Optimizing pH for soil enzyme assays reveals important biochemical functions in low pH soil

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ORIGINAL PAPER

Optimizing pH for Soil Enzyme Assays Reveals Important Biochemical Functions in Low pH Soil

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Abstract
Soil enzyme assays are often used as indicators of potential biological functions. The objective of this study was to understand enzyme activity across a range of soil pH. Soils (0–15 cm) were collected from a heathland restoration project (established 1999) on the Isle of Purbeck, UK with treatments of elemental sulphur or ferrous sulphate compared to a control, acid grassland and heathland. Enzyme assays were conducted using fluorescent substrates for β-1,4-glucosidase, β-N-acetylglucosaminidase (NAG) and phosphatase with a range of buffer pH from 3.0 to 12.0. Differences in soil pH were still evident with the control (pH 5.3) and ferrous sulphate (pH 5.2) significantly higher than elemental sulphur (pH 4.5), acid grassland (pH 4.3) and heathland (pH 4.0). The optimum buffer pH for enzyme assays varied from pH 3-4.5 for β-glucosidase, pH 4–5 for NAG and pH 4–6 for phosphatase. Comparisons using a standard MUB pH resulted in different conclusions compared to optimum pH. For example, β-glucosidase activity at pH 5 for the control was significantly higher than elemental sulphur, acid grassland, and heathland. However, there were no differences when the pH optimums were considered. Comparisons of phosphatase activity at MUB pH 6.5 resulted in higher activity in the control plots compared to the heathland, despite the heathland soils showing the highest activity at optimum buffer pH. By examining the relationships between soil pH, enzyme activity, and assay conditions, this study highlights the importance of optimizing pH in enzyme assays when comparing diverse soil types.

Keywords Phosphatase · B-1,4-glucosidase · β-N-acetylglucosaminidase · Heathlands

1 Introduction

The availability of essential nutrients in soil is intricately linked to its pH level. Low pH conditions often correspond to diminished nutrient availability, thereby influencing plant growth and ecosystem dynamics, but both plants and microbes have developed physical and biochemical mechanisms to thrive in these conditions. For example, heathlands develop on acidic and nutrient-poor soils (Roem and Berendse 2000) with ericaceous dwarf shrubs adapted to these conditions and often dominating the landscape (Diaz et al. 2011). Since the 1950s there has been a dramatic decline in lowland heaths in Western Europe, partially as a result of conversion to agricultural land (Clarke 1997; Green et al. 2007; Fagúndez 2013; Ombashi and Løvschal 2022). However, heathlands provide a unique habitat and there have been attempts to convert improved agricultural land back into heathlands. A major component of heathland restoration is manipulating the soil chemistry to reduce the pH and nutrient availability from an improved agricultural soil through acidification (Diaz et al. 2011). Although the effects of acidification treatments on soil chemistry and plant communities have been documented (Owen et al. 1999; Owen and Marrs 2000; Green et al. 2007; Diaz et al. 2011; Tibbett et al. 2019; Duddigan et al. 2020, 2024), the long-term effects on soil biology and functions is less well known. Previous studies have demonstrated effects of soil

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pH on both soil biological communities and plant communities (Dodd et al. 1994; Schuster and Diekmann 2003; Rousk et al. 2009; Griffiths et al. 2011; Tibbett et al. 2019). A decrease in soil and plant biodiversity may lead to a loss in functional capabilities in the soil (Wagg et al. 2014), thereby impacting enzyme production and stabilization in soils.

Enzymes play a vital role in biochemical processes in soil systems, where potential enzyme activity is commonly used as an indicator of soil functions. Enzymes are proteins that break down organic compounds by hydrolysis or oxidative processes (Dick and Kandeler 2005; Burns et al. 2013), with multiple forms of enzymes with the same function referred to as isoenzymes. In soils, enzymes may originate from bacteria, fungi or plants and can be present as intra- or extracellular enzymes or stabilized within the soil matrix (Burns 1982; Burns et al. 2013). Enzyme activities are sensitive to changes in soil conditions and have been used to demonstrate effects on parameters such as nutrient cycling (Nyiraneza et al. 2018), soil quality (Dick 1994; García-Ruiz et al. 2009; Giacometti et al. 2014), microbial function (Sowerby et al. 2005), soil pollution (Trasar-Cepeda et al. 2000; Lee et al. 2020) and restoration (Raiesi and Salek-Gilani 2018). In a liming study conducted by Acosta-Martínez and Tabatabai (2000), it was observed that among 14 enzymes involved in C, N, P and S cycles in soils across a variety of soil pH levels, all enzymes exhibited a significant positive correlation with soil pH seven years after treatment application, with the exception of acid phosphatase.

Potential enzyme activity for β-D-glucosidase, chitooligosaccharides (N-acetyl-β-D-glucosaminide; NAG) and phosphomonesterase (acid and alkaline phosphatases) can be used as indicators of C, N and P cycling in soils, however many enzymes play a role in these biogeochemistry cycles but were beyond the scope of this study. β-glucosidase (EC 3.2.1.21) is responsible for the hydrolysis of lignocellulose, the final and rate- limiting step in breaking down cellulose into glucose (Alef and Nannipieri 1995). Studies have found positive relationships between β-glucosidase and total C and microbial C across a range of soil types (Turner et al. 2002b). N-acetyl-β-D-glucosaminide (EC 3.2.1.30) is important in both the N and C cycles since it catalyzes the hydrolysis of chitin into amino sugars (Ekenler and Tabatabai 2004). Acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatases are non-specific phosphohydrolases that breakdown simple organic monoesters thereby releasing orthophosphate (Nannipieri et al. 2011). They are often categorized according to the assay pH, with acid phosphatases conducted at MUB pH 4.0 or 6.5 and alkaline phosphatases at pH 8.0, 10.0 or 12.0. Production of phosphatases by roots, nodules, bacteria and fungi have been well documented (Penheiter et al. 1997; Tarafdar and Claassen 1998; Tibbett et al. 1998b; George et al. 2006; Nannipieri et al. 2011; Fraser et al. 2017).

