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Measuring phenol oxidase and peroxidase activities with pyrogallol, L-DOPA, and ABTS: Effect of assay conditions and soil type

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ABSTRACT

Microbial phenol oxidases and peroxidases mediate biogeochemical processes in soils, including microbial acquisition of carbon and nitrogen, lignin degradation, carbon mineralization and sequestration, and dissolved organic carbon export. Measuring oxidative enzyme activities in soils is more problematic than assaying hydrolytic enzyme activities because of the non-specific, free radical nature of the reactions and complex interactions between enzymes, assay substrates, and the soil matrix. We compared three substrates commonly used to assay phenol oxidase and peroxidase in soil: pyrogallol (PYGL, 1,2,3-trihydroxybenzene), L-DOPA (L-3,4-dihydroxyphenylalanine), and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). We measured substrate oxidation in three soils across a pH gradient from 3.0 to 10.0 to determine the pH optimum for each substrate. In addition, we compared activities across 17 soils using the three substrates. In general, activities on the substrates followed the trend PYGL > L-DOPA > ABTS and were inversely related to substrate redox potential. PYGL and ABTS were not suitable substrates at pH > 5, and ABTS oxidation often declined with addition of peroxide to the assay. Absolute and relative oxidation rates varied widely among substrates in relation to soil type and assay pH. We also tested whether autoclaved or combusted soils could be used as negative controls for the influence of abiotic factors (e.g., soil mineralogy) on oxidative activity. However, neither autoclaving nor combustion produced reliable negative controls because substrate oxidation still occurred; in some cases, these treatments enhanced substrate oxidation rates. For broad scale studies, we recommend that investigators use all three substrates to assess soil oxidation potentials. For focused studies, we recommend evaluating substrates before choosing a single option, and we recommend assays at both the soil pH and a reference pH (e.g., pH 5.0) to determine the effect of assay pH on oxidase activity. These recommendations should contribute to greater comparability of oxidase potential activities across studies.

1. Introduction

Extracellular enzymes are the proximate agents of organic matter decomposition, and thus the activities of hydrolytic enzymes (e.g. cellulases, phosphatases, chitinases, proteases) are widely measured in ecosystem studies (German et al., 2011). In contrast, phenol oxidase and peroxidase activities have been measured in a smaller number of soil enzyme studies. Fungi and bacteria express oxidases for a variety of functions, including cellular processes and defense, as well as carbon (C) and nitrogen (N) acquisition (Sinsabaugh, 2010). Once in the soil environment, these enzymes mediate the biogeochemical processes of lignin degradation, carbon mineralization and sequestration, and dissolved organic C export.

Phenol oxidases oxidize phenolic compounds using oxygen as an electron acceptor. These enzymes include fungal laccases and prokaryotic laccase-like enzymes that typically have multiple copper (Cu) atoms at the reaction center (Baldrian, 2006; Hoegger et al., 2006). The ability of a laccase to oxidize a particular substrate is defined by the parameter k_{cat}, or the number of substrate molecules oxidized per enzyme active site per unit time. This parameter is related to the difference in redox potential (ΔE^0) between the

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first Cu in the reaction chain (designated T1) and the substrate (Xu, 1997). Electrons are transferred from the T1 Cu to T2 and T3 reaction centers and ultimately used to reduce molecular oxygen to water (Baldrian, 2006). Thus, in laccases, redox potential is a measure of the potential for a reaction center to acquire electrons. The redox potential of laccases ranges widely (450–800 mV), as do pH optima (4.0–7.5) and substrate preferences, because the polypeptide configuration that surrounds the redox center is highly variable (Baldrian, 2006). As pH increases beyond the optimum, hydroxyl ions inhibit the transfer of electrons from the T1 Cu to the T2/T3 reaction centers, which progressively reduces enzyme activity (Xu, 1997; Eichlerová et al., 2012). The redox potentials of laccases and similar enzymes are too low to directly oxidize the non-phenolic linkages of lignin. However, laccases catalyze the production of a variety of organic radicals, called redox mediators, that are able to break non-phenolic linkages and thereby depolymerize lignin (Bourbonnais et al., 1998; Leonowicz et al., 2001; Camarero et al., 2005).

Peroxidases such as manganese peroxidase and lignin peroxidase use H_2O_2 as an electron acceptor. These enzymes have Fe-containing heme prosthetic groups with redox potentials up to 1490 mV, giving them the capacity to break aryl and alkyl bonds within lignin either directly or through redox intermediates such as Mn^{3+} (Kersten et al., 1990; Higuchi, 1990; Rabinovich et al., 2004). The pH optimum corresponding to the maximum redox potential of *Phanerochaete* lignin peroxidase is about 3.0 (Oyadomari et al., 2003). Manganese peroxidase, which oxidizes Mn^{2+}, has a pH optimum of 4.5 (Mauk et al., 1998).

In soils, potential phenol oxidase activity is typically measured as the rate of oxidation of a model substrate added to soil suspensions (German et al., 2011; Burns et al., 2013). Peroxidase activity is measured as the rate of substrate oxidation in the presence of added H_2O_2 (Burns et al., 2013). Because this gross activity presumably includes both phenol oxidase and peroxidase activities, the phenol oxidase activity is subtracted from the gross activity to estimate the net peroxidase contribution. The three most commonly used substrates for phenol oxidase and peroxidase assays in soil are pyrogallol (PYGL, 1,2,3-trihydroxybenzene), L-DOPA (L-3,4-dihydroxyphenylalanine), and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sinsabaugh, 2010; Burns et al., 2013). Of these substrates, PYGL has the lowest redox potential and is therefore the most easily oxidized. PYGL redox potential decreases rapidly from 560 mV to −200 mV as pH increases from 1.5 to 8.5, meaning that PYGL vulnerability to oxidation increases with increasing pH (Gao et al., 1998; Riahi et al., 2007) (Fig. 1). The redox potential of L-DOPA is less sensitive to pH, declining from 525 to 460 mV over the pH range from 2.0 to 8.0 (Serpentinia et al., 2000). ABTS has the highest redox potential (E^0 = 1080 mV) (Bourbonnais et al., 1998), and because it has no protic groups, redox potential of ABTS does not vary with pH (Xu, 1997).

