

SOURCE AREA PROCESSES CONTRIBUTING TO
MICROBIOLOGICAL QUALITY
OF STORMWATER

by

BRAD WILSON

MARK ELLIOTT, COMMITTEE CHAIR
ROBERT E PITT, COMMITTEE CO-CHAIR
JOE BROWN
ANDREW G. GRAETTINGER
DEREK J. WILLIAMSON

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ABSTRACT

A literature review reveals a need by water managers for a model by which the regulatory impacts likely to result from stormwater runoff of fecal indicator bacteria previously defecated onto the landscape, under conditions extant within their jurisdiction, might be predicted. Though the literature provides little information as to what the needed model might look like, it also contains much in the way of relevant general sciences that, by means of logical analysis, provides a hypothetical framework by which the significance of likely parameters of such a model might be piecewise tested. I present exploratory research into the feasibility of constructing such a model. The research presented consists of a series of scoping studies, in article style, designed to separately probe the relevance, important parameters, and significance of separable putative sequential processes by which such a model might eventually be constructed, and provide guidance informing future research.

DEDICATION

This work is dedicated to the memory of Adeline and Lightnin'. In their Golden Years, they (not particularly willingly) gave up considerable, well-deserved dignity to a poop-collecting stalker.

LIST OF ABBREVIATIONS AND SYMBOLS

AGI	Acute Gastrointestinal Illness
a_w	Water Activity
BA	barely acceptable (beach classification)
BCC	Bundle of Circular Capillaries model
BMP	Best Management Practices
BOD	Biological Oxygen Demand
BWD	Bathing Water Directive
CDC	US Center for Disease Control
CFU	Colony Forming Unit
CI	Confidence Interval
CIS	Common Implementation Strategy
CSO	Combined Sewer Overflow
DNA	Deoxyribonucleic Acid
DTM	Digital Terrain Model
EC	European Community
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EPA	US Environmental Protection Agency
EU	the European Union
FC	Fecal coliforms
FE	Final Effluent

FIB	Fecal indicator bacteria
FS	Fecal Streptococci
GI	Gastrointestinal Illness
GIS	Geographic Information System
GMP	guanine monophosphate
HCGI	Highly Credible Gastrointestinal Illness
IPPC	Integrated Pollution Prevention Control Directive
LA	Load Allocation
MPN	Most Probable Number
MS4	Municipal Separate Stormwater System
MST	Microbial Source Tracking
NEEAR	National Epidemiological and Environmental Assessment of Recreational Waters
NGI	“NEEAR” Gastrointestinal Illness
NPDES	National Pollution Elimination System
NRC	National Research Council
NTAC	National Technical Advisory Committee on Water Quality
OR	Odds Ratio
PAR	Photosynthetically Active Radiation
PCR	Polymerase Chain Reaction
p.e.	population equivalents
RH%	% Relative Humidity
RNA	Ribonucleic Acid
RR	Relative Risk

RSA	reference system/antidegradation
RU	relatively unpolluted (beach category)
SAV	submerged aquatic vegetation
<i>sic</i>	“Thus,” as written
SRD	Significant Respiratory Disease
SSD	the Sewage Sludge Directive
STEC	Shiga-toxin producing <i>Escherichia coli</i>
STO	Surge Tank Overflow
STV	Statistical Threshold Value
TC	Total coliforms
TMDL	Total Maximum Daily Load
TND	the Nitrates Directive
UK	the United Kingdom
US	the United States of America
UWWT	Urban Wastewater Directive
<i>vs.</i>	<i>versus</i> , as compared to
WFD	the Water Framework Directive
WHO	the World Health Organization
WLA	Waste Load Allocation
WP	Water Potential
WQC	Water Quality Criteria
WQS	Water Quality Standards
WWTP	Wastewater Treatment Plant

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CHAPTER 1

INTRODUCTION

“Water is Life” – I can’t find the origin of the phrase, but it’s oft used and undeniably true. Water is the indispensable solvent in which reactions that maintain life take place. Water, however, is not just our life. Convincing evidence of a waterborne pathogen, a biological entity inhabiting water and capable of inducing disease, predates Koch’s formulation of the Germ Theory of Disease over a century ago.

Confirmation that *Vibrio cholerae*, a bacterium shed in the feces of cholera victims, could induce cholera in healthy consumers of water contaminated by those feces, gave rise to efforts to preclude introduction of waterborne pathogens to waters people use. From the onset of regulatory regimes governing those efforts, the compliance endpoints of regulation have been defined in terms of fecal indicator bacteria (FIB).

Early characterized waterborne pathogens were, like the cholera vector, bacteria shed to sewage with the potential to infect those who consume or contact water contaminated by that sewage. Rapid expansion in the number of such characterized vectors quickly revealed a problem. Analysis of all known potential sewage-borne pathogenic bacteria, of differing species, morphologies, metabolic capabilities and, thus, of differing required analytical methods to regulatorily ensure their preclusion from waters of human use was (and still is) infeasible. The air-tight logic of the Indicator Paradigm provides potential relief. If an indicator derives from the

same source as the pathogens of interest, and is just as or more likely than the pathogens to reach some area worthy of protection from pathogens, then absence of the indicator entering the protected area accurately or conservatively implies that no pathogens from the same source are entering either. Use of any indicator would, of course, only be justified if analysis of the indicator were easier than analysis of the pathogens.

Alas, no indicator strictly adhering to the Indicator Paradigm has been found for accurately or conservatively indicating the risk of waterborne pathogens entering waters of human use. The literature reviewed here reveals that when known waterborne pathogens were bacteria derived from sewage, and sewage was known to be a potential input to waters of human use, measurement of FIB (even if not sewage-source specific) provided useful endpoints for management of risk from known sewage-borne bacterial pathogens. Subsequent discoveries of non-bacterial waterborne human-fecal pathogens (e.g., viruses and protists, of wildly divergent post-defecation behavior vs. FIB) complicated matters but FIB from sewage still provided useful information concerning the risk to health presented by pathogens from sewage. As bioanalytical technologies improved, and measurement of FIB with improved source specificity for sewage became feasible, management of the risks posed by pathogens from sewage improved as well.

Even the FIB in current use today, however, derive from many sources other than sewage. Considerable literature exists showing a lack of correlation between FIB and known pathogens, even when both are found to be present. FIB presence does not consistently and quantitatively indicate the presence of pathogens. Moreover, findings of adaptation to and persistence of FIB in non-enteric environments show that fecal indicator bacteria do not consistently and quantitatively indicate feces. This situation logically gives rise to possible misclassification of risk. Data derived at one time or place may not be of relevance to risk

management at any other time or place. Historically, as sewage treatment and sewerage maintenance are improved, the significance of relationships between FIB and human health is diminished.

Discoveries of waterborne zoonotics, waterborne pathogens of non-sewage origins, further complicate risk management. Such discoveries actually argue against the value of source specificity in FIB, but also further diminish confidence in the value of FIB to indicate risk. Knowledge that threats to human health may originate from the feces of other animals requires that risk of those threats must be managed. Much money and effort has been expended to quantify the human-health risks represented by non-human feces and the relationship of those risks to FIB. While considerable consensus exists that animal feces represent a lesser risk to humans than do human feces, conclusive findings to that effect have not been forthcoming and, indeed, may well be impossible to find with current methods. Regulators conservatively and necessarily assume an equation of risk represented by FIB of any source.

This situation presents a quandary for water managers responsible for compliance to water-quality regulations and, again, it is the lack of source specificity of currently used FIB that creates the problem. Knowledge of the source of regulated FIB is necessary for managing them, but currently monitored FIB provide no clue as to where they come from. Much research has been devoted to FIB source-tracking methods by which managers might manage. Many studies have found the profound importance of rainfall in mobilizing non-sewage FIB to regulated waters. The literature also reveals considerable geographical variation among study sites in their FIB response to rains. What seems needed by water managers is knowledge of the processes by which FIB defecated to the landscape under their purview reach regulated receiving waters. This need would seem most important to urban water managers whose heterogeneous jurisdictions

exhibit close intermingling of potential sewage sources (leaks, overflows, etc.) and non-sewage ones (birds, pets). A model by which observed defecation patterns could be linked to the patterns of FIB release to stormwater would allow managers prioritize remediation efforts under local conditions. Such a model would logically need to encompass FIB survival in source areas between rains as well as the release of FIB to stormwater during rains. I find no evidence in the literature that such a model exists.

The literature offers little of any direct relevance to the form such a model would even take, and which parameters should be included. However considerable literature, across biological, engineering, and soil-science fields, is found that, by logical analysis, can provide a hypothetical framework by which the significance of likely important parameters might be tested.

I explored the feasibility of constructing such a model by testing for significance of logical hypotheses derived from this diverse literature. The complexity of this exploratory research, and the many source-area processes likely to be relevant to such model construction, suggested that such processes, where logically separable, should be explored separately, in a series of less refractory scoping studies. The logical chain by which FIB deposited onto sources areas survive until a rain event, are released to stormwater by a subsequent rain, and then travel down-gradient in runoff to reach regulated waters further suggest a sequential order by which the scoping studies might be defined and related to one another. I further conducted at least preliminary modeling efforts based on significant factors found in the studies to assess their adequacies and to identify needs for further research.

These considerations further suggest an appropriate structure for this dissertation as “Article Style.” This Introduction chapter and the two succeeding ones, together, compose the

“introductory material” required in this structure. Chapter 2 presents the review of literature performed here, and a statement of the need for research as suggested by the literature. Chapter 3 provides research design and experimental plan for this exploratory research, the structure of and relationships between the scoping studies.

The scoping studies themselves are presented, either as published articles or as (not yet published) article-style manuscripts written in the same style, in five sections of Chapter 4. These sections have been formatted to meet the requirements for this dissertation, with permission of the copyright holder or of the authors as appropriate. Interspersed within Chapter 4 are also sections not written to article style, and present information outside of the articles’ scope, but provide for connections and comparisons between them and perspectives concerning their importance to the overall research effort.

CHAPTER 2

LITERATURE REVIEW

2.1 Regulatory Regime

Justification for this research is enhanced by review of the historic reasons for use of fecal indicator bacteria in current regulation of environmental waters.

2.1.1. Waterborne Pathogens

While “*For its an ill Bird that will befoule her own Nest*” [*sic*] is literarily attributed to George Alsop in 1666 (Alsop, 1902), the sentiment expressed is surely much older. Intimate association with fecal material is generally regarded as distasteful.

By a general consensus, however, a rational connection between association with feces and well-being awaited the work of John Snow. As an apprentice, Dr. Snow had witnessed an early (1831) outbreak of cholera (a new disease to Europe) among miners in north Britain. As a physician, he was able to observe and investigate a new outbreak (1848-1849) in London. By spatial analysis of the geographical proximities of water sources and sewage outfalls within the city, together with access to medical records indicating the timing of the arrival of (and location of the new residence of) potential carriers previously infected elsewhere, he was able to conclude that the disease was transmitted by a waterborne “contagion.” Dr. Snow argued (by analysis of both outbreaks) that the spread of cholera could not be the result of a “miasma” (airborne exudations of death and decay, a competing contemporary theory of disease causation) because

of the differential mortality rates among closely located neighborhoods. He further argued against disease causation by “humoral” effects (imbalances in bodily fluids caused by sensory assaults) with examples of cities that had sought distant water sources, because the local ones reeked, and yet had low mortality. Dr. Snow’s analysis pointed to a contagion (a material that passes from the afflicted to others and carries the affliction with it), the transmission of which involved ingestion of human fecal material. He did note that his hypothesized contagion must be unlike most other poisons in its capability of “multiplying in the body,” confounding traditional dose/response models (Snow, 1849, and Snow 1849a). Two microscopists, Hassal and Pacini, observed and described *Vibrio cholerae*, the causative agent of cholera, in 1854. Neither identified the bacterium as a contagion at the time, and the former described its presence as a host response to infection. (Brody, *et al.*, 1999)

Robert Koch, working with other diseases (notably anthrax) devised an algorithm by which bacteria could be established as contagions. “Koch’s postulates,” have been published in several versions, but essentially if (1) a bacterium is always found in a diseased host, and if (2) that bacterium is never found in a healthy host, and if (3) that bacterium, extracted from a diseased host and maintained in pure culture, can induce the same disease when introduced into a healthy host, then that bacterium is logically the cause of the disease. Koch admitted to unanswered questions (especially concerning host “immunity” deriving from previous bacterial exposures as evidenced in Pasteur’s recent findings involving vaccination), but believed that anthrax, tuberculosis, erysipis, and tetanus had already been proven to be of bacterial contagion. He further believed that typhoid fever, diphtheria, leprosy, relapsing fever, and cholera would also eventually be conclusively attributed to bacterial infection (Koch, 1890).

Publication of Koch's postulates, and of their application to specific pathogens essentially established the "Germ Theory" of disease, and led to a steadily increasing number of bacteria recognized as health threats when present in drinking water. By the 1906 printing of *Public Water Supplies* by Turneaure and Russell, the causal relation between sewage-derived pathogens and human maladies was deemed beyond question. The early focus of US response was on separation of sewage components from drinking water. This led to wide-scale reliance on (sand) filtration of drinking water. By 1904, 10% of the US population was served by filtered water systems; the number had grown to 30% by 1914 (Craun, 1988).

Though the first major city to be fitted with a chlorine-disinfected water supply, Jersey City (and without filtration), was not so furnished until 1908, the practice spread rapidly after the introduction of liquid chlorine in 1909. By 1941, 4590 treatment plants (of 5372) nationwide included chlorination units (and most were fitted with filtration as well, McGuire 2006).

This period coincided with a large expansion of urban-sewage diversion to surface waters. The early 19th century had seen a major increase in construction of supply waterworks without installation of sanitary sewers. Many cities were served by residential cesspools, often with overflow pipes to the storm sewers. This situation was exacerbated by the 1833 introduction of the flush toilet in the US, which was eagerly adopted by those with supply waterworks in place (Tarr, *et al.*, 1984).

Shortly after general acceptance of the Germ Theory (as early as 1892, an infectious contagion, later to be identified as tobacco mosaic virus, was found in cell-free extracts, Madigan, *et al.*, 2002, p. 530), evidence began to accumulate of pathogens that were effectively invisible. For example, poliomyelitis is obviously a contagious disease. In a situation somewhat similar to that of cholera in Dr. Snow's time, the method of transmission could be traced to a

fecal-ingestion mechanism, without knowledge of the nature of contagion. Unlike that of cholera, however, the polio vector is a virus, much too small to be seen with even the best optical microscope. The identification of viruses awaited development and widespread availability of electron microscopes. Nonetheless, in the 1940s, polioviruses were found to be shed by infected patients in their stool, and hepatitis viruses were shown to be waterborne pathogens. By 2002, (Madigan, *et al.*, pp. 864-870), 16 viral diseases were considered reportable by CDC, and 19 virions were considered emergent. Unlike bacteria, viruses are obligate parasites. They are only active while inhabiting a living cell, and are otherwise inert. Though many viruses may be inactivated by chlorination, they often persist longer, especially in the inert phase, than intestinal bacteria do in the environment (NRC, 2004, pp. 36-37).

Finally, attention of late has been turned to zoonotic pathogens, those that can infect humans through water ingestion, but can also inhabit and be shed by non-human hosts. Note that several of the pathogens highlighted by Koch in his 1890 address were animal pathogens. Most are transmitted by feces-to-mouth mechanisms, but the feces are not necessarily from human sewage. Manure from wildlife or livestock may harbor organisms pathogenic to humans. One such bacterium gaining some notoriety, of late, is the O157:H7 strain of *Escherechia coli* (*E. coli*), which has infected humans through vegetables washed in water that has previously been contaminated by cattle manure. Other important organisms within this category are the shelled protozoans *Giardia* and *Cryptosporidia*, microscopic forms that are larger and more complex than bacteria with the capacity to encyst (i.e., transform to a small, armored, largely inert form that is resistant to treatment processes, NRC, 2004, pp. 36-37).

2.1.2. Indicator Species

When Robert Koch provided his postulates to the International Medical Conference in 1890, and set the foundation for the Germ Theory (Koch, 1890), he listed three bacteria likely to soon be confirmed as sewage-borne pathogens of humans. That number was soon confirmed and began growing almost immediately thereafter, and has been additionally enlarged to include non-bacteria (e.g., viruses and protozoans). The Centers for Disease Control now list over 50 reportable infectious diseases and over 40 “emerging” infectious diseases, as listed by species (Madigan, *et al.*, 2002, pp. 864-870). Moreover, even in an epidemic, not all of the population is diseased and shedding pathogens into sewage, and the pathogens may well be present only at very low concentrations. It’s also not unusual for pathogen-detection methods to be complicated, time-consuming, and different for each target species. Finally, the largest category of waterborne diseases reported every year (by CDC, the US Centers for Disease Control) is “AGI” (acute gastro-intestinal illness) of unknown etiology (Ford, 1999).

Exhaustive direct measurement of that many species, at low concentration levels, on regular basis would be burdensome, and would still not detect the unknown pathogens. Since the very beginning of water regulation in the US, monitoring and standards have been based on “indicator” species. An indicator species is an organism that is easy to test for, the presence of which would indicate the likely presence of the pathogenic species that we’re really interested in.

NRC (2004, p. 9) lists desirable attributes for indicators and for the methods by which they are analyzed (collectively, the “indicator paradigm”). For the indicator itself, they list correlation between presence of the indicator and the risk of interest (to accurately indicate presence of the pathogen), similar or greater environmental survival than the pathogen of risk (to accurately or conservatively measure persistence of the pathogen over time), similar or greater

transport through the watershed (to accurately or conservatively judge the likely areal distribution of the pathogen) and specificity as to the source (e.g., human feces but not, say, toad feces, to accurately indicate the magnitude of pathogen risk to humans). Measurement methods for the indicator should be specific to that organism, useful in a broad array of situations, precise, sensitive, and fast and easy to do.

At about the same time that Dr. Snow was trying to analyze the transmission characteristics of cholera, Dr. Theodore Escherich (a convinced contagionist), was attempting to isolate and identify the bacteria that transmitted it. He found an organism (*Escherichia coli*, or *E. coli*), but it was not the cholera organism. It was always found in large numbers in human feces and, since, has since undergone much study (as a “model organism,” due to the ease of its isolation and of its propagation in a lab) over time. (Gallup, 2010).

With detection methods available by 1900 (essentially, just microscopic bacterial examination of morphology), however, it was difficult for technicians to exclusively, rapidly, and repeatedly identify/quantify *E. coli* from amongst the multitude of organisms that inhabited sewage. As early as the 1890s, the presence of *E. coli*, together with other species within the genus *Escherichia* and several other closely related genera (the “total coliforms”), was relatively easy to confirm by a test (reported by Theobald Smith) based on a chemical analysis for end-products of lactose-fermentation (NRC, 2004, p.30). Total coliforms are always present in human feces, but are often present elsewhere (including even plants) in the environment. The first federal standard for biological quality of drinking water used in interstate commerce was set at 2.2 total coliforms/100 ml in 1914 (McGuire, 2006).

By the time water-quality criteria were extended to environmental waters (recreational uses, including swimming and bathing, fishing etc., as well as areas of shellfish harvesting), test

methods had improved to the point that measurement of a subset of total coliforms, namely the body-heat tolerant “fecal coliforms” (a smaller collection of organisms that still included *E. coli*) had become feasible (based on a consistent ability to grow at 44.5⁰ C). On basic principles (largely, apparently, expounded by Geldreich, 1956, Bacteriological Significance of Fecal Coliforms in the Environment, a referenced and relevant document that I have admittedly been unable to find), the National Technical Advisory Committee on Water Quality (NTAC) deemed that tests for fecal coliforms were superior to those for total coliforms based on the improved specificity for sewage sources (and at least environmentally correlated to fecal sources though *Klebsiella*, a fecal-coliform taxon, is well represented in non-fecal sources). The Committee further concluded (given the remaining lack of specificity for human-pathogen sources) that an epidemiological correlation between indicator concentration and actual human health effects (an expensive type of study) should form the endpoints of regulation. The Committee’s review of relevant literature revealed only two such epidemiological studies (both based on the previous total-coliform indicator) that revealed significant human-health endpoints (increases in gastrointestinal illness or GI, skin infections, or ear/nose/throat symptoms resulting from full-body contact) from freshwater (both lacustrine and riverine), which they extended to marine sources. They also found data from a follow-up study that allowed post-calculation of the fecal-/total-coliform ratio at the site of one of those studies. They applied the ratio to the endpoints and set use-based water-quality criteria as:

Recreational Waters

General uses (without regard to use related to human contact)

Average of 2000 fecal coliforms/100 ml

Maximum of 4000 fecal coliforms/100 ml

Recreational water uses other than primary contact (e.g., fishing, boating, etc)

Average of 1000 fecal coliforms/100 ml

No more than 10% of samples 2000 fecal coliforms/100 ml

Recreational water uses involving primary contact (e.g., swimming/bathing/wading)
Average of 200 fecal coliforms/100 ml
No more that 10% of samples 400 fecal coliforms/100 ml
(NTAC, 1968).

After establishment of the United States Environmental Protection Agency (EPA), new Quality Criteria for Water were promulgated in 1976. EPA discussed alternative indicators of recreational-water quality (e.g., *E coli*, *Enterococcus* spp., *Clostridium perfringes*) based on promising research reviewed. *E coli* was deemed still too difficult to specifically test for. Enterococci were lauded as a superior indicator of sewage contamination (presumably limited to only warm-blooded sources of feces, and believed unable to multiply in aquatic environments), but the lack of a standardized test method to indicate/quantify their presence was noted. *Clostridium perfringes* (*C. perfringes*), as a spore-former, was deemed too long-lived in the environment for use as a sewage-pathogen indicator. The agency retained the NTAC use of fecal coliforms as the preferred indicator and did note recent research establishing correlation between that indicator and one group of actual pathogens (*Salmonella*, spp.). EPA also noted the lack of available significant epidemiological endpoints available from marine-water research and reiterated the NTAC extension of the extant freshwater results into that arena, and all recreational-water criteria from the NTAC document were retained.

Our “current” criteria (EPA 1986, though several states retain previous coliform criteria as bases of regulation) are based on a series of “new” (initiated since 1972) epidemiological studies focused on health effects (with gastroenteritis as the primary effect). The studies included both freshwater (lacustrine) and marine sites. The studies also included sampling for a variety of potential indicator species, to allow for some direct comparison of their individual correlation

with human health affects instead of just a prevalence-based rational extension, though ratio-derived expected *E. coli* concentrations based on fecal coliform concentrations were included in the studies. (It should be noted that new analytical techniques had rendered both *E. coli* and *Enterococcus*, spp., or Enterococci, suitable as indicators from the ease-of-use perspective – Selective media incubation with specific metabolite indicators now allow for quantification of culturable cells in ~24 hrs. Both of these indicators were presumed specific to feces from warm-blooded hosts, but not exclusively from human sources).

For freshwaters, (no distinction between lacustrine and riverine), both *E. coli* and *Enterococcus*, spp. were considered acceptable predictors of gastrointestinal illness for use in setting water criteria for bathing uses. For the former (*E. coli*), a geometric mean of at least 5 samples over a 30-day period of 126/100 ml was set as the criterion, and for *Enterococcus*, spp., 33/100 ml was set. For marine waters, only *Enterococcus*, spp. sufficiently correlated with health effects, and the criterion was set at 35/100 ml (EPA, 1986).

Though new recommended criteria were announced in November 2012, I find no evidence that any state has yet incorporated these criteria into their Water Quality Standards. Nine new epidemiological studies were performed, mostly on recreational beach waters impacted by local wastewater treatment plants (though a South Carolina beach predominantly influenced by urban runoff was included), both freshwater and marine, and including a tropical site in Puerto Rico (the National Epidemiological and Environmental Assessment of Recreational Water, or NEEAR). All of EPA's NEEAR studies measured *Enterococcus*, spp. (applicable in both fresh and salty waters), though data by others directly measuring *E. coli* levels were included in the analyses. The new criteria call for a geometric mean (GM, 30-day period) not to exceed 35/100ml *Enterococcus*, spp., for all waters and 126 *E. coli*/100ml for fresh waters. The

standards also provide statistical threshold values (STVs) of 130 *Enterococcus*, spp. and 410 *E. coli* that should not be exceeded in more than 10% of samples. Finally, the new criteria introduce a new and rapid alternative test for *Enterococcus*, spp. (quantitative polymerase chain reaction, or qPCR), especially for potential use in determining beach closures, and provide action limits while noting potential interference in the test and limited experience in its application. The new criteria retain the indicators used in 1986, and continue to use incidence of gastrointestinal illnesses upon exposures as actionable endpoints (EPA 2012).

2.1.3. Stormwater Regulation

Permitting authorities (authorized states and tribes, and the EPA directly administering unauthorized entities), hereinafter “states,” are primarily impacted by federally recommended Water Quality Criteria in two programs: the National Pollution Discharge Elimination System (NPDES), and the 303(d) program establishing an obligation to set Total Daily Maximum Loads (TMDLs) for water bodies that fail (or are in danger of failing) to meet Water Quality Standards (WQS) for their intended use.

Since the 1990s, an expanding number of stormwater-runoff sources have been deemed “point sources” of pollutants, positively requiring an NPDES permit for discharges to public waters (permits to be reviewed/renewed in no more than 5 years). In phases capturing progressively smaller entities, many runoff sources (Municipal Separate Stormwater Sewer Systems or MS4s, construction sites as small as 1 acre, and industrial sites where material handling and storage operations may be exposed to weather) have been subjected to such regulation. “General” Permits, boilerplate standards based on compliance to Best Management Practices (BMPs) are available and are most frequently used by most runoff-generating entities,

but the state review/renewal process should include a determination that such standards are sufficient to protect the quality of the receiving waters (EPA, 2013).

States must also provide WQS for all waters, to be reviewed every two years. The WQS must be sufficient to support the “intended use” of the water body (the most restrictive non-consumptive use being primary contact recreation). The state may accept the federally recommended Water Quality Criteria (WQC, and there are criteria for pollutants other than bacteria) for a particular use, or may submit alternative standards with sufficient proof that such standards support the use of a particular water body (tools for development of, and thresholds for acceptability of such alternative standards are provided in Section 6, inclusive, of EPA 2012). States must also regularly monitor their water bodies to confirm that quality meets or exceeds the WQS. If a water body fails to meet any of the standards, it must be classified as a non-attainment body (and added to the “303(d) list,” named after the appropriate statute section). Classification as a non-attainment water body triggers a mandatory calculation of the TMDL of the pollutant causing the non-attainment that would bring the body back into attainment; it also requires the state to allocate portions of the TMDL to allowable discharges by individual permitted point sources (Waste Load Allocations, WLA) accounting for loads expected from unregulated sources (Load Allocations, LA, from diffuse runoff, Benham and Zeckoski, 2009). EPA’s “TMDLs to Stormwater Permit Drafts Handbook, 2008” indicates that “Currently there are thousands of Clean Water Act section 303(d) waters listed as impaired for stormwater-source pollutants such as pathogens, nutrients, sediments and metals” (EPA 2013a). By a considerable margin, the largest listed category for cause of impairment, both currently and historically, is pathogens (EPA, 2014), and generally listed and regulated based on accepted indicator organisms.

2.2 Knowledge Gaps

2.2.1. Indicator Correlation to Known Waterborne Pathogens

While choice of indicators for use in the setting of water-quality criteria has historically trended towards bacteria with better specificity to sources of human sewage, and/or to correlation with epidemiological human-health outcomes, there is a general consensus that no perfect indicator has yet been identified for all situations (e.g., see Griffin, *et al.*, 2001, and Ashbolt, *et al.*, 20010, both addressing the 1986 criteria, and anteceding promulgation of the 2012 criteria that retained the previous indicators) .

A (surprisingly) large number of failures of indicators to accurately or conservatively indicate the presence of known pathogens, when both the indicators and the pathogens were found to exist, are revealed in the literature (even limiting inclusion to the “current” indicators of recreational water quality). These discrepancies have been found in a broad variety of environmental circumstances.

In 1996, Lund evaluated *E. coli* for adequacy as Fecal Indicator Bacteria (FIB) for pathogenic *Campylobacter jejuni* (*C. jejuni*) and *Yersinia enterocolitica* (*Y. enterocolitica*), both in chlorine disinfection and in survival over time when inoculated into autoclaved (unchlorinated) oligotrophic lake water. The FIB were found to exhibit similar or slower decay than the pathogens in disinfection, but the (temperature-dependent) longevity of *Y. enterocolitica* in lake water rendered *E. coli* an inadequate indicator of its presence over time (significant survival divergence from Day 0 to 100).

A study of intertidal beach sediments in Morecambe Bay, UK (impacted by treated-sewage outfalls) found no discernable relationships between either fecal coliforms (all

subsequently identified as *E. coli*) or fecal streptococci (all Enterococci) and *Campylobacters*. No *Salmonella* was recovered from any of the three sample sites in the year-long study (Obiri-Danso and Jones, 2000).

Jiang, *et al.* (2001) studied 12 marine beaches (impacted by urban runoff) in Southern California. Concentrations of several FIB (including Enterococci) were compared to PCR-based counts of human adenoviruses. While California standards for FIB (104 CFU/ml) were exceeded at 5/12 sites, and viruses were detected (up to 7500 particles/liter) at four sites, there was no correlation between the exceedance of standards and viral presence. Jiang and Chu (2004) sampled two sites in each of 11 highly urbanized rivers in the Los Angeles area during a rainless summer. Though adenoviruses were detected in 52% of samples, and hepatitis A viruses in 76%, no correlation was found between the viruses and densities of Enterococci (nor total/fecal coliforms). The authors note that their worst ranked site (based on FIB) revealed no viruses, but the second cleanest site was virus positive.

LeMarchand and Lebaron (2003) compared FIB concentrations (including Enterococci) to those of *Salmonella* spp. and *Cryptosporidium* oocysts in effluent of nine wastewater treatment plants (WWTP), and at eight locations within the Tech River watershed (impacted by those effluents) in Southern France. There was no correlation ($p = 0.05$) between Enterococci and either *Salmonella* or *Cryptosporidium* in the effluents, and no correlation of FIB to *Salmonella* in the river samples. The authors suggested differential survival rates between indicators and pathogens in the aquatic environment.

In 2004, Horman, *et al.*, analyzing the coincidence of several known human pathogens (*Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., and Noroviruses) and several alternative indicator organisms (including *E. coli*) in Finland freshwaters (seven lakes and 15

rivers), failed to find any correlation between (culture-based) enumeration of *E. coli* and any of the studied pathogens, though presence/absence of *E. coli* was found to significantly correspond to presence/absence of at least one of the several pathogens.

A suite of alternative FIB (including enterococci, culture-based enumeration) was tested for predictive power for three known human pathogens (infectious enteric viruses, *Cryptosporidium*, and *Giardia*) in the chlorinated effluent of six wastewater-reclamation facilities in the United States (Harwood, *et al.*, 2005). No single indicator correlated to presence/absence of any pathogen, though discriminant analysis on presence/absence of the entire suite of indicators (fecal- and total-coliforms, *C. perfringens*, and F-specific coliphages, together with Enterococci) as a predictor was successful in reconstructing the presence/absence pattern of all pathogens for the majority of samples. Similarly, a six-year study of several sites in Santa Monica Bay, CA, showed no significant ($p = 0.05$) relationship between any of three FIB (total and fecal coliforms, and Enterococci) and PCR-enumerated enteroviruses, but exceedance of CA standards of any of the three FIB significantly correlated with presence of the viruses (Noble and Furman, 2001).

A study partly to “evaluate whether indicator microbes were correlated with pathogens” in a South Florida estuary (unaffected by point-source sewage effluents), was partly frustrated by a paucity of samples exhibiting the presence of pathogens (Ortega, *et al.*, 2009). One of eighteen samples was positive for reovirus, though *Cryptosporidium*, *Giardia*, and multiple enteroviruses were tested for in all samples. While regulatory guidelines for FIB were frequently exceeded (including six samples for *E. coli* and/or Enterococci) and large concentrations of alternative indicators studied were also often noted, indicator readings for the one pathogen-positive sample were deemed unremarkable.

In Brisbane, Australia Ahmed, *et al.* (2009), investigated the (binary logistic) relationship of (culture-based) concentrations of both FIB, *E. coli* and Enterococci with qPCR-derived presence/absence of three known zoonotic bacterial pathogens (*C. jejuni*, *Salmonella* spp., and enterohaemorrhagic *E. coli*, or EHEC) at a pond (receiving runoff and frequented by waterfowl) and two tidal creeks (one draining suburban/forested/grazing land, one residential), all three sites receiving input from treated wastewater. No indicator/pathogen pair was significantly ($p = 0.05$) correlated (even for the indicator *E. coli* and its component pathogenic strain EHEC). A similarly structured study by Ahmed, *et al.* (2010) found no significant correlation between either *E. coli* or Enterococci and the presence/absence of genetic markers for *A. hydrophila*, *C. jejuni*, *L. pneumophila*, *Salmonella* spp., or *G. lamblia* in harvested rainwater from 82 residential rooftops in Australia.

Though not related to our “current” indicators of recreational water quality, a final example provides a telling example of the potential health hazards deriving from the lack of FIB/pathogen specificity. A large (infecting the majority of the city’s population) outbreak of Cryptosporidiosis in Milwaukee, WI in 1993 was not recognized on the basis of ongoing FIB (coliform) monitoring of the water supply system. While an “observant pharmacist” was noting a large uptick in purchases of diarrhea medicines, “the public health community was not aware that an epidemic was occurring.” (Schaub, 2004).

Failures to find correlation between FIB and known human pathogens (or even between applicable FIB), when both are demonstrably present, are numerous (especially in the case of viral or protozoan pathogens, Henrickson, *et al.*, 2001). Boehm, *et al.* (2009) even opined that, “...most studies show a striking lack of correlation between the two.” Current criteria, based on actual health effects associated with FIB exposure (rather than actual or measurable presence of

pathogens), may demonstrate valid end-points of such criteria to the site and conditions extant during the epidemiology studies. Violations of the indicator paradigm in such studies, however, introduce “misclassification of risk” (Blumenthal, *et al.*, 2001), creating a potential bias affecting confidence in the causal nature of any association found (Craun, *et al.*, 1996). Extension of such associations to predictions of human health effects outside of the time and place of the study is of doubtful validity (Boehm, *et al.*, 2009).

2.2.2 Natural Ubiquity, Persistence, and Endemicity of FIB

Currently recommended FIB are found to be common and persistent on, to regrow on, and even naturalize to, the landscape (their non-enteric, “secondary” habitat) under many environmental conditions. Contrary to the presumptions implicit in their use as indicators of potential human fecal pathogens, FIB presence in environmental matrices may be divorced from human-fecal sources, fecal sources from homeothermic hosts, and even from feces in general.

Both *E. coli* and Enterococci were found in blood samples from Hispaniola iguanas (Maria, *et al.*, 2007). *E. coli* have been found in the nasal passages and cloacae of Costa Rican turtles (Santoro, *et al.*, 2006). *E. coli* have also been found in the digestive passages of fish (farm-raised Nile tilapia, Molinari, *et al.*, 2003). These heterothermic sources of FIB are generally considered carriers, rather than reservoirs, but *E. coli* was found to persist and grow (temperature-dependently) in rainbow-trout intestines (Del Rio-Rodriguez, *et al.* 1997). In a food-handling environment, 97% of examined local houseflies were found to harbor Enterococci in their digestive tracts (Macovei and Zurek, 2006), and association with the chitinous carapace of copepods has rendered Enterococci environmentally persistent in an aquatic environment (Signoretto, *et al.*, 2005).

In a Northern Pitcher Plant bog in Indiana, Whitman, *et al.* (2005) found both *E. coli* and Enterococci, both in mean densities exceeding the recommended WC, in accumulated fluids within the pitchers of 43 plants. The bog was described as pristine and protected (fenced, limited human with access only by boardwalk above the ombrotrophic peat, and 400 m from the nearest human residence). The genetic markers of the Enterococci were distinctly different from those of a library of human/clinical isolates (by pulsed-field gel electrophoresis). Though the authors deemed contamination by feces-contaminated insects possible, they found no correlation between FIB and arthropod densities within the pitchers, and analysis of the contents of insect traps placed inside 20 of the pitchers revealed no measurable FIB. FIB artificially inoculated to insect-free pitcher-plant fluids were found to persist (and, in the case of *E. coli*, grow) over 120 hr. in a temperature-dependent manner (at ambient 21^o and 26^o C). Mundt (1961) implicated insect carriers as a source of Enterococci on agricultural crops (not collocated with animal husbandry, and with suppression of wildlife activities by farming activities). Once inoculated, the FIB were found to multiply through the growing season (host-specifically, e.g., on corn more than tomatoes) epiphytically. The colonized crops were found to be a net source of Enterococci to the underlying soils. In similar study of wild lands (Great Smoky Mountains National Park), Mundt (1963) again found epiphytic associations between *Enterococcus* and (native) plants. In this latter study, however, the gross taxonomic-structure of the epiphytic community (by analysis available at the time, mostly species level) was found to closely correlate to the structure found in the feces of the local wildlife. Mundt concluded that suppression of local wildlife (and the resulting lack of local feces) by agricultural practices created an “artificial selection” pressure on Enterococci (mediated by dependence on floriphagous rather coprophagous insect carriers for

dissemination). Applying more modern analytical methods (PCR) to the epiphytic *Enterococcus* community in a forage-grass field (not pasture land, livestock feces were not present), Muller *et al.* (2001) identified a previously undescribed species, evolved to endemicity under environmental pressures present at leaf surfaces.

FIB association with plants was also one of the earlier indications of either naturalization or autochthonicity of *E. coli* in the tropics. A 1988 study by Rivera *et al.* measured both total- and fecal-coliform densities (with the latter being found to be 72% *E. coli* by selective-media cultivation) from accumulated rain-/overstory leachate-water in 9 bromeliads (itself an epiphyte of trees) in a cloud rain forest in Puerto Rico. Though *E. coli*-positive samples could be explained by feces from birds (of very low populations in this island ecosystem) or tree-climbing mammals (limited to rats in Puerto Rico), the authors logically concluded that presence of *E. coli* in all samples must show either FIB endemicity or extended persistence (either of which would belie any indication “recent” fecal contamination), and questioned the use of FIB in the tropics. Somewhat contemporaneously (1985), and in the same watershed (Mameyes River, Puerto Rico), Carillo, *et al.* examined the distribution of several potential FIB (including *E. coli*) at 6 sampling locations along an altitudinal gradient (upper reaches of which drained the cloud forest, and the lowest sampling point was at the effluent of a primary-treatment wastewater plant) over a year. Except for the WWTP effluent, all sampled waters were deemed oligotrophic, but all sampled waters in the study exceeded current *E. coli* WQCs. While the treated effluent exhibited the highest densities for FIB, the second highest levels were recorded at the highest elevation sampled, and all samples correlated with nutrient loads rather than potential fecal loads. *E. coli* inocula in diffusion chambers, placed at two sites upstream of the treated-sewage outfall, showed significant growth over the course of the study (72 hr.). The authors questioned the

appropriateness of using of *E. coli* (or any coliform indicator) to indicate tropical-freshwater quality. Hazen provided a (1988) review of literature and concluded that *E. coli* “can survive indefinitely in most freshwaters of Puerto Rico,” rendering the species (and other extant FIB) useless as an indicator of any fecal source, and advocated exploration of alternative indicators.

Considerable literature also exists raising questions as to the relevance of (persistent and arguably endemic) FIB in tropical soils as predictors of human-pathogen presence. Much of that literature is based on (previously recommended) total- or fecal-coliforms as indicators and is, therefore, omitted here. In response to the promulgation of the 1986 criteria based on *E. coli* and Enterococci, however, and to support the use of an alternative FIB in Hawaiian WQS, Fujioka and Byappanahalli (1996) provided a review of such literature vs. the rationale underlying the 1986 criteria to argue that the “current” FIB must also be viewed as natural bacteriological denizens in Hawaiian (and tropical) soils and, thus, inappropriate indicators of human health in tropical environments. They further presented new studies, showing that:

- 1.) *E. coli* and Enterococci are “naturally present and prevalent” in all the general soil types (though not all samples) of Hawaii;
- 2.) Natural-soil populations of Enterococci remain stable throughout a five-day study of FIB resistance to air-drying (with three-fold reduction in soil moisture), while *E. coli* populations decline with drying (but are recoverable with addition of moisture);
- 3.) Sewage application to natural soil does not result in increased FIB populations (under room-temperature laboratory, greenhouse, or secured and simulated natural conditions. Populations of both sewage-sourced FIB, however, increase dramatically with the addition of nutrients to, or prior (cobalt irradiation) sterilization of, the inoculated soil (studies ranging up to 8 days).

The authors suggested that *E. coli* and Enterococci are persistent and reproductively competent (whether natural or naturalized) components of Hawaiian soil ecosystems, limited by nutrients and microbial competition but not by recent fecal contamination. They endorsed the use of *C. perfringens* as an indicator of fecal contamination in tropical environments [Recall that *C. perfringens* had been rejected by EPA as a suitable FIB when developing the 1976 WQC due to

its potential to persist as spores. Note that as an anaerobic spore former, however, the species cannot multiply in, or naturalize to, aerobic environments but can only persist in a relatively inert form.]

Fujioka, *et al.* (1999), sampled soils in Guam (both at the bank of the Pago River, “not greatly impacted by urbanization,” and 30 m away from the bank) and found elevated FIB (*E. coli* and Enterococci) at the surface (lower densities at 18- and 36-cm depth). They also found WQC exceedances common in rivers and beaches across Guam. The authors noted the rarity of birds on the island, concluded that endemic FIB in the soil are washed into water bodies by rain causing those exceedances, and advocated the use of *C. perfringens* to indicate water quality in recreational waters.

In a dissertation, Byappanahalli (2000) expanded upon the findings of Fujioka and Byappanahalli, 1996 (above), and additionally showed: broad taxonomic and metabolic diversity of natural Hawaiian soil FIB; initial growth and extended survival (18-day study) of sewage-derived FIB on sterilized natural soils; initial growth and subsequent persistence of animal-fecal FIB on the same medium; and initial growth of pure (lux-gene) marked *E. coli* up to 6.5 log-units, followed by slow declines (though still over 1.5 log units above initial inoculum) in both nutrient-amended and unamended, sterile soils over a 68-day study (Oahu, Hawaii).

[Note: Hawaii’s current, approved WQS include “Specific criteria for recreational areas” based on *Enterococcus* densities, but separately lists “raw or inadequately treated sewage” (indicated by above long-term background densities of *C. perfringens*) as a prohibited input to such waters. Impaired status (303(d) listing) is based on the former. Beach warnings are triggered only by both. (HIDOH, 2013, and HIDOH, 2012)]

The potential of soils to provide a persistent source of FIB to watersheds is not limited to the tropics. Unc, *et al.* (2005) added sterilized sewage biosolids to natural (10-years fallow) clay soils from Ontario. Before the addition, the soils harbored undetectable *E. coli* populations (detection limit = 1 colony forming unit/g dry soil). *E. coli* growth (up to 2.3 log) was apparent within two days after biosolids addition, and elevated populations were largely maintained over the 20-day study period. Byappanahalli *et al* (2006) provided evidence of naturalization of *E. coli* to upland forest soils in the Great Lakes region (Dunes Creek watershed in Indiana). Six study “exclosures” (netting to prevent new fecal inputs), in a protected environment (Indiana Dunes State Park and National Lakeshore, with restricted human exposure) were sampled over a one-year period. Culturable *E. coli* were consistently recovered from all exclosures from March to October, and (after frozen soils thawed, albeit in lower densities) in January and February. Once recovered, densities were seen to decline progressively with desiccation of samples (up to 70 hrs), with rapid regrowth upon rehydration. *E. coli* recovered were genetically diverse (PCR analysis) and distinct from wildlife library samples tested (gulls, terns, geese, deer), providing some evidence of naturalized or native populations. Ishii *et al.* (2006) examined a diversity of soil types, present at creek shorelines and upgradient, and in a wetland, in three watersheds near Duluth, MN. Genetic diversity (PCR) of *E. coli* recovered over the one-year study was diverse across various sites but showed >92% similarities for repeat samples from individual sites (evidencing site-specific adaption/naturalization). Over 80% of isolates could not be traced to any of beaver, deer, goose, or tern sources, and genetic structure of soil samples was distinct from those obtained from nearby water sources, providing evidence that soil populations were not largely derived from recent fecal deposition or inundation. Naturalized soil isolates, selectively cultured for antibiotic resistance (as a marker) were found to grow when inoculated

into unsterilized and unamended soils from the sites from which they were originally harvested, indicating adaption to the naturally available nutrient and microbial-competitive conditions. Similarity of population structure, at individual sites, before and after winter freeze/thaw cycles confirmed that *E. coli* overwintered (at reduced numbers) and revived in the spring. The authors concluded that the bulk of *E. coli* from these sites is fully autochthonous.

Persistent populations of FIB in shoreline/beach sands are frequently found to be reservoirs of and net sources to adjacent waters. Whitman and Nevers (2003) studied a Chicago beach that had been frequently closed for exceedances of *E. coli* density criteria. The beach received sand replacement (removal of 10-15 cm surface sand, followed by replacement with upland sands demonstrably free of measurable *E. coli*) at the beginning of the bathing season, and was mechanically groomed (detritus removed) daily in-season. The authors sampled foresand (1 m above the waterline), submerged sand (at 45 cm), and offshore water along multiple (shore-perpendicular) transects, as well as gull sand (at greatest observable concentration of gulls on the beach), and they recorded meteorological conditions and swimmer counts, three times/week throughout the swimming (Memorial Day – Labor Day) season. The authors could not determine the source (gulls or over-wintered *in situ* growth) of rapid (2 wks) colonization of the replaced sand, but concluded that, once colonized, foreshore-sand *E. coli* densities are better correlated to weather (temperature/seasonality) than to new fecal sources, and that foresand (and, to a lesser extent, submerged sand resuspension by weather/wind) is a net source of *E. coli* to water. At another Great Lakes beach, Alm, *et al.* (2006) recovered, isolated, and cultivated *E. coli* from Lake Huron beach (swash-zone) sands and re-inoculated those bacteria to autoclaved sands (from the same beach) at 19⁰ C in the lab, finding more than five-order growth in two days and stable populations, thereafter, for over a month. In a follow-up, the

authors explored ambient-condition behavior of the FIB by placing similarly constructed microcosms into diffusion chambers (to allow free flow of moisture and nutrients but restrict bacterial migration in or out of the chambers) and implanting the chambers into beach sand (just above the swash zone), and sampling both the chambers and adjacent sand over 48 days (18-25^o C sand temperature, 5.7-13.2% sand moisture, 10.2 cm total precipitation). Inside the diffusion chambers, *E. coli* densities again rapidly increased (over five orders) and stabilized thereafter, while adjacent samples were stable throughout the study (the authors concluded that predation/competition explains the initial differential behavior, and a nutrient/environmental natural carrying capacity explains the long-term results). In an attempt to determine the sources of *E. coli* recovered (over two summers) from (beach, water, and sediments of) a swimming beach in Duluth, MN (characterized as impacted by two local WWTPs and seasonal waterfowl), Ishii *et al.* (2006) compared the samples (PCR-based analysis) to DNA fingerprints in a locally derived (Duluth) library containing strains obtained from wildlife (pooled beavers and deer), waterfowl (geese, gulls, terns) and treated wastewater (assumed of human source). The authors also expanded the available local library by collection of *E. coli* isolates, directly collected during the course of the study, from one of the WWTPs and from extant beach birds. They further compared their (>3600) recovered strains to fingerprints available in a statewide library containing *E. coli* isolates from dogs, cats, horses, deer, geese, chickens, ducks, turkeys, cows, pigs, goats and sheep. While 32% of the recovered strains were matched to library sources (variable relative contributions over the season, dominated by humans and waterfowl), the balance was deemed naturalized (based on inability to identify any recent host, and $\geq 92\%$ fidelity of fingerprints at each sample point over time). Twenty-four *E. coli* colonies isolated from interstitial water aseptically extracted from each of four (widely separated, across > 1 km

beachfront) foresand holes excavated into beach sand (a Lake Huron swimming beach with upstream inputs dominated by an agricultural watershed) were compared for (PCR-based) genetic relatedness by Kon, *et al.*(2007). Between-hole similarity was as low as 60%, but within-hole similarities were all >90% (and mostly = 100%), again suggesting adaption of *E. coli*. (of any source) to local sand conditions.

Solo-Gabriele, *et al.* (2000) examined a brackish (up to 10% seawater), tidewater river in (semi-tropical) south Florida (near Fort Lauderdale), and found that the intertidal riverbank soils represented a (tidally dependent) net source of *E. coli* to river water and exceeded potential contamination from local dry-weather storm-drain sources. Based on field studies, the authors followed up with laboratory studies establishing 2-3 log regrowth of (previously dried) *E. coli* in intermittently rewetted bank-soil samples within 24 hrs, followed by stable, elevated populations lasting > 70 hrs. They opined that *E. coli* possessed survival advantages over natural soil competitors/predators during desiccation and were able to bloom in rewetted soil, and that use of these organisms to indicate human-wastewater pollution is potentially flawed. Ferguson, *et al.* (2005) studied water and sediments at two frequently closed marine beaches (one protected by an artificial breakwater and impacted by stormwater runoff, the other open to ocean and potentially impacted by the Santa Ana River and/or a chlorinated sewage outfall 7.6 km offshore, and both subjected to historical FIB controls including stormwater diversion and bird exclusion during swimming seasons) in southern California. The authors found persistent enterococcal populations in intertidal sediments (and, to a lesser degree, offshore submerged sediments) to be a significant source to waters and a potentially confounding factor in use of Enterococci as indicators of recent fecal contamination. Yamahara, *et al.* (2008) found measurable Enterococci in sands nearly all (50) of 55 marine beaches in California. The authors selected one of those

beaches, Lovers Point, Monterey, a sheltered beach with tide-dominant hydrology (minimal wave-related energy) and low winds at the time of the study, to test a hypothesis that Enterococci in sand represent a diffuse source to seawater. Filtered natural seawater from the beach, trickled through a glass column packed with naturally contaminated sand from above the high-tide line, eluted nearly all the original Enterococci with about four times the estimated pore-volume in the column, and showed no measurably eluted cells with continued flushing; the authors concluded that at least some of the enterococcal cells were loosely bound to sand grains and susceptible to mobilization by seawater exposure. Collecting three samples (sand from 1.5 m above and 1.5 m below the water line, and water ankle deep) every 20 minutes over a (two tide cycles) 24-hour period at Lovers Point, the authors found that Enterococci densities in exposed sand were higher than those in submerged sand and were highest near the spring high-tide line, densities in exposed sands were lower during falling tides than in rising tides, and water densities peaked at high tide. They estimated that the total cells washed from the sand during rising tide nearly equaled the number entering the water, and that the post-peak decline in water densities could be explained assuming a nearshore-water residence time (before dilution/flush to open ocean) of between 18 and 26 hours (providing evidence that intertidal sand represents a net source to water). Studying microcosms of unaltered, unseeded sands from above the high-tide line at Lovers point and maintained in the dark at 20⁰ C, Yamahara, *et al.* (2009) found that control (unwatered) samples show unchanging enterococcal densities over a 45-day study period, but that rewetted (with filtered, high-tide Lovers-Point seawater) and gravity drained samples exhibited rapid growth in both culturable Enterococci (doubling time = 3.5 day) and total Enterococci (qPCR-measured, doubling time = 1.1 day) for two weeks (with subsequent slow decline).

Submerged sediments may also harbor persistent FIB populations. Stephenson and Rychert (1982) studied the bottom sediments and overlying waters of six streams draining livestock grazing land, with potential impacts by big-game animals, near Boise, Idaho. They found densities of *E. coli* in the sediments to consistently exceed those in the water column (2- to 760-fold), to increase during the course of summer months at resampled sites, to correlate ($r = 0.82$, but deemed “not significantly significant” due to low sample numbers) to organic content in the sediments, and to contaminate the water column upon disturbance (mechanical disturbance provided with rakes on two occasions, and by one small rain occurring during the course of the study). The authors found these patterns consistent with (temperature- and nutrient-dependent) growth of *E. coli* within the sediments providing an instream source of FIB to stream water.

LaLiberte and Grimes (1982) inoculated muddy and sandy sediments from Lake Onalaska (a lock-and-dam pool on the Mississippi River in Minnesota) with an *E. coli* isolate derived and cultured from a treated sewage outfall nearby, and incubated the microcosms (contained in cellulosic dialysis tubing) *in situ* in the lake for two 5-day periods in August and September. Control microcosms (unsterilized sediments with no inocula) maintained a background level of ~1 colony forming unit (CFU)/gram sediment throughout the study periods, unsterilized and inoculated systems harbored steady populations of 10^2 - 10^4 /gram (larger in sand) indicating persistence in the face of local predators, and inocula to autoclaved sediments rapidly grew over 2 log in the first three days (demonstrating environmental regrowth). The authors concluded that the FIB were better indicators of sediment resuspension than of recent fecal contamination.

Decrying the relative paucity of studies into environmental persistence of FIB in non-tropical regimes, and of studies investigating potential non-point sources of FIB within watersheds,

Byappanahalli, *et al.* (2003) conducted a large, three-year survey of *E. coli* concentrations across multiple environmental media (water, sediments, and soils) within the Dune Creek watershed, the primary upland source impacting waters at the (frequently closed) swimming beach in Indiana Dunes State Park (on Lake Michigan). The watershed is described as >90% natural (with 6.5% residential and 2.1% agricultural land use, both limited to upper reaches), mostly draining wetlands and seeps, though significant historic drainage ditching was evidenced by early 20th-century maps. In-water samples showed a clear increasing trend from spring/seep waters (essentially devoid of cells) to ponded waters to flowing stream waters (and, in the last, positively correlated to stream order). Soil/sediment samples showed lowest median *E. coli* densities in upland forest soils (though with extreme spatial patchiness characterized by frequent high-concentration outliers), with increasing concentrations through spring bank, stream bank, and in-stream sediments (all correlating to substrate moisture). Moreover, in-stream *E. coli* densities increased with increasing stream order, and the creek-outfall concentrations correlated significantly ($p < 0.0001$, $r = 0.52$) with those of the downstream swimming beach. The authors concluded that (natural or naturalized) persistent *E. coli* populations within sediments, divorced from exogenous (sewage, feedlot, wildlife) inputs, represented a significant non-point source to overlying waters. Pote, *et al.* constructed and maintained microcosms of pollutant-free sediments (one of high organic content, one of low) and heavily polluted (by sewage effluent) sediments overlain by continuously replenished filtered waters (either unpolluted water of negligible *E. coli* concentrations and undetectable Enterococci, or sewage-treatment effluent) from Lake Geneva, for 60 days at two temperatures (10⁰ C and 20-25⁰ C). They demonstrated a net transfer of FIB to clean overlying water at the outset, persistent culturability in all microcosms throughout the

course of the study, and significant net growth in the high-temperature treatment of the organics-containing sediments.

Considerable evidence has accumulated of environmentally persistent FIB associated with submerged aquatic vegetation (SAV). Whitman, *et al.* (2003) sampled (n = 41) beach-stranded, floating, and anchored mats of *Cladophora* (a pan-geographic, macrophytic alga inhabiting both fresh and marine waters) from 10 swimming beaches on Lake Michigan (three of which were deemed potentially impacted by local sewage sources), mostly during summer months. They found FIB ubiquitously (97% positive samples) present in high but highly variable (log-mean 5.3 +/- 4.8 and 4.8 +/- 4.5 CFU/g dry weight for *E. coli* and Enterococci respectively) densities. Concentration of both FIB was generally highest in attached algae (> floating > stranded) and higher in southern than northern sample sites. Transect sampling at two beaches (one of potential sewage impacts) revealed higher *E. coli* counts in floating mats than stranded mats, with both counts exceeding those of adjacent sand and water. Persistence of both FIB populations in algal mats exposed to direct sunlight was found to depend on the thickness of the mat (6 mm > 4 mm > 2 mm > 1mm) and was greater for Enterococci than *E. coli* (Enterococci in the thickest mat were essentially undiminished in four days of exposure). Samples of those sun-dried mats, after six months of refrigeration at 4⁰ C, exhibited 4-log growth in both FIB upon rehydration and incubation at 35⁰ C. The authors believe that *Cladophora* mats provide a suitable habitat for FIB under relevant environmental conditions, and represent a potential source to water. Byappanahalli, *et al.* (2003a) prepared leachates (filtered centrifuge supernates of *Cladophora*/lake water mixtures previously allowed to stand quiescently for 48 hours) from one of those Lake Michigan beaches, and studied their capacity to nutritionally support FIB growth in the laboratory. They found temperature-dependent (25 – 35⁰ C, the lowest temperature being

environmentally relevant at summer Lake Michigan waters) *E. coli* inoculum growth in pure leachate (greatest at highest temperatures), and they found concentration-dependent growth of *E. coli* (highest for pure leachate) inoculated into a dilution series of leachate. The authors then demonstrated growth of the *E. coli* and Enterococci naturally occurring in pure leachate (1000-fold and 100-fold respectively in 48 hours, followed by slow declines at slowing rates of decline over a week) upon elevation to incubation temperatures (35⁰ C). Comparing the capacity of *E. coli* to regrow from washed *vs.* unwashed mats (when sun-dried, refrigerated for 6 - 8 months, and then rehydrated) the authors found rapid growth in both (~ 4 log in 18 hours), but stabilization to a slightly lower density in the case of the washed samples (lower carrying capacity of the system, ~7 log CFU/g *Cladophora* for washed *vs.* 8 log CFU/g for unwashed). They concluded that the bulk of nutritional support provided by the mat derived from actual algal exudates rather than periphyton. It must be noted that *Cladophora* mats may also harbor known human pathogens. Ishii, *et al.* (2006a) examined phosphate-buffered water elutions from attached *Cladophora* mats harvested from both sides of a breakwater partially separating effluents from a polluted ditch (impacted by combined-sewage overflows, septic-field leachates, and urban and agricultural runoff) and open Lake Michigan waters at Ogden Dunes Beach (Indiana Dunes National Seashore). The authors determined the presence/absence and most probable numbers (MPN, a culture-based statistical CFU-quantification method) of *E. coli* (species confirmed by MUG, a standard assay for a species-specific metabolic product) and of four pathogenic taxa: Shiga-toxin producing *E. coli* (STEC); *Shigella*, spp.; *Salmonella*, spp.; and *Campylobacter* (all identified by PCR analysis of taxon-specific primers). However, while they found consistently high concentrations of *E. coli* and culturable quantities of *Salmonella* and *Campylobacter*, spp., there was no significant correlation between the FIB and the pathogens. The authors opined that

lower (4 log) concentrations of the pathogens, and their stronger temporal dependence on input sources, may explain the lack of correlation. They further found that qPCR analysis (which detects genetic material from dead and/or non-culturable cells) revealed a much larger (up to 36-fold) presence of pathogens than were revealed by culture-based methods. Englebert, *et al.* (2008) specifically studied the differential survival of *E. coli* and two pathogens (*Shigella* and *Salmonella*) in inoculated microcosms of *Cladophora*-mat material and/or lake water (to separately study survival of bacteria in *Cladophora*-free water, in water with *Cladophora* contact, and attached to the algae) collected from Lake Michigan (Door County, WI). The microcosms were maintained at environmentally relevant conditions (25⁰ C, with intermittent shaking, and 12-hour light/dark illumination from full-spectrum fluorescent bulbs). Attachment to *Cladophora* material provided for extended survival (with respect to either water environment) for all three taxa. All attached *Shigella* samples, however, were below detection limit (1 CFU/ml) after two days (a 7-log decline), and *Salmonella* were immeasurable after nine (5-log decline). *E. coli* densities declined ~4 log CFU/ml in nine days but stabilized and remained culturable (>100 CFU/ml) through the course of the study (48 days). The authors question the validity of *E. coli* as indicators of fecal pathogens in the presence of algal material. Byappanahalli, *et al.* (2007), performed a PCR-based analysis of DNA fingerprints of 835 *Cladophora*-associated *E. coli* isolates collected at Ogden Dunes Beach (Indiana Dunes National Seashore, and site described at Ishii, *et al.*, 2006a, above) with each other and with those (1785 unique samples) in a library representing isolates from humans, domesticated animals (cats and dogs), wildlife (beavers and deer), and waterfowl (ducks, geese, terns, and gulls). Actual *E. coli* counts were significantly higher on *Cladophora* from the “ditch” (more polluted) side than those from the “lake” side (less polluted) of the breakwater. While about 20% of the *E. coli* genotypes were shared by

populations on both sides of the breakwater, most were location-specific across the divide, and both were distinctly different from 44 samples recovered at another beach the previous year. *Cladophora*-borne *E. coli* isolates displayed genotype patterns distinctly different from those of likely sources represented in the library (< 1% chance of mis-assignment in Jackknife Analysis) and high source fidelity (97% correct reassignment).

Badgley, *et al.* (2010) used water, sediments, and submerged vegetation (*Hydrilla verticillata*, or “water thyme,” an Asian exotic considered invasive in Florida) from a freshwater lake (with a history of high enterococcal counts) in Tampa, FL, to seed paired (vegetated/nonvegetated), recirculated mesocosms (180 L). The lake was deemed to be free of sewage impacts but subject to stormwater and shorebird-feces inputs. Pairs of mesocosms were sampled for matrix-specific (water, sediment, SAV) enterococcal densities over two week periods in each of four experimental runs (April, May, July, August) conducted at ambient conditions (an open-air greenhouse at University of South Florida). Over all runs, the authors found elevated densities in the SAV (> sediments > water) and greater persistence in the SAV (>water > sediments), the latter characterized by as two-phase (initial decay with subsequent growth or slowed decay). Moreover, the enterococcal densities of all matrices (including sediments and water) in the vegetated mesocosms consistently exceeded those in the unvegetated systems (though the authors could not distinguish between SAV as a source of cells or of nutrients to the other matrices). The authors genotyped the Enterococci at initiation and near the end of all four runs and found that all examined populations were dominated (96.5%) by a single strain. Combined with the fact that the mesocosms were seeded by plants collected over a 10-month period, results allowed the authors to conclude that Enterococci were adapted to and reproductively persistent in the vegetated lake as well as in the experimental systems.

Some finite environmental survival of FIB is implicit in their selection as indicators. Their universal presence in human feces depends on infection of human newborns (e.g., see Fanaro, *et al.*, 2003 for a review). Their use to conservatively indicate the potential presence of fecal pathogens assumes their ability to survive as long as, or longer than, human pathogens deriving from the same source. Whether native or naturalized, however, naturally occurring (environmentally adapted and/or reproductively persistent) organisms are not temporally or spatially related to any fecal source. Their use to indicate pathogens from any such source introduces a potential misclassification of risk. Moreover, failure to account for significant naturally occurring FIB sources in any watershed subject to TMDL limitations would logically doom any attempt to rationally allocate bacterial loads among permittees [Note: Though not strictly a persistent source, the recognized but unaccounted capacity for the bathers themselves to locally shed significant FIB (and pathogens, not necessarily correlated) to recreational waters logically represents a similar barrier to rational allocations of waste loads among exogenous sources (Blostein, 1991, Papadakis *et al.* 1997, Kramer *et al.* 1998, Elmir *et al.* 2007)].

EPA conducted an epidemiological study of a tropical beach (Boqueron Beach, Puerto Rico, impacted by sewage outfalls and, presumably, naturally occurring FIB), as a part of the NEEAR effort (and in response to a lawsuit). In the study, swimmers were found to exhibit significantly more skin rashes than non-swimmers. No significant relationship between FIB and human-health endpoints could be established. EPA believed that the failure to find such a relationship was due to good water quality (densities of culturable Enterococci ranged from undetectable to log 2.45 CFU/100 ml over the course of the study), and interferences in qPCR

analyses (Wade, *et al.* 2010). In promulgation of the 2012 WQC, EPA notes that “the presence of FIB in water is not necessarily an indication of recent fecal contamination or potential health risk,” but concludes that “the state of the science is not developed sufficiently to distinguish environmental sources from other sources of FIB on a national basis.” Reference is made to section 6.2 for development of alternative criteria (EPA, 2012).

2.2.3 Sewage- vs. Environmental-source Disparities

The regulatory use of current FIB to equally denote pollution of potential to affect human health in all waters (whether sewage-impacted or not) has been questioned. As dominance of sewage sources in a water body is reduced, so is the correlation between indicators of sewage-borne pathogens and human health effects.

“The rationale for the use of guidelines and standards based on fecal indicator densities for indexing the health hazards in sewage polluted waters is that, under average conditions of illness in the discharging population, there is a reasonably constant indicator to pathogen ratio in the sewage and its receiving waters. Thereby, an acceptable probability of illness caused by the pathogen can be extrapolated to a given indicator density, which is then recommended as a guideline and promulgated as a standard. Such relationships appear to hold for waters receiving the discharges from relatively large municipal sewage treatment facilities. However, as the number of individuals who contribute to the source of the fecal wastes becomes smaller and smaller, the indicator-pathogen ratio will vary more and more from the average upon which the guideline or standard is based.” (Cabelli, 1983, presenting the epidemiological rationale of the 1986 WQC)

2.2.3.1 Regulatory History

A historic perspective is, again, instructive. Since the first Federal effort to regulate quality at bathing beaches (NTAC, with extension of criteria to EPA’s 1976 WC), regulatory limits have been based on large epidemiological studies of correlations between FIB and human-health effects. Available studies had been performed in the 1940s and 50s by the United States

Public Health Service (USPHS) at three water bodies (Lake Michigan, Ohio River, and Long Island Sound). At each location, total coliforms were measured at paired beaches of “low” and “high” water-quality history (with the exception of the Ohio River, where a local public swimming pool provided a surrogate high-quality beach) and compared to self-reported symptoms of swimmers. The Lake Michigan site showed no significant difference in health effects between the two beaches (median coliform densities of 91 and 181 coliforms per 100 ml) and the data were reanalyzed; significant health effects were found when three consecutive days of low coliform densities (geometric mean 43/100 ml) were compared to three days of high densities (2300/100 ml). The Ohio River beach (median 2300 coliforms/100 ml) exhibited a significant excess of gastrointestinal illness when compared to the total study population. No association between illnesses reported and water quality (medians 398 and 815 coliforms/100 ml) was found at the marine beaches. Total-coliform densities were converted to expected fecal-coliform densities on the basis of a follow-up sampling (1960s) at the Ohio River beach, finding that 18% of the former could be identified as the latter. Relevant (to this research) criticisms of the derivation of WC from these data were:

- 1.) Failure to find any difference between beaches (all findings were of differential illnesses at different times at the same beach, and with the same pollutant sources) and,
- 2.) Use of a FIB with poor specificity to sewage (*Klebsiella* spp., a subset of fecal coliforms are found in “pulp and paper mill effluents, textile processing plant effluents, cotton mill wastewaters, and sugar beet wastes, in the absence of fecal wastes”). (EPA 1986, and citing Cabelli 1983)

[Additional note: Though I could find no reference in the literature, an additional criticism might be the lack of a non-swimmer control in the re-analyses of the data, raising questions concerning evidence of any swimming-related effects. The Chicago data were based on comparison of swimmers on days of poor water quality to swimmers in good water. The Ohio River swimmers

in poor-quality water were compared to the overall study population. The only direct comparison between swimmers and non-swimmers was at the Long Island Sound sites, which found no association, at either beach.]

A series of beach studies were performed in support of development of the 1986 WC. Paired beaches (“relatively unpolluted” and “barely acceptable,” based on historic monitoring results at both, and on presence of treated-sewage outfalls at the latter), at three marine sites (New York City, Boston, and Lake Pontchartrain, LA) and two freshwater sites (Lake Erie, PA and Keystone Lake, OK), were selected (The Bathing Beach Studies). Densities of multiple FIB (including *E. coli* and Enterococci), were compared to multiple categories of self-reported illnesses (including “highly credible gastrointestinal” illnesses, HCGI, a category including diarrhea, stomachache, or nausea and fever, vomiting or debilitation, and deemed a better indication of infectious gastroenteritis, rather than a response to an irritant, than is GI when illnesses are self-reported). All findings of significant swimmer-related (*vs.* nonswimmer) GI were at “barely acceptable” (sewage-effluent impacted) beaches. High correlations between Enterococci and health effects were found at one of the marine beaches (New York City), and between both Enterococci and *E. coli* at the freshwater beaches (EPA 1986).

During the course of developing the 2012 WC (and under a mandate by a 2000 revision to the Clean Water Act, “the Beach Act,” EPA, 2010), EPA hosted an experts’ workshop to discuss risks of exposure to human *vs.* non-human fecal sources (noting a wide belief “that human feces pose a larger health risk than animal feces,” counterbalanced by recognition “that animals do harbor many bacterial and protozoan pathogens that pose a human health hazard,” and that “animal feces are often deposited in freshwater that receives no treatment.”). Expert consensus on the matter included:

- 1.) For viral pathogens, human risks were deemed non-existent or negligible (N) from agricultural animals, wildlife, and pets. Direct human risks (“fecal shedding by bathers”) were deemed low (L) or high (H) based on age of the source (children > adults). For sewage, risks were H, regardless of treatment (untreated, secondary treatment, secondary treatment with chlorine) unless secondary treatment was combined with chlorination and UV exposure (and depended on UV energy). The experts could not place a consensus risk measure on stormwater (“risk largely depends on amount of human feces present”).
- 2.) For protozoa, risks were deemed highest for wildlife (L for aquatic birds, M for “Other, e.g., deer”), followed by agricultural animals (N for poultry, M for “Other, e.g., cattle, sheep”) and pets (L). Risks from humans or human sources were unchanged from above.
- 3.) For bacterial pathogens, risks from were highest for agricultural feces (M-H for both subcategories, poultry and other), followed by wildlife (L-M for birds, M for other) and Pets (L). Humans and their sources remained unchanged except for a reduction in risk for treated sewage (M for secondary treatment, L when combined with chlorination).

Risks from secondary environments (sediment suspension and contact with beach sand) were deemed low for viruses and protozoa, and medium for bacteria (Table 5, EPA, 2007).

In 2009, EPA conducted a literature review, of extant epidemiological studies and disease-outbreak reports, to characterize relative risks from presence in recreational waters of fecal contamination from different sources. To identify potentially relevant epidemiological studies, EPA reviewed the reviews of others: three, largely meta-analytical and providing little in the way of study critique - Pruss (1998), Wade, *et al.*(2003), Zmirou, *et al.* (2003); and one - Sinton, *et al.* (1998) that was performed specifically to differentiate relative risks from exposure to feces derived from humans and non-humans (and found insufficient data for any conclusions). The Agency found mention of 40 studies. EPA further identified five more recently completed epidemiological studies (not yet summarized in a peer-reviewed summary) for a total of 45. Of the 45 studies identified, only one (Calderon, *et al.*, 1991) was specifically designed to evaluate correlation between swimmer illness and animal wastes and found no significant association between GI and FIB densities. EPA noted that Calderon’s results had subsequently been

reviewed (McBride, 1993, cited as “Comment on Calderon, R., E. Moodi, and A. Dufor, 1991. Health effects of swimmers and non-point sources of contaminated water. International Journal of Environmental Health Research, V. 1, pp. 21-31,” another reference that seems important, but that I seem incapable of acquiring) and criticized as being of insufficient sample size. The Agency also found six other studies, namely Cheung, et al. (1990), McBride, et al. (1998, ironically, later criticized by others for sample size, see below), Haile, et al. (1999), Dwight, et al. (2004), Weidenmann, et al. (2006), and Colford, et al. (2007) that even included waters not predominantly impacted by sewage sources (each separately discussed below) and found that evidence of differential risks from non-human fecal sources was “equivocal.” EPA review of waterborne-disease outbreaks involved a review of Centers for Disease Control (CDC) reports of outbreaks associated from drinking water and recreational water, the former being supplemented by a Google search for “drinking AND water AND outbreak” and the latter being supplemented by an abstract search of the DIALOG database. Review of the drinking-water outbreaks (the majority of reports), based on reviews of others covering CDC reports between 1920 and 2006, and supplemented by the Google search, leads EPA to conclude that “human illnesses can and do occur from animal-based contamination, though the data do not “enhance the current ability to quantitatively differentiate risks from animal- versus human-related pathogen sources for recreational water exposures.” Using a summary provided by Craun, *et al.* (2005) to review waterborne outbreaks from recreational waters (reported by CDC since 1978, and including outbreaks associated with “swimming pools, wading pools, spas, waterslides, interactive fountains, wet decks, and fresh and marine waters” in the definition of recreational waters), EPA found 249 relevant outbreaks. Of those outbreaks, half included information of possible source, and 90% of those were attributed to feces of ill bathers, bather overcrowding, or the presence of

diapered children (Craun, *et al.*, also estimated that 18% of the 249 outbreaks were of animal origin, though noting few were confirmed). Depending on other reviewers, EPA provides summarized information on each CDC-reported recreational-water disease outbreak from 1999-2004 at Table IV.5.1 (EPA, 2009). Review of the table reveals that, of the 144 outbreaks summarized, 2 can be characterized as outbreaks from natural waters (river, lake, pond, reservoir, ditch water) and of likely source involving (non-human) animals: one GI outbreak in a Minnesota lake, probably contaminated by geese, in which fecal coliforms were detected by monitoring; and one GI outbreak in a Washington lake, with the source attributed to “possible human feces, but duck feces could have helped to sustain contamination for a longer period of time,” where *E. coli* O157:H7 were isolated from both human and duck feces. Results from the DIALOG abstracts were presented at Table IV.6 (EPA, 2009). Of the 27 items listed in the table, three outbreaks in natural waters where human sources were not a likely source implicated are presented: one canal in France where Leptospirosis was likely from rodent urine (elevated *E. coli* was monitored but not expected to indicate urine of any source, 30.8% of 130 locally trapped rats were found to be seropositive); one lake in Canada, in which Schistosomiasis was attributed to snails (low FIB monitored, though not likely to indicate any snail excreta, ocellate cercaria detected in local snails); and one Brazilian swimming pool supplied by water from a brook (and, again, Schistosomiasis from snails). EPA, again, concludes that “recreational water outbreak literature does not appear to enhance substantially the current state of knowledge on quantitatively characterizing risks.” (EPA, 2009)

Also in 2009(a), EPA conducted a literature review of information regarding 70 known animal pathogens, and winnowed the list to 20 that, by primary host, life-cycle, etiology, and pathogenicity had the potential to be waterborne zoonotics. By review of relevance to the United

States, based on CDC disease outbreak statistics (both recreational and drinking water), the Agency further narrowed the list to six “key waterborne zoonotic pathogens”) that present potential risks in untreated recreational waters:

- Pathogenic *E. coli*,
- *Salmonella*,
- *Camplobacter*,
- *Leptospira*,
- *Cryptosporidium*, and
- *Giardia*. (EPA, 2009a)

[Note: as shown elsewhere in this review, protozoa (e.g., *Cryptosporidium* and *Giardia*) are notoriously uncorrelated to FIB, transmission of *Leptospira* is by urine contamination and unlikely to correlate to FIB, and *Salmonella* and *Camplobacter* are considered environmentally endemic by many.]

As a result of the above fact-finding efforts (and as partial settlement of a lawsuit), EPA conducted an epidemiological study at Surfside Beach, SC, impacted by diffuse sources (primarily urban runoff, with frequent rains, no upgradient septic tanks, and alcohol prohibitions and pet exclusion during swimming season) as a part of the NEEAR effort. Though swimmers experienced greater incidences of rashes, GI, and earaches than did non-swimmers, and though culturable Enterococci densities spanned a range from undetectable to log 2.81 CFU/100 ml, no significant correlation could be found between health effects and FIB densities (and qPCR measurements of Enterococci were actually inversely related to skin rash). EPA reported that the results were consistent with, but insufficient for, a conclusion of reduced risk for non-sewage sources vs. sewage ones (Wade, *et al.*, 2010).

[Note: This study (and those at all of the NEEARS beaches) included still another redefinition of the GI medical outcome measured, namely NGI (NEEARS gastrointestinal illness). NGI is less restrictive than HCGI in that one of vomiting, fever, or debilitation is not required for inclusion in NGI. In the absence of vomiting and debilitation, however, symptoms were included in the new category only if they met a more restrictive definition of diarrhea (three or more loose stools in a 24-hour period), or included nausea and stomachache. This revised GI definition was still deemed “highly creditable,” for self-reported gastroenteritis, but more likely to include viral gastroenteritis (which often presents without fever). EPA 2012]

2.2.3.2 Potentially Relevant Epidemiological Studies

EPA (2009) reviewed epidemiological studies for potential to illuminate differential swimmers’ risk from human- vs animal-feces sources, and identified only seven completed studies that were conducted on sites that included substantial non-sewage sources. EPA deemed the results of these studies equivocal, but some might be better characterized as downright contentious.

Cheung, et al. (1990) conducted a large (18,741 usable responses) prospective cohort study of nine Hong Kong beaches to determine various swimming-related risks in subtropical waters, and the relation of those risks to pollution (as measured by various indicators), for potential use in setting WC. Notably, in this review, six of those beaches were impacted by point-source sewage sources, two primarily received river runoff dominantly contaminated by pig feces, and one had both a sewage outfall and pig-related drainage. Fecal coliforms, *E. coli*, *Klebsiella* spp., fecal Streptococci, Enterococci, Staphylococci, *Pseudomonas aeruginosa*, *Candida albicans*, and total fungi, as potential indicators, were tested against self-reported GI, HCGI, earaches, eye irritations, skin rashes, respiratory illness - RI, and fever. For each potential indicator, the beaches were divided into “relatively unpolluted” (RU) and “barely acceptable” (BA) groups. Allocation of beaches into the groups was based on the threshold derived by sequentially moving the next (in terms of increasing indicator densities) beach from RU to BA to

achieve the maximum difference in the number of significant swimmer-related symptoms between the beach groups (GI, HCGI, skin rash, a combination of HCGI and/or skin rash, respiratory symptoms, and total illness incidence were considered here). The lowest indicator density that maximized the numerical difference (RU vs BA, 2 vs 5 respectively) between symptom groups showing significant swimmer-related illnesses was found in an *E. coli* threshold of 180/100 ml. The best linear correlation, between log *E. coli* at a beach and symptom group incidence at that beach ($r = .73$, $p \leq 0.05$), was for the combined category of HCGI and skin rash (though no significant correlation could be established for either symptom group separately). The authors suggested the intercept of that line (24 *E. coli*/100ml, corresponding to zero incidence of either HCGI or skin rash) and their newfound threshold of maximized difference in number of significant swimmer-illness symptom groups (180/100ml), together with an existing criterion defining 610/100 ml as “unacceptable,” to define new water quality criteria (i.e., $0 < \text{Good} < 24 < \text{Acceptable} < 180 < \text{Barely Acceptable} < 610 < \text{Unacceptable}$).

Despite the similarity in terminology, the RU and BA category definitions in this study are different than those used in the EPA Bathing Beach Studies (EPA 1986). In the EPA analysis, categories were defined on differences in historically monitored FIB and by known presence of nearby sewage sources within the watersheds of BA beaches. In Cheung, *et al.* (1990) this distinction was based solely on found FIB and found human health effects, regardless of the source of the former (presupposing an equality of sources as a predictor of risk). The approach here resulted in a pig-dominated beach (4th best water quality in terms of geometric-mean *E. coli* densities, best in terms of GI and HCGI, worst in terms of skin rash) being grouped with a mixed-source beach (best in total illnesses incidence, second best in skin rashes, second worst in FIB) in the BA category (and observation of the authors’ Figure 3 reveals that these two

beaches represent the worst outliers in the *E. coli*/combined health-effect correlation). The best beach in terms FIB density (sewage-dominated, categorized RU), however, exhibited the third worst total illnesses incidence.

The EPA (2009) review of Cheung, *et al.* (1991), noted that a significant relationship between *E. coli* densities and combined gastroenteritis and skin rash could be established, but that significant correlation between *E. coli* densities and either gastroenteritis or skin rash could not. A more recent review (Dufour, *et al.*, 2012) found sufficient reason and information to re-analyze the data. The authors grouped the beaches by predominant fecal source as described in the original article (human for six beaches, pigs for two, and mixed for one), disregarded data presented for the mixed-source beach, and analyzed the remaining data presented in the original article with respect to one (HCGI, not grouped) symptom. They found mean *E. coli* densities in the manure-dominated beaches (mean = 978/100 ml, range from 243 to 1714, n = 1326) to exceed those found at sewage-dominated beaches (mean = 187, range from 69 to 269, n = 15,116) five-fold (and Enterococci densities were about double). They determined (Fisher's Exact) the significance of swimmer-related illnesses (*vs* non-swimmers) in each group. They note the poor resolution resulting from the re-arrangement of the reduced dataset but, despite the large densities of FIB, they find the likelihood of real swimmer-related gastroenteritis at the livestock-drained beaches to be quite small ($p = 0.5246$). The potential for real illness at the sewage-contaminated beaches was more significant ($p = 0.0418$). The reviewers interpret this analysis as evidence of a lesser risk from animal- than human-feces exposures.

Calderon, *et al.* (1991), with an intent to explore swimmers risk from animal-feces exposure, studied a small (three acre) semi-rural pond in Connecticut. The pond was constructed for recreation by the damming of a river, and included a riprap barrier upstream to de-silt

incoming water. The authors surveyed the watershed to insure absence of point sources of pollution (including septic tanks). The surrounding forest was a known home to squirrels, rabbits, rodents, and deer. The authors measured FIB (*E. coli*, Enterococci, fecal coliforms) as well as *Staphylococcus*, spp. (previously shown to be shed by bathers and a potential indicator for bather density) and rainfall for 49 days (they also monitored *Pseudomonas aeruginosa*, but subsequently neglected the data due to very low presence in the water). Health data (concerning GI, headache, earache, backache, itchy eyes, skin rash, and wheezing), self-reported by 104 families with access to the pond, provided 1310 swimmer exposure (defined by full submersion) person days and 8356 non-swimmer (including non-swimming bathers) person days. FIB correlated to rainfall, and geometric means of the coliforms (double) and Enterococci (fourfold) on 16 rainy days significantly exceeded those on 33 rain-free days (Staphylococci showed no such response to rain). *E. coli* and Enterococci correlated to each other, as well as rain, and Staphylococci correlated to bather density on given days. Swimming-related GI (22.9 cases per 1000 person days vs. 2.6 for non-swimmers) was significant, with relative risk = 8.7. Noting the limited resolution of their small study to detect a dose-response curve for indicator vs. swimmers' risk and noting the strong correlations of indicators with other measured parameters (FIB with rain, Staph with bather density), the authors segregated their data into high/low exposure groups (rainy/dry weather, high/low bather density) and tested for significant differences between the pooled groups. Differences in excess GI were not significantly associated with high/low rain ($p = 0.089$), Enterococci ($p = 0.059$), *E. coli* (0.412), or fecal coliforms ($p = 0.159$), but were associated with higher bather density per day ($p = 0.011$, relative risk = 4.8) and Staphylococci ($p = 0.026$, relative risk = 2.6). The authors concluded that

swimmers' risks were better explained by exposure to other swimmers than to animal feces exposure.

The EPA (2009) review of epidemiological studies notes McBride (1993) comments to the effect that a larger study population would have revealed significant FIB-illness associations. Sinton, *et al.* (1998), in a literature review targeted to fecal-source identification in (livestock dominated) New Zealand, also note the McBride (1993) criticisms, and further add that the Calderon (1991) study focused on wildlife rather than livestock sources. The 2012 review (Dufour, *et al.*) provides no criticisms.

McBride, *et al.* (1998) conducted a prospective cohort study of relationships between self-reported exposures (defined as “unexposed” for those who did not enter the water, “paddlers” who entered the water but did not submerge their heads, and “swimmers” who submerged their heads in the water, with swimmers further subdivided into those who reported “swimming” for less than or greater than 30 minutes) to measured FIB (fecal coliforms, *E. coli*, and Enterococci) and self-reported health effects (GI, HCGI, and a combined “any GI,” respiratory illness, and “other” illnesses including ear infection, eye infection, sore throat, and skin rash) of beachgoers at seven New Zealand beaches (categorized as minimally impacted “control,” animal-waste impacted “rural,” or human-waste impacted “oxidation pond”). Absolute risks (regardless of exposure) were referenced, by beach or by exposure, to the risk exhibited by the overall unexposed population of all beaches to determine relative risks and/or risk difference in analyses (even though one of the beaches, unidentified, had “a significantly higher rate” of HGCI and respiratory illnesses reported by unexposed beachgoers). “Because epidemiological studies are often concerned with threshold effects,” the authors screened risk *vs.* indicator density relationships, only modeling such relationships if they first showed

monotonically increasing risk against increasing dummy variables representing the quartile in which an indicator density was found. FIB concentrations at impacted (non-control) beaches were described as “surprisingly low,” though occasional exceedances of extant local WC were observed in all beach categories. Means of indicator densities, calculated for each day for each beach, were pooled, and the quartile ranges for each FIB in the pooled data were derived. Usable exposure/health-effect reports (n = 3887, including 1577 exposed beachgoers, 1200 of which were swimmers) were grouped, by beach and by health effect, and absolute risks (per 1000 beachgoers) calculated. Though HCGI (when compared to Enterococci densities) was retained for further analysis because of its importance in other studies, only “any gastrointestinal illness” and “respiratory illness” (definition of both, notably, did not require associated fever) when compared to the pooled Enterococci-density quartiles were likewise retained on the basis of significant findings. Absolute risks for these three health effects were then segregated, by exposure, into groups based on the corresponding quartile of enterococcal density on the day/beach at which beachgoers were exposed. Conversion of those absolute risks into relative risks (with and without adjusting for age of beachgoer) revealed a significant ($p < 0.001$) risk ratio (RR) greater than one (1.77 – 4.77, 95% CI) for respiratory illness for all exposed beachgoers. Decomposition of those risks by exposure revealed RR respiratory illnesses significantly greater (95% CI) than one for long-time swimmers (> 30 minutes, RR = 3.31), paddlers (RR = 4.53), all swimmers (RR = 2.46), and all exposed beachgoers (RR = 2.91), though not for short-time swimmers, at all (combined) beaches when enterococcal densities were in the highest quartile. Moreover, though RRs for those exposures at lower quartiles were not significantly different from one, they rose monotonically with rising quartiles, providing some evidence of a dose-response relationship at quartile resolution. Decomposition of relative risk by

beach category revealed significant RRs for all beachgoers exposed to enterococcal densities in the highest quartile, at oxidation-pond beaches (RR = 3.17) and rural beaches (RR = 2.97), though RRs did not rise monotonically with quartiles in the latter. No RR was presented for control beaches with enterococcal densities in the highest quartile (“The upper two quartiles were combined for technical reasons”). The authors attempted to model a significant dose-response relationship between Enterococci densities and respiratory illnesses at greater than quartile resolution of the former. Their attempts failed (both at decile and actual-data definition) for inconsistency (failure to find monotonic increase of illness *vs.* source). The authors decried a less than expected sample population (n = 3877). No useful relationships could be established for other indicators (*E. coli*, fecal coliforms) or illnesses (though RR for “any gastrointestinal illness,” distinct from HCGI, of long-time swimmers exposed to third-quartile densities of Enterococci at all combined beaches was significantly different from one, at 2.04). The authors find their results consistent with an etiology involving inhalation of wave-aerosolized fecal pathogens, but note that presence of a respiratory irritant (rather than an infectious agent) could not be discounted. They conclude:

“No evidence has been found to suggest any merit in separating beachgoers’ illness risks on the basis of the type of faecal material present (i.e. from rural areas versus from oxidation ponds treating human wastes). Illness risks at the control beaches (Wenderholm and Rabbit Island) were significantly lower than at beaches believed to be impacted by oxidation pond effluent (Omanu, Raglan, Paraparaumu) and by rural runoff (Ohope and Spencerville). There was, however, no significant difference in illness risks between the two types of impacted beaches.”

They suggest that future studies of larger populations impacted by greater FIB-density ranges would reveal more significant health effects and quantifiable dose-response relationships.

The editor in me finds the authors’ wording of their conclusions troublesome, not because of any inaccuracies but because of the way they can be (and have been) misleading when uncritically cited. The use of pooled (across beaches and, therefore, fecal sources) data, both for

the quartile assignment of enterococcal densities and for defining the illness risk of the unexposed population, potentially masks beach-specific (and, therefore, source-specific) differences in exposure risk (especially considering at least one beach with “a significantly higher rate” of illness rates reported by unexposed beachgoers, and “surprisingly low” enterococcal densities at impacted beaches). The authors’ assertion that “No evidence has been found to suggest any merit in separating beachgoers’ illness risks on the basis of the type of faecal material present” is patently and factually correct, but could potentially be explained by “they didn’t look.” Likewise, “Illness risks at the control beaches...were significantly lower than at beaches believed to be impacted...” is unassailable, but the authors’ inability to derive the RR for control beaches at the highest quartile of enterococcal densities (the only quartile for which the impacted beaches showed demonstrably non-zero exposure risk) notably affects the importance of the statement. Finally, “no significant difference in illness risks between the two types of impacted beaches” cannot be disputed. The 95% CI ranges of RRs found, at rural (1.33 – 6.60) and oxidation-pond (1.81 – 5.55) beaches, overlap. However, inclusion of the lack of any evidence of a dose-response relationship (even at the quartile level of resolution of the independent variable) in rural beaches invites questions in judging potential causes of that coincidence.