Since the first report of soil enzyme activity published by Woods (1899), there have been numerous caveats associated with commonly used protocols in soil enzymology research, including not optimizing conditions for individual soils and enzymes (e.g. pH, concentrations, etc.; German et al. 2011; Margenot et al. 2018; Nannipieri et al. 2018; Margenot et al. 2023). Potential enzyme activities are assessed through an assay with a specific buffer pH, maintaining a constant temperature over a designated period, and utilizing artificial substrates that may not represent the diversity of substrates in soil to measure color or fluorescent intensity (Tibbett 2002). Traditionally a benchtop colorimetric assay utilising *p*-nitrophenol was used to determine potential activity (Tabatabai 1994; Tibbett et al. 1998a, 2000). However, Pancholy and Lynd (1972) developed a method using a florigenic substrate for lipase activity. This method was later modified to include additional enzyme assays in a microplate method using methylumbelliferyl (MUF) substrates (Freeman et al. 1995), with the advantage of higher throughput and less waste products but the disadvantage of using a smaller quantity of soil. Dick et al. (2018) demonstrated a strong correlation between traditional colorimetric and fluorescence microplate methods, particularly for phosphomonoesterase and β-glucosidase ($r=0.93$ and $r=0.81$, respectively) when evaluating the same soils across five labs, resulting in similar rankings for management treatments. The study suggests that the fluorometric microplate method is an viable alternative to the bench-scale colorimetric method, provided that the pH is optimized for each soil and each enzyme used in the assays (Dick et al. 2018). Considering that different isoenzymes may be substrate specific, this is an important consideration since enzyme activity is a function of the presence of both the enzyme and the substrate.

The pH of the buffer solution in enzyme assays can significantly affect the reaction (Frankenberger and Johanson 1982; German et al. 2011), with optimum pH values varying widely for different enzymes and soil types (Turner 2010; Puissant et al. 2019). Despite established protocols emphasizing the importance of pH optimization in enzyme assays (Burns 1982; Tabatabai 1994; Parham and Deng 2000; Turner 2010; German et al. 2011; Puissant et al. 2019), some studies use standard pH values (e.g. pH 6.5 for phosphatase or pH 5.0 for high-throughput analysis of multiple enzymes (Bell et al. 2013), or fail to report assay conditions altogether. Optimizing conditions for each fluorescent or colorimetric substrate is crucial because optimum enzyme activity can occur at different pHs for different isoenzymes (Turner 2010). This consideration is especially important when comparing diverse soil samples where both the substrate availability and the enzyme activity can influence nutrient cycling.

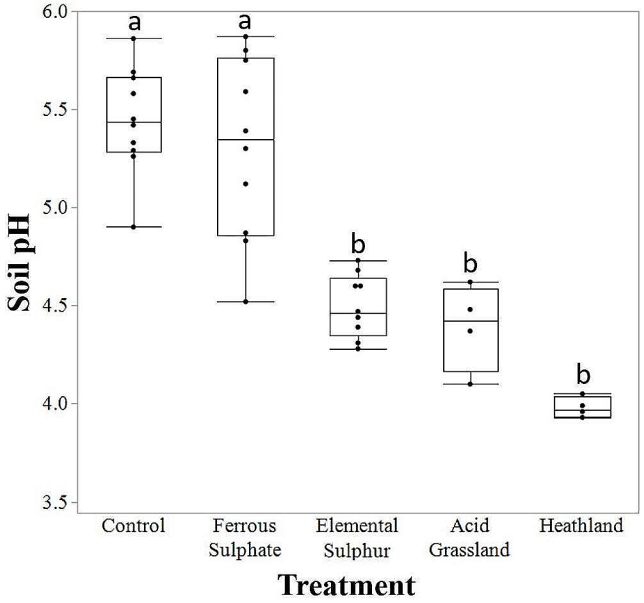

Fig. 1 Soil pH for control, ferrous sulphate and elemental sulphur treatments ($n = 10$) and reference ($n = 4$; acid grassland, heathland) plots established in 1999. Enzyme assays were conducted on three plots closest to the mean for each. Treatments with same letters represent no significant effect at $P < 0.05$ as determined by Tukey's test

The goal of the current study was to understand how potential hydrolytic enzyme activity varies across a range of soil pH and assess how the interpretation of functions for select C-, N- and P-degrading enzymes change when assay buffers are optimized for pH. Here, we focus on enzymes responsible for hydrolysis of cellulose (β-1,4-glucosidase), chitooligosaccharides (β-1,4-*N*-Acetyl-glucosaminidase), and simple phosphomonoesters (phosphatases). We hypothesized that there would be lower enzyme activity in soils with a low pH due to reduced biological functions. We also investigated if the changes in the artificially acidified soil was reflected in altered pH optimum to determine if this effect diminished overtime as the soil environment adapted. In 2017, we collected soil samples from a long-term heathland restoration experiment where acidification treatments were applied to improved agricultural land, with native heathland and acid grassland reference sites. Although the heathland restoration in this study was not successful in the acidified plots, the experiment allows a unique opportunity to study the effects of soil pH range on soil enzymes activities in a relatively small geographic area.

