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Targeting Mn-dependent peroxidase activities - considerations and optimizations

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ABSTRACT

Estimating enzyme activity is a widely applied method for understanding the activity of organisms and their impact on biogeochemical cycles. In forest soils, fungal oxidative enzymes, such as manganese peroxidases, are key regulators of carbon stocks. Here we investigate whether MBTH/DMAB assays, targeting manganese peroxidase activities, are impacted by the degree of fungal cell disruption during extraction from pure culture. Further, we assess whether substrates 2,6-DMP, ABTS, and L-DOPA can distinguish manganese-dependent peroxidase activities under conditions optimized for MBTH/DMAB. Increased mycelial disruption during enzyme extraction increased estimated manganese peroxidase activity, but also the proportion of activity presumed to be from intracellular manganese-independent peroxidases. All substrates could detect peroxidase activities, but their specificity towards manganese peroxidases varied. In particular, ABTS was more readily oxidized by manganese-independent peroxidases. We recommend that extraction methods from soil be adapted to avoid excessive release of internal peroxidases, due to the trade-off between extraction efficiency and assay specificity.

1. Introduction

Soils store a large part of the global carbon stock (Pan et al., 2011), and insight into the processes and mechanisms that regulate turnover of organic matter in the soil is vital for better understanding of biogeochemical cycles. Soil fungi and bacteria secrete extracellular enzymes that hydrolyze or oxidize organic compounds in the soil, thus contributing to regulation of carbon stocks. Generally, hydrolytic enzymes are highly specific towards target compounds of high chemical quality (*i.e.* regular repeating polymers; Bosatta and Ågren, 1991), while oxidative enzymes are less specific and can target more complex molecules with heterogeneous structures, such as lignins (Baldrian, 2008). Due to the ubiquity of extracellular enzymes in decomposition processes, high-throughput and comparably cheap enzyme assays have been developed to estimate enzyme activities. Enzyme assays are used to describe activities of microbial communities and explore links to carbon and nutrient dynamics (Sinsabaugh et al., 2002) and in particular several studies have found links between fungal communities and oxidative enzyme activity (Šnajdr et al., 2008; Bödeker et al., 2014; Entwistle et al. 2017, 2018; Kyaschenko et al., 2017a; Sterkenburg et al., 2018; Kranabetter et al., 2021; Pérez-Izquierdo et al., 2021; Jörgensen et al., 2022) with implications on the size and turnover of the soil carbon stock (Kyaschenko et al., 2017b). Because of the high diversity of enzymes, both intracellular and extracellular, it is important that assays can reliably target specific enzymes of significance in biogeochemical transformations (Nannipieri et al., 2018).

There are multiple classes of oxidative enzymes, including peroxidases (EC 1.11.1). These can be produced both intra- and extracellularly by a diverse array of soil organisms and utilize peroxides as electron acceptors. Yet, only a subset of fungi, within the class Agaricomycetes, secrete a range of ligninolytic class-II peroxidases (Floudas et al., 2012), such as manganese peroxidases (EC 1.11.1.13), lignin peroxidases (EC 1.11.1.14), and versatile peroxidases (EC 1.11.1.16). Manganese peroxidases are exclusively extracellular and oxidize Mn^{2+} to Mn^{3+}, with hydrogen peroxide (H_2O_2) as an electron acceptor. Mn^{3+}, in turn, can oxidize a wide variety of substrates including phenolic and other aromatic compounds, non-specifically (Kirk and Farrell, 1987). Exchangeable manganese has been implicated as one of the most important regulators of carbon stocks in boreal and temperate forest soils (Stendahl et al., 2017; Kranabetter, 2019; Zhang et al., 2024), suggesting that manganese-dependant oxidation of organic compounds and the fungi that produce them are fundamental to our understanding of soil carbon

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storage.

