer (Spectronic 20 Genesys; Spectronic Instruments, Inc., Rochester NY) against the reagent blank. We analyzed ammonium in soil extracts by a modified Berthelot reaction (Schinner et al., 1996) and nitrate by the cadmium reduction method (US Environmental Protection Agency, 1979).

We calculated the C:N, C:P and N:P stoichiometric imbalances between the extractable fractions and microbial biomass following Mooshammer et al. (2014) as the ratios of the extractable fractions (EOC:ETN, EOC:Pt and ETN:Pt) to the corresponding ratios of the microbial fractions (C_{micro}:N_{micro}, C_{micro}:P_{micro} and N_{micro}:P_{micro}, respectively). The stoichiometric imbalance was thus a measure of the divergence between the stoichiometries of the soil microbes and the soil organic substrates. For example, a decrease in the C:N imbalance would indicate an increasing microbial demand of C from the soil extractable fractions, whereas an increase in the C:N imbalance would indicate an increasing microbial demand of N from the soil extractable fractions.

2.6. Soil CO_2 efflux, water content and temperature

Soil CO_2 efflux (sresp) was measured at each season, prior to each soil sampling from 09:00 to 15:00 on five soil collars permanently inserted into the soil (2 cm) of each plot using a closed system with a SRC-1 soil chamber connected to an EGM-4 portable system (PP-systems, Hitchin, UK) as described by Zuccarini et al. (2020). Soil temperature (stemp) was measured simultaneously to the soil CO_2 efflux at one point close to the collars at a depth of 10 cm using a TO 15 digital soil thermometer (Jules Richard Instruments, Argenteuil, France). We determined swclab in the sieved soil subsamples by weighing before and after drying at 105 °C for 24 h.

2.7. Data analysis

The effects of season and the experimental chronic drought on swclab, stemp, microbial elemental concentrations, soil extractable elements, microbial and soil extractable stoichiometric ratios, potential soil extracellular enzymes activities and the stoichiometric imbalances were analyzed using two-way ANOVAs, with season and treatment as fixed effects and plot as a random factor (function lme in lme4 package in R v3.5.1; Bates et al., 2015; R Core Team, 2018). The data were log- or square-root-transformed prior to analysis if distributions were skewed to improve the normality of residuals. The assumptions of normality and homocedasticity were checked using Shapiro-Wilk and Levene tests, respectively. The existence of a temporal autocorrelation structure between time points (seasons) in the model residuals was checked by including an autocorrelation structure in the models and comparing the results of the mixed linear models with and without autocorrelation structure. It was not detected a significant temporal autocorrelation in any of the variables and the mixed linear models without

autocorrelation structure were the most parsimonious model.

We performed a principal component analysis (PCA) to visualize the correlations among the soil, microbial and environmental variables using correlation matrices with standardized variables. Vector variables were grouped by category (e.g. soil and microbial elements) using different colors. Cases were separated by treatment and season, and the effects of the chronic drought treatment and season were then tested for the scores of the first two components (PC1 and PC2) of the PCAs using one-way analyses of variance (ANOVAs) and Tukey HSD post hoc tests (Jolliffe 2002; Quinn and Keough 2009). Pairwise Pearson correlations between the soil, microbial and environmental variables were performed and represented in a heat map. Strongly correlated variables were clustered using the VARCLUS clustering algorithm that iteratively splits clusters of variables and reassigns the variables to clusters until no more splits are possible (SAS Institute Inc. 2018). The correlations between C_{micro}, N_{micro}, P_{micro} and swclab for each season were further explored using Pearson's correlations. The effect of season on the relationships between C_{micro}, N_{micro}, P_{micro} and swclab was tested using analyses of covariance (ANCOVAs), with season as a fixed factor and swclab as the covariate.

3. Results

3.1. Effect of chronic drought and seasonal drought on soil microclimate

Swclab was consistently lower in the chronic drought than the control treatment and was highest in winter and lowest in summer (Fig. 1a). The chronic drought treatment, however, caused higher reduction of swclab in winter than in the other seasons. The chronic drought treatment did not have main effects on soil temperature although there was a significant TreatmentxSeason interaction (Fig. 1b). Soil temperature was highest in summer and lowest in winter.

3.2. Effect of chronic drought and seasonal drought on soil microbial biomass and nutrients

The chronic drought treatment caused a significant reduction of N_{micro} (42%) and in P_{micro} (65%) in particular, and a marginally significant reduction of C_{micro} (32%) (Fig. 2a–c). These changes, however, were not reflected into significant alterations on microbial C:N, C:P and N:P ratios (Fig. 2d–f). The effect of season on C_{micro} and N_{micro} was greater than the effect of the chronic drought treatment, where C_{micro} was lowest in spring and N_{micro} was highest in winter. In contrast, P_{micro} did not change seasonally. As a result, microbial C:N ratios increase during the dry seasons (summer and autumn), C:P ratios were also lowest in spring and N:P ratios were highest in winter.

C_{micro}, N_{micro} and P_{micro} were strongly positively correlated with swclab, particularly N_{micro} (Fig. 3). The correlation between C_{micro} and swclab varied throughout the seasons ($P = 0.0051$ for the swclab × Season interaction). C_{micro} was more strongly correlated with swclab during the driest seasons of summer and autumn, with similar slopes, whereas the correlations were weakest during the wetter seasons (winter and spring, Fig. 3a). The response of N_{micro} to swclab also varied seasonally ($P = 0.007$ for the swclab × Season interaction), although slopes did not differ so much between dry and wet periods (Fig. 3b). P_{micro} was correlated with swclab similarly throughout the seasons ($P = 0.5955$ for the swclab × Season interaction, Fig. 3c).

