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BACTERIAL SOURCE TRACKING OF A WATERSHED IMPACTED BY CATTLE PASTURES

By

Celina Phelps

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Forest Products
in the Department of Forest Resources

Mississippi State, Mississippi

December 2006

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Celina Phelps

2006

BACTERIAL SOURCE TRACKING OF A WATERSHED

IMPACTED BY CATTLE PASTURES

By

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Pathogenic microorganisms introduced by cattle may be transported to distant locations via watershed runoff. *Escherichia coli, Enterococcus* spp., and *Streptococcus* spp. are a few species present in runoff from land impacted by humans, cattle, and wildlife. Initial data revealed that *E. coli* concentrations in water were greater in areas impacted by cattle than by humans. And, wildlife contributed greater concentration fluctuations than either humans or cattle. When cattle were removed from a pasture, the bacterial concentrations rapidly decreased; however, slight variations in cattle herd size did not appear to significantly influence these counts. Amplified fragment length polymorphisms (AFLP) and repeated-sequence polymerase chain reactions (rep-PCR) were molecular techniques used in this study to assess the impact of several cattle pastures on one rural communities' watershed system. Preliminary REP-PCR results estimated that 13% of *E. coli* in pasture waters originated from cows.

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CHAPTER I

INTRODUCTION

Water quality issues are present on the national and worldwide levels. Many international, federal, regional, state, and local organizations are working together to introduce better management practices and laws to protect our water resources (Cassady, 2004). Since the Clean Water Act of 1972, much has been done to improve water quality, and restrictions are more focused on preventing further impairments (EPA, 2006). Still, various daily events cause unsafe contamination of municipal, recreational, and agricultural water systems. Historical emphasis has been on controlling erosion, preventing discharge of toxic materials, and treating sewage wastewater, however, there are still numerous sources of contamination that go unchecked and unregulated. Heavy metals, thermal outlets, organic materials, and pathogenic microorganisms are some of the contaminants that continue to raise concern in many waterways (Simpson et al., 2002). Each of these may lead to long-term effects on aquatic, wildlife, and human health.

Many bacteria are naturally present in the environment, accumulating in soils and forming biofilms in water bodies. These species originate from many sources, and are not very likely to cause disease. Pathogenic microorganisms which may be introduced by humans, domestic and wild animals, or other sources, are also present in soil and water

resources. These organisms have great potential to be carried from points of pollution to distant locations where they cause illness and death. The widespread epidemics caused by pathogenic microorganisms are of grave concern due to their effects on citizens in both undeveloped and highly developed nations. Exposure to fecally-contaminated recreational water may lead to gastrointestinal disease, skin lesions, or death (Rose et al., 2001; Jamieson et al., 2002). Ingestion of microbially-contaminated water causes millions of deaths worldwide, and is also a concern in domestic rural communities that rely on well water (EPA, 2006).

Trends in agricultural practices, such as expansion of rural communities, animal confinement, and manure application have increased the likelihood of illness caused by these microorganisms (Hagedorn et al., 1999). Bacteria that have evolved resistance to specific antibiotics may be released into the environment and be passed between individuals, groups, and even separate farms. Organisms that are carried by farm animals, but that do not cause animal disease, are also introduced to the soil and water. Each of these instances provides an increased health risk to any animal or person who comes in contact with the contaminated water. Furthermore, direct contact is not necessary for exposure. Rain, streams, and even wind have the capability to transfer contaminating factors (i.e. pathogenic microorganisms) to locations far away from the initial source. Examining and understanding the relationships between impaired water bodies, pathogenic microorganisms, and sources of contamination are crucial in sustaining usable water resources (Hagedorn et al., 1999; Leung et al., 2004).

Bacterial source tracking is one method that utilizes modern technologies to identify and trace the route of contamination in many resources. Molecular methods are on the rise as the preferred systems for quick and accurate assessments. In particular, amplified fragment length polymorphisms (AFLP) and repeated-sequence polymerase chain reactions (rep-PCR) have shown great promise with respect to fecal contamination of water resources (Griffith et al., 2003; Leung et al., 2004; Meays et al., 2004). Each of these examines the specific banding patterns of DNA manipulated with specific molecular tools. AFLP applies restriction enzymes to genomic DNA, followed by amplification of sequences, to produce a species-specific fingerprint that is analyzed by capillary electrophoresis (Vos et al., 1995). Rep-PCR utilizes repeated sequences that are amplified with specific primers, to produce a fingerprint on agarose gel electrophoresis (Rademaker and Bruijn, 1998). These fingerprints are converted to a spreadsheet database and analyzed by statistical comparison for relatedness between samples.

The objectives of this study were to a) examine and analyze the concentration differences of three microorganisms (*Escherichia coli*, *Enterococcus* spp., and *Streptococcus* spp.) in water from lands impacted by cattle pastures compared to "other" sources and b) utilize molecular and statistical techniques to pinpoint the contribution from grazing cows, specifically.

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CHAPTER II

LITERATURE REVIEW

Bacterial source tracking of a watershed impacted by cattle pastures involves many divisions of research and expertise (Malakoff, 2002). First is the importance of water as a natural resource, identifying causes of impaired water, and providing adequate protection. Second is the examination of agricultural impacts, specifically from cattle production, on water quality. And, finally is the description of the many different tools and techniques used to identify the sources of contamination.

Water Quality

Water as a Resource. Water is a natural resource that has an essential role in all aspects of life. Water resources include all bodies of freshwater that may be used for human consumption, recreation, or agricultural application. These may be lakes, rivers and streams, and underground aquifers (EPA, 2006). Water present in these sources originates from seawater that has evaporated, precipitated, and flowed or seeped into the holding water body. Maintaining and protecting the world's water sources are the goals of many agencies and organizations.

Law and Regulations. In recent decades, the recognition that national waters are impaired with respect to health or conservation issues has led to the establishment of

many governmental regulations. The Clean Water Act (CWA) of 1972 sets guidelines for acceptable Total Maximum Daily Loads (TMDLs) in national waters. This Act calls for the accountability of states, territories, and tribes in identifying the presence and level of pollution in their waters (EPA, 2006). The main goals outlined were to establish all waterways as "fishable and swimmable" by 1983, and to remove all contaminant discharge by 1985 (Cassady, 2004). These goals still have not been met in all waterways, and current research and practices are working to achieve a national standard. Protection of these waters includes the assessment of several pollutants, including temperature, sediment, nutrients, organics, metals, and pathogens (Simpson et al., 2002). The Safe Drinking Water Act of 1974 established legal limits based on human health issues caused by specific water contaminants (EPA, 2006). And, the World Health Organization has established guidelines for irrigation practices to limit the concentration of microorganisms allowed in reused wastewater (Blumenthal et al., 2000). The Clean Water Act of 1972 also led to the development of the National Pollutant Discharge Elimination System (NPDES), which requires permits for discharge of point sources, such as sewage, industrial outlets, and landfills (Cassady, 2004). The overall goal of these agencies is to provide clean water for all citizens. However, enacting laws does not automatically provide results.

Generally, industrial and urban wastewater is subjected to highly developed and efficient treatment procedures to reduce and remove these contaminants and environmental effectors (Doran and Linn, 1979; Karr, 1991; Entry et al., 2002). However, most agricultural wastewater and runoff is not subject to NPDES restrictions,

and are often allowed to free-flow through ditches and channels directly into water systems (Bolstad and Swank, 1997; Ribaudo et al., 1999; Cassady, 2004). Non-point sources such as agricultural fertilizers, pesticides, animal production sites, and forest lands impact large watershed areas, are much more difficult to identify, and are the lingering cause of impaired water resources (Doran and Linn, 1979; Bolstad and Swank, 1997; EPA, 2006).

The burden of assessing pollution, establishing local guidelines, and enforcing restrictions falls to many different groups. State government, federal agencies, and independent groups all agree on the necessity of rules, but no one group can cover all the contributors to pollution (Cassady, 2004). Lax laws in one state contribute to pollution in a neighboring state's water system, outdated equipment and methods for data collection provide inaccurate results, and a general underestimation of pollution is based on the examination of only "major" facilities and water systems; the cumulative effect of these situations leads to the inadequate assessment and control of national water pollution (Cassady, 2004). According to Cassady, "thousands of facilities continue to exceed their Clean Water Act permits, [and] these facilities often exceed their permits more than once and for more than one pollutant" (2004). A 2004 estimate states that between 40% and 51% of national waterways are contaminated and classified as not safe for fishing or swimming, however a more intense examination would most likely put these numbers much higher (Cassady). In order to improve the nation's water quality, a closer examination of the water resources, sources of contamination, and methods to prevent contamination is needed (Gleick, 1998; Malakoff, 2002; Simpson et al., 2002).

Sources of Impediments. Natural events such as erosion, leaching, drought, and flooding impact water resources, and the assessment and management of these events are necessary to sustain usable water (Ribaudo et al., 1999; Entry et al., 2002). Naturally present chemicals, sediments, and microorganisms are found throughout water sources, but low-level contact with these contaminants does not usually cause health problems. Land development for urban and agricultural use has led to the introduction of much higher concentrations of these contaminants (Rose et al., 2001). Chemical, thermal, and biological alterations of water resources raise many concerns in several aspects of water quality (Karr, 1991; Simpson et al., 2002). Accumulation of chemicals and metals from industrial sites, agricultural sites, and landfills present in the water may lead to physical ailments, birth defects, and even death in wildlife and humans (Karr, 1991). Thermal outlets from industrial sites and leaching of agricultural fertilizers cause biological imbalances that may lead to a high biological oxygen demand and fish die-offs (Rose et al., 2001; Simpson et al., 2002). The presence of pathogenic microorganisms from urban wastewater, agricultural sites, and wildlife provides the potential for widespread disease and illness (Rose et al., 2001; Entry et al., 2002).

As severe water contamination continues to be an issue, technology and experience will provide the means for better management practices. One way experience has directed research is by revealing the need to track and convert non-point sources into point sources (Karr, 1991; Hagedorn et al., 1999; Simpson et al., 2002).

Bacterial Contamination. Bacterial contamination of groundwater is a key concern in developing environmental protection regulations. According to Mississippi’s

1997-98 CWA report, 31% of rivers, streams, and creeks, 8% of lakes, reservoirs, and ponds, and 66% of estuaries, bays, and coasts were found to be impaired by pathogens (Scorecard, 2006). High levels of precipitation and rising water tables provide the opportunity for microorganisms to extend from point sources and non-point sources into the public water supply (Bolstad and Swank, 1997; Rose et al., 2001). Rural communities that rely on private wells are at even greater risk; fewer regulations on water monitoring and closer proximity to agricultural sites combine to provide increased opportunity for contamination and illness (Strauss et al., 2001). Nationally, and worldwide, increased urban and agricultural development significantly affects the presence of pathogenic microorganisms in water systems.

Agriculture and Animal Production

Land Use. Agricultural lands vary greatly between regions. Food and turf crops require and produce different nutrient loads than animal production operations. Research has shown that maintaining a balance between intake and output of nutrients allows for the best long-term management of agricultural lands (Hagedorn et al., 1999; Miller et al., 2003). Conservation issues have focused on erosion and applied sources of water contamination, therefore, fertilizers, pesticides, herbicides, and other various additives fall under great scrutiny before being applied to fields (Entry et al., 2002).

Bacterial Load. Bacteria are prevalent in soil and water, and the patterns of increased contamination surrounding agricultural land demonstrate the impact of development on water resources (Niemi and Niemi, 1991; Hagedorn et al., 1999;

Jamieson, 2002). Faust (1982) found that pasture soil above the depth of 7 cm had the greatest concentration of fecal coliforms and altering the number of animals affected the concentration of these bacteria. Evans and Owens (1972) determined that pasture runoff containing fecal bacteria may be affected by the initial concentration of bacteria in and on soil and vegetation, water flow rate through the soil, and use of animal manure as large scale fertilizer.

The trend towards confined animal feeding operations (CAFOs), as opposed to large-field roaming operations, is impacting the need for better management of animal operations (Evans and Owens, 1972; Entry et al., 2002). Manure collected from CAFOs is often incorporated into a liquid and applied directly to crop fields and pastures without any treatment for pathogenic microorganisms (Evans and Owens, 1972). Vinten et al. (2004) found that the bacterial load of slurry was less than that of fresh manure, but fields with applied slurry were more likely to leach bacteria into water systems. These observations are thought to be due to die-off during storage and concentrated application of fecal material, respectively (Vinten et al., 2004). The type of bedding used in CAFOs and seasonal fluctuations in manure components may also contribute to pollution (Miller et al., 2003). As more animals are kept in less space, the potential for leaching from feedlots, housing sheds, and lagoons greatly increases, at which time, these controllable point sources become widespread non-point sources (Gagliardi and Karns, 2000). Herd size, diet, lagoons, and filter strips are a few of the tools utilized to decrease the output of pollutants (Entry et al., 2002; Miller et al., 2003). However, more effective means of control are necessary for the long term protection of water resources. The importance of

nutrient recycling conflicts with the need to prevent pathogenic contamination of large watersheds (Gagliardi and Karns, 2000).

