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THE USE OF FECAL BACTERIA IN THE ASSESSMENT OF THE ENVIRONMENTAL IMPACT BY CONCENTRATED ANIMAL FEEDING OPERATIONS ON WATER QUALITY

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THE USE OF FECAL BACTERIA IN THE ASSESSMENT OF THE ENVIRONMENTAL IMPACT BY CONCENTRATED ANIMAL FEEDING OPERATIONS ON WATER QUALITY

A Dissertation APPROVED FOR THE
SCHOOL OF CIVIL ENGINEERING AND ENVIRONMENTAL SCIENCE

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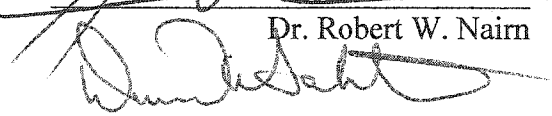

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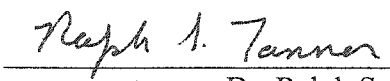

Dr. Ralph S. Tanner

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PREFACE

In recent years, the number of swine in Oklahoma has increased by 1,053% while the total number of farms reported to be producing swine has decreased from a high of approximately 8,000 in 1980 to approximately 2,800 in 1997 (Williams and Luce, 2000; Willoughby et al., 1998). These concentrated animal feeding operations (CAFOs) produce large amounts of waste, which must be stored and treated. CAFOs in temperate climates typically use clay-lined earthen basins, or lagoons, to store and treat the waste generated at these facilities. The waste, which contains valuable nutrients and organic matter, is then typically land applied as a fertilizer to cropland or pastureland (Honeyman, 1996). Over-application of these wastes to croplands can lead to nutrient enrichment of nearby lakes, streams, and aquifers.

Traditionally, increases in nutrient concentrations over background levels have been used to assess the environmental impacts of CAFOs on surface water and groundwater (Huffman and Revels, 1998; Gilliam et al., 1996; Jokela, 1992). However, many sources of pollution, including agricultural, industrial, and urban sources, can increase a water body's nutrient content. Therefore, pinpointing the specific source of ground or surface water contamination in a watershed with multiple sources of pollution can be difficult.

An alternative to nutrient enrichment studies involves the use of microorganisms for identifying nonpoint pollution sources, referred to as microbial source tracking (MST). These methods use genetic or biochemical assays to identify strains or species of bacteria that are specific to the source of contamination. Recent advances in genetic and biochemical methods of species identification have improved

the ease with which environmental samples can be analyzed for specific strains or species of microorganisms, thus making the use of microorganisms to pinpoint the source of environmental contamination more promising. This fact combined with the need for a better method to identify the source of nonpoint source pollution is the motivating force behind the formation of this project. The goal of this project is to investigate the possible uses of microorganisms in the assessment of the environmental impact of CAFO lagoons on water quality.

This goal will be achieved through the examination of three main topic areas: microbial community analysis of CAFO lagoons, fecal bacterial survivability, and MST in surface water. The first two chapters examine the species composition patterns and carbon source utilization patterns of six lagoons associated with the three swine production stages: breeding, nursery, and finishing. The next two chapters examine fecal bacterial survival under lagoon conditions and in surface water through the study of laboratory-scale mesocosms. The final three chapters describe the development and application of an MST method based on the dominant fecal bacteria species composition patterns of four known sources: cattle, human, poultry, and swine.

In Chapter One, six swine lagoons were examined for one year to determine if each swine production stage had distinct fecal bacterial species composition patterns, which could be used in future studies to determine specific sources of swine fecal pollution when incorporated into an MST method. It is hypothesized in this chapter that each stage of swine production will have unique fecal bacterial species composition patterns that can be statistically identified over time. Samples from six

lagoons were taken on a bi-monthly basis and analyzed for fecal coliform (FC) and fecal streptococci (FS) bacteria. Colonies from each fecal bacterial group were then isolated and identified using the Biolog Microbial Identification System (Hayward, CA). Species composition data were obtained from these identifications and analyzed statistically using discriminant analysis to determine if a unique species composition exists for each production stage. This has implications for the use of a phenotypic MST method based on fecal bacterial species composition because unique compositions may be detectable in surface water or groundwater and allow best management practices to be applied in the most efficient manner. This chapter was submitted for publication to *Journal of Environmental Quality* in March 2004.

Chapter Two continues the work conducted in Chapter One by examining the variability of community-level physiological profiles in six CAFO lagoons. Swine production typically occurs in multi-site facilities in which the swine are separated by age: sow facilities house sows and young until weaning, nursery facilities house swine until they reach a weight of 50 lbs, and finishing facilities house swine until they reach market weight at 250 lbs. Each stage of growth is fed a unique feed ration to promote growth. This chapter examines the effects of diet on the bacterial communities of lagoons associated with each stage of swine production. This chapter was submitted for publication to *Applied and Environmental Microbiology* in April 2004.

Chapter Three examines the stability of FC and FS populations in lagoon mesocosms. Fresh samples of cattle, poultry, and swine manure were added to tap water to create a mixture with a total solids concentration comparable to that found in

full-scale lagoons. FC and FS concentrations and species compositions were analyzed over time and analyzed statistically to determine the stability of these populations over time. Fresh waste inputs were created and added after two weeks to mimic the input of cleaning waters that full-scale lagoons receive when the barns on-site are cleaned, and the FC and FS concentrations were monitored for an additional two-week period. The stability of these bacterial concentrations is important because it directly affects how successful an MST method based on fecal bacterial species composition will be. Discriminant analysis was used to determine if the species composition of each manure source could be successfully classified. This chapter was submitted for publication to *Journal of Environmental Quality* in March 2004.

Chapter Four extends the work of Chapter 3 to surface water. Samples were taken from the lagoon mesocosms discussed above after two weeks and used to inoculate surface water samples. FC and FS concentrations and species compositions were analyzed over time to determine the decay rates for each manure source. Discriminant analysis was also performed to determine the time period in which the manure source could be successfully classified. This is important to the development of a species composition-based MST methodology because, in order to be successful, the source-specific species composition must be statistically detectable long enough to be detected by regulators. This chapter was accepted for presentation at the American Water Works Association's 2004 Annual Conference and Exposition and will be published in the conference's proceedings. It was also submitted to the peer-reviewed journal, *Journal AWWA,* for publication in February 2004.

Chapter Five discusses some of the earliest work attempted in the development of the phenotypic MST methodology. In this chapter, samples were collected over a four-month period of time and analyzed for FC and FS bacteria. FC and FS colonies were identified using the Biolog, Inc. Microbial Identification System, and the resulting identifications were analyzed via a tier testing procedure to determine which FC or FS species were the best indicator species for swine waste contamination. This chapter was not published because the methodology had changed significantly through internal review before reviewer comments were received from the journal to which it had been submitted.

Chapter Six describes the most recent work being done on the species composition MST method. In this chapter, a total of 257 species composition patterns were generated from known sources. These patterns were analyzed statistically using discriminant analysis. Discriminant analysis classified each pattern into one of four known sources (cattle, human, poultry, and swine) based on FC, FS, or fecal bacterial species composition. The average rate of correct classification for samples from all known sources was 82% for FS species composition data and 63% for FC species composition data, which indicates that such a method could be useful in identifying the major contributor to nonpoint source pollution in a watershed. This chapter was submitted for publication to *Applied and Environmental Microbiology* in March 2004.

In Chapter Seven, the MST method described in Chapter Six is used in the Turkey Creek watershed located in north-central Oklahoma and compared to a ribotyping MST method used in the same watershed. Both methods identified cattle

as the major contributor to fecal pollution in the Turkey Creek watershed. This chapter was submitted for publication to *Applied and Environmental Microbiology* in April 2004.

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CHAPTER 1:

FECAL BACTERIA SPECIES COMPOSITION PATTERNS OF SWINE LAGOONS: DIFFERENCES IN MICROBIAL COMMUNITIES BASED ON PRODUCTION STAGE

1.1 ABSTRACT

Microbial source tracking (MST) methods are currently being developed to identify major contributors to nonpoint source pollution. Most of these methods only identify the source of pollution to a specific category, such as human, cattle, or swine, but there may be situations when it is necessary to identify specific sources within a category. A study was undertaken to determine if the waste from lagoons associated with the three major swine production stages (breeding, nursery, and finishing) had distinct fecal bacterial species composition patterns that were statistically identifiable and distinct. Samples were taken from six lagoons, two from each stage, bimonthly for one year and analyzed for fecal coliform (FC) and fecal streptococci (FS). Species composition patterns were calculated and analyzed using discriminant analysis for source classification and canonical discriminant analysis for spatial plot construction. The average rates of correct classification were 63.9%, 91.7%, and 97.2% for FC, FS, and fecal bacteria species composition patterns, respectively. Spatial plots of the first two canonical variables supported these results by showing that the best separation between production stages occurred when the FC and FS data were combined to produce fecal bacteria species composition patterns. Based on the results of this study, an MST method based on fecal bacteria species composition

patterns could be used to distinguish between breeding, nursery, and finishing lagoons as sources of fecal pollution in a watershed.

1.2 INTRODUCTION

Historically, swine production has occurred on single, independently owned and operated farrow-to-finish farms with open pasture on which the swine can forage freely. Swine were bred, born, and raised on one farm until they were sold at market. Today, however, swine production has changed in order to meet the needs of consumer demand for leaner, more nutritious pork products, to increase herd health, and to decrease labor costs (NPCC, 1997). The current trend in swine production is toward multi-site production facilities that produce more swine in a smaller area and are owned by or contract with large pork-producing corporations.

On multi-site production facilities, swine are segregated based on age and raised in different locations (Harris, 2000). Generally, three production stages are used on multi-stage facilities. These production stages are the breeding stage, the nursery stage, and the finisher stage (Figure 1.1). During the breeding production stage, female swine (sows) are bred, give birth, and nurse the young. After the young are weaned, they enter the nursery stage. In this stage, the piglets are raised to a weight of 40 to 50 lbs. Once they reach this target weight at 6-10 weeks of age, swine from the nursery site are then transferred to a finisher facility where they are reared for meat consumption or as replacement breeding stock for the breeding stage. By the end of the finisher production stage, the swine generally weigh between 240 and 260 lbs. The goal of multi-site production is to eliminate the spread of pathogens by separating various age groups and thus preventing the transmission of

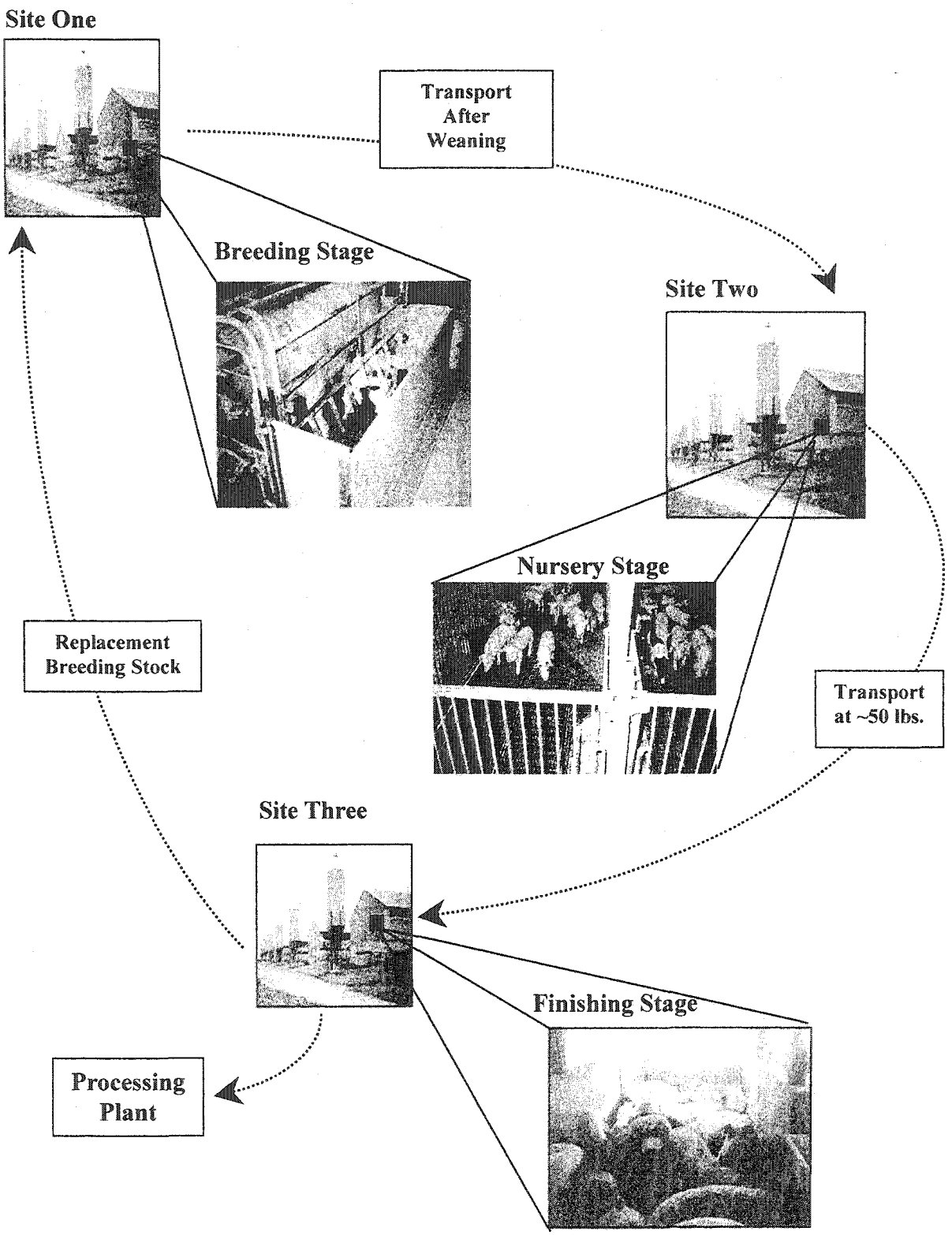


Figure 1.1: Multi-site swine production as it relates to production stage.

diseases from older swine to recently weaned piglets (Harris, 2000). This three-site production system is now commonly used by most of the major swine production corporations.

