



## Altered rainfall greatly affects enzyme activity but has limited effect on microbial biomass in Australian dryland soils

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### ABSTRACT

Drylands support a substantial proportion of the world's biodiversity and are important to food production but are sensitive to changes in rainfall regimes. Altered rainfall has been shown to impact plant growth and soil microbial activity in drylands but the longer-term effect on belowground communities and biogeochemical cycles remains uncertain. We explored how four years of reduced and increased rainfall influenced soil total and available carbon (C), nitrogen (N) and phosphorus (P) content, microbial biomass and potential extracellular enzyme activity under field conditions at six dryland sites in eastern Australia. The study coincided with a severe 3-year drought that resulted in low standing plant biomass and soil C content at all sites. Microbial biomass attributes varied considerably across sites, with rainfall treatment effects limited to decreased fungal biomass and lower fungal:bacterial ratios in semi-arid Nyngan and reduced fungal:bacterial ratios and microbial biomass C in semi-arid Quilpie in reduced treatments compared with increased rainfall plots. Similarly, available soil C, N and P varied considerably among sites, with more available N and P at four and all sites, respectively, in reduced rainfall treatments particularly when compared with increased rainfall treatments. Rainfall treatments consistently influenced enzyme activity across all sites, with higher rates in increased rainfall plots indicative of greater microbial activity and enhanced nutrient cycling. Enzymatic activity associated with N cycling showed a negative relationship with available N while enzymes associated with P cycling related positively to available C and negatively to available P. This indicates that microbes invested more in production of enzymes associated with less available nutrients. Enzyme activity was not related to microbial biomass suggesting a disconnect between biomass and enzyme production and that rainfall treatments altered the ecosystem's specific enzyme activity (activity per unit of microbial biomass). Our results suggest that altered rainfall consistently impacted dryland ecosystem function, but that microbial biomass is a poor proxy for rainfall-induced changes in soil processes.

### 1. Introduction

Anthropogenic activities have had detrimental direct and indirect effects on drylands (Höök and Tang, 2013; Barlow et al., 2016). Climatic change associated with increasing atmospheric concentrations of greenhouse gasses have resulted in significant warming, with the global mean temperature in 2019 estimated to be  $1.1 \pm 0.1$  °C above pre-industrial levels (World Meteorological Organization, 2020) and is expected, with high probability, to increase further in the future

(Intergovernmental Panel on Climate Change, 2022). Moreover, current models show high probability of widespread changes in rainfall availability, including longer periods with reduced rainfall (McLaughlin, 2011). This combined with higher temperatures will contribute to increased aridity and greater frequency of drought events (Calanca, 2007; Nam et al., 2015; Alamgir et al., 2020).

Increased aridity and longer dry spells will negatively impact plant and belowground microbial communities (e.g., fungi, bacteria), potentially disrupting vital biogeochemical cycles (Bellard et al., 2012). In the

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short term, plants respond to reduced water availability by down regulating photosynthesis to conserve water, which limits belowground C inputs thus impacting microbial resource availability (Chaves et al., 2009). Longer term water stress can result in reduced biomass production and shifts in community composition (Touchette et al., 2007; Puijalon et al., 2011; De Leonardis et al., 2012), resulting in cascading effects on soil biogeochemical cycling and belowground communities via changes in resource quantity and quality (Baquerizo et al., 2013; Maestre et al., 2015; Hu et al., 2021). In addition, water limitation can directly result in reduced microbial activity and/or in lower microbial biomass production in the longer term or under extreme drought (Jensen et al., 2003; Ren et al., 2018). Short term water stress can therefore impact microbial physiology and metabolism, resulting in altered process rates, while longer term reductions in rainfall can result in shifts in microbial community composition (Schwinning and Sala, 2004; Nielsen and Ball, 2015).

While increased aridity is a particular concern, some global circulation models predict localised increases in rainfall given the ability of a warmer atmosphere to hold more water (Kharin et al., 2007; Hirabayashi et al., 2008; Kundzewicz et al., 2014). Increased rainfall can enhance primary productivity and plant biomass, microbial activity and biomass, but the effect size is highly context dependent and influenced by mode of delivery; for example, more small events will have very different impacts from fewer but larger events (Nielsen and Ball, 2015). Prolonged periods with above-average rainfall can enhance primary productivity and promote allocation of plant resources to aboveground organs to further improve photosynthetic capability, which will have positive feedback on C inputs into soil (Xu et al., 2011; Zhang et al., 2020). In addition, increased water availability can increase microbial biomass and activity (Manzoni et al., 2014) and hence nutrient cycling and soil organic matter (SOM) decomposition (Vasconcelos et al., 2004) but can result in a reduction in soil available N due to increased uptake by plants (Austin, 2002; Knapp et al., 2008). Hence, water pulses and increased rainfall will impact plant and soil biological communities with cascading effects on biogeochemical cycling.

However, the effects of altered rainfall regimes on dryland structure and functioning remain unclear (Nielsen and Ball, 2015), with experimental rainfall manipulations often resulting in contrasting effects to those observed across aridity gradients (Yuan et al., 2017) making it difficult to predict climate change outcomes. One promising approach to assess impacts on functioning is using (potential) exo-enzymatic activity as a proxy for microbial activity. The enzymes produced by microbes are critical to nutrient cycling by catalysing processes including lignin degradation, hydrolase proteins or mineralization of phosphorus from nucleic acid (Toor et al., 2003; Caldwell, 2005; Allison et al., 2007). This makes enzymatic activity crucial to C, N and P nutrient cycling, processing and SOM formation (Burns and Dick, 2002). While the general negative effects of reduced rainfall on soil microbial biomass, nutrient pool and processes are well established (Blankinship et al., 2011), it is less clear how enzymatic activity responds to altered rainfall in drylands. One caveat is that enzymes have been predicted to accumulate during drought as has been observed for nutrients (Schimel, 2018), with some recent evidence to support this in grasslands (Ochoa-Hueso et al., 2018). Hence, interpreting enzyme activity measurements should be done with some caution.

In this study, we provide insights into the effect of altered rainfall regimes on soil biogeochemistry, microbial assemblages and potential exo-enzymatic activity at six dryland sites across a rainfall gradient in eastern Australia following four years of simulated rainfall changes. The study aimed to address three questions: 1. What are the long-term effects of altered rainfall regimes on soil total and available C, N and P given predicted changes in plant and microbial activity? 2. How does microbial biomass respond to altered rainfall regimes given predicted changes in belowground C allocation and soil water availability? 3. What are the effects of changes in rainfall regimes on enzymatic activity as a measure of microbial activity? Our main hypotheses were that i) soil C and

nutrient pools would be positively related to rainfall across sites whereas rainfall reductions would result in accumulation of available nutrients given reduced uptake by plants and microbes; ii) microbial biomass will be positively related to rainfall but moderated by soil C and nutrient contents; and, iii) enzymatic activity will be positively related to rainfall given greater C and nutrient requirements to sustain microbial biomass and activity. In addition, we expected that the effects of reduced rainfall would be greater at sites with higher historical rainfall because plant and microbial communities are less adapted to water stress, whereas the effects of increased rainfall will be greater at sites with low historical rainfall given reduced water stress (Nielsen and Ball, 2015).

## 2. Materials and methods

### 2.1. Site description and experimental design

The study was conducted at six eastern Australian dryland sites (Table 1, Fig. S1). Two sites are classified as arid (Broken Hill, Milparinka) while the other four are semi-arid (Cobar, Quilpie, Nyngan, Charleville). The vegetation type varied across sites, with Broken Hill being a shrubland dominated by *Maireana pyramidata*, Milparinka, a grassland dominated by *Astrebla lappaceae* and *Astrebla pectinate*, Cobar and Nyngan being open woodland, and Quilpie and Charleville woodland dominated by *Acacia aneura* var. *aneura*. However, at all sites, the experimental plots were positioned in open grassy to shrubby vegetation with no nearby trees. The soil in Broken Hill is brown calcareous sand, Milparinka has grey clay, Cobar and Nyngan have clay with gravel, Quilpie has red clay and Charleville has red sandy soil (Deveautour et al., 2020). The climate varies considerably across the six sites and sampling coincided with a significant drought (2017–2020; Fig. S3). Long term mean annual rainfall and temperature were collected using WorldClim2 (1970–2000), which spatially interpolates monthly climate data at high spatial resolution (approximately 1 km) registered by MODIS satellite platform (Fick and Hijmans, 2017). Weather stations were established at each site in 2016 to monitor rainfall using a rain gauge (Campbell Scientific, CS701), temperature and relative humidity (Vaisala, HMP60) and photosynthetically active radiation (PAR) (Apogee, SQ-110). Additionally, soil moisture was recorded using water content reflectometers (Campbell Scientific, CS616) in each plot at each site to verify treatment effects. Data were stored on a Campbell Scientific CR1000 control module powered by a solar panel and battery, with data transferred to Western Sydney University servers via mobile network.

