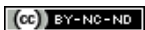


# Combination of techniques to quantify the distribution of bacteria in their soil microhabitats at different spatial scales

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

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13

14 **Abstract**

15 To address a number of issues of great societal concern at the moment, like the  
16 sequestration of carbon, information is direly needed about interactions between soil  
17 architecture and microbial dynamics. Unfortunately, soils are extremely complex,  
18 heterogeneous systems comprising highly variable and dynamic micro-habitats that  
19 have significant impacts on the growth and activity of inhabiting microbiota. Data remain  
20 scarce on the influence of soil physical parameters characterizing the pore space on the  
21 distribution and diversity of bacteria. In this context, the objective of the research  
22 described in this article was to develop a method where X-ray microtomography, to  
23 characterize the soil architecture, is combined with fluorescence microscopy to visualize

24 and quantify bacterial distributions in resin-impregnated soil sections. The influence of  
25 pore geometry (at a resolution of 13.4  $\mu\text{m}$ ) on the distribution of *Pseudomonas*  
26 *fluorescens* was analysed at macro- (5.2 mm x 5.2 mm), meso- (1 mm x 1 mm) and  
27 microscales (0.2 mm x 0.2 mm) based on an experimental setup simulating different soil  
28 architectures. The cell density of *P. fluorescens* was  $5.59\text{E}+07$  (s.e  $2.6\text{E}+06$ ) cells  $\text{g}^{-1}$   
29 soil in 1-2 mm and  $5.84\text{E}+07$  (s.e  $2.4\text{E}+06$ ) cells  $\text{g}^{-1}$  in 2-4 mm size aggregates soil.  
30 Solid-pore interfaces influenced bacterial distribution at micro- and macroscale,  
31 whereas the effect of soil porosity on bacterial distribution varied according to three  
32 observation scales in different soil architectures. The influence of soil porosity on the  
33 distribution of bacteria in different soil architectures was observed mainly at the  
34 macroscale, relative to micro- and mesoscales. Experimental data suggest that the  
35 effect of pore geometry on the distribution of bacteria varied with the spatial scale, thus  
36 highlighting the need to consider an “appropriate spatial scale” to understand the factors  
37 that regulate the distribution of microbial communities in soils. The results obtained to  
38 date also indicate that the proposed method is a significant step towards a full  
39 mechanistic understanding of microbial dynamics in structured soils.

40

#### 41 **Keywords**

42 X-ray CT; fluorescence microscopy; soil bacteria; pore geometry; soil sections; spatial  
43 distribution

44

45

## 46    **1 Introduction**

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

58    Over the years, the spatial distribution of indigenous and introduced bacteria has been  
59    studied in undisturbed or repacked soil columns, however the relationship between the  
60    bacterial spatial distribution and 3D soil architecture has not been considered (Nunan et  
61    al., 2001; Kizungu et al., 2001; Nunan et al., 2003; Dechesne et al., 2003; Pallud et al.,  
62    2004; Dechesne et al., 2005). Spatial isolation, afforded by the complexity of soil air-  
63    solid interfaces, is believed to be one of the key factors accounting for the diverse  
64    microbial communities in soils. Geometrical characteristics of the soil pore space, such  
65    as pore volume, shape, connectivity, size, and tortuosity of pathways can have an  
66    impact on microbial composition and activity in soil. They regulate the accessibility of  
67    organic matter, the diffusion of oxygen through the gaseous phase, and the diffusion of  
68    dissolved compounds through the water phase, as well as the movement of

69 microorganisms. These pore characteristics can be measured experimentally or can be  
70 estimated via non-destructive imaging.

71 Advances in the application of X-ray micro-tomography have made it possible to  
72 visualize and quantify the internal architecture of soils in three dimensions at  $\mu\text{m}$   
73 resolution. Recent studies (Kravchenko et al., 2013; Juarez et al., 2013; Wang et al.,  
74 2013; Kravchenko et al., 2014; Negassa et al., 2015) have combined X-ray tomography  
75 with other analytical methods to investigate the influence of pore geometry on  
76 distribution (Kravchenko et al., 2013; Wang et al., 2013), composition (Ruamps et al.,  
77 2011; Kravchenko et al., 2014), and activity (Ruamps et al., 2013; Juarez et al., 2013) of  
78 bacterial communities in soil. These studies show how the combination of advanced  
79 techniques can help in obtaining experimental evidence on relationships existing  
80 between microbes and physical microscale environments. Whereas the results suggest  
81 that the study of bacteria at a scale relevant to microorganisms is important, there is no  
82 clarity yet what scale that should be and if relationships and observations differ across  
83 scales.

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

92 geometry on the distribution of introduced bacteria, through co-locating 2-D thin  
93 sections within a 3-D X-ray CT volume.

94

## 95 **2 Materials and Methods**

### 96 *2.1 Preparation of soil microcosms*

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

112 To obtain the inoculum, an overnight culture of *Pseudomonas fluorescens* SBW25 was  
113 prepared in King's B medium at 23°C in the dark, washed in 1xPBS and adjusted to a  
114 specific cell density prior to inoculation using a spectrophotometer reading at

115 OD 600 nm (Thermo Fisher Scientific, UK). The cell density of *P. fluorescens* was  
116  $3.6E+07$  cells mL<sup>-1</sup> and thus  $1.8E+07$  cells were inoculated per microcosm. Additional  
117 samples were amended with 500 µL dH<sub>2</sub>O<sub>MQ</sub> instead of inoculum serving as control  
118 treatments. Three replicates per treatment were prepared and sealed in plastic bags to  
119 avoid drying of samples. The samples were incubated at 23°C in the dark for 5 days to  
120 allow bacterial growth and spread through the soil. The soil microcosms were sampled  
121 after five days for resin impregnation, as explained in the next section.

122

## 123 2.2 Fixation and dehydration of soil microcosms

124 Soil microcosms were first placed onto a hardboard covered with layers of cotton mesh  
125 to prevent loss of soil during the embedding processes. Microcosms were then placed  
126 on top of an aluminium gauze stand in a container to support the subsequent steps  
127 required for fixation and resin impregnation. To preserve the distribution of bacteria  
128 within the soil matrix, the microcosms were fixed using a 2% formaldehyde solution (v/v  
129 in H<sub>2</sub>O; 37% stock solution, Sigma Aldrich). This solution was added slowly from the  
130 sides of the container, to minimize disturbance of soil microcosms and facilitate the  
131 exchange of liquids (from bottom to top). All microcosms were completely submerged in  
132 the solution and kept overnight for fixation at 4°C. Subsequently, samples were washed  
133 in MQ distilled water for two hours, which was added the same way as the fixation  
134 solution. After washing, the samples were dehydrated with a graded series of acetone  
135 solutions (technical grade, VWR) to avoid interference with the polymerization of resin.  
136 Samples were submerged in 50% (v/v) acetone-water solution at room temperature for  
137 at least 12 hours. Subsequently a graded series of 70%, 90% and three times 100%

138 (v/v; acetone in water) was applied, each step lasting for 2 h. During the last two steps  
139 with 100% acetone, samples were kept under vacuum (280 mbar) to facilitate the  
140 complete exchange of all pores.

141

### 142 *2.3 Resin impregnation of soil microcosms*

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

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



161 was made through the microcosm to ensure a proper orientation of each block during  
162 CT scanning and subsequent fluorescence microscopy.

163

#### 164 *2.4 X-ray CT of resin impregnated samples*

165 The physical structure of resin-impregnated microcosms was obtained via X-ray  $\mu$ -CT  
166 scanning (HMX ST 225, Metris X-Tek, UK) at a resolution of 13.4  $\mu\text{m}$  per voxel. In order  
167 to visualize resin-filled pore space, samples were scanned under energy settings of 145  
168 keV and 35  $\mu\text{A}$  and 2000 angular projections. A molybdenum target and a 0.25 mm  
169 aluminium filter were used. Radiographs were reconstructed via software (CT Pro v.2.1,  
170 NIKON metrology, UK) into 3D volume datasets, which were adjusted in contrast and  
171 exported as image stacks (\*.bmp format) via volume processing software (VGStudio  
172 Max 2.2, Volume Graphics, Germany).

173

#### 174 *2.5 Preparation of polished sections for cell counting*

175 After CT scanning, polished sections were prepared for cell counting at three depths of  
176 each resin-impregnated microcosm (Supplementary Figure S1). To obtain these  
177 sections, blocks were first cut with a diamond saw (Woco 50, Conrad, Germany), then  
178 ground down to the estimated height (centre of the block and  $\pm 2.5$  mm above and below  
179 the centre) using a cup wheel grinding machine (see above). Each ground surface was  
180 subsequently polished using wet abrasive paper on a glass plate (silicon carbide,  
181 P1200) to remove grinding material and make the surface smooth. The blocks were  
182 then cleaned with cleaning solvent and exact heights were measured using a  
183 micrometre (accuracy 1  $\mu\text{m}$ ).