For any specific enzyme–substrate combination, the optimal pH for oxidation is a function of both the pH optimum of the enzyme and the response of substrate redox potential to pH. For phenol oxidases, substrates with multiple protic groups (e.g., PYGL) should have maximum oxidation rates shifted toward greater pH because the redox potential of these substrates declines with increasing pH, resulting in a greater ΔE between the T1 Cu of the enzyme and the substrate. On the other hand, enzymatic oxidation of non-phenolic substrates like ABTS should only depend on the pH optimum of the enzyme because redox potential of these substrates does not depend on pH (Xu, 1997; Fig. 1). Similar trends should apply to peroxidases.

Although PYGL, L-DOPA and ABTS are commonly used to assay the phenol oxidase and peroxidase potentials of soils, they are rarely used concurrently, which limits comparisons of oxidative activity across studies (Eichlerová et al., 2012). When comparisons have been made for particular soils or litter types, the substrates tend not to produce mutually consistent results, and absolute and relative oxidation rates vary widely among substrates in relation to soil type and assay pH (Sinsabaugh, 2010). In our experience, oxidase assays often show no detectable activity with one substrate whereas another appears to work without interference. Given the variation in oxidative enzyme activities among soils and substrates, a negative control would be useful for determining the influence of abiotic factors, such as soil mineralogy. However, negative controls for these assays have been poorly evaluated.

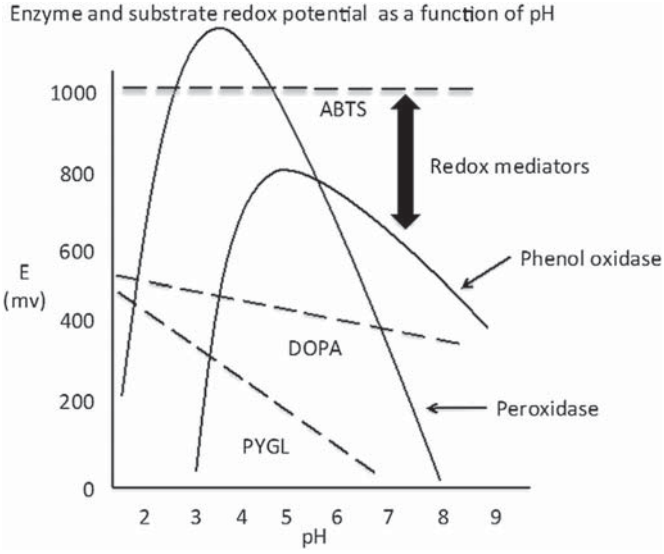

Fig. 1. Expected trends for oxidative enzyme activity based on pH optima and redox potentials of phenol oxidases and peroxidases and the redox-pH relationships of the substrates 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), L-3,4-dihydroxyphenylalanine (L-DOPA) and pyrogallol (PYGL) (Xu, 1997; Gao et al., 1998; Riahi et al., 2007). Enzyme redox potential is shown as a solid line and substrates are shown as dashed lines. The non-phenolic linkages of lignin are oxidized at redox potentials >1000 mV. Some phenol oxidases can generate soluble redox mediators whose redox potentials are comparable to those of peroxidases.

To assess the magnitude of these problems and provide methodological recommendations to investigators, we compared the oxidation of PYGL, L-DOPA, and ABTS across a range of assay conditions and soil types. We aimed to determine whether relative phenol oxidase and peroxidase activities can be predicted based on pH and the redox potential of the substrate (Fig. 1). We compared substrate oxidation rates at pH values ranging from 3.0 to 10.0 in soils from Alaska, California, and Costa Rica and evaluated the appropriateness of autoclaved and combusted soils from these sites as negative controls. We chose these soils to observe whether trends in substrate oxidation as a function of pH were consistent across soils displaying an array of different conditions (e.g., pH, moisture content, and organic matter content). We also compared oxidation rates of PYGL, L-DOPA and ABTS in 17 soils that ranged widely in bulk soil pH, organic matter content and other variables (Table 1).

2. Materials and methods

2.1. Enzyme assay protocol

Enzyme assays were performed following published microplate protocols (Gallo et al., 2004; Allison and Jastrow, 2006; Floch et al., 2007). Soil suspensions were prepared by homogenizing 1 g of

Table 1
Potential phenol oxidase and gross peroxidase activities for 17 soils measured with three substrates: PYGL (pyrogallol, 1,2,3-trihydroxybenzene), L-DOPA (L-3,4-dihydroxyphenylalanine), and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

Sample origin	Coordinates	SOM (%)	Bulk pH	Phenol oxidase			Peroxidase			Phenol oxidase	Peroxidase
				Assay pH 5.0			Assay pH 5.0			Assay pH 8.0	Assay pH 8.0
				PYGL	DOPA	ABTS	PYGL	DOPA	ABTS	DOPA	DOPA
Boreal Forest, AK[a]	63.91°N 145.73°W	16.80	5.0	N.D.	N.D.	0.44	5.48	1.66	0.83	N.D.	5.0
Hardwood Forest, OH	41.66°N 83.78°W	7.40	6.6	3.21	N.D.	0.38	15.4	12.3	0.36	7.3	49.6
Hardwood Forest, OH	41.66°N 83.78°W	7.40	6.6	N.D.	N.D.	N.D.	31.8	3.79	N.D.	9.7	32.5
Hardwood Forest, OH	41.57°N 83.86°W	4.60	6.1	6.43	0.38	0.64	19.7	8.10	1.57	17.6	30.9
Tall Grass Prairie, OH	41.65°N 83.77°W	10.40	7.2	N.D.	N.D.	1.93	N.D.	N.D.	0.54	57.9	99.4
Maple Litter Microcosm	41.68°N 83.71°W	6.20	4.5	1.72	N.D.	N.D.	7.50	0.65	N.D.	10.7	19.8
Semiarid Grassland, NM	34.40°N 106.68°W	1.80	8.2	10.8	5.75	0.39	24.2	2.17	0.22	140.0	267.0
Creosote Shrubland, NM	34.35 °N 106.69° W	2.40	7.5	5.11	2.72	N.D.	4.54	0.25	N.D.	69.5	29.6
Juniper Savannah, NM	34.43°N 105.86°W	3.70	7.2	N.D.	N.D.	0.38	5.59	0.05	N.D.	60.2	441.0
Piñon Juniper Woodland, NM	34.36°N 106.27°W	6.00	6.0	13.3	7.08	0.48	4.94	N.D.	N.D.	1.7	141.0
Ponderosa Pine Forest, NM	35.86°N 106.60°W	11.60	5.5	3.30	1.75	0.03	8.04	8.56	0.41	63.6	481.0
Subalpine Spruce Forest, NM	35.89°N 106.53°W	4.80	5.2	3.25	1.73	0.30	2.87	0.59	N.D.	32.0	497.0
Semiarid Grassland, CA[a]	33.73°N 117.70°W	3.10	6.8	N.D.	N.D.	0.50	5.53	1.79	N.D.	11.3	19.7
Trop. Rainforest, Costa Rica[a]	8.71°N 83.61°W	15.10	5.8	1.83	1.42	3.63	13.3	5.75	2.37	N.D.	N.D.
Antarctic Dry Valley, Antarctica	77.61°S 163.00°E	0.10	10.0	2.57	0.09	0.01	3.11	2.70	0.21	52.3	160.0
Antarctic Dry Valley, Antarctica	77.61°S 163.00°E	0.11	8.7	0.33	0.17	0.06	1.55	4.54	0.11	3.2	63.1
Antarctic Dry Valley, Antarctica	77.61°S 163.00°E	0.02	7.9	0.03	N.D.	0.05	7.32	N.D.	0.32	N.D.	N.D.
Mean				4.32	2.34	0.66	10.05	3.78	0.69	38.36	155.77
Standard error				1.17	0.82	0.26	2.15	0.95	0.23	10.69	47.78

