An improved Amplex Red-based fluorometric assay of phenol oxidases and peroxidases activity: A case study on Haplic Chernozem

Sajedeh Khosrozadeh1,2 | Maxim Dorodnikov3,4 | Thomas Reitz1 | Evgenia Blagodatskaya1

1Department of Soil Ecology, Helmholtz-Centre for Environmental Research – UFZ, Halle (Saale), Germany
2Department of Soil Science, College of Agriculture, Isfahan University of Technology, Isfahan
3Department of Soil Science of Temperate Ecosystems, Georg August University of Göttingen, Göttingen, Germany
4Department of Biogeochemistry of Agricultural Ecosystems, Georg August University of Göttingen, Göttingen, Germany

Abstract
The initial steps of complex organic matter degradation in soil comprise several oxidative processes catalysed by phenol oxidases and peroxidases. Commonly, their activity is determined by colorimetric approaches based on a range of substrates, making comparison between studies difficult. To improve this shortcoming, we conducted a calibration procedure for assessing phenol oxidase and peroxidase activities in soils by a sensitive fluorometric assay. This assay is based on the colourless and non-fluorescent Amplex Red® reagent as a substrate, which forms a fluorescent product, resorufin, upon oxidation. Commercially available resorufin was used as a standard for calibration. We applied Michaelis–Menten kinetics to determine functional traits of phenol oxidases and peroxidases in a case study on Haplic Chernozem from different land-use and climate treatments of a field experiment. The kinetic parameters, limit of detection and limit of quantification demonstrated a high sensitivity of the fluorometric assay to differences caused by these treatments. For the total oxidative enzymes, the catalytic efficiency (Kcat, calculated as Vmax/Km) was 30% higher in the cropland compared with the pasture plot. However, compared with soil samples collected from ambient climatic conditions, Kcat of total oxidoreductases was 60% lower in cropland exposed to elevated air temperature and drought. The phenol oxidase activity was substantially higher than peroxidase activity in cropland land use. However, higher Kcat of peroxidase than phenol oxidase resulted in 60% faster substrate turnover under both climatic conditions. In our case study, the Amplex Red-based assay enabled rapid and high-throughput analyses of phenol oxidase and peroxidase activity in soil. The sensitivity of this assay for soil enzymology applications needs further testing for a larger range of soils and environmental conditions.
1 | INTRODUCTION

Soil microbial communities decompose organic residues and soil organic matter (SOM) to satisfy their nutrient and energy demands by producing diverse extracellular hydrolytic and oxidative enzymes (Dick & Burns, 2011; Kandeler, 2007; Nannipieri et al., 2002). The decomposition of complex organic compounds begins with oxidation processes catalysed by multiple oxidoreductases, such as phenol oxidases and peroxidases (Burns et al., 2013; Sinsabaugh, 2010). Thus, these enzymes are key mediators in carbon (C) and nutrient cycling (Billings & Ballantyne IV, 2013; Dwivedi et al., 2019).

Phenol oxidases catalyse the oxidation of phenolic compounds using molecular oxygen (O₂) as an electron acceptor. Fungal laccases are the most studied phenol oxidases in soil (Burns et al., 2013). Peroxidases are enzymes that use hydrogen peroxide (H₂O₂) as an electron acceptor, and function under wide range of redox conditions. Lignin peroxidases and manganese peroxidases are prevalent in terrestrial ecosystems (Bach et al., 2013; Burns et al., 2013). In soil, phenol oxidase activity is typically measured as the rate of substrate oxidation in a soil suspension in the absence of H₂O₂ (Bach et al., 2013; Burns et al., 2013; German et al., 2011). In contrast, the cumulative activity of both phenol oxidases and peroxidases is determined in the presence of H₂O₂. The difference between both assays, that is, with and without H₂O₂, represents the net peroxidase activity (Burns et al., 2013; Sinsabaugh, 2010).

Despite the central role of oxidoreductases in decomposition processes in soils, environmental studies have so far focused on the activity of soil hydrolases, mainly due to the higher complexity of reactions catalysed by oxidoreductases along with their challenging quantitative assessment (Bach et al., 2013; German et al., 2011). The activity assays most frequently applied for a wide range of soil hydrolytic enzymes are either colorimetric using p-nitrophenol (pNP)-linked substrates (Burns et al., 2013; German et al., 2011) or fluorometric using fluorescent dye-conjugated substrates e.g., 4-methylumbelliferone (MUF) and 7-amino-4-methyl coumarin (AMC) (Marx et al., 2001). The main advantages of using fluorogenically-labelled substrates are as follows: (i) extraction and purification of the reaction products are unnecessary since they can be directly measured in a soil suspension; (ii) fluorescence measurements are very sensitive, enabling quantification and comparison of the activities of enzymatically released products at low concentrations; and (iii) various reactions can be standardised by using substrates coupled to the same fluorescence dye (e.g., MUF or AMC). In contrast, colorimetric approaches for determination of phenol oxidase and peroxidase activities are more complicated due to the free-radical, non-specific nature of the oxidation reaction under variable redox conditions, complex interactions between enzymes, assay substrates and the soil matrix (Bach et al., 2013; German et al., 2011; Sinsabaugh, 2010).

