QUANTITATIVELY TRACING BACTERIAL TRANSPORT IN SATURATED SOIL SYSTEMS

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Abstract. Three isolates of Escherichia coli were labeled by their resistance to sodium azide and, separately, to novobiocin, nalidixic acid, and tetracycline. The strains exhibited a high degree of persistence in the soil environment and were recoverable on strain specific media at levels within the 95% confidence interval of the numbers covered on nonselective media. The E. coli strains were subsequently used to evaluate the events which would occur when a septic tank drainfield became submerged in a perched water table and effluent-borne bacteria escaped into the groundwater. Field experiments were conducted by introducing the strains into horizontal lines installed into the A, B, and C horizons of a soil profile and transport was evaluated by collecting groundwater samples from 5 rows of piezometers (sampling six separate depth zones/row) located downslope from the injection lines. The major portion of subsurface transport of the bacterial populations occurred in specific zones in the soil profile and at an apparent maximum velocity of 17.0 cm min⁻¹. The maximum bacterial density in the groundwater, observed at each sampling distance downslope, was used to produce a mathematical relationship which described the overall decrease in numbers of organisms with increased distance through the soil. The potential health hazards which could occur by the subsurface transport of fecal organisms in relation to these experiments are discussed.

1. Introduction

One-third of all waterborne disease outbreaks reported in the United States from 1971 to 1976 were traced to the consumption of water from untreated groundwater sources (Craun et al., 1976; Craun, 1978). In addition, a 1970 nationwide projection for marginally treated public and private water supplies estimated that approximately 60 million consumers relied upon the absence of microbial pathogens in groundwater (Allen and Geldreich, 1975). Septic-tank/soil-adsorption disposal systems ranked highest in total volume of wastewater discharged directly into the groundwater and were also the most frequently reported source of groundwater contamination (Geraghty and Miller, 1978). However, properly installed and correctly functioning septic systems do not contribute to pathogen contamination of groundwater supplies because the soil, in this case, serves as an efficient filtering and adsorptive media. Consequently, fecal organisms are retained in the treatment zone directly adjacent to the drainfield trench where aerobic conditions contribute to rapid destruction of effluent-borne enteric pathogens (Bouma et al., 1972; Viraraghavan and Warnock, 1976; Ziebell et al., 1974).

The purification of drainfield effluent during soil percolation is the optimal condition necessary for maintenance of groundwater quality, yet improper site selection and/or

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poor installation can result in a malfunctioning of this soil percolation system. The manifestation of soil adsorption dysfunction includes surfacing of untreated effluent, incomplete treatment due to anaerobic conditions, or the subsurface escape of fecal organisms from the treatment zone. All of these conditions create potential health hazards (Reneau and Pettry, 1975; United States Environmental Protection Agency, 1973). In this study antibiotic/azide resistant strains of *Escherichia coli* were used to assess the hazard potential associated with the subsurface escape of pathogens from the treatment zone. In these circumstances, seasonally high water tables inundate the soil adjacent to the drainfield trench and rapid water movement transports the organisms with little filtering or adsorptive effects (Rahe *et al.*, 1978).

The use of resistance-labeled organisms for natural ecosystem analysis is well documented (Danso *et al.*, 1973; Habte and Alexander, 1975; Hagedorn *et al.*, 1978; Rahe *et al.*, 1978) although, in these studies, few safeguards were taken to insure that the observed results were not the consequence of spontaneous reversion of the organisms used or reduced recovery on selective media. Therefore, the *E. coli* strains used in our research were suspended in diffusion chambers *in situ* to determine survival, apparent reversion and selective recovery rates over a hypothetical maximum experimental period.

2. Materials and Methods

The bacteria used in this study were isolated from the Corvallis, Oregon, Municipal Wastewater Treatment Plant. Purified cultures were identified as *Escherichia coli* by growth and gas production in EC broth (Difco) after 24 h at 44.5°C, characteristic colonies on Difco Eosin Methylene Blue (EMB) agar, and IMViC patterns of [+ + − −]. Spontaneously-resistant mutants were selected for with the gradient plate procedure (Miller, 1972) and the three strains used in this study were all resistant to 100 μg ml⁻¹ sodium azide (Azi) and, individually, to 100 μg ml⁻¹ novobiocin (Nov), nalidixic acid (Nal), and tetracycline (Tet). These test strains were therefore designated: Nov⁻ Azi⁻ *E. coli*, Nal⁻ Azi⁻ *E. coli*, and Tet⁻ Azi⁻ *E. coli*.

