Bacteria survival experiment for assessment of wastewater reuse in agriculture

Edward Smith and Aimen Badawy

ABSTRACT

Growth and survival of a strain of *E. coli* were investigated in laboratory-scale soil columns under essentially static conditions in three Egyptian agricultural soils. One pore volume of a buffer solution of known cell concentration was applied to a set of identical columns at time zero, and individual columns were analyzed for viable *E. coli* colony forming units at times ranging from 1 hr to 7 d and at various soil depths. The resulting concentration-depth profiles yielded information that can promote proper application of wastewater reuse in agriculture and the assessment of associated health and environmental risks. Biomass growth in soil occurred over the first 2 to 3 days after application, achieving biomass production 40–70 times the number of cells applied depending on the soil. Culturable populations declined to only a few viable cells at the end of 7 days. *E. coli* growth rate and total biomass production were well correlated to the soil organic content. Indoor conditions resulted in slower but more prolonged *E. coli* growth than in outdoor experiments, verifying the determinative roles of climatic factors and soil moisture.

Key words | bacterial growth, *E. coli*, soil columns, wastewater reuse

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INTRODUCTION

Agricultural water withdrawals in Egypt represent ~85% of the total, posing large environmental challenges to the sustainability of fresh water resources in Egypt. Expansion of irrigation in the Nile basin and beyond will require innovative management of the full range of water resources to meet the increasing demand and face the associated environmental consequences. In this regard, several countries in the arid region of the Middle East practice wastewater treatment and reuse, including Egypt (Bazza 2003). However, of Egypt's total wastewater, just 19% was treated and only 6% (or one-third of the amount treated) reused based on data at the close of the millennium (UN-FAO 2000). Although precise current data is not available, several ambitious wastewater collection and treatment projects in Egypt over the past decade have increased the percentage of treated wastewater to 35–40%; however, the percent of reuse has not risen proportionally.

Unfortunately, there are also potential problems associated with wastewater reuse in agriculture, among them health risks to irrigation workers and communities with prolonged exposure to improperly treated wastewater and consumers of food crops irrigated with contaminated water. Environmental problems include surface and groundwater pollution, and partial damage to certain soils; e.g. reduction of indigenous microorganisms (Friedel *et al.* 1999) and swelling due to elevated salinity (Gross *et al.* 2005; Shigematsu *et al.* 2008). Of highest priority is addressing the threat to public health. To this end, the World Health Organization (WHO) published guidelines for the safe use of wastewater in agriculture in 1989 (UN-WHO 1989). These were subsequently revised in 2000 and most recently in 2006 (UN-WHO 2000, 2006). National guidelines for wastewater reuse in agriculture exist in Egypt, drawing on numerous national and international programs in addition

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to the WHO initiatives (US-EPA 2004). Total and/or fecal coliforms are most often used as indicators for regulatory purposes, with guidelines qualified according to reuse and exposure group restrictions, anticipated levels of exposure, types of crops or terrain to be irrigated, or even the irrigation technique to be applied. Most guidelines also assume or require a certain level of treatment prior to reuse. The guidelines typically do *not*, however, consider important site-specific elements such as the type of soil, soil organic fraction, irrigation schedule, and the impact of other system conditions on the growth, survival, and transport of eco-health parameters such as *E. coli*–this despite the extensive body of data from laboratory and field studies on the growth and survival of fecal coliforms in soil (for a review, see Gerba & Britton 1984; Foppen & Schijven 2006). This study was an attempt to improve our understanding of bacterial interactions with soil by investigating the growth and survival of a strain of *E. coli* in laboratory-scale soil columns under essentially static conditions in three Egyptian agricultural soils. The resulting concentration-depth profiles yielded information that can promote proper application of wastewater reuse in agriculture and the assessment of associated health and environmental risks.

MATERIALS AND METHODS

American Type Culture *Escherichia coli* (ATCC 25922 from the Naval American Marine Research Unit in Cairo) was obtained on a MacConkey culture plate. Aqueous buffer solutions were prepared to achieve concentrations of *E. coli* that averaged 64,000 CFU/100 ml for application in each column test. A new buffer solution was prepared prior to each experiment by dissolving one *HACH* (USA) BOD nutrient buffer pillow (KH_2PO_4, $MgSO_4$, $CaCl_2$, and $FeCl_3$) in 3 litres of distilled, deionized water. All bacterial enumeration was performed using the standard membrane filter technique for fecal coliforms, Method 9222 D from Standard Methods (1998). The only deviation was the incubation of bacteria in a thermostatically and humidity controlled air incubator rather than a water bath. In a comparative experiment with a water bath, statistical analysis using the *f*-test demonstrated that the data belonged to the same population with 95% confidence.

