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ACE protein as a candidate proxy for microbial functional processes in contrasting Australian cropping soils

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Abstract

Soil microbial processes underpin carbon and nutrient cycling, soil resilience and plant health outcomes, making them pertinent to soil health assessments. Despite this, there remains little consensus on which biological indicators to measure, how to interpret them and how to integrate results. Here, we evaluated 82 microbially relevant indicators measured at pre-sowing, in-crop and post-harvest across four field experiments in Australian broadacre cropping systems spanning contrasting soils, climates and management regimes. Multivariate profiles of six enzymes involved in carbon (C), nitrogen (N), phosphorus (P) and sulfur (S) cycling varied significantly among sampling times and sites ($R^2 = 0.72$, $P < 0.001$). Soil pH was the best single BIOENV model predictor (Spearman $\rho = 0.598$), consistent with its role as a master regulator of microbial communities and extracellular enzyme systems, while total C (TC) and soil autoclavable citrate-extractable (ACE) protein constituted the most parsimonious model ($\rho = 0.705$). Co-occurrence network inference identified soil ACE protein as the central node of consistently high importance, linking soil physicochemical properties and enzyme activities involved in nutrient cycling. Soil ACE protein showed strong and consistent correlations with chitinase and phosphatase activities across all sites and sampling times ($r = 0.62–0.76$, $P < 0.001$). While ACE protein has previously been proposed as an indicator of labile organic N and general soil health, our results show that it also tracks variation in enzyme-mediated nutrient cycling across contrasting soils and sampling times. These findings position soil ACE protein as a candidate low-cost, robust proxy for microbial functional processes.

1. Introduction

Despite the recognised importance of soil biology to C and nutrient cycling, biological assessment has received comparatively little formal guidance relative to physical and chemical testing (Lehmann et al. 2020; Fierer et al. 2021; Creamer et al. 2022). Soil health assessment has traditionally relied on chemical and physical metrics, with biological measures often added opportunistically and interpreted in isolation (Bünemann et al. 2018; Lehmann et al. 2020; Fierer et al. 2021). In addition, the wide array of biological indicators available to measure raises practical constraints. It is neither cost-effective nor necessary to measure all biological indicators to answer a research question. Indicators vary in analytical accessibility and complexity, cost, standardisation, temporal sensitivity and variability (Ritz et al. 2009). Suitable biological indicators must be analytically consistent, interpretable, sensitive enough to detect management-induced change over annual timescales, and practically accessible to growers (Benintende et al. 2015; Schloter et al. 2018). Unlike soil pH, which is accessible, cost-effective and directly translates to management guidance, relatively few biological indicators adequately meet these criteria (NRCS USDA 2019; Fierer et al. 2021). As a result, the selection and interpretation of biological indicators remain ambiguous, and no single functional proxy has emerged as broadly applicable (Schloter et al. 2018). This ambiguity undermines confidence in biological testing and limits the use of microbial diagnostics for on-farm decision-making (Norris et al. 2020; Fierer et al. 2021). Curating meaningful biological indicators therefore remains a priority. These challenges are best understood by considering the main classes of soil indicators – physicochemical context, C and nutrient pools, microbial

community composition, functional potential and realised function – and the distinct information each provides (Soil CRC 2024).

Soil physicochemical properties such as pH, electrical conductivity (EC), texture, moisture, bulk density and porosity provide environmental context for interpreting biological measures. However, microbial communities also influence physicochemical measures, with substrate transformation and secondary metabolites altering rhizosphere acidity, redox potential, ionic strength, moisture and nutrient status (Huang et al. 2013). Soil pH regulates microbial community biomass, diversity and composition as well as enzyme catalytic efficiency, all of which exhibit optimal ranges of activity (Fierer and Jackson 2006; Sinsabaugh et al. 2008; Malik et al. 2018). Electrical conductivity (EC) integrates soluble salts and ions in the soil solution and is shaped by pH, P, K, Ca^{2+}, Na^{+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, organic matter, CEC, and other soluble salts and organic ligands (Bronson et al. 2005; Wayayok et al. 2009; Peralta and Costa 2013). As such, it has been used as a proxy for nutrient status. Studies have reported positive correlations of EC with crop yield, fertiliser regimes, and decomposition of nutrient-rich substrates (Carmo et al. 2016). However, EC is temporally variable, with measures fluctuating with plant phenological stage and nutritional consumption (Bodale et al. 2021), highlighting the importance of sampling time for interpretation of results. Physicochemical indicators provide context and inform management decisions but do not capture substrate availability, quality or how effectively these substrates are processed.

Indicators of soil C and N resources include total C (TC) and N (TN), total organic C (TOC) particulate organic C (POC), permanganate-oxidisable C (POXC), hot-water-extractable and water-soluble organic C (HWEOC, WSC), char-derived C (CharC) and resistant organic C (ResC), mineral N (MinN) and C:N ratios. These measures describe the quantity, quality and stoichiometry of substrates available to microbial communities (Haynes 2005; Culman et al. 2012; Baldock et al. 2013). However, many of these fractions are operationally defined, making it difficult to resolve microbial accessibility and stabilisation mechanisms (Wander 2004; Lehmann and Kleber 2015).

Complementary biochemical and molecular tools, including phospholipid fatty acid analysis (PLFA), quantitative polymerase chain reaction (qPCR) and phylogenetic amplicon sequencing, provide indicators of microbial biomass, target-gene abundance and community composition, respectively, and have improved understanding of microbial responses to physicochemical gradients and land management (Nkongolo and Narendrula-Kotha 2020). However, limited functional inferences can be drawn from composition alone (Louca et al. 2018). For example, Bowles et al. (2014) found that potential enzyme activities involved in C and N cycling were more strongly predicted by soil physicochemical properties than by microbial community composition, suggesting that compositional attributes do not necessarily predict functional responses. Likewise, functional redundancy can decouple taxonomy from function, allowing taxonomically distinct communities to maintain similar functional profiles (Louca et al. 2018). Consequently, a shift from taxonomy- to trait-based indicators may better link resource availability with microbial function and more directly reflect soil processes and ecosystem service delivery (Krause et al. 2014; Hicks et al. 2022).