Concentrated animal feeding operations (CAFOs) are required by the Clean Water Act and the National Pollutant Discharge Elimination System to meet an effluent limitation guideline of zero discharge (Johnson et al., 1999). To meet this requirement, CAFOs in warm climates generally use lagoons to store the wastes generated. These CAFO lagoons can impact surface and ground waters. Groundwater quality can be degraded when a lagoon liner is compromised or is not adequate to prevent seepage. Surface water quality can be degraded when a spill occurs. Spills usually occur when a lagoon overflows during periods of flooding. The Clean Water Act Amendments of 1990 require lagoons to be able to contain precipitation amounts up to and including that which would occur during a 25-year, 24-hour storm (Meyer and Mullinax, 1999). However, if design volume has not been maintained through land application or precipitation exceeds the 25-year, 24-hour volume, lagoons will overflow and impact surface water quality.

One way in which the environmental impact of CAFO lagoons on surface water and groundwater quality can be monitored is through analysis of the nutrient levels in these waters (Huffman and Revels, 1998; Gilliam et al., 1996; Jokela, 1992). However, many different agricultural, industrial, and municipal wastes contain high concentrations of nutrients, and the contamination cannot be conclusively linked to any particular source. For instance, nitrate pollution can be caused by such varied sources as crop production, CAFOs, municipalities, industries, and geologic

formations (Thompson, 1996). Therefore, a more conclusive method for pinpointing the nonpoint sources of pollution is needed.

Research is currently being conducted in the area of microbial source tracking (MST) as an approach for identifying major nonpoint sources of pollution. MST methods typically use genetic or biochemical assays to identify species of bacteria or physiological characteristics that are specific to the source of contamination. MST methods currently being researched include antibiotic resistance analysis, ribotyping, and fecal bacterial species composition (Wiggins et al., 1999; Hagedorn et al., 1999; Bernhard and Field, 2000; Carson et al., 2001; Parveen et al., 1999; Evenson and Strevett, unpublished data).

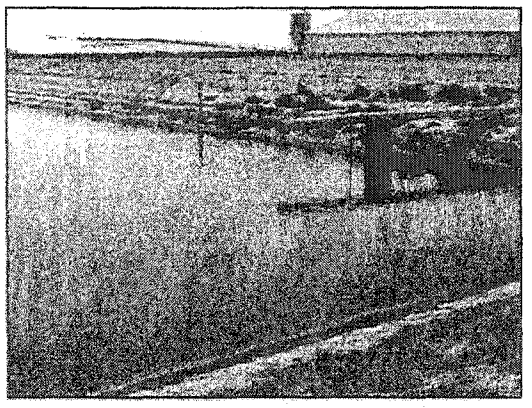
Most of these methods only identify the source of pollution to a specific category, such as human, cattle, or swine. This may be appropriate for most watersheds, but when more than one source of pollution in a category is located within a watershed, targeting best management practices may be difficult. To determine if a species composition-based MST method can be used to distinguish between sources of fecal pollution from the three major swine production stages (breeding, nursery, and finishing), a study was undertaken to examine the fecal bacterial species composition patterns in swine lagoon systems during a one year period by sampling two lagoons from each production stage (breeding, nursery, and finishing) on a bimonthly basis. The objective was to determine if each lagoon type had distinct fecal bacterial species composition patterns that were statistically identifiable over time.

1.3 MATERIALS AND METHODS

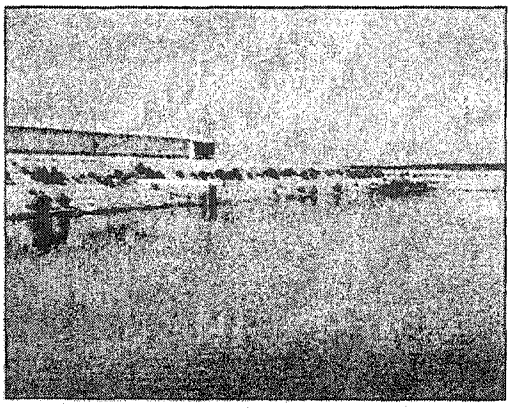
Six lagoons were selected for this study, two lagoons from each production stage: SRS1 and SRS2 were breeding stage lagoons, SRS3 and SRS4 were nursery stage lagoons, and SRS5 and SRS6 were finishing stage lagoons (Figure 1.2). All six lagoons were located within 5 miles of each other. This close proximity meant that all six lagoons were constructed in areas of similar geology and existed under similar climatic conditions. All six lagoons were owned and operated by a single entity to ensure similar operating conditions and production procedures.

Each lagoon was sampled on a bimonthly period for one year from September 2002 through July 2003. In situ water quality and weather measurements were taken at each site. In situ water quality measurements included temperature, dissolved oxygen, pH, and conductivity (Appendix 1). Weather measurements included wind speed and direction, ambient temperature, and solar radiation (Appendix 2). Weather and in situ measurements were averaged for each site for all sampling events (Table 1.1) and for each sampling event for all sites (Table 1.2). Statistical analysis of the data set was completed using analysis of variance techniques and the generalized linear model procedure (PROC GLM). Statistical Analysis Software (SAS) version 8 (SAS Institute, Inc.; Cary, NC) was used for all statistical analysis.

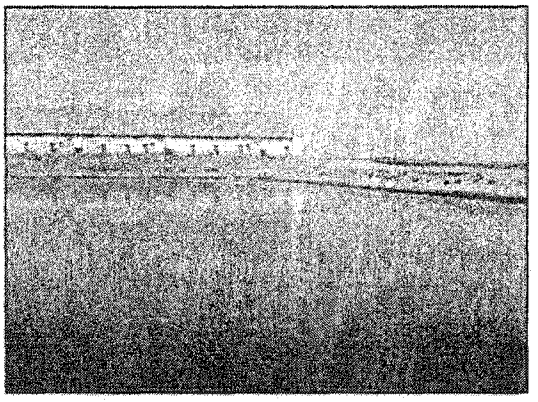
Grab samples were taken in four locations around each lagoon using a sterile grab sampler and placed in sterile 250 mL polypropylene bottles. Each grab sampler was used at a single lagoon for the entire length of the study. The samplers were also cleaned and wiped with ethanol after every sampling trip to prevent cross-contamination. All samples were stored at 4°C during transport, and the



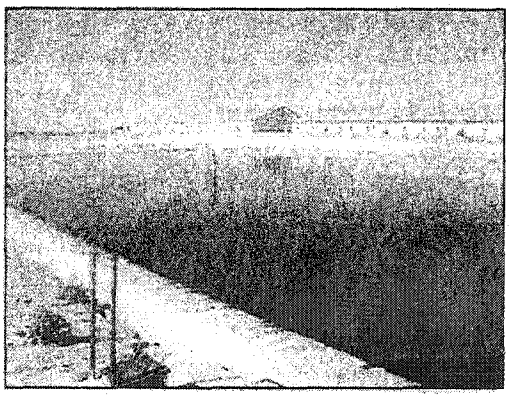
SRS1: Breeding Stage Lagoon



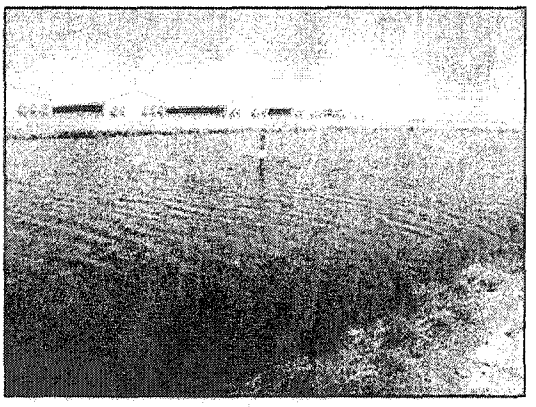
SRS2: Breeding Stage Lagoon



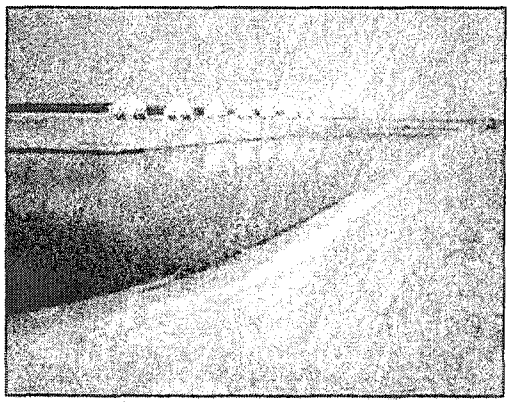
SRS3: Nursery Stage Lagoon



SRS4: Nursery Stage Lagoon



SRS5: Finishing Stage Lagoon



SRS6: Finishing Stage Lagoon

Figure 1.2: Photographs of the six research sites.

Table 1.1: Summary Statistics for SRS1 through SRS6

	SRS1 Mean ± Std Dev	SRS2 Mean ± Std Dev	SRS3 Mean ± Std Dev	SRS4 Mean ± Std Dev	SRS5 Mean ± Std Dev	SRS6 Mean ± Std Dev
Ambient Temperature (°C)	13.6 ± 12.5	13.2 ± 12.6	15.1 ± 12.0	15.2 ± 12.5	16.1 ± 12.1	16.5 ± 12.0
Solar Radiation (W/m^2)	551 ± 235	405 ± 242	655 ± 225	602 ± 231	552 ± 266	603 ± 316
Wind Speed (m/s)	6.6 ± 1.3	6.3 ± 1.3	6.2 ± 1.8	5.9 ± 1.1	6.4 ± 1.0	6.1 ± 1.8
Water Temperature (°C)	15.8 ± 6.3	16.1 ± 6.7	15.8 ± 7.1	16.2 ± 7.7	17.2 ± 7.4	16.8 ± 8.3
Dissolved Oxygen (mg/L)	0.8 ± 0.5	0.6 ± 0.4	0.7 ± 0.5	0.4 ± 0.3	0.4 ± 0.2	0.1 ± 0.03
pH	8.0 ± 0.1	7.9 ± 0.1	7.7 ± 0.6	7.8 ± 0.1	8.2 ± 0.2	8.2 ± 0.2
Conductivity (mS/cm)	7.9 ± 1.3	6.6 ± 1.4	9.2 ± 0.8	9.3 ± 0.8	12.0 ± 0.3	14.0 ± 0.6

Table 1.2: Summary Statistics for Each Sampling Event

	9/26/2002 Mean ± Std Dev	11/21/2002 Mean ± Std Dev	1/23/2003 Mean ± Std Dev	3/25/2003 Mean ± Std Dev	6/3/2003 Mean ± Std Dev	7/17/2003 Mean ± Std Dev
Ambient Temperature (°C)	14.7 ± 1.3	18.1 ± 1.0	-5.9 ± 1.7	11.9 ± 2.1	19.4 ± 0.9	31.6 ± 1.4
Solar Radiation (W/m^2)	548 ± 138	463 ± 113	192 ± 77	710 ± 185	632 ± 241	822 ± 115
Wind Speed (m/s)	6.3 ± 0.4	7.0 ± 1.0	6.0 ± 0.9	7.2 ± 0.5	3.9 ± 1.0	7.1 ± 0.4
Water Temperature (°C)	17.7 ± 0.9	11.4 ± 1.2	7.2 ± 1.3	13.0 ± 0.9	21.7 ± 0.6	26.8 ± 1.9
Dissolved Oxygen (mg/L)	0.7 ± 0.5	0.6 ± 0.5	0.7 ± 0.4	0.4 ± 0.3	NA	0.2 ± 0.1
pH	8.1 ± 0.2	NA	8.1 ± 0.2	8.0 ± 0.2	7.9 ± 0.1	7.7 ± 0.5
Conductivity (mS/cm)	9.0 ± 3.1	9.5 ± 3.2	9.7 ± 2.9	10.0 ± 2.5	10.0 ± 1.9	9.4 ± 1.9

four grab samples were mixed together in sterile 1 L polypropylene sample containers upon return to the laboratory. Each composite sample was analyzed within 8 hours of collection for fecal coliform (FC) and fecal streptococci (FS) bacteria using selective media, mFC agar for FC and mEnterococcus agar for FS (Difco Laboratories), and the membrane filtration technique as described by *Standard Methods* 9222D and 9230C, respectively (APHA, 1998). The FC and FS concentrations were recorded, and statistical analysis of the data set was completed using analysis of variance techniques and PROC GLM to determine if the mean FC and FS concentrations for each lagoon were significantly different at any time during the duration of the study.

Five colonies were randomly selected and isolated on selective media from each of the resulting FC and FS agar plates that had counts between 20 and 80 colony-forming units (CFUs). After an FC or FS colony had been isolated on selective media, an isolated colony from that pure-culture plate was transferred to Biolog Universal Growth Agar containing 5% defibrinated sheep's blood. The maximum number of transfers performed was limited to five to prevent unnecessary stress and limit metabolic changes, as specified by identification procedures set by Biolog, Inc. (Hayward, CA). The growth from the blood agar plate was then suspended in a 0.85% sterile saline solution and used to inoculate GN2 or GP2 Microplates™ according to the procedures developed by Biolog, Inc. for the identification of gram-negative and gram-positive bacteria, respectively. GN2 Microplates™ required an inoculum with a 63% transmission (T) for gram negative, enteric bacteria while GP2 Microplates™ required an inoculum with a 20%T for gram-positive cocci.

Each microplate contains 95 different sole carbon sources, which produce a metabolic fingerprint that is specific to a single species or strain of microorganism. After incubation, the patterns of positive, negative, and borderline responses were read manually and input into the MicroLog™ Microbial Identification System (Biolog, Inc.). The results obtained for each of the five colonies identified were compiled and used to calculate a fecal bacterial species composition pattern for each sample as an average percentage basis. FC, FS, and fecal bacterial species composition patterns were obtained for each lagoon sample. The fecal bacterial species composition data were obtained by combining the identification results for FC and FS to create a single dataset.

Discriminant analysis was used to analyze the species composition patterns for each sample (SAS version 8). In discriminant analysis, the PROC DISCRIM function was used to classify the fecal bacterial species composition patterns into production stage categories (prior probabilities, equal; covariance matrix, pooled). This function produces a source-by-source matrix with the number and percentage of correctly classified samples for each source located on the diagonal. Average rate of correct classification (ARCC) for each fecal bacterial type was calculated by averaging the percentages of correctly classified samples on the diagonal as reported previously by Wiggins (1996).