The EPA (2009) review of this study reports “Log-linear modeling of the results demonstrated a statistically significant association between illness and enterococci densities,” and “no significant differences in illness risks” between impacted-beach categories (noting the authors’ inability to establish satisfactory FIB dose-response relationships), and significant difference between the impacted and control beaches (without qualification). Dufour, *et al.*

(2012) noted the lack of good dose-response information and the difficulties in interpreting source-specific effects from pooled data (without explanation).

Haile, *et al.* (1999) present data from a 1995 epidemiological study conducted in Santa Monica, CA. The study examined relationships between self-reported health outcomes (two levels of HCGI, significant respiratory disease or SRD – the definition of which includes fever and/or coughing with phlegm rendering the category more “highly creditable” for infectious etiology than is RI, fever, chills, eye discharge, earache, ear discharge, skin rash, infected cut, nausea, vomiting, diarrhea, diarrhea with blood, stomach pain, cough, runny nose, cough, cough with phlegm, and sore throat) of 10,459 (usable) subjects associated with indicators (binned concentrations of total coliforms, fecal coliforms, Enterococci, total-/fecal-coliform ratio and, most relevant here, distance from a storm drain) at three beaches in Santa Monica Bay. All three beaches were primarily impacted by urban runoff at municipal separate storm system (MS4) outfalls draining to the beaches. The authors calculated RRs for exposures at various distances upcoast or downcoast from the outfalls referenced to control risks evidenced > 400 m from the outfalls, and found significant (95% CI range excluding one) RRs for swimmers at the outfalls (< 1 m upcoast or downcoast from the edge of the outfall) for fever (RR = 1.61), chills (1.60), ear discharge (2.09), cough and phlegm (1.65), and SRD (1.78). They further found that distance from the drain served as useful proxy for FIB densities.

These data have often been uncritically presented (e.g., Schiff, *et al.*, 2000, and Arnone and Walling, 2007) as evidence of significant health effects to swimmers exposed to sources other than sewage. “Uncritically” in this case cannot be construed as blameworthy; in fact it is not surprising considering the presentation in Haile, *et al.* 1999. Relevant information concerning site description is never explicitly presented in the article, and can only be found in

the author's reference (12), namely a previously published technical report of the same study (Haile, *et al.*, 1996). That earlier (hard to find) report describes the Santa Monica MS4 as historically contributing to high FIB densities and pathogenic human viruses, and prone to "sewage spills and hydraulic overload following rainstorms" intermittently leading to "discharge of primary treated sewage and floatables such as tampon applicators into storm drains," with "leaky sewer lines, illegal sewer connections, blocked sewer overflows, leaky septic tanks, and local direct human sources" contributing. It was the fact that the MS4 was not very separate and was releasing its suspect effluents without treatment that originally justified this (expensive) epidemiological study, funded by the Santa Monica Bay Restoration Project. Data from this study subsequently justified proposals for a public-awareness campaign to educate swimmers to potential hazards of untreated storm-drain effluents, increased sanitary-survey efforts, and source-control measures. The earliest citation I found that references this qualification to the study results was Colford, *et al.*, (2007). The EPA review (2009) notes the qualification, citing Colford.

Dwight, *et al.* (2004) surveyed 1873 surfers in urban North Orange County (NOC) and rural Santa Cruz County (SCC, with urban/rural difference defined by higher human population density in the former), CA, for health symptoms (SRD, HCGI, fever, nausea, stomach pain, vomiting, sinus problems, cough, phlegm, sore throat, eye redness, ear pain, and skin infection) recalled from the prior three months, water exposure, educational level, income, political outlook, and level of concern about water quality on 1 April in 1998 (an El Nino winter resulting in record high precipitation across California) and 1999 (a La Nino winter with record low precipitation in NOC and very low precipitation statewide). Though FIB data were not collected, local health-agency data showed greater total coliform densities in coastal waters in the wet year

than the dry year at both sites and (despite lower rainfall in both years) higher coliform concentrations in NOC than in SCC. Logistic regression of adjusted odds ratios (ORs) between the two counties, and stratified by year were presented. The authors found almost twice the reports of “any symptom” in NOC compared to SCC (OR = 1.85, 95% CI = 1.4 to 2.5), and higher rates for every symptom in NOC (including 95% CI ranges excluding one for HCGI, fever, stomach pain, diarrhea, sinus problems, sore throat, eye redness, and skin infection) during the 1998 winter. In 1999, the difference (NOC/SCC) in all symptoms was considerably smaller (OR = 1.17, 95% CI = 0.9 to 1.6), the number of symptoms in which NOC surfers exceeded those in SCC declined (to 6 of 12 symptoms, with an OR CI range distinct from one only for sore throat), and a general decrease in all symptom ORs was found year over year. They further found that risk of any symptom reported in either county typically increased by about 10% for each added 2.5 hours of water exposure per week. The authors concluded that release of untreated urban runoff to beach sites represents a potential comparative health risk. The EPA (2009) review of this article provides no criticisms or added insights.

Wiedeman, *et al.* (2006), conducted a randomized controlled study of associations between multiple health-effects and multiple indicators at five freshwater beaches in Germany to better inform the setting of regulatory limits on fecal exposures to bathers. Though the fecal sources to beach waters included “treated and untreated municipal sewage, agricultural runoff, and contamination from waterfowl,” the authors provide no analyses of effects based on fecal source, and provide no data from which disaggregation effects by source might be divined. The EPA (2009) review of this article concurs.

In a prospective cohort study, Colford, *et al.* (2007) recruited 8797 (usable responses) volunteers for various levels of exposure (swimmers with any water contact, swimmers who

swallowed water, and nonswimmers who did not get wet) to measured indicators (Enterococci – measured both by culture methods and qPCR, total and fecal coliforms, *Bacteroides*, spp. somatic coliphage, male-specific coliphage, and adenovirus, and norovirus) of water quality to assess the rates of medical outcomes (GI, two levels of HCGI, dermatologic symptoms, RI, and SRD). The six beaches at Mission Bay, CA, at which the study took place, had recently been found to be largely free of human-fecal inputs (< 10% as estimated by microbial source tracking, MST, techniques), and grazing livestock were absent from the watershed. Regression of ORs for exposed groups against nonswimmers revealed significant excess diarrhea and skin rash for all exposures (any water contact, water on face, water swallowed). Disaggregation by age showed that most of these symptoms (and cramps) were exhibited by younger swimmers (ages 0 to 5, or 5 to 12). No correlation could be established between illness risks and FIB densities (culturable or by PCR), somatic coliphage, pathogens (although only one pathogenic virus particle, an adenovirus was detected in one sample), or dichotomous densities (exceeding vs. not exceeding state standards) of Enterococci. Noting the absence of significant swimmer risk from HCGI and severe respiratory symptoms, the authors report the possibility that swimmer risks that were found could be attributed to salt-water irritation as well as to any infectious agent. The authors did find significant correlations between rates of HCGI, nausea, and fever, and densities of male-specific coliphage¹ but caution that that indicator was rarely detected and only when few swimmers were present. The authors conclude that lack of correlation between illness and indicators was a consequence of the paucity of human fecal material.

¹ ”Male-specific RNA coliphages are promising candidate indicators of human viruses in waters” (EPA, 2001). Further testing/typing of the coliphages, under procedures still under development, may have provided an evidentiary link between even these cautionary, small-sample effects and the “<10%” human sources to the bay (Field and Samandpour, 2007, and Stewart-Pullaro, *et al*, 2006, and see section 2.2.3.3 below).

In a prospective randomized exposure study, at an (unidentified) subtropical beach of no known domestic sewage sources, Fleisher, *et al.* (2010), studied the incidence of self-reported gastrointestinal illness (defined in a manner equivalent to NGI), acute febrile respiratory illness, eye and ear infections, and skin infections, in relation to randomly assigned exposures (bathers who spent 15 minutes in individual, 5-m wide swimming zones of ~knee-deep water, and submerged their heads three times; and non-bathers who spent 15 minutes in chairs set on plastic sheeting at a location “far from water and sand exposure”) to bather-collected enterococcal densities (i.e., subjects collected water samples from their own swimming zones prior to head immersion, and densities ranged from undetectable to 3320/100 ml over the course of the study). The usable sample of subjects was 1303 (651 bathers and 652 non-bathers). Elevated adjusted illness rates of bathers over non-bathers were established for gastrointestinal disease (OR = 1.79, 95% CI = 0.94 to 3.43, $p = 0.07$), respiratory illness (4.46, 0.99 to 20.97, 0.051) and skin illness (5.31, 2.58 to 10.96, 0.0001), and an enterococcal dose-response correlation was calculated for skin illness. That malady increased 1.46-fold (0.97 to 2.21 CI range) for each log increase in enterococcal density ($p = 0.07$). The authors note that their failure to establish adequate dose-response relationships for other illnesses “may be a result of the study sample size,” and concluded, “Our findings suggest that there is an increased risk to bathers even when using marine recreational waters with no known source of domestic sewage. We also observed a correlational dose-response association between skin illness and increasing enterococci exposure.”

Review of the eight studies presented in this section (plus the NEEARS study at Surfside, SC, discussed above) finds only seven in which human fecal sources were either largely excluded (Wade, *et al.*, 2010, Calderon, *et al.*, 1991, Colford, *et al.*, 2007, and Fleisher, *et al.*)

from the study, or where presentation of data from the study allowed for separation of swimmer risk from exposures to different (human/non-human) fecal sources (Cheung, *et al.*, 1990, McBride, *et al.* 1998, and Dwight, *et al.*, 2004). Only four of these studies revealed elevated swimmers' risks for health effects categorized as "highly credible" (easily attributable to infections) for exposures to exclusively or largely non-human fecal sources (NGI at Wade, *et al.*, 2010; HCGI at McBride, *et al.*, 1998; HCGI at Colford, *et al.*, 2007; and NGI and SRD at Fleisher, *et al.*, 2010). Moreover, none of the studies was able to establish a correlation between any highly credible risk and the density of any FIB over the range found in the study, and only one (Fleisher, *et al.*, 2010) was able to establish a satisfactory effect/dose relation between any health risk and any FIB (skin rash *vs.* Enterococci). As to questions concerning a difference between swimmer risks at human- *vs.* animal-impacted recreational waters, the results reviewed here are distinctly different from those found in the seven (non-tropical) NEEARS studies, the five Bathing Beach Studies, and Haile, *et al.*(1999), that were specifically chosen to elucidate effects traceable to known sewage sources (and all of which contribute to a conclusion that "highly credible" and infectious swimmer risks sufficiently correlate to FIB densities to allow derivation of WC that predictively reduce morbidity among users of recreational waters to acceptable levels). The difference between the two classes (human source *vs* non-human source) of epidemiological studies also provides considerable weight of evidence to a conclusion that risks associated with non-human fecal sources are less than those presented by sewage. The unique nature of study sites (with differing animal sources present at different sites), small sample populations in several of the studies, and the inability to confidently distinguish between irritant and zoonotic etiologies for many ailments, however, preclude conclusions that results (of absent significant effects) of these studies can be extended to other locales not significantly

impacted by sewage. Moreover, absence of significant effects inherently provides no quantifiable confidence in the null hypothesis.

As mentioned above, the EPA (2009) reviewed seven of these studies and concluded that results were “equivocal” concerning a lesser risk from non-sewage sources to recreational waters than from human sources. Dufour, *et al.* (2012), reviewing four of them (Cheung, *et al.*, 1990, with reanalysis, Calderon, *et al.*, 1991, McBride, *et al.*, 1998, Colford, *et al.*, 2007) concluded that those studies “do not provide evidence for associations between swimming-associated risk and exposures to bathing waters contaminated with faeces from animals or birds” and noted that other studies (outbreak investigations and case-control studies) “have not established a definitive link between water contamination and specific sources.” They then asked the question, “can the lack of excess faecal-associated illness in swimmers be taken as evidence that animal contaminated waters do not pose a health risk to swimmers?” The authors opine that the inherent differences of indicator/pathogen ratios imposed on the system by near-universal treatment of one source and near lack of treatment of the other, and the fact that swimmer access to a beach is based on indicator standards derived from the former; they conclude that any beach regularly posing a (indicator-derived) potential zoonotic risk would have no bathers to study. The authors then pose the question as to whether any epidemiological study to determine the presence/absence of risks from animal-/bird-contaminated waters can be conducted. Based on low frequency of potentially zoonotic pathogens, high indicator/pathogen ratios in untreated wastes with permission to expose humans based on the indicators, high morbidity/mortality rates of some (even if rare or not contagious through waters) potential zoonotics, and the poor ability of epidemiological study designs to quantify sporadic, episodic risk, they again conclude that it is unlikely. “Until the time when information is available to regulators for developing water quality

criteria for waters contaminated by non-human faecal wastes, they may be left with no other choice than regulating animal-polluted bathing water as if it poses the same risk as human-contaminated bathing water.”

In addition to (and possibly tangential to) the arguments (largely based on regulatory/ethical considerations) above (Dufour, *et al.*, 2012) to identify a (potentially inherent) futility in attempts to conclusively “prove” a distinction between risks from animal/human sources by epidemiological studies, one might add a geographical argument. Recall that, of the studies reviewed, only Calderon, *et al.* (1991), by exhaustive study of a small watershed, were able to exclude any possible current sources of human feces (except, of course, for the bathers themselves, an irreducible inclusion in any study of human health risks, which were found to be the most likely source of risk), and their study was subsequently criticized for small sample size that potentially masked other significant epidemiological risks. In all other studies reviewed, differential risks presented by different fecal sources were based on a presumed differential in human/animal fecal contributions (as indicated by presence/absence of known sewage sources or by higher/lower population densities) in the (larger) watersheds impacting the study sites, and only one of those (Colford, *et al.*, 2007) made any attempt to estimate (by MST methods) the magnitude of human sources to the “animal-impacted” sites. It would seem difficult to quantify relative risks presented by human/animal fecal sources, in any epidemiological study, if the relative contributions of human/animal feces impacting the study sites were unknown.

Watersheds of small enough area for exhaustive exclusion of current human-fecal sources would be unlikely to exclusively impact sites of sufficiently large recreational-water acreage to examine large-sample significance of swimmer risk. In the absence of knowledge concerning relative

human/animal fecal contributions to any site, establishment of any relative human-/animal-sourced risk at those sites would be difficult in any epidemiological study.

Difficulties in determination of differential risk presented by different sources of contamination arise, at least in part, from the lack of source specificity exhibited by current FIB.

2.2.3.3 Relative Fecal Contributions

“However, it is important to note that, irrespective of the relative risks involved, improved methods for identifying and apportioning faecal sources would assist water managers in lowering overall faecal pollution levels.” (Sinton, *et al.*, 1998)

The potential for misclassification of risk presented by the poor source specificity of currently used FIB has led to efforts to determine, by other means, where in the world the FIB found in recreational waters actually come from. The authors’ attempts to differentiate health risks posed by human- vs. animal-impacted waters, in the epidemiological studies reviewed in the preceding section, represent such an approach (though of coarse resolution).

2.2.3.3.1 Geographical Source Tracking

Many such source-tracking efforts take the form of simply monitoring for FIB across finer-grained geographical subdivisions of the source areas impacting the receiving waters, to better differentiate the likely sources of the FIB (and the delineation of source areas harboring persistent FIB populations, reviewed at section 2.2.2 provides many examples). Monitoring of FIB (with poor source selectivity) and calculation of FIB loads, at sample points selected to represent drainage and/or landscape likely to be dominated by FIB from specific fecal sources, together with concomitant FIB monitoring (and total loads implied) at receiving waters, can

produce a quantifiable geography of segregated FIB sources relative to the total FIB impacting the water body. Such efforts (at least) provide prioritized targets for source remediation to improve the microbiological quality of receiving waters.

Non-human animals often contribute far larger fecal loads to the landscape than do humans. Not surprisingly, non-point sources, non-sewage sources, and non-human sources of feces have been found to be significant sources of FIB to many receiving waters.

Weiskel, *et al.* (1996), intensively sampled potential fecal sources throughout the watershed draining to a small (2.14 km²) embayment (Buttermilk Bay) in Massachusetts with a long history of shellfishery closures due to FIB-based standard exceedances, to develop an FIB budget and to target remediation efforts to return the waters to compliance with standards for designated use. Flow and fecal coli (FC) densities (all subsequently confirmed as *E. coli*) were directly measured repeatedly at the only “outfall” from the catchment, the drain of the lobster-holding tanks at a bayside seafood facility, to determine an annual load from that source. The only other direct-pathway load considered, waterfowl fecal deposits, was estimated by frequent bird (ducks, geese, and swans) counts, together with literature values of daily per-bird FC production. Wet-weather samples were taken (30-min after onset of rain and after 6 mm of rain had fallen) from the six (intermittent, draining impervious surfaces) storm drains from the watershed, and samples were regularly drawn, over the course of the study, from the mouths of the three continuous streams. Wet weather flows from the surface-water pathways were segregated from dry (more or less than 2.5 cm rain, as measured at a nearby weather station, in the 96 hours prior to sampling). A site-specific rainfall/runoff relationship was derived by summing the runoffs from each source normalized to its sub basin-specific total rainfall (based on sub-catchment acreage). The potential for septic-system FIB to reach the bay was estimated

by sampling effluent at four systems (three cesspools, one drain-field system) and sampling groundwater along transects downgradient in the plume of each. The decay curve with distance was applied to all septic systems in the watershed. FC densities harbored in wrack (decaying mats of aquatic macrophytes, mostly eelgrass) were directly measured by serial elution from weighed samples, and the transport pathway of those organisms was quantified by sampling of water adjacent to a weighed mass (1000 kg, wet weight) of wrack at the high-tide line (for one week after placement of the wrack, and for one week after its removal). Water-column FIB densities were determined over 21 randomly selected, measured nearshore bottoms (1 m², sandy and muddy), before and after mechanical resuspension of sediments, and the differences were summed over the acreage of each bottom type. FIB from septic systems represented the largest source contribution to the watershed but, due to attenuation in groundwater, contributed less than 0.01% to the baywater load. Waterfowl defecation was the second largest watershed source and, due to direct deposition into water, was the largest (67%) contributor to baywaters FIB on an annual basis (though the authors estimated that 98% of the load from waterfowl was deposited during fall and winter months, a time of little recreation or shellfishing). Surface runoff was estimated to contribute 24% of the annualized FIB load in the bay and, moreover, showed a rainfall-driven peak in summer months; on disaggregation, the storm-drain effluents deriving from impervious surfaces contributed far more FIB annually (8×10^{12} CFU) than wet (2×10^{12}) and dry (1.8×10^{12}) streamflows combined. The authors found waterfowl an important source of FIB to water, but concluded that (at least for settings similar to this site) remediation efforts should focus on infiltration of stormwater.

Garcia-Armisen, *et al.* (2005) conducted a similar study to define “priorities for the management and sanitation efforts to improve the microbiological quality of the Seine river

estuary” in France. The estuary was defined as the tidewater segment of the Seine River, and was characterized as a water body of historically poor water quality draining a “highly anthropogenized” watershed. Major sources of FIB (FC) were identified as the upstream Seine (draining developed areas including Paris and its WWTPs, and separated from the estuary by Poses Dam, at which upstream discharge is regularly measured), seven large tributaries to the estuary proper, and the discharges of 20 smaller WWTPs (treating 758,000 inhabitant equivalents in total with the largest, Rouen WWTP, treating 555,000 equivalents). The authors also noted a maximum turbidity zone, representing an area of sediment resuspension caused by tide and salinity gradient that moved up- and downstream in response to river discharge, in the lower reaches of the estuary. They conducted five sampling campaigns at a wide variety of river discharges, as measured at the dam:

- 1.) along a longitudinal transect of the upper Seine (eight sample points from a stretch which included the effluent outfall of a large Parisian WWTP, 6.5×10^6 inhabitant equivalents);
- 2.) at the downstream discharge of the Poses dam;
- 3.) at the mouths of the seven tributaries discharging directly into the estuary;
- 4.) at the inputs and outfalls of five of the directly discharging WWTPs (representing, at 715,000 inhabitant equivalents, 94% of the treatment capacity on the estuary);

as well as 29 sampling campaigns along a longitudinal transect (eight points, over a 160 km stretch) within the estuary. Disaggregating results into low-flow ($< 500 \text{ m}^3/\text{sec}$ discharge at the dam) and high flow conditions, the authors found large and variable FC densities (3-6 log CFU/100 ml) in the upper Seine, in a spatial pattern exhibiting a lower peak at the Parisian WWTP during high-flow (highly dilutive) conditions and higher densities at the dam (due to shorter FIB residence time traveling along the transect) than during lower flows. They found less variability (3-4 log CFU/100ml) in FIB densities within the estuary, with peaks corresponding to the largest WWTP there and to the maximum-turbidity zone under all flow conditions, and with greater densities at high discharges than at low. Despite efficient removal of FIB (~99%) in the

WWTPs discharging to the estuary, the outfalls discharged high FIB densities (~ 5 log/100 ml). Densities in the tributaries varied widely (2-6 log/100 ml) with the highest densities occurring at the mouth of a small, low-discharge urban river. Relative contributions by various sources to the total load in the estuary varied widely with hydrological conditions. At times of high river discharge, the upper Seine was the dominant source (up to 92 % of the total). At lower flows, the tributaries relative contribution increased (to as high as 76%), the small estuary WWTPs became a secondary source (19 – 30%), with the upper Seine contribution dropping to as low as 5%.

Frenzel and Couvillion (2002) measured FIB (FC, *E. coli*, and Enterococci) in a survey of five streams in Anchorage, AK, primarily to assess the impact of residential development on water quality. Sample sites were chosen to encompass a spectrum of population density (sub-basin densities ranging from zero to 1750 residents/km²). Noting that the more populated catchments were more likely to be served by centralized sewer systems than by household septic systems, the authors also included sample points draining areas of similar population densities but differing in their wastewater systems, enabling a comparison between the two. No WWTP effluent drained to any sampled creek, but only the sanitary-sewered neighborhoods had separate storm-sewer drains. The authors also sampled over a March-September (locally ice-free) season to capture potential seasonal effects caused by ice-melt in the upper reaches of the watersheds. High-density (population) sites (>390 persons/km²) consistently and significantly ($p < 0.05$) produced higher densities of all measured FIB than the medium-density sites (390 > persons/km² > 39) and the low-density sites (< 39). FIB concentrations from medium-density site runoff also consistently exceeded those from low-density sites (though for Enterococci, $p = 0.0643$). Comparing only high- and medium-density sites (the only population categories for which such a comparison was possible), the sites serviced by septic systems (with sewage sources in-basin and

no stormwater conveyance) were consistently and significantly lower than those serviced by WWTPs (and MS4) for all measured FIB.

Noting a relative paucity of information concerning the relative FIB contributions by pets, Wright, *et al.* (2009), resorted to actual enumeration of various taxa (humans, dogs, birds, and ghost shrimp, the last a notably heterothermic source), together with source-specific FIB loads per shedding event, to determine the relative contributions to frequent exceedances of criteria at a Florida beach. Virginia Key is a small island bordering Biscayne Bay and east of Miami. The authors studied the easternmost 360 m of a 1.6 km swimming beach, without WWTP influence, with one bathroom facility at the westernmost end, with no impacting storm drains, and with direct runoff from a paved road running 13 m upgradient of the high-tide line. They characterized FIB/dry-weight of dog fecal deposits by collecting nine shedding events from the beach, measuring mass, moisture, and Enterococci of each fecal deposit, and segregating the results from large and small (greater or less than 9 kg) dogs; they further refined average defecation mass by following two dogs (one large, one small) for a week, collecting and weighing all deposits. Avian feces (26 samples) were collected from birds on the beach, and from native birds at a local zoo and a local wildlife rehabilitation center (all of which were fed a wild diet), and included ibis, gulls, pigeons, coots, ducks, herons, and pelicans. Nine shrimp fecal mounds were collected and analyzed, and human shedding/swim was assumed to be equal to that found in a previous study at the beach. Sixty-six panoramic views of the study area were collected over a 16-month period with an automated-pan digital camera placed 440 m from the beach to count birds (by taxon), dogs (by size), and humans (in the water). Shrimp mounds were enumerated along a 50-m transect extending perpendicularly from the shoreline. Mass balance of the sources studied showed dogs to be the largest contributors to FIB at the beach (contributing

6.3×10^{10} CFU Enterococci/panoramic image). At any given time, human shedding (1.3×10^8) and birds (7.0×10^7) accounted for relatively smaller FIB loads. Not surprisingly shrimp contributions were negligible (1.0×10^4) but were (more surprisingly?) consistent throughout the study period.

A notably large fraction of the literature found, relevant to this section, derives from two world regions – southern California, and the British Isles. The regions are worthy of separate treatment not only because of the size and scope of the studies represented, but also for the divergent regulatory contexts under which the studies were conducted.

Subsequent to the findings of Haile, *et al.* (1996 and 1999), of significant swimmer risk presented by proximity of a sewage-contaminated storm drain (see section 2.2.3.2, above) in Santa Monica Bay, CA, a bevy of large studies have been conducted in southern California. Schiff, *et al.* (2001), conducted an inventory of routine coastal (offshore, shoreline, and watershed) microbiological water-quality monitoring efforts (mostly carried out by NPDES permittees and public-health authorities) in southern California. The authors found that, due to the usage value of regional bathing beaches, about \$3 million dollars were spent per year in the region, exceeding such expenditures of the remainder of the state (~ \$0.5 million) and of the country (~\$2 million). The authors further found that individual monitoring entities in the region often used different FIB-assay techniques (limiting regional comparisons of data) and repetitively sampled a small fraction (~7% of total shoreline miles) of potentially impacted areas. The authors urged (successfully, it would seem) greater cooperation, and integration of efforts, between entities measuring water quality in the region. All of the studies cited here have been (of course) conducted under the EPA Federal regulatory regime discussed above (sections 2.1 and 2.2.3.1), and many have been conducted under the auspices of the Southern California Coastal

Water Research Project (SCCWRP). [The Santa Monica Bay Restoration Project, the sponsor of the Haile, *et al.* study (1996 and 1999), has since been renamed the Santa Monica Bay Restoration Commission (SMBRC). Cooperation and integration of operations between SCCWRP and SMBRC is governed by a complex and dizzying array of Memoranda of Agreements too numerous to recount here. Such cooperation and integration, however, is sufficiently evidenced by a common member of their governing boards (General Manager, Los Angeles County Sanitation Districts) as required by the by-laws of both organizations, and the current presence of an SCCWRP Board member on two Advisory Committees of the SMBRC (SCCWRP, 2014 and SMBRC, 2014).]

Noble, *et al.* (2000, and cited as a model study by Schiff, *et al.*, 2001, above), conducted a large survey (253 California sample sites, sampled weekly over a dry-weather, five-week period) of the 270 miles of public beaches (located within a total of 690 miles of shoreline in northern Mexico and southern California and includes Santa Monica Bay) within the Southern California Bight (SCB). The SCB is an oceanographically defined region bounded by (and influenced by a large eddy of) the California Current. The study also included a “round-robin” intercalibration effort, comparing results derived from quality-control samples distributed among 22 regional analytical labs using a variety of assays. Sample sites were randomly stratified into six categories of high/low bather use, sandy/rocky beach, and perennial/ephemeral freshwater sources (mostly storm-sewer drains). The perennial water sources (exhibiting year-round runoff) were sampled at “zero point” (mouth of flow) and/or a randomly selected point within 100 yards of the mouth. The ephemeral (seasonal-flow) streams were only sampled at the zero point, and the 81 freshwater sources accounted for 99% of gauged runoff to the Bight. The study authors also collaborated with Mexican scientists to acquire comparable data for 19 high-use sandy

beaches and for 10 (zero-point, perennial) freshwater outlet sites. The authors measured water quality of the receiving waters as either complying with or exceeding California recreational-water standards for total- or fecal-coliforms or Enterococci (and they also calculated a TC/FC ratio as a measure of contamination originating from feces of warm-blooded hosts), and estimated the impact of contamination for each stratum as the percentage of the total shoreline-miles that were non-compliant for at least one FIB standard. The authors found ~95% compliant shoreline-miles (for all measured FIB) throughout the course of the study area and period. In general, individual-strata compliance rates were not significantly different from the whole, exceedance of standards either for more than one FIB or repetitively for any was found to be rare. The notable exception was for shoreline miles impacted by freshwater sources, which showed significantly elevated non-compliance rates, especially as represented by the zero-point samples (60% exceeded monthly standards for at least one indicator, and 40% exceeded a daily compliance standard). Impacts as measured against daily limits for individual FIB differed considerably (34.2% non-compliant shoreline-mile-days for Enterococci, 24.8% for FC, and 12.0% for TC) for zero-point sites, and repetitive exceedances at sites and noncompliance for multiple FIB standards were found to be common at the freshwater sources. The authors also found that, while overall noncompliance rates of all Mexican sites were higher than those found in California (attributed to higher contributions from untreated sewage in the former), water quality in the mouths of freshwater outlets on both sides of the border were similar. Schiff, *et al.* (2000), produced a historical perspective of water quality in the SCB. Covering far more pollutants than FIB, and citing many more authors than Noble, *et al.* (2000) and Haile, *et al.* (1999), the authors made a case that a 30-year rise in the importance of non-sewage contributions to water quality in receiving waters directly derives from a 30-year history of improved treatment

of sewage. They also noted that, although local WWTPs within the Bight discharged miles offshore into deep ocean, the freshwater outlets routinely conveyed effluents from plants further upstream (e.g., with effluent discharges comprising > 95% of the dry-weather flow of the Los Angeles River); and that the volume of urban runoff in the SCB exceeded that of local WWTP effluents almost two to one. Noble, *et al.* (2003), conducted a similar study of the same region (and same sample points as Noble, *et al.* 2000) but focused on effects of rainfall. Samples were collected at the (previously sampled) 254 stratified (rocky/sandy beaches and ephemeral/perennial freshwater sources) sites during a four-hour period about 36 hours after a storm that dropped 3-7 cm of rain across the entire region. The samples were, again, distributed amongst 21 laboratories in an intercalibration effort (each lab analyzing samples from the same sites as in the previous dry-weather study). Measure of impact was, again, the fraction of shoreline miles (total and for each stratum) exceeding one or more California FIB standards for TC, FC, and/or Enterococci (plus the TC/FC ratio). In this study, noncompliance was 10-fold more widespread along the Bight than in dry weather, with a much larger fraction of total shoreline miles (58%) exceeding at least one FIB standard. Moreover, the magnitude of exceedances was far larger than was the case for the dry-weather study (e.g., 77% of samples exceeding the enterococcal threshold did so by more than one standard deviation of measurement error as determined by the round-robin intercalibration effort, and 2/3 of wet-weather exceedances were more than twice the California standard), and the overall noncompliance rate for more than one indicator at any site doubled. By strata, the point-zero, perennial-stream sources again had the greatest impact on water quality, though the fraction of noncompliant shore miles more than doubled (to 87%) over the dry-weather results. Wet/Dry differences for other strata studied were even greater, however, with wet-weather noncompliant shore miles

increasing well over five-fold for each (random-perennial and ephemeral freshwater sites, and both sandy/rocky beaches) stratum. The similarity between U.S. and Mexican shorelines impacted by freshwater sources found in the previous dry-weather study remained, but the five-fold difference in sandy-beach exceedances across the border disappeared in the wet-weather study.

In a more focused (dry-weather) study, Grant, et al. (2001) analyzed potential sources of FIB to Huntington State and City Beaches (“Huntington Beach,” in Orange County, CA, and within the SCB). The beaches had experienced frequent closures due to new (1999) state FIB standards, most frequently for excessive Enterococci densities. Unlike many watersheds discharging to the Bight, the contributing Talbert Watershed had recently undergone an extensive sanitary survey to exclude the potential for sanitary-sewage leaks. The 3400-hectare watershed was described as urbanized, with residential, commercial and light-industry districts, as well as plant nurseries, and was drained by an MS4 of three open-channel trunk lines that converged just upgradient of a 10-hectare tidewater constructed wetland (a pickle-weed dominated saltwater-marsh bird habitat which, in turn, drained through a single outlet to the ocean). The lower, tidally influenced portions of the MS4 were also fitted with eight pump stations, by which storm drainage was intermittently (weather dependently) lifted from the system conduits to the open trunk lines. In an attempt to identify the non-sewage FIB sources to, and quantify their impacts on, the beach, the authors conducted a 15-day study, the first eight days of which all of the pump stations were taken offline (with stormwater either stored in the forebays of the stations or diverted to the sanitary system), with pumps active for the last week.

The authors:

- 1.) Monitored flow (at five-minute intervals) and enterococcal densities (hourly) landward and seaward of the marsh, and at two points in the upper reaches (above tidewater limit) in the MS4,
- 2.) Measured Enterococci (hourly) within the surf zone at the inlet/outlet to the marsh and at three other locations “downstream” (with the prevailing wave direction and offshore current),
- 3.) Took hourly measurements of solar radiation (at a station 6 km away), and
- 4.) Conducted an hourly bird census of the marsh.

Additionally, the authors monitored pump-station effluent (only while pumps were online), and took samples of bird feces and vegetation in the marsh, and of sediments both in the marsh and in the surf zone (all subjected to Enterococci analyses). Finally, they conducted two ebb-tide dye studies to estimate the areal extent and dilution of the marsh plume in the offshore current, and one rising tide study to estimate tidal-flow residence time within the marsh. The authors found that the marsh was a net source of Enterococci, both to the watershed during rising tides (landward samples exhibiting significantly higher densities than those at the seaward sample point), and to the beach during ebb tides, regardless of the idleness/operation of the pump stations (and hourly samples at opposite ends of the marsh were deemed directly comparable with a found tidal residence time within the marsh of < 40 minutes). They further found that ebb-tide effluent from the marsh could explain FIB densities in the surf zone assuming no more than 2:1 dilution in the plume and near-total entrainment of the plume into the off-shore current (with both assumptions supported by their dye studies). The authors found that urban runoff did not contribute significantly to marsh FIB populations, due to the long (~7-day) residence time of runoff waters in the tidally influenced trunk lines landward of the marsh. Within the marsh, the authors found birds to be a significant (but not sufficient) source of FIB, and concluded that bird-

or runoff-derived FIB must accumulate and/or grow in the sediments and vegetation to explain marshwater densities (a conclusion supported by their sediment and plant samples).

Like Grant, et al. (2001), Schiff and Kinney (2001) focused on a small watershed and explored potential FIB source areas within that watershed, but the latter included a wet-weather analysis in the study. Mission Bay is a 4600-acre convoluted recreational water park (26 miles of shoreline), created in 1956 by dredging (mean depth = 8 feet) of a coastal wetland in San Diego, CA (and note that Mission Bay is the same site that was later deemed to be “largely free of human-fecal inputs” in the dry-weather epidemiological study of Colford, *et al.*, 2007 discussed above). Baywater mixing with ocean waters is inhibited by narrowness of the (dredged) outlet channel, and the park can be subdivided into distinct east (landward) and west (seaward) sections separated by islands and causeways that restrict circulation within the bay. The two largest tributaries to the bay (Tecolote Creek and Rose Creek) both enter the bay in its eastern section. Schiff and Kinney:

- 1.) Reviewed historic monitoring results from 20 shoreline stations, within both sections of the bay, operated by the City of San Diego (> 7300 samples, with total- and fecal-coliform analyses performed 1987-1994, and Enterococci analyses 1991-1994);
- 2.) Collected three sediment samples (one before onset of the wet season, the second “pre-storm” sample during the wet season two-weeks subsequent to a 1.1-inch rain and immediately prior to a 1.2-inch rain and the last “post-storm” sample shortly after that second rain, all at ebb tide) at each of the 17 shoreline monitoring stations that consistently had sampleable sediment;
- 3.) Collected dry weather water samples at each of the 22 storm drains showing dry-weather flow (of 89 draining to the bay), and subsequently resampled each of the 22 during wet weather;
- 4.) Sampled, three to five times during each of four storms, seven mainstream reaches between subwatersheds in the Tecolote Creek watershed and six such reaches in Rose Creek; and
- 5.) Sampled the drainage of 20 small (1-4 acres each) catchments (all lacking sewer lift stations or mains), each defined by a single land use (residential, commercial, industrial, and opens lands, the last category including parks, recreation areas, and a Navy base) in the Rose Creek watershed.

The authors' analysis of the historic monitoring data showed a clear seasonal pattern in this arid environment, with highest FIB densities in the winter (December-March) wet seasons. Annual variations ranged over two orders of magnitude between seasons, and all monthly geometric mean densities (for all combined monitoring stations and for each FIB) that exceeded California water-quality objectives over the span of the study occurred in the winter months (with enterococcal monthly exceedances for every winter in the record). For all stations disaggregated by bay section, densities in the east (landward) section were consistently and significantly higher (up to an order of magnitude for FC and Enterococci) than those in the west during the wet (winter seasons), but the differences between sections largely disappeared during summer months. During dry days (regardless of season), geometric means of FIB densities calculated by station showed no spatial relationships, but during wet days (defined as any sampling day within 48 hours following a recorded rainfall) the individual stations in the east section again showed markedly higher FIB concentrations, with local maxima at the mouths of the two (largest) tributary creeks. Significant ($p < 0.01$) Spearman rank correlations between rainfall depth and both FC and enterococcal densities were found for nearly all of the stations located in the east section of the bay. Dry-season sediment samples were nearly all below detection limits for all three FIB. In the wet season, FC and Enterococci densities typically rose two orders of magnitude (and as much as five orders at the tributary mouths) between the pre- and post-storm sample campaigns, indicating both a strong rainfall response and a lack of persistence. Of the 22 storm drains exhibiting dry-weather discharge (all less than one gallon/minute, and only one of which discharged freshwater instead of salt), half contained measurable FIB. The storm drain exhibiting both dry-weather freshwater flow and measurable FIB was found to discharge 10^4 CFU/100 ml (by a Most Probable Number, MPN analysis) Enterococci, and 10^6 CFU/100 ml

total coliforms, but did not measurably elevate nearby baywaters. All 22 of these storm drains discharged noncompliant effluents (California standards for all three FIB) in the wet-weather resampling campaign. No spatial patterns of FIB densities were discernable within the tributary watersheds. FIB densities were not significantly different between reaches of either watershed, from headwaters to the mouths, though all samples exceeded California standards for all three FIB. Despite the authors' exclusion of sewage works within their selected single land-use catchments (and despite that one such catchment was open land restricted from public access), drainage from all land use catchments also exceeded state standards. The authors remark on the lack of any apparent point source of FIB in the watershed, as well as the similarity in water quality of runoff from urban and non-urban land uses. They further observe that, despite the frequent noncompliance here, results from this study generally represent lower FIB densities than those found elsewhere in San Diego and in southern California. Citing others, they note that "large numbers of indicator bacteria may not be the result of human contamination."

In another southern California urban watershed, Stein and Tieffenthaler (2005) conducted a longitudinal (along the drainage) study of FIB (total coliforms, *E. coli*, and Enterococci) and metals in Ballona Creek (12.7 km from storm-drain outlet "headwaters" to the beach), an 80% urbanized (residential, commercial, industrial, public) watershed with no permitted wastewater discharges (though illicit sanitary discharges were suspected) and no consistent discharges from industrial activities (except for "construction, cleanup, and dewatering"). The watershed drains a 329 km² area within greater Los Angeles and reaches the Pacific at Marina del Rey (within the SCB). The authors sampled and measured the flow of 35-40 storm drains (those that were both accessible and exhibiting measurable flow at sampling time) and 12 in-river sites on three occasions between May and September (the dry season). The authors found that the three-sample

mean concentration at of Enterococci at all in-river sample points exceeded California WQS (as much as 17-fold). WQS for *E. coli* were likewise exceeded (as much as three-fold) for all but one (tidewater) point. Both (of these) FIB exhibited a similar spatial pattern (along the drainage) to that of metals, with major a major peak in concentrations in the upper reaches of the watershed (where the first few storm drains contributed to the low flow emanating from the “headwater” drain) and a lesser one in the lower reaches (where a major, not sampled, tributary joins the creek just up stream of the tidewater), but variability of in-river FIB samples (about five orders of 10) vastly exceeded that of metals samples (about five-fold). The authors noted that FIB were subject to processes (growth, die-off, random population fluctuations) not affecting chemical-species concentrations. The authors further noted that individual storm-drain FIB concentrations “consistently and uniformly exceed water quality standards in almost all locations,” but did not correlate well to in-river concentrations.

In 2003, EPA approved a “unique” proposal by the Los Angeles Regional Water Quality Control Board (the Regional Board) to allow a number of single-sample exceedances of FIB criteria in setting the bacterial TMDL for Santa Monica Bay Beaches (EPA, 2005)². Recognizing the importance of storm-drain effluents in the impairment of waters in the basin, the historic monitoring/studies establishing the ubiquitous nature of elevated FIB densities in local stormwater (from all land-uses even where the potential for significant human fecal contributions could be excluded from stormwater source areas), and the difficulties inherent in capturing and

² It is currently unclear as to whether this form of “relief” will be available subsequent to promulgation of the 2012 WQC. The general consensus (e.g., see Barash, 2012) seems to be that the equation of risk posed by elevated FIB densities, of any source, explicated in EPA 2012, would preclude such consideration, but the relevant technical support materials promised in Section 6 of EPA 2012 are still forthcoming, and at least one TDML (Diamond, 2013) with extended implementation schedule based on RSA has since been approved.

treating the entire volume of short, intense storms typical of the semi-arid region, the EPA approved short-term exceedances of single-sample criteria based on a “reference system/antidegradation” (RSA) approach, and an extended implementation schedule (the latter to allow for a time-consuming, holistic, “multi-benefit watershed approach” to stormwater remediation). While the numerical FIB WQC are not relaxed in the RSA approach, a variance for compliance with EPA’s antidegradation policies (no “backsliding”) for impaired waters is set by the lesser number of single-sample exceedances of the impaired watershed or of a similar but undeveloped watershed devoid of human-feces sources and of human-built water conveyances (the “reference,” and the EPA also recognized a “natural sources exclusion,” in which exceedances of WQC in watersheds from which any anthropogenic impact could be proven to be, or had been, excluded would not constitute backsliding). EPA granted a (wet-weather) variance for the coastal areas of Los Angeles and Ventura Counties based on reference to Leo Carillo Beach (fed by Arroyo Sequit Canyon, a watershed of ~98% open space with “little evidence of human impact,” and an 18-year implementation schedule conditional upon:

- 1.) a four-year review of the science underlying the Plan, and
- 2.) continued progress in implementation of “watershed-wide storage, and re-use and onsite treatments” of stormwaters.

EPA further warned other regulated entities seeking similar relief that selection of reference locations (“no or virtually no anthropogenic impact”) was critical to a successful review of any such proposal, and that such review would require “multiple levels of approval” (as a water-quality standards action).

Griffith, *et al.* (2006) examined the wet-weather FIB densities at six reference beaches (including Carillo Beach) in southern California. Reference beaches were defined as open beaches with breaking waves and with freshwater inputs, draining from watersheds that were at

least 93% undeveloped (with development restricted to upper reaches) and of comparable acreage to nearby urban beaches (1/2 sand beaches and three sand/cobble, three with a terminal lagoon system and three without). The authors collected samples four times (day of rain and three days thereafter) for each of at least five storms (at least 0.10 inches following at least three antecedent dry days) at each beach over two wet seasons (October through April, hereinafter “winter”), both in the wave-wash zone of mixing and immediately upgradient of the beach in the freshwater source. Samples were analyzed for FIB (total coliforms, *E. coli* and Enterococci) and salinity at all locations, and flow in the freshwater tributaries (and the authors also tested for human enterovirus, a specific marker for human feces, to test the assumption of minimal human contributions to the watersheds). Analysis of the data included:

- 1.) Comparison of the frequency of exceedances of any California standard during winter wet-weather days (defined as day of rain and three days thereafter, data collected in this study) vs. the frequency of exceedances during winter dry weather and summer dry weather (data collected routinely by local health authorities);
- 2.) Comparison of exceedances of standards by day of the (four) days defining wet weather;
- 3.) Comparison, between beaches for wet-weather FIB exceedances and for the decay rate of enterococcal densities over the four days of wet weather;
- 4.) Comparison of FIB-density responses to large vs small storms (greater or less than mean daily rainfall) and to early-season (before New Year) vs. late-season storms, and the authors further compared responses between storms that breached lagoon/sand berms (when present) to those when storms did not;
- 5.) Comparison of enterococcal densities at the beach to salinity there and enterococcal flux from the tributary stream;
- 6.) Comparison of FIB exceedances from small (<25 km²), medium, and large (>100 km²) watersheds; and
- 7.) Comparison of beach FIB densities (with lagoon systems) for berm-breaching vs. non-breaching storms.

The authors’ marker for human feces was found for four sampling days (at three beaches) over the two-year study period; the authors assumed trespass, and deleted data from those four day-beach samples (of n = 136) from further analysis. In aggregate, the frequency of wet-weather

FIB exceedances of standards (16% of wet-weather days) was more than ten-fold greater than that occurring in dry weather (regardless of wet/dry season). Wet-weather exceedance frequency varied considerably between beaches (0% to over 30%), with large-watershed exceedance frequencies about twice those of medium-acreage source areas, and more than four-fold greater than those of the small watersheds (and enterococcal densities were the cause of the majority of exceedances at all beaches). Wet-weather exceedance frequencies were greatest on the day (within 24-hours) of the rain (27%) with monotonic decay (21%, 15%, and 3% over the three days) following the storm (with Enterococci exhibiting greater persistence, and accounting for all exceedances on the third day following the rain). Early-season rains prompted a slightly greater exceedance rates (18% vs. 15%) than late-season storms, but a much greater frequency of exceedances for multiple FIB (63% of non-compliant samples vs. 33%), suggestive of at least some accumulation/persistence of FIB during the dry-season. Large storms led to exceedance frequencies about double those of small storms, with the ratio increasing to three-fold (for beaches with sand berms present) between berm-breaching storms and those that left the berm intact. The watershed-tributary sources were found to account for most of the wet-weather variability ($r^2 = 0.73$) in enterococcal densities at the wave-wash zones, and (where lagoons were present and berms were breached) beach-water quality correlated to that in the creek waters ($r^2 > 0.93$ for all FIB) about as well as they did to lagoon densities, indicating that the lagoons served more as conduits for the watersheds than as independent sources.

In an extension of the theme of characterizing reference systems, Stein and Yoon (2007) studied 22 stream reaches (in six southern California counties and 12 watersheds) to characterize natural-landscape contributions of a host of pollutants (including metals, nutrients, solids, and bacteria), in both wet and dry weather, to water quality. Sampling sites selected were at least

95% undeveloped and lacking in evident anthropogenic effects (e.g., septic tanks), regionally distributed across southern California with a representative mix of geological settings and land covers, in streams of year-round or prolonged dry-weather flow, and in catchments that had not burned in the three years prior to the study. The authors focused on third-order watersheds, which they characterized as large enough to generate reliable flow but small enough to allow for selection of homogeneous contributing drainage basins. Relevant to this review, and over a two-year period (including one of significantly above annual rainfall and one in which ~2/3 of the long-term annual average of rain fell) the authors:

- 1.) Collected three dry-weather (at least 30 rain-free days prior to sample, and in the dry season) samples along a cross-creek transect, followed by three replicates within ten minutes of the first (along with temperature, pH, dissolved-oxygen, canopy-cover, and stream-flow measurements), and
- 2.) Collected wet-weather (wet-season) samples (no measurable rainfall for three prior days, samples collected when discharge was between the times of ~10% above baseflow on the ascending limb of the hydrograph and 50% below peak flow on the falling limb), with at least four samples collected in each of 30 site/storm events.

All samples were tested for FIB (total coliforms, *E. coli*, and Enterococci). For dry-weather, across all natural sites, the authors found *E. coli* densities (geometric mean), by MPN methods, of 15.83 CFU/100 ml (12.46 > 95% CI > 20.11). The same values for Enterococci and total coliforms were 19.84 (CI 15.45 to 25.49) and 1047.83 (CI 767.82 to 1429.96) respectively. In comparison to a nearby developed urban basin (Bellona Creek, see Stein and Tiefenthaler, 2005, above), the natural-site concentrations were about two orders lower, but at least some samples exceeded California standards for all three FIB, the median Enterococcus density nearly equaled the standard, and all but one total-coliform sample were noncompliant. Dry-weather FIB densities did not correlate to any environmental factor studied, nor to runoff volume or catchment area. Wet-weather densities were higher. Natural-site values for *E. coli* (geometric

mean = 125 CFU/100 ml, 95% CI between 39.70 and 399), Enterococci (140, CI 38.80 to 511), and total coliforms (4460, CI 1510 to 13100) were all considerably higher than those for dry-weather, though the coefficient of variation was generally lower. The natural-site densities were again 2-3 orders of magnitude lower than those found in a nearby, developed watershed (Los Angeles River, brief description at Schiff, *et al.*, 2000, above), but median values for all three FIB exceeded local standards. FIB were again found unresponsive to measured environmental factors, and there was no evidence of a “first flush” (defined here as > 30% pollutant load delivered to the stream within the first 25% of rainfall volume).

Meanwhile, in the British Isles, the Bailiwick of Jersey upgraded the sewage plant at St. Helier in 1993. Though not strictly beholden to European Union (EU or its predecessor the European Community, EC) environmental regulation, Jersey has historically complied with such standards as matter of policy. Beaches in St. Aubins Bay had frequently exceeded numerical FIB standards expressed in Directive 76/160/EEC (The Bathing Water Directive, or BWD, 1976, of the European Community). In expectations of achieving “compliance” at the popular summer destination, authorities (at considerable expense) added an ultraviolet (UV) disinfection system (the “first such plant in Europe,” per Kay, *et al.*, 1999) at the (extant, secondary-treatment) treatment works, designed to achieve <200 FC/100 ml FC (well below, by an order of magnitude, the mandatory “Imperative” beachside limits, and slightly below the more stringent “Guide” standards). Initial tests showed that FIB reduction in effluents exceeded design goals, the St. Helier plant was hailed as a “model system,” and then beach monitoring revealed frequent noncompliance (even by Imperative standards) in the first year of operation (a “surprise,” per Kay, *et al.*, 1999). With recognition that noncompliant beach samples seemed to occur during and after rain events, a study was launched to examine the relative FIB contributions to the Bay

from potential watershed sources (seven streams, including the stream receiving treated effluent). Flow and FC densities were analyzed for all streams, under both rainy and fair conditions, and for sample points within the WWTP (secondary-treatment effluent, and UV-disinfected effluent). Overall results (with actual UV treatment) revealed that though the treatment-works effluents accounted for 47.4% of total discharge to the Bay, they only contributed 1.5% of the total tributary FC load. With separation of calculated FIB loads (stream/effluent, secondary/UV treatment), it was found that, (hypothetically) absent UV treatment, base-flow effluents would have been predominant (67.55% of total FC load to the Bay), followed by wet-weather stream flow (25.43%) and fair-weather stream flow (6.23%). With inclusion of UV disinfection, storm-flow FC from streams represented 67.55% of the total FIB load to the beaches, and stream flows (wet- and dry-weather combined) contributed over 96% of the total tributary FC. The (expensive) improved treatment scheme had obviously reduced total FIB loads to the beaches, but may have represented a misguided priority in attempting to achieve consistent microbiological water-quality compliance. The Jersey authorities added underground storage capacity for storm overflows (for subsequent treatment), and field surveys to explore upland source reductions (more appropriate to remediation of non-point sources, Wyer, *et al.*, 1998, and Kay, *et al.*, 1999).

The St. Aubins data “provided a first indication of the problems that could emerge” without early recognition of the importance of non-point sources in design of schemes to improve beach-water quality (Wyer, *et al.*, 1998). At a time when many similar schemes were under consideration or were already under construction (many in the United Kingdom, or UK, proper, and many at sites with only rudimentary extant sewage treatment) to achieve compliance with the existing BWD (and in anticipation of already proposed more stringent revisions), the St.

Aubins experience provided incentive for increased focus on watershed sources to bathing waters (Kay, *et al.*, 1999). One such study examined (in Wyer, *et al.*, 1998) potential FIB sources in the Staithes Beck catchment, Yorkshire, which drained (along with a smaller and intermittent stream, Gun Cutter) to Staithes Harbor on the North Sea. The 43 km² watershed was described as agricultural, with pastoral grassland and arable cropland (and moorland in the upper reaches). Crude sewage from a combined-sewer system outfell to the harbor at the mean low-tide line. Sewage samples were collected, over a seven-week (August through September, 1995) period, at two onshore locations, at the outfall (during low spring tide), and at a sewer overflow (CSO) to the harbor following rain. Riverine samples were collected at the mouths of both catchments at the high-tide line. Hourly flow measurements were derived (from continuous monitors) at the sewer-sample points, at the CSO, near the mouth of Staithes Beck, and in a culverted segment of Gun Cutter, and rainfall was measured at a reservoir within the watershed. Samples were analyzed for (culturable) TC, *E. coli*, and FS. Densities of all three FIB were significantly lowered (diluted) at sewage sample points and elevated in Staithes Beck during hydrograph events (as compared to base-flows). Flow-related changes at Gun Cutter were insignificant (Student's *t*, 95% confidence). The (untreated) sewage system contributed over 95% of the TC and *E. coli* loads to the Harbor, and almost 60% of the FS, over the course of the study. Due to greater flows and higher FIB densities during hydrograph events (~10% of the study period), however, Staithes Beck contributed more than half of the total TC and *E. coli* loads and over 80% of the FS loads during wet weather (Gun Cutter and the CSO, with minimal flow, did not contribute significantly to wet- or dry-weather FIB loads). The authors subsequently conducted a hypothetical study to examine the impact of improved sewage treatment in Staithes. Simulating an upgrade to secondary treatment with UV disinfection, substituting FIB-concentration data

derived in the St. Aubins study for those found at the sewer outfall in this study, the authors found that despite an overall 97% reduction in total TC and *E. coli* loads with a 67% reduction for FS (and an overwhelming shift in dominance of sources to Saithes Beck in all weather), consistent BWD compliance would not be achieved.

In the year following the Staithes study, the authors (Wyer, *et al.*, 1998) conducted a similar study of the Nyfer catchment, the watershed of the Afon Nyfer draining to the Irish Sea at Newport. The 119 km² watershed includes pastoral grassland and moorland, with deciduous woodland in steep-slope valleys. Newport sewage (in another combined-sewage system) was screened before discharge to a submarine outfall at the north end of a beach (Newport Sands). Samples were collected (10 weeks, July-September 1996) at the mouth of the Afon Nyfer, at the mouth of a major tributary to the Nyfer (Afon Clydach), at eight smaller streams (one draining directly to the Sea), at two inland sewer locations, and at the sewage-screening station on the coast, and analyzed for TC, *E. coli*, and FS; flow was again reported hourly and rainfall data were collected hourly at Newport. Again, sewage sources were diluted to significantly lower FIB concentrations (all three taxa), with significantly elevated levels in riverine samples (drainage-network expansion and increased sediment resuspension), in and following rainfall. Sewage (barely treated by screening) at base-flow conditions again dominated FIB loads (~60%) with very little flow (0.4% of total discharge) over the course of the study though the Nyfer (19%) and smaller streams (19%) were significant contributors. During high flow due to rain (257 hours, 15% of the study period), the Afon Nyfer contributed 69% of total FIB to the coast from this watershed. The authors' hypothetical scenario simulating treatment and disinfection again showed considerable reductions in FIB loadings (> 68% for all FIB overall, and over 97% during base-flow hours), but compliance to (even Imperative) standards was not achieved. The authors

concluded that, while treatment of sewage (where present) is certainly needed for any scheme to bring receiving waters into compliance with BWD standards, without attention to diffuse watershed sources especially during storm events any such scheme is likely to fail. Even with untreated sewage sources adjacent to beaches, non-point non-sewage sources further upgradient may be the dominant sources of FIB to bathing waters when it rains. They further noted that typical monitoring routines (focused on sewage sources and/or beach waters, during base-flow conditions) are inappropriate for the required assessment of non-point watershed sources, and they urge greater use of field surveys and modeling to provide the required information.

Kay, *et al.* (1999), review these same three studies (St. Aubins, Staithe, and Nyfer) with an eye to suggesting what form such investigations of watershed sources should take. In addition to the general conclusions of Wyer, *et al.* (1998), the authors further noted land-use distinctions between the three studied watersheds that suggested approaches to acquiring needed watershed-source information to prioritize remediation strategies in a “holistic approach” to achieve receiving-water compliance (again, noting anticipated BWD revisions forthcoming, based on World Health Organization guidance, the “WHO Draft Guidelines,” see below). For example, the authors note that, although all three of the studied watersheds included pasture lands, the Nyfer catchment had seen the largest (post-war) historic expansion in stocking densities in response to governmental subsidies (price supports and headage payments) to “less favoured” agricultural areas. The Nyfer basin had the largest herd intensity of the three study areas, and also exhibited fecal-coliform counts, in upstream areas unexposed to sewage, exceeding $10^6/100\text{ml}$. Noting that the sheep in Wales (11 million) are more numerous than the people (2.2 million), and that daily fecal-coliform generation by a sheep is five-fold greater than that of a human, the authors estimate that ovine FC production in Wales equals that which would be

produced by 55 million humans (untreated). To the authors, this information suggested a likely relationship between the percentage of improved-pasture acreage within a subcatchment and the generation of FIB therein. Their Figure 12 shows a significant correlation ($r^2 = 60\%$). The authors urge the use of land-use data (remotely acquired with field-survey confirmation), and hydrology/terrain information, combined with upland water-quality sampling, to develop models of non-point FIB generation and transport, separable by weather and even in heterogeneous (by land use) watersheds. They argue that such an approach would enhance prioritization of watershed-source remediation strategies (through identification of outlier subcatchments exhibiting significantly different FIB densities than would be predicted by such a land-use/water-quality model), and would allow for scenario modeling (in which impacts of proposed, and potentially expensive, remediation strategies could be explored before such strategies are implemented).

Crowther, *et al.* (2003), presented an effort to build such a model from data generated in a case study of a “large” (374.2 km²) rural watershed in Wales. Opining that relative importance of rural sources “inevitably” increases as progressive improvements are made in sewage infrastructure (both in treated effluent quality and in system leaks/spills/overflows), and that large-river watersheds (10²–10³ km²), by dint of their greater discharges, would inherently have greater impacts on receiving waters than smaller ones, the authors focused their efforts on:

- 1.) FIB-density variations along river networks,
- 2.) Relative importance of various land uses to generation of FIB within the catchment,
- 3.) Distance/travel time of watershed sources from the receiving waters, and
- 4.) Total FIB loads expected at the watershed outlet.

The authors further noted that, for large watersheds, exhaustive identification of sample points draining homogenous (point- or diffuse-) sources is impossible. The sparsely populated study area, contributing runoff to beaches in the Aberystwyth area of Cardigan Bay is dominated by

Afon Rheidol and Afon Ystwyth, the hydrology of the former being affected by flow regulation at two reservoirs constructed for hydroelectric-power generation and by a piped transfer of water from the upper-reach reservoir to the lower one (where the plant is located). The basin was divided into 24 sampled sub-catchments, four of which (on the mainstream Rheidol) were omitted from subsequent analysis due to suppressed hydrograph-flow responses resulting from the artificial (hydroelectric) transfer between sub-basins, leaving 243.4 km². The remaining study area (20 sampling points) covered all land-use types represented in the watershed. Samples (taken at 2-3 day intervals with additional opportunistic sampling following rains) were taken (August through September, 1999) and analyzed (in triplicate) for culturable TC, *E. coli* and Enterococci. Flow was measured at seven existing gauging stations, two more installed for the study, and by stage boards at remaining sample points. By field survey, the authors divided the watershed into six land use types:

- 1.) Improved pasture (generally intensively/moderately used for high stocking levels of dairy/livestock farming, or silage production, comprising 41.56% of watershed acreage),
- 2.) Rough Grazing (no signs of recent improvement, and generally of lower stocking levels, 31.41% of acreage),
- 3.) Woodland (mostly conifer plantations, 24.13%),
- 4.) Built-up (farmyards and camper sites, 0.97%),
- 5.) Arable (mostly barley, 0.39%), and
- 6.) Other (including waste ground, parks, recently deforested land, and water bodies, 1.54%).

The authors developed a 50-m resolution raster representation of watershed terrain (from a digital terrain model, DTM) and hydrology in a Geographical Information System (GIS). They input percentage of area represented by land-use types, by sub-catchment, and sub-catchment areas into the GIS (both in vector form) along with 50-m raster layers of the sub-catchment divides, flow paths, outlet and mean altitudes, and mean slope gradients. Additionally, the authors input (for improved-pasture cells only, the dominant land-use type) rasters of

information concerning sub-catchment slope and flow distance to the outlet. Improved pastures were found to be concentrated in lower reaches of the watershed (representing 80-90% of land use in those sub-catchments) and also at low altitudes and near sub-catchment outlets, while rough grazing land (up to 99.95% in one sub-catchment) and woodland (up to 87.5%) clustered nearer headwaters. The small amount of built-up land was not geographically clustered, but showed some collinearity with both livestock land uses. With four exceptions (three of which were at sample points affected by sub-basin transfers of hydroelectric-plant source waters) all sample points showed significantly ($p < 0.05$) elevated densities of all three FIB during high-flow conditions as compared to base-flow, generally by at least an order of magnitude and, in some cases, more than two. Nearly all high-flow samples exceeded Imperative standards, and all exceeded the Guides. By watershed location, lowest densities were typically found nearest the headwaters (mostly woodland) and on slopes of the uplands (rough grazing) with highest densities exhibited by intensively farmed lowlands. Base-flow densities at reservoir outlets were similar to those found upstream, but showed no significant response to high-flow conditions. All three FIB densities (per sub-catchment) positively correlated to percent land-use type (within the same sub-catchment) for improved pasture ($0.609 < r^2 < 0.909$), and built-up land ($0.691 < r^2 < 0.866$) and negatively correlated to rough grazing land ($-0.725 < r^2 < -0.549$) for both base- and high-flow; no significant correlation was evident for woodland or reservoir area. The authors noted the collinearity between pastures and the (very small acreage of) built-up land and the (exclusionary) paucity of pastureland in sub-catchments dominated by rough grazing, and concluded that the major source of FIB to this rural watershed was the pasture land. FIB concentrations did not significantly correlate to sub-catchment area, nor to mean slope gradient, and a negative correlation to altitude was, again, attributed to the collinear absence of improved pasture in

uplands. Examining only improved pasture, peak correlations for all FIB with flow distances occurred in the 2 km (stream-flow path) nearest the sub-catchment outlets, and in the lowest 20% of sub-catchment area, under base-flow conditions. At high flow, best fits were found with wider bands around the outlet (< 5 km stream path, < 60% of area). Multiple (step-wise, F to enter = 0.05) linear regression revealed % improved pasture to be the primary factor for all FIB under all conditions, with short flow-paths (< 1 km or < 2km) to the sub-catchment outlet more important during base-flow, and longer paths (< 5 km or entire sub-catchment) providing best fits during high flow. Correlation coefficients for high-flow conditions (73-86%) were consistently higher than those at base flow (67-82%, $p < 0.0001$ for all models). Besides providing confirmation of the importance of non-human sources to compliance in receiving waters, especially during times of high flow, the authors believe that their findings reveal the importance of reduced residence time in delivery of FIB at high-flow conditions. They further note that the method used here achieved strong and highly significant correlations between FIB runoff and land-use types in this large watershed, even in the absence of detailed knowledge of individual point fecal sources or livestock densities. Finally, noting that the one sample point (unassociated with sub-basin transfers) not showing significantly greater Enterococci in high flow was due to anomalously high base-flow concentration, the authors conclude that the approach presented here is useful for prioritizing in-basin follow-ups and remediation efforts.

As cited above, these studies were performed under the regulatory regime enforced by the EC, defined by the BWD (1976), and in anticipation of revised standards to come. This Directive required fortnightly sampling for FIB (TC and FC), and other quality parameters, of all “bathing waters” (either with explicitly authorized bathing or with frequent bathing use that was not explicitly prohibited), and specified compliance (95% of all samples, excluding those influenced

by “floods, natural disasters and abnormal weather conditions”) with Imperative (I) standards (culturable TC<10,000/100 ml, FC<2,000/100 ml) within 10 years. Though this mandatory, human-health based water-quality criterion was applied at the bathing waters, the BWD further charged Member States to “endeavour” to meet Guide (G) standards (culturable TC<500/100 ml, FC<100/100 ml, FS<100/100ml) in that time period, and opined that they “should” survey tributary watersheds to determine potential FIB sources and their likelihood to impact (“according to the distance from”) bathing waters, while establishing that compliance requirements might be waived if (without human intervention) pollutants natural in soils “enriched” the waters (providing that such a waiver did not endanger human health, BWD, 1976). Some potential bacteriological sources within tributary watersheds were regulated separately, under several other EC Directives and under quality or emissions criteria other than FIB densities. The Sewage-Sludge Directive (SSD, 1986) regulated the application of domestic-sewage treatment sludge to agricultural lands to prevent toxic accumulations of heavy metals in soils. This Directive prohibited such applications to soils that already exceeded defined safe limits of metals concentrations, set application-rate limits (based on metals analysis of the sludge) that were deemed to prevent exceedances of safe soil-metal limits, and mandated treatment (biological, chemical, or heat, to reduce fermentability and health hazards) of sludges prior to application (unless those sludges were “injected or worked into the soil). SSD exempted septic-tank sludges from EC regulation. Livestock manure (and chemical fertilizer) was regulated under The Nitrate Directive (TND, 1991). This Directive was focused on protection of drinking-water sources (both surface water and groundwater) from nitrate contamination and on remediation/prevention of eutrophication of water bodies. The Directive mandated that EC Member States establish (voluntary) codes of good agricultural practice, along with any needed

training/outreach for farmers, that would minimize the impact of nitrate pollution on all water bodies. The Directive further mandated that Member States identify “vulnerable zones,” tributary areas to drinking-water sources exceeding or in danger of exceeding drinking-water nitrate standards or areas contributing nutrients to water bodies experiencing or in danger of eutrophication. For vulnerable zones, TND required the development of mandatory “action programmes” that (at least, and relevant here):

- 1.) Determined when land application of nitrate sources (including manure shed by the livestock) must be prohibited to prevent degradation of receiving waters, and
- 2.) Provided for livestock-manure storage capacity sufficient to prevent nitrogen-compound emissions through the longest anticipated period of land-application prohibition, or
- 3.) Provided for environmentally safe disposal of livestock manure during periods of land-application prohibition.

All of these action-program provisions were to account for planned crop uptakes of nutrients. Tanneries (of capacities exceeding 12 tonnes/day), slaughterhouses (exceeding 50), disposers/recyclers of animal carcasses and wastes (10), and facilities for intensive rearing of poultry and pigs (“CFOs” in American parlance), were classified as industrial facilities and positively required a permit for their emissions (specifically including nutrients or substances creating oxygen demand when released to water, Integrated Pollution Prevention Control Directive, or IPPC, 1996). Urban wastewater (domestic sewage, whether combined with runoff/industrial wastewaters or not) was regulated under the Urban Waste Water Treatment Directive (or UWWT, 1991). This Directive mandated the provision of urban-wastewater collection systems and treatment (secondary treatment or equivalent, the latter being defined by either an effluent concentration or a percent reduction of five-day biological oxygen demand, BOD5, chemical oxygen demand, and total suspended solids) for progressively smaller (defined in terms of population equivalents, 1 p.e. = 60g of BOD5 shed per day) population centers

through 2005. The Directive allowed for exclusion of urban runoff resulting from heavy rains in the calculations of the p.e. of a population center, but also included an accelerated compliance schedule and a more stringent treatment standard (with limits on effluent nutrients as well as oxygen-demand) for “sensitive areas” (draining to drinking-water sources or to water bodies exhibiting or in danger of exhibiting eutrophication).

The anticipated revisions to BWD (1976) were based on the “WHO Draft Guidelines” (WHO, 1998. Guidelines for Safe Recreational-water Environments: Coastal and Freshwaters. Consultation Draft. World Health Organization, Geneva) in broad circulation at the time, and later codified (largely unchanged) into formal Guidelines for Safe Recreational Water Environments (“WHO Guidelines,” WHO, 2003). The Guidelines derive from a 1998 subject-matter expert meeting in Annapolis, MD, co-sponsored by WHO and EPA to critique bathing-water regulatory systems extant worldwide. This meeting led to the “Annapolis Protocol,” a proposed alternative strategy to better reflect health risk to bathers than contemporary regulatory schemes and deemed sufficiently radical as to require some testing prior to any implementation. This proposed strategy was derived as a logical outcome of an expert consensus that:

- 1.) Routine water monitoring of FIB densities (with analytical lags involved) only provides for retrospective management actions in response to (often episodic) excursions, after exposure has taken place and maybe even after such exposure has ended;
- 2.) Human sources of feces represent a greater risk to health than non-human sources, though FIB are not human-specific;
- 3.) Multiplicity of analytical techniques used for enumeration of FIB (and even multiplicity of FIB analyzed) hampers comparability of data across jurisdictions; and
- 4.) Pass/Fail criteria of water quality, in the face of a real spectrum of increasing health-effect severity with increasing sewage pollution, masks valuable information useful in prioritization of remedial actions.

The second point of consensus, involving both the importance of FIB sources and the lack of specificity of commonly used FIB to their source, was supported by (yet another) review of U.S.

outbreak reports (34 outbreaks for which etiological information was available, culled from Morbidity and Mortality Weekly Reports, 1985-1994). The majority (19), implicating *Shigella*, pathogenic *E. coli*, *Giardia*, or Cryptosporidia, occurred in shallow waters frequented by human children, and were epidemiologically traced to the bathers themselves. The remainder (where the pathogens were considered likely to derive from sources outside the bathing waters) consisted of infections by *Leptospira* (transmitted by urine), Norwalk virus, Adenovirus, and AGI of likely viral etiology (the last three deemed most likely of sewage origin). The fourth point, which also included the presumption of a differential health risk presented by different host sources of FIB, led the meeting attendees to propose a bivariate, multilevel classification system of bathing-water safety. FIB densities in the waters (“microbiological quality,” as revealed by regular monitoring) together with the degree to which human feces contributed to those FIB (“risk potential,” as revealed by sanitary survey of the tributary watershed) were both to be included in bathing-water characterization. Participants proposed that STVs of specified indicator densities (FS/Enterococci for temperate marine waters, *E. coli* or FS/Enterococci for temperate freshwaters, and *Clostridium*, spp. for tropics, to improve comparability of data across like sites) provide for quality classification of waters (five levels, A-E). They further proposed that direct sewage sources to the bathing waters (categorized by level of treatment), riverine sources (categorized by watershed population and “worst case” dry-weather flow), and bather density at the bathing waters themselves (modified by dilution potential of the waters) be combined to derive a risk classification (again, five levels, Very Low to Very High). The participants provided a matrix by which the categorization on both variables could be used to provide a primary classification (five-tier, Very Poor to Excellent) of bathing waters with respect to bather safety (WHO, 1999).

Further noting the high variability of microbiological measures, both spatially and temporally, Annapolis attendees proposed a scheme by which the primary classification of a beach could be modified (“reclassification”) based on validated success of management actions to prevent bather exposures at the places or times of elevated risks. Though spatially limited areas of elevated risk may simply be posted, fenced off, or otherwise placed off-limits for bathers, temporally limited times of excursions were deemed more complicated. Participants believed that the most important predictor for such excursions was rainfall. Rainfall, however, mobilizes both sewage (especially in the case of overflows, but also through improper connections to storm sewers) and non-human feces (through expansion of effective drainage basin). Hydrological responses of stream flow to rainfall further complicate matters, by both re-suspending in-stream sediments and increasing the effective drainage basin, by increasing dilution of pollutants present, and by reducing residence time for die-off in-stream. For both types of reclassifications (spatial and temporal), participants at Annapolis encouraged the use of computer modeling to define appropriate preventive management actions (based on aforementioned watershed-source surveys), follow-ups to confirm that actions effectively prevented bather exposure at times/places of elevated risk, and increased frequency of monitoring/sanitary-surveillance to confirm continued validity of the original parameters upon which preventive actions were based (WHO, 1999).

While no specific inclusion of the explicit Annapolis presumption that FIB of non-human origin represent a lesser exposure risk than FIB from sewage (either in governmental regulatory schemes or even in the WHO Guidelines) were found in this review, the regulatory principles espoused in the Protocol (and derived from that presumption) influenced subsequent EU Directives and spurred research into the upstream sources of FIB in bathing waters. The Water

Framework Directive (or WFD, 2000) represented an ambitious attempt to integrate all disparate aspects of European water policy into one coherent document. This (as might be expected) very long and very complex Directive generally (and relevant here):

- 1.) Defined “pollution” as the introduction of harmful heat or substances into the environment “as a result of human activity,” and defined “pollutant” as “any substance liable to cause pollution” (with specific, but not exhaustive, reference to substances regulated under SSD, TND, and UWWT);
- 2.) Defined the geographical unit of all water regulation to be a “river basin district,” with boundaries that included the whole of the watershed, affiliated estuaries and affected coastal waters, and underlying groundwater;
- 3.) Defined as “protected areas” all waters used for drinking-water abstraction or shellfish harvesting, and all waters regulated under BWD and TND;
- 4.) Mandated the establishment of “programmes of monitoring” for basin-wide characterization of all waters (to be presented in GIS format), with specified attention to surface waters (volume, level, flow, ecological and chemical status), groundwaters (chemical and quantitative status), and protected areas (with emission or water-quality standards in the underlying Directive by which the area was established taking precedence over surface- or ground-water standards where designations overlapped);
- 5.) Mandated that the characterization of waters include a review of “the impact of human activity on the status” of surface waters, with specified attention to historic data acquired under UWWT, IPPC, BWD; and TND, and
- 6.) Mandated the establishment of “programmes of measures,” required to use a “combined approach” to control point sources (through emission limits) and diffuse sources (through, at least, “best environmental practices”). “Basic measures” were required to, at least, assure compliance with standards specified in BWD, SSD, UWWT, TND, and IPPC; promote water-use policies to avoid compromising objectives specified for Protected Areas, and reduce the level of treatment required for waters abstracted for human consumption.

Among the effects of WFD was a requirement to characterize FIB sources (point and non-point, resulting from human activities) throughout any watershed tributary to bathing waters, and to render their control mandatory whenever BWD standards “are unlikely to be achieved” (WFD, 2000).

Responsibilities for implementation of WFD provisions noted here fell entirely to the individual Member States, though the European Commission (the Commission, and with assistance by a Regulatory Committee established by the Directive) would provide advice on coordination between States if needed. Implementation of the provisions was to be accomplished

under a schedule deemed ambitious (e.g., Member States were required to enact and report laws and regulations, that would lead to full compliance, within three years of publication of WFD, 2000). On the day of passage of WFD, an informal meeting of the Water Directors of the Member States agreed on a need for a common strategy, a voluntary cooperative venture to share information, and to reduce duplicative resource expenditures, among the Member States (with Commission participation) to provide (non-binding) guidance for issues of common concern. Subsequent meetings of the Water Directors developed the Common Implementation Strategy (CIS), which created a division of labor and responsibilities for the Member States into various Working Groups focused on the various perceived barriers to WFD implementation (one of which, focused on characterization and control of human impacts on basins, was co-chaired by the UK representative). The CIS also provided for information exchange between the Members, and for a network of pilot-river basins (including the Ribble Estuary basin in the UK) for large-scale testing of implementation strategies proposed by the Working Groups (CIS 2001, and CIS 2003). [While a revision of BWD to better reflect Annapolis Protocol guidance was still anticipated throughout this period and still influenced CIS deliberations (CIS 2003), and while the Commission did present proposals for such revision at least twice (neither of which were enacted by the European Parliament, Commission of the European Communities, 2002), a new BWD was not ratified until 2006. That new Directive did replace extant regulatory FIB with *E. coli* and Enterococci for classification of waters (in a multi-tier, Poor to Excellent classification system), with a transition period in which historic FC data could be considered as equivalent to *E. coli* and FS equivalent to Enterococci. The new BWD also (through a new definition of “short-term pollution”) allowed for neglect of high FIB concentrations due to rainfall in

assignment of water class so long as such excursions were predictable enough to enable reduced swimmer-exposure risk through “management measures.” BWD, 2006]

Citing the need to comply with provisions of WFD (and the need to coordinate such compliance with the still anticipated revision to BWD), Wither *et al.* (2003) presented an “initial desk study,” largely based on existing data available from historic monitoring and previous studies, into estimating a FIB budget for the watershed tributary to the Ribble Estuary to inform and guide subsequent studies. The Ribble watershed, described as a “very large” catchment (1583 km²) and containing “some extensive urban areas,” drains to the Fylde coast, a long - popular tourist destination in western UK. The eight identified Fylde beaches had suffered a long history of failures to meet (even Imperative) FIB standards of BWD 1976, and a decades-long effort to remediate coastal point sources (“involving sewerage infrastructure improvements, new secondary treatment plants, the addition of terminal disinfection and sewage storage to reduce spill frequency”) had largely been completed by this time. That effort had provided for improvements in bathing-water quality (at considerable expense, over 600 million British Pounds), but to achieve consistent Imperative compliance, and to improve towards compliance with Guide standards, attention to riverine FIB sources was deemed necessary. By the time of publication, the Ribble watershed had been identified as a CIS pilot-river basin (or “Sentinel” basin in the oft-used WHO parlance, Wyer, *et al.*, 2003, and Kay, *et al.*, 2005). The authors supplemented historic data with high-frequency (“approximately daily”) sampling of FIB (TC, FC, and FS) culturable concentrations at the tidal limits of four major rivers (Darwen, Douglas, Yarrow, and Ribble), with an additional sample point on the Douglas (just upstream of two WWTPSs that discharged very near to the tidal limit, both of which were slated for near-future upgrades to allow UV disinfection) during the bathing season of 1999. With available rainfall

and hydrograph information for each river, the FIB results for each of their samples (and for those previously supplied by others) were segregated into base-flow and high-flow categories (the latter defined by hydrograph response to rainfall), and the geometric mean for each flow category, for each FIB, and for each river presented (notably all of which vastly exceeded BWD 1976 I standards). With available river-discharge data, the authors calculated FIB loads for each river over the 1999 season, and found that the Ribble (by far the largest drainage basin) dominated riverine discharges into the estuary (wet-weather over 50% of season total discharge, dry-weather over 20%, and almost 75% of total discharge from the river). The Douglas (< 11% of season discharge), however, with its near-mouth WWTP discharges, dominated FIB loads delivered (e.g., over 50% of total FC, rising to over 75% at base flow). Recalculating loads, simulating complete disinfection of the down-gradient WWTPs on the Douglas by substitution of samples taken just upstream of those plants, drastically reduced that river's percentage contribution of FC to the estuary, but reduced the total tributary FC load to a much lesser extent. To estimate general sewage loads to the watershed, the authors first availed themselves of extant dry-weather flow records of the 53 treatment works in the basin. Plant-design full-treatment flow capacities were also available, and they had access to previous regional studies providing segregated base- and high-flow effluent quality, for all three FIB and for all treatment types present within the watershed (excepting untreated-overflow quality, only for high flow). Applying low-flow effluent quality to dry-weather treatment flows, and high-flow quality to treatment-capacity flows, the authors estimated FIB loads, by flow regime, from sewage-works effluents to the basin. They then attempted to estimate FIB loads discharged by intermittent sewage-system overflows (more problematic). Flow volume of the 574 reported overflows within the basin (mostly CSOs, but also including surge tanks and pump stations, etc.) was

virtually non-existent. The authors did have access to a previous study (Wyer, *et al.*, 1998 a,b, and not directly consulted here³) presenting a histogram comparing the percentage of 288 hourly volume observations of overflows at a Welsh treatment works to seven bins of overflows expressed as overflow/total-flow percentage. Analysis of that study provided a most likely overflow volume equal to about 25% of treatment-capacity flow, with a wide range of variability. The authors applied an additional overflow load (25% of treatment capacity flow x untreated-overflow FIB densities) to high-flow effluent estimates; noting the uncertainties introduced by use of this application, they also provide a sensitivity (to the range of values in the Welsh-plant study) analysis on overall sewage-source budgets for the Ribble watershed. The authors concluded that:

- 1.) Under contemporary conditions, the Lower Douglas WWTPs are predominant contributors to the FIB loads to the estuary, especially in dry weather, and (planned) hypothetical removal of those sources would more evenly distribute FIB loads between catchments of lesser or more upstream sewage sources and greater diffuse inputs;
- 2.) Under dry-weather conditions, 18 (of the 53) WWTP effluents contribute 99% of the sewage load to the estuary;
- 3.) Under wet-weather conditions, total loads of FIB to the estuary increase between eight- and fifteen-fold (TC and FS respectively) over dry-weather conditions, with the estimated sewerage-overflow component representing the largest part of that increase; and
- 4.) Sewerage-overflow response to rainfall represents the largest knowledge gap in modeling of the system (Wyer, *et al.*, 2003)

Noting WFD requirements for modeled characterization, at catchment scale, of both point and diffuse FIB sources, and further noting that field survey is not a feasible approach to

³ Wyer, *et al.*, 1989 a,b, not directly consulted here, are cited in the reviewed document as:

Wyer, M. D., J. Crowther, and D. Kay, 1989a. Faecal Indicator Organism Sources for the Ogwe Catchment, South Wales, Report to Dwr Cymru and the Environment Agency, CREH University of Wales, Aberystwyth, and

Wyer, M.D., J. Crowther, and D. Kay, 1989b. Faecal Indicator Organism Sources for the Ogwe Catchment, South Wales, Report to Dwr Cymru and the Environment Agency, Environment Agency R&D Technical Report E61

identification of sources in large basins, Kay *et al.* (2005) attempted to model FIB fluxes through the Ribble basin on the basis of available digital land-use/land-cover data. The authors expanded their study area to include a fifth river (The Lostock, also draining to the estuary at the tidal limit but not included in the analysis of Whither, *et al.*, 2003) and upstream/tributary subcatchments (including two major tributaries to the Ribble itself, the Hodder and the Calder). They note the dominance of the Ribble in terms of drainage area (1130 km²), with relief ranging from the Pennine hills to tidewater. The Calder, Darwen, and Douglas basins are described as containing extensive conurbations, with the rest of the basin district being “largely pastoral” (forested cover mostly in the upper reaches of the Holder). The authors sampled 41 sites, selected to include major catchment outlets, to geographically coincide with existing discharge/gauging stations where available (temporary staff gauges installed otherwise), and to represent the range of land-cover types and relief present in the district, over 44 days of the 2002 bathing season. Samples were collected at 2-3 day intervals, with more intensive opportunistic sampling during high-flow conditions following rainfall. All of the 903 samples, so collected, were analyzed for culturable *E. coli* and Enterococci (only 859 samples provided usable TC values due to an incubator malfunction) and all six samples exhibiting below detection-limit values were reported at that limit. FIB density results were segregated into base-/high-flow categories on the basis of hydrograph response to rainfall. A Digital Terrain Model (25 m resolution) was used to develop a GIS model of the hydrology (drainage network, sub-basin outlets, watershed boundaries) of the basin district. The 17 classes of remotely sensed land cover represented on an available (25 m) Landsat raster were converted to the six land-use categories previously defined by field surveys of smaller basins (e.g., see Crowther, *et al.* 2003, above) by geographical overlay comparison with the previous studies; the authors here defined a seventh, “reservoir catchment” land-use

category for pixels representing land upgradient of lakes, to account for the sedimentation and die-off of upstream-generated FIB in still water bodies. The proportional land-use profile was calculated for the catchment upstream of each of the 41 sample points (for downstream sample points, this entailed summation of lands in all the subcatchments upstream). Multiple regression (forward selection, $F=0.05$ to enter) was performed to create a model to predict (dependent variable) geometric mean of FIB density at a sample point from (predictor variables) percent land uses associated with each sample point, for each FIB and for each (base/high) flow regime. High-flow FIB densities were significantly elevated (lack of 95% confidence intervals) over those at base flow for every site sampled. High-flow geometric-mean *E. coli* densities exceeded those at base flow four- to 63-fold (mean = 15 x). Similar patterns were revealed for TC (two to 85 times higher at high flow, mean = 11 x) and Enterococci (five to 215, mean = 21 x). In the regression models, % built-up land (entered with a positive slope) was found to be the most important predictor of FIB flux in all six cases (3 x FIB, 2 x flow regimes), and the only significant predictor for base-flow TC ($r^2 = 51\%$) and *E. coli* (59%). With additional predictors (% rough grazing and/or % arable land), high-flow models of all three FIB showed greater predictive power ($59.7\% < r^2 < 68.1\%$). All models were deemed of acceptable fit (based on normal probability plot of standardized residuals) and considerable significant ($p < 0.001$). The authors noted that the importance of built-up land in this basin is dissimilar to the results derived from previous studies of smaller, more pastoral catchments (in which improved pasture was generally the best predictor). The authors believed that the use of remotely sensed land-cover provides a viable mechanism for modeling/predicting FIB fluxes deriving from all (grouped point and diffuse) sources across large, mixed-use watersheds (for which field survey would be prohibitively onerous). They believed that their findings highlight the need for attention to wet-

weather, high-flow conditions in tributary watersheds (“which may account for > 90% of organisms”), information of which has been largely lacking in historic, routine FIB monitoring programs. They finally noted that large-area studies such as this, providing an expected-value baseline for FIB flux by land-use, would assist in identifying outlier “hotspots,” source areas worthy of targeting for priority remediation. (Kay, *et al.*, 2005)

Stapleton, *et al.* (2008), building on the previous studies presented here, extended the characterization of the Ribble basin district to include FIB budgets (integrated over the 2002 bathing season, a significantly rainy season as compared to 11 years of previous data) and (most relevant here) quantitative apportionment of sources as contributors to those budgets. The authors noted the importance of % built-up areas (with associated sewerage sources) as a predictor of FIB fluxes presented by Kay, *et al.* (2005). They further noted the dominance of sewerage sources (especially treated effluent in dry-weather, intermittent overflows in wet, with significant knowledge gaps in quantification of the latter) in the (desk-study estimated) FIB budgets presented by Wither, *et al.* (2003). The final-effluent (FE) discharges of the 15 largest WWTPs in the district (and estimated to contribute >95% of effluents by Wither, *et al.*, 2003), and any associated Storm Tank Overflow (STO) points were fitted with hourly-report discharge monitors. One CSO point in the inflow sewerage network for each of the 15 WWTPs was also so fitted. Routine FIB sampling of effluent points was supplemented by opportunistic sampling in response to rainfall; overflow points were only sampled opportunistically. All (1299) samples were analyzed for culturable (membrane filtration, triplicate analyses) TC, FC, and Enterococci (with results and analyses only for FC presented “as an exemplar”). A previous analysis (Wyer, *et al.*, 1998, see above) of the response of flow through a large WWTP in response to hydrograph events had suggested a threshold, 1.74 x average dry-weather flow (dwf), to separate base (dry-

weather) flow from stormwater-surcharged flow through the plant; analysis of the data collected in this study convinced Stapleton, *et al.*, that (with manual exclusion of any events exceeding $1.74 \times \text{dwf}$ but unassociated with recent rainfall) this threshold was appropriate to define high-flow FE discharge as a component in high-flow riverine discharge. All overflow (CSO or STO) data was associated with the high-flow regime in the rivers. The authors found that:

- 1.) As waters flowed downstream, their FC concentrations increased at WWTP sites, generally increasing with additional WWTP inputs downstream, but often attenuating between such inputs,
- 2.) FE FIB concentrations showed no general difference between flow regimes, with some plants exhibiting relatively poorer performance, and some exhibiting greater dilution under high-flow conditions,
- 3.) An estimated 5.24×10^{17} FC (cfu) were delivered to the Ribble estuary (at the tidal limit) during the 2002 season,
- 4.) The majority of all FC (4.78×10^{17} cfu) were delivered to the estuary under high-flow conditions,
- 5.) About 80% of the high-flow increase in sewage inputs to the estuary (over base-flow conditions) was attributable to intermittent overflow events.