The methodology of current phenol oxidase and peroxidase activity assays differ in the type of substrates, assay conditions and incubation time. Commonly used substrates to measure the activities of phenol oxidase and peroxidase include: (1) Some substrates (e.g., PYGL and ABTS) are restricted to a narrow acidic pH range and differ in their redox potential, making them difficult to use in...
neutral and alkaline pH soils (Bach et al., 2013). (2) There may be inconsistencies between activities obtained from the oxidation of different substrates in the same soil (Bach et al., 2013), which are linked to different substrate-specific optimal oxidation conditions (e.g., pH). (3) The chemical nature of some substrates prevents targeting both phenol and peroxidase activities. For example, TMB shows slow oxidation in the absence of H₂O₂, suggesting that TMB is not an appropriate substrate for phenol oxidase (Johnsen & Jacobsen, 2008). (4) There may be a lack of suitable standards for calibration. For instance, the oxidised products of L-DOPA, TMB and ABTS are not commercially available. Instead, calibration is based on the complete transformation of L-DOPA and ABTS to the oxidised products using purified laccases or horseradish peroxidases. After filtration and enzyme removal, the respective product is used for calibration by a series of dilutions (Floch et al., 2007; German et al., 2011). However, estimation of the amount of product and its stability in time requires further investigation. (5) For some substrates, for example, L-DOPA and PYGL, a relatively long incubation period (3–4 h) is necessary, whereas an assay based on TMB and ABTS requires less than 20 and 5 min, respectively (Bach et al., 2013; Floch et al., 2007; Johnsen & Jacobsen, 2008). In addition, measurements of phenol oxidase and peroxidase activity by a colorimetric approach based on PYGL and L-DOPA failed to reveal Michaelis–Menten kinetics (Sinsabaugh, 2010) due to their ability to cleave non-specific linkages of substrates and mediate free-radical reactions requiring specific cofactors (German et al., 2011). However, kinetic parameters (\(V_{\text{max}}\) and \(K_m\)) of phenol oxidases and peroxidases were successfully estimated using ABTS (Floch et al., 2007) and TMB (Triebwasser-Freese et al., 2015) as substrates.

Consequently, there is a need for developing a suitable method and substrate for the precise and high-throughput determination of phenol oxidase and peroxidase activities in soils. Medical studies have reported an Amplex Red (10-acetyl-10H-Phenoxazine-3,7-diol; ADHP) based fluorometric assay for the estimation of peroxidase activity and H₂O₂ concentration in cells and tissues (Zhao et al., 2012). The applicability of Amplex Red for soil analyses has been partly confirmed for laccase activity in the absence of H₂O₂ addition (Wang et al., 2017). However, there are still knowledge gaps regarding the applicability of Amplex Red assay, that is, whether it can be used to determine phenol oxidase and peroxidase activities in soil simultaneously, and be sensitive enough to detect differences in activity caused by known external drivers, such as land use and climate. Thus, we developed an improved microplate assay to determine phenol oxidase and peroxidase activities in soil based on the oxidation of the colourless and non-fluorescent Amplex Red substrate to coloured, fluorescent resorufin (Figure 1). We studied whether the Amplex Red-based fluorometric assay followed Michaelis–Menten kinetics and how sensitive were the kinetic parameters of these enzymes to modelled land-use and climatic changes. The novelty of the current study is in unifying the calibration and reporting of kinetic parameters of peroxidases and phenol oxidases in soil. The establishment of a rapid and sensitive assay of phenol oxidase and peroxidase activities in the soil will strongly enhance the comparability of oxidoreductase activities in environmental studies.

2 | MATERIALS AND METHODS

2.1 | Soil and treatment selection

To evaluate the assay, we used soil samples of the Global Change Experimental Facility (GCEF), which is a large field experiment located at the field research station of the Helmholtz Centre for Environmental Research, Bad Lauchstädt, Halle (Saale), Saxony-Anhalt, Germany (51°22′60 N, 11°50′60E). The prevailing soil type is a Haplic Chernozem (total C 2.06%, total N 0.18%, pH (KCl) 7.3, pH (CaCl₂) 7.5; Altermann et al., 2005).

The GCEF comprises five land-use types that are exposed to ambient and future climatic conditions (Schädler et al., 2019). For our purpose, we selected contrasting treatments with respect to climate and land use: (i) organic farming (here referred as ‘cropland’) soil collected from plots with ambient and future climate. Future climate conditions include higher average temperatures and more intensive summer drought compared with the ambient climate (Schädler et al., 2019). (ii) We also collected soil from extensive pasture (“pasture”) under ambient climate to compare the cropland and grassland under the same climate. Pasture grassland is characterised by a great plant species diversity and represents a less disturbed agroecosystem than croplands with its typical measures, including crop rotation, soil cultivation and fertilisation (Schädler et al., 2019). This sample selection enabled us to test the sensitivity of the Amplex Red-based assay to contrasting land-use and climatic conditions, by comparing the oxidoreductase activities in soil samples obtained from ‘cropland under ambient climate’ with those of ‘cropland under future climate’ as well as of ‘pasture under ambient climate’. The topsoil samples (0–15 cm) were sieved (2 mm) and stored at 4°C before analyses.

2.2 | Fluorescence spectrum and calibration test

An analysis of the fluorescence spectrum of resorufin (50 μM resorufin sodium salt in 50 mM Trizma buffer;
pH 7.4) was conducted to determine the maximum excitation and emission wavelength. Excitation and emission spectra were recorded in scanning mode using a Varian Cary Eclipse fluorescence microplate reader (Agilent Inc., Santa Clara, CA).

For calibration, 1 mM stock solutions of resorufin and the resorufin sodium salt were prepared in 50 mM Trizma buffer (pH 7.4). The stock solutions of both resorufin and the resorufin sodium salt were separately diluted in Trizma buffer to 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40 and 50 μM. Because Amplex Red and resorufin are sensitive to light, all flasks were covered with aluminium foil, and all preparation procedures and enzyme measurements were conducted in a dark room and under red light (when possible) to prevent Amplex Red and resorufin photo-oxidation.

