X

Survival of Bacterial Indicator-Species on Impervious Environmental Surfaces in Tuscaloosa, Alabama

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Because of historic difficulties in the measurement of sewage-borne pathogens, the microbiological quality of stormwater runoff is often characterized on the basis of bacterial indicator species. These species are assumed to derive from a common (sewage) source with pathogens of interest, and to arrive in, survive in, and move through watershed environments in numbers that correlate with health risk from those pathogens. Commonly used indicator species (especially *E.coli* and Enterococcus spp.), however, may derive from sources other than sewage, and survive in the (non-enteric) environment at rates divergent from those of the pathogens they are presumed to indicate (National Research Council, 2004).

In an ongoing effort to model background (i.e., of non-sewage origin) discharges of indicator species from stormwater source areas in the Tuscaloosa, AL area, a model for the environmentally relevant survival of indicator species (*E. coli* and Enterococci) on impervious environmental surfaces is presented.

X.1 Methods

X.1.1 Bacterial Cultivation and Enumeration

A full-factorial study (2^3, Temperature/Moisture/UV-B exposure) of the indicator-species' environmental survival factors was per-

formed for each taxon (Enterococci and *E. coli*). Pet-feces slurries (1 mL) were applied to salt-passivated paving blocks and incubated in controlled environmental chambers (freezerless refrigerators fitted with BOD-controllers/heaters for temperature control, desiccant or humidifiers for moisture control, and UV-B enhanced fluorescents with Lexan panels to split the chambers into UV-exposed and UV-shielded regions) at conditions encompassing those likely to be found in Tuscaloosa, AL. Active control of temperature (40 and 90 degrees F, Cool or Warm) held the parameter steady (+/- 2 degrees) over the study period. Relative humidity (25% and 80%, Dry or Wet) varied over about +/- 4%. UV exposure was treated as present/absent (UV or Dark).

Over an extended period (about two weeks), duplicate inoculated paving blocks were subjected to mechanical biofilm disruption by consistently applied and timed toothbrush abrasion, washing the slurry debris to sample bottles and diluted to 100 mL. The most probable numbers (MPN) of surviving *E. coli* and Enterococci colony forming units (CFU) per 100 mL were measured using IDEXX methods and normalized to inoculation-date (Day 0) MPN (also acquired from brush-off samples from blocks inoculated and brushed in the same way). IDEXX methods (Colilert, Enterolert, Quantitray 2000) provide for selective incubation of the taxons of interest, and colorimetric/fluorometric indicators of viable colonies within 24 hours. MPN measurement values with three orders of magnitude ranges (1 to 2,420 CFU/100 ml) are directly available with the Quantitray 2000 units. Additional dilutions of each sample were incubated to assure that all samples were quantified over even wider ranges.

X.1.2 Breakpoint Analysis

There is considerable reason to expect that the growth or decline (change in MPN over time) of bacterial populations is a first order (log-linear) relationship and of the form:

$$log(MPN/initial\ MPN)=k*t \qquad \text{(X.1)}$$

where:

k is the net growth constant (slope of the function), and

t = time (hours).

Changes in the slope of log(CFU) versus time are likely caused by a change in environmental conditions or a change in the makeup of the subject population.

Introduction of a viable bacterial inoculant to a new (habitable) medium (batch-style) typically results in up to four distinct phases of population behavior: Lag, Exponential-growth, Stationary, and Exponential-death (Madigan, et al, 2002, pp. 144-145):

• The Lag phase is characterized as a period of adaptation to the new environment, in which little or no population growth occurs, and its length is dependent on differences between the environmental history of the inoculant and the environmental conditions of the new medium. Inoculants transferred to environments similar to their historical conditions may exhibit little or no lag time; for transfers to a very different environment, lags may be considerable. Of course, if new conditions are so foreign to members of the inoculant population as to render it uninhabitable, individual cell death may occur until remnants of the inoculant population are viable (Madigan, et al, 2002, pp. 144-145).

• In the Growth phase, the adapted (or naturally selected) population grows exponentially; population at any given time is dependent on the number of actively dividing members of the population present at previous times. Rate of growth is dependent on environmental conditions and genetic (metabolic mechanisms available) make-up of the population (Madigan, et al, 2002, pp. 144-145).

• The Stationary phase (in which the population is static) represents conditions in which available nutrients (either from the original inoculant or from release by lysis of dying cells) is balanced by buildup of refractory (and often inhibitory – waste) products (Madigan, et al, 2002, pp. 144-145).

• The Death phase (dominated by waste buildup) is exponential.

Any or all of these phases may occur (or, of course, may be missed by insufficient time-density of sampling) and both environmental conditions and the genetic makeup of the population are relevant (Madigan, et al, 2002, pp. 144-145). The four main environmental factors influencing bacterial growth are temperature, pH, and the availability of water and oxygen (Madigan, et al, 2002, p.151). For impervious, terrestrial environmental surfaces, neither

pH nor oxygen is likely to be a factor. An important factor in cell *death*, however, is that of UVB exposure (Madigan, et al, 2002, pp. 272-273), which is bacteriocidal during cell division.

Because we cultivated our samples at constant conditions, a change in slope of log(CFU) versus time must be viewed as a population change, either through induction of new enzymes in individual cells, or through natural selection in the overall population.

