

Quantitative soil zymography: Mechanisms, processes of substrate and enzyme diffusion in porous media

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Abstract

Soil membrane zymography enables 2D mapping of enzyme activities on the surface of soil samples. The method is based on diffusion of components of enzymatically-mediated reactions to/from membrane, and, thus, reflects the distribution of enzyme activities at the intact soil surface. Zymography has been already successfully implemented in numerous soil ecology applications. Here we identify two methodological aspects for further improvement and expansion of the method at micro and macro scales: first, accounting for the area of contact between the soil surface and the zymography membranes and, second, accounting for diffusion effects during the zymography procedure. We tested three methods, namely, laser-scanning, staining with a fluorescent product (e.g. MUF: 4-methylumbelliferone), and X-ray computed micro-tomography, for assessing the area of the soil surface in contact with the membranes. We quantified diffusion of MUF, enzymes and substrate between the substrate-saturated membrane and soil as well as diffusion processes during membrane zymography via HP2 software. Diffusion of the substrate from the membrane and of the MUF-product to the membrane was detected, while there were no clear evidence of enzyme diffusion to/in the membrane. According to the model simulations, the enzyme activities detected via 2D zymography probably represent only a small portion, about 20%, of the actual reactions within the soil volume that is in both direct contact and in hydrological contact with zymography membranes. This is a result of omnidirectional diffusion of reaction products. The membrane contact with the soil surface estimated by three methods ranged from 3.4 to 36.5% further signifying that only a fraction of enzymes activity is detectable in a course of 2D soil zymography.

Keywords: *In situ* 2D zymography; Soil enzyme activity; Microbial hotspot's localization; Diffusion processes

Abbreviations: MUF, 4-methylumbelliferone; WM, Water-saturated Membrane; SM, Substrate-saturated Membrane; CT, computed tomography

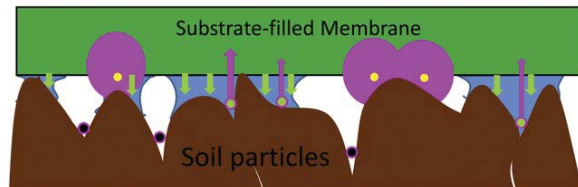
1 Introduction

Soil zymography is a new technique for *in situ* visualization and quantification of two-dimensional distribution of enzyme activities in soil and rhizosphere studies (Hoang et al., 2016; Ge et al., 2017; Liu et al., 2017; Razavi et al., 2016, 2017; Sanaullah et al., 2016; Spohn et al., 2013; Spohn and Kuzyakov, 2014). During zymography a membrane saturated with an enzyme-specific fluorogenic substrate is placed on the surface of a soil sample. Upon a contact of the substrate with soil enzymes, a fluorescent product (e.g. MUF: 4-methylumbelliferone, or AMC: 7-amido-4-methylcoumarin) is released and its presence on the membrane is then detected under UV light. The fluorescing pattern on the membrane reflects spatial distribution of active enzymes on the soil surface.

The key difference between the membrane zymography and classical measurements of enzyme activities in soil slurries is the enzyme-substrate accessibility. Classical enzyme assays maximize access of substrate to all potentially reactive enzyme sites by ensuring sample destruction and detachment followed by the release of enzymes from soil matrix to the suspension (Schimel et al., 2017). However, in intact soil, an occurrence of a contact between a substrate and an enzyme depends on the presence of transport avenues and on diffusion processes taking place within them. Membrane zymography to a certain extent emulates the diffusion processes taking place in the intact soil. Just as in soil, the fluorescent patterns on the membrane depend on diffusion. This feature is both a blessing and a curse of the method, as it enables clear visualization of the real processes occurring in soil, but substantially complicates interpretation of its results.

Interpretation of the fluorescent pattern on soil zymograms is based on the assumption that spots with high fluorescent levels correspond to localities with high enzyme activity, while spots with no fluorescence correspond to soil surface localities without enzymes. However, the observed fluorescent pattern is likely a complex outcome of diffusion of the involved chemical compounds, i.e., enzymes, substrates, and fluorescent products. Below we describe the hypothesized concept of physical processes taking place during zymography measurements.

The activity detected in a course of soil zymography is generally attributed to extracellular enzymes excreted by roots or microorganisms, which exist either immobilized on surfaces of the soil matrix and organic materials or in a free form in the soil solution (Gianfreda and Bollag, 1994; Rao et al., 2000). The immobilized enzymes are assumed to be strongly attached, thus immobile, while the free enzymes are assumed to be mobile (Nannipieri and Gianfreda, 1998). While it is presumed that both forms exist (Stotzky, 1986; Nannipieri et al., 1996), their relative sizes are generally unknown and likely dynamic, due to fluctuations in enzyme production by roots and microorganisms, and continuous biochemical enzyme degradation and their immobilization by soil particles and organic material. The soil surface in contact with zymography membrane is, typically, very uneven. Thus, as the distances between enzymes located on/near the soil surface and the membrane increase, the potential contributions of enzymes to the fluorescent signal on the membrane decrease (Fig. 1). Free enzymes in the soil solution can potentially reach the membrane by diffusion or react with dissolved substrate within the water film boundary between the membrane and soil surface. Immobilized enzymes can be broadly divided into three groups in terms of their position with respect to the membrane: 1) enzymes in direct physical contact with the membrane, 2) enzymes in hydrological contact with the membrane through water films, and 3) enzymes without any contact with the membrane. When the membrane with a substrate is placed on a soil surface, the enzymes in direct contact with the membrane (group 1) are the first to be involved in catalytic activities. The substrate readily available for this enzyme group is decomposed quickly and the released MUF immediately contributes to appearance of a fluorescent signal on the membrane. Involvement in catalyzes of the enzymes connected with the membrane through water films (group 2) depends on the time needed, first, for the substrate to diffuse from the membrane and reach the enzymes, and, second, for the released MUF to diffuse back to the membrane. The immobilized enzymes with no contact with the membrane (group 3) are unlikely to contribute to the fluorescent signal, since there are no means for the substrate and MUF transport to/from them (Fig. 1). Thus, the fluorescent signal detected on the membrane under UV-light may originate from multiple sources including immobilized and free enzymes. Upon reaching the membrane, the MUF products can also laterally diffuse within it during the incubation and thus decrease the spatial resolution of the zymogram. Higher catalytic activity of free enzymes as compared to immobilized ones (Rao et al., 2000) further complicates interpretation of soil zymograms. The same complications exist in standard fluid-based enzyme assays.



- - enzymes on contact with the membrane (group-1)
- - enzymes contacted with the membrane through water film (group-2)
- - enzymes without contact with the membrane (group-3)
- ▲ - water meniscus between soil surface and the membrane
- ↓ - substrate diffusion
- ↑ - product diffusion

Fig. 1 Hypothetical pathways of the substrate and product diffusion between the membrane and soil surface, when a membrane with substrate is incubated on the soil surface during zymography analysis.

alt-text: Fig. 1

It should be noted that diffusion, in contrast to convective flow, is omnidirectional, and rates of either vertical or lateral diffusion of substances, enzymes, and products, e.g. MUF, in soils and membranes are generally unknown. Therefore, quantification of *in situ* enzyme activity in the soil and the rhizosphere, based on membrane zymography, requires accurate assessments of diffusion pathways and rates for all involved chemicals. We assessed following diffusion pathways involved in zymography analysis: 1) diffusion of enzymes from soil into zymography membranes; 2) diffusion of MUF and substrate within the membranes; 3) diffusion of substrate from the membrane to soil; and 4) diffusion of MUF from soil to the membrane.

