Development of an agar lift–DNA/DNA hybridization technique for use in visualization of the spatial distribution of *Eubacteria* on soil surfaces

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Abstract

While microbial growth is well-understood in pure culture systems, less is known about growth in intact soil systems. The objective of this work was to develop a technique to allow visualization of the two-dimensional spatial distribution of bacterial growth on a homogenous soil surface. This technique is a two-step process wherein an agar lift is taken and analyzed using a universal gene probe. An agar lift is comprised of a thin layer of soil that is removed from a soil surface using an agar slab. The agar is incubated to allow for microbial growth, after which, colonies are transferred to a membrane for conventional bacterial colony DNA/DNA hybridization analysis. In this study, a eubacterial specific probe was used to demonstrate that growing bacterial populations on soil surfaces could be visualized. Results show that microbial growth and distribution was nonuniform across the soil surface. Spot supplementation of the soil with benzoate or glucose resulted in a localized microbial growth response. Since only growing colonies are detected, this technique should facilitate a greater understanding of the microbial distribution and its response to substrate addition in more heterogenous soil systems. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Bacterial growth; Colony hybridization; Microbial spatial distribution; Soil surfaces

1. Introduction

New approaches that allow for analysis of microorganisms on soil surfaces, their relative distribution, and activities in the environment are needed to gain a better understanding of ecological interactions and factors responsible for controlling microbial activities such as nitrogen fixation and the degradation of hazardous waste materials. In natural environments, a significant proportion of all microorganisms are associated with surfaces in complex multispecies communities. It is thought that the stationary microbial communities residing on these surfaces are primarily responsible for the degradation of xenobiotics and ammonium production for plant nitrogen uptake in both soil and aquatic systems. The environment on surfaces and in biofilms can differ considerably from that in suspension. Despite this, most of our knowledge of microbial physiology, distribution and ecology has been obtained from samples of suspended microorganisms.

Often, the relative distribution, growth and activity
of microorganisms are determined by destructive sampling of natural systems. Traditionally, the relative microbial distribution, diversity and growth in a given environment has been assessed by plate counts or most probable number on heterotrophic and/or nutrient specific media. We employed such plate counts as a method to evaluate the performance of our method. Another common approach has been to remove a representative sample and identify or characterize the bacteria within the sample via analysis of genetic material (e.g. polymerase chain reaction or DNA/DNA hybridization) or biochemical assay (Waid, 1984; Kanazawa and Filip, 1986; Linne von Berg and Bothe, 1992; Van Gestel et al., 1996; Senoo et al., 1997; Schneegurt and Kulpa, 1998; Manz et al., 1998). Recent developments have allowed researchers to develop numerous tagged probes to identify, quantify and detect different organisms and microbial activity in complex mixtures (Giovannoni et al., 1988; Linne von Berg and Bothe, 1992; Chua et al., 1996; Chalmers et al., 1997; Jones et al., 1997; Senoo et al., 1997; Huang et al., 1998; Lawrence et al., 1998; Manz et al., 1998; Moller et al., 1998; Schneegurt and Kulpa, 1998). Representative or destructive sampling may not, however, accurately reflect in situ microbial interactions, activities, and distribution.

Direct microscopic observation is a more extensive and less intrusive approach commonly used in the investigation of microbial in situ spatial distribution, structure, and function of complex microbial communities on surfaces (Waid and Suan, 1973; Chua et al., 1996; Chalmers et al., 1997; Jones et al., 1997; Huang et al., 1998; Lawrence et al., 1998; Moller et al., 1998). Most of these studies employ a combination of microscopy (e.g. confocal laser scanning microscopy, CLSM) and fluorescent tagging procedures. Specifically, these techniques have been used to observe and quantify biofilms on surfaces (Jones et al., 1997; Huang et al., 1998; Lawrence et al., 1998; Moller et al., 1998). The spatial distribution and activity of organisms within biofilms generally represents a microscale view of homogenous, relatively flat surfaces. The study of the in situ spatial distribution of microorganisms in heterogenous systems such as soil or sediment, presents more of a challenge. Most microscopic investigations of microbial distribution on soil and sediment surfaces usually involves examination of individual particle surfaces or aggregate, never entire soil surfaces (Waid and Suan, 1973; Chalmers et al., 1997).