Rainfall manipulations were established in spring 2016 to assess how altered rainfall affects vegetation, soil biota, and biogeochemical cycles, particularly carbon (C), nitrogen (N) and phosphorus (P), across a rainfall gradient in regions that vary in interannual rainfall, with northern sites generally more variable. A standard 3 × 3 m rainfall shelter design (Yahdjian and Sala, 2002), combined with rainfall collection and gravity-induced transfer of water was used to create three treatments with three replicates at each site: drought (65% rainfall reduction relative to ambient), ambient and increased rainfall (+65% rainfall relative to ambient, Fig. S2). Drought simulation shelters were equipped with rainfall-exclusion roofs to reduce incoming rainfall, with the displaced rain captured in gutters, collected in a bucket and transferred to the nearest neighbouring increased rainfall plot using polyethylene piping. The effective rate of transfer to increased rainfall plots is, however, less than 65% given inefficiencies in transfer and differ among rainfall event sizes, with small events resulting in greater proportional transfer losses in gutters, buckets and pipes while large rainfall events led to saturation of soils across all treatments. However, soil moisture sensor data confirmed appropriate treatment specific changes in soil water content for most event sizes. Ambient and increased rainfall shelters allow throughfall of rainfall but were covered by a permeable mesh bird netting as ‘mock’ roofs to control for shelter effects. The shelters are 0.8 m at their lowest point and 1.89 m at their highest point, with the highest point oriented towards the north, allowing natural

**Table 1**

Location of study sites and their climate type, mean annual precipitation (MAP, in mm year<sup>-1</sup>) and temperature (MAT, in °C), soil texture (2017) and average pH (2017). Climate data based on WorldClim2 (1970–2000; [Fick and Hijmans, 2017](#)).

Site name	Coordinates		Site characteristic					Soil pH (-)	
	Latitude	Longitude	Climate	MAP (mm yr <sup>-1</sup> )	MAT (°C)	Soil Texture (%)			
						Sand	Silt	Clay	
Milparinka	29.6 S	141.7 E	Arid	209	20	72.9	11.4	15.6	8
Broken Hill	32.0 S	141.6 E	Arid	258	18.4	62	11.9	26.1	7.3
Cobar	31.8 S	145.6 E	Semi-arid	363	18.3	68.8	7.6	23.7	6.5
Quilpie	26.6 S	144.6 E	Semi-arid	377	21.5	80.7	7.1	12.2	5.4
Nyngan	31.7 S	146.6 E	Semi-arid	405	18.5	81.6	4.2	14.2	6
Charleville	26.4 S	146.2 E	Semi-arid	494	20.4	58.5	10.4	31.1	4.9

sunlight to reach the core experimental area throughout most of the year.

Vegetation surveys were conducted annually from spring 2016 (pre-treatment) to spring 2020. Vegetation cover was determined visually for each species within a 1.5 × 1.5 m quadrat using a square PVC frame placed in the centre of the plot to avoid edge effects. To reduce impacts on the vegetation, cover was used to estimate aboveground biomass (g m<sup>-2</sup>) using allometric relationships based on allometric relationships for each species from the same sites ([Chieppa et al., 2020](#)). When species could not be reliably identified, allometric relationships for the most similar species and functional group was used instead. Standing biomass varied considerably between sites and through time, with a pronounced decrease in standing biomass coinciding with the prolonged drought ([Fig. S4](#)). However, there were no consistent differences in standing biomass among rainfall manipulation treatments.

## 2.2. Soil sampling and analyses

Soil sampling was completed each spring from 2016 to 2020; however, additional data were collected in 2020 to assess effects of longer term altered rainfall treatments on soil microbial communities and functional characteristics (e.g., biomass, enzyme activity, C and nutrient cycling). In brief, eight soil cores measuring 3.5 cm diameter and 10 cm depth were collected randomly within the same 1.5 × 1.5 m quadrat used for vegetation surveys and combined to form one composite sample per plot per time point. In total, 54 samples were collected each spring, with nine samples per site and three replicates per treatment per year. Samples were stored with ice in coolers and transported to the Hawkesbury Campus, Western Sydney University for processing immediately upon return.

Soil samples were sieved through a 2 mm sieve to remove rocks and plant material. The homogenized soil was then subsampled for individual analyses. Soil water content was estimated by oven drying ~10 g fresh soil at 105 °C for 2 days. Texture (sand, silt, clay content) was assessed using hydrometers following Robertson et al. (1999). Soil pH was measured in a 1:5 soil:water slurry using a calibrated pH meter (S20 SevenEasy Mettler Toledo). Approximately 5 g air dry soil were mixed with 25 mL distilled water in a centrifuge tube and shaken for 1 h before measurements were taken. After grinding to a fine powder, total C and N were determined by oxidative combustion using a CN analyser (TruMac, LECO Corporation, St Joseph, MI, USA) based on the Dumas method ([LECO Corporation, 2003](#)) with a combustion temperature of 1350 °C. Total P was determined on pellets made of 1 g air-dried soil mixed with 2.5 g boric acid powder using an Epsilon 4 Benchtop X-ray fluorescence (XRF) spectrometer (Malvern Panalytical, Malvern, UK).

## 2.3. Microbial biomass, PLFA and enzyme activity

Microbial and available C, N and P, and ammonium and nitrate, were quantified using the extraction method described in [Vance et al. \(1987\)](#). Microbial and available C and N was extracted from 10.5 to 11.5 g fresh soil using 33 mL 0.5 M K<sub>2</sub>SO<sub>4</sub> solution following shaking for 2 h and 4

days of fumigation. Phosphorus was extracted from 4 to 4.05 g fresh soil using 40 mL of reverse osmosis water following shaking for 3 h and 1 day fumigation. Both fumigated and unfumigated samples after shaking were filtered using ashless grade 42 quantitative filter circles (125 mm) and transferred to 70 mL containers. Both fumigated and unfumigated samples were then analysed using a TOC-L analyser (microbial and available C, N content, Shimadzu, Kyoto, Japan) and a AQ2 analyser (microbial and available P, ammonium and nitrate, unfumigated samples only, SEAL Analytical, Maquon WI, USA). Soil weights were corrected to account for moisture content, available nutrients were obtained from unfumigated results, and microbial data were calculated as the difference between fumigated and unfumigated values. No extraction efficiency correction was used to avoid artifacts due to different extraction efficiencies among sites. All data are reported in mg/kg.

The phospholipid fatty acids (PLFAs) of soil microbes were analysed based on the protocol by [Buyer and Sasser \(2012\)](#), with PLFAs extracted from ~4 g freeze-dried soil. Extractions were completed using 4 mL of Bligh-Dyer extract (consisted of 200 mL 50 mM K<sub>2</sub>HPO<sub>4</sub> in H<sub>2</sub>O, 500 mL methanol, and 250 mL chloroform). Evaporation was done using the cold solvent trap Labconco CentriVap (at 30 °C with vacuum). Neutral lipids and glycolipids were removed using chloroform and acetone, and phospholipids extracted using a 5:5:1 methanol:chloroform:water solution. Transesterification was done with 0.2 mL transesterification reagent (0.561 g KOH, 75 mL methanol, 25 mL toluene) and 0.4 mL 0.075 M acetic acid (Acetic Acid Glacial HPLC grade, 99.8%). After transesterification each sample was resuspended in 50 µL hexane and transferred into new vials and analysed using gas-chromatography (Agilent Technologies gas chromatograph). Fatty acids were summed into the following biomarker groups: Total PLFA (sum of all fatty acid groups 10:0–20:5 ω3c), gram positive bacteria (15:0 anteiso, 15:0 iso, 16:0 anteiso, 16:0 iso, 17:0 anteiso, 17:0 iso), gram negative bacteria (17:0 cyclo ω7c, 18:1 ω7c, 18:1 ω9c, 19:0 cyclo ω7c), actinobacteria (16:0 10-methyl, 17:0 10-methyl, 18:0 10-methyl), protozoa (20:2 ω6c, 20:3 ω6c, 20:4 ω6c, 20:5 ω3c), fungi (18:2 ω6c), and the putative arbuscular mycorrhizae (16:1 ω5c). Microbial PLFA data are reported in µg/g.

