

# Secondary fate of pathogenic bacteria in livestock mortality biopiles

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## Abstract

Prominent bacterial pathogens, such as *E. coli* O157:H7 and *Salmonellae* sp. (SA), reside in multiple hosts, exist in animal-borne wastes, and are excreted by animals in large volumes. They have also been linked to increasing incidence of mammalian infections. Emerging zoonoses cause ≈12-24% of global infectious diseases, thus human and animal exposure to bacterial pathogens embodies a health risk. Livestock, for example, represent vectors for and defenses in bio- or agri-terrorism situations. It is impractical, however, to monitor for all bacterial pathogens due to difficulty, time, expense, virulence, and their ubiquity in nature. Indicator micro-organisms are therefore used to equate bacterial pathogen presence. Choosing a universal indicator is therefore a foremost concern that is continuously debated in academic and public circles.

*Escherichia coli* (EC) and *Streptococcus fecalis* (SF) have historically been used as indicators of pathogens inherent in the gut of humans and warm-blooded animals. Since EC is the prominent species in the total (TC), fecal coliform (FC), and the Family Enterobacteriaceae (EB) groups, EC detection should coincide with detection of EB and TC. Likewise, TC or SA presence should elicit EB detection. Theoretically, since SF is not an EB member, no similarities in detection or ultimate fate should be observed. The crux of this research was to assess the fate of indicator bacteria in solid and liquid media associated with slaughterhouse-residual biopiles during the secondary composting phase. Traditional (membrane filtration; MF) and rapid (PetriFilm™) enumerative methods, and classical (TC, EC, SF) and non-classical (EB, SA, EC NAR) indicator bacteria, were used. This presentation will focus on the re-growth potential of these micro-organisms.

## Key Words

Biopile, compost, slaughterhouse, bacteria, pathogen, fate.

## Introduction

Bacteria may number in the millions or billions in 1 mL of freshwater or 1 g of soil, respectively. In particular, a slaughterhouse is a large source of bacteria, of which many may be pathogenic. For example, total coliform (TC) bacteria comprise ≈10% of human and animal enteric micro-organisms. This research intended to quantify the occurrence, magnitude and movement of indicator bacteria during secondary-phase slaughterhouse-residual (SLR) biopiling. To study this topic, a facility was constructed that simulated a natural environment but allowed for analysis of physical, biological, chemical, and hydrological biopile parameters.

Five indicator bacteria were monitored: Family Enterobacteriaceae (EB), TC, *Escherichia coli* (EC), nalidixic acid resistant *E. coli* (EC NAR), and *Streptococcus fecalis* (SF). Most regulatory standards employ TC and SF, and (primarily) EC due to its ecology, easy enumeration, and the virulence of certain strains. For example, Canadian compost standards require low levels of bacteria (fecal coliforms {FC}, *Salmonella* sp.) as a measure of process efficiency (CCME 2005b). Canadian drinking water standards remain at 0 TC and 0 EC cfu/100 mL (Health Canada 2008). As well, Canadian standards for agricultural water use of 1000 TC and 100 FC cfu/100 mL (CCME 2005a) correspond with WHO (2006) guidelines that permit liquid (as irrigation) to be applied to edible crops if TC levels are <1000 cfu/100 mL.

Bacterial survival patterns have been described (Lemunier *et al.* 2005). In different media, bacteria can persist from hours to years. However, different bacteria exhibit different spatial patterns due to differences in local media conditions. Pathogen survival in the environment depends on many factors, but mainly temperature. Canadian compost standards, for example, stipulate that 55°C be maintained for at least 3-15 d to ensure pathogen inactivation (CCME 2005b). Biopiling confers additional inactivation means, such as toxicity from decomposition products, and microbial antagonism and competition. Though most pathogens do not survive the thermophilic conditions in biopiles, pile homogeneity is critical since they may persist as

clusters, both in space and over time, in non-thermophilic zones. These pathogenic bacteria that survive a composting process can leach into upper soil zones. Information on the fate of bacterial pathogens during secondary phases of biopiling (ie composting) is therefore needed.

## Methods

Biopiles were formed on soil layers in three cells that were constructed in Bible Hill, Nova Scotia. The cells were reproducible in design and function, and able to collect and quantify effluent from the biopiles. Technical facility design has been described (Michitsch 2009).

Solid media (i.e. soil, sawdust, SLRs) were sampled at the start of the experiment, and analyzed for five bacterial pathogen indicators (ie EB, TC, EC, SF, EC NAR). At the end of each experiment, biopiles were sampled at three vertical locations at each of three horizontal locations, for nine total biopile samples per cell. After the biopiles were removed, soil samples at 0-10 cm and 10-20 cm depths in each cell were obtained in a 3×2 grid from the soil surface. This grid corresponded with the locations for sampling the biopiles, for 12 total soil samples per cell. The solid media samples were processed to form ≥10:1 diluent:sample mixtures to prevent particle interference during bacterial enumeration (Andrews and Hamack 2005). Biopile effluent was sampled ≈3-4× per week in E1 and E2.