The goal of this study was to develop a method to aid in the visualization of the spatial distribution of microorganisms on a macroscopic rather than a microscopic scale (i.e. that observed for biofilm development). Specifically, a method was developed to examine microbial growth and distribution on homogenous, contoured soil surfaces in response to an applied carbon source. Briefly, thin layers of soil from constructed microcosms were lifted using agar slabs and allowed to incubate. Colonies on the agar slabs were transferred to nylon membranes and subjected to colony hybridization using a 16S rDNA radiolabeled probe specific to the domain Eubacteria (Giovannoni et al., 1988). The bacterial distribution on the soil surface was visualized by autoradiography.

2. Materials and Methods

2.1. Soil microcosms

Soil microcosms were constructed using three different soil types, a Hayhook loamy sand, a 80:20 Vinton/Mt. Lemmon mixture soil and a Gila sandy loam. The specific properties of each soil are presented in Table 1. All soils were sieved (< 2 mm) then packed to the brim of sterile 2 cm diameter Petri dishes. Each soil was adjusted to a moisture content of approximately 20% by addition of either 500 mg/l filter-sterilized sodium benzoate in saline (amended microcosms) or just 0.85% sterile saline (unamended microcosms). The soils were moistened by slowly dripping the sterile solution over the entire surface. The microcosms were capped, sealed with parafilm and allowed to incubate at 25°C for 48 h. Negative controls to rule out the possibility of nonspecific binding of radiolabeled probe to soil minerals or organic matter were performed as follows. Subsamples of each soil were sterilized by autoclaving for 20 min on 3 consecutive days. Sterilized soils were aseptically packed into sterile Petri dishes and moistened with sterile saline.
Table 1
Soil properties

<table>
<thead>
<tr>
<th>Soil</th>
<th>Texture</th>
<th>% Sand</th>
<th>% Silt</th>
<th>% Clay</th>
<th>% TOC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH</th>
<th>CEC&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hayhook</td>
<td>Loamy sand</td>
<td>86</td>
<td>4</td>
<td>10</td>
<td>0.1</td>
<td>7.5</td>
<td>7</td>
</tr>
<tr>
<td>80:20 Mixture</td>
<td>Loamy sand</td>
<td>85</td>
<td>9</td>
<td>5</td>
<td>0.8</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Gila</td>
<td>Sandy loam</td>
<td>65</td>
<td>28</td>
<td>12</td>
<td>0.2</td>
<td>ND</td>
<td>15</td>
</tr>
</tbody>
</table>

<sup>a</sup> % Total organic carbon as determined by the Soil, Water, Plant Analysis Lab (SWPAL), University of Arizona, Tucson.

<sup>b</sup> Cation-exchange capacity in milliequiv./100 g soil as determined by SWPAL, University of Arizona, Tucson.

<sup>c</sup> ND not determined.

2.2. Localized soil supplementation

In order to determine whether a localized and distinct microbial response to substrate amendment on a soil surface could be visualized, a point source application of a glucose solution was added to the center of Hayhook soil microcosms. The entire soil surface was moistened with sterile saline to achieve a desired water content of approximately 20%. Glucose was subsequently supplemented directly into the center of the dish using 50 to 250 µl of a 10% glucose–0.85% saline solution. In order to reduce the extent of lateral diffusion by glucose through the porous medium, thin circular barriers were constructed from transparency plastic and placed in the center of the dish prior to packing and moistening. These soil microcosms were allowed to incubate at room temperature for 24, 48 and 144 h prior to conducting agar lifts.