Indicators of functional potential capture the capacity of these microbial communities to compete for and utilise available food sources. Microbial respiration has long been used as an integrative measure of soil microbial activity and organic C mineralisation (Anderson 1982). However, respiration alone provides limited insight into metabolic efficiency, as high or low CO_2 flux may reflect differences in microbial biomass, substrate availability or physiological activity. Normalising respiration by microbial biomass yields the metabolic quotient (qCO_2), whereas microbial C use efficiency (CUE) requires quantification of C allocation between growth and respiration (Malik et al. 2018; Azevedo et al. 2024). Potential activities of extracellular enzymes involved in C, N, P and S acquisition – including β-glucosidase (GLC), N-acetyl-β-D-glucosaminidase (NAG), phosphatase (P), arylsulfatase (S) and acetyl esterase (ACE) – provide functional indicators of microbial resource-acquisition potential (Sinsabaugh et al. 2008; Bowles et al. 2014). Assays conducted at native soil pH better approximate activity under ambient conditions, whereas assays at a common buffered pH standardise reaction conditions but may confound interpretation where enzyme pH optima differ among soils (Turner 2010; German et al. 2011; Puissant et al. 2019). While enzyme measures indicate the potential of microbial communities to depolymerise organic matter and mobilise nutrients, they are highly sensitive to short-term variation in soil moisture and temperature, lack standardisation across laboratories and have limited commercial accessibility (Burns et al. 2013; Nannipieri et al. 2018). Accordingly, enzyme measures are more suited to research applications and less practical for routine on-farm monitoring. Shotgun metagenomic approaches can link taxonomy and functional potential, but current cost, analytical complexity and computational demand preclude their use as routine soil health indicators (Fierer et al. 2021; Anthony et al. 2024). While indicators of functional potential target key soil processes, they do not necessarily translate to what happens *in situ* (Wallenstein and Weintraub 2008; Nannipieri et al. 2018).

Realised function indicators capture the outcomes of microbial activity as they accumulate in the soil matrix. These include soil aggregation and structural stability, water retention, nutrient retention and availability, C stabilisation, and accumulated organic protein pools. Potentially mineralisable N (PMN), typically measured in a laboratory after 7 (MinNT7) and 14 days (MinNT14), indexes biologically active N reserves and microbial capacity to convert organic N into plant-available forms. However, it cannot distinguish efficient microbial mineralisation from exhaustion of a finite residue pool (Schimel and Bennett 2004). Soil autoclavable citrate-extractable (ACE) protein reflects accumulated microbial- and organic matter-associated proteins and nitrogenous amino compounds, is analytically simple, and has been proposed as a proxy for labile organic N and general soil health (Moebius-Clune et al. 2016; Hurisso et al. 2018; Naasko et al. 2024). Proteins represent the largest organically bound N pool in soil, and protein depolymerisation is the rate-limiting step in N cycling (Schimel and Bennett 2004; Jan et al. 2009). ACE protein has also been linked to improved wet aggregate stability, organic matter, active C indicating roles beyond N supply (Fine et al. 2017). Its relationships with enzyme activities across contrasting soils, climates and management regimes remain poorly characterised.

Together, these substrates, microbial communities, functional traits and functions performed *in situ* then determine how effectively inputs are processed, completing the cycle and influencing the next generation of inputs, communities and functions. In this study, we surveyed 82 soil indicators

representing physicochemical context, C and nutrient fractions, microbial community composition, functional potential and realised function at pre-sowing, in-crop and post-harvest across four Australian broadacre cropping field experiments with varying management practices. Our aim was to identify biologically meaningful indicators that can serve as practical proxies for soil functional processes. We hypothesised that soil enzyme activities involved in C, N, phosphorus and sulfur cycling would be structured primarily by soil pH and C content across sites, sampling times and management regimes, and that no single additional biological indicator would provide a universally robust proxy for soil biological function. Specifically, our expectation was that, beyond pH and TC, individual biological measures would show context dependent behaviour and therefore limited potential as general indicators of soil health.

2. Methods

2.1 Field study sites.

Four field experiments spanning key grain-growing regions in New South Wales, Victoria, South Australia and Western Australia (Fig. 1) were accessed for assessment of soil physical, chemical and biological indicators. Sites targeted regionally relevant constraints (Table S1, Online Resource 1). The Birchip Cropping Group (BCG) site was established in 2018 on a Solonetz (World reference base; IUSS Working Group WRB 2022), aimed to assess the legacy effects of herbicide use. Treatments included a nil-herbicide control, two contrasting in-crop herbicide regimes and the same regimes with additional herbicide applications during the fallow period. The Minnipa Agricultural Centre (MAC) site, established in 2021 on a Calcisol (WRB), aimed to lift grain yields in calcareous soils by addressing soil chemical and biological constraints, comparing 1) typical grower practice with 2) liquid phosphorus (P) and trace elements, 3) anti-rhizoctonia fungicide, trace element chelates and liquid N and P, 4) banded P-enriched biochar with Urea, and 5) granular N and P fertiliser to match N and P content of treatment 4. The Central West Farming Systems (CWFS) site, established in 2019 on an Acrisol (WRB), aimed to improve soil physical, nutritional and biological function through increased plant diversity. Treatments compared typical practice with and without lime, and diversified rotations including pulse sequences, undersown crops and summer cover crops, all with lime applied. The Wheatbelt NRM (WNRM) sites, established prior to 2019 on an Arenosol (WRB), aimed to determine differences in soil biological activities associated with long-term adoption of regenerative agricultural practices, using two pairs of paddocks that compared regenerative practices – zero tillage, diverse rotations, cover crops, organic amendments and reduced synthetic inputs – with paired conventional systems based on monoculture grain or lupin-cereal rotations, standard fertiliser and routine chemical use. Each conventional versus regenerative pair were located over the fence from each other, while each pair was located more than 500 km apart in Newdegate and Buntine districts, respectively.

2.2 Soil sampling.

In 2022, 0–10 cm soil cores were collected using a sterilised stainless steel soil corer and composited in the field at pre-sowing, in-crop and post-harvest, with three cores per plot at CWFS and WNRM and four cores per plot at BCG and MAC. Composite samples were kept refrigerated prior to physicochemical and biochemical analyses. A total of 201 composite samples were collected and analysed. Additional composite 0–10 cm samples were collected using the Predicta® research protocol for DNA extraction and qPCR-based diagnostics (PIRSA 2018) for each plot.

2.3 Soil physicochemical and organic matter measurements.

Subsamples of the refrigerated composite soil were air-dried at 40ºC, crushed and sieved to < 2 mm for biological analyses with a portion ball-milled to < 250 µm for chemical analyses. Soil pH (1:5 $CaCl_2$) and electrical conductivity (EC) (1:5 H_2O) were measured using an automated electrode system (Rayment and Lyons 2011). Total C and N were quantified on a 0.2 g subsample of dried, ground soil by dry combustion (LECO TruMac). Organic C fractions (particulate, resistant and char C) were estimated by mid-infrared reflectance spectroscopy calibrated to reference fractions (Janik et al. 2007; Baldock et al. 2013). Permanganate-oxidisable C (POXC) was determined colorimetrically following method 6E1 (Rayment and Lyons 2011). Water-soluble and hot-water extractable C were measured sequentially on 3 g soil by TOC analysis (Aurora 1030 C TOC analyser; Ghani et al. 2003). Mineral N and potentially mineralisable N were measured in KCl extracts after aerobic incubations (0, 7 and 14 d), as described by Allen et al. (2019).