Fecal Indicators. Total coliforms may originate from many sources, including municipal sewage, wildlife, soil, and plants, and are used as a non-specific indicator of fecal contamination (Strauss et al., 2001). Fecal indicators such as *Escherichia coli*, *Streptococcus* spp., and *Enterococcus* spp. are useful in assessing the presence of fecal contamination in soil and water (Hagedorn et al., 1999; Wright et al., 2004). These organisms may not necessarily be pathogenic, but are utilized in determining the presence of highly pathogenic organisms and strains (Jamieson et al., 2002; Carson et al., 2003). However, *E. coli*, among other fecal bacteria, has been isolated from many locations where no fecal contamination has occurred, thus using fecal coliform or fecal streptococcus as "fecal indicators" should be exercised with caution (Gauthier and Archibald, 2001). These groups include many different strains which may indicate diverse sources, pathogenicity, and survivability (Faust, 1982; Mubiru et al., 2000; Jamieson et al., 2002).

Some strains of *E. coli*, *Salmonella* spp., *Campylobacter* spp., *Shigella* spp., *Cryptosporidium parvum*, and *Giardia* spp. cause disease and are associated with water discharge (Rose et al., 2001; Jamieson et al., 2002). These microorganisms pose a serious threat to humans and can remain viable in the environment for weeks to months (Gagliardi and Karns, 2000; Jamieson et al., 2002). Historically, the presence or absence of these organisms has been used to determine water quality (Doran and Linn, 1979).

However, more recent trends call for the source location, contaminant reduction, and future protection (Karr, 1991; Hagedorn et al., 1999).

Conflicting reports of the comparison of fecal indicators and pathogenic organisms (Niemi and Niemi, 1991; Meays et al., 2004) and pathogenic organisms' survivability in soil and water (Mubiru et al., 2000; Jamieson et al., 2000) naturally lead to a closer examination of contaminants, sources, and large-scale effects on human and animal health. Understanding the relationship between sources, organisms, and runoff; and differentiating the types of microorganisms present in these systems is essential in improving water quality for human use and consumption (Hagedorn et al., 1999; Leung et al., 2004).

Bacterial Source Tracking

Bacterial source tracking is the use of chemical, microbiological, molecular, and other methods to determine the source of fecal contamination (Sargeant, 1999; Simpson et al., 2002; Meays et al., 2004). Testing for the presence of chemicals, such as detergents and caffeine, has been used to determine if groundwater is contaminated by human sources; however, the concentrations of these must be very high to be effective and testing must be very close to the source (Sargeant, 1999; Meays et al., 2004). Species-specific indicators are groups of bacterial strains that tend to be more prevalent in certain animal species (Sargeant, 1999). Differing nutrients and biological requirements within the host animal or environment cause changes in the bacterial population, and the use of source tracking technology will develop the link between

bacterial strains and their normal animal host (Stoeckel et al., 2004). Many techniques have been used in tracking the source of bacterial contamination of water, soil, food products, and other resources (Griffith et al., 2003). These fall in two general methodologies, biochemical or molecular, and can be further divided into four categories: genotypic library-based, phenotypic library-based, genetic culture-independent non-library-based, and evaluation of bacterial and human viruses (Simpson et al., 2002; Griffith et al., 2003; Meays et al., 2004; Potucek, 2004; EPA, 2005).

Biochemical Methods. The biochemical, or phenotypic, methods have been in use for many years due to developing technology and an understanding of physical interactions. These methods examine the physical attributes, biochemical products, and chemical requirements of microorganisms (Leung et al., 2004; EPA, 2005). Fatty acid analysis examines the composition of bacterial membranes (Potucek, 2004). Ratio comparisons are based on host animal flora. In the past, these were used to assess human and non-human contamination; however, more recent data shows that survival rates of these bacteria differ, and the ratio method does not provide an accurate account (Hagedorn et al., 1999; Sargeant, 1999; Simpson et al., 2002; Meays et al., 2004), such that new methods are necessary. The two most common biochemical source tracking methods are antibiotic resistance analysis (ARA) and carbon utilization (Griffith et al., 2003; Leung et al., 2004; Potucek, 2004; Stoeckel et al., 2004). ARA is based on the specific resistance patterns of microorganisms present in animal populations that are exposed to or inoculated with different antibiotics (Hagedorn et al., 1999; Wiggins et al., 1999; Meays et al., 2004; Stoeckel et al., 2004). Carbon utilization patterns develop as

microorganisms adapt to the different food sources of their host animal (Stoeckel et al., 2004). Both of these require a library base of known patterns for the hosts, microorganisms, and strains of interest (Griffith et al., 2003).

Molecular Methods. Several different molecular methods have been used in bacterial source tracking, with varying success (Griffith et al., 2003; Leung et al., 2003; Meays et al., 2004). The library-based methods include Amplified Fragment Length Polymorphisms (AFLP), repetitive extragenic palindromic-PCR (rep-PCR), ribotyping, and Pulsed-Field Gel Electrophoresis (PFGE) (Griffith et al., 2003; Meays et al., 2004; EPA, 2005). The AFLP procedure utilizes species specific adapter and restriction site sequences that serve as primer target sites (Vos et al., 1995). Rep-PCR examines the fragments located between repeated sequences (most often REP, BOX, or ERIC) in genomic DNA (Rademaker and Bruijn, 1998; Meays et al., 2004). Ribotyping with one or two specific restriction enzymes examines fragment variances in the 16S ribosomal sequence, while PFGE is a more sensitive and intricate method that observes fragment variances in the whole genome (Geornaras et al., 2001; Meays et al., 2004; Stoeckel et al., 2004). The library-independent methods include host-specific PCR and t-RFLP which are specific to the host population (Simpson et al., 2002; Griffith et al., 2003; Meays et al., 2004). Host-specific PCR relies on the length differences of host-specific genetic markers in genomic DNA or 16S rDNA of intestinal microorganisms, while t-RFLP focuses on the sizes of terminal end fragments (Meays et al., 2004). PCR-based fingerprinting methods have been used for several years, however many problems have

arisen; slight temperature changes, DNA and primer concentration, DNA quality, and type of polymerase all can affect the efficiency and reproducibility (Janssen et al. 1996).

AFLP Technique. Amplified Fragment Length Polymorphism (AFLP) is a relatively new method used in bacterial source tracking. Great interest has been given to AFLP due to several features; reduced sensitivity to variations in concentration, banding patterns are always reciprocal to the number of bases, and primers are an exact match with the target site (Janssen et al., 1996). Leung et al. (2004) observed that AFLP is extremely reproducible and discriminatory in identifying closely related strains of several different bacterial species, including *E. coli*. Isolating and sequencing each strain individually would not be practical, so AFLP is preferred for this research due to its abilities to produce restriction fragments without primers specific to the nucleotide sequence and to distinguish between closely related strains (Ajmone-Marsan et al., 1997). AFLP analysis has been correlated with previous taxonomical data, and found to produce identical relatedness between species (Janssen et al., 1996; Leung et al., 2004). In comparison to multiple antibiotic resistances, AFLP has been found to be the best indicator in classifying the source of *E. coli* from fecal samples (Guan et al., 2002; Altier, 2004).

AFLP is a genotypic library-based method that can produce strain-specific fingerprints for closely related bacteria (Geornaras et al., 2001; Leung et al., 2004). AFLP is very similar to the RFLP (Restriction Fragment Length Polymorphism) analysis and consists of three basic steps: 1) digestion of DNA with restriction enzymes and ligation of specific adaptors to the restriction fragments; 2) preamplification and selective

amplification of the fragments with corresponding primers; and 3) electrophoretic separation of the products on a high resolution gel (Janssen et al., 1996; Liscum and Oeller, 2006). A core sequence, enzyme specific sequence, and selective extension are the three parts that make up an AFLP primer. Primers that anneal extend on each end of the restriction fragments so that the bases on both sides of the restriction site will be complementary; these serve as templates for PCR (Vos et al., 1995). Complex genomes most often require two-step amplification (Liscum and Oeller, 2006). Each step uses two oligonucleotide primers, one that binds to the MseI end and one that binds to the EcoRI end. During the preamplification step, neither primer is labeled. After preamplification, the product is diluted and serves as a template for the selective amplification step. One of the primers is labeled (radioactive or fluorescent dye), usually EcoRI, and the primer pairs are chosen based on the organism to be analyzed. The resulting product is a highly specific collection of DNA fragments with identifiable labels. A sequencing polyacrylamide gel (Vos et al. 1995; Ajmone-Marsan et al., 1997; Guan et al. 2002) or capillary electrophoresis (Beckman Coulter, 2001) provides a distinct pattern or “fingerprint” for each sample.

Rep-PCR Technique. The repetitive extragenic palindromic-PCR method utilizes repeated, conserved, natural sequences within the genome of bacteria to provide strain-specific fingerprints (Rademaker and Bruijn, 1998; Dombek et al., 2000). Three of these repeated sequences have been identified and employed in molecular methods, including repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and BOX sequences (Versalovic et al., 1994). Primers for the

repeated sequences are not specific for a given organism, and no information is needed about the genome of interest (Rademaker and Bruijn, 1998). When compared to Ribotyping, the two-step process of rep-PCR and gel electrophoresis allows for relatively non-complex, quick results (Carson et al., 2003). Rep-PCR is also the preferred method due to reproducibility of products and superior banding patterns (Carson et al., 2003). Rep-PCR is valuable when a large collection of possible sources is available for comparison; however, this also limits the technique to use in small localities (McLellan et al., 2003).

BOX-PCR has produced better results than REP-PCR when comparing various animal- and human-source bacterial fingerprints (Dombek et al., 2000). Johnson et al. utilized BOX-PCR to group environmental bacteria from the Mississippi River into probable sources based on fingerprint libraries of many animal and human sources from Minnesota watersheds (2002). Rep-PCR has also been used to accurately cluster the highly pathogenic O157:H7 strain of *E. coli* from various contaminated processed meat sources (Hahm et al., 2003).

The equipment, personnel, and time resources required for AFLP and REP-PCR will be utilized in the attempt to identify *E. coli* from cattle sources in environmental water samples.

CHAPTER III

METHODS AND MATERIALS

Since 2002, the Mississippi Agriculture and Forestry Experiment Station (MAFES) and the Forest Products Laboratory have been tracking the numbers of three different fecal indicator bacteria: *Escherichia coli*, *Enterococcus* spp., and fecal *Streptococcus* spp. from a watershed impacted by beef cattle production located at the MAFES Prairie Research Unit.

Water Quality

Site Description. The watershed area consisted of two fenced pastures, a wildlife-inhabited hill, and downstream runoff from the small rural town, Prairie, MS. Water flowed through natural and man-made ditches to four designated sampling sites. The sites are described as: pristine (P3), normal roadside runoff (P1A), high-load pasture (P1), and variable-load pasture (P2). The pristine site (P3) was a wooded area uphill from the grazing cattle with no human habitation. This site was used as an indicator of bacteria that are naturally present in the area, originating from inhabiting and migratory wildlife and birds. The roadside runoff (P1A) was a ditch that ran into the creek sampled in P1 and P2. This site distinguished the microorganisms that were present in water runoff before cattle contribution. This water was impacted by various animal and human

sources, as the ditch ran from the town of Prairie to the sampling site. Sampling point (P1) was located in a creek that collected from a high-load pasture consisting of 275 acres with 125 to 150 cows with calves. Sampling point (P2) was in a creek that collected runoff from a pasture consisting of 125 acres with a varying number of cows and calves. The cows were added to and removed from the pasture after a various number of days, not to be revealed until after the water samples were processed in the laboratory.

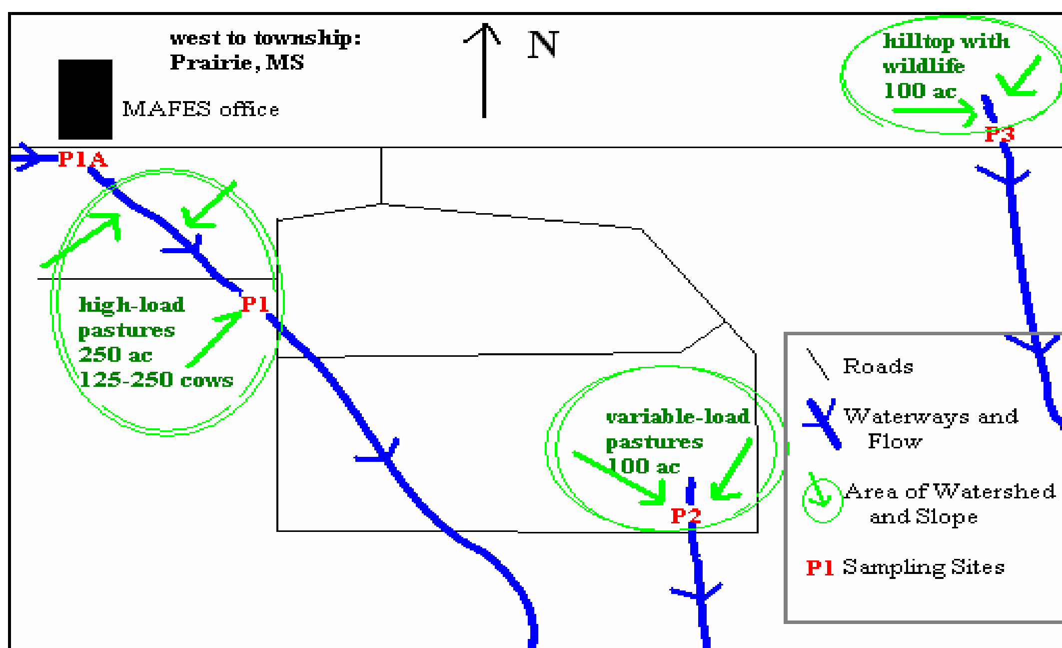


Figure 3.1 Pastures and watershed contributing to sample sites at the MAFES Research Unit in Prairie, MS.

