Title:

The relative importance of invertebrate and microbial decomposition in a rainforest restoration project

Running head:

Functioning of tropical soil communities

Authors and addresses:

1. Julian Donald (corresponding author), Centre for Research in Biosciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY. Email: Julian.donald@uwe.ac.uk
2. Iain Weir, Applied Statistics Group, University of the West of England, Coldharbour Lane, Bristol BS16 1QY.
3. Sam Bonnett, Centre for Research in Biosciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY
4. Pete Maxfield, Centre for Research in Biosciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY
5. M. D. Farnon Ellwood, Centre for Research in Biosciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY

Author contributions:

JD, MDFE conceived and designed the research; JD performed the experiments; JD, IW analyzed the data; PM, SB contributed reagents, materials and tools for microbial analysis; JD, MDFE wrote the manuscript. All authors contributed to manuscript revision.

Abstract

Tropical rainforests are increasingly disturbed by human activities. While restoration projects often succeed in replacing tree cover, they rarely manage to restore soil function. Consequently, there is an urgent need to understand the changes that occur during soil restoration. Model ecosystems such as the Eden Project present an ideal opportunity to investigate these changes. The Eden Project was built 15 years ago, its plants grown from seedlings, or sown directly into a soil made up of standardized mixtures of recycled organic material. Today, the Eden Project's rainforest biome consists of a diverse community of plants, invertebrates and microorganisms. Different areas within the biome are managed differently, allowing us to separate the relative contributions of decomposers under differing physical conditions. Litterbag experiments revealed significant differences in decomposition rates in bags of different mesh sizes. Phospholipid Fatty Acid (PLFA) analysis revealed that microbial biomass and community structure varied under different management regimes. Soil enzyme assays revealed that glucosidase activity increased in soils with more organic matter, whereas phenol oxidase activity increased in more alkaline soils. Our study takes a step towards understanding the interactions between invertebrates and microbes, and the way in which soils function during restoration.

Key words: soil, enzymes, microorganisms, decomposition, invertebrates, ecosystem function

Implications for practice:

- Studies of model ecosystems can inform the management of restoration projects.

- Artificial soils can support communities of invertebrates that contribute to decomposition and soil nutrient cycling.
- The use of PLFA analysis in combination with Hydrolitic and Oxidative enzyme assays can be used to confirm microbial community composition and functioning during soil restoration.
- Soil organic matter content and pH influence microbial enzyme activity. Regular additions of organic matter in the form of mulch will therefore help to maintain optimum conditions for microbial functioning during forest restoration projects.

Introduction

Tropical rainforest cover has declined sharply as a result of timber extraction and conversion to agriculture (Asner et al. 2009). Forest degradation causes a reduction in soil invertebrate diversity and a shift in associated microbial communities (Ewers et al. 2015; McGuire et al. 2015). Given the importance of invertebrates and microbes for soil functioning, this is likely to have consequences for nutrient cycling and plant viability (Nannipieri et al. 2003; Orgiazzi et al. 2016). Restoration projects will therefore benefit from an understanding of these neglected elements of ecosystem recovery. Microbial diversity and activity in particular have recently been proposed as the most sensitive biological indicators of differences in soil functionality (Muñoz-Rojas et al. 2016).

One way of understanding the effects of disturbance on these indicators is to perform microcosm experiments. However, to capture the reality of a dynamic ecosystem such as a rainforest, studies need to be performed on a larger scale. Using mesocosms (Bonnett et al. 2016) such as botanic gardens for restoration studies (Aronson 2014), or for simulating rainforest ecology (Donald et al. 2016), can prove useful for exploring trends in soil community dynamics. The Eden Project in Cornwall, UK, is a botanic garden housed in a restored china clay mine, which uses standardized artificial soils (termed technosols by Séré et al. (2008)) as the foundation for its plant collection. The Rainforest Biome, an enclosed hothouse, contains over 1400 species of tropical plants, in addition to a large range of native and alien invertebrate species that have colonized the site over the 15 years

since its construction. Whilst the crop pest species are well documented (see Treseder et al. 2011), only anecdotal evidence exists regarding the soil fauna likely to be involved in below-ground food webs. Determining the relative contributions of microorganisms (<0.1 mm), and soil mesofauna (0.1 to 2 mm) can confirm how different components of the soil biota are contributing to the process of decomposition during forest restoration. To this end, we ask the following questions: 1) Do invertebrates of different sizes play different roles in decomposition? 2) How does the composition of microbial communities change under varying soil types? 3) How does the activity of these microbial communities change under different management regimes?

