Percolation and Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Typhimurium in Soil Amended with Contaminated Dairy Manure or Slurry

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The effect of cattle manure and slurry application on percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium was investigated for different soil depths after the addition of water. Four treatments were chosen for the first set of experiments: (i) addition of inoculated farmyard manure on the soil surface, (ii) mixing of inoculated farmyard manure with the top 10 cm of soil, (iii) addition of inoculated slurry on the soil surface, and (iv) injection of inoculated slurry into the top 10 cm of the soil. Homogeneity of water distribution in the soil profile was confirmed by a nondestructive nuclear magnetic resonance method. Survival data were fitted to a modified logistic model, and estimated survival times were compared. In the second set of experiments, pathogen-inoculated farmyard manure or slurry was applied to soil columns with 1-month-old lettuce plants. More pathogen cells percolated to greater depths after slurry than after manure application. Survival of *E. coli* O157:H7 was significantly longer in soil with slurry than in that with manure, while survival of *Salmonella* serovar Typhimurium was equally high with manure and slurry. The densities of the pathogens were not different in the rhizosphere compared to the bulk soil with manure, while the densities were higher by 0.88 ± 0.11 and 0.71 ± 0.23 log CFU per g (dry weight), respectively, in the rhizosphere than in bulk soil after slurry application. Our results suggest that surface application of manure may decrease the risk of contamination of groundwater and lettuce roots compared to injection of slurry.

In the last 10 years food-borne disease outbreaks have increasingly been associated with the consumption of fresh vegetables and fruits contaminated with human pathogenic bacteria (3, 31). A significant number of the outbreaks were attributed to *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Bovine manure and slurry are the main environmental sources of these pathogens, with average concentrations between 10^5 and 10^6 CFU per g (dry weight) (gdw) of manure or slurry (24), but the density can be as high as 10^7 CFU gdw^-1 of manure (10).

Utilization of organic manures such as farmyard manure and slurry is the most economic and practical option for improving soil quality while providing as well an additional source of nutrients for growing plants. This is especially true for organic farms, where synthetic fertilizers cannot be used. Both organic and conventional soils can be fertilized with liquid slurry and/or farmyard manure. However, farmyard manure is more frequently used at organic farms.

The survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium is thought to be better in slurry than in farmyard manure (24; also A. V. Semenov, L. van Overbeek, N. Hidayah, A. J. Termorshuizen, and A. H. C. van Bruggen, submitted for publication) but is also dependent on the way manure or slurry is applied to agricultural fields (24). Survival of the pathogens may range from several days (turned composted manure) to more than a year (nonaerated manure) (9, 19). This broad difference in survival times is caused by various abiotic factors such as temperature (30, 38), presence of oxygen (A. V. Semenov, et al., submitted), and chemical composition (5) as well as by biological factors (e.g., microbial community composition) (5, 16, 30). The presence of plant roots is often neglected in controlled experiments although root exudates may support survival of human pathogens by providing a supply of easily available nutrients (18). Moreover, it has been shown that *E. coli* O157:H7 and *Salmonella* serovar Typhimurium may become associated with the surface of plants growing in soil amended with contaminated manure (15, 23) and may even be internalized by the plants (7, 18, 20, 32).

When microorganisms are introduced on or in soil, their movement is mainly determined by the flow of percolating water (13). Water flow and the ultimate distribution of bacteria in soil are affected by soil texture, pH, temperature, and the structure of the root system in soil (17). Like other bacteria, *E. coli* O157:H7 and *Salmonella* serovar Typhimurium are able to move through the soil profile with water after rainfall or irrigation and can even reach the groundwater (2, 21). In field experiments, 20% of *E. coli* cells applied with contaminated slurry to the field were found in drain water (37). This water can contaminate plants when it is used for irrigation. Since *E. coli* O157:H7 can survive in well water up to 65 days (1), there is a high risk that private water supplies could be contaminated with enteric pathogens.

Laboratory transport studies can mimic bacterial transport in field conditions only to a certain extent. The natural heterogeneity in field soil leads to the appearance of cracks and...
macropores through which water flow may occur while relatively homogeneous soil is commonly used in laboratory experiments. This may lead to underestimations of the movement of enteropathogens through the homogenized and possibly compacted soil. On the other hand, the presence of artificial boundaries (the so-called “wall effect”) and unexpected cracks may lead to overestimations of the movement of water and bacteria through the soil in mesocosms. The wall effect can be minimized by inserting sandpaper against the inner wall of soil columns while cracks can be minimized by careful packing of the soil. Nuclear magnetic resonance (NMR) can be used to check the homogeneity of water distribution in a soil column. NMR is a nondestructive and noninvasive spectroscopic method to measure static and dynamic water behavior in heterogeneous substrates (35). The data received from magnetic resonance images can give information about the spin density and spin relaxation values that reflect the interaction of water with the soil. These measurements have been proven to be highly correlated with water content in soils (35).

