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Continental-scale patterns of extracellular enzyme activity in the subsoil: an overlooked reservoir of microbial activity

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All authors contributed to this project by designing the cross-site study, collecting/processing samples, characterizing soils, analyzing data, or some combination thereof. E.L.A. was primarily responsible for leading this cross-site effort and for coordinating the research activities across all project personnel. Laboratory analyses were conducted by K.A., C.C., J.L.D., S.C.H., W.H.Y,

and N.C.D. Data analysis was led by N.C.D. The manuscript was written by N.C.D. and S.C.H., with critical input from all coauthors.

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ABSTRACT

Chemical stabilization of microbial-derived products such as extracellular enzymes (EE) onto mineral surfaces has gained attention as a possibly important mechanism leading to the persistence of soil organic carbon (SOC). While the controls on EE activities and their stabilization in the surface soil are reasonably well-understood, how these activities change with soil depth and possibly diverge from those at the soil surface due to distinct physical, chemical, and biotic conditions remains unclear. We assessed EE activity to a depth of 1 m (10 cm increments) in 19 soil profiles across the Critical Zone Observatory Network, which represents a wide range of climates, soil orders, and vegetation types. For all EEs, activities per mass of soil correlated positively with microbial biomass (MB) and SOC, and all three of these variables decreased logarithmically with depth ($p < 0.05$). Across all sites, over half of the potential EE activities per mass soil consistently occurred below 20 cm for all measured EEs. Activities per unit MB or SOC were substantially higher at depth (soils below 20 cm accounted for 80% of whole-profile EE activity), suggesting an accumulation of stabilized (i.e., mineral sorbed) EEs in subsoil horizons. The pronounced enzyme stabilization in subsurface horizons was corroborated by mixed-effects models that showed a significant, positive relationship between clay concentration and MB-normalized EE activities in the subsoil. Furthermore, the negative relationships between soil C, N, and P and C-, N-, and P-acquiring EEs found in the surface soil decoupled below 20 cm, which could have also been caused by EE stabilization. This finding suggests that EEs may not reflect soil nutrient availabilities deeper in the soil profile. Taken together, our results suggest that deeper soil horizons hold a significant reservoir of EEs, and that the controls of subsoil EEs differ from their surface soil counterparts.

Key words: Acid phosphatase, β-glucosidase, N-acetylglucosaminidase, critical zone, ecological stoichiometry, extracellular enzymes, microbial ecology, phospholipid fatty acids, subsoil

INTRODUCTION

Globally, soils store approximately 1,500 Pg of soil organic carbon (SOC) in the upper meter of the soil profile, with 50-67% of SOC occurring below 20 cm (Jobbágy & Jackson 2000). The persistence of this C pool is, in part, controlled by extracellular enzymes (EEs) primarily released by soil microorganisms that decompose soil organic matter (Burns *et al.* 2013). However, even though the majority of SOC occurs in the subsoil, most studies of soil microorganisms and the EEs they secrete focus on the upper soil layers (Yost & Hartemink 2020). While the age (and thus persistence) of SOC increases with depth (Trumbore *et al.* 1996; Paul *et al.* 1997; Rumpel *et al.* 2002), recent studies have shown that subsoil (>20 cm depth) C is still vulnerable to decomposition. Indeed, subsoil microbial communities have resource demands that rival those of surface soils when normalized to a microbial biomass (MB) basis (Jones *et al.* 2018). Understanding subsurface processes is critical in an age of global change because vulnerability of SOC to EE attack could be enhanced by increased temperatures or wetting/drying cycles (Schimel *et al.* 2011; Hicks Pries *et al.* 2017). Hence, if subsoils are disturbed (either physically or through altered environmental conditions), portions of the soil organic matter pool at depth could become accessible to EEs, resulting in the mineralization of significant quantities of C and nutrients. Therefore, increased understanding of EE patterns at depth could help elucidate the mechanisms of subsoil organic matter decomposition and aid in predicting how pools of SOC and nutrients will be affected by ongoing global change factors.

Because EEs both respond to and influence soil properties, the study of EEs has led to greater insights into soil C persistence (Billings & Ballantyne 2013; Birge *et al.* 2015; Dove *et al.* 2019), nitrogen (N) and phosphorus (P) mineralization (Weintraub & Schimel 2003; Waring *et al.* 2014; Chen *et al.* 2018), ecosystem development (Olander & Vitousek 2000; Selmants & Hart 2010; Turner *et al.* 2014), and microbial metabolism (Sinsabaugh & Shah 2011, 2012; Sinsabaugh *et al.* 2013). Given that the methods for measuring EE activity in soils are relatively high-throughput, inexpensive, and reproducible across laboratories (Dick *et al.* 2018), it is one of the most common soil biogeochemical measurements ("soil extracellular enzyme activity" resulted in 2,013 records using Clarivate Analytics Web of Science as of Jan. 28, 2020). However, despite the widespread measurement of soil EEs, most studies have focused on EE activities in surface horizons, with few studies exploring EE activity patterns in soil horizons below 20 cm (but see Taylor *et al.* 2002; Kramer *et al.* 2013; Stone *et al.* 2014; Taş *et al.* 2014; Schnecker *et al.* 2015; Loeppmann *et al.* 2016; Jing *et al.* 2017).

Numerous soil physical and biogeochemical properties change with depth. As organic matter (both SOC and organically bound nutrients) moves into the subsoil, it becomes increasingly more microbially processed and sorbed onto charged mineral surfaces (Rumpel & Kögel-Knabner 2010), which concomitantly increase with depth. Soil pH may also increase with depth in instances where the parent material is enriched in so-called "base" cations (i.e., calcium, magnesium, potassium, and sodium; Brubaker *et al.* 1993). These gradients in soil properties result in subsoil microbial communities that are vastly different than their surface soil counterparts (Eilers *et al.* 2012; Brewer *et al.* 2019). Soil pH (Sinsabaugh *et al.* 2008; Kivlin & Treseder 2014), substrate availability and demand (Olander & Vitousek 2000; Dove *et al.* 2019), and microbial community composition (Schnecker *et al.* 2015) influence EE activities in surface

soils. Because these factors change along soil profiles, EE activities should also change with soil depth. Two main generalizations have emerged from the few studies that have investigated EE activities in subsoils: 1) EE activities decline with depth in association with decreases in soil organic matter concentrations and decreases in microbial biomass (Taylor *et al.* 2002; Stone *et al.* 2014; Loeppmann *et al.* 2016); and 2) EE activities at depth are less responsive to surface conditions, manipulations, and management practices (Kramer *et al.* 2013; Jing *et al.* 2017; Yao *et al.* 2019). However, our ability to quantify the total EE pool and elucidate the controls on EEs in subsoils has been hindered by unstandardized ancillary measurements, assay parameters, and depths of sampling across studies (Nannipieri *et al.* 2018).