AK = Alaska, USA. OH = Ohio, USA. NM = New Mexico, USA. CA = California, USA.
Activity units are μmol h^{-1} g dry soil. Because sample size was low ($n = 1$) in most locations, no error values are reported for individual sites. At pH 5, two-way ANOVA on log+1 transformed data showed a significant effect of substrate (Phenol oxidase $F_{2,17} = 11.32$, $P < 0.001$; Peroxidase $F_{2,21} = 32.27$, $P < 0.001$) on enzyme activities, whereas site was significant for peroxidase only (Phenol oxidase: $F_{15,27} = 1.86$, $P = 0.109$; Peroxidase: $F_{16,21} = 2.48$, $P = 0.026$). N.D. = Not detectable.
[a] Denotes sample origin used in the pH gradient study.

fresh soil in 125 ml of buffer using a hand held Bamix Homogenizer (BioSpec Products, Bartlesville OK, USA). Enzyme activities were measured by combining 200 μL of soil suspension with 50 μL of substrate solution. Controls included in each microplate were: substrate + buffer, soil suspension + buffer, and buffer-only blank. For peroxidase assays, all wells received 10 μL of 0.3% hydrogen peroxide, including controls. Assays were incubated for 3 or 4 h (depending on the experiment; see below) at the recorded temperature of the site where the sample was collected. Preliminary assays revealed that incubation times of 3–4 h were sufficient to produce linear accumulation of reaction products over time. Both shorter and longer incubation times were investigated, and results from longer incubations tended to be less reliable, as the accumulation of reaction product over time often became non-linear in longer incubations. Buffers were 50 mM sodium acetate, 25 mM maleic acid disodium salt hydrate, or 50 mM sodium bicarbonate (depending on assay pH; see below). Substrates were purchased from Sigma–Aldrich (St. Louis, Missouri, USA) and solutions were freshly prepared for each trial (5 mM PYGL, 25 mM L-DOPA, or 2 mM ABTS in deionized water), following the published protocols cited above. Micromolar extinction coefficients were determined for the oxidation products of each substrate, as described in the protocols, and calculated on the basis of μmole of product with a path length of 10 mm (German et al., 2011). The extinction coefficients used in our calculations were 4.2 for PYGL, 7.9 for L-DOPA and 73 for ABTS, which are similar to values reported in the literature (Allison and Vitousek, 2004; DeForest, 2009; Saiya-Cork et al., 2002; Shi et al., 2006; Yao et al., 2009). Activity values were calculated as μmol h^{-1} g^{-1} soil. Soil replicates chosen from each location were stored after the initial collection at −80 °C and subsequently stored at −20 °C until analysis.

2.2. Activity as a function of pH

Soils were collected from Delta Junction, Alaska (63.91°N, 145.73°W), Irvine, California (33.73°N, 117.70°W), and Nicoya Peninsula, Costa Rica (8.71°N, 83.61°W). Three replicate samples were taken with a soil corer (2.5 cm diameter × 5 cm depth), at least 10 m apart, from each sampling location, individually sealed in plastic bags, and homogenized by hand before freezing. Detailed descriptions of these soils and their handling can be found in German et al. (2012), and bulk soil properties are shown in Table 1. Assays were conducted using PYGL, L-DOPA, and ABTS at pH values from 3.0 to 10.0, at 0.5 pH unit intervals, with and without the addition of 10 μL of 0.3% hydrogen peroxide. We used 50 mM sodium acetate buffer for pH values ranging from 3.0 to 5.0 and adjusted the pH with glacial acetic acid. We used 25 mM maleic acid buffer for pH values ranging from 5.5 to 7.5, and adjusted the pH with 3 M hydrochloric acid. We used 50 mM sodium bicarbonate buffer for pH values ranging from 8.0 to 10.0, and adjusted the pH with 3 M hydrochloric acid or 10 M sodium hydroxide solution. Assays were incubated for 3 h (a duration that was confirmed to produce a linear increase in absorbance over time) in the absence of light at temperatures appropriate for each site: 10 °C for Alaska, 22 °C for California, and 28 °C for Costa Rica (German et al., 2011, 2012). Absorbance was read at 3 h, and potential phenol oxidase and peroxidase activities were calculated as described previously (German et al., 2011).