Soil ACE protein was quantified on duplicate 2 g air-dried soil subsamples by extraction with 16 mL of 20 mM sodium citrate buffer (pH 7.0), autoclaving at 121°C for 30 min, and colorimetric determination of protein concentration in the clarified extract using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), referenced against the bovine serum albumin standard (Hurisso et al. 2018). This provided a bulk measure of the autoclavable, citrate-extractable protein fraction of soil organic matter, considered indicative of potentially bioavailable soil N.

2.4 Microbial community indicators.

Phospholipid fatty acid (PLFA) profiles were determined on 3 g freeze-dried soil, spiked with internal standards (C19:0 1,2-DNPC and C13:0 1,2-DTPC) prior to Bligh-Dyer extraction, solid-phase fractionation and methylation of phospholipids (Bligh and Dyer 1959). Methylated PLFAs were quantified, with a further internal standard (C10:0 methyl ester) added before analysis, by GC-MS (Agilent 5977C) against an external calibration curve (Supelco 37-compound FAME mix, CRM 47885). Total bacterial and fungal PLFA and functional groups were derived from biomarker sums (Frostegård et al. 1991). Gram-positive PLFAs were represented by 14:0 iso, 15:1 iso ω6c, 15:0 iso, 15:0 antesio, 16:0 iso, 17:0 iso and 17:0 antesio, and gram-negative by 14:1 ω5c, 15:1 ω5c, 16:1 ω7c, 17:1 ω7c, 17:0 cyclo ω7-8c, 18:1 ω7c or ω9t, 18:1 ω5c and 19:0 cyclo ω7c. Arbuscular mycorrhizal fungi (AMF) was represented by 16:1 ω5c, and general fungi by 18:1 ω9c and 18:2 ω6c. Actinobacteria comprised 16:0 10-methyl, 18:1 ω7c 10-methyl and 18:0 10-methyl, while general bacterial PLFAs comprised 14:00, 15:00, 16:00, 17:00, 18:00

and 20:00. Total PLFA was calculated as the sum of all aforementioned biomarkers, total fungi as comprised general fungi plus AMF, and total bacteria as the sum of gram-positive, gram-negative, Actinobacteria and general bacteria biomarkers. The gram-negative stress ratio was expressed as (17:0cy + 19:0cy) : (16:1 w7c + 18:1 w7c), and the saturated:unsaturated ratio as the sum of all PLFAs with Y = 0 (including branched, methyl-branched and cyclopropyl forms) divided by the sum of all PLFAs with Y ≥ 1.

DNA was extracted from samples oven-dried at 40°C upon reception at SARDI using a bead-beating protocol, with a standard amount of internal control added to each sample prior to extraction, yielding 160 µL of DNA per sample (Ophel-Keller et al. 2008). DNA extracts were diluted 1:5 prior to qPCR analysis, and an internal control qPCR test was conducted on each extract to monitor DNA extraction efficiency and PCR inhibition. Quantitative polymerase chain reaction (qPCR) assays, performed by SARDI Molecular Diagnostics, quantified six arbuscular mycorrhizal fungal (AMF) groups (kDNA copies/g soil), 15 free-living nematode guilds (kDNA copies/g soil), 6 plant-parasitic nematodes (nematodes/g soil) and 18 cereal and pulse pathogens (pgDNA/g soil).

2.5 Enzyme activities.

Activities of six enzymes involved in C, N, P and S cycling (β-glucosidase, N-acetyl-β-D-glucosaminidase, phosphatase, arylsulfatase, esterase and leucine aminopeptidase) were measured using microplate fluorometric assays based on the hydrolysis of nonfluorescent substrates into fluorescent products (Marx et al. 2001; Turner 2010; Weng et al. 2017). Enzyme assays included five 4-methylumbelliferyl (MUF)- (MUF-β-glucoside, MUF-sulfate, MUF-phosphate, MUF-N-acetyl- β-d-glucosamine and MUF-acetate) and one aminomethylcoumarin (AMC)-based (AMC-leucine) substrates purchased from Biosynth AG, Switzerland. Briefly, soil suspensions (0.5 g soil in 25 mL deionised water) were diluted 1:10, combined with modified universal buffer at pH 7 or at a pH approximating native soil pH, plus substrate, in 96-well plates, and fluorescence measured at 0, 15 and 120 min. Triplicate technical replicates were averaged and expressed per unit dry soil.

2.6 Statistical analyses.

Analyses were conducted in R version 4.2.3 (R Core Team 2023). Enzyme data were $\log_{10}$-transformed prior to analysis to reduce right-skew, stabilise variance and better satisfy normality assumptions (Fig. S1). Distributions of physicochemical measures, fungal and nematode population abundances and enzyme activities were evaluated with non-metric multidimensional scaling (NMDS) using the ‘metaMDS’ function of the *vegan* R package (v.2.7.3) (Oksanen et al. 2003) based on Euclidean distances. PERMANOVA using ‘adonis2’ function in the *vegan* R package tested for effects of Time, Site and their interaction on indicator composition. Environmental fits using the ‘envfit’ function in the *vegan* R package were calculated and projected onto enzyme NMDS ordinations. BIOENV (Best Subset of Environmental Variables with Maximum Rank) analyses using ‘bioenv’ function in the *vegan* R package was computed on log-transformed enzyme-composition distance matrices and Spearman rank correlations between biological and environmental distances to identify subsets of environmental variables that best

explained variation in enzyme composition. Mantel tests using the ‘mantel’ function of *vegan* R package were used to test the strength and significance of Spearman correlations between enzyme and environmental distance matrices.

To assess how soil conditions and substrate quality shape nutrient-cycling functions, a correlation-based co-occurrence network was constructed, combining physicochemical variables and enzyme activities measured at native soil pH and standardised pH 7. Pairwise Spearman rank correlations with P-values were calculated using ‘rcorr’ in the *Hmisc* R package (v.5.2.1) (Harrell 2023). Edges with $|r| \geq 0.4$ and $P \leq 0.05$ were retained, and an undirected network was visualised using the ‘ggraph’ function in the ggraph R package (v.2.2.1) (Pedersen 2017) in combination with the *igraph* R package (v.2.1.1) (Csardi and Nepusz 2024) using a Fruchterman-Reingold layout. Nodes were coloured by indicator class (i.e. physicochemical versus enzyme measures) and scaled by degree (number of links), while edges were coloured by nature of association (i.e. blue = negative, pink = positive) and line width represented the strength of association. Node-level topological features were calculated using the *igraph* R package to compare the degree (sum of links to other nodes), weighted degree (sum of link weights), eigenvector centrality (influence based on connections to well-connected nodes), betweenness centrality (extent to which a node connects otherwise separate groups), closeness (average distance to all other nodes), PageRank Walker (probability of visiting a node via random walks through the network), bottleneck scores (frequency of a node lying on shortest paths), Markov centrality (how quickly a node is connected to the rest of the network via random walks) and authority scores (how important a node is based on links it receives from other important neighbouring nodes) (Kleinberg 1999; Newman 2010; Masuda et al. 2017).