Quantifying diffusion is greatly complicated by the ubiquitous unevenness of soil surfaces and related water films, which introduce a large uncertainty into size (area) and quality of contact between the soil surface and the membrane, the problem being particularly substantial in well aggregated and coarse textured soils. Due to differences in sizes and shapes of soil particles and aggregates, presence of soil pores and incompletely decomposed plant residues, a soil surface is never perfectly flat, and thus the actual area in direct physical contact between the membrane and the soil may be relatively small (Fig. 1). Its size can be difficult to measure, though theoretically, on an ideally sliced dry soil surface, the contact area should be equivalent to the volumetric fraction of solid substances in soil, i.e. = [1.0 - soil porosity]. However, in moist soil the indirect contact areas, e.g. via water films, can be much larger in size than the direct contact areas (Fig. 1), but the quality of this type of contact is inferior to direct contact. By quality of the contact here we refer to spatial accuracy with which the fluorescent signal on the membrane can reflect activity of the enzyme. Due to tortuosity of diffusion pathways, such spatial accuracy resolution is expected to be much lower for indirect contact areas. The size of indirect contact depends on the soil water content and the soil water retention properties. Knowing the positions of the direct and indirect contacts on the membrane is an important prerequisite for soil zymogram's interpretation. Indeed, an absence of a fluorescent signal in a location that is in contact with the membrane indicates absence of enzymes, while absence of a signal in a location that had no or poor contact cannot be unequivocally interpreted.

The uneven soil surface, complex contacts between membrane and soil surface, potential diffusion of involved chemical compounds raise questions of: (i) how representative are the fluorescent patterns on soil zymograms of the actual enzyme activity on the soil surface, and (ii) what is the optimal incubation time for the membrane on the soil surface for accurate evaluation of enzyme activities? We posit that answers to these questions can be obtained only by experimentally based modeling of physical and biochemical processes, such as water-soil-water distribution, substrate, product and enzyme diffusion, and substrate transformation by enzymes that are the processes occurring during the membrane incubation.

Here we addressed several methodological aspects improving the method for its use in rhizosphere experiments, and importantly for zymography expansion to a wider range of micro and macro scale studies in soil ecology. Since relatively little is known about transport processes involved in enzyme activities in soil, e.g., travel-distances of enzyme from producing microbial cells to substrate, amounts of product that can return to the enzyme producing cell, etc. (Allison et al., 2011; Burns et al., 2013), we used experimentation combined with modeling to better understand and interpret the results of 2D soil zymography.

The specific objectives of the study were: 1) to quantify the diffusion pathways of substrates, enzymes and produced MUF involved in zymography analysis and enzymatic reactions; 2) to compare the methods estimating the area of the soil surface in contact with zymography membranes; and 3) quantitative estimation of *in situ* enzyme activity using zymograms and process modeling.

2 Materials and methods

2.1 Taking and processing zymography images

For soil zymography we used a Nikon D90 camera (Nikon Inc.) with a Sigma 18-250 mm f/3.5-6.3 DC Macro OS HSM lens (Sigma Corp. of America), mounted on a Rocwing Pro Copy Stand (Rocwing Co., UK). A 22W Blue Fluorescent Circline Lamp - FC8T9/BLB/RS (Damar Worldwide 4 LLC.) was used as a source of UV light. Camera settings were defined experimentally (Guber et al., 2018, in review) and provided images with a view field of 106 × 71 mm, at a resolution of 0.622·10⁻³ mm² pixel⁻¹ and flat field non-uniformity of 0.099, assessed as a coefficient of grayscale (*G*-value) variation measured in a dry polyamide membrane filter. Focal length, aperture and shutter speed were set to 250 mm, f6.3 and 1/125 s, respectively.

Hydrophilic polyamide membrane filters (Tao Yuan, China) were used in all zymography measurements (Sanaullah et al., 2016; Razavi et al., 2016). The filters are 100 μm thick with average pore size of 0.45 μm. Very low protein binding capacity and large pore size (0.45 μm) of these filters are assumed not to be restrictive to diffusion of enzymes through the filter, because average size of enzyme is 3-7 nm (Erickson, 2009). The membranes were calibrated in standard MUF solutions according to Guber et al. (2018, in review).

The images of the membranes were analyzed using ImageJ/Fiji software (Schindelin et al., 2012). Fluorescent color images were converted to 32-bit grayscale format, corrected for background *G*-values, and used for pixel-based MUF calculations.

2.2 Enzyme, substrate, and product diffusion experiments

In order to assess potential diffusion of chemicals involved in zymography, i.e., enzymes, substrate, and produced MUF, we performed a series of diffusion experiments. The experiments were conducted using intact soil cores (45 mm diameter, 25 mm height) from the top 0–10 cm layer of an agricultural plot on a well-drained Alfisol of the Kalamazoo series (mesic Typic Hapludalf) (Robertson and Hamilton, 2015). Soil texture was sandy loam (65% sand, 27% silt and 8% clay), bulk density was 1.56 g cm^{-3} , and volumetric water content at the time of measurement was equal to $\sim 0.18\text{ cm}^3\text{ cm}^{-3}$. The cores were located within acrylic sampling rings and, for the experiments, the soil above the sampling ring was cut using a microtome blade to flatten soil surface, without smearing, and to provide the best possible contact between the membrane and the soil surface.

2.2.1 Experiment 1

The goal of the first experiment was to determine whether the enzymes could diffuse from the soil into the membrane. A 4-morpholineethane sulphonic acid (MES) was added to distilled water to achieve pH 6.5 in the water prepared for the membrane saturation. Three layers of water-saturated membranes were placed one above another on the surface of the soil (Fig. 2, right panel) and incubated for 1 h. Respectively, these Water-saturated Membranes are referred to as WM-1 (directly attached to the soil surface), WM-2 (middle layer), and WM-3 (top layer). After the incubation, the membranes were removed from the surface, separated from each other, and placed in contact with membranes saturated in 6 mM solution of 4-methylumbelliferone- β -D-glucopyranoside (referred further to as the substrate) for 1.5 h. These Membranes with the Substrate are referred to as SM-1, SM-2, and SM-3. It was hypothesized that during the incubation the enzymes, i.e., β -glucosidase, would diffuse from the soil into the water-filled membranes. Upon contact between water-saturated membranes, containing diffused soil enzymes, with their substrate-saturated counterparts, MUF would be released. Then, MUFs distribution and quantity could be assessed by fluorescent patterns on the substrate-saturated membranes. Note that the substrate-saturated membrane was always placed on the side of the water-filled membrane that was not in contact with either soil surface (for WM-1 membrane) or with underlying water-filled membrane (for WM-2 and WM-3 membranes). This approach guaranteed that only the enzymes that passed through the respective membrane would contribute to fluorescent patterns.