Each combination ($2^3 = 8$ combinations of temperature, humidity, and UV exposure) of environmental conditions (treatments, combinations of environmental factors) was treated as a log-linear (first-order) segmented (with unknown break points) model of normalized MPN with respect to time, and with continuity between the segments imposed (as shown later in Figure X.3, for example). Statistical analysis of such models is not straightforward. Hudson (1966) provides a graphic algorithm (for minimization of overall sum of squares of error, SSE in the segmented model) and shows that it generally provides the maximum-likelihood estimate (MLE) of the abscissa of an unknown breakpoint (tBP); he provides no information as to how likely that estimate may be (rendering inferences impossible). Feder (1975a and 1975b) proves that, provided that the model is *identified* (i.e., includes no more hypothesized breakpoints than are present in the real population), and that no hypothesized tBP coincides with an abscissa of observation in the sample, minimization of SSE (the MLE function) converges asymptotically to the true population BP. In the unidentified case (i.e., too many BPs assumed), the MLE function becomes *indeterminate* ("estimates are not asymptotically normal"). Lerman's second condition arises because a discontinuity exists in the SSE function at each observation point, rendering it non-differentiable there, allowing for a possible true BP existing between the MLE tBP and an adjacent sample observation point (i.e., the MLE function becomes *unstationary*). For the unstationary case, he proves that, as the number of sample observations increases, minimization of SSE of a pseudocase (in which the observation point coinciding with the tBP is removed from the dataset), still converges (at a known rate) to the true BP. Lerman (1980) adapts Feder's work into a grid-search algorithm (again only for the identified case, and incorporating the pseudocase approach when necessary) in which proposed tBPs are mapped across the range of the observations and the SSE at each is determined. Progressive refinement (finer grain)

of the grid provides the tBP (minimization of the SSE versus proposed-tBP function) and also an estimate of the variance of that tBP estimate, corresponding to the range (which need not be continuous or symmetrical) of proposed-tBPs for which SSE is less than the minimum SSE plus its associated mean square of error (minSSE+MSE). Finally, Bai & Perron (1998) derive a log-likelihood ratio by which it can be determined whether addition of a new breakpoint to an identified model results in a new model which is also identified, and publish critical values for that ratio.

We found the grid-search method amenable to spreadsheet implementation. We first modeled each treatment by simple linear regression, resulting in a one-segment (R=1, no breakpoints) model. We then hypothesized a breakpoint, and searched for it by Lerman's grid method. If the resulting MLE did not coincide with an observation point, we accepted the tBP and associated uncertainty indicated by the search (e.g., see Figure X.1). We found grid-search of the (asymptotically converging) pseudocase, however, problematic for the limited number of data points we had for each treatment (typically about 35). In one case, analysis of the pseudocase resulted in the tBP jumping about 100 hours (and across multiple observation points, an impossible situation) because of the slower convergence of the smaller, highly variable dataset. In these cases we retained the grid-derived tBP and accepted the greater uncertainty inherent; we conducted a one-sided grid-search solution around the tBP to establish one side of the variance range and took the adjacent observation point as the other (e.g., see Figure X.2). Note that since we generated our grid search left-to-right (increasing t), the segment containing the discontinuity occurred between our tBP and the immediately preceding (adjacently left) observation. In both situations, the new model was tested against Bai and Perron's criteria for identification and, if it was identified, repeated the sequence. For the final model of each treatment, we numbered each tBP and intervening segments left-to-right (e.g. see Figure X.3).

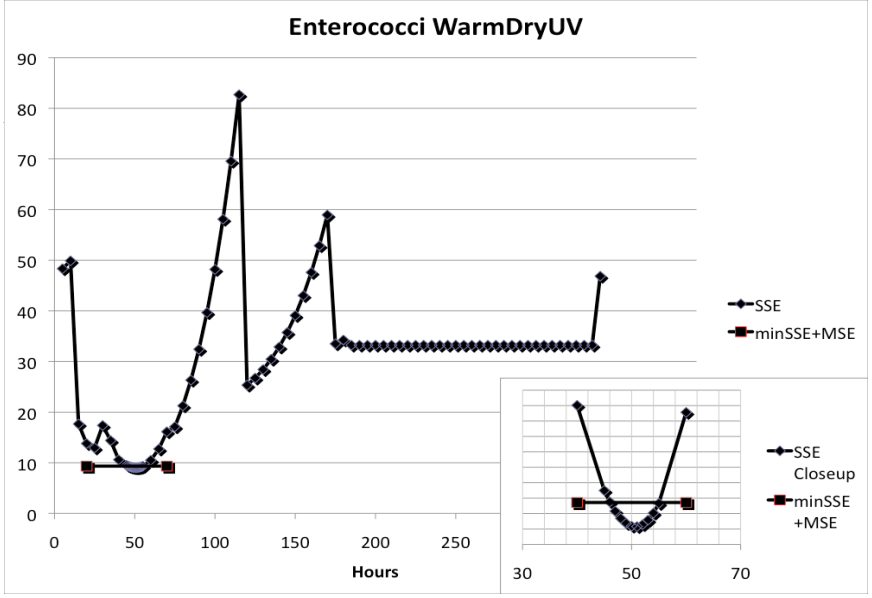

Figure X.1 Example graphic derivation of estimated tBP variance, normal case.