Spatial plots of the species composition patterns for each production stage (breeding, nursery, and finishing) were generated using canonical discriminant analysis (PROC CANDISC, SAS software). Canonical discriminant analysis derives a linear combination of variables with the highest multiple correlation with the

groups. Plotting the first two canonical variables on an x-y scatter plot provides a visual interpretation of pattern analysis. A large separation between source groups indicates highly accurate source identification.

1.4 RESULTS AND DISCUSSION

1.4.1. Site Environmental Data

In situ and weather parameters such as wind speed, temperature, and dissolved oxygen content were measured at each site on each sampling event. Each parameter measured was averaged for a single site for all sampling events and for a single sampling event at all research sites. The data are summarized in Tables 1.1 and 1.2. The averages for each parameter presented in Tables 1.1 and 1.2 were analyzed to determine if any statistical differences existed and between which research sites or sampling events these differences occurred.

Analysis of variance results for site averages indicated that ambient temperature, water temperature for each lagoon, wind speed, and solar radiation showed no significant difference between each site ($p=0.05$) (Appendices 3-6, respectively). Dissolved oxygen concentrations (DO), pH, and conductivity each showed differences between sites (Appendices 7-9, respectively). Significant differences associated with production stage were not observed for DO or pH, and despite being statistically significant, averages for both parameters for all sites were within 0.5 mg/L and 0.5 units, respectively. The lowest average DO occurred at SRS6 and was significantly less than both SRS1 and SRS3. The lowest average pH value occurred at SRS3 and was followed closely by SRS4. The average pH values for both of these sites were significantly less than the average pH at SRS5. Average

conductivity values showed the greatest statistical difference between research sites ($p=0.05$) (Appendix 9). Only SRS3 and SRS4 were not significantly different than each other. The differences in average conductivity for each site is most likely attributed to lagoon levels over the course of the study. Both SRS5 and SRS6 had been drawn down just before the first sampling event to allow for embankment repair, while SRS1, SRS2, and SRS3 had effluent removed at some point during the study for irrigation purposes (Table 1.3). The effluent removal may have affected the conductivity by raising the solids concentrations in the lagoons.

Table 1.3: Effluent Removal for Research Sites.

Site #	Volume Removed (gal)	Dates Removed
SRS1	5,143, 900	2/20 to 3/16/2003
SRS2	11,526,900	2/12 to 3/25/2003
SRS3	3,574,500	11/02 to 11/11 2002
SRS3	1,956,600	5/27 to 5/29 2003

When analyzed statistically, sampling event averages for water and ambient temperatures showed the expected seasonal differences ($p=0.05$) (Appendices 10 and 11, respectively). Average DO for each sampling event also showed seasonal differences, as indicated by the statistical difference between the DO concentrations for January and July (Appendix 12). The other months were not statistically different. This can be explained by the fact that all six lagoons were anaerobic, and the DO concentrations were only significantly affected when the effects of temperature on DO were at its most extreme, during the winter and summer. Seasonal variations in conductivity did not occur, while pH values for September and January were significantly different than for July (Appendices 13 and 14, respectively). However, as mentioned above, the range of pH values for each sampling event was tightly clustered between 7.7 and 8.1. This difference is not large enough to have a significant effect on the microbial community within the lagoons. The sampling event averages for wind speed and solar radiation also showed statistical differences (Appendices 15 and 16, respectively), but it is more likely that these differences were due to weather conditions on the day samples were taken rather than due to seasonal effects.

For both the site and sampling event averages, it is important to note that while there were statistical differences, the range of each of these parameters is not so great that it would have a significant effect on the lagoon communities. The parameters that would have the greatest effect on microbial communities, such as DO, pH, and water temperature, showed the least amount of variation between research sites.

1.4.2. Fecal Bacteria Concentrations

FC and FS concentrations were analyzed for each lagoon over a one-year period (Figures 1.3 and 1.4, respectively). From these figures, it can be seen that both the FC and the FS concentrations were within 4 orders of magnitude of each other, with the lowest concentrations for both FC and FS found in the finishing lagoons. Statistical analysis of the bacterial concentrations indicated that the FC concentrations were not statistically different between production stages over time ($p=0.05$). Only the FS concentrations for the nursery lagoons during the November sampling event were statistically different, which was 1.5 orders of magnitude higher than either the breeding or finishing lagoons and 0.3 orders of magnitude greater than the other nursery lagoon. All other FS concentrations were not significantly different ($p=0.05$). This indicates that despite seasonal variations in temperature, pH, and DO, the FC and FS concentrations remain relatively constant over time in a lagoon environment. Despite research suggesting that fecal bacterial concentrations decrease rapidly once the microbes are exposed to an environment outside the host organism such as surface water or groundwater (McFeters et al., 1974; Crane and Moore, 1986), this study indicates that fecal bacteria can survive for an extended period of time in the lagoon environment. Prolonged survival in lagoon environments may be caused by the elevated nutrient contents in these environments. Several studies have found that elevated nutrient contents can increase survival in aquatic environments (Slanetz and Bartley, 1965; Hendricks, 1972; Hirn et al., 1980).

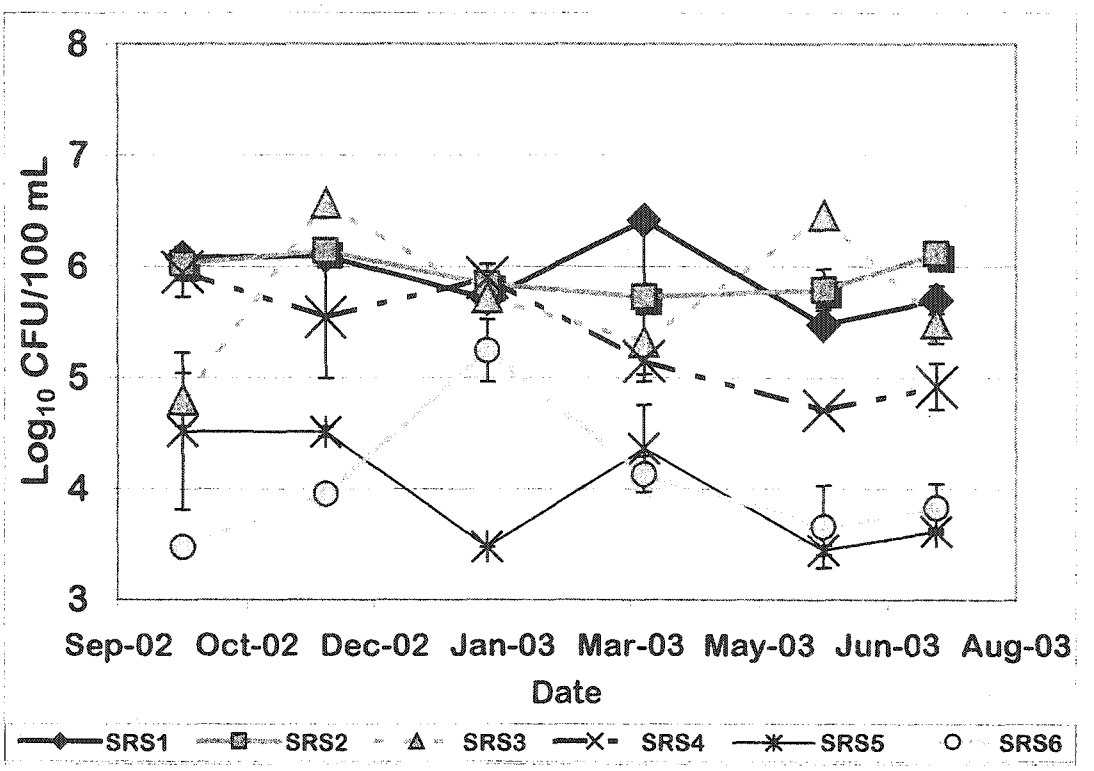


Figure 1.3: Fecal coliform concentration changes over time. Error bars represent one standard deviation of the mean of three replicates. Data points without error bars have small standard deviations with error bars masked by the symbol.

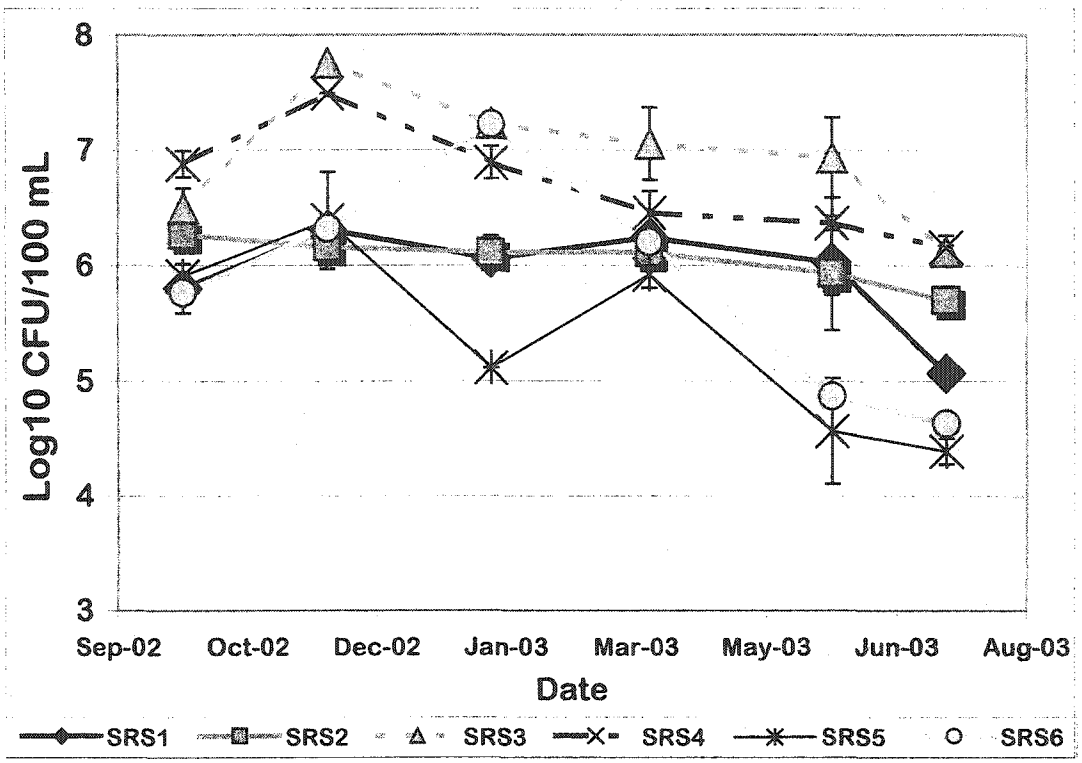


Figure 1.4: Fecal streptococci concentration changes over time. Error bars represent one standard deviation of the mean of three replicates. Data points without error bars have small standard deviations with error bars masked by the symbol.

1.4.3. Species Composition Patterns

Discriminant analysis of the FC and FS species composition data indicated that distinct species composition patterns of fecal bacteria exist for each production stage (Table 1.4, 1.5, and 1.6). The discovery of distinct species composition patterns is important for MST because this indicates that each production stage has a unique species composition pattern that can be used in an MST method based on species composition patterns to identify the major contributor to fecal pollution attributed to swine at the level of production stage. The average rate of correct classification (ARCC) for FC species composition patterns (ARCC = 63.9%) was lower than the ARCC for the FS species composition patterns (ARCC = 91.7%) and fecal bacterial species composition patterns (ARCCs = 97.2%). FC species composition patterns for each production stage were misclassified more frequently than the FS or fecal bacteria species composition patterns, with rates of correct classification of 66.7%, 41.7%, and 83.3% for breeding, nursery, and finishing stages, respectively (Table 1.4). Misclassification was largely due to the dominance of *Escherichia coli* in all manure sources. The FC species composition patterns had the least separation between the three production stages because of the dominance of *E. coli* in all three production stages; *E. coli* accounted for 63.3%, 53.3%, and 58.3% of the total FC species identified for breeding, nursery, and finishing stage samples, respectively. For this reason, an MST method using FC species composition patterns that identified *E. coli* only to the species level would not be able to adequately distinguish between the three production stages. A method using the FS or fecal bacteria species composition patterns may be more useful for source identification.

Table 1.4: Discriminant Analysis Results[1] for Fecal Coliform Species Composition

Manure Source	Breeding	Nursery	Finishing
Breeding	8 66.7	0 0.0	4 33.3
Nursery	1 8.3	5 41.7	6 50.0
Finishing	1 8.3	1 8.3	10 83.3

Table 1.5: Discriminant Analysis Results[1] for Fecal Streptococci Species Composition

Manure Source	Breeding	Nursery	Finishing
Breeding	11 91.7	0 0.0	1 8.3
Nursery	0 0.0	10 83.3	2 16.7
Finishing	0 0.0	0 0.0	12 100.0

Table 1.6: Discriminant Analysis Results[1] for Fecal Bacterial Species Composition

Manure Source	Breeding	Nursery	Finishing
Breeding	12 100.0	0 0.0	0 0.0
Nursery	0 0.0	11 91.7	1 8.3
Finishing	0 0.0	0 0.0	12 100.0

[1] The number of observations and percent classified into source are listed in each cell.

An important result of this study is the improved RCCs obtained when the fecal bacterial composition patterns were used to classify the samples into production stages (Table 1.6). The fecal bacterial species composition patterns were correctly classified more frequently than the FS species composition patterns. The RCCs for the FS species composition patterns were 91.7%, 83.3%, and 100% while the fecal bacteria species composition patterns were correctly classified at rates of 100%, 91.7%, and 100% for breeding, nursery, and finishing stages, respectively.

Misclassifications occurred in the FS and fecal bacterial species composition patterns for each production stage primarily when the swine populations in the barns were close to the ages of other production stages. For instance, the sample that was misclassified as a finishing stage pattern when it came from a nursery stage lagoon for the fecal bacteria species composition patterns was taken during a time when the swine in the barns were being transferred to a finishing facility (unpublished data). Similar results were also seen for the FS species composition patterns.