Systematic, continental- and global-scale assessments and meta-analyses of EEs in surface soils have begun to clarify controls and correlates of EE activity (Sinsabaugh *et al.* 2008, 2009; Xiao *et al.* 2018). For instance, EE stoichiometry (the ratio of C-, N-, and P-acquiring enzymes), which can represent the relative C, N, and P demand (Sinsabaugh & Shah 2012), scales at 1:1:1 (C:N:P) globally across soil, freshwater, and saltwater ecosystems, suggesting that the plasticity of microbial resource demand is somewhat constrained (Sinsabaugh *et al.* 2008, 2009). These large-scale assessments also confirm that pH, substrate availability, and microbial demand influence EE activity in surface soils (Sinsabaugh *et al.* 2008, 2009; Xiao *et al.* 2018). However, it is currently unknown if these controls in surface soils extend into the subsoil. We posit that EE activities at depth may follow different patterns than in the surface horizons given that EEs at depth are less responsive to environmental perturbations (Jing *et al.* 2017), subsoils have greater spatial heterogeneity of organic substrates than at the surface (Salomé *et al.* 2010), and the microbial communities at depth are dominated by oligotrophic microorganisms (Brewer *et al.* 2019).

To quantify EE activities and elucidate their controls throughout the soil profile, we sampled the upper meter of mineral soil at 10 cm increments in 19 soil pits across the 10 United States National Science Foundation-supported Critical Zone Observatories (CZOs; http://criticalzone.org/national/). We hypothesized that EE activities per mass of soil would decline with depth due to decreased SOC and MB concentrations; however, a significant proportion of EE activity in the top meter of soil would occur below 20 cm depth. We also hypothesized that the fundamental controls on EE activities would differ between surface and subsoil horizons due to shifting biological, chemical, and physical conditions throughout the soil profile. Specifically, as organically bound microbial resources decrease with depth, mineral sorption of both substrates and EEs will become a more dominant control of potential EE activity. Our overall goal was to quantify potential EE activity in the subsoil over a diverse set of soils, ecosystems, and climates to elucidate how EE activity mediates subsoil C and limiting nutrient availabilities.

METHODS

Site selection and sampling

Samples were collected from the network of 10 Critical Zone Observatories (CZOs, http://criticalzone.org) across the USA, which represents a wide range of hydrogeological provinces, soil orders, and vegetation types as described in Brewer *et al.* (2019). Soils were collected at peak greenness (as estimated from NASA's MODerate-resolution Imaging Spectroradiometer, or MODIS) between April 2016 and November 2016, with the exception of the Eel River CZO samples, which were collected in May 2017 (also at peak-greenness). At each CZO, we excavated two separate soil profiles ("sites") selected to represent distinct soil types

and landscape positions (Table 1). Any organic horizon was first removed, and then mineral soils were sampled in 10-cm increments with a sterile hand trowel dug into the face of each soil pit to a depth of at least 100 cm or to refusal (e.g., bedrock, hardpan, coarse regolith).

All soil samples were shipped overnight at 4 °C to the University of California, Riverside for processing. A portion of each field sample was sieved (< 2 mm), homogenized, divided into subsamples for further analyses, and frozen (−20 °C). For some soils (particularly some wet, finely textured depth intervals), sieving was impractical. These samples were homogenized and larger root and rock fragments were removed by hand. In addition, as samples from SHAL (70-100 cm depth; see Table 1 for site abbreviations) consisted almost entirely of medium-sized weathered bedrock (Cr material), soil was collected by manually crushing weathered bedrock with a ceramic mortar and pestle with this material then passed through a 2-mm sieve.

Soil physiochemical measurements

Soil pH, gravimetric water content, and clay concentration were measured using modified Long-Term Ecological Research (LTER) protocols (Robertson *et al.* 1999). Briefly, soil pH was determined in a 1:2 (weight to volume) solution using 5 g of oven-dried soil and 10 ml of Milli-Q water (Millipore Sigma, Burlington, MA, USA). The solution was measured on an Orion DUAL STAR pH meter and an epoxy combination electrode (Orion 9165BNWP Combination Sure-Flow pH Electrode; Thermo Fisher Scientific, Waltham, MA, USA). For determining gravimetric water content, approximately 7 g field-moist soil was dried at 105 ºC for a minimum of 24 h. Soil texture was measured on oven-dried and sieved soil using the hydrometer method following Gee & Bauder (2018).

Prior to soil total organic C and N analysis, soils were freeze-dried using a Savant Novalyphe-NL500 freezer dryer (Savant, Farmingdale, NY, USA) and ground to a fine powder using a roller mill. If effervescence occurred when a drop of 1 M HCl was added to a subsample of each soil sample, then inorganic C was removed from 2 g of the soil sample by twice-washing with 30 mL 0.1 N HCl (allowing the soil slurry to stand for 1 h during each wash), twice-washing with 30 mL DI, and then freeze-dried. The soil samples were analyzed for total organic C and total N by continuous-flow, direct combustion using a Vario Micro Cube elemental analyzer (Elementar, Hanau, Germany).

Microbially available orthophosphate, referred hereafter as Olsen P, was estimated by extracting 1 g of soil with 200 ml of 0.5 M $NaHCO_3$ at pH 8.5 (Olsen *et al.* 1954). This measurement includes both directly available phosphate and phosphate bound to calcium minerals that could become potentially available to microbes. Briefly, slurries were shaken for 30 min and filtered through Whatman No. 42 filters. Orthophosphate was measured colormetrically using a Lachat AE Flow Injection Auto Analyzer (Method 12-115-01-1-Q, Lachat Instruments, Inc., Milwaukee, WI, USA).