While it was shown that water is the most important dispersal factor for percolation of bacteria in different types of soil (13, 36) as well as for percolation of enteropathogens under various management practices (11), the movement and distribution of E. coli O157:H7 and Salmonella serovar Typhimurium in soil after application of manure and slurry are still unclear. It is also not clear if and how survival of enteric pathogens is influenced by the depth of the soil where they end up after transport through the soil.

The objectives of our study were the following: (i) to determine the extent of percolation of water and E. coli O157:H7 and Salmonella serovar Typhimurium from contaminated manure or slurry through a soil column, (ii) to determine the influence of application methods of manure and slurry on percolation and survival of these pathogens at different depths in a soil column, and (iii) to determine the influence of plant roots on percolation and survival of the pathogens, applied with manure or slurry, at different depths in bulk soil and the rhizosphere.

**MATERIALS AND METHODS**

**Bacteria.** E. coli O157:H7 strain B6-914 GFP-91 (where GFP is green fluorescent protein) was provided by Fratamico (8). The strain had been modified to carry an ampicillin (Sigma-Aldrich Chemie GmbH, Germany) for 50 µg ml⁻¹ ampicillin (Sigma-Aldrich Chemie GmbH, Germany) for E. coli O157:H7 and with kanamycin (50 µg ml⁻¹) for Salmonella serovar Typhimurium and was incubated at 37°C on an orbital shaker (200 revolutions min⁻¹) for 18 h. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times, and resuspended in sterile distilled water. The number of cells per milliliter of nutrient medium compared to the wild-type strain (8). Salmonella serovar Typhimurium MAE 119 (Agel/D101 saw) was obtained from Romling et al. (27, 28).

This strain carried resistance to kanamycin and gentamicin and carried the GFP gene after transformation with the PAG408 minitransposon. No differences between the wild-type of Salmonella serovar Typhimurium and its transformed form were found (27). Green fluorescence of both gfp-transformed strains was checked under UV light. Stock cultures were stored in 30% (wt/wt) glycerol at −80°C.

**Manure and slurry.** Slurry and fresh manure without urine from organically managed Frisian Holstein cows on a standard 50% grass/clover silage–50% dried grass diet were used for the experiments. Manure was mixed with straw (90% manure and 10% straw [kg/kg, dry weight]) and stored for nearly 1 month in a heap (sampling depth, 20 cm; temperature, 30 to 40°C at that depth) in a covered placement at the organic farm of L. M. M. Pool (Benninkom, The Netherlands). Slurry (50% liquid manure and 50% urine) was stored in a nonaerated reservoir (10 by 10 m) for 1 month before being used in the experiments. About 5 kg of manure from this heap as well as 5 liters of slurry from the farm reservoir was collected in June 2006 for the first set of experiments and in June 2007 for the second set; samples were homogenized and stored in closed plastic bags at 5°C for 2 weeks before the start of the experiments, which were carried out in a time span of 10 months. Chemical characteristics of farmyard manure and slurry are presented in Table 1.

**Soil.** For the experiments, sandy soil (50- to 2,000-µm sand particles, 80%; <2-µm clay particles, 4.2%) was collected at the organic experimental farm Droevendaal (Wageningen University and Research Center, The Netherlands) from a field that had not been cultivated over the last 3 years and was covered with grass. Throughout the sampled field, 10 soil subsamples (20 cm deep) were collected between the plants and mixed in June 2006 for the first set of experiments and in June 2007 for the second set. All samples were transported to the laboratory in plastic bags, thoroughly mixed, sieved through 0.5-cm mesh to remove plant parts and earthworms, and stored at 5°C until the start of the experiments.

**Tube preparation.** Fifteen (for the first set of the experiments) and 18 (for the second set) PVC-U gray tubes were prepared. Each tube was 50 cm long and 5 cm in diameter. The bottom part of each tube was closed with a cap of the same material and diameter. To prevent a wall effect, the internal surfaces of the tubes were covered with sandpaper. Four holes of 1-cm diameter each and at the same distance from each other were drilled around the tubes at heights of 13, 23, 33, and 43 cm from the top (16 holes per tube in total). The cylinders were also cut in half vertically, from the bottom to the top. That was done to allow samples to be taken from the rhizosphere and bulk soil at the end of the second set of experiments. All openings were closed with waterproof power tape at the start of each experiment. The tubes were filled with the sieved soil from the bottom to 3 cm from the top. The top 3 cm was left to add manure and slurry at the start of the experiments. The tubes were placed in a box vertically and were left undisturbed for 1 week to allow settling of the soil before manure or slurry was applied. The bulk density was 1.3 g cm⁻³.