For the purposes of source apportionment, the authors deemed diffuse sources most difficult to quantify. Decrying the impossibility of directly monitoring all such sources (especially in large watersheds) and the shortcomings of MST approaches (more below), they propose a model-based approach to estimate inputs from such sources and demonstrate its use. Noting that all sewage sources (FE or intermittent) within the district were located within the (25 m resolution) built-up areas depicted in the GIS model of Kay, *et al.*, 2005, the authors recalculated FC inputs, by subcatchment, assuming zero % built-up land across the basin district, to develop a model of “diffuse only” sources for high-flow conditions. The most important finding for this review is that the diffuse-only model predicted FC densities exceeding BWD Imperial standards for every point throughout the district, for every river (most by well over an order of magnitude of ten). The authors then proceeded to associate measured sewerage inputs (15 systems) to their associated subcatchments in the model, added in estimated (on the basis of the measured

sources) sewage loads elsewhere in the district, and calculated the proportion of loads (segregated between diffuse, FE, and intermittent overflow sources) delivered to the estuary during high-flow conditions. The authors concluded that no investment focused solely on WWTP discharges could achieve regulatory compliance, and that attention to storm-sewage discharges and reductions in diffuse-source pollution would be required to meet environmental objectives. (Stapleton, et al., 2008)

It is tempting to conclude that some implicit or presumed recognition of a lesser risk presented by FIB of non-human origin provides incentive to undertake difficult and expensive studies to quantify the magnitude of those FIB deriving from very large landscapes. Whether through regulatory relief provided by appeal to natural-background FIB densities deriving from comparable “reference systems” (as formerly practiced under the RSA scheme in California) or provided by a potential upward “reclassification” of bathing waters predictably impacted by runoff from subcatchments devoid of sewer sources, such relief does seem to have spurred efforts to conduct such studies. It should be noted, however, that knowledge of the relative contributions of FIB from various hosts, in and of itself, provides no information as to the relative risks presented by those FIB. The studies presented here do serve to show the importance of non-human contributors of FIB to regulatory compliance of waters, and the importance of rainfall in their delivery to those waters. Elimination of all sewage sources, even if possible, would not assure compliance to current regulatory regimes. Quantification of non-human FIB contributions is a necessary component in the prioritization of remedial efforts in any watershed, and some modeling approach would seem useful for such quantification in any heterogeneous, mixed-use watershed. It is only with a separate measure of risk, however (e.g., as in the

epidemiological study of Calderon, *et al.*, 1991, or in the re-analysis by Dufour, *et al.*, 2012, of data provided by Cheung, *et al.*, 1990, above), that such information provides knowledge of health effects.

This “geographic” source tracking strategy (monitoring of portions of the landscape segregated to isolate areas of homogeneous FIB sources) has the flexibility and power to resolve not only human vs non-human source contributions, but also to resolve the type of non-human source. Examining only sites known to be free of sewage sources in Tuscaloosa, AL, Shergill (2004) sampled wet-weather flow from four land-cover categories (open space, streets, parking lots, roofs), each segregated into sites deemed “prone” to pet and/or urban wildlife use (dog-walking areas for open spaces, tree cover accommodating birds and squirrels in all land covers) and “not prone” (open air, with pet exclusion for open space) in 2002-2003. The author analyzed (by Kruskal-Wallis) relatively few paired samples (~10-11 for each cover/source category) of *E. coli* and Enterococci densities, substituting the Upper Detection Limit for samples that exceeded that limit (a practice that would tend to suppress evidence of elevated densities in “prone” areas where the limit was most frequently exceeded). Despite large variability in the dataset Shergill found significantly ($p < 0.05$) elevated densities of both FIB on tree-covered roofs (as compared to “not-prone” roofs) and of Enterococci on “prone” streets (*E. coli* densities were considerably elevated for the roofs with tree cover, $p = 0.164$). The author was able to conclude that for these sites, without sewage sources but that regularly exceeded bathing-water standards in runoff, arboreal wildlife particularly contributed. In an interesting follow-up, the author then compared (Mann-Whitney test) the wet-weather source-area samples to a dilution series of wet-weather sewage effluent from the local WWTP. The lowest FIB concentration (greatest dilution) in the sewage for which the dilute sewage and the source-area samples could

be confidently ($p < 0.05$) separated as different represented a threshold concentration for source apportionment. Any runoff sample exceeding that threshold could confidently be deemed sewage contaminated (rather than only background). For “prone” streets (the source area with highest *E. coli* densities), the concentration above which runoff would unambiguously contain sewage was 3470 cfu/100 ml (MPN). The relevant value for Enterococci was found to be 18,530 cfu/100 ml. (Shergill, 2004)

2.2.3.3.2 Specific Contributions

The large quantities of FIB from non-human hosts should not be surprising considering the large number of warm-blooded animals defecating on the landscape. Recall the estimate in Kay, *et al.*, 1999, that 11 million sheep in Wales, at normal daily rates of defecation, deposited a fecal mass equivalent to that which would be produced by 55 million Welsh humans. Non-human homeotherms simply outnumber human ones, and many species eat and eliminate far larger volumes than do humans. Bartram (2012), considering only human and livestock sources of feces worldwide estimated that humans generate 14% of the total. For decades, information concerning defecation rates and fecal bacteria have been collected (much by agronomists for the former and microbiologists for the latter). Yost, *et al.*, 2011, provided a survey of recent work by others comparing the (range of) *E. coli* and Enterococci densities (cfu/gram fresh feces) for humans, bovines, swine, and poultry (and from their Table 1):

Humans: <i>E. coli</i> (10^8)	<u>Enterococci</u> : ($10^5 - 10^8$)
Bovine: ($10^5 - 10^8$)	($10^4 - 10^6$)
Swine: (10^7)	(10^8)
Poultry ($10^7 - 10^8$)	($10^6 - 10^7$)

The authors also cite work showing *E. Coli* densities of 1.2×10^4 CFU/g dry feces from Weddell seals, the enterococcal loads for a host of bird species, and information concerning alternative-indicator content in the feces of many animals.

Several researchers have used this sort of information to apportion FIB in waters to their sources. Census counts of animals present in sources areas, when combined with defecation rates and bacterial loads, serve as an estimate of the FIB contributed by each source host. One example of this kind of study (Wright, *et al.*, 2009), in which monitored FIB at a recreational beach were apportioned between birds, dogs, humans, and shrimp, has already been reviewed here (above). Such attempts, however, may be problematic. Pitt (2007) provides a very large array of data (somewhat older) concerning FIB concentrations (mostly TC, FC, and FS), and defecation rates from a wide variety of animals. The author also reviews several comparisons of fecal generation by tributary sources to monitored FIB loads within receiving waters. In a study of the Lower Rideau Watershed (Ottawa), the calculated FIB deposited by pets and birds in the tributary watershed exceeded the annual FIB in runoff by orders of magnitude. In another, dogs, cats, and rodents in New York City were estimated to generate far more FIB than ever reached the receiving waters, at least implying some attenuation or retention of FIB on the landscape. Moreover, in studies of migratory waterfowl stopping over in (often wetland) areas, results were inconsistent (sometimes the water quality improved upon flow through the area, and sometimes it deteriorated), suggesting that nesting area could serve as either a sink or a source of FIB. FIB not deposited directly into the receiving water spend some time on the land before they are washed from source areas to water bodies. Without some knowledge of FIB survival on surfaces, and of their transport across landscapes, these direct-count methods may be misleading.

2.2.3.3.3 Microbial Source Tracking

While the geographical scheme discussed above, in which the likely source host of FIB is derived from the region from which the FIB is collected, is certainly a useful technique to track the source of microbes, the term Microbial Source Tracking (MST) is most often used to refer to situations in which a tracer is collected and analyzed along with the FIB. The tracer, a secondary indicator, is presumed to provide information concerning the source of the FIB in the same sample, and may be chemical (including biochemical) or biological (including viral). Use of biochemical tracers of late has come to include separate genetic analysis of the sample FIB themselves, and is often referred to as Molecular Source Tracking.

An early use of tracers involved the use of a biological (bacterial) tracer, FS (at a time when the recognized regulatory FIB was FC), and the FC/FS ratio was believed to infer the (human vs non-human) source of FC in a sample. While the scheme was developed from several workers over time, the rationale and a demonstration of its use is presented at Geldreich, *et al.*, 1968. Previous studies had indicated that the TC/FC ratio in the fresh feces of pets (dogs and cats) and urban wildlife (rats, chipmunks, and rabbits) was consistently quite low (< 0.7), while human feces typically exhibited a 4.4 ratio and > 4.0 in domestic sewage. The authors collected stormwater from three urban locations (street gutters, a wooded hillside, and an outfall) in Cincinnati, OH, and on nearby rural one draining agricultural lands, over \sim a year for all. FC densities often (and seasonally) exceeded the General Use standards for environmental waters of the time. The FC/FS ratio was ≤ 0.70 for all seasons at all sample points. The authors recommended prohibition of pets from waterfront areas, an improved garbage collection plan, and diversion of stormwater from beaches and reservoirs (Geldreich, *et al.*, 1968). Sinton, *et al.*, (1998), in a review of methods to distinguish FIB sources, discussed the use of the FC/FS ratio.

They noted that subsequent research had determined that the speciation of FS differed between feces of humans many animals, and that the various species showed differential survival in the environment. This differential survival caused the FC/FS ratio to shift over time. The ratio started high and dropped in human-derived samples, and started low and rose for many agricultural animals. Although use of the direction of this “ratio shift” to apportion sources was noted, the authors found that use problematic.

In 1993, EPA published a “User’s Guide” for investigating, and distinguishing the source of, inappropriate discharges of pollutants into separate storm sewers. The document provided “fingerprints,” patterns of the presence/absence of various parameters (chemical, visual, or odiferous) evident in dry-weather flows by which practitioners could divine the likely source of a discharge into the sewer. Sewage sources could be differentiated from all other likely sources (potable water, industrial water, washwater) by the presence of all of fluorescence, potassium, ammonia, and distinctive odor. Sanitary-sewage could be distinguished from septic-tank effluents by the additional presence of surfactants and visible floatables in the former (Pitt, *et al.*, 1993). Sinton, *et al.*, (1998, a review focused on environmental waters) noted that ammonia nitrogen is associated with fecal-matter breakdown but also occurs in natural environments. Uric acid, likewise had been used to track human-sewage discharges in waters, but could derive from other vertebrates. The authors also review the use of

- 1.) Fecal sterols: Coprostanol is the principle human fecal sterol, but is also produced in the guts of other mammals. A “fingerprint” provided by combination of sterols defecated by mammals can be used to apportion between sources because of specific difference in diet, capacities for sterol synthesis, and sterol isomerization by anaerobic flora. The sterol profile of humans, however, is similar to that in cats and pigs. Little information on preservation of isomer profiles in the environment was available.
- 2.) Fluorescent whitening agents: The authors noted low detection limits in the analysis of fluorescence but also the very large dilution of the whitening agents in environmental waters. They further note successful tracking of septic-tank leachate

- plumes in both lakes and groundwater. While whitening agents are specific to human sources (washing waters, not necessarily sewage), other natural materials fluoresce as well, raising the potential for analytical interferences in natural waters.
- 3.) Sodium tripolyphosphate: Another component of washing powders, this tracer is prone to hydrolysis and (adsorption to sediments) settling in natural waters. Septic-tank plumes had been successfully tracked, but concentrations were often not much above contemporary limits of detection.
 - 4.) Long-chain alkylbenzenes: (Still another indirect tracer of human feces, and still another detergent component) These substances are purely synthetic and are stable in natural waters. They have been used successfully in tracing sewage through waters and sediments. (Sinton, *et al.*, 1998)

A review by Simpson, *et al.*, 2002 decried that knowledge concerning the long-term fate of caffeine, fragrance materials laundry detergent brighteners, and fecal stanols and sterols is lacking, and more work is needed to establish the host specificity of fecal sterols. Field (2004) reviews the use of tracers including caffeine, fecal sterols, brighteners and surfactants, fragrances and polycyclic aromatic hydrocarbons, and finds that all can identify recent fecal inputs. The author particularly noted that caffeine and fragrance combined was useful to identify human sewage (in developed areas), and that fecal sterols profiles were successful in distinguishing agricultural from wildlife inputs, and for distinguishing between contamination from human, dogs, and birds. The author noted, however, that after release to waters, over time the spread and persistence of the plumes of these substances were unlikely to match those of pathogens.

Sinton, *et al.*, 1998, review F-specific RNA coliphages as potential microbial source tracers. Also known as F+ or male-specific, these viruses attach to the F or “male” pilus of *E. coli* cells (and are, thus, differentiated from somatic coliphages that attach to the cell wall). F-specific coliphages may also contain DNA genomes rather than RNA. The RNA phages could be grouped into four (genetically based) serotypes by their differential sensitivity to certain antiviral sera. Research in the 1980s indicated that type I was generally isolated from the feces of non-human animals, II and III from human sewage, and IV was of mixed origins. The authors noted

that the viruses seem rare in human feces, but “paradoxically” are typically well represented in human sewage ($10^3 - 10^4$ plaque forming units, pfu, per ml). The survival of the phages in the wild, and ability to reproducibly identify type in natural matrices had not been extensively studied. Serotype identification of the phages was deemed a difficult and expensive task, but recent work had indicated that genotypic methods of analysis were feasible (Sinton, *et al.*, 1989). Embrey, 2001, noted that several researchers had also nominated F-specific RNA coliphages (regardless of serotype) as surrogate indicators of fecal risk for viral infection as well as source (based on similarity to human enteroviruses in terms of morphology). The author sampled 31 sites (10 in rural/agricultural areas, the remaining urban/suburban) along streams draining to Puget Sound, WA. Analyses of the samples included FIB, coliphage, and a suite of 63 chemical tracers (the last only at 13 sample sites) in an exercise to characterize the microbiological quality of these streams (draining to shellfisheries) and to identify fecal sources. Of the eight sites testing positive for at least one chemical source tracer, seven were urban/suburban, and the one agricultural site revealed only caffeine. Only 15 sample sites yielded detectable F-specific RNA coliphages, and only at low densities (1 – 7 isolates/site). Two of those samples serotyped to group II, and both were in urban, residential areas. Ten of type I, indicating non-human sources were about split evenly between more densely populated areas and rural ones, though the possibility of pet/wildlife influence in the former could not be ruled out. The authors opine that larger samples might have reduced the number of failures to detect the coliphages. Noble, *et al.*, 2003b, conducted an MST two-laboratory study of the ability to detect, enumerate, and correctly type coliphage samples. Natural freshwaters and “mixed matrix (filtered seawater or freshwater amended with humic acids), seeded with unlabeled slurries of various fecal samples (sewage, and feces from humans, dogs, cows, and gulls, alone or in various combinations) were sent to the

laboratories. The detection and enumeration portion of the trial portion was conducted in two phases, with the first procedure providing enumeration, followed (if the first test failed to detect coliphage) a procedure involving enrichment but only providing presence/absence results. In the first phase, workers were able to enumerate 4 of the 12 freshwater samples ($\sim 10^2 - 10^3$ pfu/100ml) and then successfully detected the presence of coliphage in three more (with disagreement between the labs for one more). In the salt or humic matrices, only two samples were initially enumerated, with six more subsequent positives. In the second phase, all samples testing positive in the first were typed (genotyping in one laboratory, sequence analysis in the other). In the genotyped freshwater analyses, all four samples seeded with sewage received a type designation (i.e., tested positive in the first phase), three of them correct (genotype II and/or III). One was mistyped as I, and a cow/gull slurry was misidentified as of human source; later analysis of the pure fecal samples revealed a minority of gull isolates also typed III. For the matrix samples, all three samples containing sewage received typing (two correctly). The third, which had been mixed with dog and cow was only typed I. One sample with human and gull feces was successfully typed III. The sequenced samples containing sewage (alone or in combination), and one with human feces in combination received the correct human-source designation. Of note was the inability of the analysts to detect coliphage deriving from individuals (human, dog cow) compared to the detection rate for bulk, composited feces (sewage, gulls), but the authors believe that risk also derives from shedding populations rather than individuals. The consistent “mistyping” of gull feces, both in pure form and in the slurries, serve as reminders that source designation by typing is a statistical construct (deserving of refinement) and not absolute (Noble, et al., 2003b). Noting recent work showing the significance of source designation by typing of F-specific RNA coliforms (with exceptions), Stewart-Pullaro, *et*

al.(2006) apply the scheme to tracking sources (and further evaluate the scheme itself) in South Carolina. Samples (117) were collected at 96 water stations in five SC watersheds. Additional samples were drawn at 10 municipal wastewater outfalls, one mobile home park, a retirement home, two hog lagoons, and litter from seven chicken houses. Feces samples were collected from a horse, a dog, a bird, and two cows. Once F-specific RNA coliphages were isolated, they were typed by both serological and genetic means. Measurable F-specific RNA coliphages were found in surface waters, wastewaters, hog lagoons, and chicken litter, with greatest concentrations in wastewaters. The authors believe that rarity of infection in individuals, and compositing phages of multiple individuals into wastewater, with possible phage replication among the high density *E. coli* in sewerages explains the phenomenon. With the exception of wastewaters (87% type II or III), type I coliphages (94% in surface waters, 70% in hog lagoons, though chicken-litter isolates were about evenly split between types I and IV) dominated the landscape. Fourteen of 96 typed surface-water samples were typed II or III. Nine of those were found to be downstream of wastewater discharges (GIS). In a 2007 review of source-tracking literature, Field and Samadpour noted the library-independent nature of the scheme, and the recent changes in favor of genotyping over serotyping. They cited recent information concerning a differential environmental survival rate among phage types, providing yet another possible explanation of greater human-sourced densities in wastewaters than in feces.

The coliphage MST scheme described is technically a subset of the class of library-independent molecular MST methods (though not a typical one). Such methods more generally compare the structure of a target gene (or genes) found in a sample, amplified by PCR, to markers, the structures of the same gene(s) found in different strains of the organism. The targets/markers are chosen to be on active segments of the genome, likely to change (base-pair

additions/deletions) over time in response to evolutionary pressures, such as adapting conditions in the gut of a specific host but to be well conserved within the adapted strain. Library-dependent schemes are, in some ways, quite the opposite. Sample organisms are grown in culture to “amplify” the DNA content, the cells are lysed, and the DNA is extracted. The DNA strands are then clipped into smaller pieces by the use of restriction enzymes, which target specific, short base-pair sequences, and the pieces are separated in to a spectrum pattern on electrophoresis gel (usually based on fragment size). The resulting DNA “fingerprint” is then compared to patterns produced in a similar way with material from other, past samples. In the MST case, the past samples would be a complete (as possible) library of fingerprints produced locally from the same species inhabiting different hosts. In most MST cases, the organism of interest is the FIB itself, though similar techniques (Meays, *et al.*, 2004). Sinton, *et al.*, 1998 did not include microbial tracers in their MST review, writing “DNA-based approaches show considerable promise for source identification, but we consider that this area of work is too new to allow a broad review.” By 2004, Mays, *et al.*, had produced a new review including comparison of seven different variations on the theme presented here. The library-independent approaches are so called because markers can be obtained from the feces of local hosts. Without PCR amplification of the sample material, library-dependent approaches must generally both culture samples and maintain an extensive library. Most of the various approaches (both categories) presented are characterized as complex, expensive or technically demanding, and some are “still early in development.” Field, 2004, stressed the need for local, exhaustive libraries for the methods dependent on them and worried that the markers of waterborne sample organisms may genetically diverge after shedding by the host.

2.2.4 FIB in Stormwater Source Areas

Section 2.2.3.3.1 establishes the importance of storm washed FIB of non-sewage origin to regulatory compliance in receiving waters. Section 2.2.3.3.2 reveals a gap in current knowledge between the load of defecated FIB to the landscape and the load of such FIB mobilized to stormwater. MST tracers, as yet, suffer from inability to fill that gap, due either:

- 1.) to differential survival and transport between the tracer and biological entities (e.g., chemical tracers),
- 2.) to insufficient source specificity of the tracer (e.g., F-specific coliphage),
- 3.) to a failure to account for biological adaptivity on the landscape (library-independent molecular MST in general), or
- 4.) to an inability to exhaustively conclude that all relevant molecular fingerprints are available for comparison (library-dependent molecular MST in general).

This situation would seem to call for exploration into:

- 1.) Quantification of relationships between stormwater source-area environmental characteristics and the net persistence (growth – death) of FIB deposited on the landscape during inter-event (between rains) periods.
- 2.) Quantification of relationships between the stormwater source-area characteristics and the mobilization and transport of surviving FIB from the source area during subsequent rains.

Though the etiological/epidemiological considerations presented thus far provide useful guidance, the first task essentially represents an exercise in ecology of microbial populations, the study of the interactions between populations and their environment. The second task is more an exercise involving processes more generally studied in engineering disciplines. The strategy applied here will involve appeal to basic principles of both the biological and engineering disciplines, often firmly established in textbooks rather than in current journals, with more recent supporting articles where relevant.

2.2.4.1 FIB Characterizations

At the grossest (least specific and, phylogenetically, most “primitive”) level of taxonomy (classification) of living things, FIB share a domain. As implied in their name, all FIB are classified as members of the *Bacteria* domain, and share some characteristics relevant to this review. All bacteria are unicellular organisms (Madigan, et al., 2002, p. 335), though cellular morphologies may vary considerably (p. 64). Single cells may cluster and/or interact, but each cell contains all needed assets for survival and for propagation. All bacteria are also microscopic, encompassing varying linear dimensions generally ranging from ~ 0.2 – 2 microns (p. 65). The bacterial genome consists of a nucleoidal chromosome, a closed loop (“circular”) structure of a single (double stranded) DNA molecule (though smaller, extra-chromosomal DNA molecules of similar structure may be present, especially in plasmids, p. 25). Bacteria are “sexually promiscuous,” in the sense that genetic material is frequently transferred from one single-cellular organism to another (by several processes, including plasmid transfer through conjugation, pp. 278-296) and may even be incorporated into the recipient’s chromosome through genetic recombination (pp. 276-278). Bacterial reproduction however, is purely asexual, by the process of binary fission (and, in a few taxa not including our FIB, by budding, pp.389-390). Binary fission is a process by which a cell’s genome is first replicated, and sufficient cell-structure components are generated to provide viability to each of the resulting duplicate chromosomes. These components are segregated within the “parent” cell and subsequently distributed between two “daughter” cells by physical division of the parent (Madigan, *et al.* 2002, pp.3-5).

The FIB under study here, however, diverge at the very next phylogenetic level of classification (taxonomically subdividing the Bacterial domain into its various phyla). *E. coli* is classified in the phylum *Proteobacteria*, while Enterococci are assigned to the phylum *Firmicutes*. This phylogenetic (presumed evolutionary) split implies meaningful differences in the genotypic (based on genetic-material composition) and phenotypic (based on observable characteristics) expectations as to how the different taxa “behave” (especially survive) under various conditions. Moreover, the FIB are further separated by at least four more hierarchical levels of taxonomic classification (four more increasingly distal “branches” in the phylogenetic “tree of life”). Phyla are divided (and presumed to evolutionarily diverge) into classes, classes into orders, orders into families, and families into genera. It is at the genus level (*Enterococcus*, spp.) that Enterococci are defined. *E. coli* is just one species in the genus *Escherichia*. Though both taxa of FIB have obviously evolved adaptations to the same primary habitat (the digestive systems of homeothermic hosts), they have apparently done so through significantly divergent development of genes, enzymes and structures (Carrero-Colon, *et al.*, 2011, pp. 25-31). Viewed in this light, it should not be surprising that FIB not only have poor environmental (secondary-habitat) correlation with the pathogens of interest, but also poor correlation between FIB (section 2.2.1). In any study of factors relevant to the environmental survival of FIB, the taxonomic identity of the FIB themselves must be considered one such factor.

2.2.4.1.1 Enterococci

Enterococci, as the name implies, are cocci, non-motile spheroid cells of small diameter (~ 0.5 – 1 micron). They can occur as single cells or in stranded (“string of pearls”) clusters. In cultures, they have been found to survive and grow at temperatures ranging from 10 – 45⁰ C, and

to survive at 60⁰ C for as long as 30 minutes. The various species typically exhibit maximum growth at an optimum temperature of ~35⁰ C. They thrive in the mammalian gut (both small and large intestines, Madigan, *et al.*, 2002, pp. 734-735), reaching densities of 10⁸/gram feces in the human colon, though they only make up about 1% of the intestinal flora and densities vary with host diet. Enterococci have also been found in matrices as diverse as soil, water, dairy products, food and plants, and been found to survive in 6.5% saline broth, at pH as high as 9.6, and show a resistance to desiccation. At least fifteen species have been described, and 23 species have been identified genomically (Carrero-Colon, *et al.*, 2011, pp. 29-30).

Carrero-Colon, *et al.* (2011, pp. 29-30), citing earlier work, describe Enterococci as lacking cytochromes and as catalase negative (with “pseudocatalase” activity), and they characterize the genus as aerotolerant anaerobes (i.e., with the capacity to detoxify active-oxygen species, but limited to a fermentative metabolism). More recent work (e.g., Winstedt, *et al.*, 2000) however, has shown both cytochrome structures and oxidase activity in Enterococci, allowing for the much greater conservation of cellular energy available through oxidative phosphorylation in oxic environments (where environmental O₂ serves as the terminal electron acceptor of catabolism). This “nascent” respiratory capacity in Enterococci has led many (though not all) researchers to characterize these organisms as facultative aerobes. Enterococci are lactic-acid (end-product) fermenters, and display a broad array of isomerases providing an ability to catabolize a wide variety of sugars and sugar moieties (Carrero-Colon, *et al.*, 2011, pp. 29-30).

2.4.1.1.2 *E. coli*

Escherichia coli represent a single species, based on genotypic similarity across the taxon, though hundreds of strains (sub-species, some pathogenic) have been described and even

engineered. As a species, *E. coli* can be characterized with greater granularity than can the genus *Enterococcus*, and as a “model organism” (Madigan, et al., 2002, pp. 316-318), the species has probably been better characterized than any other.

E. coli cells are Gram-negative rods, 0.75 – 1 micron wide and 2 – 3 microns long (Madigan, et al., 2002, p. 65). Cells occur singly or in pairs, and may be motile (by flagella, Carrero-Colon, et al., 2011, p. 25). *E. coli* colonizes the gut (especially the colon) of homeothermic hosts in densities as high as 10^9 /gram feces (sometimes exceeding 1% of bacterial biomass), and the densities in the large intestine are not greatly affected by the diet of the host. The species has been found in sewage and treated effluent, natural waters, and multiple environmental surfaces (sand, soils, sediments, p.28). In waters containing modest nutrients, *E. coli* can survive for months at temperatures as low as 15⁰ C (p. 29). *E. coli* growth in the environment is inhibited by extreme pH levels (high or low) and desiccation (Maczulack, 2011, p. 277). Like all coliforms, *E. coli* catabolizes lactose to gas at 35⁰ C and, like all fecal coliforms, can also do so at 44⁰ C (Carrero-Colon, et al., 2011, pp.27-28). The “cardinal temperatures” (minimum at which cells can grow, optimum at which growth is maximized, and maximum above which growth cannot occur) are listed as 8⁰ C, 39⁰ C, and 48⁰ C, respectively (with minor variation by strain and complexity of growth medium, Madigan, et al., 2001, pp. 151-152).

In anoxic environments, *E. coli* is capable of either lactic acid or mixed acid fermentation pathways (the latter of which catabolizes a portion of the substrate structure to gaseous end products, Madigan, et al., pp. 120-122 and pp. 377-378). *E. coli* is catalase positive and oxidase negative (lacking cytochrome c), though its universal inclusion of cytochrome b provides for use

of environmental O₂ as a terminal electron acceptor in oxic environments⁴ (Carrero-Colon, *et al.*, 2011, p. 25, and Madigan, *et al.*, pp. 128). Wild-type *E. coli* strains show few requirements for growth factors (illustrating considerable anabolistic flexibility) and are not limited to sugars as substrates for catabolism (deriving energy from amino acids and organic acids as well, Madigan, *et al.*, pp. 378).

2.2.4.2 Bacterial Population Dynamics

The unicellular nature and the shared (phylum-level) reproductive process of bacteria (binary fission) mechanistically impose a particular mathematical form upon changes in bacterial populations. While a single cell may simply persevere, any change in existential status is limited to a binary “choice,” namely double or die. Net population growth, simply the sum of status changes of the individual cells in the population, depends only on the number (or density) of cells present and the likelihood that any cell will divide or disappear. Net growth of bacterial populations is inherently exponential. This implies that the logarithm of cell numbers in a population changes linearly with respect to time (Madigan, *et al.*, 2002, pp. 142-144, and note the similarity of this log-linear form to 1st order chemical reactions, Reynolds and Richards, 1996, pp. 7-8). Population changes depend only on the concentration of one “reactant” (cells) and on a rate-of-change constant imposed by environmental conditions. The rate constant of growing cell populations under given conditions is often alternatively expressed as “generation time” (the average lag between one cell division and the next) or “doubling time” (the time to double a population of cells).

⁴ The fact that both taxa under study here achieved facultative aerobic competence, but that each did so using completely different organelles (used here in the primitive sense, as intracellular structures not necessarily membrane-bound) and enzyme systems, again underscores the need to treat them separately in any study of their ability to adapt to environments.

Living cells, however, differ from simpler chemical reactants when exposed to a change in environmental conditions. In the case of cells, the reactant itself may change in response to a change in conditions, either through selection of the most viable sub-population at the new conditions or through the cell-by-cell induction of previously unexpressed genes more appropriate to survival at the new conditions. In the case of cells, the reactant may react to environmental changes in ways that change the local environment, through release of cellular components to extracellular space. The mechanistically appropriate instantaneous rate constant of change in a microbial population may not be constant in time.

Much work investigating the response of bacteria to a change in environmental conditions has been through inoculation of batch culture media in the laboratory. Such studies provide a general pattern (the “Growth Cycle”) of time-dependent population response to a new environment (in a closed system). Five sequential phases of this pattern are identified, each of which may or may not be exhibited in any given circumstance, and the causative factors of each phase are well established:

- 1.) Lag Phase – Upon inoculation to a fresh culture medium (even if that medium is conducive to inoculant growth), exponential growth of a population does not generally begin immediately. Inoculated bacteria often suffer a period of suppressed reproduction, the length of which is dependent on the history the inoculum and the degree of change in conditions involved in the transfer to the new medium. Bacteria previously undergoing exponential growth and transferred to a new medium providing the same or similar conditions, for example, may experience only a short lag or none at all. Cells previously in some other phase of the growth cycle, however, may experience a lengthy lag as the cells are forced to re-synthesize depleted cellular components needed to re-initiate log-linear growth, even when transferred to a similar culture medium. Exponentially growing cells, transferred to a medium of changed composition or to a medium exposed to different environmental conditions will likewise experience a lag as the new enzymes or structures required to exploit the new environment are synthesized. While it must be noted that these growth-cycle phases derive from closed-system batch studies, this initial lag phase occurs early in the cycle, when the cell population is small and bacterial alteration of the medium is minimal.

- 2.) Exponential Phase - If and when enough cells from the inoculum have adapted to the new, post-transfer circumstances, log-linear growth may begin with a rate constant determined by the cells' ability to exploit components of the new medium under the new culture conditions.
- 3.) Stationary Phase – The closed nature of the batch system, through limited availability of some nutrient essential for reproduction and/or a significant presence of some cellular waste product which inhibits reproduction, subsequently brings about a stationary phase (i.e., rate constant ~ 0) in which cell death is roughly balanced by cell fission and the population remains stable. In the case of nutrient limitations this phase is characterized by “cryptic growth,” where cell death and lysis release nutrients for recycling, allowing still viable cells to grow and divide. This phase also gives rise to the concept of “carrying capacity” (see below) in environmental biology, defining a population that can be sustained by an environment dependent on the “openness” of that environment to nutrient inflow and waste outflow.
- 4.) Death Phase – Build up of inhibitory waste products in a closed system may bring about a situation in which cryptic growth can no longer balance cell death (i.e., reduced carrying capacity of the system). Death phase is characterized by exponential decline, generally with a decay rate of lesser magnitude than the growth rate previously seen in the Exponential Phase. (Madigan, *et al.*, 2002, pp. 144-151)

Many of the phases in the growth cycle have been seen in environmental studies already reviewed (especially at section 2.2.2).

The existence of a Stationary Phase in the population dynamics of batch systems has given rise to interest in factors contributing to maintenance of steady-state populations in continuous, flow-through systems (probably at least as important to our ecological interests here as the batch-study findings). Numerous batch studies reveal that both (exponential) population growth rate and the net yield of such growth are quite sensitive to the nutrient availability in the system at low levels of nutrient. At higher levels of available nutrients, net growth becomes relatively insensitive to nutrient concentration and only the (stationary population) yield is significantly affected. In the laboratory, continuous-culture studies are typically carried out in a “chemostat,” a culture vessel of fixed volume in which the flow-through volume of fresh medium through the system (dilution rate) and the nutrient concentration of that fresh medium can be controlled. A chemostat allows for separate manipulation (unavailable in batch studies) of

population growth rates (through manipulation of the dilution rate) and the equilibrium population densities (through manipulation of the concentration of some growth-limiting nutrient in the diluent). Though chemostat studies are limited to liquid systems, they show that stable microbial populations can be maintained (by balanced cell growth and cell loss) across wide variations of dilution rates (“openness” of the system to input nutrients and shed wastes and cells), and that the densities of those stable populations depends mainly on the instantaneous nutrient availability with the system (Madigan, *et al.*, 2002, pp. 148-151).

The ecological focus of this proposed research centers on bacteriological behavior subsequent to fecal “inoculation” of landscapes (the secondary habitat) by populations originating in digestive systems of homeothermic hosts (the primary habitat). This review of basic principles of microbial population dynamics illustrates that any model of such behavior should be of log-linear form. The characteristics that differentiate living cells from other chemical entities participating in 1st-order processes further imply that exploration of such models must account for the potential serial phases of the biological response to changes in environmental conditions. These considerations suggest the use of log-linear segmented regression with unknown breakpoints.

2.2.4.3 Environmental Survival Factors

Besides the obvious (discussed above) need for available nutrients allowing for survival of bacteria in any environment, Madigan *et al.* (2002, p. 151) list the four major environmental factors affecting microbial growth as temperature, pH, availability of water, and oxygen. The authors further name (at pp. 272-273) exposure to ultraviolet radiation as a meaningful environmental inhibitor to bacterial growth.

Besides the (maybe not so obvious, but also discussed above) inhibitory effects of waste products released by cells but remaining in the cells' local environments, the authors (at p. 636) also name bacterial creation of biofilms as a mechanism by which cells can change (and, in this case, enhance) their survival likelihood by release of cellular products to their local environs.

Each of these environmental factors is discussed separately below but, due to the importance of the change in conditions to the response of microbial populations moved between environments, a brief description of the FIB primary habitat is considered first.

2.2.4.3.1 The Homeothermic-Gut Environment

Homeothermy (the “warm-blooded” lifestyle) is practiced by a wide variety of animals to regulate core body temperatures internally. Internal temperature regulation allows for maintenance of temperature-sensitive biological functions at or near optimal conditions in the face of varying temperatures in the surrounding environment. Homeothermy enhances survivability at a cost, especially in terms of nutritional energy requirements. Homeothermic mechanisms include synthesis of insulative structures (fur, feathers, blubber layers), muscular actions (goose bumps, shivering), and environmentally triggered enzyme cascades that increase metabolic rates solely to create heat. There are additional costs to water balance, both in heating mechanisms (which increase evaporation) and cooling (perspiration, Brody, 1945, pp. 264-265).

These mechanisms to regulate internal temperatures, with some variable efficiency⁵, are exhibited across many animal species (essentially birds and mammals). The temperatures maintained vary by species, but are much more constant through time than are those in the

⁵ The efficiency of homeothermic mechanisms is somewhat dependent on body size and the ratio of radiative surface area to heat-generating body mass. Many small homeotherms, and many (of any size) in or near polar regions must migrate or hibernate in winter. (Brody, 1945, pp. 264-265)

surrounding environment. Across most homeothermic species, regulated body temperatures range between ~ 36 and 43° C. This typical range can be further divided between mammals ($38^{\circ} \pm 2^{\circ}$) and birds ($40^{\circ} - 43^{\circ}$, Brody, 1945, pp. 265-266).

FIB, by adapting to survival in the core-body gut of homeotherms, gain a measure of temperature stability there, sharing the bounty deriving from the (energy-wise) spendthrift lifestyle of their hosts; the long-term viability of the FIB, of course, still requires the ability to survive in the (more variable) external environment long enough to colonize (infect) new hosts.

The broad diversity of hosts (almost all of the birds and mammals) to which FIB (and many other endosymbiotic microbes) have adapted implies an ability to adapt to a diversity of host digestive-system structures. Though the temperature throughout the digestive tract of a homeothermic host remains quite constant, other factors important to survival of microbial symbionts (e.g., pH and oxygen) vary widely by location within a gut ecosystem.

The enteric flora (the community of endosymbionts inhabiting the gut) represents a huge number and wide diversity of organisms. The number of (overwhelmingly unicellular and almost all bacterial) floral cells in a single gut (as high as 10^{13}) vastly outnumbers the (larger, eukaryotic) cells of the host organism (typically on the order of 10^9 , Kraus and Khafipour, 2011, p.1). Molecular methods reveal that ~ 75 - 80% of gut inhabitants have not yet been cultured, and imply a much larger species and metabolic diversity in the flora than is available to a single-species host. The large number of organisms in the flora implies at least a commensal form of symbiosis (in which the floral community enjoys the warm, wet, and dark environment of the gut at no added cost to the host organism). Much work, however, suggests a mutualistic symbiosis, in which both participants (flora and host) benefit. Most of the diverse floral organisms in the gut express catabolic (especially fermentive) functions unavailable to hosts, and digest

macromolecules (refractory to animal enzymes) into soluble chemical species that the host can absorb and utilize. Many floral participants express anabolic functions lacking in animal systems, and synthesize cell components that can then be captured by the host through digestion of floral cells. In general, any member of the enteric flora that successfully colonizes any ecological niche of the host gut will provide competition for that niche against its colonization (infection) by any host pathogen subsequently introduced (Willing and Jansson, 2011, pp.1-40, and Maczulak, 2011, p. 256). These mutualistic associations seem to have been developed over eons. Molecular methods have identified over 100 bacterial phyla present in the environment. Broad studies of enteric flora of homeothermic hosts find only 17 of these phyla present. Moreover, only five of the detected phyla (*Firmicutes*, *Baracterioides*, *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Fusobacteria*) represented over 95.5% of the floral sequence fingerprints found across all hosts studied. This belies random colonization of the gut by environmental taxa, and implies that floral species have evolved to this primary habitat and that the gut has evolved to enhance successful colonization of the most host-beneficial symbionts. The biogeography of the gut is a product of coevolution, in which the survivability of both host and microbes is relevant (Willing and Jansson, 2011, pp.42-45).

The simplest homeothermic gut type is exemplified by mammalian carnivores, though some omnivores (e.g., humans) and herbivores (pandas) share the structural features described here. The carnivore structure consists of a single tube (with or without organ-specific sacculations) lacking diverticuli. Digestive organs present here are sequentially arranged, proximal to distal (head to tail) in the order: mouth (oral cavity), esophagus, gastric stomach, small intestine, and large intestine (colon). The oral cavity provides some digestive enzymes (e.g., salivary amylase), but primarily provides for physical disaggregation (mastication, or

chewing, by action of teeth and jaws) of bulk foodstuffs into smaller particles to accelerate digestive processes downstream. The esophagus, in the carnivore model, mostly serves as a simple conduit to the stomach, but is included as a separate organ because of potential digestion-related alterations that may occur in alternative digestion-system models (discussed below). The stomach is the primary organ of digestion in this carnivorous model. This is the point at which the host secretes major, generalized digestive enzymes (e.g., pepsinogen) and enzyme co-factors (esp. hydrochloric acid, HCL) into entrained (masticated) food, and converts large macromolecules (including many entrained microbial cells) in food into more soluble/absorbable/usable moieties. The stomach also (by virtue of pH ~2) exerts some selective pressure on potential floral organisms ingested along with the food. Though some microorganisms can “hide” from the local acidic environment within food particles, and passage through the stomach is generally rapid, subsequent survival depends on, at least transient, tolerance to very low pH. The digested foodstuffs (and any undigested, refractory components) and surviving microbes pass sequentially into the small intestine, along with host glandular secretions (notably bicarbonate) to enhance host absorbance of digesta. Increased pH in the small intestine (typically 4 – 5) allows for surviving (acid and oxygen tolerant) microbes (notably, for this proposed study, facultatively aerobic Enterococci) to grow and reach significant densities. The multiplying cells in the lumen of the small intestine compete with the host for nutrients and progressively (distally) raise the pH and lower the oxygen content of the digesta. By the time the digesta and entrained microbes reach the colon, the material present is largely devoid of host-digested nutrients and the food material is mostly made up of refractory components that the host has no capacity to degrade (due to the competitive use by both host and flora). By the time the digesta and entrained microbes reach the colon, the pH in the lumen has reached near neutrality

(pH ~ 7) due to previous aqueous-phase material exchanges with the host. By the time the digesta and entrained microbes reach the colon, the intestinal lumen is essentially devoid of oxygen, due to and maintained by the action of facultative aerobes (which, with approach to pH neutrality now includes significant *E. coli*). By the time the digesta and entrained microbes reach the colon, conditions are favorable for the growth of entrained obligate anaerobes, and for the full array of fermentative metabolic processes available to those taxa (Madigan, *et al.*, 2002, pp. 733-735, Krause and Khafipour, pp. 1-7, and Willing and Jansson, 2011, pp. 45-47).

The obligate anaerobes viably present at this point in the digestive system have obviously been selected by their ability to survive the oxic and acidic conditions present in more proximal sections of the digestive system. This largely limits the colonic anaerobic community to taxa that can sporulate (and see discussion of Clostridia as potential alternative FIB in section 2.2.2 above). The presence and maintenance of colonic anaerobes also depends on a microbe-microbe symbiosis with the facultative aerobes present (including *E. coli* and Enterococci) that also have been selected by passage through the digestive system. Because of the great energy conservation potential provided by oxidative phosphorylation (*vs.* substrate phosphorylation), the facultative aerobes preferentially use molecular oxygen as their terminal electron acceptor when it is available (reducing it to water), and create and maintain the anoxic conditions in the colon. Fermentative processes (in which no external electron acceptor is available) inherently provide less energy to the microbe than do oxidative processes and, in a closed system, fermentation is subject to poisoning by local buildup of waste products. The very inefficiency of fermentation, however, gives rise to a microbe-host symbiosis. Fermentation waste products (especially the organic acids), because of their relatively reduced condition, can provide significant energy to the host when these soluble acids are absorbed into the oxic host body and metabolized.

Absorption of these acids by the host, and out of the colonic lumen, allow for continued metabolic activity by anaerobes (Madigan, *et al.*, 2002, pp. 113-131).

Even with coevolutionary selection of microbes, the colonic population of anaerobes makes available a wide variety of catabolic mechanisms to derive energy from substances that resist degradation by stomach acids and enzymes. Colonic anaerobes (and facultative aerobes practicing fermentative metabolism) also provide an array of biosynthetic mechanisms unavailable to homeothermic hosts. When the products of those syntheses are soluble (notably some host-essential vitamins produced by fermentation in *E. coli*, Maczulak, 2011, p. 257), they contribute to additional evolutionary pressures favoring symbiotic lifestyles. When the products are contained in the anaerobic cell they are of course (at least in this “simple” carnivore model), lost by shedding of the cells to feces. The colon provides stable environmental conditions for cell growth, regular (if not constant) inflow of nutrients, and regular shedding of waste products and inhabitant cells. These conditions combine to maintain the colonic flora populations at a carrying capacity analogous to the Stationary Phase of the growth cycle, and have led to a characterization of the colon as a natural chemostat (Madigan, *et al.*, 2002, p. 735).

The evolutionary success of the “simple” structure of the carnivore digestive system is made possible by the “carnivorous” diet of the host animals expressing that system. Animals eating animals can generally extract most of the (absorbable, monomeric) nutrients required for host survival/growth by the digestive breakdown of polymeric structures present in animal-sourced foodstuffs. This generalization is obviously limited to host-required (“essential”) nutrients that survive host digestive processes (especially in the stomach), and some symbiotic flora is required for fermentative reconstitution (in this case, colonic) of chemical species (e.g., vitamins) lost in gastric digestion. This generalization can be further extended to animals with

diets (other than carnivorous) otherwise rich in the sugars, starches, fats, and proteins that are easily digestible by animal digestive enzymes (e.g., fructivores, seed-eaters, and some omnivores). The most common source of organic carbon (required for all heterotrophic life) on terrestrial landscapes, however, is cellulosic plant fiber (generally also containing hemicelluloses and lignins). Cellulose, like starch, is a polysaccharide. Cellulose, unlike starch, contains a beta-1,4 polymeric linkage that sterically renders it virtually immune to environmental (hydrolytic) degradation. In the absence of enzymatic action, the environmental half-life of cellulose has been estimated to be “several million years” (and human use of cellulosic materials, from cotton fabrics to structural lumber, would certainly serve as evidence of the rot-resistance, even within actual environmental systems, of this particular polymer). The evolution of cellulases (enzymes capable accelerating the degradation of cellulose) has occurred only rarely, in very few biological taxa (a situation attributable to the poor solubility and large molecular size of cellulose). The cellulases that do exist in nature do serve as catalysts in the sense that they accelerate (through lowering of activation energy) cellulose degradation, but even enzymatic degradation of cellulose can generally be regarded as slow. While some eukaryotes (e.g., termites and some nematodes, in cooperation with their gut flora) can express some cellulases, even in oxic environments (a few fungi), most exploitation of this environmentally prevalent carbon source is by action of bacterial anaerobes (Wilson, 2011). It should be noted here that at least one strain of *E. coli* has been engineered (Lawrence Berkley National Laboratory) to express cellulase (Yarris, 2011). For the most part, however, the utility of FIB (and any other facultative aerobes in digesta, identified or not) in this regard is limited to the establishment and maintenance of anoxic conditions.

An ability to make use of (environmentally prevalent and biologically underutilized) cellulose as a metabolic substrate provides both evolutionary and ecological advantages to organisms *vs.* organisms lacking such ability. An ability to make timely use of cellulose as a metabolic substrate requires expression of genes unavailable in homeothermic-animal (FIB-host) genomes. While evolutionary modifications to the prototypical mammalian-carnivore model have been found to have no real connection to utilization of cellulose⁶, most such modifications do arise as a consequence of the advantages provided by efficient application of cellulases to cellulosic diets, and involve symbiotic relationships between the FIB-harboring host and the host-harbored FIB. As discussed above, the limited fermentative capacity available in the carnivore system is located in the colon, distal to the gastric stomach (i.e. “hindgut” fermentation, though limited by retention time and limited contact between cellulases and their substrate). Many herbivores (especially grazers) have achieved enhanced efficiencies in hindgut fermentation, increasing retention time in the colon through its sacculation. Muscular constrictions in the colon slow the movement of digesta between intervening sacs in which colonic foodstuffs and flora enjoy long contact. This modification (digesta-filled colons of horses and elephants can approach 9% of total body weight) allows for increased capture of soluble nutrients by the host. As in the carnivore model, however, potential nutrients that are insoluble

⁶ Co-evolution of the avian gizzard (a muscular stomach chamber), together the crop (an esophageal sacculation) and a loss of dentition and associated jaw musculature, has been attributed to evolutionary pressures accompanying a lifestyle including powered flight. By diminution of a heavy, toothed snout, far forward of the wings, and by its replacement with a more-medially located, grit-containing gizzard to manage pre-gastric mastication (“chewing”), birds acquire aerodynamic efficiencies. The geometry of these developments precludes development of foregut rumination, limiting birds to hindgut fermentation. A need to minimize the weight of long-retained foodstuffs further limits significant cecal development to a few, mostly folivorous birds (Louchart and Viriot, 2011).

(including the floral cells themselves) are lost to the host at defecation (Kraus and Khafipour, 2011, pp. 2-5).

Hindgut fermentation is further enhanced by the development of ceca, diversionary sacs (dead-end “cul de sacs”) of the digestive system in which digesta can be stored for extended time outside the normal flow through the colon (and while the proximal end of the human colon is termed a cecum due to the presence of the vermiform appendix, it is generally considered an evolutionarily vestigial one). While a cecum, in and of itself, still cannot capture insoluble nutrients, many cecal fermenters have also adapted coprophagic behaviors. Coprophages generally secrete considerable mucous (polysaccharides of limited solubility) into cecal digesta. When the cecal “pellet” is (muscularly) ejected into the colon, the floral cell mass largely survives passage to defecation, and is re-ingested by the coprophage. The host can then capture insoluble materials and cell mass (now largely devoid of indigestible cellulose) through normal digestion processes (stomach and small intestine) with wastes passed on to the colon (for a second time) for defecation (Krause and Khafipour, 2011, pp. 2-5 and 12-13).

Some homeotherms have evolved alternative foregut adaptations to more efficiently capture the biosynthetic (often less soluble) products of floral fermentative metabolism (and cecal-fermenting coprophages, by use of a recycle loop, must be considered to represent a transitional form of foregut fermentation from an engineering perspective). These adaptations may involve sacculations of the stomach, with proximal sacs in which floral fermentation of digesta is encouraged prior to gastric digestion. Further modifications to the prototypical digestive-system model include development of true multi-chambered stomachs, with muscular flow control of digesta between chambers (especially in the true ruminants). Proximal sacs/chambers provide for long contact (retention time) between foodstuffs and flora before the

digesta ever enter the gastric (low pH, enzyme enriched) stomach (or stomach chamber). Foregut fermentative structures can be quite large (e.g., well over 15% of host body mass for cattle, sheep and camels). The long retention time with mixed flora allows for facultative aerobes (including FIB) to create and maintain an anoxic environment suitable for obligate anaerobes. The near-neutral pH in the foregut, conducive to production of bacterial cellulases, is often supplemented by secretion of bicarbonate in the host-animal saliva. The long retention time also allows for long contact between cellulosic materials and (kinetically slow) cellulases. Foregut fermentation allows for host-animal utilization of all digestible (now largely cellulose-free) foregut-fermentation products by prototypical (gastric-stomach digestion and small-intestine absorption) processes. Foregut fermentation, however, has the potential of denying the host animal access to animal proteins already in the diet. Pre-gastric fermentation allows fermentative bacteria to convert “essential” (to the animal host, e.g. mothers-milk) materials from animal-diet sources to materials more useful for bacterial growth. Foregut fermenters have generally evolved muscular structures (e.g., the reticular groove) by which some foodstuffs (including chewed cud, in the case of true ruminants) can be shunted directly to the gastric stomach from the oral cavity, bypassing foregut-fermentative structures (Krause and Khafipour, 2011, pp. 2-5 and 7-12).

The rumen (collectively, pre-gastric stomach chambers in true ruminants) has been (like the colon) likened to a chemostat, in which enteric flora are maintained in a “Stationary Phase” over a broad range of dilution rates (Madigan, *et al.*, 2002, p.658). In the case of rumen (or any other foregut-fermented discharges), however, the fermentative cell mass subsequently moves to the host’s gastric stomach for capture of nutrients (now rich in digestible starches and proteins and poor in cellulose) in that acid/enzyme environment. Passage through the stomach obviously

selects flora in foregut fermentative systems just as was seen in hindgut systems and much of the floral community fails to viably reach the intestines. Prior to this section, the homeothermic gut has been identified as the “primary habitat” for FIB. The gut, however, presents multiple, sequential, very different environments, all of which apply selective pressures on floral organisms.

The focus of this research allows for some simplification of a complex situation. The interest here is on the post-defecation behavior of FIB on terrestrial landscapes. All relevant organisms are discharged to the outside environment in feces from the colon (*via* anus/cloaca). Whether previously fermented in foregut structures or captured for recycle by cecal coprophages, only colonic discharges that eventually reach the external environment matter here.

As discussed above in this section, the homeothermic host (including its colon) provides FIB an environment of much more constant temperature than would be found on the landscape. Moreover, homeothermic body temperatures ($\sim 36 - 43^{\circ}\text{C}$, above) are not vastly different from optimum-growth temperatures ($\sim 35 - 39^{\circ}\text{C}$) and are well within the cardinal temperatures ($\sim 10 - 48^{\circ}\text{C}$) found for FIB in culture (section 2.4.1.1). Also already discussed, the colonic environment is anoxic due, at least in part, to the facultative capacities of the FIB themselves, and is near pH-neutral due to multiple host-digesta exchanges of soluble materials having taken place more proximally in the gut. Ultraviolet B (UVB) radiation is attenuated in the most surficial ~ 200 microns of human skin (UCSF, 2007); it is generally (safely) assumed that FIB in the colon are completely sheltered from that potentially growth-inhibiting factor.

As further discussed above, the colon is a hindgut-fermentative organ through which the host homeotherm gains access to soluble products of floral fermentation. By the time digesta are defecated, they are relatively depauperate in such potential nutrients. As a hindgut-fermentative

organ, however, the colon is incapable of capturing insoluble nutrients (including the floral cell mass itself). Feces, when deposited on the landscape, typically includes $\sim 1/3$ (of wet weight) bacterial cell mass (Madigan, *et al.*, 2002, p.735). While facultative FIB are significantly and universally present in homeothermic feces ($\sim 10^8 - 10^9$ /gram feces, 2.4.1.1 above), they are vastly outnumbered by other floral organisms ($\sim 10^{10} - 10^{12}$ /gram, Willing and Jansson, 2011, p.45), most of which have been identified as obligate anaerobes ($\sim 10^{10} - 10^{11}$ /gram, Madigan, *et al.*, 2002, p. 735), and most of which would be expected to die (absent timely sporulation) upon defecation to an oxic landscape. The homeothermic host defecates that which it can no longer use by way of colonic processes. As evidenced by the various foregut-fermentative adaptations noted above, however, feces often contains considerable nutritional material accessible by organisms capable of oxidative phosphorylation.

Not unrelated to its soluble-nutrient absorbing function, the colon also contributes to host water balance, a function the efficiency of which varies widely across species, diets, and environments (Krause and Khafipour, 2011, p. 4). Much research into the moisture content of feces has been carried out by agronomists interested in the use of manures as soil amendments. One such study (Akhtar, *et al.*, 2013) serves to illustrate the wide range of variability here. The authors sampled manures of cows (grazing foregut fermenters), horses (grazing hindgut fermenters), goats (arid-adapted, browsing foregut fermenters), and chickens (omnivorous hindgut fermenters). The herbivorous manures ranged from $\sim 26 - 31\%$ moisture content, with (not surprisingly) goat feces at the lower end of that range. Chicken manure was far drier (7.7%). All manures were found to be moister environments than those that would be expected for FIB deposited to environmental surfaces (absent ongoing rain), and even the moisture content of chicken manure exceeds the field capacity of some agriculturally productive soils.

2.2.4.3.2 Temperature

In a sense, biology can be viewed as a sub-discipline in the science of chemistry (itself a sub-discipline of particle physics). The viability and reproduction of a cell is sustained by a multitude of biochemical reactions that provide for the generation of useful energy and for synthesis of necessary cellular structures. The rates of these reactions depend on kinetics, the likelihood that reactants will collide (a concentration parameter) and will collide with sufficient energy to overcome the meta-stable state of the reactants and allow for their combination into a new, more stable state (the “activation energy” of reaction). In cells, both of these parameters are temperature dependent.

Chemical reaction rates typically increase with increasing temperatures (absent thermal decomposition of the reactants or of any catalysts involved in the reaction). Reaction rates are commonly modeled with the Arrhenius Equation, which posits a relationship between a rate constant (for given conditions), that is dependent on the activation energy of a reaction system, and the absolute temperature at which the system reacts. Empirical application of the Arrhenius Equation across varying temperatures allows for calculation of Q_{10} , the relative change in the rate constant k of a system in response to a 10°C rise in temperature. Over the range of biosphere-relevant temperatures (a narrow range in terms of absolute temperatures), Q_{10} of cell-mediated reactions has been found to be fairly constant. Reynolds and Richards (1996, pp. 9-10), for example, report the Q_{10} for water-/wastewater-treatment processes (environments in which FIB commonly thrive in the non-enteric environment) as 1.072.

The situation is somewhat complicated by cellular production/use of enzymes, proteinaceous catalysts (especially inside the cells themselves). The catalytic nature of enzymes, by way of their conformationally complex macromolecular structure, influences the geometry of

collisions between substrates (in an enzyme's "active site") to the effect that activation energies are reduced. Individual enzyme-catalyzed reactions have long been modeled with Michaelis-Menten kinetics, which posits a rectangular-hyperbolic function of effective reaction rate to available substrate. The Michaelis-Menten model is mechanistic, and considers a reversible reaction between enzyme [E] and substrate [S] reactants in mass-balance equilibrium with an enzyme-substrate complex [ES] with a second, irreversible, reaction that consumes [ES] and produces [E] and enzymatic product [P]. Total (bound and unbound) enzyme in the system, [E] + [ES], is considered a constant, and the hyperbola can be completely described by the asymptotic limit V_{\max} ([E] to [P] conversion rate in a system where extant enzymes [E] are constantly saturated for substrate) and the "Michaelis constant" K_m (the substrate concentration at which [E] to [P] conversion occurs at a rate $\frac{1}{2} V_{\max}$, Reynolds and Richards, 1996, pp. 33-34, and Fabritz, 1995, Appendix A). Many catabolic (intracellular) reactions are characterized by $Q_{10} \sim 2$ over biologically relevant temperature ranges (Atlas, 1984, pp. 339-340).

Extension of enzymatic kinetics to cell/biomass growth is more tenuous, but provides some comfort that the latter can be viewed as a sum of the former. Monod kinetics were derived from a purely empirical exercise, an attempt to curve-fit a function of cellular growth to available nutrients. Much work (since the 1940s) on single-species culture (in both batch and continuous, flow-through, systems) on defined media has led to a broad acceptance that cell/biomass growth rate is dependent (again, as a rectangular-hyperbolic function) on the concentration of a "limiting nutrient" (nutrient of most limited supply in relation to the nutrients of need for growth). In the Monod model, shape of the hyperbolic function is defined by the "maximum" growth rate (empirically defined as the asymptote of the growth-rate vs. limiting-nutrient function) and the

limiting-nutrient concentration at which $\frac{1}{2}$ the maximum growth rate occurs (Grady, *et al.*, 1999, pp. 77-80).

The complex macromolecular conformation of enzymes is, in itself, meta-stable with respect to temperature and, above some threshold temperature, an enzyme is spontaneously and irreversibly “denatured” (conformationally rearranged into a catalytically inert structure). This bidirectional (sometimes enhancing and sometimes inhibiting) nature of temperature on cellular metabolism imposes a biphasic effect of temperature on cellular growth and reproduction. Within the temperature ranges of stable enzyme structures, biochemical reaction rates would be expected to increase monotonically with temperature. Above the temperature at which enough important enzymes are denatured to reduce the overall (amalgamated across many bioreactions) metabolic rate-constant enhancement to zero (optimum growth temperature), however, the sum of reaction rates declines with temperature. At the temperature that denatures some enzyme crucial to cell growth, growth ceases (maximum growth temperature); due to the irreversible nature of protein denaturation, exceedance of the maximum growth temperature is generally fatal to cells (Madigan, 2004, pp. 151-152, and Atlas, 1984, pp. 339-342).

It must be noted at this point that, despite mechanistic arguments for a log-linear growth/death rate for microbial populations over time at given set of environmental conditions (2.2.4.2, above), there is no such argument for a simple relationship between the growth-rate constant of a population and the environmental conditions at which that constant is expressed (and this caution applies to environmental factors other than temperature). The (oft-used) extension of single-reaction (thermodynamically based, “Arrhenius-like”) behavior to (mechanistically derived, log-linear) microbial-population growth rates provides a cautionary example. The empirical findings of relatively constant Q_{10} for FIB-mediated WWTP processes

across wide ranges of temperatures, and the empirical findings Monod-modeled growth similar in form to Michaelis-Menten kinetics both provide empirical evidence that microbial growth can be modeled on the basis of some (known or unknown) limiting reaction. Other researchers (e.g., Ratkowski, *et al.*, 1982) have suggested better empirical fits for growth-rate constants achievable with a power (square) transformation of absolute-temperature change, rather than the Arrhenius exponential model, across a wide variety of temperature-adapted microorganisms (psychrophilic, mesophilic, and thermophilic), particularly in between minimum and optimum growth-rate temperatures, and especially in the absence of lag-phase growth. Some researchers have recently concluded that best fits are achieved with power transformation of temperature differences between minimum-growth and optimum-growth conditions and a factor accounting for more Arrhenius-like behavior (which may derive from temperature-dependent stability of globular proteins) above optimum-growth temperatures (Ratkowski, *et al.*, 1983, Zweitering, *et al.*, 1991, and Ratkowski, *et al.*, 2005).

The concentration of important reactants/substrates within cells (both globally and locally) is also influenced by the cells (by biosynthesized structures, especially the cell membrane). Reaction rates, and even the direction of reversible reactions, are determined by disequilibrium concentrations of “reactants” vs. “products” of those reactions. Diffusion (or active transport) into (or production in) cells (or intracellular structures) of reactants drives (by mass action) an increase in products. Diffusion out of (or destructive use within) cells/structures of products likewise drives an increase in production (Reynolds and Richards, 1996, pp. 3-5). The minimum growth temperature of a cell is typically that at which some essential nutrient cannot be imported from the extracellular environment at rates that can support necessary metabolic processes (by “gelling,” or reduced transport rates through the cell membrane).

Generally, the minimum growth temperature is qualitatively different than the maximum.

Whereas the maximum limit of growth implies an irreversible limit for viability, the minimum may only imply a (temporary) lack of access to extracellular nutrients, an inability to maintain a cross-membrane reactant/product gradient (e.g., the proton-motive gradient needed for oxidative phosphorylation), or simply insufficient reaction rates for growth. Low temperatures do not, of themselves, denature enzymes. Absent the expansion of water accompanying its freezing (progressively below 4⁰ C for pure water), which may disrupt structures affecting substrate/product gradients, temperatures below the minimum temperature for growth do not necessarily imply loss of viability for a cell (Madigan, 2004, pp. 151-152, and Atlas, 1984, pp. 339-342). While lower temperatures reduce cellular growth rates, they can enhance cellular preservation (Atlas, 1984, p. 342).

Both taxa of FIB under consideration here are classed as “mesophiles,” a class that is defined as organisms displaying optimum growth temperatures between 20 and 50⁰ C (see 2.4.1.1, above), which should not be surprising considering their primary adaptation to the homeothermic gut. As a further consequence of evolutionary adaptation, and of the mechanisms limiting growth, the temperature ranges of growth for organisms are asymmetrical; the optimum growth temperature is invariably nearer the maximum than the minimum (Madigan, 2004, pp. 151-152, and Atlas, 1984, pp. 342-344).

It must be noted here that the material presented in this section so far only addresses the temperature dependence of the growth rate of bacteria, and that the cardinal temperatures of taxa are derived from laboratory culture studies. Reactions leading to cell death (e.g., waste, toxin, or inhibitor production in closed systems) may also be enhanced by temperature rise and, for overall population changes, and a negative Q₁₀ is possible. Also, though the fecal flora is

dominated by bacteria (up to 10^{11} cells/gram), FIB are defecated to the landscape along with many other potential competitors and/or predators, and differential temperature response of members of a complex community must be considered (Willing and Jansson, 2011, p. 39). Protozoans, for example, can also exhibit facultative aerobism (Madigan, *et al.*, 2002, p. 484), and their predation is deemed the primary cause for FIB die-off in the environment (Whitman, *et al.*, 2011, p.120).

The many large studies (section 2.2.2, above) finding persistent/growing FIB populations in tropical soils (e.g., Fujioka and Byappanahalli, 1996), and finding genetic evidence of winter-preserved, spring-recoverable FIB populations in temperate soils (e.g., Ishii, *et al.*, 2006) provide support for expectations presented here; over the range of environmental conditions typically found, FIB growth is encouraged by higher temperatures, but even freezing conditions (absent other adverse factors) are not necessarily fatal. Further, they show the importance of competition and predation (which may overwhelm growth rates) on the net survival of FIB on the landscape (e.g., Alm, *et al.*, 2006). Madigan, *et al.*, 2004, lists temperature as an important (“if not *the* most important”) environmental factor affecting microbial growth (p.151). Environmental temperatures would be expected to influence the carrying capacity for FIB in stormwater source areas. Moreover, the difference between environmental temperatures and those of the homeothermic gut may well play a role in the time required for FIB to adapt to landscapes subsequent to defecation, and must be examined here. Finally, as Gallagher, *et al.* (2012), point out, temperature (important as it is) is only one factor affecting cell growth, and may well interact with other factors affecting growth.

2.2.4.3.3 Oxygen

Madigan, *et al.* (2002) list the presence of oxygen as an important environmental factor determining the environmental distribution of microorganisms (p.151). As discussed above (section 2.2.4.1), the availability of molecular oxygen as a terminal electron acceptor provides for enhanced catabolic energy conservation in organisms capable of its use. Conversely, the potential for generation of toxic oxygen species (especially free-radicals) may be inhibitory or fatal to organisms lacking the necessary enzymes for detoxification. Both taxa of FIB under consideration here are “facultative” in the sense that they can both use and tolerate oxygen as well as survive its absence.

The environmental distribution of oxygen is largely a function of its limited solubility in and diffusion through water (Madigan, *et al.*, 2002, p. 161, and Atlas, 1984, p. 346). Very deep waters, bottom sediments, and flooded muck soils (and colons) can provide environments in which microbial respiration depletes available oxygen faster than new oxygen can diffuse to replace it, creating an anoxic environment. Mitsch and Gosselink (2007, pp. 173-174) note that oxygen diffusion through aqueous solutions is about “10,000 times slower than oxygen diffusion through a porous medium such as a drained soil.” However, they further note that, despite the typically flooded nature of wetland soils, an oxic layer remains on the surface of submerged sediments, “sometimes only a few millimeters thick.”

Interest here is in survival of FIB in stormwater source areas, terrestrial surfaces between rainfalls. The heterogeneity of such environments and the small size of microbial cells force consideration of microenvironments. Madigan, *et al.*, (1984, p. 635) consider a single 3-mm (radius) soil particle for which microelectrode data has provided an oxygen-concentration contour map. The authors note that “for a typical 3-micron rod-shaped bacterium,” 3-mm scales

to “human experiences over a distance of 2 km!” They find that even obligate aerobes could actively respire at a depth of over 2-mm into such a particle, with obligate anaerobes thriving a very short distance away in its center.

While wet microenvironments (of very short diffusion lengths) may occur in drained soils, surfaces, and biofilms (see below), even such environments should be driven towards oxygen equilibrium with the bulk, unchanging, overlying atmosphere. The troposphere (literally and etymologically “turnover zone”) to which terrestrial environments are exposed is well mixed with respect to the “permanent gases” (stable and non-condensable at atmospheric conditions), a classification of tropospheric components that includes oxygen (at a steady 21% by volume, NCSU, 2013). In an ideal gas mixture (a good approximation for air at total barometric pressures less than a few atmospheres) at one atmosphere pressure, the volume fraction of oxygen equals its mole fraction and (in atmospheres) partial pressure (Dalton’s Law, Geankoplis, 1983, p.10-11). Components of such a mixture should follow, to a good approximation for partial pressures less than an atmosphere, Henry’s Law, which posits a temperature-dependent “constant” ratio between gas-phase concentration and equilibrium liquid-phase solubility (Maddox, 1973, p. 14-3). Though oxygen availability may vary within source areas, such availability is itself expected to be only a function of temperature. Though the taxa of FIB under study here may differ in their response to a change from the anaerobic colon to the aerobic landscape, that differential response would be a function of the taxon itself. Oxygen as an independent factor relevant to the landscape survival of FIB can safely be neglected in this research.

2.2.4.3.4 Water Activity

“All life is aquatic” (oft quoted maxim of, apparently, unknown source), and biochemical reactions take place in an intracellular aqueous environment. Maintenance of a suitable intracellular environment for life-sustaining metabolic activities is derived through the presence of a hydrophobic cytoplasmic membrane. The membrane exhibits very low solubility for (largely hydrophilic) macromolecular biochemicals and for charged chemical species (the latter also allowing for maintenance of the transmembrane proton gradient needed for aerobic respiration). The membrane prevents “leaking” (passive permeation loss) of vital cellular constituents to the extracellular environment. The membrane does, however, allow for relatively free diffusion of small, even polar, molecules (notably water) and is, thus, deemed semipermeable. Aquaporins, proteinaceous membrane-spanning channels allowing for free transport of whole solutions (solute as well as solvent), are available to cells of all taxonomic domains. Activation of such channels (with obvious leakage implied) seems limited to (hypo-osmotic) conditions in which the membrane is in danger of physical disruption by turgor (Madigan, *et al.*, 2002, pp. 69-70).

The (in/out) direction of water diffusion through the cytoplasmic membrane is controlled by transmembrane differences in water activity (a_w , “ratio of the vapor pressure of the air in equilibrium with a substance or solution to the vapor pressure of pure water,” Madigan, *et al.*, 2002, p. 159). In aqueous environments (including lab cultures, the historic source of most relevant studies), and inside the cell itself (also an aqueous environment), any transmembrane difference in water activity is primarily a function of osmolarity, the degree to which water molecules are already interacting with existing solutes and, thus, less available to dissolve others. Water flows from regions of high water activity (low solute concentration) to regions of low water activity. Taxon-specific tolerance to the dehydrating effects of low environmental water

activity is generally deemed a function of a cell's ability to actively import or to synthesize intracellular "compatible solutes (or osmoprotectants)," water-soluble materials that lower cytoplasmic water activity without inhibiting biochemical activities. Though any solute (notably sugars, oligopolysaccharides, proteins, etc., within the cell) may affect osmolarity, much characterization of microbial species' tolerance ranges has been based on salt tolerance (easily measured halophilicity) in aqueous extracellular environments. On that basis, *E. coli* are classified as nonhalophilic, and Enterococci are deemed halotolerant (and see section 2.2.4.1., and the need to resuscitate desiccated *E. coli* in Byappanahalli, *et al.*, 2006, above). Most microbes require, and most agricultural soils provide, environmental water activities of at least 0.90 to maintain metabolic activity⁷. Like the effect of temperature (discussed above), conditions providing less than the minimum requirements may (without disruption of structures, e.g. denaturation of enzymes) allow for renewed viability upon rehydration (Madigan, *et al.*, 2002, p. 138 and pp. 159-161, and Atlas, 1984, p. 349).

Like oxygen (discussed above), environmental surfaces exposed to the atmosphere, onto which FIB might be defecated, should be driven towards equilibrium with the bulk a_w in the troposphere. Unlike oxygen, however, water vapor is not an atmospheric "permanent gas." Water vapor, at typical tropospheric conditions, exists well below its critical temperature and may even encounter its triple point. Condensation, freezing, sublimation, and evaporation in the atmosphere (phase changes of water) are major contributors to weather, and keep the tropospheric permanent gases well mixed. Water vapor in the atmosphere overlying terrestrial environments shows considerable geographical and temporal variability (0-4% by volume/mole fraction, NCSU, 2013), and must be considered as a meaningful environmental variable here.

⁷ Some halophilic bacteria can grow at culture-measured a_w as low as 0.75 (Madigan, *et al.*, 2002, p. 160), and the wilting-point a_w of plants is generally above 0.99 (Cobos, 2012).

In the atmosphere, a_w numerically reduces to relative humidity (RH%, Atlas, 1984, p. 349). Simple equation of halophilicity/osmotolerance (aqueous measures water-deficit tolerance) with xerotolerance (general resistance to drying) would lead (and has often led) to a conclusion that a naked bacterial cell suspended in the atmosphere should quickly (with very limited diffusion lag imposed by the nanometer-scale cytoplasmic membrane) reach water-equilibrium with the surrounding medium (i.e., cease growth before the overlying atmosphere dries to ~ 90 RH%). Such an assumption, however, would also lead to a conclusion that plants can't grow below atmospheres of less than ~99 RH%.

The potential for phase changes in water (and aqueous solutions), on and within environmental surfaces (which would, here, also include transpiration from vegetated soils in the water-activity balance) at relevant environmental conditions is as important to this research as is such potential in the atmosphere. While temperature has been characterized by some as the most important environmental factor affecting bacterial survival, water (with phase-change complexities) is most difficult to analyze here. Soils, for example, harbor pockets and films of liquid water, even when exposed to a less than water-saturated atmosphere (Madigan, *et al.*, pp. 638-640). Moreover, a bacterial cell is not just a bag of saltwater. Though a lack of intracellular, membrane-bound organelles is a defining characteristic of prokaryotes, macromolecular structures of varying hydrophilicity serve to compartmentalize intracellular water content and activity (Madigan, *et al.*, pp. 90-96). These intra- and extra-cellular microenvironments are important, especially with respect to potential buffers and lags imposed by serial equilibria between a drying atmosphere and the desiccation of metabolically important structures within a cell. In real environments, osmotic transfer of water across the cytoplasmic membrane represents just one of the important and sequential water transfers.

Much of the relevant literature discussing the “water relations” of transport through porous media is presented by agronomists and soil scientists (often discussing water relations with plants, too), and traditionally in terms of water potential (WP). WP and a_w are interconvertible (for systems compared to the same reference conditions) by:

$$RT/V_w \times \ln(a_w) = \text{WP},$$

where R is the universal gas constant, T is absolute temperature, and V_w is the partial molal volume of water (this form of the conversion reports WP in units of pressure). For a reference system fully saturated by pure water ($a_w = 1$), $\text{WP} = 0$. For a system of lower water activity than this reference, at the same temperature/pressure conditions as the reference, WP is obviously negative. WP is thought to provide greater numerical resolution than a_w in the biologically relevant ranges of the energy state of water (and for perspective and by my calculations⁸, $a_w = 0.99$ corresponds to $\text{WP} = -1.4$ MPa, and $a_w = 0.9$ is equivalent to $\text{WP} = -14$ MPa). Water still flows down-gradient with respect to WP in the event of any disequilibrium (Papendick and Campbell, 1981, pp.1-3).

Papendick and Campbell (1981, pp. 1-2) describe WP in porous media as the sum of WP_{osm} , WP_{mat} , WP_{grav} , WP_{press} , and WP_{ob} , where:

WP_{osm} = osmotic potential due to solutes in water (and always negative),

WP_{mat} = matric potential due to the adsorptive and capillary effects of phase interfaces imposed by inclusion of water in a matrix including solid and gaseous components (and always negative),

WP_{grav} = gravitational potential attributable to the elevation difference between a system and a reference,

WP_{press} = pressure potential due to a difference between overall (gas or hydraulic) pressure between the system under study and the pressure at which the reference system was characterized, and

WP_{ob} = overburden potential caused the weight of overlying matter in a nonrigid porous system.

⁸ I used V_w at $4^{\circ}\text{C} = 1.8016 \times 10^{-5} \text{ m}^3/\text{mole}$, and T at $20^{\circ}\text{C} = 293.15\text{K}$

In this framework of the various components of potential, a planktonic cell immersed in a bulk aqueous salt solution is seen to be a special case imposed by the semipermeable cell membrane. In a parcel of bulk aqueous solution of limited (cell-diameter) elevation extent and under atmospheric pressure, WP_{grav} and WP_{ob} are negligible in any comparison of the WP inside a cell and the WP immediately outside it. Additionally, the miniscule quantity of cell-wall solids adjacent to the bulk solution (potentially contributing to local extracellular WP_{mat}) should, likewise, be negligible. A water-potential balance on the system becomes equilibration of the sums (of $WP_{\text{osm}} + WP_{\text{press}}$) inside and outside the cytoplasmic membrane, and the WP_{press} of the bulk extracellular solution is essentially unchanging. In the absence of barriers to diffusion, all solution components are expected to follow Fickian behavior, and move from high to low component concentrations in the face of any component-concentration gradient. In the presence of a semipermeable cytoplasmic membrane, however, movement of many “solute” components of aqueous solutions is inhibited while “solvent” (water) movement is not (in a free-energy sense, water gradients derive from the “dilution” of water by the “solutes,” Kramer and Boyer, 1995, p. 33). Water-potential balance across the membrane can only be achieved by water movement through the membrane and only achieves equilibrium in a cell/aqueous environment when (relatively unchanging) osmotic potential of the bulk extracellular environment is balanced by the sum of important potentials ($WP_{\text{osm}} + WP_{\text{press}}^9$) within the cell (Papendick and Campbell, 1981, p2). The WP_{osm} of a bulk extracellular solution of known composition (e.g., culture

⁹ Admittedly, the cell wall does provide a finite WP_{ob} component on the total water potential of the intracellular environment as the membrane swells against, or shrinks from the wall under changing WP_{press} . The effect is most pronounced early in the dehydration process and depends on the elasticity of the cell wall (discussed for plant cells at Kramer and Boyer, 1995, pp. 61-63). The analysis presented above apparently assumed a rigid cell wall. The cell-wall structure, as a supporting structure for the membrane, also obviously represents a threshold capacity for membrane containment of WP_{press} , exceedance of which would result in membrane rupture, cell death, and remixing of all cellular components into the surrounding medium

medium) can be easily calculated (well-estimated) by the Van't Hoff equation and, even in cases where solute concentration is unknown, easily measured in an osmometer (Kramer and Boyer, 1995, pp.33-35).

The water relations of soils and surfaces on which FIB might be deposited are more complex. While $WP_{\text{osm}} + WP_{\text{press}}$ may still largely define the intracellular face of the cytoplasmic membrane, the extracellular environment here is dominated (between rains) by WP_{mat} , which affects both equilibrium hygroscopy and water retention during dehydration. The water molecule is held together by strong (110 kcal/mol) covalent bonds, and is rarely (less than one in 10^7 molecules) ionized. The spatial arrangement of those bonds, however, provides for a strong dipole effect (asymmetric distribution of partial charges). The structure of ice crystals is defined by weaker and more ephemeral hydrogen bonds (~ 3 kcal/mol) between the oxygen atom of one molecule and a hydrogen atom of another that arise (without full intermolecular electron-pair sharing) from dipole interactions between molecules. Melting of ice only breaks $\sim 14\%$ of the hydrogen bonds and, even at temperatures of incipient boiling, the potential hydrogen bonds in liquid water remain $\sim 70\%$ intact. Those bonds are ephemeral (half-life $\sim 10^{-10}$ sec) as mobile (“solvent” water) molecules constantly trade places with hydrogen-bonded (“solute” water) molecules, but they impose a structure (the geometries of which are yet to be elucidated) on bulk liquid water. Water-water hydrogen bonding (cohesion) in the bulk (all three dimensions) liquid is greater than that at a liquid/vapor interface (where such bonding is only dimensionally available within a half-space, creating a surface tension), which in turn, is greater than that in the bulk vapor phase (no hydrogen bonding). Moreover, water can form hydrogen bonds (adhesion) with, and reduced potential at, solid (even insoluble) surfaces, depending on their hydrophilicity (“wettability,” largely based on the presence of polar moieties within the macromolecular

structure of the surface and, in soils, mostly dependent on the presence of surficial oxygens or hydroxyls). Both cohesion and adhesion of water molecules contribute to liquid retention in porous media, even in the face of drying/draining conditions. Capillarity (a combination of adhesive/cohesive forces) in an unsaturated porous medium may even draw underlying liquid water (e.g., water table in soils) upwards, against gravity, into the vadose zone. The sum of these cohesive and adhesive forces creates matric hygroscopy, a localized equilibrium between liquid water (or aqueous solution) and a less-than-saturated (vapor) atmosphere. Both the cohesive and adhesive forces affecting water in an unsaturated porous medium are affected by the structure and texture of the medium (Kramer and Boyer, 1995, pp. 16-41 and pp. 84-114).

Papendick and Campbell (1981) note that theory is lacking for accurately predicting a relationship between water content and water potential from soil properties (the highly non-linear “characteristic curve”), necessitating use of empirical models. They further note that the permanent wilting point of many multicellular plants (whose multicellular roots must span both air-dried and water-filled microenvironments) occurs at soil-water contents where the measured bulk soil-water potential corresponds the measured osmotic potential in their tissues (and for many plants, $-15 \text{ bars} = -1.5 \text{ MPa} = 0.989 a_w$, which would also agree with a mechanistic analysis of cells in saltwater), but that such an analysis has proved to be largely useless for analysis of the water relations of microorganisms. The authors also note that soils exhibit hysteresis, differential soil-water content/potential relationships under wetting *vs.* drying conditions (which favor water-content retention in the face dropping water potential during drying cycles) that are presumably attributable to matric forces. Finally, they attribute soil-water retention (during draining/drying) at “high” matric potentials (above -1 bar , $= -100 \text{ KPa}$, $= 0.9993 a_w$) to capillary (adhesive and cohesive) effects (and dominated by soil structure and

porosity), while they find that at lower potentials, soil texture (adhesive surface effects of soil particles) is dominant (pp. 4-7).

Tuller and Or (2003, pp. 1-20) examine assumptions underlying historic derivations of soil-water characterization curves, and find them wanting. The traditional “Bundle of Cylindrical Capillaries” model (BCC, and apparent in the above discussion by Papendick and Campbell, 1981, see pp. 7-9) ignores purely adhesive/adsorptive effects due to angularities/non-circularities of pore spaces in unsaturated non-porous media. The BCC model assumes that pore spaces in porous media fill or empty like an assorted collection of laboratory capillary tubes, based on their circular cross-section diameter, and that water-content retention can be modeled against an “effective” pore diameter (D_e , and for perspective, $0.99 a_w$ corresponds to ~ 2 microns, through which a bacteria would barely fit, Papendick and Campbell, 1981, p. 4). Examining pores of polygonic cross sections, Tuller and Or find that only the inscribed circle within the polygon exhibits capillary behavior (both in filling and draining). During the filling (imbibition) of a polygonic pore under increasing potential, vapor first condenses (adhesively) onto flat surfaces, and accumulates (cohesively) into corners. It is only when corners fill to the point that a circular dry cross section remains (and in the case of extreme angularity, this represents a condition where a very large portion of the pore is already liquid-filled) that a spherical meniscus can form and spontaneously fill the remainder of the pore by capillary action. Likewise, on draining, considerable adhered liquid remains in the pore at water potentials far below those that capillary “drain” the pore.

Sophocleus (2010) focuses on capillarity to mechanistically explain (in an “easily understood manner” much appreciated here) matric forces leading to reduced water potential. In a critical and synthetic review of literature, the author provides force-balance and free-energy

analyses to show that, in capillary rise against gravity, it is the adhesive forces at the wetting face in the pore that balances gravity and the cohesive forces within the bulk liquid physically drag the liquid up behind the wetting front. The reduced potential within the liquid phase represents an actual negative pressure (tension). The pressure difference across the meniscus is maintained by the differential matric forces across it (even in the absence of a physical membrane) and any solutes or cells fully embedded in the liquid phase would also experience that tension.

The upshot of this admittedly tedious review is that microscopic cells embedded within liquids retained by matric forces would only experience water losses due to the osmotic potential within the same liquid, even if the overlying atmosphere exposes the porous medium to a much lower potential. The RH% in the air is an important variable in the survival of FIB on environmental surfaces, but (in a very complex way) so is the nature of the surface.

Noting the historic preponderance of osmotic-system studies into the water relations of cells, and the ~25-yr absence of a critical review of literature relevant to (ecologically significant) air-drying of bacteria, Potts (1994) provided the latter. Potts presented a (difficult to confirm, given existing technologies) hypothetical mechanism (involving the unique kinetic nature of the liquid/glass transition of trehalose solutions, as well as their capacity to form hydrogen bonds with many macromolecules) with the potential to explain anhydrobiosis (survival and capacity for rehabilitation upon rehydration after desiccation sufficiently extreme to strip all liquid/mobile water from a cell), a phenomenon that typically occurs well outside the environmental conditions relevant to this research. Much of the literature reviewed, however, and Potts' analysis of it in support of that hypothesis is relevant here.

Webb, *et al.* (throughout the 1960s, and not directly consulted in this review of a review) conducted extensive experiments in which bacterial cultures were atomized into aerosols (mean

droplet diameter ~ 10 microns) suspended in atmospheres of varying RH%. The authors calculated (after one hour of suspension) death (decay) rate constants for a variety of bacteria. Onset of cell mortality for drying-sensitive species (e.g., *E. coli*) occurred as the RH% approached the estimated intracellular water content (“RH” $\sim 80\%$, WP ~ -31 MPa). Onset of cell death for drying-tolerant species (e.g., *Staphylococcus aureus*) likewise occurred at a humidity similar to the internal water, but with much lower decay rates exhibited under drier atmospheres. Noting that such studies involve very rapid drying (allowing no time for *de novo* synthesis of compatible solutes, and restricting cellular response to cell shrinkage), Potts (1994) compared these results to osmotic studies and to protein-solution studies. *E. coli* in osmotic media have been seen to lower their growth rate, in a linear fashion, with increasing osmotic stress until the extracellular potential reaches (extrapolated) -4.64 MPa ($a_w \sim 0.97$), where growth ceases. This potential is equivalent to that in which “free” water is removed from protein-water solutions, leaving only (matric, hydrogen-bonded) water “bound” to the protein. The amount of bound water in ternary protein solutions (water-protein-cosolvent) can diverge widely from that found in binary (water-protein) solutions. For instance, in a 1M solution of glycine (a “compatible solute”), 0.54 g of water is bound to 1 g of lysozyme (vs. 0.2 g in a binary water-lysozyme solution). The compatibility of solutes has been found to be a function of their ability to act as cosolvents of proteins (and other macromolecules). In a cell, kosmotropes (“water structure builders”, notably including many soluble sugars, and which by the effective diameter of their interactions with the proteins vs that of water) sterically create a (matric) surface tension (even in the absence of a physical membrane) around the bound-water film and increase its volume. While chaotropic solutes (“structure breakers”) also reduce the intracellular WP_{osm} and, thus, tend to draw water into the cell, they competitively bind to the protein molecule at the expense of

its hydrating layer and can denature the protein (i.e., incompatible solutes). Dehydration behavior for both rapidly air-dried cells and for osmotically dried cells is readily explained by the potentials at which:

- 1.) Intracellular free water is stripped from the cell (resulting in a growth-rate minimum), and
- 2.) Bound water hydrating important macromolecules is subsequently perturbed (and the onset of cell-death from dehydration of important macromolecules).

The death rate of cells under rapid-drying conditions in which the bound-water is perturbed is also readily explained by the long-held “preferential-exclusion hypothesis,” that cell viability depends on the extent to which the intracellular location of reduced osmotic potential provided by the action of extant compatible solutes is physically separated (at angstrom scales) from the intracellular location of the hydration layers of bound water around important biomolecules (with the greater volume of bound water in the presence of more compatible solutes taking longer to strip away at any given drying potential) (Potts, 1994).

Expanding the review (Potts, 1994) to studies of bacterial responses to drying mechanisms other than aerosolization, however, reveals that how (and, most importantly, how rapidly) cells are air-dried also affect viability upon desiccation. In one study for instance, when 25-microliter aliquots of *E. coli* on wetted paper filters (originally wetted to at least 8 mg water/10⁸ cells) were air dried under varying atmospheres, the viability (of this notoriously drying-sensitive species) increased monotonically with the time to reach air-dried status for the filter (out to 13 hrs, the maximum drying time studied). The more time it takes for water efflux to exhaust intracellular free water, the longer a cell has to synthesize and accumulate compatible solutes in response to that efflux (certainly relevant to cells immersed in matrixly bound water films and droplets within unsaturated porous media exposed to a drying atmosphere). Moreover, under such slow drying conditions, cells are found to accumulate a narrow subset of potential

compatible solutes, namely the non-reducing disaccharides trehalose and sucrose. In cell-preservation studies, these sugars have been found to provide protection of viability not only in freeze-thaw cycles (mechanistically comparable to osmotic stresses, in which any compatible solute should be effective), but also in preservation by freeze-drying (more comparable to extensive air-drying) in which bound water of macromolecules is necessarily perturbed (and, thus, unlike traditional osmotic-stress experiments). The preferential-exclusion mechanism cannot be operative in the latter. These observations led Crowe, *et al.*, (again, many references, none of which were directly consulted here) to propose an alternative “water-replacement” hypothesis of desiccation protection; polyhydroxyl molecules, by dint of their capacity to form hydrogen bonds, may (when sterically possible) replace the bound water of hydration around macromolecules and, thus, stabilize their structure in the face of bound-water removal.