Phospholipid Fatty Acid Analysis

We used phospholipid fatty acids (PLFAs) to determine differences in the microbial biomass (MB) and the ratios of fungal to bacterial biomass. Briefly, total lipids were extracted using 10 ml of methanol, 5 ml chloroform, and 4 ml of a 50 mM phosphate buffer (pH = 7.4) from 5 g of lyophilized soil (White *et al.* 1979; DeForest *et al.* 2004). To determine analytical recovery, phospholipid 19:0 (1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine) and 21:0 (1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine) standards (Avanti Polar Lipids, Inc., Alabaster,

AL, USA) were added during the extraction phase (DeForest *et al.* 2012). Polar lipids were separated from other lipids using silicic acid solid-phase chromatography columns (500 mg 6 ml^{-1}; Thermo Scientific, Waltham, MA, USA), and the separated polar lipids were converted to fatty acid methyl esters (FAME) through methanolysis (Guckert *et al.* 1985). The resulting FAMEs were separated using a HP GC-FID (HP6890 series, Agilent Technologies, Inc. Santa Clara, CA, USA) gas chromatograph, and peaks/biomarkers were identified using the Sherlock System (v. 6.1, MIDI, Inc., Newark, DE, USA). External FAME standards (K104 FAME mix, Grace, Deerfield, IL, USA) were used to determine concentrations. The sum of all detected 14–19 C-length PLFAs was used to calculate MB because longer PLFAs can be indicators of mosses and higher plants (Zelles 1999). Ratios of fungal to bacterial biomass (fungi:bacteria) were calculated by dividing the amount (mol) of the fungal biomarker 18:2ω6c by the sum of all other microbial biomarkers (i.e., mol 18:2ω6c /(mol MB – mol 18:2ω6c)).

Extracellular enzyme activity

We measured potential EE activity (i.e., activity not limited by substrate concentrations) of α-glucosidase (AG), β-glucosidase (BG), cellobiohydrolase (CB), β-xylosidase (BX), N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), and acid phosphatase (AP) fluorometrically following Bell *et al.* (2013). Briefly, an 800 µl soil slurry consisting of 2.75 g of field-moist soil in 91 ml of 50 mM sodium acetate buffer (pH = 5.5) was incubated with 200 µl of each of the 100 µM 4-methylumbelliferone (MUB)-linked or 7-amido-4-methylcoumarin (AMC)-linked substrates (only LAP was AMC-linked) in 96-deep well plates. After a 3-h incubation at 20 °C, plates were centrifuged, and the supernatant was transferred to black, flat-bottom 96-well plates. Fluorescence was measured on a Tecan M200 Pro (Tecan Group Ltd.,

Männedorf, Switzerland) using an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

The choice of buffer pH in EE activity assays depends on the research question (Burns *et al.* 2013) and, as such, we decided to use a consistent pH of 5.5 for all soils assayed similar to other cross-site soil EE studies (DeForest 2009; Dick *et al.* 2018). The intensity of florescence of MUB is pH dependent (Mead *et al.* 1955), therefore comparisons across sites must be done at a consistent pH to avoid attributing biological phenomena to the chemistry of the florescent substrate. A buffer pH of 5.5 was chosen because this is within the range of soil pH for most of our sites (Table 1) and the range of pH optima for our enzymes (4.0-6.5, as determined in a variety of biomes; Parham & Deng 2000; Niemi & Vepsäläinen 2005; Turner 2010; Min *et al.* 2014). Nevertheless, we recognize that our buffer pH may not be indicative of the native soil pH (or pH within the microsites in which EEs operate), which may reduce our ability to quantify *in situ* EE activity.

The enzymes, AG, BG, BX, and CB are involved in the degradation of organic C, and total C-acquiring enzyme activity (C_{sum}) was operationally defined as the sum of these four enzyme activities. The enzyme, NAG is involved in releasing N-acetylglucosamine from aminopolysaccharides such as chitin and peptidoglycan, and LAP catalyzes the hydrolysis of leucine residues at the N-terminus of peptides and proteins. Both NAG and LAP are considered N-acquiring enzymes and were similarly summed to define the variable N_{sum}, which we use as a proxy for N acquisition by decomposition. Acid phosphatase is involved in releasing phosphate from monoester bonds, representing a P-mineralizing enzyme (Burns *et al.* 2013). This suite of EEs, while not inclusive of all relevant enzymatic substrates, represents many of the most frequent hydrolytic reactions during decomposition of organic matter (Sinsabaugh & Shah 2012).

Furthermore, these EEs have been extensively studied across numerous surface soils (Sinsabaugh *et al.* 2008, 2009) to which we can compare with our deep-soil measurements.

Extracellular enzyme activities were expressed per soil mass (mmol EE activity kg^{-1} soil h^{-1}), SOC (mmol EE activity kg^{-1} SOC h^{-1}), and MB (mmol EE activity kg^{-1} MB h^{-1}). These latter two variables are called SOC-normalized and MB-normalized, respectively in this paper. We also measured the ratio of C-, N-, and P-acquiring enzymes. Because EEs mediate nutrient acquisition for soil microorganisms, they can be used to determine relative nutrient demand (Olander & Vitousek 2000; Sinsabaugh & Shah 2012). Hence, we used C_{sum}:N_{sum}, C_{sum}:AP, and N_{sum}:AP as proxies for C:N, C:P, and N:P relative demand ratios, respectively.

Statistical analysis

All statistical tests and visualizations were conducted in R (R Development Core Team 2008) using the lme4 (Bates *et al.* 2015) and MuMin (Barton 2018) packages. We used mixed-effects models with site as a random effect to examine the relationship between depth, SOC, MB, clay, and fungi:bacteria and EE activity (expressed on soil mass, SOC, and MB bases). We similarly used mixed-effects models with site as a random effect to examine the effect of soil stoichiometry (using ratios of SOC, total N, and available P) on enzyme stoichiometry. These models were conducted on the complete dataset, the surface soil dataset (depth ≤ 20 cm), and the subsoil dataset (depth > 20 cm) to determine differences in the controls of EE activities between the surface and subsoils. Because we did not characterize the horizonation of the sampling pits, we *a priori* chose 20 cm to represent the subsoil because most EE studies do not sample below this depth. However, we also conducted our analysis using a 30 cm threshold, and the overall interpretation remained unchanged (see Appendix 1). Therefore, for clarity, we report results

using only the 20 cm threshold for the subsoil. To denote the variance explained by the models, we report the marginal R^2 value, which expresses the increase in explained variance by including the fixed effect(s) (Nakagawa & Schielzeth 2013). We also used ANOVA and Pearson's correlation to determine if the fraction of EE activity below 20 cm differed by soil order and if the aggregate surface and subsoil EE activities were correlated, respectively. We assessed significance at the $\alpha = 0.05$ level and marginal significance at $\alpha = 0.10$. If significant differences were detected, we used Tukey's Test of Honest Significant Differences to determine which soil orders were significantly different.

We used QQ-plots and scale-location plots to inspect normality and homoscedasticity, respectively. Because many of the mixed-effects models failed to meet parametric assumptions, all dependent and independent continuous variables were natural log-transformed and re-analyzed. The resulting models, along with the ANOVAs, met the assumptions of parametric tests. For visualization purposes, data are left untransformed unless otherwise stated.