**Inoculation of manure and slurry.** Bacterial inocula were prepared in Erlenmeyer flasks containing 150 ml of fresh LB broth (twofold dilution) with 50 µg ml⁻¹ ampicillin (Sigma-Aldrich Chemie GmbH, Germany) for E. coli O157:H7 and with kanamycin (50 µg ml⁻¹) for Salmonella serovar Typhimurium and were incubated at 37°C on an orbital shaker (200 revolutions min⁻¹) for 18 h. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times, and resuspended in sterile distilled water. The number of cells per milliliter of suspension was determined using a spectrophotometer, and the optical density of 0.7 at 630 nm in a 1-ml cuvette was equal to 1 × 10⁵ CFU ml⁻¹. Prepared inocula were added with a pipette to manure or slurry and mixed thoroughly within a double layer of plastic autoclavable bags for 5 min.

**Setup of the experiments.** Two series of experiments were carried out to investigate percolation and survival of E. coli O157:H7 and Salmonella serovar Typhimurium at different depths in the soil profile either in the presence or

### Table 1. Chemical characteristics of manure and slurry used in both sets of the experiments

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dry matter content (%)</th>
<th>pH</th>
<th>Organic matter (g/kg)</th>
<th>N-NO₃ (g/kg)</th>
<th>N-NH₄ (g/kg)</th>
<th>DON (g/kg)</th>
<th>DOC (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manure</td>
<td>35.2</td>
<td>9.1</td>
<td>74.75</td>
<td>0.00</td>
<td>1.00</td>
<td>0.17</td>
<td>5.02</td>
</tr>
<tr>
<td>Slurry</td>
<td>5.5</td>
<td>8.5</td>
<td>60.93</td>
<td>0.00</td>
<td>0.60</td>
<td>0.37</td>
<td>12.48</td>
</tr>
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</table>
absence of plants. For the first set of the experiments, separate experiments were carried out with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium while for the second set both pathogens were used simultaneously.

Four treatments were chosen for the first set of experiments: (i) addition of inoculated farmyard manure on the soil surface, (ii) mixing of inoculated farmyard manure with the top 10 cm of the soil, (iii) addition of inoculated slurry on the soil surface, and (iv) injection (by syringe) of inoculated slurry into the top 10 cm of the soil.

In the second set of experiments, 1 week after the tubes were filled with soil, one seed of iceberg lettuce (*Lactuca sativa* L. cv. Tamburo) was sown per tube. Farmyard manure or slurry was applied to the soil tubes with 1-month-old lettuce plants. Two treatments were compared: (i) addition of inoculated farmyard manure on the soil surface (manure was located at least 0.5 to 1 cm distance from the lettuce stem) and (ii) injection (by syringe) of inoculated slurry into the top 10 cm of the soil (at the same distance from the lettuce stem).

To have the same dry weight of amendments as well as the same concentration of manure and slurry (10%, kg/kg) for each treatment, 15 g of inoculated manure or 75 ml of inoculated slurry was added to the tubes with soil according to the treatments outlined above. Moreover, to equalize the amount of water added to the tubes as well as to mimic the influence of rainfall on percolation of the pathogens, 90 and 30 ml of water were added to the soil with manure and slurry, respectively, using an infusion pump (Harvard Apparatus) with a flow of 2.2 ml min⁻¹.

Control tubes for chemical measurements were prepared with noninoculated manure or slurry. All tubes were transported to the Uniform ( Wageningen University and Research Center, The Netherlands) greenhouse and maintained at 16°C with 19 h of supplemental light and a relative humidity of 50%. Each treatment had two replicates in each experiment. For the first set of experiments, each experiment was repeated three times, and for the second set experiments were repeated twice.