The increased number of variables used to classify samples into production stages may account for the increased RCCs for the fecal bacteria species composition patterns and is supported by spatial plots for each set of species composition patterns. Plots of the first two canonical variables (Can1 and Can2) are presented in Figures 1.5, 1.6, and 1.7 for all three sets of species composition patterns (FC, FS, and fecal bacteria, respectively). These plots show Can1 on the x-axis and Can2 on the y-axis. The most distinct separation between production stages occurred in the fecal bacteria

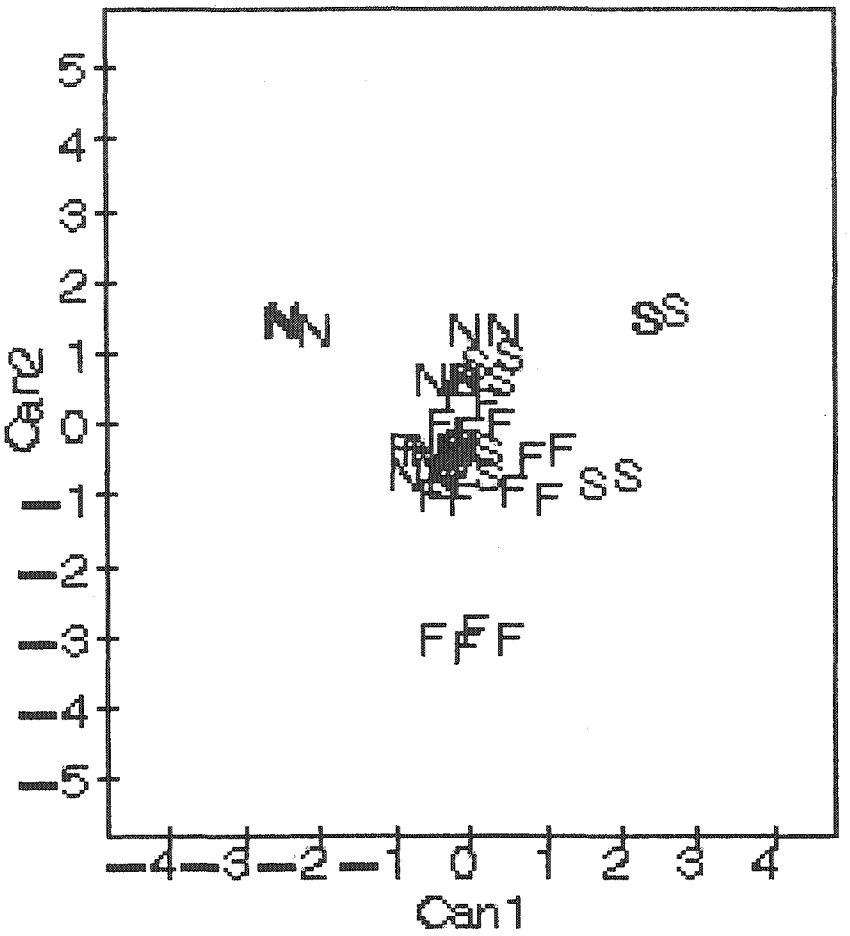


Figure 1.5: Spatial plots of FC species composition patterns. Position of patterns is indicated by the following symbols: breeding (S), nursery (N), and finishing (F). Can1 is on the x-axis, and Can2 is on the y-axis.

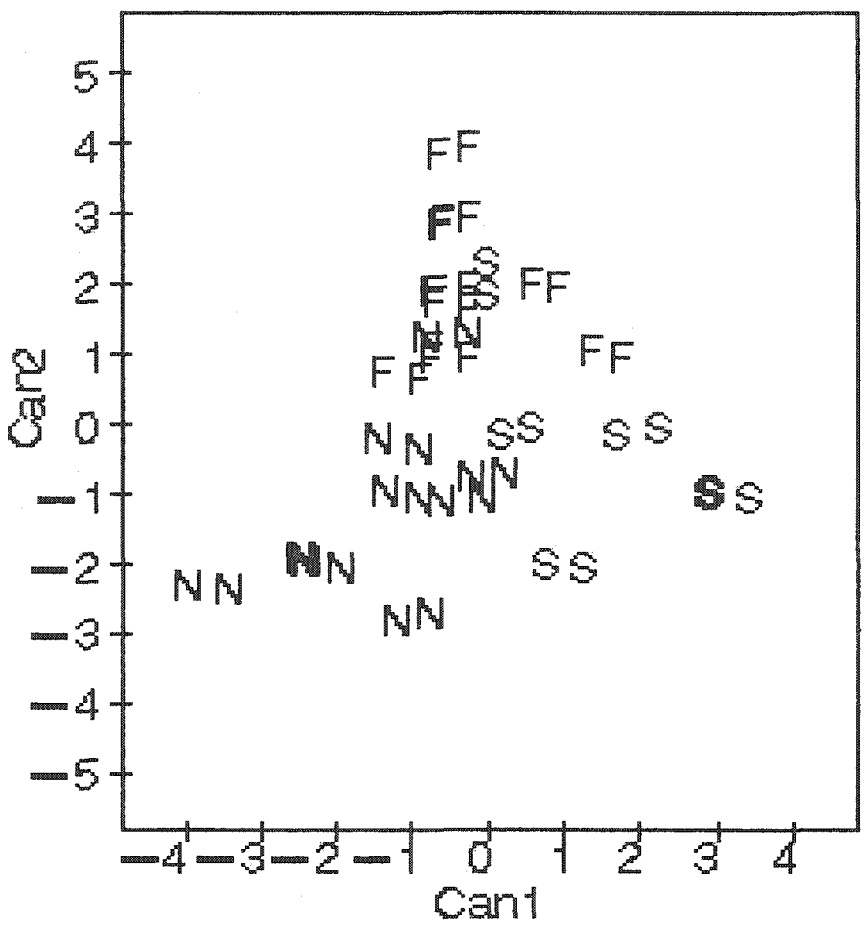


Figure 1.6: Spatial plots of FS species composition patterns. Position of patterns is indicated by the following symbols: breeding (S), nursery (N), and finishing (F). Can1 is on the x-axis, and Can2 is on the y-axis.

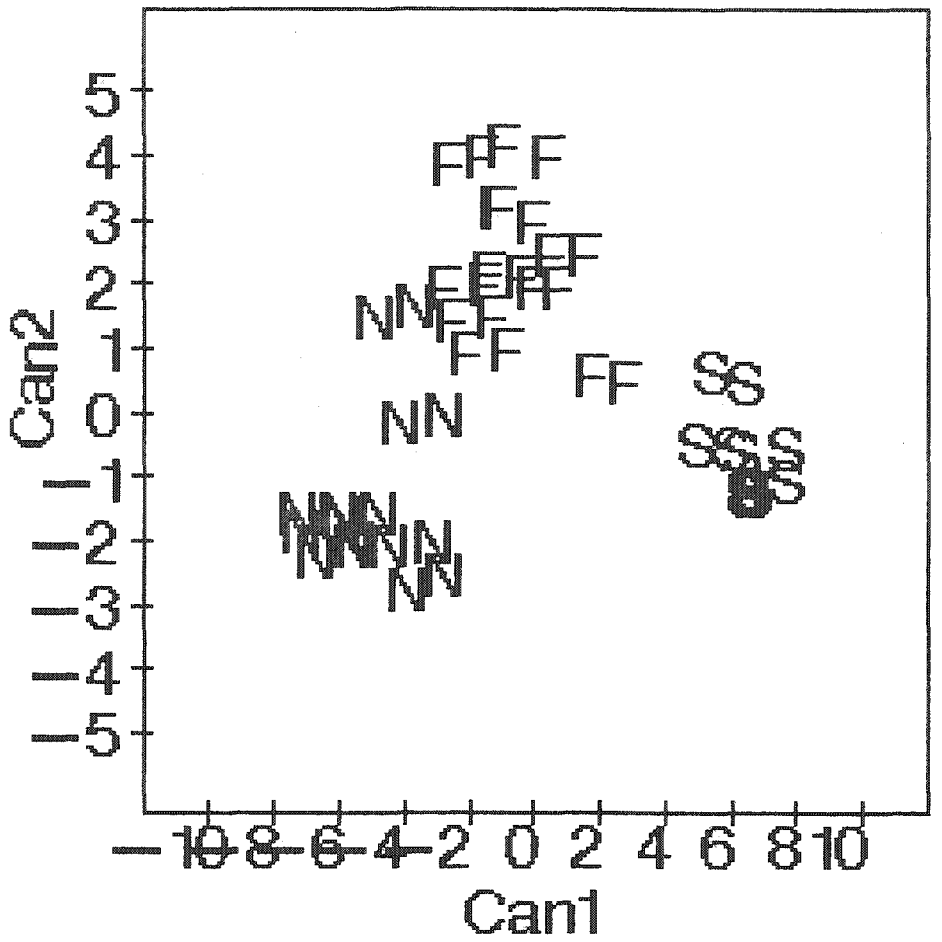


Figure 1.7: Spatial plots of fecal bacteria species composition patterns. Position of patterns is indicated by the following symbols: breeding (S), nursery (N), and finishing (F). Can1 is on the x-axis, and Can2 is on the y-axis.

database. These spatial displays support the discriminant analysis results and provide a useful method to visualize the distinct separation between each production stage.

The distinctness of these species composition patterns over time is important because it shows lagoon effluent does not have to be freshly deposited in order for an MST method based on these patterns to be effective, as is the case with FC/FS ratios and other methods (McFeters et al., 1974; Edwards et al., 1997). Lagoons have unique species composition patterns that may be used as a method for pinpointing the production stage of fecal pollution in a watershed where multiple swine sources exist.

1.5 CONCLUSIONS

Based on the results of this study, an MST method based on fecal bacteria species composition patterns could be used to distinguish between breeding, nursery, and finishing lagoons as sources of fecal pollution attributed to swine in a watershed. Further work needs to be done to determine if the differences between species composition patterns could be determined in surface water with other contributing sources.

1.6 ACKNOWLEDGMENTS

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CHAPTER 2

COMMUNITY LEVEL PHYSIOLOGICAL PROFILES OF SWINE LAGOONS: DIFFERENCES IN MICROBIAL COMMUNITIES BASED ON PRODUCTION STAGE

2.1. ABSTRACT

Community-level physiological profiles have been used by several researchers to investigate the physiological diversity in soils, surface waters, and landfill leachate through the examination of carbon source utilization patterns. In this study, Biolog Microplates™ were used to obtain carbon source utilization patterns for microbial communities found in swine lagoons. It is hypothesized that these carbon source utilization patterns for swine lagoons will be unique to a specific swine production stage. Carbon source utilization patterns were obtained by inoculating GN2 Microplates™ with diluted, processed samples from six swine lagoon systems associated with specific production stages. Digitized images of each plate were taken at 24-hour intervals, and pixel data obtained from these images was used to establish carbon source utilization patterns. Average well color development ranged from –26 to 116, with the highest values associated with the two nursery lagoons. Principal component analysis was used to determine that differences between these patterns did exist based on production stage. Plots of the first two principal components showed separation between the production stages and indicated that production stage, and the associated dietary differences, did impact the microbial community structures in each lagoon system. Further analysis of carbon source utilization patterns via canonical discriminant analysis showed a high degree of separation between production stages.

Based on these results, carbon source utilization patterns may prove to be a quick, inexpensive, and accurate method for identifying nonpoint source pollution attributed to swine at the production stage level.

2.2. INTRODUCTION

The current trend in swine production is towards multi-site production facilities that produce more swine in a smaller area and are owned by or contract with large pork-producing corporations. On multi-site production facilities, swine are segregated based on age and raised on different locations (Harris, 2000). Generally, three production stages are used on multi-stage facilities. These production stages are the breeding stage, the nursery stage, and the finisher stage. The goal of multi-site production is to eliminate pathogen transfer by separating various age groups and thus preventing the transmission of diseases from older swine to recently weaned piglets (Harris, 2000). This three-site production system is now commonly used by most of the major swine production corporations.

These production facilities, also known as concentrated animal feeding operations (CAFOs), generate large amounts of wastes, which are typically stored in lagoon systems in temperate climates. Lagoon systems are designed to treat and store a specific amount of wastewater. Effluent from these systems is typically land applied to crop- or pastureland and serves as a low-cost fertilizer. Benefits of lagoon systems include decreased labor and investment requirements and increased storage time to allow land application to occur at appropriate times for crop production (Hermanson, 1975).

Because land application is an important use of lagoon effluent, CAFO waste can have a negative environmental impact on groundwater and surface water quality through nonpoint source pollution. When CAFO lagoon effluent is over-applied on a small area of land, the nutrient concentrations often exceed crop uptake levels and the retention capacity of the soil (Honeyman, 1996; Boyd, 1994). If this occurs over a period of several years, it can lead to a build up of nutrients in the soil. These nutrients along with bacteria and other organic materials can then be leached from the soil during the next period of precipitation and transported to the nearest body of water where it can lead to a decrease in water quality through nutrient enrichment and eutrophication (Boyd, 1994).

Many different agricultural, industrial, and municipal wastes contain high concentrations of nutrients, and water quality impairment cannot be conclusively linked to any particular source simply through nutrient enrichment studies. For instance, nitrate pollution can be caused by such varied sources as crop production, CAFOs, municipalities, industries, and geologic formations (Thompson, 1996). Therefore, a more conclusive method for pinpointing the nonpoint source of pollution is needed.

Research is currently being conducted in the area of microbial source tracking (MST) as an alternative to traditional uses of fecal bacteria. MST methods typically use genetic or biochemical assays to identify species of bacteria or physiological characteristics that are specific to the source of contamination. Methods currently being researched include antibiotic resistance analysis, ribotyping, and fecal bacterial species composition (Wiggins et al., 1999; Hagedorn et al., 1999; Bernhard and Field,

2000; Carson et al., 2001; Parveen et al., 1999; Evenson and Strevett, in press). Most of these methods, however, only identify the source of pollution to a specific category, such as human, cattle, or swine. This may be appropriate for most watersheds, but when more than one source of pollution in a category is located within a watershed, targeting best management practices may be difficult.

Community-level physiological profiles (CLPPs) have been used by several researchers to examine microbial community structure in soils, landfill leachate, coastal marine waters, and freshwater samples (Garland and Mills, 1991; Yao et al., 2000; Miethling et al., 2000; and Röling et al., 2000). These studies have used Biolog Microplates™ (Biolog, Inc., Hayward, CA) to assess the microbial communities' abilities to utilize a range of carbon sources, thus providing a way to investigate the physiological diversity of microorganisms. Differences in the carbon source utilization patterns are interpreted as differences in the active microorganisms associated with each microbial community.