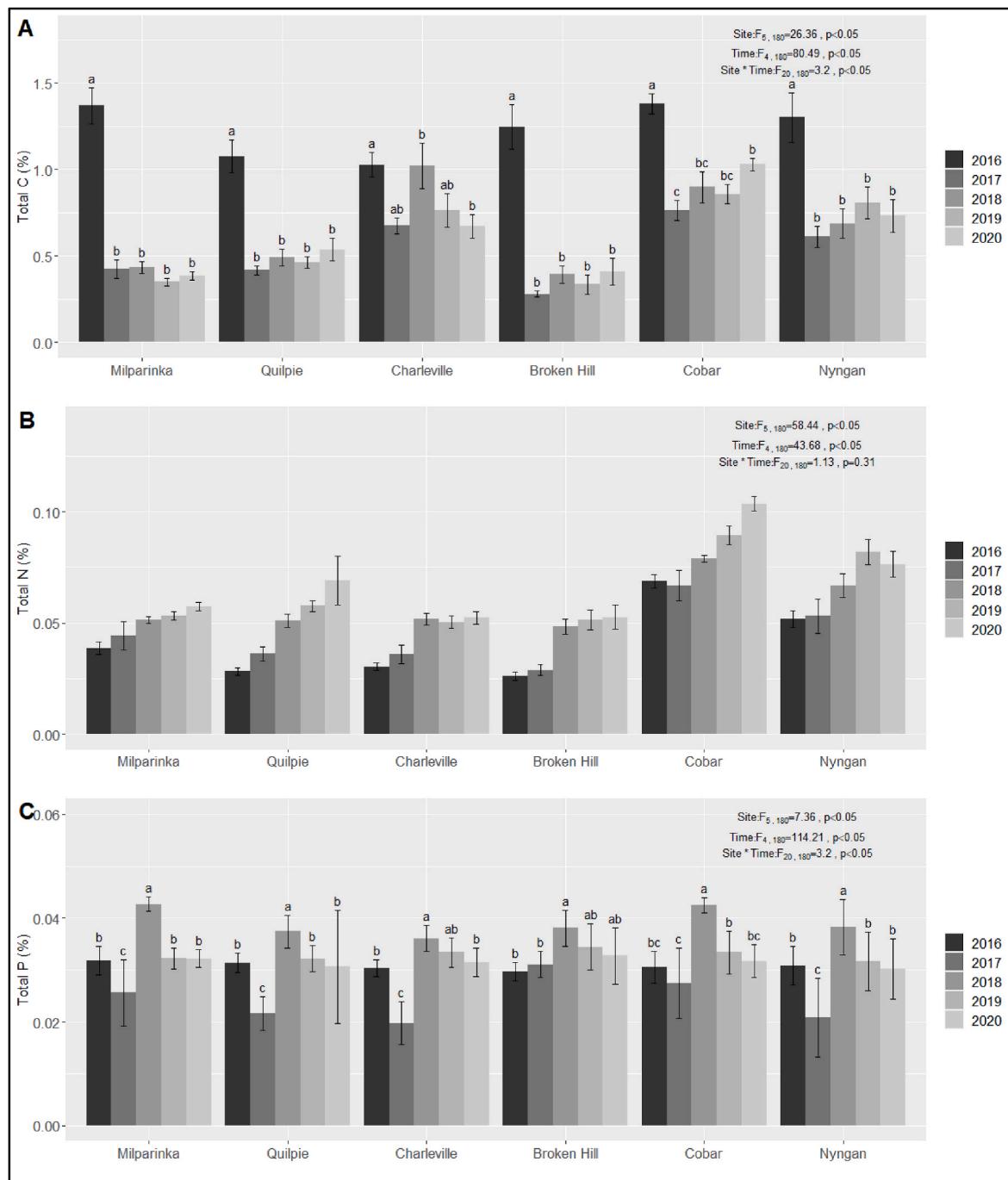
Soil potential extracellular enzyme activities were measured using fluorometry as described in [Bell et al. \(2013\)](#). We analysed seven enzymes representing C cycling (b-1,4-glucosidase, b-D-cellubiosidase, a-glucosidase, b-xylosidase), N cycling (b-1,4-N-acetylglucosaminidase, leucine aminopeptidase) and P cycling (phosphatase). Assays were prepared using 2.75 g fresh sieved soil homogenized in 91 ml of 50 mM buffer and blended for 1 min. All sites used the same buffer. Additional control replicates of soil slurries with 4-methylumbelliflferone (MUB) and 7-amino-4-methylcoumarin (MUC) standard curves were included with each sample. Substrates were incubated for 3 h at 25 °C. After incubation samples were centrifuged for 3 min and moved into 96-well plates using a multichannel pipette. Plates were analysed using TECAN Infinite M200 microplate fluorometer. Obtained fluorometric values are reported in nmol activity g/dry soil/hr.

## 2.4. Statistical analyses

All data were analysed for normality using the Shapiro-Wilk normality test (Royston, 1982). Non-normally distributed data were transformed using the square root function. Data were then divided into two groups: explanatory variables (MAP, one-year rainfall calculated as cumulative site-specific rainfall for the twelve months prior to the 2020 sampling date, three-months rainfall calculated as cumulative site-specific rainfall for the three months prior to the 2020 sampling date, MAT, standing biomass, sand, silt, clay, pH, total C, available C, total N, available N, ammonium, nitrate, total P, available P, C:N ratio,

C:P ratio, N:P ratio) and response variables (total PLFA, gram positive, gram negative, actinobacteria, protozoa, arbuscular mycorrhizae, fungi, fungi/bacteria ratio, microbial C, microbial N, microbial P, and enzyme C, N and P representing the sum of enzymes associated with C, N and P cycling, respectively). We used MAP, one-year and three-month rainfall as explanatory variables as certain biological variables respond to rainfall at different time scales. Specifically, microbial biomass is likely associated with longer term rainfall whereas enzyme activity may be more closely related to short term changes in rainfall.

First, we assessed whether response variables varied among sites and with treatment (increased, ambient, reduced), and year where



**Fig. 1.** Soil C (A), N (B) and P (C) content across sites for 2016–2020. Bars represent standard error ( $n = 9$ ). 2016 represent baseline data with samples collected. Test statistics for significant differences among sites, treatments, through time, and their interaction based on three-way ANOVA. Letters above bars indicate differences among years across all sites where significant interaction site x time effects were observed. Milparinka and Broken Hill are arid whereas Quilpie and Charleville (QLD) and Cobar and Nyngan (NSW) are semi-arid ecosystems.

applicable, across whole dataset, including their interaction, using the ANOVA function in the “stats” R package (Chambers et al., 2017). Tukey’s honest significant difference (Tukey’s HSD) post-hoc tests were conducted if significant site by treatment or time interactions were found. Post-hoc test was conducted within the site treatment or time dataset, depending on interaction. Relationships among response and explanatory variables were then assessed in two steps. First, to reduce the number of variables for further analyses, we evaluated the effect of explanatory variables on response variables using the random forest importance test with the “randomForest” R package (Breiman, 2001). The random forest test was conducted using 1000 trees. The random forest importance test provides insight into which explanatory variable is the most important for predicting the outcome of response variables but does not account for the fact that treatments were nested within sites. Secondly, because random forests tests does not account for the hierarchical nature of the data (i.e., treatments nested within sites), the relationships between explanatory variables identified as most important and response variables were further assessed using the linear mixed-effects model (LME) from the “nlme” R package with both site and treatment included as random effects (Laird and Ware, 1982; Lindstrom and Bates, 1988). The ANOVA function in R from the “stats” R package was used to inspect how well the linear mixed effect model results fit the data. Lastly regression graphs were produced for significant connections to evaluate how strong variables are coupled. All R statistic were conducted using R (R Foundation for Statistical Computing) ver. 3.6.1 (2019-07-05) with R-Studio 2022.07.01 Build 554 "Spotted Wakerobin" Release (7872775e, 2022-07-22) for Windows.