2.3. Potential negative controls for phenol oxidase and peroxidase assays

Approximately 10 g of soil from Alaska, California, and Costa Rica were either autoclaved for 30 min at 121 °C or combusted at 550 °C for 3 h to destroy oxidative enzymes. Combustion destroys enzymes by oxidizing and removing all organic compounds, including enzymes, from soil. Autoclaving is often used to permanently denature proteins using heat and pressure (Sinsabaugh, 2010), although it is possible that mineral-stabilized enzymes could withstand autoclaving (Stursova and Sinsabaugh, 2008). These 'negative control' soils were assayed at pH 5.0 using 50 mM sodium acetate buffer under the same temperature conditions as

the fresh soils using PYGL, L-DOPA, and ABTS as substrates. Absorbance was read at 30 min intervals for 3 h, and the absorbance was regressed against time for each substrate and soil collection location. Linear relationships between absorbance and time were taken as oxidative activity of a soil against a particular substrate.

2.4. Oxidase activity in diverse soils

We collected soils from different ecosystems to compare the behavior of each substrate in assays with different soil properties. One soil sample ($n = 1$) was collected by corer or auger (to a depth of 10 cm) from 17 locations, representing a diverse range of vegetation and soil types (Table 1). There was no plot-scale replication within sites because samples were collected as part of a broad survey for a different study. Following collection, all soil samples were frozen at −20 °C until analyzed. For all soils, assays were conducted at pH 5 using 50 mM sodium acetate buffer and at pH 8 using 50 mM sodium bicarbonate buffer. For each substrate, assays were conducted with and without addition of hydrogen peroxide. Assays were read in a spectrophotometer at 4 h; detailed kinetic analyses were not conducted for this part of the study. Readings were adjusted for absorbance due to substrate and homogenate controls, as described in the assay protocol section.

2.5. Statistical analysis

To measure the effects of pH on substrate oxidation in soils from Alaska, California, and Costa Rica, we performed linear regression analyses to obtain slope, R^2 values, and associated P-values for the dependence of oxidation on pH. We used two-way ANOVA to examine the effects of soil collection site and assay substrate on measured phenol oxidase and peroxidase activities in diverse soils. To satisfy normality assumptions, data were log+1 transformed before running the ANOVA. P-values below 0.05 were considered significant.

3. Results

3.1. Activity levels across a pH gradient

Phenol oxidase—PYGL oxidation was most readily detected at pH values ranging from 6 to 7 (Alaska and California), but was also detected under more acidic conditions in Costa Rican soils (Fig. 2 A–C). At pH > 7.5, PYGL rapidly auto-oxidized, making activity calculations impossible under alkaline conditions with this substrate. Overall no significant trends in PYGL oxidation with respect to pH were found at any of the sites. In Alaskan soil with a native pH of 5.0, L-DOPA oxidation was limited to pH values ranging from 3.5 to 7.0, whereas oxidation was detected under more alkaline pH values (5.0–9.0) for soils from California and Costa Rica (Fig. 2 A–C). Similar to PYGL, no significant linear trends in phenol oxidase activity with pH were found for L-DOPA. ABTS oxidation rates decreased significantly ($P < 0.05$) with pH in Alaskan and California soils, and ABTS was not oxidized above pH values of 7.5 in any soil (Fig. 2 A–C; Table 2).

Peroxidase—When using PYGL as a substrate, peroxidase activity was detectable between pH values of 3.0 and 7.5, even when phenol oxidase activity was undetectable in a particular soil (Fig. 2). With PYGL there was a significant negative relationship between net peroxidase activity and assay pH for soils collected from Alaska and Costa Rica (Table 2; Fig. 2D–F). In California soils, the relationship between activity and pH switched from negative to positive at pH 5.5 ($P = 0.01$; Table 2; Fig. 2E). For L-DOPA, there were positive relationships between peroxidase activity and pH above pH 6.0 (Fig. 2 D–F). This trend was significant for Alaska and California soils (Table 2). Peroxidase activities were measurable with ABTS in Alaskan soils at pH values under 6.0, but were near zero in Costa Rican and Californian soils (Fig. 2). In Alaskan soil, there was no significant relationship between pH and peroxidase measured with ABTS.

3.2. Potential negative controls for phenol oxidase and peroxidase assays

The oxidative activity of autoclaved and combusted soils varied with soil and substrate. For Alaskan soils, autoclaved samples readily oxidized PYGL and L-DOPA with and without peroxide, showing steady increases in absorbance over time, with R^2 values $\geq$0.75 (Table 3). Moreover, the slopes of these lines were greater than those of the native soil, showing that greater oxidation was detected in the potential negative controls than in the soils containing active oxidases and peroxidases. ABTS was not oxidized by autoclaved Alaskan soils in the absence of peroxide, but peroxide addition provided a linear increase in absorbance over time against this substrate, again, with the autoclaved soils showing greater activity than the native soil. Autoclaved Californian and Costa Rican soils showed no oxidative activity against any substrate in the absence of peroxide, but showed activity against PYGL and L-DOPA in the presence of peroxide, with R^2 values $\geq$0.65 (Table 3). The autoclaved Costa Rican soils had lower slopes than the native soils in the presence of peroxide. ABTS was not oxidized by autoclaved Californian or Costa Rican soils.

Combusted Alaskan soil showed oxidative activity only against PYGL without peroxide, but oxidized all three substrates in the presence of peroxide, with R^2 values $\geq$0.85 (Table 3). As with autoclaved soils, the oxidation shown by the combusted soils in the presence of peroxide exceeded the oxidation observed in the native soils. In contrast, combusted Californian soils showed no oxidative activity against PYGL, and variable oxidation against ABTS, all in the absence of peroxide. L-DOPA was oxidized by combusted Californian soils, with or without peroxide, with R^2 values $\geq$0.66, and all three substrates were oxidized in the presence of peroxide (Table 3). Oxidation rates in combusted Californian soil exceeded oxidation rates in the native soils with added peroxide. With the exception of ABTS in the presence of peroxide, combusted Costa Rican soils readily oxidized all substrates, with R^2 values $\geq$0.90 (Table 3). In contrast to the other soils, the combusted Costa Rican soil had lower oxidative activity than the native Costa Rican soils with added peroxide.

See Supplemental Tables S1, S2, and S3 in the online version of this article for the complete dataset on the autoclaved and combusted soils.