Scatter plots with linear regressions were generated using the ‘ggplot’ function in *ggplot2* R package (v.4.0.3) (Wickham 2016), and pairwise Pearson correlations using ‘cor.test’ function in the R *stats* package (R Core Team 2023) were used to explore bivariate relationships between soil ACE protein and enzyme activities. All tables were generated in Microsoft Excel v.2605 Build 16.0.20026.20166 (Microsoft Corporation 2023). Pairwise relationships among C fractions were assessed using linear regression and visualised as a correlation matrix with effect size and significance notation using the ‘ggpairs’ function in the *GGally* R package (v.2.2.1) (Schloerke et al. 2020). Treatment effects on individual soil variables within each site were assessed with one-way ANOVA using the ‘aov’ function in the R *stats* package, with ANOVA effect size (η^2) and significance notation presented as a tile heatmap using *ggplot2*.

3. Results

3.1 Treatment effects on biological indicators.

Treatment effects on individual soil variables within sites were generally modest relative to effects of site location and sampling time (Fig. S5). Where significant treatment effects were detected, they were largely restricted to WNRM and CWFS. At WNRM, soil pH, β-glucosidase measured at pH 7 (GLC),

phosphatase measured at native soil pH (PspH), *Didymella pinodes/Phoma pinodella* qPCR abundances (Fun_DidPin_DNA) differed significantly ($P < 0.05$) by treatment. At CWFS, *Pratylenchus neglectus* qPCR abundances (Nem_PrtNeg_DNA) and leucine aminopeptidase measured at pH 7 (LEU) differed by treatment. However, all other variables did not differ significantly by treatment within sites. In addition, several variables, particularly fungal and nematode qPCR groups, had low detection levels (Fig. S5).

3.2 Site and time controls on soil biological indicators.

Distributions of all soil biological indicators showed strong separation by site and sampling time (Fig. S2A-D). Site and time together explained more than half of the variance in physicochemical variables, fungal and nematode communities, and enzyme activities ($R^2 = 0.55–0.72$, $P < 0.001$, PERMANOVA).

3.2.1 Physicochemical variables and carbon fractions.

Site and sampling time explained 54.9% of the variation in physicochemical variables ($P < 0.001$, PERMANOVA; Fig. S2A). Within sites, observations clustered by sampling time, with BCG, CWFS and WNRM observations associated with HWEOC and WSC. WNRM had additional clusters with ACE protein and POXC. In contrast, MAC was associated with resistant C forms (ResC, CharC), TC, TOC and pH. In terms of correlations between C fractions, TC, TOC, POC, CharC and ResC showed strong positive pairwise correlations ($r = 0.65–0.97$, $P < 0.001$), while HWEOC and WSC showed weaker or negative relationships with resistant C fractions (Fig. S3).

3.2.2 Fungal and nematode populations.

Site and sampling time explained 58.7% of the variation in fungal population abundances ($P < 0.001$, PERMANOVA; Fig. S2B). Fungal population abundances at CWFS shifted most over time, while fungal communities at BCG and MAC remained relatively consistent across sampling times. WNRM demonstrated high variability across samples. Site and sampling time explained 72.2% of variation in nematode population abundances ($P < 0.001$, PERMANOVA; Fig. S2C). The MAC and BCG sites had the most distinct nematode communities. *Pratylenchus thornei* was significantly enriched at MAC, while BCG had greater abundances of *Pratylenchus neglectus*, *Tylenchinae* tests 1 and 6 and *Mesorhabditinae*. Nematode population abundances at CWFS, BCG and MAC shifted most over time, while WNRM demonstrated high variability across samples.

3.2.3 Enzyme activities and environmental drivers.

Site and sampling time explained 72.4% of variation in enzyme activities, indicating the strongest site- and time-dependent structuring of the indicator classes assessed ($P < 0.001$, PERMANOVA; Fig. 2A; Fig. S2D). Enzyme activities at BCG, CWFS and MAC shifted most throughout the year, whilst WNRM showed more mixed enzyme compositions across sampling times. Enzyme vectors for chitinase (NAGspH) and chitinase standardised to pH 7 (NAG), phosphatase (PspH) and phosphatase standardised to pH 7 (P) showed elevated responses at CWFS and WNRM. Leucine aminopeptidase (LEUspH) corresponded to

MAC, while both β-glucosidase measures (GLC, GLCspH), both arylsulfatase measures (S, SspH) and esterase (ACEspH) corresponded to BCG (Fig. 2A).

Enzyme activities measured at native soil pH versus standardised pH 7 were highly correlated for chitinase, phosphatase, β-glucosidase and leucine aminopeptidase (Pearson r = 0.70–0.95, P < 0.001; Fig. S4), with median values similar between assay conditions. Esterase activities under the two assay conditions were positively correlated (Pearson r = 0.317, P < 0.001).

Envfit analysis identified multiple soil physicochemical variables as significantly associated with enzyme composition (Table S2). BIOENV analyses across all sites, times and treatments identified soil pH as the single best individual predictor of enzyme composition (Spearman ρ = 0.598; Fig. 3C, Table S3). TC coupled with soil ACE protein alone increased explanatory power (Spearman ρ = 0.705) with marginal increases attained from a 6-variable model comprising soil pH, TC, ACE protein, ResC, C:N ratio and MinN (Spearman ρ = 0.71; Figs. 2B,3C).

3.3 Soil ACE protein links physicochemical properties and enzyme activities.

A co-occurrence network was inferred to analyse connections and importance of soil physicochemical variables and enzyme activities. Within this measured-variable network, soil ACE protein occupied a central position (Fig. 3A) and achieved the universally highest ranking across a suite of centrality and importance metrics (Fig. 3B). Among physicochemical nodes, soil ACE protein attained the highest degree, eigenvector centrality, closeness centrality, PageRank, bottleneck score, Markov and authority score (Fig. 3B).

Soil ACE protein showed strong positive correlations with chitinase and phosphatase activities across all sites (Pearson r = 0.76 and 0.62, respectively, P < .0001; Fig. 4; Fig. S6). Across sampling times, these associations remained positive for both native pH and pH 7 assays, with strongest correlations at pH 7 (Fig. 4; Fig. S6). Relationships with β-glucosidase were moderate and more site-dependent, with significant correlations observed at BCG and MAC (Pearson r = 0.35, P < 0.001), but weak and non-significant relationships at CWFS and WNRM (Pearson r = 0.1, P > 0.05; Fig. 4). Positive correlations with esterase activity were also observed at MAC (Pearson r = 0.55 at native pH and 0.31 at pH 7) and WNRM (r = 0.67 and 0.64, respectively). In contrast, relationships with arylsulfatase varied among sites, with positive, negative or non-significant associations (Fig. S6).