**Sampling procedure.** Samples (approximately 0.5 g) were collected at depths of 10, 20, 30, and 40 cm after 1, 3, and 5 days (and additionally after 7 and 21 days for the first set of experiments) and at a distance of 2, 4, 6, and 8 cm from the root tip 7 days after manure or slurry application (only for the second set of experiments). During the sampling time, both rhizosphere and bulk soil samples were collected at the same depths. The rhizosphere samples weighted, on average, 0.4 g and the bulk soil samples weighed 0.5 g. All samples were put in preweighed dilution tubes with 4.5 ml of 0.1% peptone buffer and weighed. Samples were vortexed and sonicated for 30 s (Branson 5200; 120-W output power and 47 kHz). Tenfold dilution series were prepared with sterile distilled water, and 50 μl of the two highest dilutions per sample was plated in duplicate on sorbitol-MacConkey agar (Oxoid) with ampicillin (50 μg ml⁻¹) for *E. coli* O157:H7 and on Luria-Bertani agar with kanamycin (50 μg ml⁻¹) for enumeration of *Salmonella* serovar Typhimurium. After the addition of approximately 20 sterile glass beads per petri dish, stacks of several plates were repeatedly shifted in different directions to allow the glass beads to spread the inoculum over the surface of the plate. Fluorescent bacterial colonies were counted under a Nikon Epifluorescence microscope (Eclipse 650; Nikon, Tokyo, Japan) with a 465- to 490-nm excitation filter and a long-pass emission filter (500 nm) after incubation at 37°C for 24 h. Fluorescent colonies made up 95% of all colonies on a plate. The number of fluorescent CFU was calculated per gram of dry soil. The calculated detection limit was 100 CFU gdw⁻¹.

**NMR.** An NMR instrument that can measure 1H was used to determine the movement and distribution of water in a nondestructive and noninvasive way in soil columns with noninoculated manure and slurry on the surface or incorporated in the top 10 cm. The NMR system consists of an Avance console (Bruker, Karlsruhe, Germany) and a superconducting magnet with a 50-cm vertical free bore (MagneX, Oxford, United Kingdom), which generates a magnetic field of 3 T (128-MHz proton frequency). A radio frequency coil was used for detection of the signal. The vertical bore of the magnet allows the measurement of soil tubes in the vertical direction. A remote climate control unit was used to maintain optimal conditions. For the measurements a pulsed-field gradient with stimulated echo and multispin echo was used. The data received from the magnetic resonance images gave information about the spin density and spin relaxation values (*T₁* and *T₂*) reflecting the interaction of water with the soil. *T₂* measurements are highly correlated with the water content of the soil (35); *T₁* relaxation time was determined for every centimeter of the soil columns since the echo train was adjusted for every pulsed-field gradient step. The NMR signal intensity of an additional tube with water was used for calibration. Fifty slices (every 1 cm) were measured per soil tube. A technical discussion of the mathematical and physical properties of the NMR instrument can be found elsewhere (14, 34, 35).

**Chemical characterization.** Fresh samples of soil at depths of 10, 20, 30, and 40 cm after manure and slurry application from the control pots were analyzed at the start and at the end of the experiments. The pH was measured in water suspension of 1:2.5 (v/v) with an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO₃⁻), ammonium (NH₄⁺), and total dissolved N content were determined colorimetrically in a solution of 0.01 M CaCl₂ with an AutoAnalyzer II (Technicon Instrument Corporation, Tarrytown, NY). Dissolved organic nitrogen (DON) was calculated as the difference between total dissolved N and the amount of nitrogen present as NH₄⁺ and NO₃⁻. Dissolved organic carbon (DOC) was measured by a carbon analyzer in a soil extract of 0.01 M CaCl₂. Dried (24 h at 40°C) manure and slurry samples were ground and used to measure total carbon and total nitrogen by the Dumas method, followed by detection by a Fisons element analyzer type EA 1108 (Thermo Finnigan Italia S.P.A., Milan, Italy). Water content was measured by comparison of the fresh and dried (40°C for 24 h) weights of samples.