RESULTS

Whole profile soil properties among sites

Soil organic C, total N, available P, and fungi:bacteria decreased while clay percentage increased with depth across the CZO network (all: $p < 0.001$, Fig. S1A-E). Across all sites, soil pH increased slightly with depth (on average 0.1 pH units over 1 m, $p = 0.028$, Fig. S1F).

Distribution of extracellular enzyme activity is related to microbial biomass and organic carbon throughout the top meter of soil

For all assayed EEs, EE activity per mass of soil declined logarithmically with depth ($p < 0.001$, Fig. 1 and S2), with the strongest decline for NAG ($\beta = -0.223$) and the weakest for AG ($\beta = -0.109$). However, about 50% of the total-profile EE activity kg^{-1} soil in the top meter occurred below 20 cm (Fig. 2A). The proportion of the EE activity below 20 cm differed by the soil order for many of the assayed EEs (Table S1 and S2). Mollisols had about a 1.5 times greater percentage of the sum of C- and N-acquiring EE activity kg^{-1} soil below 20 cm than Inceptisols or Ultisols ($p < 0.05$ for all comparisons, Fig. 2B). For AP, the 39% higher proportion in the subsoil for Mollisols compared to Inceptisols was only marginally significant ($p = 0.063$). Neither mean annual temperature (MAT) nor precipitation (MAP) significantly correlated with the proportion of EE activity below 20 cm ($p > 0.05$, Fig. S3 and S4).

There were also differences in the percentage of MB and SOC in the subsoil among soil orders (MB: $p < 0.001$, SOC: $p = 0.013$), with Mollisols having an almost two times greater proportion of MB and SOC below 20 cm than Inceptisols (MB: $p = 0.006$, SOC: $p = 0.013$; Fig. 2B). While the proportion of MB below 20 cm was significantly higher in Mollisols compared to Ultisols (about 1.5 times greater, $p = 0.001$), the difference in the proportion of SOC below 20 cm between Mollisols and Ultisols was only marginally significant ($p = 0.057$).

Microbial biomass-normalized EE activity increased with depth for all enzymes (Fig. S5; all: $p < 0.05$). The strongest increases were for LAP and AP, which increased six- and seven-fold, respectively, from the 0-10 cm to the 90-100 cm depth, while NAG and BG increased by 85% and 103%, respectively. Throughout the top meter, over 80% of MB-normalized EE activity occurred below 20 cm (Fig. S6A). However, because the proportion of MB below 20 cm also varied among soil orders, the proportion of MB-normalized EE activity below 20 cm was consistent among soil orders for most assayed EEs (AG: $p = 0.333$, BG: $p = 0.175$, CB: $p =$

0.278, BX: p = 0.211, NAG: p = 0.027, LAP: p = 0.537, AP: p = 0.048; Fig. S6A). Nevertheless, the proportion of MB-normalized NAG activity below 20 cm was 15% greater in Ultisols compared to Inceptisols (p = 0.025), and the proportion of MB-normalized AP activity below 20 cm was 17% greater in Ultisols compared to Mollisols (p = 0.042).

There were inconsistent patterns of EE activity normalized by SOC with depth. N-acetylglucosaminidase normalized by SOC decreased with depth (p = 0.004); AG, LAP, and AP increased with depth (AG: p = 0.016, LAP: p = 0.002, AP: p < 0.001); and BG, CB, and BX did not change with depth (BG: p = 0.322, CB: p = 0.344, BX: p = 0.198; Fig. S7). Similar to the proportion of MB-normalized EE activity below 20 cm, the proportion of EE activity normalized by SOC below 20 cm averaged about 80% and did not differ among soil orders (all: p > 0.1; Fig. S8).

With a few exceptions, aggregate EE activity (per mass of soil, MB, and SOC) below 20 cm correlated with the aggregated activity in the upper 20 cm (Table S3). On average, these correlations were strongest for SOC-normalized EE activities and weakest for MB-normalized EE activities. As such, aggregate surface soil AG, CB, and BX activity normalized by MB was not correlated with respective aggregate activities in the subsoil (p > 0.05).

Controls on extracellular enzyme activity throughout the top meter of soil

Consistently, MB, SOC, and fungi:bacteria were better predictors of EE activities per mass of soil than pH or clay concentrations (Table S4). This was generally consistent among surface soil- and subsoil-only datasets except for fungi:bacteria, which was only a strong predictor in the surface soil (Table S5).

Normalized by MB, soil pH was generally not a significant predictor of the assayed EE activities (Table S6). This pattern was mostly consistent among surface soil- and subsoil-only datasets, with the exception of surface soil CB ($p = 0.023$) and subsoil LAP ($p = 0.042$, Table 2). In contrast, normalized by SOC, soil pH had a variable effect on EE activities. In the surface soil, pH was positively correlated with BG ($p = 0.001$), CB ($p = 0.002$), BX ($p = 0.042$), and LAP ($p = 0.004$, Table 2). However, in the subsoil, pH was negatively correlated with CB ($p = 0.025$) and AP ($p < 0.001$), and positively correlated with LAP ($p < 0.001$, Table 2).

When EE activities were normalized per unit MB, clay concentrations and fungi:bacteria were generally correlated positively with EE activities (Table S6). When surface and subsoil EE data were analyzed separately, the effect of clay concentrations and fungi:bacteria on MB-normalized EE activities was more often significant in the subsoil (Table 2).

Relating soil and extracellular enzyme stoichiometries throughout the top meter of soil

When considering soils from all depth increments, only $soil_{C:N}$ and $EE_{C:N}$ were correlated (C:N: $p = 0.013$, C:P: $p = 0.292$, N:P: $p = 0.276$), but this negative correlation between $soil_{C:N}$ and $EE_{C:N}$ was relatively weak (marginal $R^2 = 0.038$; Fig. S9). However, using the surface soil-only dataset, all soil and EE stoichiometries were negatively correlated (C:N: $p = 0.003$, marginal $R^2 = 0.268$; C:P: $p = 0.002$, marginal $R^2 = 0.193$; N:P: $p = 0.004$, marginal $R^2 = 0.260$; Figure 3). In the subsoil, these correlations decoupled such that none of the stoichiometries were significantly correlated (C:N: $p = 0.288$, C:P: $p = 0.358$, N:P: $p = 0.282$; Fig. 3). Split amongst 10 cm increment sample depths, negative correlations between soil and enzyme stoichiometry were generally significant ($p < 0.05$) only in the upper soil layers (Fig. S10).