While no significant treatment differences on ACE protein content were observed within sites, protein content differed across sites (P < 0.001). CWFS and WNRM exhibited the highest soil ACE protein content (5.05 ± 0.08 and 5.0 ± 0.29 mg g^{-1} soil, respectively) compared with BCG (3.12 ± 0.06 mg g^{-1}) and MAC (2.62 ± 0.06 mg g^{-1}) (Fig. S7).

4. Discussion

Here, we characterised physicochemical measures, C and nutrient fractions, microbial community biomass and composition, enzyme-mediated processes and resulting soil functional outcomes across four sites spanning contrasting soils, climates and management systems. This analytical design addressed calls for cross-site validation as a prerequisite for adopting biological indicators (Fierer et al. 2021). Our findings demonstrated that soil pH, TC and soil ACE protein structured enzyme composition across contrasting sites, confirming that organic matter quantity and quality underpin microbial functional capacity (Lehman et al. 2015) while soil pH shapes catalytic efficiency (Sinsabaugh et al. 2008). Biomass and taxonomic indicators (PLFA, qPCR groups) captured additional information on bacterial, fungal, nematode and pathogen communities and, while useful for sites with disease-related research questions, were less directly interpretable as routine indicators of soil function. These findings align with growing evidence that functional measures such as enzyme activities and labile N proxies are more meaningful indicators of soil biological processes than taxonomic descriptions or operational groups alone (Schloter et al. 2018; Fierer et al. 2021).

4.1 Soil ACE protein as an indicator of microbial functions.

Soil ACE protein was repeatedly selected in best-subset BIOENV models, displayed strong envfit loadings and, along with TC, constituted the most parsimonious physicochemical predictors of enzyme composition across all sites and sampling times, consistent with ACE protein as a key indicator of soil health and organically bound N (Hurisso et al. 2018; Naasko et al. 2024). Of all physicochemical variables, soil ACE protein occupied the most central and important node in the measured-variable co-occurrence network linking physicochemical measures and enzyme activities, achieving the highest ranking across the suite of importance and centrality metrics. Strong positive relationships were then observed between soil ACE protein and individual enzyme activities involved in nutrient cycling, with the most consistent results across sites and times observed between soil ACE protein and chitinase and phosphatase, respectively. These strong positive associations reflect active microbial investment in nutrient acquisition, specifically, chitinase targeting chitin-bound N and phosphatase mobilising organic P (Sinsabaugh et al. 2008; Daunoras et al. 2024). The close coupling of soil ACE protein content and chitinase enzymes may additionally reflect suppression of pathogenic fungi and nematode eggs via chitinase-mediated depolymerisation of chitin in fungal cell walls and nematode eggshells (Duo-Chuan 2006; Gortari and Hours 2008). The relationship with β-glucosidase was more site-dependent, likely reflecting the greater sensitivity of C-acquisition enzymes to local C substrate availability and microbial stoichiometric demands (Sinsabaugh and Follstad Shah 2012; Geisseler and Scow 2014). Associations with the remaining enzymes were more context dependent. Positive relationships with esterase at MAC and WNRM suggest that ACE protein can also covary with hydrolytic activities beyond N and P acquisition, although this relationship was not consistent among sites. The mixed relationships with arylsulfatase may reflect differences in substrate specificity, as this enzyme hydrolyses sulfate esters rather than protein-bound sulfur (van Loo et al. 2018). Its activity may therefore vary independently of ACE protein content. Leucine aminopeptidase activity was associated with the alkaline Calcisol at MAC, where the median soil pH of 8 was within the reported optimum range (pH 7–9) for this enzyme (He et

al. 2026). This contrasts with its relatively low ACE protein content, suggesting that pH-related controls may partly explain the weak protein-enzyme association. The inconsistent and non-significant relationship between soil ACE protein and microbial biomass PLFAs, including PLFA functional groups, suggests that soil protein is not a by-product of microbial growth or biomass but rather a shift in microbial function. Microbial communities adjust C allocation between growth and enzyme production depending on nutrient stoichiometry and CUE (Malik et al. 2018). Together, these patterns suggest that soil ACE protein represents organic N availability, N and P acquisition and the investment of organic resources back into the soil organic matter pool. Further research is required to evaluate links between soil ACE protein content and microbial CUE.

Unlike enzyme assays, which measure potential activity under optimised laboratory conditions (German et al. 2011), soil ACE protein reflects the accumulated products of microbial biosynthesis and organic matter processing under field conditions (Hurisso et al. 2018), positioning it as an indicator of realised rather than potential function. While enzyme activities are sensitive indicators of microbial processes, they are operationally challenging, exhibiting high fluctuation with soil temperature and moisture, lacking methodological standardisation across laboratories, and lacking widespread commercial availability. Enzyme activities typically fluctuate widely across sampling dates at the same site simply due to soil temperature and moisture variability, which complicates temporal and spatial comparability, consistency of measurements and threshold development (Wallenstein and Weintraub 2008; Steinweg et al. 2012). Fierer et al. (2021) report that suitable biological indicators should maintain stability within a temporal window; that is, responsive to management over seasonal to annual timescales, but not so sensitive as to fluctuate over days to weeks. Soil ACE protein represents an accumulated proteinaceous pool that is more temporally stable than enzyme activities or mineral N, positioning it closer to this window of sensitivity (Hurisso et al. 2018; Naasko et al. 2024). The assay is technically straightforward, uses inexpensive reagents and standard laboratory equipment, and generates a quantitative index (Moebius-Clune et al. 2016; Hurisso et al. 2018). Previous studies have shown that soil ACE protein responds readily to management, with higher values under practices that build soil organic matter (e.g. manure, cover crops, reduced tillage) and lower values under more intensive, disturbance-based systems (Naasko et al. 2024). Our results extend previous work, which proposed soil ACE protein as an indicator of labile organic N (Hurisso et al. 2018; Hurisso and Culman 2021), to show that it also captures variation in enzyme-mediated N and P cycling. Here, we present a novel finding that strengthens the utility of soil ACE protein as a functional soil health indicator. Accordingly, we propose that soil ACE protein is a promising low-cost proxy for nutrient-cycling capacity and the extent to which plant-derived inputs are transformed into stable, biologically active soil organic matter. As these conclusions are drawn from a subset of Australian soil types and climatic regimes, extending measurements across more soils, land uses and seasons will be essential to establish interpretive thresholds for practitioners.

4.2 Functional limitations of community composition as a soil health indicator.

Community-level indicators and population biomarkers based on PLFA and qPCR, respectively, provided useful contextual information on bacterial, fungal and nematode populations, but their direct relationships with enzyme composition and soil ACE protein were largely weak and inconsistent. This exemplifies the widely reported challenge of inferring microbial function from community composition in heterogeneous field systems, particularly given overriding influences of seasonal dynamics and management history on both taxonomic and functional indicators (Bowles et al. 2014; Louca et al. 2018; Fierer et al. 2021). Indeed, there is no ideal soil microbial community against which field soils can be benchmarked, and higher microbial diversity is not inherently indicative of better soil health (Fierer et al. 2021). This highlights the limited utility of community composition metrics for routine assessment and reinforces the case for functional indicators with more meaningful interpretation.