### 3.1. Changes in edaphic variables through time and in response to rainfall manipulations (2016–2020)

Edaphic variables differed considerably among the six sites before the treatments were imposed (i.e., 2016) but there were no systematic pre-treatment differences in edaphic variables among plots designated for the rainfall manipulations. In addition, there were no treatment, or treatment by site and treatment by year, effects observed (Figs. S5A–C). Accordingly, we proceeded to test for site by year effects only using two-way ANOVAs, with Tukey’s HDS post-hoc tests for differences among years within sites. A significant drop in total C content was observed across all sites in 2017 (Fig. 1A), coinciding with the onset of the drought, with C content remaining low throughout 2018–2020 at all sites except semi-arid Charleville compared to 2016. By contrast, total N increased gradually throughout the study in most sites through time (Fig. 1B). Additionally total P varied across sites and time with higher P content in 2018 than 2016. (Fig. 1C). This resulted in corresponding shifts in soil stoichiometry with strong reductions in soil C:N ratios, and significant reductions in soil C:P ratios, throughout the study across all sites (Table S1). In addition, significant differences between sites and treatments were observed for available N and P in 2020 with most sites presenting higher content in reduced rainfall treatments compared to ambient and increased treatments (Fig. 2B and C). The observed pattern was consistent across most sites, but particularly large effects were observed for available N in semi-arid Charleville and Cobar and in arid Broken Hill. Additionally, high available P content was observed in Milparinka and Broken Hill. There were no differences in available C among treatments (Fig. 2A).

## 3. Results

### 3.2. Variation in microbial biomass and microbial C, N, P content across sites and treatments (2020 only)

PLFA markers and microbial biomass C, N and P (Table S3) varied significantly among sites (Table S4); however, only fungal PLFA mass and the fungal/bacterial ratio, based on PLFA markers differed among rainfall treatments, showing an increase with increased rainfall, driven

mainly by differences between reduced and increased rainfall treatments in semi-arid Nyngan and Quilpie (only fungal/bacterial ratio) (Fig. 3A and B). Microbial biomass C presented significant effects of treatment; however, post-hoc analysis within sites showed a significant increase only in semi-arid Quilpie (Fig. 3C).

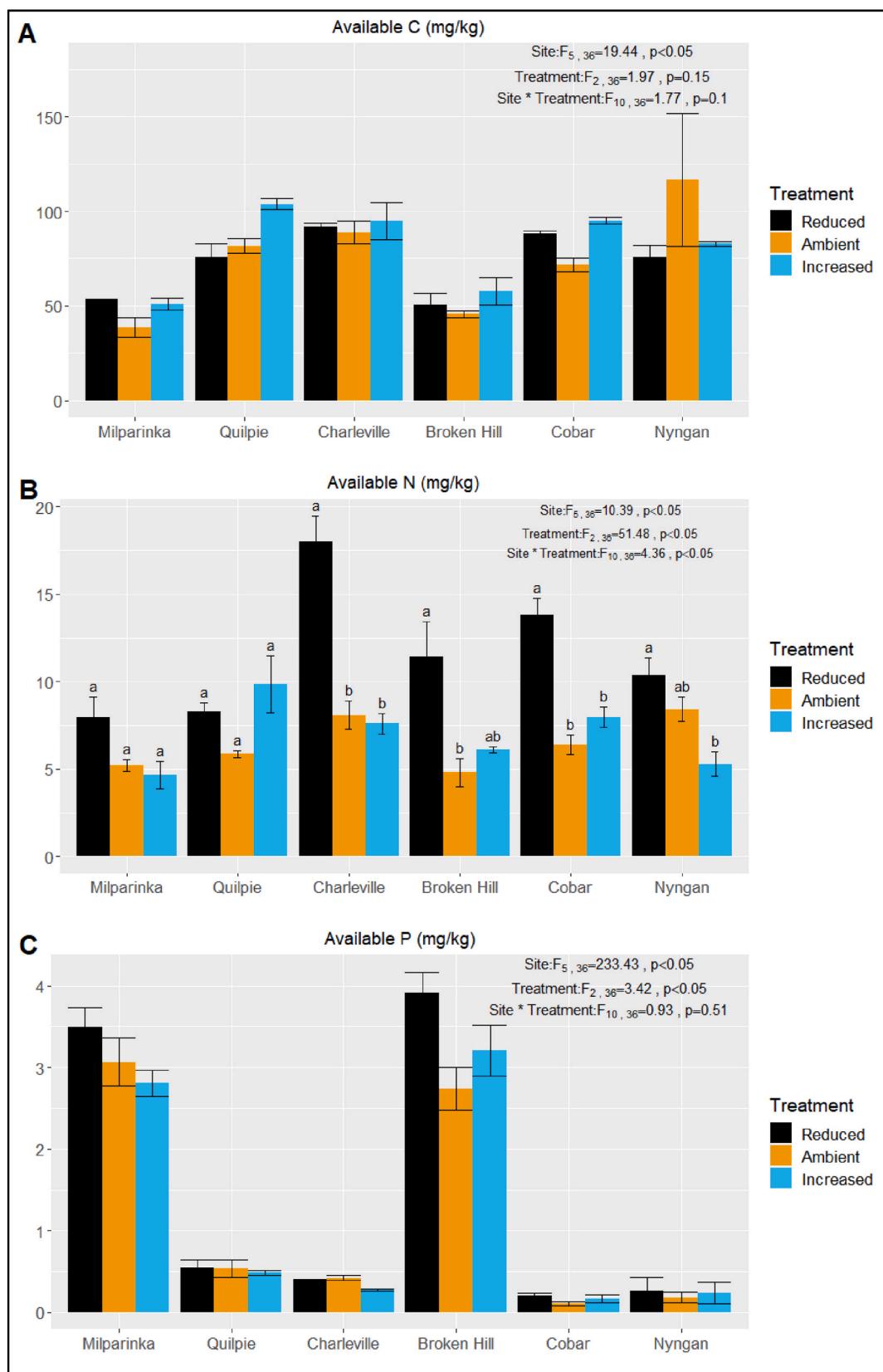
Random forest importance tests (Table S5) indicated that total PLFA, arbuscular mycorrhizae and protozoa were strongly related to pH (13.55, 21.7 and 7.97 IncMSE, respectively); however, total PLFA was also closely linked to one-year rainfall, mean annual temperature, total N, C:N and N:P ratio. Protozoa were also found to be closely linked with one-year rainfall and silt whereas arbuscular mycorrhizae were related to long-term rainfall, one-year rainfall, three-month rainfall, mean annual temperature, silt, total C, total N, C:N and N:P ratio. Gram positive bacteria were closely related to one-year rainfall (16.79 IncMSE), although other variables such as mean annual temperature, pH, total C, total N, available P, C:P and N:P ratio were given high scores. Gram negative bacteria and actinobacteria were both closely related to mean annual temperature (16.39 and 17.39 IncMSE, respectively) but gram negative bacteria were also closely related to one-year rainfall, pH, total C, total N, C:N and N:P ratio, whereas actinobacteria were also related to available P. Fungi, and the fungal:bacterial ratio, were most strongly related to total N (16.94 and 17.11 IncMSE, respectively) with fungi having a strong connection also to one-year rainfall, pH, total C, available P, C:N, C:P and N:P ratio whereas the fungi:bacterial ratio was strongly related to one-year rainfall, sand, total C, total N, total P, available P and N:P ratio. Microbial C showed the strongest connection to three-month rainfall (23.02 IncMSE) with additional strong scores to one-year rainfall, mean annual temperature, pH, available C, nitrate, available P and C:N ratio. Microbial N was most closely related to C:N ratio (15.11 IncMSE) but relationships were also identified with one-year rainfall, total C and total N. Microbial P showed strong dependency on one-year rainfall (11.01 IncMSE), while rainfall long term, pH, nitrate were also identified with a high score.