Methods

Site details

The Eden Project is a unique botanic garden, opened in 2001, situated on a 105 ha site within a decommissioned china clay quarry near St Austell, Cornwall, UK (50.3601°N, 4.7447°W). The Eden Project is made up of an outdoor garden, and two large enclosed biomes (Fig. 1). Eden's rainforest biome, one of the largest greenhouses in the world, stands 50 m tall and covers an area of 15,590 m^2. Over 1400 plant species are housed within an effectively sealed environment, under the following controlled climatic conditions (mean ± SD): air temperature (21.07 ± 2.8°C) soil (20.04 ± 0.8°C), humidity (97.4 ± 3.6%).

Soil within the biomes was developed in partnership with the University of Reading, using sand recycled from the local china clay industry, composted bark, green waste from the surrounding area, and lignitic clay as a by-product of Devon's ball clay industry. This mixture provides the optimum amounts of trace nutrients, cation exchange capacity, and was able to bind the soil mixtures together (Table 1). The soils have since been managed with the addition of a compost mulch mix, composed of green waste collected onsite. Applications of mulch have varied across the biome, with a resulting range in soil organic matter content. Soil invertebrate diversity is much lower than that of a tropical rainforest, nevertheless the site does have an abundant community of mesofauna (0.1-2

92 mm) and macrofauna (>2 mm), most notably white-footed ants (*Technomyrmex albipes*), Australian
93 cockroaches (*Periplaneta australasiae*) and Suriname cockroaches (*Pycnoscelus surinamensis*)
94 (Treseder et al. 2011).

95 Soil functioning was tested at 12 points across the rainforest biome, selected in order to capture the
96 largest variation in horticultural management regimes and soil conditions across the site.

97 **Leaf litter decomposition**

98 Leaves were cut from the nitrogen-fixing mimosoid legume *Samanea saman*, a tree chosen due to its
99 presence within the biome, a relatively fast rate of decomposition, and its high nutrient value
100 (Schilling et al. 2016). Individual leaflets were stripped from their petioles and dried for 48 hours in
101 an oven at 50°C.

102 Leaf litter bags measuring 20 x 15 cm were prepared using a nylon mesh and a glue gun. Two mesh
103 sizes of 2 x 2 mm (large) and 0.8 x 0.8 mm (small) were used to include or exclude mesofauna. Each
104 bag was filled with 3 g of dried leaves, before being stapled shut.

105 At each of the 12 sites, three bags of each mesh size were placed on the ground and covered with a
106 mulch layer to simulate the leaf litter layer of a forest soil. Six bags per site across 12 sites gave 72
107 bags in total. The bags were arranged around a central stake to facilitate orientation; alternating
108 from large to small avoided microclimate effects.

109 At three intervals (3, 5, 7 months), one bag of each mesh type per site was retrieved, placed into a
110 paper bag and dried at 50^{O}C for 48 hours. Once dry, great care was taken to separate invasive roots
111 and soil from the leaf litter before reweighing it.

112 **Soil collection**

113 At each site, four replicate samples of soil were collected from the corners of a 1 m quadrat. Any
114 ground litter was removed, and using a trowel, approximately 50 cm^3 of soil was taken from

between 0-5 cm depth and transferred into 20 x 28 cm zip lock polythene bags. These were then placed in a cool box before being transferred to the laboratory, where they were stored at 4°C, and opened regularly to allow the soils to respire. Prior to analysis, the soil was homogenized by being passed through a 5mm mesh. A sub sample of approximately 10 cm^3 was taken from the same sites, and stored in glass vials at -20°C.