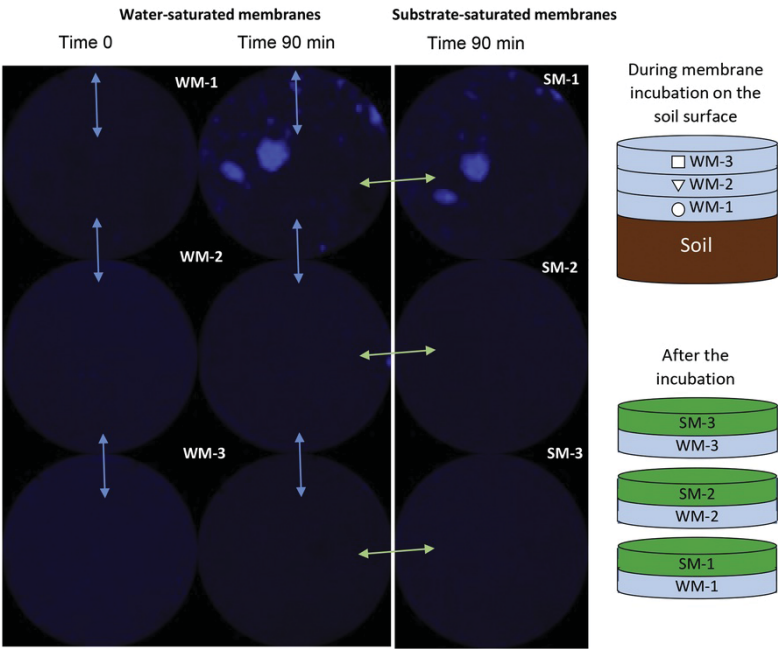


Fig. 2 Visualization of forward and downward diffusion of MUF and enzymes between and within membrane and soil by zymography. Example of Water-saturated Membranes (WM) and Substrate-saturated Membranes (SM) after the water-saturated membranes were removed from the soil surface and kept for 90 min in contact with the substrate-saturated membranes. As shown on the insert right, WM-1 was in direct contact with the soil, while WM-2 and WM-3 were placed on each other above WM-1. The figure clearly shows the diffusion of the substrate from SM-1 to WM-1 and backward MUF diffusion for the membrane contacted soil surface. Diffusion of enzymes through WM-1 to WM-2 and WM-3 was not detected (absence of MUF spots at SM-2 and SM-3) during 90-min membrane incubation of the soil surface. Blue arrows mark directions of potential diffusion during incubation, top arrows mark diffusion exchange with soil. Green arrows mark directions of potential diffusion after incubation between the membranes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alt-text: Fig. 2

During incubation of water-filled membranes a plastic bag filled with fine sand with a load of approximately 6 g cm^{-2} was placed on the top of them to ensure good contact between the membranes and the soil surface, yet without significant pressure. The incubations were conducted at a constant temperature of 20°C . During contact of water-filled and substrate-filled membranes they were kept in transparent polyethylene envelopes, to prevent drying and capillary water flow within the membranes. Fluorescent images of both water-filled and substrate-filled membranes were obtained and the amounts of MUF on both types of membranes were calculated as described in Section 2.1.

2.2.2 Experiment 2

The second experiment aimed at exploring diffusion of substrate and MUF between/within the membranes. Three membranes saturated in 6 mM substrate solution were placed on the soil surface with the same settings as those used for the first experiment (Fig. 3 right panel), and are referred to as Substrate-saturated Membranes SM-1, SM-2, and SM-3, respectively. After the incubation, the membranes were removed from the soil, separated from each other and placed in transparent polyethylene envelopes. Then, the membranes were photographed under UV light over a 2 h period every 10 min. We hypothesized that if MUF content in the membrane increased during the 2 h after the membrane was removed from the soil surface, it would indicate that enzymes were present within the membrane. Indeed, if the enzymes were present within the membrane itself, it would lead to MUF release by the enzymes beyond that achieved when the membrane was located directly on the soil surface. Increases in MUF contents in the membranes that were not in contact with soil would directly demonstrate that enzymes diffused from the soil surface into the membranes. On the other hand, lack of change in MUF content levels after the membrane was removed from the soil surface would suggest that any MUF present within the membrane originated in the soil and that it had diffused into the membrane when it was in contact with the soil surface. This experiment was conducted in triplicate.

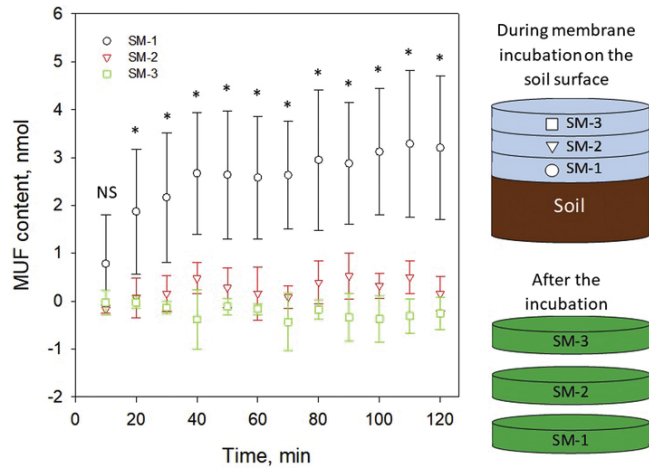


Fig. 3 Differences in MUF contents between time zero and measured time in the substrate-saturated membranes during 120 min after the membranes were removed from the soil surface. Colors denote three measurement sets of membranes. Relative positions of the membranes are shown on the insert. MUF production continued only in SM-1 membranes indicating presence of enzymes only in the membrane that directly contacted the soil surface. Stars mark times when SM-1 was significantly higher than SM-2 and SM-3 ($p < 0.05$), SM-2 and SM-3 were not significantly different from each other at any of the studied times. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alt-text: Fig. 3

2.2.3 Experiment 3

The goal of the third experiment was to test whether the MUF produced in the soil in a course of enzymatic reactions with the substrate would return (diffuse back from the soil) to the membrane. We assumed that the substrate diffuses from the membrane into the soil, whereas the product (MUF) diffuses in soil omnidirectionally. We followed the protocol used in the Experiment 1 with the only difference that 2 h prior to placing water-saturated membranes on the soil surface, the surface was subjected to zymography procedure. That is, a substrate-saturated membrane was placed on the soil surface and incubated for 1 h. MUF content was measured in the water-saturated membranes after 60 min incubation on the soil surface.

We hypothesized that a portion of MUF released by the enzymes during zymography would remain in the soil for a long time. It is also possible that the substrate amounts diffusing into the soil from the membrane during zymography are high enough to maintain the enzymatic reactions in soil even after the membrane is removed. In the case of repeated zymography on the same soil surface, this MUF can diffuse from the soil into the subsequent membrane, and thus obscure the results of the

latter zymography.

2.3 Experiment 4

The goal of the fourth experiment was to estimate the area of contact between the membrane and the soil surface. Despite a broad implementation of soil zymography, there is no standard procedure for detection of the contact area between zymography membranes and the soil surface. We tested three approaches to measure/estimate the membrane-soil surface contact area, namely: (i) soil surface topography estimation with a laser scanner, (ii) estimation of the membrane's contact with the soil surface via staining the surface with MUF solution, and (iii) estimation of the membrane's contact with the soil surface using X-ray computed micro-tomography (CT) scanning.

The experiment was conducted in the University of Kassel (Germany). Due to the length of X-ray scanning and limited access to the X-ray facility, only one soil sample was processed using all three methods, this sample is referred to as the main sample. An intact soil core was prepared and subjected to zymography analysis using the procedures described above. We obtained a zymogram of β -glucosidase for the main sample over the course of a 1-h incubation. β -glucosidase zymograms were also obtained for 10 additional soil cores. However these cores were not subject to X-ray or laser scanning.