2 Materials and Methods

2.1 Experimental Design and Soil Sample Collection

Soil samples were collected from a heathland restoration field trial that was initiated in 1999 near Wareham, Dorset, UK (2°4'W, 50°39'N). Plots for the experimental treatments (50 m x 50 m) were arranged with 10 reps across two contiguous farms, where the area had been converted from heathland to improved grasslands in the 1950s. The predominant soil type in this area is Endogleyic Albic Carbic Podzols, with some Arenic Mollic Gleysols also present (FAO soil classification system). The experimental acidification treatments included ten replicate (50 × 50 m) plots of: (1) control with no amendments, (2) powdered ferrous sulphate (Dried Copperas™ [EA West, Grimsby, UK], $Fe^{II}SO_4$, 21% Fe, 11% S), and (3) pelletised elemental sulphur (Brimestone 90™, 90% S). Four reference plots were established on adjacent fields for both acid grassland and native heathland (50 m x 50 m). For this study, three of the ten replicates were used for each treatment that had a soil pH value closest to the mean soil pH (Fig. 1), as well as three plots from the reference heathland and acid grassland plots. For more details on the experimental setup please see Green et al. (2007).

Plant communities assessed in 2014 did not differ significantly for the control, ferrous sulphate and elemental sulphur treatments for composition of grasses (mean 60%) (Tibbett et al. 2019). Control and ferrous sulphate treatments had 25% legumes while they only comprised of 7% in the elemental sulphur, with heather and shrubs present in the elemental sulphur plots only (representing < 2%). The cover allocated to forbs was 23% in the elemental sulphur plots. The heathland reference plots were dominated by *Calluna vulgaris*, while the acid grassland plots were dominated by grasses.

Soil samples were collected in June 2017 using a gauge auger (d = 2.5 cm, 0–15 cm), with 25 cores taken following a 'W' shape across the plot and combined into one composite sample. After mixing thoroughly, a subsample was placed in a cooler and frozen within 4 h of sampling for enzyme analysis. A subsample was used to determine gravimetric water content and the remainder sieved (< 2 mm) and air-dried for chemical analysis.

2.2 Soil Chemical Analysis

Soil pH was measured in soil slurry (2.5:1 H_2O to soil ratio) after shaking for 15 min at 120 rpm (Rowell 1994). Extractable soil nutrients (P, Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, K^+, Mg^{2+}, Mn^{2+}, S) were determined using a Mehlich3 protocol (Mehlich 1984) and analysed by inductively coupled

plasma optical emission spectrometer (Perkin Elmer 7300 Dual View). Soil was ground to 0.2 mm prior to analysis of total C and N by ignition (Thermo Scientific Flash 2000 CN Analyser, Thermo Fisher Scientific, Massachusetts, USA).

2.3 Soil Enzyme Assays

Potential soil enzyme activities were analysed on frozen soil samples using a range of pH buffers, as described by Turner (2010), except that 1 mM NaN_3 was not added to the assay. The 4-methylumbelliferone (MUB) fluorescent substrates 4-MUB-β-D-glucoside, 4-MUB-N-acetyl-ß-D-glucosaminide and 4-MUB phosphate (Sigma-Aldrich, UK) were used for the β-glucosidase, NAG, and phosphatase assays, respectively. Modified universal buffer solutions were adjusted with 0.1 M HCl or 0.1 M NaOH to pH 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 9.0, 10.0, and 12.0. For each enzyme, the soil sample was split onto two 96 well plates as pH 3.0–6.0 and pH 6.5–12.0 with six technical replicates included for each buffer pH. As well as the soil blank and one sample as the substrate blank (with the substrate added after the incubation period). Each plate also included a standard curve for each soil at each pH to account for 'quenching'. Briefly, 2 g of frozen soil were brought to room temperature and homogenized in 200 mL of DI H_2O. Samples were blended for 1 min on high using a hand blender followed by 10 min on a stir plate. Homogenized soil suspensions were added to 96 well (0.4 mL) black clear bottom plates with a multichannel pipette (50 uL soil homogenate, 50 uL buffer solution, 100 uL of substrate). Final substrate concentrations in the assays were 200 µm for phosphatase and 100 µm for NAG and β-glucosidase). Plates were incubated at 37 °C with gentle shaking at 100 RPM for 1 h. Plates were removed and 50 uL of 0.5 M NaOH were added to the wells and substrate added to the soil and substrate blanks. Plates were read 1 min after addition of NaOH at 360 nm and 460 excitation on a SpectraMax Ix3 (Molecular Devices, California) using the auto-gain function since each plate contained the standard curve.

2.4 Statistical Analysis

Statistical analysis was completed using Genstat (18th Edition, VSN International LTD, UK). All variables were tested for homogeneity of variance using Bartlett's test and for normality using the Shapiro-Wilk test and transformed when required. The significance of the effect of treatment was tested with one-way analysis of variance (ANOVA). Significant differences among treatments were determined using Tukey's post-hoc test ($P < 0.05$). Pearson correlation coefficients were calculated for optimal enzyme activity and soil parameters with the significance represented by a two-sided test of correlations different from zero in Genstat.

3 Results

3.1 Soil Chemical Properties

Differences in the soil chemical properties were still apparent 17 years after the field study was initiated and acidification treatments were applied (Table 1). The elemental sulphur plots had significantly lower soil pH than the control and ferrous sulphate, while the native heathland plots were lower than all others at pH 4.0. Total C in the control plots were lower at 33.31 g C kg^{-1} compared to 46.28 g C

Table 1 Chemical properties for soil (0–15 cm) collected from control, ferrous sulphate, elemental sulphur, acid grassland and heathland plots