3.3. Effect of chronic drought and seasonal drought on soil nutrients, potential enzyme activities and microbial to soil stoichiometric imbalances

Six years after the experiment establishment, TOC had higher values in control compared to chronic drought plots, while the opposite happened for TN, although differences were not statistically significant (Table 1). The effect of chronic drought on the soil ammonium in the same soil samples changed across seasons. Ammonium decreased in response to chronic drought during the wettest seasons (winter and spring), whereas it increased during the driest seasons (summer and autumn). Soil nitrate had lower values in chronic drought plots compared to control plots during spring.

ETN and Mn were significantly higher in the chronic drought than the control treatment (Table 1). EOC, ETN and Mn were consistently highest in summer in both treatments, whereas K and Mo were highest in winter and Pt was lowest in autumn. Soil extractable ratios were only significantly affected by season, with highest C:N and C:P values in summer (Table 1) and lowest N:P ratios in winter.

Chronic drought decreased potential ure, prot and acp enzyme activities and seasonal drought caused a declines in akp activities in autumn (Table 1).

The stoichiometric imbalance between the soil extractable fractions and microbial biomass was affected by seasonal but not by chronic drought (Fig. 4). The C:N imbalance was lower during summer and autumn, as a result of the lower C:N ratios in the extractable fraction relative to the C:N ratios of microbial biomass during the drier seasons (Fig. 4a). In contrast, the N:P imbalance increased from the wettest seasons (winter and spring) to the driest seasons (summer and autumn,

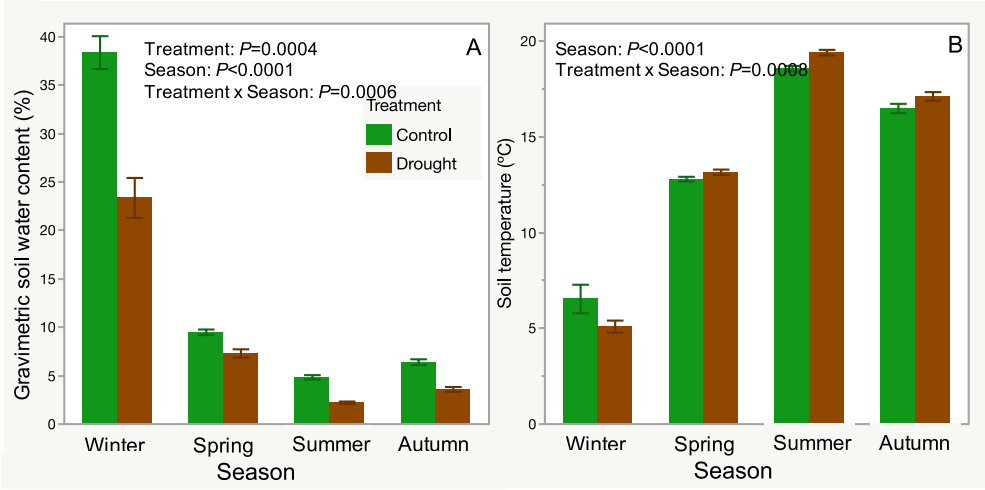

Fig. 1. Soil microclimatic variables in the treatments and seasons measured in 2014 (16 years after the beginning of the experiment). Mean (±standard error) of A) gravimetric soil water content and B) soil temperature. Chronic drought samples are in brown, control samples are in green. Significant effects of treatment and season are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