Enumeration methods were previously described (Michitsch 2009). In both experiments, TC and EC were enumerated using the HACH™ mColi-Blue (Hach Company 2000) and PetriFilm™ E. coli/Coliform Count Plate (3M Canada) methods. EC NAR enumeration was adapted from established methods (Jamieson *et al.* 2004). EB and SF were enumerated in final media samples in E1, and for the entirety of E2. The PetriFilm™ Enterobacteriaceae Count Plate method (3M Canada) was used for EB enumeration. SF was enumerated using USEPA (2005) Method 1600 and sparingly by APHA Method 9230 C (Eaton *et al.* 1995).

Enumeration data were normalized by a Log10 transformation and analyzed over time (repeated measures) and in space (cell or biopile location) using Proc GLM and Proc CORR (SAS 2008). For the effluent samples, enumerations were not analyzed in space due to conglomeration of liquid from each cell into single samples.

## Results

### *Daily bacteria loads*

Individual indicator loads (ie EC, SF) were significantly different between cells and over time ( $P \leq 0.01$ ;  $r^2 = 0.57-0.77$ ), and between the indicators themselves ( $P \leq 0.01$ ;  $r^2 = 0.77$ ). However, EB, TC, and EC loads ( $P \leq 0.01-0.05$ ) were positively correlated with one another in individual cells. Little EC NAR was detected, but when detected it was positively correlated with EB ( $P \leq 0.05$ ), and TC and EC ( $P \leq 0.01$ ). In contrast, SF was negatively correlated with EB and EC ( $P \leq 0.01$ ), and EC NAR ( $P \leq 0.05$ ) in some cells.

### *Cumulative loads and preferential flow*

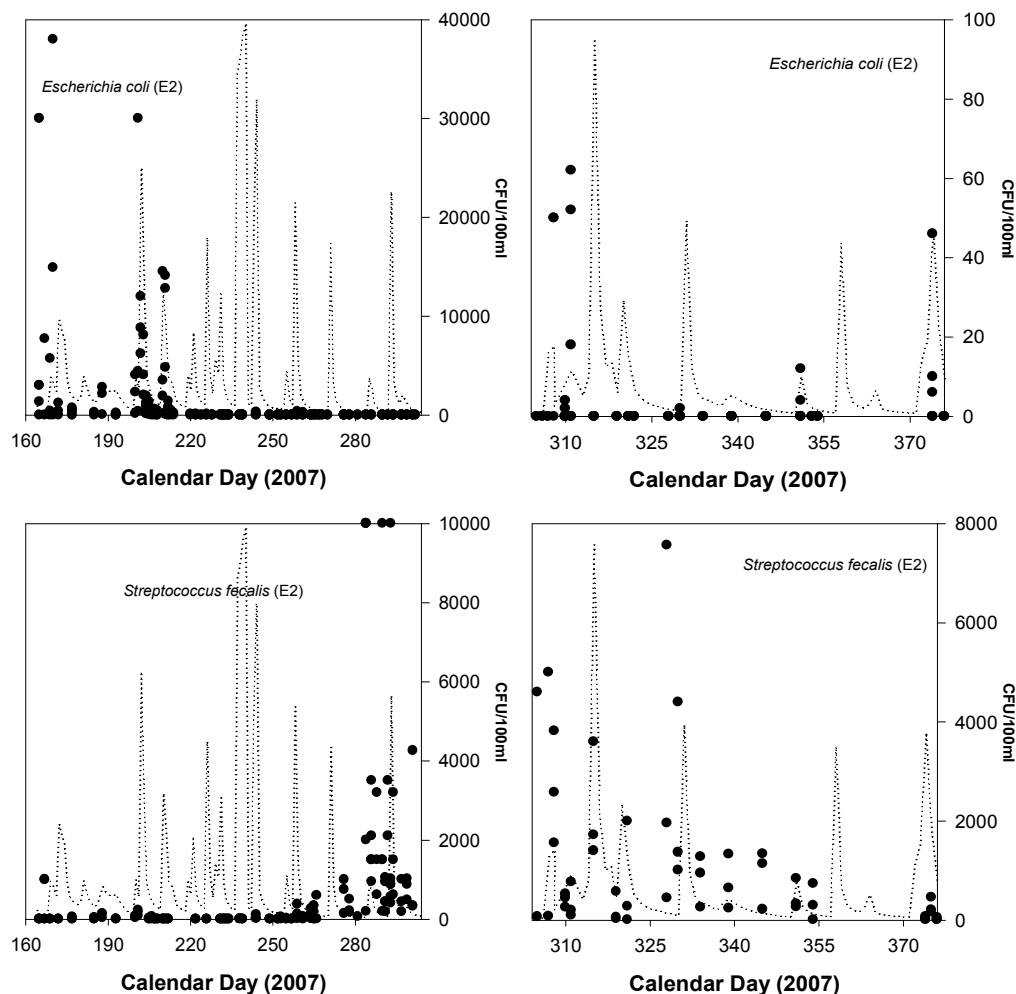
Cumulative EC and SF loads corresponded more closely with precipitation event occurrence than cell flow rate. For example, cumulative SF loads following CD 240 in E2 (Figure 1) increased on days when effluent increased following a precipitation event. However, the magnitude of these increased loads did not correspond in magnitude with the associated increase in effluent amount or precipitation event size, but rather to the occurrence of the precipitation event. This trend was supported using inoculated EC NAR as a tracer (data not shown), which suggested bacterial transport via preferential flow.

