Received: 28 February 2022 | Accepted: 23 March 2022

DOI: 10.1002/sae2.12015

RESEARCH ARTICLE

JOURNAL OF SUSTAINABLE AGRICULTURE AND ENVIRONMENT
Open Access
Global Initiative of Crop Microbiome and Sustainable Agriculture

Microbial nutrient limitation and catalytic adjustments revealed from a long-term nutrient restriction experiment

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: PA 2377/1-1; Bundesministerium für Bildung und Forschung, Grant/Award Numbers: 031B0508H & 031B0508F, BOMA 03037514

Abstract

Introduction: Microbial abundance and activities in soils are predominantly determined by soil carbon (C), nitrogen (N) and phosphorus (P) availability. Much research has focused on the effects of soil N than P availability on soil microbial biomass and enzyme activities as sensitive proxies of microbial ecophysiology highlighting the need to investigate how microbes will respond to P availability in soil, especially in cropping systems.

Materials and Methods: The effect of P fertilisation on microbial biomass-C, -N and -P, and the kinetic parameters (maximal velocity [V_{max}], Michaelis constant [K_m] and catalytic efficiency [K_a]) of β-1,4-glucosidase (BG; C-acquiring), leucine-aminopeptidase (LAP; predominantly N-acquiring) and acid phosphomonoesterase (PHO; P-acquiring) were measured in a nutrient-poor agricultural soil (devoid of fertiliser application since 1942).

Results: This study showed that P fertilisation led to a 65% and 56% increase in microbial biomass-N and -P, respectively, indicating severe P limitation and inefficient N acquisition by microbes without P availability. Increased K_a values of LAP with P fertilisation further hint toward the production of efficient isoenzymes to avoid resource tradeoffs for nutrient acquisition.

Conclusions: Overall, these results decipher microbial metabolic and catalytic adjustments mediated by soil P availability. Increased microbial biomass-N and -P with P fertilisation indicated microbial N and P colimitation that was partly overcome by the production of efficient enzymes for N acquisition with P fertilisation. We argue to incorporate microbial enzyme activities as a response to different management strategies to better inform us about soil biogeochemical cycles in cropping systems.

KEYWORDS
catalytic efficiency, enzyme activity, kinetic parameters, microbial biomass, phosphorus fertilisation

1 | INTRODUCTION

Like in any other ecosystem, heterotrophic soil microbes are fundamental to biogeochemical cycles in agroecosystems as their metabolism and growth regulate the mineralisation of various elements. However, the community composition and functioning of soil heterotrophic microbes are sensitive to various environmental factors including soil pH (Malik et al., 2018; Rousk et al., 2009), temperature (Razavi et al., 2015), atmospheric CO_2 concentration (Keane et al., 2020), availability of resources (Clayton et al., 2021) and land-use intensity (Tischer et al., 2019). This has direct implications for energy and nutrient fluxes, such as carbon (C), nitrogen (N) and phosphorus (P) fluxes, in agroecosystems with consequences for crop production and soil C sequestration. Therefore, it becomes crucial to identify microbial growth constraints mediated by nutrient limitation as well as their potential strategies for nutrient acquisition from soils. In this regard, the determination of microbial biomass and activities of enzymes involved in rate-limiting steps of soil organic matter (SOM) turnover and nutrient mineralisation have been used as sensitive proxies to infer microbial responses to environmental factors (Rosinger et al., 2019; Sistla et al., 2012).