Three irrigated agricultural soils were selected for the study. Soil 1 is a slightly clayey, slightly loamy sandy soil from an irrigated field at the Red Sea coast 350 km southeast of Cairo. Soil 2 is a sandy desert soil from South Tahrir City reclaimed for agricultural use using disinfected manure. Soil 3 is also from a land reclamation project in Sadat City, but is a more clayey, fine-textured soil. Based on the FAO-Unesco Soil Map, all three soils could be classified as Calcisols, although Soil 1 has a Leptosol component, and Soil 3 may have some associated Luvisols (FAO 1993). Prior to experimental investigations, the soil was sieved through a U.S. Standard No. 10 sieve to remove stones, sterilized by autoclaving in a glass beaker the day before use, cooled to 22°C in an incubator with the beaker sealed by aluminum foil, then placed in sterilized polyurethane containers until use. Some relevant soil properties are listed in Table 1.

Biological analysis of the soil samples on fecal media (m-fc agar, GCC Diagnostics, UK) indicated the presence of both fecal and non-fecal bacteria. Therefore, the soil samples were sterilized prior to use as noted above. This sterilization was found to have no impact on the organic matter content, and Fourier Transform Infrared (FTIR) spectra recorded between 4,000 and 400 cm^{-1} of sterilized versus unsterilized soil indicated no observable change in organic functional groups (Coates 2000).

Table 1 | Properties of test soils (Bowles 1992)

Parameter	Units	Soil 1	Soil 2	Soil 3
Soil texture	–	Loamy sand	Sand	Sandy clay loam
Bulk density	g/cm^3	1.48 ± 0.04	1.73 ± 0.04	1.27 ± 0.02
Particle density	g/cm^3	2.48 ± 0.07	2.63 ± 0.05	2.27 ± 0.04
Porosity	%	40.3 ± 0.9	34.2 ± 1.0	43.9 ± 1.0
Organic fraction	%	0.57 ± 0.02	0.45 ± 0.02	1.47 ± 0.03

Bacterial survival experiment

These experiments were conducted in the soil columns depicted in Figure 1 below. The columns were made from API drinking water grade PVC pipe and fitted with a plexiglass porous disc and stainless steel mesh screen to support the soil. Packing of the columns such that the bulk density was equivalent to that of the field soil and to prevent layering and creation of macropores has been detailed previously (Smith & Hegazy 2006). The soil depth in each column was 5 cm. Upon preparation of the bacterial buffer solution and the soil columns, the experiment was initiated by applying a volume of buffer solution equivalent to one pore volume (for the given soil type) onto the surface of each of seven soil packed columns used in an individual experiment. This amount was manually applied by pouring the buffer solution in a slow and even manner so that no ponding and no bottom leakage were observed. The first column of each experiment served as a reference column and was thus analyzed as soon as buffer solution application was completed (actually, within 60 minutes of buffer solution application). The remaining six columns were assessed after 12, 24, 48, 72, 120 and 168 hours, successively.

The final mass of the column was measured in order to immediately calculate the average water fraction (F_W) which was assumed to be evenly distributed throughout the short soil column. Determination of the profile of fecal coliform counts in the soil was performed using a modification of the method described by Abu-Ashour *et al.* (1998). Five consecutive one centimetre segments were collected and analyzed from each column. This was achieved by removing the lower supporting clip (at the bottom end of the column) and applying pressure to the supporting disc to move the packed-soil upwards. A sterile cutting knife was used to cut off the soil segments as each centimetre became exposed. Each segment was contained in a separate foil plate, weighed, and then manually blended to achieve homogeneity. For quantitative assessment of bacteria in each depth segment, 10 g of the blended sample were added to separate 250-ml sterile polyurethane containers. Deionized, distilled water (DDW) was added to make 100 ml in each container according to:

$$V_{DDW} = 100\,\text{ml} - 10 \times F_W$$

where V_{DDW} is the volume of DDW added and the last term represents the water contributed by the 10 g sample. The containers were then sealed and placed on a shaker at 400 rpm for approximately 20 minutes, with the assumption that the vigorous shaking results in bacteria recovery from the soil. It is difficult to perform a true recovery test since the initiation of the experiment and segregation of the soil segments requires a minimum of 20 minutes, during which cell growth has likely commenced. Tests with Soil 1 yielded a cell "recovery" of 130–140% of the number of cells applied in one pore volume during this initial period. Duplicate plate counts (at each segment) were obtained *via* serial dilution followed by filtering through a membrane filter which was then placed on the supporting media and incubated at 44.5°C for 24 hours prior to enumeration.