Soil organic matter and pH

Loss on ignition was used as a proxy for soil moisture and organic matter content (Heiri et al. 2001). 5 g of soil from each sample was placed into a crucible and transferred into an oven at 105 °C for 24 hours, weighed, and then placed into a furnace at 450 °C for a further 12 hours before being reweighed.

pH was tested on a 50 ml solution of 10 cm^3 soil dissolved into deionized water using a benchtop Jenway 3510 pH meter and electrode.

Hydrolase enzyme activity

100 µM Methylumbelliferyl-ß-D-glucopyranoside (MUF) substrate solutions were prepared for the enzyme glucosidase, along with a MUF standard (DeForest 2009). 100 µm of each soil solution (1:5 wet soil to deionised water) was pipetted out onto a 96 well plate, with three wells for each soil sample. One contained a soil blank with deionised water (250µl), one with the MUF substrate (150 µl), and one with the MUF standard (150 µl) in addition to wells containing a blank of deionised water, and reference wells for the MUF substrate and MUF standard. The reaction was left active for one hour before 50 µl of 1M sodium hydroxide was added to terminate the reaction. The plate was then transferred to a BMG Labtech Fluostar Optima Fluorometer plate reader to record levels of fluorescence. An average of three sub-samples was calculated for each sample. A single extreme outlier, likely caused by an error in fluorescence detection, was removed. The data were then converted to give glucosidase activity (µmol MUF g^{-1} $hour^{-1}$) as outlined by DeForest (2009).

Oxidative enzyme activity

0.75 ml of soil solution (1:5 wet soil to deionised water) was pipetted into two Eppendorfs for each soil sample. 0.75 ml of deionised water was added to one, whilst 0.75 ml of a 10 mM solution of L-3,4-dihydroxyphenylalanine (L-DOPA) was added to the other. These were incubated at room temperature for one hour before being centrifuged at 10000 rpm for five minutes. 300 µl of the resulting supernatant was pipetted onto a clear microplate and transferred to a BMG Labtech Fluostar Optima Fluorometer plate reader to measure the absorbance at 460nm. Phenol oxidase activity per sample was calculated by comparing the L-DOPA solution with that of the water blank. An average of three sub-samples was calculated for each sample. The data were then converted to give phenol oxidase activity (µmol dicq g^{-1} $hour^{-1}$) as outlined by DeForest (2009).

Phospholipid Fatty Acid analysis

Soils that had been frozen upon collection were then freeze-dried and ground into a fine powder. 500 mg of this powder was added to 2.8 ml of a 2:0.8 ratio of methanol:water solution in a 7 ml Precellys homogenization tube, and homogenized at 1000 rpm (2 x 10 s). Samples were transferred to pyrex centrifuge tubes with 1.35 ml of chloroform, vortexed (30 s), and sonicated (15 minutes), before centrifugation (3000 rpm for 5 minutes). The supernatant solution was transferred into a 30 ml glass vial, and the soil sample re-extracted with Bligh-Dyer solution (2 x 3 ml). The organic and aqueous phases were separated by the addition of water (1 ml) and chloroform (1 ml), and centrifuged at 3000 rpm for 3 minutes. The organic (bottom) layer was removed and the aqueous layer re-extracted with chloroform (3 x 2 ml). The sample was blown down under nitrogen and the total lipid extract (TLE) was then stored at -20°C.

The TLE was further separated using column chromatography following the method described by Dickson et al. (2009). The sample was washed through with 5 ml of a 99:1 chloroform:acetic acid

solution to separate out neutral fractions, 20 ml of acetone to separate out the glycolipids, and the remaining phospholipids were washed out using 6 ml of methanol.