184

## 185 *2.6 Alignment of polished sections and image processing*

186 A stereomicroscopic image of each polished section representing an individual layer of  
187 resin impregnated microcosm was taken and used to find the corresponding layer in the  
188 image stack of CT data (Supplementary Figure S2 (A, B)). Image stacks were rotated to  
189 match the orientation of the stereo microscopic images, which corresponded to the  
190 orientation of the virtual counting grid applied in cell counting. The selected CT image  
191 was then cropped to the region of interest (where bacterial cells were counted) in Image  
192 J v1.47 (<http://rsbweb.nih.gov/ij/>) (Supplementary Figure S2 (C)). The cropped region of  
193 interest was then thresholded using the indicator kriging segmentation method (Houston  
194 et al., 2013).

195

## 196 *2.7 Analysis of pore geometry*

197 The pore architecture of each microcosm was analysed at three different scales in 2D,  
198 hereafter referred to as microscale, mesoscale, and macroscale. The areas selected for  
199 the analysis of pore characteristics at each scale in individual microcosms are depicted  
200 in Fig. 1. The microscale corresponds to each field of view of size  $0.2 \times 0.2$  mm, the  
201 mesoscale is associated with a field of view of size  $1.0 \times 1.0$  mm, and finally the  
202 macroscale encompasses the region of interest of size  $5.2 \times 5.2$  mm (Fig.1). In 2D,  
203 each slice was analysed with a thickness of one voxel.

204 Pore geometry was also analysed at the macroscale in 3D to get a broader perspective  
205 on the relationship between pores and bacteria. For this, the neighbouring 476 slices,  
206 above and below the plane, were used to calculate a measure of pore geometry in 3D.

207 The size of the area analysed at each scale is described in Table 1. A macro was  
208 recorded in ImageJ v1.47 (<http://rsbweb.nih.gov/ij/>) to crop images at the different  
209 scales analysed. The segmented images were then evaluated by software developed  
210 in-house (Houston et al., 2013a). This software was used to quantify pore  
211 characteristics, like porosity, connectivity, and the area of solid-pore interfaces of the  
212 pore volume, based on voxel data obtained from CT-scans. The porosity was calculated  
213 as the volume fraction occupied by pores, whereas connectivity was determined as the  
214 volume fraction of pore space that is connected with the external surface of the image  
215 volume. The surface area of solid-pore interfaces was estimated using Minkowski  
216 functionals, and expressed in relation to the area of solids directly connected to the pore  
217 space (Houston et al., 2013b).

218

## 219 *2.8 Enumeration of bacteria in polished sections*

220 To enumerate bacteria, a drop of an anti-fading medium containing  $1.5 \mu\text{g mL}^{-1}$  DAPI  
221 stain (Vectashield H-1200, Vector Laboratories, USA) was applied on top of the  
222 polished surface of blocks, which was covered afterwards with a cover slip ( $24 \times 32$   
223 mm, Menzel Gläser, Germany). Bacterial cells were evaluated with a fluorescence  
224 microscope (Axioscop 2, Carl Zeiss, Germany) equipped with an Hg vapour lamp (HBO  
225 103 W/2, Osram, Germany) using a 63x objective lens (Plan-Neofluar, Carl Zeiss,  
226 Germany). DAPI-stained cells were detected with an appropriate fluorescence filter set  
227 (F46-000, AHF, Germany) and counted manually using an ocular with an integrated  
228 squared grid reticle ( $10 \times 10$ ,  $1.25 \text{ mm}^2$ ; Carl Zeiss, Germany). Cell counts were  
229 obtained at counting spots arranged on a grid of  $6 \times 6$  fields of view with distance of 1

230 mm in x- and y-direction respectively resulting in a total area of  $5.2 \times 5.2$  mm per  
231 polished section (Fig. 1). The location of the starting point for each analysed layer was  
232 chosen by placing each polished block on a reference slide and following the coordinate  
233 system on the microscopic stage. Thus, the same position of the virtual counting grid  
234 could be applied for each block and layer. Cell counts were extrapolated from cell  
235 counts per area of field of view to cells per gram of dry soil by assuming a focus depth  
236 of  $4 \mu\text{m}$  during fluorescence microscopic observation.

237

### 238 *2.9 Statistical analysis*

239 Statistical analysis was performed using SPSS software version 21. A mixed effect  
240 linear model (assuming normal distribution) was applied to investigate differences in soil  
241 pore characteristics between treatments, with treatments as fixed factor and three  
242 individual microcosms per treatment as error term for treatment. To comply with the  
243 normality assumption, the porosity and connectivity measures were transformed using  
244 the probit function. Data relative to the solid-pore interfacial area met the normality  
245 assumption.

246 A generalized mixed-effect Poisson model with log-link function was used to investigate  
247 significant differences in cell numbers between different treatments, with treatment  
248 taken as a fixed factor. The effect of soil pore characteristics such as porosity,  
249 connectivity, and solid-pore interfacial area, on the distribution of bacteria was also  
250 determined by a Poisson model with treatment as a fixed factor. The size of the  
251 analysed scale was introduced as an offset variable in the Poisson model.

252

### 253 3 Results

#### 254 3.1 Pore geometry of resin impregnated soil

255 Among the two different aggregate sizes, a distinguishable difference in visible soil  
256 pores larger than minimum size of 13.4  $\mu\text{m}$  was evident by visual inspection of the 2D  
257 sliced images derived from the analysed layer (Fig. 2). An increase in the size of pores  
258 with increasing diameter of aggregates was clearly visible. The visual difference was  
259 however not apparent among the quantitative measures of the pore geometry analysed  
260 at different scales.

261 The three studied scales differed in terms of porosity and solid-pore interface between  
262 the two treatments. The average values of soil pore characteristics at different scale for  
263 each treatment are presented in Table 2. In terms of porosity the samples analysed at  
264 microscale were not significantly different ( $p = 0.929$ ), with average porosity of 20.8% in  
265 1-2 mm and 19.2% in 2-4 mm sized soil aggregates. The average solid-pore interfacial  
266 area was slightly higher in 2-4 mm than in 1-2 mm aggregate size treatment (Table 2),  
267 however the difference was not statistically significant ( $p > 0.05$ ). In samples analysed  
268 at mesoscale 2D, even though the average porosity was slightly higher in 2-4 mm  
269 (21.5%) than in 1-2 mm (19.3%) aggregate soil (Table 2), the difference was not  
270 statistically significant ( $p > 0.05$ ). However, the average solid-pore interfacial area  
271 between treatments was statistically significant ( $p < 0.001$ ), with 0.036  $\text{mm}^2$  in 1-2 mm  
272 and 0.041  $\text{mm}^2$  in 2-4 mm sized soil aggregates. At macroscale 2D, soil porosity was  
273 very similar and not significantly different between the two treatments. The average  
274 solid-pore interfacial area was higher in 2-4 mm (1.070  $\text{mm}^2$ ) than 1-2 mm (0.967  $\text{mm}^2$ )  
275 aggregate size treatment, however no significant difference ( $p > 0.05$ ) was observed.

276 In samples analysed at the macroscale in 3D, even though the differences in average  
277 soil porosity between the two treatments was very minor, with average porosity of  
278 20.9% in 1-2 mm and 20.0% in 2-4 mm sized soil aggregates, the difference was  
279 statistically significant ( $p < 0.001$ ). Soil connectivity was also significantly different ( $p <$   
280  $0.001$ ) between treatments, with an average connectivity of 96.16% in 1-2 mm and  
281 94.29% in 2-4 mm aggregate sized soil. However, the solid-pore interfacial area among  
282 different aggregate size treatments was not significantly different, with  $8.05 \text{ mm}^2$  in 1-2  
283 mm and  $7.72 \text{ mm}^2$  in 2-4 mm aggregates sized soil ( $p > 0.05$ ).