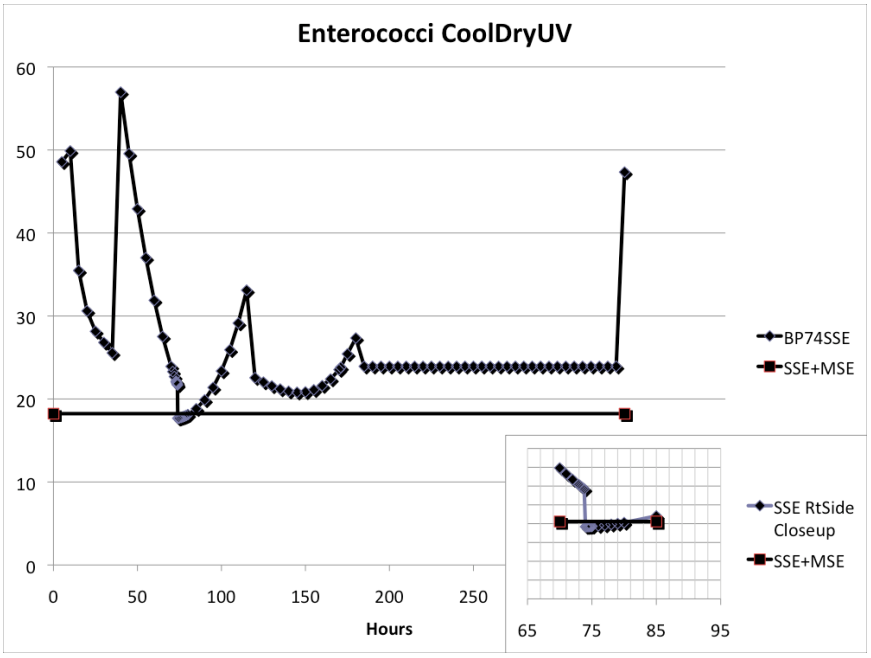

Figure X.2 Example right-sided graphic derivation of tBP variance, discontinuity on the left.

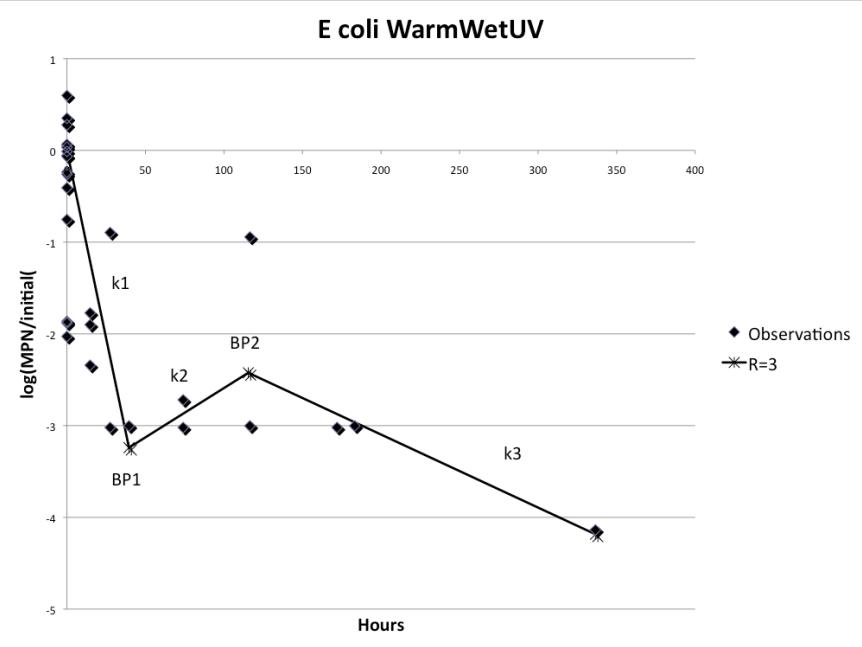

Figure 3 Example of a 3-segment (R=3) treatment model with segment and breakpoint designations

X.1.3 Environmental Factors

Each taxon (*E. coli* and Enterococci) was subjected to traditional (pooled variance) factorial analysis to rank the importance of the environmental factors (Temperature, Humidity, and UV exposure coded as 1=Shaded/0=Exposed, plus their interactions) to the abscissa of each breakpoint and to the slope (k) of each intervening segment. tBPs, their associated uncertainties, and the k of each segment were derived directly from the breakpoint analysis above. Variance of each k was determined from log(MPN/initial MPN)/t=k for each nonzero t in the segment.

This exercise was rendered problematic by the fact that different treatments (even within a taxon) differed in the number of tBPs revealed, from R=1 (one segment with no BP), to R=3 (three segments with two BPs). Orthogonality of the contrasts was achieved by generation of *artificial* BPs within segments without breakpoints but for which tBPs were revealed in corresponding

segments of other treatments. Transparency of the artificial points to the factorial analysis was achieved by assigning to them abscissae equal to the weighted average of revealed tBPs, and by assigning them zero variance. The k values of the new segments (on each side of the artificial BP) generated by this action were held to be equal, but the number of observation points (n) and the variance associated with those points were distributed (n-weighted) between the new segments.

Environmental-factor effects on tBP or k values were deemed important if their standard errors (SE) led to conclusions of at least 90% confidence (reasonable, considering the small sample sets) that the effects were not zero, although confidence in the importance of effects was much higher (and noted) in some cases. Conclusions that effects were not zero were reached when the calculated confidence interval (CI) was smaller than the calculated effect:

$$CI=SE*t(a) \quad \text{(X.2)}$$

where

t(a) is the Student's t-table return for the appropriate degrees of freedom and

(a) corresponds to the p-value resulting in the reported confidence level (i.e., alpha).