Potts (1994) found direct evidence of the water-replacement mechanism limited to *in vitro* studies of non-cellular (i.e., macromolecular) solutions (proteins and/or phospholipids), though neutron-scattering studies of water structure in whole *Artemia* cysts were deemed “consistent with” the hypothesis. Potts further opined that confirmation of the mechanism in active whole cells would represent a major technological challenge. More important to my review of this review, however, Potts shows that:

- 1.) Under matric stress, even drying-sensitive bacteria such as *E. coli* accumulate the sugars,
- 2.) Intracellular presence of the sugars provides enhanced structural stability to proteins and nucleic acids in the face of drying environments, and
- 3.) When secreted from the cell (and, thus, can associate with both faces of the lipid bilayer), trehalose enhances the structural integrity of drying plasma membranes as well.

Potts further finds that bacterial cells, in the face of drying conditions, secrete copious quantities of much larger (higher molecular-weight, polyhydroxyl) carbohydrates. These extracellular

polysaccharides provide a mechanism by which the cells can manipulate the matrix potential of their immediate extracellular environment (1994).

Biofilms, the matrices housing Potts' extracellular polysaccharides, have long been studied but are, as yet, poorly understood. Much early research (and, indeed, much current research as well) into bacterial biofilms has been focused on their nuisance nature and on their control or eradication. Atlas (1984, pp. 118-120) discusses bacterial capsules and slimes (two of the now recognized morphologies of the more generic term "biofilm") with respect to their importance to human health. Capsules (characterized as of extremely variable composition, depending on the species of bacteria generating them, though generally consisting of some mixture of polysaccharides and proteins) are presented primarily as a virulence factor of pathogens (e.g., capsular bacteria resist the adherence to, and phagocytosis of, bacterial cells by immune-system blood cells). Slime layers (or glycocalyxes) are differentiated from capsules by their more tenuous binding to the cells that generate them. Atlas notes that slimes seem to protect cells from nutrient and/or water losses, and may serve as an attachment mechanism for aquatic bacteria, but focuses more on their importance as the major component of dental plaque. Madigan, *et al.* (2002, pp. 636-638 and p. 707) discuss general ecological aspects of biofilms in somewhat more detail:

- 1.) Environmental surfaces may adsorb nutrients, and may even be (e.g., in the case of leaf litter) important sources of nutrients, so an ability to attach to such surfaces provides advantages to growth;
- 2.) Biofilms are complex, multilayered matrices of secreted polysaccharides (spatial details of which are analyzable by scanning laser confocal microscopy); and
- 3.) Biofilms are multicellular constructs, "cooperatively" built by actions of many cells (at least intraspecifically) "coordinated" through quorum sensing of secreted molecules that "signal" to a cell what other cells have done.

The authors (even in this chapter on microbial ecology), however, find the most significant consequences of biofilm formation as impacting the medical and industrial fields. In the former, the authors note an increase in virulence and a protection from antibiotics and antiseptics provided to biofilm-producing pathogens, and a tendency of biofilm-producing organisms to infect medical implants. In the latter (where biofilm formation is often referred to as biofouling), the need to control the tendencies of biofilms to foul and even corrode pipelines, heat-exchange equipment, and marine structures, and to harbor and protect (from disinfection) pathogens in water-supply systems is deemed “big business.”

It has been known, at least since 1943 (from the work of Claude Zobell, and others not directly consulted in this review of a review), that environmental water samples typically had more bacterial cells adhered to the walls of the sample container than cells suspended in the bulk liquid (Hall-Stoodley, *et al.*, 2004). In a situation analogous to the traditional dependence on *in vitro* osmotic studies to define the more general drying-tolerance of species, however, historical practices have tended to create a sort of a blind spot to the ecological significance of microbial adhesion. A traditional reductionist approach, studying behaviors of single-species populations (to improve behavioral resolution), derived from easily maintained stock cultures (which enhances repeatability of studies), inoculated into homogeneous (easily measurable) and often liquid (for easy cell dispersal, especially of planktonic cells) defined media of nutritionally rich compositions (to assure survival over the course of a study) have proved invaluable in feathering apart the intricately interconnected, intracellular biochemical reactions by which a cell maintains itself. Such an approach, however, ignores (and even renders immeasurable) many extracellular environmental realities. In nature, microbes are generally associated with surfaces, and generally not present as monocultures but are associated with mixed-species assemblages. Proximity to

surfaces generally induces microbes to produce and secrete extracellular polymeric substances (EPS or, with the embedded cells, biofilms) that enhance physical association with those surfaces in a gene-expression based change accompanied with an alteration in phenotype (from a motile, planktonic form to a sessile one). Planktonic bacterial cells, freely swimming/floating in liquid are actually found to be quite rare (and even free-floating environmental cells are often embedded in “flocs,” EPS matrices of compositions similar to those of surficial biofilms, see Hall-Stoodley, *et al.*, 2004). Moreover, reliance on stock, reliably cultivable monocultures in *in vitro* studies necessarily neglects any real (*in vivo*) effects of many species contributing to, and ecologically competing within, environmental biofilms. This *in vitro/in vivo* dichotomy is enhanced by the very methods by which stock cultures are derived; reliably cultivable monocultures in *in vitro* are reliably cultured only in the absence of ecological pressures to produce EPS. Worse, the monoculture-propagation regime may not only allow for evolutionary drift to ecologically maladapted phenotypes (Gilbert, *at al.*, 1993), but may also encourage it (even researchers interested in surficial behavior, such as geneticists wishing to isolate colonies on an agar plate are unlikely to propagate stock strains with a history of spontaneously reverting to a motile phenotype and actively crawling/gliding/moving across the spread-plate, see Kearns, 2010). While much of the earliest research into mechanisms by which biofilms are formed and maintained was focused on their eradication from medical and industrial settings (the latter increasingly expanding into hygienic surfaces in food processing, see Chmielewski and Frank, 2003), the findings in those fields has more recently forced an evaluation of biofilm importance in more general ecological settings.

Biofilms represent a “significant and incompletely understood” adaptive life mode of prokaryotes. Early adoption of the biofilm lifestyles is evidenced by fossils over three billion

years old that are morphologically consistent with recognized biofilm structures and by existence of biofilm-competent taxa throughout both Bacteria and Archaea (Hall-Stoodley, *et al.*, 2004), the latter implying that the adoption of this adaptation occurred before the evolutionary diversion between the two oldest phylogenetically recognized domains (Madigan, *et al.*, 2002, inside front cover). The observation that “the vast majority of bacteria” inhabiting both “natural and pathogenic ecosystems” inhabit “matrix-enclosed communities attached to surfaces” exemplifies the significance of the biofilm lifestyle (Costerton, 2009). The fact that the lifestyle is incompletely understood derives not only from the relatively recent recognition of its importance, but also from the extreme variability and complexity of the composition and morphological structure of the EPS matrix (“the dark matter of biofilms”), both of which vary both on the multispecies consortium of organisms inhabiting the biofilm and on the organisms’ collective responses to conditions across the broad array of environments in which the matrices are constructed (Flemming and Wingender, 2010). The organisms construct and/or alter the EPS in a coordinated manner (called “sentient” by Costerton, 2009, and explored in depth from a social-theoretical perspective of collective decision-making by Ross-Gillespie and Kummerlie, 2014), facilitated by secretion of “signal” molecules from a cell (some of which have been identified) that are recognized by others, even (in an evolutionarily conserved system, or one that has been redistributed by horizontal gene transfer) by others of different species (Costerton, 2009). By (multi-cellular and even multispecies) “quorum sensing” of generally recognized signals, the consortium of unicellular organisms within a biofilm can exhibit collective behaviors more commonly (historically) associated with the behaviors of multi-cellular organisms. The “behavior” of a biofilm is mediated by collective secretions of various EPS components (which include not only Potts’ exopolysaccharides, but also proteins including exoenzymes capable of

exopolysaccharide depolymerization, lipids with various hydrophobicities, and extracellular nucleic acids which include horizontal gene-transfer agents, Flemming and Wingender, 2010). One such “behavior,” common to many biofilms of many differing compositions in the face of many environmental pressures is a recognizable biofilm “life cycle,” the ontogeny of which seems to include:

- 1.) Approach (active or passive) to, contact with, and subsequent (induced) adherence/attachment to a surface by cells; and
- 2.) Cell-cell interactions that, through quorum-sensing/EPS-secretion mechanisms, organize the biofilm into a three-dimensional structure such that zones of species-specific favored habitability (based on access to nutrients, water, oxygen, proximity to potential symbionts, etc.) exist; leading to
- 3.) Microcolonies of species in favored microenvironments, in which (by close proximity) horizontal gene transfer is favored; creating
- 4.) Discrete pockets of species of enhanced genetic diversity that, upon dispersal from the biofilm, are more likely to be capable of colonizing new environments (Kaplan, 2009).

Each of these features would obviously have profound significance in any study of FIB survival on and release from source-area terrestrial surfaces. Despite the “incompletely understood” nature biofilms, their potential effects must be at least considered whenever a biofilm-competent cell can attach to a surface and initiate biofilm formation. Both taxa of FIB under study here and, indeed, most species that they would be defecated with or that they would encounter subsequent to defecation actively secrete and alter biofilm components (Romling, *et al.*, 2005, and Kearns, 2010). Initial attachment of a cell involves an intricate interplay of its van der Waals attraction to and electrostatic repulsion from the surface. In most natural ecosystems, especially in the case of a “conditioned” surface (with adsorbed organics) the free-energy barrier to attachment is of small enough dimensions to be bridged by cellular appendages (e.g., fimbriae and pilli, Gilbert, *et al.*, 1993). The adsorption of organics to a surface depends, at least in part, on the texture of the surface, but nanometer-scale topography of even polished abiotic surfaces is of sufficient roughness to allow for conditioning of water/solid interfaces (Whitehead and Verran, 2009).

What is known or can be inferred of biofilms will often be included (below) in development of the various features of this research. Most important in this section is the capacity of EPS components (especially exopolysaccharides, though some extracellular proteins are also implicated, Flemming, 2011) to adsorb (by hydrogen bonding) and retain large quantities of water, creating matrix reservoirs available to embedded microbes in the face of atmospheric drying of media.

In 1992, Roberson and Firestone isolated and cultured a strain of soil *Pseudomonas* selected (“for its mucoid colony appearance”) from an agricultural field in (Mediterranean climate) California. The culture was inoculated to sterile sand, subjected to a wet/dry cycle (wet = -0.025 MPa, dry = -1.5 MPa), rewetted (at Day 0), and then split into an experimental group of samples (dried in desiccators containing LiCl) and a control group (in similar “desiccators” containing distilled water to maintain high RH%) and analyzed (in triplicate samples of each group) for cell (CFU) counts, water content, protein content, electron-transport activity, and total carbohydrate daily until the experimental group reached “dry” status (-1.5 MPa, which occurred on the third day). The authors also isolated and concentrated the exopolysaccharide content from a colony of the same pseudomonad and determined the moisture release curves (water content vs. potential relationships) for both the purified carbohydrate (used for calculating the potential from the content measured in the above, three-day comparison), for a carbohydrate-amended sand (same type of sand used above), and for the sand alone. Relevant results of the drying vs. control (3-days in desiccators) run included:

- 1.) The matric potential of samples in the control “desiccators” remained constant (~0.025 MPa) throughout the study period, while the desiccating samples significantly ($p \leq 0.01$) diverged to lower potentials beginning at Day 1, at a rate of divergence that increased daily, and culminated in $WP_{\text{mat}} = -1.46$ MPa at Day 3;

- 2.) Cellular metabolism of the two groups (as measured by electron-transport activity) declined over the study period (with diminishing nutrient) at essentially the same (reported $p \leq 0.43$) rate;
- 3.) Both groups exhibited an increase in protein content, and a decrease in polysaccharide content between Day 0 and Day 1 of the study period, implying that both groups were anabolizing the former (and growing) while catabolizing the latter (as nutrient) in response to the rewetting at Day 0;
- 4.) After Day 1 (and measurable divergence of WP_{mat} between the groups), control-group protein and CFU growth remained high, while the desiccating-group samples exhibited slowing cell division, with increased exopolysaccharide production (at the apparent expense of proteinaceous structure).

The authors further showed (through microscopy) that control (continuously wetted) cells were, at most, interspersed in a fibrous network of exopolysaccharide material, while cells in the desiccating group, and the sand grains to which they were attached, were covered by, and embedded in “thick” layers of large-molecule carbohydrates. The moisture-retention curves generated by Roberson and Firestone further inform that:

- 1.) The exopolysaccharide materials harvested from these pseudomonads showed high affinity for water at all water potentials (at $WP_{mat} = -0.5$ MPa, the material held 10 times its own dry weight as adsorbed water while, at -1.5 MPa, the ratio was still ~ 5 and, for perspective, the authors provide that medium-textured soils at $WP_{mat} = -1.0$ typically only retain 4-10% of their dry weight as adsorbed water); and
- 2.) The rates of water-content loss, with respect to drying time, of naked and carbohydrate-amended sand were not significantly different; but
- 3.) With respect to time of drying (and water-content reduction), even small exopolysaccharide amendments roughly doubled the time (*vs.* unamended sand) required to produce a meaningful reduction in WP_{mat} ; and thus
- 4.) For given drying potentials, water content was considerably greater in amended sand *vs.* sand ($p < 0.01$).

The authors (Roberson and Firestone, 1992) find these results consistent with a hypothesis that:

- 1.) Cells, in response to falling matric potential, down-regulate synthesis of structures and cell growth, and redirect their metabolism towards the synthesis and secretion of polysaccharides; and thus
- 2.) Cells actively increase the available-water retention of their immediate (micro-) environment in response to drying of the general habitat.

A review by van Loodstrecht, *et al.*, 1990, into myriad effects of surfaces/interfaces on microbial activity (growth) finds no evidence of “direct” (e.g., an alteration of membrane permeability affecting substrate importation) effects of a nearby surface on a cell, itself, that affected activity. All growth modifications reviewed here were found best explained by “indirect” effects of the surface upon the immediate extracellular microenvironment (e.g. local alterations to “substrate availability, pH buffering, water activity,” or an increase in the extracellular stability of horizontal gene-transfer constructs).

The review of Potts (1994, which includes the work of Roberson and Firestone, 1992) highlights difficulties in analyzing the structure of many exopolysaccharide-rich components around a cell and of elucidating any structural response of such components to drying. Many such structures collapse during preparation for electron microscopy, and many even become electron-translucent in the case of desiccated cells. However, Potts does find that, in many species and under many different conditions of drying, the exopolysaccharides harvested from colonies are universally wet (“engorged with water,” with water content sometime exceeding 99% by weight). Noting the minimal water solubility of these polymers, the reviewer concludes that the water/exopolysaccharide mixtures must represent gels, in which liquid water is restrained against mixing by the structure of (the small quantity of) polysaccharide. With fewer studies of fewer species available, Potts further finds evidence that these gel-forming polymers are indeed synthesized inside the cell and actively exported, with a contemporaneous change of cellular morphology to a non-motile and desiccation-resistant phenotype, in response to drying. This last implies complex coordination of many systems (either by changes in expression of many genes, or by modulation of the activities of many enzymes). Of particular importance in my review, Potts finds that, in enteric bacteria, production of an important capsular component is

induced much more strongly under matric stresses than under osmotic assaults, and the many “genes necessary for this synthesis are scattered around the *E. coli* map.” In at least one system, three synthetic glycolipids (derivatives of polysaccharides) offered cryptoprotection (in a manner consistent with the water-replacement hypothesis) to cell membranes (under freeze-thaw cycles) at concentrations ~100 times lower than that of secreted free trehalose to achieve the same level of protection, at least suggesting that the size (molecular weight) of the carbohydrate moiety (mono-, di-, or polysaccharide) is not critical to drying tolerance. Recall that Potts’ review was focused on evidence in support of the hypothesis that anhydrobiotic survival was mediated through water replacement (with, as yet, limited evidence) by glass-forming (potentially not provable with current technologies) carbohydrates. Relevant to my review (and without need for recourse to proof of Potts’ main hypothesis) Potts finds that:

- 1.) Bacteria in general (and enteric bacteria in particular) respond to desiccation by a coordinated diversion of metabolism from growth to EPS production;
- 2.) EPS components, especially the exopolysaccharides, retain large quantities of water even in the face of low and dropping external matric potentials;
- 3.) The coordinated diversion of metabolism to EPS production/secretion of the cell is accompanied by a phenotypic change to a desiccant-resistant cell;
- 4.) Some potential EPS components have shown efficacy in providing desiccation resistance to some macromolecular structures. (Potts, 1994)

The coordinated nature of metabolic-diversion and phenotypic-conversion events during drying, together with Roberson and Firestone’s evidence (1992) of a reversion of the former (metabolic diversion) on rewetting, hints that the reversion may be similarly coordinated. More recent reviews of biofilm research (e.g., Flemming and Wingender, 2010, and Flemming, 2011) confirm all of Potts’ relevant findings presented above, and the desiccation resistance of cells embedded in EPS (of their own making or made by others) is generally expressed as a given. Moreover, multiple exoenzyme systems, by which cells can alter the architecture of previously

secreted EPS structures (e.g., for affecting biofilm density and for creation of water-filled pores or voids within biofilm structure in response to film depth and to environmental conditions) have been elucidated (see Hall-Stoodley, *et al.*, 2004). Possible mechanisms by which reversal of biofilm adaptations to drying conditions may be reversed, in a manner coordinated across the multiple gene-expression and/or enzyme-activity required for viable dispersal of cells out of the biofilm, are only very recently and very tentatively being worked out (and will be discussed, to the limited extent known, below).

In the meantime, and important here, biofilms have recently been found crucial to the survival of microlithic microbial communities in arid environments. The hygroscopy of EPS allows the mixed-microbe assemblages to adsorb water in the morning (typical daily RH% maximum, and at which atmospheric moisture may even condense in dews or fogs) and the water-retention characteristics provide available water (during more drying conditions and until the next wetting event) to communities living (under rocks and pebble pavements) in both hot and polar deserts (Chan, *et al.*, 2011). Additionally, FIB in the colon have also been found to be embedded in biofilms (Macfarlane and Dillon, 2007, and recall drying stresses within the colon discussed in 2.2.4.3.1 above). In some ways, digesta and associated flora in the colonic lumen could be considered a floc, sloughed from surficial films, somewhat preconditioned to drying tolerance and already armed with water-retentive EPS. Upon discharge, FIB and EPS in feces (which really should, in my opinion, be considered a real and tertiary habitat for FIB) join another, extant biofilm-competent assemblage.

Direct effects of air drying on aerosolized bacterial cells have been found to affect growth of those cells in a reasonably linear manner until a threshold of no growth, corresponding to the

point of equilibrium between RH% and cellular free water is achieved, occurs. Under more stressful drying conditions, at which bound water is stripped from the cells, cell death increases with decreasing RH% in a linear manner at a rate consistent the compatibility of extant intracellular solutes. Such a system should exhibit a fairly simple three-phase, but monotonic, relationship between net survival and atmospheric RH%. FIB (biofilm-competent bacteria), defecated to terrestrial landscapes (generally unsaturated porous media), should still exhibit a monotonic, positive relationship between net growth and RH% but with many, many more step discontinuities, each corresponding to conditions at which matric potentials exerted by the drying atmosphere exhaust the (adherent, capillary, adsorbent) hygroscopic and/or water-retentive capacities of the various media (whether the matric-potential discontinuity occurs at a membrane or not, and many of which seem dependent on microscopic structure) across which water is transferred from important cell structure. While individual taxa obviously differ in drying tolerance, a recent meta-analysis of 33 studies into the activity (as measured by heterotrophic-respiration and/or by nutrient-mineralization rates) of entire soil microbial communities across a range of climates and soil types and sampling depths found two distinct thresholds of activity cessation based on from which soil layer (mineral soil or surface litter) the samples were harvested. In the mineral-soil studies, the water potentials at which bacterial activities ceased meaningfully clustered at about -14 MPa, consistent with the point at which free flow of nutrient solutes becomes inhibited by the draining of water-filled pores. In surface litters, however, growth was maintained to stresses of about -36 MPa, where cells were expected to experience onset of actual dehydration (Manzoni, *et al.*, 2012).

2.2.4.3.5 Nutrients

Effects of nutrient availability on net growth of microbes has been discussed in considerable detail above (section 2.2.4.2). In terms of both batch-system Growth Cycle, and of the (flow-through) chemostat carrying capacities, the growth, maintenance, or decline of FIB populations is intimately dependent on the bioavailable nutrients in their environment. Recognition of the mature biofilm nature (immediately above) of colon contents (discussed at 2.2.4.3.4) and excreta provides fodder for logical conclusions concerning the nutrient availability to FIB defecated to the oxic landscape. Feces typically consist of ~ 1/3 bacteria by weight (2.2.4.3.4, and see Madigan, et al., 2002, p. 735). The remainder consists of EPS (bioavailable nutrients with the action of exoenzymes under conditions otherwise conducive to growth, section 2.2.4.3.4, and see Flemming, 2011) with adsorbed water solutions of fermentation-resistant organics (which would become bioavailable to facultative FIB upon exposure to an oxic atmosphere, section 2.2.4.3.1). While considerable evidence exists that the organic content of soils plays a major role in the long-term establishment of native FIB strains on the landscape (section 2.2.2), the survival of FIB upon defecation should not be subject to nutrient limitations until the organics with which they are already closely associated are exhausted. The possibility of disintegrating fecal mass depositing FIB-containing detritus into large enough and pure enough water-filled microenvironments in pervious surfaces as to dilute away associated nutrients cannot logically be excluded, and should be checked in this research. A hypothesis that extant organics on the surface prior to the defecation event is a significant factor contributing to FIB survival between the time of defecation and the time of a rain, however, would seem inappropriate.

Unc, *et al.*, (2006) examined the soil populations of *E. coli* in soils, subsequent (for 20 days) to amendment by application of sewage biosolids, at four soil depths (0-10, 10-30, 30-50, and 50-70 cm). Soil samples were from a fallow clay soil (Ontario) to which no wastes or fertilizers had been applied for at least 10 years, isolated from standing water bodies, without notable wildlife exposure, and containing naturally occurring and measurable *E. coli* populations. Half of the soil (S_S) was subjected to 3 days of repetitive autoclaving and confirmed sterile by failure to produce any growth on several nutrient agars. Half of the biosolids (B_S), from treated, dewatered sewage sludge, were similarly sterilized, confirmed sterile, and then autoclaved for an additional 10-14 days. The authors stored four soil/biosolid mixtures (sterile soil and sterile biosolids, $S_S B_S$; fresh soil and fresh biosolids, $S_F B_F$; and sterile soil with fresh biosolids, $S_S B_F$, all three as controls: and an experimental mixture consisting of fresh soil and sterile biosolids, $S_F B_S$), for each soil depth (from which the soil samples were harvested), for 20 days at room temperature while maintaining all mixtures at the calculated field capacity of the source soil. The samples were analyzed for culturable *E. coli* MPN at intervals over the course of study.

- 1.) $S_S B_S$ mixtures exhibited no measurable *E. coli*, at any soil depth, at any time in the study.
- 2.) At Day 0, both mixtures including fresh biosolids ($S_F B_F$ and $S_S B_F$) exhibited nearly equal MPN values ($\sim \log 1.5/\text{gram dry soil}$) at all soil depths implying that fresh biosolids contained many more *E. coli* CFU than the fresh soil. This implication is supported by very low ($< 1 \text{ MPN/gram}$ at all depths) *E. coli* densities for $S_F B_S$ at Day 0.
- 3.) Subsequent to soil amendment, *E. coli* densities in the fresh-biosolid mixtures diverged, with no significant change ($p > 0.05$) in the sterile-soil mixture over the course of the study. This would seem to imply that the *E. coli* populations in the applied fresh biosolids were already, at the time of application, stationary at the carrying capacity provided by the associated nutrients. The $S_F B_F$ mixture, however, showed rapid (though depth-dependent) growth and, by the fourth day, the densities were significantly ($p < 0.01$) higher (by up to four orders) than those at Day 0. The densities found in the $S_F B_F$ mixtures stabilized at the new, higher level after Day 4, as if the addition of biosolid organics had provided for a new, higher carrying capacity for at least some subset of the cells in the mixture.

- 4.) The S_FB_s mixtures (devoid of applied *E. coli*) also showed rapid (though depth dependent) growth (up to 2.62 orders) over the first few days of the study. Evidence of this new, higher carrying capacity waned (in a depth dependent manner) over subsequent days of the study period, but at all depths, Day 20 densities were greater than those found at Day 0 by at least 1.5 orders.

2.2.4.3.6 pH

In 1952, Allen, *et al.*, provided a study into the death rate of (sewage- or river-derived) *E. coli* (then still called *Bacteria coli*) and *Enterococcus faecalis* (then still called *Streptococcus faecalis*) in solutions (of autoclaved water, without added organics) buffered (with “very dilute phosphate”) to several levels of pH (~5, 6, 7, and 8). The authors found that the survival (defined as the zero-intercept of the log of the surviving percentage of original cell count) of both taxa varied (from ~ 100 to over 400 hrs) in a pH-dependent manner. Survival time (surprisingly?) was shortest in the central range (~6 – 7 pH), with enhanced survival under conditions diverging from neutrality.

Textbooks typically characterize FIB as neutrophiles, with optimum growth in the range $6 < \text{pH} < 8$ (as expected for organisms primarily adapted to life in the colon). Atlas (1984, pp. 352-353) offers cardinal pH values for well-studied *E. coli* (minimum = 4.4, maximum = 9.0, optimum between 6 and 7). Enterococci may survive across a somewhat broader range (section 2.2.4.1.1) but should also be primarily adapted for the colonic environment. Atlas (1984, pp. 352-353) further describes the mechanism by which pH affects survival in bacteria as the disruption of proteins. Attractions and repulsions between charged moieties influence both the three-dimensional structure of enzymes and the catalytic functions at their active sites. Madigan, *et al.* (2002, pp.158-159), note that even extremophiles, surviving in solutions of very low or high pH, maintain intracellular pH very near to neutrality. Most organisms exhibit growth over an environmental range of 2-3 pH units. Which 2-3 units depend on the integrity and transport

functions of the cytoplasmic membrane and their ability to maintain near neutrality inside the cell (e.g., membrane lipids of alkiliphiles may even dissolve in an environment “acidified” to pH neutrality).

Universal presence of FIB in homeothermic colons implies some mechanism to survive passage through gastric stomachs (typical pH ~2), and an ability to “hide” in some diffusion-limited aggregate has been proposed (section 2.2.4.3.1). In 1980, Diderichsen (not directly consulted here, but reviewed in Beloin, *et al.*, 2008, pp.249-291) identified the *flu* gene in *E. coli*, mutations of which varied surface properties of cells. Wild-type *flu* encodes Antigen 43 (Ag43), a membrane protein found to be important to biofilm structure. Ag43 is not implicated in the initial attachment of cells to surfaces, and its production is actually competitively inhibited (by Type 1 fimbriae production) while attachment processes are ongoing. Ag43 is expressed (in response to many sensed environmental signals) during biofilm maturation (three-dimensional structure building) and promotes cell-cell adhesion. In solution, Ag43 expression leads to the formation of flocs, aggregates of cells (not only the *E. coli* but also others when present) and diffusion-limiting EPS, and promotes sedimentation (Beloin, *et al.*, 2008, pp.249-291). One environmental signal that strongly encourages Ag43 expression is low pH, and the resulting flocculation has been implicated in protection and rapid passage of FIB through the stomach (Goller and Romeo, 2008, pp. 37-66). Another sensing system, the two-component *cpxRA* system has been found to promote biofilm development (affecting both cell-surface and cell-cell interactions) in response to elevated pH (Beloin, *et al.*, 2008, pp.249-291).

The diffusion-limiting nature of EPS serves not only to minimize washout of nearby nutrients (and other useful substances, e.g., exoenzymes and signaling molecules) available to a cell, but also locally restricts intrusion of harmful substances (Beloin, *et al.*, 2008, pp.249-291).

While few individual EPS components have been successfully isolated and identified, their collective effects on diffusion through the biofilm is well-studied. Biofilm characteristics as diverse as the resistance of embedded cells to disinfectants to the maintenance of diverse microenvironments suitable for microcolonies of diverse species in close proximity to each other result from the presence of the EPS (Chmielewski, and Frank, 2003). Even in aquatic biofilms, steep pH gradients often stably exist (Flemming, 2011), and adaption to diverse pH conditions on the landscape is considered an important selection pressure influencing the evolution of the biofilm lifestyle (Stoodley, *et al.*, 2004).

Following reasoning similar to that expressed in section 2.2.4.3.5 (nutrients) above, an expectation that extant pH of source-area surfaces has a significant impact on the survivability of FIB (defecated as cells embedded in biofilms saturated with pH-neutral solutions which crumble, under drying conditions prior to a rain, into cell/EPS aggregates that may be incorporated into existing, diffusion-limited biofilms) would seem misguided. However, the possibility of a significant effect, at least on pervious surfaces, must be checked in this research.

2.2.4.3.7 Ultraviolet Radiation (UV)

In 1930, Gates showed that a lethality peak (lower incident-energy dose required for a given cell-death response) coincided with an absorbance peak in spectral studies of UV-radiation exposures of whole cells (*S. aureus* and, as now characterized, *E. coli*). That such a coincidence (in a narrow wavelength band of ~ 260-270 nm with available resolution) was evident overturned the contemporary paradigm (apparently based on a long history of studies involving ionizing radiation) that cell death correlated inversely and monotonically with electromagnetic wavelength. Gates found these findings consistent with even earlier work (Bang, S., 1905, and

not directly consulted here, but cited by Gates) that there is a wavelength-dependent bactericidal “effectiveness,” and concluded that some intracellular structure that preferentially absorbs certain wavelengths, to its detriment, must be crucial to cell survival (and, as a side note – Francis Crick was 14 yrs old at the time of Gates’ publication, and James Watson was 2). Gates’ effectiveness maximum (260 – 270 nm) is deemed “ecologically not relevant.” Ever since the earth’s atmosphere was sufficiently oxygenated to provide fodder for a significant stratospheric ozone layer (~1.5 billion years ago), all solar UVC (the UV band of wavelengths below ~ 280 nm) has been “quantitatively absorbed” before it reached the earth’s surface (Sinha, *et al.*, 2002).

Calkins and Barcelo (1982) note that Gates’ and others’ findings presaged the now-recognized importance of DNA (as the repository of cellular genetic information and a biologically relevant target of UV action). They also recognize the differential effectiveness (Relative Biological Effectiveness, or RBE) by which specific wavelengths may affect the stability of DNA. Finally, the authors incorporate laboratory-derived RBE of various wavelengths (largely limited to the range of emissions from mercury-vapor lamps, 254-365 nm) and the actual spectrum of solar UV wavelengths reaching the earth’s surface to create “Action Spectra,” of UV biocidal action (effectiveness per unit energy at a given wavelength, multiplied by insolar UV energy flux at that wavelength). Spectra of *E. coli* and several other species all showed distinct action peaks in the wavelength range of ~ 305-315 nm (near the upper wavelength limit of the UVB band), though (by inspection of the authors’ Figure 2) measurable action on *E. coli* extended well into the (longer-wave) UVA band as well.

A textbook discussion of UV effects on microbes discusses the extreme lethality of UVC (centered on ~260 nm) and its utility (with easy generation by mercury-vapor lamps) in lab sterilization (Madigan, *et al.*, 2002, pp. 272-274, p. 174, and p. 700). The authors further discuss

“one well-established” mechanism by which UV radiation affects cells, namely its capacity to generate pyrimidine dimers in DNA molecules. Ultraviolet is not considered ionizing radiation; the energy of a UV quantum is insufficient to disrupt covalent bonds. Shortwave UV radiation, however, is sufficiently energetic to disrupt the hydrogen bonds (intermolecular dipole interactions not involving shared electron pairs) between the complimentary bases inhabiting opposing strands of the DNA molecule. At the UVC absorption peak (~ 260 nm) of DNA, quanta are of sufficient energy to disrupt all such bonds and (with bactericidal consequences) “melt” DNA into its component strands. There are UV wavelengths in the UVB band, both of sufficient energy and arriving in sufficient fluxes through the atmosphere (the ozone layer screens UVB less efficiently than UVC), able to disrupt some base-pair hydrogen bonding in an environmentally relevant manner. The pyrimidine (adenine-thymine) pairs, being only double bonded, are more susceptible to such disruption than are purine pairs (held together by three hydrogen bonds between the strands). Moreover, if two pyrimidine bases (especially two thymines) occupy adjacent sites on a single strand, the hydrogen bonds may competitively rearrange to bind adjacent bases on the same strand before the bonds between strands are re-established. These single-strand dimers, unless repaired, render the transcription and replication mechanisms of the cell incapable of performing their functions. Various repair mechanisms have evolved across many species. Some require light to be active, some involve excision and replacement of the dimer (with possible reading errors leading to a mutation), but all such repair systems require time. Failure to transcribe a gene would only affect viability of a cell if it were the only copy of a gene crucial to survival in the cell’s genome. Failure to replicate the genome during cell division (the replication fork becomes irreversibly locked upon encountering a dimer), however, halts the cell-division process and renders the cell unculturable.

Madigan, *et al.* (2002, p. 164), also note the potential (in the presence of light and oxygen) for the photochemical conversion of triplet (“normal”) oxygen to singlet oxygen (a rearranged spin-pairing of outer electrons) of sufficient energy to induce many deleterious reactions within a cell. UVA flux to the earth’s surface is virtually unhindered by the ozone layer (which is largely transparent to this band). UVA quanta, however, are of insufficiency energy to disrupt the strand-bridging hydrogen bonds in DNA, and their spectrum of absorption by DNA is generally (unless the flux is such that significant intracellular heat is generated) irrelevant to cell survival. The *E. coli* action spectrum of Calkins and Barcelo (1982, at their Figure 2), however, does show evidence of significant deleterious effects well into the UVA range (even with, by inspection, potential evidence of a secondary action peak at about 350 nm). The UVA band (along with visible-light bands) has been found to induce DNA damage (and damage to other cellular structures) in an indirect way. Many cellular or environmental substances (“photosensitizers”), in the presence of oxygen and light, generate singlet oxygen that (until quenched) has sufficient reactivity to induce secondary photoreactions damaging to cell structures (and, in the case of DNA, may result in strand breaks, by disruption of phosphodiester bonds in the structurally important “backbone” of the molecule). These secondary effects do not depend on the direct absorbance by DNA, but on the absorbance of UVA (and light) by the various photosensitizing structures and the presence of oxygen (Sinha and Hader, 2002).

Most *in situ* studies of FIB survival response to UV have been performed in aquatic or marine systems (e.g., Davies-Colley, *et al.*, 1994, and Sinton, *et al.*, 1994), and have been unable to find any significant correlation between measured UVB flux and cell death. In the former (Davies-Colley, *et al.*, 1994), for example, the authors found that the depth-dependent decay rate of FIB (in this case, FC and Enterococci from sewage) correlated most strongly with extinction

(with depth, in seawater) of 360-nm radiation (well into the UVA band). The authors of this study did note that correlation between cell inactivation and 360-nm exposure does not necessarily mean that such correlation is caused by 360-nm radiative effects; the correlation could equally represent combined effects of two causal mechanisms occurring at two absorption peaks on either side of 360 nm. They further note that the extinction of radiation with depth in water is wavelength dependent, with shorter wavelengths (e.g., UVB) disappearing to water absorption at much shallower depths. The authors deemed UVB irradiation an insignificant contributor to FIB inactivation in waters.

I find very few studies that have captured (or even could have captured) the wavelength-dependent bi-phasic mechanistic effect of UV radiation on landscape bacteria (though the 2002 review by Sinha and Hader, and that by Rastogi, *et al.*, 2010, imply its general acceptance by those dates). Slieman and Nicholson (2000) exposed dormant endospores of a mutated strain of *Bacillus subtilis*, lacking known UV-damage repair mechanisms, and exposed them (under air) to:

- 1.) Artificial UVC germicidal radiation (predominantly 254-nm);
- 2.) Artificial UVB radiation (290-320 nm);
- 3.) Full-spectrum sunlight (near noon, Tucson, AZ), containing both UVA and UVB (and other) components; and
- 4.) Full-spectrum sunlight filtered by a glass plate known to block all UVB (< 325 nm).

The exposed spores were de-coated, lysed, and analyzed for accumulation of known UV-exposure products:

- 1.) “Spore photoproduct,” a photoproduct unique to UV-exposed endospores, not relevant to the FIB research, and ignored hereinafter;
- 2.) Pyrimidine dimers;
- 3.) Single-strand DNA breaks; and
- 4.) Double-strand DNA breaks.

The spores exposed to germicidal UVC accumulated all of the photoproducts studied, as did the spores exposed to full-spectrum sunlight. The UVB-irradiated spores, however, exhibited only the dimers, and showed no evidence of accumulation of strand breaks. Conversely, the spores exposed to sunlight with the UVB portion of the spectrum blocked accumulated no pyrimidine dimers, but accumulated both single-strand and double-strand breaks in the DNA backbones. Muela, *et al.* (2000), examined responses of *E. coli* in stirred flasks (of very limited radiative transmission paths) of (sterilized) river water (DOC = 6.89 mg C/L) exposed to (artificially generated, wavelength segregable) sunlight components (including UVB, UVA, and photosynthetically active radiation, or PAR, a large component of visible-light insolation) over 48 hours. The authors' full-spectrum "sunlight" (produced by combined exposure from three types of specialized light bulbs of defined emission spectra) was designed to mimic the relative band contributions of their locally (Barcelona) measured insolation, but was (in terms of total radiative flux) only about 1/3 of outdoor sunlight. The authors exposed their flasks to full-spectrum (simulated) sunlight, UVB band only, PAR only, and UVA only, and analyzed responses through the study period. Responses measured were total cell count (by microscopy of stained cells), viable cell count (by growth in cell size, as measured by optical microscopy, by glucose utilization, and by epifluorescence of an indicator specific to active electron transport), and culturable cell count (by CFU count on two different enriched agars). No (irradiation) treatment was found to significantly reduce the total cell count over the study period. Full-spectrum "sunlight" was found to induce a general loss of viability (with a six-hour lag period for electron-transport activity), but glucose uptake was only diminished to about 0.01% of the Hour 0 measurement over 48 hours. Culturable cells, however, crashed under the full-spectrum treatment, with counts falling to below 0.01% in six hours, and to below detection limits

(0.000001% of the Hour 0 count) by 24 hours. The UVB-irradiated flasks were found to most closely duplicate the full-spectrum results. Viable cell counts declined, but remained measurable for most measurements for some time (with glucose uptake > 0.1% of the Hour 0 measurement at the end of the study). Again, culturability crashed (< 0.000001%) by 24 hrs. Both PAR and UVA treatments were quite different. For both of these treatments, all measures of viability and measures of culturability followed slow and parallel declines (and all remaining at or above 1% of Hour 0 numbers). The authors found that UVB irradiation specifically induces a “viable but not culturable” state in *E. coli*. The longer wavelengths of insolation (UVA and PAR) seem to exert a more general deleterious effect on *E. coli* survival, in which non-culturable cells are not culturable because they are also not viable.

It would seem that any examination of the environmental effects of UV radiation on FIB survivability on landscape surfaces must take into account the effects of all wavelength bands of insolation¹⁰ to account for the action of mechanisms that repair UV-induced damage; a simple light/dark experiment would be insufficient. The UVB band is the (apparently exclusive) cause of sunlight generation of pyrimidine dimers in DNA, but actions of (at least some) repair mechanisms are dependent on availability of visible light. The UVA band (along with visible light) has been implicated in more general damage to (DNA, lipid, and proteinaceous components of) cells through secondary photoproducts. The quenching of these secondary products (especially singlet oxygen) is accomplished by either the cell damage itself, or by

¹⁰ Thankfully, fluorescent lights that are designed to mimic the relative flux/wavelength spectrum of incident insolation, if not the total (across the spectrum) flux are available. Indoor gardeners, naturalist indoor tanners, and (especially) terrarium owners that raise reptiles indoors have created a market and suppliers, of late, have responded.

taxon-specifically evolved photoprotectants (the latter of which are often evolved in habitual presence of, and activated by, light, see Madigan, *et al.*, p. 164 and pp. 553-554).

The recent recognition that most environmental bacteria are associated with biofilms provides a potential complication to the conclusions above, and many reviewers (e.g., Hall-Stoodley, *et al.*, 2004) have implicated UV exposure (on the “primitive earth”) as an evolutionary pressure that selected for the biofilm lifestyle. Many studies have focused (again) on aquatic (receiving water) systems where the (monotonically wavelength-dependent) extinction of UV in water cannot be accounted for, and where the fully saturated water content of biofilms must be assumed. In one study (Schultz-Fademrecht, *et al.*, 2008), the authors examined the inactivation/time of FIB (FC and Enterococci) derived from sewage, in a shallow (0.1m, a short transmission path) microcosm (a circulating flume filled with local river water), portions of which were exposed to (various intensities of) artificial full-spectrum sunlight and portions of which were shaded (a light/dark setup). The flume bottom was populated with biofilm-coated (average thickness ~ 20 microns as determined confocal-laser microscopy) stones from the same (Isar, Germany) river. In the 30-hr study, the authors found the introduced FIB were rapidly (beginning at less than 1 hour) and significantly (preferentially) harbored in the biofilm. By the end of the study, FC concentration in the biofilm was 10^3 -fold higher than that of the bulk liquid, and the ratio for Enterococci was even higher. The authors did find that the biofilm (in this shallow system) offered significant UV protection (as evidenced by smaller decay constants) to both taxa at all exposure fluxes measured, though population declines still occurred in all exposed treatments. The authors’ use of full-spectrum sunlight, however, provided no opportunity to resolve the differential effects by wavelength band.

Elsari and Miller (1999) note a lack of tools allowing for non-invasive study of important cellular functions within biofilms, and present one (in a study that appears to be unique in the literature). The authors constructed a whole-cell biosensor by fusion of the *recA* gene of *Pseudomonas aeruginosa* to the *lux* operon of *Vibrio fischeri*. The *recA* gene has been shown inducible by DNA damage. The *lux* gene codes for Luciferase, which fluoresces at 490 nm. When this fused operon is introduced, by plasmid, into whole *Pseudomonas* cells, the resulting probe gives a visible signal of DNA damage introduced in the cell by various insults to which it is exposed. The authors suspended cultures of their probe into an alginate matrix and extruded 4-mm beads of the resulting mixture. As an aside, alginate was one of the first (and is still one of the few) EPS polysaccharides isolated and characterized, is highly conserved and expressed by many microbial species, and is commercially available (produced by separation from brown-algae cultures). The authors found their 4-mm beads to block UV transmission in a band-dependent manner. The beads were most opaque to UVC, transmitting a maximum of 13% of incident radiation from a germicidal bulb with peak emission at 254 nm. They were more translucent to (also artificially generated) UVB and UVA, transmitting up to about a third (31% and 33% respectively) of incident exposure to those bands. The authors incubated their beads under measured (pulsed) exposures to the three UV bands, and monitored the bioluminescence signal for DNA damage for 20 hours. They also measured viable cell counts, before and after irradiation, for each treatment. Exposures to UVC and UVB (the latter calibrated to cover the range of environmentally relevant solar fluxes, i.e., from 0 to average daily clear-sky dose) produced similar patterns of bioluminescence over time (30-minute lag before onset of luminescence, with peak brightness at 5-6 hours). Despite the reduced transmission of UVC through the beads, however, the UVC exposures produced much more DNA damage than did

UVB exposures at similar (externally provided) doses. Both provided for a lesser reduction in cell viability at all doses when compared to liquid cultures similarly dosed. Notably, however, and despite the photoprotective effect provided by the (simulated) biofilm, cell survival of the UVB-irradiated fell to 50% at very low doses ($\sim 50 \text{ J/m}^2$), and fell to about 10% at doses approximating average daily insolation (125 J/m^2) in that band. The authors found no elevated bioluminescence, indicating no elevated DNA damage, over the 20-hr study period, for the UVA-irradiated *Pseudomonads* (again, with doses calibrated to cover the range between 0 and average daily insolation dose in that band). The authors found that, despite the similar ($\sim 1/3$ incident) transmissions of UVB and UVA through the alginate, that opacity was sufficient for effective photoprotection from the less-energetic UVA band. The authors proceeded to run a second study, involving effects of UVA in the presence of psoralen. Psoralen is plant-derived, and unlikely to be produced intracellularly in bacteria. It is, however, highly diffusible and commercially available (used in combination with UV irradiation in medical procedures), and is a known photosensitizing chromophore. The authors note that extracellular psoralen, produced by local plants but diffusing into bacterial cells, has been implicated in photosensitization of *Pseudomonads* in marine environments. Cell- and psoralen-containing alginate beads produced no elevated bioluminescence in the absence of light over the period of study (as did UVA exposure in the absence of psoralen in the previous study). Such beads did, however exhibit a two-peak response over time in the presence of various exposures to light. The first (and smaller) bioluminescence peak was exhibited immediately, without lag, in the first hour of exposure. The second (larger and, with respect to time, much broader) peak, in all exposures, occurred 10-14 hours after irradiation. The magnitude was greatest at highest UVA dose (again calibrated to approximate the average daily insolation dose), and lowest at ambient (indoor) light (indoor

lighting, absent selection of specialized bulbs, is notoriously lacking in UV emissions). Notably, the peak (average daily insolation dose) luminescence response in the (UVA-psoralen) study (normalized to cell concentration) is almost an order of magnitude lower than that of the UVB-study peak (similarly normalized). The authors find some difficulty explaining the two-peak response to radiation-psoralen combinations, but surmise that it involves the “SOS response.” SOS is one of the UV-repair mechanisms discussed above (and in Madigan, et al., 2002, pp. 273-274). It is an inherently error-prone (mutagenic), multi-gene response whose name derives from its last-gasp nature (it is only induced upon failure of other responses to fix the problem, and one of its inducers is presence of *recA* products). Elasri and Miller note that even in the absence of bioluminescence between the peaks in this system, cell death is occurring (data not shown). They believe that the first (no-lag) luminescence peak represents DNA damage immediately caused by singlet oxygen generated by photosynthesizer in the immediate vicinity of the chromosome, and immediately repaired by SOS. While cell death continues from damage to other structures throughout the cell, the bioluminescence signal (indicating damage to the chromosome) does not rise again until the (larger number of) singlet oxygens, generated elsewhere in the cell, diffuse to the chromosome.

Contrary to the rationale, based on diffusion limitations in biofilms, presented above (2.2.4.3.5&6), an assumption that most or all environmental FIB exist in biofilms provides no rational excuse to neglect UV as an effector of cell survival. While some biofilm components do demonstrably provide UV shielding to embedded cells, they apparently do not obviate solar-UV influence in any band. The apparent dominance of UVB-induced mechanisms affecting landscape bacteria does, however, lead to logical questions concerning the independence of UV exposure as a significant contributor to deleterious effects on cells. Rates of pyrimidine-dimer

generation and repair only influence cells in the act of dividing. Deleterious UV influence of this mechanism should interact with any other (growth-promoting) factors that lead towards reproductive fission.

2.2.4.4 Source-Area Export

Recognition that surficial biofilms represent the “principle mode of life of bacteria within the environment” obviously renders the “life cycle” of the biofilm lifestyle, and especially mechanisms leading to dispersal from that film, important to study of the release from and transport out of source areas by FIB (Webb, 2007 and see discussion at section 2.2.4.3.4). I find three different mechanisms in the literature potentially relevant here, any or all of which may lead to the release of FIB from landscape surfaces. Any of these mechanisms has at least the potential to explain the frequent lack of intra-storm first-flush behavior found in section 2.2.3.3.1, above. The literature is silent concerning which (if any) of these mechanisms are most likely at work in the case of land-defecated FIB, but the assumption of each mechanism logically provides testable hypotheses of its activity.

2.2.4.4.1 Seeding Dispersal

“Seeding dispersal” represents the most complex mechanism presented here, requiring active coordination between multiple cells (Webb, 2007). Webb, *et al.* (2003), present the observed features of seeding dispersal as a developmental sequence by which monospecific microcolonies, arising from chemical gradients imposed by the diffusion-limiting character of EPS, change to a specialized dispersal-competent compartment within the film. Largely lacking knowledge of the specific signal molecules involved, the authors describe an oft-observed

sequence of events exhibited by many cell assemblages in many environments. In response to stresses (nutrient starvation serves as the authors' primary example here):

- 1.) Cells synthesize and export EPS, while synthesizing and (intracellularly) storing polysaccharide lyases (catabolic enzymes);
- 2.) Deep in the film, starvation (or some unknown signal triggering programmed cell death) causes death in a subset of the microcolony population;
- 3.) Dead cells lyse and release the lyases which quickly and catalytically depolymerize the surrounding exopolysaccharides;
- 4.) In the new, enclosed aquatic environment (caused by lyase action on the exopolysaccharides), enriched with nutrients (from both lysed cells and depolymerized EPS), the remaining cells revert to both growth/reproduction metabolism (*vs.* EPS production) and the planktonic morphological phenotype (*vs.* sessile).

The final-stage reversion in metabolism and morphology would obviously call for concerted action by a huge number of cellular enzymes. The aquatic "seed," upon disruption, disperses planktonic cells to the environment, leaving a microscopically visible "shell" (a rim scar) on the surface of the biofilm.

After the publication of > 40 microbial genomes, Galpern, *et al.* (2001) searched the then available databases for readable genes containing sequences expected to encode conserved domains analogous to known prokaryotic two-component signal transduction systems (i.e., predicted to provide for proteins of similar structure and enzymatic activity). One pair, the GGDEF domains (with a Glycine-Glycine-Aspartine-Glutamine-Phenylalanine motif conserved at about positions 114-118 in domains of about 180 amino residues) and the EAL domains (Glutamine-Alanine-Leucine motifs at positions ~ 29-31 of ~240, Romling, *et al.*, 2005, and Galperin, *et al.*, 2001) were deemed "still poorly characterized domains," but a few studies had implicated examples of the former (GGDEF) as a diguanylate cyclase, with capacity to catalyze the reaction of two guanine monophosphate (GMP) molecules to form a cyclic dimer (c-diGMP) implicated in the synthesis and extracellular export of cellulose in an *Acetobacter* species (since renamed *Glucoacetobacter xylinum*). The EAL domain had been found in a protein that

influenced virulence in one organism (*Bordatella pertussis*) and was also found in association with cellulose metabolism in *xylinum* where it was identified as a putative phosphodiesterase, which might cleave c-diGMP. The authors hypothesize that the GGDEF and EAL domains must represent a two-component signal-transduction system acting on c-diGMP as a secondary messenger and that, due to its presence in many species and in multiple copies/species (e.g., *E. coli* contains 19 sequences coding for GGDEF, 17 for EAL¹¹), must be an important system.

Simm, *et al.* (2004) note a huge expansion of discoveries of genomes (now numbering 93 available) containing many sequences likely to code for GGDEF domains (691 found) and EAL domains (503). The authors, introducing plasmids into a mutant serovar of *Salmonella enterica*, proved (pretty convincingly) that presence of each of these two domains were both sufficient and necessary for competitively producing or degrading the c-diGMP dimer in this serovar. Moreover, the authors showed that the expression of GGDEF *vs.* EAL proteins led to opposite phenotypic changes, with GGDEF inducing EPS production, adhesion and clumping behavior, and loss of motility. Finally, the authors showed that introduction of their plasmids (expressing competence for the production of either GGDEF- or EAL-domain proteins) elicited similar responses in a nosocomial *Pseudomonad* strain and non-pathogenic *E. coli*.

Over 2200 proteins have now been found that (putatively, by sequence) contain GGDEF and/or EAL domains. Such proteins (those that have been isolated and characterized) have been found to contain a sensory domain that, by binding to some environmentally relevant molecule, induces action of the GGDEF and/or EAL domain to affect the relative intracellular

¹¹ Genomic sequencing of enterococcal species has lagged behind that of *E. coli* by many years. I do note, however, that at least one putative c-diGMP phosphodiesterase, identified by its predicted EAL domain, has recently been entered into the UniProt database. (Palmer, *et al.*, 2010, and U1DZU2 – U1DZU2_ENTGA, 2013)

concentrations of c-diGMP or its monomers (proteins have been found that contain both GGDEF and EAL domains, and can differentially induce those domains depending on the binding status of the sensory domain). Many of these proteins have not yet been isolated, so the included sensory domains remain largely uncharacterized. Differential actions of such systems in response to oxygen, pH, temperature, nutrient status, and light, however, have been established. The actions of such systems on cellular concentrations of c-diGMP *vs.* its components is well established. Upregulation of GGDEF domains is found to increase cell-cell communications, fimbrial (attachment structures) and EPS production, biofilm formation and conversion to sessile phenotypes. Increased EAL action induces production of motility structures and increased growth-based competitiveness (Romling, *et al.*, 2005).

No evidence of c-diGMP modulation activity (leading to seed formation), or of preferential seed rupture, in response to rewetting of film-bound FIB is found in this review of the literature (and there's no evidence that anybody has ever looked). Lack of evidence, however, does not allow for exclusion of seeding dispersal as a possible mechanism of FIB release on terrestrial surfaces in response to a rain event; most of the (many) relevant proteins in the *E. coli* genomic arsenal have yet to be isolated, much less examined for their sensory-domain specificity, and most of the (potential) enterococcal domains have yet to be searched for. If seeding dispersal is the exclusive mechanism for FIB mobilization in the face of a rain event, one would expect release of strictly unattached (to particles or EPS) FIB of strictly planktonic phenotype (and, in the case of *E. coli*, strictly unicellular and motile cells). Such a hypothesis would be easily testable (at least for *E. coli*) by "maceration" (application of shearing forces, e.g., by a blender) calibrated (to the studied system) to maximize disaggregation of any potential cellular flocs or clusters without increasing loss of cell culturability. In seeding dispersal, at least

for *E. coli* (recall that planktonic Enterococci may exist in the multicellular “string of pearls” morphology), macerated runoff samples would show no significant increase in CFU density compared to unmacerated samples. Note that an assumption of seeding dispersal would not dictate an assumption of “first-flush” behavior (where contaminants are quickly flushed from and exhausted from surfaces by rain onset). In fact, an assumption that FIB are released from the biofilm matrix to the extent that it is wetted (as in this mechanism) would logically lead to expectations that CFU released from that matrix would increase as the wetting front proceeded through the matrix at least (with possible lags afterwards, in the case of seed formation) until the entire biofilm matrix is saturated; macerated samples (analyzed for densities of either FIB taxon) of runoff resulting from seeding dispersal should be responsive to rain depth.

Biofilm retention of FIB on the landscape, released by this mechanism or any other discussed here, also raises the question as to whether FIB defecated since the last rain are exhausted by the current one.

2.2.4.4.2 Cell Division

Like seeding dispersal, the effect of cell division on release of cells (called “erosion” by some reviewers, e.g., Kaplan, 2009) is deemed “active” dispersal, in that cell behavior directly and mechanistically induces release. Unlike the previously discussed mechanism, however, this one is rather simple and straightforward as biofilm processes go. Further, this mechanism has been found relevant to the landscape systems under study here (one example at Roberson and Firestone, 1992, discussed above). Upon wetting, cells at the surface of biofilms (which may, of course, include the walls of water-filled pores in complexly structured biofilms) revert to growth-/reproductive-competent metabolism. The act of cell division at the surface has been seen to

physically push a daughter cell into the overlying water. It is not known whether the evicted cells remain floc-competent (e.g., for *E. coli*, AG43 expression persists), but chemostat studies of *E. coli* (Allison, *et al.*, 1990), *Pseudomonas aeruginosa* and a *Vibrio* strain (see Gilbert, *et al.*, 1993) suggest that cells released under this mechanism lose capacity to attach, as well as to produce EPS. The review by Gilbert *et al.* (1993, and which cites the work of Allison *et al.*, 1990) further finds evidence that, at least in the cases of *E. coli* and *Pseudomonads*, that the apparent loss of attachment capacity of the released daughter cells is not a function of general biomass-growth rate in the chemostats, but specifically the frequency of cell-division events. In either case, the rate of cell release, so long as the surface of the biofilm remains wetted, should depend only on the number of wetted surficial cells and their doubling times under extant wetted conditions. Once the entire biofilm surface is wetted (creating the potential for a brief initial lag based on rain depth), the rate of total-cell release from a surface should remain constant until the surface dries. Macerated samples (to remove any effects of possible flocculation subsequent to release of floc-competent cells) may well show a cell-density (CFU/volume) response to (diluting) rain intensity over time but total cell release (density x intensity) should not increase with either time or accumulated rain depth.

2.2.4.4.3 Sloughing

“Sloughing” is one of the longer studied and better-understood mechanisms of cell dispersal from biofilms. Sloughing has been found to be relevant to the terrestrial landscapes in focus here (though, like just about every topic discussed in this review, the bulk of *in situ* studies are performed in aquatic environments). Unlike the previously discussed mechanisms of dispersal, sloughing is generally deemed “passive.” Though cell density in the biofilm is

important in any examination of the environmental effects of dispersal here, the mechanism itself depends only on hydration status of the EPS and on the physical response of that EPS to shearing forces imposed by the overlying water (the latter of which would obviously include raindrop impacts on terrestrial source areas).

Rewetting of dried EPS leads to their re-hydration, and a swelling of the biofilm (reversing previous shrinking during drying). This swelling effectively dilutes the EPS (especially the exopolysaccharides that are the presumed dominant contributors to structural integrity of biofilms) and decreases the number of effective nonspecific binding sites between EPS moieties, thus reducing biofilm resistance to physical disruption (i.e., the gel becomes more liquid). Rewetting dried EPS by rain on a terrestrial landscape involves a contemporaneous increase in disruptive forces (from both overland flow and impacting rain, and based on reviews by Hall-Stoodley, *et al.*, 2004, and Flemming and Wingender, 2010).

Sloughing, as the exclusive mechanism of the dispersal of FIB from terrestrial stormwater sources areas would logically imply a possibility that the release of FIB subject to rain would respond to both rain depth and to rain intensity. Sloughing should be expected to increase cell dispersal in response to rain depth, as the wetting front advances through the biofilm, sequentially converting more and more (cell-containing) biofilm volume into a less shear-resistant structure (at least until the biofilm is saturated to full depth). At the same time, the increasingly shear-sensitive (and cell containing) biofilm is subjected to (both runoff-shear and raindrop-impact) generated physical stresses that are dependent on rain intensity.

Note that assumption of any or all of these biofilm mechanisms as significant contributors to the rain-induced release of FIB from stormwater source areas implies an

underlying assumption that all or most of the FIB in those sources areas were associated with biofilms in the first place.

2.3 Literature Review and Need for Further Research

The current regulatory regime for microbiological quality of environmental waters (as highlighted at section 2.1, above, of this review) arose from knowledge that sewage-borne bacterial pathogens, when ingested, inhaled, contacted, and/or otherwise introduced to a suitable host had the capacity to cause infectious diseases in humans. This purely etiological knowledge prompted efforts to preclude entry of sewage-borne pathogens into waters likely to provide exposure. Preventing ingestion of the offending bacteria is a logical way to prevent the diseases they cause. Technological challenges in detection and enumeration of those sewage-borne pathogens, however, led to regulatory regimes in which compliance was measured on the basis of fecal indicator bacteria (FIB), indicators of sewage contamination and assumed to indicate the likely presence of sewage-borne pathogens. Any etiological (causative) link between an indicator and disease incidence logically depends on the extent to which the chosen indicator adheres to the indicator paradigm, which posits that indicators positively correlate to pathogens upon release to the environment and that indicators and pathogens “behave” (survive and move) similarly subsequent to release. Failures of a chosen indicator to comply with assumptions implicit in the indicator paradigm present a possible misapplication of risk, and a potential bias in any study comparing the presence of the indicator to the incidence of diseases.

Not surprisingly, no “perfect” indicator exists. Though there has been a historic trend (as analytical technologies advanced) toward indicators exhibiting greater and greater specificity to the (originally) presumed primary source of waterborne bacterial human pathogens (sewage),

currently used indicators still derive from a multitude of non-sewage sources. Moreover, discoveries of human pathogens of other than bacteriological etiology (e.g., viruses and protists) has led to interest, by regulatory authorities, in managing potential pathogens with environmental survival and transport patterns divergent from those of FIB. Finally, discovery of zoonotic organisms, with the capacity to cause human disease but to derive from non-human sources, has forced authorities to consider fecal sources other than human as potential causes of human disease. All of these factors represent violations of the original indicator-paradigm construction, and inclusion of these factors in any regulatory scheme that makes use of indicators selected under that paradigm introduces potential bias. Continued use of the default regulatory FIB derived on the basis of the original paradigm results in potential under-protection from sources of known risk (highlighted at section 2.2.1 of this review) and possibly misguided protection from sources of unknown risk (2.2.2).

Of necessity (not unlike the need to rely on FIB in the first place), regulators have turned to epidemiological studies (studies of disease patterns, with or without etiological assumptions) as the primary tool to determine appropriate microbiological quality standards for environmental waters. Review of such studies (2.2.3) allows for a clear division of the results of such studies, based on the extent to which etiological assumptions deriving from the indicator paradigm can be applied. Most of the (expensive and few) large epidemiological studies have been performed in recreational waters with tributary sources of (treated and/or untreated) human sewage and, thus, include some etiological linkage in epidemiological patterns found correlating FIB densities in waters to infectious diseases in bathers. These studies provide for confident estimates of the risk presented to bathers in such waters containing FIB, and are the basis of FIB limits in bathing waters that present acceptable risk. The (even fewer) studies providing information by which FIB

of non-human origin (or of environmentally persistent FIB of any ultimate source) could possibly be correlated with infectious human health risk (despite frequent exceedances of FIB-based extant guidelines) are, at best, inconclusive. While subject-matter expert consensuses of a lesser etiological risk presented by FIB from non-human (*vs.* human) feces are continually reported (as recently as Bartram, 2012, and Suresh, *et al.*, 2012), the differential results of these two groups of epidemiological studies do not, and probably cannot with current state of knowledge and regulatory structures, provide for unequivocal conclusions of differential risk to bathers (see section 2.2.3.2, above). These results do, however, highlight the need for inclusion of FIB deriving from specifically non-human source, which have demonstrably led to exceedances of regulatory guidelines, in any responsible compliance program.

While control of FIB of non-human origin would seem important to microbiological quality compliance, the methods and strategies for effective control of these often diffuse and episodic sources differ greatly from those appropriate for control of sewage discharges. Multiple studies reviewed here, in which attempts have been made to apportion FIB according to sources, illustrate the importance of wet-weather flows and stormwater in the delivery of FIB of non-human origin to water bodies. Attempts to treat, and/or disinfect such huge and intermittent discharges are infeasible or prohibitively expensive¹². Remediation of non-human FIB is often best accomplished by methods that enhance infiltration, settlement or sedimentation within the watershed, or otherwise inhibit transport across the landscape (Kay, *et al.*, 2012, and see Clary, *et al.*, 2010). Entities charged with responsibilities for microbiological compliance of waters must account for and control FIB of non-sewage origins. They must be able to apportion and

¹² Wholesale disinfection of runoff could also create potentially significant unintended ecological consequences by elimination of the landscape transport of non-FIB biota, and widespread dissemination of disinfection byproducts

locate, by human/non-human source and by likelihood to affect water bodies (especially *via* runoff), FIB within watersheds in order to prioritize and select appropriate remediation strategies.

While the source of FIB has no real effect on limits of regulation, it certainly matters to watershed managers responsible for managing compliance with those limits. Much research (as presented here) has been focused on attributing the FIB found in the monitoring of waters to the (host) source from which they derive. In a historical sense, the results of such efforts mimic the differential results found in epidemiological studies. Untreated-sewage point sources often originally dominated as tributary sources of FIB to non-compliant monitored waters. In exercises like attempts at compressing a water balloon, control of the system at one point often led to emergence of problems at another. Better treatment of sewage resulted in an increase of the relative importance of sewerage failures, overflows/leakages/inappropriate connections from sewage-containing systems to the less regulated environment, as sources of FIB to monitored waters and to non-compliance therein. Sewerage improvements, in turn, led to an increase in the relative importance of non-human (often diffuse) sources of FIB in (still extant) non-compliance. In a source-apportionment sense, parallels also exist. Studies have repeatedly shown that, with removal of human sources (either by comparison to reference systems or by GIS subtraction of human sources), non-human sources of FIB, regardless of the risk they present, will contribute to regulatory non-compliance in many monitored waters. Any strategy relying on only sewage controls (improved sewage treatment and/or sewerage infrastructure improvements) is likely insufficient to assure compliance due at least to landscape bacteria of non-human sources.

The needed apportionment of FIB among sources is complicated by the default choice of the FIB themselves in the WQC (EPA, 2012). *E. coli* and Enterococci are inherently ill-suited to

source-apportionment due to lack of specificity for sources likely to be found in relevant watersheds. Water managers are tasked with the responsibility to achieve compliance to standards based on FIB, the enumeration of which fails to provide the information required to plan/design compliance programs. Source-tracking methods, used in conjunction with regular FIB monitoring, would seem required. EPA (2012, at Section 6.1) does offer (and encourages use of) additional tools (other than FIB monitoring) by which water-quality managers can gather site-specific information concerning the sources of FIB. The first of these tools, namely the sanitary survey of tributary watersheds, serves to catalog and locate potential FIB sources, sanitary or otherwise, in catchments tributary to water bodies, along with frequent observations of environmental conditions that may affect the likelihood that found sources will affect monitored water bodies. The sanitary survey, though often time/labor intensive, requires little in the way of the expensive analytical infrastructure or specialized technical expertise called for in application of molecular MST approaches. The sanitary survey must logically be considered a type of (as generally termed here) geographical source-tracking technique (which has proven to demonstrate the importance of non-human FIB sources in watersheds to the frequency of non-compliance in monitored waters, and the importance in rainfall in effecting the episodic non-compliance they can cause, see section 2.2.3.3.1, above).

The source of FIB certainly matters, and some sort of source tracking, to at least allow an apportionment between sewage and non-sewage sources, would seem necessary for compliance planning in basins with significant non-human sources of FIB. Such planning, however, would also seem to require some measure of the actual impact such sources have on the receiving waters. EPA (2012, section 6.1.1) extols the sanitary survey, in combination with regular FIB monitoring, as a source of information by which the magnitude of impacts of sources found in

the survey might be modeled, accounting for environmental conditions (section 6.1.2). EPA deems this combination of identification/location of FIB sources and conditions that might episodically affect delivery of those FIB to regulated waters important in the prioritization of targets for, and the strategies for remediation of, sources important to compliance planning. This review (especially see section 2.2.3.3.2) reveals that enumeration of FIB deposited on the landscape, even with the source of those FIB identified, is insufficient without knowledge of what happens to those FIB subsequent to deposition. EPA believes that any modeling of processes by which deposited FIB actually enter water must account for site-specific conditions (including weather, climate, seasonality) likely to affect such processes. The Technical Support Material, promised by EPA (2012, section 6.1.2) and providing guidance on the structure of modeling efforts required for such an approach, appears to be still forthcoming.

Retention of *E. coli* and Enterococci as default (WQC) FIB in the regulation of recreational waters (and, by extension through TDMLs, to tributary sources), together with the explicit presumption of equal risk to bathers presented by all FIB (regardless of source), would appear to render any consideration of differential risk presented by different host sources of FIB moot, at least considering the current state of knowledge¹³ (EPA, 2012). Even neglecting potential for risk differential, however, water managers are faced with a dilemma regarding compliance. That dilemma derives from a knowledge gap between what FIB sources can contribute to noncompliance and the significance of what such sources actually do contribute to noncompliance. That gap, in turn, derives from (at least in part) our lack of knowledge as to what happens to FIB between their deposition on the landscape and their transport to water.

¹³ EPA, 2012 does provide for site-specific, scientifically defensible (to be approved by EPA) alternative WQS (as opposed to default WQC) on the basis of epidemiological studies or Quantitative Microbial Risk Assessments (the latter involving exhaustive quantification of chains of potential-source defecation/fecal-source infectiousness to humans/likely delivery to humans).

Identification of the important parameters affecting the survival of FIB in, and the transport of FIB from, stormwater source areas would (together with source identification by sanitary survey) allow for the site-specific modeling of source-specific impacts needed by managers to prioritize compliance actions.

CHAPTER 3

HYPOTHESES AND EXPERIMENTAL PROCEDURES

The overarching goal and focus of this research is to provide water managers with tools to prioritize mitigation strategies for compliance with existing regulations pertaining to FIB (fecal indicator bacteria). The literature reviewed here reveals that such tools should include some methods by which water managers could derive some expectations as to the likely effects of natural-background (of other than sewage origin and, often, of unsewered transport) regulated FIB deposited on the landscape within their jurisdictions and mobilized to receiving waters by rainfall. Convenience to managers would be enhanced if the use of such tools only required expertise in, and performance of, monitoring and survey activities already expected under existing regulations.

A model of the processes by which FIB defecated on stormwater source-areas survive until they are mobilized by subsequent rainfall and by which they are released from those source areas for transport to receiving waters, and the (transport-relevant) physical form in which they are released would seem useful. Such a model would provide managers with a mechanistic (currently missing) link between survey observations and monitoring results. It would allow for causal separation of varying monitoring results into components of differential survival of FIB (e.g., seasonal temperature or local water-retention characteristics) and of differential observed defecation of FIB (e.g., temporal bird migrations, or the geography of pet walking). It would allow for predictability of regulation-relevant watershed responses to observed defecation rates,

and provide information important to mitigation planning. Once natural-background patterns of runoff were established for a given locale, unexpected increases in monitored FIB densities could be viewed as a “hotspot,” where search for an unintended sewage release would likely prove more useful than would an increase in resources towards stormwater-focused mitigation (though the work of Shergill, 2004, reviewed at 2.2.3.3.1 indicates that such a “spot” might need to be pretty hot to unambiguously reveal itself).

While the literature reviewed here provides ample evidence of the need for some sort of model to link observations of landscape FIB deposition to their effects downstream, it also provides little information concerning how such a model might be constructed. The literature also provides evidence that construction of a useful model might be dauntingly complex, if feasible at all.

My plan here was to provide exploratory research, piecewise, into model components suggested in the literature, in a series of scoping studies. Those studies are designed to test for the appropriateness of those suggested components for inclusion in model construction and to provide guidance for needed further research.

3.1 Research Design

3.1.1 Environmental Survival

The numerous examples of FIB persistence on the landscape (see section 2.2.2), and the seasonal first-flush phenomenon in regions with distinct wet/dry seasons (2.2.3.3.1) suggest the need to include interevent (between rains) survival of FIB as a component in construction of any model (see generalization at Figure 1).

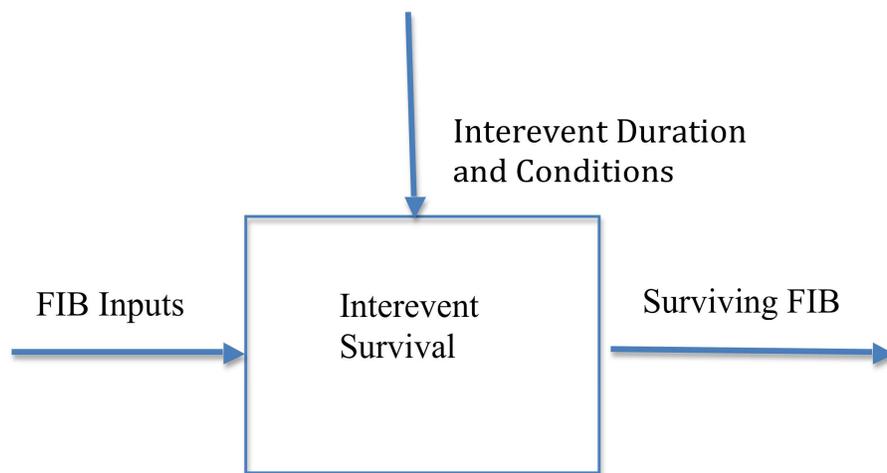


Figure 1 Generalized Interevent Block,

The reviewed literature (at 2.2.4.3, inclusive) provides support for a hypothesis that the interevent (over time, between rains) survival of FIB in stormwater source areas is significantly dependent on five factors, namely:

- 1.) The nature of the organism itself (either *Escherichia coli*, or *Enterococcus*, spp.),
- 2.) The nature of the unsaturated porous medium (across a broad range of potential structures and textures including impervious pavements of varying porosities and hydrophobicities and soils of various compositions and porosities),
- 3.) Absolute temperature (over a biologically and environmentally relevant range),
- 4.) Water activity (primarily driven, through potentially complex series equilibria interactively influenced by the nature of the unsaturated porous medium, by RH% of the overlying atmosphere), and
- 5.) UV exposure (expected to include likely interactions with both other significant survival factors and other insolation bands).

The literature also provides little certainty over the likely significance of two other factors.

Environmental pH, and the availability organic nutrients are important survival factors. FIB, however, are deposited onto the landscape in feces, a biofilm matrix rich in embedded organics and soluble species. In the face of this uncertainty, the potential significance of the landscape presence of these substances, at least pervious surfaces required checking.

Moreover, these factors cannot generally be expected to linearly influence cell survival. This situation would logically best be modeled by a full factorial study (see Box, *et al.*, 2005, esp. Chapter 5) of seven dimensions and multiple levels for each effector, a daunting task. Each sampling event, over time corresponding to interevent duration, would require analysis of multiple samples, numbering (3?, 4?) multiple levels of exposure for each factor to capture nonlinearities of influence raised to the power of seven, and would far exceed incubator space available to this (or, likely, any) researcher.

Thankfully, one of the hypothetically significant factors under study can be seen as strictly binary (either *E. coli* or Enterococci). Another, temperature, over the limited range corresponding to an overlap between biologically relevant (from minimal growth to optimum growth) and environmentally relevant temperatures, has been found to be approximately linear in influence on cell survival (implying that interpolation between two levels would provide quantitatively useful modeling information, 2.2.4.3.2). Water activity, is expected to exhibit monotonic influence over cell survival, which would imply that two levels of study should be sufficient for at least establishing the significance of its influence. Due to likely interactive thresholds imposed by microenvironmental factors, however, it may only provide qualitatively useful (interpolated) modeling information. Matric and osmotic components of thermodynamic “activity” have potential, at varying scales, to affect everything from availability of liquid environmental water, water and nutrient transfer into or out of cells, and the vital stability of intracellular structures (2.2.4.3.4). Finally, some factors (e.g., UV exposure and surface) are expected to exhibit their influence so rife with interactions (with other hypothesized growth factors and, in the case of UV, other insolation bands) that only a binary consideration (at

relevant endpoints) would provide a meaningful measure of significance, and would provide the most useful measure of (dominantly interactive) effects on cell survival (2.2.4.3.70).

I reduced the scope of this exploratory research to an analysis of two levels of each factor:

- 1.) Organism – Either *E. coli* or Enterococci (an inherently binary factor);
- 2.) Surface – Either impermeable pavement (concrete) or porous (sandy) soil, expected to span the gamut of (at least the porosity-based) likely interactions with water activity (and also, the gamut of likely influence on biofilm formation, see below);
- 3.) Temperature – Examined by endpoints of low (near minimum growth, but above freezing) or high (near cardinal optimum of growth), and which is expected to provide useful modeling-relevant information by interpolation;
- 4.) RH% - Examined by endpoints of low or high, encompassing most of the range expected in temperate climes, and which may provide useful modeling-relevant information by interpolation;
- 5.) UV – Either present (artificial full-spectrum “sunshine”) or absent (the same spectrum with UV selectively filtered out);
- 6.) pH – Either neutral or acidic on pervious surface (adjusted with added dilute distilled white vinegar and/or dilute baking soda; and
- 7.) Nutrients – Either present (in the form of added bioavailable sugars from dilute molasses) or “absent” (with at least volatiles removed by heating) on the sandy-soil matrix.

This reduced-scope system (full 2^7 factorial = 128 analyses per sampling event) is still daunting in terms of required incubator space, especially if any experimental replication is considered. It was, however, expected to preserve, at least, an ability to determine the significance of any factor and all possible interactions (in contrast to a fractional factorial design that may overlook important information).

Further reductions in analytical logjams were accomplished by breaking the study into more digestible bites. I analyzed the survival of FIB in source areas as a collection of ($2^2 = 4$) separate factorial studies. The separate studies were divided on the basis of organism and source-

area surface. Analytical methods for enumerating the two FIB taxa under study here (unsurprisingly) differ (e.g., see Enterolert™ and Colilert™, both undated). While the testable significance of taxon as a factor was obscured by the separation of treatments, such separation allows for modeling more appropriate to actual use. A permeable-/impermeable-surface separation should be seen more as a convenience for this research, and its testable significance would likewise be obscured, but it also allowed for the needed check for significance of extant pH and nutrients on pervious surfaces.

Finally, I examined the likely effect (which can be alternatively viewed as a shift in life-cycle stage, or as a change in carrying capacity, see 2.2.4.2) of defecation from the colon (see section 2.2.4.3.1) on the subsequent landscape survival of FIB. For each treatment, net cell survival over time (Interevent Survival, in Figure 1) was modeled as a log-linear regression with unknown breakpoints (by maximum-likelihood methods). This model form was suggested by standard textbook models of microbial population dynamics (2.2.2.4.2). Testing the adequacy of this model was assumed to be testable by residuals analysis, and assumed that any required alterations to the model would be revealed by the residual behavior. Those assumptions (especially the latter) were found faulty in the heavily censored datasets encountered in the pervious-surface studies, and are discussed in the Results and Discussion section below (4.1.2).

Both the time at which any breakpoint was evident, and the intervening slopes (over time) between found breakpoints, were examined for significance of factors and their interactions in four separate full-factorial analyses:

- *E. coli* on impervious surface and subjected to two levels of each of three factors (temperature, RH%, and UV exposure, the last represented as a presence/absence factor with non-UV bands of full-spectrum insolation applied to both levels);
- Enterococci on impervious surface and subject to two levels of each of three factors (temperature, RH%, and presence/absence of UV exposure);

- *E. coli* on pervious surface and subject to two levels of each of five factors (temperature, RH%, presence/absence of UV exposure, presence/absence of organics, and acid/neutral pH); and
- Enterococci on pervious surface and subject to two levels of each of five factors (temperature, RH%, presence/absence of UV exposure, presence/absence of organics, and acid/neutral pH);

Each of these analyses was judged against a hypothesis that temperature, RH% and UV exposure are all (independently or in interaction) significant factors affecting FIB survival.

3.1.2 Washoff

The ambiguity of findings of FIB first-flush behavior, and the absence of any signs that even long, heavy rains reveal source limitation of surficial FIB (Sections 2.2.2 and 2.2.3.3.1) both suggest likely importance of a component concerning the mechanisms by which rainfall mobilizes extant FIB to stormwater in any modeling effort (illustrated, in general fashion, at Figure 2).

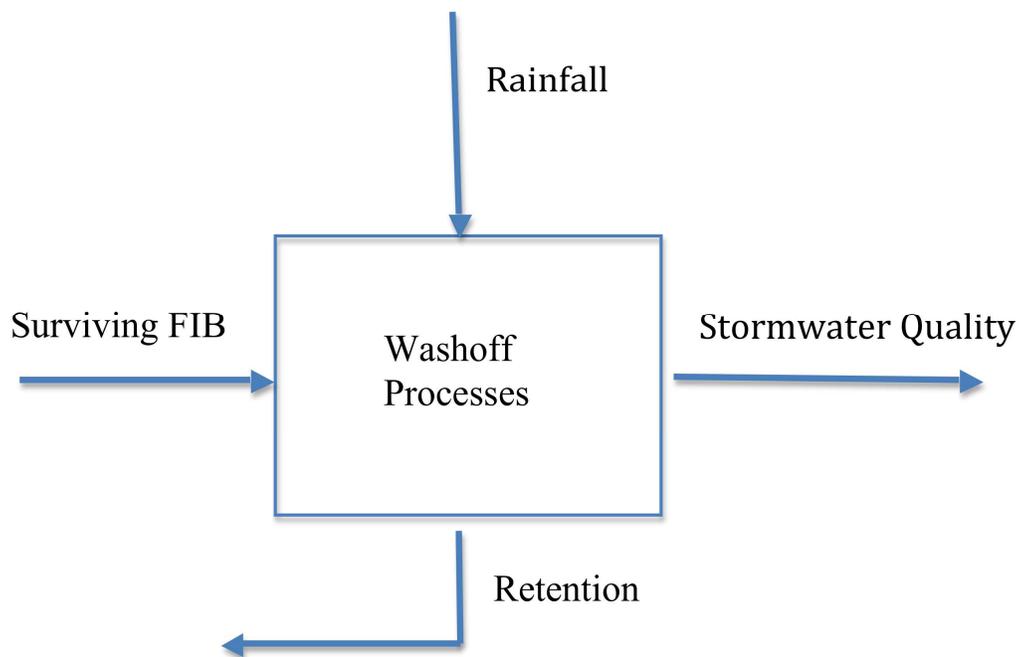


Figure 2 Generalized Washoff Block.

Section 2.2.4.4, inclusive, provides considerable evidence that washoff processes are dominantly biofilm dispersal mechanisms, and includes three such mechanisms that *could* operate on terrestrial environments, namely:

- 1.) Seeding Dispersal
- 2.) Surficial Cell Division (or erosion), and
- 3.) Sloughing.

Literature reviewed here, however, provides no useful information concerning which (or, indeed, if any) of these mechanisms are significant contributors to the release of FIB from rain-wetted terrestrial surfaces. In the absence of definitive information in the reviewed literature, I started with some assumptions and then proceeded to test the logical outcomes arising from those assumptions.

The first such assumption is:

Assumption A – Prior to a rain, FIB are *present* on the landscape in predominantly the filmbound (either to particle or to the gross-surface) state.

This assumption, well supported in the literature, leads to a logical conclusion that:

FIB are *released* from source areas, in response to wetting by rain, by one or more of the biofilm-dispersal mechanisms identified above.

Assumption A is probably not directly testable at landscape scales. Any feasible microscopic examination capable of discerning the state of source-area FIB would likely leave much of any source area unexamined. A finding that the state of FIB released from a source area, however, failed to conform to the state expected from *any* of the identified dispersal mechanisms would obviously be cause for re-examination of Assumption A.

Any testing of all possibilities allowable under the logical conclusion arising from Assumption A remains dauntingly complex. Some simplifying organizational structure for such testing can be imposed by my Assumption B, below:

Assumption B – Most FIB released in response to rain are “sloughed” as passively dispersed aggregates (flocs or filmbound particles).

Unlike A above, this assumption is generally *not* supported by the reviewed literature. This assumption could be viewed as an “educated guess,” in that (in light of the implied underlying assumption that most landscape FIB are biofilm-bound) sloughing *has* been found to be operational in environmentally relevant systems and would seem likely to release greater numbers of FIB than would erosion (which also *has* been found relevant here). That guess, however, would seem barely distinguishable from wild speculation. The reason I proceeded under this assumption is that sequential testing/confirmation of hypotheses that logically arise from it could allow for specific rejection of alternative biofilm-based mechanistic assumptions, and could provide confirmation of the underlying (A) assumption of ubiquitous environmental biofilms.

Assumption B, of the significance of sloughing, implies that released FIB in stormwater are generally embedded in shear- and/or impact-disrupted biofilm fragments, possibly multicellular CFU, and possibly responsive to both rain depth and rain intensity. Assumption B also logically leads to a conclusion that:

- 1.) FIB are not released solely by seeding dispersal, and
- 2.) FIB are not released solely by cell-division/erosion.

If (but decidedly not only if) FIB were not filmbound prior to a rain (filmbound was assumed in A), they would be released, in response to rain, as unattached cells of fully

planktonic form. Unattached, to either particles or surfaces, bacterial cells (of specific gravity very nearly equal to unity) would be expected to reveal themselves through rapid flushing from the landscape in response to rain. Recalling (Section 2.4.1.1.2) that planktonic *E. coli* generally exist in unicellular (sometimes paired) morphology, such a violation of Assumption A would also result in a finding that disaggregation/maceration of a subsample did not materially elevate (and would no more than double) *E. coli* CFU density above that of a split, raw (not macerated) subsample. Finally, the unattached nature *and* the planktonic morphology (single-cell or in pairs) of *E. coli*, unexpected under the assumption, provide another testable endpoint. The size of planktonic *E. coli* (Section 2.4.1.1.2) implies that single or paired cells would all pass through a 10-micron filter and all be retained on a 0.45-micron filter, *and* that such a filtered fraction would not experience a meaningful (more than 2 x) elevation of CFU density for that taxon under maceration. Whether filmbound particles or cell-containing biofilm flocs (the latter including disaggregated fecal material), such elevation under maceration implies cells of sessile morphology amongst the CFU released.

It should be noted here that though testing of these expected outcomes may confirm the validity of Assumption A, such testing alone cannot logically lead to its rejection. Such a rejection of A could only be justified by exhaustive rejection of all three identified biofilm-dispersal mechanisms (each of which can mimic one or more of the outcomes expected under an Assumption A violation). Also worthy of note, if no biofilm-dispersal mechanism(s) *were* found operational in rain-wetted source areas (*i.e.*, if Assumption A were rejected through the sequential testing of Assumption B outcomes below), the possibility of biofilm *retention* of some FIB, throughout the course of a rainstorm could also be confidently rejected. Graphically, in such a case, no retention output of Figure 2 must be tied in as supplementary FIB input at Figure 1.

If seeding dispersal were the sole biofilm-dispersal mechanism, in contradiction of Assumption B, such a contradiction should reveal itself. As in possible Assumption-A violations above, this situation should result exclusively in unattached runoff FIB of fully planktonic morphology (with no increased *E. coli* CFU in response to maceration). Unlike the above-discussed situation, however, this case should show monotonically increasing FIB densities (with possible lags and an eventual plateau) over time and/or rain depth as the biofilm is progressively wetted and seeded in a rain event. Moreover (and related), since operation of this mechanism implies active reversion of sessile bacteria to planktonic morphology in response to rain, there is no reason to even expect either a first-flush response or exhaustion of FIB over the course of a rain event (Section 2.2.4.4.1).

If (contrary to Assumption B), erosion were the sole biofilm-dispersal mechanism operating in the rain-mediated release of FIB from source areas, the situation would be expected, again, to reveal itself (Section 2.2.4.4.2). The literature suggests, but does not conclusively establish, that FIB released under this mechanism are released in planktonic form. It does, however, establish that the cell-by-cell release rate is only dependent on the number of dividing FIB cells on the wetted surface of the biofilm and on the doubling time of cells under conditions of the wetted surface. If erosion were the sole biofilm-dispersal mechanism of release, the product, of macerated FIB density multiplied by rain intensity, should remain constant throughout the period that the biofilm surface remains fully wetted.

It is these considerations, rather than any clear expectations provided in the literature, that provide a possible hypothetical framework (below) by which significance of rain-induced alternative release mechanisms might be compared. It is from a joint assumption of ubiquitous biofilms and of a specific a specific biofilm-dispersal mechanism that testable expectations

concerning releases consequent to those assumptions arise. The three modeling efforts described below were designed to serve both as scoping studies to sequentially test those hypothetical expectations, and to reveal descriptive information concerning dispersal forms and patterns of potential use in future research.

3.1.2.1 CFU release, a screen for operational mechanisms

The above analysis raises the possibility that CFU releases (“washoff processes” at Figure 2) are dependent on both accumulated rain depth *prior* to sampling and the rain intensity *at the time* of sampling. The former could be due to the rainwater influx required to fully hydrate any biofilms on the surface (rendering more film susceptible to disruption), *or* to a flush (or even complete exhaustion) of FIB-containing components on the surface. The influence of rain depth, as an independent factor, on the release of CFU depends on the number of potentially removable CFU-forming entities (either free cells or FIB containing fragments) present on the surface when a releasing event (raindrop impact or runoff shear) occurs, a number that may well change over the course of a storm. The independent influence of rain intensity would depend only on the effectiveness of (shear-/impact-related) forces exerted on extant potentially removable (susceptible) CFU. The possibility for interaction between rain depth and intensity seems apparent.

I subjected three closely collocated suburban source-area surfaces to a simulated, extended rain event. The three surfaces were a roof, a street, and a lawn in a neighborhood with considerable but spotty tree cover, abundant urban wildlife (mostly birds and squirrels), and a large domestic pet population (leash-law for dogs, though not universally observed). All three of these surfaces had previously yielded measurable densities of FIB under natural rainfall

conditions in previous monitoring projects (see Pitt, *et. al*, 2003), and were expected to again. The failure of one of these surfaces (the roof) to significantly provide measureable FIB densities in runoff necessarily requires further discussion (and is presented, in more relevant detail below in Results and Discussion, at Section 4.2.1).