### *Liquid media*

Large reductions of EB, TC, and EC were observed in cell effluent as biopiling progressed (Figure 1). EC NAR inoculations on CDs 200 and 259 coincided with elevated EB, TC, and EC (CD 200 only) detections. Exclusion of these outliers yielded exponentially decreasing trends. An exponential increase of SF was also observed in cell effluent. During the decrease in EB, TC, and EC levels, <500 cfu/100 mL of SF was detected in cell effluent. However, an exponential increase of SF to a maximum of  $\approx 10^4$  cfu/100 mL was observed from  $\approx$ CD 275 onward once EB, TC, and EC population levels had stabilized.

Due to these trends, significant differences were found for EB, TC, EC, and SF counts over time, and when compared to one another ( $P \leq 0.05-0.01$ ;  $r^2 = 0.55-0.88$ ). At the  $P \leq 0.10$  level, TC and EC counts in E1 were correlated in all cells, as were EB, TC, and EC ( $P \leq 0.01$ ). For SF, however, the nine total comparisons

between SF and the three EB indicators (i.e. 3 contrasts by 3 cells) resulted in three negative, one positive, and five insignificant correlations at the  $P \leq 0.05$  level.



**Figure 1.** *Escherichia coli* and *Streptococcus fecalis* levels (cfu/100 mL) in biopile effluent. Primary (left hand side) and secondary biopiling phases are shown.

## Conclusion

Due to an established hierarchy, detection of EC elicits the detection of TC, as does TC for EB. As well, EC NAR mimics EC behaviour but is not present in the environment, thus it is used as a tracer of pathogenic bacteria that should similarly elicit EB, TC and EC detection. Since SF is not a member of the Family Enterobacteriaceae, its detection should not mimic the fate of EB or its members. These trends were observed in this research.

It was found that large (>15 mm) and intense (1-4 d) precipitation events generated nearly 90% of indicator bacteria loads. It was also found that annual indicator bacteria loads comprised <0.01% of their levels contributed by the input media (Michitsch 2009), thus, biopiling the SLRs inactivated or retained the microorganisms with much success. Members of the EB group and SF exhibited distinctly different transport behaviour, which was a novel finding that suggested FC:FS ratios should not be used to monitor for pathogen presence. Resurgent SF populations as EC levels declined suggested that re-growth and persistence may occur under certain conditions, such as high moisture content and lower temperature. However, SF levels reduced exponentially in biopile effluent during secondary-phase biopiling.

Though EC declined to 0 cfu/100 mL, EB (E2 only) and TC (E1 and E2) were still detected in effluent samples at the end of biopiling. This suggested that EB and TC persisted. Conversely, the exponential increase of SF in E2 suggested that it also persisted during adverse conditions until the biopile environment became hospitable for its growth. Statistical analyses confirmed these growth trends. As well, the inconsistent correlations for SF with the other indicators highlighted the different survival capabilities of these families of bacteria.

The presence of the indicator bacteria in some final biopile and soil samples confirmed their survival and persistence in spite of osmotic stress, temperature extremes, pH fluctuations, predation, etc. Some bacteria likely became dormant, entered a viable-but-not-culturable (VBNC) state, inhabited protective biofilms, or hid within particle cores. Though EB and TC are ubiquitous in nature, the presence of SF in final biopile samples supported this reasoning.

In summary, pathogenic bacteria may persist in different media for long times in viable or VBNC states, with the potential to re-grow. In this research, the SLRs and inoculated EC NAR represented large sources of indicator bacteria, whereas the soil was void of EC and SF. However, exponentially decreasing populations of EB, TC, and EC were observed in cell effluent. This highlighted the suitability of biopiling for pathogen inactivation. Indicator presence in final biopile and soil samples, however, suggested their persistence but not migration from the media. As well, SF was observed to exponentially re-grow once other indicator species declined and under less ideal environmental conditions. This highlighted the differences in survivability between the indicator bacteria. The behaviour of EC NAR supported inactivation as the primary process in the biopiles. However, the biopiles constituted continual sources of the indicator bacteria due to persistence in isolated locations and changes in dominant species. This suggested that tertiary biopiling phases and secondary methods should be performed to ensure pathogen degradation and that nutrient reserves have been depleted to prevent re-growth.

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