

# Interconnectivity of habitats in soil: combining X-ray micro tomography and thin sectioning to reveal fungal-soil structure interactions

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**Abstract:** The extreme heterogeneity and interconnectivity of the 3-dimensional pore space within soil makes it a unique habitat for the diverse microbial population and has a pivotal role in microbial interactions. Manipulation and quantification of the 3-dimensional pore space and the spatial distribution of micro-organisms is therefore essential if we are to fully understand microbial interactions. Here we pack soil microcosms at different bulk-densities to manipulate soil structure and use x-ray micro tomography and soil thin sections to analyse the effect on the connectivity of the pore volume and on fungal exploration.

**Keywords:** pore-size, x-ray micro-tomography, habitat, thin sections

## Introduction

Multitrophic interactions in soil depend on the interconnectivity of habitats, and not just on the interactive capability of organisms. Water movement, diffusion of molecules and microbial movement in soil are key features of the soil, regulating microbial interactions. Too often we fail to recognise (or exploit) the complexity of the soil habitat in order to provide effective management. To date, the microscopic environment in soil has remained largely hidden as techniques that operate at scales relevant to microbes are only scarcely available. Much progress has been made recently using biological thin sections within which the spatial distribution of micro-organisms can be observed in a 2-dimensional transect through soil. However, it is difficult to infer connectivity between habitable micro-sites in soil from 2-D slides as this can only truly be quantified in 3 dimensions. In this paper we present how novel X-ray micro-tomography (to characterise non-destructively the soil structure and pore volume) can be linked with thin sectioning, to reveal structure-biology interactions at microscopic scales. Exemplified by the fungus *Rhizoctonia solani* we show how interconnectivity of habitats (pore volume) in soil changes with the bulk-density and regulates the spatial distribution of fungal hyphae. We discuss its importance for interactions in soil.

## Material and methods

### *Sample preparation*

Soil aggregates of an arable sandy loam were packed into polypropylene cylinders (6 cm diameter, 5 cm high), to attain bulk densities of 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m<sup>-3</sup>. At each bulk density the air-filled pore volume was 0.17. Full details can be found in Harris et al (2004). Each sample was inoculated with *Rhizoctonia solani*, introduced with discrete nutrient sources placed in a line going through the centre of the sample. After incubation at 23 °C for 5 days fungal hyphae were stained and the soil impregnated with resin. Thin sections (6.2 cm<sup>2</sup>

and 30  $\mu\text{m}$  thick) were taken from a horizontal slice through each sample. Blocks on either side above and below the section were used for 3-D scanning of the soil structure.

#### ***Visualisation and quantification of 3-dimensional pore space in soil***

The 3-dimensional pore space was visualised with an X-TEK benchtop micro-tomography system (Johnson *et al.*, 2007, [www.simbios.ac.uk](http://www.simbios.ac.uk)). Samples were scanned using a molybdenum target, X-ray source settings of 155kV and 25  $\mu\text{A}$ , and an aluminium filter (0.25 mm) to reduce beam-hardening artefacts. Ring artefacts were minimised during the acquisition of angular projections, which were also corrected for field flattening. CT datasets were collected using 1169 angular projections and then reconstructed using a filtered back projection algorithm with a resolution of 73.9  $\mu\text{m}$ . Beam-hardening corrections were applied during the reconstruction. All 3D volumes were converted using VGStudioMax v.1.2.1 into 260 x 260 x 525 image stacks with voxel-thick slices. Binary data sets were produced by thresholding the grey-scale image stacks in ImageJ. The choice of the threshold parameter was based on the analysis of the histogram region corresponding 5 voxels.

A region of interest, measuring approximately  $2 \times 2 \times 4$  cm, was subjected to a quantitative analysis of the 3D pore structure using in-house developed software. Specifically we quantified: (i) the total 3-D pore volume (total volume within which biotic interaction occur), (ii) the pore-size distribution (related to size-exclusion based on pore diameter), (iii) the connectivity of the pore volume (characterises the volume of soil within which interactions can occur), and (iv) the spectral dimension of the pore space (a measure of pore volume interconnectedness). For each property we quantified how these are affected by bulk-density of the soil.

