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Scaling Down the Analysis of Environmental Processes: Monitoring Enzyme Activity in Natural Substrates on a Millimeter Resolution Scale

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Natural environments often show high levels of spatial heterogeneity. With a methodology based on the immobilization of fluorescent substrates, the distribution of extracellular enzymes can be studied at a 2.3-mm resolution with a detection limit of 1.8 nmol · h^{-1} · cm^{-2}. The method is applicable to environmental samples such as wood, litter, soil, or fungal colonies.

The determination of enzymatic activities is a simple approach to the study of environmental processes mediated by fungi and bacteria. Thus, enzyme activities have been interpreted as indirect measures of microbial biomass, rhizosphere effects, soil productivity, or mineralization potential of naturally occurring substrates or xenobiotics (2). Fluorogenic 4-methylumbelliferyl (MUB)- or 4-amidomethylcoumaryl-labeled enzyme substrates have been introduced for measuring the activity of a wide variety of hydrolytic enzymes, including exoglucanases, phosphatases, peptidases, and several others, with sufficient sensitivity based on the fluorescence of reaction products (2, 21).

The spatial dependence of environmental variables, such as enzyme activities, has been previously studied at various levels of resolution, for example, in the soil ranging from centimeters to kilometers (7). While the picture of the spatial distribution of environmental variables provides meaningful results at any scale, depending on the question asked and sample size chosen, both the decreasing sampling distance and sample size result in a decrease of spatial autocorrelation range due to the presence of spatial heterogeneity at different scales (9). In several environments, the extent of such spatial heterogeneity is high, even at scales smaller than a few centimeters. This was documented in the case of spatial distribution of phyllosphere or litter-associated fungi (15), decomposing wood colonized by saprotrophic basidiomycetes or ascomycetes (4), enzyme activity variation within the litter and soil horizons (1, 6, 20), or the distribution of soil bacteria. Bacterial diversity in soils on a millimeter scale (11) and communities of *Archaea* recovered as spatially independent 0.1-g subsamples from within a single 20-mm-diameter soil core varied in their composition (14). The microbial biomass content and community composition, as well as the rates of microbe-catalyzed processes, have been demonstrated to vary considerably over a scale of several millimeters at the soil-litter interface (16–18). Additionally, lichen soil crusts show spatial heterogeneity at a comparable resolution (22). The understanding of the variability of microbe-catalyzed processes at such scales has been hindered by the limitations of sample size requirements for the analysis. Previous efforts of dense sampling of enzyme activity were obtained at a resolution of centimeters (19) or limited to measurements along linear transects (12). Here, we show a fluorimetric MUB-based enzyme assay suitable for the study of small-scale distribution of extracellular hydrolytic enzymes of fungi and bacteria over surfaces of various substrates.

Samples (fungal colonies growing on agar, thin slices of colonized wood, soil, or decaying leaf litter) were fixed into plastic plates and overlaid with a 1% low-melting-point agarose in a 50 mM Na-acetate buffer supplemented with appropriate MUB substrates immediately before application (45°C). After brief chilling at 4°C to solidify the agarose overlay, fluorescence was read at 40°C using a multimode microplate reader, Infinite M200 (TECAN, Austria), by scanning the surface of the gel at a rectangular 2.3- by 2.3-mm grid for 5- to 10-min intervals over a period of 30 to 120 min. The data were visualized in Origin 8 (Originlab, MA), and the geostatistical analysis (variogram construction and map construction by kriging) was performed in Surfer 8 (Golden Software, Inc., CO). Linear fitting was used to determine the relationships between fluorescence and MUB concentration and between fluorescence increase and the activity of purified β-glucosidase applied at various concentrations on the surface of an agarose gel and dried at room temperature under vacuum (see the text in the supplemental material).

The amount of MUB linearly correlated with detected fluorescence ($P < 0.0001$), and the recorded increase of fluorescence corresponded well with the activity of purified β-glucosidase applied to the gel surface ($P < 0.0001$; Fig. 1). The detection limit of the method was determined to be 1.8 nmol · h^{-1} · cm^{-2} as 3× the maximal background fluorescence change.

Visualization of enzyme activity was carried out on (i) colonies of saprotrophic basidiomycetous fungi on malt agar (Fig. 2a and b); (ii) thin sections of a dead *Betula pendula* branch colonized by fungi with a fruit body of *Fomes fomentarius* (Fig. 2c and d); (iii) *Quercus petraea* leaves decaying *in situ* (Fig. 2e and f); and (iv) profiles of *Quercus* sp. forest topsoil collected with a soil slicer (10) (Fig. 1). The results show that even at the scale of a few square centimeters, enzyme activity varied considerably; the coefficients of variation (CV = SD/mean) of enzyme activities in fungus-colonized wood were 0.31 ± 0.10 for cellobiohydrolase, 0.40 ± 0.08