Treatment	Control	Ferrous sulphate	Elemental sulphur	Acid grassland	Heathland
pH_{H2O}	5.3 ± 0.11a	5.2 ± 0.15a	4.5 ± 0.04b	4.3 ± 0.06bc	4.0 ± 0.01c
	g kg^{-1}				
Total C	33.31 ± 0.24b	36.69 ± 2.62ab	35.29 ± 2.19ab	37.25 ± 1.89ab	44.48 ± 3.47a
Total N	1.75 ± 0.09a	1.84 ± 0.15a	1.59 ± 0.11a	1.64 ± 0.12a	1.44 ± 0.35a
	mg kg^{-1}				
P*	16.63 ± 1.66a	15.89 ± 0.77a	18.53 ± 3.87a	11.87 ± 3.87ab	4.92 ± 0.53b
Al^{3+}	81.01 ± 19.50bc	76.24 ± 18.32c	131.0 ± 5.43ab	109.7 ± 3.44bc	175.5 ± 5.07a
Ca^{2+}	826.5 ± 74.19a	823.9 ± 53.90a	503.8 ± 105.7b	431.7 ± 49.60b	217.7 ± 38.84b
Cu^{2+}	5.53 ± 0.42c	5.44 ± 1.30c	8.61 ± 1.52bc	39.00 ± 8.85a	26.75 ± 4.49ab
Fe^{3+}	192.9 ± 16.71ab	244.3 ± 33.76a	157.8 ± 16.71ab	269.7 ± 29.94a	112.00 ± 1.48b
K^+	25.98 ± 2.30b	34.81 ± 2.19ab	33.43 ± 6.64ab	44.27 ± 2.42a	38.21 ± 2.49ab
Mg^{2+}	74.92 ± 7.42a	80.27 ± 11.80a	45.57 ± 7.42b	63.75 ± 2.65ab	87.08 ± 11.80a
Mn^{2+}	5.03 ± 1.25a	4.93 ± 0.48a	3.22 ± 0.48ab	1.33 ± 0.15b	0.64 ± 0.11b
S	6.07 ± 0.64ab	5.97 ± 0.72ab	5.52 ± 1.24ab	8.00 ± 0.34a	4.11 ± 0.53b

Mean values ($n = 3$) are presented ± standard error. Means with same letters within a soil property represent no significant effect at $P < 0.05$ as determined by Tukey's test

*Mehlich3 extractable nutrients

kg^{-1} in the native heathland plots. There were no significant differences in soil N but the highest values were found in the ferrous sulphate (1.84 g N kg^{-1}) and control (1.75 g N kg^{-1}) plots.

Available P, as represented by Mehlich3 extraction, showed the highest concentrations in the elemental sulphur (18.53 mg P kg^{-1}), control (16.63 mg P kg^{-1}) and ferrous sulphate (15.89 mg P kg^{-1}) plots, while the heathland soils had significantly lower available P at 4.92 mg P kg^{-1} (Table 1). Mehlich3 extractable Ca^{2+} was also significantly higher in the control and ferrous sulphate treatments, corresponding with higher pH in these treatments. The Al^{3+} content was significantly higher in soil from the heathland compared to control and ferrous sulphate plots.

Despite the addition of ferrous sulphate as $Fe^{II}SO_4$, there was no significant difference in Fe^{3+} concentrations in the ferrous sulphate soil compared to the control and elemental sulphur treatment at the time of sampling (Table 1). However, the ferrous sulphate (244.3 mg kg^{-1}) and the acid grassland (269.7 mg kg^{-1}) values were significantly higher with more than double the Fe^{3+} concentration compared to the heathland (112 mg kg^{-1}). The use of ferrous sulphate and elemental sulphur as acidification agents did not lead to any significant effect on S compared to the control at 17 years following application but the acid grassland did have higher S than the heathland.

3.2 Soil Enzymes across MUB pH

The potential enzyme activities for β-glucosidase, NAG, and phosphatase varied widely for the treatments depending on the pH of the MUB used in the assays (Fig. 2). For the β-glucosidase assays, the highest activity for the elemental sulphur, acid grassland and heathland treatments was at MUB pH 3.0 or less and decreased as the pH became less acidic. However, it is possible that the optimum activity could occur in assays below pH 3.0 but we were unable to maintain a consistent pH below this value. For all others assays for β-glucosidase, NAG and phosphatase the potential activity increased above assay pH 3.0 and leveled off by pH 12.0.

The post hoc Tukey tests for the data presented in Fig. 2 showed that potential β-glucosidase activity differed significantly at MUB pH 3.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 8.0 (Table 2). However, the acid grassland and heathland soil had significantly higher β-glucosidase activity at MUB pH 3.0, followed by no difference at 4.0, and then a change to the control and ferrous sulphate treatments having significantly higher activity (Fig. 2; Table 2). Although peak enzyme activity may have occurred at MUB pH < 3.0, we could not acquire a stable analysis below these values and used the MUB pH 3.0 for the comparisons. The potential enzyme activities in the heathland plots were consistently lower than the control for β-glucosidase assays with MUB pH 4.5 to 6.5.

There were no differences among treatments for potential NAG activity for MUB pH 4.0–7.0 and 9.0–12.0 (Table 2). At MUB pH 3.0, the soil from elemental sulphur plots showed significantly lower activity than the heathland soils, despite the heathland having a lower soil pH. At MUB pH 8.0 and 9.0, NAG activity for the control and ferrous sulphate treatments were significantly higher than the elemental sulphur treatment.

As with the β-glucosidase activity, the treatment effect varied with the pH conditions for the phosphatase assay. However, we were unable to determine optimum β-glucosidase values for the elemental sulphur, acid grassland and heathlands since the highest values were at MUB pH 3.0. At MUB pH 3.0-4.5, the heathland soil showed significantly higher potential phosphatase activity than the control, ferrous sulphate and elemental sulphur treatments (Table 2; Fig. 2). At pH MUB 5.0 to 6.0 there were no differences, followed but the opposite trend where the control plots were significantly higher than soils from the heathland.