284

### 285 3.2 Visualisation and quantification of bacterial distribution in soil

286 Under UV excitation, bright blue signals of the stained *Pseudomonas* cells were  
287 detected on impregnated samples. Although soil particles and resin exhibited blue  
288 autofluorescence as well, the stained cells were easily distinguishable against the  
289 background (Supplementary Figure S3). DAPI-stained *Pseudomonas* cells appeared  
290 evenly spread mainly on the surface of the clay-humus complexes or at solid-pore  
291 interfaces. Very few (1-3) cells were observed in a resin-filled pore area surrounding the  
292 soil particles. No DAPI signals were detected in negative control samples of sterilized  
293 soils without inoculum. Visual comparison of cell density in each analysed layer of a  
294 treatment was carried out to determine treatment effects (Fig. 3). Cell density ranged  
295 from 25 to 700 cells per counting spot in the treatment with 1-2 mm aggregate sizes  
296 compare to 0 to 650 cells per counting spot in the treatment with aggregate size of 2-4  
297 mm. In general, the cell numbers of both treatments differed between different counting  
298 spots on each analysed layer. Therefore, the result showed a variation in the number of

299 cell counts between different treatments. The cell density of *Pseudomonas* was 290.8  
300 (s.e=13.4) cells mm<sup>-2</sup> in 1-2 mm and 303.7 (s.e=12.7) cells mm<sup>-2</sup> in 2-4 mm soil  
301 aggregates. These numbers correspond to 5.59E+07 (s.e 2.6E+06) cells g<sup>-1</sup> and  
302 5.84E+07 (s.e 2.4E+06) cells g<sup>-1</sup>, respectively, in columns packed with 1-2 mm and 2-4  
303 mm soil aggregates.

304

### 305 3.3 Influence of soil pore geometry on bacterial distribution at different scales

306 To examine the relationship between soil pore geometry and bacterial cell counts, the  
307 cell density (no. of cells mm<sup>-2</sup>) of *Pseudomonas* was plotted against soil porosity and  
308 solid-pore interfacial area analysed at different scales (Fig. 4, SI Fig. S4). In Figure 4,  
309 more data points are plotted in the microscale and mesoscale graphs compared to the  
310 macroscale graphs. This is because each data point in the graphs corresponds to the  
311 analysis of a counting spot of individual layer in each replicate of a respective treatment.  
312 Therefore, there is a noticeably wider spread in the cell density values in the graphs  
313 showing data at the microscale and mesoscale, compared to the macroscale.

314 At microscale 2D, the values of the solid-pore interfacial area ranged from 0.000-0.008  
315 mm<sup>2</sup> for the 1-2 mm aggregate size and 0.000-0.010 mm<sup>2</sup> for the aggregate size 2-4  
316 mm. The cell density ranged between 0-1600 cells mm<sup>-2</sup> (Fig. 4a & b). The influence of  
317 soil porosity and solid-pore interfacial area on the distribution of *Pseudomonas* cells  
318 varied between treatments (Table 3). At the microscale, the influence of soil porosity on  
319 *Pseudomonas* cell distribution was statistically significant ( $p = 0.001$ ), showing a slight  
320 reduction ( $\beta = - 0.0301$ ) in cell density with increasing porosity, for samples made up of  
321 2-4 mm. No significant trend was found for samples with aggregates 1-2 mm., However,

322 the influence of solid-pore interfacial area on the distribution of *Pseudomonas* cells was  
323 statistically significant in both aggregate size treatments (Table 3), but showed a  
324 contrasting effect with a decrease for aggregates sized 1-2 mm ( $\beta = -19.203$ ) and an  
325 increase for aggregates sized 2-4 mm ( $\beta = 16.417$ ) aggregates. In samples analysed at  
326 the mesoscale in 2D, the solid-pore interfacial area ranged from 0.00-0.10 mm<sup>2</sup> for the  
327 1-2 mm aggregate size and 0.00-0.012 mm<sup>2</sup> for 2-4 mm aggregates. The cell density  
328 ranged between 0-1600 cells mm<sup>-2</sup> (Fig. 4 c & d). Compared to the microscale, at the  
329 mesoscale only soil porosity in samples made up of 1-2 mm aggregates significantly  
330 influenced ( $p = 0.030$ ) the distribution of bacterial cells, showing a small decrease ( $\beta = -$   
331 0.051) in the cell density with increasing porosity. For samples made up of 2-4 mm  
332 aggregates, distribution of *Pseudomonas* cells was not significantly influenced by  
333 porosity or the solid-pore interfacial area.

334 At the macroscale, cell density refers to the average of cell counts over 36 counting  
335 spots in each analysed layer. For samples analysed at the macroscale in 2D, solid-pore  
336 interfacial area ranged from 0.5-1.0 mm<sup>2</sup> in the 1-2 mm aggregate size and 0.5-2.5 mm<sup>2</sup>  
337 in the 2-4 mm sized aggregates. The mean cell density ranged from 0-500 cells mm<sup>-2</sup>  
338 (Fig. 4 e & f). The influence of soil porosity on *Pseudomonas* cell distribution was  
339 statistically significant ( $p = 0.000$ ) in both treatments, with a decrease ( $\beta = - 0.849$  for 1-  
340 2 mm and  $\beta = -0.794$  for 2-4 mm) in cell density with increasing porosity. The influence  
341 of solid-pore interfacial area also showed statistically significant influence on distribution  
342 of *Pseudomonas* cells for both sized aggregates. In samples analysed at the  
343 macroscale in 3D, between the two treatments the soil porosity of the analysed area  
344 ranged from 10-30%, connectivity of pores ranged from 90-100% and solid-pore



345 interfacial area ranged from 1.2-2.5 mm<sup>2</sup> (Fig. 5). In both treatments, the distribution of  
346 *Pseudomonas* cells was significantly influenced by porosity, connectivity, and solid-pore  
347 interfacial area. However, these effects show contrasting influence when compared for  
348 the two aggregate sizes. For aggregate size 1-2 mm, porosity, connectivity and soil-  
349 pore interface have negative effect on the cell density. Whereas for aggregate size 2-4  
350 mm, these three parameters show positive effect (Table 4).

351

## 352 **4 Discussion**

### 353 *4.1 Bacterial distribution*

354 In this study bacteria were visualized with the intercalating DNA stain DAPI. This stain  
355 has been used to visualize indigenous bacteria in resin-impregnated soil samples  
356 before (Li et al., 2003; Eickhorst & Tippkötter, 2008), and as a counter-stain in  
357 undisturbed soil samples (e.g., Eickhorst & Tippkötter, 2008). No DAPI signals were  
358 detected in control samples, which confirms that the autoclaving procedure successfully  
359 sterilized the soils and that the bacteria that are visualized in inoculated samples were  
360 those introduced artificially. *Pseudomonas* cells were observed at solid-pore interfaces.  
361 The very few cells observed in the pore space were most likely cells closely connected  
362 to solid-pore interfaces above or below the targeted soil pore. This observation is no  
363 surprise and is inherent to the impregnation method because if, as is likely based on  
364 earlier experiments (Vandevivere and Baveye, 1992), there had been cells in the lumen  
365 of pores, they would have been removed or forced onto the surfaces during the  
366 exchange of liquids for the fixation and dehydration of the samples.

367 In order to investigate the impact of the different treatments during sample preparation  
368 (fixation, washing, and dehydration), a separate series of soil microcosms was tested  
369 for cell removal during these steps (Supplementary data S5). The results of this test  
370 showed that relative cell losses ranged from -1.26% after fixation to -0.25% after  
371 dehydration for *Pseudomonas* cells which is a negligible proportion and shows that the  
372 majority of bacteria were attached to the surfaces throughout the preparatory  
373 treatments. For non-autoclaved samples, relative cell losses were even lower (by  
374 approx. 100 times), suggesting that the observed cell losses during preparation are a  
375 result of the inoculation of cells in this experiment.

376 In polished sections, *Pseudomonas* cells were observed to be distributed as single cells  
377 through the soil matrix. White et al (1994) also observed a similar distribution of  
378 *Pseudomonas fluorescens* stained cells throughout the soil pore network. This kind of  
379 pattern was different than for indigenous bacteria that were observed in the form of  
380 small clusters or microcolonies constituted by cells of identical or different morphologies  
381 (Nunan et al., 2001; Li et al., 2004; Eickhorst & Tippkotter, 2008). Raynaud and Nunan  
382 (2014) also observed an aggregated pattern in distribution of indigenous bacteria in thin  
383 sections of soil. This suggests that the distribution of bacteria in soil is an effect of  
384 extrinsic (pore size and organic matter) and intrinsic (reproduction by binary fission)  
385 processes in soil. Differences in the distribution pattern can also be related to how  
386 bacteria spread and access nutrient sources in soil. A different response between  
387 species can be expected in their relationship with the soil architecture. In this study,  
388 although based on visual inspection, it seemed that the introduced bacteria were

389 homogeneously mixed within the samples, a heterogeneous distribution in cell counts  
390 between different counting spots was observed.