2.1. Survival Recovery Evaluations

The survival characteristics of the *E. coli* strains were determined using membrane filter chambers (McFeters and Stuart, 1972). Cells were grown in the nutrient constituents of M–FC broth (Difco) for 16 to 18 h at 37°C, washed twice by centrifugation (4000 × g for 10 min) in a filter-sterilized soil extract, and diluted to the desired population density. The chambers, bounded by Millipore membrane sheets (HAWP 304 FO, 0.45 μm pore size, Millipore Corp., Bedford, MA) were filled with the bacterial suspensions, submerged into a beaker of freshly collected groundwater and, after a 1 h equilibration period, the first sample was removed. The chambers were then transported to a field site and immersed (in a previously constructed trench) into the water table so that subsurface water movement provided a constant exchange of the soil solution bathing the cells.
Samples were taken every 12 h for the 84 h duration of the experiment and one-half ml samples were removed from each chamber and immediately diluted in cold phosphate buffer (American Public Health Association, 1975) with 0.1% peptone. The temperatures recorded during the sampling periods ranged from 9 to 13.5°C.

Enumeration was initiated within 15 min of sampling and a comparison of the effects of using selective vs nonselective media was determined by parallel platings from the same dilutions. A modification of the single drop plating technique (Miles and Misra, 1938) was used to determine total surviving cells. Five 0.025 ml drops were dispensed (Oxford Micro-Doser Pipette) on pre-dried plates of Tryptic Soy Agar (Difco) with 0.3% yeast extract (TSYA), and the strain specific (SS) M–FC agar containing rosolic acid, 100 µg ml⁻¹ Azi, and 100 µg ml⁻¹ of the appropriate antibiotic (SS–MFC). All plates were incubated for 12 to 14 h at 37°C and colonies were counted with the aid of 7× to 45× magnification. The reduction in bacterial numbers on the SS–MFC media as compared to TSYA was taken to be a measure of the apparent reversion of an organism to a non-resistant state and/or the inability of a cell to tolerate selective media. This assessment was termed the selective recovery rate.

A second membrane filter chamber experiment was conducted in the laboratory with cell suspensions prepared as described. The three E. coli strains were combined and diluted 100× in both a sterile and nonsterile soil solution and each suspension was placed in a single filter chamber. The chambers were submerged in a beaker of recently collected groundwater and the contents of the beakers were mixed continuously while the temperature was maintained at 15 to 18°C. Samples from both chambers were collected daily for four days with the bathing solution changed after each sampling. Enumeration of the individual strains was accomplished by the single drop technique on SS–MFC media. Also, an identical sample volume was passed through a membrane filter (Millipore HAWG 025 00) and placed on SS–MFC media (1.0% agar). By comparing bacterial counts derived from these two techniques the recovery rate by membrane filtration was obtained.

2.2. SITE DESCRIPTION AND WATER TABLE CHARACTERISTICS

The experimental site was located on a hillslope soil (upper backslope position) in Benton Co., Oregon, with a 14% northeast facing relief. The surface horizon was a dark brown silty clay loam which overlaid a dark reddish brown silty clay to a depth of 34 cm. The subsoil was a dark brown heavy clay from approximately 35 to 60 cm in depth. This was underlain by a substratum of reddish and yellow-brown saprolite which graded into CaCO₃ cemented sandstone at 100 cm. The thickness of the saprolite varied from a few cm to 70 cm over the experimental site and the depth to lithic sandstone varied from 70 to 150 cm from the surface. The soil series was described as a moderately well-drained transition between the Philomath and Dixonville series.

The elevated water table varied in depth with distance downslope from 55 cm at the farthest upslope sampling line to 85 cm at the farthest downslope sampling position.
height of the perched water table in the soil profile was well within the range of the water
table monitored at this location during the winter of 1975–76 by Hammermeister (1978).