3.3 Relationship between Soil pH and Optimal Enzyme Activity

The results of statistical tests depended on whether we compared the results at (1) the standard protocol MUB pH or (2) the optimum pH based on the individual treatments that varied in soil pH from 4.0 to 5.3 (Fig. 2; Table 2). Potential β-glucosidase activity at MUB pH 5.0 was significantly higher in the control ($P = 0.01$) than the soils with pH 4.5 (elemental sulphur), 4.3 (acid grassland) and 4.0 (heathland). However, when the pH optimums were considered, the heathland and acid grassland soils were nominally higher but these differences were not significant (Table 3). All of the MUB pH optimums for β-glucosidase were below the pH 5.0 recommend in the high-throughput enzyme assay protocol, with two of the treatment optimums at pH 4.5 and the other three at 3.0 (Table 3).

For the phosphatase assays there was no difference in phosphatase activity at MUB pH 5.0 but at pH 6.5, which is often used for phosphatase assays, the control showed significantly higher activity compared to the heathland plots ($P = 0.02$; Table 2). In contrast to these results, at MUB pH 4.0 (often used for acid phosphatase assays), the heathland was significantly higher than the control, ferrous sulphate and elemental sulphur plots ($P < 0.001$). These results corresponded with the MUB pH optimum comparisons where the heathland was significantly higher at MUB pH 3.0 (86.1 μmol $MU^{-1}g^{-1}hr^{-1}$), compared to the control plot at MUB pH 6.0 (41.0 μmol $MU^{-1}g^{-1}hr^{-1}$; $P = 0.04$; Table 3). The

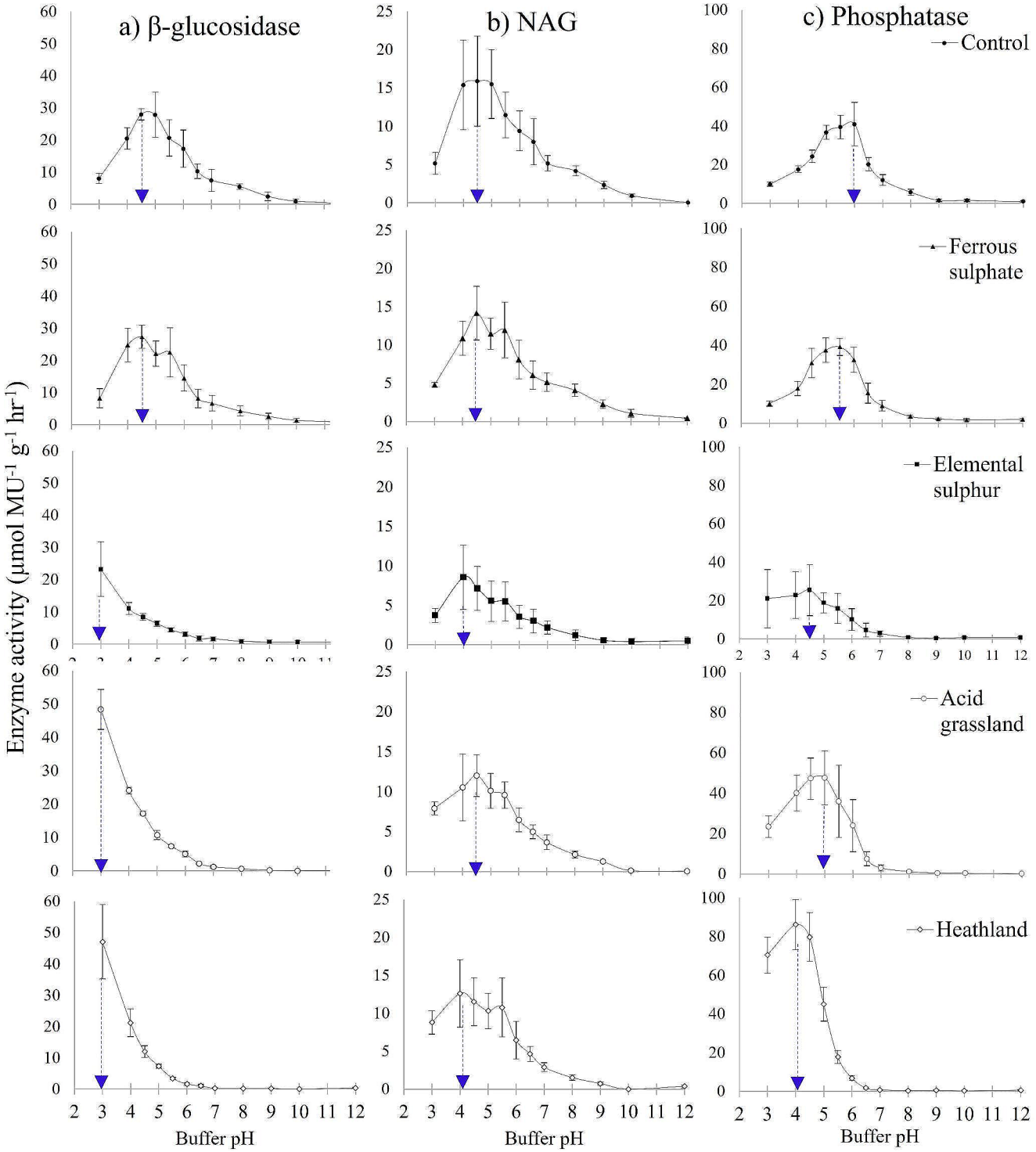

Fig. 2 Potential enzyme activity for (**a**) β-glucosidase, (**b**) N-acetyl-β-D-glucosaminidase (NAG) and (**c**) phosphatase activities at a range of MUB pH for soil samples from control, ferrous sulphate, elemental sulphur, acid grassland and heathland samples with standard error bars ($n = 3$). Blue arrows indicate buffer pH optimum

standardized MUB pH 5.0 value may be most applicable for NAG where the optimum buffer pHs were 4.0–5.0 and the statistical differences were consistent across this range (Tables 2 and 3). Optimum pH for enzyme assays varied from MUB pH 3.0-4.5 for β-glucosidase, 4.0–5.0 for NAG and 4.0–6.0 for phosphatase.