In this study, Biolog Microplates™ were used to obtain carbon source utilization patterns for microbial communities found in swine lagoons. It is hypothesized that these carbon source utilization patterns will be unique to a specific swine production stage. If these patterns exist, these carbon source utilization patterns may prove to be a quick, inexpensive, and accurate method for identifying nonpoint source pollution attributed to swine to the production stage level.

2.3. MATERIALS AND METHODS

2.3.1 Sample Sources, Collection, and Preparation

Six lagoon systems were selected for this study, two lagoons fed by wastes from each production stage: SRS1 and 2 were breeding stage lagoons, SRS3 and 4 were nursery stage lagoons, and SRS5 and 6 were finishing stage lagoons (Figure 1.2). All six lagoons were located within 5 miles of each other. This close proximity meant that all six lagoons were constructed in areas of similar geology and existed under similar climatic conditions. All six lagoons were owned and operated by a single entity to ensure similar operating conditions and production procedures.

Each lagoon was sampled in November 2002, January 2003, and March 2003. Grab samples were taken in four locations around each lagoon using a dedicated sterile grab sampler and placed in sterile 250 mL polypropylene bottles. All samples were stored at 4°C during transport. Upon return to the laboratory, the four grab samples were mixed together in sterile 1 L polypropylene sample containers and were analyzed for heterotrophic bacteria using R2A agar (Difco Laboratories) as outlined by *Standard Methods* 9215 (APHA, 1998).

Samples were processed using a modified version of a procedure previously reported by Guckert et al. (1996) for community analysis of activated sludge microbial habitats. Thirty milliliters of each composite sample were aseptically transferred to sterile centrifuge tubes containing 0.3 mL each of filter-sterilized solutions of 0.1% w/v sodium pyrophosphate (SPP) and 0.1% v/v Tween 80. The samples were then homogenized using a laboratory blender for 10 seconds and centrifuged at 700xg for 5 minutes at room temperature to separate cells from any

solids in the samples. Victorio et al. (1996) found that the combination of SPP, Tween 80, and homogenization recovered the greatest number of suspended cells from mixed liquor samples, like the lagoon samples collected in this study. After centrifugation, the supernatant was transferred to clean, sterile tubes and diluted with a filter-sterilized phosphate buffer to standardize the inoculum density for each sample. Standardization of inoculum density was important because it can have confounding effects on CLPP and prevents adequate estimation of community dynamics (Garland, 1997).

2.3.2 Preparation of Microplates™

The diluted supernatant was used as an inoculum for triplicate GN2 Microplates™ (Biolog, Inc; Hayward, CA), which were used to evaluate the metabolic responses of each lagoon microbial community. Each of the wells of the GN2 Microplates™ was inoculated with 150 μL of the diluted supernatant. The 96-well GN2 Microplates™ allow for the simultaneous testing of a microbial community's ability to utilize 95 separate carbon sources (Table 2.1) through a unique redox technology involving the use of a tetrazolium dye. With this assay, wells in which the carbon source can be oxidized by the microbial community turn purple, indicating a positive reaction, due to the concomitant reduction of a tetrazolium dye present in each well (Bochner, 1989). Negative wells, in which the microbial community cannot utilize the carbon source, remain clear.

Table 2.1: Sole Carbon Sources in GN2 Microplates™

Carbon Sources		
Polymers	**Carboxylic Acids**	**Amino Acids**
α-cyclodextrin	acetic acid	D-alanine
dextrin	cis-aconitic acid	L-alanine
glycogen	citric acid	L-alanyl-glycine
Tween 40	formic acid	L-asparagine
Tween 80	D-galactonic acid lactone	L-aspartic acid
	D-galacturonic acid	L-glutamic acid
Carbohydrates	D-gluconic acid	glycyl-L-aspartic acid
N-acetyl-D-galactosamine	D-glucosaminic acid	glycyl-L-glutamic acid
N-acetyl-D-glucosamine	D-glucuronic acid	L-histidine
adonitol	α-hydroxybutyric acid	hydroxy L-proline
L-arabinose	β-hydroxybutyric acid	L-leucine
D-arabitol	γ-hydroxybutyric acid	L-ornithine
cellobiose	p-hydroxy phenylacetic acid	L-phenylalanine
i-erythritol	itaconic acid	L-proline
D-fructose	α-keto butyric acid	L-pyroglutamic acid
L-fructose	α-keto glutaric acid	D-serine
D-galactose	α-keto valeric acid	L-serine
gentiobiose	D, L-lactic acid	L-threonine
α-D-glucose	malonic acid	D, L-carnitine
m-inositol	propionic acid	γ-aminobutyric acid
α-D-lactose	quinic acid	
lactulose	D-saccharic acid	**Aromatics**
maltose	sebacic acid	urocanic acid
D-mannitol	succinic acid	inosine
D-mannose		uridine
D-melibiose		thymidine
β-methyl-D-glucoside		
D-psicose		**Amines**
D-raffinose	**Brominated Chemicals**	phenylethylamine
L-rhamnose	bromosuccinic acid	putrescine
D-sorbitol		2-aminoethanol
sucrose		
D-trehalose		**Alcohols**
turanose		2,3-butanediol
xylitol	**Amides**	glycerol
	succinamic acid	
Esters	glucuronamide	**Phosphorylated Chemicals**
methylpyruvate	alaninamide	D, L-α-glycerol phosphate
mono-methyl succinate		glucose-1-phosphate
		glucose-6-phosphate

2.3.3 Data Collection

Following inoculation and an initial (0 hour) scan, GN2 Microplates™ were incubated at 25°C without agitation. Digitized images of each plate were obtained using a Hewlett-Packard HP ScanJet 4400C flatbed scanner for the 72 hours at 24-hour intervals. The digitized images were then analyzed using ERDAS Imagine 8.3 (ERDAS, LLC; Atlanta, GA) according to a modified version of a method previously described by Garland and Mills (1991). ERDAS, a geographical information system software package selected for its ability to handle large digital images, was used to isolate a central pixel within each well of the Microplates™. The pixel values associated with the green scanning band were converted into gray-scale intensity values as a measure of the color response in each well.

2.3.4 Data Analysis

The gray-scale intensity value for the A1 well (water control well) of each Microplate™ was subtracted from the gray-scale intensity values of all other wells to yield corrected raw data and account for any color development that may have been due to the use of carbon in the inoculum. Average well color development (AWCD) was then calculated for each Micropolate™ according to the following formula described by Garland and Mills (1991):

$$AWCD = [(\Sigma(C - R)]/95 \quad (1)$$

where C is the gray-scale value of the A1 well and R is the gray-scale value of each of the 95 sole carbon source response wells.

The corrected raw data were then transformed by dividing the corrected raw data by the AWCD for the Microplate™ (Garland and Mills, 1991):

$$\text{Transformed data} = (C - R)/\ [(\Sigma(C - R)]/95 \qquad (2)$$

Garland and Mills (1991) showed that data transformation of each gray-scale intensity value reduced the influence of inoculum density and rate of color development differences on the classification of samples. The transformed data were then used for all statistical analyses.

2.3.5 Statistical Analysis

The AWCD for each lagoon system was recorded over the incubation period. Statistical analysis of the data set was completed using analysis of variance techniques and the generalized linear model procedure (PROC GLM) to determine if the mean AWCD for each lagoon was significantly different at any time during the duration of the study. Statistical Analysis Software (SAS) version 8 (SAS Institute, Inc.; Cary, NC) was used for all statistical analyses.

The relationships among samples on the basis of the values of the transformed data were determined by principal component analysis (PCA) using SAS version 8. PCA allows a large number of variables to be reduced to a few principal components that account for most of the variance between variables. Plotting the first two principal components (Prin1 and Prin2) in an x-y scatter plot provides useful information on the distribution of the data and the relationships among samples. PCA provides a means by which microbial samples can be compared on the basis of the pattern of carbon source utilization for each sample.

Once PCA analysis showed that each production stage's microbial community had different carbon source utilization patterns, these patterns were analyzed via canonical discriminant analysis to determine if these patterns have the potential to be used to identify the source of unknown samples. Spatial plots of the carbon source utilization patterns for each production stage (breeding, nursery, and finishing) were generated using canonical discriminant analysis (PROC CANDISC, SAS software). Canonical discriminant analysis derives a linear combination of variables with the highest multiple correlation with the groups. Plotting the first two canonical variables on an x-y scatter plot provides a visual interpretation of pattern analysis. A large separation between source groups indicates highly accurate source identification.

2.4. RESULTS AND DISCUSSION

AWCD averages for each lagoon system were calculated for the first sampling event, and AWCD values ranged from –26 to 116, with the highest values associated with the two nursery lagoons. The color development patterns over the 72-hour incubation period are displayed in Figure 2.1. Only the microbial community from SRS1 showed no lag in color development. All other samples had a lag period of 24 hours before color development began in the response wells. This lag period is not unusual. Previous studies using CLPPs for surface water, groundwater, and activated sludge also showed a lag period of approximately 24 hours before color development (Choi and Dobbs, 1999; Guckert et al., 1996). After the initial lag period, color development in all samples increased linearly over the next 48 hours. The maximum AWCD was 116 and occurred in the two nursery lagoons.

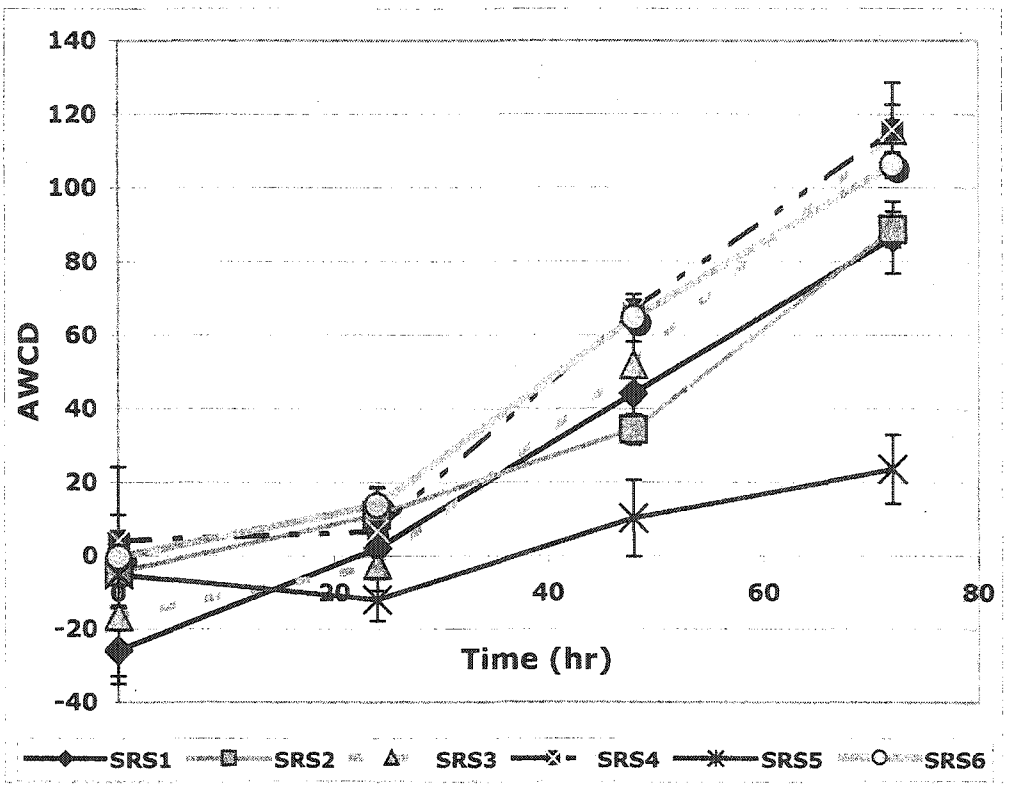

Figure 2.1: Average well color development (AWCD) for two breeding (SRS1 and SRS2), two nursery (SRS3 and SRS4), and two finishing (SRS5 and SRS6) lagoons in GN2 Microplates™ over a 72-hour incubation period.

Analysis of variance of the mean AWCD values showed no significant differences for any of these samples.

Principal component analysis of the transformed AWCD data after 72 hours of incubation was used to determine if differences existed amongst the samples based on production stage. Plots of the first two principal components (Prin1 and Prin2) showed separation between the production stages and indicated that production stage did impact the microbial community structure in each lagoon system (Figures 2.2-2.5). Figure 2.2 shows the PCA results for all three production stages. In this figure, samples from the finishing lagoons had lower coordinate values than samples from either the nursery or breeding lagoons for Prin1. Samples from all three production stages overlapped toward the center of this figure.

In order to better visualize the distinctness of the carbon source utilization patterns for each production stage, carbon source utilization patterns were analyzed via PCA for two production stages at a time. The results of PCA on carbon source utilization patterns for the breeding and nursery production stages are presented in Figure 2.3. Figure 2.4 displays the results of PCA for the breeding and finishing production stages while Figure 2.5 displays the same results for the nursery and finishing production stages. From these figures, it can be seen that the carbon source utilization patterns for the breeding production stage are distinctly different from both the nursery and finishing production stages. This is indicated by the small number of overlapping points on Figures 2.3 and 2.4. The carbon source utilization patterns for the nursery and finishing production stages show less separation and distinction.