Paddocks Site Description. The research unit also consisted of six fenced, bermed paddocks (1.5-2% slope) with a runoff weir and automatic water collector at the lowest point. Cattle stocking rate, age and type of cattle, and amount and type of groundcover were some of the variables that were examined at these sites. Sites PB3 and

PB6 were control sites on which no cows or calves were placed. Sites PB1 and PB4 each received 4 cow/calf pairs, while sites PB2 and PB5 each received 2 cow/calf pairs. The exact composition of each site was controlled by MAFES personnel, and was not revealed until laboratory analysis was complete.

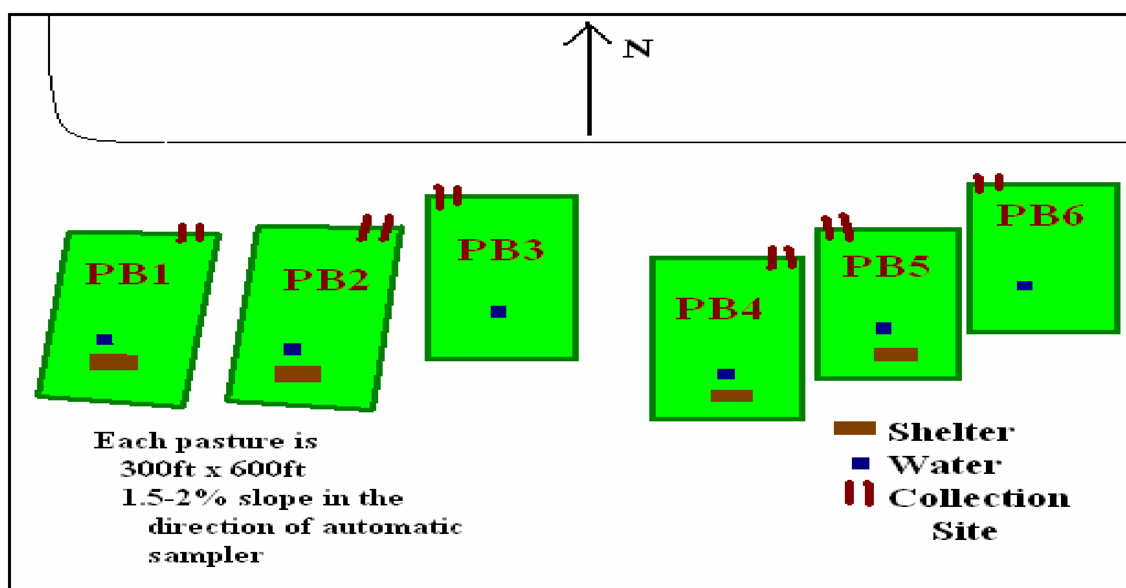


Figure 3.2 Paddock layout at the MAFES Research Unit in Prairie, MS.

Sampling Procedure. Water samples were collected from all ten sites when precipitation fell and provided adequate runoff. Adequate runoff was defined as the production of flowing (not stagnant) water through the collection site ditches. MAFES personnel collected the samples from P1, P1A, P2, and P3 by hand, with whirl-packs attached to a reach-pole. Samples at the paddock sites were gathered from the automatic collectors and transported in whirl-packs. All samples were immediately placed on ice and transported to the laboratory.

Sample Processing. Once in the lab, water samples were transferred into sterile beakers, logged in, and processed. Information collected for the log include the date, sample number, dilutions for each sample, and a physical description of each sample, which accounted the volume, color (manual visual assessment of "yellowness"), and turbidity (manual visual assessment of "cloudiness").

Processing began with Standard Method 9222 (APHA, 1998). Millipore filters (0.2 um) and a vacuum filtering system were used to collect bacteria from water samples. A Millipore filter was placed on the filter screen, and the vacuum apparatus was assembled. Sterile distilled water was poured into the vacuum beaker (100 ml) and the appropriate amount of sample (depending on dilution) was swirled in. The dilutions varied between 0.1 ul/ml and 100 ul/ml, which were obtained by adding between 100 ul and 10 ml of sample water to the water in the vacuum beaker. Dilutions were chosen for each sample, individually, based on previous bacterial counts, to provide plates with 30-300 colonies. Thus, the dilutions varied for each sample, depending on the assumed bacterial load and amount of sample received.

The vacuum filtering system is designed to pull water through, leaving the bacteria from each sample on the Millipore filter paper. Each paper was placed on a plate of selective media (Difco, Kansas City, MO): Standard Method 9230C (APHA, 1998) utililizes mE medium to assess *Enterococcus* spp. and m *Enterococcus* (FS) medium to assess *Streptococcus* spp., and EPA Method 1603 (2002) utilizes membrane-thermotolerant *Escherichia coli* agar (mTEC) medium to assess *E. coli*. All samples were filtered at three dilutions, with each being placed on all three types of media. For

example: The sample from P1 may have been diluted at 0.1, 0.5, and 1.0 ul/ml; three filter cycles were completed at each dilution; and each filter paper was placed on one of the three types of media. One complete set of water samples provided: ten samples X three dilutions X three media plates = 90 unique plates.

mTEC plates were placed in a water bath at 44.5°C for 24 hours, mE plates were placed (inverted) in a water bath at 41°C for 48 hours, and FS plates were placed (inverted) in an incubator at 37°C for 48 hours. After their respective times, all plates were counted for total number of positive colonies, which were then calculated into the number of bacteria in 100 ml of runoff water. The calculation was obtained by multiplying the number of colonies counted by 100 ml of water, and dividing by the dilution factor. For example, if 25 colonies were counted on the 0.5ml plate: 25 X 100 / 0.5 = 5000 bacteria in 100 ml of water sample.

Bacterial Source Tracking

Sampling Procedure. Fecal samples were collected directly from cow patties at the Prairie station, using sterile cotton swabs. Fresh cow patties were sampled randomly from three sites, PB1, PB3 and PB2. Site 1 and 3 each contained four cows and four calves and site 2 contained two cows and two calves. The swabs were immediately placed on ice and returned to the laboratory. Between 25 and 50 unique samples were collected from the Prairie cows in July 2004.

Sample Processing. In the laboratory, fecal and water samples were suspended in sterile NanoPure water and filtered at three dilutions (.1, 1, and 10 ul/ml), following

the same Standard Method 9222D (1992) as previously described. Filters were placed on mTEC media and incubated at 44.5ºC for 24 hours. The positive colonies (yellow) were subcultured in EC with methyl-umbelliferyl-ß-glucronide (MUG) broth at 44.5ºC for 24 hours (Freier and Hartman, 1987; Bej et al., 1991). Each positive (growth and fluorescence) sample was streaked for isolation onto Tryptic Soy Agar (TSA) and incubated at 35ºC overnight. For confirmation, a single colony was resuspended in Brilliant Green (BG) broth at 44.5ºC overnight, then at 35ºC for 48 hours (Donovan et al., 1998). The positive (growth and gas) samples were resuspended in TSB (Tryptic Soy Broth) to produce good growth, split into four Eppendorf tubes, and spun down to produce a pellet. The medium was removed from each tube, and glycerol was added to two of the four tubes for cellular preservation. All tubes were stored in a -80ºC freezer.

DNA Extraction. DNA was extracted by the Advanced Preparation protocol, as described by Invitrogen (2004). The Advanced Preparation procedure started with 1ml of bacterial culture spun down in 1.5 ml microcentrifuge tube. All media was removed and the samples were air dried at room temperature for 5 minutes. Degradation of cellular membranes was obtained by the addition of 1ml of TES sucrose (8% [wt/vol] sucrose, 50 mM NaCl, 20 mM Tris, 1 mM EDTA, and 1 mg/ml lysozyme). The sample was vortexed, transfered to a 2 ml tube, and incubated at room temperature for 5 minutes. Membrane components were separated by mixing with 100 ul of 10% sodium dodecyl sulfate (SDS). Next was 800 ul of phenol:chloroform:isoamyl alcohol (25:24:1), followed by aggressive mixing, and centrifugation at 12000 x g for 15 minutes, separating the DNA into an aqueous phase, which was then transferred into a new 1.5 ml

microcentrifuge tube. DNA was precipitated by the addition of 50 ul of 3.0M sodium acetate and 600 ul of cold isopropanol. The tubes were inverted to mix well and placed in a -70°C freezer for 15 minutes (or may stay overnight in -20°C freezer). Pellets were formed by centrifugation at 12000 x g at 4° C for 20 minutes. Supernatant was discarded and 500 ul of 70% EtOH was added, followed by centrifugation at 12000 x g at 4° C for 10 minutes. Supernatant was discarded, this time followed by air drying for 15 minutes at room temperature. The pellet was dissolved in 500 ul of TE (10mM Tris-HCl, 1mM EDTA), 2.5 ul of RNase A (20mg/ml) was added, and the samples were incubated at 37° C for 10 minutes.

DNA was further purified by mixture with 500 ul of phenol:chloroform:isoamyl alcohol and centrifugation at 12000 x g for 10 minutes. The aqueous phase was transferred into a new 1.5 ml tube and the precipitation (addition of sodium acetate and isopropanol and 15 minutes in -70°C freezer) and pellet-formation (20 minutes centrifugation, discard of supernatant, addition of EtOH, 10 minutes centrifugation, discard of supernatant, and air drying at room temperature) steps were repeated. After the complete drying of all samples (a hot block was used to speed up drying time), the pellets were dissolved in 100 ul TE (10mM Tris-HCl, 1mM EDTA) and stored in a -70ºC freezer.

AFLP Method

AFLP Procedure. The AFLP procedure followed Applied Biosystems' (Foster City, CA) AFLP Microbial Fingerprinting Protocol (2005). *EcoR1* and *Mse1* digestion enzymes were ordered through New England Biolabs (Ipswich, MA) and T4 Ligase was ordered through Promega (Madison, WI). Applied Biosystems' kits provided adaptor pairs and selective primers for EcoR1 and Mse1 and also Core Amplification Mix, which contains the necessary buffer, nucleotides, and polymerase to perform the amplification steps.

Adaptor pairs were annealed by heating in a 95°C water bath for 5 minutes, cooled to room temperature for 10 minutes, and spun at 1400 x g for 10 seconds. The enzyme master mix was prepared (for 100 samples) by combining 10 ul of 10X T4 DNA Ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 20 ug/ml BSA), 10 ul of 0.5 M NaCl, 100 U of *Mse1*, 500 U of *EcoR1*, 100 U of T4 DNA Ligase, and sterile distilled water to 100 ul. These were mixed, centrifuged for 10 seconds, and stored on ice until use. Next, the restriction-ligation master mix was prepared (for 100 samples) by combining 100 ul of 10X T4 DNA Ligase buffer, 100 ul of 0.5 M NaCl, 50 ul of 1.0 mg/ml BSA, 100 ul of Mse1 adaptor, 100 ul of EcoR1 adaptor, and 100 ul of the enzyme master mix (from previous step). Microcentrifuge tubes were labeled for all samples, and 5.5 ul of the restriction-ligation master mix was placed into each. Sample DNA (0.01, 0.1, and 1.0 ug concentrations) was added, in addition to sterile distilled water, to equal 5.5 ul of DNA/water. Samples were mixed, and incubated in an Eppendorf Mastercycler thermal cycler (with a heated lid) for 2 hours at 37°C. After

incubation, the restriction-ligation products were diluted by adding 189 ul of TE (20 mM Tris-HCl and 0.1 mM EDTA) buffer to each sample tube. Diluted samples were stored in a -20°C freezer.

Preselective amplification was performed to purify the DNA segments to be sequenced. For each sample, 0.5 ul of EcoR1 + 0 preselective primer (5' –GAC TGC GTA CCA ATT C- 3'), 0.5 ul of Mse1 + C preselective primer (5' –GAT GAG TCC TGA GTA AC- 3'), and 15 ul of Core Amplification Mix were placed in labeled PCR reaction tubes. 4 ul of sample DNA (diluted from digest/ligation product) was added to the 16 ul of amplification mixture. Amplification was achieved by thermal cycling at 72°C for 2 minutes, followed by 20 cycles of 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 2 minutes. Preselective amplification products were then diluted 1:20 by mixing 10 ul preselective amplification product in 190 ul TE buffer (20mM Tris-HCl and 0.1mM EDTA) and placed in a -20°C freezer.

Selective amplification master mix was prepared for each sample by combining 0.5 ul of EcoR1+0 selective primer, 0.5 ul of Mse1+C selective primer (fluorescent dye-labeled), and 7.5 ul of Core Amplification Mix in labeled PCR reaction tubes. 1.5 ul of sample DNA (diluted from preselective amplification product) was combined with the 8.5 ul of amplification mixture. Amplification was achieved by thermal cycling at 94°C for 2 minutes, followed by 31 cycles of 94°C for 20 seconds, 66°C for 30 seconds (providing a 1°C decrease with the first 11 cycles), and 72°C for 2 minutes. A final hold at 60°C for 30 minutes allowed for adequate annealing, after which, samples were stored at 4°C.