X.1.4 Model Construction

The important environmental effects (main effects and interactions) on k and tBP values, derived above, were used to model those parameters as a function of environmental factors:

$$\textit{Model Parameter} = \textit{Mean(Parameter)} + \textit{Sum of (Effects of Environmental Factors)} \quad \text{(X.3)}$$

where

Parameter is the treatment k or BP (artificial or not) entered into the tables of contrast for the factorial analyses,

Mean(Parameter) is the treatment-weighted mean for that *parameter*, and

Effects of Environmental Factors (EEF) are the adjustments to *Mean(Parameter)* attributable to each important environmental-factor. For 2-level factorial, effects are of the form:

EEF = [Product(EF-MEF)(1/2 environmental effect)]/Product(REF)*

where

EF is the value of that environmental factor for an observation point

MEF is the mean of that environmental factor amongst observation points

REF is the range (High value - Low value) of an environmental factor amongst observation points

X.2 Results and Discussion

X.2.1 *E. coli*

Results from breakpoint analysis of the *E. coli* dataset (Figure 4) are complex. One treatment (Warm/Wet/Dark) showed no significant tBP (not even a lag), and also exhibited the smallest initial decline. Two treatments (Warm/Dry/UV and Warm/Wet/UV) showed two tBPs each, with an initial decline, a rebound of growth, and a subsequent second decline. Cool treatments were nearly indistinguishable from each other, and resulted in more rapid declines than Warm/Shade treatments. All treatments exhibiting BPs showed slower declines later in the study period than in the initial die-off.

Warm conditions in general, and Warm/Wet/Dark in particular, most closely match the primary habitat (gut of warm-blooded animals) of our enteric bacteria, and would likely impose the least stringent adaptation requirements. The fact that only Warm/UV treatments elicit regrowth and three-phase behavior suggests an interaction. While UVB is not strictly ionizing radiation, it is of sufficiently high frequency to rearrange bonds in complex biomolecules. In particular, UVB causes dimerization of adjacent thymine units within the *E. coli* genome (and other bacteria, as well - repair mechanisms are species specific). UVB does not kill a quiescent cell; it interrupts the cell-division process by preventing replication of the genome. UVB only kills cells that are otherwise capable (by warm conditions) of, and are in the act of reproducing (Wulff & Rupert, 1962).

Figure X.4 – E. coli BP models

The factorial-analysis results (Table X.1) are likewise complex, especially in terms of the timing of the breakpoints. Such complexity should not be unexpected considering that even the number of breakpoints is treatment specific. The fact that only k1 shows any significant evidence of influence by environmental factors may imply adaptation (either at cellular or population level) for later segments.

Our model for *E. coli* survival is therefore:

*log(MPN/initial MPN) = k1*t for times <= tBP1* (X.4)

where

k1 = -0.108 + (H - 52.5)*0.000551
+ (T-65)*(H-52.5)*0.0000203

T is degrees F

H is %relative humidity, and

*tBP1(hours) = 21.6 + (T-65)*0.0209 + (H-52.5)*0.0293*
*- (S-0.5)*1.95* + (T-65)*(H-52.5)*0.00229
- (T-65)*(S-0.5)*0.0503
- (H-52.5)*(S-0.5)*0.0560
- (T-65)*(H-52.5)*(S-0.5)*0.000506

where

S is a Shade Code (1 = Shade, 0 = Exposed)

for times > tBP1 and <= tBP2,

*log(MPN/initial MPN) = k1*tBP1 + 0.002214*(t-tBP1*)

where

tBP2 = 80.71 - (T-65)*0.0924 + (H-52.5)*0.163
+ (T-65)*(H-52.5)*0.00326 - (H-52.5)*(S-0.5)*0.163
- (T-65)*(H-52.5)*(S-0.5)*0.00326

and for times >tBP2

*log(MPN/initial MPN) = k1*tBP1 + 0.00221*(tBP2)*
- (0.00501)(t-tBP2*)

Table X.1 Important (alpha, (a), <=0.1.

E. coli	k1				
Main Effects	Effects	SE(Effect)	t(a)	CI(effect	df=17
Humidity	0.061	0.00055	2.6	0.060	(a)=0.01
Interactions					
TempHumid	0.11	0.024	2.9	0.070	(a)=0.01
E. coli	BP1				
Main Effects	Effects	SE(Effect)	t(a)	CI(effect	df=224
Temperature	2.1	0.33	2.6	0.87	(a)=.005
Humidity	3.2	0.33	2.6	0.87	(a)=.005
ShadeCode	-3.9	0.33	2.6	0.87	(a)=.005
Interactions					
TempHumid	12.6	0.33	2.6	0.87	(a)=.005
TempShade	-5.0	0.33	2.6	0.87	(a)=.005
HumidShade	-6.2	0.33	2.6	0.87	(a)=.005
TmpHumShd	-2.8	0.33	2.6	0.87	(a)=.005
E. coli	BP2				
Main Effects	Effects	SE(Effect)	t(a)	CI(effect	df=37
Temperature	-9.2	1.05	2.8	2.9	(a)=.005
Humidity	17.9	0.80	2.8	2.2	(a)=.005
Interactions					
TempHumid	17.9	0.80	2.8	2.25	(a)=.005
HumidShade	17.9	0.80	2.8	2.2	(a)=.005
TmpHumShd	17.9	0.80	2.8	2.2	(a)=.005

The model presented does not fully account for variability in the observations (R^2 is only 0.42, and see Figure X.5) of the full *E. coli* dataset. It does, however, offer improved correlations with, and better balance between, under-predictions and over-predictions than would be provided by a simple linear regression of the same dataset (compare Figures X.6 and X.7). Residuals of the model are reasonably well behaved, providing some comfort in the pooled-variance methods used here (Figure X.8).