The experiments were performed under various environmental conditions. The first set was conducted outdoors to simulate (in this case, summer) conditions of sun exposure and temperature as in actual irrigation practice. Although an individual experiment required a total of seven days for completion, the entire set of experiments was conducted over a period of 3 months (mid-June to mid-September). The temperature ranged from a maximum of 31.7 to 38.2°C during day time to a minimum of 26.4 to 30.8°C at night in this period. The interval between sunrise and sunset ranged from 14 hours to 11.2 hours, and relative humidity ranged from 40 to 56 percent. The second set of experiments was conducted in the laboratory where temperature was maintained at 22°C with a relative humidity of 52 percent. The indoor columns were mounted next to a large (closed) window, providing a significant amount of natural light in

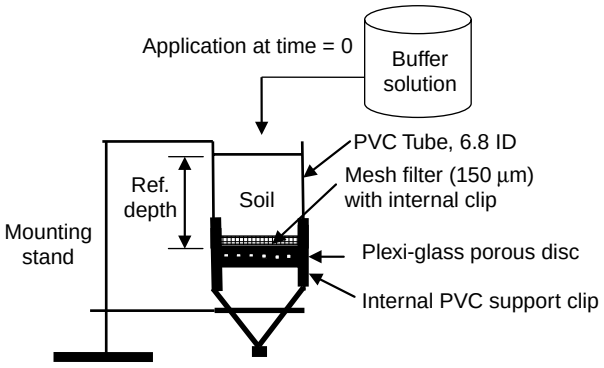

Figure 1 | Soil column setup for bacteria survival test.

addition to artificial lighting; however, the surrounding buildings blocked direct sunlight during portions of the day.

RESULTS AND DISCUSSION

As anticipated, bacterial growth and survival was a function of environmental conditions. *E. coli* concentration versus depth for Soil 1 is shown in Figure 2 to illustrate typical deviations in bacteria counts for a given sample. The data compare concentrations at 24 hours after application of buffer solution of outdoor versus indoor conditions, and indicated more rapid growth in the outdoor case. Note that the *E. coli* concentration units in Figure 2 are per 100 ml of water used in the method for extraction and enumeration of bacteria, not per 100 ml of soil and/or water in the column itself. The same applies to the relative concentrations in Figures 3–5.

Figures 3–5 depict relative *E. coli* concentration (i.e. viable cells relative to the concentration in the applied buffer solution of 64,000 CFU/100 ml) versus depth over a one-week period for the three test soils in outdoor conditions. The profiles of Soils 1 and 2 are very similar in terms of the timing of growth and death of cells, and even the relative concentrations attained with time. Although the depth profiles of Soil 3 follow the same pattern with respect to time as the other two soils, bacteria concentrations versus time are considerably greater in Soil 3. Soil 3 has a notably higher organic content than Soils 1 and 2. The strong functional relationship of adsorption of *E. coli* and organic content of the test soils and other

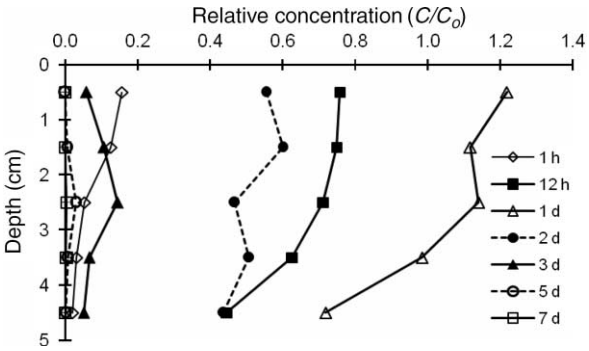

Figure 3 | *E. coli* survival profile in soil 1 (outdoors).