Based on the random forest importance tests, we assessed relationships between biological markers and one-year rainfall, three-month rainfall, mean annual temperature, pH, total C, total N, available P, C:N ratio, C:P ratio and N:P ratio using linear mixed effects models. Linear mixed effect models (Table S8) supported the findings that total PLFA was positively related to total C (Fig. 4A), total N (Fig. 4B), C:P ratio (Fig. S6A) and N:P ratio (Fig. S6B). Gram positive bacteria were positively related to total C (Fig. 4C), total N (Fig. 4D), C:P ratio (Fig. S6C) and N:P ratio (Fig. S6D). Gram negative bacteria were positively related to total C (Fig. 4E), total N (Fig. 4F), C:P ratio (Fig. S6E), N:P ratio (Fig. S6F), and weakly related to C:N ratio (not shown,  $R^2 < 0.2$ ). Actinobacteria were positively related to total C (Fig. 4G) and total N (Fig. 4H). Arbuscular mycorrhizae were positively related to total N (Fig. 4I), and weakly related to total C, C:N ratio and C:P ratio ( $R^2 < 0.2$ ). Protozoa were only related to available P (not shown,  $R^2 < 0.2$ ). Microbial C was most closely and negatively related to three-month rainfall (Fig. 5A) and pH (Fig. 5B), and microbial P was most closely related to one-year rainfall (Fig. 5C) and available P (Fig. 5D).

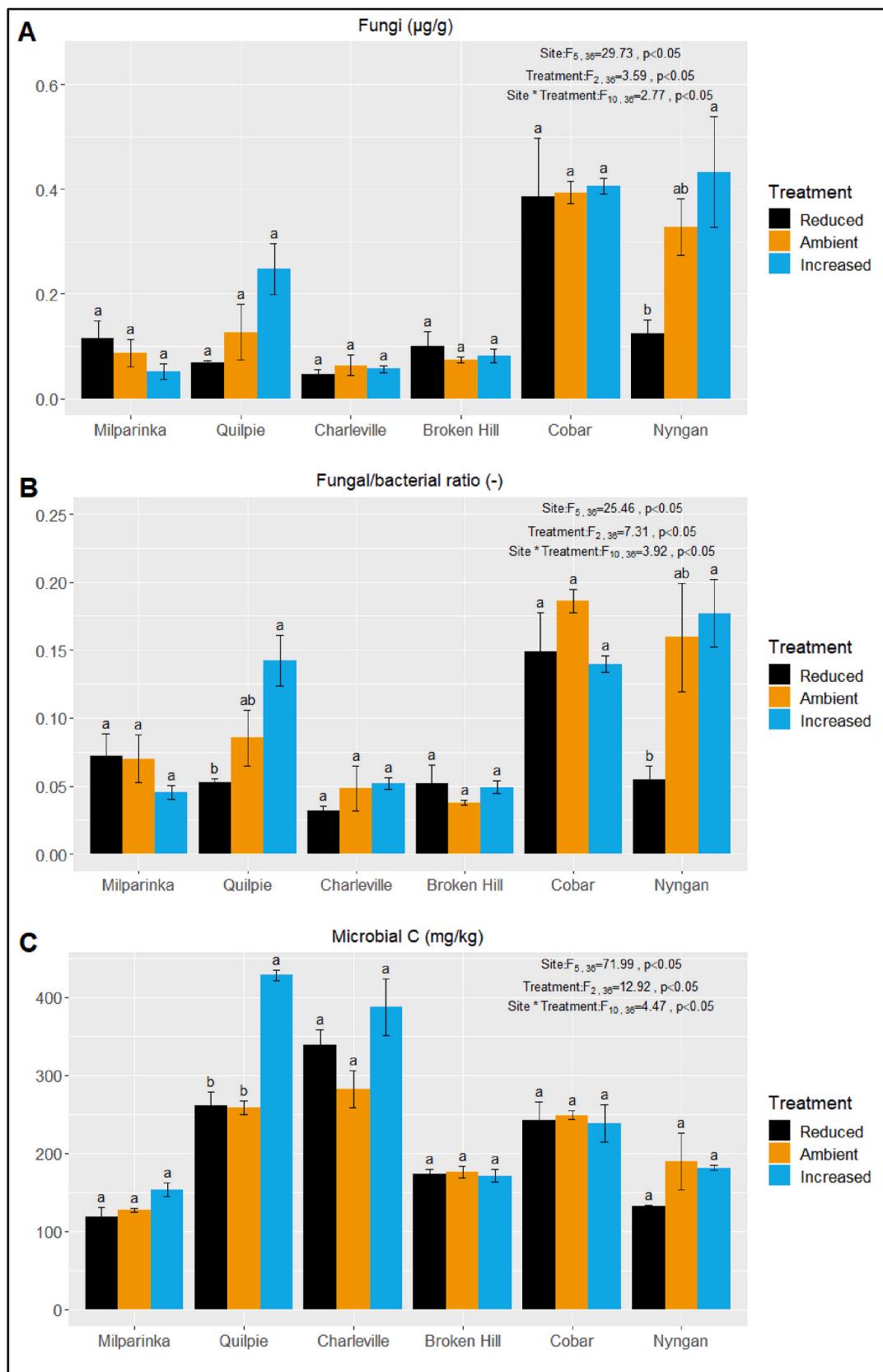
### 3.3. Variation in enzymatic activity across sites and treatments (2020 only)

The potential activity of enzymes associated with C, N and P cycling all varied substantially among sites and with treatment, but the treatment effect differed across sites (Table S4). In most cases, the increased rainfall and reduced rainfall treatments differed most strongly with increased rainfall treatments showing higher enzymatic activity compared to reduced rainfall treatments (Fig. 6A–C). However, the effect size of increased rainfall differed between sites, with semi-arid Nyngan presenting the highest N-associated enzyme content in increased rainfall plots. Similarly, high P-associated enzyme content was observed in semi-arid Cobar.

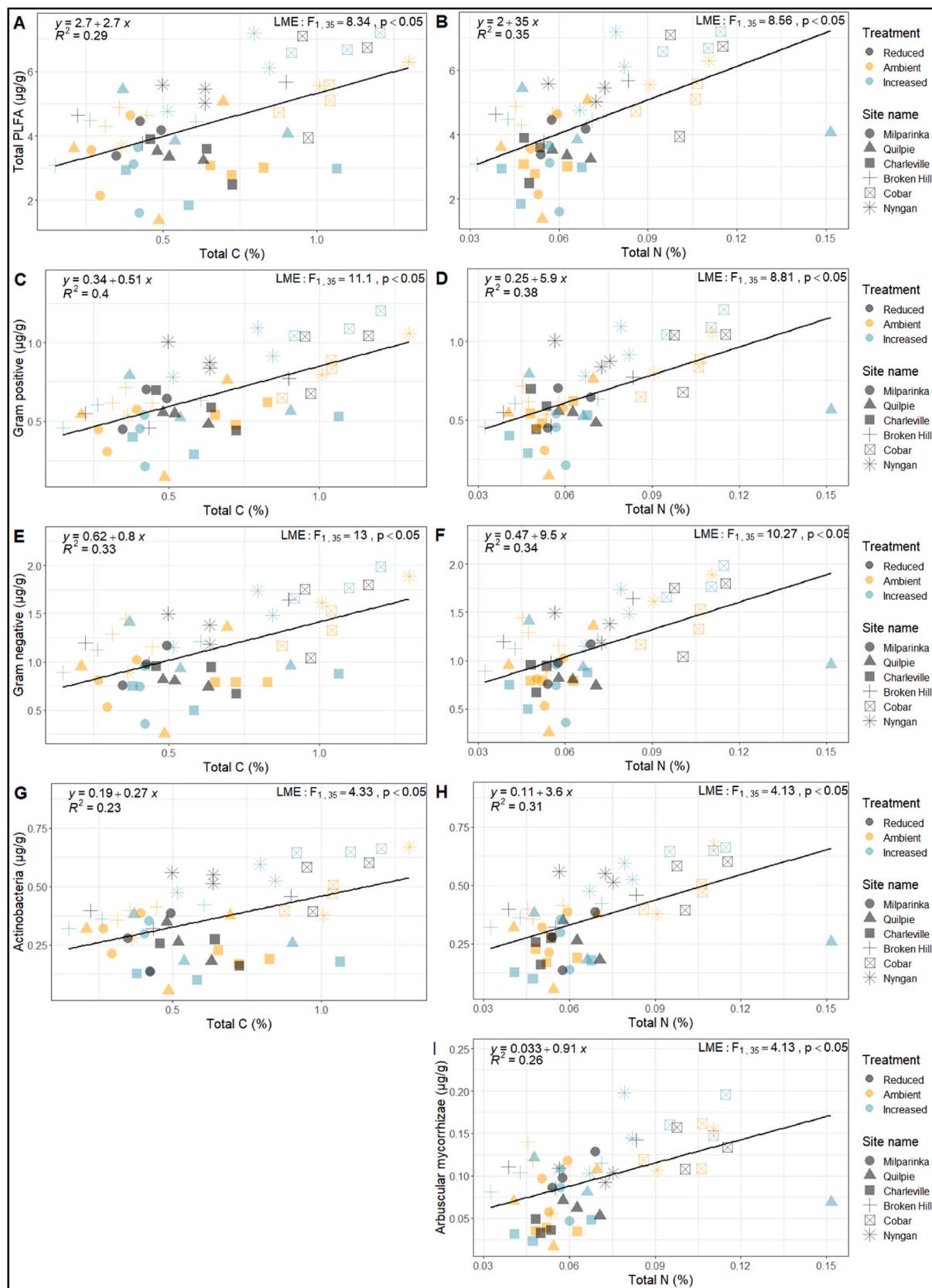
Random forest importance test showed strong relationships between