Taxonomic profiling remains most powerful when combined with targeted functional indicators and in scenarios where specific taxa-function relationships have been validated. For instance, Predicta®B diagnostics for soilborne pathogens in Australian grain systems serves as a farmer-ready tool to profile disease risk (Ophel-Keller et al. 2008; PIRSA 2018). Shotgun metagenomics can bridge taxonomy and function by profiling functional genes alongside community composition, but current costs, analytical complexity and high computing demands limits its suitability as a routine soil health indicator (Fierer et al. 2021; Anthony et al. 2024). In this study, nutrient-cycling functions were strongly associated with soil ACE protein rather than with microbial community attributes. While taxonomic indicators are valuable for capturing microbial community composition, diversity and disease risk, function-oriented measures provide more direct and interpretable information for routine soil health assessment and grower decision support (Schloter et al. 2018; Fierer et al. 2021).

4.3 Limited effects of field site treatments.

Treatment effects on soil biological indicators were modest and largely confined to a subset of variables at individual sites, consistent with the dominant structuring role of site and sampling time identified. While greater treatment effects were anticipated, geographic location, sampling time and field variability have also exerted greater influences in other studies. For example, a year-long, multi-timepoint field study found that temporal effects explained 30% of variation in microbial community composition compared with just 2% for treatment (Joos et al. 2025), consistent with reports that spatial and technical variability in agricultural soils frequently exceed treatment effects (Jeanne et al. 2022) and that climate and inherent soil properties are primary drivers of microbial biogeography (Xue et al. 2024). The treatments applied across the BCG, CWFS and MAC included herbicide regime variations at BCG, liming and increased plant diversity via intercropping and cover cropping at CWFS, nutrient and amendment strategies to overcome calcareous soil constraints, especially limited P availability and high pH at MAC. These incremental changes, or combinations management modifications, may have weakened treatment effect signals. Similarly, WNRM compared two pairs of neighbouring sites, with each regenerative paddock located over the fence from its conventional counterpart, and the two site pairs separated by more than 500 km. Importantly, the two pairs were in distinct geographic locations driving greater physicochemical similarity between pairs than between practices. Where significant treatment

effects were detected, they tended to involve variables with high short-term sensitivity, such as specific fungal and nematode populations and enzyme activities at WNRM (Fig. S5).

The relatively short duration of some experimental treatments at the time of sampling may also have limited biological response. At the MAC site, the primary management objective was to alleviate nutrient constraints, particularly P limitation, that are characteristic of Calcisols, where high pH and free calcium carbonate promote P sorption and reduce plant-available P. Consistent with this, MAC was associated with resistant C forms (ResC, CharC), TC, TOC and pH, reflecting a highly alkaline carbonate-rich Calcisol with limited reactive mineral surfaces to build mineral-associated OC, such that resistant C fractions were dominant (Schmidt et al. 2012). Conventional grower practice typically addresses this constraint using inorganic P fertiliser inputs, which, while effective in the short term, do not necessarily build soil P-supplying capacity or biological function over time. In contrast, the amendment-based treatments trialled at this site intended to target longer-term improvements in nutrient cycling and soil biological activity, with benefits expected to accrue progressively rather than within a single season. Given that the MAC site was established only one year prior to sampling, the modest treatment effects observed here are consistent with the longer timeframe typically required for these strategies to influence P availability and microbial function. Over an extended trial period, sustained improvements in P cycling and soil biological activity would be expected to translate into detectable increases in plant biomass and, ultimately, yield. A short-term fertiliser-only strategy is less likely to deliver these outcomes on its own.

Herbicide application regimes and frequency at BCG did not elicit detectable shifts in biological indicator profiles, which is reassuring given that herbicide impacts on soil biology are a common concern among growers. The BCG and CWFS sites were established earlier in 2018 and 2019, respectively, and compared herbicide application frequency at BCG and crop diversity and liming at CWFS. These treatments represented relatively modest contrasts in management intensity rather than the pronounced, sustained contrasts that are typically needed to elicit detectable shifts in biological indicator profiles. This is consistent with other long-term studies showing that increasing crop rotation diversity alone often fails to alter soil microbial community structure, even several years after establishment (Struijk et al. 2026). At CWFS, liming produced broad, significant shifts across nearly all indicator classes, while increasing plant diversity through crop rotation, cover cropping and intercropping affected mainly pathogen-specific DNA markers, with little change to the majority of chemical, biochemical or PLFA-based indicators. These findings are consistent with this broader pattern, whereby inherent site factors and sampling timing often explain a greater proportion of variation in soil health indicators than management alone (Norris et al. 2020; Fierer et al. 2021). Together, these results suggest that, at least over the timeframes and treatment contrasts examined here, herbicide regimes and plant diversity treatments are not major drivers of biological indicator variation relative to site and sampling time effects, though stronger treatment contrasts, such as liming, may be needed to elicit more consistent, detectable responses.

5. Conclusions

We initially expected soil pH and C pools to mediate enzyme activities and that no single biological indicator would reliably proxy soil biological function across contrasting soils, management practices and sampling times. Our multi-indicator analysis broadly confirmed the central role of pH and total C but also revealed that soil ACE protein serves as an unexpected core indicator. ACE protein was strongly and consistently associated with chitinase and phosphatase activities, occupied a central position in the co-occurrence network and alongside pH and C pools, was associated with multivariate variation in enzyme activities. However, weaker associations with leucine aminopeptidase and site-dependent relationships with arylsulfatase indicate that ACE protein does not uniformly track enzyme activities involved in N and S acquisition. Collectively, these results show that ACE protein integrates aspects of organic matter status, N and P cycling, and microbial investment into soil, highlighting its potential as a practical functional indicator while still requiring validation beyond the studied systems.

Declarations

Author Contribution

M.R. and L.V.Z. designed the project. M.R. led the project, secured funding and obtained the dataset. K.W. analysed the data, wrote the manuscript and prepared all tables and figures. All authors reviewed and edited the manuscript.