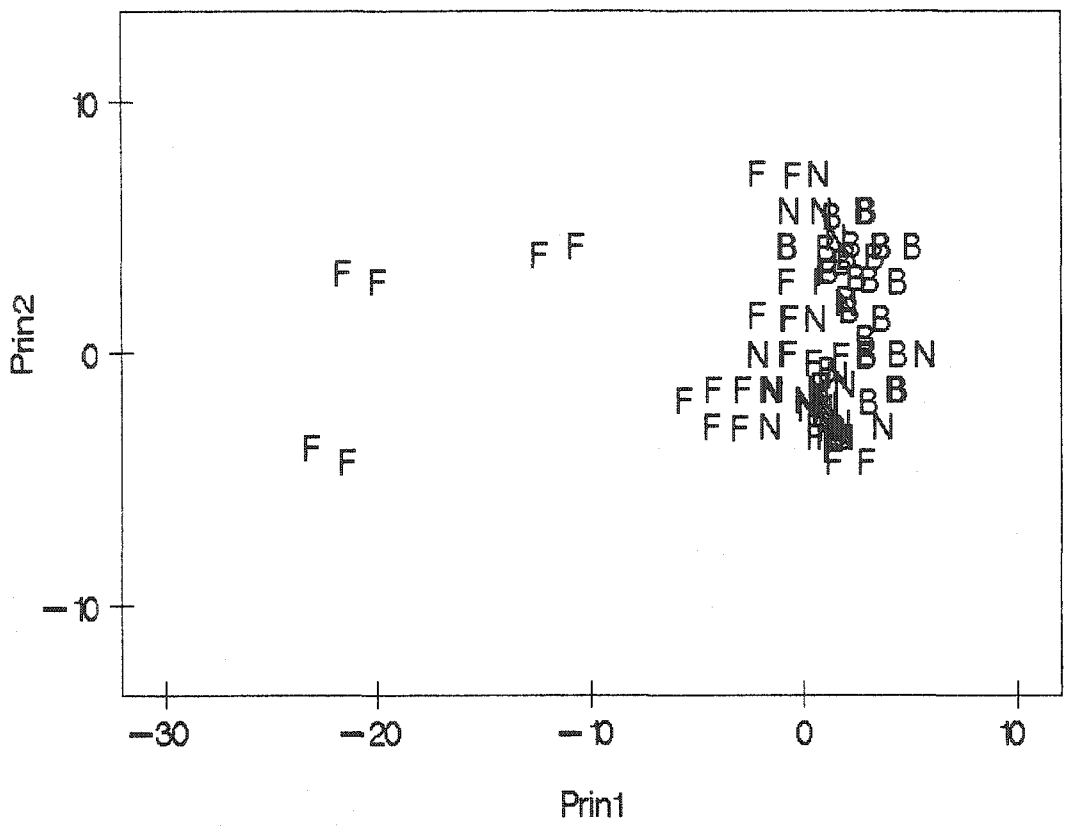

Figure 2.2: Multivariate classification of swine lagoons associated with the breeding (B), nursery (N), and finishing (F) stages of swine production based on carbon source utilization in GN2 Microplates™ at 72 h. Scores for the first and second principal components (Prin1 and Prin2, respectively) are plotted.

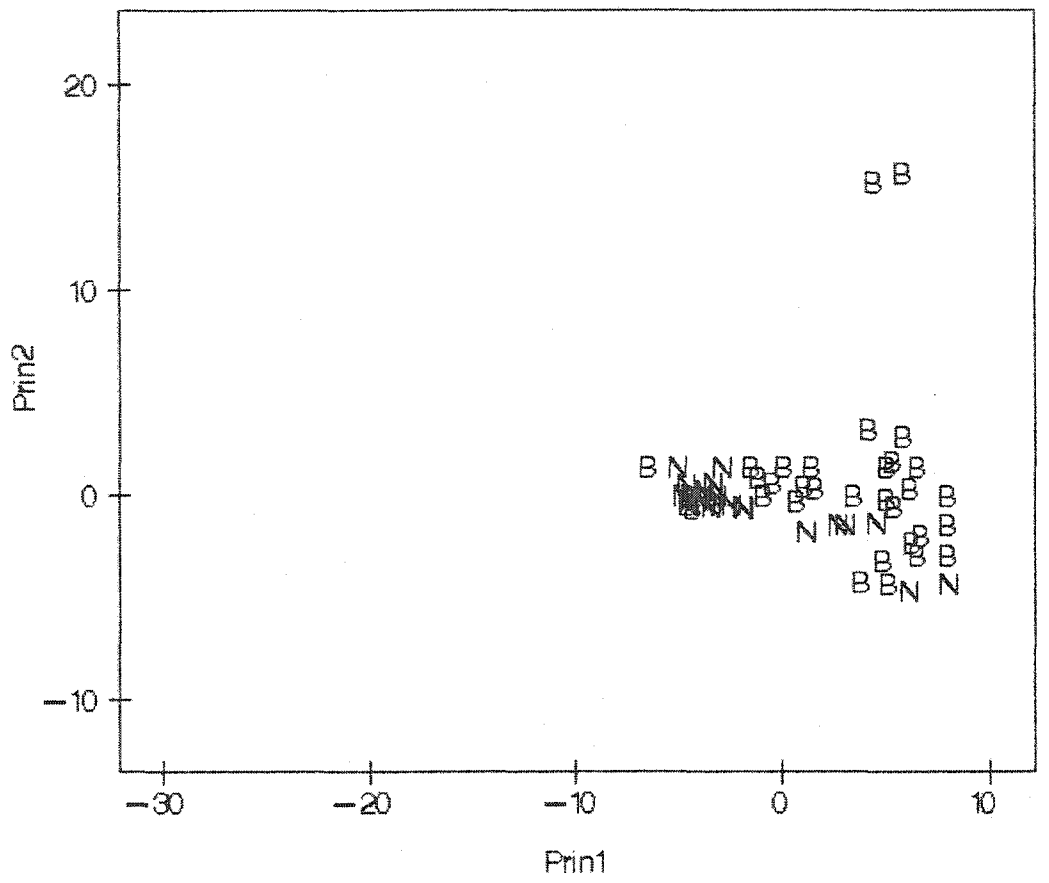

Figure 2.3: Multivariate classification of swine lagoons associated with the breeding (B) and nursery (N) stages of swine production based on carbon source utilization in GN2 Microplates™ at 72 h. Scores for the first and second principal components (Prin1 and Prin2, respectively) are plotted.

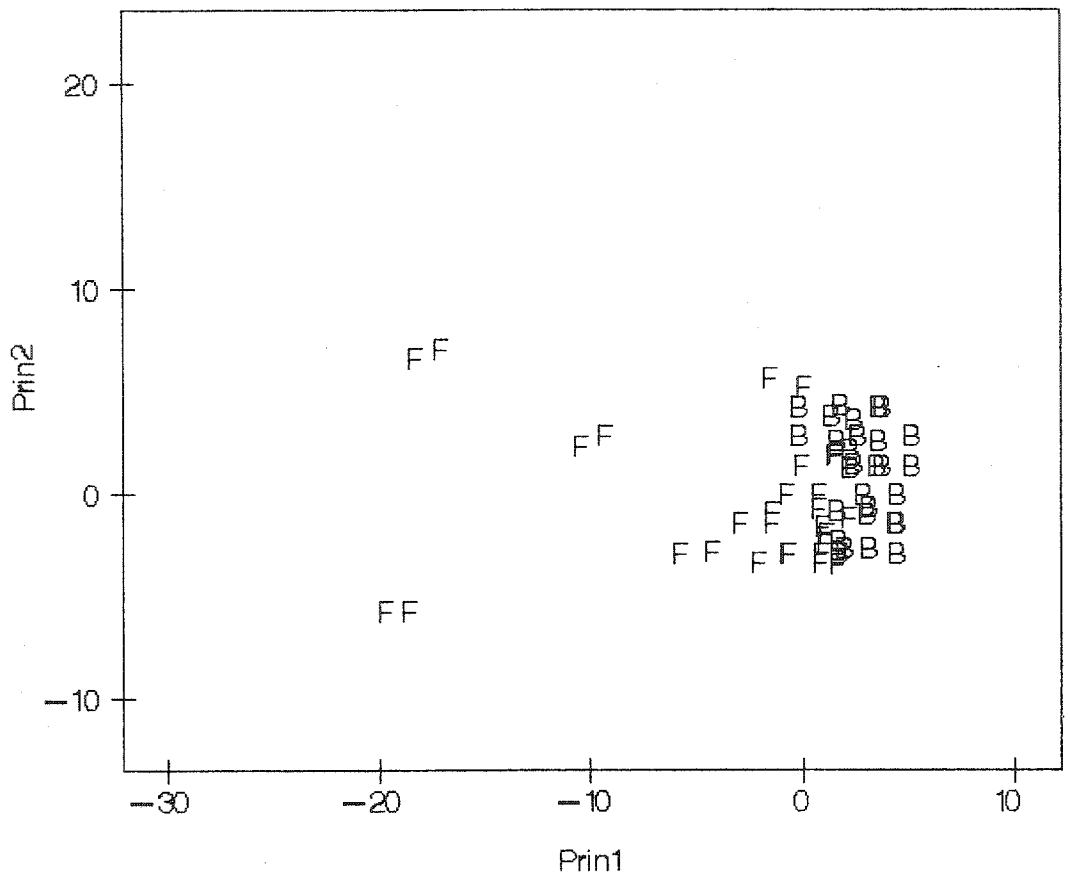

Figure 2.4: Multivariate classification of swine lagoons associated with the breeding (B) and finishing (F) stages of swine production based on carbon source utilization in GN2 Microplates™ at 72 h. Scores for the first and second principal components (Prin1 and Prin2, respectively) are plotted.

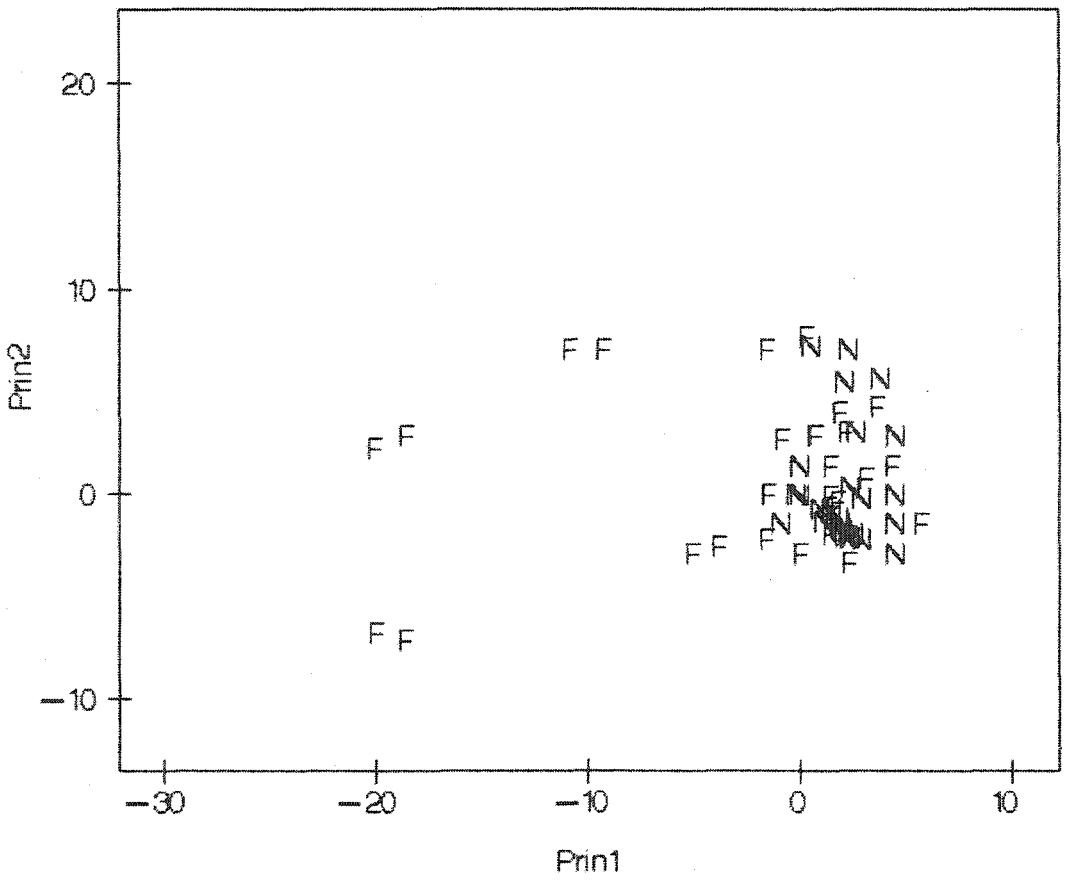

Figure 2.5: Multivariate classification of swine lagoons associated with the nursery (N) and finishing (F) stages of swine production based on carbon source utilization in GN2 Microplates™ at 72 h. Scores for the first and second principal components (Prin1 and Prin2, respectively) are plotted.

The similarity between carbon source utilization patterns for these two stages may be attributable to the age of the swine supplying waste to these lagoon systems. During the January sampling event, swine in SRS3 and SRS4 were in the process of being transferred to a finishing facility while swine in SRS5 had just been transferred to this finishing facility (unpublished data). Similar activities were also occurring during the March sampling event at SRS3, which was again in the process of transferring swine to a finishing facility. The age of the swine during these two sampling events were similar and may account for the similarity of the carbon source utilization patterns.

The functional basis for the differences in carbon source utilization patterns for each production stage was determined through the evaluation of correlation values between each carbon source and the first two principal components (Garland and Mills, 1991). Table 2.2 lists the correlation values for carbon sources that had greater than 0.15 or less than –0.15 of their variance explained by Prin1 or Prin2. Those carbon sources with the greatest correlation values listed in Table 2.2 consist primarily of polymers, carbohydrates, carboxylic acids, and amino acids. Variability in the first principal component was classified by a high utilization of L-fructose and two amides, succinamic acid and glucuronamide and a low utilization of the polymers, Tween 40 and Tween 80, carboxylic acids, amines, and amino acids. A high utilization of carbohydrates, with the exception of xylitol, carboxylic acids, and the polymers, dextrin and glycogen, and a low utilization of the amino acids and amines characterize the variability in the second principal component. The majority of the carbon sources in the GN2 Microplates™ were utilized by the microbial

communities in all three production stages as indicated by the low correlation values for all 95 carbon sources.

Further analysis of carbon source utilization patterns via canonical discriminant analysis showed a high degree of separation between production stages. Plots of the first two canonical variables (Can1 and Can2) are presented in Figure 2.6 for all three sets of carbon source utilization patterns (breeding, nursery, and finishing, respectively). These plots show Can1 on the x-axis and Can2 on the y-axis. Distinct separation between production stages was evident. The spatial displays support the PCA results and provide a useful method to visualize the distinct separation between each production stage.

The distinctness of these carbon source utilization patterns over time is important because it shows lagoon effluent does not have to be freshly deposited in order for an MST method based on these patterns to be effective, as is the case with FC/FS ratios and other methods (McFeters et al., 1974; Edwards et al., 1997). Lagoons have unique carbon utilization patterns that may be used as a method for pinpointing the source of fecal pollution in a watershed where multiple swine sources exist.