3.3. Oxidase activities in diverse soils

Consistent with our pH gradient analysis, rapid auto-oxidation precluded assays with PYGL at pH 8 in any soil. Moreover, at pH 8, ABTS was not oxidized in any soil with or without peroxide. Thus, L-DOPA was the only substrate suitable for assays at both pH 5 and 8. For soils with measurable phenol oxidase activity at both pH values, oxidation rates at pH 8 were 93.90 ± 65.77 (mean ± SEM) times greater than rates at pH 5. With the exception of two soils that showed no activity at either pH, soils with no detectable activity at pH 5 showed measurable activity at pH 8 (Table 1). With peroxide addition, L-DOPA oxidation at pH 8 was 776.42 ± 673.21 times greater than activity at pH 5 for soils with measurable activity at both pH values. Furthermore, soils with no detectable peroxidase activity at pH 5 displayed activity at pH 8, except one soil (from the McMurdo Dry Valleys, Antarctica) that had no activity at either pH. At pH 5,

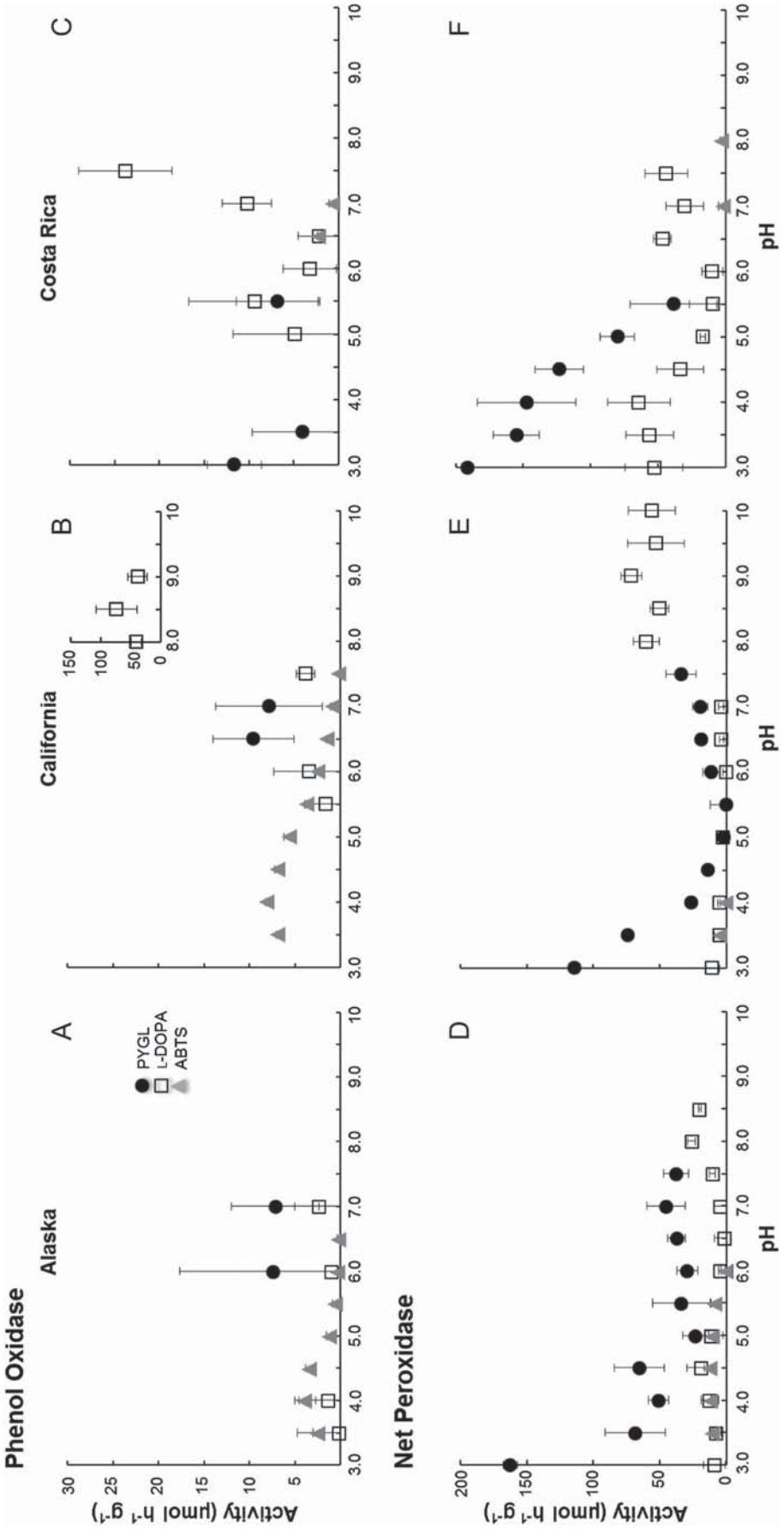

Fig. 2. Phenol oxidase (Panels A–C) and peroxidase (Panels D–F) activities as a function of pH in soils collected from Alaska, California and Costa Rica. Activities were determined using pyrogallol (PYGL), L-3,4-dihydroxyphenylalanine (L-DOPA) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrates. Values are mean ± SEM ($n = 3$ cores for each enzyme and pH). The inset shows high phenol oxidase activities measured using DOPA in California soils at pH 8–9.

all substrates provided useable data (Table 1). Using two-way ANOVA, we detected significant effects of substrate ($F_{2,17} = 11.32$, $P < 0.001$), but not site ($F_{15,17} = 1.86$, $P = 0.109$) on phenol oxidase activities. Mean phenol oxidase activities varied inversely with substrate redox potential with a PYGL:L-DOPA:ABTS activity ratio of 7:4:1 (see Supplemental Table S4 in the online version of this article for activity ratios for each site). However, four of the 17 soils showed activity toward ABTS, but not toward L-DOPA or PYGL, which have lower redox potentials. Gross peroxidase activities differed by substrate ($F_{2,21} = 32.27$, $P < 0.001$) and site ($F_{16,21} = 2.48$, $P = 0.026$), and were also inversely related to substrate redox potential; mean peroxidase activity ratios (PYGL:L-DOPA:ABTS) were 15:5:1. One soil showed activity toward ABTS, but not toward L-DOPA or PYGL.