The regression analysis between the optimal enzyme activity and soil pH for each sample demonstrates a negative

Table 2 Tukey HSD test for soil enzymes at different buffer pH

Treatment	Modified universal buffer pH											
	3.0	4.0	4.5	5.0	5.5	6.0	6.5	7.0	8.0	9.0	10.0	12.0
	β-glucosidase											
Control	b	a	a	a	a	a	a	a	a	a	a	a
Ferrous sulphate	b	a	a	ab	a	a	ab	a	ab	a	a	a
Elemental sulphur	ab	a	b	c	b	b	b	a	bc	a	a	a
Acid grassland	a	a	b	bc	b	ab	b	a	bc	a	a	a
Heathland	a	a	b	c	b	b	b	a	c	a	a	a
	N-acetyl-β-D-glucosaminide											
Control	ab	a	a	a	a	a	a	a	a	a	a	a
Ferrous sulphate	ab	a	a	a	a	a	a	a	a	a	a	a
Elemental sulphur	b	a	a	a	a	a	a	a	b	b	a	a
Acid grassland	ab	a	a	a	a	a	a	a	ab	ab	a	a
Heathland	a	a	a	a	a	a	a	a	ab	ab	a	a
	Phosphatase											
Control	b	b	b	a	a	a	a	a	a	a	a	a
Ferrous sulphate	b	b	b	a	a	a	ab	a	ab	a	a	a
Elemental sulphur	b	b	b	a	a	a	ab	ab	b	a	a	a
Acid grassland	ab	ab	ab	a	a	a	ab	ab	ab	a	a	a
Heathland	a	a	a	a	a	a	b	b	b	a	a	a

The same letter within a column represent no significant difference at $P < 0.05$

Table 3 Treatments ranked in order of decreasing soil pH with corresponding enzyme activity at optimum MUB pH for β-glucosidase, N-acetyl-β-D-glucosaminide (NAG) and phosphatase for soils collected from control, ferrous sulphate, acid grassland, elemental sulphur and heathland plots

Treatment	Soil pH	β-glucosidase (μmol MU^{-1} g^{-1} hr^{-1})	MUB pH optimum	NAG (μmol MU^{-1} g^{-1} hr^{-1})	MUB pH optimum	Phosphatase (μmol MU^{-1} g^{-1} hr^{-1})	MUB pH optimum
Control	5.3	28.0 a	4.5	**15.9 a**	**4.5**	41.0 ab	6.0
Ferrous sulphate	5.2	27.3 a	4.5	14.1 a	4.5	39.2 ab	5.5
Elemental sulphur	4.5	23.2 a	3.0	8.5 a	4.0	25.4 b	4.5
Acid grassland	4.3	**48.4 a**	**3.0**	12.0 a	4.5	47.6 ab	5.0
Heathland	4.0	47.1 a	3.0	12.6 a	4.0	**86.1 a**	**4.0**

Means ($n = 3$) with same letter within a column represent no significant effect at $P < 0.05$ as determined by Tukey's test

relationship for β-glucosidase and phosphatase (Fig. 3), although these relationships were not significant ($P = 0.13$ and $P = 0.24$, respectively). The optimum NAG activity with soil pH showed an insignificant slightly positive relationship ($R^2 = 0.03$, $P = 0.58$).

3.4 Optimal Enzyme Activity and Soil Properties

All soil chemical properties except Al and Mn had a negative relationship with optimal β-glucosidase activity, although only the negative correlation with S was significant ($P = 0.05$; Table 4). The results from the NAG assays showed mostly positive correlations, where the relationship with soil available P was significant at $P = 0.03$. The relationship among phosphatase and soil properties demonstrated a negative relationship for pH, total C, total N, Cu, Fe, Mg and S, with significant correlations for Cu and S at $P = 0.04$ and $P = 0.02$, respectively.

4 Discussion

4.1 Soil Chemical Properties

The application of acidification treatments in an attempt to restore improved agricultural land to heathland has long-lasting effects on the soil chemistry. Our results demonstrate that after 17 years the pH of soil in the elemental sulphur treated plots was still lower than the control and ferrous sulphate treated plots, in association with higher Ca^{2+} and Mg^{2+} values. The pH units did increase from pH 3.0 in the upper soil profile of the elemental sulphur plots in the year following application (Green et al. 2007), compared to pH 4.5 in 2017. Soil pH reductions are widely associated with a loss of base cations. As seen in the control and elemental sulphur plots, the higher soil pH was associated with an increase in plant available P, as well as extractable Ca^{2+}. Soils with low pH can result in Al saturation (Goulding 2016) and a pH of < 5 can result in toxic levels of Al in soil solution and affect