2.3. Agar lifts

An “agar lift” is a thin layer of soil that is lifted onto an agar slab. For this work, agar lifts were performed using the agar medium R<sub>A</sub> (Becton Dickinson, Cockeysville, MD, USA). The agar was poured into a Petri dish and allowed to solidify. The agar was inverted over the soil surface and gently pressed down such that individual soil particles came in contact with and adhered to the agar. The dish was gently lifted from the soil surface. Loose soil particles remaining on the agar medium were removed by gently tapping the outer side of the inverted Petri dish. The agar lifts were incubated agar-side up for time periods ranging from 0 to 24 h. This incubation period allowed for growth of bacteria, found on and around the soil particles, to a density high enough to be detected with the oligonucleotide probe. The next step was to perform a conventional colony lift on the agar slab and finally, the lifts were probed with a eubacterial oligonucleotide probe. The methodology described herein is summarized in a simplified version (Fig. 1).

2.4. Colony lift and hybridization conditions

As with conventional molecular colony hybridization methodology, bacterial colonies that grew on and around soil particles embedded into the agar slab were transferred onto positively charged nylon membranes (Hy bond-N<sup>+</sup>, Amersham, UK) (Sambrook et al., 1989). The membranes were allowed to dry overnight before the DNA was immobilized by UV cross-linking. Any soil remaining on the membrane up to this stage in the procedure was gently brushed off with a soft bristle brush, and the membranes were vigorously agitated in 2×SSC for approximately 3 min at room temperature. This solution was poured off prior to DNA/DNA hybridization. Hybridization was performed using an 18-mer universal eubacterial 16S rDNA gene probe (Giovannoni et al., 1988) previously end-labeled using [<sup>32</sup>P]ATP and T4 polynucleotide kinase (Promega, Madison, WI, USA) (Hammer et al., 1997). Membranes were prehybridized at 42.5°C for 30 min in Rapid-hyb buffer (Amersham, UK). Between 2.5 and 5 µCi probe was allowed to hybridize overnight at the optimal hybridization temperature (42.5°C). A single rinse was performed with 5×SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min to rinse off any unbound probe. Two stringency washes were performed at the hybridization temperature for 15 min with 1×SSC–0.1% SDS. The membranes were exposed to X-ray film at −80°C with an intensifying
screen (Fisher, Pittsburgh, PA, USA) for approximately 24 h depending on the specific activity of the probe. To test the reproducibility of the hybridization signal, all membranes were stripped of original probe and reprobed as described previously (Hammer et al., 1997).

2.5. Bacterial enumeration

The culturable indigenous microbial population in all soils was enumerated immediately after performing an agar lift. Duplicate 1 g samples were taken from each soil and were serially diluted and plated on two media: R\textsubscript{2}A (Difco, Detroit, MI, USA) and benzoate medium [per liter: 10 g agar (Becton Dickinson), 3.27 g Bushnell Haas broth (Difco), 250 mg filter-sterilized sodium benzoate]. Heterotrophic (R\textsubscript{2}A) cell counts were performed after 24 h of incubation at room temperature, whereas benzoate degrader counts were performed after 2 weeks of incubation at room temperature.

2.6. Microbial growth and mineralization in the presence of glucose

Two additional experiments were performed to help validate probe results obtained from glucose-amended Hayhook soil microcosms. In the first, the change in colony forming units (cfu)/g oven dry soil as a function of time was determined following glucose amendment of Hayhook soil microcosms. Triplicate 1 g soil samples were taken every 24 h for 10 days, serially diluted in sterile saline, plated on R\textsubscript{2}A agar medium and enumerated after 24 h of incubation at room temperature. In the second, glucose mineralization was determined by quantitation of $^{14}\text{CO}_2$ evolved during growth on uniformly labeled $[^{14}\text{C}]$-glucose (Sigma, St. Louis, MI, USA) (Zhang and Miller, 1994). Briefly, triplicate 20 g samples of Hayhook soil were placed into 250 ml screw cap Erlenmeyer flasks designed for the collection of $^{14}\text{CO}_2$. The flasks were amended with $[^{14}\text{C}]$-glucose–0.1 N CaCl solutions to a final concentration of 10, 1, or 0.1% glucose (0.05 $\mu$Ci/flask).
The moisture content was adjusted to approximately 20%. The flasks were periodically flushed through a series of traps to collect evolved $^{14}$CO$_2$ (Zhang and Miller, 1994). Radioactivity was quantified using a Packard (Meriden, CT, USA) Tri-Carb liquid scintillation counter.