Ratios of activity with versus without added peroxide ranged widely by substrate. Excluding soils with no oxidase activity, ratios of oxidation rates with peroxide to those without peroxide were generally lowest for ABTS (median 1.9) followed by PYGL (median 3.1) and L-DOPA (median value 4.5 at pH 5 and 3.2 at pH 8). At pH 5, four of five soils with no measurable phenol oxidase activity on PYGL showed activity with added peroxide. For L-DOPA, six of eight soils with no measurable phenol oxidase activity showed peroxidase activity; one soil (Piñon Juniper Woodland, NM) showed phenol oxidase activity, but no detectable activity with added peroxide. For ABTS, three soils showed no activity with or without peroxide, whereas four soils that showed phenol oxidase activity had no detectable activity with peroxide addition. At pH 8, one of three soils with no phenol oxidase activity on L-DOPA showed activity with added peroxide.

Phenol oxidase activities observed using PYGL and L-DOPA were highly correlated across the data in Table 1 ($r = 0.90$, $P = 0.001$), whereas activities on ABTS were not correlated with those for PYGL ($r = 0.07$, $P = 0.838$) or L-DOPA ($r = 0.05$, $P = 0.908$). No patterns were apparent for gross peroxidase activity: activities measured with PYGL and L-DOPA were not significantly correlated ($r = 0.37$, $P = 0.179$), and other substrate combinations also showed poor correlations (PYGL vs. ABTS: $r = 0.31$, $P = 0.423$; L-DOPA vs. ABTS $r = 0.12$, $P = 0.786$).

4. Discussion

Oxidation rates for commonly used phenol oxidase and peroxidase substrates depended on assay pH and soil type. Both PYGL and ABTS were unsuitable for assays at alkaline pH because of auto-oxidation with PYGL, and prohibitively high redox potentials with ABTS. L-DOPA appears to be the only substrate that is suitable for use across a broad range of pH values. Eichlerová et al. (2012) also observed that L-DOPA was oxidized across a broader range of pH values than ABTS. However, L-DOPA was not oxidized in all our samples (even if other substrates were), and its oxidation was sometimes negatively affected by peroxide addition. We even observed variation in our ability to detect the same enzyme under the same conditions—for example, native AK and CA soils showed phenol oxidase activity on PYGL at pH 5.0 in our negative control study (Table 3) but not in our pH study (Fig. 2A–B).

It is possible that our detection limits were better in the negative control study because absorbances were consistently measured over multiple time points. In order to improve detection limits, shorter assay incubation times of 5 min or 1 h have been used for ABTS and PYGL (Allison and Jastrow, 2006; Floch et al., 2007). In our preliminary assays, reaction rates for ABTS and PYGL remained linear over 3–4 h, but longer incubation times produced inconsistent results, possibly because reaction product accumulation rates are more likely to become non-linear with longer incubations.

Table 2
Slope, R^2 and associated P-values for regressions of potential activity against pH for phenol oxidase and net peroxidase; activity was measured with PYGL, L-DOPA and ABTS in soils from Alaska, California, and Costa Rica. Only significant trends in substrate oxidation with respect to pH are reported along with the pH range over which the trend occurred.

Location	Substrate	pH	R^2	P-value	n	Trend
Phenol oxidase						
Delta Junction, AK	ABTS	3.5–6.5	0.73	<0.01	7	Decreasing
Irvine, CA	ABTS	3.5–7.5	0.94	<0.01	9	Decreasing
Net peroxidase						
Delta Junction, AK	PYGL	3.0–7.5	0.43	0.04	10	Decreasing
Delta Junction, AK	DOPA	6.0–8.5	0.73	0.03	6	Increasing
Irvine, CA	PYGL	3.0–5.0	0.92	0.01	5	Decreasing
Irvine, CA	PYGL	5.5–7.5	0.94	0.01	5	Increasing
Irvine, CA	DOPA	6.0–10.0	0.75	<0.01	8	Increasing
Nicoya Peninsula, CR	PYGL	3.0–5.5	0.96	<0.01	6	Decreasing
Nicoya Peninsula, CR	DOPA	3.0–5.5	0.76	0.02	5	Decreasing

Despite difficulties in measuring oxidase activities consistently across substrates and soil types, our data generally support the predictions illustrated in Fig. 1. Average oxidase activities across soil types at pH 5.0 were highest for PYGL, which has the lowest redox potential, and lowest for ABTS, which has the highest redox potential (Table 1; Table S4). In our pH study (Fig. 2), oxidative responses were consistent with the interplay between the pH optima of the enzymes and pH effects on substrate redox potential.

4.1. Assay pH

The patterns of ABTS oxidation rates as a function of assay pH were similar to those reported by Xu (1997), Floch et al. (2007), and Eichlerová et al. (2012). Xu (1997) suggested that oxidation of non-phenolic substrates such as ABTS declines as pH increases due to inhibition of the T1 reaction center of laccase. Eichlerová et al. (2012) attributed this inhibition to hydroxyl ions. The decrease in ABTS oxidation with increasing pH in the presence of hydrogen peroxide may also be explained by the low pH optimum of peroxidases. For instance, the pH optimum for lignin peroxidase is 3.0 (Marquez et al., 1988), so one would expect lower rates of ABTS oxidation in the presence of peroxide at pH values greater than 3.0. Moreover, the oxidation of ABTS can be decreased by fulvic acids, humic acids, and low molecular mass compounds extracted from soils (Eichlerová et al., 2012), which may partly explain difficulties in using ABTS with bulk soil homogenates.

For PYGL, there was no significant relationship between oxidation and assay pH in the absence of hydrogen peroxide, mostly because activities were often undetectable. Similar to PYGL, no significant trends were found for L-DOPA, but activity substantially increased at high pH in Californian and Costa Rican soils. This result was surprising given that phenol oxidase activities are generally inhibited under alkaline pH. However, pH optima for certain laccase isozymes have been reported up to pH 7.5 (Bollag and Leonowicz, 1984). This observed increase in L-DOPA oxidation might reflect favorable pH conditions for particular laccase isozymes in the soil. It is difficult to conclude whether this increase in oxidative activity with L-DOPA is a function of the enzyme or the substrate because we could not evaluate phenol oxidase activity under high pH with the other substrates due to auto-oxidation of PYGL and the high redox potential of ABTS.