Most assays of oxidative enzymes are colorimetric and estimate potential enzyme activity by utilizing substrates that produce dyes when they are oxidized by an enzyme. By measuring the difference in dye formation (absorbance at a substrate specific wavelength) between samples incubated with or without H_2O_2 in the reaction, peroxidase activity is distinguished from that of phenol oxidases (such as laccases EC 1.10.3.2). To further distinguish manganese-dependent peroxidase activity, assays can be adapted to exploit that manganese peroxidases rely solely on oxidation mediated through Mn^{3+} (Glenn and Gold, 1985; Baldrian et al., 2000; Arnstadt et al., 2016a). Manganese-dependent peroxidases can thus be targeted by comparing rates of product formation between reactions where a manganese source is present, or where free manganese ions are inhibited through the addition of a chelator. This approach, with inclusion of $MnSO_4$ or EDTA, has previously been used for methods based on the coupled oxidation of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) and 3-(dimethylamino)benzoic acid (DMAB; Ngo and Lenhoff, 1980; Daniel et al., 1994), and for 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Arnstadt et al., 2016a). Similar adaptations for 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) and 2,6-dimethoxyphenol (2,6-DMP) have not been developed. Comparisons of enzyme assay substrates has largely been focused on ABTS and L-DOPA (Sinsabaugh, 2010; Bach et al., 2013), with very few studies including MBTH/DMAB or 2–6,DMP (Castillo et al., 1994; García-Esquivel et al., 2021).

Typically, enzyme assays are done in buffer solutions under optimal pH and temperature conditions, with non-limiting concentrations of reagents to maximize the assay efficiency. Since H_2O_2 is crucial for the functioning of peroxidases, we test whether the concentration of H_2O_2 in the reaction affects reaction rates. Moreover, samples are often finely homogenized when doing assays, which may release "stabilized" enzymes that were chemically or spatially immobilized (Wallenstein and Weintraub, 2008; Buckley et al., 2019). Thus, one important caveat is that these assays measure "potential activities" of enzymes and are likely to overestimate the actual enzyme activities (Wallenstein and Weintraub, 2008; Nannipieri et al., 2012). In addition to the release of stabilized extracellular enzymes, the buffer choice and homogenization process could also release intracellular enzymes (Nannipieri, 2006). Despite this issue often being mentioned in papers that review enzyme assay protocols (Nannipieri et al., 1996; Nannipieri, 2006; Fornasier et al., 2011; German et al., 2011), there has been little investigation into the extent that sample homogenization method influences enzyme activity. Therefore, we assess the potential interference by the presence of intracellular peroxidases released during cell lysis by measuring enzyme activity from pure culture of a manganese peroxidase-producing saprotrophic fungus at different levels of mechanical intensity during extraction. Finally, we evaluate whether the inclusion of $MnSO_4$ or EDTA to specify manganese-dependent peroxidases could also be used under the conditions optimized for MBTH/DMAB with the substrates 2, 6-DMP, ABTS, and L-DOPA by measuring activities of horseradish peroxidase and a commercial manganese peroxidase, and from pure culture of a saprotrophic fungus.

We expected that (i) higher extraction intensity would increase the estimated enzyme activities in extracts from a cultured saprotrophic fungus. Further, we expected that (ii) fungal cell lysis due to mechanical disruption of living mycelium would release internal peroxidases, thus increasing the proportional contribution of manganese-independent peroxidases to total peroxidase activity. We also expected that (iii) all substrates could be used to distinguish between manganese-dependent and -independent peroxidase activity. Specifically, we expected that the manganese peroxidase would oxidize the substrates *only* when manganese was present, while manganese-independent peroxidases would oxidize the substrates regardless of manganese presence.