2.3 Phenol oxidase and peroxidase activities assay

An Amplex Red stock solution was prepared by dissolving 25 mg Amplex Red in dimethyl sulfoxide (DMSO, Zhou et al., 1997; Wang et al., 2017), and then deionised water was added to obtain a final concentration of 50 mM Amplex Red. To prevent multiple freeze–thaw cycles, 100 μl aliquots of the stock solution were pipetted into separate micro-Eppendorf® tubes and frozen at ~20°C. Immediately before use, the thawed Amplex Red stock solution was diluted in 50 mM Trizma buffer (pH 7.4) in glass tubes to prepare a concentration series (0.01, 0.05, 0.1, 0.25, 0.5, 1 and 2 mM).

After dilution, the substrates were bubbled with inert gas (N2) in the dark for 5 min. Thereafter, the glass tube lids were closed tightly. The reaction mixture containing 50 μl soil suspension (1:20 w/w soil in water, 60 s sonication), 100 μl substrate solution with corresponding concentration and 50 μl of Trizma buffer was pipetted into microplate wells. The soil suspension was stirred while pipetting. Controls, only containing substrate and buffer were included for each microplate. For the total oxidative enzyme activity assay, all wells, including the controls, received 10 μl of 0.3% H2O2 (Figure 2 and Figure S1) while phenol oxidase activity was determined in another microplate without addition of H2O2 (Figure S1). Soil samples were measured in three replicates for each Amplex Red concentration. A standard calibration curve was recorded by replacing the Amplex Red substrate with resorufin sodium salt. A scheme of the microplate layout is shown in Figure 2. After pipetting, the microplate was wrapped in aluminium foil to minimise light exposure. The enzyme assay and resorufin standard measurements were conducted at 530 nm excitation and 585 nm emission using a Varian Cary Eclipse microplate reader (Agilent Inc., Santa Clara, CA) with FLUOTRAC™ microplates (96 well). In a pre-test, the fluorescence signal development over time (5 min steps) was determined for 60 min in order to determine the optimal incubation time. The auto-fluorescence signal of Amplex Red (Amplex Red + buffer) development was stable during the first 10 min; thereafter, the variability of the signal increased (Figure S2) indicating that multiple (more than three times) reading of the same plate affected the precision of measurement. According to the obtained results, fluorescence intensity was finally measured 10 min after substrate addition for all studied samples. The slope of a linear regression of the fluorescence intensity versus standard resorufin concentrations (1.25–10 μM, Figure 2) was used to calculate the oxidoreductase (with H2O2) and phenol oxidase (without H2O2) activities in nmol resorufin g soil−1 min−1. The peroxidase activity was estimated by subtraction of phenol oxidase activity from total oxidative activity.

All reagents were of analytical grade and purchased from Sigma–Aldrich, Merck KGaA, Darmstadt, Germany: Amplex Red (10-acetyl-10H-Phenoxazine-3,7-diol; ADHP) in the form of the commercial product called Ampliflu™ Red, resorufin sodium salt (C12H6NNaO3), resorufin (7-hydroxy-3H-phenoxazin-3-one, C12H7NO3), Trizma buffer (Trizma-base + Trizma hydrochloride), dimethyl sulfoxide (DMSO) and H2O2.

2.4 Enzyme kinetic parameters

The phenol oxidase and peroxidase kinetics were described by the Michaelis–Menten model, whereby the reaction rate (ν) was a function of substrate concentration
with a saturating pattern. The kinetic parameters ($K_m$ and $V_{\text{max}}$) were determined for each enzyme type using the Michaelis–Menten equation:

$$v = \frac{V_{\text{max}} [S]}{K_m + [S]},$$

(1)

where $v$ is the reaction rate, $V_{\text{max}}$ is the maximum reaction rate, $[S]$ is the substrate concentration and $K_m$ is the Michaelis–Menten constant, that is, substrate concentration at $\frac{1}{2}V_{\text{max}}$, indicating the enzyme affinity to a substrate (German et al., 2011). Here and below, the estimated values for $V_{\text{max}}$ and $K_m$ represent apparent parameters because enzyme adsorption to SOM and clay particles affects the measured kinetic parameters and induces changes in comparison with the purified enzyme (Marx et al., 2001; Sinsabaugh et al., 2014). $K_m$ represents the potential substrate availability and enzyme affinity to a substrate, whereas $V_{\text{max}}$ can be interpreted as the activity of extracellular enzymes in soil solution and on soil particles. $K_m$ and $V_{\text{max}}$ values were estimated by non-linear regression using the ModelMaker 3 (Cherwell Scientific., 1999). Although the kinetic parameters $K_m$ and $V_{\text{max}}$ are usually independent and weakly correlated in assays with purified enzymes, in soil suspensions, these parameters may appear to be positively interrelated due to microbial community functions or measurement methodology (Tischer et al., 2015; Triebwasser-Freese et al., 2015). Therefore, we estimated the enzyme catalytic efficiency ($K_{\text{cat}}$), which has been suggested as an eco-physiological indicator (Tischer et al., 2015), as the ratio of $V_{\text{max}}$ to $K_m$ in mmol$_{-}\text{resorufin} \cdot \mu$mol$^{-1}$ Amplex Red min$^{-1}$. The turnover time ($T_t$) of the substrates was calculated at $S = K_m/10$ from the following equation: $T_t (<\text{min}) = (K_m + S)/V_{\text{max}}$ (Tischer et al., 2015).