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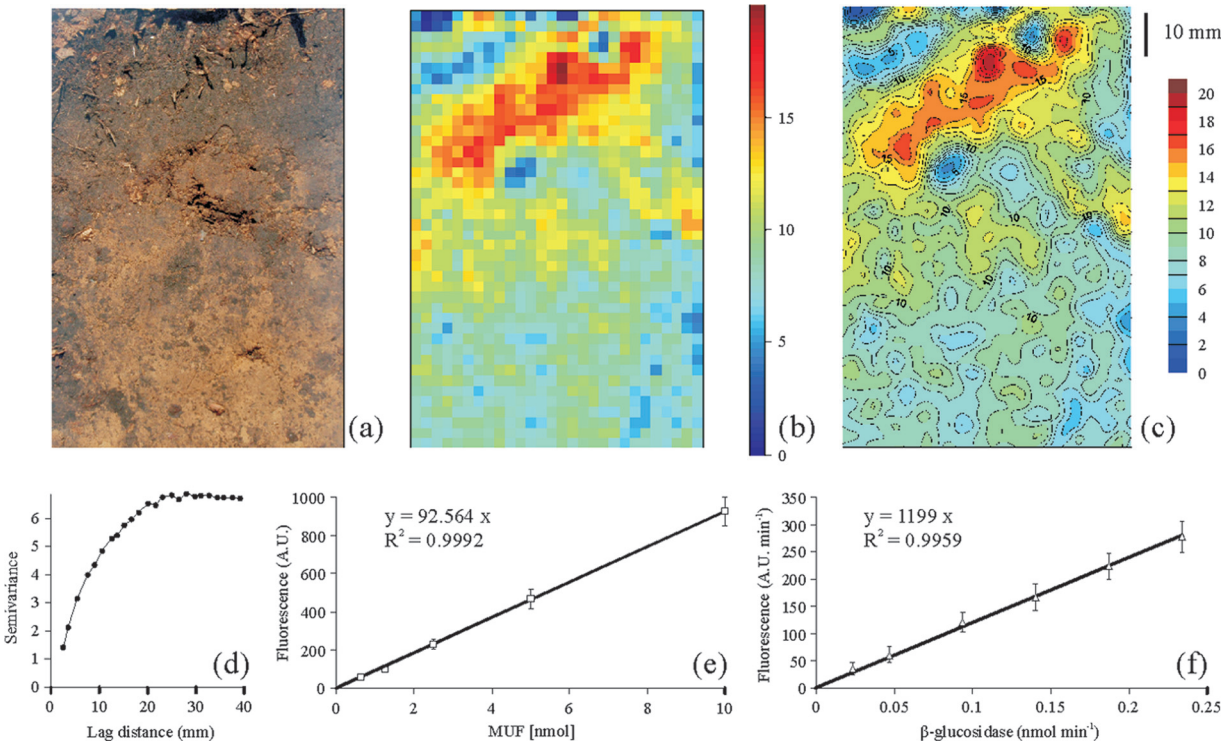

FIG 1 Distribution of β-glucosidase in the *Quercus* forest soil profile. (a) The soil profile of *Quercus* forest soil after litter removal (80-mm width by 120-mm depth); (b) measured values of β-glucosidase activity in nmol · h^{-1} · cm^{-2}; (c) krigged map of the distribution of β-glucosidase; (d) the plot of semivariance against distance; (e) linear fit of the observed fluorescence against the amount of MUF ($n = 6$ samples); (f) linear fit of the observed fluorescence change against the activity of purified β-glucosidase from almonds (Sigma, MO; $n = 6$ samples).

for β-glucosidase, 0.52 ± 0.21 for *N*-acetylglucosaminidase, and 0.56 ± 0.23 for β-xylosidase. In the decomposing leaves, the CV were 0.39 ± 0.08 for *N*-acetylglucosaminidase, 0.45 ± 0.12 for β-xylosidase, and 0.41 ± 0.22 for cellobiohydrolase. Even within single fungal colonies, the enzyme activities are unevenly distributed (Fig. 2).

In decaying wood, the enzyme activities spatially autocorrelated in a range of <30 mm (12 to 32 mm for cellobiohydrolase,