soil-water environments has been demonstrated previously (Rogers & Logan 2000; Guber *et al.* 2007; Smith & Badawy 2008). Whether the enhanced growth mechanism is a result of concentration of bacteria at organic sites via adsorption, or subsequent availability of the soil organic matter for utilization is not clear. What is clear is that enhanced growth and survival of viable cells is a function of the soil organic content. Moreover, being a finer and more clayey-textured soil, water retention was observed to be greater and over a longer period in Soil 3, constituting more favorable conditions for growth (Abu-Ashour *et al.* 1994). Soil texture, in particular the presence of clays, and the associated moisture profile are known as determinative factors in fecal coliform survival in topsoil (Dong *et al.* 2002; Trevisan *et al.* 2002). Water drains more freely through sandy soils (Soils 1 and 2) than through those having more clay (Soil 3). This also implies more bacteria loss via faster advective transport from the topsoil in Soils 1 and 2 versus Soil 3 (Malkawi & Mohammed 2003). In fact clayey soils tend to have higher organic content than sandy soils owing to

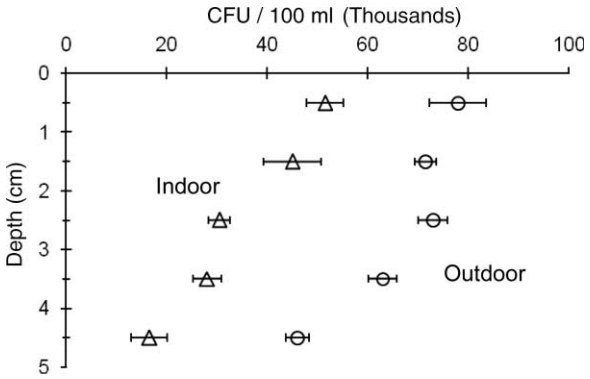

Figure 2 | *E. coli* survival profile in soil 1 after 24 hours (outdoor vs. indoor).

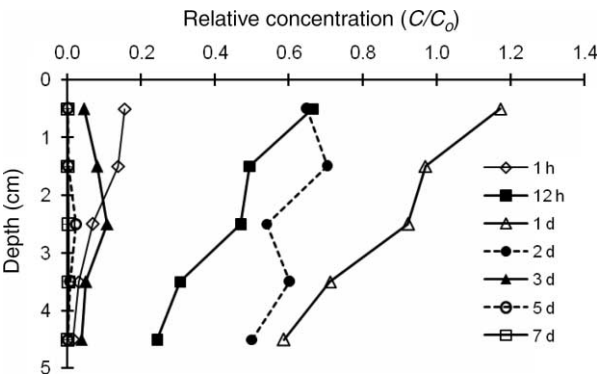

Figure 4 | *E. coli* survival profile in soil 2 (outdoors).

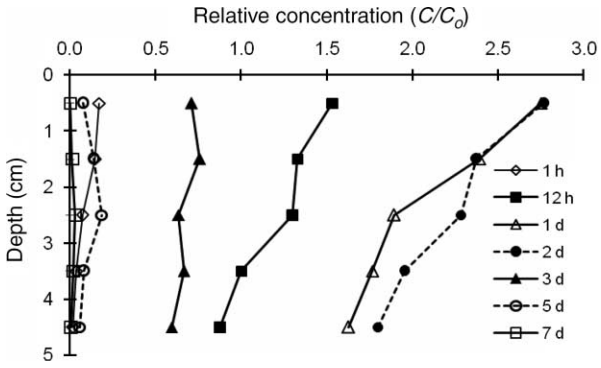

Figure 5 | *E. coli* survival profile in soil 3 (outdoors).

a lower aeration level, meaning less decomposition of associated organic matter over time (Cornell University 2007).

The profiles of the three soils revealed that maximum concentrations occurred at the surface (top cm) during the period of bacteria growth. This may be due to the greater availability of oxygen near the surface as well as some immediate adsorption and concentration of bacteria at the point of application of buffer solution. However, as the experiment proceeded to the *E. coli* death phase (after 1 day for Soils 1 and 2; after 2 days for Soil 3 in the outdoor experiment), inactivation of cells at the surface was faster relative to the rest of the column, presumably due to increased exposure to solar radiation resulting in disinfection and drying (O'Toole *et al.* 2008;). Surface concentrations reached zero within 5 days in Soils 1 and 2, while a small though measurable concentration was still observed in Soil 3 after 7 days. Once again, soil texture and moisture retention likely played an important role. The indoor *E. coli* concentration-depth profiles, although not presented here, followed the same pattern as the outdoor treatment except that the growth rate was slower as indicated in Figure 2. However, the growth period was prolonged; from 1 to at least 2 days for Soils 1 and 2, and from 2 to 3 days for Soil 3. Furthermore, cell concentrations did not reach zero in any of the indoor columns even after 7 days. Another difference was that surface concentrations continued to be the highest in the profile until the 5th day. The slower but prolonged growth period can be attributed to the relatively moderated, and lower, temperature in the indoor environment and associated prolonged retention of moisture versus drying at the surface. In addition, less direct ultraviolet (UV) light penetration mitigates bacteria die-off due to solar disinfection, especially near the surface. This is consistent with other studies that indicate that *E. coli* die off rates increase with temperature and desiccation (Trevisan *et al.* 2002; Foppen & Schijven 2006). Field tests, however, suggest that intensity of solar (especially UV) radiation is more effective in coliform inactivation than elevated temperature (Manios *et al.* 2006).