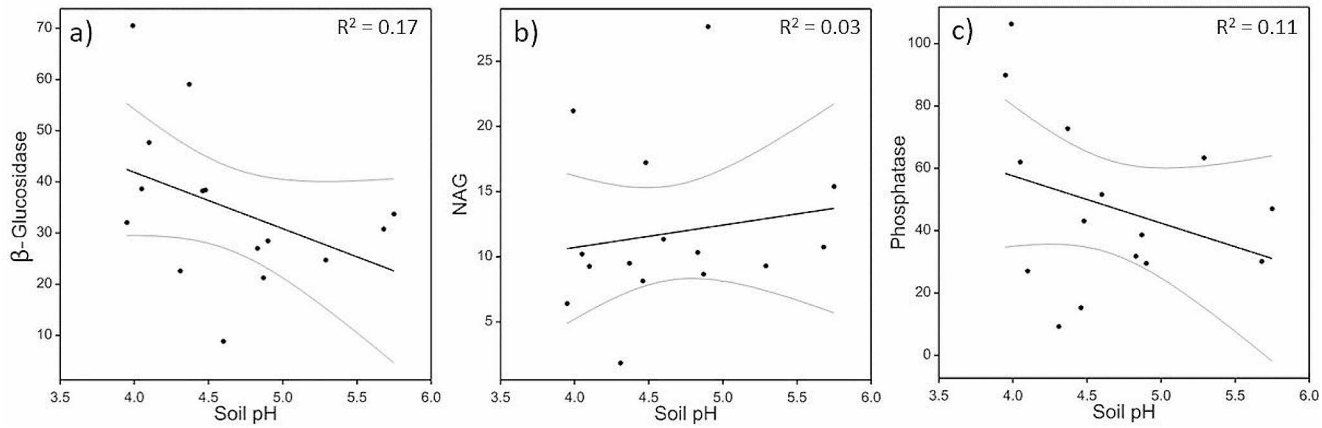

Fig. 3 Relationship between soil pH and potential enzyme activity for (**a**) β-glucosidase, (**b**) N-acetyl-β-D-glucosaminidase (NAG) and (**c**) phosphatase in soils across a pH range. Grey lines represent 95% confidence limits

Table 4 Pearson correlation coefficients for optimal enzyme activity of β-glucosidase, N-acetyl-β-D-glucosaminide (NAG), and phosphatase and soil properties

	β-glucosidase		NAG		Phosphatase	
	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value
pH_{H2O}	-0.41	0.13	0.16	0.58	-0.32	0.24
Total C	-0.34	0.22	-0.27	0.33	-0.30	0.29
Total N	-0.37	0.17	0.04	0.90	-0.28	0.31
P*	-0.04	0.90	**0.56**	**0.03**	0.09	0.75
Al	0.17	0.55	0.11	0.70	0.03	0.91
Ca	-0.12	0.68	0.03	0.91	0.10	0.73
Cu	-0.44	0.10	-0.39	0.15	**-0.54**	**0.04**
Fe	-0.15	0.60	-0.07	0.81	-0.27	0.34
K	-0.17	0.55	0.09	0.75	0.21	0.46
Mg	-0.38	0.17	-0.36	0.19	-0.11	0.70
Mn	0.06	0.84	0.16	0.57	0.22	0.42
S	**-0.52**	**0.05**	0.13	0.65	**-0.58**	**0.02**

* Mehlich3 extractable nutrients

plant and organism survival and growth (Singh et al. 2017). When comparing soils across the pH range, we report more than double the concentration of extracted Al^{3+} in heathland plots compared to the control and ferrous sulphate plots. The addition of elemental sulphur also increased the extractable Al^{3+} compared to the ferrous sulphate plots. Although we did not assess possible negative effects on the biotic communities in this study, it is possible that plants that thrive in low pH soil have evolved with mechanisms to combat toxic effects, such as root excreted chelating agents or restricting Al^{3+} uptake to the root epidermis and outer cortex (Silva et al. 2000; Kochian et al. 2004; Vardar and Ünal 2007; Singh et al. 2017).

Despite the sustained effect on soil pH, there was not a long-lasting effect of ferrous sulphate or elemental sulphur on extractable Fe^{3+} or S. It is possible that a large portion of the elemental sulphur was oxidized through chemical and biochemical processes after 17 years. Nor and Tabatabai (1977) reported that as much as 75% of elemental sulphur had been oxidized in field soils during a 70-day incubation at 30˚C. In addition, the S concentrations reported in our study are potentially bioavailable S from Mehlich3 extractions rather than total S.

4.2 Relationship between Enzyme Activity and Soil Properties

Contrary to our initial hypothesis, we observed the highest potential enzyme activity for β-glucosidase and phosphatase in the acid grassland and heathland plots, corresponding with the lowest soil pH values (4.3 and 4.0, respectively). There was a negative relationship between soil pH and β-glucosidase and phosphatase activity, but these correlations were not significant when the assays were conducted at the optimum pH. Since the soils were collected 17 years after the acidification treatments were applied, it's possible that the soil biology has adapted the modified envimilar findings were reported by Turner et al. (2002b), who found

no relationship between soil pH and β-glucosidase (buffer pH 6.0) in 29 grassland soils across England and Wales with a range of pH (4.7–6.8). In contrast, Giacometti et al. (2014) reported a significant positive correlation between β-glucosidase activity and pH, but a significant negative with phosphomonoesterases in a long-term study comparing organic compared to mineral N fertilizer applications. Brockett et al. (2012) also found a positive correlation between pH and β-glucosidase, cellobiohydrolase, beta-1,4-xylosidase, and NAG at seven forest sites across western Canada when all enzymes assays were conducted at buffer pH 5.0 (Brockett et al. 2012). In a global study encompassing 1154 data points across various ecosystems, Sinsabaugh et al. (2008) found a positive association between β-glucosidase, NAG and phosphatase with soil pH (4.1–8.7). Notably, the assays were conducted at pH 5.0 for acid soils and at pH 8.0. for the alkaline soils. It would be interesting to determine if trends remained the same when optimized for buffer pH across such a diverse set of soil samples. The mean optimum pH values reported in our study were 3.6 for β-glucosidase, 4.5 for NAG and 5.0 for phosphatase. These values are similar to those reported by Turner (2010), with optimum pH means 3.9 for β-glucosidase, 4.2 for NAG, and 4.5 for acid phosphomonoesterase for seven tropical rainforest soils. The optimum pH values are lower than the buffer pH used in the studies mentioned above.