DISCUSSION

Our continental-scale sampling efforts show that microbial activity at depth is non-negligible, and the relative proportion of EE activity (kg^{-1} soil) at depth depends predominately on soil development (i.e., soil order; Fig. 2B). Although replication of each soil order was relatively small (n = 4 - 5), this finding was strikingly consistent despite large gradients in MAT and MAP for each soil order (e.g., MAP spanned an order of magnitude for Inceptisols; Table 1). Our analysis shows that climate is an unlikely driver of the relative vertical distribution of EE activity. Instead, this phenomenon is likely due to changes in the vertical distribution of substrate (organic C) and MB among these soil orders (Batjes 1996; Fig. 2B), which strongly correlate with EE activity (Sinsabaugh *et al.* 2008; Table S4). Hence, we show that SOC and MB are the strongest controls of EE activities throughout the soil profile.

We hypothesize that increases in the MB-normalized EE activities at depth suggest an accumulation of EEs chemically stabilized on mineral and organic surfaces. While MB-normalized EE activity is often related to the relative activity of the microbial community or differences in metabolic strategies among microbial taxa (Boerner *et al.* 2005), we alternatively hypothesize that the increase in MB-normalized EE activity is due to EE stabilization, namely the sorption of the EEs onto clay or organic matter particles that impedes EE degradation (Sarkar *et al.* 1989, Burns *et al.* 2013). Because EE activities are often measured in a salt-buffered soil slurry that disrupts the stabilization of EEs (as is the case in our study), EE activity assays generally measure both active and stabilized EEs (Burns *et al.* 2013). We hypothesize that higher subsoil MB-normalized EE activities is primarily a product of EE stabilization instead of differences in the metabolic capabilities of the microbial community for three reasons. First, MB-normalized respiration (i.e., microbial metabolic quotient), which is another measure of the

relative activity of the microbial community, generally does not increase with depth (Dominy & Haynes 2002; Fang & Moncrieff 2005; but see Lavahun *et al.* 1996). Secondly, the relative abundance of fungi, which produce more EEs per unit MB than bacteria (Romaní *et al.* 2006), decreased with depth. Finally, the decoupling of soil stoichiometry and EE stoichiometry at depth suggests that EE activities are not responsive to altered nutrient availabilities. Taken together, these results suggest that the physiochemical process of EE stabilization, a largely abiotic process, is the major control of EE activity in the subsoil.

Extracellular enzyme stabilization as a major mechanism in the subsoil is corroborated by our finding that the influence of clay concentration on MB-normalized EE activity is higher in the subsoil than the surface soil (Table 2). Furthermore, we may have underestimated EE activity in high clay soils because clay can increase the pH optima of EEs 1 - 2 pH units (McLaren & Estermann 1957; Ramírez-Martínez & McLaren 1966). Whereas many EEs have native pH optima between 4.0 - 6.5 (Parham & Deng 2000; Niemi & Vepsäläinen 2005; Turner 2010; Min *et al.* 2014), an increase of two pH units would be significantly higher than the pH of our assay buffer (pH = 5.5). Therefore, we conclude that EE stabilization is a major process when microbial activity is relatively low and clay concentrations are relatively high, which is often the case in subsurface soil layers.

Extracellular enzyme stabilization may be partially responsible for the muted treatment effects on subsoil EE activity commonly found throughout the literature (e.g., Kramer *et al.* 2013; Jing *et al.* 2017; Yao *et al.* 2019). When the stabilized EE pool is significantly greater than the active EE pool, the ability to detect changes in the active pool is decreased. For example, if we assume that there is negligible EE stabilization in the surface soil and that the actualized MB-normalized EE activity *in situ* is constant throughout the soil profile, our results show that at

least 29-71% of the assayed MB-normalized EE activity at depth can be attributed to stabilized EEs across our study sites, depending on the EE (Equation 1).

(1) $$Z = ((Y - X)/Y) * 100$$

X = Average MB-normalized EE activity in surface soil

Y = Average MB-normalized EE activity in subsoil

Z = Percent MB-normalized EE activity in subsoil attributed to stabilized EEs

This equation calculates the difference between MB-normalized EE activity in surface and subsoil as a percentage of the MB-normalized EE activity in the subsoil and, adhering to the aforementioned assumptions, represents the percentage of MB-normalized EE activity in the subsoil attributed to EE stabilization. This calculation likely represents the lower bound of the estimated stabilized MB-normalized EE activity because any stabilization in the surface soil (X), would increase Z, and the relative proportion of fungal biomass, which release comparatively more EEs than bacteria per unit MB (Romaní *et al.* 2006), decreased with depth. Nevertheless, this implies that if the stabilized EE pool is resistant to treatment effects in experiments (e.g., Kramer *et al.* 2013; Jing *et al.* 2017; Yao *et al.* 2019), the ability to detect significant changes in microbial activity at depth using EE assays is also reduced by at least 29-71%. In instances where the magnitude of the treatment effect is modest, it is unlikely that a significant change in subsoil EE activity will be detected. However, this should not necessarily be interpreted as a lack of microbial response, and caution should be exercised in interpreting the effect of a surface manipulation or treatment on subsoil EE activity.

The discrepancy between soil and EE stoichiometry at depth may also be caused by the increased discontinuity of substrates in the subsoil and the reduced ability of the microbial community to respond to changes in resource availability (Allison *et al.* 2007). This would prevent subsoil microorganisms altering their EE stoichiometry to different nutrient conditions. Resource availability is typically higher in surface soils than in subsoils (Salomé *et al.* 2010). Recent work in soil enzymography show that C-degrading EE activities are enriched only 0.5-2.0 mm from C-rich rhizodeposits (Ma *et al.* 2018). The EE assays that we and most other researchers use disrupt the spatial arrangement of EEs and substrates such that our results express bulk EE activities and bulk resource concentrations, which may not be representative of more localized heterogeneity in resources.

Our finding that aggregated surface soil EE activity (normalized by mass of soil, MB, or SOC) generally correlates with aggregated activity in the subsoil suggests that it may be possible to extrapolate EE measurements at the surface into deeper layers. Interestingly, MB and SOC, which we demonstrate correlate with EE activity (per mass of soil), did not follow these same patterns. It is possible that high concentrations of EEs in the surface soils percolated into the subsoil. However, correlation of surface and deeper EE activities, instead, could be due to similarities in microbial community composition throughout the soil profile. Indeed, microbial community composition assessed by 16S rRNA gene sequencing of these same soils showed a stronger effect of soil location than soil depth (Brewer *et al.* 2019). This finding provides further evidence of the linkages between microbial community composition and metabolic strategies in soils (e.g., Schnecker *et al.* 2015) and demonstrates association between surface soils and subsoils.