2.3. BACTERIAL TRANSLOCATION STUDIES

Transport of *Escherichia coli* through the soil was measured by introducing the resistance-
labeled strains into the soil profile through injection lines located in depths which
generally corresponded to the A, B, and C horizons of the soil. The organisms were
subsequently recovered from piezometers located at various distances and depths down-
slope from the injection lines. Details on the installation of the experimental site were
described previously by Rahe *et al.* (1978). Briefly, three 9.15 m injection lines were
installed (0.5 m apart) at 12, 30, and 60 cm depths in the experimental site with the 12 cm
deep line occupying the farthest downslope position while the 60 cm deep line was
installed upslope from the other two. Thirty-six piezometers were installed on the experi-
mental site to depths of 12, 30, 60, 110, 150, and 200 cm in lines at distances of 2.5, 5.0,
10.0, 15.0, and 20.0 m downslope from the A horizon injection line. The 12 and 30 cm
deep piezometers sampled a 10 and 15 cm zone in the soil while the remaining piezo-
meters each sampled a 20 cm zone. The installation sequence of the six piezometers at
each sampling line was randomized. One additional sampling line which served as a
control was installed 2.5 m upslope from C horizon injection line.

Inoculum for the field experiments was prepared identically for each strain. A 1200 ml
volume of the nutrient constituents of M–FC broth was inoculated (10% v/v) and
incubated for 14 to 16 h at 37°C. The optical density (Bausch & Lomb Spectronic
20,520 nm) of a 10 fold dilution of each culture was measured for determination of the
initial inoculum levels and the individual cultures were divided among six 500 ml
aspirator bottles for transport to the field and injection into the soil. Inoculation of the
field site was accomplished by attaching an aspirator bottle to each of the injection line
access ports. The *Nov' Azi' E. coli* was injected into the A horizon, the *Nal' Azi' E. coli*
was injected into the B horizon, and the *Tet' Azi' E. coli* was injected into the C horizon
of the experimental site.

Samples were vacuum extracted through the downslope piezometers at 20, 40, 60, 90,
120, 150, 190, 230, 290, and 350 min post-injection and control samples collected just
prior to injection (designated time zero). Samples were recovered in 250 ml bottles,
transported to the laboratory and stored no longer than 24 h at 4°C before analysis. All
samples from the field experiment (350 min duration) were collected before examination
of the samples was initiated. Enumeration of the tracer bacteria present in each water
sample was accomplished by the membrane filtration method described above.

3. Results

3.1. BACTERIAL SURVIVAL AND RECOVERY

The resistance-labeled *E. Coli* strains possessed a high level of persistence in the physio-
chemical environment of the soil solution as determined by both field and laboratory
Fig. 1. The survival of resistance-labeled *E. coli* strains. The cells were washed and suspended in a soil extract and were contained within membrane filter chambers placed in a saturated soil at a field location (using a high bacterial density) or in a laboratory setting (with a low bacterial density). Symbols: (A) Nov$^r$ Azi$^r$ *E. coli*, (□) Nal$^r$ Azi$^r$ *E. coli*, (O) Tet$^r$ Azi$^r$ *E. coli*. The open characters indicate that the suspending solution was sterile while the closed characters indicate that the suspending solution was nonsterile.

Survival patterns (Figure 1). Also apparent were the effects of interactions with the associated microbiota when a nonsterile soil solution was used as the suspending medium. The *E. coli* populations in this case survived in parallel with those in the sterile soil solution for up to 48 h, after which a rapid decrease in the nonsterile populations reduced the number of cells to below detection limits by the 96 h sampling.

During the field survival studies the assumption was made that the arithmetic mean of the results obtained from TSYA counts represented the actual number of viable bacteria present in the sample (Table I). Therefore, at every sampling the mean of the results of parallel plating on SS–MFC agar was compared with the TSYA determined population density. The mean selective recovery rates of SS–MFC relative to the TSYA counts of all samples taken over the 84 h duration of the experiment were: 102.3% for the Nov$^r$ Azi$^r$ *E. coli*, 93.3% for the Nal$^r$ Azi$^r$ *E. coli*, and 104.2% for the Tet$^r$ Azi$^r$ *E. coli* (Table I). The counts obtained on SS–MFC for all three tracer strains in all samples were well within the 95% confidence limits of the TSYA counts. The recovery rate using membrane filter methods was determined by comparing the arithmetic mean of the counts from SS–MFC single drop platings with the mean of the membrane filter counts. For all samples over the entire 96 h survival experiment, the membrane filtration technique recovered 97.5% of the Nov$^r$ Azi$^r$ *E. coli*, 98.2% of the Nal$^r$ Azi$^r$ *E. coli*, and 96.7% of
TABLE I
The maximized survival and recovery rates of the three resistance-labeled *E. coli* strains under field and laboratory conditions