Sensitivity of the method was described with the limit of detection and limit of quantification. The limit of detection (LOD) is defined as the smallest concentration of product that can be reliably detected. The limit of quantification (LOQ) is the lowest concentration that can be quantitatively measured. Calculation of these parameters is based on the standard deviation of the y-intercept ($\sigma$) and on the slope (S) of the calibration line (International Conference on Harmonisation, 2005):

$$\text{LOD} = \frac{3.3 \sigma}{S}; \quad \text{LOQ} = \frac{10 \sigma}{S}.$$
considered as dependent values. The error propagation was calculated when the mean values were used for determining peroxidase activity, as well as $K_{\text{cat}}$ and $T_t$ values (Meyer, 1975).

The effects of land-use (cropland vs. pasture) and climatic conditions (ambient vs. future) on kinetic parameters of total oxidative and phenol oxidase, and peroxidase were analysed with Cohen’s ANOVA (Huck & Malgady, 1978) based on mean values and their standard deviations.

Cohen’s ANOVA is based on comparison of mean square errors of the variables:

\[
\text{MS}_{\text{error}} = \frac{\sum_{i=1}^{N} (\text{SD}_i)^2}{n},
\]

where $\text{MS}_{\text{error}}$ is mean square of error within treatments, $n$ is the number of treatments, and SD is the standard deviation. Sum of mean squares (MSS) between treatments was calculated using Equation (3).

\[
\text{MSS} = \frac{n \sum_{t=1}^{N} (Y_{t0} - Y_{00})^2}{t - 1},
\]
where $n$ is number of replicates, $t$ is the number of treatments, $Y_{i0}$ is the mean value of each treatment, $Y_{00}$ is total mean value.

The $F$ ratio was obtained by dividing the sum of means squares (MSS) by the mean square of error (MS$_{error}$). The calculated $F$ ratio of Cohen’s-ANOVA was compared with Tables of the $F$ Distribution at 0.05, 0.01 and 0.001 levels for significance determination (Beyer, 2017). All figures were prepared with R version 4.0.3 (R Development Core Team, 2020) in R Studio.

3 | RESULTS

3.1 | Resorufin spectrum and calibration

The fluorometric analysis of resorufin showed a sharp excitation spectrum with a maximum at 572 nm and a shoulder at $\sim$530 nm (Figure 3a). The emission maximum was at 585 nm (Figure 3b). Since the excitation maxima at 572 nm overlapped with the emission spectrum and considering that the wavelength filters for the plate reader usually exhibit bandwidths of 10–35 nm, we
selected 530 nm for excitation and 585 nm for emission in the assays.

The fluorescence signal of the Amplex Red oxidation product increased rapidly during the first 10 min, then slowed down between 10 and 25 min and increased linearly thereafter (Figure 4a). We chose the first 10 min as the optimum incubation time to minimise the effect of photo-oxidation of Amplex Red (see Section 4).

The fluorescent intensity of the resorufin sodium salt increased linearly within the concentration range 0–20 μM (Figure 4b, black dashed line), but thereafter the fluorescent signal followed a saturation pattern with increasing concentration (Figure 4b, blue squares). In contrast to the sodium salt, the fluorescence of resorufin followed a linear correlation over a narrower range of concentrations (0–10 μM) and had a non-linear pattern afterward (Figure 4b, brown circles). Thus, the concentration range showing a linear increase for resorufin (0–10 μM) should be used for calibration.

### 3.2 Phenol oxidase and peroxidase activity

The calculated LOD and LOQ values of Amplex Red assay were 1.03 and 3.12 nmol resorufin g<sub>soil</sub>−1 min<sup>−1</sup>, respectively. Enzyme activities in the presence of H<sub>2</sub>O<sub>2</sub> were below the LOQ for the two lowest substrate concentrations (0.4 and 2 μmol Amplex Red g<sub>soil</sub>−1), while in the absence of H<sub>2</sub>O<sub>2</sub>, enzyme activities were below the LOQ for the three lowest substrate concentrations (0.4, 2 and 4 μmol Amplex Red g<sub>soil</sub>−1). Therefore, after excluding these Amplex Red concentrations from the data set, a further fitting to the Michaelis–Menten equation was performed (Table S1). Both full and reduced data sets of total oxidative and phenol oxidase activities fitted well to Michaelis–Menten kinetics (Table S1).

The activity of phenol oxidase and peroxidase fitted well to Michaelis–Menten kinetics. The total oxidative and peroxidase activities were higher in the soil from pasture compared with that from cropland. In contrast, phenol oxidase activity in the cropland was slightly higher than in the pasture plot (Figure 5a). The V<sub>max</sub> value of peroxidase in the pasture was nearly 2.5 times higher than in the cropland plot, whereas the difference in V<sub>max</sub> of phenol oxidase between land-use types was small but still significant (Figure 5b, Table S2). Similarly, K<sub>m</sub> value of peroxidase was 4.3 times higher in soil from pasture than in that from cropland (Figure 5c, Table S2). The K<sub>m</sub> value of phenol oxidase was 10% higher in pasture as compared with cropland. The catalytic efficiency (K<sub>cat</sub>) of total oxidative enzymes, peroxidase and phenol oxidase was nearly 1.3, 2 and 1.2 times higher in pasture than in cropland plot, respectively (Table 1).