AFLP Analysis. A Beckman Coulter CEQ 2000CL DNA Analysis System was used for fragment analysis; results would have been further analyzed with DNASTAR (DNASTAR, Inc.) software. Statistical analysis would have been used to show the relationship between agricultural cattle load and fecal coliform contamination in water runoff.

BOX- and REP-PCR Method

BOX- and REP- Protocol. The BOX- and REP-PCR procedure initially followed Rademaker and Bruijn's described protocol (1998). BOX A1R, REP 1R, and REP 2I primers were ordered through Sigma-Genosys (St. Louis, MO) and *Taq* DNA Polymerase and dNTP mix were purchased from Promega (Madison, WI). Gitschier Buffer (5X) was prepared following the standard: 1 M $(NH_4)_2SO_4$, 1 M Tris-HCl (pH 8.8), 1 M $MgCl_2$, and 0.5 M EDTA (pH 8.8), each prepared and autoclaved separately (Rademaker and Bruijn, 1998).

The described procedure was altered to replace Gitschier Buffer with Promega's 10X Mg-free Buffer and $MgCl_2$ adjusted to the described concentrations. To simplify the procedure, only REP primers (REP 1R and REP 2I) were used for all samples, and DMSO was removed from the final reactions.

The reaction master mix was prepared (for each sample) by combining 0.4 ul of BSA (adjusted for 10 mg/ml), 3.125 ul of dNTP mix (adjusted for 10 mM mix), 1 ul of primer 1 (BOX A1R), 1 ul of primer 2 (REP 2I), 0.4 ul of *Taq* DNA Polymerase, 2.5 ul of 10X Mg-free Buffer, 6.7 ul of $MgCl_2$,and sterile distilled water to 24 ul (8.875 ul). 1

ul of each sample was placed into a tube containing the 24 ul of reaction master mix. These were mixed and placed in a thermal cycler (with a heated lid) following Rademaker and Bruijn's protocol (1998) of 95°C for 2 minutes, followed by 30 cycles of 94°C for 3 seconds, 92°C for 30 seconds, 40°C for 1 minute, and 65°C for 8 minutes. The samples were held for a final 8 minutes at 65°C, then stored at 4°C.

BOX- and REP- Analysis. Samples were run on a 2.5% high resolution agarose gel for 16 to 18 hours at 60 V (2 V/cm, dependant on distance between electrodes). The banding patterns were captured by camera and examined with GelPro software. Fragment patterns were entered into an Excel spreadsheet and converted to binary data. The simple matching function of SYSTAT was used to generate similarity indices.

CHAPTER IV

RESULTS AND DISCUSSION

The collection and analysis of *Escherichia coli* (EC), *Enterococcus* spp. (EN), and fecal *Streptococcus* spp. (FS) from water runoff of cattle pastures demonstrates the impact of grazing animals on water contamination. The amount of rainfall and time between rainfall events are also considered with respect to bacterial counts.

Four initial sites were evaluated for human, cattle, and other animal sources of contamination. The roadside runoff (P1A) of a rural town was used for comparison of human and other animal contribution. A pristine site (P3) revealed the impact of native and migratory wildlife. A high-load pasture (P1) and variable-load pasture (P2) provided values for contamination from cattle. The raw counts were averaged for each sampling date, and included the time period from March 21, 2002 to May 10, 2006.

Six separate sites (PB1 through PB6) were also utilized to express trends related to addition and removal of cow/calf pairs in controlled paddocks. The raw counts were averaged for each sampling date, and cover the time period of March 2, 2004 to May 10, 2006.

For all figures, please note the variations in bacterial counts located on the y axis. Since the data covers such an extensive time line, some counts were also averaged

seasonally (Spring-March through May, Summer-June through August, Fall-September through November, and Winter-December through February).

Water Quality

Pasture Sites by Season, EC. (Figure 4.1) With respect to EC counts, site P1A tends to track P1, in lower concentrations, which is expected, since P1A feeds into P1. This demonstrates the presence of these organisms in water originating from a source prior to cattle. Site P2 counts are lower than P1 on most sampling dates. This is expected since more cows contribute to P1 runoff than P2. Site P3 reveals more fluctuations and peaks in the fall season (September through November) and tends to fluctuate more dramatically than other sites, presumably from migratory wildlife. Further sampling at site P3 from wildlife and birds could help explain the seasonal trends.

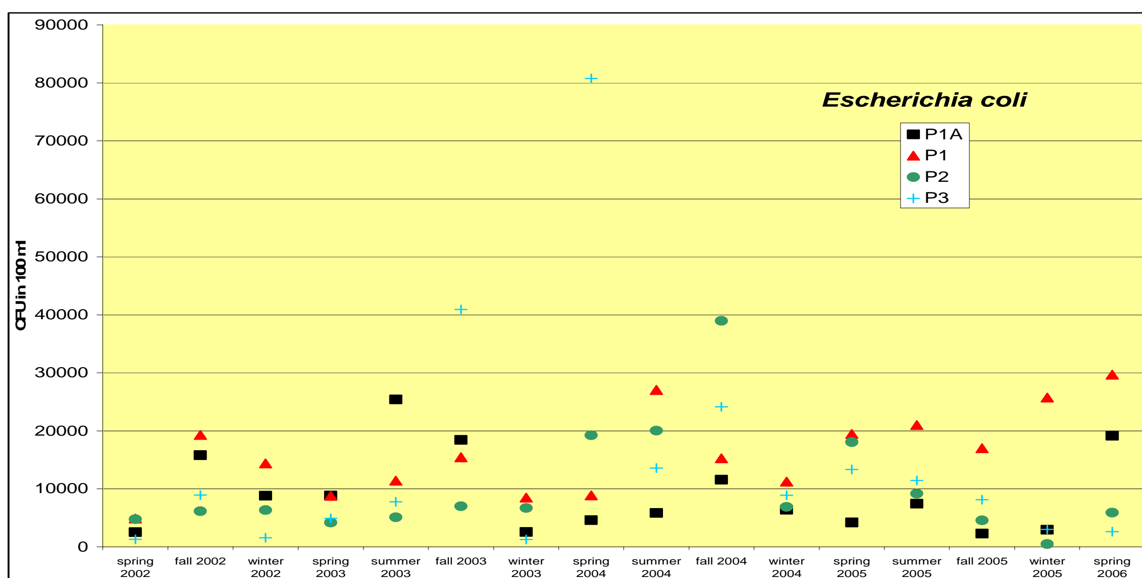


Figure 4.1 ***Escherichia coli*** **counts averaged by season over a four-year period, given in CFU per 100 ml of sample water.**

Pasture Sites by Season, EN. (Figure 4.2) Observations of EN express the same trend of P1A tracking P1 at lower concentrations. EN have been found to maintain better survival rates over land than the other species. These survival rates may lead to the observed higher concentrations at P2 versus P1. The P1 pastures consist of approximately 250 acres, which may provide ample time for all three species to die off. The P2 pastures cover approximately 100 acres, allowing enough time for EC and FS to die off, while EN survive, and are seen in greater numbers at the sampling site.

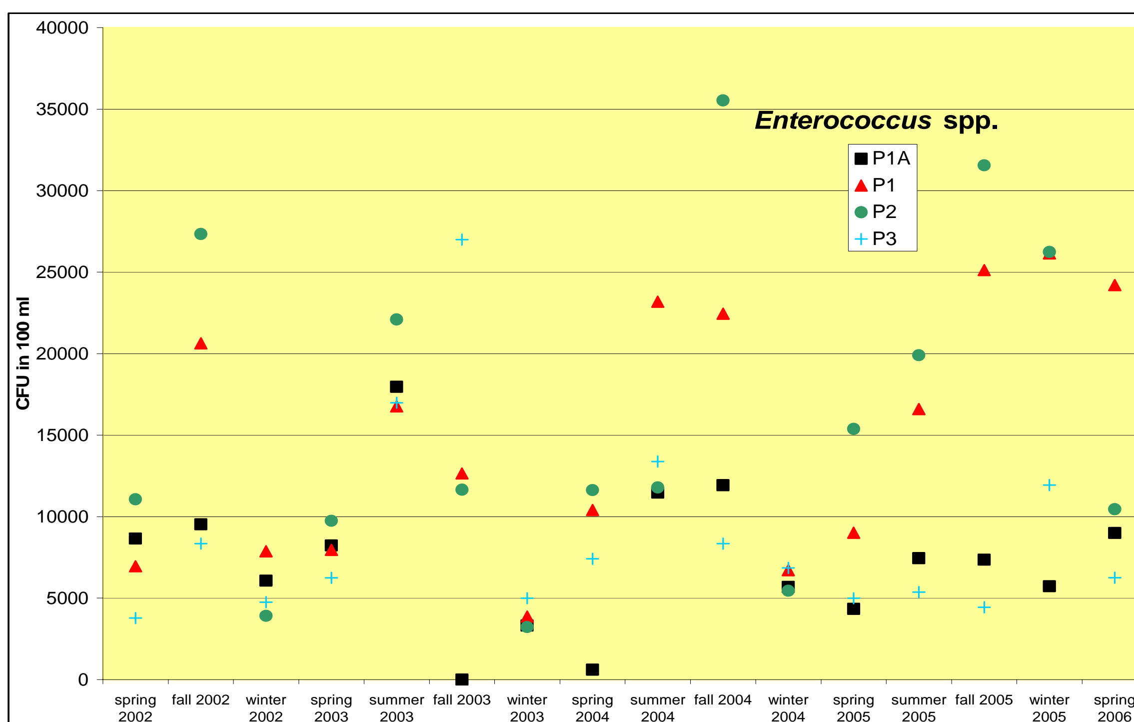


Figure 4.2 ***Enterococcus*** **spp. counts averaged by season over a four-year period, given in CFU per 100 ml of sample water.**

Pasture Sites by Season, FS. (Figure 4.3) When considering FS, the four sites also follow the same trends. P1 and P2 track the closest with the highest concentrations, P3 tracks slightly lower, and P1A provides the lowest concentrations. The constant presence of FS from the roadside (P1A) and pristine (P3) sites may indicate this organism as a "background species" in the cattle impacted sites. These results suggest that FS may not be ideal fecal indicators, as they may be predominantly contributed from some "other" source.