2. Materials and methods

2.1. Enzyme solutions

Recombinant manganese peroxidase from *Phanerochaete chrysosporium* (CAS: 114995-15-2, Sigma-Aldrich, Burlington, MA, USA) was dissolved in ultrapure water and diluted to a stock concentration of 1 U ml^{-1}. Horseradish peroxidase (CAS: 9003-99-0, Sigma-Aldrich, Burlington, MA, USA) was dissolved and diluted in ultrapure water to a stock concentration of 1000 U ml^{-1}. Both stock solutions were further diluted to be used in the assays (two concentrations): manganese peroxidase diluted to 0.008 and 0.005 U ml^{-1}; and horseradish peroxidase diluted to 0.4 and 0.1 U ml^{-1}.

Peroxidases were extracted from pure cultures of *Hypholoma fasiculare* (isolate: MUCL 047611), which was selected as a representative of a filamentous manganese peroxidase-producing decomposer that does not secrete manganese-independent peroxidases (Ruiz-Dueñas et al., 2021). The cultures were grown in 9.6 cm petri dishes with *ca.* 2 g of dried and autoclaved *Pinus sylvestris* L. needles (that were collected when senesced but still attached to branches) and 25 ml of liquid Modified Melin-Norkrans medium (Marx, 1969) for 7 weeks in darkness at 20 °C. We aimed to create a gradient in extraction intensity by adding *ca.* 30 ml of 50 mM sodium acetate buffer (pH 5) to the petri dishes and agitating the culture to different degrees using three different methods. The first level was "soaking" by adding extraction buffer and leaving the culture undisturbed for *ca.* 1 min. The second level was "shaking" where extraction buffer was added and the Petri dish was gently shaken on a vortex at setting 7 (Scientific Industries Vortex-Genie2, Fisher Scientific, Hampton, NH, USA) for *ca.* 1 min without creating visible disruptions to the mycelium. The rationale for this treatment was that it would increase the contact between extraction buffer, mycelium and needles without damaging the mycelium. The final level was "fine homogenization" where the culture was thoroughly homogenized with extraction buffer using a dispersing mixer (T 25 digital ULTRA-TURRAX, IKA--Werke GmbH & Co. KG, Staufen, Germany) for *ca.* 1 min. There were three biological replicates per treatment (soaking, shaking, and homogenization). All enzyme extracts were diluted to a final volume of 50 ml with sodium acetate buffer. A schematic of the experimental set up is shown in Fig. S1.

2.2. Assay procedure

Substrate solutions were prepared in a 5:5:2:2 volumetric ratio with 100 mM sodium lactate buffer (pH = 4.5), 100 mM sodium succinate buffer (pH = 4.5), 1 mM $MnSO_4$ *or* 2 mM EDTA and a colorimetric substrate: 20 mM ABTS, 25 mM L-DOPA, 5 mM 2,6-DMP, *or* 1 mM MBTH/50 mM DMAB (1:1 volumetric ratio). All chemicals were purchased from Sigma-Aldrich (Burlington, MA, USA).

Assays were run with four technical replicates with 50 μl of enzyme solution made from either 10 μl of the diluted commercial enzymes and 40 μl of 50 mM sodium acetate buffer (pH = 5), or 50 μl of the *H. fasiculare* enzyme extract with 140 μl of substrate solution (with or without $MnSO_4$). Extracts from *H. fasiculare* were centrifuged at 2500 rpm for 5 min, and only the supernatant used in assays to avoid interference by mycelia. Either 10 μl of 2.5 mM H_2O_2 (0.125 mM in final reaction) or an additional 10 μl of sodium acetate buffer (to control for non-peroxidase activity) were added to the reaction (Table S1). The H_2O_2 concentration was optimized based on the rate of coupled MBTH/DMAB formation with two concentrations of manganese peroxidase and a range of H_2O_2 concentrations (2.5 mM; 0.5 mM; 0.25 mM; 0.125 mM; 0.063 mM; 0.038 mM, 0.019 mM, 0 mM). Samples were incubated at room temperature in transparent, flat bottomed 96 well plates (Immunoplate, unbinding, SPL Life Sciences, Seoul, Korea) for 30 min in an fluorescence/absorbance spectrophotometer (Tecan Spark, Tecan Group Ltd. Männedorf, Switzerland) with absorbance measured every 30 s at 420 nm (ABTS), 450 nm (L-DOPA), 468 nm (2,6-DMP) or 590 nm

(MBTH/DMAB).