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

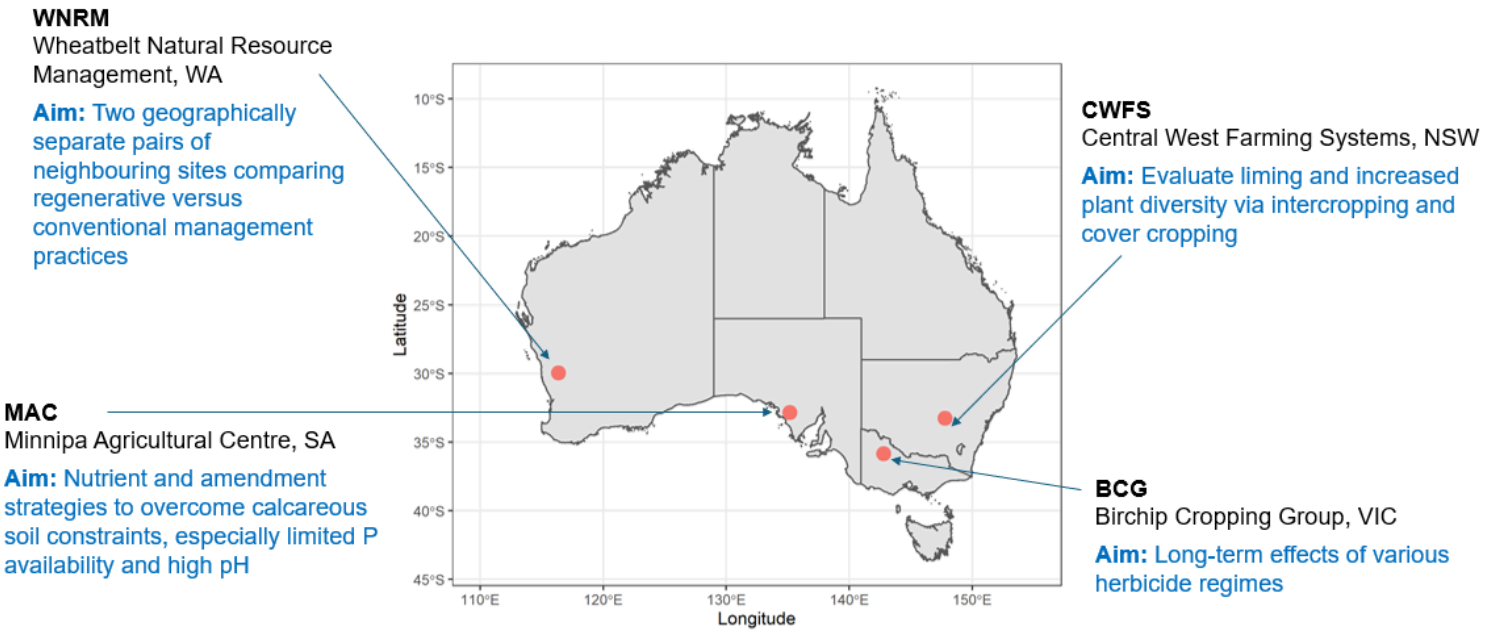


Figure 1

Geographic locations of Birchip Cropping Group (BCG), Central West Farming Systems (CWFS), Minnipa Agricultural Centre (MAC) and Wheatbelt NRM (WNRM) case study sites alongside research focus of each site

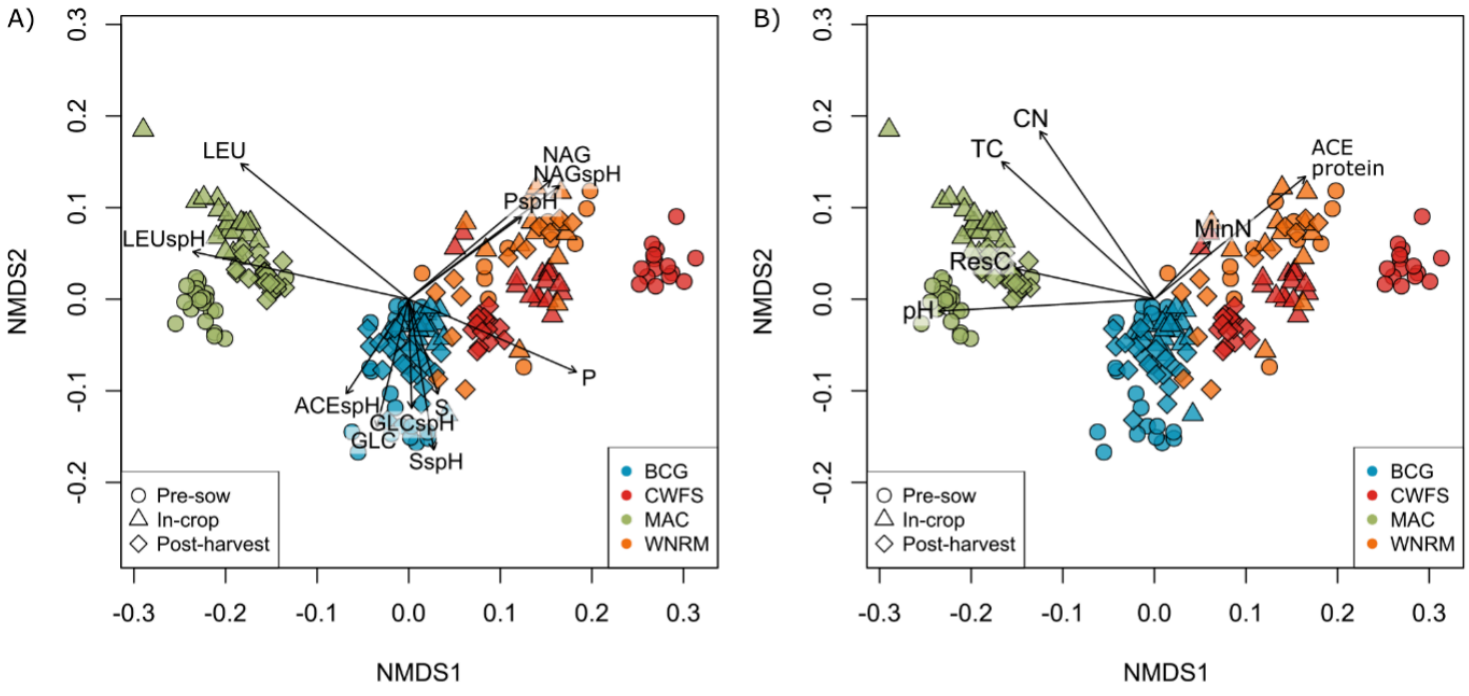
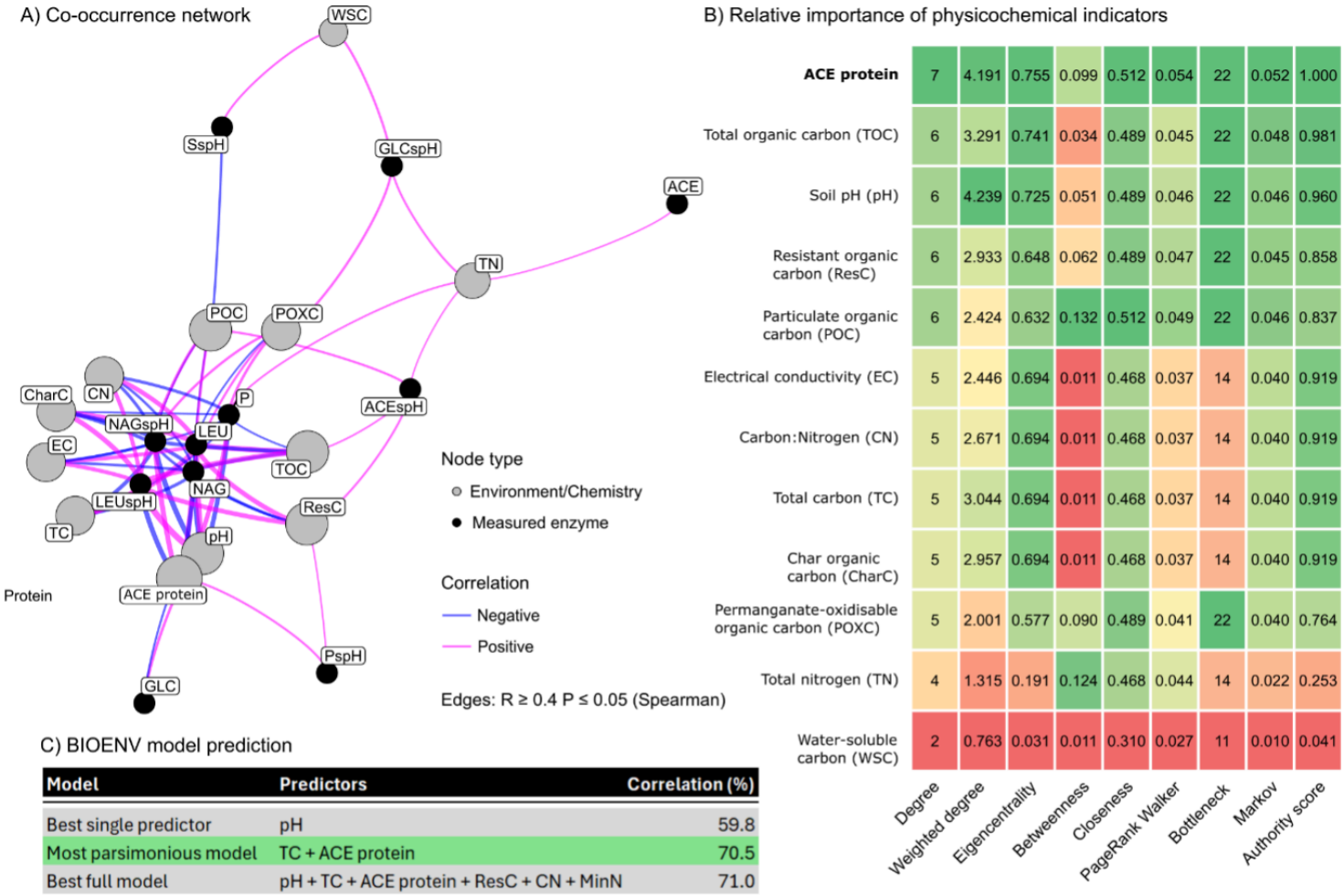