The first approach to quantifying the contact area was based on measurements of soil surface topography using a NextEngine 3D Laser Scanner (NextEngine, Inc., Santa Monica, California) and is referred to as laser scanning. The scanner uses a set of laser beams to hit the soil surface from different angles. Each point from the soil surface is automatically positioned by a laser-light sensor in a 3D coordinate system at a nominal resolution of 60 μm (Uteau et al., 2013). The soil surface was scanned at three angles to eliminate the effect of light shading, and then the three images were combined and reconstructed using NextEngine ScanStudio software (NextEngine, Inc., Santa Monica, California) to derive a 3D map of surface topography. The mean length of reconstructed triangles was 60 μm . The coordinate system was adjusted, and soil surface was interpolated for a rectangular $25 \times 25 \mu\text{m}$ grid using in-house developed MatLab code to match the spatial orientation and resolution of the soil zymogram.

While the laser scanning resulted in a detailed soil surface map, it could not provide direct information on which portions of the surface would be in contact with the membrane upon its placement on the surface. The general considerations are that the contact will take place at the local peaks of soil particles or micro-aggregates (Fig. 1). The peaks vary greatly in their heights and frequency of occurrence on the soil surface, and which peaks will be in contact with the membrane depends on the membrane's flexibility, thus, on the extent of its bending in following the soil surface. Since flexibility of the membrane is unknown, we explored a range of elastic properties of the membrane and its ability to flex in contacting the peaks. The "Maximum Filter" of ImageJ software was applied to the soil surface image, representing elevation of soil particles, with radius values r_{max} varying from 50 μm to 500 μm with 50 μm increments. Small and large r_{max} values corresponded, respectively to high and low membrane flexibility. Subtracting the original surface image from the filtered image generated a map of elevation differences $Z(r_{\text{max}})$ between the local peaks and surrounding pixels within the area defined by the radius of the "Maximum Filter". Zero $Z(r_{\text{max}})$ values corresponded to the locations of peaks on the map.

Not only the sites of direct surface-membrane contact but also the sites connected to the membrane via water films contribute to zymography (Fig. 1). Thus, we also explored the role of hypothesized water menisci that could be formed around soil surface peaks. For that, the maps of differences in elevations obtained with different radius values $Z(r_{\text{max}})$ were further processed to account for the water films that can be formed by liquids between soil particles and the membrane at sufficient soil moisture levels. These films can potentially increase the contact area and serve as diffusion pathways for the substrates and products. To account for the contact area via the water films S_{wm} , we introduced thickness of water films Z_{wm} into the $Z(r_{\text{max}})$ maps. All pixels around the local peak were included into the contact area if the differences in the elevations between the local peak and these pixels $Z(r_{\text{max}})$ were smaller than the film thickness Z_{wm} . The thickness of water films can vary depending on soil characteristics and soil moisture level, therefore we explored a range of Z_{wm} values from 10 μm to 100 μm with a 10 μm increment.

The second approach to estimating the contact area, further referred to as MUF-staining, was based on staining and visualization of MUF solution on the soil surface. The membrane saturated in a 6 mM MUF solution was placed on the soil surface and kept in contact with the soil for 1 min using a sand-filled bag. The bag provided a load on the membrane of approximately 6 g cm^{-2} . Then the membrane was removed, and the soil surface was photographed under UV light. The bright areas on the images indicated the localities on the soil surface that received MUF from the membrane, and thus could be regarded as being in contact with the membrane. Short contact time (i.e. 1 min) was chosen to reduce MUF diffusion within the soil. These measurements were conducted both on the main and on 10 additional soil cores to assess variability of contact area determined by this method.

The third approach used X-ray CT tomography to estimate the area of contact between the soil surface and the membrane. Experimental setup of X-ray microtomography scanner (Zeiss Xradia Versa 520) located in Department of Soil Science, University of Kassel is shown in Fig. S1 of supplemental materials. The membrane was saturated in 10% solution of CaI_2 , and then followed exactly the same procedures as those used during membrane placement on the soil surface for zymography analysis. The solution of CaI_2 was used in order to enhance the contrast of image gray-scale values between added liquid, soil, and membrane. The soil core with the membrane on its surface was scanned at energy of 80 KeV/7W. Total of 801 X-ray projections were taken and reconstructed to a 50 μm voxel sized tomogram. The contact area was defined using ImageJ software as the voxels that belonged to both soil and the membrane.

The results of contact area measurements obtained using the three approaches were combined with the β -glucosidase zymogram. We examined the percentage of the membrane area that was in contact with the soil surface (A_{con}), the percentage of enzyme-active area within the membrane (A_{enz}) and enzyme-active area within the membrane-soil contact area ($A_{\text{con}}^{\text{enz}}$) for the three approaches tested in this study:

$$\begin{aligned}
A_{con} &= 100 \cdot S_{con} / S_{mem} \\
A_{enz} &= 100 \cdot S_{enz} / S_{mem} \\
A_{con}^{enz} &= 100 \cdot (S_{enz} \cap S_{con}) / S_{enz}
\end{aligned}
\tag{1}$$

where S_{con} is the membrane-soil contact area (cm²); S_{mem} is the membrane area (cm²); S_{enz} is area of the enzyme-active zones on zymogram (cm²).

2.4 Experiment 5

The goal of this experiment was to assess the enzyme activity using two components: zymograms and modeling. A membrane saturated in 6 mM substrate solution was placed and kept on the soil surface for 2 h. The membrane and the soil surface were covered by transparent polyethylene and photographed under UV light every 5 min during the first hour of incubation, and at 10-min intervals during the second hour. The experiment was conducted using two replicates. The experimental data were further processed and combined with the modeling.

For the modeling we used HP2 software (Šimůnek et al., 2012), which simulates transport of multiple chemicals with mixed equilibrium/kinetic biogeochemical reactions in two- and three-dimensional variably-saturated porous media (soils). We used the HP2 software solely for illustration purposes, thus solved a 2-D problem, skipped model calibration and validation steps and used the model parameters estimated from open data base. A 2-D modeled domain (10 mm width, 30 mm height) represented a vertical cross-section of a zymography setup, and can be viewed as a simplified modeled version of Fig. 1. The model mimicked a 0.1 mm thick membrane placed above a sandy-loam soil with a 0.03 mm water film between the membrane and soil surface (Fig. 4). For simplicity, the enzymes were set in a single node located on the soil surface. Initial concentration of the substrate in the membrane was 6 mM and zero in the water film and soil. The initial concentration of product (MUF) was set to zero for the entire domain. Zero flux conditions were used at the boundaries of the domain. The HP2 software simulated diffusion of the substrate from the membrane into the soil, substrate catalysis by the enzymes and product diffusion. The diffusion coefficients for the substrate and MUF in free water were set to $0.5 \cdot 10^{-4} \text{ mm min}^{-1}$ and adjusted by the model for the pore tortuosity (Millington and Quirk, 1961). Based on the results of the enzyme diffusion experiment (see below), the enzymes were assumed not to diffuse. MUF production was modeled using the Michaelis-Menten equation (Johnson and Goody, 2012) with the parameters taken from Razavi et al. (2015):

$$\frac{dP}{dt} = \frac{V_{\max} S}{K_m + S}
\tag{2}$$

where P and S are concentrations of the product and substrate, respectively [$\mu\text{mol g}^{-1}$]; $V_{\max}$ ($0.2 \mu\text{mol h}^{-1} \text{g}^{-1}$) is the maxim rate of enzymatic activity; K_m ($16.5 \mu\text{mol g}^{-1}$) is the half saturation constant; and t is time (h).

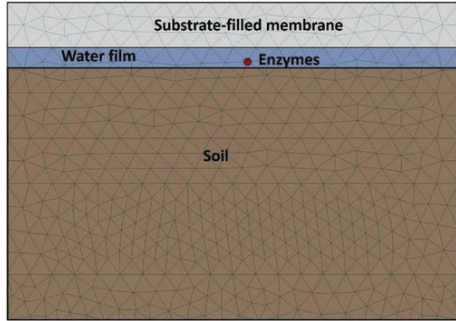


Fig. 4 The HP2 simulation domain with mesh and material distribution.