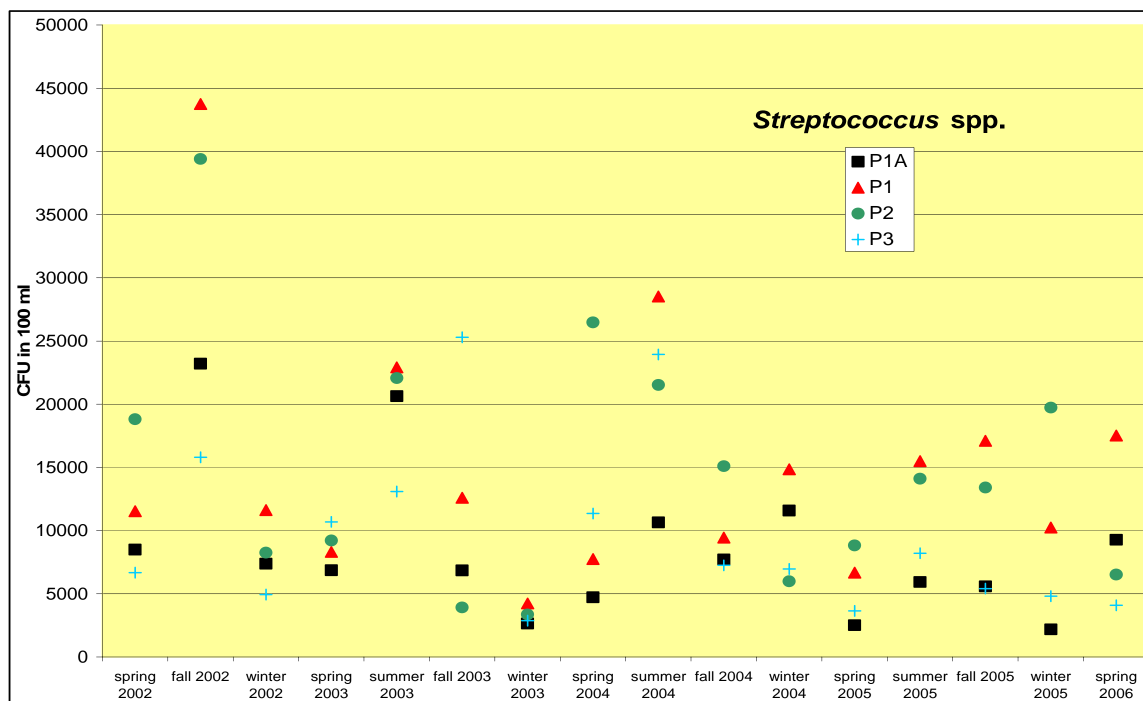
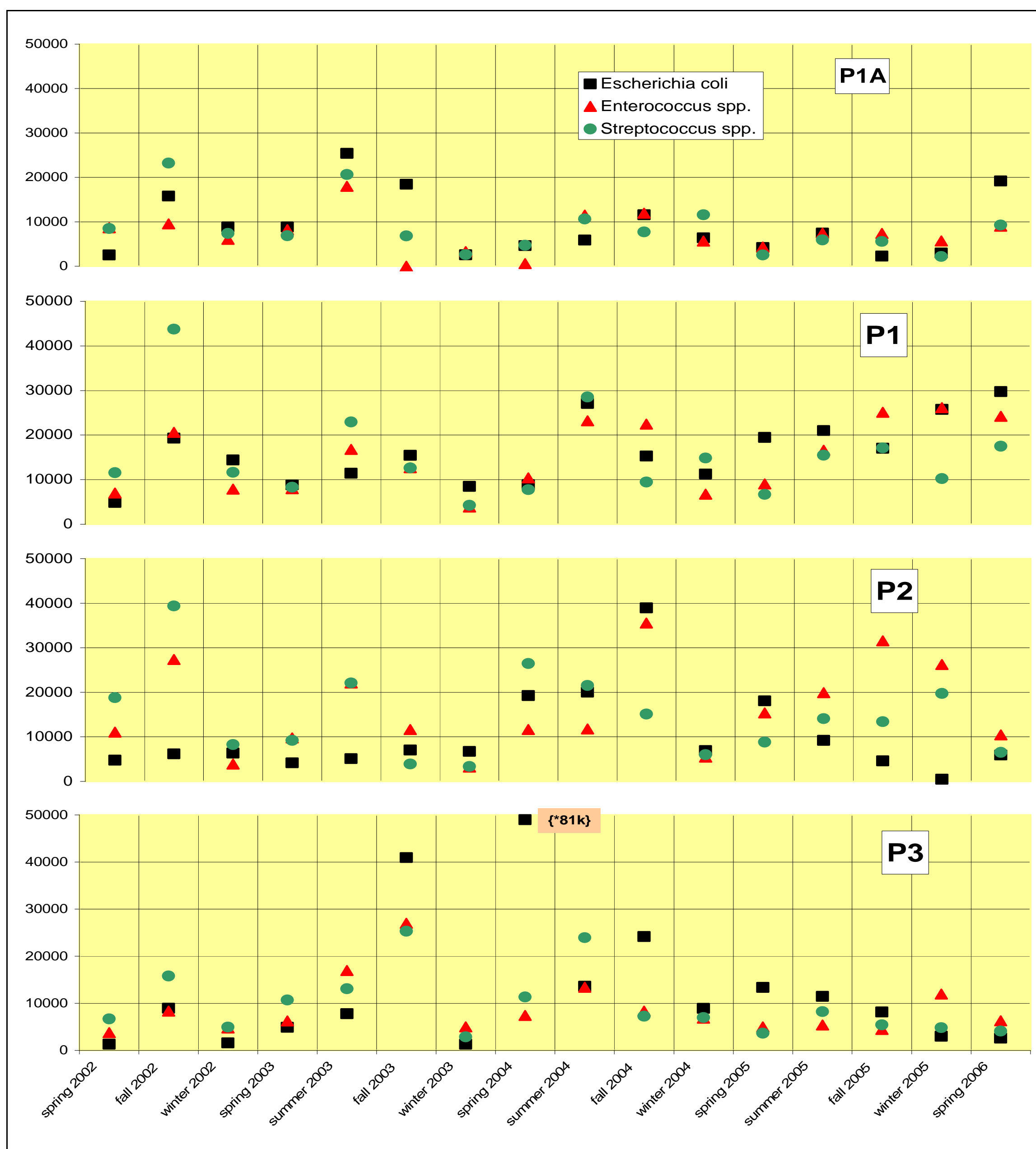


Figure 4.3 ***Streptococcus* spp. counts averaged by season over a four-year period, given in CFU per 100 ml of sample water.**

Pasture Sites by Season. When comparing the four individual sites, P1A demonstrates the fewest and lowest average of peaks (Figure 4.4). At this site, the high counts for EC and FS are seen in the first two years, and only reach the 25,000 cfu/100 ml level. No seasonal trends are recognized, however there is a definite and constant presence of all examined species. Collections further upstream from P1A, such as in front of the elementary school, church, or fire station could identify the specific sources of contamination and also the survival rates of the three bacterial species.

P1 expresses generally more and higher average of peaks, and the three bacteria basically follow independent trends across the years. P2 exhibits many more peaks above the 25,000 cfu/100 ml level, providing the most frequent and highest average of peaks compared to the other three sites. Some seasonal trends may be seen with respect to EN and FS counts. These bacteria tend to decrease in the winter, remain low in the spring, peak in the summer, and decrease again in the fall. An examination of the stocking rates and dates of the pastures contributing to P1 and P2 would aid in understanding the correspondence between cattle loads, land area, and bacterial counts.

P3 provides very similar averages to P1A, with a few exceptions. Seasonal trends are observed in the fall and summer months (fall 2002, fall 2003, and summer/fall 2004), further suggesting the contribution from migratory animals. The constant presence of all three species contributes to the thought that a considerable portion of bacterial contamination originates from some “other” source than cattle. Across all sites, the winter counts provide the lowest counts, followed by spring/summer counts. Fall expresses the highest counts, especially in the cattle and pristine sites.



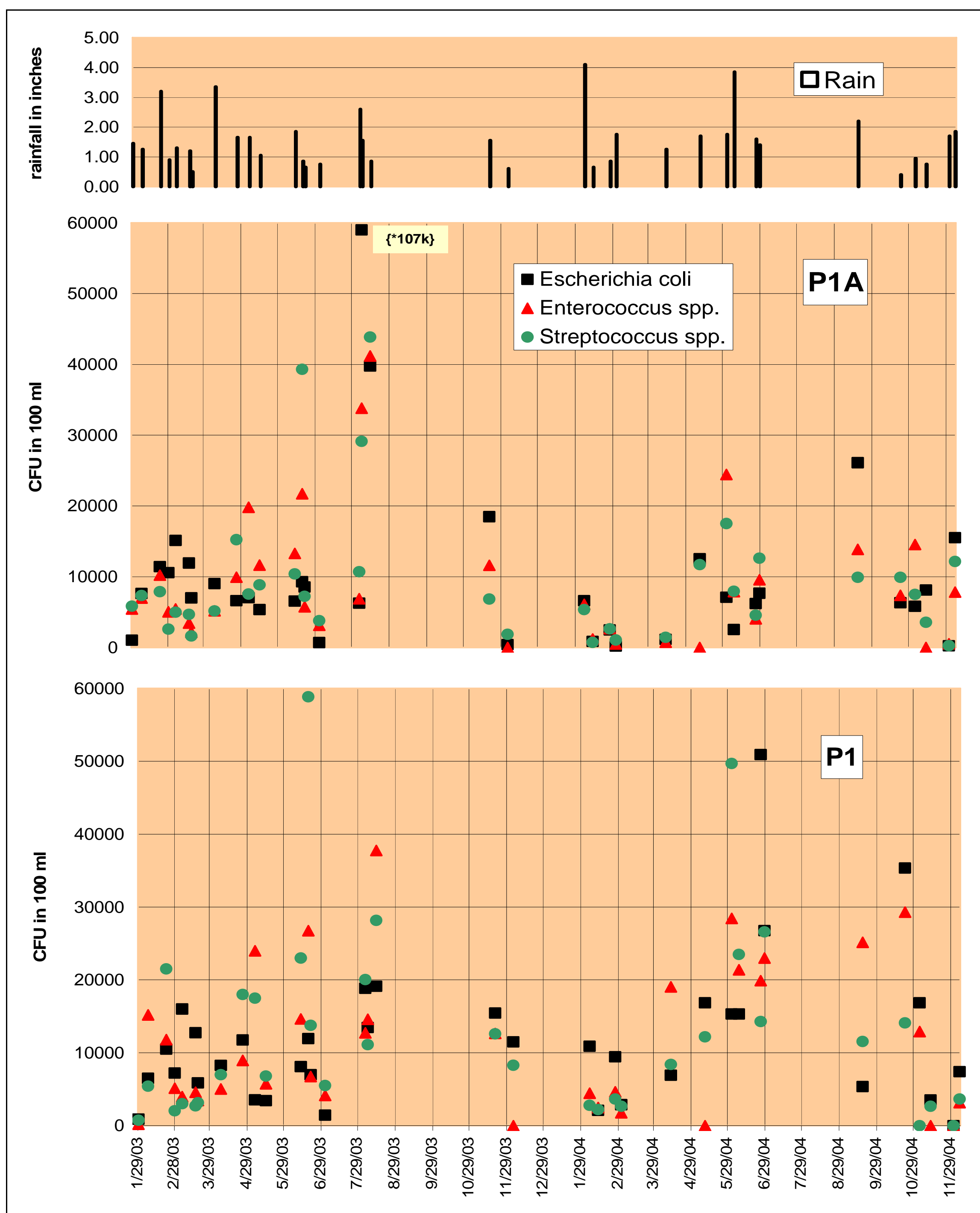
P1A (roadside runoff), P1 (high-load pastures, 250 acres), P2 (variable-load pastures, 100 acres), and P3 (pristine, wildlife)

Figure 4.4 Bacterial counts of four sites, averaged by season over a four-year period, given in CFU per 100 ml of sample water.

Pasture Sites with Rain. A closer examination of a two-year breakdown of individual sampling dates reveals many more fluctuations than the graphs of seasonal averages (Figure 4.5). The ditch sampled at site P1A flows into the creek sampled at site P1, so these were compared along with the amount of rainfall. Examination of P2 and P3 did not reveal exceptional results (not shown). Trends expected with respect to amount of rainfall and time between rainfall events might include higher peaks after longer periods without rain or after greater rainfall events. No trends were observed for these expected outcomes, or for contrary results.

Site P1A expresses nearly constant low levels of bacterial contamination. The majority of sampling points fall below 20,000 cfu/100 ml, with the exception of a few obvious peaks. The extreme EC peak seen in August 2003 demonstrates the impact of outside contributors. Further examination of the waterway may have revealed a soiled baby diaper or domestic animal fecal deposit. These possible explanations are supported by the increased counts of EN and FS on this sampling date and the next. Overall, the virtually constant presence of all three species may provide “background” contamination going into site P1.

P1 demonstrates many more peaks above 20,000 cfu/100 ml, but the majority is still below that level. EN and FS also tend to provide higher peaks than EC. Also noted is the absence of the extreme peaks that were seen in P1A in August 2003. These data reveal the contributions of cattle and “other” sources on sampling location, frequency, and bacterial contamination.



Rainfall in inches, P1A (roadside runoff) and P1 (high-load pastures, 250 acres)

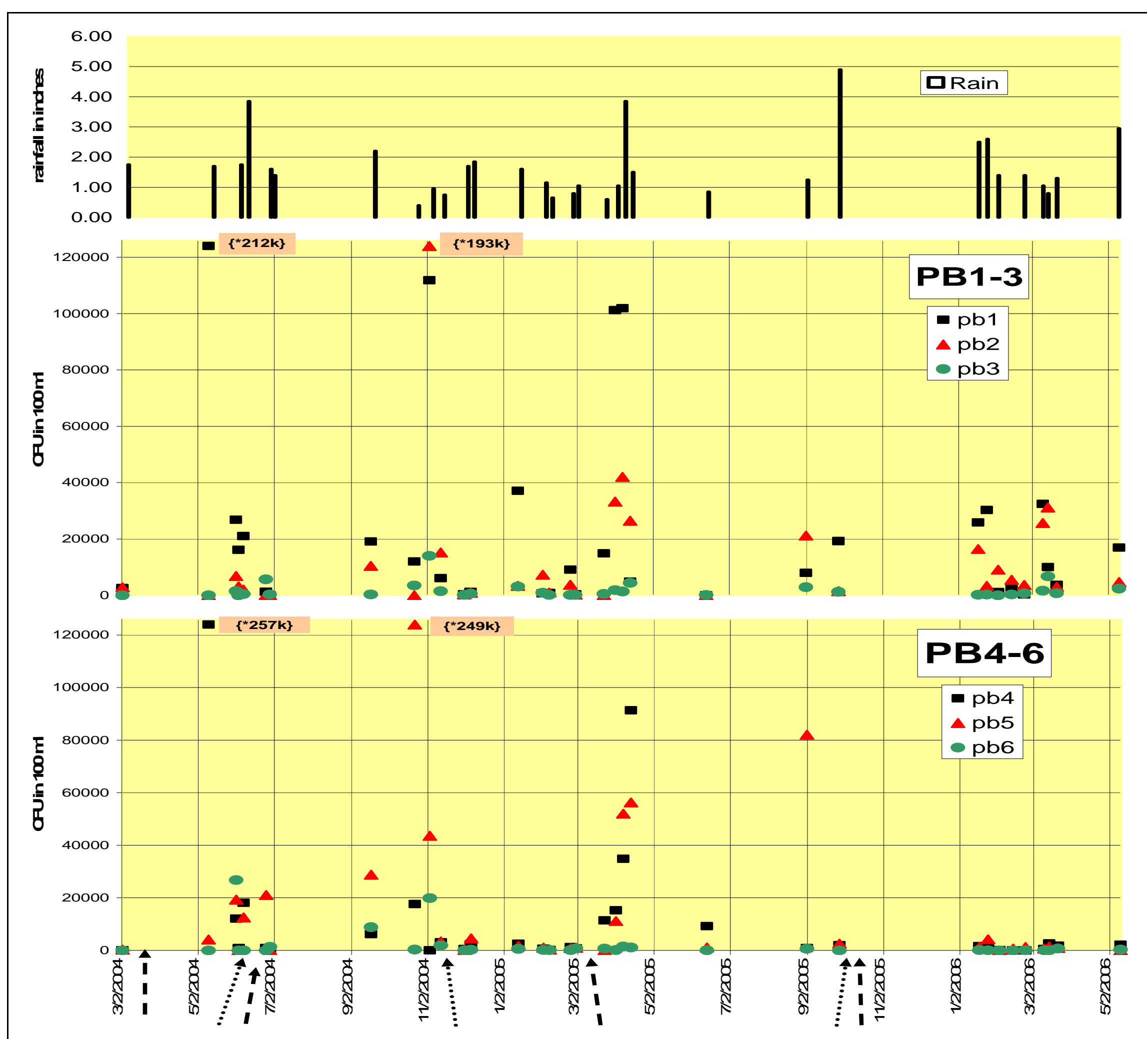
Figure 4.5 Amount of rainfall and bacterial counts of two sites, over two-year period, given in CFU per 100 ml of sample water.