The comparison of the soil samples obtained from the different climate treatments indicated a significant reduction of phenol oxidase and peroxidase activities in the cropland exposed to future climatic conditions compared with that exposed to ambient climate (Figure 6a). V<sub>max</sub> of total oxidative, phenol oxidase and peroxidase activities was 1.4, 1.3 and 1.8 times higher in the samples obtained from ambient than those obtained from future climatic conditions, respectively (Figure 6b, Table S3). K<sub>m</sub> for the total oxidative enzymes and phenol oxidase were 44%–33% lower, respectively, in the cropland from ambient than from future climate plots. Therefore, after excluding these Amplex Red concentrations from the data set, a further fitting to the Michaelis–Menten equation was performed (Table S1). Both full and reduced data sets of total oxidative and phenol oxidase activities fitted well to Michaelis–Menten kinetics (Table S1).

|                     | Cropland-ambient | Cropland-future | Pasture-ambient |
|---------------------|------------------|-----------------|-----------------|
|                     | Total oxidative  | Phenol oxidase  | Peroxidase      |
| Total oxidative     | Mean K<sub>cat</sub> | 2.07            | 0.71            | 1.65            |
|                     | Mean T<sub>t</sub> | 0.53            | 1.54            | 0.67            |
|                     | Mean K<sub>cat</sub> | 1.59            | 0.60            | 0.96            |
|                     | Mean T<sub>t</sub> | 0.69            | 1.83            | 1.15            |
| Std K<sub>cat</sub> | 0.1              | 0.01            | 0.08<sup>a</sup> | 0.05             | 0.02             | 0.04<sup>a</sup> | 0.02             | 0.03             | 0.03<sup>a</sup> |
| Std T<sub>t</sub>  | 0.03             | 0.01            | 0.02<sup>a</sup> | 0.07             | 0.13             | 0.12<sup>a</sup> | 0.01             | 0.09             | 0.07<sup>a</sup> |

Abbreviation: Std, standard deviation.

<sup>a</sup>Error propagation was calculated for peroxidase K<sub>cat</sub> and T<sub>t</sub>.
4 | DISCUSSION

4.1 | Implementation of the Amplex Red-based assay

The excitation and emission maxima of resorufin detected at 572 and 585 nm were similar to those reported for resorufin produced by pure horseradish peroxidase (Zhao et al., 2012). The resorufin fluorescence signal was detected at pH 7.4 (Figure 3), when Amplex Red is completely transformed to resorufin (Lefrançois et al., 2016). This corresponds to the pH was used for pure horseradish peroxidase assay (Zhao et al., 2012; Zhou et al., 1997) and is above the optimum pH for pure laccase, which was nearly 6.4 (Wang et al., 2017). As Amplex Red has two phenolic hydroxyl groups (Dębski et al., 2016), it occurs predominantly in the neutral form at a neutral to slightly acidic pH, whereas at alkaline pH (>8.5), the monoanionic and/or di-anionic species predominate (Dębski et al., 2016; Lefrançois et al., 2016). The low solubility of Amplex Red and partial degradation of resorufin at highly acidic conditions (Lefrançois et al., 2016) might lead to a strong decline in fluorescence and thus cause a restricted applicability of Amplex Red at pH < 5 (Bueno et al., 2002; Flamigni et al., 1989). Amplex Red is stable at neutral pH and difficult to oxidise in the
absence of oxidoreductases and high-energy light (Dębski et al., 2016). Therefore, based on appropriate calibration, it is possible to use our assay to determine oxidoreductase activities at a neutral to slightly alkaline soil pH.

As extracellular enzymes are functioning at soil pH, which does not necessarily correspond to enzymes optimum, the assays of enzymatic activity need to be adjusted to environmental conditions, particularly pH (German et al., 2011). Therefore, a comparison of oxidative activity in soils differing in pH requires resorufin-based calibrations at corresponding pH for each soil. Functional properties of purified enzymes, however, are different from those of large diversity of soil iso-enzymes, for example, laccases and other phenol oxidases with different optimum pH (Sinsabaugh, 2010). Therefore, enzymatic assays under natural pH are more realistic to mirror oxidation of organic substrates in soil after appropriate calibration (German et al., 2011). The applicability of Amplex Red at a neutral to slightly alkaline soil pH is a clear benefit in comparison to most commonly used substrates, such as TMB, PYGL and ABTS, which are not suitable for soil pH > 5 (Bach et al., 2013). Another advantage of Amplex Red is it can be used to simultaneously measure phenol oxidase and peroxidase activities, avoiding the need for various substrates commonly used in colorimetric assays (Bach et al., 2013) and allowing comparison of the oxidative activity between different soils.

The very fast enzymatic Amplex Red oxidation by soil oxidoreductases (Figure 4a) was similar to Amplex Red oxidation by purified laccase and horseradish peroxidase (Wang et al., 2017; Zhou et al., 1997). We suggest a short incubation time (e.g., 10 min) (Figure 4a), in line with that recommended for soil laccases (Wang et al., 2017). However, optimum incubation time has to be adapted for each soil. The sensitivity of Amplex Red to visible/UV light may have two opposing consequences: reduction of the fluorescence signal due to resorufin transformation to non-fluorescent dihydroresorufin (Lefrançois et al., 2016) or increase in resorufin signal due to Amplex Red photo-oxidation caused by the high-energy light source of the microplate reader during fluorescence detection (Zhao et al., 2012). This was confirmed in our time-test assay since the increase of resorufin fluorescence slowed down after 10 min and then further increased after about 25 min (Figure 4a). We interpreted the latter pattern as Amplex Red photo-oxidation by multiple readings from the same plate, which might result in an overestimation of the oxidative activity. Therefore, we recommend preventing exposure of Amplex Red to the light by avoiding time-series measurement in the same microplate and by using a short incubation time. In contrast to colorimetric methods, which require a longer incubation to reach the sensitivity threshold, a short incubation time is advantageous for the Amplex Red fluorometric assay at appropriate concentration in the soil suspension.