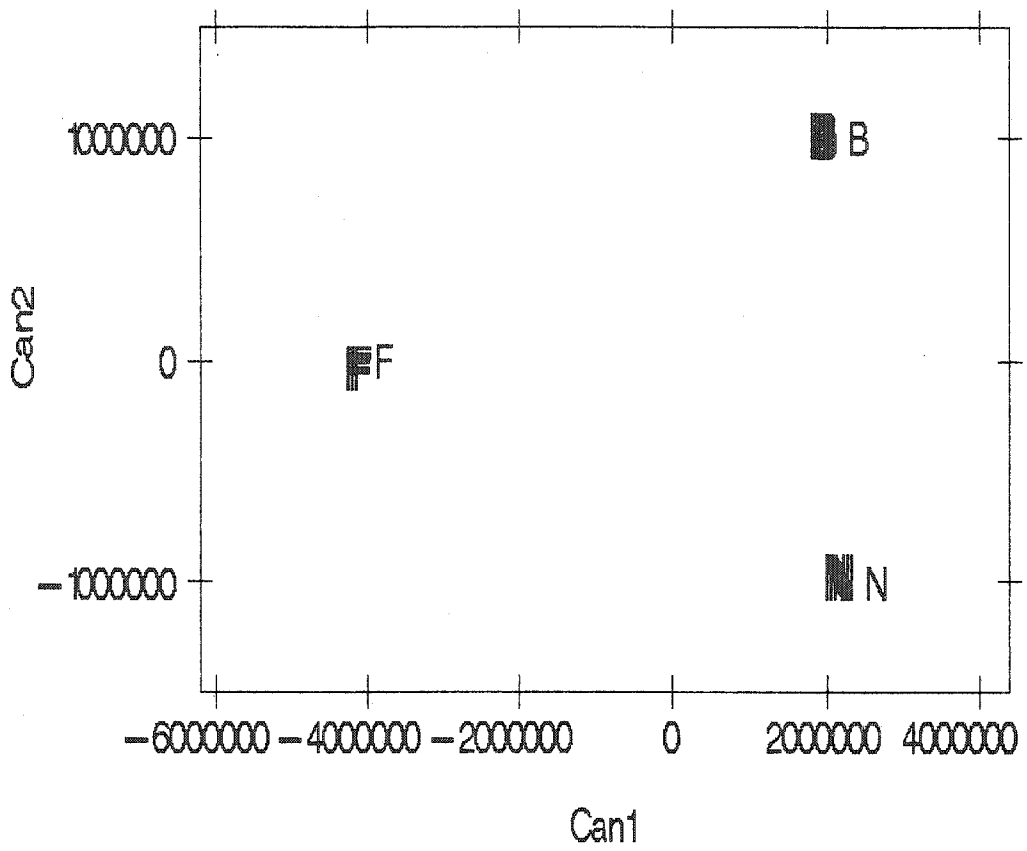

Figure 2.6: Spatial plots of carbon source utilization patterns for swine lagoons associated with the breeding (B), nursery (N), and finishing (F) stages of swine production based on carbon source utilization in GN2 Microplates™ at 72 h. Can1 is on the x-axis, and Can2 is on the y-axis.

Table 2.2: Correlation of Carbon Source Variables to PCs for Analysis of Lagoon Samples.

PC1		PC2	
Carbon Source[a]	r^b	Carbon Source	r
Polymers		**Polymers**	
Tween 40	-0.189	dextrin	0.178
Tween 80	-0.186	glycogen	0.194
Carbohydrates		**Carbohydrates**	
L-fructose	0.158	L-arabinose	0.190
		D-galactose	0.201
Esters		maltose	0.164
methylpyruvate	-0.186	D-mannose	0.173
		β-methyl-D-glucoside	0.156
Carboxylic Acids		sucrose	0.183
β-hydroxybutyric acid	-0.155	D-trehalose	0.206
D,L-lactic acid	-0.190	xylitol	-0.161
malonic acid	-0.161		
propionic acid	-0.185	**Carboxylic Acids**	
succinic acid	-0.160	cis-aconitic acid	0.156
		citric acid	0.155
Amides		D-galacturonic acid	0.165
succinamic acid	0.163	D-glucuronic acid	0.168
glucuronamide	0.152		
		Amino Acids	
Amino Acids		L-glutamic acid	0.161
D-alanine	-0.189	L-leucine	-0.162
L-alanine	-0.185	L-ornithine	-0.163
		L-threonine	-0.161
Amines			
2-aminoethanol	-0.168	**Amines**	
		phenylethylamine	-0.176
		putrescine	-0.159

[a]All other carbon sources not listed in this table had correlation coefficients of <0.15 and >-0.15.

[b]r, Pearson's regression coefficient

2.5. CONCLUSIONS

Based on the results of this study, an MST method based carbon source utilization patterns could be used to distinguish between breeding, nursery, and finishing lagoons as sources of fecal pollution attributed to swine in a watershed. Carbon source utilization patterns may prove to be a quick, inexpensive, and accurate method for identifying nonpoint source pollution attributed at swine to the production stage level. Further work needs to be done to determine if the differences between carbon source utilization patterns could be determined in surface water with other contributing sources.

2.6. ACKNOWLEDGMENTS

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CHAPTER 3

FECAL BACTERIAL SURVIVAL IN LAGOON MESOCOSMS

3.1. ABSTRACT

One approach to treating animal waste is to retain it in a treatment lagoon; however, it is uncertain how the concentrations of fecal coliform (FC) and fecal streptococci (FS) in these systems are affected by storage. To better understand how storage time affects fecal bacterial concentrations and species composition in a lagoon system, this study determined the changes in the concentration of FC and FS bacteria in simulated cattle, poultry, and swine lagoon mesocosms. Fresh manure slurry was added to the lagoon mesocosms after 14 to 21 days to determine if fecal bacterial concentrations changed, as would occur in a plug-flow system. Analysis of variance results indicated that cattle FC concentrations were significantly different than poultry and swine FC concentrations while the poultry FS concentrations were significantly different than cattle and swine FS concentrations ($p=0.05$). The secondary addition of wastes to the mesocosms did not significantly alter the concentrations in each mesocosm ($p=0.05$). Species compositions were correctly classified for each known source throughout the first 14 days of incubation under lagoon conditions. The average rate of correct classification was 85%, 100%, and 100% for FC, FS, and fecal bacterial species compositions, respectively. This indicates that a microbial source tracking method based on species composition patterns could be used successfully to identify major contributors to nonpoint source

pollution even when the source of pollution is waste that was aged under lagoon conditions.

3.2. INTRODUCTION

Concentrated animal feeding operations (CAFOs) are required by the Clean Water Act and the National Pollutant Discharge Elimination System to meet an effluent limitation guideline of zero discharge (Johnson et al., 1999). As a result, waste management strategies have been developed to help CAFO owners and operators meet this goal. Waste management strategies vary depending on climate, topography, and hydrology and involve solid or liquid waste management systems. Most operations in Oklahoma use a liquid waste management system in which wastes are pumped, flushed, or gravity-fed to clay-lined earthen basins, or lagoons (NPPC, 1997).

As early as 1914, coliform bacteria were used as an indicator of fecal contamination of drinking water (Gerba, 2000). Fecal coliform (FC) and fecal streptococci (FS) bacteria are commonly used as indicators of fecal contamination both in drinking water and surface water because they are more clearly associated with contamination by fecal matter. It has even been suggested that the FC/FS ratio can indicate the source of the fecal contamination because mammalian species have unique FC/FS ratios, although recent research has downplayed the usefulness of this ratio due to its instability over time (Gerba, 2000).

Research is currently being conducted in the area of microbial source tracking (MST) as an alternative to traditional uses of fecal bacteria. MST methods typically use genetic or biochemical assays to identify species of bacteria or physiological

characteristics that are specific to the source of contamination. Methods currently being researched include antibiotic resistance analysis, ribotyping, and fecal bacterial species composition (Wiggins et al., 1999; Hagedorn et al., 1999; Bernhard and Field, 2000; Carson et al., 2001; Parveen et al., 1999; Evenson and Strevett, unpublished data).

The authors are currently developing an MST methodology involving the use of fecal bacterial species composition, and a critical question not addressed in current literature is the stability and distinctness of fecal bacterial populations over time as the wastes are stored in a lagoon system. To understand how FC and FS bacterial concentrations and species compositions are affected by storage in lagoon systems, a mesocosm study was performed to monitor the changes in fecal bacterial concentrations and species composition over time.

3.3. MATERIALS AND METHODS

To examine the changes in FC and FS concentrations and species composition over time in lagoon systems, laboratory-scale mesocosms were established for cattle, poultry, and swine manure. Laboratory conditions were used in this study to limit the complex effects of environmental factors found in the field. Temperature was held at 25°C, and light exposure was limited to overhead fluorescent light because both temperature and sunlight have been shown to affect fecal bacterial die-off rates (McFeters et al., 1974; Van Donsel et al., 1967; Fujioka et al., 1981; Davies-Colley et al., 1997).

The lagoon mesocosms were set up in sterile 2L polypropylene containers and created by mixing fresh manure from a single known source in 1.5 L of untreated

groundwater to create a manure slurry. The manure slurries for each source had a total solids (TS) concentration of 11 g TS/L, as measured using *Standard Methods* 2540 B (APHA, 1998). The manure slurries were designed to mimic a waste lagoon in which manure is flushed from the animal housing units using tap water on site. Once inoculated, the mesocosms were mixed thoroughly, and the zero hour samples were taken. Mesocosms were stirred continuously throughout the length of the experiment to limit the settling of solids or bacteria.

Initial FC and FS concentrations in each mesocosm were determined using selective media, mFC agar for FC and mEnterococcus agar for FS (Difco Laboratories), and the membrane filtration technique as described by *Standard Methods* 9222D and 9230C (APHA, 1998). The mesocosms were sampled every 24 hours after inoculation for a 14-day period. Fresh manure slurry was added to each mesocosm after the initial 14-day period to determine the effects of periodic additions of fresh manure on FC and FS concentrations. Fecal bacterial concentrations were monitored for an additional 7 to 14 days after inoculation with fresh manure slurry. The concentrations in each replicate mesocosm were averaged together and reported as CFU/100 mL ± one standard deviation. The initial concentrations and the concentrations immediately before and after the secondary addition of fresh manure slurry were analyzed statistically using the general linear model procedure (PROC GLM) and Duncan's multiple range test for concentration as specified by Statistical Analysis Software (SAS) version 8 (SAS Institute, Inc., Cary, NC). PROC GLM was used because it can account for unbalanced data, which cannot be accounted for using the ANOVA procedure.

The dominant species composition in each mesocosm was determined for the first fourteen days of the experiment. Five colonies from either the FC or the FS membrane filtration plates were randomly selected, isolated on selective media, and identified for each replicate mesocosm. The resultant identifications were compiled and used to create FC, FS, or fecal bacteria species composition patterns. The fecal bacteria species composition patterns were created by combining the FC and FS identification results. Colonies were isolated from FC and FS plates containing between 20 and 80 colony-forming units for the 0, 24, 48, 72, 96, 144, 192, 264, and 336-hour samples. After a colony had been isolated and a pure plate obtained, an isolated colony from the pure plate was transferred to Biolog Universal Growth Agar containing 5% defibrinated sheep's blood. The maximum number of transfers performed was limited to five to prevent unnecessary stress and limit metabolic changes, as specified by identification procedures set by Biolog, Inc. (Hayward, CA).

The growth from the blood agar plate was then diluted and used to inoculate GN2 or GP2 Microplates™ according to the procedures developed by Biolog, Inc. (Hayward, CA) for the identification of gram-negative and gram-positive bacteria, respectively. Each microplate contains 95 different sole carbon sources, which produce a metabolic fingerprint that is specific to species and strains of microorganisms. After incubation, the patterns of positive, negative, and borderline responses were read manually and input into the MicroLog™ Microbial Identification System (Biolog, Inc.). The results obtained for each colony identified were then compiled and used to calculate fecal bacterial species composition as an average percentage basis from the three replicates.

The species composition data for each source over time were analyzed statistically using SAS version 8. Discriminant analysis using the PROC DISCRIM function was used to classify the fecal bacterial species composition data into source categories (prior probabilities, equal; covariance matrix, pooled). This function produces a source-by-source matrix with the number and percentage of correctly classified samples for each source located on the diagonal. Average rate of correct classification (ARCC) for each fecal bacterial type was calculated by averaging the percentages of correctly classified samples on the diagonal.

3.4. RESULTS AND DISCUSSION

The FC and FS average concentrations are presented in Figures 3.1 and 3.2, respectively. Concentrations of FC and FS for all manure sources increased initially then began to stabilize approximately 48 hours after inoculation. Analysis of variance (ANOVA) for FC concentrations indicated that the overall concentrations for the swine and poultry lagoon mesocosms were significantly greater than those in the cattle mesocosms ($p=0.05$). The overall FC concentrations in the poultry and swine lagoon mesocosms were not statistically different ($p=0.05$). Overall, FS concentrations in the poultry lagoon mesocosms were significantly greater that those in the cattle and swine lagoon mesocosms, which were not significantly different from each other ($p=0.05$). Although the FC and FS concentrations may have been significantly different between manure sources, the concentrations within each source remained within one order of magnitude. This is important because it indicates that FC and FS concentrations remain relatively constant over time in a lagoon environment. Despite research suggesting that fecal bacterial concentrations decrease

rapidly once the microbes are exposed to an environment outside the host organisms, such as surface water or groundwater (McFeters et al., 1974; Crane and Moore, 1986), this study indicates that fecal bacteria can survive for an extended period of time in the lagoon environment. Prolonged survival in lagoon environments may be caused by the elevated nutrient contents in these environments. Several studies have found that elevated nutrient contents can increase survival in aquatic environments (Slanetz and Bartley, 1965; Hendricks, 1972; Hirn et al., 1980).