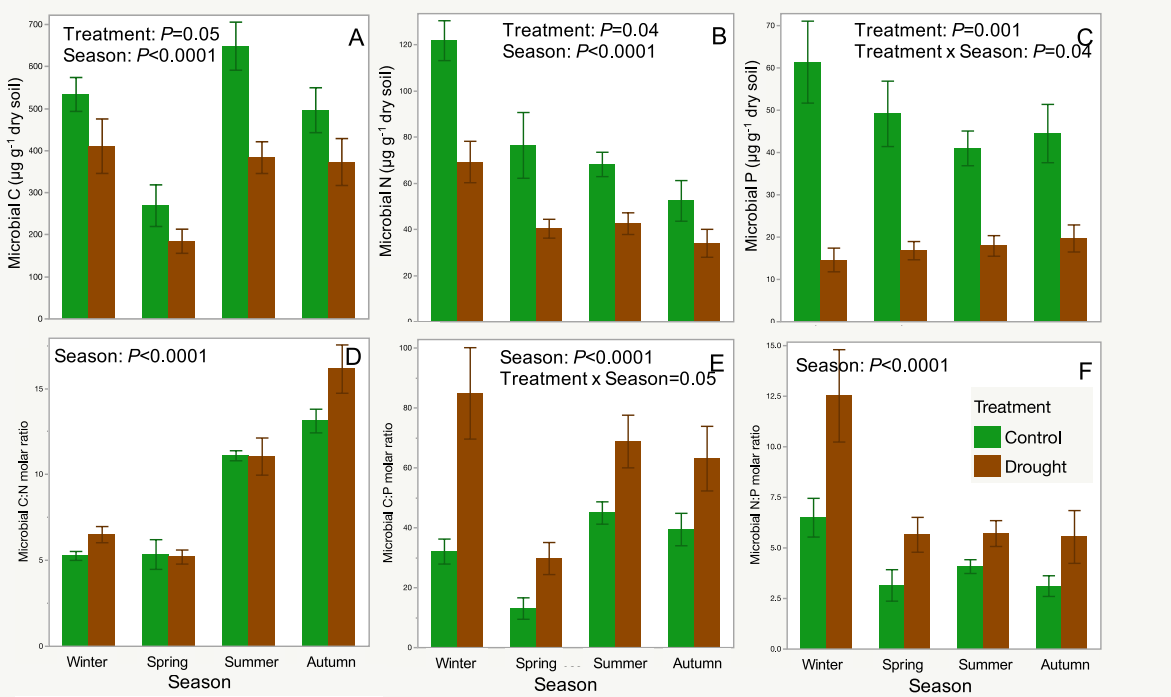

Fig. 2. Elemental composition of microbial biomass in the treatments and seasons measured in 2014 (16 years after the beginning of the experiment). Mean (±standard error) of microbial A) carbon, B) nitrogen and C) phosphorus concentrations and microbial D) C:N, E) C:P and F) N:P molar ratios. Chronic drought samples are in brown, control samples are in green. Significant effects of treatment and season are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

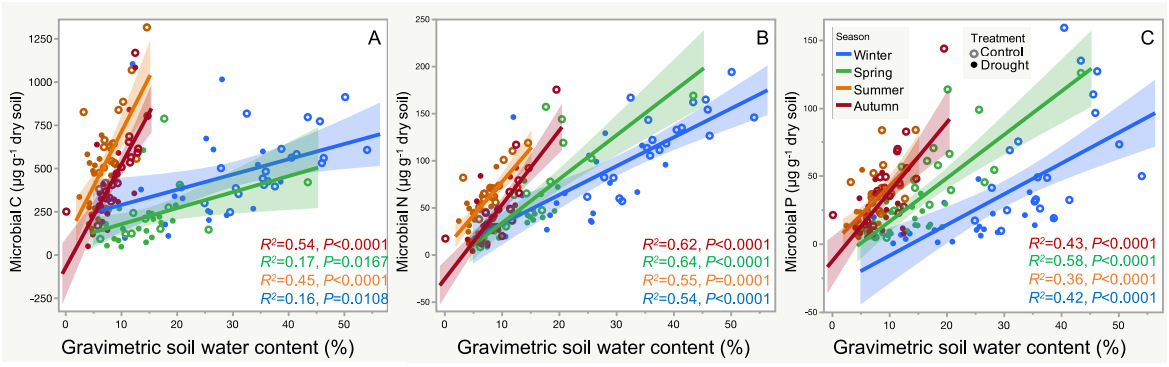

Fig. 3. Correlations between microbial C, N and P concentrations and soil water content measured in 2014 (16 years after the beginning of the experiment). *P* values and R^2 coefficients for each season are indicated. Shaded areas are the 95% confidence intervals of the regression lines. Chronic drought samples are represented by filled dots, control samples are represented by empty dots. Different seasons are represented by different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4c), and the C:P imbalance was highest in spring (Fig. 4b).

3.4. Multivariate effects of chronic drought and seasonal drought

The first (PC1) and second (PC2) principal components of the PCA performed with the soil and microbial variables explained 26.6 and 17.2% of the total variance, respectively (Fig. 5). The extracellular enzymatic activities akp, prot, ure, bgl, N_{micro} and P_{micro} contributed more to PC1, and the C:P and N:P ratios, EOC and ETN contributed more to PC2 (Fig. 5c). Samples differed between the chronic drought and control treatments along PC1 ($P < 0.0001$). The chronic drought samples had the lowest scores in PC1 and thus are associated with the lowest extracellular enzymatic activities, N_{micro} and P_{micro}. The samples also differed seasonally along PC1 ($P = 0.0002$), with higher scores in winter and spring than in summer and autumn. Samples also differed between the chronic drought and control treatments along PC2 ($P < 0.0001$), with the highest scores and therefore extractable C:P and N:P ratios, EOC and ETN in the chronic drought treatment and in summer and the lowest scores in the control treatment and in winter.

Iterative variable clustering allowed us to identify groups of variables that were strongly correlated, explaining 70.1% of the total variation in the data (Fig. 6). N-acquiring enzymes, bgl, akp, N_{micro}, Mo_{micro} and Mn_{micro} were strongly positively correlated and constituted a well distinguished cluster (cluster 1). Soil ETN and the C:P and N:P ratios formed a second strongly correlated cluster of variables (cluster 2). A third cluster was composed of Pt, Pi and Po. The microbial C:N ratios, swclab, stemp and sresp were also strongly correlated, although the correlations were positive between microbial C:N and stemp and between sresp and swclab and negative between the other combinations (cluster 4). The microbial C:P and N:P ratios were also strongly

Table 1
Soil elemental concentrations and potential enzymatic activities in the control and experimental drought treatments across seasons. All variables were measured 16 years after the start of the experiment (2014), except TOC, TN, NH_4^+ and NO_3^- that were measured 6 years after the start of the experiment (2005). *P* values for the two-way ANOVAs are included in the last three columns. Significant differences at $\alpha = 0.05$ are in bold. Values are means ± standard errors. Different letters indicate significant differences among seasons within treatments according to one-way ANOVAs, * indicates significant differences between treatments within seasons according to one-way ANOVAs. Concentrations are in % for TOC and TN, μg g^{-1} of dry soil for the rest of soil fractions and in μmol g^{-1} of dry soil h^{-1} for potential enzyme activities. TOC: Total organic carbon; TN: Total nitrogen; EOC: Extractable organic carbon; ETN: Extractable total nitrogen; Pt: Extractable total phosphorous; ure: urease activity; prot: protease activity; bgl: β-glucosidase activity; acp: acid phosphatase activity; akp: alkaline phosphatase activity.