A relatively high soil concentration in the suspension (1:20 w/w soil in water) was used in our study to increase the signal intensity of the assay considering that this suspension was additionally diluted by a factor of four in the micro-well. In contrast to hydrolytic enzymatic assay, with incubation of microplates for 2–3 h, the described assay for oxidative enzymes is very short. Thus, the recommended 10 min of incubation could be insufficient for highly diluted suspension to produce resorufin in the concentration above the instrumental detection limit. We, therefore, considered an experience of Wang et al. (2017) who used Amplex Red as a substrate for laccase activity assay in 1:20 soil suspension. Similarly, ABTS- and L-DOPA - based assays of phenol oxidative activity required low diluted suspensions of soil (Floch et al., 2007) and peat samples (Williams et al., 2000). We also considered a concern of Deng et al. (2017) who criticised 1:120 diluted suspension for enzymatic assays and stated that small size of soil sample may increase analytical error due to the heterogeneous nature of soil samples and enzymes interaction with mineral surfaces (Sinsabaugh, 2010).

Currently available colorimetric methods are often based on different oxidation products, making it difficult to compare phenol oxidase and peroxidase activities across different studies. Thus, the possibility of standardising oxidative assays by calibration with the commercially available standard resorufin is a clear benefit. Also, some of these products cannot be quantified in soils due to a lack of appropriate standards (German et al., 2011; Sinsabaugh, 2010). The fluorescent intensity of resorufin indicated an initial linear increase up to a concentration of 10 μM (for resorufin) and 20 μM (resorufin sodium salt); (Figure 4b). Due to its broader linear range, the use of the resorufin sodium salt is preferable in comparison to resorufin. Moreover, the storage and handling of the resorufin sodium salt are more straightforward than for resorufin. In both cases, calibration with resorufin or the resorufin sodium salt enables enzyme activities to be calculated from the resorufin production rate. This is a clear improvement to previous approaches using Amplex Red as a substrate without calibration (Wang et al., 2017).

### 4.2 Sensitivity of the assay for detection of changes induced by external drivers

The developed microplate assay was able to detect low amounts (nmol range) of enzymatically produced resorufin in the Haplic Chernozem (Figures 5 and 6). It was impossible to compare the LOD and LOQ values estimated in our study (e.g., LOQ = 3.12 nmol resorufin
tures or purified enzymes should be considered with caution because no calibration was applied to the latter assay (Wang et al., 2017). The LOQ values reported for microplate assay of hydrolytic enzymes (5.26–7.31 nmol MUF g⁻¹ min⁻¹) were below the LOQ in our study. The exclusion of these Amplex Red concentrations from the data set for Michaelis–Menten fitting did not significantly affect the $V_{\text{max}}$ and $K_m$ values (Table S1) but slightly decreased fitting quality ($R^2$). Therefore, a sufficient number of substrate concentrations above the LOQ is required to estimate the kinetic parameters of the enzymes to ensure a reliable fitting. It is worth to note that the sensitivity of Amplex Red assay will be dependent on environmental and laboratory conditions. Therefore, a robust comparison study of the Amplex Red-based assay across soil types and laboratories is required to further validate its use in comparative analyses. The Amplex Red approach enabled the effects of land-use and climatic conditions on the same soil type to be distinguished. Both peroxidase and phenol oxidase strongly varied between different land-use (Figure 5) and climate treatments (Figure 6). A response of enzymatic Amplex Red oxidation to land management was also reported by Wang et al. (2017). In contrast, colorimetric substrates showed a high inconsistency in results for phenol oxidase and peroxidase assays in the same soil type (Bach et al., 2013).

The $V_{\text{max}}$ and $K_m$ values of soil phenol oxidases and peroxidases obtained in our study were markedly different from kinetic parameters of pure oxidoreductase enzymes, such as laccase (Wang et al., 2017). Generally, the $V_{\text{max}}$ and $K_m$ determined in soil are very different from microbial functioning in pure culture (Tischer et al., 2015). Such differences are likely caused by enzyme and substrate interactions with the soil matrix by their adsorption on colloidal and mineral soil compounds, which potentially increase $K_m$ and decrease $V_{\text{max}}$ (Kandeler, 1990; Marx et al., 2005; Tischer et al., 2015; Wu et al., 2014). In addition, enzyme kinetics parameters estimated by substrate saturation curve are valid only in the short term until less than 15% of the added substrate are metabolised (Panikov et al., 1992). Unreasonably long incubation time can result in underestimation of $V_{\text{max}}$ and overestimation of $K_m$ (Panikov et al., 1992).

Therefore, the kinetic parameters determined in pure cultures or purified enzymes should be considered with caution or even re-considered for new generation models on SOM turnover. In general, $V_{\text{max}}$ of phenol oxidase dominated over peroxidase activity under the cropland land use, whereas an opposing pattern was observed for the pasture. However, peroxidase demonstrated a higher affinity to its substrate (lower $K_m$ values) than phenol oxidase, resulting in a higher catalytic efficiency ($V_{\text{max}}/K_m$) for the former (Table 1). Thus, under substrate limitation typical for natural soil conditions, a 40%–60% faster substrate turnover would be mediated by peroxidases versus phenol oxidases (Table 1).

The kinetic parameters of both phenol oxidase and peroxidase in the Haplic Chernozem also strongly differed between the cropland plots exposed to different climates. In the samples obtained from future climatic conditions (i.e., higher temperatures and summer drought), a lower activity (lower $V_{\text{max}}$) and lower substrate affinity (higher $K_m$) was observed than in ambient cropland plot (Figure 6b,c). This lower $K_m$ from ambient conditions might be related to iso-enzymes produced by different microbial communities, which would increase the efficiency of substrate oxidation (Tischer et al., 2015). As a consequence, the selected enzyme system and SOM degradation mechanism of a soil microbial community (Loeppmann et al., 2016) might be different under ambient and future climatic conditions.