To enable comparison between substrates, and enzymes, standard curves were prepared using Mn(III) acetate to convert absorbance at given wavelengths to Mn^{3+} equivalents in μmol amounts. Separate standard curves were prepared for each enzyme assay substrate. Mn(III) acetate was prepared in 50 mM sodium malonate (pH 4.5) immediately before assay. The standard curves were established with the same reagents and reaction conditions as all other enzyme assays. Dye formation was immediate and substrate-specific absorbance for each concentration of the Mn(III) acetate gradient was measured at a single time-point.

2.3. Data handling

Absorbance at each time point of the 30 min incubation was calculated as the average absorbance of the four technical replicates. To test the effect of extraction intensity on the rate of manganese-independent peroxidase activity, the proportion of activity in reactions without $MnSO_4$ out of the total peroxidase activity (with H_2O_2 present) was calculated for all extraction methods and substrates. Reaction rates were calculated based on the initial linear phase of the incubation, between 0 and 10 min. The data were checked for inconsistency in the linear reaction and measurements that were >1.5 standard deviations from the median of technical replicates were removed. One-way ANOVAs were used to assess whether there were differences between the different extraction methodologies in 1) the proportion of manganese-independent enzyme activity and 2) the reaction rate of manganese-dependant enzyme activity. Tests were done for each substrate separately. To test the sensitivity of the substrates, one-way ANOVAs were used to evaluate whether the reaction rate of manganese-dependent enzyme activity varied between the different substrates, for each extraction method.

Data handling was done using the Tidyverse package (Wickham et al., 2019) and figures were drawn using ggplot2 (Wickham et al., 2020) and patchwork (Pedersen, 2022).

3. Results

Addition of different concentrations of H_2O_2 affected the rate of product formation when using the MBTH/DMAB assay with pure manganese peroxidase from *P. chrysosporium* (Fig. 1). We observed inhibited product formation when H_2O_2 concentration exceeded 0.125 mM in the reaction. The same optimal H_2O_2 concentration was observed for both concentrations of manganese peroxidase.

The rate of manganese-peroxidase activity, *i.e.* the extraction efficiency, increased with more intense extraction of *H. fasiculare* grown on pine needles (ANOVA; F-value = 23.12; df = 2; p = 0.002; Fig. 2A–C), The average proportion of manganese-independent enzyme activity was over 15-fold higher in the thoroughly homogenized extract (0.27 ± 0.03) in comparison to the soaking treatment (0.017 ± 0.01) and 2-fold higher than in comparison to the shaking treatment (0.09 ± 0.03; ANOVA; F value = 64.52; df = 2; p = <0.001; Fig. 2D). Similar indications of release of intracellular peroxidases with fine homogenization were also observed for 2,6-DMP and L-DOPA, but ABTS had a high proportion of manganese-independent enzyme activity regardless of extraction intensity (Fig. S2 and S3A; Table S2). Further, the rate of manganese-dependant enzyme activity varied between substrates, with estimated activity being higher for MBTH/DMAB than the other substrates (Fig. S3B, Table S3).

Manganese peroxidase from *P. chrysosporium* oxidized MBTH/DMAB, L-DOPA, and 2,6-DMP at a rate at least two orders of magnitude higher in the presence of manganese than with EDTA (Table 1, Fig. S4). ABTS was oxidized both in the presence and absence of manganese, but more so with manganese. Horseradish peroxidase oxidized all substrates regardless of presence of manganese.