Variable	Treatment	Season				*P*		
		Winter	Spring	Summer	Autumn	Season	Treatment	Season × Treatment
TOC	Control	1.78 ± 0.10	–	–	–	-	0.0822	–
	Drought	2.28 ± 0.13	–	–	–			
TN	Control	0.25 ± 0.02	–	–	–	-	0.1214	–
	Drought	0.18 ± 0.02	–	–	–			
TOC:TN	Control	9.71 ± 1.18	–	–	–	–	**0.0002**	–
	Drought	18.78 ± 2.21	–	–	–			
NH_4^+	Control	4.28 ± 0.27^{a}*	3.30 ± 0.54^{a}	0.96 ± 0.22^{b}	2.89 ± 0.45^{a}*	**<0.0001**	0.6598	**<0.0001**
	Drought	2.23 ± 0.13^{a}*	2.28 ± 0.10^{a}	2.01 ± 0.28^{a}	4.92 ± 0.28^{b}*			
NO_3^-	Control	1.46 ± 0.72^{a}	6.82 ± 1.83^{b}*	1.34 ± 0.77^{a}	2.39 ± 0.45^{a,b}	**0.0214**	0.5935	**0.0024**
	Drought	2.35 ± 0.17	1.73 ± 0.65*	1.28 ± 0.22	2.02 ± 0.36			
EOC	Control	308.25 ± 82.26^{a}	441.87 ± 65.26^{a,b}	646.22 ± 116.65^{b}	439.49 ± 79.51^{a,b}	**<0.0001**	0.1423	0.9852
	Drought	507.99 ± 96.92^{a}	652.58 ± 110.00^{a,b}	829.16 ± 108.56^{b}	569.55 ± 85.30^{a,b}			
ETN	Control	32.02 ± 5.51	43.67 ± 5.27	53.92 ± 8.79	44.43 ± 7.35	**0.0008**	**0.0475**	0.7557
	Drought	45.06 ± 7.49^{a}	72.59 ± 10.87^{b}	78.82 ± 8.95^{b}	66.06 ± 9.30^{a,b}			
Pt	Control	57.40 ± 5.27^{a}	57.31 ± 7.58^{a}	57.56 ± 7.31^{a}	39.52 ± 4.64^{b}	**0.0001**	0.4440	0.4629
	Drought	44.27 ± 6.33^{a,b}	39.37 ± 6.14^{a,b}	49.16 ± 6.73^{a}	34.97 ± 6.57^{b}			
C:N	Control	9.93 ± 0.66^{a}	11.59 ± 0.55^{a}	13.72 ± 0.31^{b}	11.36 ± 0.40^{a}	**<0.0001**	0.8865	**0.0020**
	Drought	12.17 ± 0.72	11.63 ± 0.54	12.44 ± 0.52	10.78 ± 0.38			
C:P	Control	13.55 ± 2.61^{a}	24.94 ± 3.97^{b}	34.18 ± 4.72^{b}	29.38 ± 4.69^{b}	**<0.0001**	0.2001	0.1266
	Drought	33.42 ± 5.47^{a}	58.06 ± 8.52^{b}	53.99 ± 8.22^{b}	43.00 ± 5.56^{b}			
N:P	Control	1.30 ± 0.16^{a}	2.06 ± 0.27^{a,b}	2.48 ± 0.33^{b}	2.59 ± 0.37^{b}	**<0.0001**	0.1391	0.3833
	Drought	2.58 ± 0.35^{a}	4.83 ± 0.58^{b}	4.38 ± 0.62^{b}	4.33 ± 0.46^{b}			
K	Control	460.41 ± 39.83^{a}	373.47 ± 42.37^{a,b}	299.40 ± 17.47^{b}	265.38 ± 23.21^{b}	**<0.0001**	0.0913	0.3733
	Drought	337.85 ± 31.65	320.24 ± 20.44	279.73 ± 17.95	252.42 ± 26.05			
Mn	Control	1643.74 ± 383.98^{a,b}	2648.34 ± 493.35^{b,c}	3230.16 ± 525.37^{c}	963.71 ± 141.72^{a}	**<0.0001**	**0.0010**	0.8877
	Drought	3488.47 ± 704.78^{a,b}	3617.99 ± 847.40^{a,b}	4155.81 ± 272.14^{a}	1978.87 ± 364.19^{b}			
Mo	Control	26.27 ± 2.05^{a}	16.80 ± 0.81^{b}	21.51 ± 1.11^{a}	16.73 ± 0.78^{b}	**<0.0001**	0.4519	0.0702
	Drought	22.71 ± 1.67	20.39 ± 1.37	22.20 ± 1.44	19.54 ± 1.32			
ure	Control	6.25 ± 0.40^{a}*	5.83 ± 0.63^{a,b}	4.85 ± 0.51^{a,b}	4.40 ± 0.50^{b}	0.0940	**0.0009**	0.2211
	Drought	3.16 ± 0.30*	3.52 ± 0.29	3.05 ± 0.39	3.35 ± 0.43			
prot	Control	0.22 ± 0.03	0.27 ± 0.03	0.27 ± 0.02	0.24 ± 0.02	0.0602	**0.0319**	0.9591
	Drought	0.17 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.18 ± 0.02			
bgl	Control	11.57 ± 1.42^{a}	9.76 ± 1.13^{a,b}	8.53 ± 0.97^{a,b}	7.28 ± 0.64^{b}	0.2438	0.3230	0.0733
	Drought	6.95 ± 0.81	8.01 ± 0.66	8.22 ± 0.98	7.09 ± 0.79			
acp	Control	30.13 ± 2.12*	27.88 ± 2.30	27.87 ± 1.59*	26.15 ± 1.94*	0.5755	**0.0035**	0.3875
	Drought	16.56 ± 1.22*	17.97 ± 1.16	14.08 ± 2.02*	17.21 ± 1.15*			
akp	Control	26.63 ± 2.63	24.23 ± 3.23	24.25 ± 1.62	19.09 ± 1.61	**0.0442**	0.1710	0.4507
	Drought	18.79 ± 2.23	21.28 ± 2.23	17.99 ± 1.75	14.99 ± 1.40			