Microbial enzyme production and secretion, for instance, are linked to the availability of limiting nutrients in the soil where higher availability of N or P in the soil leads to a reduction in the production of microbial enzymes which are catalysing N and P containing organic compounds in soil (Sinsabaugh et al., 2014; Turner & Joseph Wright, 2014). These results are consistent with evolutionary-economic theory as well as from empirical studies suggesting that microbes avoid resource tradeoffs for enzyme productions when nutrients are already present in the available forms for microbes to take up (Allison et al., 2011; Malik et al., 2019). Various studies have shown the effect of N limitation on microbial ecophysiology including adjustments in microbial biomass and secretion of enzymes to acquire N from soil (Cusack, 2013; Kumar et al., 2018; Luo et al., 2019). However, relatively few investigations have been made in the context of microbial response to P limitation, especially from cropping systems. Given that P availability is fundamental for microbes as P is directly involved in the synthesis of nucleic acids (both DNA and RNA) and adenosine triphosphate (ATP), thereby regulating microbial functioning and metabolism (Dilly & Nannipieri, 2001; Landi et al., 2006), it becomes important to investigate microbial investments in growth and enzyme productions under P limitation with consequences for SOM mineralisation and release of nutrients which can be taken up by crops.

The present study, therefore, focused on investigating microbial responses to soil P availability. Our overarching hypothesis was that in P-limited soils, the availability of mineral P (via fertilisation) triggers microbial metabolism and leads to its uptake in microbial biomass and consequently downregulation of P-acquiring enzymes without affecting their C and N acquisition strategies. As various enzymes are involved in more complete mineralisation of SOM from complex to simpler forms, we focused mainly on measuring the kinetic parameters of three key enzymes, which are involved in the terminal breakdown of organic compounds to simpler forms as their activities represent the microbial bottleneck for the uptake of their products. For example, the breakdown of polymeric cellulose to oligomeric units (cellobiose) is catalysed by cellobiohydrolases and their product (i.e., cellobiose) acts as a substrate for glucosidases (i.e., β-1,4-glucosidases), which breakdown cellobiose to monomeric units (glucose). Without further breakdown, microbes can then directly take up these monomeric units (glucose). Therefore, instead of measuring the kinetic parameters of cellobiohydrolases, we focused on measuring the kinetic parameters of β-1,4-glucosidases. Similarly, we measured the kinetic parameters of leucine-aminopeptidase (LAP) and acid phosphomonoesterase (PHO) as 'terminal' enzymes.

2 | MATERIALS AND METHODS

Topsoil (0–20 cm) was collected from the control plots (without any fertiliser application since 1942) of a long-term experiment at Dikopshof of the University of Bonn, Germany. The soil was classified as Luvisol with 11.8% sand, 71.2% silt and 17% clay with a total C (7.8 g kg^{-1}), total N (0.74 g kg^{-1}), C:N ratio (10.5), CAL-extractable P (23.2 mg kg^{-1}) and soil pH of 6.48 (also see Kumar et al., 2019). Freshly collected soil was passed through a 2 mm sieve and 1.5 kg of sieved soil was preincubated for 3 days followed by 45 days of main incubation period in open-top Polyvinyl chloride (PVC) pots in a climate chamber. Pots were placed randomly in the climate chamber to avoid position-specific bias. As the soil was both N and P limited at the same time and as the main aim of this incubation study was to investigate the soil P limitation effects on microbial functioning, we avoided co-limitation of N and P in soil for microbes. To avoid N limitation for microbial growth, all pots received mineral N fertiliser (KNO_3 at the rate of 120 kg N ha^{-1}), whereas only half of the pots received mineral P fertiliser (KH_2PO_4 at the rate of 60 kg P ha^{-1}) to manipulate microbial P availability. The rate of N and P fertilisers was as per common usage in agricultural soil in the area. As we used open-top PVC pots which are susceptible to water evaporation, we maintained the soil moisture level at 70% water holding capacity with distilled water throughout the incubation period. Further, to avoid short-term apparent effects of fertilisers and to investigate microbial response after the exhaustion of added fertiliser's nutrient elements, pots were destructively harvested after an incubation period of 45 days. Microbial biomass -C and -N (MBC and MBN) were measured by using the chloroform fumigation-extraction method with modification (Vance et al., 1987). The differences between extracted C and N from fumigated (24-h fumigation) and nonfumigated fresh soil samples by using 0.05 M K_2SO_4 were used to calculate MBC and MBN, respectively. Extraction efficiency factors K_{EC} (0.45) and K_{EN} (0.54) were used to determine MBC and MBN, respectively (Joergensen & Mueller, 1996; Wu et al., 1990). For microbial biomass P (MBP), direct chloroform fumigation with anion-exchange membrane (AEM) method was used (Brookes et al., 1982; Bünemann et al., 2012; Yevdokimov et al., 2016). For this, AEMs (6.25 cm long and 1.5 cm wide) were charged within 0.5 M $NaHCO_3$ for 24 h by continuous shaking, while