The model-derived parameters applied to our experimental conditions are presented in Table X.2. All treatments exhibit an initial lag/die off, the rate of which depends on the temperature and humidity. Notably, the Warm/Wet conditions (those most like the enteric habitat, and exerting the least pressure for adaptation) show the lowest initial rate (k1) of decline, but all inoculants had declined 2-3 orders of magnitude within a day or so. The duration of the decline appears quite variable (19 to 27 hours), but should be interpreted with caution. Recall that the BP analysis resulted in several tBPs that coincided with the first (earliest) observation point. Though the values listed in the table represent the best estimates for predictive purposes, they must be viewed mechanistically as the latest likely time for the change. The true BP1 may have occurred before the first observation. The insensitivity of k2 and k3 to environmental factors imply that all adaptive mechanisms available to the inoculant population had been implemented prior to the (and caused the) first breakpoint. The two phase behavior subsequent to BP1 could be attributed to waste buildup in these batch systems or to accumulation of UV-generated Thymine dimers (and review of the warm-treatment behaviors in the original BP analysis, Figure X.4, suggests that both factors are involved).

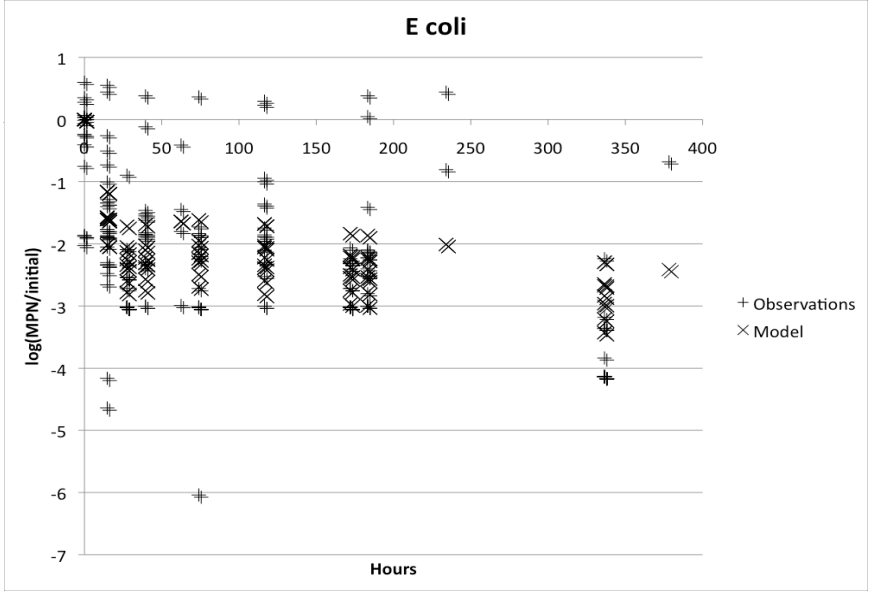

Figure X.5 Overlay of model predictions on observations, all treatments combined

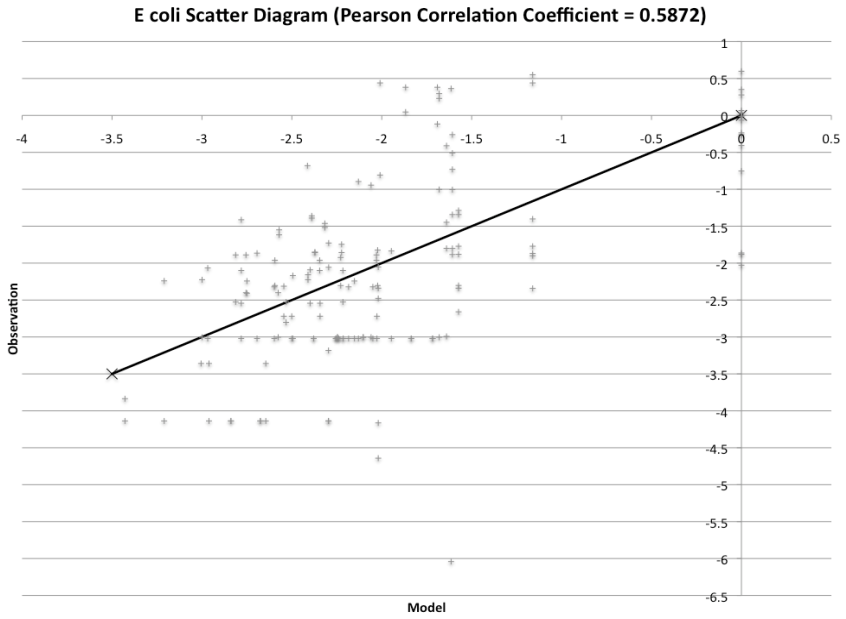

Figure X.6 Observations vs. Model. Line is Observation = Model Prediction

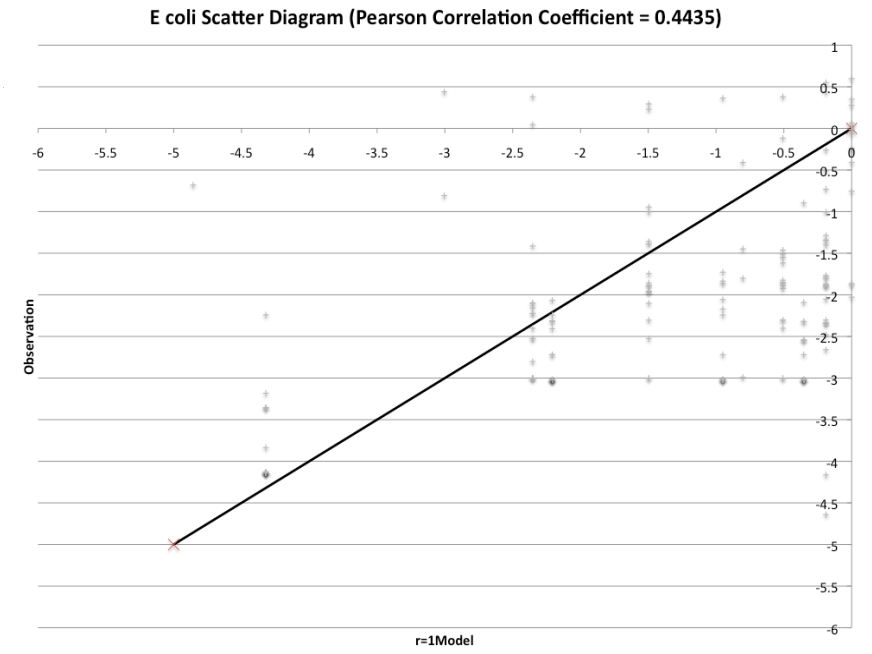

Figure X.7 Observations vs. predictions of linear regression without environmental factorial