An acid catalyzed derivatization method was used to prepare the phospholipid fraction for analysis. Here, a solution of hydrogen chloride in methanol (5% w/v) was created by dripping 2.5 ml of acetyl chloride slowly into 26 ml of anhydrous methanol, chilled in an ice bath to control the exothermic reaction. 1.9 ml of this solution was added to each lipid sample, along with 10 µl of a known C_{18} alkane standard. The sample was heated at 60 °C for two hours in a sealed tube. Once cool, 1 ml of water was added and the fatty acid methyl esters (FAMEs) were extracted into hexane (3 x 1 ml). Water was removed using a column of sodium sulphate, and the resulting solvent was evaporated at 40°C under nitrogen. FAMEs were re-dissolved in 30 µl of hexane. 1 µl of the resulting solution was analyzed using gas chromatography. This was performed using a Hewlett-Packard Series 5890 Series II gas chromatograph (*Agilent Technologies UK Ltd., Edinburgh, UK*) equipped with a flame ionization detector using helium carrier gas (pressure of 10 psi). The lipid concentrations were analyzed using a Varian VF23ms (*Varian BV, Middelburg, The Netherlands*) 50% cyanopropyl equivalent fused-silica column (30 m x 0.25 mm x 0.25 µm). The temperature program for fatty acid derivatives was 40°C (2 min) to 100°C at 15°C min^{-1}, to 240°C at 4°C min^{-1} (held for 20 min). Detailed chromatograms were produced for each sample and the total microbial biomass per sample was calculated relative to the standard. The peaks displayed on the resulting chromatogram were identified as either fungal or bacterial as specified by Frostegård & Bååth (1996), allowing fungal:bacterial ratios to be calculated for each soil sample.

Statistical analysis

Two-way ANCOVA was used to control for the effects of soil pH while comparing the treatment effects of mesh size and time on leaf litter weight loss. We used linear mixed-effects modelling to assess the fixed effects of soil organic matter content on glucosidase and of pH on phenol oxidase; both modelled with random intercepts for the sample sites. Visual inspection of residual plots did

not reveal any obvious deviations from homoscedasticity or normality. Models that differed in the random effects specification were compared by likelihood ratio tests. The significance of terms in the fixed-effects specification was assessed by standard linear regression conditional *F*-tests. Averages of soil moisture, organic matter content, pH, glucosidase and phenol oxidase activity were calculated for each site. Pearson's correlations of these with soil microbial biomass and fungal:bacterial ratios were assessed for significance using *t*-tests of correlation. All analyses were carried out in the R programming language and environment (R Development Core Team, 2014) with the nlme software package (Pinheiro et al. 2016) being used for the linear mixed-effects modelling.

Results

Leaf litter decomposition

The amount of leaf litter lost from the litter bags was significantly greater in the large mesh treatment at the 5-month interval (Fig. 2). Soil pH had a significant effect on leaf litter decomposition ($F_{1,65}$ = 27.29, p <0.001). Having partitioned this effect ANCOVA revealed that, at the sample mean pH of 7.34, both exclusion ($F_{1,65}$=4.71, p= 0.034) and time ($F_{2,65}$ = 37.06, p <0.001) had significant main effects on litter weight loss. There was no significant interaction between time and exclusion treatment ($F_{2,65}$=0.77, p= 0.466).

Glucosidase activity

As soil organic matter increased, so too did glucosidase activity ($F_{1,33}$ = 5.18, p = 0.030; Fig. 3). The relationship between soil glucosidase activity and soil organic matter (% loss on ignition) showed significant variation in intercepts across sites; SD = 95.59, $\chi_1^2 = 19.34$, p < 0.001. When this sample site variation was taken into account, fitted linear relationships showed that as soil organic matter increases so too does glucosidase activity (Fig. 3).

Phenol oxidase activity

As pH increased, so too did phenol oxidase activity ($F_{1,35}$ = 11.01, p = 0.002; Fig. 4). The relationship between soil phenol oxidase activity and pH showed significant variation in intercepts across sites; SD = 0.00082, $\chi_1^2 = 28.43$, $p < 0.001$. When this sample site variation was taken into account, fitted linear relationships demonstrated that phenol oxidase activity increases under more alkaline conditions (Fig. 4).

Phospholipid Fatty Acid analysis

Sites around the biome varied in their soil microbial biomass (mean = 31528.76 ng/g of soil, SD = 9319.17, n =12), and fungal: bacterial ratios (mean = 0.13, SD = 0.04, n = 12).

Microbial biomass was correlated significantly with phenol oxidase activity across the biome (r_9=0.63, p = 0.03), and fungal:bacterial ratios correlated significantly with glucosidase activity (r_9=0.91, p < 0.001), (Table 2).