**Statistical analysis.** The number of fluorescent colonies per petri plate was expressed as CFU gdw⁻¹, and standard deviations were calculated for every treatment, time, and depth combination. To describe the decline in CFU over time (for experiment 1), log-transformed data were fitted (separately for each replication) to a modified logistic function by nonlinear regression (Gauss-Newton method): *C* = *a* / (1 + *e*⁻*m* *t*⁻¹), where *C* is the log CFU gdw⁻¹ at time *t* (days), *a* is the upper asymptote (CFU gdw⁻¹), *m* is a parameter for the shoulder (days), and *t* is a slope parameter for the rate of change (day⁻¹) (SAS System for Windows, version 9.1; SAS Institute Inc., Cary, NC). The upper asymptote was kept constant as 7 log CFU gdw⁻¹. This model was selected since the regression coefficient (*R²*) was the highest for all treatment and depth combinations compared to other tested models (linear, exponential, Weibull, or logistic). Moreover, this model had shown excellent fits for decline data of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium during previous studies (6, 30). For the second set of experiments, 1 week after the tubes were filled with soil, four treatments were chosen for the first set of experiments: (i) addition of inoculated farmyard manure with the top 10 cm of the soil, (ii) injection (by syringe) of inoculated slurry into the top 10 cm of the soil, (iii) addition of inoculated manure or slurry into the top 10 cm of the soil, and (iv) inoculated slurry added directly to the soil. The performance of both nonlinear and linear models was assessed by calculating the significance level and *R²*. In addition to the model parameters, the time needed to reach the detection limit (2 log CFU gdw⁻¹) was calculated (survival time in days). A two-sided *t* test was used to distinguish differences in estimated survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (unpaired *t* test) as well as in population density between rhizosphere and bulk soil (paired *t* test). The influence of type of manure or slurry application and sampling depth on the estimated survival times of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was assessed by general linear model and mixed model procedures (SAS System for Windows, version 9.1; SAS Institute Inc., Cary, NC) separately for each pathogen for the first set of experiments and for both pathogens together for the second set. Treatment (combination of manure type and application method) and soil depth were considered fixed effects while repetition of the experiments and replicate within each experiment were considered as random effects in the mixed model. A split-plot design was used with depth as the subplot and treatment as main plot factors. In the experiments with lettuce, both pathogen and treatment were considered main plot factors, and depth again was considered a subplot factor. Stepwise multiple regressions were conducted by the REG procedure (SAS System for Windows, version 9.1, SAS Institute Inc., Cary, NC) to determine to what extent the measured chemical parameters could explain variation in survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. Regression analyses were conducted on normalized data to avoid possible nonlinear relations. The following parameters were included in the analysis: survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (days), organic matter (percent), log N-NO₃, log N-NH₄, DON (mg kg⁻¹), DOC (mg kg⁻¹), and log pH. Variables in the regression models were considered significant at the level of 0.1. Models were restricted to a maximum of two parameters. Multiple regressions were conducted for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium separately for the experiments without lettuce and together for the experiments with lettuce.

**RESULTS**

**NMR and soil water content.** Visual analysis of NMR images of water intensity showed an absence of soil cracks as well as absence of a wall effect for all treatments (Fig. 1). Therefore, it was assumed that the distributions of soil and water in the tubes were generally homogeneous. Moreover, the water content determined after drying of soil samples at 40°C was very similar to the water content determined by NMR (Fig. 2).

**Chemical characteristics of the soil.** In the first set of experiments, concentrations of N-NO₃, DON, and DOC signifi-
significantly ($P < 0.05$) increased with depth at the beginning as well as at the end of the experiment, while N-NH$_4$, pH, and organic matter were equally distributed during 21 days of the experiments. Incorporation of manure and injection of slurry led to significantly higher concentrations of all four soluble compounds (N-NO$_3$, N-NH$_4$, DON, and DOC) in upper layers of the soil tubes than treatments where manure and slurry were spread on the soil surface.

Comparison of chemical characteristics for slurry and manure treatments at the beginning of experiments in the second set showed significantly higher concentrations of N-NO$_3$ and DON at a 10-cm depth after injection of slurry while DOC was higher at 40 cm after application of manure than after an injection of slurry. At the end of the experiments, N-NH$_4$ was significantly higher in manure treatments for the first 30 cm, and the concentration of N-NO$_3$ was lower than with the slurry treatments.

**Percolation of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil columns without plants.** The initial calculated inoculum densities of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were 7.8 and 8.1 log CFU gdw$^{-1}$ of soil (for upper 10 cm), respectively, both for manure and slurry treatments. One day after the application of manure (spread on the soil surface or mixed in the top 10 cm of soil) and slurry (spread on the soil surface or injected into 10 cm of soil) followed by rainfall simulation, the pathogens and water were distributed throughout the soil tubes (Fig. 2). In general, the distributions of the pathogens for the different treatments were similar to each other (the highest density at 10 cm and the lowest at 40 cm). The most pronounced difference between the densities of the pathogens at the top (10 cm) and at the bottom (40 cm) of the soil columns was for treatments with applications of manure (on average, 4.1 and 3.3 log CFU gdw$^{-1}$ for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, respectively),

![Representative examples of water distribution (intensity of gray shading represents water content) at depths of 10 to 23 cm in a soil tube with manure applied on top of the soil after 1 day for the first set of the experiments as measured by NMR.](image1)

**FIG. 1.** Representative examples of water distribution (intensity of gray shading represents water content) at depths of 10 to 23 cm in a soil tube with manure applied on top of the soil after 1 day for the first set of the experiments as measured by NMR.