Paddock Sites with Rain, EC. (Figure 4.6) Sites PB1 and 4 each contain 4 cow/calf pairs, PB2 and 5 each contain 2 cow/calf pairs, and PB3 and 6 are the control sites and do not contain any cows/calves. On 3/23/04, cows were placed on the paddocks; they were removed on 6/30/04. On 7/1/04, a new set of cows/calves were placed, they were removed on 11/9/04. On 3/8/05, a third set of cows/calves was placed; they were removed on 10/20/05. The final set of cows (no calves) was placed on 10/25/05.

Examination of sites PB3 and 6 reveals practically nonexistent levels of EC contamination. A few peaks around 20,000 cfu/100 ml may be seen, but are not significant to the overall trends seen at the other sites. This is to be expected, since sites PB3 and 6 are the control sites without cows. Furthermore, wildlife does not contribute as greatly to these sites, as there are few trees or bushes for habitat. Sites PB1 and 4 were expected to express the greatest peaks, but a few peaks at PB2 and 5 surpass those seen at PB1 and 4. One possible explanation is the discrepancy of distance between shade, food, water, and samplers. Some trends of increased counts after (but not immediately) addition of cows/calves are seen, but not across all sites. Observations of cow/calf behaviors at each site may assist in explaining these differences. The time between removal and addition of cows/calves is not significant for the 6/30/04 and 7/1/04 or 10/20/05 and 10/25/05 stocking dates. However, the four month period between removal on 11/9/04 and addition on 3/8/05 demonstrates the loss of viable bacteria across all sites, with the exception of one sampling at site PB1. The 10/25/05 addition of cows provided counts that were lower than all other sampling periods of sites with cow/calf pairs. This

is expected, as the number of stocked cattle was reduced by half and the time period was during the winter and spring seasons.

Overall, the amount of rainfall and time between rainfall events does not appear to affect bacterial counts. This holds true for all species and sites (data not shown).



Rainfall in inches, PB1-3, and PB4-6

Figure 4.6 Amount of rainfall and *Escherichia coli* counts for six sites, over a two-year period, given in CFU per 100 ml of sample water.

Bacterial Source Tracking

Sample DNA was initially extracted following both the Advanced Preparation protocol, as described by Invitrogen (2004), and the QIAGEN DNeasy tissue kit (Valencia, CA). DNA concentrations estimated by a UV fluorometer and gel electrophoresis revealed the Advanced Preparation protocol as the better method for genomic DNA extraction.

AFLP Procedure. The preliminary protocol employed restriction digest and adapter ligation as described by Invitrogen (2004), followed by preamplification, selective amplification, and CEQ sample preparation as described by Beckman Coulter (2001). Several variations in DNA concentration, digest enzymes, gel parameters, buffer composition, and amplification times and temperatures were assessed for application to the protocol. At all combinations, gel electrophoresis results (not shown here) did not yield the expected results, and the entire procedure was altered to the Applied Biosystems protocol (2005). By providing all necessary adaptors and primers, this system allowed for a more cohesive protocol. As described in the AB protocol (2005), gels were run at specific steps in the procedure. Results (not shown) would occasionally provide the expected outcome, and those samples were continued through the process. However, reproducible products were never accomplished.

To alleviate some of the concerns about DNA concentration, all samples were quantified with a NanoDrop (Wilmington, DE) for more accurate values. Based on the NanoDrop readings, nearly all samples were within the acceptable range of purity. Samples that had previously been examined by gel electrophoresis were then subjected to

fragment analysis. Results (not shown) yielded very few and very small peaks. Again, DNA concentrations, buffer components, and primer parameters were assessed and altered, yet results did not accomplish the expected outcome. AFLP is generally considered to be a temperamental procedure. The results of this process indicate that AFPL is not reliable for use on a large scale expansion of this experiment.

REP- PCR Procedure. The preliminary protocol utilized the Rademaker and Bruijn (1998) protocol. Both BOX and REP primers were used and all other procedure components were identical. Gel results (not shown) for various adjustments of buffer, DMSO, and primer concentrations were examined, but did not produce any banding patterns. The adjusted protocol, utilizing Promega's 10X Mg-free buffer and $MgCl_2$ was attempted along with the removal of DMSO. Gel results (Appendix A) revealed that some component of Gitschier Buffer was interfering with the PCR reaction. The removal of DMSO also appeared to enhance the visible banding patterns. Finally, to simplify the procedure, and reduce the over-usage of dNTP mix, only REP primers were used for all samples.

Following the adjusted protocol, and regardless of the sample DNA concentration, nearly all PCR reactions produced readable banding patterns on 2.5% high resolution gels. Digital photographs utilized ultraviolet light and exposure filters to capture the observed banding patterns (Appendix A). These were analyzed with GelPro software and converted to Excel spreadsheets to provide fragment size fingerprints for all samples (Appendix B).

The spreadsheet data was converted to binary code and further analyzed by the simple matching function of SYSTAT (Figure 4.7). For the purpose of this research, some samples were removed from the database to simplify and clarify the cluster analysis results. Replicate samples were averaged and questionable gel patterns were discarded to reduce the opportunity for error.

Variables 1 through 22 represent the cattle manure-derived samples, Variables 23 and 24 represent water-derived samples (from P1A, before cattle pastures), and Variables 25 through 37 are the water-derived samples (from P1, after cattle pastures). This analysis generated a cluster that includes all manure samples except for two outliers—Variables 9 and 22. This discrimination could be due to the original gel electrophoresis examination or extreme strains of EC. Out of the 15 "unknowns," (from water samples) only two fell within the cattle cluster. Based on these preliminary results, approximately 13% of the EC in the pasture waters originated from the cows. Neither of the two samples that came from P1A (before cattle pastures) was grouped in the cattle cluster. Furthermore, these two samples were greatly unrelated. Analysis of a larger collection of samples would be necessary in order to validate these estimates and results.

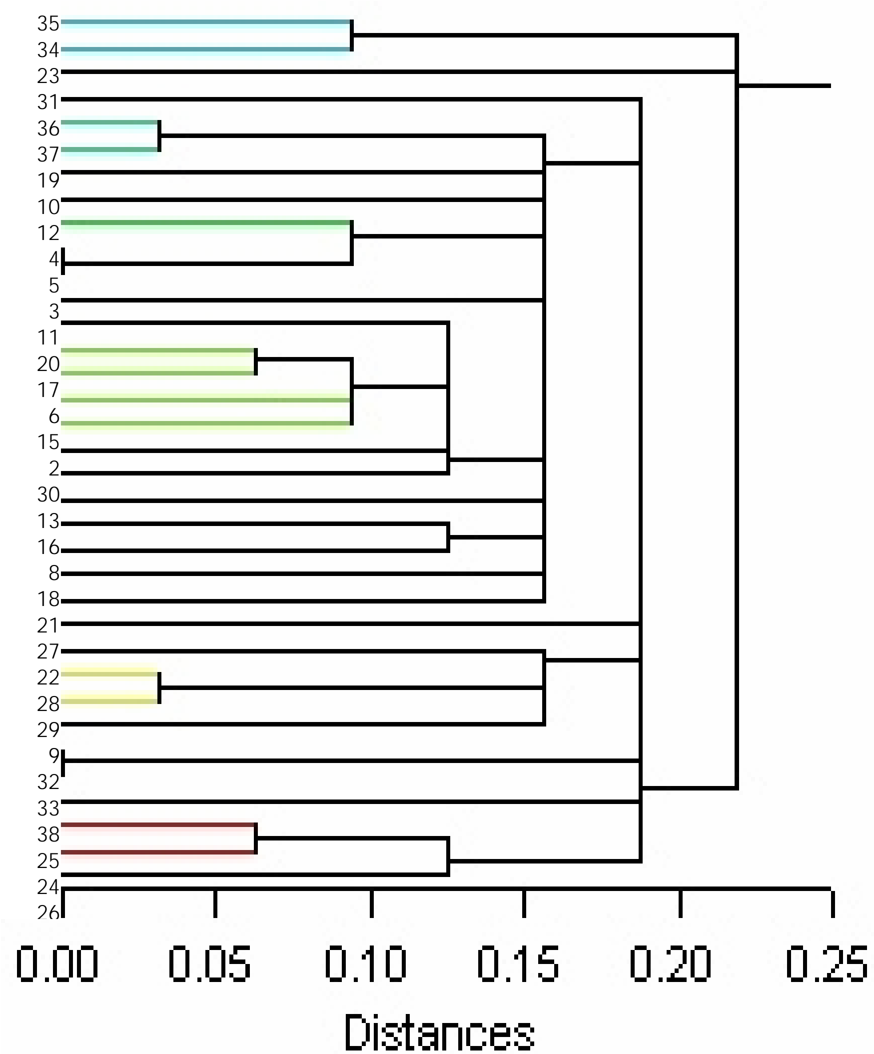


Figure 4.7 Simple matching cluster analysis of cattle manure-derived and water-derived EC samples, based on REP-PCR fingerprints.

CHAPTER V

CONCLUSION

The examination and analysis of the many factors contributing to water contamination is a historically challenging task. For many years, fecal indicator bacteria have been used to determine the overall quality of water for municipal, recreational, and agricultural use. However, recent trends are moving away from this method and placing more emphasis on specific source tracking of the larger watersheds contributing to water resources. New techniques are developed, tested, and determined to be useful or not. The branches of water quality studies spread and overlap across many different fields, and this research has attempted to link the physical attributes of land management to the molecular techniques of microbiology.

Results of this study have contributed to the general assumption that bacteria are universally present in water and on land. Contributing factors such as grazing cattle, migratory and indigenous wildlife, and human development impact the quality of water resources in differing magnitudes. The "fecal indicator" bacteria included in this research, *E. coli* (EC), *Enterococcus* spp. (EN), and *Streptococcus* spp. (FS), reveal the need for developing and applying more exact methods for source tracking.

Water Quality

All three species displayed two general trends. These were a) the constant presence of microorganisms and b) site P1 tracking site P1A very closely. EC trends demonstrated higher peaks and averages than the other species, and had distinct fall spikes at the pristine site, presumably caused by migratory animals. EN expressed higher counts at P2 than P1, perhaps due to survivability rates. And FS provided the most "background" presence, seen in the constant low levels at P1A and P3. Examination of the site differences revealed P1A and P3 as maintaining the lowest averages and fewest peaks. The key differences between these sites were the isolated extreme peaks at P1A and the seasonal trends at P3. P1 and P2, the cattle sites, provided the highest averages and most frequent peaks, with P2 tending to be higher. However, the "washout" effect must be considered since these data have been averaged by season.

Some design elements that might have contributed greatly to this project would have been to expand the sampling sites to include locations further upstream (from P1A) in the town to help pinpoint the source of isolated extreme peaks. More collection sites downstream (from P1 and P2) could help explain the discrepancies between distance, time, and survivability rates of the bacteria (especially EN). A pre-described schedule and record of the stocking rate and dates would have aided in explaining the trends seen at P1 and P2. And, steady sampling of the sites, regardless of rainfall events, would have provided regular data sets to be used in statistical analysis.

Results of the paddock sites reveal the partial impact of grazing cattle on fecal contamination of water runoff. The control sites expressed practically nonexistent levels

of EC, while the presence of EN and FS further supported these bacteria as being introduced from "other" sources. Increased EC counts following the introduction of cows would suggest that cattle contribute greatly to contamination, however these increased counts are not seen at all sites, or on all introduction dates. The removal of cows was seen to reduce the bacterial counts to practically nonexistent, yet after the final introduction, some counts never did return to the high peaks seen on earlier sampling dates. Overall, the data reveals the need for a larger-scale experimental design. Animal behavior, land components, scheduled introduction/removal of cows, and steady sampling dates could provide more consistent and analytical results.

With respect to rainfall (time and volume), some trends were expected. Rainfall events that occurred after a longer period of time (>4 weeks) or with greater volume (>1 inch) might be expected to yield increased bacterial counts in the runoff, as the bacteria had more time to accumulate in the manure and soil or there was more water to move the bacteria. However, neither the time between rainfall events, nor volume of rainfall impacted the counts.