These results have significant implications for wastewater reuse in agriculture via irrigation and in the assessment of both health and environmental risks. For instance, Figures 3–5 indicate that bacterial contamination was at its highest levels within 24–48 hours from application, achieving concentrations more than one order of magnitude higher than in the applied water. Therefore, field workers should avoid handling irrigated soil entirely during this period. Moreover, complete bacterial inactivation required 5 to 7 days from the time of irrigation. Irrigation cycles may need to be structured accordingly to allow enough time for natural die-off of bacteria since more frequent application could saturate the adsorption capacity of the soil, thereby promoting leaching of substantial amounts of bacteria

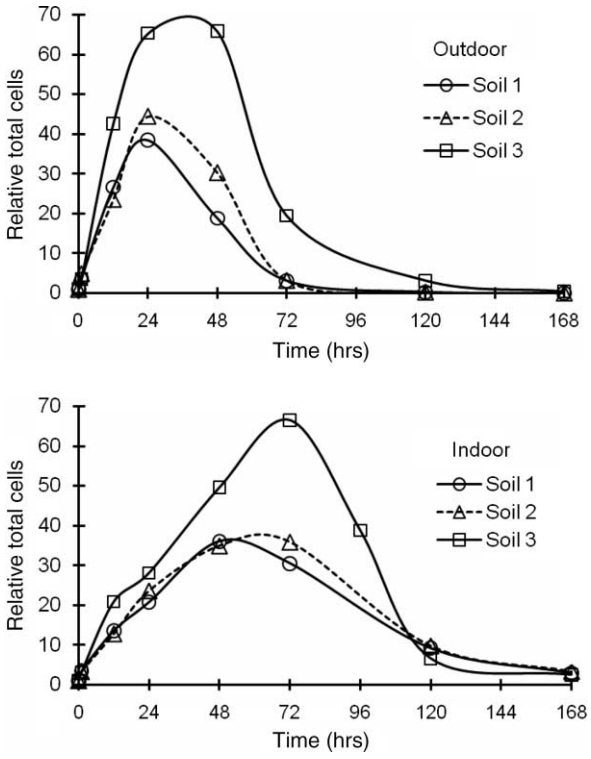

Figure 6 | *E. coli* survival versus time in three test soils (top–outdoors; bottom–indoors).

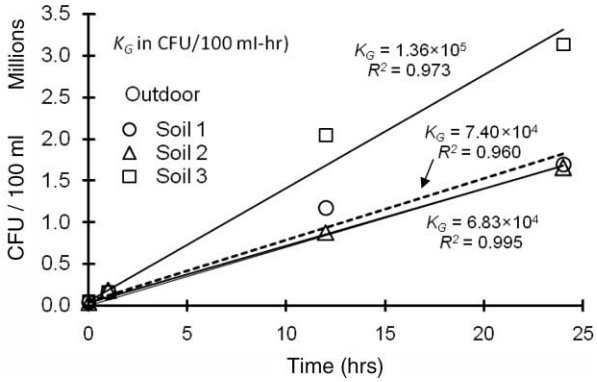

Figure 7 | Estimation of zero-order growth constant for three test soils (outdoors).