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th><em>E. coli</em> strain</th>
<th>Initial density</th>
<th>Final density</th>
<th>Duration (h)</th>
<th>Relative recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field site(^c)</td>
<td>Nov(^r) Azi(^r)</td>
<td>5.8 × 10^8</td>
<td>4.2 × 10^8</td>
<td>84</td>
<td>102.3</td>
</tr>
<tr>
<td></td>
<td>Nal(^r) Azi(^r)</td>
<td>2.4 × 10^8</td>
<td>1.8 × 10^8</td>
<td></td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>Tet(^r) Azi(^r)</td>
<td>6.5 × 10^8</td>
<td>3.2 × 10^8</td>
<td></td>
<td>104.2</td>
</tr>
<tr>
<td>Laboratory(^d)</td>
<td>Nov(^r) Azi(^r)</td>
<td>8.5 × 10^5</td>
<td>1.4 × 10^5</td>
<td>96</td>
<td>97.5</td>
</tr>
<tr>
<td></td>
<td>Nal(^r) Azi(^r)</td>
<td>6.9 × 10^5</td>
<td>1.2 × 10^5</td>
<td></td>
<td>98.2</td>
</tr>
<tr>
<td></td>
<td>Tet(^r) Azi(^r)</td>
<td>4.3 × 10^5</td>
<td>3.4 × 10^5</td>
<td></td>
<td>96.7</td>
</tr>
</tbody>
</table>

\(^a\)Bacterial counts relative to TSYA counts.
\(^b\)Bacterial counts relative to SS-MFC direct plating counts.
\(^c\)Individual suspension of *E. coli* strains in membrane filter chambers.
\(^d\)Mixed suspension of all *E. coli* strains in a membrane chamber.

the Tet\(^r\) Azi\(^r\) *E. coli*; also well within the 95% confidence limits of the numbers derived from direct plating.

3.2. BACTERIAL TRANSLOCATION STUDIES

Computer-generated three-dimensional plots which displayed the translocation of resistance-labeled *E. coli* in the groundwater of the experimental site were constructed using, as the axes, times in minutes after injection, bacterial counts/ml, and the depths of the six sampling zones (Figures 2a to 2e). The individual plots depict the results from the five sampling lines located at increasing distances downslope. The translocation patterns of the individual strains injected into the A, B, and C horizons were of such similarity that only the data from the B horizon inoculations are shown.

Prior to inoculation of the site, no background antibiotic-resistant *E. coli* were detected from any of the piezometers. After injection into the profile at a depth of 30 cm (B horizon) the tracer strain was first detected in the 40 min sample from the 300 and 550 cm downslope sampling lines (Figures 2a, b). This initial appearance occurred at both distances in the 90 to 110 cm zone. Bacterial transport in the 90 to 110 cm zone continued downslope through the 1050 and 1550 cm sampling lines to the 2050 cm downslope position where the labeled strain was found in the 130 to 150 cm zone (Figures 2c, d, e). The bulk of bacterial translocation for this soil series occurred almost exclusively within these specific zones in the soil and in all cases the bacterial counts showed an increase over time to a maximum value (which differed for each distance downslope). The instances where the transport was detected in zones either above or below the specific zone in which maximum movement rate and bacterial numbers occurred demonstrated a vertical widening of the zone of influence in the soil profile. However, the reduced rate of trans-
port in these zones along with the greatly reduced bacterial counts obtained suggested that this effect was only secondary and influenced a comparatively small portion of the total number of \textit{E. coli} cells.