The simulated rainwater applied consisted of de-chlorinated (sodium thiosulfate added) tap water. The water was delivered through three separately piped pairs of commercial drip-irrigation emitters. Drip-irrigation emitters are designed to mimic natural raindrop size and rainfall distribution patterns. The emitters chosen (RainbirdTM, SQH) were designed to each cover half of an eight-foot square (4' x 8'), from an edge of that half-square, at a nominal delivery rate of ~1/2"/hr, when supplied with water between 20 and 50 psi (see Rainbird, undated). Each pair of emitters was connected to a separate output line of a valved garden-hose manifold. The manifold was supplied by the simulated rainwater from a booster pump (garden-hose fittings) *via* a garden-hose pressure-regulator (SanningerTM, 25 psi) and flow-meter (in series) connection. The booster pump, in turn, was fed from a coiled garden hose sunk into a 30-gallon tub prefilled and dosed (with kitchen measuring spoon) with sodium thiosulfate, and illustrated in the next section below). The manifold output was also fitted with a garden-hose pressure gauge for monitoring/diagnostic purposes (Figure 3). All emitters and piping were affixed to a wooden, 8' x 8' frame, with adjustable legs for leveling on the landscape (Figure 4). This system was not expected to duplicate the kinetic effects of natural rainfall intensity; even realistically sized raindrops (generally considered range-bound ~ 1-5 mm) should not be expected to reach terminal velocity before impact from this artificial apparatus (and see Pitt, *et al.*, 2007, esp. pp. 175-178). This approach was, however, expected to provide better control of (an albeit ersatz) “intensity” for this exploratory research than would be achievable with any

natural rain. Details of construction are below, and hydraulic characterization, at each surface studied, of this “rainframe” are presented as an appendix at Section 4.2.1 (Results and Discussion).



Figure 3 Rainframe Manifold.



Figure 4 Rainframe on Lawn. (during hydraulic characterization, and before installation of tarp/canopy to provide shade to simulate cloud cover.

In an attempt to provide separable effects of wetted time, rain depth, and rain “intensity” (the last defined here by the usual depth/time ratio), I applied the simulated rainfall in the order of:

- 1.) Nominal ½” intensity for 20 minutes,
- 2.) Nominal 1” intensity for 20 minutes, and
- 3.) Nominal 1½” intensity for 20 minutes,

and I repeated the sequence three times (for a three-hour simulated heavy-rainfall event) for each surface. The timing of the initial 20-minute exposure was not begun (stopwatch was reset) until the visible onset of runoff from the surface. For each 20-minute segment of the study period, a sample was drawn at midway (10-minutes subsequent to initiation of each new intensity regime). At the initiation of each 20-minute segment of the study period, measured cumulative volumetric flow was recorded, to provide for a measure of the average intensity for each segment and cumulative depth at each sampling event. Each sample was halved, by cone splitter, to allow separate measurements of the two FIB taxa under study here. Each half was then split to allow for separate, comparative measurements of FIB densities in raw (as is) sample vs macerated sample (all density measurements by IDEXX™ methods).

Resulting datasets were screened per the following hypothetical structure:

- MPN of macerated *E. coli* CFU are significantly elevated relative to those found in the raw, unmacerated subsample. Favorable finding of this condition would imply that *E. coli* are not exclusively present in their planktonic form, but significantly present in the sessile, filmbound state. (which, again, may include sloughed fecal fragments) This finding, in turn, would allow for rejection of seeding dispersal as the sole mechanism of *E. coli* release from the landscape. Noting that planktonic *Enterococci*, spp., can exist in the multicellular, “string of pearls” morphology, such a finding for that taxon would not be as directly conclusive. A finding that most *E. coli* were present in a source area in the filmbound state, however, would provide for a strong presumption that co-located *Enterococci* were similarly filmbound.

- The product of macerated-sample CFU multiplied by the rain “intensity” at the time of sampling is not constant throughout the rain event (continuously wetted period). Favorable finding of this condition would allow for confident rejection of cell-division/erosion as the sole mechanism of FIB release from the source area.

Valid finding of both of the hypothesized conditions above would provide for a conclusion that sloughing is at least a significantly contributing mechanism to rain-induced release of FIB from source areas. Such a conclusion forces recognition that any modeling effort must account for both rain depth (possibly expressed in a manner bifurcated over time) and intensity as possible significant effectors of FIB densities in runoff. It also raises the possibility of some biofilm retention of FIB on the surface that was not completely sloughed, even in the face of this simulated heavy “rain.” This last contingency was tested for with somewhat destructive testing by use of a high-pressure (household “pressure-wash”) garden-hose nozzle at the end of each rain simulation.

This study was deemed likely to require some extended storage of samples in the field before a return to the lab for their analysis. It also required some optimization of the maceration parameters to provide maximized CFU disaggregation without causing cell death. Both of these preliminary studies are presented as appendices at section 4.2.1 (Results and Discussion, below).

3.1.2.2 CFU Form, Modeling Efforts

Assumptions implicit in the above analysis include the potential that the form of multicellular aggregates released from source-area by rain may be of flocs or filmbound mineral particles (or some combination of both). The nature of these aggregates would be of use to anyone interested in the entrainment/resettlement behavior of them downgradient of their release from biofilms. Pitt, *et al.* (2007, p. 3, and citing others not directly consulted here) inform that

median particle size distributions in stormwater are limited in span (by the inability of rain/runoff to suspend large particles). In a study of (actively disturbed) construction sites, even during “high intensity rains” (> 1 inch/hour), median particle size was found to be 8.5 microns and only 10% of particles exceeded 20 microns. While not directly addressing FIB densities in runoff, these considerations imply a need for some information concerning nature/form of aggregates released by washoff processes in this exploratory research. Even if an FIB-containing CFU is dislodged by raindrop impact, it will not reach downstream, regulated water-bodies if it is intercepted or if it resettles before reaching that water-body (see Figure 5).

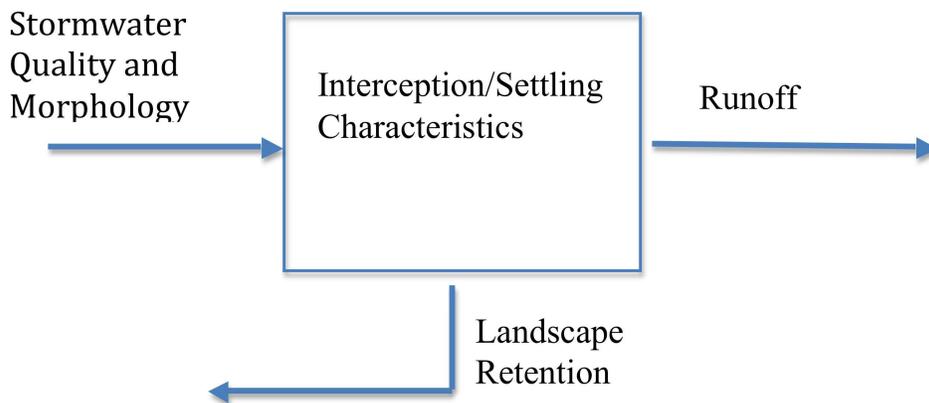


Figure 5 Post-release source-area processes.

Downgradient interception or shear disruption by landscape features is logically a function of the size and density of any FIB-containing aggregate released by wash-off processes. Whether or not any FIB-containing aggregate is or remains entrained by runoff is a function of

its settling characteristics. In light of these considerations, I modeled the form/morphology of runoff FIB CFU in two studies.

3.1.2.2.1 CFU Form, a filter cascade

This study was designed as a largely descriptive study into the three-dimensional size of FIB-containing aggregates in runoff likely to affect their likelihood of reaching regulated water-bodies. It does, however, include some element of hypothesis-testing/screening concerning mechanisms by which such aggregates enter runoff to begin with. If all *E. coli* CFU released from a source area were of planktonic form (motile and unattached, generally unicellular with some paired cells), all such CFU would be expected to pass through a 10-micron filter, and to exhibit no more than a 2 x elevation of CFU when macerated. Such discrete bacteria CFU would be readily transported to receiving waters.

Considering the focus here on continued mobilization of FIB, once released from the source-area surface (presumably by fully kinetic raindrop impact), a natural rainfall was opportunistically chosen for study. The dense leading edge of the weather-radar signature of the chosen storm provided expectations of sufficient (~ 4L) runoff, early in the event, from the lawn studied in 3.1.2.1 above (and corresponding to the surface/timing of maximum CFU densities identified in that study, see section 4.2.1, below).

Well-stirred bulk sample was churn-split to subsamples providing for:

- 1.) Whole (unfiltered) oven-dried solids determination,
- 2.) Whole, raw (not macerated) FIB-density determinations (both taxa, with dilutions to target the three-order reading window available to IDEXX enumeration), and
- 3.) Whole, macerated FIB-density determinations.

Remaining bulk water sample was then sequentially poured (or, when necessary, suction-filtered) through:

- 1.) A 250-micron screen, with the retentate reserved for solids determination; and with the remaining filtrate subject to a
- 2.) 106-micron screen, segregated as above; and with the remaining filtrate to a
- 3.) 45-micron filter. Retentate from this separation step was reserved for solids determination as directly above. Filtrate from this separation was subjected to subsampling as in the “whole” analysis (above, to provide for a +45-micron FIB determination) and the remainder poured to a
- 4.) 20-micron, segregated as in step 3 directly above, and sequentially to a
- 5.) 10-micron filter, then a
- 6.) 5-micron filter and, finally, a
- 7.) 0.45-micron filter.

FIB analysis of the filtrate of the 0.45-micron filter is added as a diagnostic control. Any finding of measurable FIB (especially of *E. coli*) in this fraction would indicate some sort of failure in my sterile technique. Relatedly, this overall sequence (albeit contorted and complex) was necessary to assure sufficient pre-sterilized sample splitters and filtrate-receiving vessels through its entire course.

All results from this scoping study (and like all such studies discussed here) are presented (section 4.2.2.1) for the potentially useful information important to further research. The data set arising here is only tested against the hypotheses that:

- Not all measurable *E. coli* CFU reach the 10-micron filtrate fraction in this sequential cascade (violation of which would imply that such CFU were in fully planktonic morphology) and
- No measurable *E. coli* reach the 0.45-micron filtrate fraction (violation of which would imply some sort of cross-contamination between fractions).

3.1.2.2.2 CFU Form, a settling study

This study is an entirely descriptive one; no hypothetical structure is imposed. It seems important, however, to this exploratory research into the feasibility of modeling natural-background of source-area FIB in regulated waters released from source areas by rain. While the

study directly above (3.1.2.2.1) provides size-binned information of interest here (raw and macerated CFU densities and total solids), this study directly addresses settling rates of whole CFU. As in the case of size-dependent interception above, any resettlement of dense FIB-containing aggregates back to the landscape would prevent them from reaching down-gradient waters (and graphically relegating them to FIB inputs at Figure 1, for a subsequent interevent period).

A simple settling chamber was fabricated from a generic (no brand name available) 5-gallon aquarium (Figure 6). The tank was fitted with three separatory funnels to draw (by vacuum hose) fixed volume samples from three different depths with minimal disturbance. A meter stick was also affixed to measure surface draw-down over the study period.

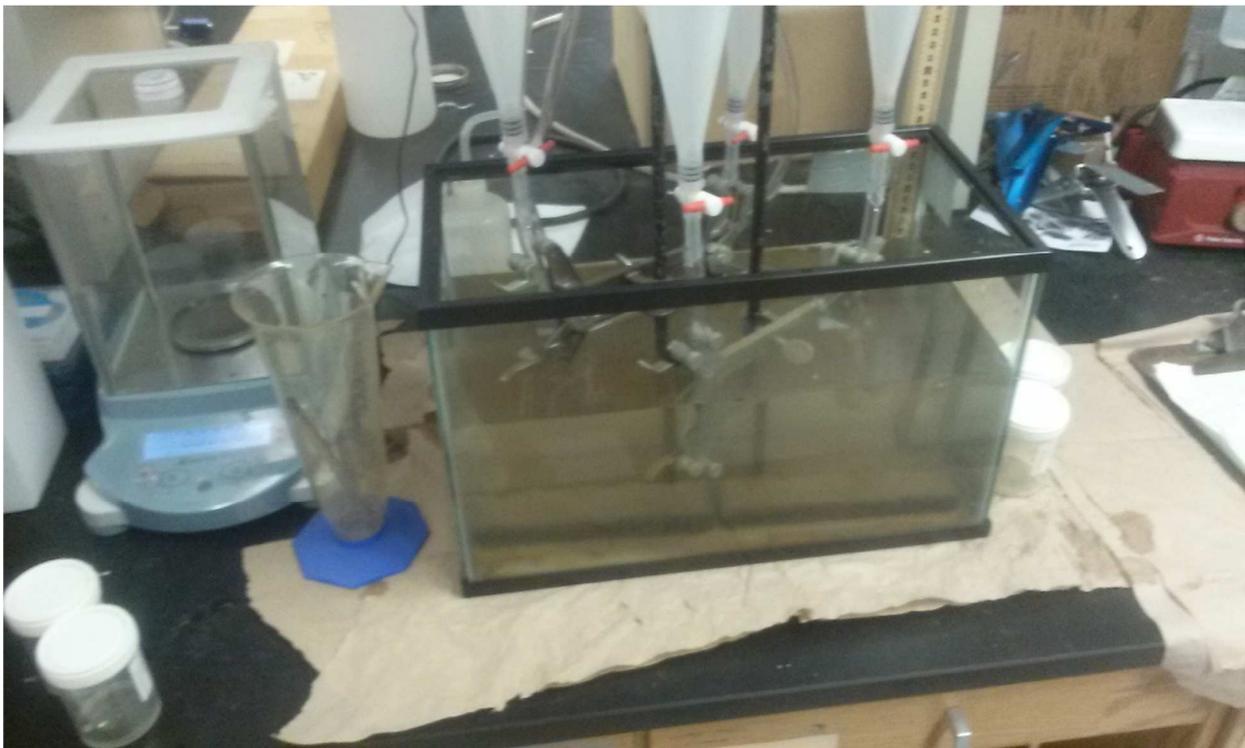


Figure 6 Settling Tank.

Runoff from a rainstorm (chosen by the same parameters as that in 3.1.2.2.1, above) was collected and diluted to fill the tank. Samples were drawn over an 8-hour study period and

analyzed for FIB densities (both taxa). Data were used to graphically construct isoconcentration lines plotted against depth vs time in the usual manner for flocculant sedimentation for pilot data (see Davis and Cornwell, 2008, pp. 278-280), and presented in section 4.2.2.2, Results and Discussion.

3.2 Experimental Plan

3.2.1 Landscape Survival of FIB

Hypothesis 1

Temperature, RH%, and UV irradiation are all (independently or interactively) significant factors affecting net survival of FIB, subsequent to deposition on and prior to rain-washoff from stormwater sources areas.

3.2.1.a Prediction 1.a

Temperature, RH%, and UV presence/absence (the last in the presence of other insolation bands) are all (independently or interactively) significantly contributing factors to net survival of both E. coli (species) and Enterococcus (genus) on impervious surfaces.

3.2.1.a.1 Research Activities

I inoculated simulated pavements (small concrete paver blocks) with slurries of pet feces (of known age, and dilution-optimized for minimization of non-detects in MPN analysis) and exposed them, over a reasonable inter-rain period, to artificially-produced differential environments (2³) of low/high temperature, low/high RH%, and presence/absence of UV exposure (both levels of the last with presence of full-spectrum visible light). Intermittently over

the study period (and including at Day 0, immediately subsequent to inoculation), simulated pavements were washed (new pavements for each time period), with physical biofilm disruption (a timed and consistently applied toothbrush scrub), and the washoff eluents were analyzed for MPN CFU density of FIB. Each sampling point (in time) for each taxon (*E. coli* or Enterococci) was replicated.

3.2.1.a.1.1 Slurry preparation

Pet-feces slurries were prepared from the feces of either of a mother-son pair (of shared genetics and shared diets, to, hopefully, reduce variability of fecal FIB) of basset hounds (Adeline and Lightnin') to which I had regular access (and the capacity to note time of defecation). Slurries were prepared using distilled water to avoid introduction of chlorine from tap water. Dilution of slurries was optimized to maximize the likelihood of samples being in the quantification range for the MPN analysis through preliminary research into FIB survival within the fecal mass (which should be considered a real, tertiary habitat for FIB). The model derived from this preliminary (method-development) research was not expected to be generally applicable to feces from other sources, but is included in an appendix of section 4.2.1, in Results and Discussion below.

3.2.1.a.1.2 Simulated Pavement

The pet-derived fecal slurries were applied (immediately, to quickly relieve any potential osmotic stress due to slurring in distilled water) onto (commercially widely available, 2" x 4" x 2", each chiseled in half for this study) cement patio-paving blocks. Such blocks are of raw, sawn-cement construction. In this study, the blocks were artificially "weathered" (by overnight

soak in dilute table-salt brine, followed by tap-water rinse, and by full air drying to off-gas chlorine) before inoculation (see Carroll, 1962).

3.2.1.a.1.3 Interevent Period

I analyzed (MPN) replicated (for each taxon, and for each environmental treatment) samples over about a two-week period. This period was expected to reasonably represent typical interevent periods (of absent rain) in temperate, humid environs and (more importantly) should encompass a sufficient time-span to show any adaptive changes by the FIB subsequent to defecation onto the landscape.

3.2.1.a.1.4 Differential Environmental Exposures

Environmental chambers were constructed from four freezerless refrigerators. All four were fitted with thermometers, humidity gauges, and UV-enhanced (designed to mimic the natural insolation spectrum) fluorescent bulbs, and divided into UV-exposed and “UV-dark” (the latter, though, still exposed to visible-light wavelengths) sectors by (translucent but UV-opaque) Lexan™ panels (with a very sharp cutoff of transmission for insolation wavelengths < 400 nm). Two chambers were fitted with (commercially available, child-care) room humidifiers and two with (commercially available, painter-supply) calcium-chloride desiccators, to create low/high RH% separation. Each of the RH%-separated groups were further subdivided into low-temperature chambers (controlled only by the refrigerator thermostat, set to and stabilized at ~ 40°F) and high-temperature chambers (controlled by thermostatically controlled, and commercially available units, BOD Cubators™, specifically sold for conversion of refrigerators into chambers appropriate for BOD analysis, set to and stabilized at ~ 90°F). This environmental-

chamber system allows for a full factorial experiment set of 2^3 conditions (temperature, RH%, UV presence/absence) of environmental exposures to inoculated simulated pavements over the study period (see Figure 7).

Warm (90 ⁰ F) Dry (~30% RH)	Warm (90 ⁰ F) Moist(~85% RH)
UV Shielded	UV Shielded
Cool (40 ⁰ F) Dry (38% RH)	Cool (40 ⁰ F) Moist(~85% RH)
UV Shielded	UV Shielded



Figure 7 Environmental Chambers. Left Panel = 2^3 factor exposures. Right Panel = Example Chamber (Warm/Moist, with BOD Cubator and humidifier installed).

3.2.1..a.1.5 Sample Collection

Over the course of the “interevent” study period, replicate pavers (for each taxon and for each set of environmental conditions) were subjected to biofilm-disruptive washoff. Most biofilm-disruption literature is focused on elimination of the nuisance presented by biofilms, and no chemical treatment was found that preserved viability of the embedded cells. I used a traditional physical biofilm-disruption method, namely a toothbrush. I applied three, one-minute each, toothbrush scrubs of consistent pressure, with intervening wash-bottle rinses of the brush and the pavers. Preliminary research into this method revealed less than total recovery of applied CFU, but a very consistent fractional recovery of the total applied (as measured from CFU

densities in defined volumes of slurry prior to inoculation). Possibly worthy of note here was the greater recovery (~80%) of Enterococci than of *E. coli* (25-30%), a phenomenon that might be attributable to toothbrush shearing of the multicellular “string of pearls” morphology in the former. All washoff eluents were collected into sterile sample bottles for MPN determination.

3.2.1.a.2 Analyses

3.2.1.a.2.1 MPN Determination

FIB density in washoff was determined by IDEXX™ methods, which use pre-mixed selective defined-substrate media containing fluorophores activated by binding to taxon-specific metabolic products. Mixtures of *E. coli* or Enterococci samples and the provided reagents, incubated (for 24 hours) in “Quanti-trays” (trademarked assemblages containing 97 separable sub-sample cells of varying volumes), provided for high-resolution determination the MPN of the targeted taxon over a limited (~ three-order, CFU/100 ml greater than or equal to 1 and less than or equal 2,419.6) range (Enterolert™, Colilert™, and IDEXX Quanti-tray/2000™, all undated).

3.2.1.a.2.2 Treatment-specific Modeling, with Breakpoint Analysis

The mechanism by which FIB reproduce (binary fission) implies that net growth of populations should be of 1st order (log-linear) form over time:

$$\log (\text{MPN}_t / \text{initial MPN}) = k \times t$$

where:

k	=	net growth constant (slope of the function), and
t	=	time (hours).

A change in growth-/death-relevant conditions (e.g., defecation to the landscape), however, may (with potential lag-times) impose a change in slope of the function as cells (by adaptation) or

populations (by selection) respond to those changes (see 2.2.4.2, above). This situation suggests that the net growth of each taxon at each set of environmental conditions would best be described by segmented log-linear regression of unknown (abscissae, time of) breakpoints, an approach that (surprisingly) is anything but straightforward. Hudson (1966) provides (and proves validity of) a graphic algorithm by which the maximum-likelihood estimator (MLE) of the abscissa of a hypothesized unknown breakpoint (tBP) in time-series data can be determined as the time at which the overall sum of squares of error (SSE), summed over the two linear segments separated by the found breakpoint, is minimized. Hudson, however, provides no information concerning how likely it is that the MLE represents a true breakpoint (BP) present in the underlying data, and application of the algorithm necessitates prior knowledge (or assumption) that some true BP exists. Feder (1975a and 1975b) proves that, provided the model is identified (i.e., contains no more tBP than there are BP in the system), and that no tBP coincides with the abscissa of an observation in the dataset, then minimization of SSE (the MLE function) converges asymptotically to the BP. Feder finds that in the unidentified case (i.e., too many BP assumed), MLE function becomes indeterminate, and estimates are not asymptotically normal. The author's second condition arises because a discontinuity exists in the SSE function at each observation point in the dataset, rendering it non-differentiable there. At such points, the MLE function becomes unstationary, allowing for a true BP existing anywhere between the tBP (found with the MLE) and an adjacent observation. For the latter (unstationary) case, Feder proposes use of a "pseudocase," a dataset from which the offending observation has been removed, and shows that SSE minimization of the pseudocase converges to the BP, at a rate dependent on the number of remaining observations (and, of course, assuming that the pseudocase doesn't provide a tBP coinciding with yet another remaining observation). Lerman (1980) adapts Feder's work into a

grid-search algorithm (suitable for implementation in a spreadsheet) that, again, requires an identified system, and adopts the pseudocase into the algorithm when called for. In Lerman's method, a researcher maps proposed tBP across the span of abscissae of the observed dataset, and calculates the SSE at each tBP. Progressive (finer grain) refinement of this mapped tBP grid allows the researcher to narrow down the location (abscissa) of the MLE tBP by minimization of SSE vs. tBP. Lerman's exercise also provides an estimate of the variance of the MLE tBP found. The variance corresponds to the range of abscissae (not necessarily continuous or symmetrical) in which SSE is less than the sum of the minimum SSE (min SSE, by which the MLE tBP was located) and its associated mean square of error (MSE). Finally, Bai and Perron (1998) derive a log-likelihood ratio by which it can be determined whether the addition of a new assumed breakpoint to an identified model results in a another identified model (and publish critical values for that ratio).

I modeled (in a spreadsheet) each taxon for each treatment as an unsegmented (i.e., one-segment, $R = 1$) log-linear regression of net MPN change ($MPN_t/\text{initial MPN}$) vs. time (hours), and determined the SSE. I then tested a hypothetical $R = 2$ model with grid-search by Lerman's methods to determine minSSE, and with application of Bai and Peron's log-likelihood ratio to test whether (at 90% confidence) the two-segment model is warranted. If $R = 2$ was accepted, I then tested hypothetical $R = 3$ models, with grid-method subdivisions of each $R = 2$ segment, and so on until further subdivision was not warranted. For each taxon-/treatment-specific model, the abscissa of each found significant MLE tBP was noted, and the slopes of any intervening segments resulting from segmentation were calculated, for purposes of graphing and visualization. The variance of any found significant MLE tBP (as estimated by the abscissae range over which $\text{minSSE} + \text{MSE}$ exceeds SSE) were retained for further analysis.

3.2.1.a.2.3 Environmentally Significant Effectors

Each taxon (*E. coli* and Enterococci) was subjected to traditional (pooled variance) full-factorial (2^3) analysis (see Box, *et al.*, 2005, pp. 173-215) to rank the importance of the environmental factors (temperature, humidity, and UV exposure coded as 1 = shaded and 0 = exposed, plus their interactions) to the abscissa of each breakpoint and to the slope (k) of each intervening segment. MLE-derived tBPs, their associated uncertainties, and the k of each segment were derived directly from the breakpoint analysis above. Variance of each k was determined directly from $\log(\text{MPN}_t / \text{initial MPN}) / t = k$ for all observations at nonzero t in the segment.

3.2.1.a.3 Critical Test 1.a

Confirmation of Prediction 1.a. was found if temperature, RH%, and UV absence were all (as independent factors or as participants in interactions) found significant contributors (at 95% confidence) to adaptation-time lags (as evidenced by BP timing) and/or net growth (as evidenced by intervening slopes). Significance of any (independent or interactive) factor on a given parameter (k or MLE tBP) was concluded when the confidence interval ($\text{CI} = \text{SE} \times t(\alpha)$) was smaller than the calculated effect for that factor, where:

SE = Standard Error of the effect of that factor,
t(α) = the Student's t-table return for the appropriate degrees of freedom, and
(α) = some alpha corresponding to at least 95% confidence that the effect of the factor is not 0.

3.2.1.a.4 Modeling Effort 1.a

Design of this modeling effort assumes successful confirmation of Prediction 1.a (*via* Critical Test 1.a., at 3.2.a.3, above). Actual modeling here included only and all factors found significant in the Critical Test. The model was of segmented form, where:

- Segment one was defined as a line from the origin (0,0) and extending along slope $MPN_t/\text{initial MPN} = k_1$ (as determined from significant factors found in the factorial study) until the abscissa (time) of BP₁ (as determined from significant factors found in the factorial study) was reached;
- The origin of the second segment (if a likely breakpoint was found to be present) was the ordinate defined by slope k_1 at the abscissa of BP₁, the segment was extended at k_2 until BP₂ was reached; and
- So on until the end of the study period.

The models produced were checked using residual analyses comparing the predicted and the observed datasets for goodness of fit, and are presented with descriptive statistics.

3.2.1.b Prediction 1.b

Temperature, RH%, and UV presence/absence (the last in the presence of other insolation bands) are all (independently or interactively) significantly contributing factors to net survival of both E. coli (species) and Enterococcus (genus) on pervious surfaces.

[This prediction is based on literature suggestions and the hypothetical structure presented above.

The potential significance of extant landscape pH and nutrients was also checked here.]

3.2.1.b.1 Research Activities

The research activities here largely parallel those presented in section 3.2.a above, though uncertainties concerning the likely significance of environmental surface factors (extant pH conditions and bioavailable organics) required examination, generating a 2⁵ factorial study here. Alternative FIB extraction methods were used as it was obviously not possible to use the same

toothbrush sampling methods as used for the concrete blocks. Finally, a large number of non-quantified datapoints (above or below detection limits) here required alternative (censored-regression) analytical methods for deriving treatment-specific models.

I inoculated sterilized soils with slurries of pet feces and exposed them, over a reasonable inter-rain period, to artificially produced differential environments using a full-factorial 2^5 study design for low/high temperature, low/high RH%, and presence/absence of UV exposure, neutral/acidic pH, and presence/absence of bioavailable organics. Intermittently over the study period (and including at Day 0, immediately subsequent to inoculation), the test soils were washed, with physical biofilm disruption (timed shaking by hand, with subsequent timed settling period before elution), and the washoff eluents analyzed for MPN CFU density of FIB.

3.2.1.b.1.1 Slurry Preparation

Slurries were prepared as described in 3.2.a.1.1, above, but from an alternative fecal source. The deaths of Adeline and Lightning' forced a reliance on feces (of relatively poorly definable age) from dogs (of more variable parentage) boarded overnight in the kennel of a local veterinarian.

3.2.1.b.1.2 Soil Material Preparation

Bulk sandy soil with some included clay was mined from the just below the visible demarcation between the A and B layer (the latter lacking any visible sign of darkening by humic organics) of a local eroding creek bank undercutting a vegetated surface, and baked (450⁰ F) in a home kitchen oven for three hours. This treatment was presumed to drive off all but the most refractory soil organics. It also served to sterilize the soil (at least the absence of measurable FIB

was confirmed). Before inoculation with FIB slurries, the baked soil was divided into differentially amended portions to simulate the environmental-surface factors (pH and organics). One portion remained as is, amended only with distilled water to simulate a surface lacking available organics and of neutral pH prior to fecal deposition (pH was checked with a commercial soil pH meter while wetted). One portion was amended with (serially diluted with distilled water to achieve ~ 6.5 pH) distilled grain vinegar (a commercial product devoid of organics except acetic acid, which Enterococci cannot metabolize, and which *E. coli* can metabolize only by an energetically unfavorable pathway under duress, see Gottschalk, 1985, pp. 99-103). This acidified portion was also checked, while wetted, by pH meter. Organics were added to the third soil portion through amendment with (serially diluted to achieve ~5% soil-water organics) molasses. Molasses is a commercially available concentrated mixture of naturally derived sugars (mono- and di-saccharides, quintessentially bioavailable) with trace quantities of a wealth of micronutrients and cofactors. The 5% soil-water organic content is at the high end of conditions normally found in natural mineral soils, but was expected to provide usable organics throughout the study period, even in the absence of an actively recirculating extant soil flora. This concentration was also found to, coincidentally, acidify the applied “soil water” to 6.5 pH, and this soil portion provided for an organics-present/acidic model. Finally, a portion of the organics-amended soils was acid-neutralized with (again, requiring much serial dilution) baking soda, a commercially available, and fully non-nutritive formulation of sodium bicarbonate, to provide an organics-amended/pH-neutral model (and see USDA Reports 02053, 19304, and 18372, all undated). Each simulated soil was air-dried (laboratory hood), and divided into 1-tbsp pervious-surface microcosms in standard aluminum weighing dishes, before inoculation.

3.2.1.b.1.3 Interevent Period

Study period was ~ 2-weeks in length, as described at section 3.2.a.1.3

3.2.1.b.1.4 Differential Environmental Exposures

Differential environmental surface factors (pH and organics) were provided through the soil-amendment activities described, at 3.2.b.1.2 above. The more general environmental-factor (temperature, RH%, and UV) differentiation was generated *via* the environmental chambers described at 3.2.a.1.4.

3.2.1.b.1.5 Sample Collection

Boehm, *et al.* (2009), provide a large survey of (22) oft-used methods for quantitative release of FIB (*E. coli*, and Enterococci) from sandy matrices. The authors conclude that the simplest method that achieves consistent, repeatable, high extraction recoveries from sandy matrices is:

- 1.) Two minutes of vigorous hand shaking of the sample in buffered or deionized water, followed by
- 2.) 30 seconds of settling time before elution.

At interim times in the study period, I collected exposed samples according to the following procedure:

- 1.) Sluice samples from the weighing dish, with a (distilled-water) wash bottle, into sterilized sample bottles;
- 2.) Immediately add the appropriate (IDEXX™) defined-substrate medium (Colilert™ or Enterolert™) to quickly relieve any potential osmotic stress;
- 3.) Fill the sample bottle (with distilled water) to a premarked (preliminary, method-development researched) line to account for displacement of 1-tbsp of sandy soil, so as to allow elution of the 100-ml liquid sample required for MPN analysis;
- 4.) Vigorously shake the bottle for two minutes;

- 5.) Allow the sample to settle for 30 seconds; and
- 6.) Decant the liquid to a Quanti-tray™ for MPN analysis.

3.2.1.b.2 Analyses

3.2.1.b.2.1 MPN Determination

The MPN of FIB CFU was determined as previously described at section 3.2.1.a.2.1 above. All such analyses were carried out by IDEXX methods.

3.2.1.b.2.2 Treatment-specific Modeling, with Breakpoint Analysis

The presumption of the validity of a log-linear regression model with unknown breakpoints, well established in the literature of microbial population dynamics, and described at length in section 3.2.1.a.2.2 above, was maintained here. Likewise, the sequential determination of the appropriate number of appropriate breakpoints (by methods of Bai and Perron, 1998), with supply of input parameters for that determination by the spreadsheet-implemented grid-search algorithm (of Lehrman, 1980) was repeated here. Required inputs for that latter (grid-search) analysis, however, were not available by spreadsheet calculation from a problematic dataset encountered here.

The change of fecal source (to kennel dogs, providing feces of more uncertain FIB content) and the expansion of this study to necessarily include five potential factors combined to create many (about 1/3 for *E. coli*, fewer for Enterococci) censored (above, or below the detection limits of IDEXX enumeration) datapoints. Moreover, this censoring was not stochastic, but was mechanistically imposed by the detection limits. Such censoring is “nonignorable” (per Little and Rubin, 1987, pp. 8-13) and requires *censored-regression* analysis to avoid bias; no Ordinary Least Squares analysis performed *only* on the uncensored data can be expected to

produce unbiased results (pp. 221-223). The analysis and consequences of non-ignorable censoring are more clearly and relevantly discussed, with the actual censored dataset in hand, at 4.1.2 below.

Censored-regression analysis is an inherently iterative, MLE procedure, available in some computerized statistical applications. For each tBP in the (Lehrman) grid-search method, the MLE slopes and variances of those slopes for adjoined segments were determined in the Censored-Regression model of EViews8™, with found slopes input to the grid-search spreadsheet. Upon optimization of each treatment-significant model (MLE breakpoint per Lehrman grid-search, number of breakpoints per Bai and Perron significance), the found censored-regression parameters (slope and variance) provided required inputs to the subsequent factorial analysis of environmentally significant effects.

3.2.1.b.2.3 Environmentally Significant Effectors

Except for the necessary expansion of scope to a 2⁵ factorial, and for the substitution of censored-regression slope parameters (directly above), the procedure discussed in 3.2.1.b.2.3 above was duplicated (and see Box, et al., 2005, pp. 173-215).

3.2.1.b.3 Critical Test 1.b

Confirmation of Prediction 1.b. was found if temperature, RH%, and UV absence all (as independent factors or as participants in interactions) were found to be significant contributors (at 95% confidence) to adaptation-time lags (as evidenced by BP timing) and/or net growth (as evidenced by intervening slopes). Significance of any (independent or interactive) factor on a

given parameter (k or MLE tBP) was concluded when the confidence interval ($CI = SE \times t(a)$) was smaller than the calculated effect for that factor.

3.2.1.b.4 Modeling Effort

Design of this modeling effort, again, assumed successful confirmation of Prediction 1.b. The design would seem sufficiently flexible, however, to accommodate any found violations of the underlying assumptions (*e.g.*, a finding of significant effects by the presence of extant organics). Actual modeling here included only and all factors found significant in the Critical Test. The model is of segmented form, as in 3.2.1.a.4. Any model produced was checked against the observed dataset for goodness of fit, and presented with descriptive statistics.

The obvious weaknesses (especially in terms of interpolation between, or extrapolation beyond only two levels of effectors examined) in this approach are recognized. Only the significance of some factors' effects found here over the ranges studied can be quantitatively extended to more general circumstances. Even assuming monotonicity of the significant effects here can, at best, only provide qualitative predictive interpolations, over the studied range, elsewhere.

3.2.2 Landscape Export of FIB

Hypotheses 2:

2.a. *FIB, on landscape source areas and between rains, exist primarily within biofilms.*

2.b. *FIB are released from landscape source areas, in response to rainfall, primarily as passively sloughed aggregates (flocs and/or film-bound particulates).*

3.2.2.1 Predictions 2

3.2.2.1.1 Prediction 2.1

CFU densities of E. coli in macerated subsamples of runoff are more than double those found in as-is subsamples (as collected) split from the same samples.

E. coli, exhibiting planktonic morphology, generally exist unattached and unicellularly (or, occasionally, in the case of recently divided cells, in not-yet-separated pairs, section 2.2.4.1.2). Confirmation of Prediction 2.1 allows for conclusive rejection of exclusive operation by any mechanisms resulting in release of *E. coli* of exclusively planktonic form (*i.e.*,

- flush of planktonic *E. coli* extant before onset of rain, and
- seeding dispersal of filmbound *E. coli* in response to rain).

3.2.2.1.2 Prediction 2.2

CFU of E. coli from raw (not macerated) samples do not all pass through a 10-micron filter.

E. coli, exhibiting planktonic morphology, generally exist unattached and unicellularly or, occasionally, in the case of recently divided cells, in not-yet-separated pairs. Single or paired cells of planktonic *E. coli* should pass the filter (section 2.2.4.1.2). Confirmation of Prediction 2.2 allows for conclusive rejection (independent of confirmation of Prediction 2.1 above) of exclusive operation by any mechanisms resulting in release of *E. coli* of exclusively planktonic form (*i.e.*,

- flush of planktonic *E. coli* extant before onset of rain, and
- seeding dispersal of filmbound *E. coli* in response to rain).

3.2.2.1.3 Prediction 2.3

The product of the FIB CFU density of macerated samples, multiplied by the intensity of rainfall at the time of sampling, is not constant over the course of a rain event.

Daughter cells are released singly from biofilms by each cell-division of a surficial cell in the erosion mechanism (section 2.2.4.4.2). The total number of such released cells over time (cell density x diluting runoff rate) is expected to remain constant so long as the surface remains wetted. After onset of runoff from the wetted surface, runoff is expected to be proportional to rain intensity. Confident confirmation of Prediction 2.3 allows for rejection of cell-division erosion as the sole mechanism of FIB release from source-area surfaces.

These hypothetical predictions are not based on any expectations supported in my literature review, but do jointly provide a framework for screening of potentially relevant mechanisms, for release of FIB to rainwater runoff from source areas, that are presented in the literature. Such screening is based on that either:

- Confirmation of Predictions 2.1 and 2.3, or
- Confirmation of Predictions 2.2 and 2.3,

logically leads to a conclusion that passive sloughing meaningfully contributes to the release of extant FIB to runoff in response to rain. This finding, in turn, logically leads to a conclusion that both rain depth and rain intensity must both be considered in any modeling efforts, with potential changes in those parameters over time in any rain event.

3.2.2.2 Research Activities

3.2.2.2.1 Simulated Rain Exposure

Predictions 2.1 and 2.3 both depend on conditions that may change over the course of a rain event. To test these predictions I exposed each of three surfaces (a collocated roof, lawn, and street) to a three-hour simulated heavy rainfall.

Simulated rainwater consisted of tap water, de-chlorinated with sodium thiosulfate (ProLine™, sold for aquarium use). Sufficient water for this extended rain simulation required two-tank staging of supply/de-chlorination. While one tank fed the pump (Ultitech™, 1-HP) another was filling and equilibrating with dosed thiosulfate (see Figure 8).

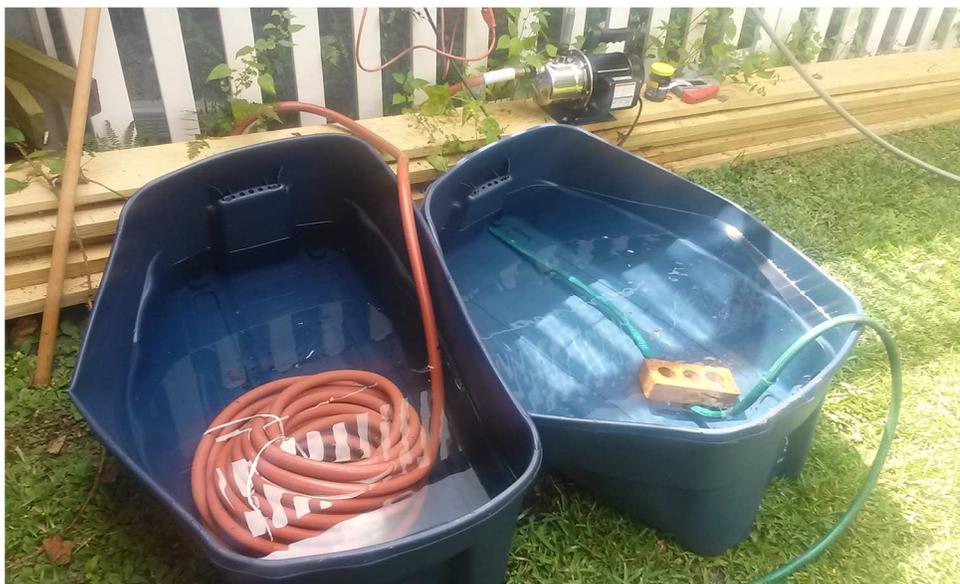


Figure 8 Simulated Rainwater Feed System. Tank on left provides de-chlorinated feed while right-hand tank fills/de-chlorinates.

De-chlorinated feed was pumped to the “rainframe” (shown at Figure 4, above, with details below) *via* a lawn (Orbit™) garden-hose manifold (Figure 3, above). Inlet to the manifold was fitted with a garden-hose pressure regulator (Senninger™, 25-psi) and flowmeter (Save A

Drop™, providing flow rate and cumulative flow volume) in series (Figure 9). One outlet tap of the manifold was also fitted with a generic (no brand name available) garden-hose pressure gauge, purchased at Lowes™, for diagnostic/monitoring purposes.



Figure 9 Feed-manifold Inlet Fittings. during flow-meter calibration
Remaining outlet taps of the manifold were variously split- (hose tees) or series-connected to three separate pairs of drip-irrigation emitters (Rainbird™ SQH). These emitters are designed to mimic natural raindrop size (~ 1-5 mm diameter) and distribution over a 4' x 8' rectangle from an edge of that rectangle, across a broad range of fed pressure, at ~ 0.5 inch/hour nominal equivalent precipitation rate (see “H” in Figure 10). Worthy of note here, these emitters are *not* expected to provide the full surface-disruptive impact of equivalent natural-rain intensity (which includes components of rain-drop size, precipitation rate *and* delivery of drops at terminal velocity).

SQ Nozzle Performance					
<i>4 feet throw @ 6" height above grade</i>					
Nozzle	Pressure psi	Throw Radius ft.	Flow gph	Flow gpm	Precip. Rate w/no overlap in/h
Q 	20	4.0	6.4	0.11	0.64
	30	4.0	7.4	0.12	0.74
	40	4.5	7.4	0.12	0.59
	50	4.5	7.4	0.12	0.59
H 	20	4.0	10.2	0.17	0.51
	30	4.0	12.2	0.20	0.61
	40	4.5	13.7	0.23	0.54
	50	4.5	13.7	0.23	0.54
F 	20	4.0	20.0	0.33	0.50
	30	4.0	24.2	0.40	0.61
	40	4.5	27.3	0.46	0.54
	50	4.5	27.3	0.46	0.54

Performance data taken in zero wind conditions

Figure 10 SQH Performance. (Reprinted directly from <http://www.rainbird.com/landscape/products/sprayNozzles/SQseriesNozzles.htm>, undated)

The three pairs of emitters were affixed to a wooden, 8' x 8' frame, in three orientations. Two pairs were focused inwardly from opposite sides of the square frame. The third was focused out (oppositely) from the center. Leveled, on a flat slab, the so fitted frame was preliminarily tested for even "rainfall" distribution of coverage of the square (by coefficient of variation, of 4 x 4 = 16 sample points) under various valving scenarios. On the basis of these preliminary studies (in an Appendix of section 4.2.1), the centrally placed pair of emitters was chosen as the best pair (lowest overall coefficient of variation) for use as the low-flow (nominal 0.5-inch/hr) intensity and a best "uphill" orientation (up and right in Figure 11, based on lowest coefficient for the uphill tier) was selected.

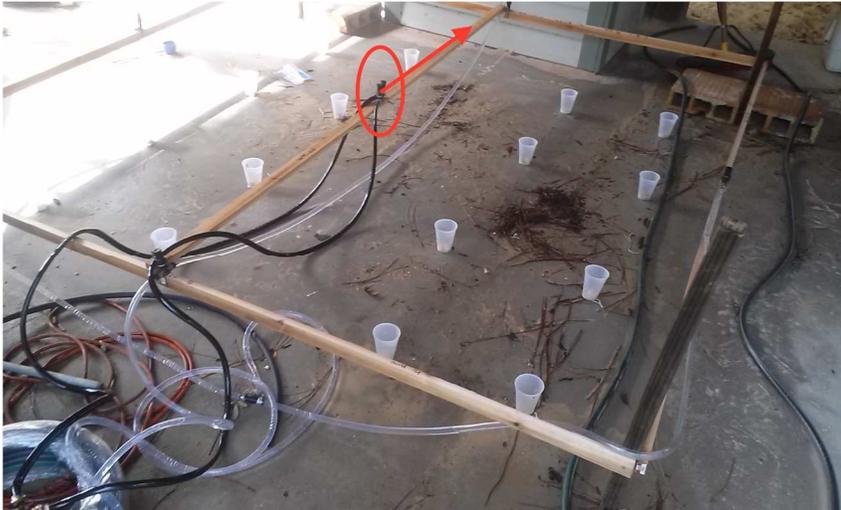


Figure 11 Rainframe, preliminary hydraulic characterization. Oval = Low-flow emitter pair, Arrow = uphill.

Valving at the manifold allowed for control of three flow-rate settings corresponding to nominal “intensities” (equivalent precipitation rates):

- 1/2” inch/hour, with central emitters on;
- 1” inch/hour, with side emitters added; and
- 1½” inch/hour, with up- and down-hill emitters valved in.

The flowmeter at the manifold (with a pre-calibrated correction) allowed for calculation of actual intensity and cumulative depth at the time of each sampling event. A plastic lined trench (or roof gutter, etc) allowed for direct collection of runoff samples from the downhill border of the frame. One hour before each sampling run (or each hydraulic-characterization run), a tarp/cover was installed in an attempt to better simulate the evaporation regime of a cloudy sky (Figure 12).



Figure 12 Example Collection Trench.

As previously discussed (3.1.2.1, above) I applied the simulated rainfall in the order of:

- 4.) Nominal ½” intensity for 20 minutes,
- 5.) Nominal 1” intensity for 20 minutes, and
- 6.) Nominal 1½” intensity for 20 minutes,

and I repeated the sequence three times (for a three-hour simulated heavy-rainfall event) for each surface. The timing of the initial 20-minute exposure was not begun (stopwatch was reset) until the visible onset of runoff from the surface. For each 20-minute segment of the study period, a sample was drawn at midway (10-minutes subsequent to initiation of each new intensity regime). At the initiation of each 20-minute segment of the study period, measured cumulative volumetric flow was recorded, to provide for a measure of the average intensity for each segment and cumulative depth at each sampling event. This sequence provided for separability of wetted time, cumulative depth prior to sampling, and intensity at time of sampling as potential factors affecting CFU densities in surface runoff.

Each sample was halved, by cone splitter, to allow separate measurements of the two FIB taxa under study here. Each half was then split to allow for separate, comparative measurements of FIB densities in raw (as is) sample vs macerated sample (all density measurements by IDEXX™ methods).

Resulting datasets were screened per the following hypothetical structure:

- MPN of macerated *E. coli* CFU are significantly elevated relative to those found in the raw, unmacerated subsample. Favorable finding of this condition would imply that *E. coli* are not exclusively present in their planktonic form, but significantly present in the sessile, filmbound state. This finding, in turn, would allow for rejection of seeding dispersal as the sole mechanism of *E. coli* release from the landscape. Noting that planktonic *Enterococci*, spp., can exist in the multicellular, “string of pearls” morphology, such a finding for that taxon would not be as directly conclusive. A finding that most *E. coli* were present in a source area in the filmbound state, however, would provide for a strong presumption that co-located *Enterococci* were similarly filmbound.
- The product of macerated-sample CFU multiplied by the rain “intensity” at the time of sampling is not constant throughout the rain event (continuously wetted period). Favorable finding of this condition would allow for confident rejection of cell-division/erosion as the sole mechanism of FIB release from the source area.

Valid finding of both of the hypothesized conditions above would provide for a conclusion that sloughing is at least a significantly contributing mechanism to rain-induced release of FIB from source areas. Such a conclusion forces recognition that any modeling effort must account for both rain depth (possibly expressed in a manner bifurcated over time) and intensity as possible significant effectors of FIB densities in runoff. It also raises the possibility of biofilm retention of FIB, not sloughed, even in the face of this simulated heavy “rain.” This last contingency was tested for with somewhat destructive testing by use of a high-pressure (household “pressure-wash”) garden-hose nozzle at the end of each rain simulation.

3.2.2.2 Morphology of CFU Released

The potential that CFU densities in runoff samples may actually represent multicellular aggregates of flocs or filmbound particulates has obvious implications for their effects on compliance of receiving waters downgradient. Shear disaggregation of such an assemblage, into more than one survival-/reproductively-competent assemblage, during post-release transport to receiving waters would increase the actual (compliance-relevant) effect on those waters. Cell-death within such an assemblage would not affect viability of the CFU until all cells within the CFU had died. Presence of mineral (soil) particles within a floc/aggregate would affect settling and consequent transport behavior between its release from a source area and its arrival at regulated waters. Even assurances that potential FIB CFU on the landscape survived the interevent period, and were subsequently released from the surface in response to a rain event, these considerations force some attention to whether or not such entities would ever reach a regulated water body.

These considerations force some attention to the form/morphology of any liberated CFU. Such attention, in turn, suggests use of a real rain, with the realistic kinetic energy applied to the landscape by raindrop impact at terminal velocity.

I explored various factors likely to affect either interception or resettling behavior in two, largely descriptive, scoping studies below: a filter cascade, focused on size and multicellularity of CFU; and a settling study. The first of these, also provides size-dependent information, independent of that obtained in the Simulated-Rain study (3.2.2.2.1, above) concerning mechanisms of release of FIB from source areas.

3.2.2.2.1 A Filter Cascade

Logically expected outcomes of organizing assumptions presented at section 3.1.2 above provided that confirmation of Prediction 2.1 (above at section 3.2.2.1.1) allows for confident rejection of sole operation of any FIB-release mechanism (of those found in the literature) resulting in fully planktonic *E. coli* CFU (i.e., flush of extant planktonic cells, or seeding dispersal) in response to rain. That logic, however, does not work in reverse. Failure to find confirmation of Prediction 2.1 does not allow for rejection of mechanisms leading to potential release of multicellular CFU: the literature suggests that erosion is *likely* to result in unicellular CFU, and that sloughing is perfectly capable of producing the same result.

Logically expected outcomes of organizing assumptions presented at section 3.1.2 above, however, also provided that confirmation of Prediction 2.2 (above at section 3.2.2.1.2) allows for confident rejection of sole operation of any found FIB-release mechanism resulting in fully planktonic *E. coli* CFU. The size-dependency of tests for Prediction 2.2 requires information not available from the simulated-rain study above, suggesting examination here. The logic here, though, is just as one-way as that presented above; failure to confirm Prediction 2.2 still does not allow for rejection of any identified FIB-release mechanism. Such, it would seem (alas), is the nature of exploratory research.

The research-activity procedures performed here were entirely compliant with the research-design steps explicated at section 3.1.2.2.1 above. Results are presented at section 4.2.2.1, below.

3.2.2.2.2 A Settling Study

This study is entirely descriptive, and only meant to inform future research. No hypothetical structure or analysis is imposed. The research-activity procedures performed here were entirely compliant with the research-design steps explicated at section 3.1.2.2.2 above. Results are presented at 4.2.2.2, below.

CHAPTER 4

RESULTS AND DISCUSSION

A series of scoping studies follows. Most of the material here is presented in article style, reformatted to meet requirements of this dissertation. Some explanatory sections outside the articles/manuscripts are interspersed, with appropriate identification, when necessary to describe linkages between studies, and their interrelated positions within this overall research effort.

4.1 Survival Studies

4.1.1 Impervious-surface survival of FIB

4.1.1.1 Survival of Bacterial Indicator Species on Environmental Impervious Surfaces

A published article (Wilson, B.M. and R.E. Pitt. 2013. "Survival of Bacterial Indicator-Species on Environmental Impervious Surfaces. "*Journal of Water Management Modeling* R246-14. doi: © CHI 2013) is reprinted, in its entirety and verbatim but reformatted to meet requirements of this dissertation, with permission of CHI (Appendix, below).

SURVIVAL OF BACTERIAL INDICATOR SPECIES ON IMPERVIOUS ENVIRONMENTAL SURFACES

Bradford M. Wilson and Robert Pitt

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 the health risk from those pathogens. Commonly used indicator species (especially *Escherichia coli* and *Enterococcus* spp., or enterococci), 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).

Field and Samadpour (2007) provide a critical review of both the *indicator paradigm* (our current reliance on fecal indicator bacteria, FIBs) and an alternative monitoring regimen utilizing fecal source tracking (FST) methods. While noting the inadequate state of the art for direct measurements of pathogens, the authors find deficiencies in the correlations of FIBs to specific pathogens, and of FIBs to epidemiological measures of human health. They ascribe the deficiencies in the indicator paradigm to its inability to identify the source hosts of environmental FIBs. Landscape survival of FIBs and the ratio of FIBs to human pathogens deposited on the landscape are dependent on the source of the feces. More specifically, though zoonotic infections from non-human sources occur, correlation between human health threat and FIB presence suffers when major fecal sources other than sewage are present. (The authors

proceed to find current state FST methods alone also deficient: they propose a multi-level combination of expanded source and epidemiological surveys, and pathogen, FIB and FST testing, while noting the expense and the laboratory retooling that would be required for such an approach.)

Continued reliance on the use of FIBs to manage the microbiological risk of environmental waters would be better informed by knowledge of the nonhuman contributions of FIBs to stormwater. In an ongoing effort to model background (i.e. of non-sewage origin) discharges of indicator species from stormwater source areas in which the presence of sewage contamination can be precluded, in the Tuscaloosa, Alabama area, a model for the environmentally relevant survival of indicator species (*E. coli* and enterococci) on impervious surfaces within the environment is presented.

14.1 Methods

14.1.1 Bacterial Cultivation and Enumeration

A full factorial study (2^3 , temperature/moisture/UVB exposure, the latter being ultraviolet B radiation) of the indicator species' environmental survival factors was performed 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 commercial biological oxygen demand, BOD, controllers and heaters for temperature control, desiccant or humidifiers for moisture control, and UVB 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. The raw concrete paving blocks had been prepared by an overnight soak in mild brine (0.25 cup, 62.5 mL, table salt into 40 gal, 151.4 L, trash can of tap water), followed by thorough tap water rinse and air drying, to provide an

unreactive, passive surface. Slurries were produced by blending dog feces with distilled water (to assure microbiological purity and the absence of bactericidal components) and immediately applied (with a 3 mL sterile disposal syringe) to the passive blocks (to quickly relieve any potential osmotic stress of the distilled water). No additional nutrients (other than fecal materials) were added. Active control of temperature (40 °F and 90 °F, 4.4 °C and 32 °C, cool or warm) held the parameter steady (± 2 °F, 1.1 °C) over the study period. Relative humidity (25% and 80%, dry or wet) varied over about $\pm 4\%$. UV exposure was treated as present or 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 (three scrubs of 1 min each, with intervening wetting between scrubs), washing the slurry debris into sample bottles and dilution to 100 mL (with distilled water). Method development comparisons of wash-off MPN to inoculant MPN showed incomplete but consistent (within 95% confidence bands of MPN measurement) recovery of the inoculant by this abrasion–rinse technique. Washed-off samples were immediately mixed with defined substrate formulations (Colilert or Enterlert) for relief of osmotic stresses. The most probable numbers (MPN) of surviving *E. coli* and enterococci colony forming units (CFU) per 100 mL were measured using IDEXX (IDEXX Laboratories, Inc.) methods and normalized to the inoculation date (Day 0) MPN (also acquired from brush-off samples from blocks inoculated and brushed in the same way). IDEXX reagents (Colilert and Enterolert) provide for selective incubation of the taxons of interest, and colorimetric and fluorometric indicators of viable colonies within 24 h. These are recognized water assays under *Standard Methods for the Examination of Water and Wastewater* (Eaton et al., 2005:sections 9223 and 9230b, respectively). MPN measurement values

with three orders of magnitude ranges (from 1 MPN/100 mL to 2 420 MPN/100 mL) are directly available with the reagents when used in conjunction with Quantitray 2000 units. Additional dilutions of each sample were incubated to ensure that all samples were quantified over even wider ranges.

14.1.2 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, arising as the sum of binary fission and death of individual cells (both dependent on the number of viable cells at any given time). This pattern, well established in textbooks currently in use (e.g. Madigan et al, 2002:142) is of the form:

$$\log (MPN / \text{initial } MPN) = k \times t \quad (14.1)$$

where:

k = net growth constant (slope of the function), and
 t = time (hours).

Changes in the slope of $\log(MPN)$ 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., 2002:144–5):

- *Lag Phase*

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: 144–5).

- *Growth Phase*

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 the genetic (metabolic mechanisms available) composition of the population (Madigan et al., 2002:144–5).

- *Stationary Phase*

The stationary phase (in which the population is static) represents conditions in which available nutrients (either from the original inoculant or from release by the lysis of dying cells) is balanced by a buildup of refractory (and often inhibitory) waste products (Madigan et al., 2002: 144–5).

- *Death Phase*

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: 144–5). The four main environmental factors influencing bacterial growth are temperature, pH, and the availability of water and oxygen (Madigan et al., 2002: 151). For terrestrial environmental surfaces, oxygen is unlikely to be a factor. For dry weathered pavements (without liquid moisture, between rains), pH is likewise probably unimportant. An important factor in cell death, however, is that of UVB exposure (Madigan et al., 2002: 272–3), which is bactericidal, especially during cell division.

Because we cultivated our samples at constant conditions, a change in slope of $\log(MPN)$ versus time must be viewed as a population change. Population change may arise 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 below in Figure 14.3, for example).

The 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 the algorithm 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; 1975b) proves that, provided the model is identified (i.e. includes no more hypothesized breakpoints than are present in the real population), and if no hypothesized *tBP* coincides with an abscissa of observation in the sample, then minimization of *SSE* (the MLE function) converges asymptotically to the true population breakpoint (BP).

In the unidentified case (*i.e.* too many BPs assumed), the MLE function becomes indeterminate (estimates are not asymptotically normal). Feder'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). The exercise also provides an estimate of the variance of that *tBP* estimate, corresponding to the range (which need not be continuous or symmetrical) of proposed *tBPs*. The range includes all time in which

SSE is less than the minimum SSE plus its associated mean square of error ($\text{minSSE} + \text{MSE}$). Finally, Bai and Perron (1998) derive a log-likelihood ratio by which it can be determined whether the 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 (see Figure 14.1 below). We found grid search of the (asymptotically converging) pseudocase, however, problematic for the limited number of data points we had for each treatment (typically ~ 35). In one case, analysis of the pseudocase resulted in the tBP jumping about 100 h (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 (see Figure 14.2). 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 (see Figure 14.3).

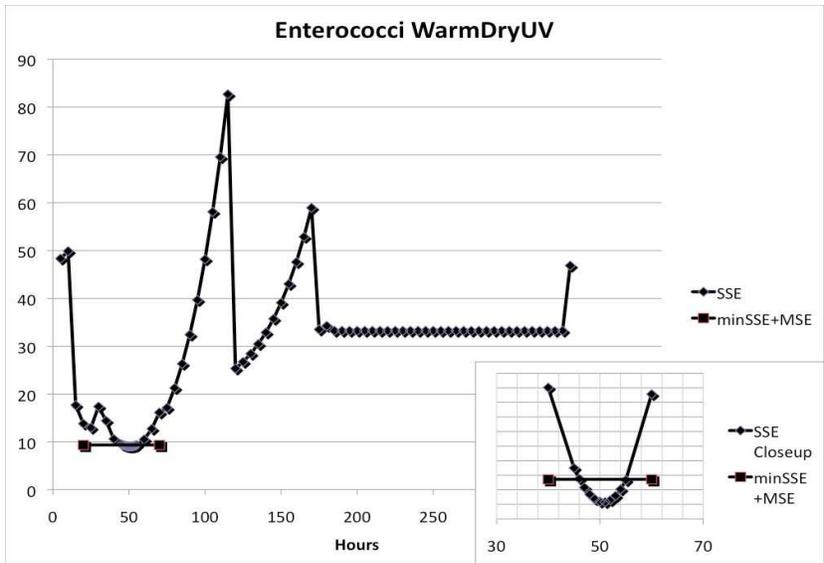


Figure 14.1 Example of graphic derivation of estimated tBP variance, normal case (with closeup inset).

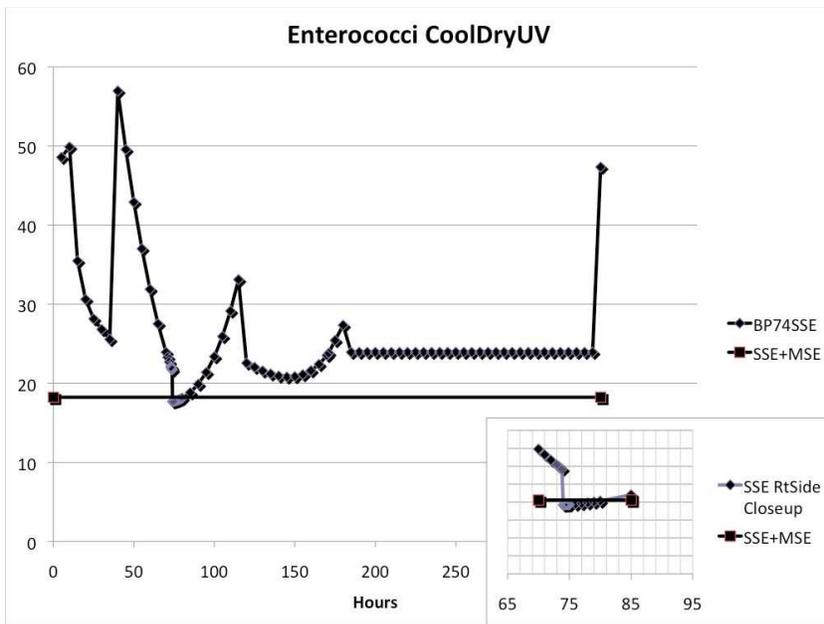


Figure 14.2 Example of right-sided graphic derivation of tBP variance, discontinuity on the left (with closeup inset).

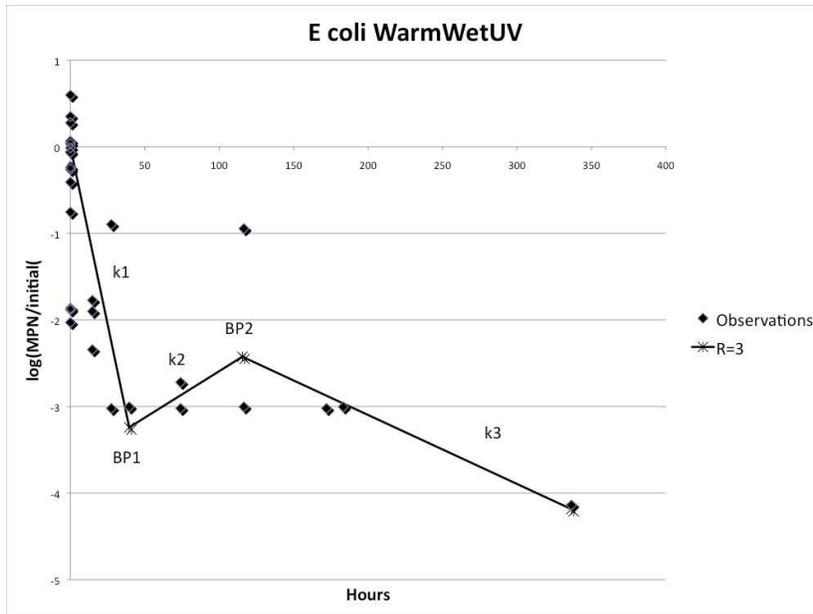


Figure 14.3 Example of a three-segment ($R = 3$) treatment model with segment and breakpoint designation.

14.1.3 Environmental Factors

Each taxon (*E. coli* and the 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 and 0 = exposed, plus their interactions) to the abscissa of each breakpoint and to the slope (k) of each intervening segment. The tBPs, their associated uncertainties, and the k of each segment were derived directly from the breakpoint analysis above. The variance of each k was determined from $\log(MPN / \text{initial } MPN) / t = k$ 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 the 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:

$$CI = SE \times t(a) \quad (14.2)$$

where:

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

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

14.1.4 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:

$$\text{Model Parameter} = \text{Mean (Parameter)} + \text{Sum of (Effects of Environmental Factors)} \quad (14.3)$$

where:

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

Mean(Parameter) = treatment weighted mean for that parameter , and

$\text{Effects of Environmental Factors (EEF)}$ = adjustments to Mean(Parameter) attributable to each important environmental factor.

For 2-level factorial, effects are of the form:

$$EEF = [Product(EF-MEF) \times (1/2 \text{ environmental effect})] / Product(REF) \quad (14.4)$$

where:

EF = value of that environmental factor for an observation point,

MEF = mean of that environmental factor amongst observation points, and

REF = range (high value to low value) of an environmental factor amongst observation points.

14.2 Results and Discussion

14.2.1 *E. Coli*

Results from breakpoint analysis of the *E. coli* dataset (Figure 14.4 below) are complex. One treatment (warm/wet/dark) showed no significant tBP (not even a lag), and also exhibited an absence of any initial accelerated 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 (the 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 (the primary bactericidal band in sunlight) is not strictly ionizing radiation, it is of sufficiently high frequency to rearrange bonds in complex biomolecules.

Most importantly, UVB causes dimerization of adjacent thymine units (and other photoproducts) within bacterial genomes that inhibit the progression of (both RNA and DNA) polymerases. An unrepaired lesion within a gene prevents transcription of that gene. Each

unrepaired lesion also stops replication of the entire genome during fission. (Wulff and Rupert, 1962; Sinha and Hader, 2002). Hospitable (e.g., warm) conditions prompting greater cell growth and division might increase UV sensitivity until repair mechanisms are induced or tolerant strains are selected for.

The factorial analysis results (Table 14.1) 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.

Table 14.1 Important alpha, $(\alpha) \leq 0.1$.

E. coli k

Main Effects	Effects	SE(Effect)	t(a)	CI(effect)	df=17 (a)=0.01
Humidity	0.061	0.00055	2.6	0.060	
Interactions					
Temp/Humid	0.11	0.024	2.9	0.070	(a)=0.01
<i>E. coli BP1</i>					
Main Effects	Effects	SE(Effect)	t(a)	CI(effect)	df=224 (a)=.005
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					
Temp/Humid	12.6	0.33	2.6	0.87	(a)=.005
Temp/Shade	-5.0	0.33	2.6	0.87	(a)=.005
Humid/Shade	-6.2	0.33	2.6	0.87	(a)=.005
Temp/Humid/Shade	-2.8	0.33	2.6	0.87	(a)=.005
<i>E. coli BP2</i>					
Main Effects	Effects	SE(Effect)	t(a)	CI(effect)	df=37 (a)=.005
Temperature	-9.2	1.05	2.8	2.9	(a)=.005
Humidity	17.9	0.80	2.8	2.2	(a)=.005
Interactions					
Temp/Humid	17.9	0.80	2.8	2.25	(a)=.005
Humid/Shade	17.9	0.80	2.8	2.2	(a)=.005
Temp/Humid/Shade	17.9	0.80	2.8	2.2	(a)=.005

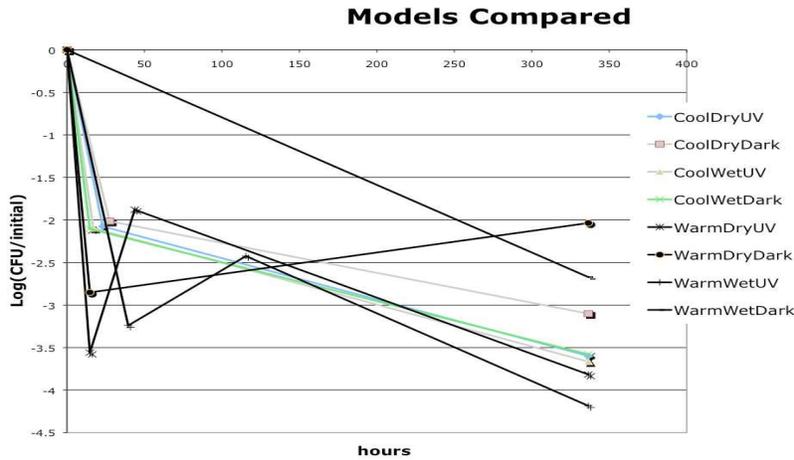


Figure 14.4 *E. coli* BP models; CFU determined by MPN (IDEXX) methods.

Our model for *E. coli* survival is therefore, for times $t \leq tBP1$:

$$\log(MPN / \text{initial } MPN) = kI \times t \quad (14.5)$$

where:

$$kI = -0.108 + (H - 52.5) \times 0.000551 + (T - 65) \times (H - 52.5) \times 0.0000203 \quad (14.6)$$

where:

T = temperature (°F)

H = %relative humidity, and

$$tBP1 \text{ (hours)} = 21.6 + (T - 65) \times 0.0209 (H - 52.5) \times 0.0293 - (S - 0.5) \times 1.95 + (T - 65) \times (H - 52.5) \times 0.00229 - (T - 65) \times (S - 0.5) \times 0.0503 - (H - 52.5) \times (S - 0.5) \times 0.0560 - (T - 65) \times (H - 52.5) \times (S - 0.5) \times 0.000506 \quad (14.7)$$

where:

S = shade code (1 = shade, 0 = exposed)

Our model for *E. coli* survival is, for times $t > tBP1$ and $t \leq tBP2$:

$$\log(MPN / \text{initial } MPN) = k1 \times tBP1 + 0.002214 \times (t - tBP1) \quad (14.8)$$

where:

$$tBP2 = 80.71 - (T - 65) \times 0.0924 + (H - 52.5) \times 0.163 + (T - 65) \times (H - 52.5) \times 0.00326 - (H - 52.5) \times (S - 0.5) \times 0.163 - (T - 65) \times (H - 52.5) \times (S - 0.5) \times 0.00326 \quad (14.9)$$

Our model for *E. coli* survival is, for times $t > tBP2$:

$$\log(MPN / \text{initial } MPN) = k1 \times tBP1 + 0.00221 \times (tBP2) - (0.00501) \times (t - tBP2) \quad (14.10)$$

The model presented does not fully account for the variability in the observations (R^2 is only 0.42, and see Figure 14.5) of the full *E. coli* dataset. It does, however, offer improved correlations with, and better balance between, under- and over-predictions than would be provided by a simple linear regression of the same dataset (compare Figures 14.6 and 14.7, and note closely concentric trending in Figure 14.5). Residuals of the model show little evidence of any trend over time, providing some comfort in the pooled variance methods used here (Figure 14.8).

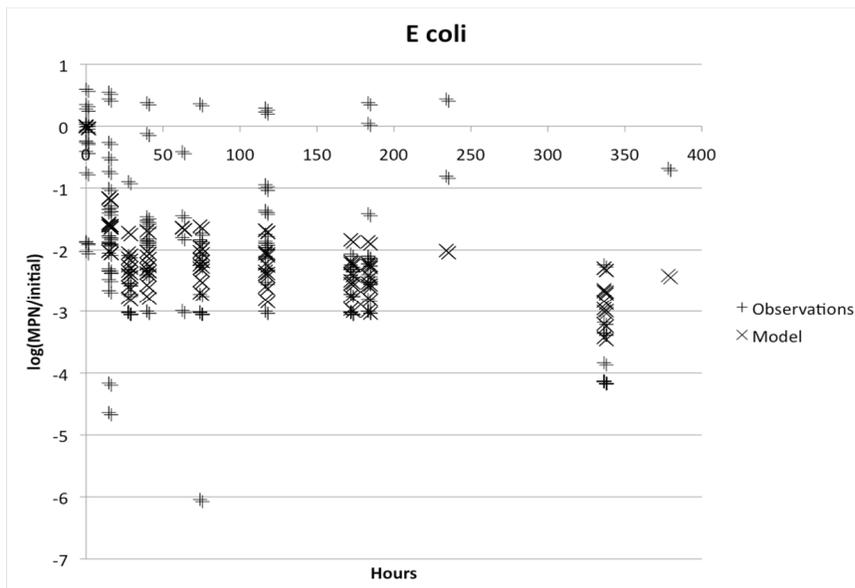


Figure 14.5 Overlay of model predictions on observations, all treatments combined.

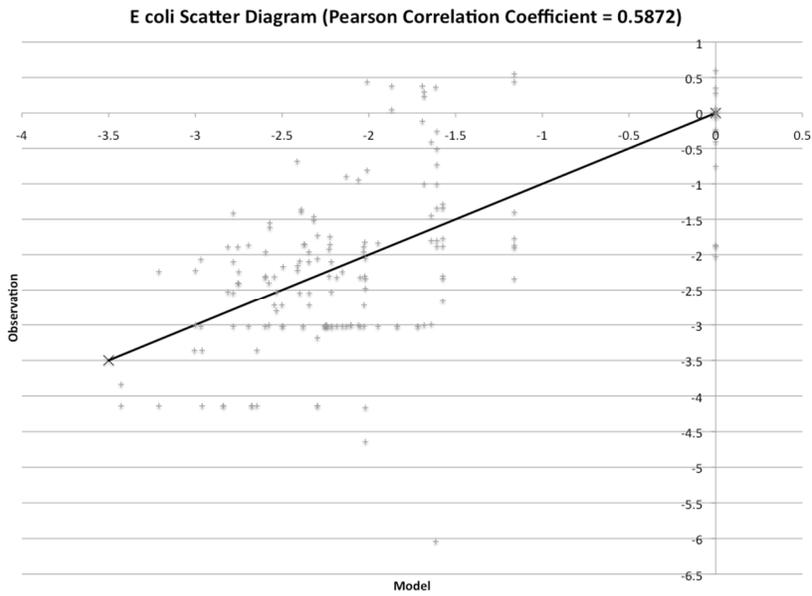


Figure 14.6 Observations vs model; line is observation = model prediction.

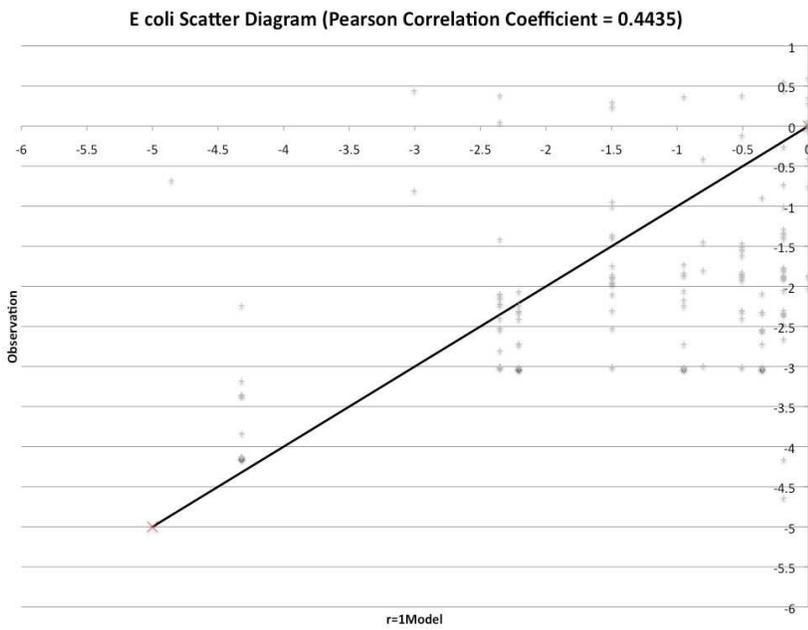


Figure 14.7 Observations vs predictions of linear regression without environmental factorial.

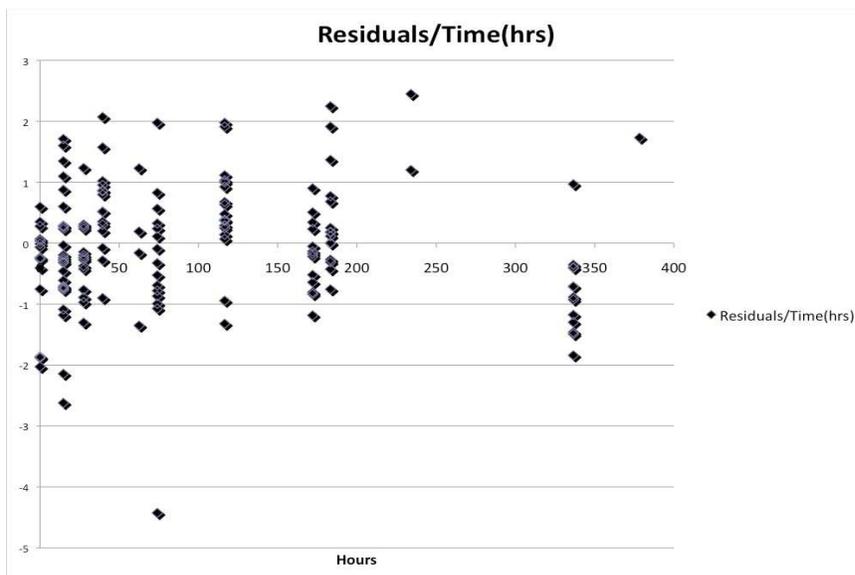


Figure 14.8 Residuals vs time for the presented *E coli* model.

The model-derived parameters applied to our experimental conditions are presented in Table 14.2 below. All treatments exhibit an initial lag or 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 (k_1) of decline, but all inoculants had declined from two to three orders of magnitude within a day or so. The duration of the decline appears to be quite variable (19 h to 27 h), 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 insensitivities of k_2 and k_3 to environmental factors imply that all adaptive mechanisms available to the inoculant population had been implemented prior to (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 a review of the warm treatment behaviors in the original BP analysis, Figure 14.4 above, suggests that both factors are involved).

Table 14.2 *E. coli* modeled parameters, applied to experimental conditions.

	k1 (1/hours)	BP1 (hours)	k2 (1/hours)	BP2 (hours)	k3 (1/hours)
Cool/Dry/UV	-0.109	21.6	0.00221	76.8	-0.00501
Cool/Dry/Dark	-0.109	22.1	0.00221	79.0	-0.00501
Cool/Wet/UV	-0.107	21.3	0.00221	83.5	-0.00501
Cool/Wet/Dark	-0.107	19.4	0.00221	81.2	-0.00501
Warm/Dry/UV	-0.137	20.4	0.00221	71.0	-0.00501
Warm/Dry/Dark	-0.137	19.1	0.00221	77.8	-0.00501
Warm/Wet/UV	-0.0787	27.1	0.00221	91.2	-0.00501
Warm/Wet/Dark	-0.0787	22.0	0.00221	84.5	-0.00501

14.2.2 Enterococci

Treatment analyses of the breakpoints is less complex for enterococci than for *E. coli* (see Figure 14.9), although some disparity as to number and tBP values per treatment appears here as well.

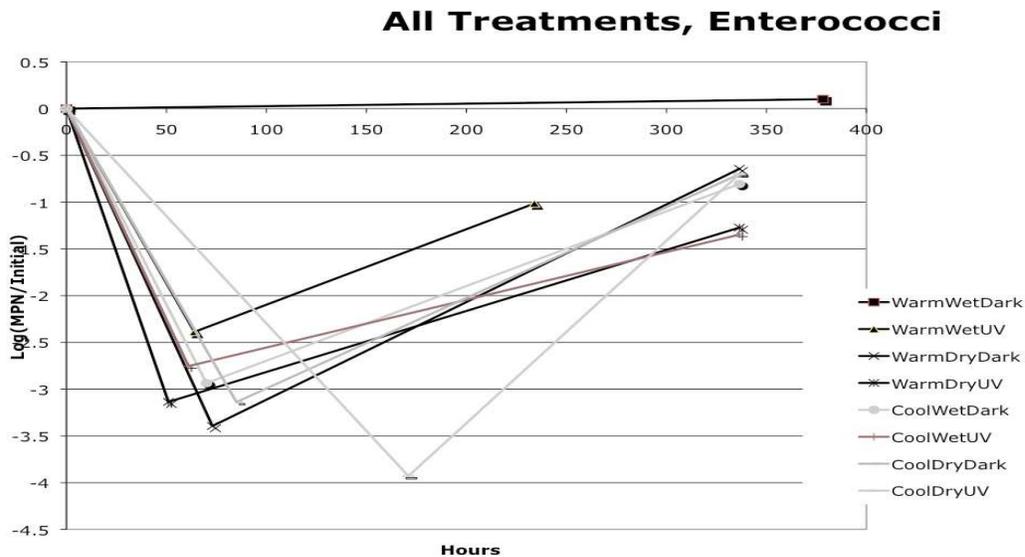


Figure 14.9 Enterococci BP models.

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 higher taxon provides an overall greater potentiality of adaptive capacity (natural selection acting differently on distinct species or strains in different conditions) and a greater likelihood of genes for UVB damage 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 2-week period. It also should be noted that no population is in decline at the end of the study period. As a final note, our study was unable to distinguish bacterial lysis from other fecal components of the inoculant slurry as nutrients for growth. Factorial analyses (Table 14.3) 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 14.3 Important factors per enterococci 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
Shade Code	0.015	0.11	0.010		(a)=0.1
Interactions					
Temp/Humid	0.020	0.011	1.7	0.019	(a)=0.05
Temp/Shade	-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
Shade Code	11.2	0.31	2.7	-0.84	(a)=0.005
Interactions					
Temp/Humid	8.7	0.31	2.7	0.84	(a)=0.005
Temp/Shade	1.2	0.32	2.7	0.85	(a)=0.005
Humid/Shade	-5.3	0.32	2.7	0.84	(a)=0.005
Temp/Humid/Shade	-4.3	0.32	2.7	0.85	(a)=0.005

Our model for enterococci survival is therefore:

$$\log(MPN / \text{initial } MPN) = k1 \times t \text{ for } t \leq tBP \quad (14.11)$$

where

$$k1 = -0.0356 + (H - 52.5) \times 0.000137 + (S - 0.5) \times 0.00727 + (T - 65) \times (H - 52.5) \times 0.00000372 - (T - 65) \times (S - 0.5) \times 0.00771 \quad (14.12)$$

and

$$tBP = 68.74 - (T - 65) \times 0.881 - (H - 52.5) \times 0.0483 + (S - 0.5) \times 5.59 + (T - 65) \times (H - 52.5) \times 0.00158 + (T - 65) \times (S - 0.5) \times 0.0119 - (H - 52.5) \times (S - 0.5) \times 0.0483 - (T - 65) \times (H - 52.5) \times (S - 0.5) \times 0.000784 \quad (14.13)$$

and for $t > tBP$:

$$\log(MPN / \text{initial } MPN) = k1 \times tBP + 0.00652 \times (t - tBP) \quad (14.14)$$

Comparison of the model with observations (Figure 14.10) 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 14.11 and 14.12, and closely concentric trending in Figure

14.10). Residual plot, again, provides no evidence of increase or decrease over time (Figure 14.13).

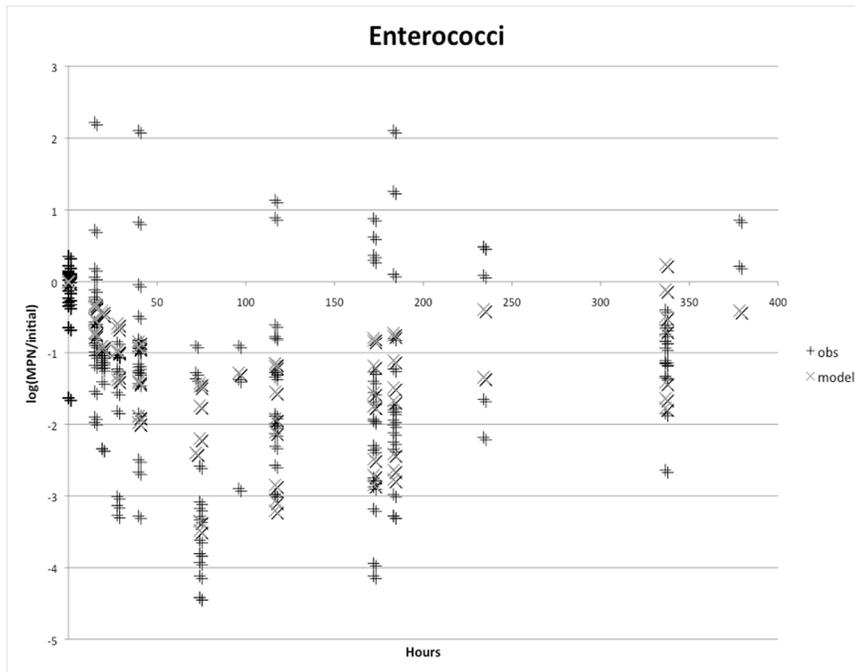


Figure 14.10 Enterococci, observations vs model comparison.

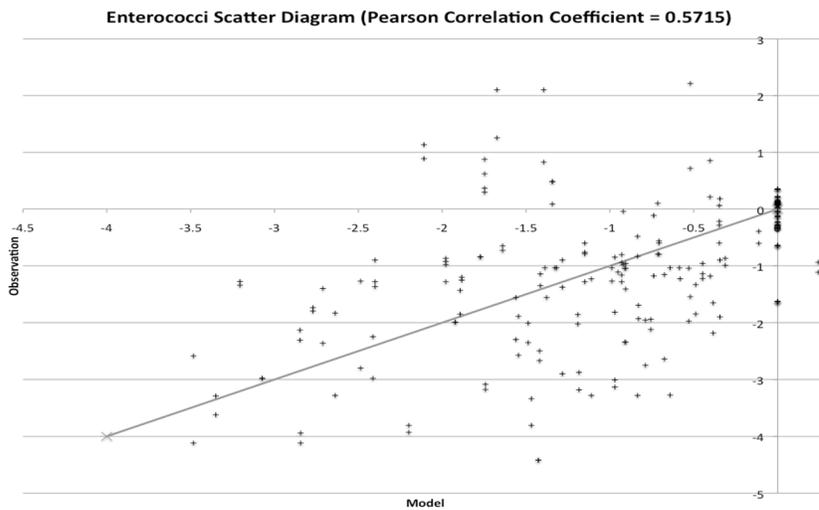


Figure 14.11 Model Predictions vs observations; line shows observation = prediction.

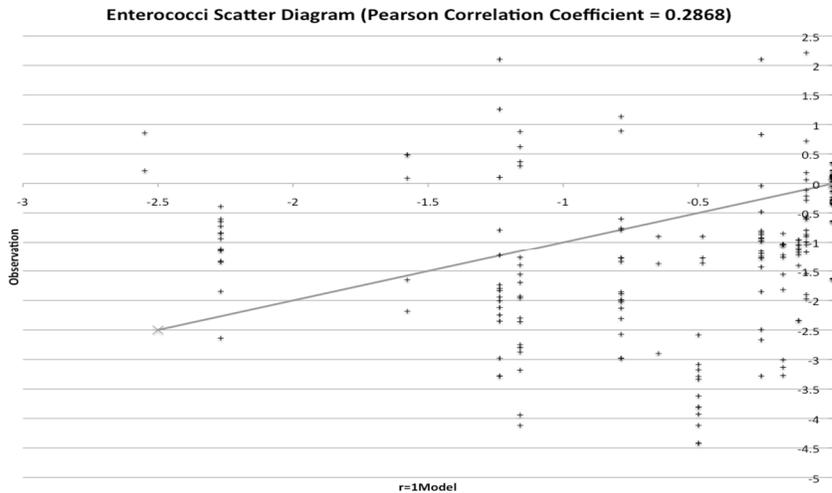


Figure 14.12 Paired observations vs predictions from a simple linear regression; line displays observation = prediction.

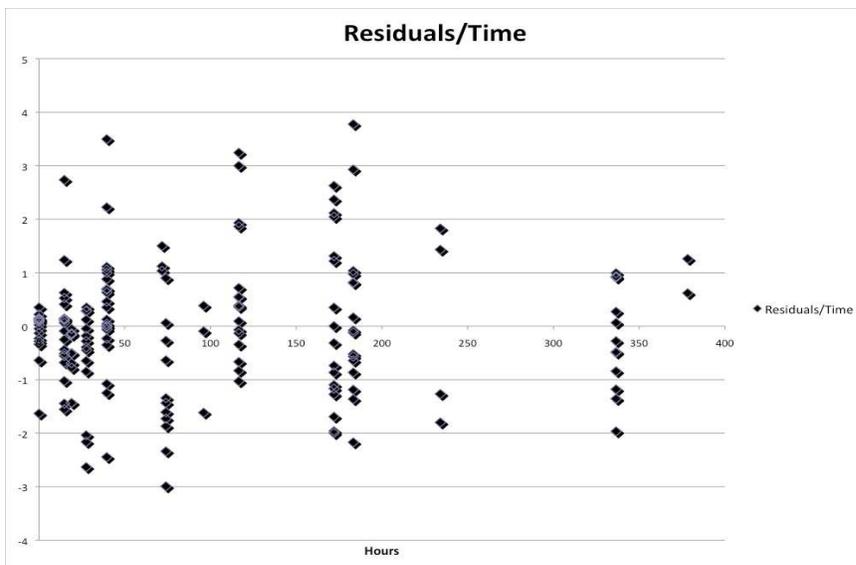


Figure 14.13 Residuals, over time, of the proposed model.