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The model generated output for the total MUF production, the substrate and MUF contents in the membrane at 1 min intervals for the 2-h incubation experiment. Measured and simulated dynamics of MUF contents were used to assess the enzyme activity as changes of MUF content in the membrane per unit of time and per area of membrane. Since, a single image is typically used in membrane zymography, a cumulative approach was used in calculations. For this approach, the measured values of MUF content were divided by the incubation time counted from the moment when the membrane was placed on the soil surface (time zero). For comparison, we used a differential approach, i.e. every two subsequently measured MUF contents were divided by the time interval between the two measurements. The differential approach was also applied to the simulated total MUF production and MUF contents in the membrane.

2.5 Statistical analyses

Data analyses were performed using SAS. PROC MIXED procedure was used to conduct F-tests for treatment comparisons in diffusion experiments. Measurements conducted at each experimental unit, multiple times, were treated as repeated measures and analyzed accordingly using REPEATED statement of PROC MIXED; while individual membranes/samples in such analyses were regarded as random effects (Milliken and Johnson, 2009). When treatment effects were found to be statistically significant at $p < 0.05$ they were followed by t-tests for comparisons among individual treatment pairs. When relevant, the treatment means were compared to zero using t-tests in LSMEANS outputs of PROC MIXED.

3 Results

3.1 Diffusion of enzymes, substrates and products

3.1.1 Experiment 1

In the first diffusion experiment the presence of MUF was only detected in those water-saturated membranes that were in direct contact with the soil surface (WM-1 membranes). No MUF was detected in the second and third water-saturated membranes placed above each other on the first membrane (WM-2 and WM-3 membranes) (Fig. 2 and Table 1). The substrate-saturated membranes in contact with the WM-1also contained MUF, but the fluorescence intensity of the MUF spots in the SM-1 ($G \sim 92$) membrane was considerably less than the intensity in the WM-1 membrane ($G \sim 107$). MUF content in the membranes of the second (WM-2, SM-2) and third (WM-3, SM-3) layer did not differ from zero within the measurement accuracy of the method. Changes in MUF content occurred during the incubation only in the WM-1 membranes, the membranes that were in direct contact with the soil.

Table 1 Average MUF contents (nM) and their respective standard errors in Water-saturated Membranes immediately after they were removed from the soil surface (WM_{t0}) and after 90 min contact between WM and Substrate-saturated Membranes (SM_{t90}). Note that at time t₀ the WM membranes have not received substrate, thus could not have any MUF produced, the reported values reflect the zero background. Total MUF is calculated as (WM_{t90} + SM_{t90}) - WM_{t0}. Stars mark the columns where WM1 was significantly different from WM2 and WM3 ($p < 0.05$). WM2 and WM3 were not different from each other for any of the studied variables ($p > 0.8$). Mean MUF values that were significantly greater than zero ($p < 0.05$) are shown in bold.

Membrane	WM _{t0}	WM _{t90}	SM _{t90}	Total MUF
WM1	0.07	2.41	1.54	3.88
WM2	0.10	0.09	0.05	0.04
WM3	0.09	0.02	0.04	−0.03
Std error	0.03	0.34	0.16	0.52
	NS	*	*	*

These results showed that the enzymes did not diffuse into membranes WM-2 and WM-3 through membrane WM-1, but were attached to WM-1 and produced MUF after the membrane removal from the soil surface and being placed in contact with the substrate saturated membrane SM-1.

3.1.2 Experiment 2

In the second experiment MUF was detected in all substrate-saturated membranes, after the 1-h incubations on the soil surface (Fig. 3). However, MUF contents differed significantly depending on the position of the substrate-saturated membrane in respect to the soil surface in the order SM-1 $\gg$ SM-2 > SM-3. When the membranes were removed from the soil, further increases in MUF content were observed only in the SM-1 membranes, but not in the SM-2 and SM-3 membranes (Fig. 3). The increase in the MUF content in the SM-1 membranes lasted for 40–60 min after their removal from the soil surface. These results showed that the enzymes did not diffuse into membranes SM-2 and SM-3 through membrane SM-1, but small soil particles with enzymes were attached to SM-1 and continued producing MUF even after the membrane removal from the soil surface.

3.1.3 Experiment 3

The substrate diffusion experiment revealed that relatively large quantities of residual MUF remained in the soil after the substrate-saturated membrane was removed. MUF was detected in all three water-saturated membranes that were placed on the soil surface 2 h after the substrate-saturated membrane was removed (Fig. 5). MUF content in the WM membranes decreased with the distance from the soil surface, and the WM membranes contained in total approximately three times the amount

of MUF when compared with the substrate-saturated membrane used in prior zymography (Fig. 5).

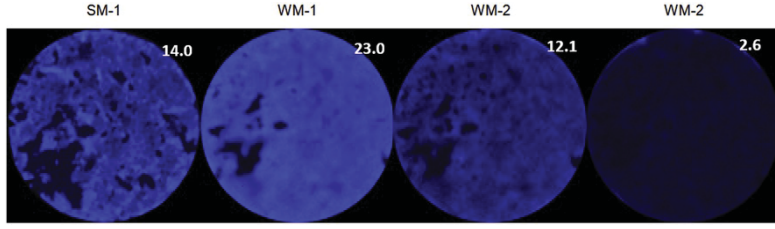


Fig. 5 Example of a substrate-saturated membrane from soil zymography (SM-1) and of water-saturated membranes (WM) that were placed as three layers on the same soil surface 2 h after the substrate-saturated membrane was removed from the soil surface. Membrane WM-1 was in direct contact with the soil, while WM-2 and WM-3 were placed on each other above WM-1. Values next to each membrane are the total MUF contents, nmol.

alt-text: Fig. 5

The result suggests that only a fraction of the MUF diffuses back from the soil to the membrane during actual zymography measurement, thus enzyme activity estimated from zymography images may considerably underestimate the real activity in the soil.

3.2 Experiment 4

The β -glucosidase activity measured prior to the surface contact area measurements was only detected on 3.1% of the membrane surface. In the additionally measured 10 soil cores, this area constituted 1.1%-6.1% of the membrane surface. Despite the relatively small total area on the main sample, there were 546 spots of high fluorescence, which could be interpreted as locations with high enzyme activity. In the additional cores, the numbers of spots with high enzyme activity ranged from 101 to 753. The sizes of such individual spots varied greatly, from $339 \mu\text{m}^2$ to 2.84 mm^2 .

As illustrated by estimates based on laser-scanning, the size of the contact area depends on the settings of potential membrane flexibility (as represented by “max filter” radius) and potential thickness of water menisci (Fig. 6a). As expected, the lower was the membrane flexibility setting, i.e., the greater r_{max} values, the lower was the contact area A_{con} . While the greater was the assumed thickness of the water menisci, $\log_{10}(Z_{\text{wm}})$, the greater was A_{con} . The size of the enzyme-active zones from the areas in contact with the soil surface, $A_{\text{con}}^{\text{enz}}$, almost increased linearly with increasing A_{con} (Fig. 6b).