391 Dechesne et al. (2005) also showed that the distribution of introduced bacteria was  
392 more heterogeneously distributed than that of indigenous bacteria. Other results have  
393 also shown a non-random distribution of microorganisms in soil (Nunan et al., 2003;  
394 O'Donnell et al., 2007; Young et al., 2008). We stress that although the technique we  
395 develop here has generic validity, the specific interrelationships that are found between  
396 aspects of pore geometry and bacterial distribution may therefore be a result of the  
397 system we deployed. It is, for example, reasonable to expect that when bacteria are  
398 randomly mixed with soil, as is the case in our experiments, time will need to elapse for  
399 a different relationship to develop. When bacteria are mixed through soil, connectivity of  
400 the pore space may not be a factor contributing to the distribution. However,  
401 connectivity of pore space is required for bacteria to move through soil. The fact that we  
402 still observe relationships in our results can be explained by the fact that *Pseudomonas*  
403 is expected to spread over significant distances under these experimental conditions  
404 (Juyal et al., 2018). In this study, Juyal et al (2018) also showed that the rate of  
405 growth depends on the soil structure. There is a complex number of factors influencing  
406 bacterial distribution, ranging from physical (pore geometry), to nutritional and biological  
407 factors (differences in motility and attachment). Some studies have related the variation  
408 in bacterial distribution to a range of factors like organic matter content, soil moisture  
409 content, aggregate size classes and their location within aggregate, and pore size class  
410 (Franklin and Mills, 2009; Kravchenko et al., 2014; Or et al., 2007; Ruamps et al., 2011).  
411 The dominant processes however remain to be identified, but the technique developed

412 here offers real opportunities to disentangle these processes as for the first time 2-D  
413 thin sections are placed within a 3-D geometry. Among different aggregate size  
414 treatments, a significant difference in *Pseudomonas* cell density was observed.  
415 Samples with 2-4 mm aggregate size had higher cell density compare to samples with  
416 1-2 mm aggregate size. Similar kinds of differences in numbers of bacterial populations  
417 have been reported by past studies related to different soil particle sizes or aggregate  
418 fractions (Ranjard and Richaume, 2001; Sessitsch et al., 2001).

419

#### 420 *4.2 Influence of pore geometry on bacterial distribution*

421 The key goal of this article was to develop a methodological approach to analyse the  
422 effect of pore characteristics on spatial patterns of bacteria at scales associated with  
423 microhabitats. The approach consists of combining 2D and 3D methods to gain  
424 quantitative information on the relationship between pore characteristics and bacteria  
425 introduced in soil. It is known from previous research that the spatial distribution of  
426 bacteria is not random at fine scales and their location in soil is dependent on factors  
427 like substrate availability, soil water, and pore size distribution (Nunan et al., 2003;  
428 Ruamps et al., 2011). Along the same lines as what we attempt in this article, Hapca et  
429 al. (2011, 2015) developed a statistical method to combine 2D SEM-EDX data with 3D  
430 X-ray tomography images to generate the 3D spatial distribution of chemical elements  
431 in soil. Progress has been made combining techniques to analyse the relationship  
432 between soil pore characteristics and microbial community distribution and their activity  
433 in soil. For example, Kravchenko et al. (2013) studied the effect of intra-aggregate pore  
434 geometry on the distribution of *E. coli* in macro-aggregates. They used culture-based

435 methods (colony forming unit method) to enumerate *E. coli* distribution in aggregates  
436 and X-ray tomography to quantify pore architecture of intact aggregates from different  
437 managements. In our study, microscopic examination of polished sections was used to  
438 quantify bacteria in soil. The advantage of this method used over the culture- and non-  
439 culture-based approaches is that the use of impregnated soil samples made it possible  
440 to characterize the *in situ* relationship between bacteria and soil features without  
441 destroying the samples. Another advantage of this methodology was the use of X-ray  
442 CT to quantify pore architecture in the same layer.

443 The relationship between pore geometry and bacterial cell density was analysed at  
444 different scales. The scale at which observations are made is often determined by  
445 technology alone, but here we quantified the effect of pore geometry at the scale at  
446 which microbes actually live and interact with their surrounding environment and also if  
447 the effect is specific to that scale or variable at large scales. From the published  
448 literature, it appears that opinions concerning what range of microscales needs to be  
449 considered depending upon the individual microorganism under study, the microbial  
450 process of interest, and also to some extent on the tools available for the studies  
451 (Grundmann, 2004). Therefore, the scales used in this study have been defined based  
452 on the appropriate scales of the applied techniques, i.e., computed tomography  
453 (macroscale in this study) and fluorescence microscopy (microscale in this study).  
454 Analysis at different scales has been carried out by others to study the spatial pattern of  
455 either indigenous bacterial population (Nunan et al. 2002) or microbial activity (Gonod,  
456 2006) from meter to micro-meter scales. These authors identified spatial structures of  
457 bacterial populations at microscale in topsoils and at large and microscale in subsoils.

458 They related this difference in spatial pattern at different depths to transport of nutrients  
459 through soil (Nunan et al., 2002). Therefore, it is noted that different significant effects  
460 are found depending on the spatial scale of analysis. This confirms that the spatial scale  
461 of observation is an important aspect to be considered when doing this type of analysis,  
462 but it also raises the question of what might be causing this effect and how best to  
463 proceed. Therefore, we need to fully understand the spatial variability of soil microbes at  
464 different scales.

465 In this study, the analysis at each scale was done in 2D and 3D for two key reasons.  
466 First, the connectivity of pores, which is an important parameter in relation to transport  
467 of nutrients and bacteria cannot be determined in 2D, and second, the degree of  
468 tortuosity of the pore space is different in 2D compared to 3D. In our experiments, no  
469 significant difference in the pore characteristics in 2D and 3D between different  
470 aggregate size treatments was observed, but it should be noted that a part of the pore  
471 volume, associated with sub-resolution pores, could not be detected by the X-ray  
472 scanner due to limitation of the scan resolution, which was selected so as to enable us  
473 to scan entire microcosms. Therefore, the conclusions made here are based on the  
474 proportion of pores actually observed (i.e., pores larger than CT-scan resolution of  
475  $>13.4 \mu\text{m}$ ). This fact had an effect on the analysed solid-pore interfaces as well, where  
476 many data points in the microscale and mesoscale data were observed at zero (Fig. 4).  
477 The respective cell counts were observed on the portion of pore volume that was not  
478 detected by X-ray CT. Despite this issue, an influence of pore characteristics on  
479 *Pseudomonas* distribution at different spatial scales (macro-, meso- and microscale in

480 this case) was supported by the data. But the effect was quite variable across the three  
481 scales analysed over different dimensions in each treatment.

482 Samples analysed in two dimensions (2D) at macroscale showed a significant effect of  
483 porosity on *Pseudomonas* cell distributions in both treatments but at mesoscale and  
484 microscale this was not the case as the solid-pore interface showed no significant effect  
485 on the distribution of *Pseudomonas* cells in all treatments. This difference between the  
486 two scales could be due to the size of the sample as the information is constrained at  
487 this scale. Therefore, to avoid this problem of sample size used for pore soil architecture  
488 determination, the analysis was done in 3D where a bit of the surrounding area of the  
489 3D soil environment was considered. The results showed that at macroscale, all three  
490 pore characteristics exhibited a significant effect on the *Pseudomonas*-inoculated  
491 treatment. This difference in analysis between two dimensions could be that in 2D the  
492 information of pore characteristics information is constrained to the 2D-single plane from  
493 3-D pore geometry. The results show that there was no general relationship between  
494 pore geometry and bacterial counts and this varied with the spatial scale and  
495 dimension, therefore measuring and identifying whether a relationship exists are tightly  
496 linked to identifying the 'appropriate spatial scale'. The appropriate scale is needed to  
497 help understand the development of the microbial spatial patterns and to determine the  
498 factors that regulate and maintain soil biodiversity and microbial community function in  
499 soil. We advocate that the use of mechanistic models that include explicit description of  
500 microbial dynamics and soil architecture, such as those developed by Portell et al.  
501 (2018), will be required to advance our understanding of complex interrelationships at  
502 these scales and will offer an evidence base for identification of the scale dependence

503 of relationships between soil structure and bacterial distribution. Data sets as provided  
504 in this study will be imperative towards further development and testing of such models.

505

506

## 507 **5 Conclusion**

508 In this paper, a methodology is presented to determine the effect of pore geometry on  
509 the distribution of bacteria at a range of spatial scales. The data presented in this paper  
510 suggest that porosity, connectivity, and solid-pore interfaces influence the distribution of  
511 bacteria in soils at macroscales. The development of the method presented here is a  
512 significant step towards understanding how bacterial distribution is affected by soil  
513 architecture in various applications and experimental conditions (e.g., packed  
514 microcosm systems or undisturbed natural soil samples). Our research also raises  
515 several issues regarding the “appropriate” spatial scale at which to carry out analyses.  
516 This question is crucial, and in the absence of a general trend, the scale containing the  
517 most representative information, within practical limits, should be selected for further  
518 analysis. For a combination of techniques this may require to sample at different spatial  
519 scales. The information obtained using this approach can lead to new frameworks to  
520 model the distribution of bacteria in a 3D soil environment, which in due course, should  
521 result in more accurate predictions of, e.g., biophysical processes driving C dynamics in  
522 a range of situations (e.g., Falconer et al., 2015; Portell et al., 2018).