The model-derived parameters applied to our experimental conditions are presented at Table 14.4. 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 by two or three orders of magnitude in the initial period.

Table 14.4 Enterococci modeled parameters, applied to experimental conditions.

	k1 (1/hours)	BP (hours)	k2 (1/hours)
Cool/Dry/UV	-0.0501	70.0	0.00652
Cool/Dry/Dark	-0.0235	76.7	0.00652
Cool/Wet/UV	-0.0477	66.5	0.00652
Cool/Wet/Dark	-0.0211	70.5	0.00652
Warm/Dry/UV	-0.0359	63.2	0.00652
Warm/Dry/Dark	-0.0479	70.4	0.00652
Warm/Wet/UV	-0.0233	64.0	0.00652
Warm/Wet/Dark	-0.0353	68.6	0.00652

The insensitivity of k_2 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.

14.3 Conclusions

We developed the models presented here in support of an ongoing effort to model source area processes contributing to the 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* spp.) were found to be quite persistent (especially under environmental conditions that most closely approximate enteric conditions) on impervious surfaces subject to the extreme Tuscaloosa, Alabama 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 the 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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4.1.1.2 Appendices

The following appendices are not part of the above, reprinted article. They are presented here to provide continuity and integration within this dissertation

Appendix 1 FIB survival in feces

Considering the notoriously large variability historically found in environmental microbiological sampling efforts, together with the limited (three orders of magnitude) measurable span available in IDEXX numeration, preliminary research was conducted into the survival of FIB in feces (which should be viewed as a tertiary habitat for landscape FIB). A pair of basset hounds of some shared genetics (a mother/son pair) and shared diet provided FIB inoculants in this study. They also provided fecal material of precisely measurable age, in that I could record time of deposition. Feces were stored outdoors, sheltered from rain, at uncontrolled ambient conditions. Aged feces were slurried (distilled water) and analyzed at times ranging from about three hours after deposition (in transit to lab) to ~120 hours (the time at which feces had generally dry-crumbled or become fly-blown), to provide knowledge necessary to target sample dilutions likely to provide detectable observations. Results are presented at Figures 14.14 and 14.15 below.

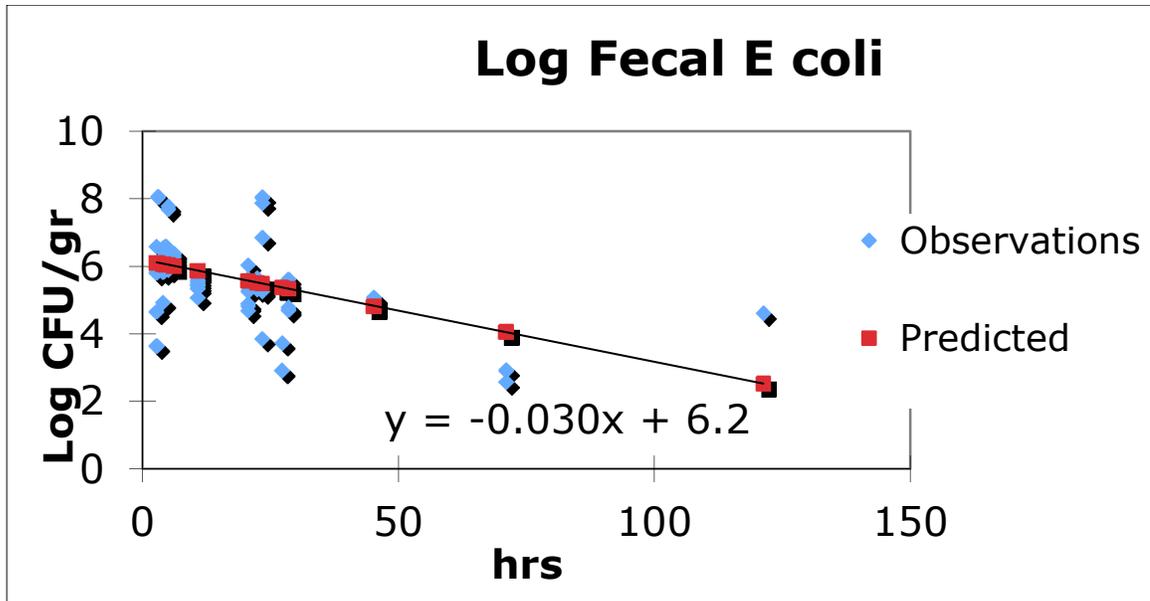


Figure 14.14 Log Fecal *E. coli*. n = 54, $r^2 = 0.26$

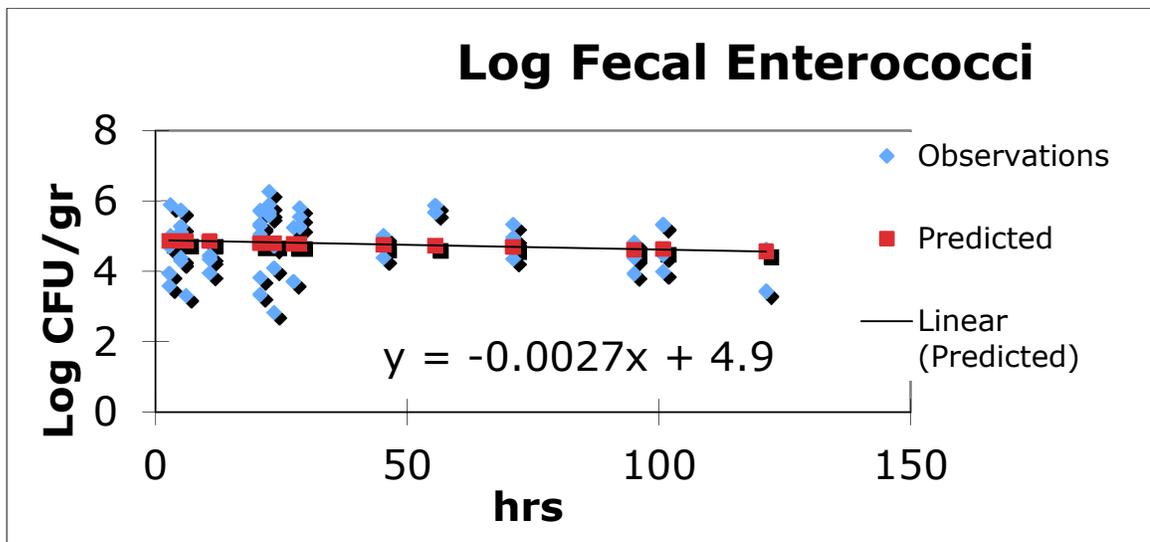


Figure 14.15 Log Fecal Enterococci. n = 54, $r^2 = 0.014$

These figures should not be seen as generally applicable. Fecal sources here were specifically chosen to reduce variability in measured FIB. The remaining variability displayed, however, does provide a cautionary perspective for further research.

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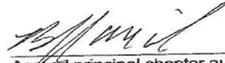
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Tuscaloosa, Alabama

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Cc: Brad Wilson <brad4d@hotmail.com>; Robert Pitt <rpitt@eng.ua.edu>; Dr. Elliott, UA <melliott2@bama.ua.edu>; Karen Finney <karen@chiwater.com>; Nandana Perera <nandana@chiwater.com>;

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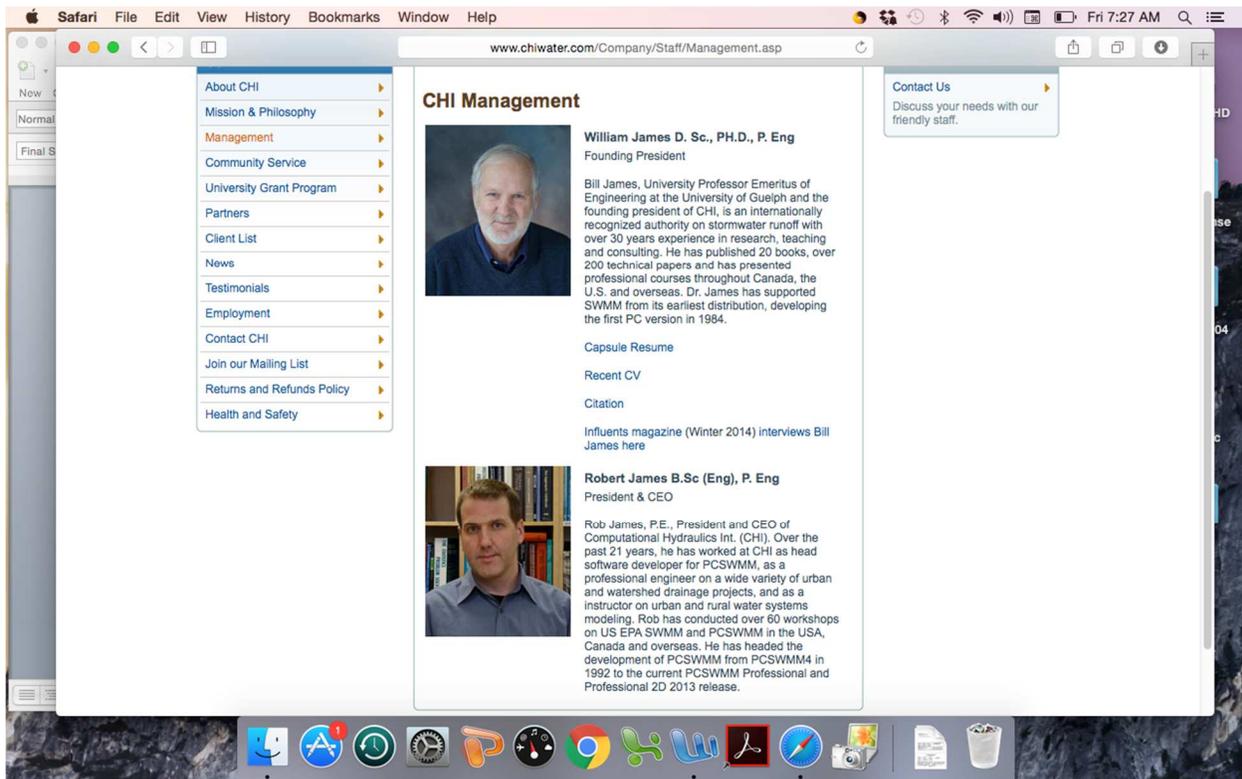


Figure 14.18 CHI Principles

4.1.2 Survival of Bacterial Indicator Species on Pervious Surfaces

An article-style manuscript is presented. As in the previous section, this article was originally written to the CHI style guide. Unlike the previous, however, it has not yet been submitted for publication. It is reprinted here in its entirety, and reformatted to conform to the requirements of this dissertation, with permission of the authors.

4.1.2

SURVIVAL OF BACTERIAL INDICATOR SPECIES ON PERVIOUS ENVIRONMENTAL SURFACES

Bradford M. Wilson, Robert Pitt, and Mark Elliott

A literature review revealed a need, by watershed managers, for some method of estimating the natural background (of non-sewage origin) of stormwater fecal indicator bacteria inputs to downstream waters. The same review, however, provided little information concerning the model parameters or even structure by which such estimation might be accomplished.

The study presented here investigates the survival of fecal indicator bacteria in stormwater source areas, between rains, on pervious surfaces. It represents the second in a planned series of scoping studies exploring the feasibility of constructing a model by which natural-background inputs might be predicted from watershed observations. Comparisons between this study and the previous, a similar study conducted on impervious surfaces, are also presented where relevant to the overall modeling effort.

4.1.2.1. Introduction

Due to difficulties in direct measurement of waterborne pathogens, the microbiological quality of waters is typically characterized on the basis of fecal indicator bacteria (FIB). FIB are assumed to derive from a common (historically sewage) source with pathogens of interest, and to arrive in, survive in, and move through watershed environments in numbers that correlate with the risk from those pathogens (the *indicator paradigm*). Commonly used indicators, however, also derive from sources other than sewage or even feces, and survive in the environment at rates

divergent from those of pathogens they are presumed to indicate (National Research Council, 2004).

Considerable expert consensus exists that FIB from non-human sources represent lesser correlative risk to human health than do those deriving from sewage (the *species barrier*). Much effort and money has been expended to confirm this assertion, but results are deemed equivocal by many regulatory authorities (*e.g.*, see Dufour, *et. al.*, 2012, and EPA, 2012) and the source of FIB is not considered relevant under many water-quality criteria (WQC).

Knowledge of the source of FIB, however, remains important to achieving *compliance* with WQC. Managers of tributary watersheds require knowledge of source, especially sewage vs. non-sewage, to manage/prioritize strategies for compliant contributions to downstream waters. Tools for mitigation of sewage effluents differ, for instance, from those relevant to managing squirrel-derived fecal material in stormwater runoff.

The study presented here explores the survival of commonly used FIB, between rains, on pervious stormwater-source surfaces. It is part of a series of scoping studies to explore the potential for modelling expected FIB in runoff from watershed observations of non-human fecal sources, a series that explores mechanisms of FIB release, and the form of such releases in response to rain, as well as interevent FIB survival. This article also presents comparisons and contrasts between this study and a previous work (Wilson and Pitt, 2013, which was focused on FIB survival on pavements), and conclusions concerning needs for future work.

4.1.2.2. Methods

4.1.2.2.1 Bacterial Cultivation and Enumeration

A full factorial study (2^5 , Temperature/Moisture/UV exposure/acidity/organics, the last three coded as presence/absence, and where UV = ultraviolet radiation, and see Box, *et al.*, 2005)

of environmental survival factors was performed separately for each of two FIB taxa: *Enterococcus* spp. (or Enterococci) and *Escherichia coli* (or *E. coli*). As in our previous, impervious-surface study, we hypothesized that the first three factors would be found significant to survival, but expanded this current study to include the last two, soil-water characteristics, for consideration.

Sandy loam from the B-horizon, underlying a well vegetated and undercut creek-bank, was dried and sterilized (at least to undetectable levels of tested FIB) in a home oven. This treatment (450^o F = 232^o C, for three hours) was also assumed to remove any bioavailable organics from the soil. The sterile soil was split to provide for four experimental soil-water treatments:

- Distilled water (and hereinafter *H2O* or unamended, to achieve an amendment of acidity- and organics-absent characteristics);
- Serially diluted molasses (*Mol*, in distilled water) to achieve 5% bioavailable organics, and which serendipitously provided pH=6.5;
- Serially diluted distilled grain vinegar (*Acid*), to achieve pH 6.5 and absent added organics (except for acetic acid which is not bioavailable to Enterococci, and is only available by an energetically unfavorable metabolic pathway to *E. coli*); and
- Serially diluted molasses (organics amended), as above, augmented with serially diluted baking soda (*MolB*) to achieve pH neutrality.

Each soil split was saturated with the simulated soil waters, checked for pH (+/- 0.05 pH units by lawn-soil pH meter) and allowed to air-dry in a laboratory hood. Each simulated soil type was further split into 1-tablespoon (1 tbsp = 14.8 mL) aliquots to serve as pervious-surface experimental microcosms. Each such microcosm was inoculated with 1 mL of pet-feces and distilled-water (dog feces obtained from a local veterinarian, and discussed with greater detail and relevance at 4.1.2.2.2, below), slurried in a 1-L laboratory blender (WaringTM) at low speed

(18,000 rpm) for two minutes. All such microcosms were distributed between four controlled-environment chambers. Each such chamber was a freezerless refrigerator fitted with:

- Commercial biological oxygen demand (BOD) controllers/heaters (BOD-cubator™) or the refrigerator-installed thermostats for temperature control;

- Desiccants (DampRid™) or nursery humidifiers (Johnson and Johnson™) for moisture control; and

- UV-enhanced fluorescent tubes (StarLites™), designed to mimic the insolation spectrum. Lexan™ panels, very nearly UV-opaque, were used to split the chambers into UV-exposed and UV-shaded regions.

Active control of temperature (40^o F and 90^o F, 4.4^o C and 32^o C, cool/low or warm/high) held the parameter steady ($\pm 2^{\circ}$ F, 1.1^o C) over the study period. Relative humidity levels (25% and 80%, dry/low or wet/high) varied over about $\pm 4\%$. UV exposure was coded as present or absent (UV exposure as shade-absent, dark as shade-present).

Intermittently (targeting at Days 0, 1, 2, 3, 5, with intervals subsequently expanding to ~ 4 days) over the multiple study period exposure periods (about two weeks), exposed microcosms were subjected to mechanical biofilm disruption by the method provided by Boehm, *et. al.*, 2009. Those authors conducted a comparative study of 22 methods for extraction of FIB (*E. coli* and Enterococci) from sandy matrices. They found that two minutes vigorous shaking, by hand, of samples in deionized (we used distilled) water, allowed to settle for 30 seconds, provided repeatable, high FIB recoveries in the eluent. Washed-off samples were immediately mixed with defined-substrate formulations (Colilert/Enterolert™) for relief of osmotic stresses. The logarithms of 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 the average inoculation-date (Day 0) values. IDEXX reagents (Colilert and Enterolert) provide for selective incubation of the taxa of interest, and colorimetric and fluorometric indicators of

culturable CFU in 24 h, and are recognized water assays under Standard Methods for the Examination of Water and Wastewater (21st Edition, Eaton *et. al*, 2005, sections 9223 and 9230b, respectively). MPN measurement values with three orders of magnitude range (from 1 to 2420 MPN/100 mL) are directly available with the reagents when incubated in Quantitray 2000TM units. We applied historical knowledge to arrive at likely estimates of appropriate sample dilutions to achieve results within the detection limits.

4.1.2.2.2 Analyses

Breakpoint Analysis

At a given set of conditions, bacterial populations are expected to grow or decline in a first-order (log-linear) manner:

$$\text{Log (MPN/initial MPN)} = k*t \quad (1)$$

where:

k = net growth constant (slope), and

t = time (here, in hours).

This expectation arises from the nature of bacterial reproduction (*binary fission*) where viable population change is the sum of cells that double less the cells that die. Bacterial cells, however, are more complex and adaptable than more mundane chemical species. Faced with a change in environmental conditions, as would be expected when deposited on landscape surfaces, bacterial populations may undergo sequential changes in k (up to four phases of the *growth cycle*, described in detail in the literature review) in response to new surroundings (Madigan, *et. al.*, 2002, pp. 142-151).

These considerations suggest that analysis of processes under study here should take the form of segmented log-linear regression with unknown breakpoints, and with imposition that the

regression is continuous at the breakpoint. Lerman (1980) provides a grid-search algorithm, implementable in a spreadsheet (ExcelTM used throughout), by which the maximum likelihood estimation (MLE) of the breakpoint abscissa of such a regression might be determined. The MLE breakpoint (BP) is the point at which the sum, of the Sum of Squares of Residuals (SSEs) of both segments joined at that point, is minimized. In Lerman's procedure, trial breakpoints (tBPs) are mapped across the range of the observations and the sum of SSEs at each tBP is determined. Progressive refinement (finer grain) of the grid provides the MLE BP, where the ordinate of the graphed function is minimized. Lerman's method also provides an estimate of the variability associated with the MLE BP. Specifically, a horizontal line drawn at the summed SSE at its minimum, plus the Mean Squared Error (MSE) of the likelihood function calculated at the SSE minimum, intersects the likelihood function at points providing the basis for an estimated confidence interval around the found BP (illustrated, *e.g.*, at Figure 4.1.2.1, reprinted from Wilson and Pitt, 2013 with permission of the authors). Bai and Perron (2003) show that MLE breakpoints can be sequentially added/inserted into existing regression models (*e.g.*, into a purely linear regression, or into either segment of a 1-breakpoint model, *etc.*) to derive a log-likelihood ratio (with calculated critical values) by which such any added BP might be deemed spurious.

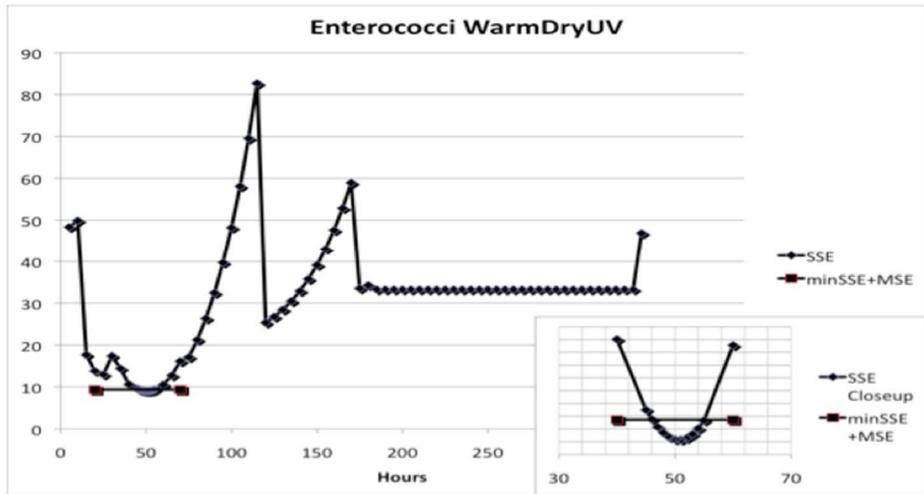


Figure 4.1.2.1 Example breakpoint analysis of a treatment (reprinted from Wilson and Pitt, 2013).

Censored Data

The experimental methods outlined thus far parallel those used in the previous, impervious-surface study as much as possible, in order to allow legitimate comparisons between the works. The dataset derived here, however, was problematic in that a considerable fraction of results were non-quantifiable (either above or below the detection limits, ADL and BDL respectively, of the IDEXX enumeration system). Besides the notoriously large general variability of microbiological measurements, two factors contributed.

First, we lost our primary FIB sources subsequent to the earlier study. Adeline and Lightnin', a mother/son pair of basset hounds of shared diet had provided fecal material of reduced measurement variability and of precisely measurable age. Their deaths forced us to rely on feces from the kennel of a local veterinarian, derived from dogs of unknown parentage and diet, and deposited at unknown times within about a fourteen-hour (overnight) window. This had the effect of reducing the value of our knowledge base in our effort to target sample dilutions likely to fall within the detection range of our enumeration methods. Additionally, our expansion

to examine the soil-water factors in the current (2^5) study reduced our capacity to introduce multiple dilutions to the available incubator space for MPN measurement.

These non-detects represent *nonignorable* gaps in the dataset, in that they are not random deletions but are mechanistically imposed by *censoring* of the dependent-variable *observations* at the detection limits (DL) of our measurement system. These deletions represent *partial information* that must be accounted for to avoid biased analysis (Little and Rubin, 1987, pp. 8-14). This complication is only exacerbated by our assumed need to model FIB survival at conditions of a given treatment as segmented and log-linear, and is exemplified at Figure 4.1.2.2, the organics-amended and pH-neutralized (MolB) treatment exposed to warm/dry/shade-present conditions. Simple inspection of the WarmDryDark series, together with the censored point (a BDL, *i.e.*, *left-censored*, observation, and plotted here as half the lower detection limit, LDL, for the dilution analyzed, and labeled as $\frac{1}{2}$ LDL) reveals that the abscissa (or even the significant presence) of any breakpoint in the system is dependent on to which segment the censored point is assigned. Clearly, and every bit as important, is the *extent* to which that point is censored (by how much the *actual* MPN, if uncensored, would exceed the detection limits) and how that censored point might effect the MLE slope of the segment to which it is assigned.

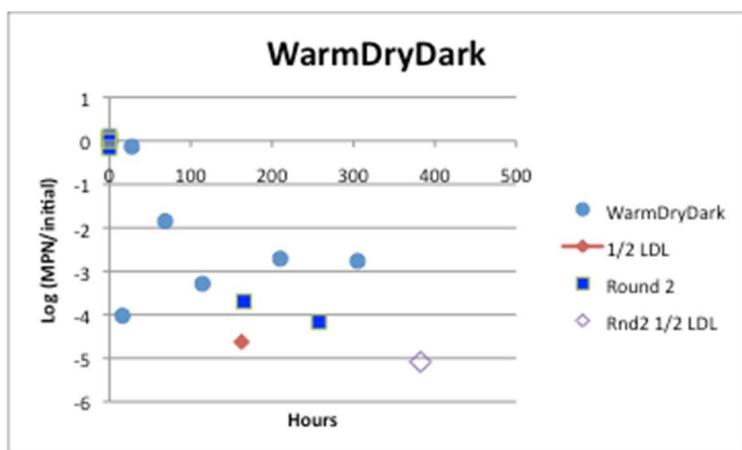


Figure 4.1.2.2 An example (*E coli*, molasses and bicarb – *MolB* - amended) treatment dataset. LDL = Lower Detection Limit.

The specific mechanism leading to the missing (censored) values is *known*, in that we know (from sample-dilution information and IDEXX detection limits) both the value at which missing observations were censored, and the values (DL) at which measurable observations would have been censored (Little and Rubin, 1987, pp. 218-223). Our knowledge of the mechanism of censoring suggests use of a *censored regression* model for each segment of each treatment. Such a model (sometimes named a generalized Tobit model after the special-case prototype) is an inherently iterative method accounting for both the distribution of quantifiably measured observations and the probability of any observation actually being quantifiably measured (*i.e.*, the *partial* information available here). Censored-regression modeling is available in some commercial statistical packages (e.g., EViews8™, which was used here).

The censored-regression approach provides MLE of regression parameters (the slope and its variability) of interest here, as they *would have been* modeled from the *latent variables* (*i.e.*, the dependent-variable observations as if they had not been censored). Specific inputs required are the abscissae of all observations, the ordinates and potential censoring limits of all quantifiable observations, and the actual DL at which censored observations were actually

censored (all available from our censored dataset). Use of such a method, however, complicates (through denial of an array of measurable residuals) traditional goodness-of-fit quantifications for any modeling efforts going forward. This complication is discussed in more detail below (subsequent to presentation of Figure 4.1.2.3 which will aid in illustration). It also deserves a more extensive illustration, after presentation of both full datasets, which appears as an endnote at section 4.1.2.5, below.

In an effort to improve the quality and quantity of information available for our censored-regression analyses, we repeated our data-collection effort as outlined at 4.1.2.2.1 above. We used leftovers of the split, amended-soil simulants (still stored in the indoor-ambient laboratory hood) and the same settings on the environmental chambers as before. We again normalized all MPN measurements to their Day 0 (inoculant) values, to preserve comparability between the two sampling runs. We targeted elution/analyses timing in this second round to bracket non-detects in the first, and targeted dilutions for analysis based on updated information from the first round. This resulted in additional quantified observations (*e.g.*, Round 2 at Figure 4.1.2.2) and additional censored observations (Round 2, $\frac{1}{2}$ LDL at Figure 4.1.2.2). We combined the two resulting datasets into one. For each experimental treatment in the combined dataset, and for each tBP in our grid search (Breakpoint Analysis, above), we calculated the censored-regression estimated slopes and SSEs in EViews8. Parameters from these analyses were pasted into our breakpoint-analysis spreadsheet for determination of MLE BP abscissae (in hours), significance (with a standard requiring 90% confidence that the null hypothesis, of no real breakpoint, is rejected), and estimated variances. Ordinate of the MLE breakpoint was found by imposition of continuity between adjoining segments. An illustrative example of a treatment, in which a

significant breakpoint was found (and the same treatment presented at Figure 4.1.2.2), is shown at Figure 4.1.2.3.

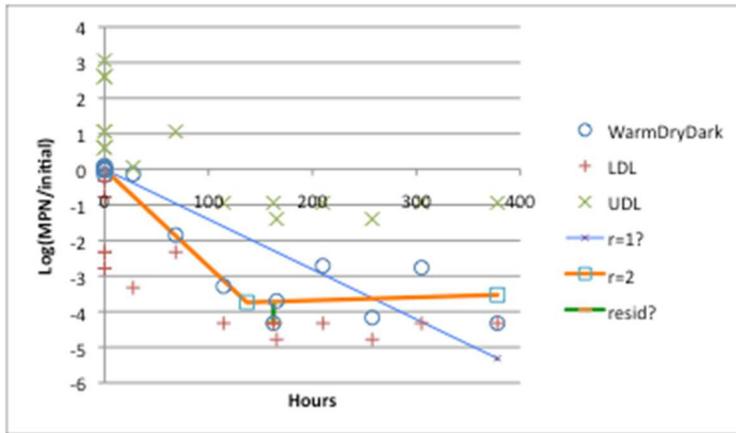


Figure 4.1.2.3 An example (*E. coli*, organics-amended, pH-neutralized) treatment breakpoint model in which UDL = Upper Detection Limit, and LDL = Lower Detection Limit for each observation. $r=1?$ is the (null) no-breakpoint censored-regression model, and $r=2$ is the found two-segment model, resid? provides an example of an unquantifiable residual.

The goodness-of-fit complication arising from use of censored-regression methods results directly from the *partial* nature of information available in the censored dataset, and is worthy of some discussion here. It can be illustrated by returning our attention to Figures 4.1.2.2 and 4.1.2.3, above. In Figure 4.1.2.2, we plotted the BDL, left-censored, observations, at ordinates corresponding to $\frac{1}{2}$ of their (LDL) censoring points, conforming to a fairly conventional graphical representation of BDL data. In Figure 4.1.2.3, we plotted those same points *at* their point of censoring (with the censoring LDL graphically superimposed). This latter representation also conforms to a common graphical convention for BDL data and, additionally, conforms to inputs required for the censored-regression iterative algorithm. *Neither* of these plotting conventions reveals any kind of known (or even knowable) ordinates for the latent-variables underlying these censored observations. Ordinates of the latent-variable underlying these BDL observations are bound on only one side (by the LDL). Similarly, latent-variable ordinates

corresponding to ADL (right-censored) observations are unbound above the Upper Detection Limit (UDL). While the likelihood function (which includes the distribution of censoring DL, EViews8, 2013, Censored Regression Models, esp. Eq. 27.27), maximized in the censored-regression model, provides the MLE parameters of interest, it leaves any particular censored *observations* in the dataset only partially observed. The *extent* of censoring for any such observation remains unknown.

Any attempt to equate the observed LDL of a left-censored observation in either of these figures with a fully quantifiable observation (as would be available in an Ordinary Least Squares, OLS, analysis), frustrates any meaningful determination of a traditional residual. Even though we have at least an MLE of the modeled projection from any observation, we only partially know the latent-variable observation on which that projection is based (see the *resid?* series at Figure 4.1.2.3). Moreover, the needed inclusion of censored-observation DL in the dataset frustrates any meaningful determination of the average observation (y_{avg}) of the set and, thus, a well-defined residual attributable to even a fully quantifiable observation. In OLS analyses, we would have the luxury of equating the total deviation of an observation from the average ($y_i - y_{avg}$) as the sum of the model deviation from the average ($y_{model} - y_{avg}$) and observation's deviation from the modeled projection (the residual, $y_i - y_{model}$). In our censored system, we are denied the *expectation* that a residual should equal zero.

Such, it would seem, is the nature of censored-regression analyses. Each censored observation available in the dataset is an inherently biased individual estimate (because of the *partial* nature information provided) of the latent-variable observation upon which we base our MLE projection from that censored observation. Moreover, any set of residuals based only on the fully quantifiable (uncensored) observations likewise introduces systemic bias to analyses, in that

we are ignoring the *nonignorable* partial information that *is* available in the censored data (Little and Rubin, 1987, pp. 8-14) that provides the overall MLE model parameters, and that makes recourse to censored-regression models necessary in the first place. We are denied the goodness-of-fit tools (*e.g.* graphical analysis of residuals, and the Correlation of Determination, r^2 , that is based on those residuals) typically available in OLS analyses (and we have illustrated these difficulties for the complete datasets at 5.1.2.5 below). We need to rely on more qualitative tools, largely based on graphical analysis of correlative scatterplots (performed here in StatPlus for Mac™).

Factorial Analysis

Parameters derived from the above treatment analyses served as inputs to a traditional factorial analysis (see Box, *et. al.*, 2005, Chapter 5) to determine the significance of effects (at 95% confidence level) of each environmental factor studied (Table of Contrasts below) for each studied FIB taxon. Treatments exhibiting significant breakpoints in the grid-search analysis provided two censored-regression estimated slopes (of segments joined at the found breakpoint) and estimated variances of those slopes. No treatment in the current study exhibited more than one significant break. Abscissae of the found breakpoints, and the MLE variance around the breakpoint were derived from the grid-search exercise itself. Treatments showing no significant breakpoint were necessarily considered with an imposed breakpoint at the abscissa corresponding to the n -weighted average of those that were found, and with equal slopes derived from the censored-linear regression applied to the joined segments so imposed, to avoid bias in the factorial analysis.

Treatment	T	H	S	O	A	TH	TS	TO	TA	HS	HO	HA	SO	SA	OA	THS	THO	THA	TSO	TSA	TOA	HSO	HAS	HOA	SOA	THSO	THSA	THOA	TSOA	HSOA	THSOA		
CoolDrUVH2O	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	-1	
WarmDryUVH2O	1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1	1
CoolWetUVH2O	-1	1	-1	-1	-1	-1	1	1	1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	-1	-1	1	-1	1	
WarmWetUVH2O	1	1	-1	-1	-1	1	-1	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	1	1	1	1	1	1	-1	1	1	1	-1	-1	-1	-1	
CoolDryDarkH2O	-1	-1	1	-1	-1	1	1	1	1	-1	1	1	1	1	1	-1	-1	-1	1	1	-1	1	1	-1	1	-1	1	-1	1	-1	-1	1	
WarmDryDarkH2O	1	-1	1	-1	-1	-1	1	-1	-1	-1	1	1	-1	-1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	1	1	-1	1	-1	-1	
CoolWetDarkH2O	-1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	-1	1	-1	1	1	1	1	1	-1	-1	-1	1	1	1	1	1	-1	-1	1	-1	
WarmWetDarkH2O	1	1	1	-1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	-1	-1	-1	1	-1	-1	1	-1	-1	1	1	-1	-1	1	1	1	1	
CoolDrUVMol	-1	-1	-1	1	-1	1	1	1	1	-1	1	1	1	1	-1	-1	1	-1	1	-1	1	-1	1	-1	1	1	-1	1	-1	-1	-1	1	
WarmDryUVMol	1	-1	-1	1	-1	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	1	1	-1	1	1	-1	-1	
CoolWetUVMol	-1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	1	-1	-1	1	-1	-1	
WarmWetUVMol	1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	1	1	
CoolDryDarkMol	-1	-1	1	1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	-1	1	-1	-1	1	1	-1	1	1	-1	1	-1	1	-1	-1	1	-1	
WarmDryDarkMol	1	-1	1	1	-1	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	-1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1	1	
CoolWetDarkMol	-1	1	1	1	-1	-1	-1	1	1	1	1	-1	1	-1	-1	-1	1	-1	1	-1	1	1	-1	-1	-1	-1	-1	1	1	1	-1	1	
WarmWetDarkMol	1	1	1	-1	1	1	1	1	-1	1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	-1	1	-1	-1	-1	1	-1	-1	-1	-1	-1	
CoolDrUVAcid	-1	-1	-1	-1	1	1	1	1	-1	1	1	-1	1	-1	-1	-1	-1	1	-1	1	1	1	-1	1	1	1	1	-1	-1	-1	-1	1	1
WarmDryUVAcid	1	-1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	-1	1	1	-1	1	-1	-1	-1	1	1	1	1	-1	1	1	1	-1	-1	
CoolWetUVAcid	-1	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	-1	-1	1	-1	-1	-1	1	1	1	-1	-1	1	-1	1	-1	1	-1	-1	-1	
WarmWetUVAcid	1	1	-1	-1	1	1	-1	1	-1	1	-1	1	1	-1	-1	-1	1	-1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	
CoolDryDarkAcid	-1	-1	1	-1	1	1	-1	1	-1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	-1	1	-1	-1	-1	-1	1	-1	1	1	-1	
WarmDryDarkAcid	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	1	
CoolWetDarkAcid	-1	1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	-1	-1	-1	1	-1	1	-1	1	-1	1	-1	-1	-1	1	-1	1	-1	-1	1	
WarmWetDarkAcid	1	1	1	-1	1	1	1	-1	1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	-1	1	-1	-1	-1	1	-1	-1	-1	-1	-1	
CoolDrUVMolB	-1	-1	-1	1	1	1	1	-1	-1	1	-1	-1	-1	1	-1	1	1	1	1	-1	1	1	-1	1	-1	-1	-1	-1	1	1	1	-1	
WarmDryUVMolB	1	-1	-1	1	1	-1	-1	1	1	1	-1	-1	-1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	-1	-1	1	-1	-1	1	1	1	
CoolWetUVMolB	-1	1	-1	1	1	-1	1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	-1	-1	1	
WarmWetUVMolB	1	1	-1	1	1	1	-1	1	1	-1	1	1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	-1	-1	-1	-1	-1	1	-1	-1	-1	
CoolDryDarkMolB	-1	-1	1	1	1	1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	-1	-1	-1	-1	-1	-1	-1	1	1	1	-1	-1	-1	1	
WarmDryDarkMolB	1	-1	1	1	1	-1	1	1	1	-1	-1	-1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	-1	-1	1	-1	-1	1	-1	-1	-1	
CoolWetDarkMolB	-1	1	1	1	1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	-1	
WarmWetDarkMolB	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 4.1.2.1 Table of Contrasts for 2⁵ factorial. T = Temperature (+ is 40°F, - is 90°F), H = % Humidity (+ is 90% RH, - is 25% RH), S = Shade Code (+ is UV shading present, - is shade absent), O = Organics Code (+ is organics amended, - is no added organics), A = Acidity Code (+ is acid amended, - is no added acidity, Letter combinations represent interactions.

4.1.2.3 Results and Discussion

4.1.2.3.1 *E. coli*

Segmented regression models of all *E. coli* (censored) treatments are presented at Figure

4.1.2.4. A rather striking feature, clearly visible in the figure, is the surprising paucity of significant breakpoints found (twelve out of 32 treatments). Additionally, it is notable that there are almost as many treatment models (five) exhibiting apparent loss of fitness over the study period as those showing adaptive improvement (seven). Finally, worthy of note here, six of the seven treatments revealing successful adaptation were found with the *MolB* (organics amended, pH-neutralized) soil-water simulant. These features represent profound differences in FIB responses as compared to those displayed in the previous, pavement study, and will receive discussion below in Section 4.1.2.4.

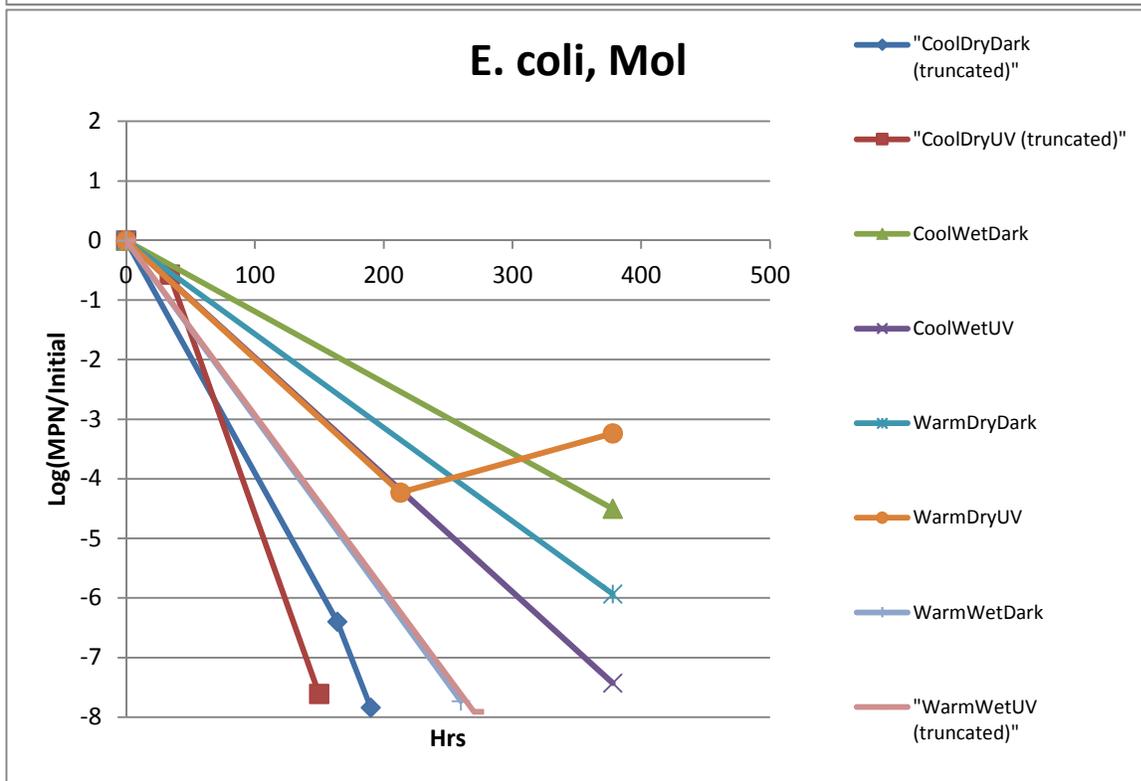
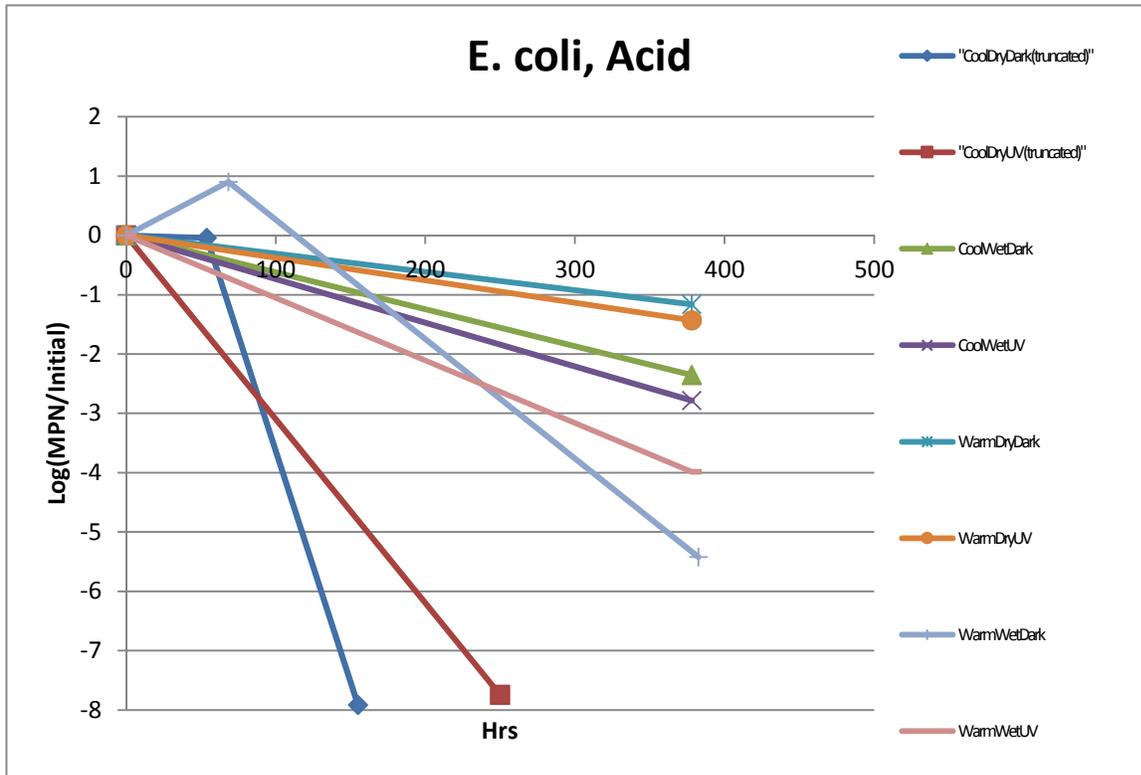


Figure 4.1.2.4 *E. coli* treatment models, panels separated by soil amendments for clarity.(Part 1/2).

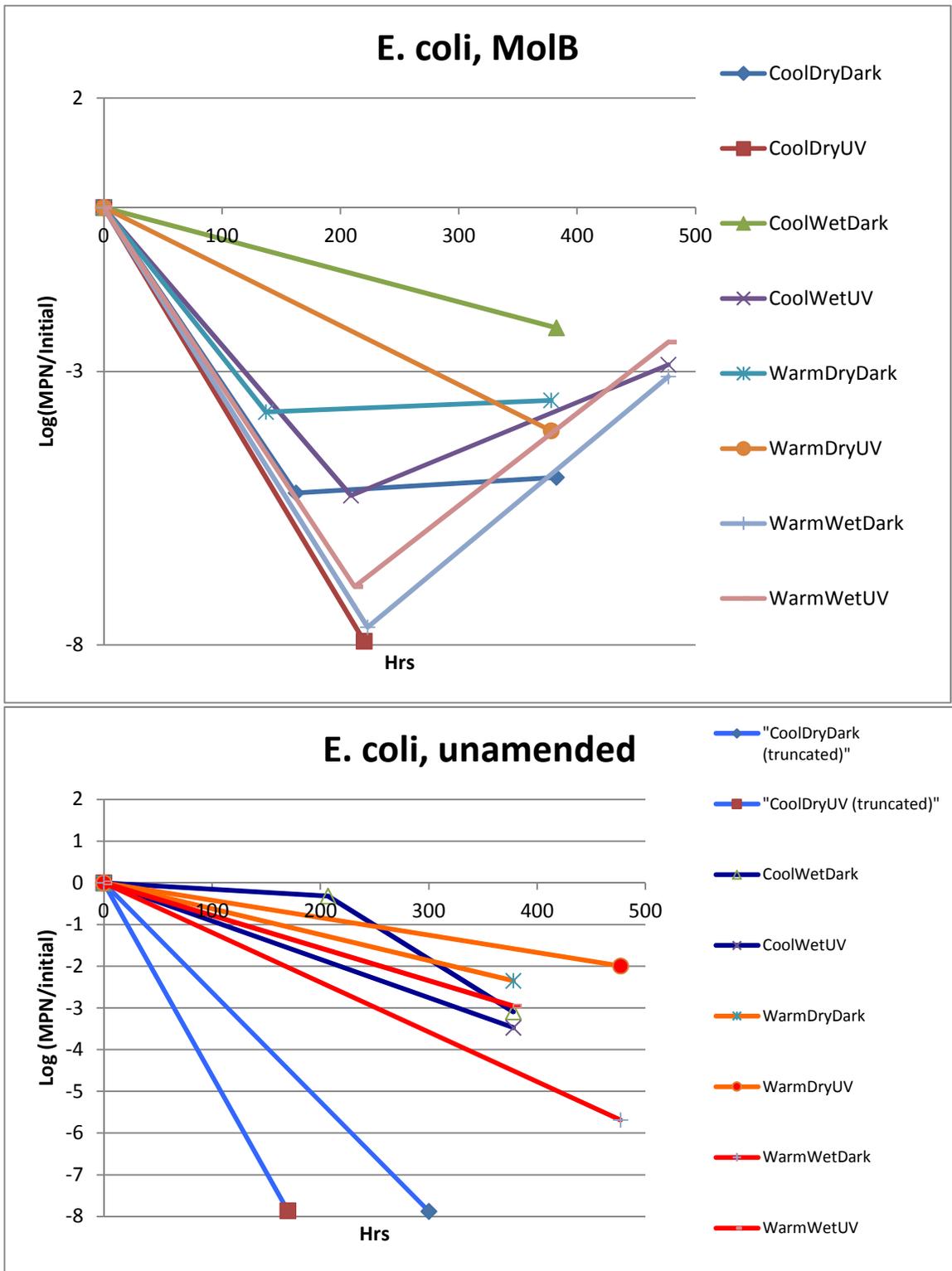


Figure 4.1.2.4 *E. coli* treatment models, panels separated by soil amendments for clarity. (Part 2/2). “unamended” = only distilled water added, *Acid* = vinegar amended, *Mol* = molasses amended, *MolB* = molasses and baking-soda amended (see 2.1, above).

An important question arising at this stage of analysis, however, is whether or not any factorial modeling effort of this dataset would result in a segmented system. With dummy breakpoints imposed on unsegmented treatment models at the n-weighted average abscissa of the found breakpoints, n-weighted $k1$ (the slope exhibited prior to the average breakpoint hour) and the subsequent slope $k2$ are very nearly equal (~ -0.017). A pooled-variance t-test for difference of the adjoined slopes provides little evidence (at $p = 0.997$) for rejection of the null hypothesis (that the slopes do not significantly differ and that no real breakpoint exists in the overall model). Consequently, the parameters (MLE slopes and variances) derived from the censored, unsegmented treatment models served as inputs for factorial analysis, and provided for unexpected results that (again) must be discussed below (Section 4.1.2.4). None of the studied factors, nor any of their interactions, was found to be a significant contributor (at our 95% confidence threshold) to patterns of *E. coli* survival on pervious surfaces. By far, the largest factorial effect found, namely that of the Temperature/Humidity interaction, provides less than 50% confidence that it is not zero (and see Figure 4.1.2.5). The overall MLE survival model derived is:

$$\text{Log (MPN/initial MPN)} = -0.017 * t \text{ [hrs]} \quad (2)$$

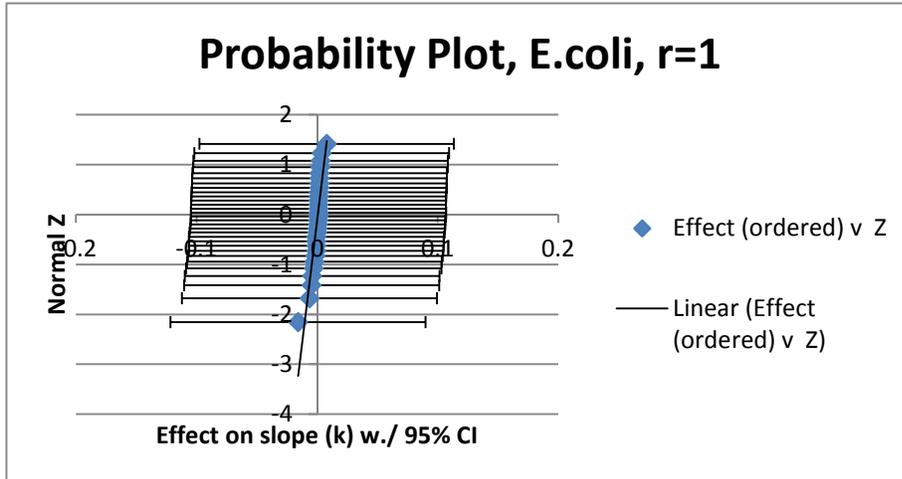


Figure 4.1.2.5 Normal Probability Plot of (rank ordered) effects on *E. coli* survival-rate constant. Confidence intervals of effects are from factorial apportionment of pooled (MLE) treatment variances.

A plot comparing modeled predictions and observations is presented at Figure 4.1.2.6, and provides an illustrative example of problems inherent in quantifying goodness-of-fit for models of censored datasets. The censoring points (DL) of observations have been superimposed on the figure.

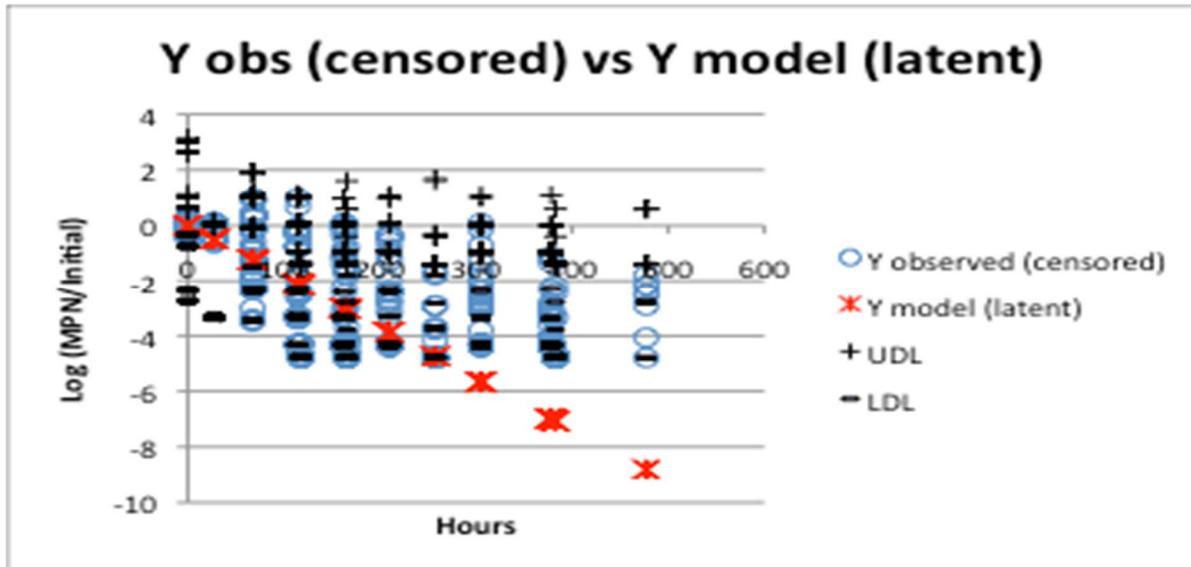


Figure 4.1.2.6 Observed (as censored) vs. modelled survival, *E. coli*. UDL = Upper Detection limit, LDL = Lower Detection Limit.

A large number (133 out of 336) of observations here are censored one way or another and presented here at their respective detection limits. Of the 133 such censored observations,

most (121) are left censored (*i.e.*, at the LDL) while only 12 are right censored. The limit to which we could concentrate our pet-feces slurries and still read the IDEXX fluorometric indicators played a part. Right-censored observations could be followed by a subsequent analysis of a more dilute sample. Adjusted follow-up for left-censored observations, however, was limited by the LDL of an undiluted sample. Many of the LDL shown at Figure 4.1.2.6 (and, indeed, *most* of those plotted at greater than 350 hours) represent treatments exhibiting left censoring at no dilution, and for which follow-up analyses were terminated (subsequent BDL results would be *ignorable* in the sense of Little and Rubin, 1987, pp. 8-14).

With our limited knowledge of the extent to which censored observations are censored, we are denied recourse to the coefficient of determination (r^2) available in typical ordinary least-squares analyses. In an asymmetrically censored dataset such as this one, a simple equation of censoring limits with measurably observed data could well result in a regression sum of squares (SSR) exceeding the total sum of squares (SST), and an r^2 much greater than unity (and the lead author here stubbornly confirmed that this was so for this dataset). Moreover, with the MLE slope (based on the latent variables) projecting well outside the array of observations (including censoring limits) a residuals plot does not (nor should it be expected to) conform to OLS standards for visualization of goodness of fit,

We are restricted to a more qualitative goodness-of-fit analysis of this system, graphically illustrated at Figure 4.1.2.7. The presented scatter-plot of the predicted *vs.* observed (the latter, again, including detection limits for censored points) dependent variable ($Y=\log(\text{MPN}/\text{initial})$) provides some confidence in that the series are positively correlative. The magnitude (Pearson Correlation Coefficient is only 0.63) of that correlation, however, severely limits that confidence.

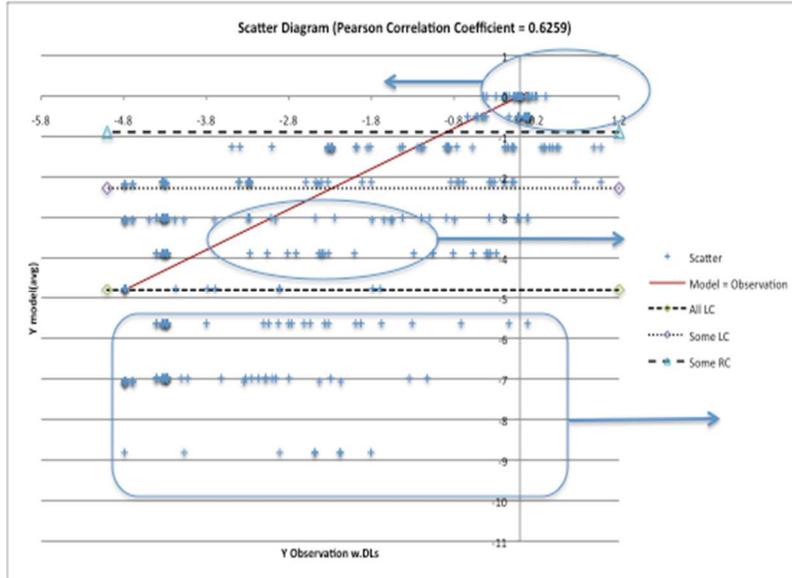


Figure 4.1.2.7 Model vs. Observed (as censored) Scatter Plot, *E. coli* survival, where lc=left censored, rc=right censored. All points within rectangle-marked bands have been shifted by censoring bias. Some points within oval-marked bands have been so shifted (see text).

Like the residual analyses discussed above, this measurement of a linear relationship between the censored observations and the latent-variable predictions is distorted by bias in the former. The scatterplot, however, provides an opportunity for visualization of that bias. The minimum point at which any observed dependent variable (Y , the log of normalized survival) was censored was found at $Y = -4.79$. Every point in Figure 4.1.2.7 that lies below the *All LC* horizontal (and boxed by a rounded rectangle in the figure) in the figure represents a modeled prediction that is displaced (graphically to the right), to some unknown extent, away from the point at which an uncensored latent-variable observation would have been plotted, and away from the perfect correlation line (*Model = Observation* on the figure) by censoring bias. Likewise, some points plotted below the *Some LC* horizontal (and marked with an oval in the figure, the maximum found left-censoring point $Y = -2.275$) have been so displaced, and points plotted above -0.891 (*Some RC* on the figure) have been displaced in the opposite direction.

While it is easy to conclude that the correlation coefficient found is *at least* as positive as it would have been without censoring, there is also considerable reason to suggest that it would have been more largely positive without censored data.

Censoring the *predictions* at the points at which they *would have been* censored, if they had been measured with the same method and at the same dilution as their corresponding observations, provides for a less biased comparison (Figure 4.1.2.8). Though the inherent bias from censored observations remains, they are compared to model predictions with similar bias imposed. It even provides a system that yields a *meaningful* r^2 (in that $0 \leq r^2 \leq 1$) but it only *means* the extent to which our modeling effort explains the censored dataset (and not the underlying latent variable). Such an exercise yields $r^2 = 0.69$ (with an implied Pearson coefficient of 0.83), which must be viewed as a maximum limit to any measurement of fit. Clearly, by any measure, considerable variability exists in the actual behavior of *E. coli* on pervious surfaces that is not explained by our modeling efforts.

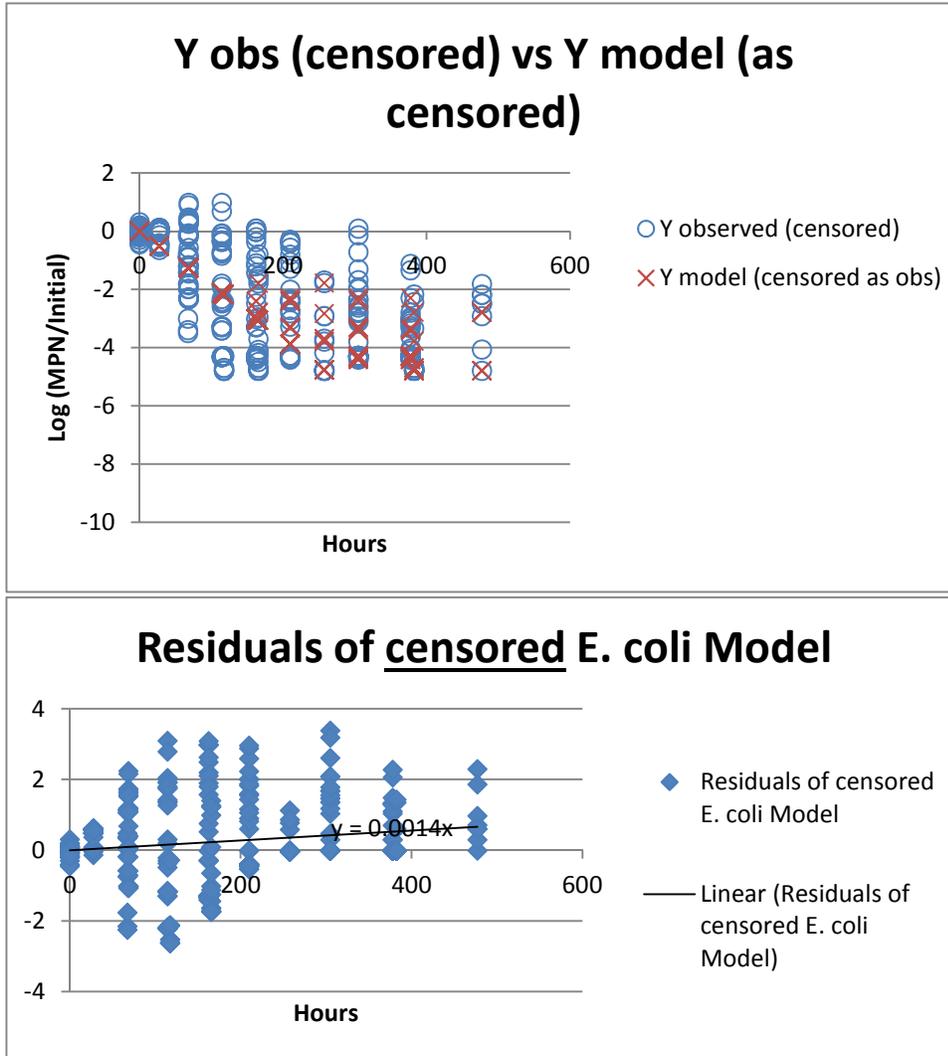


Figure 4.1.2.8 Model vs observations (both series censored) of *E. coli* survival on a pervious surface.

4.1.2.3.2 Enterococci

Figure 4.1.2.9 shows results of the (grid-searched) MLE segmented-regression Enterococci treatment models derived from the array of censored-regression segment slopes. The rarity of treatments exhibiting significant breakpoints (four of 32, and all four of which were exposed to Warm Dry conditions) is, again, apparent. Unlike the *E. coli* studied above, however, Enterococci present adjoining segments of sufficiently different slopes to warrant breakpoint modeling of the overall system. With the dummy breakpoints imposed, we can confidently reject

the null hypothesis (that the n-weighted $k_1 = -0.0062$ and $k_2 = 0.0021$ do not significantly differ and that no breakpoint likely exists, $p < 7 \times 10^{-6}$).

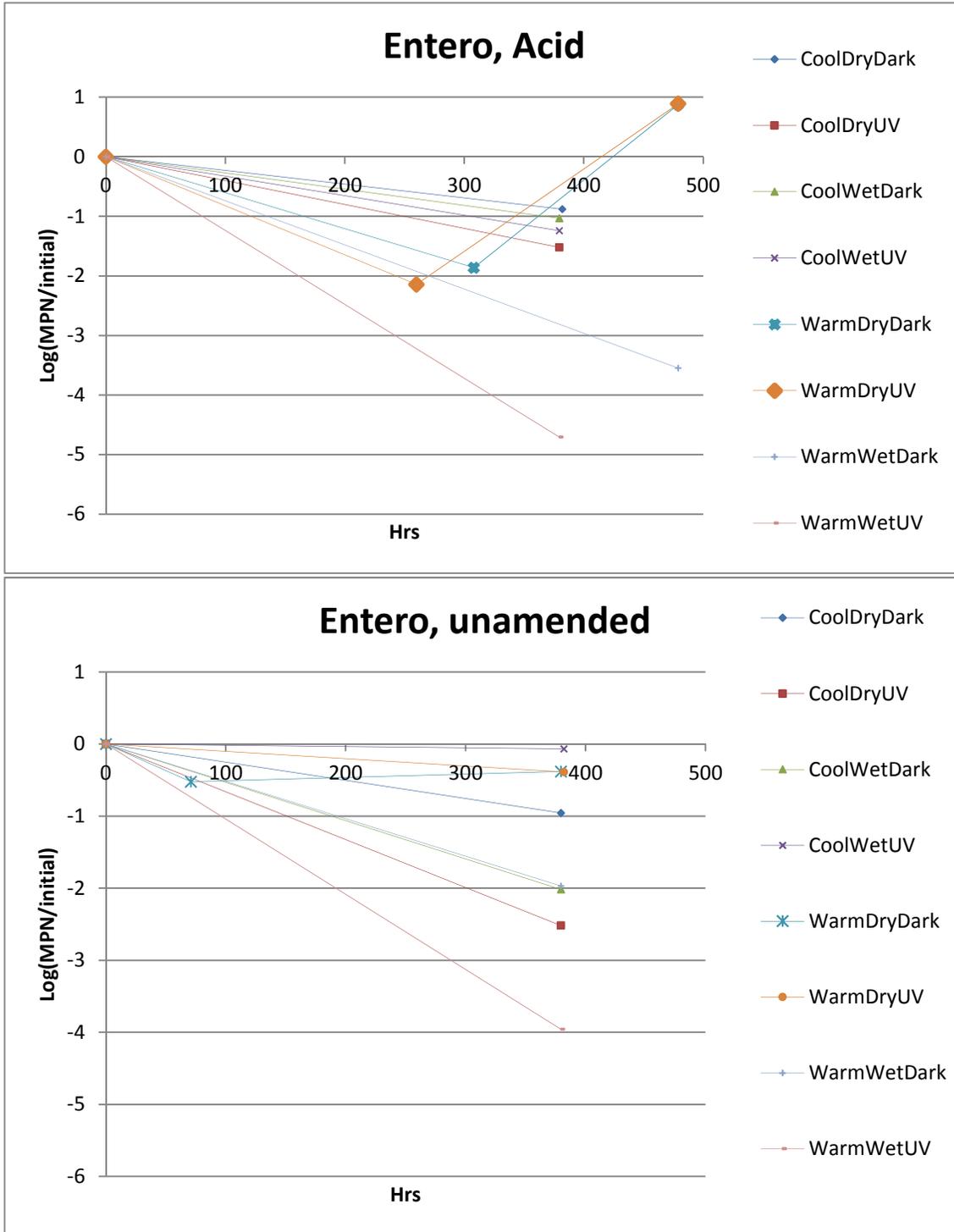


Figure 4.1.2.9 Enterococci treatment models. (Part 1 / 2).

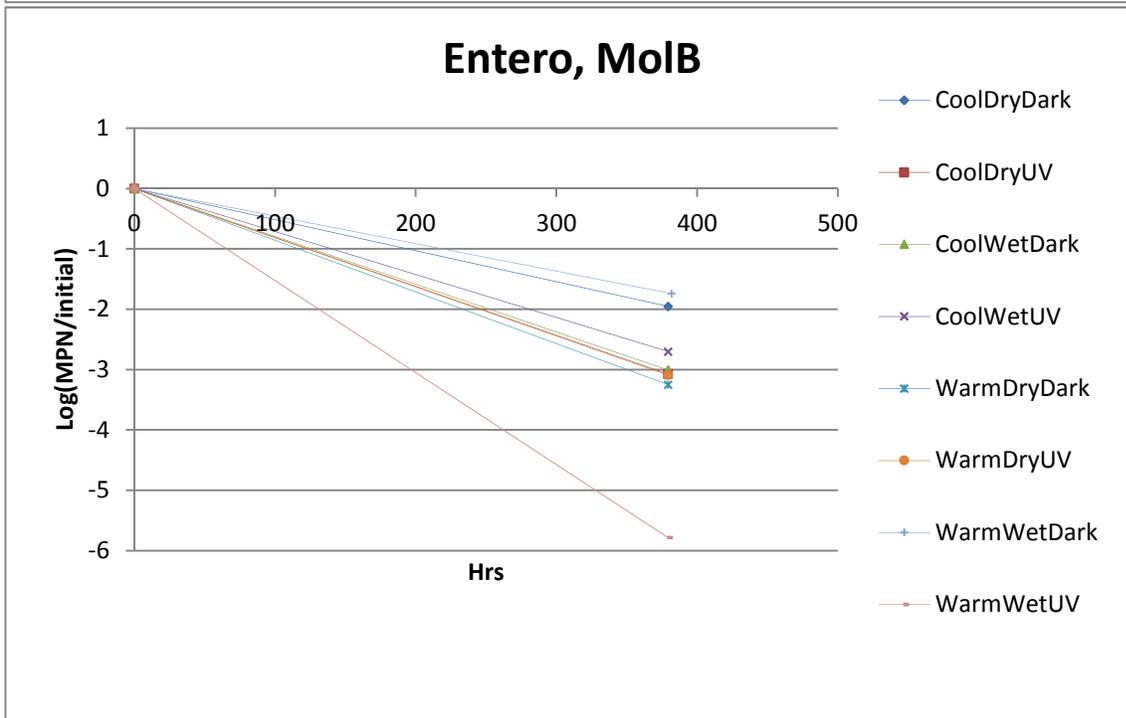
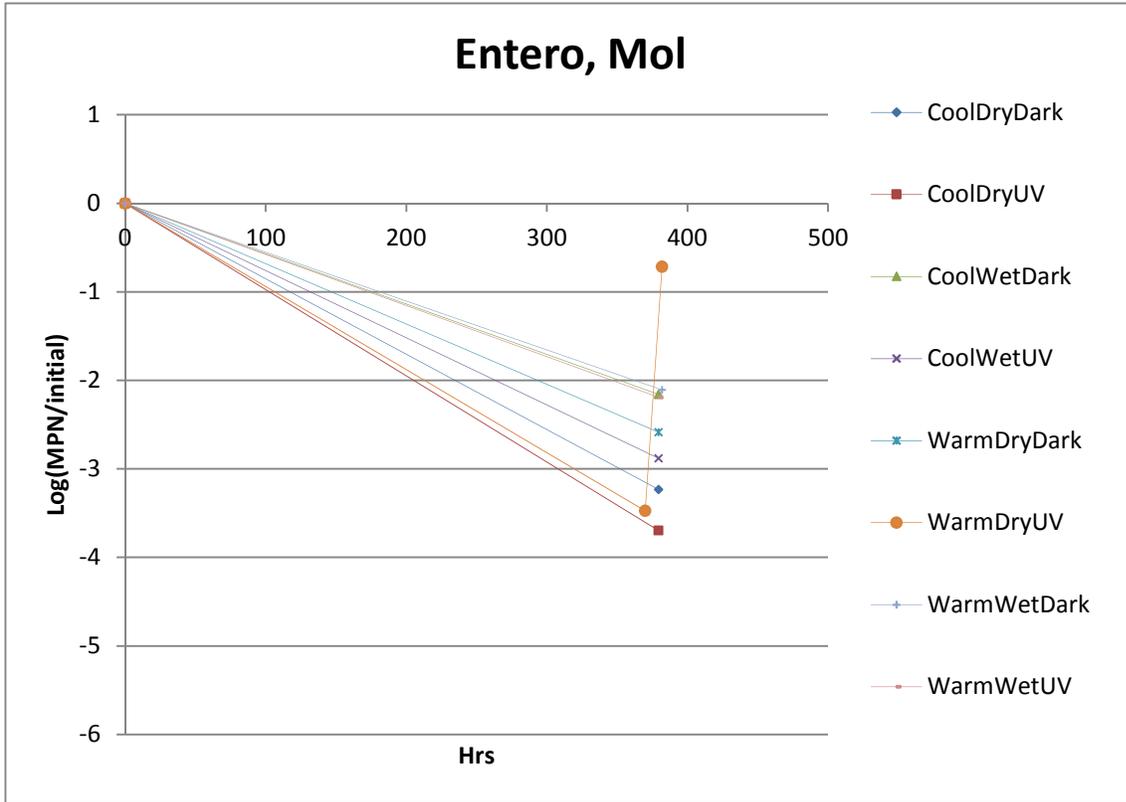


Figure 4.1.2.9 Enterococci treatment models(Part 2/2) . *H2O* = distilled water amended, *Acid* = vinegar amended, *Mol* = molasses amended, *MolB* = molasses and baking-soda amended

Factorial analysis of the system reveals no significant effects on either segmented slope from any main factor nor from any interaction studied, a finding confirmed by normal probability plot Figure 4.1.2.10).

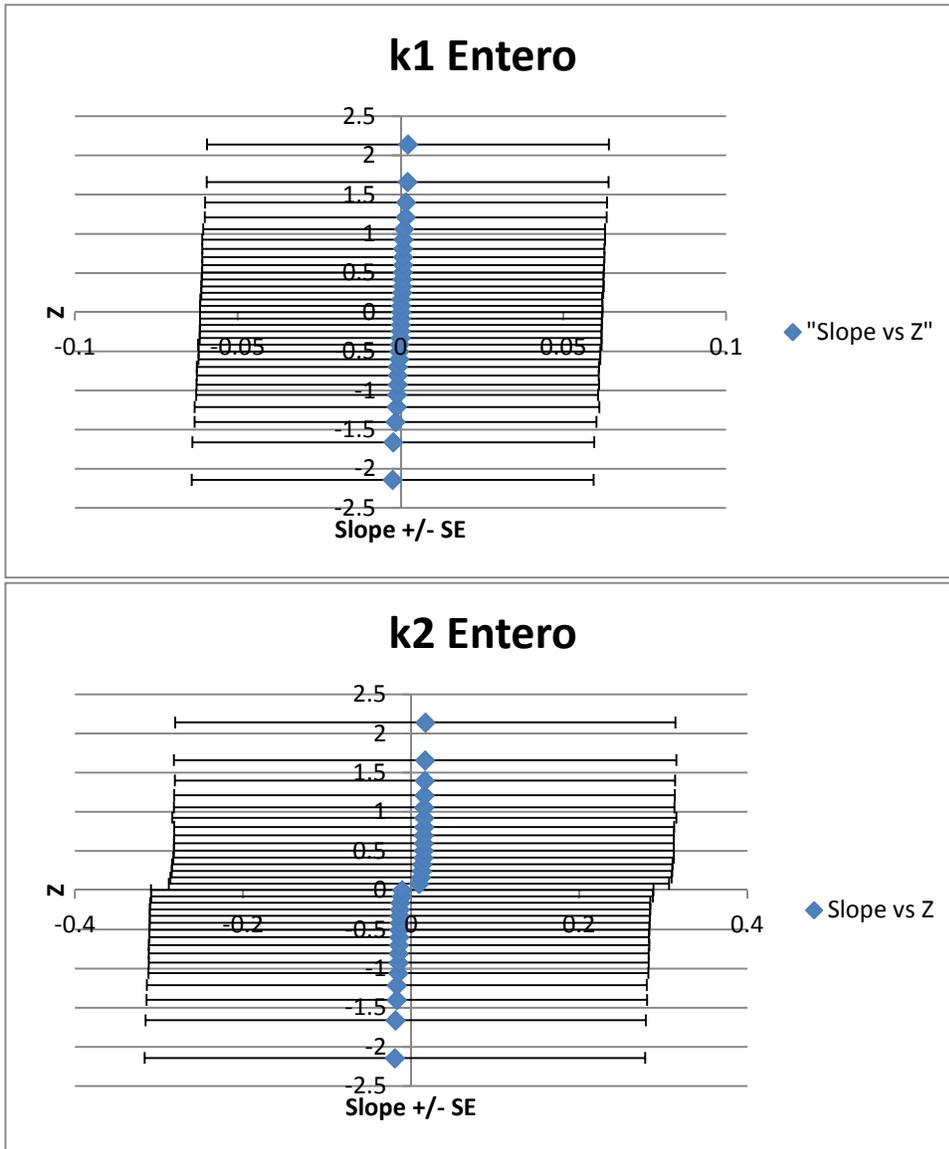


Figure 4.1.2.10 Normal Probability Plots of (rank ordered) effects on Enterococci survival-rate constants. Confidence intervals from factorial apportionment of pooled treatment variances.

Factorial analysis of effects on the breakpoint abscissa (hour), however, is more complex and problematic. At our 95% confidence threshold, the analysis identified a rather daunting 24 significant effectors (the three present/absent-coded main variables, and their interactions

including the full five-way interaction) influencing the timing of the breakpoint. The problematic nature of the results is well illustrated at Figure 4.1.2.11.

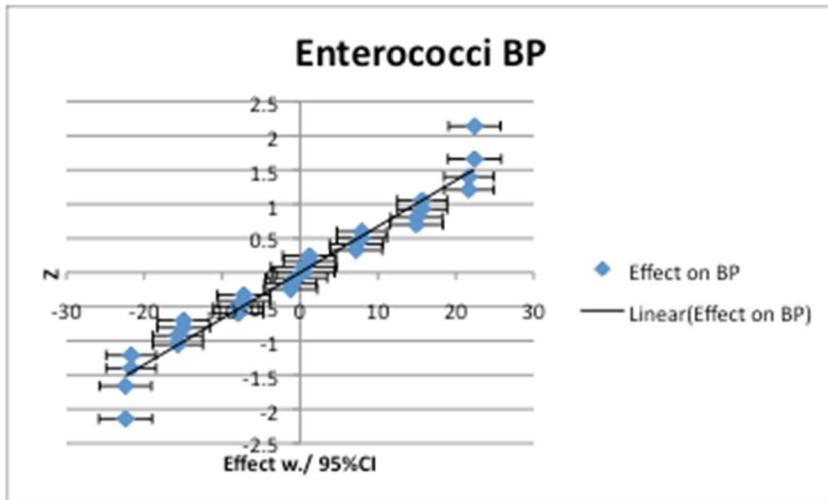


Figure 11 Normal Probability Plot of (rank ordered) effects on the breakpoint abscissa of an Enterococci survival model. Confidence intervals derived from factorial apportionment of pooled treatment variances.

The first feature of note here is the marked discrepancy between the number of factors found significant in the factorial analysis and the number graphically denoting significance in the figure. Visual observation of the figure only shows only two factors with clear separation from the diagonal line plotted from the whole set. Another striking feature of the figure is that it (visually/graphically, and without any regard to apparent significance) seems to consist of 14 couplets, each consisting of a pair of points of similar magnitude (of effect) and of variability, symmetrically surrounding a three-point core (the effects of temperature, humidity, and their two-way interaction) at the origin. While not shown here, readers should note that points in each such pair are of exactly the same magnitude and variability, to the precision provided by our EXCEL™ printout (nine significant figures) and the symmetry of abscissae is also as exact. While each couplet is artificially separated vertically (the ordinal Z axis) by the inability of the EXCEL SORT™ function to account for ties, and while this feature of figure construction may

well account for some of the apparent discrepancy, the situation suggests the presence of collinearity amongst the effectors. The rarity of found significant BPs amongst the treatments (4 out of 32) makes considerable collinearity here an expectation (if it were possible to visualize a response surface of so many dimensions, it would include 28 *coincidental* observations).

The proper handling of collinearity amongst effectors has been a source of some contention in the regression-statistics community for decades (*e.g.*, see Friedrich, 1982, and Allison, 2012). Some researchers argue that only the full model, with collinear terms included, provides unbiased descriptive statistics of the system. Others decry the instability of such models when used in predictions, and urge the deletion of terms with problematically *high* Variance Inflation Factors (VIF, and researchers have variously defined *high* as exceeding values across the range of 2.5 to 10). Being primarily interested in predictive modeling here, we chose the latter approach. Note that both camps in this ongoing debate agree that neither approach lends itself well to analysis of causality.

We calculated VIF of all studied factors and interactions by Equation 3, a spreadsheet-friendly form for which we (lacking residuals) have MLE parameters:

$$\text{VIF}_j = S_{x_j}^2 (n - 1) SE_{b_j}^2 / S^2, \quad (3)$$

where:

$S_{x_j}^2$ = variance of predictors of the *j*th parameter

$(n-1) SE_{b_j}^2$ = variance of the *j*th effect (factorial apportionment of pooled MLE treatment variances), and

S^2 = pooled MLE treatment variances.

[This formulation of the VIF defining equation and nomenclature here was provided, without derivation, by ProfTDub, 2010. An extended algebraic derivation of a fully compatible formulation can be found at Gujarati, 2004, pp.342-353, with special attention to that author's Equation 10.5.4.]

Results confirmed considerable collinearity between factors/interactions. Setting our rejection standard at $VIF > 10$, we retained only seven predictors deemed not problematically high. Temperature and Humidity, and all interactions including them, showed VIF well exceeding a thousand. The intersection of the set of unproblematic variables with the set of variables showing significant factorial effects trims our breakpoint model to a relatively manageable six parameters (Shade, Organics, pH, and three of their interactions).

Our resulting model for survival of Enterococci on a pervious surface is:

$$\text{Log(MPN/initial)} = -0.00620 * t \text{ for times } \leq BP$$

where

t = time [hours]

BP = breakpoint abscissa [hours]

$$= 254 + ((S - 0.5) * -7.84) + ((O - 0.5) * 7.44) + ((A - 0.5) * 3.97) + ((S - 0.5) * (A - 0.5) * 10.8) + ((O - 0.5) * (A - 0.5) * -11.2) + ((S - 0.5) * (O - 0.5) * (A - 0.5) * -3.61) \text{ [hours]}$$

S = Shade Code (= 1 for shade present, = 0 for shade absent, UV exposed)

O = Organics Code (= 1 for organics amended, = 0 for organics limited to fecal contribution)

A = Acidity Code (= 1 for acidity present, = 0 for acidity absent);

and

$$\text{Log(MPN/initial)} = ((t - BP) * 0.00209) + (-0.00620 * BP) \text{ for times } > BP. \quad (4)$$

This model, compared to the (as censored) observations is presented at Figure 4.1.2.12. The Enterococci dataset ($n = 294$) is not as heavily censored (32 censored points) as that of *E. coli*, above, and more points are right censored (26 points) than left. The reduced set of considered effects on the breakpoint abscissa did not harm correlative goodness of fit. The positive correlation between the model and the censored dataset (0.54, see Figure 4.1.2.13), which again must be considered a minimum measure of the latent-variable situation, compares favorably with that found when all BP factors were considered (not shown, but also 0.54). As compared to the *E. coli* system above, this dataset is apparently less censored in terms of the frequency, and apparently in terms of the magnitude of censoring, as well. No MLE predictions

fell outside the minimum LC point (-4.53) or the maximum RC point (1.74), the horizontal bands in which *all* plotted points had been shifted by censoring bias. The implied Pearson coefficient of the censored dataset *vs.* the model censored as the dataset (Figure 4.1.2.14), which must be considered as a maximum limit for the underlying, latent-variable situation, only improves to 0.64. Clearly, some significant source(s) of variability in the survival of FIB on terrestrial surfaces remain(s) unaddressed here.

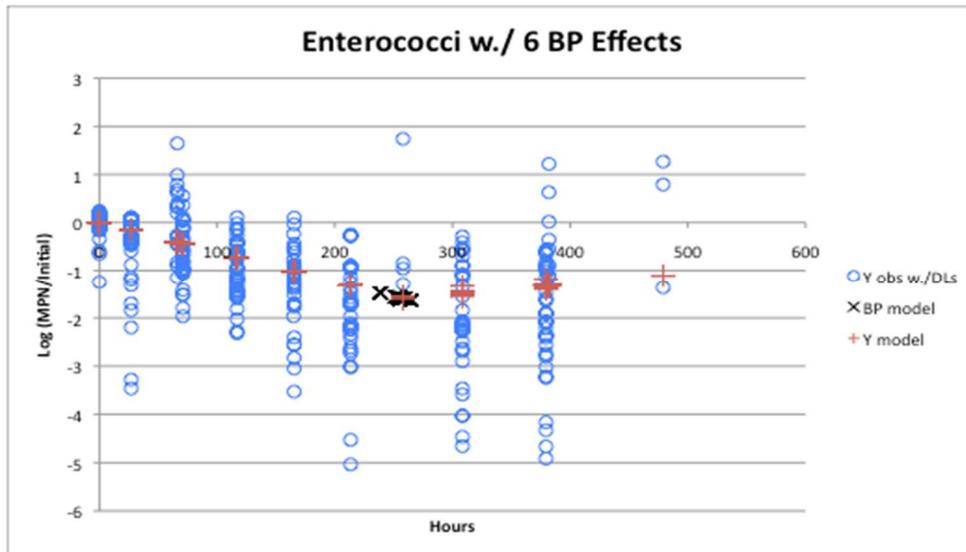


Figure 12 Enterococci model *vs.* (censored) observations. Breakpoint factors trimmed to six, by VIF criteria (as defined in the text).

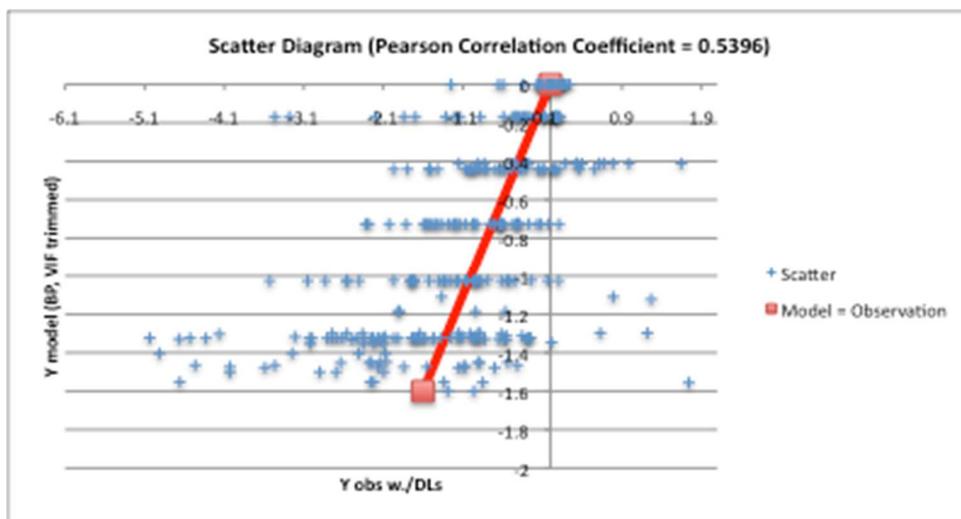


Figure 4.1.2.13 Model *vs.* Observed (as censored) Scatter Plot, Enterococci.

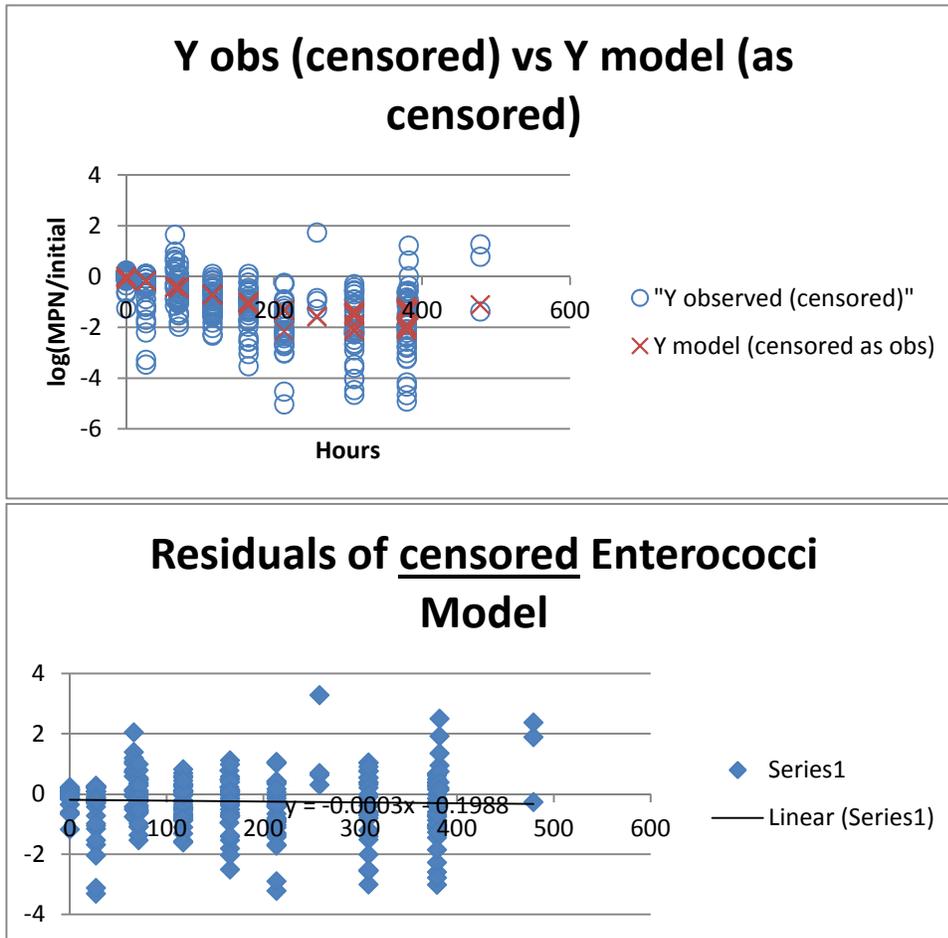


Figure 4.1.2.14 Model vs. observations (both series censored) of Enterococci

4.1.2.4. Comparisons and Conclusions

The survival models presented here (and in the previous study, Wilson and Pitt, 2013) are clearly weak in terms of goodness-of-fit and of explanatory power. Their utility would be obviously enhanced by identification of significant sources of variability not considered here. Despite shortcomings of both, comparisons of the two provide some useful information concerning future research efforts.