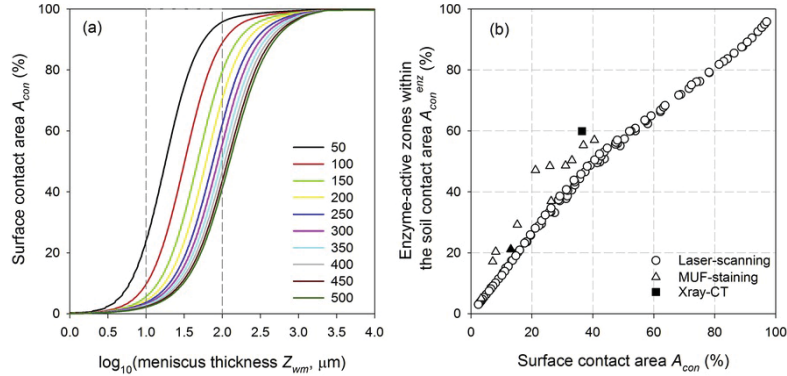


Fig. 6 (a) Exploring the effect of potential membrane flexibility (as represented by “max filter” radius) and potential thickness of water menisci on the size of the contact area between the soil surface and the membrane (A_{con}) using laser scanned soil surface topography data. Black dash vertical lines correspond to the range of surface contact areas that is reported on the adjacent part (b). (b) The relationship between the size of the surface contact area (A_{con}) and the size of the enzyme-active area ($A_{\text{con}}^{\text{enz}}$). Presented are (i) data from the range of potential membrane flexibilities (“max filter” radius) and potential sizes of water menisci explored using laser scanned surface of the main sample and reported on (a) (open circles), (ii) data from MUF-staining of the main sample (black triangle) and of the additional samples (open triangles), and (iii) data from X-ray CT-scanning of the main sample (black square).

alt-text: Fig. 6

The contact area A_{con} obtained via MUF-staining was equal to 13.1% in the main sample and varied from 3.4% to 40.5% in the additional samples (Fig. 6b). The contact area included 21.2% of the area with detected enzyme activity, i.e., 78.8% of detected enzyme activity was out of the contact area. The relationship between A_{con}^{enz} and A_{con} based on the MUF-staining of additional cores, followed the same trend as the laser-scanning data with slightly higher percentage of enzyme-active zones, while the MUF-staining data from the main sample were very close to the laser-scanning results (black triangle in Fig. 6b).

The X-ray CT method produced a much higher contact area than the MUF-staining approach (36.5%). The contact area included 60% of the area with detected enzyme activity (Fig. 6b).

3.3 Experiment 5

The MUF content modeled using HP2 software with parameters taken from literature were remarkably consistent with the MUF measurements in the membrane (Fig. 7a). A non-linear increase of MUF content in the membranes was observed during the 2-h incubation experiment for the measured data of both replications and for the modeled values. A slow increase of MUF contents within first 45 min of the incubation was followed by a relatively fast increase between 45 and 75 min of the experiment, and by a decrease after 75 min (Fig. 7a). Estimates of enzyme activity calculated for 45–75 min using the differential method highly oscillated (Fig. 7b), likely because this range of measured MUF contents corresponded to the breakpoint in the piecewise MUF calibration (Guber et al., 2018, in review). We showed that the intensity of fluorescence increased rapidly with increasing MUF membrane concentration until the breakpoint on the calibration curve. Further increases in MUF concentration resulted in only minor changes in the membrane fluorescence intensity.

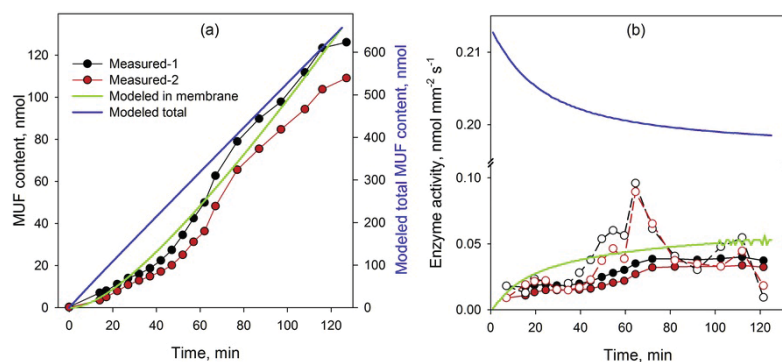


Fig. 7 Measured and simulated dynamics of MUF content (a), and estimated enzyme activity (b). Symbols in (b) denote cumulative (closed) and differential (open) calculations of enzyme activity. Blue lines correspond to the modeled amounts of MUF (a) and of enzyme activity (b) both within the membrane and within the soil adjacent to the membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alt-text: Fig. 7

The enzyme activity estimated from simulated MUF contents were slightly higher than those estimated from the measurements using the cumulative approach and fluctuated in agreement with those from the differential approach (Fig. 7b). For the cumulative calculations, estimated enzyme activity increased from $14.3 \pm 0.7 \text{ nmol mm}^{-2} \text{ s}^{-1}$ for time interval 20–45 min to $32.8 \pm 0.2 \text{ nmol mm}^{-2} \text{ s}^{-1}$ for the time interval 75–120 min (Fig. 7b).

The MUF contents modeled for the whole system, which is both in the membrane and in the adjacent soil, were considerably higher than those both observed and modeled in the membrane (Fig. 7a). Total MUF production simulated in the soil and on the surface of membrane during the experiment was 5 times greater and enzyme activity was 4 times greater than those estimated from MUF contents measured in the membranes (Fig. 7a and b).

The results of modeling clearly showed that enzyme activity estimated from zymography images considerably underestimated the enzyme activity in the soil layer on the contact with the membrane.

4 Discussion

4.1 Enzyme, substrate and product diffusion

The first diffusion experiment (*Experiment 1*) demonstrated that the diffusion of enzyme, i.e. β -glucosidase, from soil into the membranes was negligible when compared with the substrate and MUF diffusion. MUF was detected only in the water saturated membrane that was in direct contact with the soil surface (Fig. 2). Consequently, it can be assumed that either enzymes themselves and/or enzyme producing microbes were attached to the surface of WM-1 membranes, either directly or along with soil particles. Much higher contents of MUF observed in WM-1 compared with SM-1 membranes (Table 1) suggested that no enzyme movement took place between WM-1 and WM-2 as well, and all MUF detected on WM-1 and SM-1 membranes was produced solely in WM-1 and then diffused to SM-1. Given the small size of the enzyme molecules, $\sim 130 \text{ \AA}$ (13 nm) (Sanz-Aparicio et al., 1998), it is unlikely that the thickness of the

inert membrane (100 μm) and the membrane pore size (0.45 μm) restricted enzyme diffusion. Thus, strong attachment of enzymes and/or enzyme producing microorganisms to soil particles and minimal presence of free enzymes capable of diffusion in the solution of the studied soil are the likely causes for the observed results.

The second diffusion experiment (*Experiment 2*) further demonstrated that there was appreciable MUF diffusion among the membranes, while, consistent with the first experiment, the diffusion of enzymes through the membranes was not observed. As in the first experiment, enzymes (and possibly enzyme producing microbes) with/without soil particles were attached to the soil-contacted surface of SM-1 membranes, leading to MUF production. It is important to note, that activity of enzymes attached to SM-1 lasted for 40-60 min after the membranes were separated from the soil surface. Summarizing results of the two experiments: fluorescent patterns on soil zymograms are produced by diffusion of substrate from a membrane to immobilized soil enzymes followed by subsequent diffusion of produced MUF back to the membrane. It should also be noted that absence of enzyme diffusion (or minor diffusion) during the relatively short duration of zymography makes possible identification of enzyme locations using 2D soil zymography. Otherwise, interpretation of zymography images would be extremely difficult, if possible, due to continuously changing spatial patterns in enzyme activity.