The sampling times which corresponded to the first appearance of tracer bacteria in each sampling line as compared to the apparent downslope distance traveled by the bacteria are plotted in Figure 3. The linear relationship between the time of first appearance and distance traveled established the apparent maximum velocity of bacterial transport through the site at 17.0 cm min\(^{-1}\), and this velocity remained constant over the entire site. In comparison, the relationship between the sampling times which corresponded to the appearance of the maximum bacterial counts vs the downslope distance (Figure 3) increased disproportionately with distance. This observation was characteristic of the hydrodynamic dispersion phenomena observed in liquid-porous systems which (with constant velocity) lowered the rate of increasing counts with additional distance. The time of first appearance and time of maximum peak development from the farthest...
Fig. 2. The B horizon injection plots showing the saturated zones of translocation of \textit{E. coli} through the soil profile at increasing distances downslope. The open planes represent the magnitude of the populations recovered (y-axis) at each sampling time (x-axis) from the soil depths sampled (z-axis). Symbols: (0 - 0 or O - - - O) indicate zones where a water sample could be recovered, ( - - - ) marks the location of the water table at each distance downslope.

The upslope sampling line did not fit well into this relationship. Also, inspection of the data plots (Figure 2a) indicated that the movement patterns at this distance (300 cm) were split between the 90 to 110 and 130 to 150 cm sampling zones. This evidence can be used to suggest that the passage of the maximum rate of bacterial flow occurred either vertically between the detection zones (i.e., in the 110 to 130 cm zone), or was directed away from the individual sampling piezometers by the horizontal spatial variability of
Fig. 3. The first appearance where the tracer strains could be detected (open characters) and first appearance of the maximum bacterial numbers (closed characters) of the resistance-labeled *E. coli* strains (for all depths) at increasing distance downslope. The symbols are located in terms of specific sampling times at the various sampling distances downslope relative to the horizontal injection of: (△) Nov<sup>+</sup> Azi<sup>+</sup> *E. coli* in the A horizon, (□) Nal<sup>-</sup> Azi<sup>-</sup> *E. coli* in the B horizon, (○) Tet<sup>-</sup> Azi<sup>-</sup> *E. coli* in the C horizon.

the soil system. In either case, the maximum flow was intercepted farther downslope as suggested by the close agreement of the recovery times among subsequent sampling lines.

The maximum bacterial concentrations in the groundwater decreased with distance downslope from the injection line (Figure 4). The individual points were determined

![Graph showing bacterial transport](image)

Fig. 4. The percent reduction with distance downslope of the tracer *E. coli* strains injected into the soil profile of the experimental site. Symbols: (△) A horizon injection, (□) B horizon injection, (○) C horizon injection.
from the mean value (N) of the maximum bacterial counts observed over the range of sampling times beginning with the initial appearance of the maximum value, and continuing through to the final sampling. Subsequently, all mean maximum values were divided by the injection density of the individual tracer strains (Ne) to normalize for injection density differences and obtain the percent reduction. Therefore, the variation in the three relationships observed in Figure 4 must have resulted from differences in the horizontal locations of injection of the three individual strains relative to the zone of transport. The vertical distance in the soil profile between the depth of injection (12, 30, and 60 cm) and the zone of maximum transport was thought to largely affect the numbers of organisms that entered into that zone. However, there was no apparent correlation between the maximum numbers observed downslope and the depth of injection. This indirectly suggested that structural variations with depth in the soil profile could also influence the ability of the organisms to leave the injection depth and enter the zone of maximum transport.

The reduction of maximum bacterial counts as a function of distance downslope for the tracer strains injected into the A, B, and C horizons was represented by logarithmic equations (Table II). These equations were generated from the mean maximum values (N), and the relationships fitted to the transformed values by linear regression analysis.

### Table II

<table>
<thead>
<tr>
<th>Linear transformation</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>A horizon injection:</td>
<td></td>
</tr>
<tr>
<td>log (cells/ml) = 8.57 - 1.91 log (distance)</td>
<td>0.97**</td>
</tr>
<tr>
<td>B horizon injection:</td>
<td></td>
</tr>
<tr>
<td>log (cells/ml) = 8.79 - 1.66 log (distance)</td>
<td>0.88*</td>
</tr>
<tr>
<td>C horizon injection:</td>
<td></td>
</tr>
<tr>
<td>log (cells/ml) = 9.00 - 1.81 log (distance)</td>
<td>0.93**</td>
</tr>
</tbody>
</table>

*a Determined as the arithmetic mean of the maximum bacterial densities observed from the time of first appearance of the maximum numbers of bacteria and continuing to the final sample.