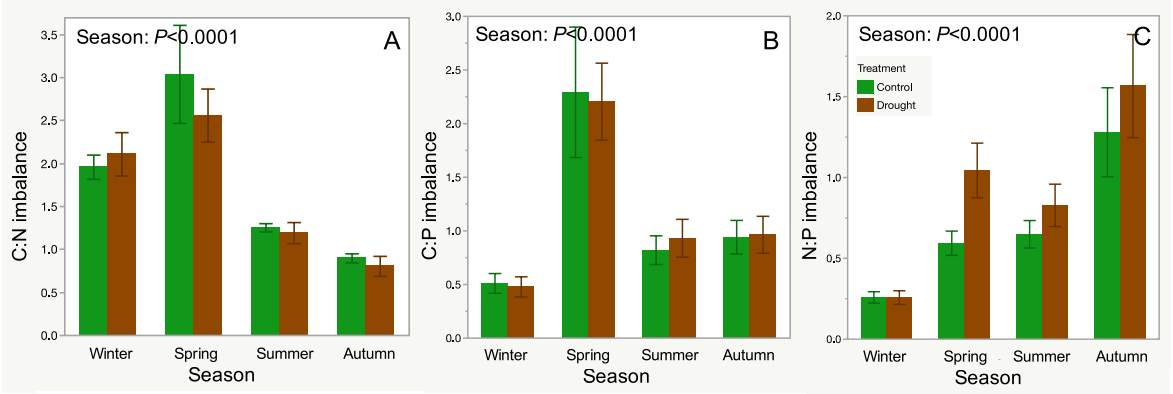

Fig. 4. Stoichiometric imbalances between the soil extractable fractions and soil microbes measured in 2014 (16 years after the beginning of the experiment). Mean (±standard error) of A) C:N imbalance, B) C:P imbalance, C) N:P imbalance. Stoichiometric imbalances are calculated as the ratio of soil extractable stoichiometric ratios to the respective ratios of microbial biomass. Chronic drought samples are in brown, control samples are in green. Significant effects of treatment and season are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

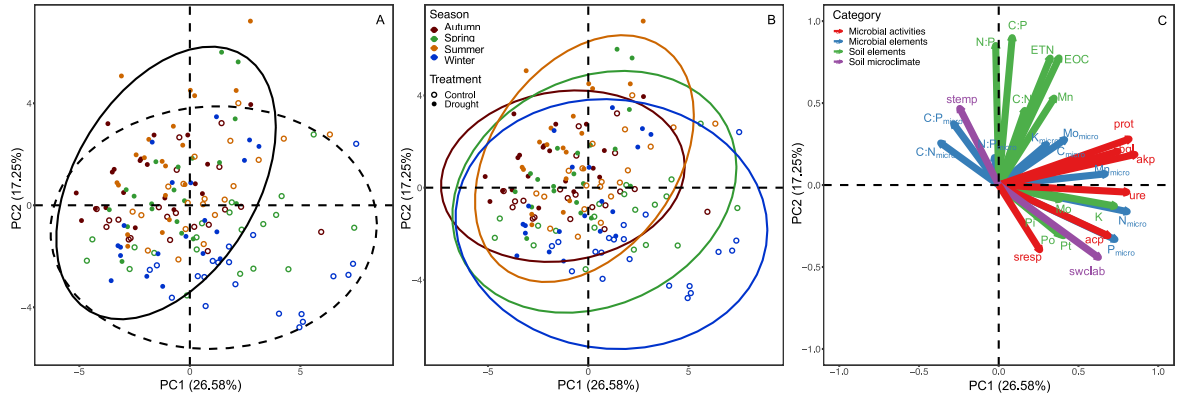

Fig. 5. Two-dimensional representation of the areas defined by the first two principal components (PC1 and PC2) of the principal component analysis of season, treatment, microbial activities (soil respiration and enzymatic activities), soil extractable fractions, microbial elemental concentrations and soil microclimatic variables. All variables included were measured in 2014 (16 years after the beginning of the experiment). A) Values assigned to each soil sample for PC1 and PC2 grouped by treatment. B) Values grouped by season. C) Relative contribution of each variable to PC1 and PC2. Chronic drought samples are represented by filled dots, control samples are represented by empty dots. Different seasons are represented by different colors. Vector colors correspond to the variable categories. The percent contributions to the total variability are in parentheses. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