Our previous, impervious-surface study of *E. coli* survival provided a two-breakpoint (three-segment) factorial model that, despite its low predictive power (Pearson coefficient = 0.59), provided a better fit than an alternative (no-breakpoint) linear model (0.44). It was the

poor fit of the previous model that, in part, prompted us to include the two additional (soil-water) factors for study in our current effort. Our findings of multiple breakpoints with subsequent rising-slope (regrowth) segments in the *MolB* (organics-amended, pH-neutralized) treatments (Figure 4.1.2.4, bottom panel) here suggest that inclusion of those factors in the pavement study may have provided a better fit. *E. coli* is the more metabolically fastidious of our studied FIB.

Also, in the previous, pavement, study, the first segment (prior to the first found breakpoint hour) provided evidence that our hypothesized environmental factors (temperature, moisture, UV) were significant contributors to the initial decline in populations, as would be expected in a lag phase induced by the transfer of an FIB population from an enteric environment to the landscape. Subsequent segments in the previous model showed reductions in the number of significant environmental effects on slope, and an eventual reduction in the steepness of decline. All of these features are in agreement with expectations of a population of bacteria adapting to a new environment.

The pervious-surface study presented here, however, provides an unsegmented (no breakpoints) model, also of poor fit ($0.63 \leq$ likely latent-variable Pearson coefficient ≤ 0.83), but providing no significant evidence of adaptation. An obvious question here relates to causes of the difference in model structure between the studies. The oft-opined problematic nature of the dataset in this study provides one possible answer. Figure 4.1.2.15 presents a comparison between the previous (2^3 pavement) study treatments and the *H2O* (lacking organic or acidity amendments) treatments from the current study (a 2^3 subset). These are likely the most relevant presentations for comparison in that they both represent tests in which no soluble substances, other than those already present in the fecal inoculant, have been introduced. The figure reveals that several treatments on pavement expressed significant breakpoints very early in the study,

within the first 24 hours of environmental exposure. Our expansion to a 2⁵ study here denied us an opportunity to detect such points. Our incubators were full, for enumeration of our initial Day 0 samples during that period. No timely partial-day exposure samples could be analyzed. Many treatments in the pavement study, however, also expressed additional breakpoints later in the study period, and all that expressed any points ended the study with a smaller decay slope than the initial slope (evidencing adaptation to environmental conditions). While our expansion of the (2⁵) scope in the current study may have masked some breakpoints, there is some evidence that the FIB behavior on soils is different than that on pavements.

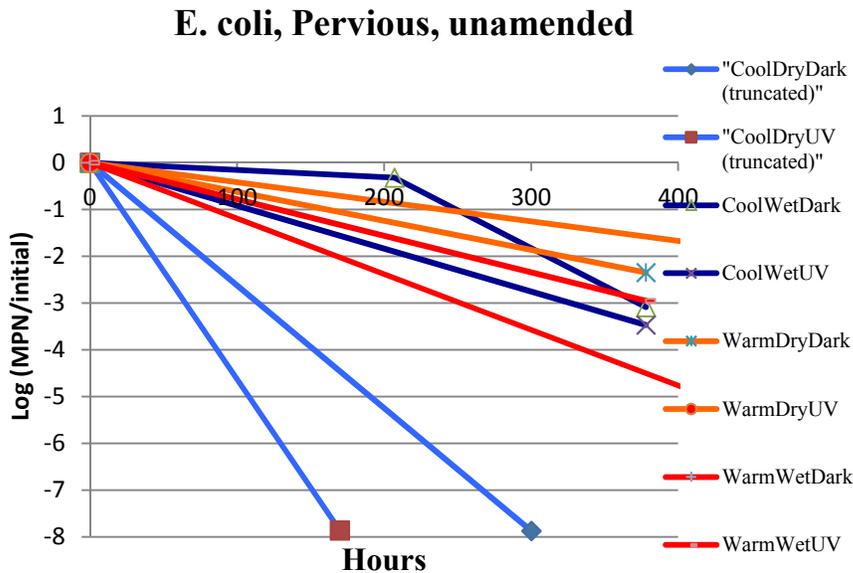
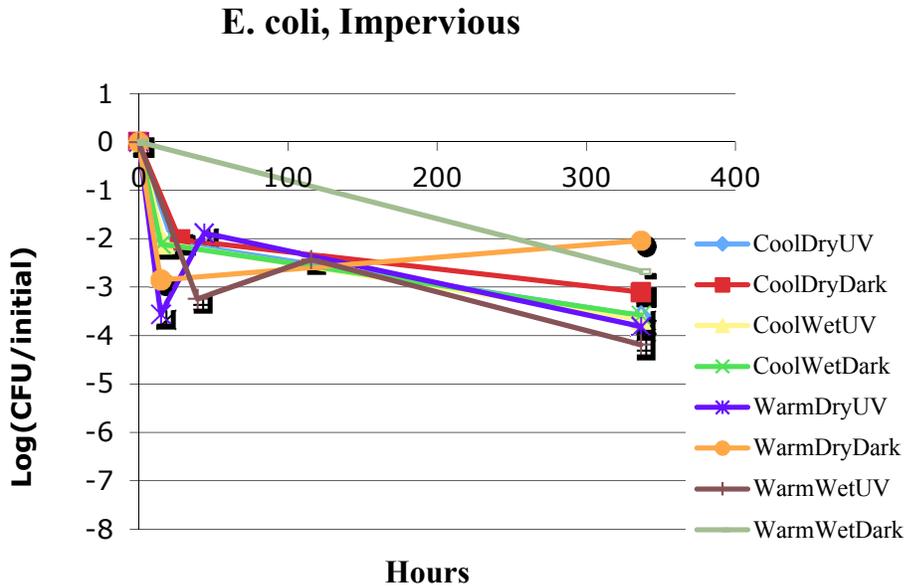


Figure 4.1.2.15 Unamended *E. coli* treatments compared

Our experimental plan, and our use of breakpoint analysis, was based on standard, textbook concepts of how populations of bacteria grow/decline and adapt. It also included an unstated, underlying assumption that our inoculants would behave as *populations*, and thus behave according to classic population dynamics. We have here some evidence that this might not be the case on pervious soils.

Populations are defined as organisms of shared genetics exposed to a given *habitat*. Soils differ from pavements in the number and diversity of *microenvironments*. Researchers armed with an array of microelectrodes have even found a gradient of a substance as diffusive as oxygen, ranging from completely anaerobic (0% oxygen) to fully atmospheric (21%) within a single soil particle (Madigan, *et al.*, pp. 635). While we might expect temperature to be homogeneously applied across our experimental microcosms, other factors would not. Pervious surfaces are pervious, and harbor pore spaces and shaded surfaces. UV-exposed microcosms include sheltered pores, and bacteria within such pores should not be expected to behave in concert, as a population, with more exposed cells, even in the same sample. Likewise, adsorbed and capillary water, and any substances dissolved therein, would provide a very heterogeneous landscape, even in a 1-tablespoon environment. Such considerations force some attention to concepts of bacterial *microecology* (and see Madigan, *et al.*, 2003, pp. 634-642). Such considerations, however, also lead towards realization that the mechanistic model of net survival provided classic microbial population dynamics may not be applicable in all situations. Moreover, the literature provides little (none found here) as to alternative survival models of general applicability.

An *incoherent* response of an inoculated sample to an array of microenvironments would be expected to mask, by a broadening of the range of observation abscissae involved, breakpoints that might have been revealed by a more concerted response from a population. Such a situation might, though, reveal itself by a concave-upward array of residuals of an unsegmented linear model. Our censored datasets in the current pervious-surface study frustrate any attempt to rigorously analyze the effect that microenvironments may have had on our results. We must take a more qualitative approach to our analysis. Figure 4.1.2.16 shows our *E. coli* dataset, edited to

only include actual (uncensored) observations, and the modeled points that are derived from those observations. Both series have also been edited to only include the first observational abscissa (hour) subsequent to the n-weighted average BP found (which serendipitously coincided with the first modeled point that exceeded censor points). At least visually/graphically, evidence of an upwardly curved observational series, underlying this linear (no breakpoint) model, seems fairly apparent.

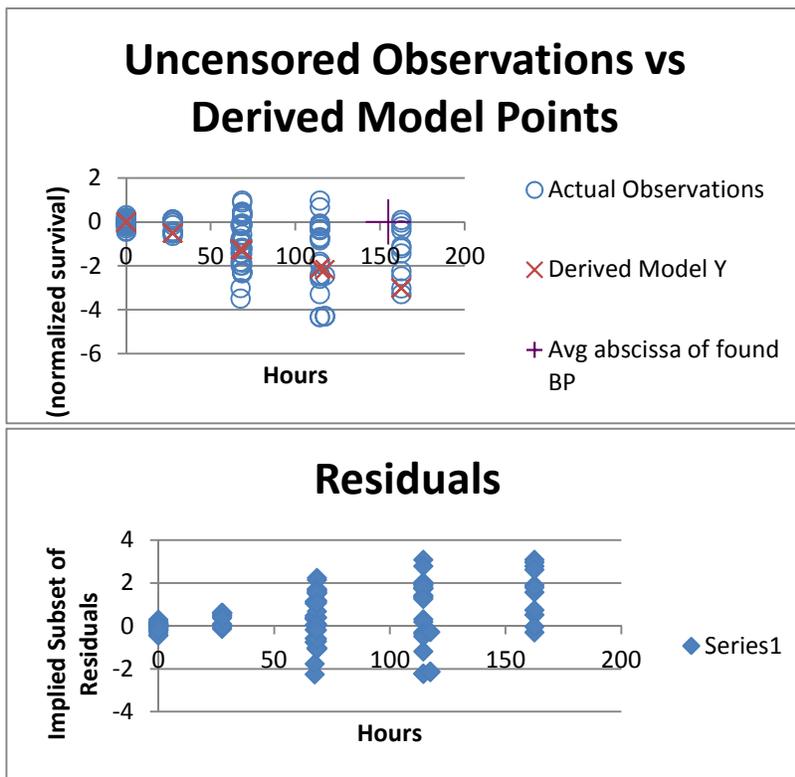


Figure 4.1.2.16 *E. coli*, edited to actual observations up to and including the found average breakpoint. Upper panel = observations vs model, lower panel = residuals.

Additional evidence, at least suggestive of masked adaptation, is provided at our Figure 4.1.2.8, in which the effect of censoring bias on *E. coli* residuals has been artificially removed from this linear (no breakpoint) model. Upward concavity of residuals is even visually in evidence in *each* segment (prior to and subsequent to the found overall breakpoint abscissae, ~

255 hours) of the Enterococci model with censoring bias artificially removed (Figure 4.1.2.14, bottom panel).

It should be noted here that effects of microenvironment heterogeneity on our system models was considered and intentionally not directly accounted for in our experimental plan here. Microhabitat sampling seemed infeasible and unlikely to contribute to our overall interest in the *bulk* release of FIB from stormwater source areas to runoff. Moreover, we assumed that residuals analysis of our study would reveal any failure of the population-dynamics model, and would provide clues to what appropriately curved alternative models might look like. Though not conclusive, evidence here suggests that this might represent a flaw in our plan. This scoping study was sufficient, even with the problematic (heavily censored) dataset, to reveal considerable evidence of that flaw through the contrived *pseudoresidual* analyses of the overall models presented. It was our inability to find significant breakpoints in the *treatments*, however, that led, mathematically, to the apparent masking of adaptive behaviors in both models presented in this study (overall for *E. coli*, and segment-wise for Enterococci).

It would seem that the *simplest* fix to this situation would be to remove the needed recourse to censored-regression methods with their inability to supply an unbiased set of residuals, a *fix* requiring a rather daunting expansion of incubator space (and, notably, beyond the resources available here and, likely, most anywhere). In our previous pavement studies, we had incubator capacity to simultaneously house undiluted eluent, together with two additional dilutions, for each (2^3) sample analyzed, for each of the two FIB taxa under study here. With available 1:100, and 1:10,000 dilutions, together with the three-orders detectable range available in the IDEXX enumeration, this dilution scheme provided a seven-order span in which no censoring would occur. Even with our more variable fecal source available for this study, the

same dilution scheme produced no need to account for censoring in our Day O analyses here. With (hypothetical) incubator-expansion to allow such a scheme in these (2^5) studies, we would have had a full set of fully observable *observations*, and fully definable *residuals* by which to more accurately judge goodness-of-fit for the overall models presented here. Just as important here, such a scenario would have supplied a set of fully definable residuals for each *treatment* model. Any left-censored observation subsequent to the first such seen would be *ignorable* by definition (in the sense provided by Little and Rubin, 1987). It is only by a full set of definable residuals (unavailable from any censored-regression treatment) for the *treatments* that inadequacies of the breakpoint model due to microenvironmental diversity would have revealed themselves, and would have defined appropriate alternatives. Future research is best informed by these considerations.

It should finally be noted here that microenvironmental heterogeneity should also be expected to suppress *contrasts* between sample treatments and suppress the measured factorial significance of predictor and interactive effects, as well. Best example of this is embodied in the implied factorial contrast between shade-present and shade-absent treatments. In the system of the microenvironment-rich sample microcosms presented by this study, there are many portions of the sampled shade-absent microcosm that are, in actuality, shade-present. Many individual bacteria, and many CFU subject to our analyses, in shade-absent treatments likely responded as if they were (and, in all actuality, probably *were*) in shade-present microenvironments. Such suppression of contrasts, however, should be expected to reflect actual conditions related to the bulk runoff from environmental source areas.

4.1.2.5 Endnote: Censoring consequences illustrated

The oft-neglected consequences of nonignorable censoring, and the complications they present to analyses seemed worthy of extended illustration. We present here a discussion of the analytical difficulties encountered due to such censoring in our particular datasets.

One method sometimes used to avoid such complications is to ignore it. We have intentionally done so, for illustration purposes, in Figure 4.1.2.17. In the figure, we have regressed (with the Excel Trendline™ function) only the quantifiable observations in our *E. coli* dataset, neglecting all censored data there. While this model, with no found breakpoints and no found significant environmental factors affecting survival, provides very little explanatory power (r^2 only ~32%, top panel of the figure), the observed residuals derived (bottom panel) show little cause for concern for the legitimacy of this measurement. For this effort, where the regressed model is derived from the same data as those that provide the residuals, there is little observable reason to disbelieve an expectation that remaining error of the regression is zero (though there might be some visual evidence of a hidden break). Though there may be some reason to question a constant variance throughout the study period, any visual evidence of heteroskedasticity is not particularly glaring. Some might find the residuals presented here as fairly well behaved.

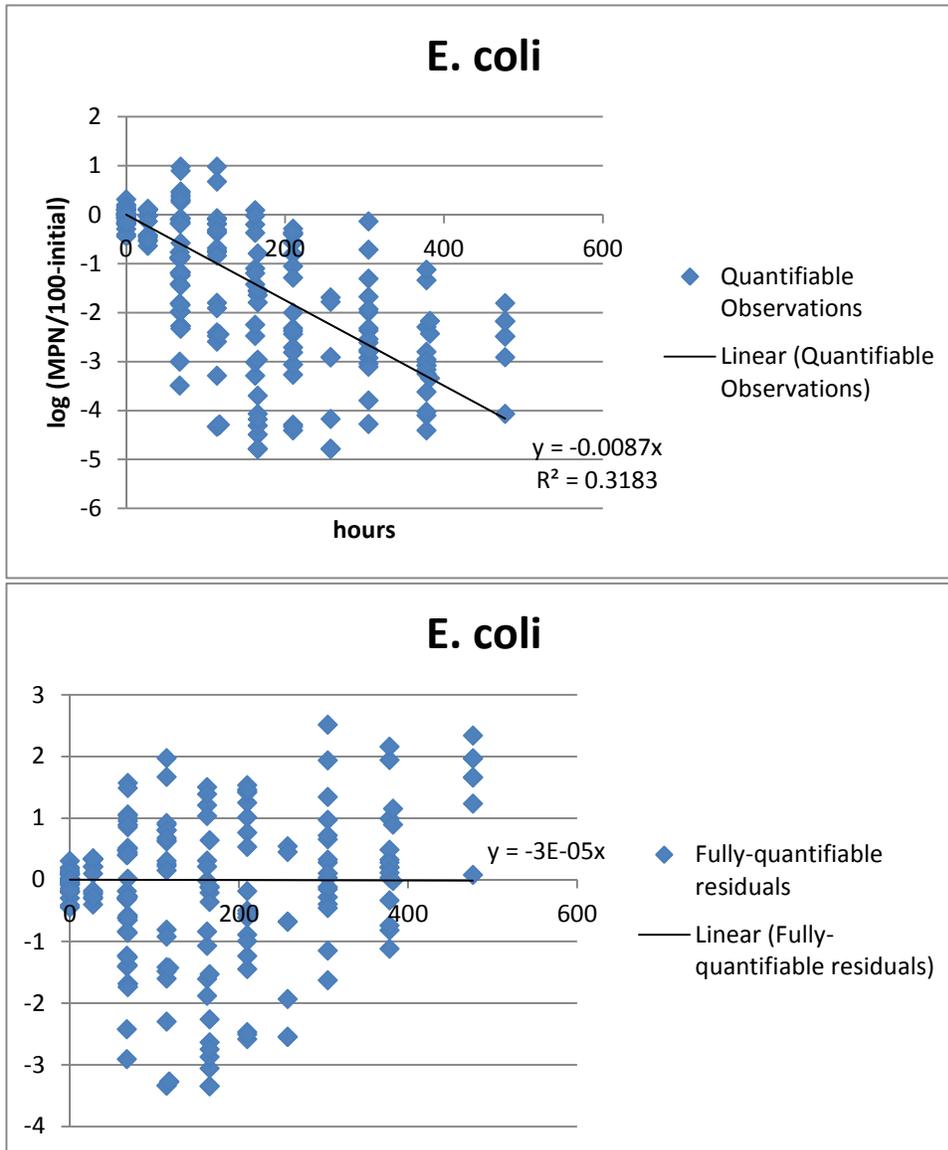


Figure 4.1.2.17 A Modeling Effort Limited to Fully Quantifiable Values in the *E. coli* Dataset. Trend lines drawn from OLS regression.

We know, however, from Little and Rubin (1987, esp. pp. 8-13) that the model described in the figure above, in fact, presents a biased view of what the FIB under study were actually doing. This bias arises from our ignorance of the nonignorable (mechanistic) partial information present in the dataset. We can get an estimation of that bias by performing a censored-regression analysis, which provides an MLE of the regressed slope of *E. coli* survival that would have been exhibited had the observations not been censored (the *latent* dependent variable). We can see

from Figure 4.1.2.18, below (top panel), that bias is considerable. The predicted log decline of CFU over time in the censored model is twice as great as that found in the OLS model above. This is because the two models regress two different datasets. In the censored regression, the maximum-likelihood influence of the unquantified (and unquantifiable) observations is considered. The *E. coli* dataset here contained many DL violations, and the array of those violations was asymmetrically dominated by BDL observations (LDL distribution in the figure for illustration). The influence of those BDL observations (many largely negative, occurring early in the study period) creates a steepening of the decal curve relative to the OLS regression.

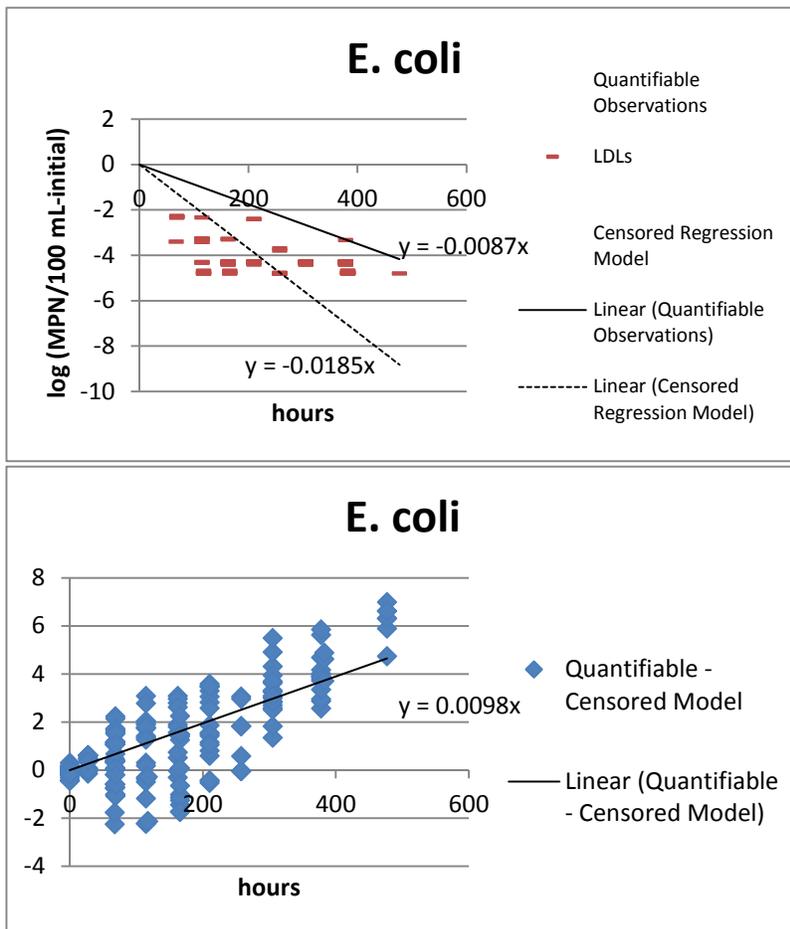


Figure 4.1.2.18 Censored-regression modeling effort, and “residuals.” Asymmetric censoring.

Just as important to our discussion here is the effect that censored regression has on residuals. The censored-regression model does assume constant variance (estimable by “scale factor” in Eviews8, and see Little and Rubin, 1987, pp. 218-223). It provides no expectation, however, that residuals calculated from the model equal zero and, thus, no expectation that a graphic display of such variability would be centered on a horizontal line (Little and Rubin, 1987, pp.259-262). Graphically, the increased steepness of the censored-model decline (top panel) is *not* mirrored by any change of the array of values representing the quantifiable observations. In the bottom panel of the figure above, we calculated the censored-model expectations for the quantifiable observations and calculated the “residuals” they represent in the usual way (ordinate of the observation minus ordinate of the model expectation). The array shows little evidence of heteroskedasticity. A line mentally drawn across the bottom of this spread might even be seen to intersect the origin at an ordinate a little below -4 (the approximate value of the LDLs for our observations at no dilution in our two experimental runs). The slope of this array, however, is far from zero, as should be expected from a dataset as asymmetrically censored as this one.

A similar analysis was conducted on our Enterococci dataset (Figure 4.1.2.19) and a comparison to the figure above is worthwhile. Our Enterococci data were much less, and more symmetrically, censored than those of *E. coli*. Moreover, a modeled breakpoint was found at ~255 hours that improved symmetry of variability, as well. The resulting residuals array (bottom panel in the figure) observably deviates much less from the traditional (OLS) expectation of zero-slope than does that of *E. coli* above. From a mechanistic perspective, however, this observation from our censored-regression “residuals” is no more meaningful than is the steep slope encountered above.

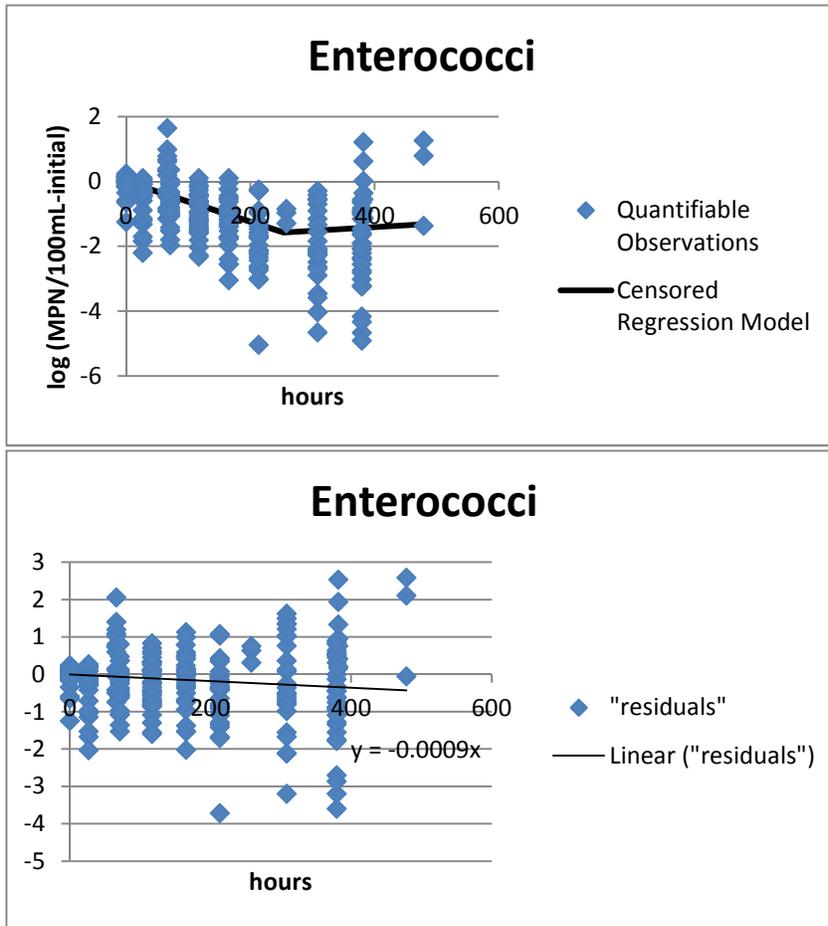


Figure 4.1.2.19 Censored-regression modeling effort, and “residuals.” Breakpoint found.

Authors

Bradford M. Wilson, Ph.D. student, University of Alabama, and to whom inquiries should be directed. Contact: brad4d@hotmail.com, (205) 394-2350, (251) 517-7392, 13040 Dixie Rd./Fairhope, AL. 36532

Dr. Robert E. Pitt, Emeritus Cudworth Professor of Urban Water Systems, Civil Construction and Environmental Engineering Dept., Environmental Institute, University of Alabama

Dr. Mark Elliott, Assistant Professor, Civil Construction and Environmental Engineering Dept., Environmental Institute, University of Alabama

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4.2 Washoff Studies

4.2.1 CFU Release, a screen for operational mechanisms

An unpublished article- (CHI-) style manuscript is presented. It is formatted to meet the requirements of this dissertation with permission of the authors. Preliminary and method-development studies are appended.

4.2.1

INDICATOR BACTERIA RELEASES FROM URBAN SURFACES, A SCREENING STUDY OF OPERATIONAL MECHANISMS

Bradford M. Wilson, Robert Pitt, and Mark Elliott

A literature review revealed a need by watershed managers for some method of estimating the movement of stormwater fecal indicator bacteria (FIB) to regulated downstream waters. The same review, however, provided little information concerning model parameters or structure by which such estimations might be accomplished.

The study reported in this paper consists of investigations into the mechanisms by which FIB are dispersed from stormwater source areas in response to rainfall. It represents one in a series of scoping studies performed to explore the feasibility of constructing a model by which urban FIB inputs might be predicted from watershed observations.

4.2.1.1 Introduction

Due to difficulties in direct measurement of waterborne pathogens, the microbiological quality of waters is typically characterized on the basis of fecal indicator bacteria (FIB). FIB are assumed to derive from a common (historically sewage) source with pathogens of interest, and to arrive in, survive in, and move through watershed environments in numbers that correlate with the risk from those pathogens (the *indicator paradigm*). Commonly used indicators, however, also derive from sources other than sewage or even feces, and survive in the environment at rates divergent from those of pathogens they are presumed to indicate (National Research Council, 2004).

Considerable expert consensus exists that FIB from non-human sources represent lesser correlative risk to human health than do those deriving from sewage (the *species barrier*). Much effort and money has been expended to confirm this assertion, but results are deemed equivocal by many regulatory authorities (*e.g.*, see Dufour, *et. al.*, 2012, and EPA, 2012) and the source of FIB is not considered relevant under many water-quality criteria (WQC).

Knowledge of the source of FIB, however, remains important to achieving *compliance* with WQC, and will become increasingly important if future studies make better links between sources of FIB and human health risks. Managers of tributary watersheds require knowledge of FIB sources, especially sewage *vs.* non-sewage, to manage/prioritize strategies for compliant contributions to downstream waters. Tools for mitigation of sewage effluents differ, for instance, from those relevant to managing squirrel-derived fecal material in stormwater runoff.

The study presented here explores the mechanisms by which FIB may be released to stormwater in response to rainfall. Which, of the several possible, mechanism(s) are operational on source-area surfaces have the potential to affect both the morphology of released FIB and the pattern of their release.

4.2.1.2 Materials and Methods

4.2.1.2.1 Study Area

Three closely collocated study sites were chosen in a suburban residential neighborhood in Tuscaloosa, AL. The roof, lawn, and adjoining street of a single residence were expected to provide separation of surface-related effects with minimal heterogeneity of environmental influences. The neighborhood provides considerable (but spotty) tree cover, ample urban wildlife (mostly birds and squirrels, with occasional rabbits and one groundhog sighting), and a

considerable pet presence (leash law in place, though not universally complied with). All rainfall data here were taken from the available Preliminary Monthly Climate Data (the “F6 Product”) for Tuscaloosa Regional Airport (TCL), provided by the National Weather Service, and are hereinafter referred to as “TCL F6.” TCL is ~2.5 miles from the study area as measured by ruler on a GoogleMaps™ printout.

4.2.1.2.2 Simulated Rain

Simulated rainwater consisted of de-chlorinated (sodium thiosulfate added) tap water. The water was delivered through three separately piped pairs of commercial drip-irrigation emitters. Drip-irrigation emitters are designed to mimic natural raindrop size and rainfall distribution patterns. The emitters chosen (Rainbird™, SQH) were designed to each cover half of an eight-foot square (4' x 8'), from an edge of that half-square, at a nominal delivery rate of ~1/2"/hr, when supplied with water between 20 and 50 psi. Each pair of emitters was connected to a separate output line of a valved garden-hose manifold (Orbit™). The manifold was supplied by the simulated rainwater from a booster pump (Ultitech™, 1-HP, garden-hose fittings) *via* a garden-hose pressure-regulator (Sanninger™, 25 psi) and flow meter (SaveADrop™) in series connection. The booster pump, in turn, was fed from a coiled garden hose sunk into a 30-gallon tub prefilled and dosed (with kitchen measuring spoon) with sodium thiosulfate. The manifold output was also fitted with a garden-hose pressure gauge for monitoring/diagnostic purposes. All emitters and piping were affixed to a wooden, 8' x 8' frame, with adjustable legs for leveling on the landscape (and, hereinafter, our “rainframe”). This system was not expected to duplicate the kinetic effects of natural rainfall intensity; even realistically sized raindrops (generally considered range-bound ~ 1-5 mm) should not be expected to reach terminal velocity before

impact from this artificial apparatus. This approach was, however, expected to provide better control of “intensity” for this exploratory research than would be achievable with any natural rain (especially considering the area is suffering in a significant drought period), and did realistically simulate any effects attributable to rain depth.

4.2.1.2.3 Rain Application

In an attempt to provide separable effects of wetted time, rain depth, and simulated-rain “intensity” (the last defined here by the usual depth/time ratio, and all of which are important in this screening study), we applied the simulated rainfall in the order of:

- 7.) Nominal ½” intensity for 20 minutes,
- 8.) Nominal 1” intensity for 20 minutes, and
- 9.) Nominal 1½” intensity for 20 minutes,

and repeated the sequence three times (for a three-hour simulated heavy-rainfall event) for each surface. The timing of the initial 20-minute exposure was not begun (stopwatch was reset) until the visible onset of runoff from the surface. For each 20-minute segment of the study period, a sample was drawn at midway (10-minutes subsequent to initiation of each new intensity regime). At the initiation of each 20-minute segment of the study period, measured cumulative volumetric flow was recorded, to provide for a measure of the average intensity for each segment and cumulative depth at each sampling event. Each sample was halved, by cone splitter, to allow for separate measurements of the two FIB taxa under study. Each half was further split to allow for separate, comparative measurements of FIB densities in raw (as is) sample (straight and 1:100 diluted) *vs* macerated (1:100 diluted) sample. This split/dilution scheme was designed to fit within available incubator space and to maximize likelihood of FIB-density measurements

within detection limits of our numeration method (Most Probable Number, MPN of Colony Forming Units, CFU, by IDEXX™ methods). This scheme failed for the detection-limit criterion in several instances, requiring modified analytical approaches detailed in the Results and Discussion section below.

4.2.1.2.4 Hypotheses and testing

An extensive literature review (essentially a “review of reviews” including Hall-Stoodley, *et al.*, 2004; Kaplan, 2010; and Mcdougald, *et al.*, 2012; with all relevant referenced citations therein) provides a consensus view that the vast majority of environmental bacteria exist within a biofilm matrix (which include fecal matter). While much of this biofilm literature is focused on environments not relevant here (*e.g.*, biological and biomedical surfaces, environmentally exposed manufactured surfaces, and aquatic environments), it also provides three relevant biofilm-dispersal mechanisms by which FIB might be released from terrestrial surfaces by rainfall, namely (and using terminology from Kaplan, 2010):

- Seed Dispersal,
- Cell Division, and
- Sloughing.

The literature, together with the morphological characteristics (Madigan, *et al.*, 2002, pp. 65 and 734-5) of our FIB taxa of interest here, also provide for mechanistic outcomes by which the putative mechanisms might be judged for actual relevance.

If, contrary to consensus, most FIB were not biofilm-bound, the following outcomes would be expected (with some exceptions):

- *E. coli* would show no significant (and no more than 2x) elevation in CFU density in response to maceration. This expectation arises from the morphology of the planktonic

- form of this taxon (single or occasionally paired cells). No such telltale signal can be expected from Enterococci, which can exhibit multicellularity in the planktonic form, but a finding of the absence or presence of one taxon in the filmbound state would allow for a strong presumption that the other, from collocated samples, was similarly situated; and
- Both taxa of FIB should be rapidly flushed from the landscape. This expectation arises from the full exposure of planktonic (not attachment competent) cells (of near neutral buoyancy) to overlying stormwater flow. In the literature, this mechanism is deemed *passive* in that no action by the cells themselves is involved, but only the fluid/particle dynamics.

A finding of greater than two-fold *E. coli* CFU elevation in response to maceration, relative to raw (not macerated), would allow for rejection of the flushing of extant planktonic cells as the sole mechanism of FIB dispersal. Failure to find such elevation, however, does not confirm operation of such flushing. The logic here is not reversible. All of the putative mechanisms are capable of producing unicellular CFU.

If seeding dispersal were the sole mechanism of FIB release from extant biofilm, we would expect:

- *E. coli* would show no significant (and no more than 2x) elevation in CFU density in response to maceration. This expectation arises from the morphology of the planktonic form of this taxon (single or occasionally paired cells). No such telltale signal can be expected from Enterococci, which can exhibit multicellularity in the planktonic form, but a finding of the absence or presence of one taxon in the filmbound state would allow for a strong presumption that the other, from collocated samples, was similarly situated; and
- FIB density would rise monotonically (with unknowable lags and plateaus) through time of continuous wetting of the biofilm throughout a rain event. This expectation arises from the well-established cycle mechanics of seeding development in which fluid-filled pockets develop within the film, cells within the pockets convert to planktonic morphology, and planktonic cells are released to the environment as the “seed” bursts. Though the mechanics are well-established, the intricate quorum-sensing of specialized signal chemicals that direct the cycle are, as yet, largely unknown. Seeding dispersal is an *active* mechanism, dependent on concerted cell actions.

A finding of greater than two-fold *E. coli* CFU elevation in response to maceration, relative to raw (not macerated), would allow for rejection of the dispersal of seed-borne planktonic cells as the sole mechanism of FIB release from the surface.

If Cell Division/erosion were the sole mechanism of FIB release from extant biofilm, we would expect that the product of the CFU of macerated samples multiplied by the rain intensity

at the time of sampling would remain constant over wetted time. This expectation arises from the mechanics of this dispersal scheme, in which cells at the biofilm surface divide in response to wetting, and eject one daughter cell (per fission event) to the overlying water. The release rate of total individual cells (cell density x overlying volumetric flow rate) should only be a function of the (active) doubling time of the FIB at conditions of the wetted surface.

A finding of greater than two-fold *E. coli* CFU elevation in response to maceration, relative to raw (not macerated), and a non-constant cell-release rate, forces a consideration of some contribution of (passive) sloughing of filmbound CFU in response to the kinetic energy and shearing potential of overlying stormwater. Such consideration in turn forces consideration that the release of FIB CFU from source areas may be a function of both rain depth and rain intensity under conditions that may change over wetted time of the surface.

Our hypotheses in this effort are:

1. MPN of a macerated subsample *E. coli* CFU is significantly elevated relative to that found in the split raw (not macerated) subsample, and
2. The product of macerated-sample CFU multiplied by the rain intensity at the time of sampling is not constant throughout the rain event.

This hypothetical structure is a screening tool by which the contributions of various putative release mechanisms might be explored/compared. While there is considerable consensus that FIB are (prior to rain) extant on environmental surfaces in the filmbound state, the literature is silent as to which biofilm dispersal mechanism(s) may be operational. Testing of these hypotheses depend more on logical endpoints of measurements than on statistical measures of confidence.

4.2.1.2.3 Results and Discussion

4.2.1.2.3.1 Roof

The *E. coli* data from our roof study (late summer, Tuscaloosa, AL), displayed at Figure 4.2.1.1, were problematic in that 9/12 sampling events produced no readable results (all below the detection limit, DL = 1 MPN/100mL, even as straight, undiluted samples). Moreover, the few readable results were poorly separated from BDL measurements (95% confidence intervals, derived from the IDEXX MPN enumeration procedure, are shown in the bottom panel of the figure). Finally, no macerated subsamples returned readable results. Though this last feature (recall that macerated samples were analyzed at 1:100 dilution) should not be surprising given the extremely low enumeration values for even straight raw samples, these results together render this dataset unsuitable for testing the above hypotheses.

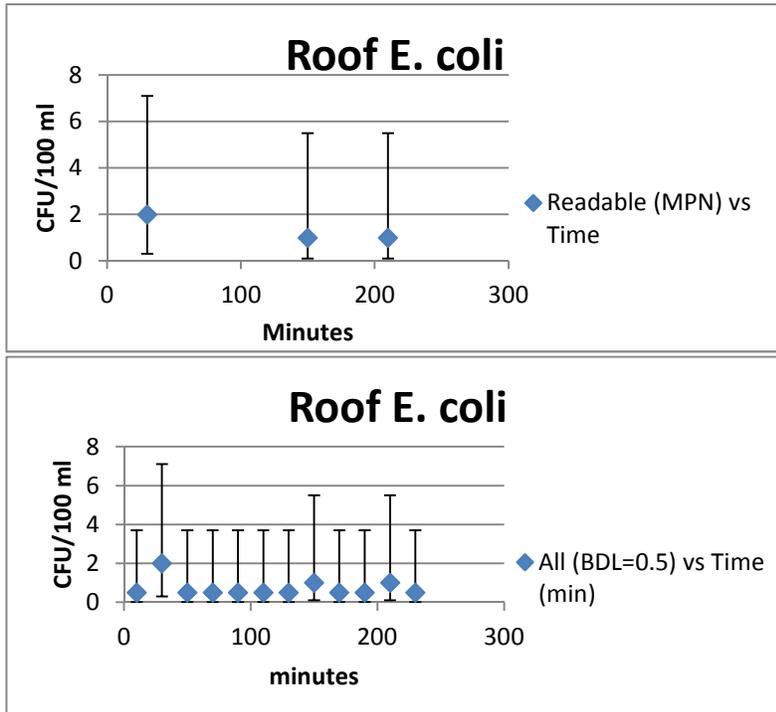


Figure 4.2.1.1 Roof, *E. coli* vs time. Below Detection Limit (BDL) charted at $\frac{1}{2}$ lower detection limit. 95% confidence limits from IDEXX Quantitray2000™ enumerations.

Our Enterococci data from the roof study were less problematic, with 8 of the 12 samples providing quantifiable results (see Figure 4.2.1.2, below). We had no data from any macerated samples (1:100 diluted).

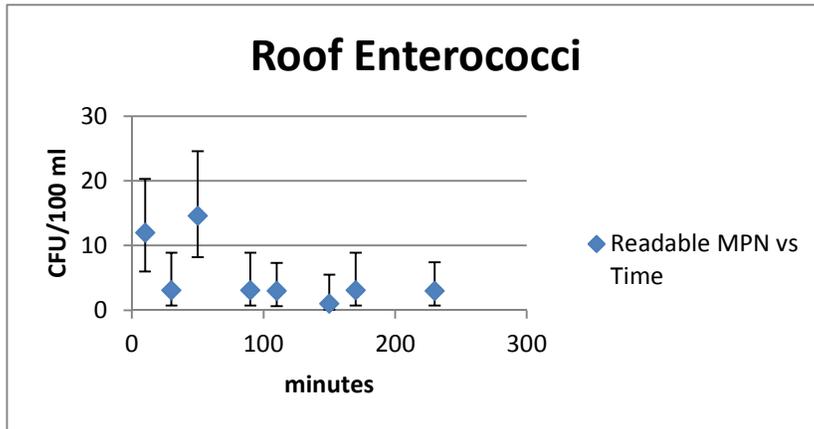


Figure 4.2.1.2 Roof Enterococci vs Time. 95% confidence limits from MPN determination.

While not usable to test the above hypotheses for the roof runoff FIB values, this dataset does present sufficient information to provide suggestions for future similar research. This study site was chosen on the basis of previous, unrelated research (Pitt, *et al.*, 2013) in which this roof produced greater FIB densities than those found in the current study. The previous work, however, did include marked differences in protocol from this current study. First, the previous work only involved opportunistic sampling of natural rains, after runoff was sufficiently established for easy sampling in bulk. Second, the previous work involved sampling from the downspout of the roof, rather than directly from the edge of the roof eave (before entrance to the gutter, as was done in the current study). Either of these might represent a flaw in our site selection for this study. The vegetated overstory had also been removed subsequent to the previous work, but we had no way to check for the potential significance of this additional potential factor. Loath to waste any (even partial) information available of potential value in our overarching exploratory research, we designed a brief follow-up study.

The current (focus of this article) study (with simulated rain) was performed after a three-day rainless interevent period subsequent to a 0.15” rainfall. The next natural rain was sampled on the same roof (though on the opposite side of the ridge from our simulated washoff study). For the follow-up study, the interevent dry period from the same 0.15” rainfall had been extended to 11 days. This follow-up sampling was performed during the first ten minutes of a 0.28” rainfall (entire rain contained in one hourly report at TCL, F6), a rainfall that was described in field notes as “dying to a drizzle within 15 minutes.” By any measure, this follow-up sampling fell well within the intensity range of our current (simulated-rain) study. Follow-up samples, from roof-eave edge and from downspout effluent were each diluted (1:2) for separate analysis by taxon. Results are shown graphically in Figure 4.2.1.3.

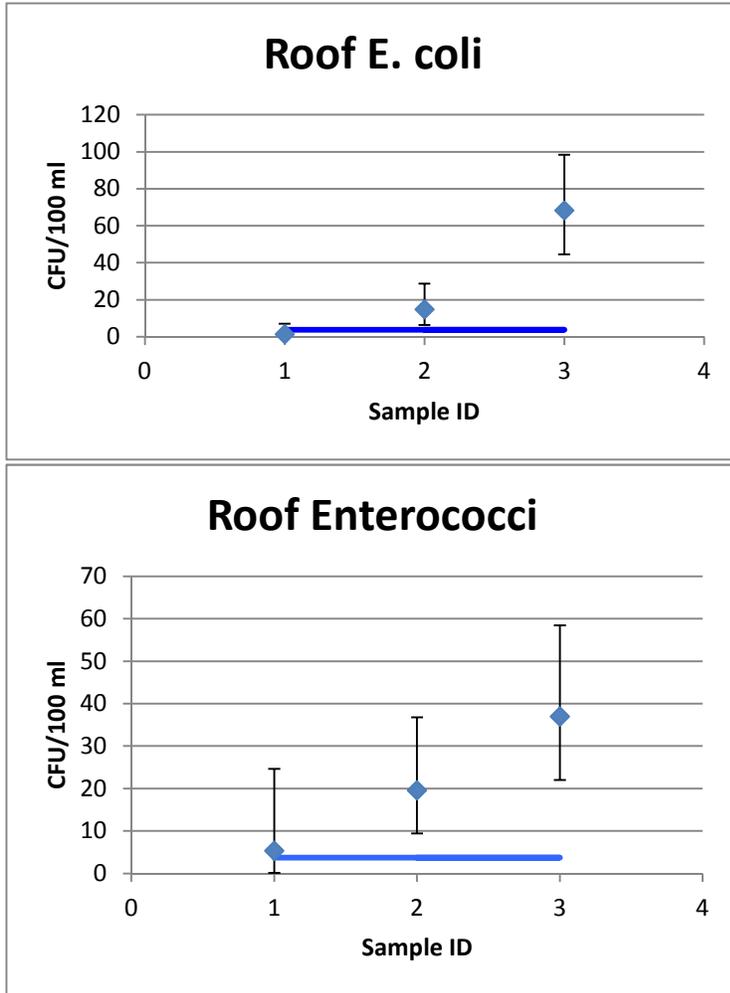


Figure 4.2.1.3 Washoff vs Follow-up. Sample IDs: 1=Washoff (eaves) readable mean from prior simulated rain tests; 2=Follow-up (natural rain), eaves; 3=Follow-up (natural rain), downspout, horizontal line = Upper Confidence limit of BDL. All 95% confidence limits from MPN determination.

The significance of these follow-up results is twofold. Important to this particular study, the elevated FIB densities captured from the roof eaves relative to those obtained from the simulated-rain study (which was also sampled as runoff from the eave-edge, before entrance to the gutter) is an indication of the unrealistic nature of our simulated rain-intensity surrogate. While the greater control over rainfall-rate equivalence allowed by our simulated rain was important to our study here, there did seem some trade-off with the kinetic energies available from terminal-velocity natural-drop impacts to dislodge FIB. Important to the overall goal of our

research here is the even greater elevated densities obtained from natural-rain samples from the downspout vs those from the natural-rain samples from the roof eaves; absent any expectation of birds and squirrels preferentially defecating into the downspouts or gutters, it provides some evidence of interevent source-area retention of FIB from a previous rain.

4.2.1.2.3.2 Lawn

Our simulated-rainfall examination of rain-induced FIB releases from a pervious surface was performed on the fourth day after a 0.30" rainfall (late summer, Tuscaloosa, TLC F6). Measured coefficient of variation (0.46) of simulated-rainfall distribution over the course of this examination was slightly larger than found in our preliminary hydraulic characterization (0.31-0.35, performed on a level slab, and presented in the Appendix, below) of the rainframe.

Though including information not directly related to hypothesis testing here, our sampling scheme provided for general characteristics of our simulated rainfall vs the nominal design parameters. The example presented in the Appendix represents this study (see esp. Figure 4.2.1.2.23).

4.2.1.2.3.2.1 *E. coli*

Figure 4.2.1.4 presents a graphic representation of our findings relevant to testing of Hypothesis 1.

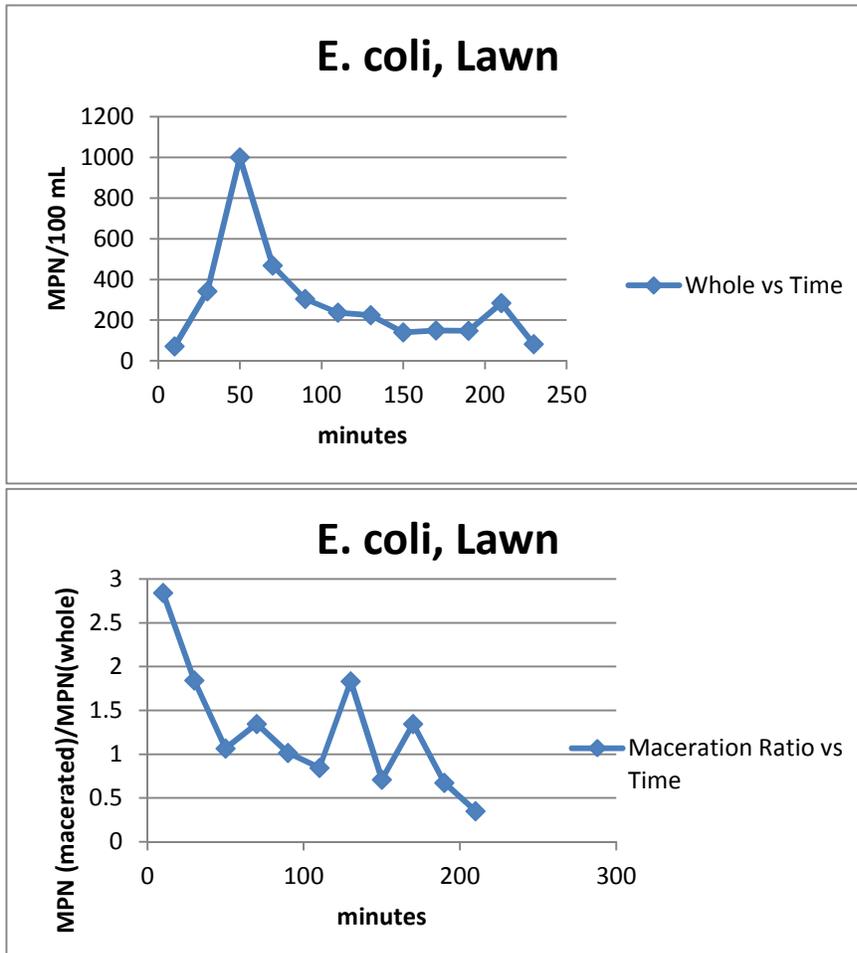


Figure 4.2.1.4 *E. coli*, lawn vs time, with multicellularity signal.

The figure above (and all presented here) represents a treatment in which the surface was continuously wetted over the course of the study period. The presence of a maceration ratio (MPN of macerated subsample/ MPN of raw subsample) in excess of 2 (~ 2.85 in the first, leftmost data point of the bottom panel of the figure) allows for rejection of two putative release mechanisms (namely passive flush of extant planktonic cells, and active dispersal of seeds) as sole processes by which *E. coli* was released in response to our simulated rainfall. Other samples showing elevated response to maceration provide further evidence of some other operational mechanism(s). This finding does not logically allow us, however, to reject either mechanism as a contributing factor. Further inferences are not so categorical. Also worthy of note from this

figure is the presence of some maceration ratios with values less than unity. Our “optimized” maceration procedure (see Appendix below) does seem to kill some cells in the blender.

In the particular case of extant (prior to rain) planktonic *E. coli*, we do have, at least, a qualitative measure of the significance of relative potential *E. coli* CFU contributions to the total over the course of a rain. The expectation of a first-flush response (4.2.1.2.4 above) from such CFU (cells or cell pairs) provides a further expectation that their total contribution would quickly diminish over wetted time. Our observations here of increasing CFU densities out to about an hour of rainfall (and over an inch of depth) while the signal for multicellularity was in decline provides some support for the literature consensus that most extant FIB were indeed filmbound before the rain. Any definition of “most” here is, however, not quantifiable with information available.

Our analysis of Hypothesis 2 is shown at Figure 4.2.1.5. The macerated CFU density multiplied by rain intensity is a measure of total cell-release rate. The top panel of the figure is the same as that in the figure above, and provides perspective of the time course of the rain and the results that any monitoring effort would capture. The failure of the bottom-panel plot to even remotely resemble a horizontal line allows for categorical rejection of cell division/erosion as the sole mechanism by which *E. coli* were dispersed by this rain. This, of course, does not, by itself, logically exclude the possibility of erosion as a contributor. This measure does, however, provide for an estimate of the extent of such a contribution. Further, in this special case, we are serendipitously provided a measure of the extent to which seeding (the only planktonic-cell source likely to be operating late in a rainfall) may contribute to *E. coli* release in this rain. The mechanism of cell-division release implies that FIB are released as single cells (whether planktonic or sessile). The expectation that erosion results in a constant cell-release rate over

wetted time implies that the minimum rate (found at 190 minutes in the figure) represents the maximum rate that could possibly be held constant over a rain. An assumption that all of the raw (not macerated) CFU found in our 190-minute sample were unicellular generally provides a maximum number of CFU that could possibly be attributable to erosion over the rain, and the product of that raw CFU density multiplied by the intensity at 190 minutes provides an “erosion potential,” a general limit to the CFU-release rate that could be attributed to cell-division.

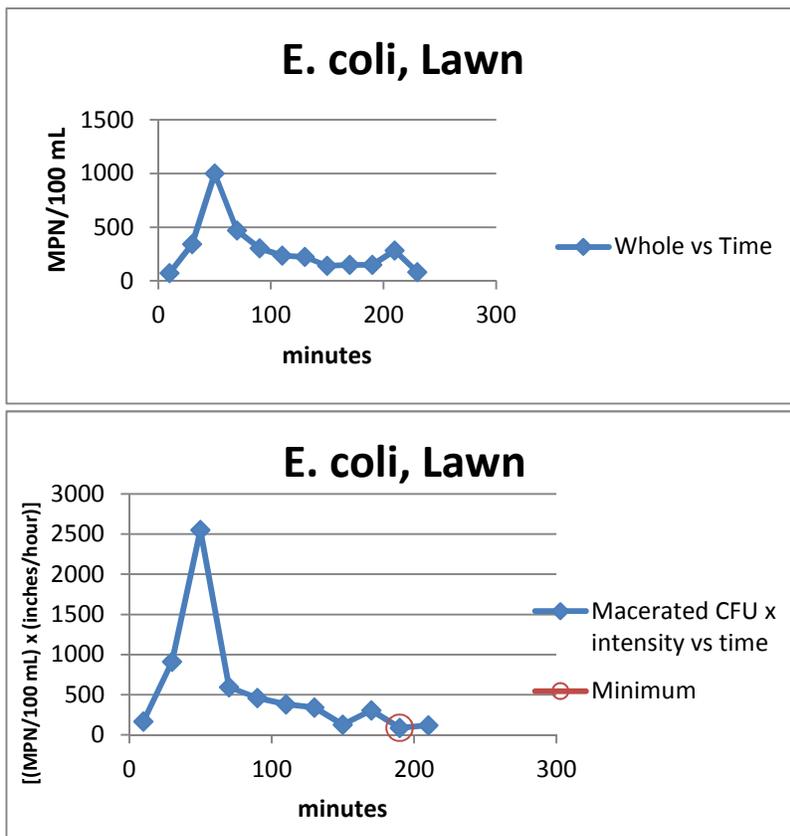


Figure 4.2.1.5 *E. coli* vs Time, with Cell-release rate signal,

In this special case, the planktonic nature of seeding-dispersed *E. coli*, together with the finding of the minimum cell-release minimum so late in the rain event, combine to make this erosion-potential measurement more restrictive. While the cell-signals leading to lags and plateaus in rates of seeding dispersal are poorly understood, the literature provides that the rate increases monotonically (or at least does not decline) over wetted time. In this particular case,

the “erosion potential” as defined above becomes what we’ll call a “planktonic potential,” a maximum limit to the contribution of the sum of both active-dispersal (seeding and erosion) mechanisms (see Figure 4.2.1.6).

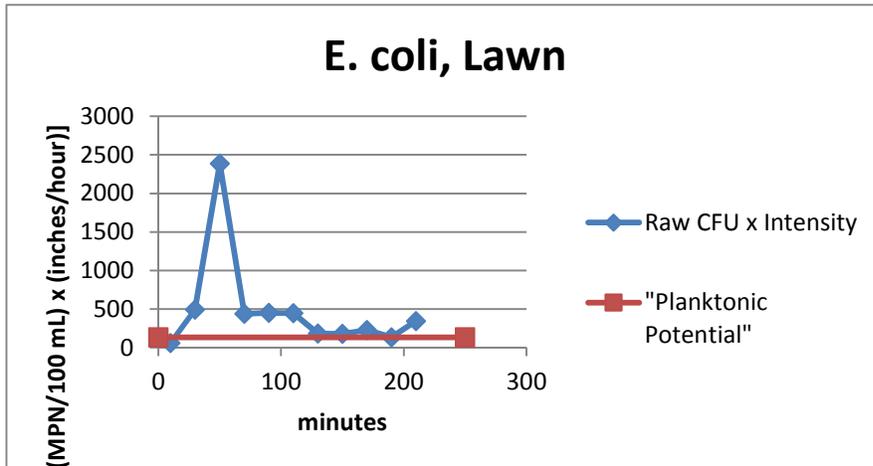


Figure 4.2.1.6 *E. coli* vs time, with active/passive release separation.

While we cannot quantify the individual components here, it would seem clear that passive-release mechanisms (sum of extant planktonic flush and biofilm sloughing, above the “planktonic potential” line in the figure) account for much of the CFU release of *E. coli* from this pervious surface. This implies a need to examine potential effects of depth and intensity with possible changes over wetted time in any modeling efforts.

While the data presented here may seem insufficient (12 data points over a 3-hour, >5-inch, simulated rain, with minimal replication) for any proper modeling effort, it seemed important to at least explore the conclusions derived from this screening exercise for a preliminary goodness of fit. This (*E. coli*, Lawn) dataset was chosen for this rudimentary exercise because it was (frankly) the easiest to work with. This section provides the most complete dataset, the most thorough testability of hypotheses, and an unexpected field observation that revealed a presumptive breakpoint. Relevant portions of the development of this model-building effort are presented.

The strategy here was to model the found CFU densities as a function of rain depth and/or rain intensity, with the potential for changes in wetted time or depth. This strategy represents, in essence, a focus on the passive-release mechanisms (pre-rain extant FIB flush, and sloughing, with the latter accounting for an unquantifiable “most” of the results). It also relegates the unknown (and, in the case of seeding dispersal, currently unknowable) active-release mechanisms to generators of noise.

Our first finding of note was an apparent correspondence between an apparent discontinuity in a plot of MPN/depth vs wetted time, and the apparent extinction of visible cloudiness of our samples (Figure 4.2.1.7 and, upon this observation, we regretted not having thought to have turbidimeter handy). The visible cloudiness (bottom panel in the figure) waned over the first three (in time) sampling events, even though this was a period of increasing intensity in our simulated-rain application schedule, and disappeared by the fourth. The observed cloudiness was easily suspended by hand swirling the sample containers and did not disappear for over a half-hour of quiescent settling. This observation provided a potential mechanistically explainable breakpoint for our modeling effort here. We don't know (nor can we know with available data) the composition/form of the suspended material (flocs?, cells filmbound to organics or clay particles?, partially disaggregated fecal material?) but the coincidence of maximum cloudiness (first sample in time) with the maximum signal of multicellularity (Figure 4.2.1.4, above) argues against the flushing of (pre-rain) extant planktonic *E. coli* as a major contributor. Timing of the apparent discontinuity was difficult to ascertain in this low-resolution dataset, but was somewhere between 50 and 70 wetted-surface minutes (1.16 – 1.72 inches of simulated rain depth). We proceeded on an assumed breakpoint at 60 minutes (1.44” rain depth). Worthy of note here is that there is no reason to impose continuity on the breakpoint model as we

proceed here. In fact, the mechanistic defensibility of this assumed breakpoint, based on potential extinction of some available CFU (easily suspended) class argues against such an imposition.

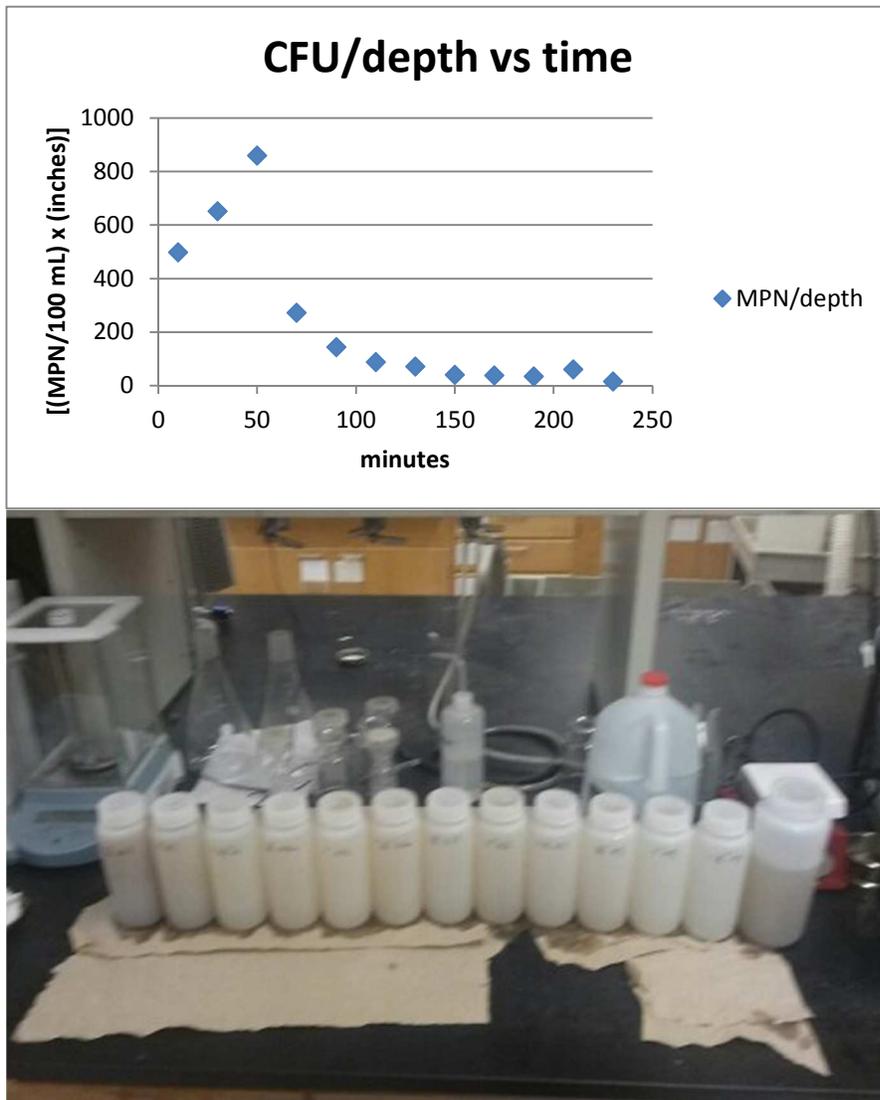


Figure 4.2.1.7 *E. coli*, lawn, discontinuity identification. No common scale, but sample order (in time, left to right) is preserved. Rightmost vessel in bottom panel = final washoff.

With the assumed breakpoint imposed, the segments adjoined thereby were each examined for sensitivity to depth or intensity (no potential interactions were considered in this rudimentary effort with this dataset). Best fit for both adjoined segments was found against depth (see Figure 4.2.1.8). The so adjoined segmented models were combined and a goodness of fit

was calculated (in Excel™, Figure 4.2.1.9). While some “cherry picking” of datasets to model here is obvious (and admitted), results would seem to provide some confidence in the mechanistic-screening strategy presented.

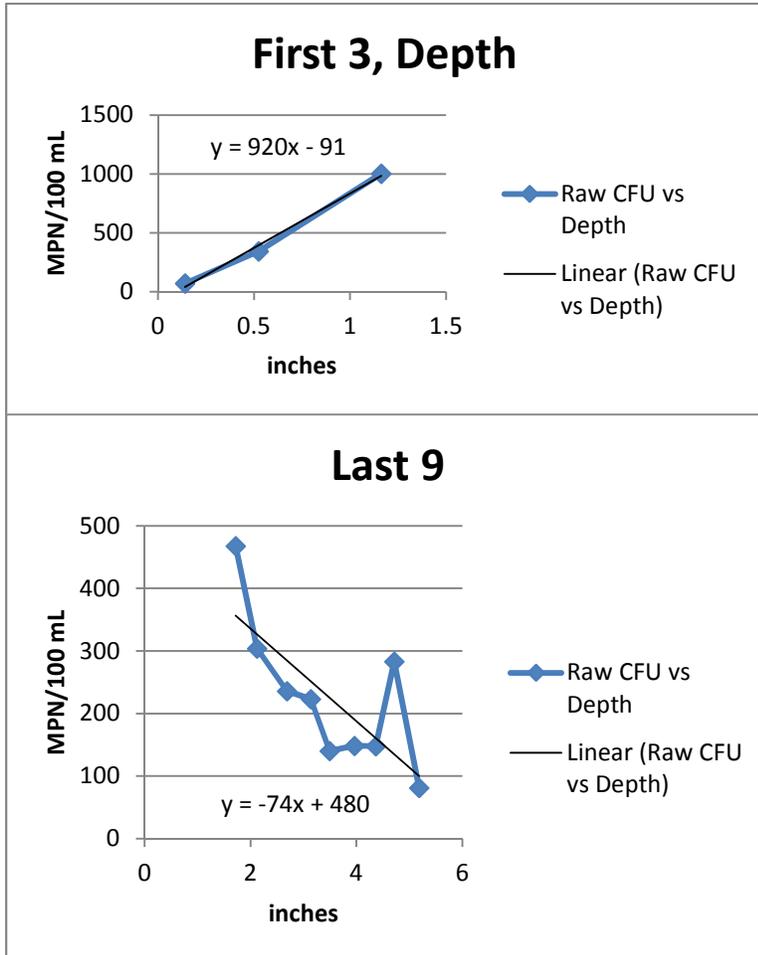


Figure 4.2.1.8 Segment Models, *E. coli*, Lawn. Upper Panel = 1st 3 (in time) sampling events, Bottom Panel = Last 9 sampling events, not to common scale.

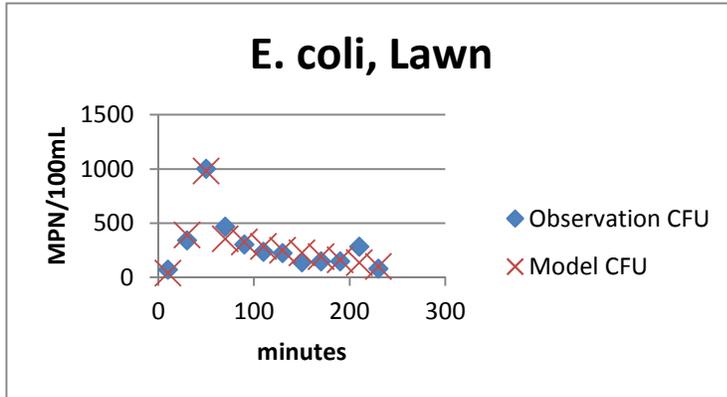


Figure 4.2.1.9 Segmented Model, *E. coli*, Lawn. n = 12, $r^2 = 0.93$

As a final note for this section, the posited (though not individually quantifiably knowable) significance of the examined biofilm-dispersal mechanisms important to *E. coli* release from this lawn imply a potential for post-rain biofilm retention of FIB. Such retained FIB may well survive and be available for dispersal by subsequent rains (as was at least hinted at by the follow-up study of the roof-downspout above). At the end of the data collection effort presented in this section, the study site was “swept” with a household “pressure wash” fitting affixed to our rainframe manifold (with booster pump). The pressure-wash fitting had been preliminarily characterized as providing about 10 inches/hour to about a one square foot area (with an observation that droplet size and speed of impact both considerably exceeded those encountered in natural rainfalls). While not strictly destructive sampling, it was as close as we were willing to get for the lawn under study here. After the simulated, ~ 5 – inch rainfall modeled here, a five-gallon sweep of the studied surface released (mean of two readable dilutions) runoff of 1,192 CFU/100 mL (and see cloudiness of rightmost vessel, bottom panel, Figure 4.2.1.7 above). Clearly, *E. coli* FIB from the lawn washoff tests are not source limited.

4.2.1.2.3.2.2 Enterococci

Though Enterococci are poor candidates for testing of Hypothesis 1, due to their potential to express considerable and unpredictable multicellularity even in the planktonic morphology, Figure 4.2.1.10 is presented for perspective. The most important feature in this figure here is that every CFU found in stormwater monitoring has the potential to (upon shearing downstream) produce upwards of ~ 1.8 CFU in regulated waters. Our only recourse here, of some necessity, is the presumption that Enterococci collocated with *E. coli* on the landscape, prior to a rain event, share (between taxa) a naked and planktonic state or filmbound and sessile state. Our relevant conclusions above were that neither passive flush of extant (pre-rain) FIB, nor seeding dispersal of FIB can be a sole operating mechanism for release of FIB from stormwater source areas. Our observations here (rapidly the rising CFU densities out past 70 minutes, > 1.7 inches of rain depth under multiple simulated intensity regimes) belie any first-flush behavior and provide evidence that “most” (unquantifiable) extant Enterococci were (pre-rain) filmbound.

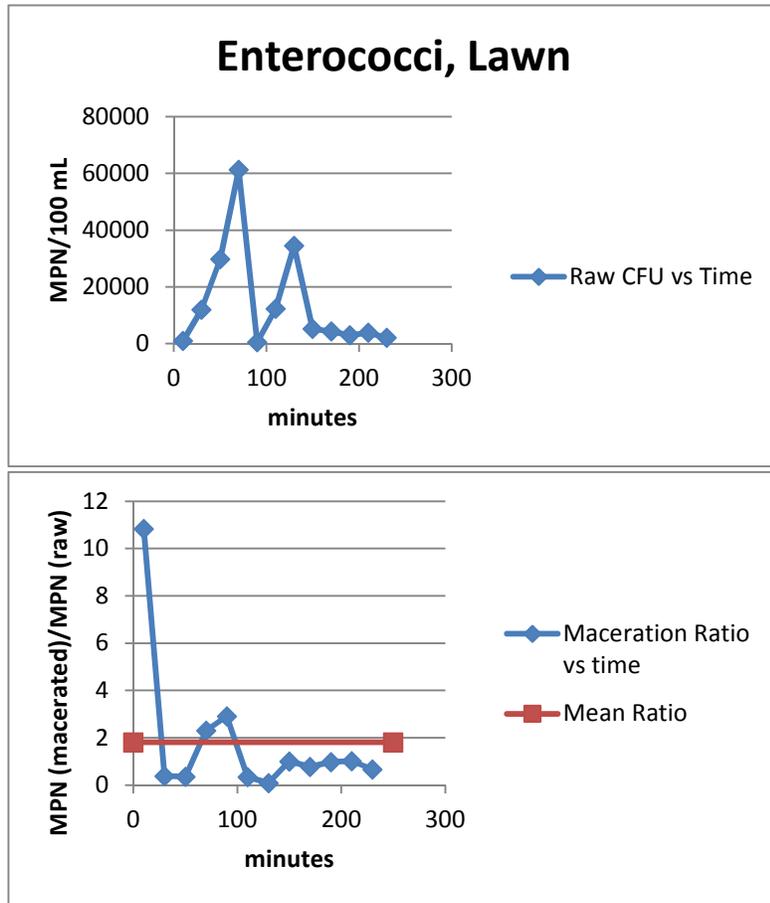


Figure 4.2.1.10 Enterococci, Lawn. Mean maceration ratio (bottom panel) = 1.8.

Hypothesis 2 testing, concerning cell-division/erosion potential, is presented at Figure 4.2.1.11. While information here is considerable, it is not as defining as that found in the case of *E. coli* above (Figure 4.2.1.5). The absence of any semblance of a horizontal line in the plot still allows for categorical rejection of cell-division/erosion as the sole mechanism by which these FIB are dispersed in response to rain. The identification of a minimum in the cell-release rate (at 230 minutes) still provides for a calculation of an “erosion potential,” a maximum limit of CFU contribution that can be attributable to cell-division/erosion. We still have a minimum cell-release rate that is near the end of our 3-hour rain, and that defines a clear maximum limit to total cell-release rate that might be attributable to active-release mechanisms in general. Without any

expectation of unicellular planktonic Enterococci, we are logically denied any way to translate our “erosion potential” to a “planktonic potential” as we did above.

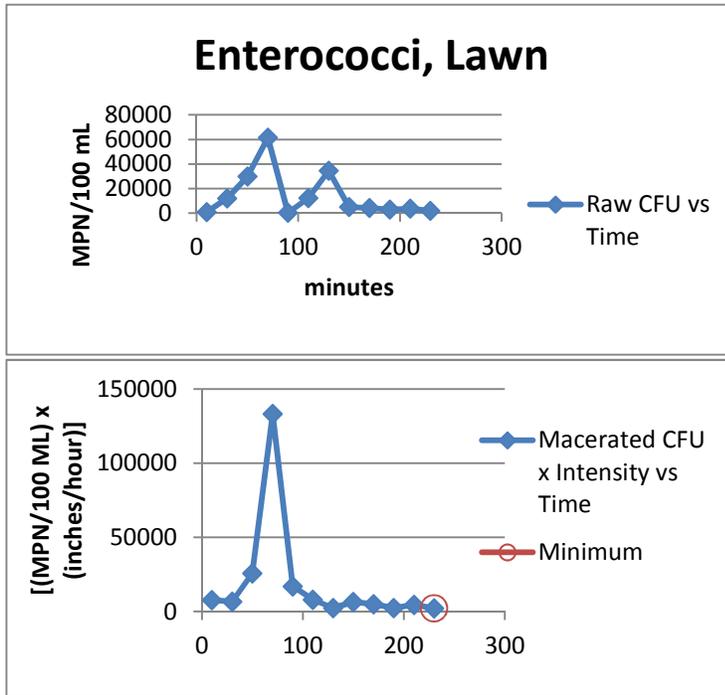


Figure 4.2.1.11 Enterococci, Lawn, total cell-release rate

Figure 4.2.1.12 shows the best mechanistic segregation available for this Enterococci dataset. The potential for multicellular morphology in planktonic Enterococci reduces our ability to isolate active from passive release mechanisms. The area below the “erosion potential” line in the figure represents the maximum contribution of active cell division. Such potential contribution is likely small enough to be treated as noise in any modeling effort. The ample area above that line represents all contributions from passive release mechanisms but also may include some unknowable contribution from seeding dispersal. Our necessary presumption of shared (planktonic vs sessile) state, on the shared landscape that provided our *E. coli* data above, allows us to conclude that the upper area does not represent *only* active seeding contributions and even that it *mostly* represents contributions from passive mechanisms. We can only assume,

however that seeding contributions are limited enough to safely treat as noise in any modeling efforts.

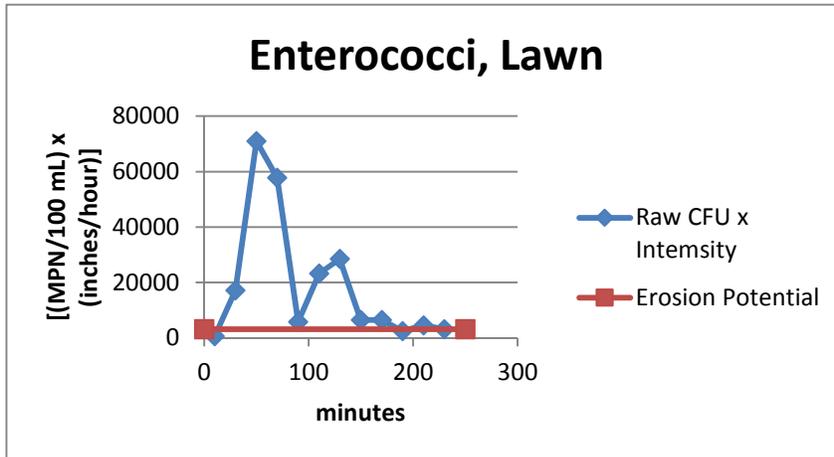


Figure 4.2.1.12 Relative contributions to Enterococci CFU

A rudimentary modeling effort was performed, following the strategy suggested in the *E. coli* section above. An obvious discontinuity is found at about 80 minutes in a time plot of CFU/depth (Figure 4.2.1.13). This breakpoint candidate is found one sampling event later than that found in the *E. coli* effort above. A second candidate appears at ~ 140 minutes, but we risk ignoring it for this effort (future research in this arena would be best informed by a turbidimeter more sensitive than our eyeballs). Depth-based modeling (Figure 4.2.1.14) of the adjoined segments was again found to be a better fit than modeling by intensity. The resulting segmented model is presented at Figure 4.2.1.15.

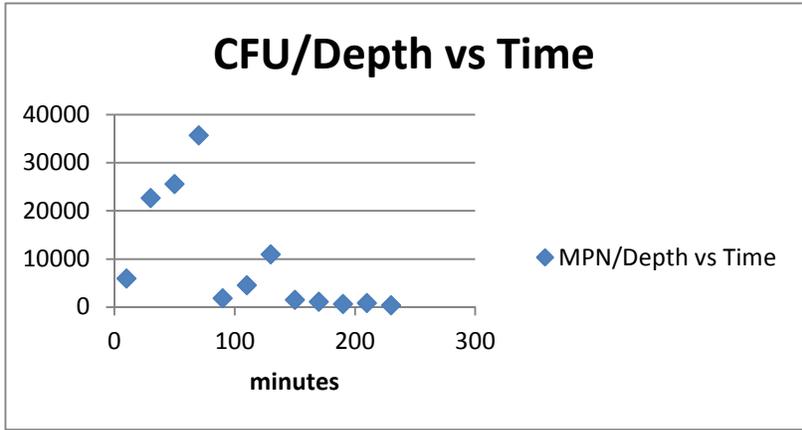


Figure 4.2.1.13 Enterococci, Lawn, breakpoint identification.

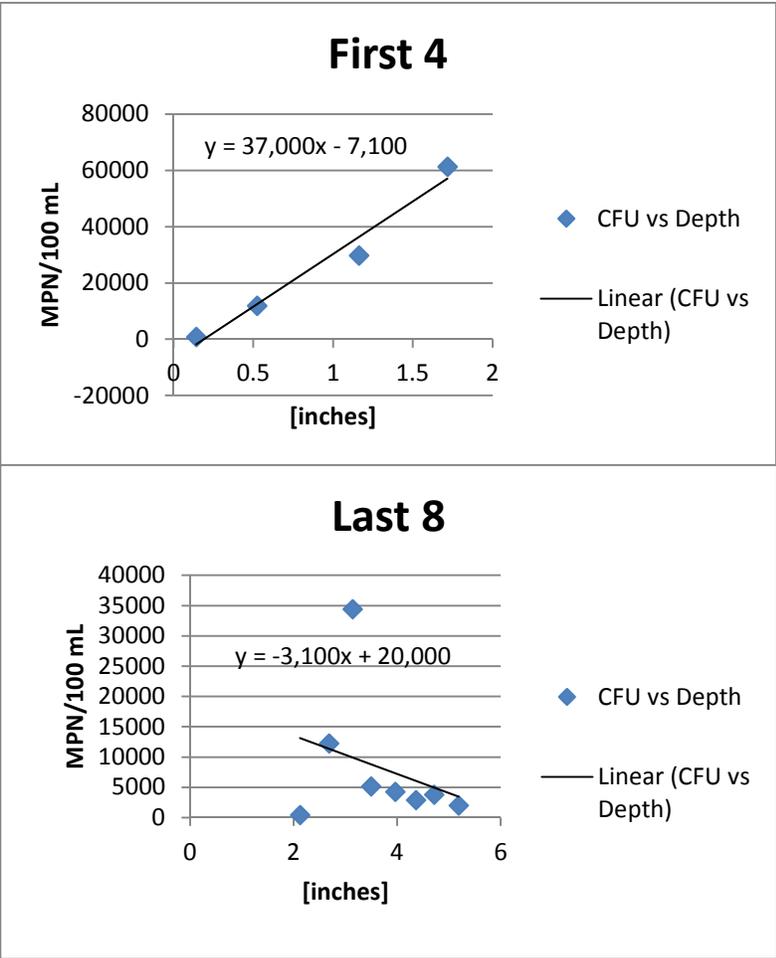


Figure 4.2.1.14 Enterococci, Lawn, segment models. (no common scale)

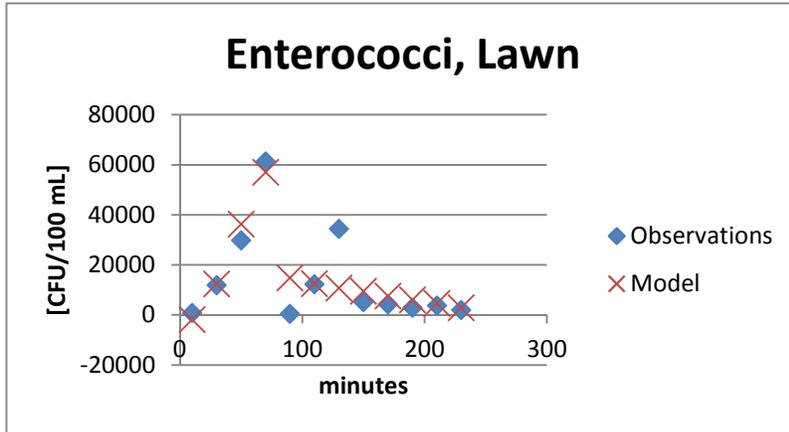


Figure 4.2.1.14 Enterococci, Lawn, segmented model. n=12, $r^2=0.79$

Goodness of fit ($r^2 = 0.79$) resulting from this modeling effort suffered relative to that found in the *E. coli* study above. Whether from unaccounted noise in the current dataset or from our negligence of a candidate breakpoint in this simple effort, the results do provide some comfort with our presumption that “most” uncertainty can be captured by effects of passive release mechanisms.

Our post-study, final five-gallon washoff by sweeping motion with the pressure-wash nozzle yielded 4,690 CFU / 100 mL Enterococci, after 3 hours exposure to five inches of simulated rain, again indicating that Enterococci are not source-limited on lawn surfaces.

4.2.1.2.3.3 Street

Our street washoff studies (early autumn, Tuscaloosa) were performed on the third rainless day after a 0.06-inch rain (TCL F6). Characterization of simulated-rain distribution run contemporarily with the studies found a coefficient of variation = 0.46 on this sloped street. Intermittent and incipient emitter clogs may have contributed to the poor distribution, and certainly contributed to lower than nominal measured rain intensity. 2.5” of simulated rainfall was delivered over the three-hour study period.

4.2.1.2.3.3.1 *E. coli*

Washoff data of *E. coli* in stormwater from our street study site are presented at Figure 4.2.1.15. Only 5 of the 12 sampling events yielded readable results (all others BDL) and no readable maceration data were obtained (1:100 dilutions). Like the roof studies above, the low MPN values of this dataset was surprising

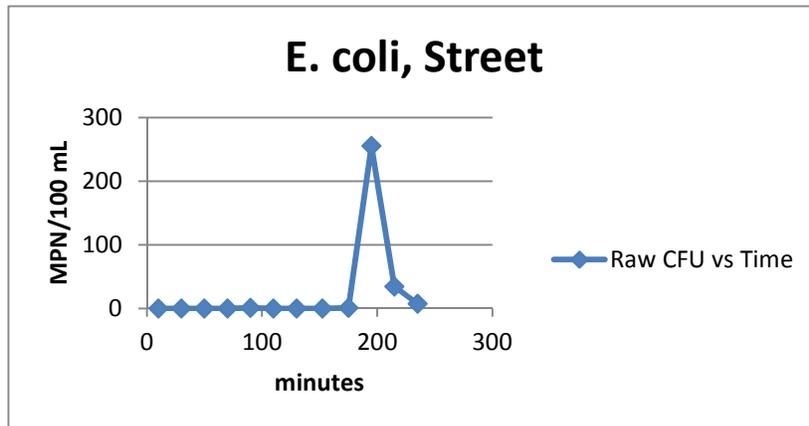


Figure 4.2.1.15, *E. coli*, Street, CFU vs wetted time.

Visual observation of sample bottles revealed no hint of cloudiness (Figure 4.2.1.16). Considerable settleable solids (all black) were produced in high-intensity samples and especially in the final washoff.



Figure 4.2.1.16 Sample Vessels, in wetted-time order. (1st on left, post-study washoff at right).

This observation may at least help to explain our results here, and may supply some aid in interpretation of the lawn studies above. The early stages of both of those studies appeared to be dominated by easily suspended particulates exhibiting considerable multicellularity. Distinct breakpoints found seemed to coincide with exhaustion of those particulates, and the regimes following the breakpoints exhibited reduced FIB densities and reduced evidence of multicellularity. The results above could be consistent with either particulate sloughing that masked a contemporary low-density release regime until exhaustion of particulates, or to a low-density regime that revealed itself only after some wetted time of exposure. While far from conclusive, the few data here at least suggest a wetted-time lag before onset of FIB release. This might further suggest that sloughing of easily suspended filmbound solids dominate releases if they're available, and that shearing of more extended surficial films is enhanced over time by wetting and swelling.

This street site, like the roof site discussed above, has yielded larger FIB densities in past research efforts. Those previous efforts, however, involved sampling of heavy, natural rainfalls, selected for heavy runoff (typically about ankle deep in the street gutters). No such runoff was acquired here (nowhere close), and sampling was difficult.

Whatever the reasons, the dataset acquired here could not be used to test the stated hypotheses.

Our final, post-study washoff with pressure-wash nozzle, after three hours of exposure to 2-1/2" of simulated rain yielded 70 CFU/100 mL (average of two readable dilutions).

4.2.1.2.3.3.2 Enterococci

We acquired 11 readable raw samples and six readable macerated samples from our 12 sampling opportunities. As stated before, Enterococci samples are unsuitable for testing of Hypothesis 1. We lack any information concerning that hypothesis from the *E. coli* study above that could be presumptively applied here. Figure 4.2.1.17 presents multicellularity information for samples with readable maceration enumerations.

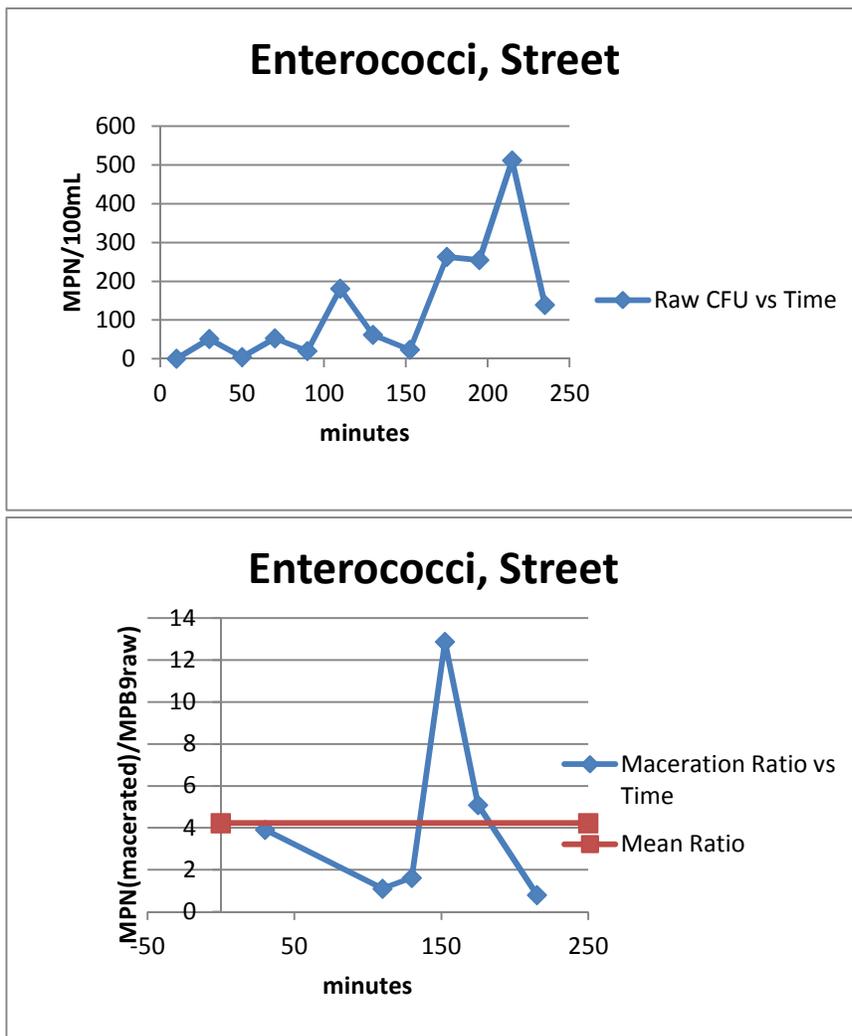


Figure 4.2.1.17 Enterococci, Street, multicellularity

Worthy of note in the figure (bottom panel) is the average raw CFU has the potential to introduce 4.2 viable CFU downstream. Also note (top panel) that, in the absence of visible

suspended particulates in these samples, an increase in CFU densities seems to begin after about a 90-minute lag.