4. Discussion

When performing the assays to measure potential enzyme activities, concentrations of reagents in the assay are optimized to not be limiting, *i.e.* the rate limiting step should be the enzyme concentration in the extract. We observed that the highest H_2O_2 concentrations, and even a H_2O_2 concentration just 2x the optimal condition, reduced the initial rate of activity and the end-point absorbance (Fig. 1). Presumably, too high a concentration of H_2O_2 may damage the enzyme or reduce Mn^{3+} back to Mn^{2+} before it oxidizes the substrate (Wariishi et al., 1992). At the same time, concentrations below the optimum may have caused depletion of the H_2O_2 and thus reduced the end-point absorbance of the assay. Although this test was done in a pure enzyme solution, the optimal concentration of 0.125 mM falls within the range mentioned in other studies that assay peroxidases in pure culture and environmental samples (0.010–0.250 mM; Table S4). Still, it is advisable to optimize concentrations when assaying potential peroxidase activities.

Increased mechanical disruption of *Hypholoma fasiculare* mycelia grown on pine needles increased the rate of manganese peroxidase activity, suggesting that insufficient extraction can result in underestimation of potential manganese peroxidase activity. It is likely that the hydrophobic mycelium made the efficiency of the soaking treatment low

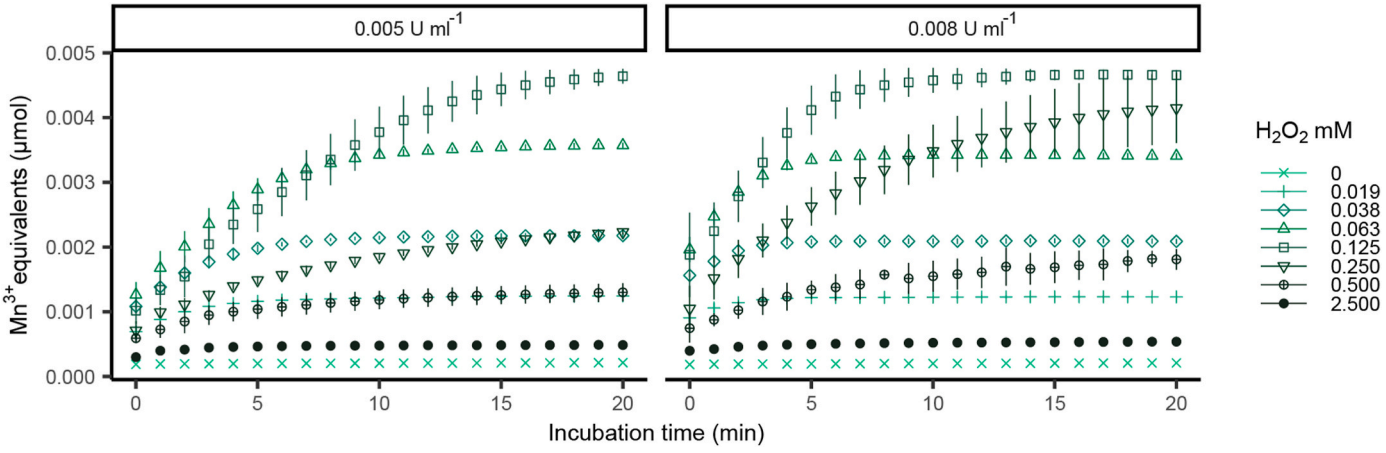

Fig. 1. Mn^{3+} equivalents (μmol) formed over time in reactions with manganese peroxidase from *Phanerochaete chrysosporium* at two concentrations (0.005 U ml^{-1} and 0.008 U ml^{-1}) with varying H_2O_2 concentrations added to initiate the reaction. Points represent average absorbance of four technical replicates, with lines indicating standard deviation of the mean, colors/shapes correspond to different concentrations of H_2O_2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