3. Results and discussion

3.1. Agar lift methodology

The primary objective of this work was to develop a method to allow visualization of the bacterial spatial distribution on soil surfaces. During the development of this method, it was observed that agar lifts required an incubation period to allow visualization of microbial distribution on the different soil surfaces. Fig. 2 shows the effect of incubation time on agar lifts from two different soils, Hayhook (Fig. 2A) and the 80:20 mixture soil (Fig. 2B). Each autoradiograph is of a soil microcosm surface roughly 2 cm in diameter. Soil microcosms were either unamended but moistened with saline (Fig. 2(A and B), columns U) or amended over the entire surface with a 500 mg/l benzoate solution (Fig. 2(A and B), columns A) and then incubated for 48 h. Agar lifts were taken and incubated for different intervals of time, namely 0 (data not shown), 6, 12, 18 and 24 h. Agar lifts taken from

Fig. 2. An autoradiograph depicting the effects of increasing agar lift incubation time (6–24 h) prior to colony hybridization. (A) Hayhook agar lifts; (B) 80:20 mixture agar lifts, (U) unamended soils, (A) soils amended with 500 ppm sodium benzoate. All microcosms were incubated for 48 h at room temperature prior to agar lift.
unamended soils required 18 h (Fig. 2B, column U) to 24 h (Fig. 2A, column U) of incubation prior to colony lifts to clearly visualize the bacterial distribution on the soil surface. In contrast, agar lifts taken from soil microcosms amended with benzoate, required less incubation time, 6 h to 12 h (Fig. 2A and B), columns A, respectively). The requirement for growth prior to detection of bacterial colonies is most likely due to the limited sensitivity of the eubacterial 16S rDNA probe. The probe’s sensitivity was predetermined, by dot blot analysis of lysed *Pseudomonas* cells, to be roughly 10⁷ cells or 8 ng purified genomic DNA (data not shown). Giovannoni et al. (1988) report similar data. Theoretically, one bacterial cell contains roughly 10 femtograms of DNA (Neidhart et al., 1983). Using this value, 8 ng of DNA would correspond to approximately 10⁶ cells. Thus, for a “microcolony” to be detectable, it must contain 10⁶ cells. The results presented in Fig. 2 clearly demonstrate that an incubation period is essential for allowing visualization of the microbial spatial distribution with the eubacterial 16S rDNA probe. Since all soils contain differing numbers of microorganisms in different physiological states, it then follows that the incubation time for agar lifts will vary and must be optimized for each soil tested.

The autoradiographs shown in Fig. 2 demonstrate that eubacterial colony distribution was not uniform over the entire surface of the soil. There were areas with intense signal suggesting that some soil particles or surfaces are more conducive to microbial colonization and growth than others. Areas without any signal at all correspond to areas of the agar medium that did not come in contact with the soil surface. This was due to unavoidable irregularities found on the soil surface. The results shown in Fig. 2 also indicate that benzoate supplementation resulted in an overall increase in intensity of probe signal, suggesting that growth was occurring in the amended soils. In Fig. 2B at 18 h, however, the intensity in the probe signal from the amended soil surface appears similar to that of the unamended 80:20 mixture soil. This was most likely due to an incomplete or inefficient transfer of colonies, resulting in a “smearing” of the probe signal from this particular agar lift. These results appear to corroborate traditional studies wherein soils amended with a carbon source tend to have increased cell counts.