exchanging HCO_3^- for Cl^-. The AEMs were stored at room temperature with deionized water before use. For P extraction, fresh soil subsamples (3 g dry weight equivalent) were weighed in a 50 ml falcon tube filled with 30 ml deionized water, and one AEM was added to the falcon tube. Thereafter, 300 µl chloroform was added to the falcon tube followed by 24 h shaking at 150–160 rev min^{-1}. After 24 h, the AEM was removed with tweezers and carefully washed with deionized water four to five times to remove any adhering soil particle and quickly immersed in another falcon tube containing 45 ml 0.25 M H_2SO_4 and shaken for 3 h to elute P from AEM. The amount of P in solution was then determined colourimetrically using malachite-green dye by using a 96-well transparent plate in a plate reader (Victor 3 1420-050 Multilabel Counter; PerkinElmer) at 630 nm (Yevdokimov et al., 2016). The same procedure was followed for another soil subsample except for chloroform fumigation. The difference between extracted P from fumigated and non-fumigated soil samples was used to calculate MBP with an extraction efficiency factor K_{EP} of 0.4 (Brookes et al., 1982). Extracted C, N and P from nonfumigated soil samples were used as proxies for dissolved organic-C (DOC), dissolved-N (DN) and dissolved-P (DP), respectively. Total mineral N in soil was extracted with 0.05 M KCl.

Potential maximal activities of β-1,4-glucosidase (BG; C-acquiring), LAP (predominantly N-acquiring) and acid PHO (P-acquiring) enzymes were measured by using fluorogenically labelled artificial substrates (Kumar et al., 2021; Marx et al., 2001). For BG and PHO, 4-methylumbelliferon (MUB)-based fluorogenic substrates were used, whereas for LAP, 7-amino-4-methycoumarin (AMC)-based fluorogenic substrate was used. Briefly, soil suspensions were made by vigorous