523

524

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532

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642

1 **Figure captions**

2 **Figure 1**

3 Diagrammatic representation of spots where bacterial cells were counted in the given  
4 area of interest under the fluorescent microscope. Top row: greyscale images after CT-  
5 scanning for each scale (left: macroscale, centre: mesoscale, right: microscale;  
6 resolution: 13.4  $\mu\text{m}$ ). Bottom row: corresponding thresholded images. The grey squares  
7 in the bottom row represent each counting spot of size 0.2 x 0.2 mm. The distance  
8 between each counting spot was set to 1 mm. Grid in the microscale image (bottom  
9 right) represents the raticle grid used for cell enumeration in a single field of view.

10

11 **Figure 2**

12 Visual comparison of grey scale (left) and thresholded (right) images of the physical  
13 structure of soil with aggregate size 1-2 mm (A) and 2-4 mm (B).

14

15 **Figure 3**

16 Visual comparison of two-dimensional stereomicroscope images (left) and cell counts  
17 (right). One analysed layer is exemplarily shown for each treatment; (A) *Pseudomonas*  
18 *fluorescens* inoculated in packed 1-2 mm soil aggregates and (B) *Pseudomonas*  
19 *fluorescens* inoculated in packed 2-4 mm soil aggregates.

20

21 **Figure 4**

22 Relationship between bacteria cell density and soil-pore interface at microscale (A, B),  
23 mesoscale (C, D) and macroscale (E, F) in 2D in soil with aggregates of size 1-2 mm  
24 (left column; A, C, E) and 2-4 mm (right column; B, D, F). Data points in the graph  
25 represent individual counting spots per treatment (microscale and mesoscale) and  
26 means of each layer per treatment (macroscale;  $\pm$ SE,  $n=3$ ).

27

28 **Figure 5**

29 Relationship between mean bacteria cell density and porosity, connectivity and soil-pore  
30 interface at macroscale in 3D in soil with aggregates of size 1-2 mm (white dots) and 2-  
31 4 mm (grey dots). Data points in the graph represent individual analysed volumes of  
32 each replicate per treatment. Data are means  $\pm$ SE ( $n=3$ ).

33

34

35



36 **Tables**

37 **Table 1**

38 Physical dimensions of the region of interest (ROI) analysed for pore structure at  
39 macroscale, mesoscale, and microscale in 2D and 3D.

Scales	Dimensions	Physical dimension of ROI	
		(mm)	(voxel)
Microscale	2D	0.2 × 0.2	15 × 15
Mesoscale	2D	1.0 × 1.0	77 × 77
Macroscale	2D	5.2 × 5.2	400 × 400
	3D	6.2 × 6.2 × 6.2	476 × 476 × 476

40

41

42 **Table 2**

43 Average values of soil porosity and soil-pore interface analysed at macroscale,  
 44 mesoscale and microscale in 2D. Mean cell counts  $\pm$ SE are presented. Superscript  
 45 letters indicate significant differences between aggregate size and scales ( $p < 0.01$ ).

Scale	<i>n</i>	Porosity (%)		Soil-pore interface (mm <sup>2</sup> )	
		1-2 mm aggregates	2-4 mm aggregates	1-2 mm aggregates	2-4 mm aggregates
Microscale 2D	364	20.82 $\pm$ 1.86 <sup>a</sup>	19.26 $\pm$ 1.78 <sup>a</sup>	0.001 $\pm$ 0.000 <sup>a</sup>	0.002 $\pm$ 0.000 <sup>a</sup>
Mesoscale 2D	364	19.37 $\pm$ 0.96 <sup>a</sup>	21.50 $\pm$ 1.11 <sup>a</sup>	0.036 $\pm$ 0.001 <sup>b</sup>	0.041 $\pm$ 0.001 <sup>b</sup>
Macroscale 2D	9	21.05 $\pm$ 2.28 <sup>a</sup>	21.08 $\pm$ 2.21 <sup>a</sup>	0.967 $\pm$ 0.038 <sup>c</sup>	1.070 $\pm$ 0.097 <sup>c</sup>

46

47

48 **Table 3**

49 Results of the Poisson model analysis on influence of pore structure on distribution of  
 50 bacteria in soil with different aggregate sizes at microscale, mesoscale, and macroscale  
 51 in 2D. Numbers reported in the table are the p-values and coefficient values ( $\beta$ ) are the  
 52 estimation of the fixed coefficients (porosity and soil-pore interface) in the test model of  
 53 the analysis.

Scales	Treatments	Porosity		Soil-pore interface	
		p-value	Coefficient $\beta$	p-value	Coefficient $\beta$
Microscale 2D	<i>Pseudomonas</i> inoculated in soil with aggregate sizes 1-2 mm	0.469	0.006	0.027	-19.203
	<i>Pseudomonas</i> inoculated in soil with aggregate sizes 2-4 mm	0.001	-0.0301	0.025	16.417
Mesoscale 2D	<i>Pseudomonas</i> inoculated in soil with aggregate sizes 1-2 mm	0.030	-0.051	0.297	0.962
	<i>Pseudomonas</i> inoculated in soil with aggregate sizes 2-4 mm	0.609	-0.009	0.187	-0.931
Macroscale 2D	<i>Pseudomonas</i> inoculated in soil with aggregates sizes 1-2 mm	0.000	-0.849	0.025	-0.536
	<i>Pseudomonas</i> inoculated in soil with aggregate sizes 2-4 mm	0.000	-0.794	0.001	-1.439

54

55

56 **Table 4**

57 Results of the Poisson model analysis on influence of pore structure on distribution of  
 58 bacteria in soil with different aggregate size at macroscale in 3D. Numbers reported in  
 59 the table are the p-values and coefficient values ( $\beta$ ) are the estimation of the fixed  
 60 coefficients (porosity and soil-pore interface) in the test model of the analysis.

Scales	Treatments	Porosity		Soil-pore interface		Connectivity	
		p-value	Coefficient $\beta$	p-value	Coefficient $\beta$	p-value	Coefficient $\beta$
Macroscale 3D	<i>Pseudomonas</i> inoculated in soil with aggregates sizes 1-2 mm	0.009	-1.640	0.007	-0.170	0.039	-0.548
	<i>Pseudomonas</i> inoculated in soil with aggregate sizes 2-4 mm	0.001	3.061	0.000	0.339	0.000	2.583