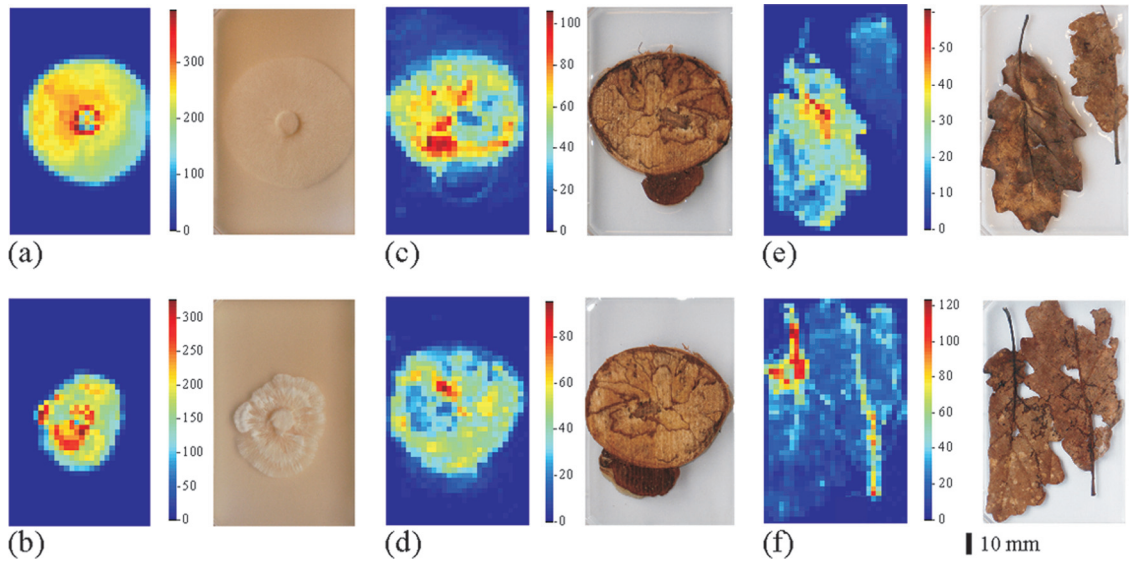

FIG 2 Distribution of hydrolytic enzymes across fungal colonies, fungus-colonized wood sections, and decaying leaves. Distribution of β-glucosidase (a) and phosphomonoesterase (b) across the colonies of the saprotrophic basidiomycete *Hypholoma fasciculare* on malt extract agar, β-glucosidase (c) and cellobiohydrolase (d) over a cross-section of a *Betula pendula* branch colonized by wood-decomposing fungi, including *Fomes fomentarius* (fruit body), *N*-acetylglucosaminidase (e), and cellobiohydrolase (exocellulase) (f) over the surface of *Quercus petraea* leaves decomposing for 10 months on the forest floor surface. Enzyme activities are expressed in nmol · h^{-1} · cm^{-2}.

β-xylosidase, β-glucosidase, and *N*-acetylglucosaminidase). The cross-section of the log showed borderlines between different species or genets of fungal colonizers (4), and high or low enzymatic activity was often associated with a particular patch colonized by a specific fungus (Fig. 2c and d), the spatial autocorrelation of the enzyme activity being thus largely affected by the size of these patches. The spatial autocorrelation in leaves ranged from 29 ± 21 mm for β-xylosidase, over 43 ± 19 mm for cellobiohydrolase, and up to 62 ± 16 mm for *N*-acetylglucosaminidase. This roughly corresponds to the sizes of areas in living plant leaves colonized by a single dominant fungal species (13), and we can hypothesize that the distribution of certain enzymes reflects the presence of their producers at this spatial scale.

Soil is a highly heterogeneous environment, and a link between the heterogeneity of soil physicochemical properties and microbial abundance has been demonstrated on a centimeter scale (8). When sampled at a coarse scale with 16-cm^2 samples analyzed over a 144-m^2 site, the CV of β-glucosidase activity in *Quercus* sp. forest mineral soil was 0.29 (20). In the same soil, where 0.053-cm^2 samples were collected over an area of only 0.0048 m^2, the CV was very similar (0.24 ± 0.06). This shows that enzyme activity is highly variable, even within a few square centimeters. The soil properties, microbial biomass, and enzyme activities of the soil from this study showed spatial autocorrelation in a range of tens of centimeters to more than 1 m if 16-cm^2 samples were analyzed (3); the autocorrelation range of β-glucosidase activity was 92 cm. Here, we show that the autocorrelation range drops to 21 ± 4 mm with a sample size reduction to 0.053 cm^2. To determine whether the current sample size and sampling frequency were appropriate for the estimation of the range, the data sets were resampled (i) by reducing the sampling depth to 1/4 by the analysis on 0.053-cm^2 samples taken at a double distance and (ii) by increasing the sample size to 0.212 cm^2 using the mean activities of four adjacent squares. These analyses delivered range estimates of 22 ± 4 mm and 20 ± 3 mm, respectively, showing that the sampling distance and sample size did not represent limitations for the reliable estimation of this important parameter.

There is a recent report on a method of enzyme measurement at a submillimeter scale in marine sediments (5). Unfortunately, due to the requirement of a foil adhesion onto the sample surface, this method is not applicable for dry samples with uneven surfaces. In terrestrial environments, enzyme activity distribution was previously studied using a Plexiglas window in soil with filter paper containing enzyme substrates that enabled the visualization of the presence or absence of enzymes but not the quantification of activity (6). At present, the lowest sample size used for the analysis of enzyme activity distribution was used for leaf discs of immersed litter, where 0.20-cm^2 samples were analyzed individually on a 2-cm grid (19). The method described here has an improved resolution and is technically more feasible, because whole surfaces are analyzed at once. The obvious limitation compared to classical methods of sampling is the 2-dimensional sampling that quantifies enzyme activity associated with surfaces rather than a volume of the sample. Despite this, the approach seems to be useful for the study of spatial distribution of hydrolytic enzymes in environmental samples with sufficient sensitivity. In addition, this work opens the possibility for future targeted sampling of microbial community composition under enzyme activity hot spots, an approach that can potentially increase our knowledge of microbial community structure-function relationships.

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