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Combination of techniques to quantify the distribution of bacteria in their soil microhabitats at different spatial scales

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ABSTRACT

To address a number of issues of great societal concern at the moment, like the sequestration of carbon, information is direly needed about interactions between soil architecture and microbial dynamics. Unfortunately, soils are extremely complex, heterogeneous systems comprising highly variable and dynamic micro-habitats that have significant impacts on the growth and activity of inhabiting microbiota. Data remain scarce on the influence of soil physical parameters characterizing the pore space on the distribution and diversity of bacteria. In this context, the objective of the research described in this article was to develop a method where X-ray microtomography, to characterize the soil architecture, is combined with fluorescence microscopy to visualize and quantify bacterial distributions in resin-impregnated soil sections. The influence of pore geometry (at a resolution of 13.4 μm) on the distribution of *Pseudomonas fluorescens* was analysed at macro- (5.2 mm × 5.2 mm), meso- (1 mm × 1 mm) and microscales (0.2 mm × 0.2 mm) based on an experimental setup simulating different soil architectures. The cell density of *P. fluorescens* was 5.59×10^7 (SE 2.6×10^6) cells g^{-1} soil in 1–2 mm and 5.84×10^7 (SE 2.4×10^6) cells g^{-1} in 2–4 mm size aggregates soil. Solid-pore interfaces influenced bacterial distribution at micro- and macroscale, whereas the effect of soil porosity on bacterial distribution varied according to three observation scales in different soil architectures. The influence of soil porosity on the distribution of bacteria in different soil architectures was observed mainly at the macroscale, relative to micro- and mesoscales. Experimental data suggest that the effect of pore geometry on the distribution of bacteria varied with the spatial scale, thus highlighting the need to consider an "appropriate spatial scale" to understand the factors that regulate the distribution of microbial communities in soils. The results obtained to date also indicate that the proposed method is a significant step towards a full mechanistic understanding of microbial dynamics in structured soils.

1. Introduction

Soil microorganisms play a vital role in soil ecosystem processes, and their location is restricted to the conditions provided by micro-habitats, whose properties vary, among other factors, due to the large spatial heterogeneity of soils (Vos et al., 2013). Bacteria tend to aggregate in their habitats and form what has been referred to as "microbial hotspots". Hotspots are zones in which the biological activity is much faster and intensive compared to average soil conditions (Kuzyakov and Blagodatskaya, 2015). However, little is known about what controls the spatial distribution of bacteria in soil. Studying the spatial patterns at the microscale could help to determine the factors controlling microbial community and activity. Subsequently, this data and knowledge of the relevant factors could help in the development of predictive models that would foster the understanding of bacterial contributions to soil functions.

Over the years, the spatial distribution of indigenous and introduced bacteria has been studied in undisturbed or repacked soil columns, however the relationship between the bacterial spatial distribution and 3D soil architecture has not been considered (Nunan et al., 2001; Kizungu et al., 2001; Nunan et al., 2003; Dechesne et al., 2003; Pallud et al., 2004; Dechesne et al., 2005). Spatial isolation, afforded by the

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complexity of soil air-solid interfaces, is believed to be one of the key factors accounting for the diverse microbial communities in soils. Geometrical characteristics of the soil pore space, such as pore volume, shape, connectivity, size, and tortuosity of pathways can have an impact on microbial composition and activity in soil. They regulate the accessibility of organic matter, the diffusion of oxygen through the gaseous phase, and the diffusion of dissolved compounds through the water phase, as well as the movement of microorganisms. These pore characteristics can be measured experimentally or can be estimated via non-destructive imaging.

Advances in the application of X-ray micro-tomography have made it possible to visualize and quantify the internal architecture of soils in three dimensions at μm resolution. Recent studies (Kravchenko et al., 2013; Juarez et al., 2013; Wang et al., 2013; Kravchenko et al., 2014; Negassa et al., 2015) have combined X-ray tomography with other analytical methods to investigate the influence of pore geometry on distribution (Kravchenko et al., 2013; Wang et al., 2013), composition (Ruamps et al., 2011; Kravchenko et al., 2014), and activity (Ruamps et al., 2013; Juarez et al., 2013) of bacterial communities in soil. These studies show how the combination of advanced techniques can help in obtaining experimental evidence on relationships existing between microbes and physical microscale environments. Whereas the results suggest that the study of bacteria at a scale relevant to microorganisms is important, there is no clarity yet what scale that should be and if relationships and observations differ across scales.

In this general context, the aim of this article is to develop a procedure that can be used to quantify the influence of pore geometry on the spatial distribution of bacteria in soil. This was achieved by integrating 2-D fluorescence microscopy with 3-D X-ray tomography techniques. The specific objectives of this study are (i) to quantify using X-ray micro-tomography, the pore geometry of resin-impregnated soil microcosms representing different soil architectures (aggregate sizes); (ii) to quantify bacterial distributions in polished sections of resin-impregnated soils; and (iii) to determine if there is an effect of the scale of observation, by analysing the influence of pore geometry on the distribution of introduced bacteria, through co-locating 2-D thin sections within a 3-D X-ray CT volume.