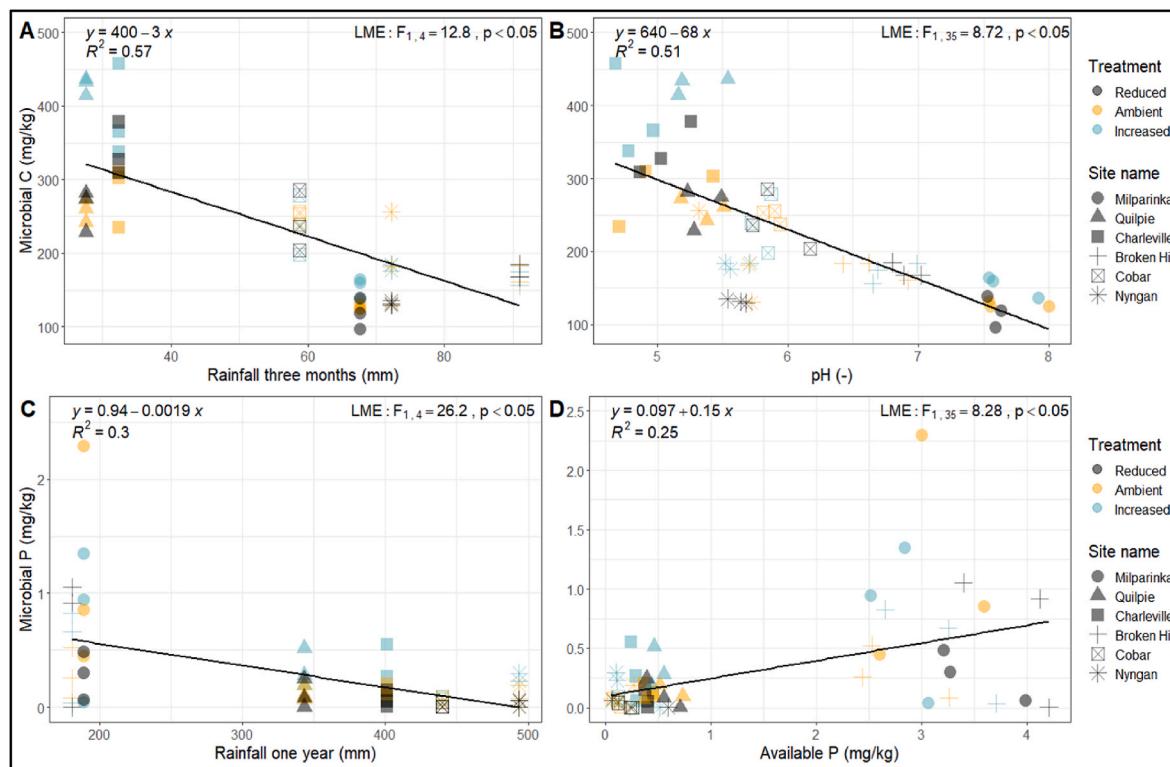
**Fig. 2.** Available C (A), N (B) and P (C) content across sites and treatments for 2020. Bars represent standard error ( $n = 3$ ). Test statistics for significant differences among sites, through time, and their interaction based on two-way ANOVA. Letters above bars indicate differences among treatments across all sites where significant interaction site x treatment effects were observed. Milparinka and Broken Hill are arid whereas Quilpie and Charleville (QLD) and Cobar and Nyngan (NSW) are semi-arid ecosystems.



**Fig. 3.** Average fungal PLFA marker concentration (A), fungal/bacterial PLFA markers ratio (B) and chloroform fumigation derived microbial C (C) across sites and treatments. Bars represent standard error ( $n = 3$ ). Test statistics for significant differences ANOVA. Letters above bars indicate differences among treatments across all sites where significant interaction site x treatment effects were observed. Milparinka and Broken Hill are arid whereas Quilpie and Charleville (QLD) and Cobar and Nyngan (NSW) are semi-arid ecosystems.



**Fig. 4.** Relationships between total PLFA and key PLFA markers and soil C (A, C, E, F) and N (B, D, F, H, I) content across all sites and treatments. Test statistics for linear mixed effects models with site and treatment included as random effects are presented.



**Fig. 5.** Relationships between microbial biomass C (A, B) and microbial biomass P (C, D) to and explanatory variables across all sites and treatments. Test statistics for linear mixed effects models with site and treatment included as random effects are presented.

enzymes associated with C cycling and ammonium (11.79 IncMSE), which is indicative of available N more broadly (Table S6). Three-month rainfall had the highest score for enzyme activity associated with N cycling (14.93 IncMSE), where mean annual temperature, pH, ammonium and available N identified were also relevant. Soil pH ranked highest for enzyme activity associated with P cycling (16.56 IncMSE), although other variables such as long-term rainfall, one-year rainfall, available C, available N, nitrate and available P also showed high scores.

Based on random forest importance test the following explanatory variables were picked for the linear mixed model: long-term rainfall, one-year rainfall, three-month rainfall, pH, available C, ammonium, available N and available P. Linear mixed effect models (Table S9) supported that N-associated enzymes were positively related to three-month rainfall (Fig. 7A), pH (Fig. 7B) and negatively to available N (Fig. 7C). P-associated enzymes were positively related to one-year rainfall (Fig. 7D) and available C (Fig. 7F), negatively to pH (Fig. 7E) and available P (Fig. 7G), while the relationship with available N was low ( $R^2 < 0.2$ ). The relationship between C-associated enzymes and available N was also weak ( $R^2 < 0.2$ ).

#### 3.4. Linkages between enzyme activity and microbial biomass

The random importance test (Table S7) indicated that C-associated enzymes were most closely linked to fungal biomass (7.44 IncMSE). N-associated enzymes were most closely linked to microbial C (21.66 IncMSE) but were also related to gram positive, actinobacteria, arbuscular mycorrhizae and microbial P. P-associated enzymes were most strongly linked to fungal biomass (15.83 IncMSE) but were also related to several other markers including total PLFA, protozoa, arbuscular mycorrhizae, fungal/bacterial ratio and microbial C.

Given the variable outcomes of the random forest tests, we used linear effects models to evaluate relationships of enzymes with all microbial biomass markers to account for site and treatment effects; however, no significant relationships were found between enzyme