Discrepancies in the effect of soil pH between MB-normalized and SOC-normalized EE activities likely reflects the impact of soil pH on SOC stabilization and how well our bulk MB and SOC measurements correlate with microbial-available SOC. In numerous ecosystems, low soil pH is associated with greater SOC stabilization due to an increased charge of clay minerals and Al- and Fe-oxyhydroxides resulting in an increase of their sorption capacity (Rasmussen *et al.* 2018). Therefore, increasing pH (and decreasing SOC stabilization) likely reflects greater available SOC as a fraction of total SOC, which would result in higher SOC-normalized EE activities. Soil pH was not a significant mediator of MB-normalized activities possibly because our MB measurements better reflect the available SOC pool, given that microbial growth is generally substrate-limited (Jones *et al.* 2018). These results highlight the interactions between SOC, MB, and soil stabilization of microbial substrates and products and provide further evidence for the strong effect of soil stabilization in regulating EE activities throughout the soil profile.

Taken together, our results suggest that the relative importance of the different controls on EE activities change with depth. We summarize this in a conceptual model, where the active EE pool is controlled by microbial EE production (proximately influenced by MB and resource demand), and the stabilized EE pool is primarily influenced by EE stabilization onto clay particles (Figure 4). Because MB and resource demand decrease with depth as C becomes more limiting and clay concentrations increase, the subsoil total EE pool is maintained because of the relatively large proportion of stabilized (sorbed on soil colloids) EEs that decay slower than unstabilized (present in the bulk soil solution) EEs. Understanding how soil texture affects EE stabilization and decay dynamics is a critical knowledge gap in enzyme-explicit microbial models (e.g., Schimel & Weintraub 2003; Manzoni *et al.* 2016; Abramoff *et al.* 2017; Sulman *et*

al. 2018). For instance, Schimel *et al.* (2017) estimated EE decay dynamics in multiple soils by measuring EE activities for weeks after sterilization. While these soils varied in texture, there did not appear to be a consistent pattern between soil texture and EE decay, possibly because of changes in other edaphic factors (i.e., moisture, substrate, etc.). Future work should systematically study EE decay and its relation to multiple edaphic factors including clay concentration to test our proposed conceptual model.

Overall, the suite of EEs studied here exhibit similar patterns with depth across a wide range of sites and represent a diverse set of biochemical reactions. Hence, we posit that these patterns are robust and may be applicable to other EEs released by soil microorganisms. Our findings imply that the vast majority of EE studies are missing a large portion of the total EE activity in soils, and that the unmeasured subsoil EE activity varies in its response to environmental conditions. Nevertheless, if undisturbed, extrapolating surface soil EE values into the subsoil may be appropriate. As numerous other experiments have shown (Blume *et al.* 2002; Taş *et al.* 2014), ignoring subsoils, and exclusively focusing on surface soils, can limit our ability to understand whole-profile EE-dynamics and soil C storage.

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TABLES

Table 1: Characteristics of the 19 study sites across ten Critical Zone Observatories (CZOs).

Site	CZO	Latitude	Longitude	$pH_{1:2(water)}$[1]	Elevation (m)	MAP (mm)	MAT (°C)	Parent Material	Soil Order	Vegetation
AGRI	Christina	39.8622	-75.7834	4.55 – 5.07	105	1145	12	fluvium	Inceptisol	Forest
BSLT	Reynolds Creek	43.1171	-116.7258	6.09 – 6.64	1917	479	6.3	basalt	Mollisol	Shrubland
CTNA	Catalina-Jemez	32.4293	-110.7610	4.72 – 5.31	2100	840	12	granite	Entisol	Forest
FLUD	Christina	39.8625	-75.7830	4.71 – 5.11	113	1145	12	quartzite	Ultisol	Forest
GARN	Shale Hills	40.6949	-77.9199	3.24 – 4.29	554	1050	9.5	sandstone	Inceptisol	Forest
GOOS	IML	40.4374	-88.5552	7.08 – 7.51	250	1000	11	fluvium	Mollisol	Cropland
GRNT	Reynolds Creek	43.1927	-116.8105	5.65 – 7.55	1565	616	7	granite	Mollisol	Shrubland
HARD	Calhoun	34.6064	-81.7234	4.91 – 5.34	183	1250	16	gneiss	Ultisol	Forest
ICAC	Luquillo	18.2814	-65.7909	4.05 – 4.34	690	5000	19	quartzite	Inceptisol	Forest
LVRD	Luquillo	18.3237	-65.8185	4.27 – 4.77	343	3456	23	volcaniclastic	Oxisol	Forest
MDRN	Eel River	39.7294	-123.6419	--	487	1500	12	sandstone	Alfisol	Forest
MEAD	Boulder Creek	40.0210	-105.4796	5.54 – 5.68	2642	519	5.1	gneiss	Mollisol	Grassland
NSLP	Boulder Creek	40.0125	-105.4690	4.29 – 5.06	2521	519	5.1	gneiss	Inceptisol	Forest
PINE	Calhoun	34.6074	-81.7228	4.88 – 5.72	184	1250	16	gneiss	Ultisol	Forest
PRAR	IML	40.4275	-88.6032	5.88 – 7.88	250	1000	11	loess	Mollisol	Cropland
PROV	Southern Sierra	37.0675	-119.1950	4.77 – 5.28	2016	1200	8	granite	Inceptisol	Shrubland
SCST	Catalina-Jemez	32.4263	-110.7612	5.49 – 6.11	2100	840	12	schist	Entisol	Forest
SHAL	Shale Hills	40.6640	-77.9064	4.19 – 4.78	282	1050	9.5	shale	Ultisol	Forest
SJER	Southern Sierra	37.1088	-119.7314	--	405	513	16.4	granite	Alfisol	Grassland

[1]pH was not measured on MDRN and SJER soils because of limited soil collected.

Table 2: Marginal R^2 values for mixed-effects models with soil clay concentration, pH, or fungi:bacteria ratio as the sole fixed effect, and soil pit as a random effect on extracellular enzyme (EE) activity normalized by microbial biomass (MB) or soil organic carbon (SOC) concentration in surface- (≤ 20 cm) and sub-soils (> 20 cm) across all sites. Key: α-glucosidase (AG), β-glucosidase (BG), cellobiohydrolase (CB), β-xylosidase (BX), N-acetylglucosamine (NAG), leucine aminopeptidase (LAP), and acid phosphatase (AP) activity. Bolded values represent a significant (α = 0.05) effect and +/- signifies the direction of the effect (surface soil: n = 29, subsoil: n = 114).