In the presence of hydrogen peroxide, PYGL oxidation decreased from pH 3.0 to 5.5, as predicted based on the pH optimum ($\sim$3.0) of the enzyme (Figs. 1 and 2). This pattern was also observed with L-DOPA in Costa Rican soil. At higher pH, auto-oxidation made enzymatic activity determination impossible with PYGL, whereas peroxidase activities on L-DOPA increased at pH values above 6.0 in

Table 3
Equations and R^2 values for significant ($P < 0.05$) regressions of net absorbance units against time for phenol oxidase and net peroxidase in native, autoclaved, and combusted soils from Alaska, California, and Costa Rica. Assays were conducted at pH 5.0 and read at 30 min intervals over a period of 3 h. Time interval represents the entire period over which positive net absorbance was observed. N.D. = Not detectable. N.S. = Not significant.

Location	Substrate	Equation	R^2	*P*-value	Time interval (h)	*n*
Native soil: phenol oxidase						
Delta Junction, AK	PYGL	0.018x + 0.020	0.90	<0.01	0.0–3.0	7
Delta Junction, AK	DOPA	N.D.	N.D.			
Delta Junction, AK	ABTS	N.D.	N.D.			
Irvine, CA	PYGL	0.006x + 0.001	0.87	0.02	1.0–3.0	5
Irvine, CA	DOPA	N.D.	N.D.			
Irvine, CA	ABTS	0.123x + 0.168	0.93	<0.01	0.0–3.0	7
Nicoya Peninsula, CR	PYGL	N.D.	N.D.			
Nicoya Peninsula, CR	DOPA	N.D.	N.D.			
Nicoya Peninsula, CR	ABTS			N.S.	0.0–3.0	7
Autoclaved soil: phenol oxidase						
Delta Junction, AK	PYGL	0.035x + 0.0381	0.95	<0.01	0.0–3.0	7
Delta Junction, AK	DOPA	0.016x + 0.014	0.94	<0.01	0.0–3.0	7
Delta Junction, AK	ABTS	N.D.	N.D.			
Irvine, CA	PYGL	N.D.	N.D.			
Irvine, CA	DOPA	N.D.	N.D.			
Irvine, CA	ABTS	N.D.	N.D.			
Nicoya Peninsula, CR	PYGL	N.D.	N.D.			
Nicoya Peninsula, CR	DOPA	N.D.	N.D.			
Nicoya Peninsula, CR	ABTS	N.D.	N.D.			
Combusted soil: phenol oxidase						
Delta Junction, AK	PYGL	0.032x − 0.0283	0.97	<0.01	1.0–3.0	5
Delta Junction, AK	DOPA	N.D.	N.D.			
Delta Junction, AK	ABTS	N.D.	N.D.			
Irvine, CA	PYGL	N.D.	N.D.			
Irvine, CA	DOPA	0.006x + 0.011	0.66	0.05	0.5–3.0	6
Irvine, CA	ABTS			N.S.	0.0–3.0	7
Nicoya Peninsula, CR	PYGL	0.084x − 0.012	0.94	<0.01	0.5–3.0	6
Nicoya Peninsula, CR	DOPA	0.033x − 0.001	0.90	<0.01	0.0–3.0	7
Nicoya Peninsula, CR	ABTS	0.131x + 0.10	0.93	<0.01	0.0–3.0	7
Native soil: net peroxidase						
Delta Junction, AK	PYGL	0.045x + 0.097	0.77	<0.01	0.0–3.0	7
Delta Junction, AK	DOPA	0.043x + 0.093	0.76	0.02	0.5–3.0	6
Delta Junction, AK	ABTS	0.065x + 0.023	0.96	<0.01	0.0–3.0	6
Irvine, CA	PYGL	0.012x + 0.026	0.89	<0.01	0.5–3.0	6
Irvine, CA	DOPA	0.018x + 0.007	0.94	<0.01	0.5–3.0	6
Irvine, CA	ABTS	N.D.	N.D.			
Nicoya Peninsula, CR	PYGL	0.134x + 0.269	0.90	<0.01	0.5-3.0	6
Nicoya Peninsula, CR	DOPA	0.153x + 0.164	0.77	<0.01	0.0–3.0	7
Nicoya Peninsula, CR	ABTS	−0.009x + 0.033	0.84	0.03	0.5–3.0	5
Autoclaved soil: net peroxidase						
Delta Junction, AK	PYGL	0.089x + 0.172	0.75	<0.01	0.0–3.0	7
Delta Junction, AK	DOPA	0.075x + 0.195	0.95	<0.01	0.5–3.0	5
Delta Junction, AK	ABTS	0.166x + 0.423	0.76	0.01	0.0–.0	7
Irvine, CA	PYGL	0.022x − 0.006	0.95	<0.01	1.0–3.0	5
Irvine, CA	DOPA	N.D.	N.D.			
Irvine, CA	ABTS	N.D.	N.D.			
Nicoya Peninsula, CR	PYGL	0.068x + 0.044	0.84	0.01	0.5–3.0	6
Nicoya Peninsula, CR	DOPA	0.053x + 0.074	0.65	0.03	0.0–3.0	7
Nicoya Peninsula, CR	ABTS	N.D.	N.D.			
Combusted soil: net peroxidase						
Delta Junction, AK	PYGL	0.138x + 0.073	0.96	<0.01	0.5–3.0	6
Delta Junction, AK	DOPA	0.070x + 0.041	0.85	0.01	0.5–3.0	6
Delta Junction, AK	ABTS	0.176x + 0.130	0.87	<0.01	0.0–3.0	7
Irvine, CA	PYGL	0.117x + 0.014	0.97	<0.01	0.5–3.0	6
Irvine, CA	DOPA	0.074x + 0.026	0.99	<0.01	0.5–3.0	6
Irvine, CA	ABTS	0.134x + 0.025	0.93	<0.01	0.5–3.0	6
Nicoya Peninsula, CR	PYGL	0.055x + 0.011	0.97	<0.01	1.0–3.0	5
Nicoya Peninsula, CR	DOPA	0.032x + 0.014	0.93	<0.01	0.5–3.0	6
Nicoya Peninsula, CR	ABTS	N.D.	N.D.			