#### ***Visualisation of fungal mycelium in situ***

Biological thin sections (30  $\mu\text{m}$  thick) were analysed from the same samples to obtain high resolution mapping of mycelia distribution and 2-D pore space in soil (for detail see Harris *et al.*, 2003). First we quantified fungal densities in microscopic images (0.44  $\text{mm}^2$ ) at high magnifications that are essential to visualise fungal mycelium. In order to allow quantification of mycelia distribution at larger scales, we summarised the quantitative analysis to presence or absence of fungal hyphae in the microscopic sections. The distribution of mycelium within the larger sections (6.2  $\text{cm}^2$ , equivalent to the size of a fungal colony) was subsequently quantified as the proportion of sites occupied by the fungus as a qualitative measure of mycelia density in different soil samples. Further details of the fungal structure interactions in these sections can be found in Harris *et al.* (2003).

## **Results and discussion**

#### ***Effect of bulk-density on pore volume and connectivity***

The geometrical constraints of the pore volume within soil are difficult to prescribe experimentally, even within controlled experimental systems. Thus we reverted to surrogates of these structural measures, namely aggregate-sizes, bulk-density, and air-filled porosity. This enabled us to manipulate soil structure and pore-volume within limits as an experimental variable in replicated microcosms, packed at bulk-densities ranging from 1.2 to 1.6  $\text{Mg m}^{-3}$ . We then used x-ray micro-tomography to quantify the precise effects of our experimental variables on the geometrical characteristics of the pore volume, here shown for the two extreme densities of 1.2 (Fig. 1a) and 1.6 (Fig. 1b).

The pore volume changed quantitatively, exemplified here by the following 3 characteristics: (i) with increasing bulk-density the pore volume decreased; (ii) with

increasing bulk-density the pore-size distribution shifted with a decrease mainly in the fraction of larger inter-aggregate pores ( $> 300 \mu\text{m}$ ) while the fraction of smaller pores increased; (iii) the connectivity of the pore space reduced drastically with increasing bulk-density. Particular noteworthy and important for biotic interactions was the difference in connectivity of the pore volume, as shown for the largest pores in each soil sample (Fig. 1d). At low bulk-densities (1.2 – 1.4) almost the entire pore volume is part of 1 single pore, occupying 75-95% of the pore volume. At higher densities, areas in the pore volume were isolated from each other, or connected through pore necks smaller  $70 \mu\text{m}$ , with the largest pore occupying  $<20\%$  of the pore volume. The connectivity determines the probability that two organisms occur in the same (connected) pore space, a pre-requisite for interaction to occur. Isolate pores not part of this large cluster are of equal importance as they may provide safe-havens against antagonists introduced into soil. This illustrates that exclusion of the pore volume is not just a function of the pore diameter but relates to connectivity of the pore volume, and that largest pores can be shielded from invasion.

#### ***Effect of bulk-density on fungal exploration***

The highest resolution at which we can quantify microbial distribution in soil is currently obtained destructively using biological thin sectioning. At microscopic scales (i.e.  $0.77 \times 0.58 \text{ mm}$ , the only scale at which fungal mycelium can be detected in soil thin sections) fungal hyphae explore the space between aggregates (Fig. 2a). Consecutive microscopic images enabled to quantify the spatial distribution of mycelium within an area of  $27 \times 23 \text{ mm}$  which is a relevant length scale for fungal colonies (Fig. 2b,c). A clear correlation existed between distribution of fungal mycelium and soil structure with fungal hyphae predominantly exploring the larger well connected pore volume in soils packed at low densities. This highlights the importance of the connectivity of the large pores for fungal exploration.

#### ***Combining biological thin sections and X-ray tomography***

Combination of the thin sections with the connectivity data from the 3-D structure does raise interesting questions related to the mechanisms involved in fungal exploration. At low BD, the pore volume was well connected with pores of diameters of at least  $73.9 \mu\text{m}$ , sufficiently large for fungi to penetrate. Nevertheless a large part of this volume was not exploited by the fungus. This is consistent with previous findings that fungi follow larger pores and surfaces in soil. By combining the sections with further analysis of the pore volume addressing the relationship between connectivity and pore diameter, it may now be possible to refine our understanding of the way fungi spread through soil, with important consequences for colonisation of organic matter in soil and interactions with plants and other microbes.