	MB-normalized[1]			SOC-normalized[2]		
EE	Clay	pH	fungi:bacteria	Clay	pH	fungi:bacteria
Surface soil						
AG	< 0.001	0.015	0.016	0.002	0.023	0.016
BG	< 0.001	0.135	0.013	0.001	**+0.366**	0.008
CB	0.003	**+ 0.119**	0.052	0.002	**+0.301**	0.002
BX	0.004	0.076	0.011	< 0.001	**+0.161**	**-0.095**
NAG	0.067	< 0.001	**+0.298**	0.011	0.122	**+0.146**
LAP	0.004	0.024	0.007	**+0.020**	**+0.142**	0.039
AP	0.004	0.024	< 0.001	**+0.102**	0.009	**-0.226**
Subsoil						
AG	**+0.097**	0.013	0.019	0.019	0.073	0.005
BG	0.063	0.017	**+0.171**	0.001	0.001	0.014
CB	0.043	0.007	**+0.037**	< 0.001	**-0.106**	0.001
BX	**+0.094**	0.002	**+0.146**	0.002	0.035	0.001
NAG	0.020	0.005	**+0.080**	0.032	0.075	0.006
LAP	0.001	**+0.088**	0.001	0.009	**+0.086**	< 0.001
AP	**+0.142**	0.081	0.002	0.210	**-0.455**	0.002

[1] Enzyme activity per unit microbial biomass
[2] Enzyme activity per unit soil organic carbon

FIGURES

Figure 1: Distribution of activity of α-glucosidase (AG), β-glucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CB), N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), and acid phosphatase (AP) per soil mass as a function of depth throughout the top meter of soil across sites. See Table 1 for site abbreviations.

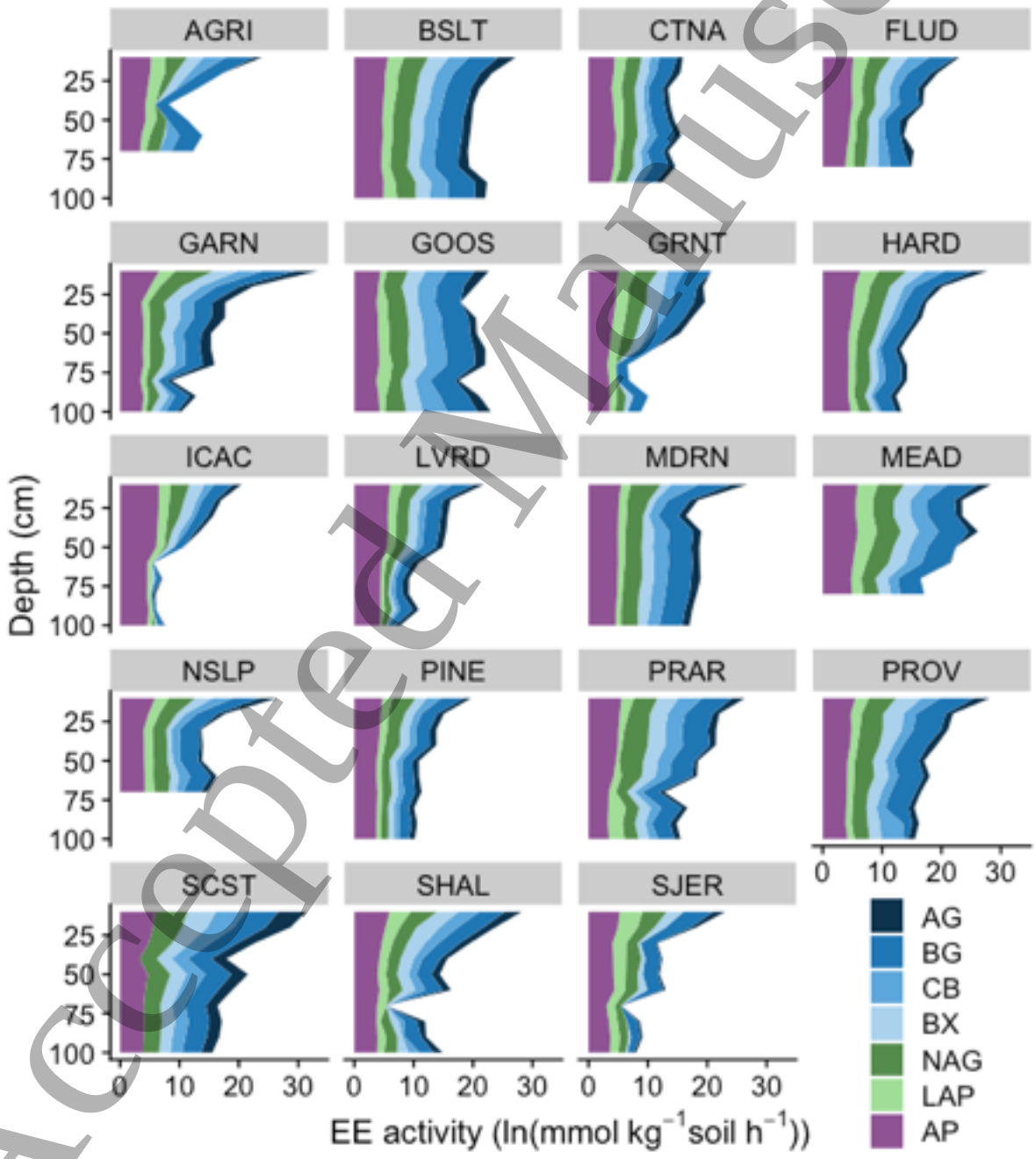

Figure 2: Percentage of α-glucosidase (AG), β-glucosidase (BG), cellobiohydrolase (CB), β-xylosidase (BX), N-acetylglucosamine (NAG), leucine aminopeptidase (LAP), and acid phosphatase (AP) activity below 20 cm in the top meter (A); and proportion of soil organic carbon (SOC), microbial biomass (MB), sum of C-degrading enzymes (C_{sum} = AG + BG + CB + BX), sum of nitrogen-mineralizing enzymes (N_{sum} = NAG + LAP), and acid phosphatase (AP) below 20 cm in the top meter of soil among soil orders (B). Error bars show ± one standard error of the mean (Figure panel A: n = 19; Figure panel B: Inceptisol: n = 5, Mollisol: n = 5, Ultisol: n = 4).