5 | CONCLUSIONS AND OUTLOOK

We applied Amplex Red as a substrate to improve fluorometric approach for the determination of oxidative enzyme activities in the Haplic Chernozem. The approach enabled phenol oxidase and peroxidase activities to be distinguished by the oxidation of Amplex Red either in the presence or absence of H₂O₂. We confirmed the applicability of the method at a neutral to slightly alkaline soil pH and for a short (e.g., 10 min) incubation time. Data comparison between different treatments using the assay is simplified by the possibility of standard calibration based on commercially available resorufin, which is the product of Amplex Red oxidation. The activity of both phenol oxidase and peroxidase followed Michaelis–Menten kinetics, and kinetic parameters ($V_{\text{max}}$ and $K_m$) were sensitive to differences in soil properties caused by land-use and climatic conditions. Further studies on a broader range of soils and varying environmental conditions should support the sensitivity and reliability of the Amplex Red-based assay.

ACKNOWLEDGEMENTS

This work was done at Helmholtz-Centre for Environmental Research – UFZ and financially supported by this research centre. We thank the staff of the Bad Lauchstädt Experimental Research Station (especially Ines Merbach...
and Konrad Kirsch) as well as Martin Schädler for their work in maintaining the plots and infrastructures of the Global Change Experimental Facility (GCEF), and Harald Auge, François Buscot, Stefan Klotz and Martin Schädler for their role in setting up the GCEF. The first author greatly appreciated her scholarship to Isfahan University of Technology, Isfahan, Iran.

Open access funding enabled and organized by Projekt DEAL.

**AUTHOR CONTRIBUTIONS**

Sajedeh Khosrozadeh: Conceptualization (equal); data curation (lead); formal analysis (equal); investigation (lead); methodology (lead); project administration (equal); resources (lead); software (lead); validation (equal); visualization (equal); writing – review and editing (equal). Maxim Dorodnikov: Data curation (lead); formal analysis (lead); software (lead); validation (equal); visualization (equal); writing – review and editing (equal). Thomas Reitz: Project administration (equal); visualization (equal); writing – review and editing (equal). Evgenia Blagodatskaya: Conceptualization (lead); data curation (lead); formal analysis (equal); investigation (lead); methodology (lead); project administration (lead); resources (lead); software (supporting); supervision (lead); validation (lead); visualization (lead); writing – review and editing (lead).

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available in the supplementary material and from the corresponding author upon reasonable request.

**ORCID**

Sajedeh Khosrozadeh https://orcid.org/0000-0003-0154-7596

**REFERENCES**

Altermann, M., Rinklebe, J., Merbach, I., Körschens, M., Langer, U., & Hofmann, B. (2005). Chernozem—soil of the year 2005. *Journal of Plant Nutrition and Soil Science*, 168, 725–740. https://doi.org/10.1002/jpln.200521814

Bach, C. E., Warnock, D. D., Van Horn, D. J., Weintraub, M. N., Sinsabaugh, R. L., Allison, S. D., & German, D. P. (2013). Measuring phenol oxidase and peroxidase activities with pyrogallol, L-DOPA, and ABTS: Effect of assay conditions and soil type. *Soil Biology and Biochemistry*, 67, 183–191. https://doi.org/10.1016/j.soilbio.2013.08.022

Beyer, W. H. (2017). *Handbook of tables for probability and statistics* (2nd ed.). CRC Press. https://doi.org/10.1201/9781351073127

Billings, S. A., & Ballantyne, F., IV. (2013). How interactions between microbial resource demands, soil organic matter stoichiometry, and substrate reactivity determine the direction and magnitude of soil respiratory responses to warming. *Global Change Biology*, 19, 90–102. https://doi.org/10.1111/gcb.12029

Bueno, C., Villegas, M. L., Bertolotti, S. G., Previtali, C. M., Neumann, M. G., & Encinas, M. V. (2002). The excited-state interaction of resazurin and resorufin with amines in aqueous solutions. *Photochemistry and Photobiology*, 76(4), 385–390. http://doi.org/10.1562/0031-8655(2002)076<0385:esitoo>2.0.co2

Burns, R. G., DeForest, J. L., Marxsen, J., Sinsabaugh, R. L., Stromberger, M. E., Wallenstein, M. D., Weintraub, M. N., & Zoppini, A. (2013). Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biology and Biochemistry*, 58, 216–234. https://doi.org/10.1016/j.soilbio.2012.11.009

Cherwell Scientific. (1999). *Model maker. 3.1 MMAN 1*. Cherwell Scientific Publishing Ltd.

Dębski, D., Smulik, R., Zielonka, J., Michałowski, B., Jakubowska, M., Dębowska, K., Adamus, J., Marcinek, A., Kalynaranaman, B., & Sikora, A. (2016). Mechanism of oxidative conversion of Amplex® red to resurufin: Pulse radiolysis and enzymatic studies. *Free Radical Biology and Medicine*, 95, 323–332. https://doi.org/10.1016/j.freeradbiomed.2016.03.027

Deng, S., Dick, R., Freeman, C., Kandeler, E., & Weintraub, M. N. (2017). Comparison and standardization of soil enzyme assay protocols for meaningful data interpretation. *Journal of Microbiological Methods*, 133, 32–34. https://doi.org/10.1016/j.mimet.2016.12.013

Dick, L. K., Jia, G., Deng, S., & Dick, R. P. (2013). Evaluation of microplate and bench-scale β-glucosidase assays for reproducibility, comparability, kinetics, and homogenization methods in two soils. *Biology and Fertility of Soils*, 49(8), 1227–1236. http://doi.org/10.1007/s00374-013-0820-8