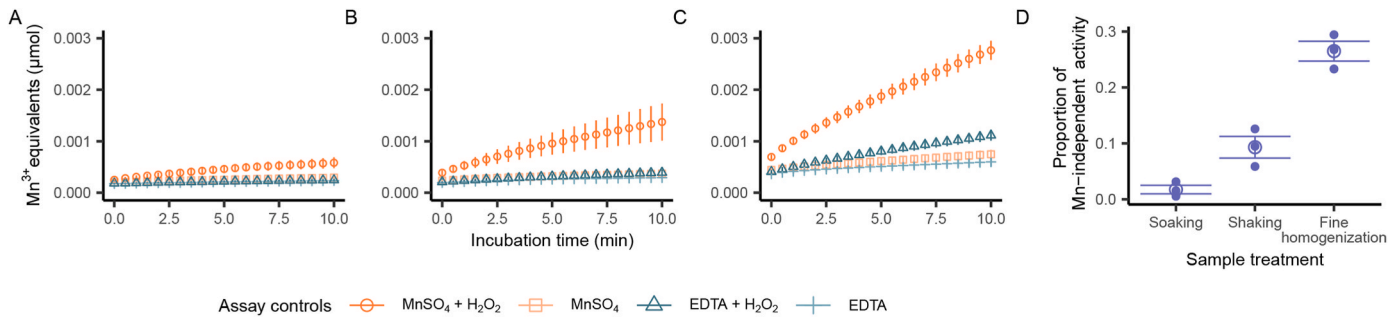

Fig. 2. Peroxidase activities (Mn^{3+} equivalents (µmol) formed per min) in cultures of *Hypholoma fasiculare* grown on sterilized senesced pine needles, assayed with MBTH/DMAB. Enzymes were extracted in 50 mM sodium acetate buffer at different extraction methodologies; (A) soaking the mycelium, (B) gently shaking the mycelium on a vortex (no visible tears in the mycelium) or (C) fine homogenization of mycelium and needles with a dispersing mixer. Points represent average absorbance of three biological replicates with four technical replicates with lines indicating standard deviation of the mean. (D) The proportion of Mn-independent activity relative to total peroxidase activity at the varying levels of extraction intensity of *Hypholoma fasiculare* grown on pine needles in pure-culture. Hollow coloured points represent means of replicates with standard error bars (n = 3).

Table 1
Reaction rates (Mn^{3+} equivalents formed (µmol) per min) of commercial manganese peroxidase (MnP) and horseradish peroxidase (HRP) with four different substrates in the presence of either $MnSO_4$ or EDTA. Reaction rates are calculated as the average slope of four technical replicates within the first 5 min of the reaction being initiated by addition of 0.125 mM H_2O_2.

		Reaction rate (µmol/min)	
		$MnSO_4$	EDTA
MBTH/DMAB	MnP	2.32×10^{-04}	5.61×10^{-06}
	HRP	1.67×10^{-04}	1.71×10^{-04}
ABTS	MnP	2.03×10^{-05}	1.10×10^{-05}
	HRP	7.24×10^{-04}	8.05×10^{-04}
L-DOPA	MnP	3.43×10^{-04}	2.00×10^{-06}
	HRP	9.16×10^{-06}	3.87×10^{-06}
2,6-DMP	MnP	1.14×10^{-04}	8.20×10^{-08}
	HRP	3.19×10^{-06}	2.95×10^{-06}

since the extraction buffer barely came in contact with the needles, where one would expect the highest peroxidase activity (Keiluweit et al., 2015). The shaking treatment allowed for more contact between needles and extraction buffer, while fine homogenization allowed for full contact between needles and extraction buffer. While enzyme activities measured from soaked and shaken samples were low, they only captured manganese-dependent oxidation of the substrate, as indicated by a positive reaction rate only when $MnSO_4$ was present (Fig. 2A–B).