Our findings are consistent with general considerations of the cost:benefit ratio for microbial enzyme producers that favors slow diffusion of enzymes away from their producer's cells (Allison, 2005; Allison et al., 2011; Burns et al., 2013) and with observations that activity of free enzymes is relatively minor as compared to immobilized enzymes (Kandeler, 1990). The absorption of the produced enzyme molecule on the soil/substrate surface in close proximity to the producer likely maximizes the product return, while prolonged diffusion of the enzyme in a free form decreases it.

Experiment 3 demonstrated that substantial amounts of the substrate diffused into the soil during the 1-h zymography membrane incubation, and continued to be catalyzed by the soil enzymes. MUF produced in soil during the following 2 h was then diffusing into water-saturated membranes, subsequently applied on the soil surface. Notably, the MUF amount diffused into the water-saturated membranes 2 h after zymography considerably exceeded the amount of MUF in the substrate-saturated membranes during zymography itself (Fig. 5). This finding has important implications for conducting zymography analysis. Specifically, in line with Razavi et al. (2016), it suggests that repeated zymography on the same soil surface, i.e., measurements of 2 or more enzymes from membranes sequentially applied on the same soil surface is not recommended. MUF produced in the soil after earlier incubations with substrate membranes might diffuse into subsequently applied membranes and affect their fluorescent patterns. However, the magnitude of this process likely varies depending on soil characteristics, thus, preliminary tests for the presence and magnitude of this phenomenon should be conducted prior to making decisions regarding repeated zymography applications in a given soil.

4.2 Contact area between the membrane and soil surface

All three approaches for assessing the contact area between soil and membrane (*Experiment 4*) provided useful information for improving interpretation of zymography maps, but each approach had its own pros and cons. The laser scanning approach produced a detailed map of the actual soil surface. However, knowledge of soil surface topography alone is not sufficient to determine the contact with the membrane. The necessary prerequisites are the information on the flexibility of the membrane and on the thickness of water films around soil peaks in contact with the membrane. These two parameters can greatly affect the contact area (Fig. 6a).

The MUF-staining approach detected from 5% to 57% of the A_{enz} (Fig. 6b), but a large fraction of the enzyme zones were not detected (Fig. 8b). One of the reasons is attachment of soil particles to the substrate-saturated zymography membrane during incubation and their subsequent removal from the soil surface along with the membrane. These particles represent the localities where the contact between the soil and the membrane was the greatest and where direct contacts between the enzymes and the membrane were most likely (Fig. 1). Yet, gaps left upon their removal on the soil surface are erroneously classified by MUF-staining as sites, where there was no contact between the soil and the substrate membrane. This is probably the greatest shortcoming of the MUF-staining surface determination method. Additional possible explanation of discrepancies between zymograms and MUF-stained soil surface maps is MUF diffusion into the soil or dilution by the soil solution that reduced luminescence intensity of applied MUF solution below the detection level. However, the MUF-staining method has another important shortcoming. MUF solution, diffused from the membrane into the soil, can reside in the soil for hours and days. This will make problematic subsequent MUF-based measurements of the enzyme activity on the same soil surface and might even affect measurements on new surfaces of the same sample (e.g. for 3D zymography), which could have been subjected to MUF diffusion. Perhaps, it is thus worth exploring other chemicals, detectable at different wave lengths for repeated zymography.

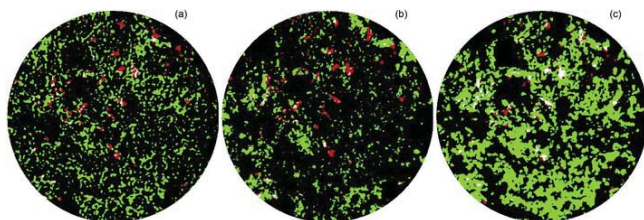


Fig. 8 Maps of contact area between soil surface and the membrane (green), enzyme-active area beyond (red) and within (white) the contact area obtained using the laser-scanning (a), MUF-staining (b) and X-ray CT (c) approaches. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alt-text: Fig. 8

The X-ray CT approach is the best experimental method for determination of the contact area because it accounts both for direct contacts between the membrane and the soil surface as well as for the indirect contacts via water menisci. It should be noted that in this study the MUF-staining method was applied prior to CT-scanning, thus the removal of the soil particles mentioned above as the problem of the MUF-staining method negatively contributed here to the X-ray CT performance as well. Despite this, contacts determined via the X-ray CT approach captured the presence of 60% of the area with detected enzyme activity (Figs. 6b and 8c). The performance is expected to be even better if the zymography membrane were not removed from the surface prior to CT scanning, as should be done during the actual use of X-ray CT for surface contact determination. However, the shortcoming of the X-ray CT approach is that it is much more time and cost consuming than laser scanning and, especially, MUF-staining approach. Moreover, an additional concern for using X-ray CT occurs in the experiments where multiple zymography readings are planned from the same soil sample, since repeatable scanning can have a negative effect on microbial activity, including new enzyme production.

Among the three methods, the laser-scanning approach appears to be the option with the highest potential in terms of balancing accuracy, costs, and negative effects on subsequent experimentation and zymography measurements. Surface topography measured by the laser scanner prior to zymography can provide accurate information not only on the distances between the membrane and the soil surface, but also point to potential localities of short-term hydraulic contacts. Some of the hydraulic contacts might exist only for a short period of time after the saturated membrane is placed on the soil surface; however, this time could be sufficient for movement of substrate and MUF.

4.3 Assessment of enzyme activity based on MUF measurements and modeling

Despite the fact that the HP2 model was used exclusively for illustration of diffusion processes occurred during zymography without any prior calibration and validation, it accurately described the dynamics of MUF changes in the membrane during incubations (Fig. 7a). This result indicates that the model can be used for gaining further insights into the processes taking place during zymography.

The modeling provided an understanding of the temporal dynamics of fluorescent patterns in the membrane. Specifically, the initially slow increase (0-15 min time interval in Fig. 7a) followed by a nearly linear increase of MUF content was caused by MUF diffusion from the substrate-saturated membrane to the enzyme area through the water film, and backward MUF diffusion to the membrane. Due to substrate and MUF diffusion, such a time lag between the substrate release from the membrane and MUF appearance on the membrane will always be present. This lag was clearly observed in the experimental data during the first 20 min of incubation (circles in Fig. 7a).

The diffusion affected not only MUF travel time to the membrane, but also the amount of MUF that reached the membrane and, thus, what was detected in the course of zymography. MUF contents in the membranes obtained experimentally as well as simulated by the model were much smaller than the total simulated MUF contents generated by the enzymes in the layer of soil adjacent to the membrane (Fig. 7a). Though, the percentage of total generated MUF that reached the membrane increased with time during modeling, only 20% of the total generated MUF reached the membrane after 2 h (Fig. 9), and the enzyme activity detected in the membrane was 4 times lower than that the total activity simulated during the 2-h experiment. The low fraction of MUF reaching the membrane was caused by diffusion of MUF into surrounding soil. This fraction could be even smaller, since the model did not account for MUF adsorption by soil particles and organic material, the magnitude of which is generally unknown.