Discussion

Forest restoration projects depend upon the successful restoration of their soils, and this can only be achieved by reducing the uncertainty surrounding soil functional processes. Our work has confirmed the effects of management regime on the standardized soils of the Eden Project, highlighting its use as a model for forest restoration. Specifically, we have shown that the addition of mulch results in a matrix of varying pH and organic matter content, which in turn governs microbial activity. Moreover, by using exclusion treatments, we have confirmed the roles played by invertebrates and microbes in decomposition under different management regimes.

While the species richness of the Eden Project is lower than that of a tropical rainforest, the biome does support communities of decomposers. These range from insects such as ants and cockroaches (Treseder et al. 2011) to other arthropods, and microorganisms known to be highly abundant and key to the decomposition of organic matter in natural soils (Orgiazzi et al. 2016). After five months, significantly more leaf litter had disappeared from the large-mesh bags, we suspect as a result of the

colonization and movements of the mesofauna. Indeed, the ability of soil animals to fragment organic matter and redistribute microbes throughout the leaf litter is known to be an important component of soil food webs (Soong and Nielsen 2016). However, while our results confirm the importance of the mesofauna to decomposition in the intermediate stages of our experiment, the lack of any significant differences in decomposition rates after seven months confirms the relative importance of microbe mediated decomposition.

Our results support the notion that microbial communities drive the bulk of nutrient cycling in below ground food webs (Swift et al. 1979). The amount of *Samania saman* leaves lost from our litterbags was equivalent to that of a study of decomposition using the same species in a dry forest in Costa Rica (Schilling et al. 2016). In this study, leaf litter decay rates were shown to be positively correlated with measures of fungal community structure and soil fertility. In our study, fungal:bacterial ratios and microbial biomass correlated positively with phenol oxidase and glucosidase activity respectively, demonstrating the links between soil conditions, microbial community structure and function. Furthermore, the importance of soil conditions was underpinned in our study by the significant effect of soil pH on leaf litter decomposition. pH and organic matter content also contributed to the activity of microbial extracellular enzymes, catalyzing the cycling of nutrients within the soil. These trends mirror the findings of others who have studied global gradients of pH and soil carbon (Sinsabaugh 2010; Hendriksen et al. 2016), indicating that this managed artificial soil displays the same patterns of functioning as natural soils.

Mulching intensity has resulted in changes to the microclimatic, chemical and physical properties of the Eden Project's soils, and may have had a greater effect on soil functioning than plant inputs such as leaf litter or root exudates, which are known to influence soil microbial communities (Nemergut et al. 2010). This conclusion is supported by studies citing soil organic carbon as a key indicator for tropical soil fertility (Joergensen 2010). The addition of manure to a tropical technosol in a microcosm experiment (Neina et al. 2016) has been shown to increase microbial functioning, but

such studies remain limited in their ability to reflect the complexity of soil dynamics in natural ecosystems.

Although they are intensively managed, and in spite of their artificial nature, we have shown that the soils of the Eden Project house communities of invertebrates and microbes that contribute to organic matter decomposition. Our results support the view that a wide range of ecosystem processes depend upon communities across multiple trophic levels (Soliveres et al. 2017). Whilst a more comprehensive study of a suite of enzymes is required to gain a direct indication of soil quality (Trasar-Cepeda et al. 2008), our results nonetheless provide an insight into the forces influencing microbial structure and functioning in an artificial soil. Model systems such as the Eden Project prove useful in simulating complex tropical forest dynamics, albeit under controlled conditions, which are absent from more traditional microcosm studies. To our knowledge, this study is the first to demonstrate how adaptive management of a technosol can promote increases in soil enzyme activity, and modify microbial biomass and community composition. Most importantly, our study highlights the potential of artificial soils to facilitate a functional soil community under tropical conditions.