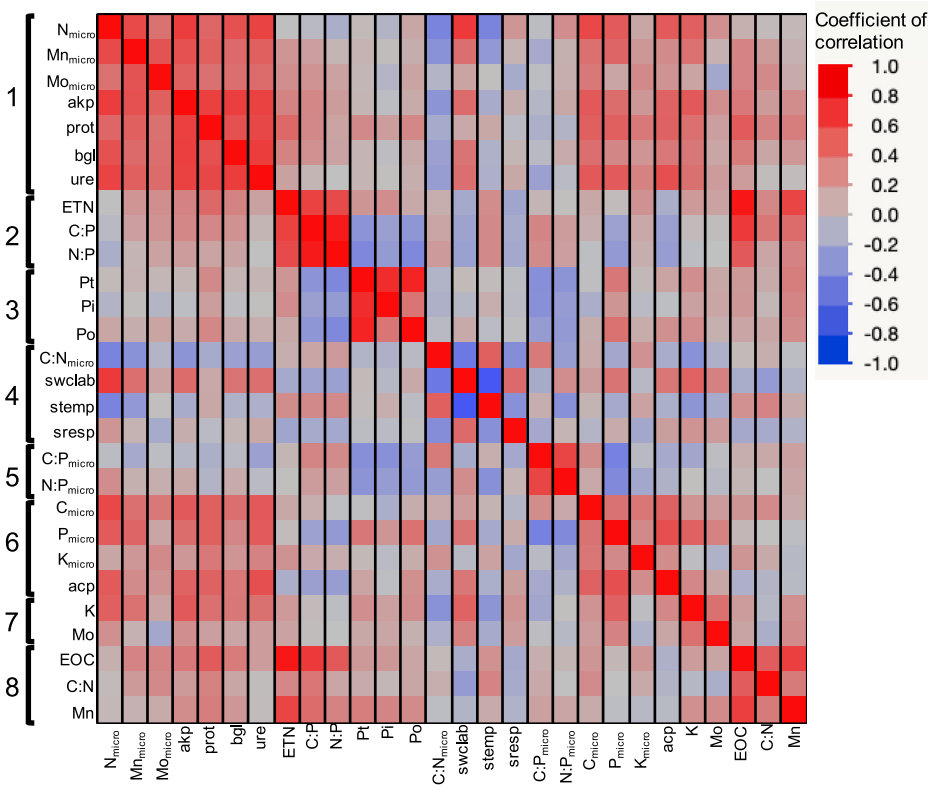

Fig. 6. Heat map of pairwise correlation coefficients between the soil, microbial and environmental variables measured in 2014 (16 years after the beginning of the experiment). The colors represent the coefficient of correlation between each pair of variables, where red represents a positive correlation and blue represents a negative correlation. The brackets and numbers indicate clusters of strongly correlated variables. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

positively correlated (cluster 5). C_{micro} was strongly correlated with P_{micro}, K_{micro} and acp (cluster 6). Extractable K and Mo were also strongly correlated (cluster 7), as were EOC, C:N and Mn (cluster 8).

4. Discussion

4.1. Effect of chronic drought on the C:N:P stoichiometry of microbial biomass and soil

Chronic drought altered soil N availability and the capacity of soil microbial community (as a whole) to retain N and P. Soil ETN increased in the chronic drought treatment (Table 1), particularly in summer, consistent with previous studies of the effects of chronic drought on extractable N (He and Dijkstra 2014; Matías et al., 2011). This result, and the consistently higher values of EOC (although not statistically significant) and TOC (marginally significant) in the chronic drought plots compared to control plots (Table 1), are evidences of the accumulation of organic matter in upper soil layers. The chronic conditions of low water availability may have impaired substrate mobility and accessibility (Borken and Matzner 2009; Moyano et al., 2013), thus reducing the release of enzymes from soil microbes. Persistent decreases in the potential mineralization of N and P and the depolymerization activities of soil microbes (decrease in prot, ure and acp in our study; Table 1) may also slow the rates of SOM decomposition and the capacity

of microbes to uptake N and P (Dijkstra et al., 2015). The impaired microbial SOM mineralization and substrate accessibility under chronic drought most likely caused the significant and persistent reductions in microbial N and particularly microbial P and the marginal reductions of microbial C in chronic drought plots across all seasons (Fig. 2), supporting first and second hypotheses. These data, together with previous results from the same site (Asensio et al., 2021), provide clear evidences of restricted microbial activity and N and P retention in the microbial biomass pool under chronic drought and of the accumulation of organic matter in the soil.