The use of impregnated blocks offers additional benefits which will help to overcome technical and ecological problems related to scale and resolution. By cutting the impregnated blocks into smaller sections, additional scans can be obtained with resolutions up to  $1 \mu\text{m}$  to explore the full range of multi-scale heterogeneity that fungi encounter as they colonise soil. Thin sections of the current samples were obtained prior to scanning, but with future experimental design it will be possible to align the sections exactly in the 3-D pore volume and to obtain additional information on the spatial distribution of water.

#### **Conclusions**

By combining thin sectioning with the 3-D characterisation of the pore space we showed that the connectedness of pore volume in soil can be quantified non-destructively and manipulated within microcosms in order to study the effect of pore geometry on fungal colonisation.

Further exploitation and development of the combination of these techniques may reveal the mechanisms by which fungi explore structured soil and will introduce rich opportunities to manipulate the soil environment to our advantage, i.e. to identify ways that optimise the interactions between fungal pathogens and their antagonists.

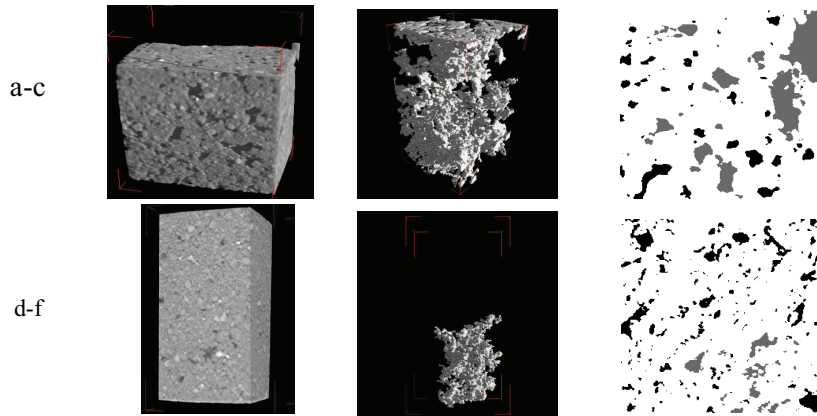


Fig. 1. Examples of 3-D visualisations of soil structure obtained by X-ray micro-tomography for a loam based soil packed at 1.2 (a-c) or 1.6 (d-f)  $\text{Mg m}^{-3}$ . Pores packed at different bulk-densities differed considerably in connectedness of the pore volume, as shown by the volume belonging to the largest pore (c, e). Pore volume exclusion occurs as pores become isolated from the largest connected cluster (e,f). Legend e,f: black: solid; grey: connected largest pore; white: isolated pores.

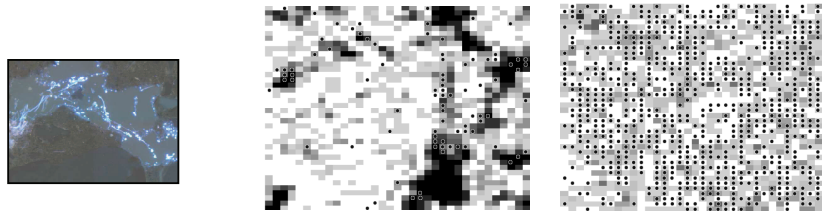


Figure 2. Soil fungal interactions visualized in thin soil sections at different spatial scales. At microscopic scales, fungal hyphae spread predominantly in the pore space between aggregates (A:  $0.44 \text{ mm}^2$ ). At larger scales ( $6.2 \text{ cm}^2$ , approximately confirm size of a fungal colony) fungal exploration is mediated by the soil structure: in a loosely packed soil (BD  $1.2 \text{ Mg m}^{-3}$ , b) sparse fungal colonies are formed, spreading predominantly through the larger pore voids; in compacted soil (BD  $1.6 \text{ Mg m}^{-3}$ , c) denser fungal colonies are formed. Location of fungus represented by a dot.

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