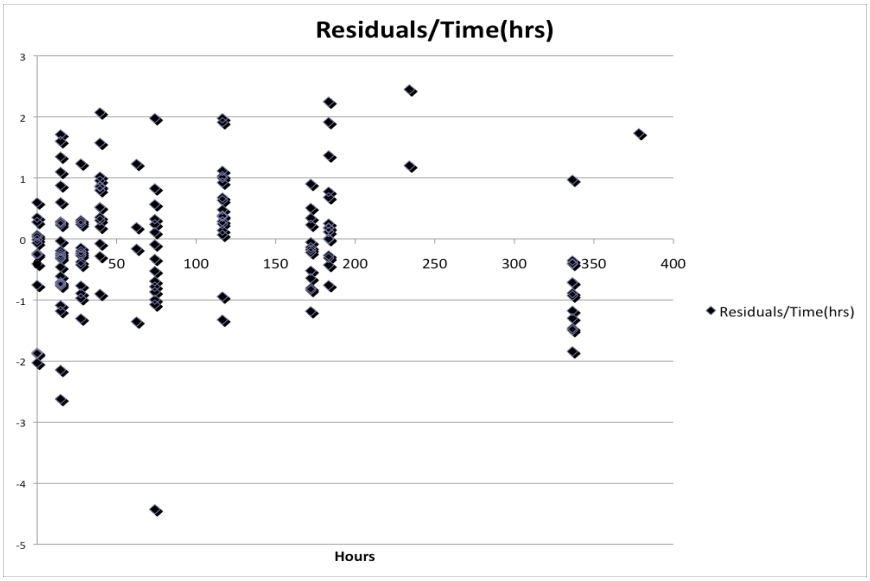

Figure X.8 Residuals vs. time for the presented *E coli* model

Table X.2 E coli modeled parameters, applied to experimental conditions

	k1 (1/hours)	BP1 (hours)	k2 (1/hours)	BP2 (hours)	k3 (1/hours)
CoolDryUV	-0.109	21.6	0.00221	76.8	-0.00501
CoolDryDark	-0.109	22.1	0.00221	79.0	-0.00501
CoolWetUV	-0.107	21.3	0.00221	83.5	-0.00501
CoolWetDark	-0.107	19.4	0.00221	81.2	-0.00501
WarmDryUV	-0.137	20.4	0.00221	71.0	-0.00501
WarmDryDark	-0.137	19.1	0.00221	77.8	-0.00501
WarmWetUV	-0.0787	27.1	0.00221	91.2	-0.00501
WarmWetDark	-0.0787	22.0	0.00221	84.5	-0.00501

X.2.2 Enterococci

Treatment analyses of the breakpoints is less complex for Enterococci than for *E. coli* (see Figure X.9), although some disparity as to number and tBP values per treatment appears here as well. The Warm/Wet/Dark treatment shows no evidence of a breakpoint (even a lag). It also displays a slope statistically indistinguishable from zero. The clear trend of greater net survival in Warm treatments seen in the *E. coli* analysis is not evident here, and the timing of breakpoints in treatments (where they occur) is less varied than occurred for *E. coli*. Our assay in this case is sensitive to metabolic signals for an entire genus rather than a single species. One is tempted to argue that the greater genetic diversity of the larger taxon provides an overall greater range of adaptive capacity (natural selection favoring different species/strains at different conditions) and greater likelihood of genes for UVB-repair mechanisms within the initial inoculant. Remarkably, when regrowth phases are recognized, none of the treatments show a net decline of more than about one order of magnitude over a two-week period. It also should be noted that no population is in decline at the end of the study period.

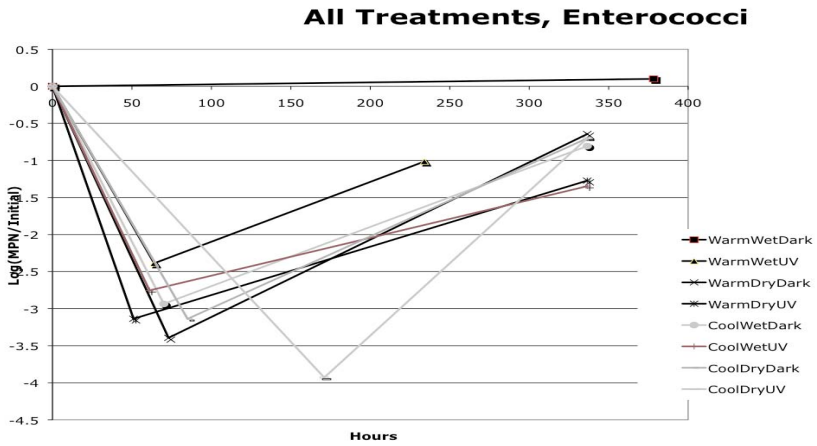

Figure X.9 Enterococci BP models

Factorial analyses (Table X.3) for Enterococci were also simpler than for *E. coli*, but again showing greater complexity for tBP values than for intervening segments. As for the *E. coli* analyses, k values become insensitive to environmental factors subsequent to the tBP, implying capacity for adaptation to the secondary (non-enteric) habitat.