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Table 1- Technosol mix in the Rainforest Biome at the Eden Project

	Sand	Organic Component	Lignitic Clay
Subsoil	65%	25%	10%
Topsoil	25%	65%	10%

Table 2: Correlations of Eden Project soil microbial biomass and fungal : bacterial ratio with soil physicochemical conditions and extracellular enzyme activity. Significance (d.f. = 10 in each case) is indicated as follows: n.s. = not significant (p>0.05), * *p*< 0.05, ** *p*< 0.01, * *p*< 0.001.**

	Microbial Biomass	Fungal Bacterial
Soil moisture	0.14 n.s.	-0.168 n.s.
Soil organic	0.37 n.s.	-0.024 n.s.
pH	0.452 n.s.	-0.19 n.s.
Phenol Oxidase	0.63 *	-0.296 n.s.
Glucosidase	0.09 n.s.	0.91***
Fungal bacterial	-0.181 n.s.	

Figure 1: The Eden Project Rainforest Biome from construction to completion. Photo a) courtesy of Eden Project, b, c, d) Julian Donald

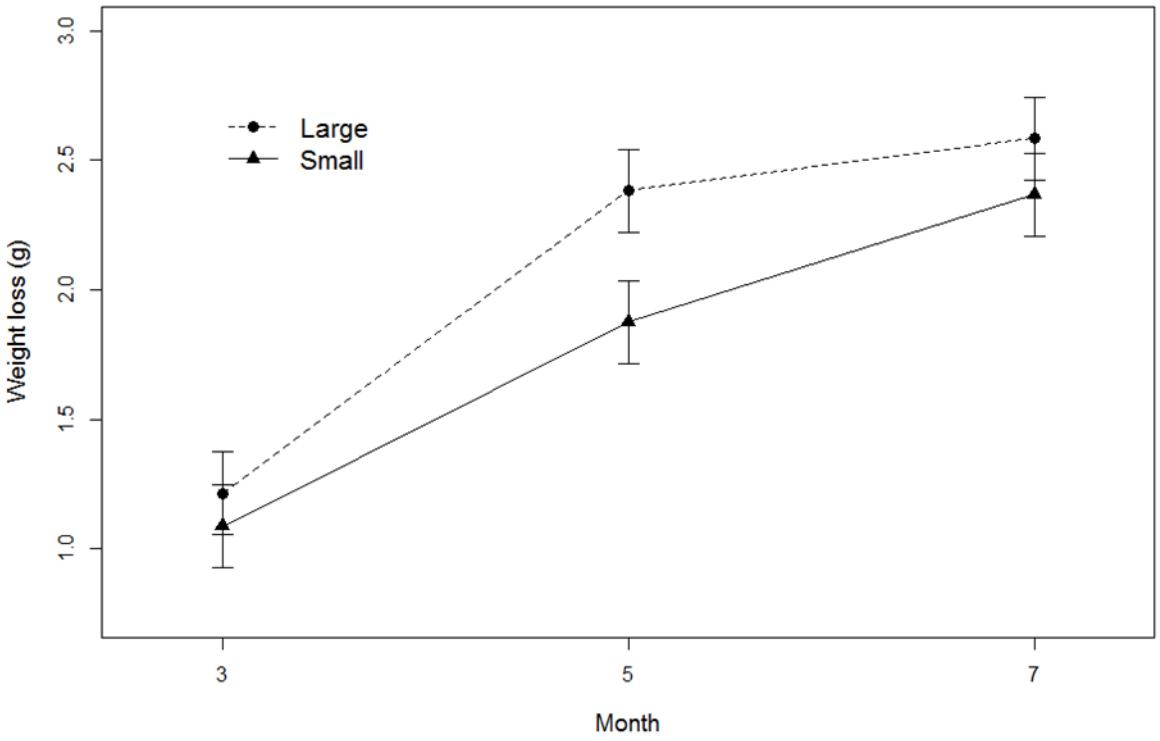

Figure 2: Mean weight loss of leaf litter in litter bags after 3, 5 and 7 months in the Rainforest Biome of the Eden Project (*n* = 12, SE ±1). The two exclusion treatments were 0.8 x 0.8 mm (small) and 2 x 2 mm (large), evaluated at the sample mean soil pH value of 7.34.