61

### Figures

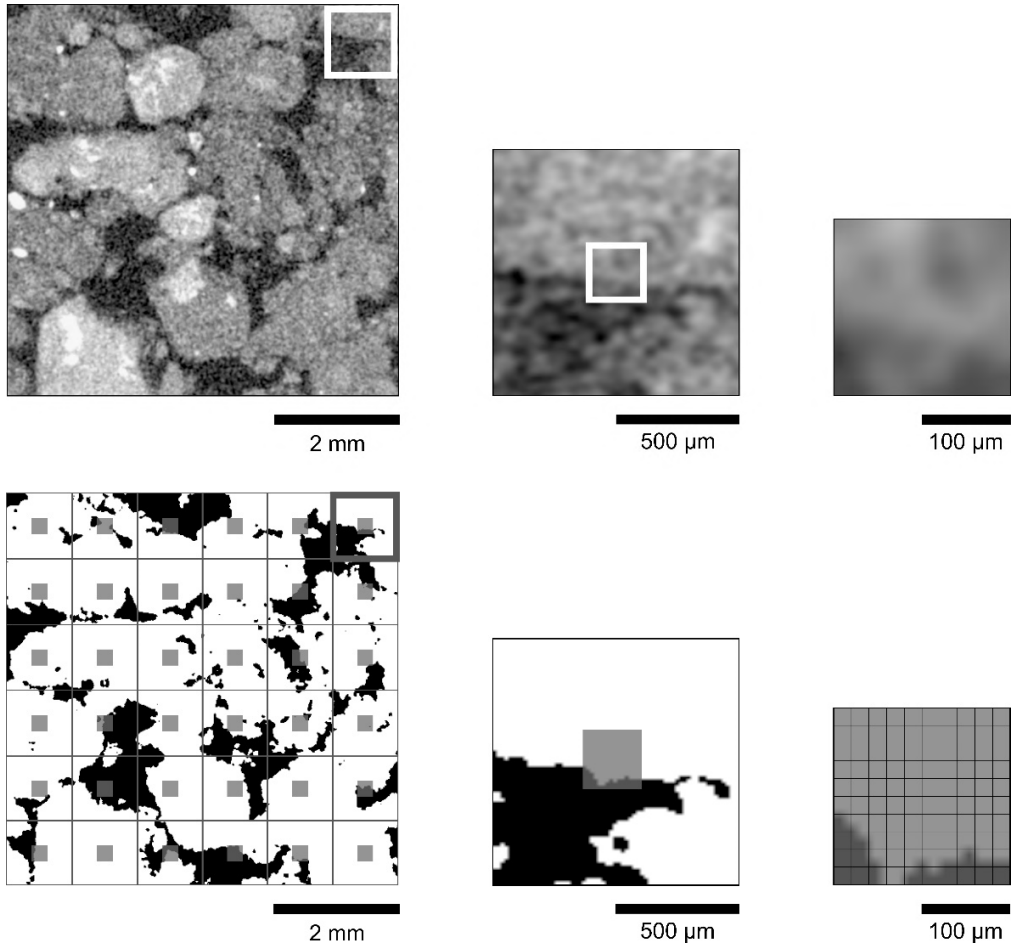
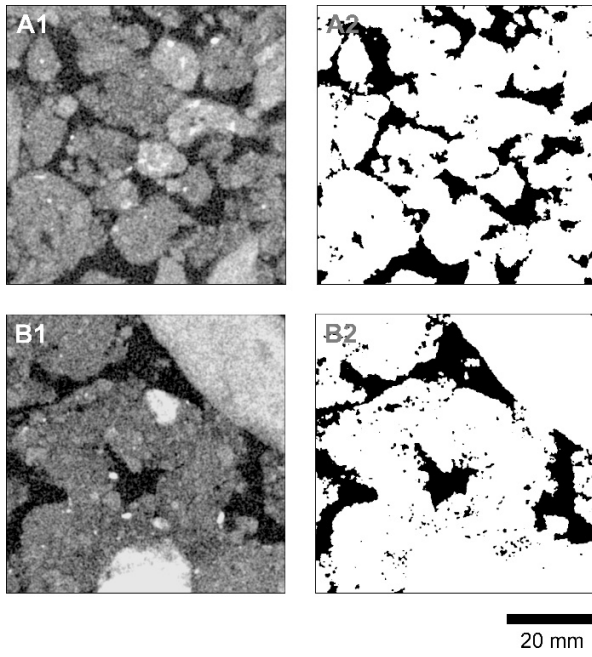
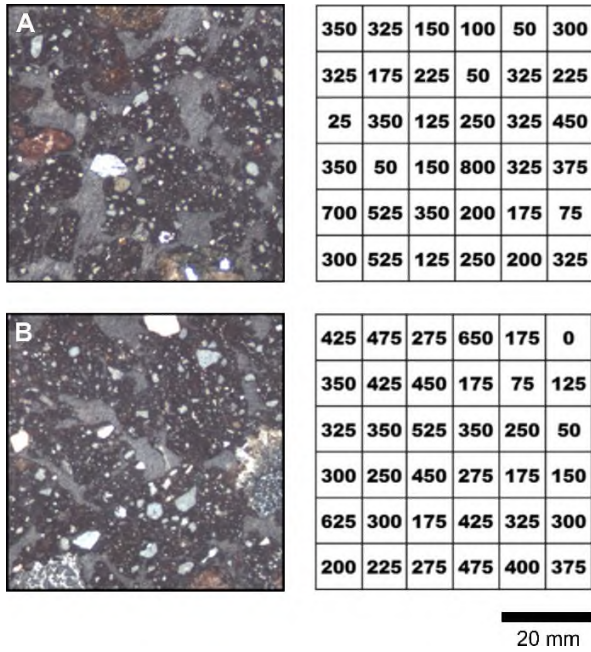