Table X.3 Important factors per Enterocci factorial analysis

Enterococci	k1				
Main Effects	Effects	SE (Effect)	t(a)	CI(effect)	df=56
Humidity	0.015	0.011	1.3	0.014	(a)=0.1
ShadeCode	0.015	0.11	0.010		(a)=0.1
Interactions					
TempHumid	0.020	0.011	1.7	0.019	(a)=0.05
TempShade	-0.077	0.011	2.7	0.030	(a)=0.005
Enterococci	BP				
Main Effects	Effects	SE (Effect)	t(a)	CI(effect)	df=233

Temperature	-8.8	0.31	2.7	0.84	(a)=0.005
Humidity	-5.3	0.32	2.7	0.84	(a)=0.005
ShadeCode	11.2	0.31	2.7	-0.84	(a)=0.005
Interactions					
TempHumid	8.7	0.31	2.7	0.84	(a)=0.005
TempShade	1.2	0.32	2.7	0.85	(a)=0.005
HumidShade	-5.3	0.32	2.7	0.84	(a)=0.005
TmpHumShd	-4.3	0.32	2.7	0.85	(a)=0.005

Our model for Enterococci survival is therefore:

$$log(MPN/initial\ MPN) = k1*t \text{ for } t<=tBP \qquad (X.5)$$

where

$$k1 = -0.0356 + (H-52.5)*0.000137 + (S-0.5)*0.00727 + (T-65)*(H-52.5)*0.00000372 - (T-65)*(S-0.5)*0.00771$$

and

$$tBP = 68.74 - (T-65)*0.881 - (H-52.5)*0.0483 + (S-0.5)*5.59 + ((T-65)*(H-52.5)*0.00158 + (T-65)*(S-0.5)*0.0119 - (H-52.5)*(S-0.5)*0.0483 - (T-65)*(H-52.5)*(S-0.5)*0.000784$$

and for t>tBP,

$$log(MPN/initial\ MPN) = k1*tBP + 0.00652*(t-tBP)$$

Comparison of the model with observations (Figure X.10) makes it apparent that there are other sources of variability than the environmental factors analyzed here (and R^2 is only 0.59). However, the model again provides closer (and more balanced) agreement with the data than does a simple regression (Figures X.11 and X.12). Residual behavior provides no reasons for concerns as to methods (Figure X.13).

The model-derived parameters applied to our experimental conditions are presented at Table X.4. All treatments, again, exhibit an initial decline, with all three environmental factors (Temperature, Humidity, and UV Exposure) contributing (either as main effects or within interactions). The rates of decline, however, are only about half of those suffered by *E. coli*. None of the BPs for these

populations coincided with initial observations, and the adaptation phase of these inoculants lasted about three days. Even with the slower rates of decline, most inoculants had been reduced 2-3 orders of magnitude in the initial period. The insensitivity of k2 to environmental effects, and the fact that it is *positive* (indicating net growth) implies that these organisms adapt to impervious environmental surfaces quite well. By the end of the study period (about two weeks) all inoculants had rebounded to about 10% of their original populations.

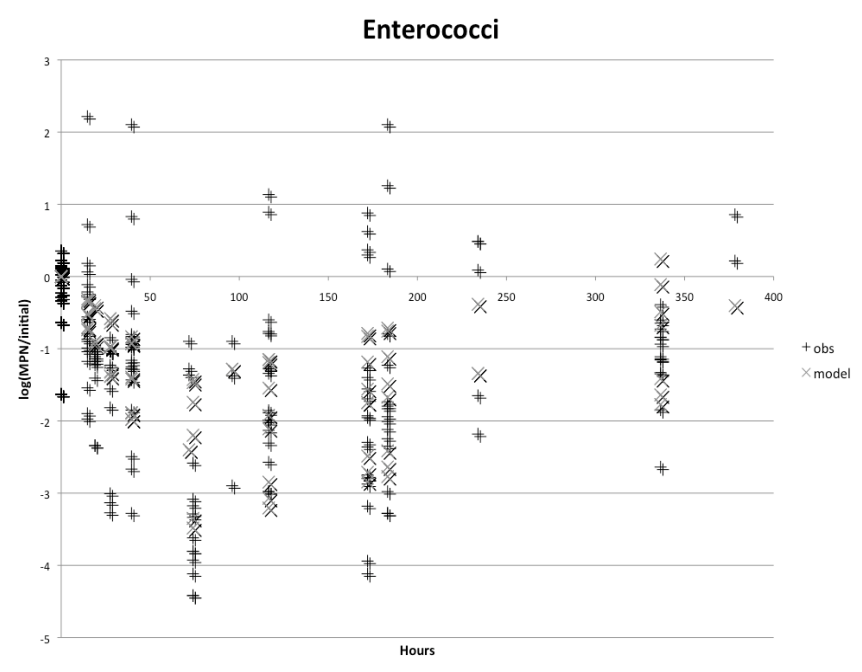

Figure X.10 Enterococci, Observations vs Model comparison.