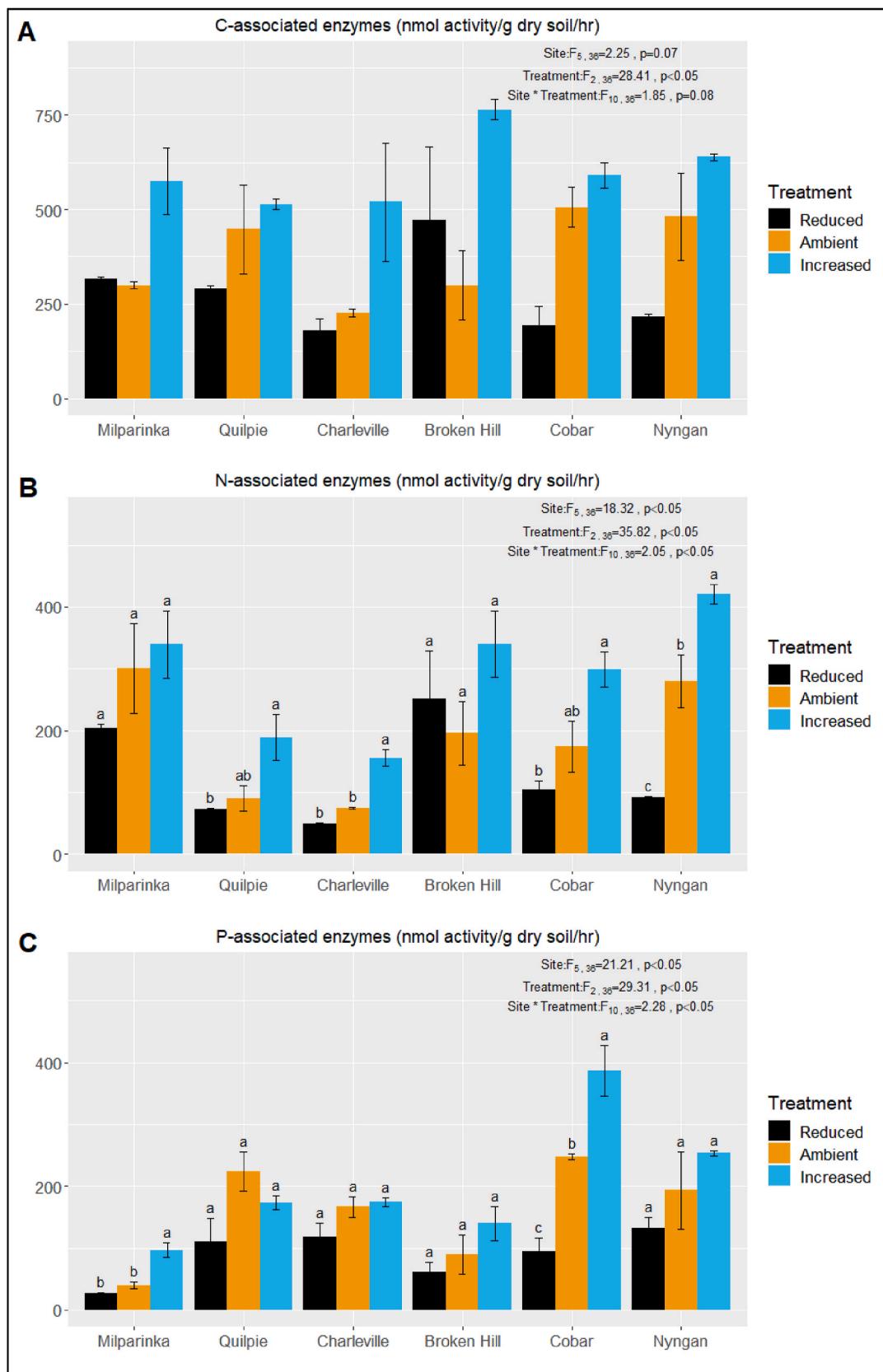
activity and microbial biomass markers (Table S10).

## 4. Discussion

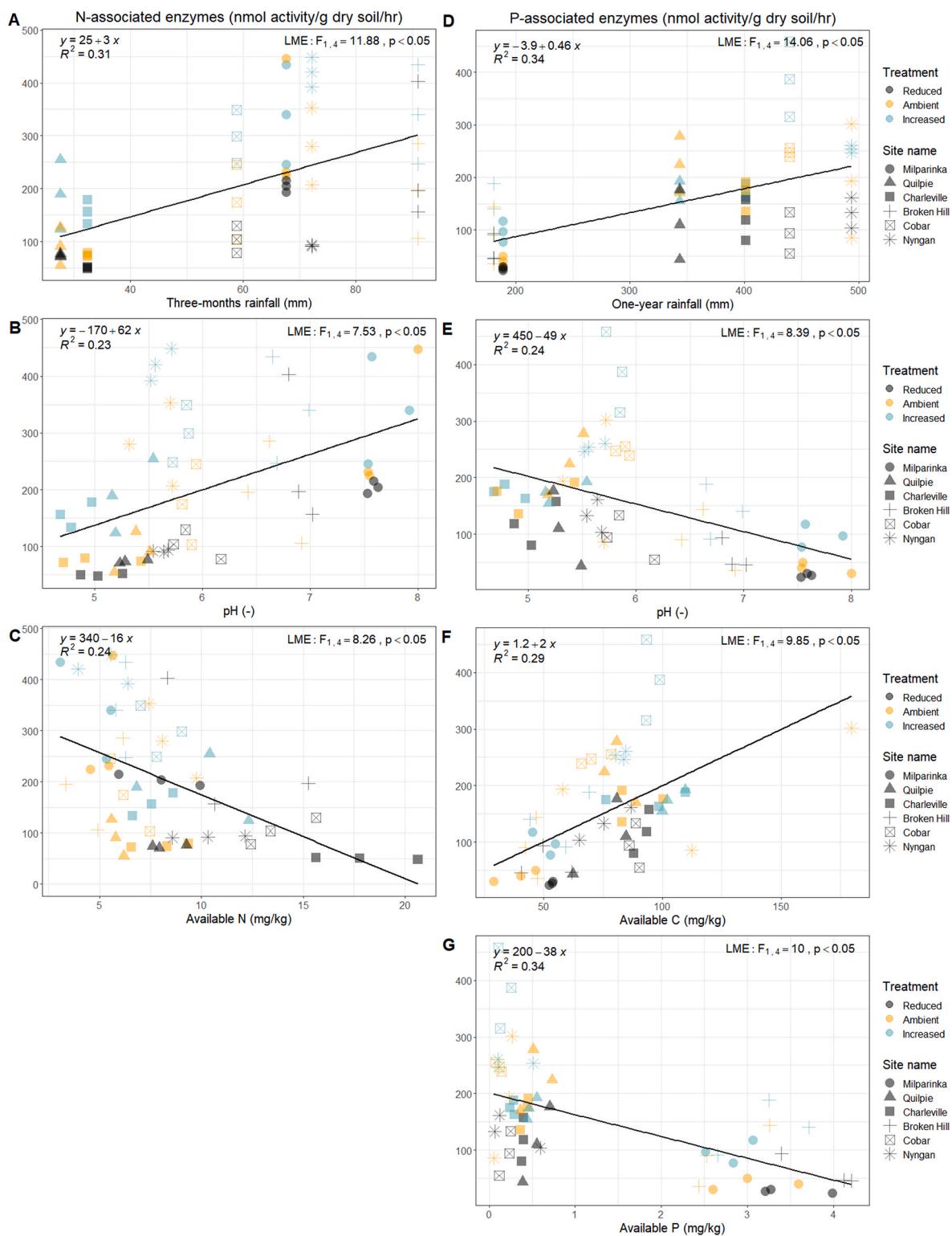
### 4.1. Contrasting shifts in soil C and nutrients in response to contemporary drought but limited effects of experimental rainfall manipulation

Our results showed substantial and consistent changes in soil total C and nutrient content through time across the six sites but limited effects of rainfall manipulations, while treatment effects were observed on nutrient availability in 2020. Specifically, we observed a large reduction in soil total C content after the relatively wet 2016, most likely caused by the coinciding prolonged drought that persisted throughout 2017–2019 (Bureau of Meteorology, 2020), while total N increased progressively through time and total P content showed no consistent pattern. This result partially supports our first hypothesis in that the reduction in precipitation associated with the onset of the prolonged drought strongly affected soil C but had limited effects on soil total N and P content. Previous studies have reported drought-induced reductions in soil total C, N and, to a lesser degree, P content resulting in decoupling of biochemical cycles (Delgado-Baquerizo et al., 2013; Jiao et al., 2016); hence, the observed reduction in soil C content is consistent with earlier findings while the increase in soil N content is somewhat surprising but support the theory that drought cause decoupling of C, N and P cycles. The contrasting responses of C and N is likely because soil C is lost through microbial respiration with little plant-derived C inputs to compensate while soil N is less impacted and can increase through time because of reduced plant uptake of N and an accumulation of mineral and microbial biomass N (Nielsen and Ball, 2015). By contrast, soil P dynamics are primarily regulated by physical and chemical processes and have been found to respond less to water availability (Delgado-Baquerizo et al., 2013).

Soil available C, N and P differed across sites in 2020, with a tendency for greater C availability at the sites with higher rainfall whereas P



**Fig. 6.** Average enzyme C (A), N (B), P (C) activity across sites and treatments. Bars represent standard error ( $n = 3$ ). Test statistics for significant differences ANOVA. Letters above bars indicate differences among treatments across all sites where significant interaction site x treatment effects were observed. Milparinka and Broken Hill are arid whereas Quilpie and Charleville (QLD) and Cobar and Nyngan (NSW) are semi-arid ecosystems.