Bacterial Source Tracking

AFLP has previously been documented as a difficult and erratic procedure. Many variations of the protocol were attempted, and all results revealed that this would not be feasible for use on this project.

The described REP-PCR protocol was altered for use in this laboratory, and has successfully yielded reliable data. Gel electrophoresis has revealed reproducible banding

patterns for cattle and water specific EC. Analysis and conversion of these banding patterns by the GelPro software into spreadsheet format reveals some possible relatedness between the strains obtained from cattle sources versus those from water samples. The extension of PCR results via fingerprinting techniques, including binary coding and statistical analysis, have established this procedure as applicable for tracking of EC contamination in water runoff from cattle. A distinct cluster was produced by cattle manure-derived EC, and only two of the water-derived EC fell within that cluster.

The greatest obstacle to large scale application of fingerprinting libraries is that individual databases would have to be developed for each site and species to be examined. Future research including design elements such as sampling from a wider variety of possible sources and the isolation of EC from water on sampling dates would increase the accuracy of water quality and bacterial source tracking techniques in best land management practices.

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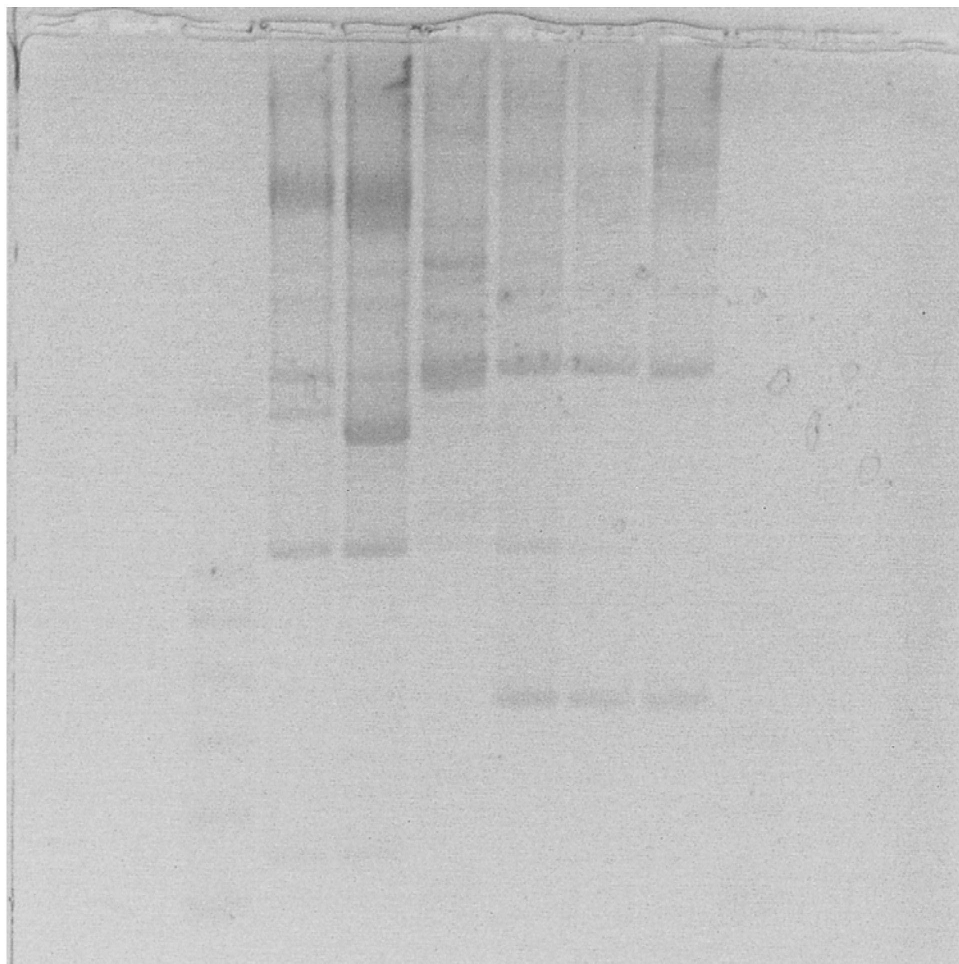
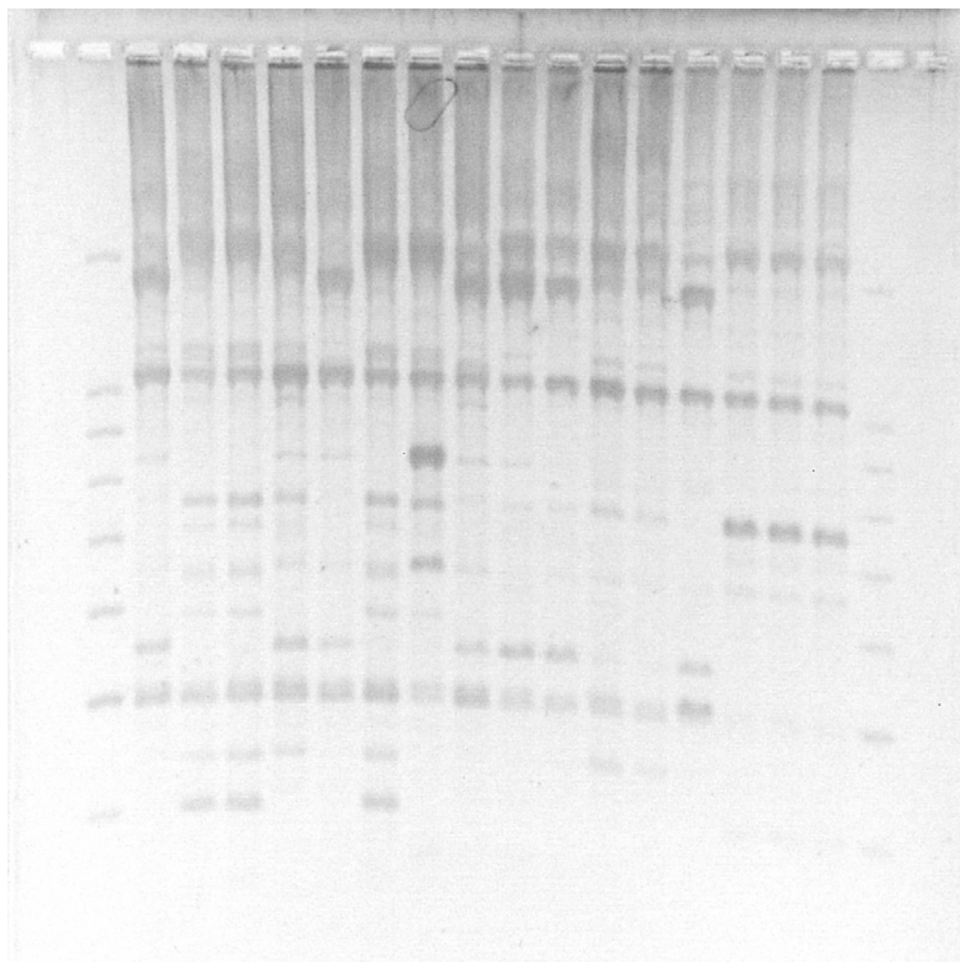
APPENDIX A

GEL ELECTROPHORESIS IMAGES

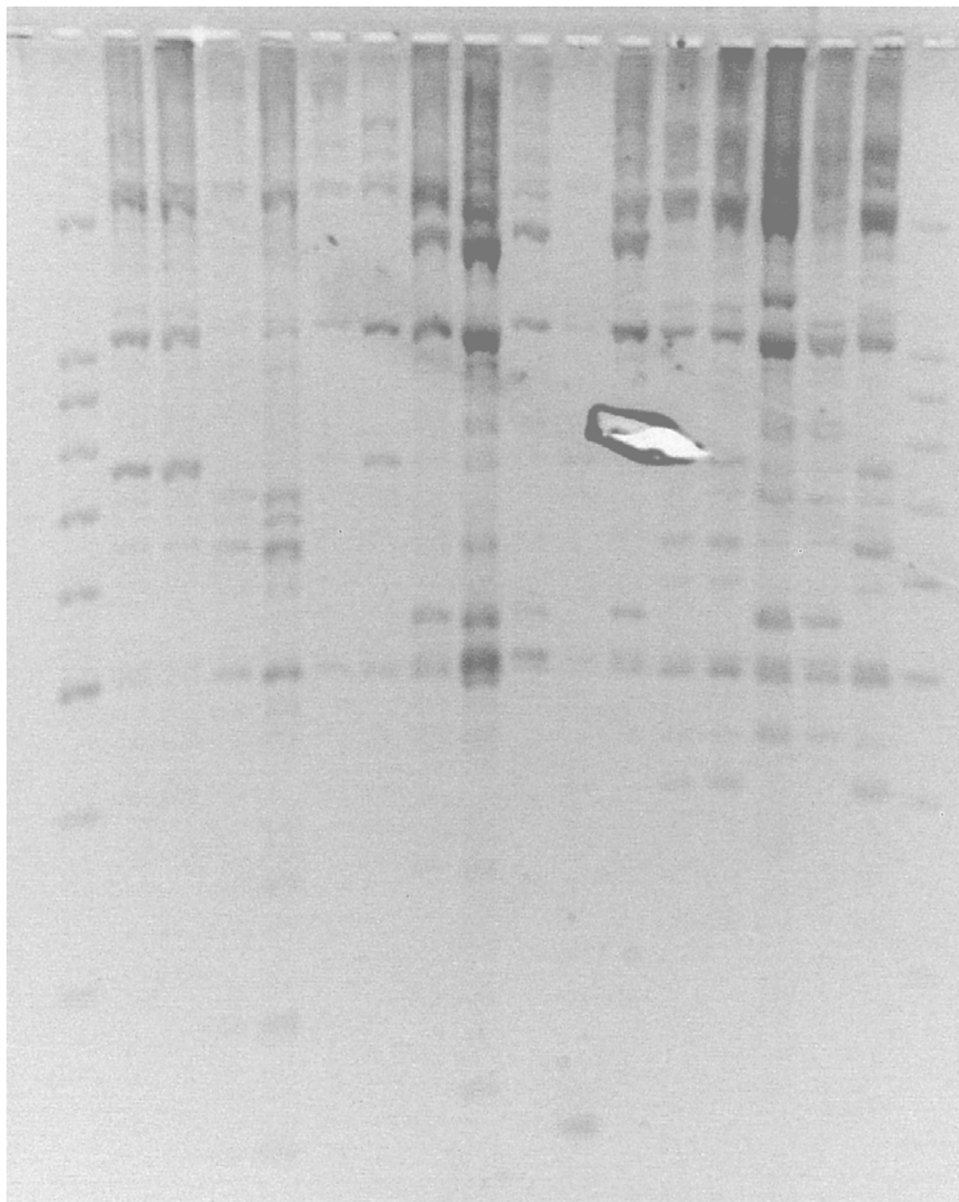
A: Three samples with three combinations of buffer and DMSO. From right to left: Samples A, B, and C with Gitshier buffer and no DMSO, samples A, B, and C with Promega 10X buffer and no DMSO, and samples A, B, and C with 10X buffer and DMSO.



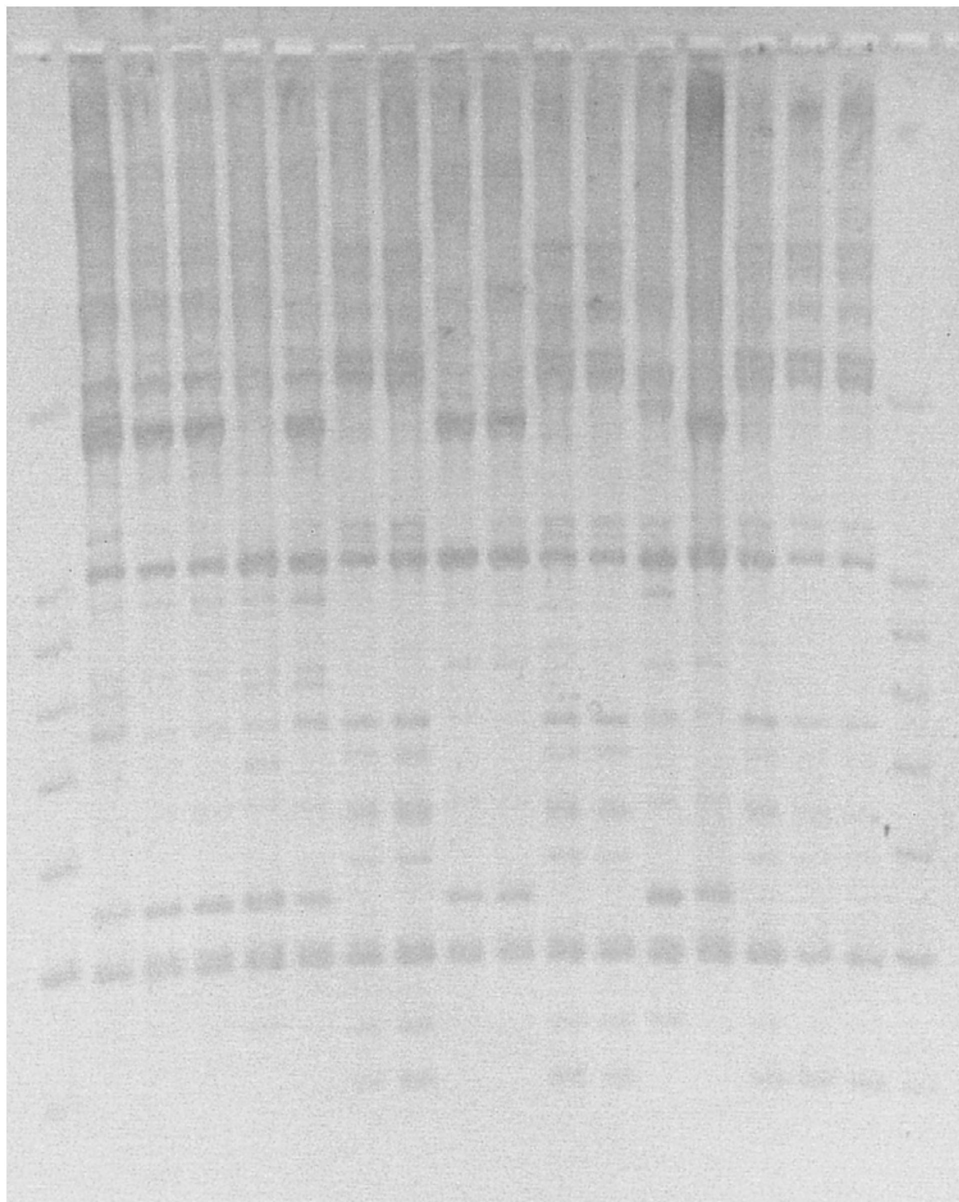
A: Gel run on 10/5/06 from manure derived EC.