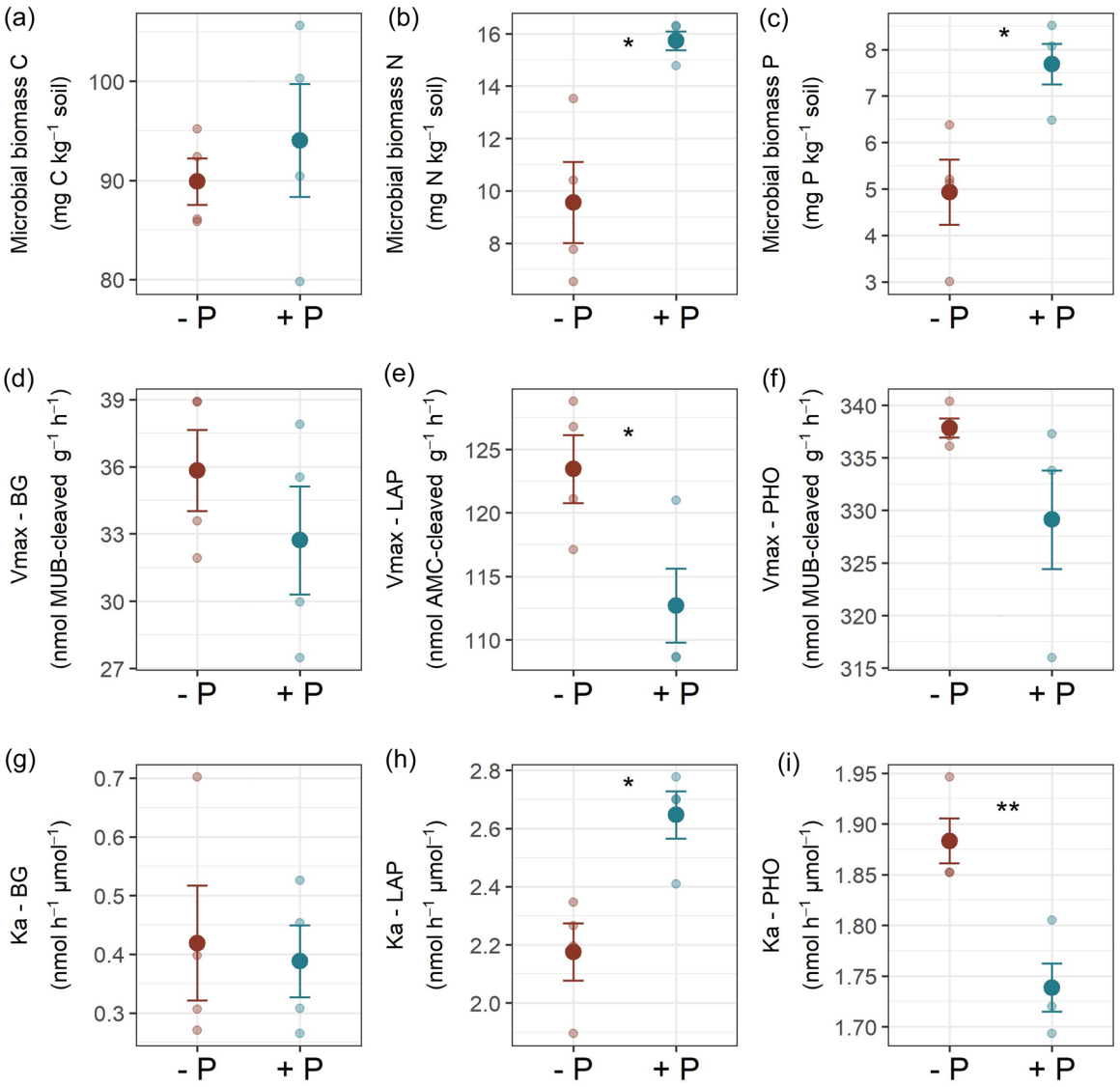

FIGURE 1 Microbial biomass-C, -N, -P (mg kg^{-1} soil) (a–c), maximal potential activity (V_{max}; nmol substrate cleaved g^{-1} soil h^{-1}) of β-1,4-glucosidase (BG), leucine aminopeptidase (LAP), acid phosphomonoesterase (PHO) (d–f) and catalytic efficiency (K_a; $nmol^{-1}$ μmol^{-1}) of BG, LAP and PHO (g–i) as affected by absence (–P) and presence (+P) of P fertilisation. Values are the means and standard errors. Small dots represent individual replicates ($n = 4$). Significant difference at $^*p < 0.05$ and $^{**}p < 0.01$ from t test, respectively

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shaking of 1 g fresh soil in 50 ml sterile water for 1 h. A 50 µl soil suspension with 50 µl MES (for MUB-based substrates) or TRIZMA (for AMC-based substrate) and 100 µl of substrate solutions of different concentrations were dispensed into a black 96-well microplate (PureGrade™, GMBH + Co KG) and gently shaken. Microplates were fluorogenically measured at 360 nm excitation and 450 nm emission wavelengths after an incubation period of 2 h with a fluorometric plate reader (Victor 3 1420-050 Multilabel Counter; PerkinElmer). Fluorescence was converted to the amount of AMC or MUB using specific standards and enzyme activities were expressed as nanomoles AMC or MUB cleaved per hour per dry weight equivalent soil (nmol g^{-1} dry soil h^{-1}). To avoid, pH-mediated variation during measurements, the pH of MES and TRIMA buffers was adjusted to that of soil samples. The kinetic parameters for each enzyme were calculated using the Michaelis-Menton equation as follows:

$$v = \frac{V_{max} \times [S]}{K_m + [S]},$$

where v is the reaction rate, $[S]$ is the substrate concentration, K_m is the substrate concentration at which the reaction rate is half of its maximum, and V_{max} is themaximum velocity of enzyme–substrate reaction. Microbial biomass-specific enzyme activities were calculated as a ratio of V_{max} and microbial biomass (V_{max} – BG activity/MBC; V_{max} – LAP activity/MBN, and V_{max} – PHO activity/MBP). Enzymatic catalytic efficiency (K_a) was calculated as a ratio of V_{max} and K_m for each enzyme (Tischer et al., 2015). Results presented in graphs are mean of four replicates ± standard errors. Data visualisation and statistical analyses were performed with R (Team R Development Core, 2018) using ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2020), corrplot (Wei & Simko, 2021), FactoMiner (Lê et al., 2008) and factoextra (Kassambara & Mundt, 2020) packages.

3 | RESULTS AND DISCUSSION

We showed that P fertilisation did not affect MBC whereas MBN and MBP increased by 65% and 56%, respectively (Figure 1a–c). Next, the V_{max} values of LAP and PHO enzymes decreased with P fertilisation (Figure 1d–f), and similar trends were observed on their specific activities (Figure S1). Interestingly, the catalytic efficiency (K_a) of the LAP enzyme increased by 22% whereas that of PHO decreased by 8% with P fertilisation (Figure 1h,i). Next, P fertilisation led to a decrease in mineral N in soil (Figure 2a). Principal component analysis (Figure 2b) and correlation matrix (Figure 3) revealed a negative correlation between the V_{max} values of BG, LAP and PHO to MBC, MBN and MBP, respectively. Further, P fertilisation did not affect the elemental ratio in microbial biomass and DOC:DN and DOC:DP except for a decrease in DN:DP ratio (Figure S1).

As expected, P fertilisation alleviated microbial P limitation and led to microbial uptake of fertiliser P. Consequently, a strong decrease in catalytic efficiency, as well as a slight decrease in V_{max} values of PHO enzyme, were observed. These findings are in agreement with previous studies showing negative feedback mechanisms on PHO activity with P fertilisation (Bilyera et al., 2021; Spohn et al., 2015; Turner & Joseph Wright, 2014). Resource availability in soil modulates microbial trait responses including nucleic acid and intracellular protein synthesis, cellular maintenance, osmotic adjustments and production of enzymes and signalling cues (Roller & Schmidt, 2015). Generally, these traits correlate (either positively or negatively) as a function of soil abiotic conditions (i.e., nutrient availability) where higher resource investments in one trait result in lower resource investments in another trait (e.g., cellular growth vs. production of enzymes for nutrient acquisition). Therefore, as with P fertilisation, microbial growth is not P limited and the higher trade-offs for synthesising and secretion of PHO enzymes by microbes may exceed the associated benefits thereby downregulating their investments for P acquisition. Our results, therefore, are following the evolutionary-economic theory of microbial resource costs and benefits for their growth and survival (Allison et al., 2011).

Intriguingly, MBN and K_a values of LAP increased with P fertilisation indicating that microbes were unable to acquire N under P limitation highlighting co-limitation of N and P. Lower MBN and higher mineral N in soil without P fertilisation hints that microbes were not able to acquire mineral N without uplifting their P limitation. As P is required as a building material for nucleic acid (DNA and RNA) synthesis and energy compounds (i.e., ATP) (Bilyera