![Comparison of chemical characteristics for slurry and manure treatments at the beginning of experiments in the second set showed significantly higher concentrations of N-NO$_3$ and DON at a 10-cm depth after injection of slurry while DOC was higher at 40 cm after application of manure than after an injection of slurry. At the end of the experiments, N-NH$_4$ was significantly higher in manure treatments for the first 30 cm, and the concentration of N-NO$_3$ was lower than with the slurry treatments.](image2)

**FIG. 2.** Comparison of two methods for measurement of water content by the common procedure (bold solid line) and NMR (thin solid line) in a soil profile with manure spread on the soil surface (A), manure mixed with the top 10 cm of soil (B), slurry spread on the soil surface (C), and slurry injected into 10 cm of soil after 1 day of the experiment 1 (D). Dashed lines represent density of *E. coli* O157:H7 (squares) and *Salmonella* serovar Typhimurium (diamonds) after 1 day for the first set of the experiments. Horizontal bars represent standard errors.
which was significantly (P < 0.05) higher than the difference in densities of the pathogens between top and bottom after application of slurry (2.2 and 1.8 log CFU gdw⁻¹ for E. coli O157:H7 and Salmonella serovar Typhimurium, respectively).

Survival of E. coli O157:H7 and Salmonella serovar Typhimurium in soil tubes. After the first day, E. coli O157:H7 and Salmonella serovar Typhimurium declined in all treatments and at all depths (10, 20, 30, and 40 cm) (Fig. 3 and 4). There were significant (P < 0.01) interactions between manure/slurry treatment and soil depth (P < 0.01). When manure was applied on the soil surface, E. coli O157:H7 was not detected both after 7 days at a 30-cm depth and after 1 day at a depth

![Graphs showing survival of E. coli O157:H7 and Salmonella serovar Typhimurium in soil tubes.](http://aem.asm.org/)

FIG. 3. Average density of E. coli O157:H7 during 21 days at depths of 10, 20, 30, and 40 cm in a soil inoculated with manure spread on the soil surface (A), manure mixed with the top 10 cm of soil (B), slurry spread on the soil surface (C), and slurry injected into 10 cm of soil (D). Vertical bars represent standard errors.

FIG. 4. Average density of Salmonella serovar Typhimurium during 21 days at depths of 10, 20, 30, and 40 cm in a soil inoculated with manure spread on the soil surface (A), manure mixed with the top 10 cm of soil (B), slurry spread on the soil surface (C), and slurry injected into 10 cm of soil (D). Vertical bars represent standard errors.
of 40 cm. In the treatment with manure incorporated into the first 10 cm of soil, the density of *E. coli* O157:H7 was below the detection level after 1 day at a 40-cm depth. In both manure treatments, *Salmonella* serovar Typhimurium showed a similarly poor survival at a 40-cm depth after 1 and 3 days before the detection limit was reached.

In the case of slurry, *E. coli* O157:H7 was detected both after surface application and injection at all depths during 21 days of the first set of experiments, while *Salmonella* serovar Typhimurium survived better at a depth of 10 to 30 cm than at 40 cm, where it survived for only for 5 to 7 days.

When log CFU values of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were regressed over time using the modified logistic survival model, there were significant fits (P < 0.05) for all treatments, depths, and replicates, with an average pseudo-$R^2$ of 0.92 ± 0.02 and 0.96 ± 0.03, respectively, for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (Fig. 5). Estimated survival time of *Salmonella* serovar Typhimurium was significantly (unpaired test; P < 0.05) higher than that of *E. coli* O157:H7 at all depths except at 40 cm. Average survival time of *E. coli* O157:H7 at all depths was significantly higher after application of slurry (29.7 ± 1.8 days) than after manure application (17.8 ± 5.8 days). For *Salmonella* serovar Typhimurium, there was no overall difference between manure and slurry (P > 0.05), but injection of slurry significantly increased survival (42.5 ± 5.5 days) compared to surface application of manure (25.7 ± 6.5 days). The longest survival time was estimated for a 10-cm depth when manure was incorporated into the top 10 cm of soil (50.1 ± 5.0 days for *E. coli* O157:H7 and 65.7 ± 5.3 days for *Salmonella* serovar Typhimurium) (Fig. 6). The mixed model showed a significant effect (P < 0.01) of the manure/slurry treatment and sampling depth as well as an effect of the interaction between treatment and sampling depth on the estimated survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium.

Stepwise multiple regression was performed to identify factors (N-NO$_3$, N-NH$_4$, DON, and DOC) that can explain the observed variation in survival times of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium which were normally distributed (Shapiro-Wilk test, $P = 0.78$ and $P = 0.95$, respectively). N-NH$_4$ was the best predictor for survival time of *E. coli* O157:H7 applied to soil with manure according to the following equation: survival time = $-270.33 (±99.37) [P < 0.05] + 129.75 (±43.81) \times (N-NH_4) [P < 0.05, R^2 = 0.69]$. Increasing concentrations of N-NH$_4$ resulted in a significantly higher survival time of the pathogen. No significant factors were associated with survival time of *Salmonella* serovar Typhimurium.