Alaskan and Californian soils (Table 2). This pattern suggests that even as pH values increasingly exceed the enzyme optimum, L-DOPA oxidizes more readily due to declines in substrate redox potential. Under neutral and alkaline conditions, the catechol group present in L-DOPA may increase the formation of hydroxyl radicals in the presence of hydrogen peroxide and iron and potentially contribute to enhanced substrate oxidation (Iwahashi et al., 1989). The same pattern probably occurs with PYGL (i.e. Fig. 2E), but this substrate reaches low enough redox potentials to auto-oxidize, thereby precluding activity measurements at high pH values in most soils.

4.2. Negative controls

In general, autoclaving and combusting soils did not prevent oxidative reactions, meaning that these treatments cannot be used

as negative controls (Carter et al., 2007; Stursova and Sinsabaugh, 2008; Sinsabaugh, 2010). Autoclaved soils displayed no phenol oxidase activity in Californian and Costa Rican soils, but showed elevated activity in Alaskan soil. In the presence of peroxide, substrate oxidation was observed in nearly all autoclaved and combusted soils, except with ABTS (Table 3). Given that the majority of autoclaved and combusted soils oxidized PYGL, L-DOPA, and ABTS, oxidative activity should probably be considered a whole soil property rather than a measure of specific enzyme concentrations. This interpretation is also relevant to our pH study, in which mineral catalysis of substrate oxidation may have occurred at alkaline pH values due to reduced redox potentials of PYGL and L-DOPA.

Substrate oxidation in sterilized treatments could be explained by minerals present in the soil such as Fe(II), which actively participates in redox cycling and can generate reactive oxygen species through the Fenton reaction (Chacon et al., 2006; Hall and Silver, 2013). Manganese oxides are also produced biotically and abiotically in soils, and may contribute to soil organic matter oxidation (Spiro et al., 2009). Recently, Hall and Silver (2013) used L-DOPA to assess phenol oxidative activity of Fe(II) in tropical soils and reported a linear increase in L-DOPA oxidation with increasing soil Fe(II) concentrations. The authors also used autoclaved soil to destroy enzymatic activity and found little change in rates of L-DOPA oxidation. Thus abiotic reactions in soil probably contribute to substrate oxidation, especially with L-DOPA and PYGL.

Oxidation rates and linearity over time were generally lower in native soils relative to combusted soils, potentially due to the presence of antioxidant compounds in native soil organic material (Rimmer and Smith, 2009). Our high-temperature treatments likely caused changes in soil physical and chemical properties that increased the availability of reactive mineral species to participate in redox reactions (Trevors, 1996). Consistent with this idea, the clay-sized fraction of soils, which is low in organic matter and rich in minerals, can show higher oxidative activities than organic-rich soil fractions (Allison and Jastrow, 2006).

4.3. Soil survey

For most of the soils analyzed, oxidative enzyme activities were consistent with the redox characteristics of the substrates. Furthermore, activity measurements based on PYGL and L-DOPA were more similar to one another than to measurements based on ABTS (Table 1; Fig. 1). However, phenol oxidase activities in five of the 17 soils were inconsistent with our hypothesized dependence on redox potential because ABTS was oxidized at a higher rate than PYGL or L-DOPA (Table 1). This pattern could occur if PYGL and L-DOPA are more subject to chemical interference than ABTS. We also observed several instances in the soil survey and the negative control experiment where peroxide addition unexpectedly reduced oxidative activity (Tables 1, S1–S3). Such a reduction could occur if peroxide addition increases the reactivity of chemical compounds that interfere with the assay. Alternatively, inconsistencies in our soil survey dataset could have occurred because we did not optimize incubation times and check for reaction linearity in every soil type, as we did with our Alaskan, Californian, and Costa Rican soils (German et al., 2011).

The relatively high frequency of inconsistent results for phenol oxidase and peroxidase assays is a cause for concern and probably contributes to the high coefficients of variation typically reported for these activities (Sinsabaugh et al., 2008). There are many potential sources of interference including substrate sorption, dimerization, and condensation of reaction intermediates with other organic molecules, as well as the existence of catalytic cycles involving minerals (Spiro et al., 2009; Sinsabaugh, 2010; Eichlerová et al., 2012). There is also the potential for synergistic interactions involving natural redox mediators and minerals, as was observed with the autoclaved and combusted soils. Unfortunately, it is difficult to identify indicators of these effects *a priori*. Within our data, there is no discernible pattern to these inconsistencies in relation to soil pH, SOM concentration, or site characteristics.

One way to test for interference in oxidase assays is to add known amounts of commercially available oxidases (e.g. tyrosinase) and/or peroxidases (e.g., horseradish peroxidase) to soil slurries and examine the oxidation rates of the different substrates (Allison, 2006). If activity does not increase linearly with the concentration of added enzyme (under saturating substrate concentrations), then side reactions within the soil environment may prevent the accurate measurement of potential oxidase and peroxidase activity in bulk soil samples. If this is the case, oxidases could be isolated from soil prior to assays, but then there may be challenges in extrapolating the assay results to the soil environment (Eichlerová et al., 2012).

4.4. Conclusions

We found that three substrates commonly used in oxidase assays each revealed distinct trends with respect to pH and soil type. Thus measuring soil oxidase activity remains a challenge. Our results lead to several recommendations for overcoming this challenge and improving oxidase techniques. For research questions that require standardized comparisons across multiple soil types, we recommend conducting assays with multiple substrates at a reference pH (e.g. 5.0) to obtain a more complete profile of oxidation potentials and identify potential sources of interference. Furthermore, by standardizing assay conditions and verifying reaction linearity, inconsistencies due to methodological differences can be minimized, allowing for a sharper focus on the oxidative variation across soil types due to enzymatic or abiotic factors. For studies that focus on a single system, researchers should evaluate multiple substrates at an assay pH that approximates the soil pH before proceeding (Burns et al., 2013). Our data on autoclaved and combusted soils suggest that assays with common oxidase substrates detect oxidative potential of the entire soil matrix rather than just potential enzyme activity (Sinsabaugh, 2010). Minerals probably contribute to this oxidative function, particularly at neutral to alkaline pH. Therefore measured oxidation rates should not necessarily be interpreted as a metric of enzyme abundance in the soil. Overall, our findings and recommendations should help generate more reliable assays of soil oxidase activities that improve our understanding of soil carbon cycling in ecosystems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.08.022.

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