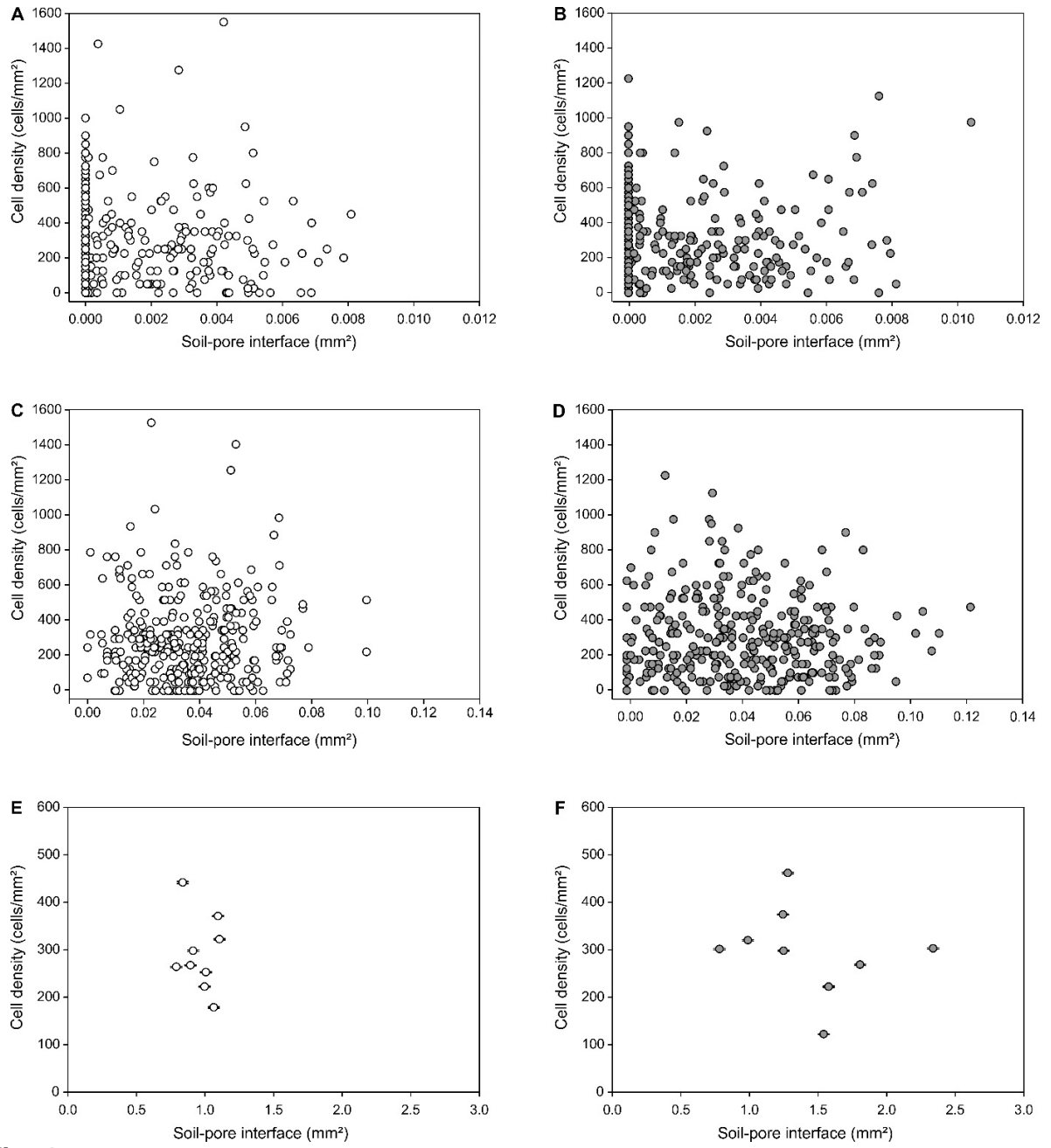
Fig. 1



**Fig.2**

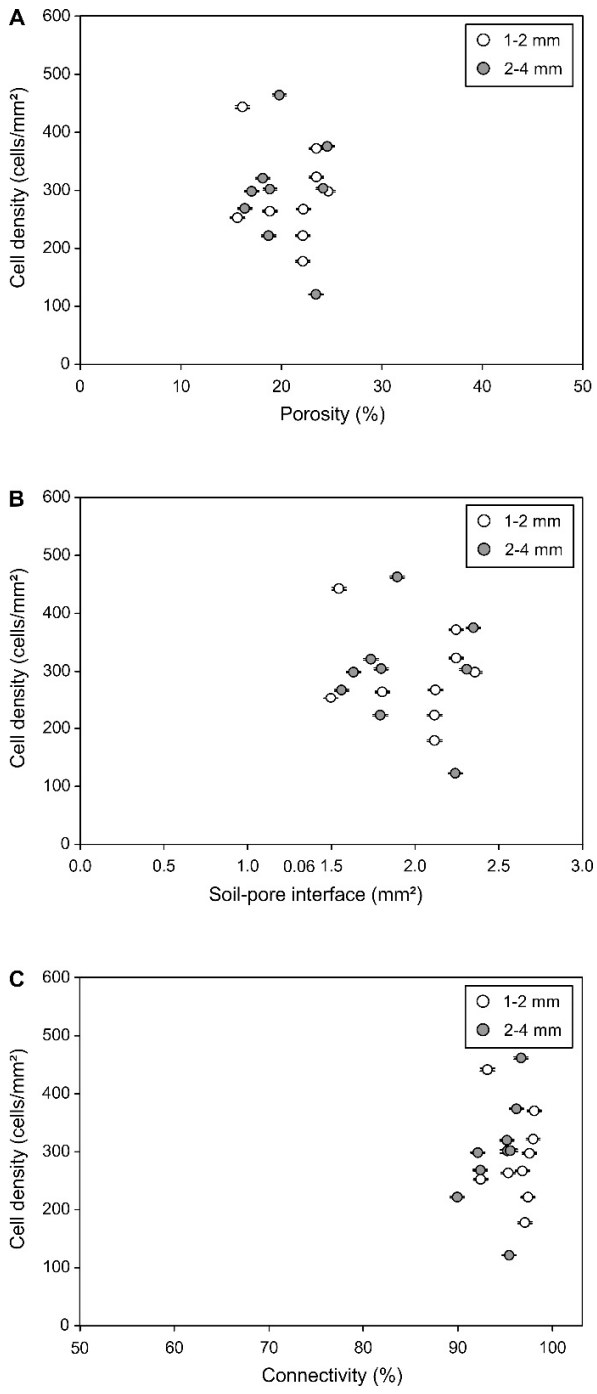


**Fig.3**



**Fig .4**





**Fig. 5**

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

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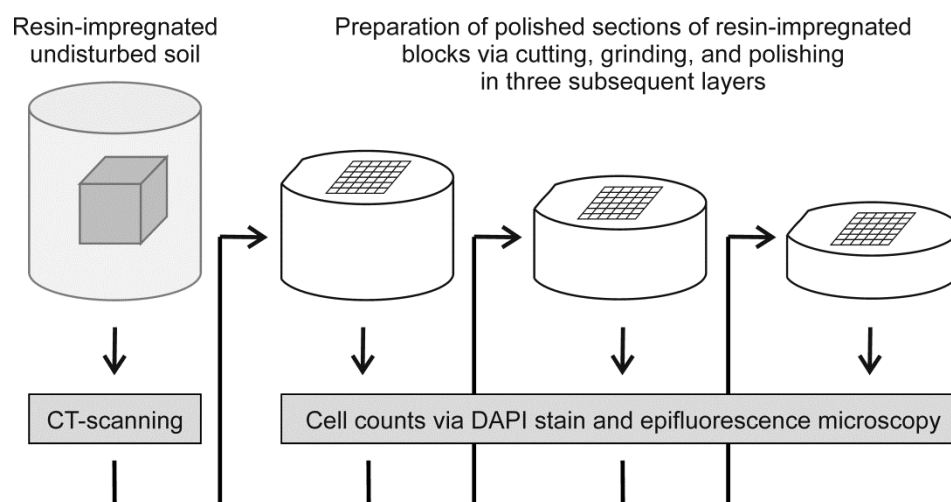
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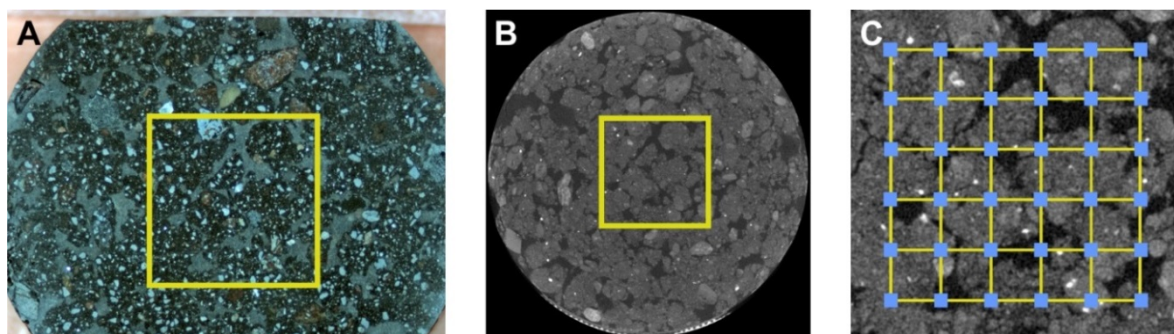
<sup>f</sup> AgroParisTech, Université Paris-Saclay, Thiverval-Grignon, France

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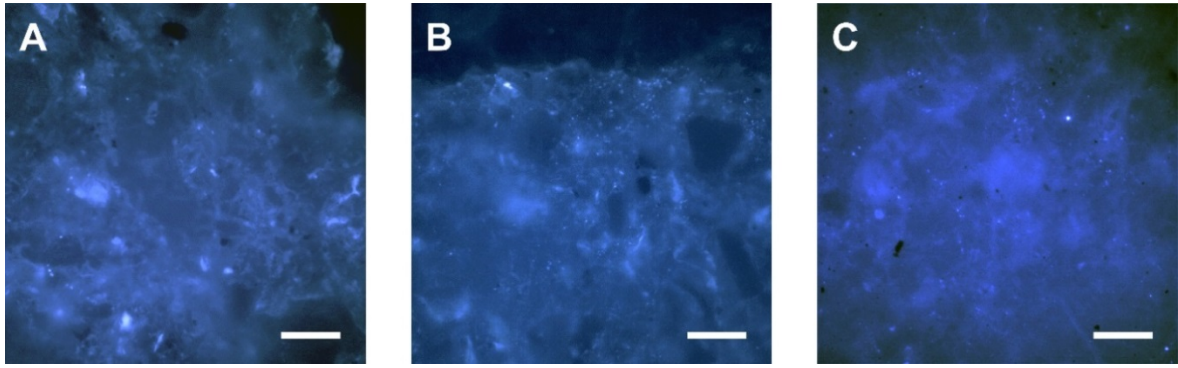
## Supplementary information



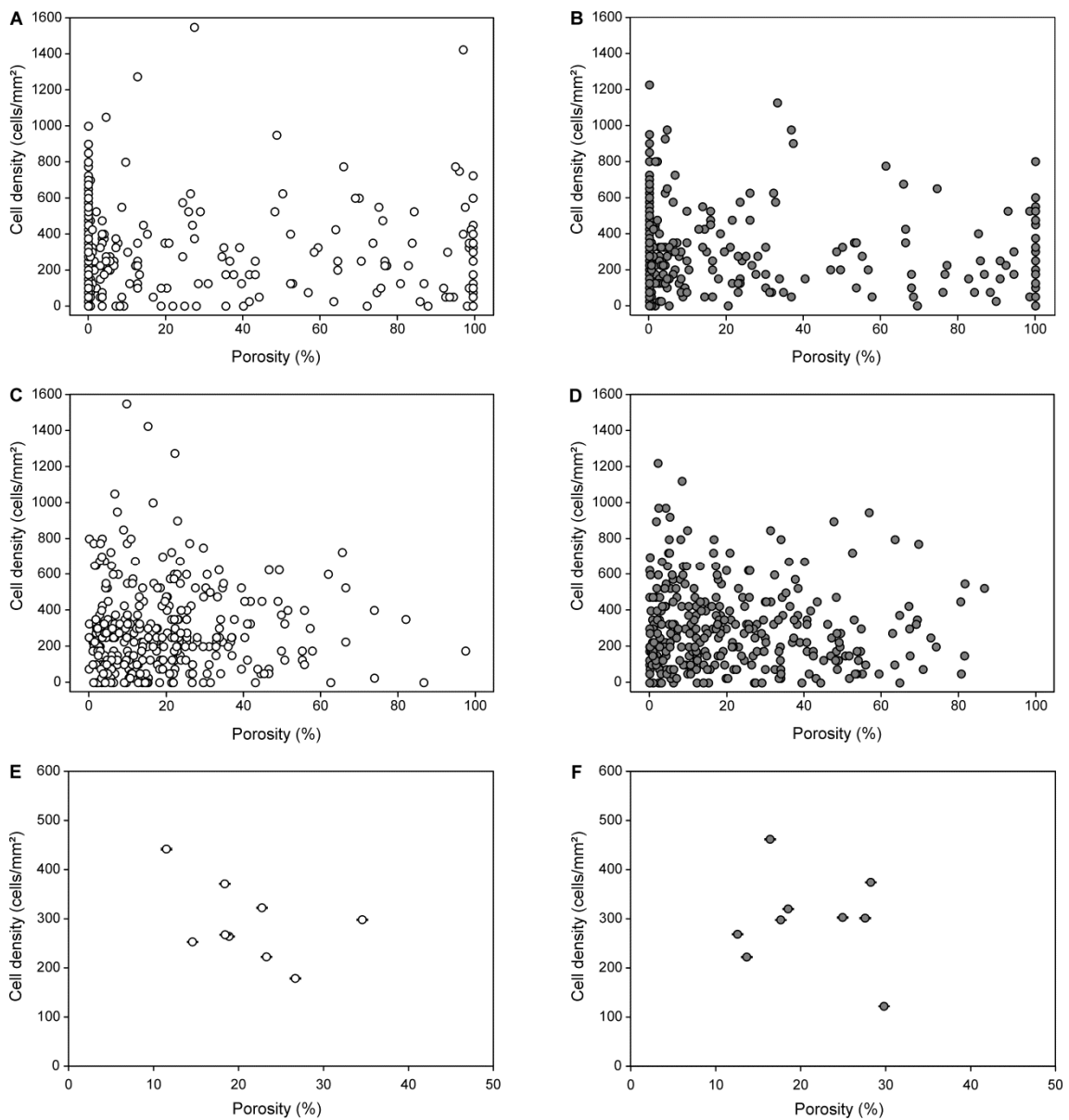
**SI Fig. S1** Preparation of three polished sections (layers) from an impregnated soil sample after CT-scanning. The distance between each layer was 2.5 mm. The frames in the diagram represent the counting area (e.g. 5.2 × 5.2 mm).