Figure 2

Non-metric multidimensional scaling (NMDS) ordination of soil enzyme activities by site and sampling time with significant ($P < 0.001$) A) enzyme activity vectors and B) soil physicochemical variables



C) BIOENV model prediction

Model	Predictors	Correlation (%)
Best single predictor	pH	59.8
Most parsimonious model	TC + ACE protein	70.5
Best full model	pH + TC + ACE protein + ResC + CN + MinN	71.0

Figure 3

A) Co-occurrence network of physicochemical variables (grey nodes) and enzyme activities (black nodes) where edges represent significant Spearman correlations (|r| ≥ 0.4, P ≤ 0.05), B) heatmap of node-level centrality metrics for physicochemical variables, and C) BIOENV model performance summarising the best single predictor, the most parsimonious model and the best full explanatory model of observed enzyme activities

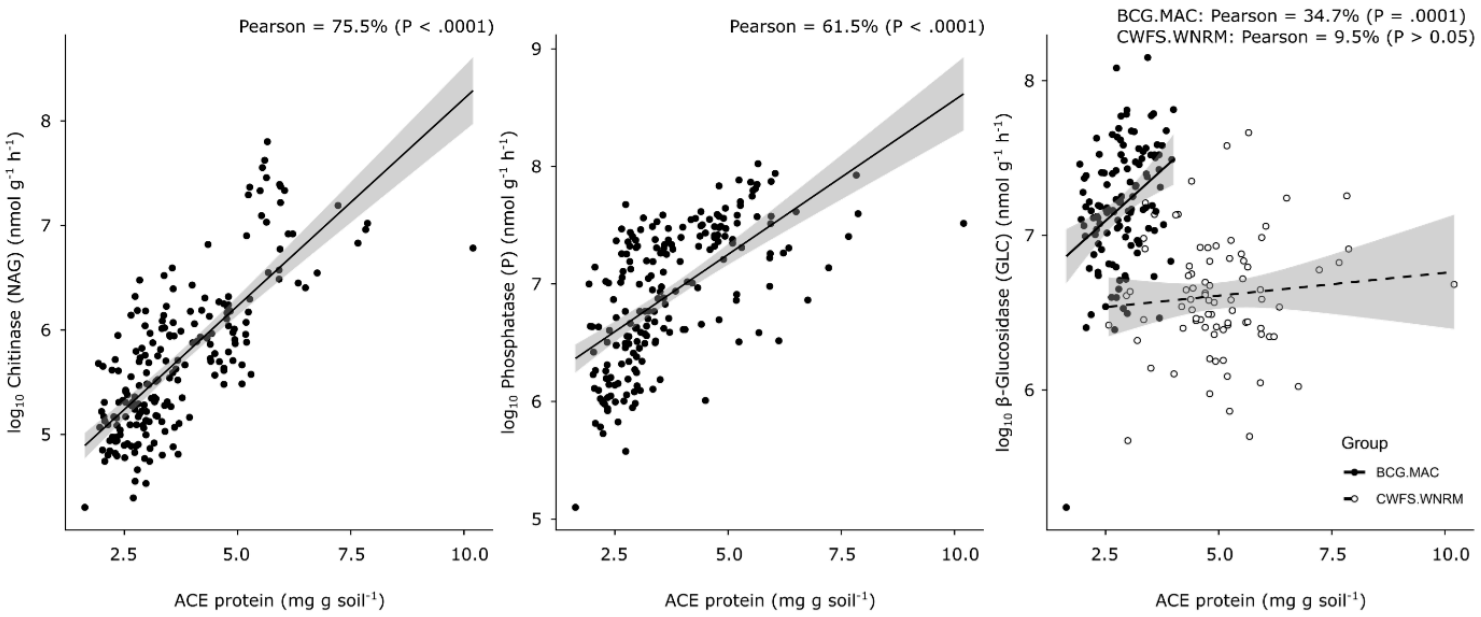


Figure 4

Relationships between ACE protein and chitinase, phosphatase and β-glucosidase activities (log_{10} nmol g^{-1} h^{-1}) measured at standardised pH 7

Supplementary Files

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- OnlineResource1.pdf