Despite the evidences of lower SOM mineralization under chronic drought, soil ammonium accumulated during the driest seasons (summer and autumn) (Table 1). The lowest N stored in the microbial biomass pool, together with the lower plant biomass growth and N storage under chronic drought in the same sites (Sardans et al. 2008a, 2020), may explain the accumulation of NH_4^+ in chronic drought plots during the driest seasons. By contrast, chronic drought caused a reduction of soil NH_4^+ during the wettest seasons (spring and winter) and of NO_3^- during spring, the period of highest plant growth in this Mediterranean ecosystem (Ogaya and Peñuelas, 2004). The lower amount of mineralized N released from microbial biomass probably contributed more to the lower availability of NH_4^+ and NO_3^- during spring than the increase in plant N uptake, since total vegetation N storage was lower under chronic drought conditions compared to control plots (Sardans et al. 2008a, 2020). Moreover, a recent study in the same sites shows that both plant and soil have lost substantial amounts of N under chronic drought conditions compared to control plots (Sardans et al., 2020). An impaired capacity of the microbial biomass pool to retain N due to chronic drought conditions may therefore reduce plant N availability during the growing period and increase the risks of N losses from the ecosystem.

Unlike N, extractable P compounds did not accumulate in the soil, but even decreased (although not significantly) (Table 1), consistent with consistent with previous results showing lower potential phosphatase enzymatic activity (Table 1, Sardans and Peñuelas 2005; Asensio et al., 2021). Phosphorous generally has low mobility in Mediterranean soils, where it can precipitate with carbonates, mainly at the early and intermediate stages of soil chronosequences, and with Fe/Al–P minerals in older soils (Carreira et al., 1997; Sardans and Peñuelas 2013). The accumulation of SOM under chronic drought may also increase the adsorption capacity of soil and further reduce the mobility of P in these soils (White 1981; Missong et al., 2018). However, despite total soil P did not decrease significantly in response to chronic drought, microbial P decreased drastically compared to microbial N and C (Fig. 2c), leading to consistent but not significant trends toward higher microbial C:P and N:P ratios across all seasons (Fig. 2e and f). Physiological adaptations of the microbial community to save P under the conditions of the low P availability exacerbated by chronic drought may account for the drastic decrease in microbial P (Merchant and Helmann, 2012). Recent evidence has shown that microorganisms can minimize their P requirements when soil P availability is low by switching from phospholipids to betaine lipids when building biomass (Warren 2020). This mechanism could account for a decrease of around 10% in the amount of P required for microbial growth. Other P sparing and recycling strategies, such as the increase in the ribosome and phospholipids degradation (Merchant and Helmann, 2012) or shifts to microbial communities towards more efficient P recycling have been described (Bergkemper et al., 2016), although still poorly investigated in soils that undergo chronic drought. The generally low availability of soil P in Mediterranean soils may therefore be exacerbated by chronic drought conditions, causing physiological adaptations or community shifts in soil microbes to save P and imbalances in soil microbial P stoichiometry.

The role of microbial biomass in retaining N and P in the soil is important in Mediterranean ecosystems (Qiu et al., 2009; Aponte et al., 2010), because microbial biomass might represent a reservoir of nutrients for vegetation during periods of lower activity (e.g. summer drought) that are generally released later during periods of growth (Blackwell et al., 2010; Schaeffer et al., 2017), although its contribution has not been quantified in Mediterranean ecosystems. A lower capacity of SOM decomposition and nutrient retention in the microbial biomass pool during unfavorable periods for plant growth (Fig. 2), together with an increasing frequency of torrential and irregular rains and higher rates of erosion, can lead to the reduction of assimilable soil nutrients for plants during the plant growing season (Table 1, Bloor and Bardgett, 2012; Peñuelas et al., 2018). Mediterranean ecosystems are exposed to frequent and short cycles of drying and rewetting. Soil rewetting rapidly activates soil microbes and the mineralization of organic compounds accumulated during seasonal drought, releasing nutrients to the soil solution (Barnard et al., 2020; Borken and Matzner 2009). Microbes later activate the synthesis of proteins and growth (Barnard et al., 2013), which immobilizes microbial nutrients (Gao et al., 2016; Yevdokimov et al., 2016). Plant uptake generally takes longer to begin after soil is rewetted (Wraith et al., 1995), so microbial nutrient immobilization can represent a buffering mechanism to ensure the availability of nutrients to plants during periods of growth (Bardgett et al., 2005; Qiu et al., 2009). Long-term drought, however, may reduce the N and P stored in the microbial biomass pool and the supply of mineralized forms of these nutrients during favorable periods for vegetation.

Strategies of the soil microbes to overcome drought may have also contributed to the smaller effects of chronic drought on microbial C and N compared to microbial P. Chronic drought requires the development of microbial adaptations, such as the accumulation of osmolytes to overcome water stress and the energy limitation imposed by low accessibilities of organic substrates (Schimel et al., 2007, Gutiérrez-Girón et al., 2014) or the creation of resistance structures (e.g. cell-wall peptidoglycans) to resist osmotic stress (Marchus et al., 2018; Yadav et al., 2018). Specifically, bacteria typically use C- and N-rich amino compounds as primary osmolytes (Csonka 1989), whereas fungi use C-rich polyols (Witteveen and Visser 1995) that do not contain N. Soil microbes can thus increase their total cytoplasmic constituents under conditions of extreme water stress, by as much as 30–40% of total C for both bacteria and fungi and by nearly 60 and 20% of total N for bacteria and fungi, respectively (Schimel et al., 1989). Microbes also have additional C costs to provide energy for the synthesis of osmolytes, which can represent two-to three-fold the C cost for the simple accumulation of C compounds (Schimel et al., 2007). These increasing C demands of soil microbes under chronic drought conditions were also indicated by the enzymatic activities, with a higher production of C-catalyzing enzymes relative to P- and N-catalyzing enzymes (Asensio et al., 2021), pointing to an increase in microbial C limitation. The need to develop mechanisms of drought resistance together with the lower mobility and accessibility of organic substrates in the chronic drought treatment probably increased the relative demand of C-catalyzing enzymes and the C costs of acquiring resources. The equivalent accumulation of C- and N-rich compounds as a mechanism to resist drought stress may account for the lack of effect of chronic drought on the microbial C:N ratios and may have contributed to the lower sensitivity of microbial C and N to chronic drought compared to microbial P.