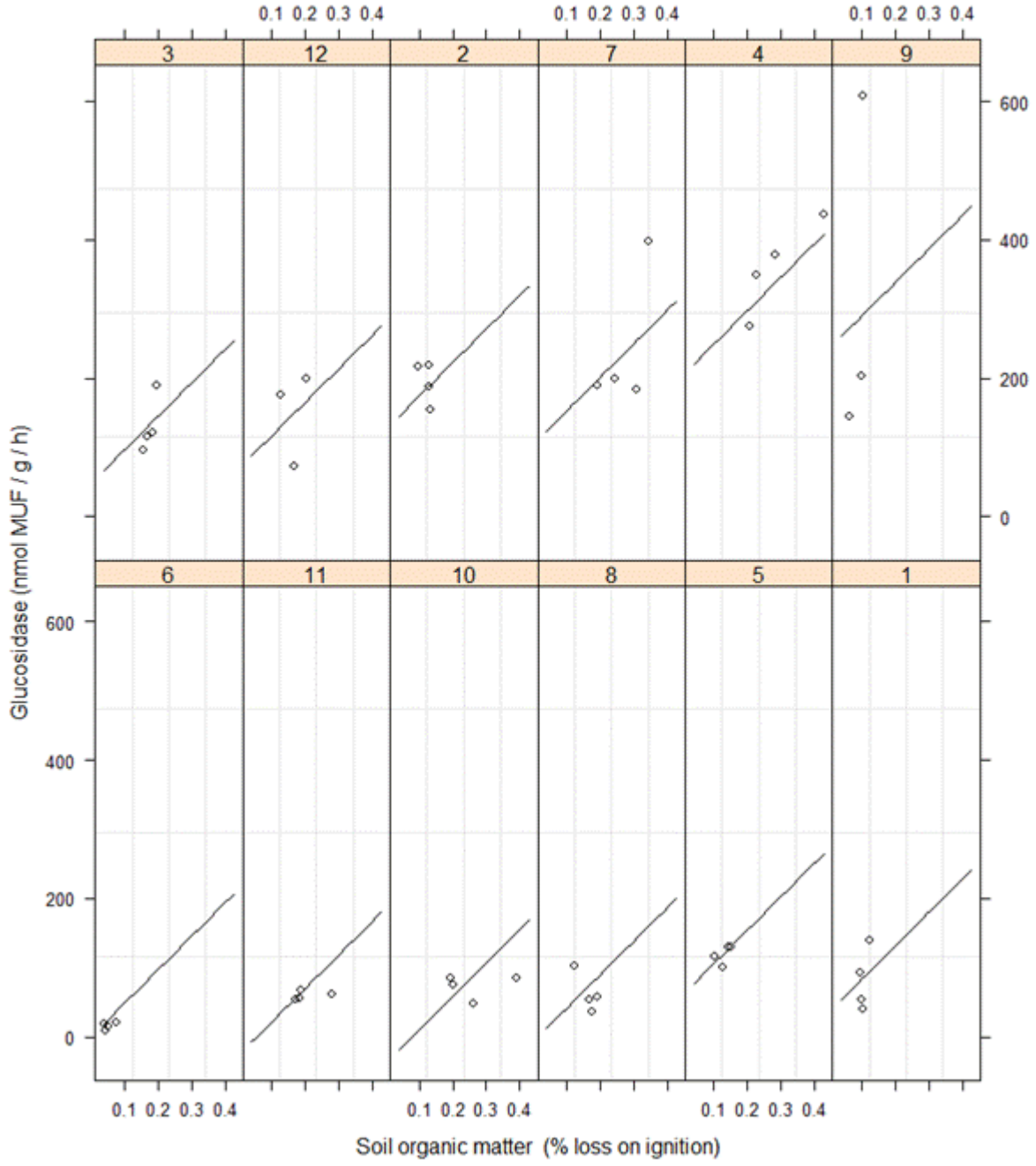

Figure 3: Glucosidase activity versus soil organic matter content for the 12 sites sampled across the Rainforest Biome. The fitted linear relationships are shown from a mixed-effects model with soil organic matter as a fixed effect and a random effect for the intercept. It can be seen that as soil organic matter increases, so too does glucosidase activity.