**SI Fig. S2** Alignment of stereomicroscope image (A) with CT scanned image (B). Yellow frame represents the area of interest where bacteria were counted. The blue frame (C) represents each counting spot of size 0.2 × 0.2 mm. The distance between each spot was set to 1 mm.



**SI Fig. S3** Microscopic images of polished soil sections showing DAPI-stained *Pseudomonas fluorescens* cells under UV excitation filter. Micrographs show the distribution of *Pseudomonas* cells in the soil matrix (A), soil-pore-interface (B), and aggregate surface (C). Scale bar: 20  $\mu\text{m}$ .



**SI Fig. S4** Relationship between bacteria cell density and soil porosity at microscale (A, B), mesoscale (C, D) and macroscale (E, F) in 2D in soil with aggregates of size 1-2 mm (left column; A, C, E) and 2-4 mm (right column; B, D, F). Data points in the graph represent individual counting spots in analyzed layers of each replicate per treatment (microscale and mesoscale) and means of each layer and replicate per treatment (macroscale;  $\pm\text{SE}$ ,  $n=3$ ).

## SI S5: Test of cell removal during sample preparation

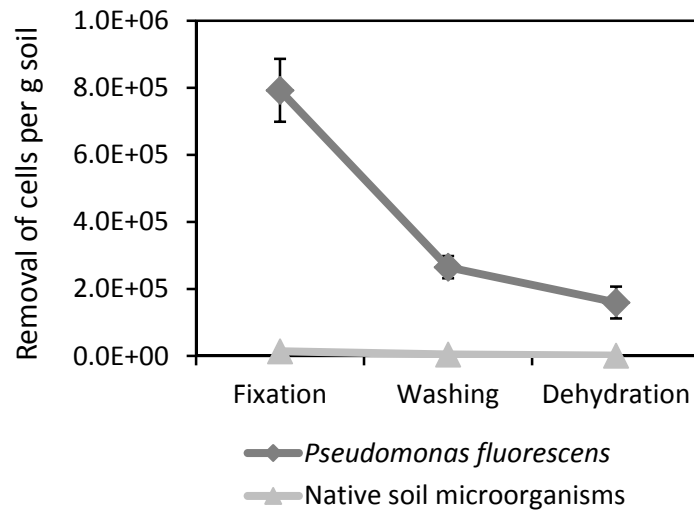
### Methodology

In order to test for cell losses during the procedures of fixation, washing, and dehydration, a set of five additional microcosms (1-2 mm aggregate size) has been packed and incubated (see main text). These microcosms were fixed, washed, and dehydrated as described in the Materials and Methods. During this procedure, each microcosm was placed in individual glass beakers to quantify the cell losses per individual microcosm. After fixation, washing in MQ distilled water, and dehydration in 50% (v/v) acetone-water solution the respective solutions were sampled after each step (5 mL each) and transferred on polycarbonate filters (0.2  $\mu\text{m}$ , Millipore). Small pieces were cut from these filters, amended with DAPI stain (Vectashield H-1200, Vector Laboratories, USA) and observed under a fluorescence microscope (see main text). Microbial cells in the tested solutions were enumerated as cells per mL solution and resulting numbers were extrapolated to cells per g soil by using the soil weight of each microcosm. Filters containing the pure solutions for each treatment served as control. An additional set of microcosms packed with non-autoclaved soil aggregates (1-2 mm) has been prepared to test for cell losses of the native soil microorganisms.

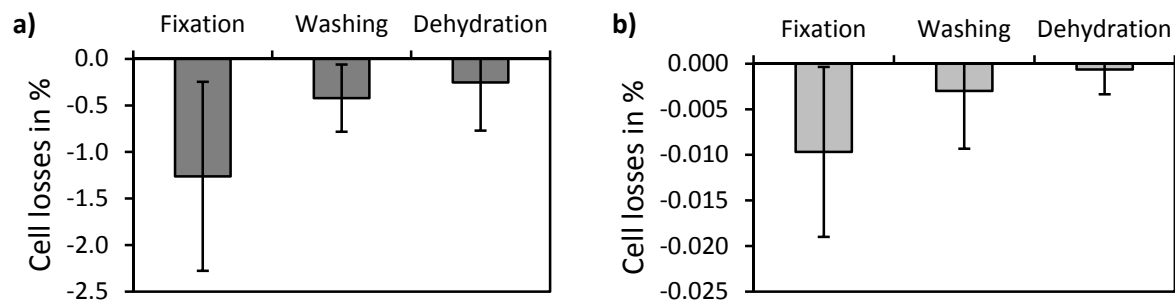
### Results

Extrapolated cell numbers counted after fixation, washing, and dehydration in the respective solutions are presented in Fig. A4. Cell losses were highest after the first treatment of fixation resulting in  $7.9 \times 10^5 \pm 9.4 \times 10^4$  cells per g soil for microcosms inoculated with *Pseudomonas fluorescens* and  $1.4 \times 10^4 \pm 2.0 \times 10^3$  cells per g soil for microcosms containing native soil microorganisms. Cell losses decreased in the subsequent treatments of washing ( $2.6 \times 10^5 \pm 3.3 \times 10^4$  cells per g soil and  $4.3 \times 10^3 \pm 1.4 \times 10^3$  cells per g soil) and dehydration ( $1.6 \times 10^5 \pm 4.8 \times 10^4$  cells per g soil and  $9.3 \times 10^2 \pm 5.9 \times 10^2$  cells per g soil) for *Pseudomonas fluorescens* and native soil microorganisms respectively.

In order to evaluate the effect of cell losses during sample preparation the proportion has been estimated based on the total number of cells in the two tested types of soil microcosms (*Pseudomonas fluorescens*:  $6.3 \times 10^7 \pm 5.1 \times 10^6$ ; native soil microorganisms:  $1.4 \times 10^8 \pm 1.3 \times 10^7$ ). For soil microcosms inoculated with *Pseudomonas fluorescens* relative cell losses ranged from -1.26% after fixation to -0.25% after dehydration (Fig. A5a). For soil microcosms with non-autoclaved soil relative cell losses were approx.. 100 times lower ranging from -0.01% after fixation down to -0.001% after dehydration (Fig. A5b).



**Fig. S5** Removal of microbial cells after the treatments of fixation, washing, and dehydration from packed soil aggregates (1-2 mm) inoculated with *Pseudomonas fluorescens* and non-autoclaved packed soil aggregates (1-2 mm; native soil microorganisms). Cell numbers were enumerated in the respective solutions and extrapolated to g soil. Error bars: standard error ( $n = 5$ ).



**Fig. S6** Losses of cells given as percentage based on the total number of cells in the two tested types of soil microcosms. **(a)** Packed soil aggregates (1-2 mm) inoculated with *Pseudomonas fluorescens*; total cell counts  $6.3 \times 10^7 \pm 5.1 \times 10^6$  **(b)** Packed non-autoclaved soil aggregates (1-2 mm) representing the native soil microorganisms; total cell counts  $1.4 \times 10^8 \pm 1.3 \times 10^7$ . Error bars: standard error ( $n = 5$ ).