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Soil zymography as a powerful tool for exploring hotspots and substrate limitation in undisturbed subsoil

Julian Heitkötter*, Bernd Marschner

Department of Geography, Chair for Soil Science and Soil Ecology, Ruhr-Universität Bochum, Universitätsstr. 150, 44801 Bochum, Germany

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ABSTRACT

It is widely accepted that soil microorganisms are not evenly distributed but are often concentrated in spatially segregated hotspots that are characterized by higher substrate availability compared to the surrounding bulk soil. However, microorganisms outside of hotspots may be in a dormant or inactive state, since they have depleted all available substrates within their vicinity. So far, the knowledge about the spatial distribution and dynamics of microbial activity in subsoil is very scarce, since most available data has been acquired from either homogenized soil samples or as bulk signals from undisturbed soil cores. In this study, we introduced a new incubation approach combining soil zymography and substrate addition on undisturbed soil core surfaces. We mapped three extracellular enzymes (β-glucosidase, chitinase and acid phosphatase) on a subsoil sample from 60 cm depth and analyzed their activity-patterns using different geostatistical and spatial analyses. After initial enzyme mapping, the soil was homogenously sprayed with ^{14}C glucose as model substrate and incubated for 14 days. Soil zymography was suitable for detecting hotspots in undisturbed soil, making up a proportion of 2.4% on average of the total area. Consequently, microbial-driven biogeochemical processes can be expected to be limited to small areas in this subsoil, while the major part of the soil volume is not contributing. Glucose additions considerably increased enzyme activities up to 900% in initial non-hotspots, while the effect was far lower in initial hotspots. These results show that microorganisms in the subsoil outside of hotspots can be activated and release enzymes when substrate is supplied. Thus, dormant or inactive microorganisms outside of hotspots are able to contribute to SOC mineralization when substrate limitation is overcome, thus most likely inducing positive priming effects. Our results clearly demonstrate the benefits of combining enzyme mapping with substrate additions on undisturbed soil to gain new insights about microbial hotspots and C-cycling in subsoils using spatial analyses. In contrast to traditional incubation experiments, this method gives high spatial information about microbial activity, allowing a more differentiated interpretation of incubation results.

1. Introduction

It is widely accepted that microorganisms in soil are not evenly distributed but are often concentrated in spatially segregated hotspots that are characterized by high substrate inputs compared to the surrounding bulk soil (Kuzyakov and Blagodatskaya, 2015; Tecon and Or, 2017). According to Kuzyakov and Blagodatskaya (2015), hotspots are mainly found in the rhizosphere, detritussphere, biopores and on aggregate surfaces, where the input of labile C boosts microbial activity and C turnover in soil. Substrate availability is heterogeneous in space and time since the "hosts" of hotspots, such as roots or preferential flow paths, are not homogenously distributed and seem to appear even patchier and more important for fresh SOM input in deep soil layers (e.g. Heinze et al., 2018; Leinemann et al., 2016). Therefore, active microorganisms in soils are found in a non-random, patchy distribution of microsites (Tecon and Or, 2017). Consequently, microbial-driven biogeochemical processes can be expected to be limited to small areas, while the major part of the soil volume is not contributing. It is assumed that less than 5% of soil is occupied by active microorganisms, while considerably lower amounts were found in subsoils (Nunan et al., 2003; Nannipieri et al., 2003). However, microorganisms outside of hotspots may be resting in a dormant or inactive state, since available substrates within their vicinity have been depleted by them. Especially, substrate limitation is suggested to be a major factor limiting microbial activity and controlling C turnover in subsoils (Heitkötter et al., 2017; Tian et al., 2016).

So far, the knowledge about the spatial distribution and dynamics of microbial activity in subsoil is very scarce, since most available data has been acquired from either homogenized soil samples or as bulk signals from undisturbed soil cores, limiting the interpretation of results. For

* Corresponding author.
E-mail address: Julian.Heitkoetter@rub.de (J. Heitkötter).

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instance, microbial substrate limitation is investigated by mixing isotopic-labeled substrates into soil and comparing the evolved SOC-derived CO_2 to an unamended sample, i.e. priming experiments (Kuzyakov et al., 2000). However, soil disturbance, as conducted in classical incubation studies by mixing substrates into soils, will provide microorganisms access to SOM, which would never have been reachable in the undisturbed subsoil environment, especially in subsoils as indicated by increased SOC-mineralization rates in disturbed subsoils compared to undisturbed samples (Salomé et al., 2010; Wordell-Dietrich et al., 2016; Ewing et al., 2006). Therefore, new incubation approaches are required to obtain high spatial and temporal information about microbial activity in undisturbed soil. The use of imaging techniques would allow a more differentiated interpretation for gaining new mechanistic understanding in the complex soil matrix. This spatial understanding of microbial processes can further serve as an input parameter for improving carbon models.