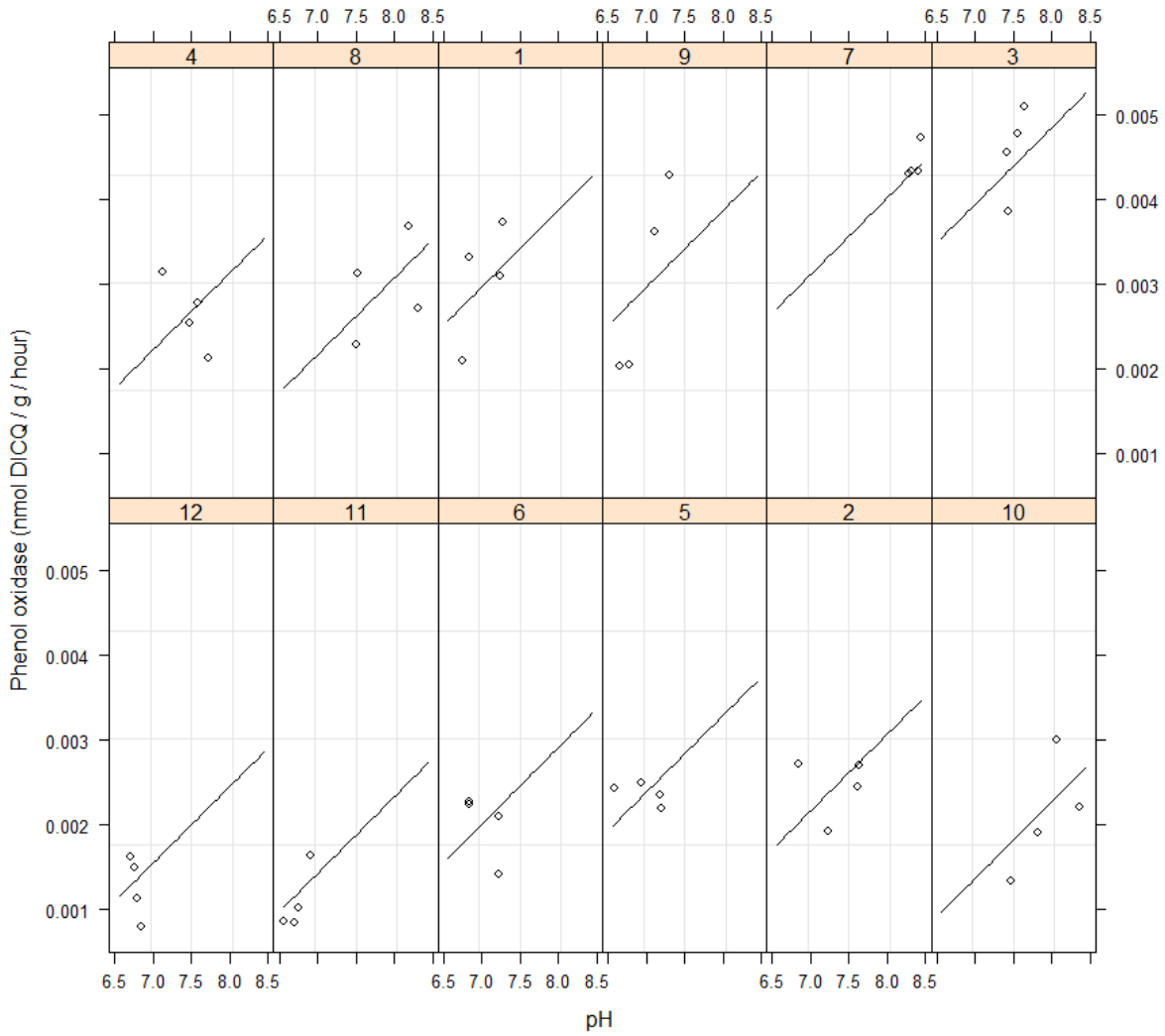

Figure 4: Phenol oxidase activity versus soil pH for the 12 sites sampled across the Rainforest Biome. The fitted linear relationships are shown from a mixed-effects model with pH as a fixed effect and a random effect for the intercept. It can be seen that as soil pH increases, so too does phenol oxidase activity.