Percolation of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil columns with lettuce plants. In the second set of experiments, manure and slurry were inoculated with *E.
coli O157:H7 and Salmonella serovar Typhimurium at 6.6 and 7.1 log CFU gdw⁻¹ of soil, respectively, and applied to soil tubes with 1-month-old lettuce plants. The densities of the pathogens after 1 day in the top 20 cm of the soil columns were similar to those observed in the first set of the experiments without lettuce plants. However, neither of the pathogens was detected at depths of 30 and 40 cm.

Survival of E. coli O157:H7 and Salmonella serovar Typhimurium in soil tubes with lettuce plants. Survival of the pathogens was determined during the first week after application of manure or slurry (Table 2). Density of E. coli O157:H7 significantly decreased at depths of 10 and 20 cm when manure was spread on the soil surface. A significant decline was also observed at a 20-cm depth when inoculated slurry was injected. In contrast, changes in the density of Salmonella serovar Typhimurium were not significant although they tended to be lower after 5 days of manure or slurry application than on the first day. Samples at 30- and 40-cm depths remained negative for both pathogens for the duration of the experiments. Due to the limited number of samplings (n = 3), instead of nonlinear regression, linear regression was performed to check main trends in E. coli O157:H7 and Salmonella serovar Typhimurium survival (Table 2). No significant relationships were found between decline rates and chemical soil characteristics. The mixed model showed a significant effect (P < 0.001) of the manure/slurry treatment, sampling depth, and pathogen, as well as the interactions between treatment, pathogen, and sampling depth on the estimated survival time of E. coli O157:H7 and Salmonella serovar Typhimurium (Table 2).

Seven days after inoculated manure and slurry were added to the soil tubes, bulk soil and soil from the rhizosphere of lettuce plants were collected at 2, 4, 6, and 8 cm from the root tip. When manure was spread on the soil surface, no significant (P > 0.05) differences in the density of E. coli O157:H7 or Salmonella serovar Typhimurium were found between rhizosphere and bulk soil (Fig. 7). However, in treatments with injected slurry, densities of E. coli O157:H7 and Salmonella serovar Typhimurium were significantly higher (by 0.88 ± 0.11 and 0.71 ± 0.23 log CFU gdw⁻¹, respectively) in rhizosphere than in bulk soil.

### DISCUSSION

The distributions of E. coli O157:H7 and Salmonella serovar Typhimurium were affected by the type of the substrate which was added (manure or slurry), by the method of its application (e.g., spread on the soil surface or incorporation into the soil), and by the presence of lettuce roots. An effect of several factors has been shown in previous works (2, 11, 12, 21, 37); however, none of these research experiments has tested the influence of these parameters simultaneously. The survival of E. coli O157:H7 was on average 1.39 ± 0.12 times shorter than survival of Salmonella serovar Typhimurium for all treatments. A similar difference in survival for the pathogens was found in previous experiments (6, 30). For the first time, we showed that application of slurry led to higher density and longer survival of the pathogens at the bottom of an unplanted soil profile than application of manure. However, in soil with lettuce plants, the pathogens did not move beyond the rooting depth after one simulated rainfall event. Moreover, the densities of E. coli O157:H7 and Salmonella serovar Typhimurium were significantly higher in the rhizosphere of lettuce plants than in bulk soil after application of slurry, while this was not the case when manure was added.

The possibility that human pathogens reach the groundwater under different soil conditions had already been studied during

<table>
<thead>
<tr>
<th>Pathogen and soil treatment</th>
<th>Sample depth (cm)</th>
<th>Log CFU gdw⁻¹ on:</th>
<th>Decline rate (log CFU/day)</th>
<th>Estimated survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td></td>
<td></td>
<td></td>
<td>0.08*</td>
</tr>
<tr>
<td>Manure</td>
<td>10</td>
<td>5.96 (±0.32) A</td>
<td>5.14 (±0.28) B</td>
<td>4.93 (±0.13) B</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.06 (±0.09) A</td>
<td>4.30 (±0.29) B</td>
<td>4.39 (±0.12) B</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Slurry</td>
<td>10</td>
<td>5.64 (±0.31) A</td>
<td>5.21 (±0.54) A</td>
<td>5.06 (±0.36) A</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.10 (±0.08) A</td>
<td>4.24 (±0.20) B</td>
<td>4.10 (±0.08) B</td>
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<td></td>
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<tr>
<td></td>
<td>40</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Salmonella serovar Typhi-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>murium</td>
<td>10</td>
<td>6.30 (±0.21) A</td>
<td>5.91 (±0.25) A</td>
<td>6.00 (±0.12) A</td>
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<tr>
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<td>4.90 (±0.36) A</td>
<td>4.54 (±0.31) A</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Slurry</td>
<td>10</td>
<td>5.51 (±0.28) A</td>
<td>5.27 (±0.32) A</td>
<td>4.93 (±0.33) A</td>
</tr>
<tr>
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<td>20</td>
<td>4.56 (±0.17) A</td>
<td>4.21 (±0.26) A</td>
<td>3.84 (±0.22) A</td>
</tr>
</tbody>
</table>