However, in the more thoroughly homogenized extract, product formation also occurred in the absence of $MnSO_4$, suggesting that disruption of the mycelium released intracellular manganese-independent peroxidases into the extract (Fig. 2C). The potential release of intracellular peroxidases during sample preparation could contribute to the total measured peroxidase activity and be misinterpreted as extracellular peroxidase activity. Hence, there seems to be a trade-off between extraction efficiency and release of internal peroxidases. However, the degree to which this trade-off is important in more complex samples (e.g. soil samples) is still uncertain, since mycelial densities may vary widely on small spatial scales. Release of intracellular peroxidases could potentially be more of a concern when biomass of soil organisms is high as it tends to be in organic horizons or in soils with high organic matter content (Bastida et al., 2021). Currently, the standard methods for measuring manganese peroxidase activities from soil are based on intensively homogenized "soil slurries" (Daniel et al., 1994; Saiya-Cork et al., 2002) and it is likely that intracellular peroxidases from a range of soil organisms are released during sample preparation (Nannipieri, 2006). Therefore, it would be advisable to interpret total peroxidase activities carefully, since some of the measured enzymes may not originate from the soil matrix, and this may lead to overestimation of the potential for oxidative decomposition in soil samples. Manganese peroxidases are, however, *only* extracellular and the manganese-dependent activity could, therefore, be assumed to be less affected by soil homogenization. Given its sensitivity to the lowest enzyme concentration presented here (the soaking treatment), the MBTH/DMAB assay could likely be performed without fine homogenization of samples to minimize noise from internal peroxidases. Yet, too little homogenization could result in an underestimation of potential enzyme activity, as enzymes may be occluded in soil aggregates. Regardless of substrate choice, extraction methods should be selected based on what is of main interest in a given study and then standardized across samples to avoid variation due to different extraction intensities.

When using the same assay conditions as optimized for MBTH/DMAB, both 2,6-DMP and L-DOPA were oxidized by manganese peroxidases from *P. chrysosporium,* only in the presence of $MnSO_4$, but neither were readily oxidized by horseradish peroxidase (Fig. S4). In contrast, ABTS was readily oxidized by horseradish peroxidase but seemed comparably stable against oxidation by manganese peroxidase (Fig. S4). ABTS has a high redox potential relative to the other substrates (Bach et al., 2013), suggesting that although it can be oxidized by both enzymes, the ABTS radical is susceptible to being reduced back to its colourless state. ABTS assays have been adapted to target manganese peroxidases in coarse woody-debris samples (Arnstadt et al. 2016a, 2016b), but this did not work with our assay conditions. However, other studies have also reported limitations of ABTS as a substrate, including interference with tannic acid (Terrón et al., 2004) and unsuitability for alkaline soils (Bach et al., 2013). Further, Glenn and Gold (1985) noted that ABTS was more readily oxidized by horseradish peroxidase than by manganese peroxidases from *P. chrysosporium*. We observed a high proportion of manganese-independent enzyme activity from extracts of needle cultures, suggesting ABTS could be oxidized directly by manganese peroxidase or by redox mediators other than manganese. This issue is likely more of a concern in soil samples which may be more chemically complex than the fungal-needle cultures used in this experiment. Given these observations, it seems reasonable to conclude that ABTS is not an ideal substrate for measuring manganese peroxidases in soil, but may still be suitable for estimating phenol-oxidase activity (Floch et al., 2007).

While there are apparent limitations of enzyme assays (more thoroughly addressed in German et al., 2011; Nannipieri et al., 2018 and references therein) they are still informative and widely used, since alternative methods, such as microdialysis or other means of sampling soil solution in pore spaces *in situ* are less developed and mainly focus on small spatial scales (Buckley et al., 2019; Levakov et al., 2021). We, therefore, urge awareness of the effects of sample preparation, substrate choice, and H_2O_2 concentration when conducting substrate-based enzyme assays and for thorough reporting of modifications made to assay protocols during optimization. This would help improve our

interpretations of the relationship between potential enzyme activities and biogeochemical processes, and lead to better estimations of the contribution of fungal peroxidases to carbon cycling.

CRediT authorship contribution statement

E.E. Packard: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **K. Jörgensen:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2025.101492.

Data availability

Data and code to reproduce the results of this study will be available on Dryad and Zenodo upon acceptance. Until then, it is available for review on GitHub (https://github.com/KMJorgensen/PeroxidaseAssayTest).

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