### 3.2. Microbial response to supplementation

In order to verify the response to supplementation observed in Fig. 2, soil microcosms of three different soil types were constructed. The microcosms were either left unamended, amended with 500 mg/l benzoate or constructed with sterile soils. All microcosms were adjusted to 20% moisture content and allowed to incubate for 48 h at room temperature prior to performing agar lifts. Agar lifts were incubated for 24 h. Fig. 3 compares the eubacterial spatial distribution on the three different soil surfaces. As mentioned, sterile controls were performed to determine whether nonspecific or cross-reactive binding of the 18-mer oligonucleotide to soil minerals or organic matter occurred during hybridization. As seen in Fig. 3, row C, the probe did not appear to react significantly with any of the sterile soils tested. The slight probe signal observed in the Gila soil may have been due to contamination of the soil subsequent to sterilization or to aerosol contamination while performing the actual agar lift. These controls verified that DNA remaining from dead cells in sterile soils did not significantly contribute to probe binding, and that the eubacterial probe did not significantly bind to soil minerals or organic matter.

The agar lift results from the amended and unamended soil microcosms shown in Fig. 3 were compared to heterotrophic and benzoate degrader counts taken from the same microcosms (Table 2). For all three soils, the mean heterotrophic counts in unamended soil microcosms were similar ranging from 5.4 · 10⁶ to 5.6 · 10⁷. The signal intensity observed in the autoradiographs were also similar for the unamended microcosms (Fig. 3, row B). However, for soil microcosms amended with benzoate, the results were more complex (Fig. 3, row A). A slight decrease in the mean heterotrophic counts was observed for the Hayhook amended microcosm while an increase was observed for both the Gila and 80:20 mixture soils. Agar lift results showed a definite increase in probe signal intensity for both the Hayhook and mixture soils, but only a slight increase in signal intensity was observed for the Gila soil. Clearly, there was little agreement between agar lift probing results and heterotrophic plate counts. In contrast, a more pronounced relationship was observed when comparing agar lift results to benzoate
Fig. 3. An autoradiograph comparing eubacterial distribution in three sterile, unamended and benzoate-amended soils. Agar lifts were incubated for 24 h prior to colony hybridization. (A) Microcosms were amended with 500 mg/l benzoate and incubated for 48 h prior to agar lifts. (B) Microcosms of unamended soils moistened with sterile saline and incubated for 48 h prior to agar lifts. (C) Microcosms were made with soils that were sterilized by autoclaving 3 consecutive days, moistened with sterile saline and incubated for 48 h prior to agar lifts.

3.3. Localized microbial response to glucose supplementation

An experiment was performed to demonstrate that the technique allows visualization of a localized microbial response to any substrate amendment. Hayhook soil microcosms were amended directly in the center with a 10% glucose solution. Fig. 4 compares the effect of glucose supplementation on the microbial response following a 6 day (Fig. 4A) and 24 h (Fig. 4B) soil microcosm incubation period. Agar lifts were only allowed to incubate for the predetermined 24 h prior to colony hybridization and autoradiography. No distinguishable increase in
Table 2
Viable and benzoate degrading bacterial cell counts for duplicate unamended and amended microcosm soil samples

<table>
<thead>
<tr>
<th>Soil/supplementation</th>
<th>24 h Viable cell counts (cfu/g dry soil)</th>
<th>Benzoate degrading cell counts (cfu/g dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hayhook</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unamended</td>
<td>$5.4 \cdot 10^6$</td>
<td>$3.7 \cdot 10^3$</td>
</tr>
<tr>
<td>Amended*</td>
<td>$3.4 \cdot 10^6$</td>
<td>$3.8 \cdot 10^4$</td>
</tr>
<tr>
<td>80:20 Mixture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unamended</td>
<td>$5.6 \cdot 10^7$</td>
<td>$2.8 \cdot 10^5$</td>
</tr>
<tr>
<td>Amended</td>
<td>$1.8 \cdot 10^8$</td>
<td>$3.4 \cdot 10^6$</td>
</tr>
<tr>
<td>Gila</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unamended</td>
<td>$2.1 \cdot 10^7$</td>
<td>$1.1 \cdot 10^6$</td>
</tr>
<tr>
<td>Amended</td>
<td>$3.9 \cdot 10^7$</td>
<td>$2.8 \cdot 10^6$</td>
</tr>
</tbody>
</table>

* All soil microcosms were amended over the entire surface with a 500 mg/l benzoate solution.