Soil properties, environmental conditions, vegetative composition and soil biodiversity can all have an effect on potential enzyme activity. Soil pH has been demonstrated to have a strong relationship with bacterial communities (Rousk et al. 2010; Griffiths et al. 2011), with Lauber et al. (2009) suggesting that soil pH can be a predictor of bacterial community structure at continental scale. While Rousk et al. (2010) found bacterial relative abundance and diversity to be positively correlated with pH, there was only a weak relationship with fungal diversity. In another study, Rousk et al. (2009) did not see a significant correlation in bacterial or fungal PLFAs across a pH gradient but there was a shift in the fungal: bacterial PLFA ratio to be more fungal dominated at low pH. They concluded that neutral or alkaline conditions favour bacterial growth while lower soil pH favours fungal growth. Excluding values for soils below pH 4.5, the authors also found a significant correlation between respiration and soil pH, indicating and increase in microbial activity at higher pH (Rousk et al. 2009). Although we did not assess changes to the soil biological communities in this study, samples collected from the same site in 2014 and 2016 revealed changes in the soil biota in plots where acidification treatments were applied (Tibbett et al. 2019; Duddigan et al. 2020). There was a reduction in nematode and rotifer abundance and earthworm biomass in those plots, while in areas where heather was able to re-establish there was lower arbuscular mycorrhizal colonization of grasses, but an increase in the ericoid mycorrhizas where heather was present (Tibbett et al. 2019).

Despite accumulating evidence supporting the role of soil biodiversity in influencing the productivity and stability of ecosystems, establishing a direct link between diversity and function remains a challenging task. Assuming decreased diversity or a community shift at low pH, this may be associated with a diminished functional capacity of the soil. However, as demonstrated in our study, the conclusions are highly dependent on the chosen methodology. The use of molecular techniques has allowed some insight into the relationship between soil properties, microbes and potential enzyme activity. For example, Nicol et al. (2008) demonstrated distinct communities of ammonia oxidizing bacteria and archaea structure in acid and neutral conditions, with different contributions among the communities to ammonia oxidations using AmoA gene copy and transcript analysis. Puissant et al. (2019) assessed differences in bacterial and fungal communities and the pH optimum of enzymes involved in C-, N-, and P- cycling using soil from long-term study that was maintained at pH 5 or pH 7 for more than 100 years. They found a strong impact of buffer pH on enzyme activity that was specific to the individual enzyme, irrespective of the soil pH. The pH optimum did tend to shift towards the pH of the soil (i.e. 5 or 7), a trend that was also apparent in our study. Although it is not possible to verify the origin of the enzymes in typical assays, using metagenomics Puissant et al. (2019) did demonstrated a shift in bacteria harbouring β-glucosidase genes with an increase in Acidobacteria in the soil with pH 5, while the pH 7 soils demonstrated an increase in Actinobacteria abundance. Our current results suggest a potential pH adaptation of microbial communities and hence a cache of soil enzymes better suited to native and experimentally adjusted soil pH. The dominance of β-glucosidase gene harbouring Acidobacteria sequences in the soil maintained at pH 5 demonstrates the adaptation of biological communities to function at a range of soil pH. Synthesis of both NAG and phosphatases have been demonstrated to be at least partially regulated by N and P availability in soils (Wanner 1996; Fraser et al. 2015; Zhang et al. 2016; Fujita et al. 2018). For example, the phosphate (Pho) regulon present in some bacteria controls phosphatase production and is regulated by phosphate-starvation (Wanner 1996).

In addition to microbial responses to soil pH, plant communities have also evolved with adaptive mechanisms to thrive in a range of soil conditions. Heathlands are characterised by low soil pH, low available nutrients and the presence of heather species. At our site, the heathland plots were dominated by *C. valgaris* where there was low available P, which may have contributed to the increased phosphatase

activity driven by plant excreted enzymes or differences in the biological community not measured here. A recent study by Duddigan et al. (2024) at these same sites reported higher carbon stocks in the native heathland compared to the restored, which may impact both substrate availability and microbial processes. Plants in acidic environments have evolved to survive and even thrive under these conditions (Diaz et al. 2006; Kleijn et al. 2008; De Graaf et al. 2009). Increased acid phosphatase activity has been demonstrated in association with *C. vulgaris* and mycorrhizal endophytes (Pearson and Read 1975). This has also been reported in field studies where phosphomonoesterase activity (assayed at MUB pH 6.0 with pNPP as a substrate) exhibited the highest activity across the growing season in a blanket peat soil dominated by *C. vulgaris*, compared to an acid grassland soil and a calcareous grassland soil (Turner et al. 2002a). It was noted that the blanket peat phosphatase values were high in comparison to reports of other soils but they did not find a significant correlation between soil pH and phosphomonoesterase activity (Turner et al. 2002a). Besides the plant and biological communities themselves, potential enzyme activity in heathland systems may be primed by N depositions, in addition to limited available P in the system. Pilkington et al. (2005) also found that N deposition influenced N and P cycling in an upland *Calluna* moor. Through a combination of field and lab incubations, the authors demonstrated that N additions stimulated phosphomonoesterase activity both in the soil and on the root surface of *Calluna* seedlings.

5 Conclusion

Our study demonstrates how using a standardized MUB pH (e.g. 5.0 or 6.5) underestimated function as represented by potential enzyme activity in the low pH soil, thereby highlighting the importance of optimizing pH when conducting enzymes assays. The MUB pH optimum varied among the treatments, as well as the individual enzymes, where the pH optimums were 4–4.5 for NAG and 4.0–6.0 for phosphatase, and highest at 3.0–4.5 for β-glucosidase (although the optimum could not be determined). The acid grassland and heathland plots resulted in the highest potential enzyme activities for β-glucosidase and phosphatase, despite having the lowest soil pH. This highlights that ecosystems adapted to low nutrient levels with no inputs may be particularly dependent on hydrolytic and oxidative enzymes. Caution must be taken to ensure that results are not a limitation of laboratory methodology when reporting reduced functional capacities across diverse soils.

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Declarations

Conflict of interest Tandra Fraser is a guest editor for Journal of Soil Science and Plant Nutrition and the peer-review process for this article was independently handled by another member of the journal editorial board.

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