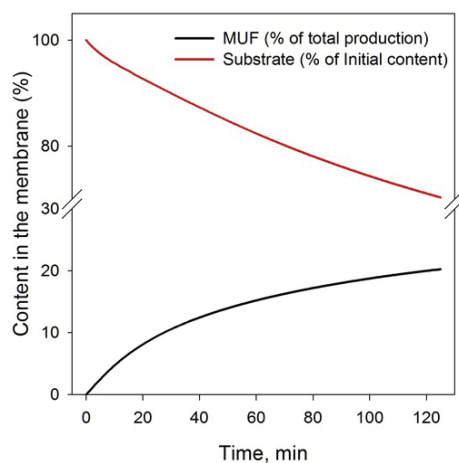


Fig. 9 Simulated changes in MUF and substrate contents in membrane. The lines reflect the diffusion of the substrate from the membrane to the soil (red line) and the backward diffusion to the membrane of the MUF enzymatically split in the soil (black line). Only approximately 20% of the total produced MUF will be recovered on the membrane after 1–2 h. This amount corresponds to approximately 25% of the substrate diffused from the membrane to the soil. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alt-text: Fig. 9

This finding provides some insights regarding the amounts of product that can potentially return to an enzyme producer. According to our simulation of MUF returned to the entire surface of the membrane, the amount that will return to the individual producer's cell is likely to be much less than 20% of the overall production. Thus, often observed immobilized enzyme remaining active for long time periods (Dick and Tabatabai, 1987; Schimel et al., 2017) might be the necessary prerequisites for maximizing benefits to the producer.

Incubation time was an important factor for assessment of the enzyme activity. First, due to the time lag between substrate diffusion from the membrane and MUF appearance in the membrane, the enzyme activity estimates during first 20 min of incubation are unreliable. However, MUF aggregation upon increasing MUF levels within the membrane resulted in less accurate MUF calibration for high MUF contents; this produced less accurate MUF estimations after 45 min zymography. Therefore, approximately 30 min (from 20 to 50 min) of the experiment were optimal for assessment of enzyme activity in the studied soil. This time interval could differ for different substrate concentrations, soils and enzyme activity. Soil characteristics and soil water contents, in particular, affect the diffusion rates and thus the optimal measurement time.

The enzyme activity assessed from the MUF measurements in the membranes differed for the two calculation methods. While the differential method can be considered as the most accurate assessment of the rates of enzyme activities, its results were not consistent in time (Fig. 7b). The high values encountered in the range of 40–60 min incubation time were likely an artifact associated with the piecewise calibration of MUF images, and with probable overestimation of MUF contents for $G > 84$, where the MUF calibration curve has very low slope. The cumulative method provided more time-stable estimates of the enzyme activity. However, it should be realized that its accuracy was also negatively affected by artefacts associated with piecewise calibration, even though it was not visually obvious from the plots (Fig. 7b).

It should be noted, that in this study the HP2 model was used to define pitfalls of the *in-situ* enzyme zymography, rather than for accurate assessment of the enzyme activity. For this reason, we used very rough model assumptions, i.e. localized all enzymes in one simulation point on the soil surface, used model parameters obtained for different soil and different incubation conditions. Therefore, the results of simulation could differ for different soils and enzyme distributions on soil surfaces. Moreover, accurate modeling of zymography cannot be done without model calibration and validation on the experimental data. For this reason we cannot assess the magnitude of underestimation of the enzyme activity using traditional 2D soil zymography. However, the results of the HP-2 simulations indicated the importance of the diffusion processes occurred during soil zymography and the necessity in their modeling for accurate *in-situ* estimation of enzyme activity.

5 Conclusions

Based on visualization and diffusion experiments, the following general conclusions regarding enzymatic and diffusion processes during zymography are suggested:

- Immobilized enzymes, that is either the enzymes or microorganisms attached to mineral surfaces of soil particles, constituted the majority of active enzymes measured during the relatively short time required for the 2D soil zymography. This makes possible interpretation of zymography images and determination of enzyme locations on the soil surface and their activity.
- The absence of free enzymes and the strong attachment of immobilized enzymes or microbial cells to mineral surfaces were the likely reasons for the absence of diffusion of enzymes in liquids of the studied system. Enzyme diffusion was not observed along a 100 µm distance (membrane thickness) despite relatively long, 1–2 h, durations of diffusion experiments. It should be noted that our experiments only addressed enzyme transport by diffusion and it remains to be seen whether enzymes can be detached from the soil surface and transported by convective water flow in soil pores.
- Diffusion of the substrates and products of enzyme catalysis preclude accurate estimates of enzyme activity in soil based solely on 2D zymograms, while the size of the soil volume (3D), where the enzymatic reactions visualized on the 2D zymograms take place, remains unknown.
- Enzyme activities detected via 2D zymography represent only a portion (~20% based on model estimates) of the actual reactions that take place within the soil volume in both direct and in hydrological contact with zymography membranes. This is a result of omnidirectional diffusion of reaction products with only a small portion of them reaching the membrane.
- Current zymography protocol provides useful information for comparisons of relative enzyme activities in different soils or on plant root surfaces, but its estimations of actual *in-situ* enzyme activities are likely not very accurate.
- We suggest that a solution to correct identification of the contributing soil volume and to estimation of actual enzyme activities within it can be found in combining 2D zymography measurements with estimation of the contact area and the contributing volume via laser scanning and with 3D modeling of diffusion and reaction processes taking place during zymography via HP2 software.

Acknowledgement

This research was partly funded by the National Science Foundation's Long-Term Ecological Research Program (DEB 1027253), by the National Science Foundation's Geobiology and Low Temperature Geochemistry Program (Award no. 1630399), by the Department of Energy Great Lakes Bioenergy Research Center (DOE Office of Science BER DE-FC02-07ER64494), by Michigan State University's AgBioResearch (Project GREEN), and by Michigan State University's Discretionary Funding Initiative. The work was supported by DAAD- German Academic Exchange Service' program "Research Stays for University Academics and Scientists, 2017" (57314018) and by the Research Award from Alexander von Humboldt Foundation. Contributions of BSR and EB were motivated and supported within the framework of the priority program 2089, funded by the DFG- Projects Nr. 403670038 and Nr. 403664478, respectively. This work has also been conducted in part as a preparatory study for the recently launched DFG priority program 2089 Rhizosphere spatiotemporal organization—a key to rhizosphere functions PE 1523/10-1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2018.09.030>.

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

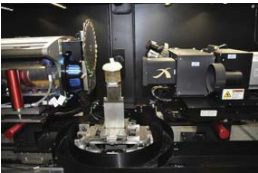


Fig. S1 X-ray microtomography setup for contact area study

alt-text: Fig. S1

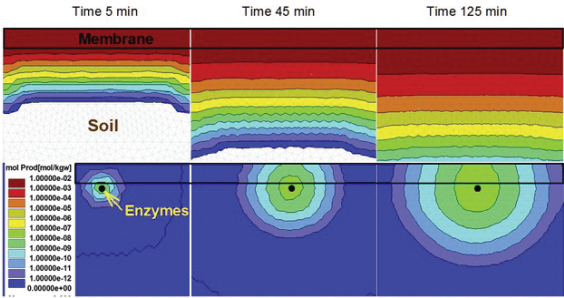


Fig. S2 Simulated patterns of the Substrate (top slides) and MUF (bottom slides) distributions in the membrane placed on the soil surface and within the soil adjacent to the membrane 5, 45 and 125 min after the membrane with substrate was placed in contact with the soil.

Download the full simulation movie from: <https://msu.edu/~akguber/experiments.html>

alt-text: Fig. S2

Highlights

- We studied processes occurring in the soil and the membrane during 2D soil zymography.
- We modeled product and substrate diffusion in the soil/membrane.
- Zymography underestimates enzyme activity due to product diffusion losses in soil.