4.2. *Effect of seasonal drought on the C:N:P stoichiometry of microbial biomass and soil*

Unlike chronic drought, seasonal drought caused marked but transient fluctuations in the C:N:P stoichiometry of the soil microorganisms among seasons (Fig. 2d–f). Microbial C:N ratios increased during the dry seasons to much higher values (C:N = 11.1–14.6) than those reported for temperate forests (C:N = 8.6) and global averages (C:N = 7.6) (Xu et al., 2013). Seasonal shifts toward a microbial community more tolerant to drought stress, with the increasing dominance of fungi compared to bacteria (Strickland and Rousk 2010; Preece et al., 2019), together with the primary accumulation of C-rich osmolytes in fungi and the synthesis of resistance structures to cope with water stress (Kakumanu et al.,

2019), may have contributed to the higher microbial C:N ratios and lower respiration rates during periods when water was limited. This was also indicated by the cluster of strong correlations among microbial C:N, microbial respiration and soil water content (Fig. 6). In contrast, soil extractable C:N ratios (C:N = 11.0–13.1) were lower than for temperate forests (C:N = 18.7) and globally (C:N = 16.4) (Xu et al., 2013). The increase in microbial C:N during the drier seasons consequently lowered the C:N imbalance between the extractable soil fractions and the microbes (Fig. 4a), representing an increase in the microbial C limitation in summer and autumn. An increase in C limitation of soil microbes also impairs the capacity of the microbial community to retain N (Marañón-Jiménez et al., 2019), as it is shown by the lower pool of microbial N during the driest seasons (Fig. 2), which may contribute to a lower availability of mineralized forms of N during the vegetation growing season (Table 1).

The progressive increase in the N:P imbalance from the wetter to the drier seasons also indicated an increase in the microbial P limitation relative to N (Fig. 4c). This finding is consistent with previous evidence of the higher production of P- versus N-catalyzing enzymes during seasonal drought at the same study site (Asensio et al., 2021). Given the lower mobility and solubility of soil P compared to soil N (He and Dijkstra 2014), decreases in the availability of soil water may lead to larger restrictions in microbial P uptake compared to microbial N uptake and stimulate more the microbial production of P-catalyzing enzymes than the production of N-catalyzing enzymes. This Mediterranean forest indeed had two distinct periods of water (Fig. 3) and nutrient (Fig. 4) limitations for soil microbes. Microbial biomass depended greatly on the availability of water and on the accessibility of C and P (Figs. 3a and 4a and c) during the dry periods (summer and autumn), whereas microbes shifted from C to N limitation during the wet periods (spring and winter) (Fig. 4a).

5. Conclusions

Both seasonal and long-term periods of chronic water limitation caused reductions in the size of the N and P pool stored in microbial biomass, partially supporting our first hypothesis. The lower mineralization activity under both seasonal and chronic water limitation led to the accumulation of C- and N-rich organic compounds in the upper soil layers. The responses of microbial biomass C and nutrients, however, differed between the seasonal and chronic droughts. Chronic drought reduced microbial biomass C, N and particularly P substantially and persistently across seasons, in agreement to our second hypothesis. The lower N pool in microbial biomass most likely was accompanied by a lower availability of mineralized forms of N during the vegetation growing season (spring), in agreement to our third hypothesis. Chronic drought may exacerbate the low mobility of P in soil due to its adsorption in accumulated SOM. The low soil P availability probably triggered microbial community shifts or physiological adaptations to spare P, which reduced particularly the size of the microbial P pool stored. Nonetheless, microbial C:N ratios remained unaltered by chronic drought, likely associated with the equivalent accumulation of C- and N-rich osmolytes by microbes. In contrast, soil microbes experienced transitory changes in the C:N:P stoichiometry in response to seasonal drought, increasing their C content relative to N content. Therefore, while microbial P is more sensible to chronic water stress than microbial N and C, the last are more closely coupled to seasonal fluctuations of water availability. These findings are consistent with the idea that microbial adaptations to seasonal drought mainly involve changes in microbial C and N contents, whereas chronic water limitation drastically and persistently impairs the P stored in the microbial biomass pool.

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.

Acknowledgments

This research was supported by the Spanish Government grant PID2019-110521GB-I00. The European Research Council Synergy grant ERC-SyG-2013-610028 IMBALANCE-P, and the Catalan Government grant SGR 2017-1005. SMJ was supported by the Serra Hunter fellowship Programme from the regional government of Catalonia.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108515.

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