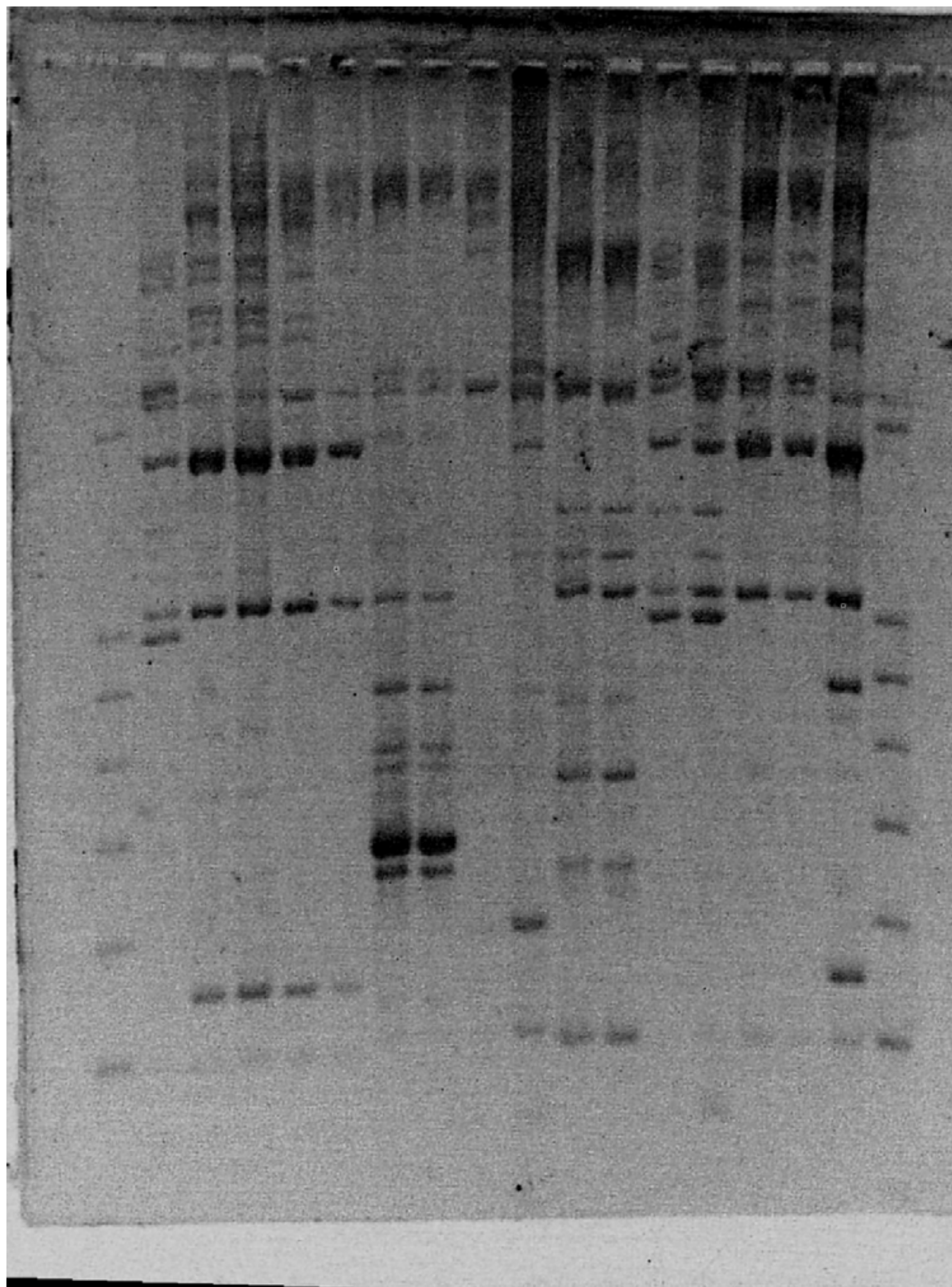
A: Gel run on 10/7/06 from manure derived EC.



A: Gel run on 10/13/06 from manure derived EC.



A: Gel run on 10/19/06 from water derived EC.



APPENDIX B

SPREADSHEET RESULTS OF GEL FINGERPRINTS

B: Size standard ladder in left column and sample numbers in top row.

	1	3	4	5	42	42	43	43	22	23	23	24	24	25	25	26	26	27
											1911	1918						
																	1828	
				1705	1705				1740	1740				1774	1760	1753		1784
								1692										
1500	1562	1527	1548	1514		1500	1500	1534	1568	1534	1527	1534			1500		1597	1552
	1418				1418								1486	1493		1473		
								1390	1377	1384	1397	1390	1342	1384	1384	1384		
	1178	1158	1164	1185		1158	1144		1144		1123	1110					1172	
1000	1048	1068	1075	1062	1062	1062	1048	1055	1041	1048	1014			1075	1041	1021	1060	1082
				991				974				996	987	978	961	957		
900																		
				856	860		860	852										
800	848								840									
	773	767	773	776		773	767		755	755			797				779	782
700		724	727	721		724					748	739		721	709	709		
	668	660	660	665		660	665							670	673		662	664
600		600	600			610	605						633	628	623	615		
				570	566													
	564							557	557	555	549		540					
500	504	506	509	511	506	509	506	504	500								509	513
		453	453			452				495	498	489	492	485	482	474		
400		408	410	456		408					440							
							377							388	387	381		
300																		
200																		

B: Size standard ladder in left column and sample numbers in top row.

	29	32	33	40	1	5	5	5	1	2	1	1	1	3	L	4	5	42
																2405	2238	2238
																2179		
			1985			1970											1994	1988
				1866					1851	1828	1806			1985		1839	1869	1839
			1791	1731		1784							1746	1746				1702
	1627		1642	1619		1634	1612	1642				1612	1619			1619	1619	1631
1500		1582			1597	1530			1560	1567	1522		1507					
					1455		1470		1418			1470	1134	1493	1470	1476	1470	
						1381										1351		
										1201		1239				1202		
		1119	1134	1112	1127		1134	1112	1112		1194			1157		1089		1101
1000						1060				1067	1097	1052	1060	1037	1007	1006	1095	
																	997	990
900															910			
						865						854	854				877	863
800											800				819	840		
		732	793	796		789		796				771		789		783	792	789
700	736	695										721	729	721	707			
	664	662				656				669	672	667	667	659			683	683
600		608								626	618			608	618			
			585		580	576	587		580			576	576			570	572	572
							541		535									
500	515	522	522	524	526	524	524	530		524	524	515	517	515	511	507	502	511
		490				464					462	464	464	453				
400		389								421	422			416	407			
		360			367	369												
300		284													311			
200		216				252		228										

B: Size standard ladder in left column and sample numbers in top row.

	42	43	43	44	44	45	45	1	2	3	4	5	6	L	29	32	32	33
	2226	2214		2417	2208	2208	2387			2393		2357	2339					
			1994	1994			1994	1994	2185									
	1988		1940	1905			1935	1923			1976	1952	1946					
	1833	1815	1827		1845	1869		1815	1815		1893	1887	1875		2076			
				1798			1792					1780	1774			1743	1757	
	1643	1643	1679	1673	1637	1625	1613	1667	1595		1661	1643	1643				1604	1674
1500	1500								1500		1565	1589	1577		1569	1576		
		1488	1470	1464	1470	1458	1446			1446	1446			1458	1417			1479
			1202	1214			1202											
			1155	1161		1190	1101	1185	1179		1173	1173	1155					
1000	1089	1089	1089	1107	1107	1089	966	1083	1071	1077	1060	1065	1054		1035	1069	1097	
	990	993				983			979					978				
900				903			900									900	922	
	880	880			887	877			863	870	893			883			857	
800	857	850					820								800	843		
	789	792	794	789	794	789	781	778	789	786	775	769	767	776		762	779	
700	744		753	747	733	728	742	742			728	717			745			
	686	686	674	676	671	664	676	667	679	669	667	655		675				
600			621	619			619	619			605	610	600					
	576	578			578	576			572	570				583				
																556	566	
500	515	517	517	520	517	517	515	517	509	507	500	502				505	510	
			457	461			456	459	449				498	483	493			
400			411	410			408	410			403	405						
													398	393				
300																		
200																		

B: Size standard ladder in left column and sample numbers in top row.

	33	40	40	41	41	42	42	43	43	44	L	45	46	w1	w2	w3	w5	w6
	2118	2139	2132	2146				2160	2132	2174			2134		2122			
			1931															
	1896			1875	1875							1811	1854		1841			
						1701										1750	1780	1787
	1639	1688		1660	1660					1611		1622		1628		1634	1634	1628
1500		1507	1521	1514	1500	1583	1576		1576		1500		1506	1506	1537	1518		
	1486						1424					1488						
				1361														
										1250								
	1132	1118	1125	1132	1118	1139	1104					1122	1146	1177		1177	1165	1195
1000					1014			1090	1076	1056	1067							
														960		980		
900											932	936						
				887	896													
800			813								826						826	
				790	797	790	790	786	776	766								
700						738	738	731	731	710	724							
				681	686	675	667	672		656								
600						614	614	614	608		630					609	626	617
	580		583	590	583							585		596				
		534	534							563								555
500				527	520	522	517	522	517	505	519							
		463				465	461	457	457									
400						415	415	415	413									
300																		
200																		

B: Size standard ladder in left column and sample numbers in top row.

	w7	w8	w9	w10	w11	w12	w13	w14	w15	w16	w17	w18	w19	w20	w21	w22	w24	w25
													2098	2067	2073			
								1909		2037	2085		1945	1927	1957			
										1884		1811		1823	1811		1860	
							1787	1780		1780	1793							
	1646	1652	1616		1640		1677									1634	1671	1671
1500	1524	1524				1585												
						1439	1421	1415	1470	1457	1476	1470	1476	1470	1463		1494	
	1183	1189			1159	1110				1116	1116	1122	1140	1140	1152	1122	1140	1189
1000			1024	1018			1055	1061	1073									
	976	992																
900							900			919	937	937		930	941			922
						870												
800			822	837														
700																		
600	602	615																
								578			574	569	567		580			
					549	555	554			565						539		
500														520				
			498															
400																		
300																		
200			234	238														

B: Size standard ladder in left column and sample numbers in top row.

	w26	w27	w29	w30	w31	w32	w33	w34	w35	w36	w37	w38	w39	w40	w41	w43	w44
				2185	2292												
				2089	2179												
				1976	2060	2179				2167		2292	2280				2125
				1905	1952	2077	2101	2149	2155	2071	2101	2214	2220			2113	1958
			1958	1839	1923	1917				1994		1940	1929	1929	1911	1982	1899
	1811	1811	1893	1798	1821	1821								1756	1744	1857	1821
				1732	1792	1750		1667			1679			1673	1661	1661	1738
	1640	1634	1625	1601	1738	1601	1601	1625	1631	1619	1607	1619	1607	1619	1613	1607	
1500	1537		1577		1613				1506								1595
			1417	1417	1417	1435	1440	1494			1458			1476	1470	1470	1435
												1292	1298	1298	1286		
	1134	1128		1113	1131						1179	1179	1173	1071	1167		
1000			1012	1006	1024	1030	1036	1048	1060			1071	1054	1000	1071	1060	1054
			957												993		
900																	
								887	887		881	868	868				887
800					819			800	800								845
								776	773			762	776				768
700				743	732			743	738								
								682	684			668	661				
600								657	652		600						645
				546	544	543	546	543	539								552
500								504	506	509	509	506	504		507	504	507
				483	489	487											
400															441		
300																	
200																	