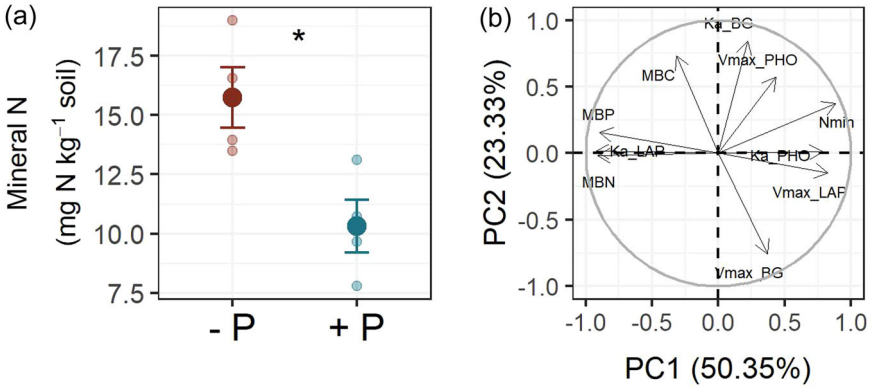

FIGURE 2 (a) Soil mineral N (mg N kg^{-1} soil) as affected by absence (−P) and presence (+P) of P fertilisation. Values are the means and standard errors. Small dots represent individual replicates ($n = 4$). *Significant difference at $p < 0.05$ from t test. (b) Principal component analysis showing the distribution of various microbial parameters

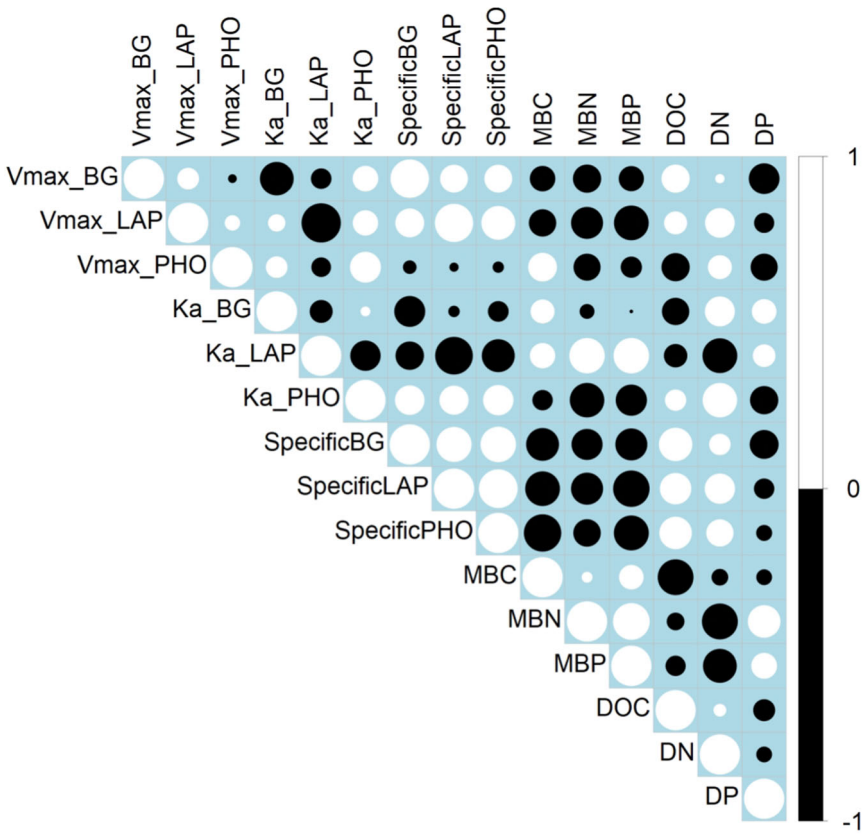

FIGURE 3 Correlation matrix of measured variables where size of the sphere is indicating the strength of correlation and white and black colour indicate positive and negative correlation, respectively. DN, total dissolved N, DOC, dissolved organic C; DP, total dissolved P; G, β-1,4-glucosidase; K_a, catalytic efficiency (V_{max}/K_m); LAP, leucine-aminopeptidase; MBC, microbial biomass C; MBN, microbial biomass-N; MBP, microbial biomass P; PHO, acid phosphomonoesterase; Specific, specific enzyme activity (V_{max}/microbial biomass); V_{max}, maximum velocity of enzyme-substrate reaction