**Fig. 7.** Relationships between N (A, B, C), and P (D, E, F, G) associated enzymes and explanatory variables across sites and treatments. Test statistics for linear effect models with site and treatment included as random effects are shown.

availability was greater at the most arid sites (Broken Hill, Milparinka). This thus supports our first hypothesis as a reduction in rainfall resulted in accumulation of ‘available’ nutrients in soil. This conforms with previous results indicating that increasing aridity results in decoupling of biogeochemical cycling (Delgado-Baquerizo et al., 2013). In addition, significant treatment effects were observed, with greater N and P availability in the reduced rainfall treatments across all sites. No

treatment effect was observed for available C which contrasts previous research (Chapin et al., 2002), but might be related to the drought impact on plant biomass and associated reductions in C inputs. The increase in N and P availability under reduced rainfall is most likely due to reduced uptake by plants and microbes resulting in accumulation of mineral forms in the soil (Hartmann et al., 2013).

#### 4.2. Effects of altered rainfall regimes on microbial biomass

Four years of altered rainfall regimes had relatively little impact on microbial PLFA markers and microbial biomass C, N and P content except for a decrease in fungal biomass and lower fungal:bacterial ratios in semi-arid Nyngan, and reduced fungal:bacterial ratios and microbial biomass C in semi-arid Quilpie, in reduced treatments compared with increased rainfall plots. These results thus only partially support the second hypothesis in that rainfall treatments had limited effects on microbial biomass. Hence, microbial biomass appeared to be highly resistant to altered rainfall regimes and would be a poor indicator of climate change impacts on biochemical cycling. Similarly, other studies found no effects of long-term reductions in precipitation on soil microbial biomass or community composition (Cruz-Martínez et al., 2009; Landesman and Dighton, 2010). Surprisingly, altered rainfall only influenced fungal PLFA markers, and through this fungal:bacterial ratios, despite fungi being considered more drought resistant. However, the coinciding prolonged and very severe drought that persisted throughout most of NSW from 2017 to 2019 caused a substantial reduction in vegetation standing biomass and soil C content that likely moderated the effects on fungi, which largely rely on plant-associated C inputs (Zeilinger et al., 2016). Conversely, bacteria may have entered a dormant state in which they would show high resistance to the imposed rainfall treatments and rely on periods with greater soil water availability for activity (Schimel et al., 2007; Manzoni et al., 2014).

PLFA markers were closely related to soil C and nutrient pools across all sites and treatments following a prolonged and very severe drought. Previous studies have reported similar findings illustrating the importance of C and nutrient pools (Reed et al., 2011; Fanin et al., 2015). This suggests that total C and nutrient pools are good indicators of microbial biomass in drylands with resource availability and quality governing nutrient cycling-biomass responses to drought periods. Hence, climate-change induced shifts in pool sizes and stoichiometry would have cascading effects on microbial biomass in the longer term. By contrast, microbial C, N or P were not related to soil total or nutrient pools, except for a negative relationship between microbial P and available P. This result contrast to some studies that found strong relationships between microbial pools and nutrients addition (Soong et al., 2018; Chen et al., 2019). In addition, we found that microbial C was significantly and negatively interacting with three-months rainfall. This suggest that microbial C accumulated during periods with reduced water availability, possibly because of lower microbial activity and hence reduced C losses associated with respiration (Tucker et al., 2013). Microbial P was negatively related to one-year rainfall implying that phosphorus accumulates over longer time periods. This result is contrary to the expectation of decreasing microbial nutrient pools with increasing aridity in drylands (Reynolds et al., 2007; Vicente-Serrano et al., 2012). The difference may be due to the difference in time frames and the conditions observed during our study. Additionally, we found a strong negative relationship between microbial biomass C and soil pH across all sites which suggest that the lower soil pH will cause reduction in microbial biomass C. Other studies have indicated that microbial C pools are related to soil pH with microbial C pools lower in more acidic condition due to shifts in mineralization rates (Motavalli et al., 1995; Kemmitt et al., 2006).

#### 4.3. Effects of altered rainfall on potential exo-enzymatic activity

In contrast to microbial biomass, altered rainfall regimes had a strong effect on potential enzymatic activity, indicating high sensitivity of enzymes to rainfall manipulation in drylands. The effects of rainfall manipulations on enzyme activity (especially enzymes related to N and P acquisition) were consistent across all sites but was particularly noticeable when comparing increased and reduced rainfall. Several studies have indicated that reduced soil water availability negatively impacted enzymatic activity (Sardans and Peñuelas, 2005; Hammerl

et al., 2019), while studies focussing on increased rainfall have shown no significant effects (McDaniel et al., 2013; Ladwig et al., 2015). By contrast, a recent study showed increasing enzymatic activity under dry conditions (Ochoa-Hueso et al., 2018). This suggests variation in enzymatic activity response to water manipulation which most likely depends on historic rainfall regimes and associated available nutrients. Our findings support the studies reporting negative effects of reduced rainfall but further show a positive effect of increased precipitation, suggesting strong sensitivity of microbial activity to altered rainfall regimes in drylands. This suggests that microbes inhabiting drylands can adjust the production of exoenzymes per unit of biomass (specific enzyme activity) thereby regulating dryland soil processes despite extreme reduction in water availability.

The enzyme activity results indicate that microbial organic matter degradation was constrained by water availability but moderated by nutrient availability. Specifically, microbes invest more resources to produce enzymes to acquire resources that are in short supply, particularly N and P in our study. P-associated enzymes also showed a positive relationship to C availability suggesting that microbes that are not C limited can allocate more resources to acquire P in Australian drylands. Previous studies have similarly reported significant relationships between enzyme activity and available nutrient pools (Asmar et al., 1994; Li et al., 2021). Moreover, N-associated enzymes were positively related to three-month rainfall whereas P-associated enzymes were more closely related to one-year rainfall, suggesting that N-associated enzymes are driven by short-term pulses while P-associated enzymes respond over longer time frames. C-associated enzymes were not related to any of the variables measured while previous studies have found strong relationships with nutrient pools (Sinsabaugh et al., 2008; Banerjee et al., 2016). Additionally, we found a weak negative relationship between P-associated enzymes and pH. A similar result was reported in a previous study (Turner and Blackwell, 2013), and may be the result of differences in adsorption and stabilization of nutrients on mineral surfaces.

We found no significant relationships between enzymatic activity and microbial biomass for any of the PLFA markers or microbial biomass C, N and P. Previous work has reported positive relationships between enzyme activity and microbial biomass (Ren et al., 2017; Yang et al., 2017). However, our study was conducted following a severe drought, which most likely influenced biomass growth-enzymatic activity relationships. This suggests that severe drought decouples biomass production and enzyme activity. Hence, dryland microbes might favour regulation of their enzymatic activity (especially those associated with N and P acquisition) and processing of available nutrient pools overgrowth during prolonged drought. These results, therefore, support our third hypothesis in that rainfall treatments strongly influenced potential enzyme activity associated with N and P acquisition further moderated by nutrient availability. However, these results should be interpreted with care as other factors may play a role. For example, there is strong evidence that abiotic enzymes can be stabilised in soil and potential enzyme activity may thus not be related to biological processes (Nanipieri et al., 2018).

#### 5. Conclusions

Our study explored relationships between microbial biomass, enzymatic activity and edaphic variables at six dryland sites exposed to long term rainfall manipulation and a severe prolonged drought. Soil C, N and P content and microbial PLFA markers showed surprisingly little response to the imposed rainfall treatments. Still, soil C and N content changed substantially through time associated with a coinciding prolonged drought that may have resulted in significant water stress across all rainfall treatments resulting in relatively limited differentiation among rainfall treatments. More rainfall was received across all sites in 2020, with significant rainfall treatment effects observed for soil N and P availability with greater availability in the reduced rainfall treatments likely due to accumulation of mineral forms. In addition, potential exo-

enzymatic activity was greatly impacted by rainfall treatments, with higher activities in the increased rainfall treatments, which suggests greater sensitivity of microbial activity than biomass to altered rainfall. There was no relationship between enzyme activity and microbial biomass suggesting decoupling of activity and biomass production, at least following a prolonged drought. These findings provide new insights on how microbial activity responds to soil water availability in drylands.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2023.109277>.

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