Hypothesis 2 testing was carried out (Figure 4.2.1.18) for the six samples that yielded results within detection limits. Cell-division would seem to contribute very little to the overall release of Enterococci here. We have no information with which to apportion the remainder.

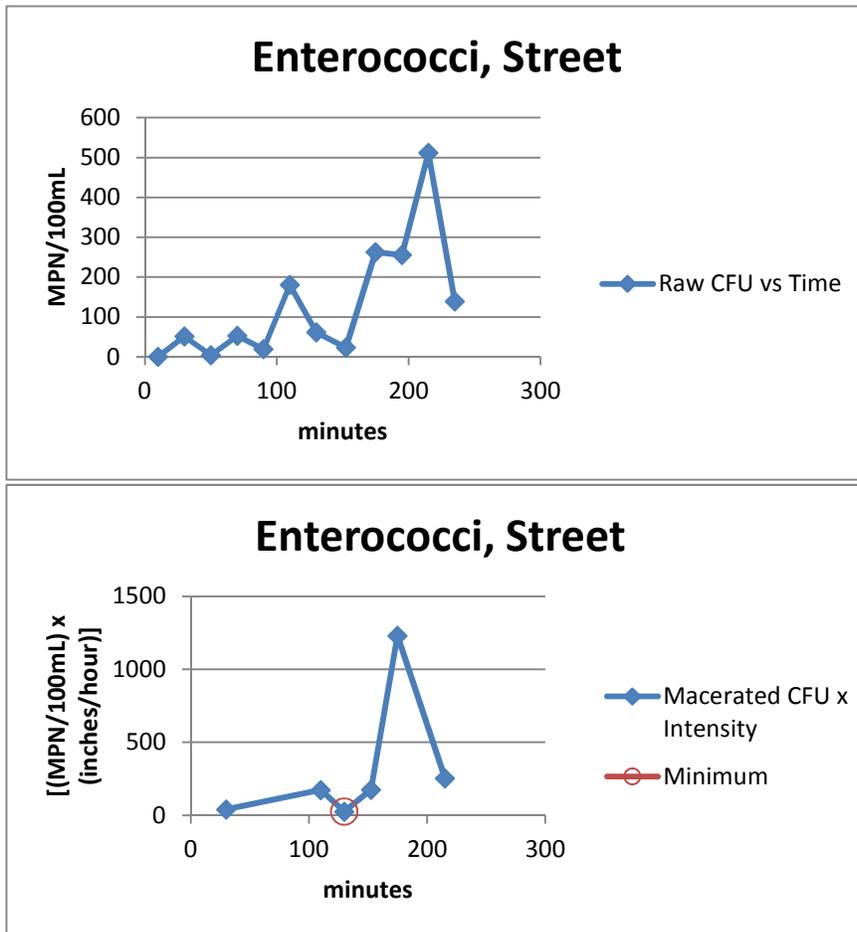


Figure 4.2.1.18 Enterococci, Street, cell release rates

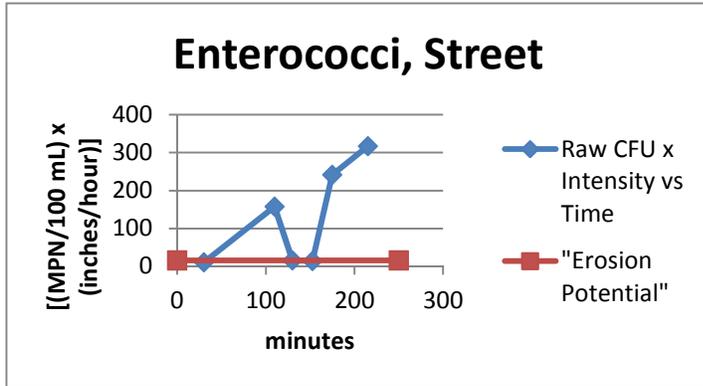


Figure 4.2.1.19 Enterococci, Street, cell-division contribution

4.2.1.3 Conclusions

Where sufficient data were available, the modeling efforts presented here indicate that contributions of passive releases (flushing of extant planktonic cells and washoff/sloughing of clumps of filmbound bacteria, likely dominated by the latter) are of a sufficiently large fraction of the total CFU released in a rain that neglecting the unknown (or unknowable) effects on runoff patterns by active dispersal mechanisms leaves sufficient information for useful models. The modeling efforts presented here are not of general utility but show that for runoff modeling of individual sites, attention to rain depth and intensity, with the possibility of breakpoints, should be sufficient.

We see convincing information that effects of sloughing on FIB-runoff patterns can be usefully split into two modes. When easily suspended filmbound particulates are present, they seem to be dominant influences early in a rain and until their extinction. The importance of sloughing from surficial films seems to arise later in the rain after sufficient wetting time. Confirmation of this asserted pattern, however, must be relegated to future research.

We see no evidence of the extinction of landscape FIB even under long (three-hour), heavy (multi-inch) rains. After completion of our simulated-rain studies, we unfailingly found FIB CFU released by the influence of a pressure-wash hose nozzle.

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- McDougald, Diane, Scott A. Rice, Nicolas Barraud, Peter D. Steinberg, and Staffan Kjelleberg, 2012. Should we stay or should we go: mechanisms and ecological consequences for bioflim dispersal, *Nature Reviews: Microbiology*, V. 10, pp.39-50.

4.2.1.2 Appendix, preliminary studies

This appendix presents information tangential to, but not directly germane to the article above. It further provides perspective and continuity to this overall dissertation.

Flowmeter Calibration

Use of a lawn-irrigation, garden-hose connected cumulative-flow meter (Save A Drop™, 0.1-gallon resolution readout) was chosen here for convenience in the field. Its use here as the primary source of information concerning both equivalent applied rain depth and intensity prompted concerns over its accuracy. A simple, but traceable calibration was performed.

The input train for the rainframe manifold was assembled as for use in this study, in the order: source water > booster pump > garden hose > pressure regulator > flowmeter > manifold (and refer to Figure 9, at 3.2.2.2.1 above). A five-gallon bucket (Homer's All-Purpose Bucket™) was filled to the 4-1/2 gallon level (by use of a household, graduated, 2-quart measuring cup). The bucket was marked at the 4-1/2 gallon level (permanent marker) and emptied. The bucket was then refilled to the mark by use of the (zeroed flowmeter) fitted manifold (one outlet tap valved wide open), and the meter output recorded. A calibration correction factor was calculated as:

$$\begin{aligned}\text{Flow [actual]} &= (4.5[\text{actual}]/4.2[\text{gauge}]) \times \text{Flow [gauge]} \\ &= 1.07 \times \text{Flow [gauge]}\end{aligned}$$

Maceration Optimization

The study above made use of a laboratory blender (Waring™ model #HGB2WTG4) to test for multicellularity of FIB CFU released from source areas. The vortex shear of a blender has long been recognized (*e.g.*, see Lindahl and Bakken, 1995) as an efficient method for disaggregation of soil-cell assemblages. The downside of such shear, and the potential for cavitation and for heating of the sample, is that it may lead to lysis and death of individual cells. The purpose here was to maximize the degree of disaggregation of CFU found in the study areas (as measured by elevation of MPN above that found in the raw sample) within limits of over-treatment leading to increased mortality (declining MPN).

Runoff was captured opportunistically from two rain events and from two of the closely collocated study sites. One was from the residential lawn, though (for this natural rain) some roof runoff from up-gradient was necessarily included. The lawn sample was collected after a rather short interevent period (only two days after a previous rain of 0.23”). The street sample (again, necessarily including runoff from up the street, but isolated from any pervious-surface runoff) was similarly collected subsequent to a short dry period (two days after a 1.03” rain). All rainfall data here (and throughout this dissertation) are taken from the available Preliminary Monthly Climate Data (the “F6 Product”) for Tuscaloosa Regional Airport (TCL), provided by the National Weather Service, and are hereinafter referred to as “TCL F6.” TCL is ~2.5 miles from the study area as measured by ruler on a GoogleMaps™ printout.

Both samples were split (cone splitter) and analyzed for both FIB taxa here, after varying intervals of continuous exposure to blender shear at both low and high speeds (18,000 and 22,000 rpm, respectively) and all results were normalized to raw (zero minutes of exposure) values. Results are shown below at Figure 4.2.1.2.20 below.

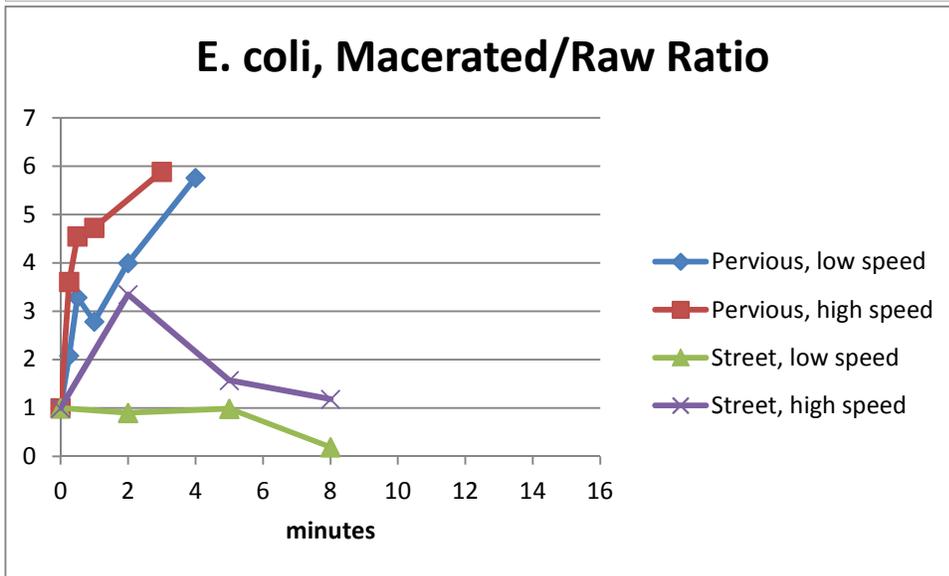
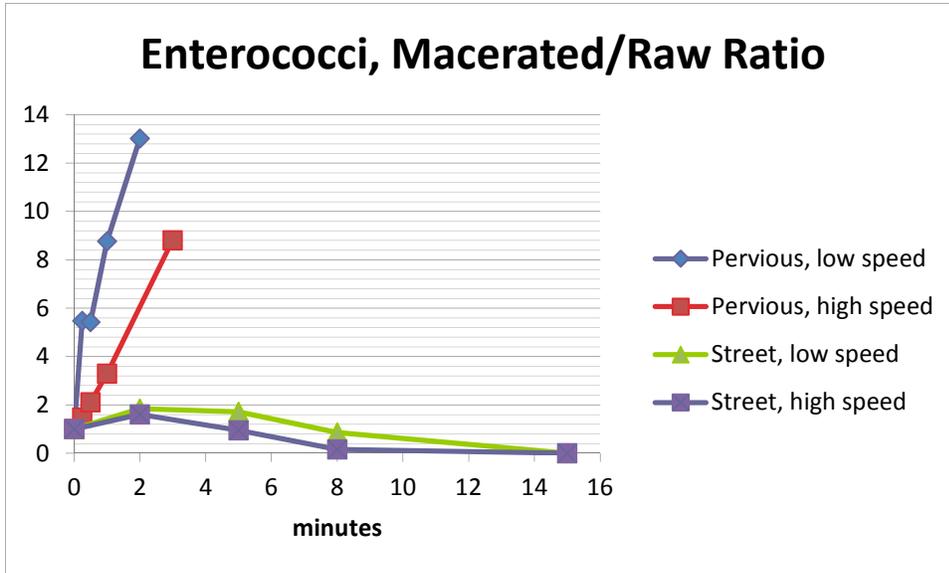


Figure 4.2.1.2.20 Maceration elevation of normalized MPN CFU vs time of maceration. Vertical axes not to common scale.

Examination of the figure above reveals considerably different responses to maceration by both taxa, depending on surface. In order to, as best possible, provide for direct comparisons, some compromise was required in this treatment “optimization” exercise. Focusing on *E. coli* (lower panel of the figure, greater resolution for clarity, and upon which the hypothetical structure of this study is largely based), samples from the pervious (lawn) surface show no diminution of MPN over times of maceration out to 3-4 minutes. Analyses of the subsequent

samples from the street surface were intentionally stretched to somewhat longer intervals to expand the search for such diminution, but found it early (2-5 minute range, with a much clearer mortality signal at low-speed). A treatment of 3-minute exposure to low-speed shearing was chosen for the study, with full knowledge that such treatment may well mask the full multicellularity of some CFU and may well include the onset of some cellular lysis.

Sample Preservation

The above study was expected to require some extended storage of samples in the field before their return to the lab for analysis. The literature reviewed here (see extended discussion at 2.2.4.3.2 above and, in particular, Atlas, 1984, p. 342) provides evidence of a long-held broad consensus that chilling such samples (near cardinal minimum-growth temperatures, *e.g.*, ice chest for FIB samples) provides best preservation of original bacterial densities during storage and/or transport. Higher temperatures run risk of either accelerating growth- or mortality-inducing reactions or, competitively, both. Applicability of chilling for preservation to the taxa measured and the surfaces studied here was preliminarily studied.

Street runoff from the study area was opportunistically sampled during a natural rain event. The street in the study area was the only surface producing sufficient runoff for sampling during this “Trace” rainfall (preceded by three consecutive days of 0.1 – 0.15”/day, intermittent rains, TCL F6). The bulk sample was split (churn splitter) into two 1-L treatment (ambient vs refrigerated) aliquots and two (one for each FIB taxon under study here) 100-mL subsamples for immediate (exposure hours = zero) analyses. The 1-L bulks were then each separated (cone splitter) into 10 individual IDEXX™ sample bottles (pre-sterilized, polycarbonate, 120 mL), for exposure, over ~ two-day period, to either indoor ambient or refrigerated conditions (the latter at

36⁰ F). Indoor ambient laboratory conditions were not in any way controlled by this researcher, but subject to the vagaries of air-conditioning and lighting prevalent in August, Tuscaloosa, AL. Intermittently, samples (with 1:100 and 1:10,000 dilutions) were analyzed for FIB densities by IDEXX methods (Colilert™, Enterolert™, and Quatitrays 2000™). Results, with observations presented as averages whenever two or more dilutions produced meaningful measurements, are presented below at Figure 4.2.1.2.21.

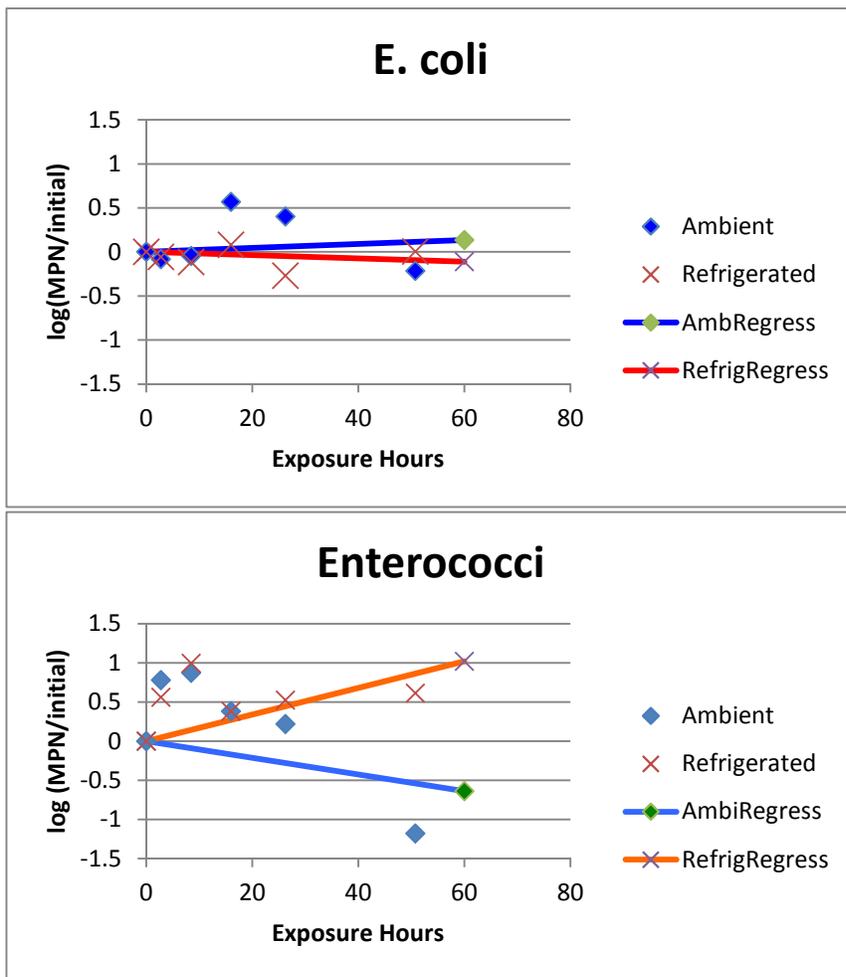


Figure 4.2.1.2.21. AmbiRegress = log-linear regression line for indoor-ambient treatment, RefrigRegress = log-linear regression for refrigerated treatment.

A notable feature is the lesser deviation from original density-measurement preservation for *E. coli* (relative to Enterococci) under both (refrigerated vs ambient) treatments. Not shown in

the figure is the very small (close to zero) regression slopes for both (0.00225/hour for Ambient, -0.00182/hour for Refrigerated). The Refrigerated-treatment produced much lower variability (a four-fold increase in r^2 relative to Ambient) though neither treatment was very good in that regard ($r^2 = 0.125$ for Refrigerated). Regressed slopes for preservation of original densities of Enterococci were different from those found with *E. coli*, both quantitatively and qualitatively. Quantitatively, slopes (roughly ten-fold larger) were different from zero (-0.0107/hour for Ambient, and 0.0170/hour for Refrigerated) and, qualitatively, the direction of the slopes (positive/negative) were reversed (positive for Refrigerated). The Refrigerated treatment for Enterococci did (as with *E. coli*) result in about a four-fold increase in r^2 (though only to 0.493). None of the treatments showed (and not shown here) any evidence of diurnal-cycle behavior. Samples collected in the above study were packed in an iced chest for storage and transport.

“Rainframe,” Preliminary Hydraulic Characterization

Use of drip-irrigation emitters attached to an adjustable wooden frame, to apply controllable simulated rainfall to sources areas in this study required preliminary characterization of the assembly to determine its optimal configuration and orientation on the landscape. This preliminary characterization was performed on a shaded, level concrete slab (pictured at Figure 11 above, and presented schematically at Figure 4.2.1.2.22 below. Sky was partly cloudy, with absent to moderate breezes from the southeast (lower right corner in the schematic) during this characterization.

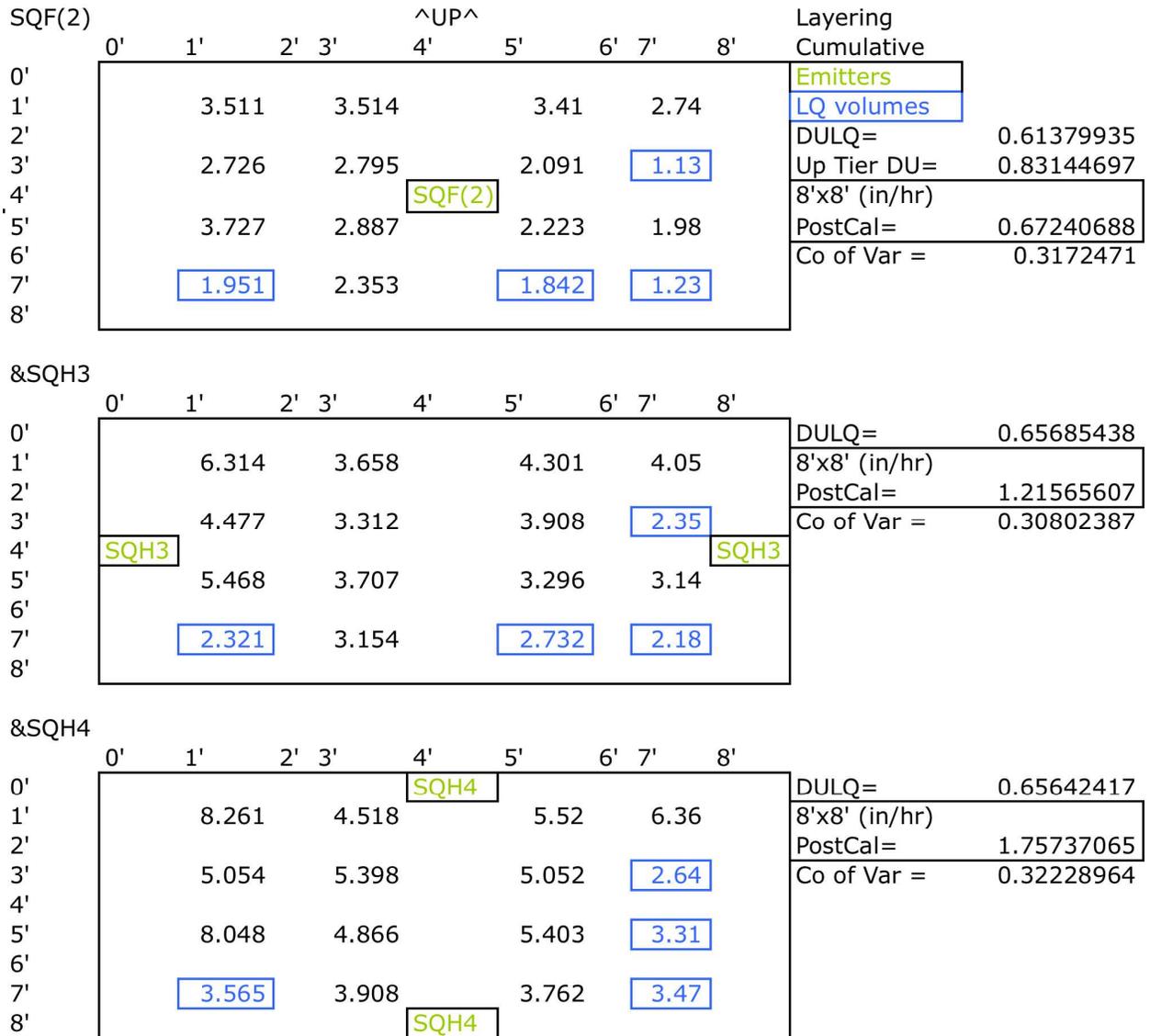


Figure 4.2.1.2.22. Schematic view of preliminary hydraulic characterization. DU=Distribution Uniformity Coefficient (by LQ = low-quarter method, see text), Co of Var = Coefficient of Variation.

The Distribution Uniformity Coefficient (by low-quarter method, DULQ) is a commonly used measure of coverage adequacy in irrigation-system designs. Its calculation is:

$$DU = \text{LQ average} / \text{Overall average} \quad [\text{depth or volume}]$$

where:

LQ average = the mean of the lowest ¼ of measured values, and

Overall average = the mean of all measured values.

The three separately plumbed pairs of drip emitters (chosen to each provide nominal ½” / hour equivalent rainfall rate with the 8’ x 8’ wooden frame to which they were affixed) were each evaluated for DULQ for comparisons. The best found (highest DULQ = 0.62) configuration (emitters in the center, SQF(2) in the figure) was chosen for the stand-alone nominal ½” /hour intensity simulator (actual calibration-corrected flow-rate equivalent to 0.67 “/hour). Note that this DULQ represents “poor” coverage for landscape-irrigation systems (“good” > 70%, and designs are typically targeted for at least 85%) but is more common in drip-irrigation (Rainbird a, undated and Growcom, undated).

DULQ comparison was also used to determine the most appropriate orientation of the frame on landscape surfaces. The Distribution Uniformity Coefficient was computed for each edge of the chosen stand-alone valving configuration, with the best (highest value, DULQ = 0.83) chosen as the “uphill” edge (top panel in the figure above). The other two emitter-pair configurations were then sequentially superimposed to derive characterizations for the nominal 1” and 1 ½” /hour configurations. DULQs, together with a more generally applicable and traditional measure of variability (Coefficient of Variability) were calculated anew, along with calibration-corrected equivalent rainfall-rate.

In-study simulated-rain characterization, an example.

Though not directly involved in hypothesis testing or analyses in this study, data collected under the sampling and analysis scheme here provide for a characterization of general rainfall parameters for each simulated storm, and for comparison with their respective nominal design parameters. Only the measured, as-simulated parameters were used in the study. An

example comparison to the nominal parameters (for the pervious lawn surface) is presented in Figure 4.2.1.2.23).

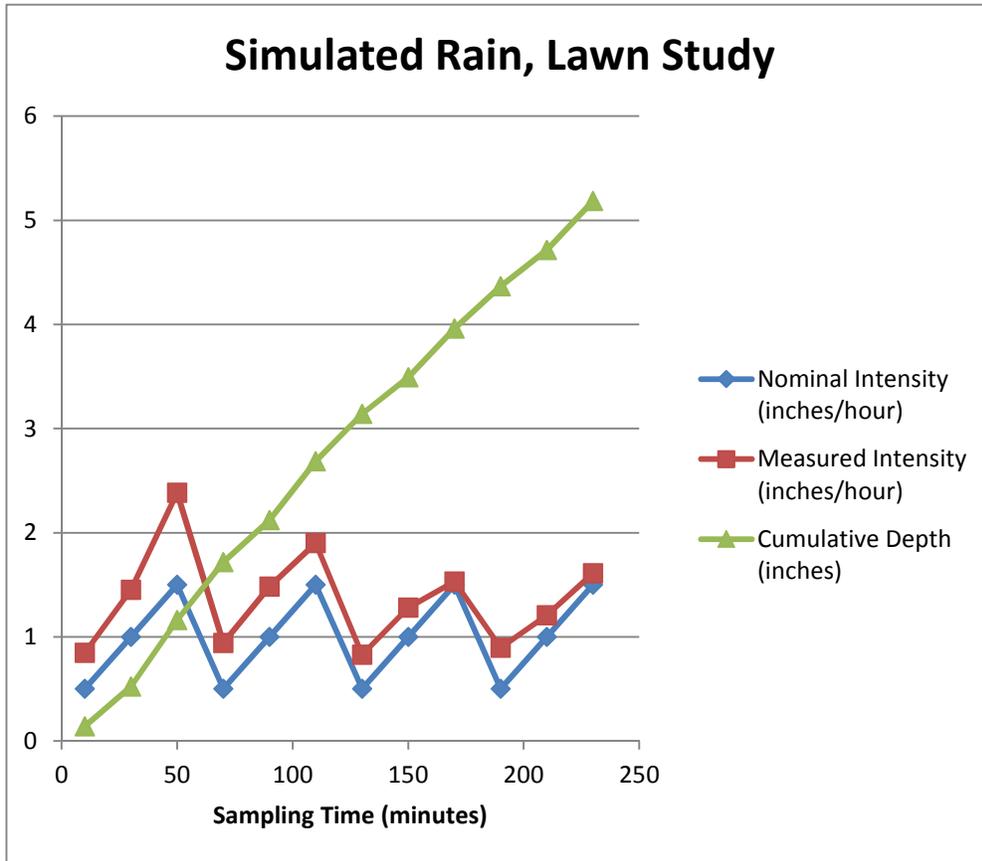


Figure 4.2.1.2.23 A Simulated Rainfall Characterization. Lawn studies.

4.2.2 CFU Characteristics, Size and Particle Affiliations

An unpublished article- (CHI-) style manuscript is presented. It is formatted to meet the requirements of this dissertation with permission of the authors.

4.2.2

CFU CHARACTERISTICS, SIZE AND PARTICLE AFFILIATIONS

Bradford M. Wilson, Robert Pitt, and Mark Elliott

A literature review revealed a need, by watershed managers, for some method of estimating the natural background (of non-sewage origin) of stormwater fecal indicator bacteria (FIB) inputs to regulated waters downstream. The same review, however, provided very little information concerning model parameters or structure by which such estimation might be accomplished.

This study consists of investigations into the form (size, and degree of aggregation) in which FIB are dispersed from stormwater source areas in response to rainfall. The size of the colony forming units (CFU) of FIB, and their affiliation with denser mineral particles, are deemed likely to affect settling and interception behavior downstream. The extent of multicellularity exhibited by CFU is likely to have an effect on their impact on the microbiological quality of regulated receiving waters. This study is one in a series of scoping studies performed to explore the feasibility of constructing a model by which natural-background inputs might be predicted from watershed observations.

4.2.2.1 Introduction

Due to difficulties in direct measurement of waterborne pathogens, the microbiological quality of waters is typically characterized on the basis of fecal indicator bacteria (FIB). FIB are assumed to derive from a common (historically sewage) source with pathogens of interest, and to arrive in, survive in, and move through watershed environments in numbers that correlate with

the risk from those pathogens (the *indicator paradigm*). Commonly used indicators, however, also derive from sources other than sewage or even feces, and survive in the environment at rates divergent from those of pathogens they are presumed to indicate (National Research Council, 2004).

Considerable expert consensus exists that FIB from non-human sources represent lesser correlative risk to human health than do those deriving from sewage (the *species barrier*). Much effort and money has been expended to confirm this assertion, but results are deemed equivocal by many regulatory authorities (*e.g.*, see Dufour, *et. al.*, 2012, and EPA, 2012) and the source of FIB is not considered relevant under many water-quality criteria (WQC). Knowledge of the source of FIB, however, remains important to achieving *compliance* with WQC. Managers of tributary watersheds require knowledge of source, especially sewage *vs.* non-sewage, to manage/prioritize strategies for compliant contributions to downstream waters. Tools for mitigation of sewage effluents differ, for instance, from those relevant to managing squirrel-derived fecal material in stormwater runoff.

The study presented here explores the physical size and the multicellularity of FIB CFU released by a heavy rainfall from a suburban residential lawn, and the association of such CFU with mineral particles. It consists of sequentially pouring a stormwater runoff sample through a cascade of screens/filters of decreasing pore size. The whole (unfiltered) runoff, and the filtrate of each barrier were analyzed for mineral particulates (dried solids), raw (not macerated) CFU (of both *Escherichia coli*, or *E. coli*, and *Enterococcus*, spp., or Enterococci), and macerated (sheared apart in a laboratory blender to reveal cell-count) CFU. By subtraction, these parameters in the filtrates were segregated into size-defined “bins” (each bounded by the pore

sizes of the cascade barriers), and the fractional contribution of the contents of each bin to the parameters found in the whole, unfiltered subsample were calculated.

4.2.2.2 Materials and Methods

4.2.2.2.1 Study Site

Previous (unpublished) research of the effects of simulated rainfall on various landscape surfaces revealed the importance of FIB-containing particulates to microbiological stormwater quality, and that the effects of such particulates was greatest early in a rain onto a pervious surface. That research also found that simulated-rain intensity was not as efficient in the generation of FIB as was that of natural rain.

The same surface studied previously, a suburban lawn, was chosen for this study. The neighborhood provides considerable (but spotty) tree cover, ample urban wildlife (mostly birds and squirrels, with occasional rabbits and one groundhog sighting), and a considerable pet presence (leash law in place, though not universally complied with).

A natural rain, opportunistically chosen for a radar signature indicating heavy, leading-edge rainfall likely to provide considerable depth of rain early in the storm. Runoff from the lawn was collected with a pre-sterilized dustpan, and composited into a sterile five-gallon pickle jar. Sample was kept on ice until morning (about five hours) before analysis. The rain was of 1.1-inch depth, with a three day rainless interevent period. Rainfall data here were taken from the available Preliminary Monthly Climate Data (the “F6 Product”) for Tuscaloosa Regional Airport (TCL), provided by the National Weather Service, and are hereinafter referred to as “TCL F6.” TCL is ~2.5 miles from the study area as on GoogleMaps™. This lawn was not a distinct source area, but received a minor contribution from an adjacent roof.

4.2.2.2.2 Filter Cascade

The collected runoff was diluted seven-fold before dividing. This initial dilution was to clarify the turbid sample somewhat, for FIB enumeration, and to provide sufficient volume for this study. The diluted sample was subsampled (churn splitter) to provide five “straight” and unfiltered 100-mL volumes for analysis: raw (not macerated) separate determination of *E. coli* and Enterococci densities, macerated density determination of both taxa, and solids determination. The four biological samples were then sequentially further diluted 100-fold twice (to 1:100, and 1:10000) to assure results within detection limits (three orders of magnitude) of IDEXX™ most probable number (MPN) enumeration FIB CFU. The whole sample was sequentially poured through a 250-micron screen and then a 106-micron screen, with subsamples for solids split from the filtrates of each screening (the -250 and the -106 samples). The -106 filtrate was sequentially poured (or suction filtered when necessary) through filters (in order of 45-micron, 20-micron, 10-micron, 5-micron, and 0.45-micron pore sizes) with each filtrate split for the same five analyses as performed on the initial undivided sample noted above.

4.2.2.2.3 Analyses

This study is largely descriptive, providing information useful for any modeling effort relating to predicting environmental impacts of stormwater from watershed observations (the overarching goal of the exploratory research of which this study is a part). Subtraction of the various parameters measured here from the sequential filtrates allows for determination of

fractional contributions segregated by size-graded bins (each bounded by the pore sizes of the sequential screens/filters used) of those parameters relative to those found in the whole sample.

CFU-density of FIB was determined using IDEXX methods; Colilert™, Enterolert™, with Quantitray™. While those CFU results may not always represent single viable cells, maceration (using a Waring™ blender) was expected to shear multicellular CFU into fragments of fewer cells, and to provide an estimated single-cell density. A comparison of the two densities, similarly analyzed (in what we call here, the maceration ratio = Macerated-CFU density/Raw-CFU density) provides a measure of the extent of multicellularity of the released CFU, and their potential to impact the quality of down-gradient waters. Size binning of these measurements provides information concerning their transport and subsequent separation as the dislodged CFU are transported to the receiving waters, and if they may be intercepted *en route*.

Solids determination here was by drying, for a week, in a 225⁰ F oven, and weighing on an analytical balance. Dry solids mostly provide an estimate of mineral content. Soil particles are generally characterized by specific gravity of about 1.5 to 2.5. Bacterial cells are of nearly neutral buoyancy (specific gravity close to 1.0), and typically consist of only ~20% dry-weight solids (Pitt, *et al.*, 2007, p. 301, and Bratbak and Dunda, 1984). Though this study provides no information concerning the extent to which cells are attached to the mineral particulates, the size-binned contributions of both CFU and dry solids provides limits concerning settling of CFU out of the stormwater flow.

This study does have one hypothetical component, namely information relevant to determination of which mechanism(s) are operational in the release of FIB from the landscape to the overlying stormwater. The mechanism of release is important to the overall goals of the

exploratory research of which this study is a part. Which mechanism(s) cause the release have implications concerning the, over time of a rain event, patterns of the response to rain.

There are four such mechanisms found in our literature review with the potential to effect FIB dispersal from terrestrial surfaces, namely:

- Passive flushing of extant planktonic *E. coli* cells by dynamics of overlying runoff and/or raindrop impact;
- Active dispersal of planktonic *E. coli* cells by seeding dispersal;
- Active dispersal of *E. coli* cells of unknown (planktonic vs sessile) morphology
- Passive dispersal of sessile *E. coli* cells by sloughing.

Important to the discussion here is that, due to the nature of planktonic *E. coli* cells, the information collected in this study has potential to allow confident rejection of the first two potential mechanisms as sole causes of FIB release in modeling efforts going forward.

Planktonic cells are generally not attachment competent, and planktonic *E. coli* are even actively motile. A conversion to sessile morphology involves an exchange of motility organelles (flagella in *E. coli*) for those allowing attachment to surfaces (fimbriae). Planktonic morphology of *E. coli* is unicellular (occasionally paired). Moreover, the size of planktonic *E. coli* cells is such that pairs would pass through a 10-micron filter and even unicellular CFU would be retained on the 0.45-micron filter. A finding of *either* of:

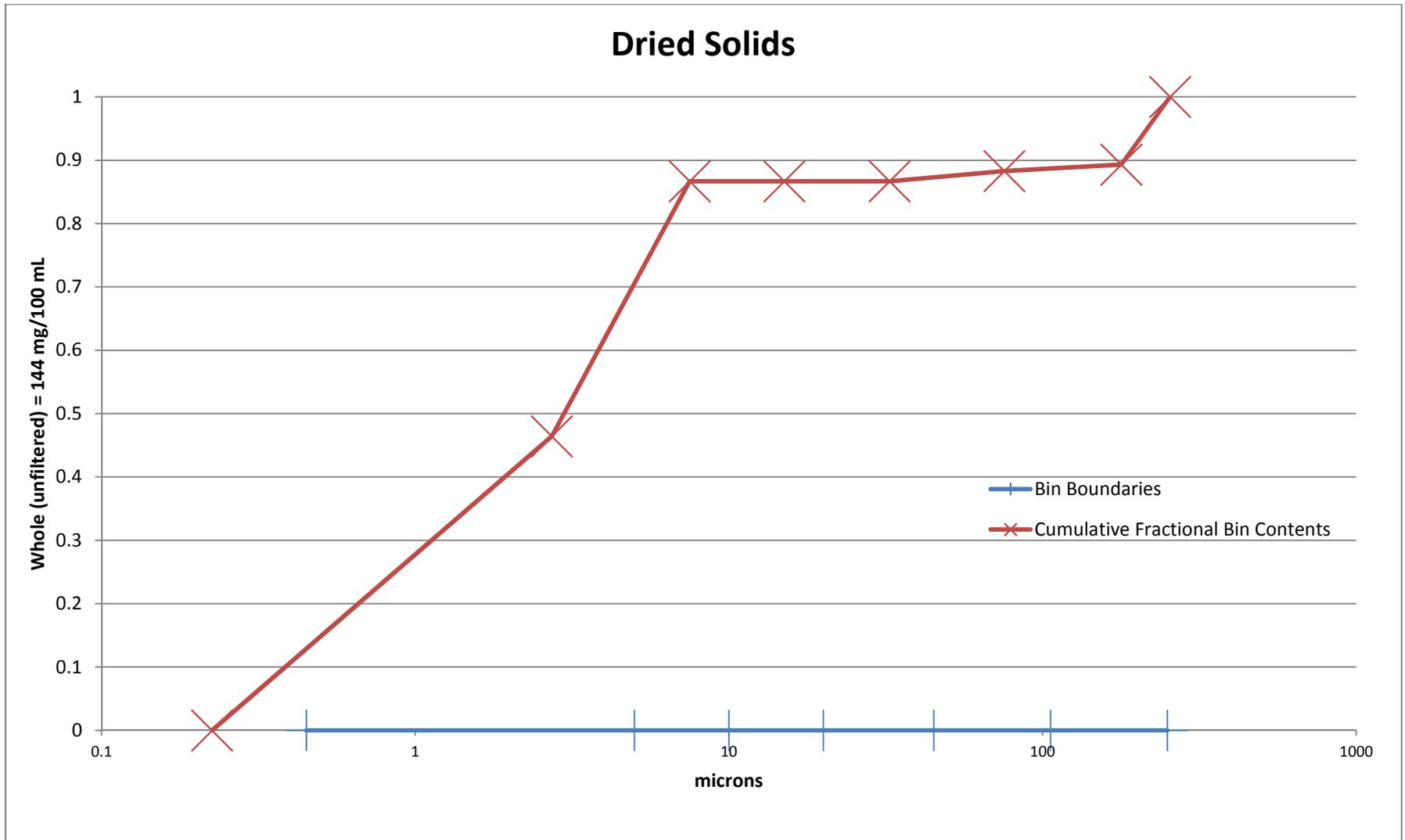
- *E. coli* CFU are found outside the bins corresponding pore-size boundaries;
- *E. coli* CFU within those bins exhibit a maceration ratio exceeding 2.0;

would allow for confident rejection of both flushing and seeding as sole mechanisms of FIB release (Mcdougald, *et al.*, 2012).

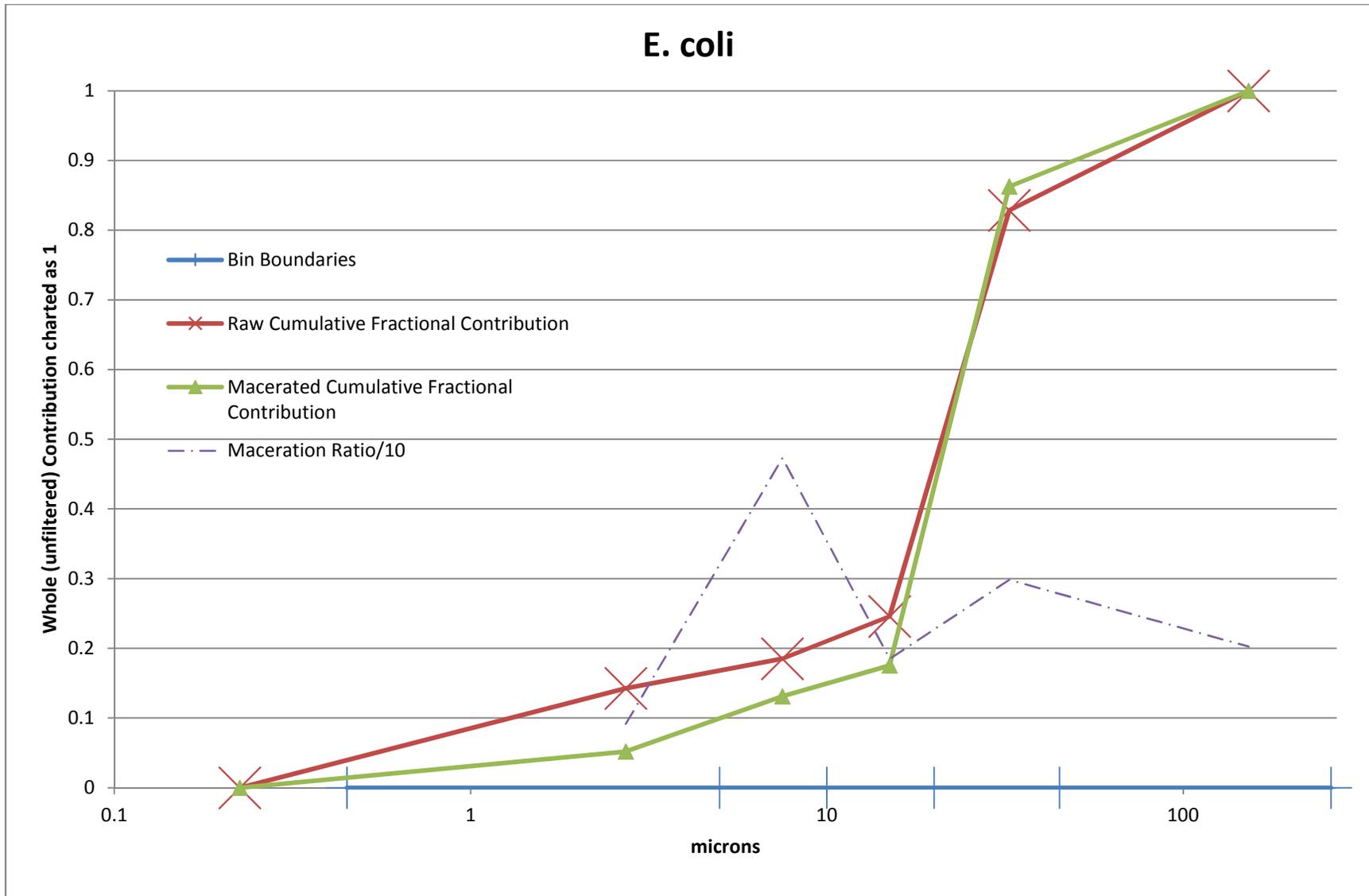
4.2.2.3 Results and Discussion

4.2.2.3.1 Descriptive Study

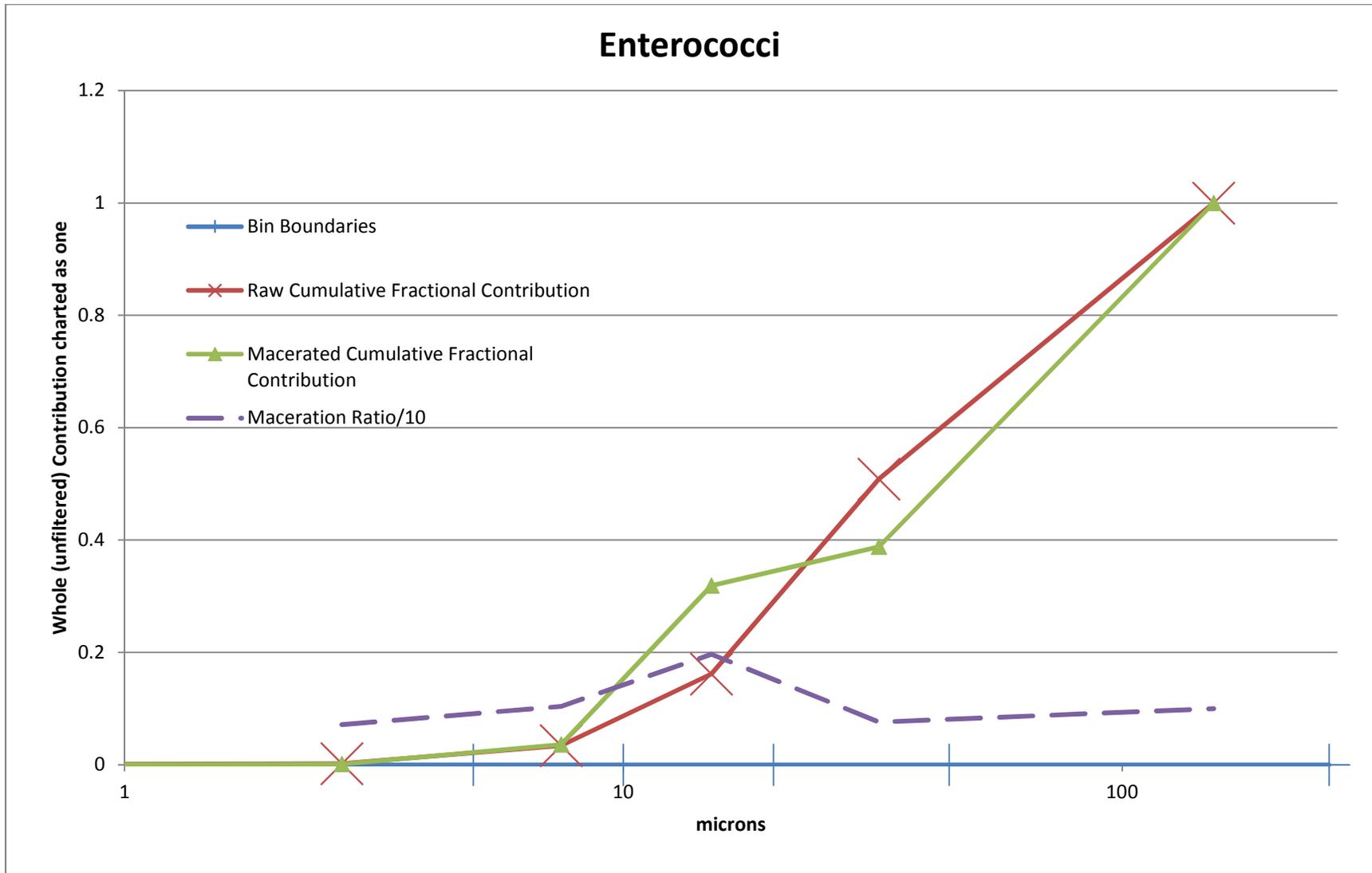
Information related to the results of this study is presented at Figures 4.2.2.1-3.



4.2.2.1 Size-binned Fractional Contributions to Whole-sample solids. Large-end boundary of >250-micron bin set arbitrarily at 260.



4.2.2.2 Size-binned Fractional Contributions to *E. coli*. Whole-sample CFU. Raw Whole CFU = 1251112.5/100 mL, Macerated Whole CFU = 316500, Large end of >45-micron bin arbitrarily set at 260.



4.2.2.3 Size-binned Fractional Contributions to Enterococci Whole-sample CFU. Raw Whole CFU = 30825/100 mL, Macerated Whole CFU = 32100, Large end of >45-micron bin arbitrarily set at 260.

There are a few observations of note here. Almost 90% of all solids (the major source of mass here) are found in bins with an upper boundary of 10 microns, with about half falling through the 5-micron filter. This agrees with expectations of soil particle size distributions erodible by rainfall (Pitt, *et al.*, 2007, p. 3). CFU of *E. coli* follow a similar pattern, though with a slightly higher size cutoff (about 30 μm), in a range of elevated maceration ratio. This may imply small multicellular CFU filmbound to mineral materials with at least a chance of settling out of relatively quiescent runoff. About half of the raw Enterococci CFU were retained on the largest-pore (45 μm) filter in a region of low maceration ratio and low mineral content. The “fluffy floc” morphology this seems to imply may make such CFU prone to interception in sufficiently tortuous flow paths, but they would not be expected to settle very fast. Neither FIB taxon studied here seems to form large multicellular CFU, either flocs or filmbound particles, in the size regions largely lacking easily suspended particles.

4.2.2.3.2 Hypothesis Testing.

We examined the data presented above with an eye to a hypothesis that the two FIB release mechanisms resulting in exclusive release of planktonic *E. coli*, as discussed above, are not sole mechanisms of FIB dispersal on this landscape. A failure to find *either* of:

- *E. coli* CFU are found outside the bins corresponding to pore-size boundaries from 0.45 to 10 μm ; or
- *E. coli* CFU within those bins exhibit a maceration ratio exceeding 2.0;

would allow for retention of the null, that either flushing or seeding, or some combination of the two, might cause all release releases of *E. coli*. Confident rejection of the null would provide a strong presumption that collocated Enterococci were also released, at least in part, by mechanisms allowing for sessile morphologies (Kaplan, 2010).

A graphic of this hypothesis testing is presented at Figure 4.2.2.4, a focused presentation of material already shown in Figure 4.2.2.2 above.

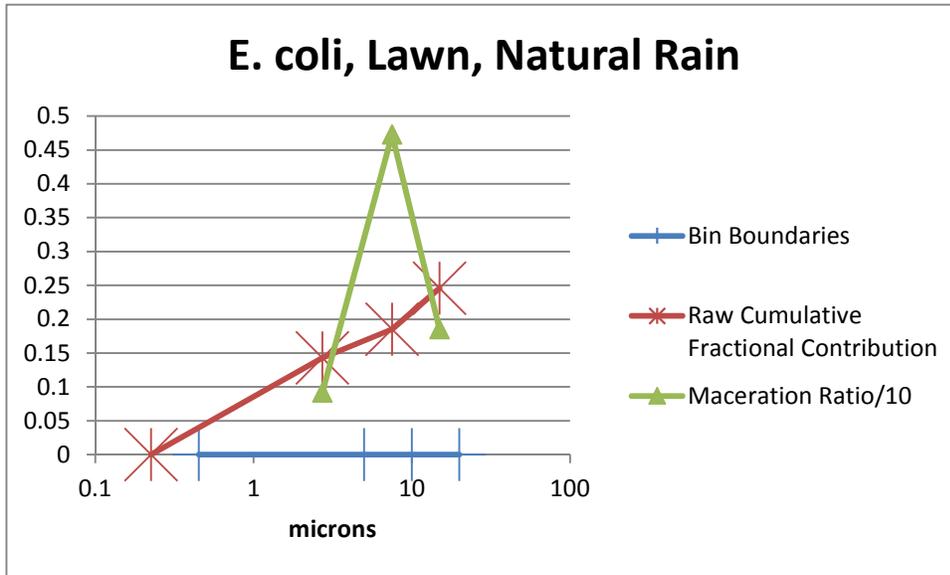


Figure 4.2.2.4 Hypothesis Testing.

The figure represents a close-up view of the relevant (0.45-5, and 5-10 microns), and adjacent bins, presented at Figure 4.2.2.2. The first relevant observation of note here is the presence of significant found *E. coli* CFU in the adjacent (to the right, 10-20 microns) bin. This finding alone is sufficient for logical acceptance of our hypothesis. Further evidence is provided by the elevated maceration ratio present in the relevant bins (and separately calculated to average over 2.8 across the bins). The finding that neither flushing nor seeding can explain this dataset without contributions from other mechanisms is of value to any modeling efforts in support of the goals of the exploratory research, especially in the case of seeding. While the mechanisms of seeding dispersal are well established, the putative causative effectors (concerted quorum sensing of specialized intercellular signal chemicals) have not been elucidated (McDougald, et al., 2012)).

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4.2.3 CFU Characteristics, a settling study

An unpublished article- (CHI-) style manuscript is presented. It is formatted to meet the requirements of this dissertation with permission of the authors.

4.2.3

CFU CHARACTERISTICS, A SETTLING STUDY

Bradford M. Wilson, Robert Pitt, and Mark Elliott

A literature review revealed a need, by watershed managers, for some method of estimating the natural background (of non-sewage origin) of stormwater fecal indicator bacteria (FIB) inputs to regulated waters downstream. The same review, however, provided very little information concerning model parameters or structure by which such estimation might be accomplished.

This study consists of investigations into the form in which FIB are dispersed from stormwater source areas in response to rainfall. The settling behavior of colony forming units (CFU) of FIB is deemed likely to affect their probability of reaching regulated waters down-gradient, and their fate and transport behavior in the receiving water. Specifically, most stormwater models consider FIB “die off” as the mechanism for their disappearance from the water column. The research conducted during this dissertation attempts to provide mechanistic information that can help provide alternative explanations for FIB population changes, such as aggregation/disaggregation and settling characteristics, in addition to the environmental factors affecting their survival on urban surfaces. FIB CFU that precipitate out of sheet flow or of ponded waters will not affect regulated waters during a rain event, but may survive an interevent rainless period to provide an unaccounted for, by any watershed observation, source to a subsequent rain. This study is one in a series of scoping studies performed to explore the feasibility of constructing a model by which natural-background inputs might be predicted from watershed observations.

4.2.3.1 Introduction

Due to difficulties in direct measurement of waterborne pathogens, the microbiological quality of waters is typically characterized on the basis of fecal indicator bacteria (FIB). FIB are assumed to derive from a common (historically sewage) source with pathogens of interest, and to arrive in, survive in, and move through watershed environments in numbers that correlate with the risk from those pathogens (the *indicator paradigm*). Commonly used indicators, however, also derive from sources other than sewage or even feces, and survive in the environment at rates divergent from those of pathogens they are presumed to indicate (National Research Council, 2004).

Considerable expert consensus exists that FIB from non-human sources represent lesser correlative risk to human health than do those deriving from sewage (the *species barrier*). Much effort and money has been expended to confirm this assertion, but results are deemed equivocal by many regulatory authorities (*e.g.*, see Dufour, *et. al.*, 2012, and EPA, 2012) and the source of FIB is not considered relevant under many water-quality criteria (WQC). Knowledge of the sources of FIB, however, remains important to achieving *compliance* with WQC. Managers of tributary watersheds require knowledge of source, especially sewage *vs.* non-sewage, to manage/prioritize strategies for compliant contributions to downstream waters. Tools for mitigation of sewage effluents differ, for instance, from those relevant to managing squirrel-derived fecal material in stormwater runoff.

The study presented here explores the settling behavior of FIB CFU released by a heavy rainfall from a suburban residential lawn. Because our previous research (unpublished) suggests that such CFU likely contain sessile (attachment-competent) FIB cells, affiliated with other FIB

cells and/or mineral-soil particles, we designed a study capable of capturing evidence of flocculant (Type II) settling in contrast to discrete settling. This study follows relevant portions of the traditional analogous procedures for settling-column analysis (see, *e.g.*, Crites and Tchobanoglous, 1998, pp. 279-282) except that we generate %CFU removal curves rather than the usual %suspended-solids removal curves. This study design was implemented twice, once for each of the two FIB taxa under study (*Escherichia coli*, or *E. coli*, and *Enterococcus*, spp., or Enterococci). Finally, this study is mostly descriptive and meant to provide guidance, potentially of generalization, for future research.

4.2.3.2 Materials and Methods

4.2.3.2.1 Study Site

Previous research conducted earlier as part of this dissertation study that examined the effects of simulated rainfall on various landscape surfaces, revealed the importance of FIB-containing particulates to microbiological stormwater quality, and that the effects of such particulates were greatest early in a rain.

The same surface studied previously, a suburban lawn, was chosen for this study. The neighborhood provides considerable (but spotty) tree cover, ample urban wildlife (mostly birds and squirrels, with occasional rabbits and one groundhog sighting), and a considerable pet (mostly dog) presence (leash law in place, though not universally complied with).

A natural rain, opportunistically chosen for a radar signature indicating heavy, leading-edge rainfall likely to provide considerable depth of rain early in the storm, was selected for this study. Runoff from the lawn was collected with a pre-sterilized dustpan, and composited into a sterile five-gallon glass jar. The sample was kept on ice or refrigerated (three days) until

processing and analyses. This extended storage was necessitated by contemporary and predicted weather (with no new rains foreseeable), and may have affected settling characteristics to some unknown extent. The stored sample was vigorously swirled in its container daily, in an attempt to re-separate any loosely bound aggregates that might have formed in the interim. The rain was of 1.1-inch depth, with a preceding three-day rainless interevent period. Rainfall data here were taken from the available Preliminary Monthly Climate Data (the “F6 Product”) for Tuscaloosa Regional Airport (TCL), provided by the National Weather Service, and are hereinafter referred to as “TCL F6.” TCL is ~2.5 miles from the study area as measured by ruler on a GoogleMaps™ printout. The lawn study area runoff also received minor runoff contributions from an adjacent roof.

4.2.3.2.2 Settling Tank

A simple settling chamber was fabricated from a generic (no brand name available) 5-gallon glass aquarium (Figure 4.2.3.1). The tank was fitted with two separatory funnels to draw (by vacuum hose) fixed volume samples from two different depths with minimal disturbance. A meter stick was also attached to the tank to measure surface draw-down over the study period.



Figure 4.2.3.1 Settling Tank, during preliminary characterization.

The tips of the two sample taps consisted of pre-sterilized glass burettes, fixed in place (five and ten cm above the base of the tank) by the burette clamps and extending above the fill line. A graduated-cylinder calibrated line corresponding to a 100-mL fill was drawn onto both funnels with waterproof marker.

The tank base was 21 cm by 49 cm (height = 25 cm). The expected draw-down of the sample in the tank resulting from each 100-mL sample withdrawal was calculated to be 0.12 cm. The actual draw-downs during the study were measured and recorded for monitoring/diagnostic purposes.

4.2.3.2.3 Data Collection and Analysis

The bulk sample of runoff was diluted 1:14 to best acquire at least one measurement above the lower detection limit of IDEXXTM enumeration methods (ColilertTM and EnterolertTM

media using Quantitray2000™ trays) within the planned spread of further serial dilutions. The diluted bulk sample was immediately churn-split for two separate taxon-specific analyses (*E. coli* and Enterococci), and the well-mixed remainder poured into the settling tank (resulting measured level = 18.0 cm +/- 0.05), and our stopwatch was set. Prior to settling, an aliquot of the 1:14 diluted bulk sample was further diluted one-hundred fold twice (1:100 and 1:10,000); This spread of further serial dilutions was to best acquire at least one measurement below the upper detection limit. Where more than one dilution provided measurable results, the results were averaged for analysis.

The settling tank was subsequently sampled, for both taxa and for both depths at the following intervals:

- 2-minute settling;
- 10-minute settling;
- 20-minute settling;
- 60-minute settling;
- 120-minute settling;
- 480-minute settling; and

all samples were serially diluted to target readable results. All results were analyzed by IDEXX methods. Where more than one dilutions provided measurable results, the results were averaged for further analysis.

All data collected were segregated as to taxon and sampling depth over time and subjected conventional settling-column analysis for the generation of isopercent CFU-removal curves.

4.2.3.3 Results and Discussion

During dataset assembly we noticed four unexpected and possibly anomalous data, two among the 16 *E. coli* samples, and two in Enterococci. These sample results each showed a momentary rise in CFU density with settling time, with one even rising above the density measured in the mixed, unsettled sample. Each such data point represented an apparent negative % Removal over time. Each such point occurred early in the study period. Each of these data represented an average of at least two widely divergent dilution-corrected FIB-density values. Without sufficient data density to diagnose the large variability among the variously diluted subsamples, and lacking recourse to repeat it, we chose to smooth the unexpected results in a manner akin to moving-average smoothing using variability measures available from the MPN method of CFU-density enumeration.

The Most Probable Number method, traditionally performed using serial dilutions, essentially consists of subdividing a sample into small enough parts to reveal binomial distributions of presence/absence of microbes amongst multiple subsamples. In IDEXX' enumeration procedures, subsampling is accomplished by (assumed) random subdivision of each sample into 97 sealed cells of known volume, each with selective fluorophores that signal presence of the microbe of interest upon incubation. The presence/absence pattern of 97 subsamples provides the MPN of FIB CFU in the sample *and* an estimate of *how* probable (in the form of 95% confidence intervals) that number is.

The spread of the confidence intervals relative to the found MPN estimate is inherently greater in the case of low MPN estimates. Our examination of our deviant data points (exemplified at Figure 4.2.3.2 for graphic illustration) revealed that each of them consisted of an

average of two readable dilutions, dominated by the uncertainty of a low MPN magnified by greater dilution.

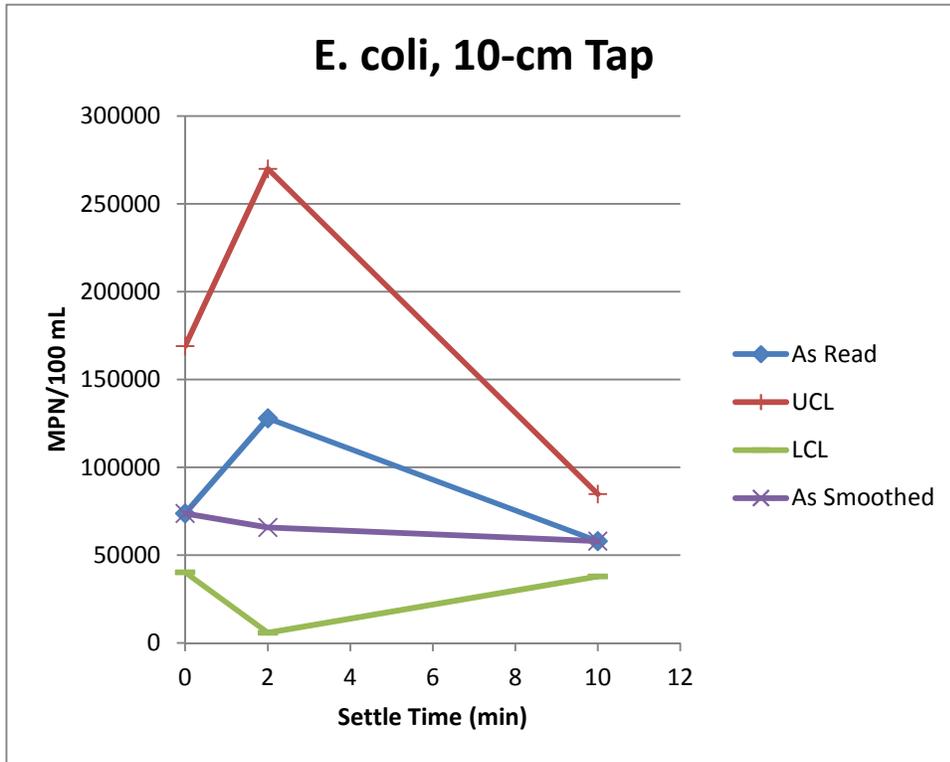


Figure 4.3.2.2 First Three Data Points, *E. coli* time series 10-cm above tank base. UCL = Upper Confidence Limit, LCL = Lower Confidence Limit, 95% CLs from MPN determination (see text).

Figure 4.3.2.2 illustrates the situation in which the two-minute sample exhibited considerably greater CFU density than did the mixed, unsettled sample (the latter graphed at zero settling time). The two-minute sample value is an average of two dilution-corrected values, namely a 1:100 dilution (MPN = 866.4, 95% confidence interval 583.8 to 1245.4) and a 1:10,000 dilution (MPN = 16.9, confidence interval 9.4 to 27). The latter, low-certainty, high (dilution-corrected) value was the more influential of the two subsamples in producing the high average of the sample. Akin to traditional moving-average smoothing, we substituted the mean of the two adjacent (Settling Times at zero and 10 minutes) values and found that the substituted value still

fell within the combined confidence interval. With all series so smoothed, the resulting datasets appeared relatively well behaved, though a clear separation of series on the basis of depth of sampling, especially at early times in the series, was not particularly evident (see Figure 4.2.3.3).

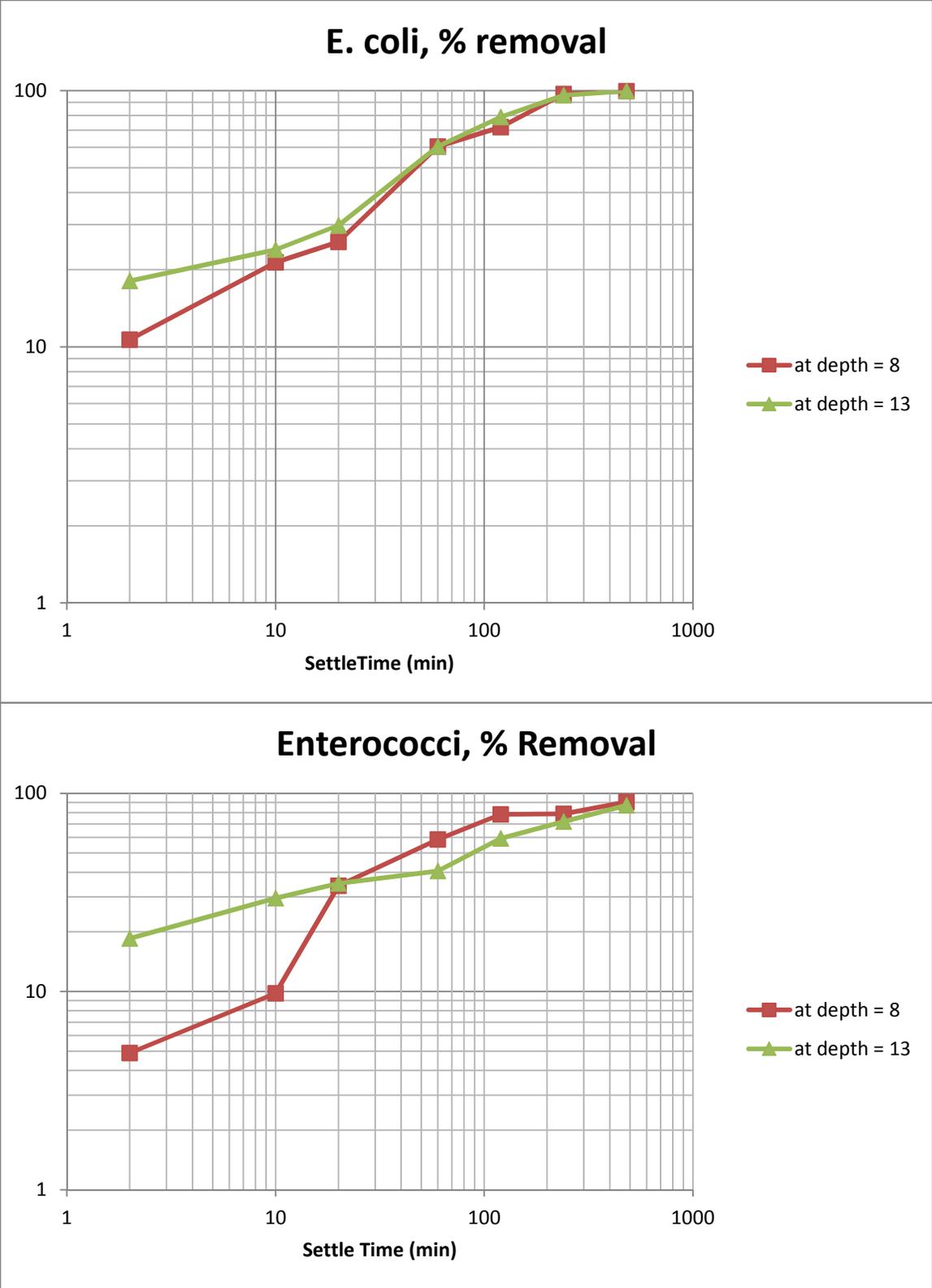


Figure 4.2.3.3 Time series % CFU removal separated by taxon and depth of sampling.

We proceeded to extrapolate isopercent CFU-removal curves, plotted against settling time and depth, in a manner analogous to traditional generation of isopercent particle-removal curves.

Our results are presented at Figures 4.2.3.4&5. Note that all curves are truncated before any extrapolated point fell below the 18-cm depth of our settling tank.

Figure 4.2.3.4, the *E. coli* curves, presents results consistent with expectations of a mixture of filmbound mineral particles and potentially flocculant CFU, the latter being sessile (attachment-competent) cells and/or cell/biofilm aggregates. The curves all seem to have a break point at two minutes. The implied settling velocity at this location for the 10% curve is about 0.1 cm/sec and would not be inconsistent with some significant portion of that 10% of CFU settling discretely, as if they were mineral particles of diameters in the microns to tens of microns range. The reduced downward slope of the 10% curve subsequent to two minutes would seem to imply some remnant of that fraction of CFU is of lesser density; a diagonal drawn from the origin to the 20-minute depth of the 10% Removal curve implies that by that time, some fraction of that 10% of total CFU is only dense enough to settle at ~0.01 cm/sec. The visible flattening of all curves presented here, along with a subsequent downward bending (not shown in the truncated graphic series, but clear in the fact that they're so truncated, and in the full spreadsheet representations) are consistent with Type II settling, though no signal of such presents itself at depths of quiescent settling shallower than about four centimeters.

The well-mixed, unsettled, *E. coli* densities found from this rain on this surface (initial-dilution adjusted MPN/100 mL = 103,000) is not expected to be of general use. Each source-area surface must be separately characterized. The behavior as presented here, representing fractional settling behaviors of CFU actually mobilized from a source area by rain, may be of more general value.

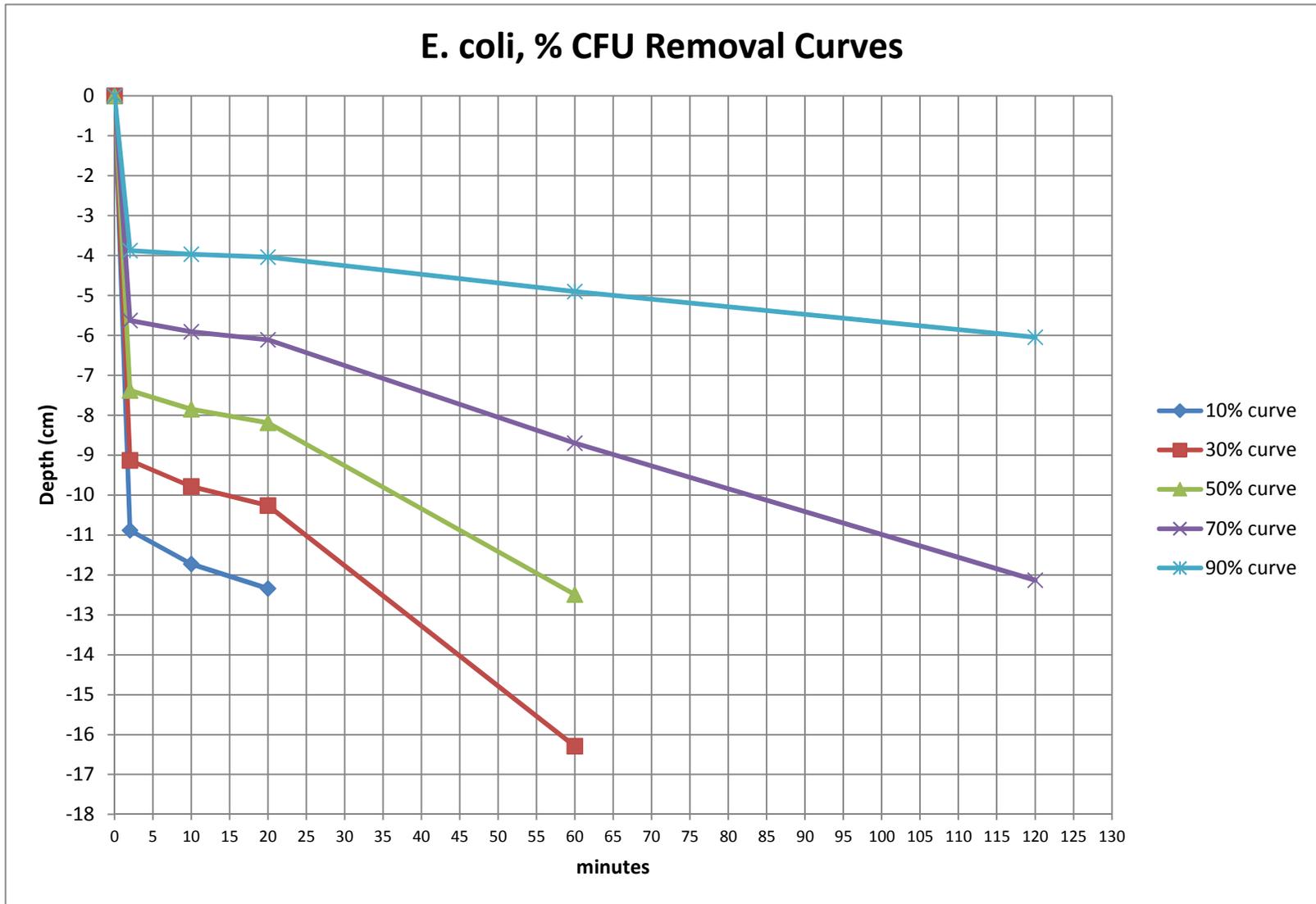


Figure 4.2.3.4 *E. coli* CFU Settling Behavior. Series truncated by depth.

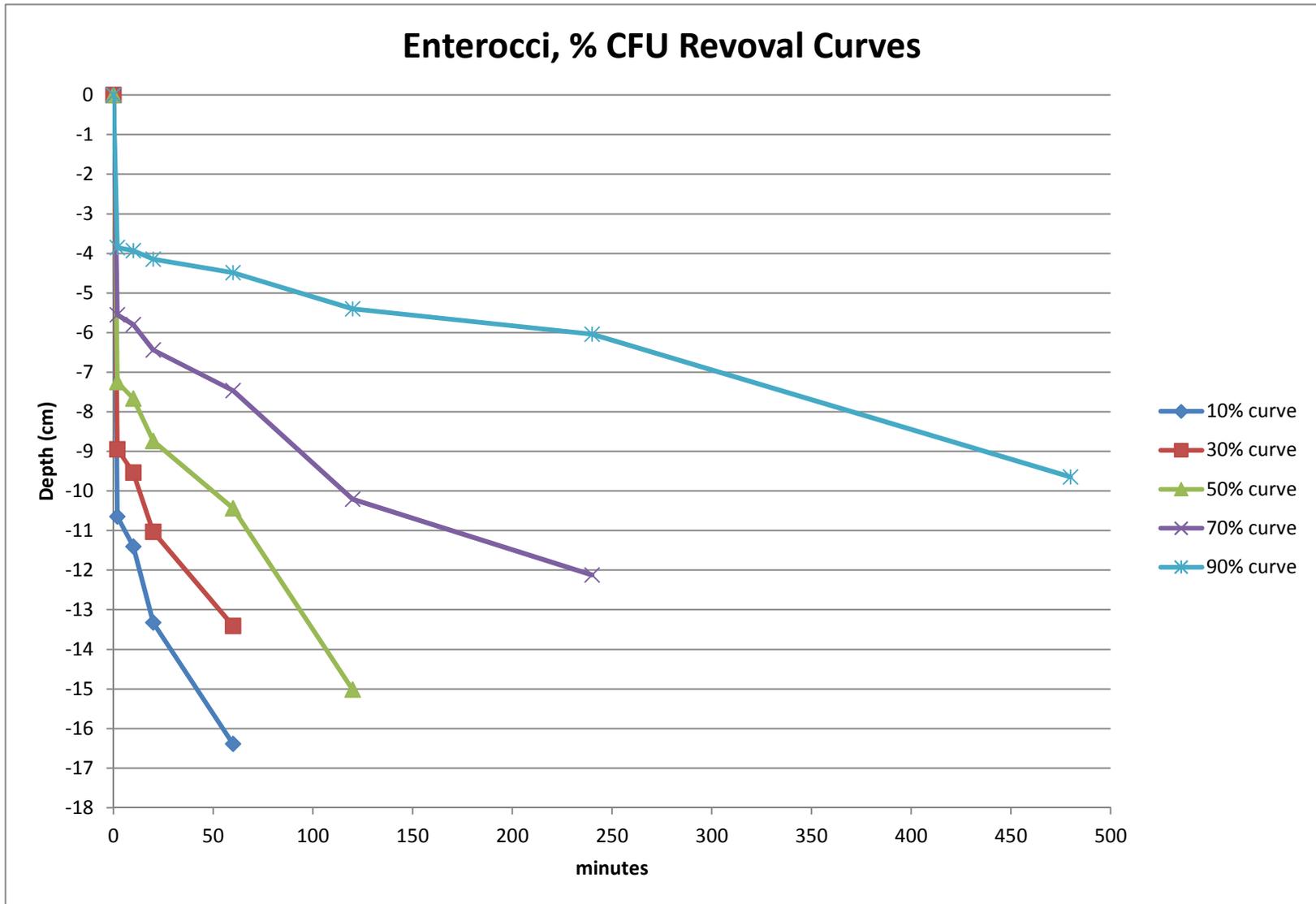


Figure 4.2.3.5 Enterococci CFU Settling Behavior. Series truncated by depth. Note: 90% curve is not artificially truncated.

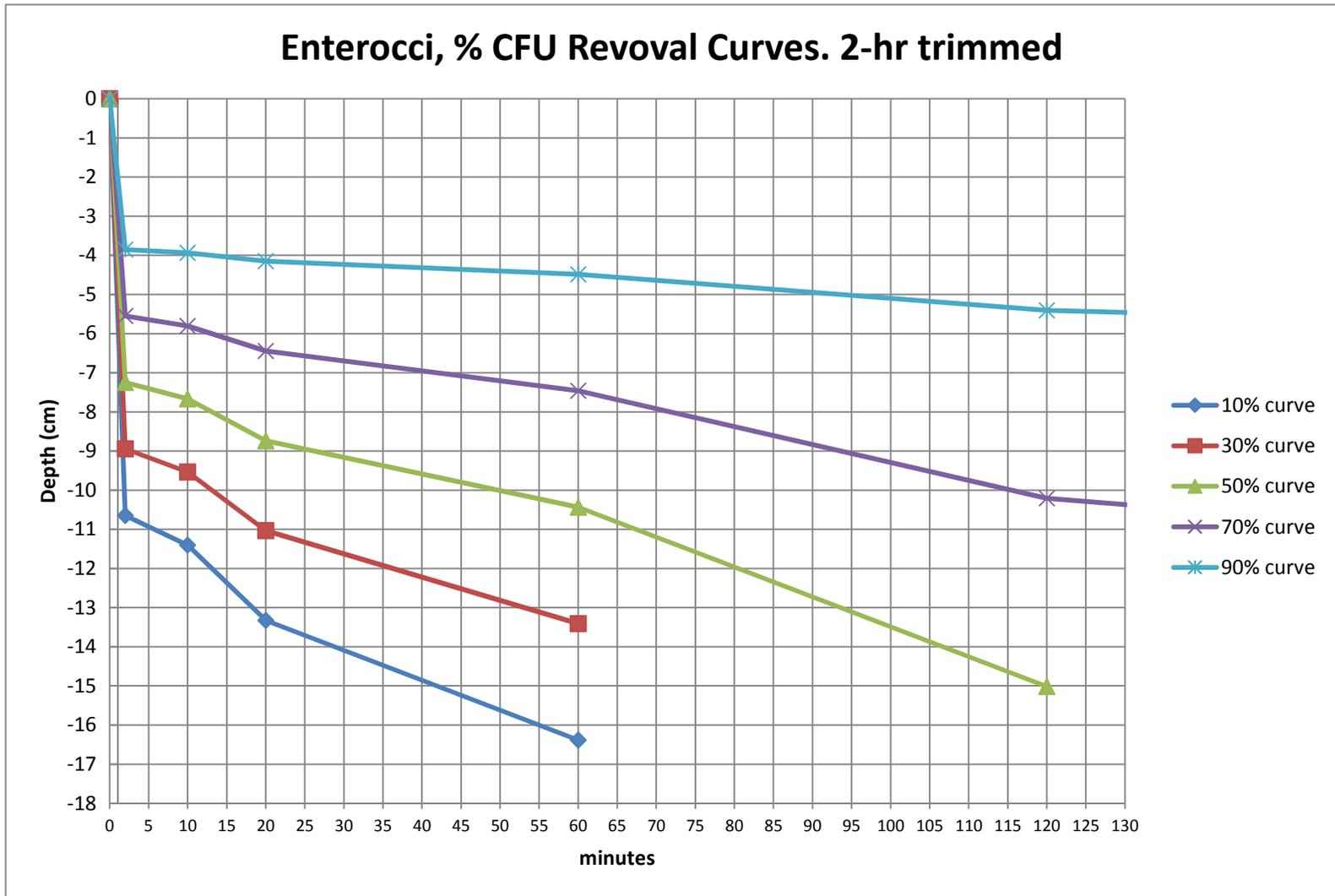


Figure 4.2.3.6 Enterococci CFU Settling Behavior. Series truncated by depth and at 2-hours of settling time

Figure 4.2.3.5 provides evidence that, as in the case of *E. coli* discussed above, Enterococci CFU released by a rain event exhibit a sharp break in settling velocity by the time of our second (two-minute) sampling. This, again, could well be attributed to a shift in dominance from (early on) rapidly settling CFU with mineral-containing components to CFU of greater organic (cellular and/or biofilm) content. The 90% CFU removal curve here was not truncated due to depth. The last sample of our study was extrapolated to not only remain within the depth range represented by our 18-cm settling tank, but also within the depth range actually sampled (to 13 cm initial depth). There is not as much evidence of onset during settling of Type II behavior at any depth, by particulates of any composition, as was exhibited by *E. coli* above. There is evidence, however (90% curve at 480 minutes) that up to 10% of our Enterococci CFU are settling at no more than ~ 0.0003 cm/sec, and would likely remain suspended for a long time under any flow conditions.

We can pin down in somewhat greater detail, the conclusions reached so far by artificially truncating our Enterococci data graphic at the same settling-time limit presented for *E. coli* at Figure 4.2.3.4 above. This close-up view (Figure 4.2.3.6) provides better resolution for graphical determination of settling times of interest, and for more relevant comparisons, by readers, of *E. coli* vs Enterococci settling behaviors. Figure 4.2.3.6 reveals that, again, most mineral-like settling CFU are rapidly dropped out of any system incapable of keeping particulates of ~ 0.1 cm/sec settling velocities suspended (see the 2-minute break at the 10% curve). Some considerable, less likely to settle, particulates also remain. In any system capable of suspending particulates of ~ 0.0006 cm/sec (see the 120-minute point in the 90% curve in the figure), about 10% of CFU originally mobilized by the rainfall keep going wherever the runoff water goes.

Readers should note that our focus the 10% and 90% curves in the graphic-analysis examples presented do not preclude use of others. This study is an analysis of fractional settling rates of the whole sample. This focus does, however, represent the most narrowly bounded conclusions on settling behavior relative to the whole sample available here. The sharp flattening bends in all curves, early in the study period, allow for a conclusion that some very-rapidly settling component is falling out of our sampling depth range. The bend at the 10% curve allows us to conclude that < 10% of the total CFU are falling at the rate exceeding that indicated by the breakpoint. Likewise, any point on the 90% curve allows us to conclude that no more than 10% of the total are settling at a rate exceeding that indicated by the point selected.

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CHAPTER 5

CONCLUSIONS AND IDENTIFIED NEEDS FOR FUTURE RESEARCH

As might be expected in exploratory research such as this, necessarily fragmented into such disparate scoping studies, the overarching conclusion here is that more work needs doing before a useful model can be constructed by which water managers can meaningfully predict the compliance-related impacts on downstream regulated waters from observations of the watershed under their purview.

Due to the article-style structure of this dissertation, in which the disparate scoping studies are presented, each of the five articles/article-style manuscripts presented provides meaningful conclusions and identifies needs for future research relevant to the scope of the studies discussed. Some findings, relevant to the overall research effort attempted here, can only be revealed by comparisons or synopses of the various chapters.

For instance, Section 4.1.1 concerning interevent FIB survival on pavements, provides for the conclusions that the assumed segmented breakpoint model, based on conventional microbial population-dynamic principles, is sufficient to reveal the relevance of all hypothesized significant factors, based on conventional population-focused ecological principles. The modeling effort presented in that section also showed, however, that any model incorporating that structure with those factors would be of much greater use if additional relevant factors could be identified, because considerable, unaccounted variability remained.

A comparison between the results of Sections 4.1.1&2 (especially see 3rd panel of Figure 4.2.1.2.4 in the latter) provides eminent candidates, worthy of future research, for those additional relevant factors (namely, the soil-water amendments), especially for the more fastidious FIB taxon under study (*E. coli*). That comparison, however, also provides meaningful cautions as to how any such future research in this arena should be conducted. Studies presented in Section 4.1.2, by themselves, provide little in the way of confident conclusions. The data collected there were problematic for analyses due to censoring, and to the limited goodness-of-fit measures available from the MLE methods by which they are appropriately modeled. Any future research into additional factors relevant to *E. coli* interevent survival on impervious surfaces should be designed to avoid such censoring. Fractional designs (no significant high-order interactions were convincingly revealed in 4.1.2), or plans to provide much incubator space should be considered.

In turn, though any stand-alone findings from 4.1.2 are inconclusive at the very best, a comparison to 4.1.1 is informative. The former, pervious surface studies revealed at least some evidence (by admittedly tortuous *pseudoresidual* analyses) that FIB survival on soils may not be governed by microbial population-based assumptions. The literature reviewed here provides no clues as to alternative survival patterns (to the breakpoint model) that *incoherent* FIB responses to microenvironments might express. I can think of no way to even explore such potential alternative survival patterns without a full set of meaningful (uncensored) residuals. This admitted failure in my Experimental Plan here should inform future research efforts.

Section 4.2.1, the primary screen for operational mechanisms contributing to mobilization of extant FIB in response to rain, provides reinforcement of the cautions for planning to avoid any nonignorable censored data. Significant and meaningful conclusions were

found (especially notable, that a focus on passive-release mechanisms provided sufficient information for useful modeling efforts concerning FIB dispersal) for the pervious, lawn surface. My inability to acquire a dataset for full hypothesis testing on the street and (especially) roof, however, severely limited extensions of those significant findings to the other surfaces. The additional release-mechanism selective findings presented in 4.2.2 (primarily the size and particle-affiliation study) provided supporting evidence for the significance of the passive-release mechanisms on pervious surfaces, but provided no support for extension to other surfaces.

Finally, in terms of the overarching goals of the exploratory research here, the relevance and full linkage of the putative sequential modeling blocks (Figures 1, 2, and 5) is supported. The final-washoff findings provided in Section 4.2.1 imply that not all extant FIB on environmental surfaces are mobilized by even heavy and high-intensity rains. Some retained FIB on the landscape (graphically, the retention path shown at Figure 2) remains as a potential unobserved FIB input (Figure 1) for the next rain. The potential for FIB, once mobilized by rain, to be intercepted (Section 4.2.2) or to resettle (4.2.3) on the landscape (see Figure 5) implies that such bacteria must also be considered potential inputs to Figure 1.

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