*b Distance in cm.

**Significant at P = 0.005.

*Significant at P = 0.01.

4. Discussion

Any tracer material suitable for application in the soil system should be: (1) physically similar to the material being simulated, (2) stable throughout the period of testing, (3) exclusively selectable from a heterogeneous sample, and (4) detectable in low concen-
trations. The three *E. coli* strains tested in these studies can be compared favorably against the above criteria as demonstrated through their survival patterns and ability to respond to selective recovery.

Knowledge of survival and recovery characteristics is essential for the application of resistance-labeled organisms to ecosystem analysis. Previous research using membrane filter chambers to determine *E. coli* survival (Faust *et al.*, 1975; McFeters *et al.*, 1974; McFeters and Stuart, 1972) and recovery on selective media (Bissonnette *et al.*, 1975) have indicated rapid death rates and greatly reduced recovery after equivalent periods and in physiochemical environments similar to those in our study. However, when care was taken to reduce the stress placed upon the *E. coli* strains in preparation for survival studies (unpublished data) and in recovery from natural environments (Klein and Wu, 1974), significant increases in percent survival and recovery over time were realized. The *E. coli* tracer experiments in our study were not designed to simulate the survival of fecal organisms naturally introduced into the soil environment, but were conducted only to determine if survival and recovery of specific bacterial strains could be maximized for a particular purpose.

This study utilized resistance-labeled strains of *E. coli* to present a phenomenological view of the characteristics of bacterial transport through a saturated soil system. In a similar fashion, effluent-borne bacterial populations from septic systems are also thought to move through the soil in zones of maximum hydraulic conductivity and at rates limited by: (1) the ability of the soil to conduct water, and (2) the hydraulic gradient or slope of the system. Granted, these characteristics will vary from soil to soil and from site to site, however, unifying principles do exist. Reneau (1978) and Viraraghavan (1978) determined the total and fecal coliform densities in the groundwater downslope from three operating septic systems inundated by high water tables. Although the data were based on the numbers of bacteria/100 ml and resulted from naturally occurring populations, the results described similar reductions in bacterial numbers with distance downslope from the drainfield tile line as in our study. Therefore, even though initially large reductions in bacterial numbers occur as the populations enter the soil system (Figure 4), once the organisms reach a highly conductive zone, relatively long distances are necessary for the further reduction of bacterial densities. Effluent-borne pathogens in these circumstances have the opportunity for rapid transport either horizontally to surface receiving waters, or vertically to aquifers through saturated recharge pathways. Our evidence indicated that serious contamination of individual water supply systems could occur when a high density of housing units with individual on-site waste disposal systems is located in soils which are generally unsuitable due to the occurrence of seasonally-perched water tables.

In sparsely populated areas, the subsurface escape of fecal organisms from the treatment zone of a septic system drainfield is highly localized and rapidly diluted in the groundwater system. However, groundwater that meets current coliform standards and is considered safe against outbreaks of bacterial disease may not be protected against low levels of virulent pathogens (National Academy of Sciences, 1977). Therefore,
proper septic system location and design, especially in areas subjected to elevated water tables and saturated flow conditions which make fecal organism transport possible, is necessary for the adequate protection of potable water sources.

Many states have adopted standards establishing the minimum soil depth to bedrock or highest level reached by a water table in which a septic system can be installed (Baker, 1978). Such standards are based on little or no information concerning the maximum depth of unsaturated soil necessary for proper treatment under all environmental conditions as might exist at any particular location. Results reported here indicated that the maximum bacterial densities recovered downslope in one soil series varied little from injection into the A horizon (12 cm depth) vs injection into the C horizon (60 cm depth) with a water table approximately 50 cm from the surface, and emphasized the need for a large zone of unsaturated soil around waste treatment systems to prevent the release and rapid transport of large populations of fecal microorganisms.

Acknowledgments


References