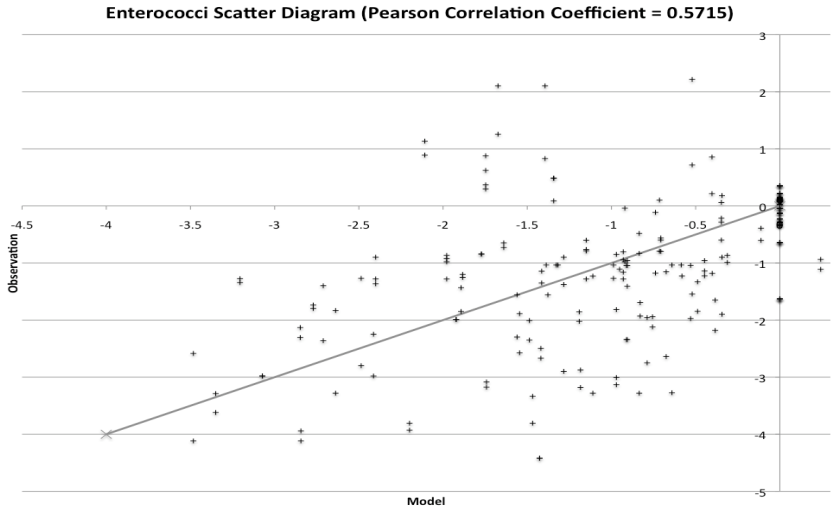

Figure X.11 Model Predictions vs Observations. Line shows Observation=Prediction

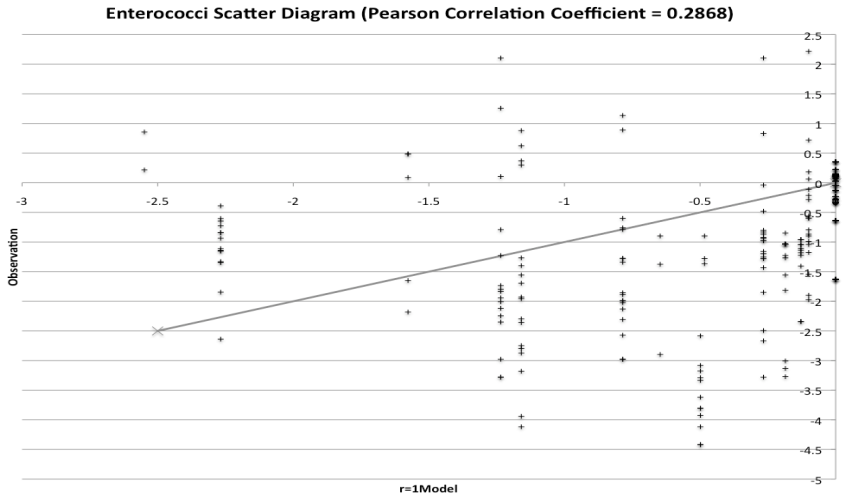

Figure X.12 Paired Observations vs predictions from a simple linear regression. Line displays Observation=Prediction

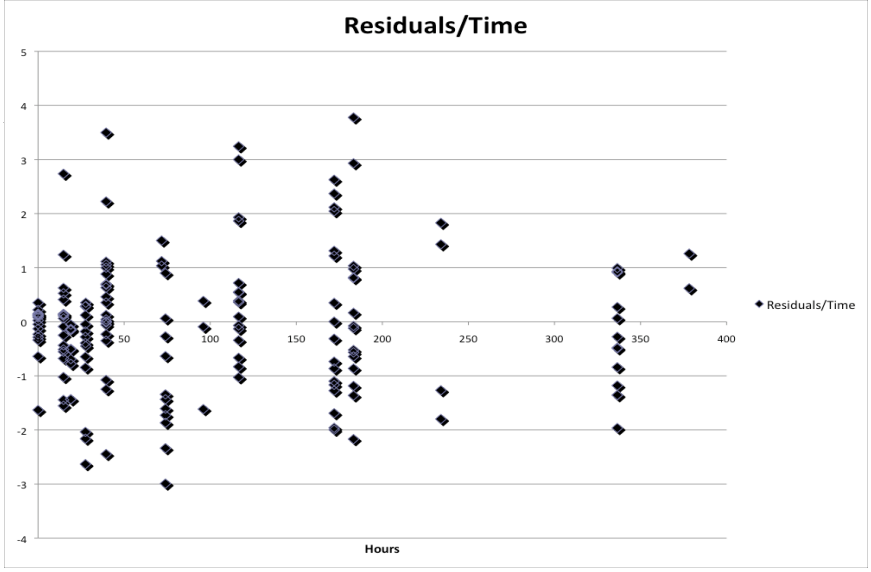

Figure X.13 Residuals, over time, of the proposed model

Table X.4 Enterococci modeled parameters, applied to experimental conditions

	k1 (1/hours)	BP (hours)	k2 (1/hours)
CoolDryUV	-0.0501	70.0	0.00652
CoolDryDark	-0.0235	76.7	0.00652
CoolWetUV	-0.0477	66.5	0.00652
CoolWetDark	-0.0211	70.5	0.00652
WarmDryUV	-0.0359	63.2	0.00652
WarmDryDark	-0.0479	70.4	0.00652
WarmWetUV	-0.0233	64.0	0.00652
WarmWetDark	-0.0353	68.6	0.00652

X.3 Conclusions

We developed the models presented here in support of an ongoing effort to model source-area processes contributing to background (i.e., of non-sewage origin) presence of fecal indicators in stormwater. Together with a planned similar study of survival on pervious surfaces (soils), they should contribute to a mass-balance link between fecal deposition on the landscape and biological stormwater quality.

Others, however, may find the work of interest. The studied indicator organisms (especially Enterococci) were found to be quite

persistent (especially under environmental conditions that most closely approximate enteric conditions) on impervious surfaces under the extreme Tuscaloosa, AL, environmental conditions. Moreover, under most conditions studied, the rate of disappearance of these organisms from the landscape slowed (or even reversed), rendering short-term studies of their survival (or even simple regression of long-term studies) unreliable in predicting their environmental fate.

We hope that risk analysis of stormwater exposures, and efficient search for sources of indicators species in runoff will be better informed by this work.

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