2. Materials and methods

2.1. Preparation of soil microcosms

A sandy loam soil was collected from an experimental site, Bullion Field, situated at the James Hutton Institute, in Dundee, Scotland. The soil (5.4% SOM, C/N: 16.4, pH ($CaCl_2$): 6.1, electrical conductivity: 49 μS cm^{-1}) was dry-sieved and sterilized by autoclaving twice at 121 °C and 100 kPa for 20 min with a 24 h interval time. Sieved aggregates of 1–2 mm and 2–4 mm size of this soil were used to prepare microcosms. These microcosms consisted of soil aggregates, packed in steel rings (16 mm inner diameter and 17 mm height, 3.4 cm^3 volume) at a defined bulk-density of 1.3 g cm^{-3}, and watered to reach a state with 40% water-filled pores. The moisture content was adjusted to 0.15 cm^3 g^{-1} by adding sterilized dH_20_{MQ} 48 h prior to packing. In each microcosm, 5.09 g of soil aggregates was inoculated with 500 μL of the bacterial suspension, mixed well to ensure an even distribution of the bacterial inoculum, and packed using a pushing rod. Control samples were packed in a similar manner except that sterile dH_20_{MQ} was used instead of the cell suspension. Three replicates per treatment for each sampling day were prepared, and the microcosms were sampled destructively four times.

To obtain the inoculum, an overnight culture of *Pseudomonas fluorescens* SBW25 was prepared in King's B medium at 23 °C in the dark, washed in 1 × PBS and adjusted to a specific cell density prior to inoculation using a spectrophotometer reading at OD 600 nm (Thermo Fisher Scientific, UK). The cell density of *P. fluorescens* was 3.6 x 10^7cells mL^{-1} and thus 1.8 x 10^7cells were inoculated per microcosm. Additional samples were amended with 500 μL dH_20_{MQ} instead of inoculum serving as control treatments. Three replicates per treatment were prepared and sealed in plastic bags to avoid drying of samples. The samples were incubated at 23 °C in the dark for 5 days to allow bacterial growth and spread through the soil. The soil microcosms were sampled after five days for resin impregnation, as explained in the next section.

2.2. Fixation and dehydration of soil microcosms

Soil microcosms were first placed onto a hardboard covered with layers of cotton mesh to prevent loss of soil during the embedding processes. Microcosms were then placed on top of an aluminium gauze stand in a container to support the subsequent steps required for fixation and resin impregnation. To preserve the distribution of bacteria within the soil matrix, the microcosms were fixed using a 2% formaldehyde solution (v/v in H_2O; 37% stock solution, Sigma Aldrich). This solution was added slowly from the sides of the container, to minimize disturbance of soil microcosms and facilitate the exchange of liquids (from bottom to top). All microcosms were completely submerged in the solution and kept overnight for fixation at 4 °C. Subsequently, samples were washed in MQ distilled water for 2 h, which was added the same way as the fixation solution. After washing, the samples were dehydrated with a graded series of acetone solutions (technical grade, VWR) to avoid interference with the polymerization of resin. Samples were submerged in 50% (v/v) acetone-water solution at room temperature for at least 12 h. Subsequently a graded series of 70%, 90% and three times 100% (v/v; acetone in water) was applied, each step lasting for 2 h. During the last two steps with 100% acetone, samples were kept under vacuum (280 mbar) to facilitate the complete exchange of all pores.

2.3. Resin impregnation of soil microcosms

A 2 L volume of impregnation mixture was prepared for a subset of up to 9 microcosms by adding 1300 μL of accelerator (0.95‰ (v/v) 1%-Cobalt Octoate accelerator, Oldopal, Büfa, Germany) and 2600 μL of hardener (1.9‰ (v/v) cyclohexanone peroxide, Akzo Nobel, Germany) to 1.4 L of polyester resin (Oldopol P50-01, Büfa, Germany), and 600 mL of acetone added as a thinner. The resulting mixture was mixed well and was kept under vacuum (240 mbar) to remove gas bubbles, until it was added to the samples.

Acetone was evacuated from the container with the soil samples, and the latter were then placed into a desiccator equipped with a tube and valve connected to the resin mixture container. Resin was then added drop by drop under vacuum (240 mbar), with the drops placed immediately next to the microcosms to allow an infiltration with resin from the bottom to the top to ensure that the pores of the soils were filled with resin mixture as completely as possible. Shortly before reaching the surface of the microcosms (after approx. 40 min) the addition of resin was stopped for a while and vacuum was increased (200 mbar) for 1 h. Finally, the remaining mixture was added to cover the sample completely with resin. Samples were left at room temperature under a hood for polymerization of the resin, which lasted 7 weeks. Resin impregnated samples were then cut, removed from steel rings, and the bottom and top were parallel ground on a cup wheel grinding machine (MPS2 120, G&N, Germany). Finally, a vertical cut was made through the microcosm to ensure a proper orientation of each block during CT scanning and subsequent fluorescence microscopy.

2.4. X-ray CT of resin impregnated samples

The physical structure of resin-impregnated microcosms was obtained via X-ray μ-CT scanning (HMX ST 225, Metris X-Tek, UK) at a resolution of 13.4 μm per voxel. In order to visualize resin-filled pore space, samples were scanned under energy settings of 145 keV and