et al., 2021), P fertilisation may have overcome these limitations allowing microbes to synthesise new RNA transcripts for enzyme synthesis. A decrease in LAP activity but its better catalytic efficiency indicates microbial production of efficient isoenzymes for N uptake with minimal resource investments. Our results are supported by a recent study from a long-term grassland experiment that was subjected to elevated CO_2 concentration in the atmosphere (Keane et al., 2020). Keane et al. (2020) found that P addition in soil resulted in a decrease in microbial P limitation but increased microbial N demand. Similarly, we also found an increase in microbial N demand as evident from the higher catalytic efficiency of LAP accompanied by a decrease in soil mineral N, indicating enhanced microbial N uptake with P fertilisation. It is also important to note that N and P fertilisation applications may have manipulated stoichiometry of soil substrates leading to altered microbial community composition. As various microbial taxa produce enzymes targeting the same substrate with distinct biochemical potential (isoenzymes), the catalytic efficiency of such enzymes is likely to vary depending on substrate quality and nutrient availability in soil (Tischer et al., 2015). Altered community composition under P fertilisation would likely have selected for microbial taxa which produce LAP enzymes with better catalytic efficiency with lower resource investments (Hartman & Richardson, 2013; Shahbaz et al., 2017; Shi et al., 2020). However, these assumptions need to be tested in future research by linking microbial community composition, activities of various enzymes, and their C and nutrient use efficiency under fluctuating nutrient availability by considering temporal dynamics of microbial community composition and functioning to nutrient availability. Next, no response of P fertilisation on MBC and kinetic parameters of BG enzyme suggests that microbes were predominantly N and P limited instead of C. This possibly relates to regular C inputs in the field from plant residues to microbes and simultaneous exhaustion of soil N and P due to plant uptake thereby driving microbes toward more N and P than C limitation.

4 | CONCLUSIONS

To conclude, the microbial nutrient limitation could be operationally identified in the presented study as soil microbes responded strongly to the added P in soil. An increase in microbial biomass N and P accompanied by better catalytic efficiency for LAP with P fertilisation indicated microbial N and P co-limitations and their ecophysiological adjustments for N and P acquisition from the soil. Unresponsiveness of microbial biomass C and the potential activity of BG enzyme to P fertilisation suggested a stronger nutrient than C limitation for microbial growth. In future studies, however, measuring the activity of various enzymes involved in different rate-limiting steps and their link to microbial ecophysiological traits (including their investments in nutrient acquisition vs. growth) and nutrient use efficiency need to be further investigated.

AUTHOR CONTRIBUTIONS

Amit Kumar conceived the idea and designed the experiment with Johanna Pausch. Amit Kumar performed the experiment, collected and analysed data and drafted the manuscript. Johanna Pausch refined the manuscript and both the authors contributed and agreed to the final version of the manuscript.

ACKNOWLEDGMENTS

We thank Karin Schmidt for laboratory assistance. We would also like to thank Michael Bonkowski and Sergey Blagodatsky as well as two anonymous reviewers for their helpful suggestions that contributed to improving the quality of this manuscript. The field site is maintained within the BonaRes project (BOMA 03037514) of the Federal Ministry of Education and Research (BMBF) Germany. Authors acknowledge funding support from BMBF (project: 031B0508H and 031B0508F) and the German Research Foundation (PA 2377/1-1) Kazem Zamanian, Sabine Julia Seidel and Hubert Hüging are thanked for soil collection. Open Access funding enabled and organized by Projekt DEAL.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings are available within the article in the figures and Supporting Information Materials. The raw data are available from the corresponding author, upon request.

ETHICS STATEMENT

The authors acknowledge having followed the ethical policies of the journal.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kumar A, Pausch J. Microbial nutrient limitation and catalytic adjustments revealed from a long-term nutrient restriction experiment. J Sustain Agric Environ. 2022;1:142–148. https://doi.org/10.1002/sae2.12015