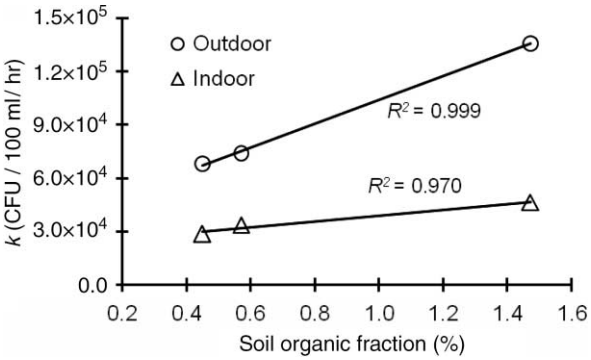

Figure 9 | Relationship of zero-order growth constant to organic fraction of test soils.

to deeper layers of the soil and increasing the risk of groundwater contamination (Trevisan *et al.* 2002; Fine & Hass 2007; Guber *et al.* 2007).

A mass balance calculation for each soil segment yielded the total viable cells for a given column. This was then plotted for a given soil versus time as relative total cells; i.e. the cells in the pore volume of the buffer solution applied to the column at time zero of the experiment. These plots are depicted for outdoor and indoor conditions for the three soils in Figure 6. The rapid growth of bacteria in the topsoil during the first 24 hours after application and eventual inactivation has been observed in previous investigations involving fecal coliforms (De Araujo *et al.* 2003; Malkawi & Mohammad 2003; Manios *et al.* 2006). Many of the observations from the concentration-depth profiles are more evident in Figure 6, namely: (1) the similarities in *E. coli* growth and inactivation between Soils 1 and 2; (2) the much higher biomass production in Soil 3–up to 70 times the number of cells initially added to the column; and, (3) the slower but more prolonged growth under indoor conditions. Figure 6 also indicates that despite the altered kinetics, the total biomass production in indoor conditions approaches that of the outdoor experiment.

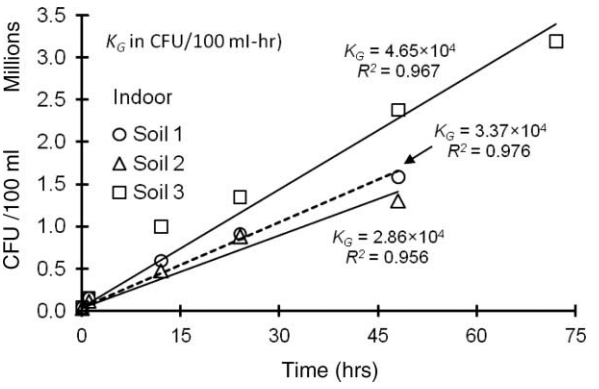

Figure 8 | Estimation of zero-order growth constant for three test soils (indoors).

The growth portion of the curves in Figure 6 was then used to estimate zero-order growth constants, K_G. These are presented in Figures 7 and 8 for outdoor and indoor conditions, respectively. Although only three soils were tested in this work, Figure 9 illustrates the potentially strong correlation between soil organic content and the zero-order growth constant. These constants may find possible utility as an independent estimate of the bacterial growth parameter for mass transport modeling of *E. coli* in soil for quantitative risk assessment of wastewater reuse applications. This research should be expanded to capture a wider range of soil organic fractions. For the same soil, future investigations should examine seasonal impacts as well as the impacts of variable input concentration of bacteria and other background constituents in irrigation water such as dissolved organic matter and salt concentrations. Moreover, although this work isolates *E. coli* in order to investigate soil-bacteria interactions for the target organism, it is recognized that real systems contain a host of other organisms that will impact these interactions (Franklin *et al.* 2001; Gantzer *et al.* 2001; Foppen & Schijven 2006). Accordingly, future studies are proceeding toward this end. Collectively, these efforts should aid in the development of wastewater reuse guidelines that account for important site-specific variables.

CONCLUSIONS

1. *E. coli* concentration–soil depth profiles were generated from short soil columns to which a single pore volume of bacterial buffer solution was applied. The resulting profiles indicated that biomass growth occurred over the first 1 to 3 days after application, achieving biomass production 40–70 times the number of cells applied depending on the soil. Natural decline of culturable populations proceeded to only a few viable cells surviving at the end of 7 days.
2. Indoor conditions resulted in slower but more prolonged *E. coli* growth than in outdoor experiments, verifying the determinative roles of climatic factors and soil moisture on bacterial growth and survival in soil.
3. *E. coli* growth in the soil columns showed a strong functional relationship to the soil organic content; i.e. a higher soil organic fraction resulted in greater total biomass production and more rapid growth rate of bacteria. Soil texture, in particular the presence of clays, and the associated moisture regime are also important variables in promoting the growth and survival of coliform bacteria in topsoil.

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