Probe signal was observed in the vicinity of supplementation for soil microcosms incubated for 24 h (Fig. 4B) or 48 h (data not shown). However, there was a clear increase in hybridization signal in the vicinity of supplementation from microcosms incubated for 6 days (Fig. 4A). It was even possible to observe a qualitative increase in signal from the microcosms that were amended with higher concentrations of glucose (compare Fig. 4A, from left to right).

Although a similar lag period has been observed for soil microorganisms (6 days) in response to glucose supplementation (Jutras, 1994), two further experiments were performed to confirm this lag in Hayhook soil. In the first experiment, cells were enumerated following supplementation with 0.1%,

![Fig. 4A](image1)

![Fig. 4B](image2)

Fig. 4. Microbial response to a point source application of glucose after (A) a 6 day (144 h) soil microcosm or (B) a 1 day (24 h) soil microcosm incubation period prior to performing an agar lift. A 10% glucose–saline solution was applied to the center of each microcosm to deliver a total mass of glucose equal to 0.005 g, 0.01 g, and 0.025 g (from left to right). Hayhook soil was used for this experiment.
Fig. 5. Culturable cell counts on (A), and mineralization of (B), 0.1, 1.0 and 10% glucose by the indigenous Hayhook soil microbial population. Both experiments was performed in triplicate. Error bars represent the standard deviation from the mean.

4. Conclusions

The agar lift technique offers several advantages in comparison with traditional approaches to visualization of microbial spatial distribution on soil surfaces. In comparison to microscopic methods, it allows a larger sample size and sample area to be viewed at one time and allows visualization of the relative distribution of culturable soil microorganisms on a macroscopic scale. Another advantage of the agar lift technique is that it is a relatively straightforward, and simple procedure. As long as the soil does not require prolonged incubation or acclimation periods, probing results may be obtained in less than 2 days. In comparison to destructive sampling techniques for enumeration or nucleic acid characterization, the agar lift method only removes a fine layer of soil from the surface being sampled. Thus, the surface can be sampled repeatedly to allow a temporal evaluation of distribution and activity. It should also be noted that while the method developed herein was employed on model soil surfaces (i.e. soils were sieved and repacked into microcosms), its use could easily be extended to undisturbed soil surfaces. Further, the technique could be used on any surface for which a macroscopic visualization of microbial
distribution is desired including household surfaces, pipeline surfaces, or leaf surfaces to name a few.

As with any other method, there are some inherent limitations to the agar lift technique. This technique does not detect all cells on the soil surface due to the necessity for enrichment of the bacteria on an agar medium. Since many cells are nonculturable, they will not contribute to the probe signal. Limitations may also occur during both transfer steps of the procedure. First, it is difficult to uniformly lift the soil onto the agar medium especially if the surface of the soil is not perfectly level. Second, there may be an inefficient transfer of colonies from the agar surface to the nylon membrane which can result in a muted or blurry signal. Finally, all soil lifted onto the membrane must be carefully brushed and rinsed off the membrane prior to probing. Any soil left on the membrane may significantly skew the results. A more intense signal, for example, was observed for unwashed membranes when compared to washed membranes (data not shown). Unlike the CLSM and fluorescent probing images (Lawrence et al., 1998), the agar lift technique does not provide a three-dimensional image of microorganisms on a soil surface. The results are simply two-dimensional imprints of the culturable microorganisms found on soil surfaces. Once a lift has been performed on a particular surface, some soil particles are removed such that results from a second lift, taken from the same surface, may differ significantly from the first.

This methodology may serve as a useful research tool for determining microbial growth and distribution patterns in soil and other heterogeneous systems. The technique may be especially useful for determining microbial growth response to substrate flow in heterogeneous systems. Even though the technique requires a cultivation step, it still allows for easy application of a large variety of culture medium and conditions (i.e. single-carbon source media and anaerobic incubation). Future work should address the effect soil properties have on oligonucleotide hybridization and the application of genera-specific or microbial activity-specific probes to aid in predicting the biodegradation potential of contaminants in subsurface porous medium. Densitometry and image analysis techniques could also be used to make the method more quantitative.

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