In recent years, soil zymography received increased attention in soil research as indicated by rising numbers of publications using this method. Soil zymography is an in situ technique for determining the spatial activity of extracellular enzymes in soils (Spohn et al., 2013) and became popular since it is a nondestructive method for mapping enzymes at high spatial resolution (Ma et al., 2017; Spohn and Kuzyakov, 2014; Hoang et al., 2016), also allowing repeated measurements on the same sample. Since microbial C and nutrient acquisition is largely determined by extracellular enzymes that are actively excreted to catalyze the degradation of SOM (Burns et al., 2013), enzyme activities are suitable as proxy for microbial activity and enzymatic-driven C-turnover. The flow cell sampling method developed by Krueger and Bachmann (2017) provides soil slices with intact surfaces, allowing analysis of surface properties and processes with high spatial resolution. Therefore, the flow cell concept may introduce a new possibility for analyzing the spatial distribution of microhabitats in soil at the mm-scale and opens up opportunities for new types of incubation experiments. Since microbial hotspots are suggested to be sufficiently supplied with substrate (Kuzyakov and Blagodatskaya, 2015), areas outside are most likely substrate limited. Newly developed spraying devices provide the possibility to add liquids evenly on surfaces (Stoeckli et al., 2014). Such devices can be used to spray substrate on soil slices to determine the effect on enzyme activities in hotspots and surrounding soil. Further, phosphor imaging seems to be an appropriate tool to analyze the spatio-temporal distribution of ^{14}C labeled substrates in the course of time, allowing quantitative interpretation. Phosphor imaging in soil science has been successfully applied to determine the spatial distribution of CH_4 assimilation in soil cores (Stiehl-Braun et al., 2011) or was combined with soil zymography to relate enzyme activities to photosynthates (Spohn and Kuzyakov, 2013).

In this study, we wanted to test if the application of soil zymography on undisturbed soil is suitable for gaining spatial information about microbial hotspots in soil, since up to now all zymography studies have been conducted in artificial soil systems (e.g. Ma et al., 2017; Spohn and Kuzyakov, 2014; Hoang et al., 2016). Here, we mapped three extracellular enzymes (β-glucosidase, chitinase and acid phosphatase) on a subsoil sampled at 60 cm depth and analyzed their activity-patterns using different geostatistical and spatial analyses. After initial enzyme mapping, the soil was homogenously sprayed with ^{14}C glucose as a model substrate to determine if microbial activity patterns can be found outside of hotspots when the hypothesized substrate limitation is removed.

2. Material and methods

2.1. Soil sampling

The sampling site is located in the "Grinderwald" forest (52° 34′ 22″ N, 9° 18′ 51″ E), Lower Saxony, Germany. The soil is a Dystric Cambisol (IUSS Working Group WRB, 2014) developed on glaciofluviatile sandy deposits from the Saale glaciation (Bundesanstalt für Bodenforschung, 1973) covered by a 100 yr old beech (*Fagus sylvatica* L.) forest stand. In this study, the sampling technique developed by Krueger and Bachmann (2017) was applied to obtain undisturbed samples from 60 to 71 cm depth with 110 mm height, 70 mm length and 5 mm width. The sampling technique consists of a sampling frame and a drilling device for pushing the frame slowly and controlled into the soil to obtain an undisturbed soil block. The sides of the frame consist of parallel stacked rectangular spacers (5-mm thickness), which are fixed by screws between two parallel acrylic glass plates. The block was then sliced with a sharpened 0.3-mm thin stainless steel panel which was inserted vertically between the rectangular spacers. With one sampling four subsamples were obtained.

2.2. Zymography

Soil zymography was performed similar to Spohn and Kuzyakov (2014) to localize hotspots and to identify the spatial distribution of enzyme activities on the surface of the flow cells before and after incubation. We determined the activity of acid phosphatase (Pho), β-glucosidase (β-glu) and chitinase (Chiti) using fluorescent methylumberrliferyl (MUF)-substrates. 4-MUF-ß-D-Glucoside (β-glu), 4-MUF phosphate disodium salt (Pho) and 4-MUF-N-Acetyl-ß-D-Glucosaminide (Chiti) were dissolved in millipore water to the desired concentration of 12 mmol. Cut polyamide membranes (Sartorius Stedim, Göttingen, Germany) were soaked in respective substrate solutions. 1-mm-thick agarose gels (1% Agarose) were carefully transferred to the flow cells and subsequently covered with substrate-soaked membranes. For each enzyme, the samples were incubated for 1 h at 20 °C in the dark. For all four flow cell replicates, the following order of enzyme mapping was maintained throughout the experiment: Pho, β-glu, Chiti. After each incubation, the agarose gel was changed. The MUF becomes fluorescent under UV-light when substrates are enzymatically hydrolyzed. Thus, the membranes were photographed after incubation in a gel documentation system (Biostep GmbH, Burkhardtsdorf, Germany) equipped with epi-UV illumination at 365 nm and a Canon EOS-700D equipped with a fixed focal length lens (F/1.8) and a 420 nm filter. Further, a control was set for each substrate by placing a substrate-soaked membrane on an agarose plate and incubating it under the same conditions as described above. After incubation, these membranes were photographed under UV-light to determine the autofluorescence of each substrate. The calibration was conducted following Spohn and Kuzyakov (2014). Membranes were cut into pieces of 4 cm^2 and soaked in solutions of MUF of different concentrations (0, 35, 70, 130 and 200 µmol). These were then photographed under UV light. The amount of MUF on an area basis was calculated from the volume of solution taken up by the membrane, the concentration of the solution and the size of the membrane. The relation between gray values and concentrations was described by a linear fit ($R^2 = 0.98$).

2.3. Incubation procedure

A laboratory incubation study was performed for 14 days at 20 °C. Glucose was used as a model substrate, which is commonly used for investigating priming effects in soils, since it is a main compound of root exudates (Kuzyakov, 2010). Additionally, one subsample received water serving as control. An overview of the incubation approach can be found in the supplementary material (Fig. 1).

After the initial zymography measurement, ^{14}C uniformly labeled glucose (Perkin Elmer, Waltham, USA) was applied to the soil surface at rate of 140 µg C cm^{-2}, corresponding to a 10 day dose of the C input by roots into the subsoil at the Grinderwald (Tückmantel et al., 2017). The ^{14}C glucose was mixed with unlabeled glucose to obtain the required concentrations and desired radioactivity of 1000 Bq $µl^{-1}$. The experiment was conducted with three experimental replicates (using three subsamples). The substrate (or the water for the control) was sprayed