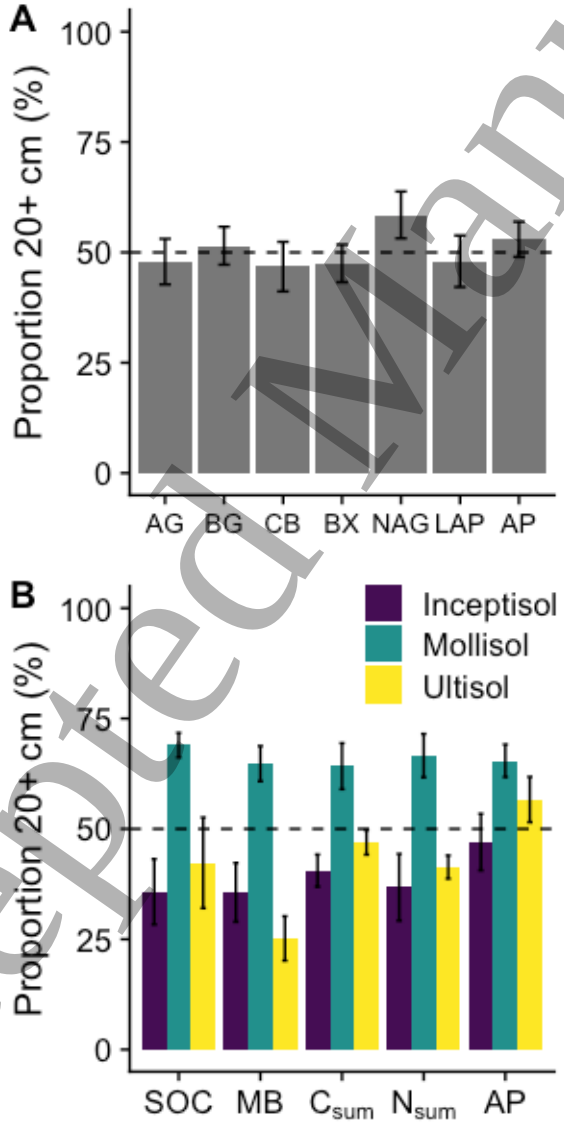

Figure 3: Correlations between soil and extracellular enzyme (EE) stoichiometry (i.e., the ratio of elements by mass and extracellular enzyme activities that target these same elements) of carbon (C), nitrogen (N), and phosphorus (P) in surface (≤ 20 cm depth) and subsoils (> 20 cm depth across all sites). Blue lines show significant (α = 0.05) mixed-effects models of the relationship between soil and EE stoichiometry (site was used as a random effect; lines were not drawn where correlations were not significant). Gray ribbons show the standard error of the model. Data points represent individual soil samples (depths within each pit). Note the scales of the axes differ among plots.

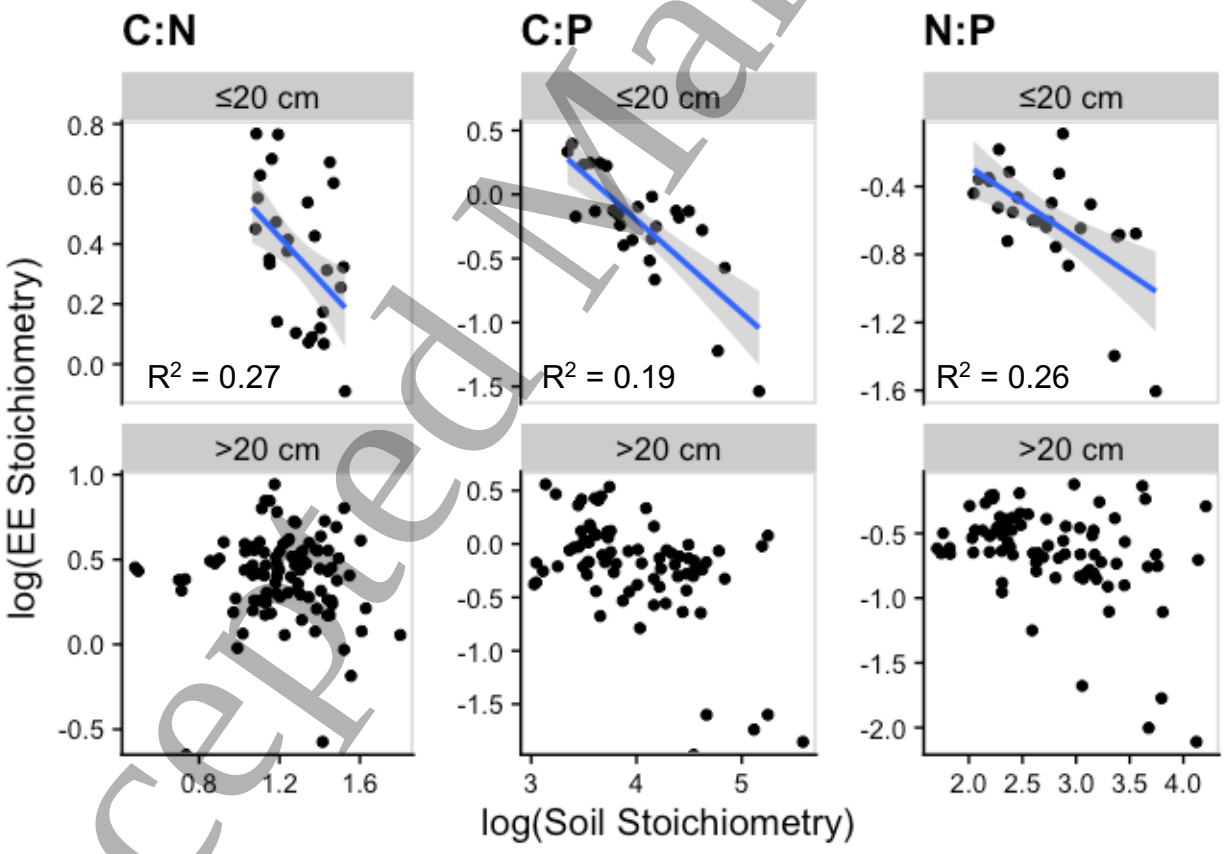

Figure 4: Conceptual model of changing controls on extracellular enzyme activity (EEA) between surface soil and subsoil. Solid lines represent fluxes and dashed lines represent moderating controls. Boxes represent pools or concentrations, and other shapes represent moderating variables. Blue parameters represent microbial parameters, and green boxes represent edaphic variables such as substrate (including carbon [C] and nutrients) and clay concentrations. The differences in the size of boxes between the surface and subsoil represent the relative size of the pool, and differences in the thickness of arrows between the surface and subsoil represent the hypothesized relative magnitude of the flux or control. A portion of the substrate pool is available to microbial biomass (MB) and is moderated by clay concentration and active EEA. Substrate availability moderates substrate demand. Bacterial biomass, fungal biomass, and substrate demand influence active EEA. Additionally, our conceptual model incorporates stabilized EEA (i.e., EEs sorbed onto clay particles), which is primarily influenced by clay concentrations. At depth, the impact of clay on substrate availability and stabilized EEA increases, while the absolute impact of substrates and microbial properties (i.e., microbial biomass and substrate demand) decreases.