* a Inoculated manure was spread on the soil surface, or slurry was injected into 10 cm of soil in the second set of experiments.
  b The same letter is placed next to values whose differences were significant (P < 0.05) among sampling times within each variable. ND, not detected (below the detection level of 100 CFU gdw⁻¹).
  c *, significant (P < 0.05) fit to a linear model. NA, not applicable.
previous experiments (11, 22, 25, 37). Such soil factors as porosity, surface area, bulk density, and macropore structure play an important role in the leaching potential of the introduced bacteria by their influence on adsorption and gravitational movement with water (2, 36). However, for organic substrates such as manure and slurry, the adsorption and desorption behavior of bacteria are due not only to differences in physical characteristics of the substrate but also to biophysical properties of the organic matter (22). Experiments on the percolation of *Pseudomonas fluorescens* also showed that application of an adsorption substrate (such as bentonite clay) to the soil may increase survival of *P. fluorescens* and reduce transport to deeper layers (13). In our experiments, it is likely that some of the organic matter in the slurry moved down the soil profile while manure particles moved less readily. This may explain why the pathogen concentrations at the bottom of the soil columns were higher after slurry application than after use of farmyard manure. Attachment of the pathogens to manure particles in the upper soil layers probably led to reduced percolation to deeper soil layers. Movement of nutrients as well as movement of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium may have been more pronounced in the case of slurry application so that the pathogens could survive better in lower soil layers in the presence of easily available compounds from slurry.

It has previously been reported that *E. coli* O157:H7 can survive longer in soil in the presence of rye and alfalfa roots (12). While manure and slurry characteristics such as chemical composition and microbial community have an important influence on survival of the pathogens, the effect of the rhizosphere can also significantly affect survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. During our experiments, the rhizosphere effect after slurry application seemed to be more pronounced since slurry initially had two times higher concentrations of dissolved organic nitrogen and carbon than manure. The nitrate concentrations in the upper 10 cm of soil were also higher after slurry than manure application. This difference may enhance secretion of root exudates which could increase survival of the pathogens (26). High densities of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in the rhizosphere may increase the risk of internal contamination of plant products (18).

In previous percolation studies with *P. fluorescens*, bacterial transport was dependent on the intensity of water flow and the number of water applications (33). A limitation of our experiments was that water was applied only once (equal amount of water for all treatments) at one flow rate only. However, we were more interested in determining the influence of the manure or slurry application method on the distribution and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil columns. Another limitation was that relatively small soil columns were used, which may not be representative of field conditions due to the influence of soil cracks and wall effects on water flow (13). However, only in such controlled...
of days after application. Moreover, the average survival time is lower with farmyard manure than slurry as the contamination of surface and groundwater with Salmonella serovar Typhimurium if slurry or manure is applied close to surface water. Although the Food and Agriculture Organization/World Health Organization standard for the use of reclaimed water for agricultural purposes allows the presence of fecal coliforms, according to European Union requirements for drinking water, no detectable fecal coliforms should be found in 100 ml of a water sample. In the case of E. coli O157:H7, safety rules should be even more strict due to its very low infective dose (4). The highest risk of pathogen movement through the soil profile is immediately after slurry application since even a small amount of rainfall can lead to drainage of water carrying significant numbers of E. coli O157:H7 and Salmonella serovar Typhimurium organisms to the groundwater. This risk is less with farmyard manure than slurry as the pathogens were not detected at a 40-cm depth within a couple of days after application. Moreover, the average survival time of E. coli O157:H7 at all depths in the soil columns was 1.67 times higher for treatments with slurry than with manure. These findings argue for surface application of farmyard manure (often used at organic farms) rather than injection of slurry. At conventional farms, slurry is more often used than solid manure. In The Netherlands, injection of slurry is mandatory to reduce ammonia emission (26), and this is practiced both in grasslands and arable fields (including vegetable fields). Unfortunately, this practice is associated with relatively high risks of percolation and survival of enteric pathogens in soil.

The presented research is part of a large research program (5, 18, 29, 30) to quantify the risks of spread of E. coli O157:H7 and Salmonella serovar Typhimurium from manure via soil to lettuce plants. The results will contribute to the development of a simulation model for the spread and survival of these two pathogens in soil and in the rhizosphere of lettuce plants after application of contaminated manure or slurry.

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