Dick, R. P., & Burns, R. G. (2011). A brief history of soil enzymology research. In R. P. Dick (Ed.), *Methods of soil enzymology* (pp. 1–19). Soil Science Society of America, Inc. https://doi.org/10.2136/sssabookser9.c1

Dwivedi, D., Tang, J., Bouskill, N., Georgiou, K., Chacon, S. S., & Riley, W. J. (2019). Abiotic and biotic controls on soil organo-mineral interactions: Developing model structures to analyze why soil organic matter persists. *Reviews in Mineralogy and Geochemistry*, 85, 329–348. https://doi.org/10.2138/rmg.2019.85.11

Flamigni, L., Venuti, E., Camaioni, N., & Barigelletti, F. (1989). A spectroscopic investigation of the temperature and solvent sensitivities of resorufin. *Journal of the Chemical Society, Faraday Transactions 2*, 85(12), 1935–1943. http://doi.org/10.1039/f29898501935

Floch, C., Alarcon-Gutiérrez, E., & Criet, S. (2007). ABTS assay of phenol oxidase activity in soil. *Journal of Microbiological Methods*, 71, 319–324. https://doi.org/10.1016/j.mimet.2007.09.020

German, D. P., Weintraub, M. N., Grandy, A. S., Lauber, C. L., Rinkes, Z. L., & Allison, S. D. (2011). Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology and Biochemistry*, 43, 1387–1397. https://doi.org/10.1016/j.soilbio.2011.03.017

Huck, S. W., & Malgady, R. G. (1978). Two-way analysis of variance using means and standard deviations. *Educational and*
Psychological Measurement, 38, 235–237. https://doi.org/10.1177/003164748303800204

International Conference on Harmonisation. (2005). International conference on harmonisation of technical requirements for the registration or pharmaceuticals for human use. Validation of analytical procedures: Text and methodology. ICH-Q2(R1). Geneva.

Johnsen, A. R., & Jacobsen, O. S. (2008). A quick and sensitive method for the quantification of peroxidase activity of organic surface soil from forests. Soil Biology and Biochemistry, 40, 814–821. https://doi.org/10.1016/j.soilbio.2007.10.017

Kandeler, E. (1990). Characterization of free and adsorbed phosphatasess in soils. Biology and Fertility of Soils, 9, 199–202. https://doi.org/10.1007/BF00335808

Kandeler, E. (2007). Physiological and biochemical methods for studying soil biota and their function. In E. A. Paul (Ed.), Soil microbiology. ecology and biochemistry (Third ed., pp. 53–83). Academic Press. https://doi.org/10.1016/B978-0-08-047514-1.00007-X

Lefrançois, P., Vajrala, V. S. R., Arredondo, I. B., Goudeau, B., Kandeler, E. (2007). Exploring the enzymatic landscape: distribution and kinetics of exo-enzymes in rhizosphere and detritusphere. Soil Biology and Biochemistry, 42, 111–118. https://doi.org/10.1016/j.soilbio.2005.09.020

Marx, M.-C., Kandeler, E., Wood, M., Wermbel, N., & Jarvis, S. C. (2005). Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle-size fractions. Soil Biology and Biochemistry, 37(1), 35–48. http://doi.org/10.1016/j.soilbio.2004.05.024

Meyer, S. L. (1975). Data analysis for scientists and engineers. J. Wiley.

Nannipieri, P., Kandeler, E., & Ruggiero, P. (2002). Enzyme activities and microbiological and biochemical processes in soil. In R. G. Burns & R. P. Dick (Eds.), Enzymes in the environment: Activity, ecology, and applications (pp. 1–35). Marcel Dekker Inc.

Remmler, P., Schulz, E., & Auge, H. (2019). Investigating the consequences of climate change under different land-use regimes: A novel experimental infrastructure. Ecosphere, 10, e02635. https://doi.org/10.1002/ecs2.2635

Sinsabaugh, R. L. (2010). Phenol oxidase, peroxidase and organic dynamics of soil. Soil Biology and Biochemistry, 42, 391–404. https://doi.org/10.1016/j.soilbio.2009.10.014

Tischer, A., Blagodatskaya, E., & Hamer, U. (2015). Microbial community structure and resource availability drive the catalytic efficiency of soil enzymes under land-use change conditions. Soil Biology and Biochemistry, 89, 226–237. https://doi.org/10.1016/j.soilbio.2015.07.011

Träibwasser-Freese, D. J., Thrayill, N., Preston, C. M., & Gerard, P. G. (2015). Catalytic kinetics and activation energy of soil peroxidases across ecosystems of differing lignin chemistries. Biogeochemistry, 124, 113–129. https://doi.org/10.1007/s10533-015-0086-3

Williams, C. J., Shingara, E. A., & Yavitt, J. B. (2000). Phenol oxidase activity in peatlands in New York state: Response to summer drought and peat type. Wetlands, 20, 416–421. https://doi.org/10.1672/0277-5212(2000)020[0416:POAOFC].2.CO;2

Zhao, B., Summers, F. A., & Mason, R. P. (2012). Photooxidation of Amplx red to resorufin: Implications of exposing the Amplx red assay to light. Analytical Biochemistry, 421, 342–348. https://doi.org/10.1016/j.ab.2013.10.016

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Khosrozadeh, S., Dorodnikov, M., Reitz, T., & Blagodatskaya, E. (2022). An improved Amplx Red-based fluorometric assay of phenol oxidases and peroxidases activity: A case study on Haplic Chernozem. European Journal of Soil Science, 73(2), e13225. https://doi.org/10.1111/ejss.13225