The secondary addition of fresh manure slurry did not appear to affect the concentrations of FC or FS bacteria in any of the lagoon mesocosms (Figures 3.1 and 3.2). Statistical analysis of the mean FC concentrations for cattle, poultry, and swine lagoon mesocosms at the beginning of the experiment, immediately before the addition of fresh manure slurry, and immediately after this secondary waste addition indicated that only the mean FC concentrations for the swine mesocosms immediately before the secondary addition were significantly different than the other mean FC concentrations ($p = 0.05$) (Table 3.1). Similar analysis of the mean FS concentrations for the lagoon mesocosms (Table 3.2) indicated that none of the mean FS concentrations were significantly different than the others ($p = 0.05$), thus the secondary addition of manure slurry did not significantly alter the fecal bacterial concentrations of lagoon mesocosms. The stability of the fecal bacterial concentrations is important because it indicated that the fecal bacterial populations were not impacted by the secondary addition of waste. If it is assumed that geospatial parameters during scale up remain constant, the results herein also indicate

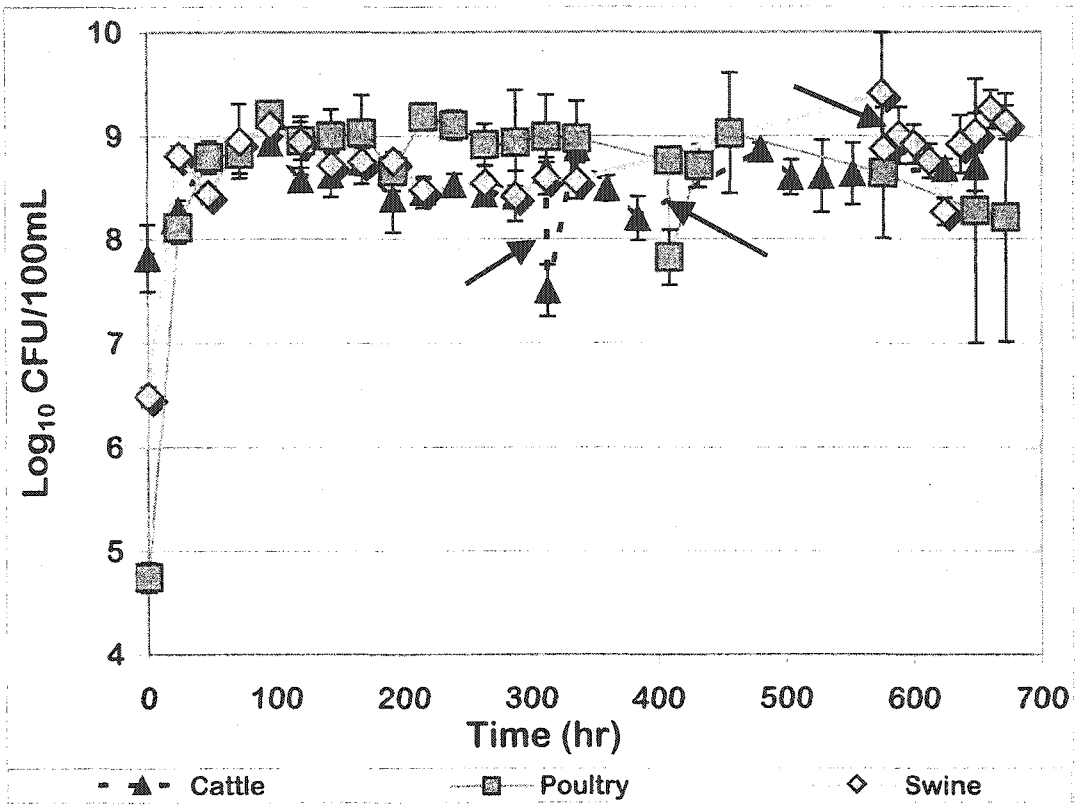

Figure 3.1: Fecal coliform decay patterns. Error bars represent one standard deviation of the mean of three replicates. Data points without error bars have small standard deviations with error bars masked by the symbol. The arrows indicate the secondary addition of fresh manure slurry.

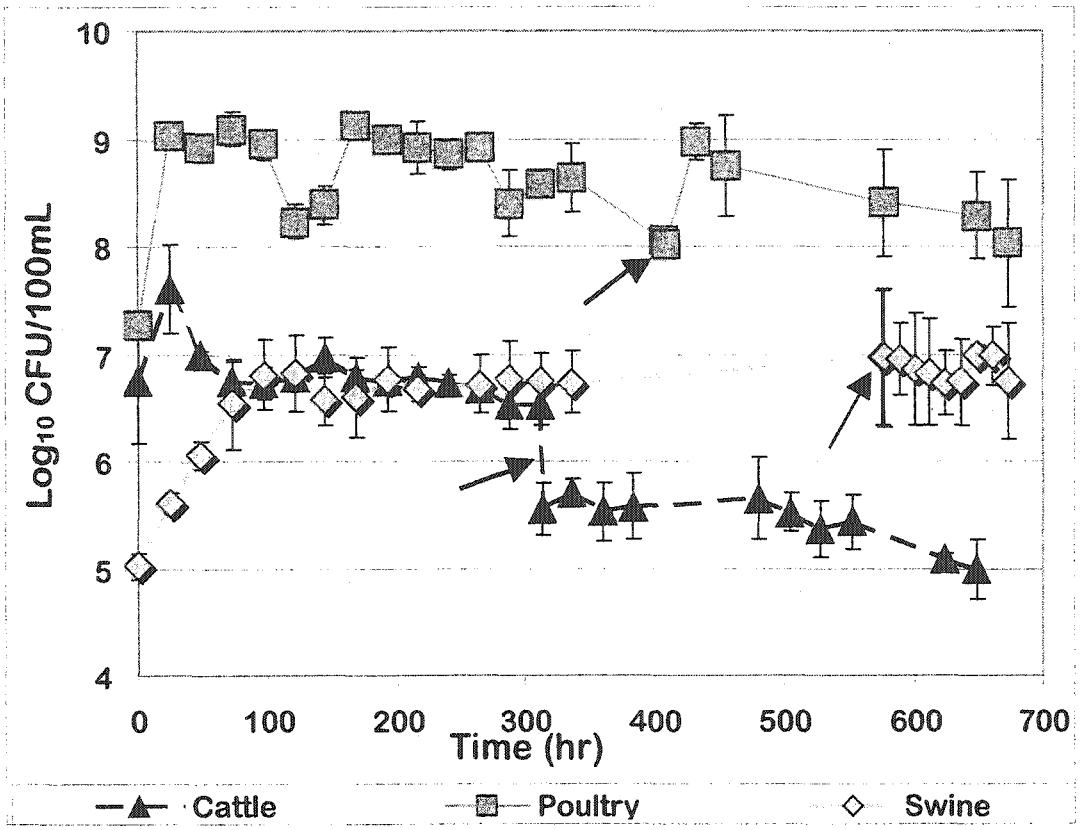

Figure 3.2: Fecal streptococci decay patterns. Error bars represent one standard deviation of the mean of three replicates. Data points without error bars have small standard deviations with error bars masked by the symbol. The arrows indicate the secondary addition of fresh manure slurry.

Table 3.1: Analysis of Variance for Fecal Coliform Concentrations

Manure Source	Sample ID	N	Mean	Duncan Grouping	
Cattle	0 hr	3	8.1E+07		B
Cattle	Before	3	4.8E+08		B
Cattle	After	3	3.6E+07		B
Poultry	0 hr	3	5.8E+04		B
Poultry	Before	3	5.9E+08		B
Poultry	After	3	7.8E+07		B
Swine	0 hr	3	3.1E+06		B
Swine	Before	3	4.6E+09	A	
Swine	After	3	7.5E+08		B

†"Before" indicates the average concentration one hour before the secondary addition of fresh wastes to each mesocosm. "After" indicates the average concentration one hour after the secondary addition of fresh waste to each mesocosm.

‡ Means with the same letter are not significantly different.

Table 3.2: Analysis of Variance for Fecal Streptococci Concentrations

Manure Source	Sample ID	N	Mean	Duncan Grouping
Cattle	0 hr	3	8.7E+06	A
Cattle	Before	3	3.6E+06	A
Cattle	After	3	4.0E+05	A
Poultry	0 hr	3	1.9E+07	A
Poultry	Before	3	1.2E+08	A
Poultry	After	3	1.1E+08	A
Swine	0 hr	3	1.1E+05	A
Swine	Before	3	1.8E+07	A
Swine	After	3	1.8E+07	A

[†]"Before" indicates the average concentration one hour before the secondary addition of fresh wastes to each mesocosm. "After" indicates the average concentration one hour after the secondary addition of fresh waste to each mesocosm.

[‡] Means with the same letter are not significantly different.

that fecal bacterial populations in treatment lagoons are not significantly altered by continuous waste input. Freitas and Burr (1996) reported that animal waste decomposition is initially done by bacterial species excreted from the animal's gut and that these species dominate during early periods of decomposition. In lagoons with continuous waste inputs and high organic loading, fecal bacterial communities remain fairly steady and nominal influxes do not disrupt the quasi-equilibrium of the community (Saqqar and Pescod, 1991). Therefore, an established lagoon should have unique dominant species compositions that can be identified that are not affected by continuous input of wastes.

Discriminant analysis of the FC and FS species composition data indicated that distinct species composition patterns of fecal bacteria exist for each manure source examined (Table 3.3, 3.4, and 3.5). The discovery of distinct species composition patterns is important in MST because this indicates that each manure source has a unique species composition pattern that can be used to identify the major contributor to fecal pollution even from an aged manure source. The average rate of correct classification (ARCC) for FC species composition patterns (ARCC = 85%) was lower than the ARCC for the FS and fecal bacterial species composition patterns (ARCCs = 100%). FC species composition patterns for each manure source were misclassified more frequently than were the FS or fecal bacteria species composition patterns. This misclassification was largely due to the dominance of *Escherichia coli* in all manure sources. For this reason, an MST method using FS or fecal bacteria species composition patterns may be more useful for source identification.

Table 3.3: Discriminant Analysis Results for Fecal Coliform Species Composition

Manure Source	Cattle	Poultry	Swine	Total
Cattle	9 100.0	0 0.0	0 0.0	9 100.0
Poultry	1 11.1	7 77.8	1 11.1	9 100.0
Swine	1 11.1	1 11.1	7 77.8	9 100.0
Total	11 40.7	8 29.6	8 29.6	27 100.0

† The number of observations and percent classified into source are listed in each cell.

‡ The average rate of correct classification for this analysis was 85%.

Table 3.4: Discriminant Analysis Results for Fecal Streptococci Species Composition

Manure Source	Cattle	Poultry	Swine	Total
Cattle	6 100.0	0 0.0	0 0.0	6 100.0
Poultry	0 0.0	9 100.0	0 0.0	9 100.0
Swine	0 0.0	0 0.0	9 100.0	9 100.0
Total	6 25.0	9 37.5	9 37.5	24 100.0

† The number of observations and percent classified into source are listed in each cell.

‡ The average rate of correct classification for this analysis was 100%.

Table 3.5: Discriminant Analysis Results for Fecal Bacterial Species Composition

Manure Source	Cattle	Poultry	Swine	Total
Cattle	6 100.0	0 0.0	0 0.0	6 100.0
Poultry	0 0.0	9 100.0	0 0.0	9 100.0
Swine	0 0.0	0 0.0	9 100.0	9 100.0
Total	6 25.0	9 37.5	9 37.5	24 100.0

† The number of observations and percent classified into source are listed in each cell.

‡ The average rate of correct classification for this analysis was 100%.

For both the FS species composition data and the fecal bacterial species composition data, all three manure sources had rates of correct classification (RCCs) of 100%. This indicates that FS and fecal bacterial species composition data for each manure source were correctly classified throughout the entire length of the study. This is important because these data indicate that FS and fecal bacterial species composition patterns exist for each manure source that can be correctly classified even in aged manure. An MST method based on FS or fecal bacterial species composition can be useful in identifying major contributors to nonpoint source pollution even if the source of the pollution is a lagoon. The stability and distinctness of these species composition patterns over time is important because it shows that the waste does not have to be freshly deposited in order for an MST method based on

these patterns to be effective as is the case with FC/FS ratios and other methods (McFeters et al., 1974; Edwards et al., 1997).

The RCCs for FC species composition were lower than those for FS and fecal bacteria in all manure sources except cattle (RCC= 100%). Both poultry and swine FC species composition had an RCC at 77.8%. In order to determine why the poultry and swine species composition patterns had lower RCCs than the cattle species composition patterns, it was important to determine which samples were misclassified. This was done by systematically removing species composition patterns from the swine and poultry sources. When the species composition data for the 264- and 336-hour samples for the poultry lagoon mesocosms were removed from the SAS dataset and reanalyzed, the RCC increased to 100%. *E. coli* accounted for 80% of both poultry samples that were misclassified. The RCC for the swine mesocosms was increased to 85.7% when the species composition data for the 336-hour sample was removed from the SAS dataset and reanalyzed. This indicated that the stability of the FC species composition patterns begins to degrade as the wastes were aged over two weeks and that dominance by *E. coli* can adversely affect the classification rates of FC species composition patterns. To be truly useful, an MST method using FC species composition patterns should be able to identify strains of *E. coli*. A study performed by McFeters et al. (1974) indicated that FC, as a group, showed less uniformity between the die-off rates for individual species. FC species composition patterns may have degraded over time in these aged manure systems because individual FC species die-off at different rates.

An important result of this study was the increased stability of FS over FC indicators. Discriminant analysis results for FS species composition patterns are presented in Table 3.4. All three sources had RCCs of 100%, indicating that the FS species composition patterns were correctly classified for the entire 14-day period. Unlike the FC species composition patterns, the FS species composition patterns remained intact and statistically identifiable. Thus, an MST method based on FS species composition may be more useful in identifying the major contributor to non-point source pollution because the dominant FS species in each waste source persist longer in the environment and remain in a constant ratio to one another longer than the dominant FC species.

3.5. CONCLUSION

FC and FS bacteria are capable of survival under the conditions encountered in the initial days after deposition and flushing into lagoons. The secondary addition of wastes to the mesocosms did not significantly alter the concentrations in each mesocosm. Dominant species composition patterns were correctly classified for each known source throughout the first 14 days of incubation under lagoon conditions. This indicates that a microbial source tracking method based on species composition patterns could be used successfully to identify major contributors to nonpoint source pollution even if the source of the pollution was aged under lagoon conditions.

3.6. ACKNOWLEDGMENTS

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CHAPTER 4

FECAL BACTERIAL DECAY RATES AND PATTERNS IN SURFACE WATER MESOCOSMS

4.1. ABSTRACT

Traditional uses of fecal bacteria as a method to identify the major contributors to nonpoint source pollution have recently been replaced by novel alternative methods such as microbial source tracking (MST). A critical question not adequately addressed in MST literature is the stability and distinctness of fecal bacterial populations once they have entered surface water. Therefore, this laboratory-based study was designed to determine if cattle, swine, and poultry manure had distinct populations of fecal coliform (FC) and fecal streptococci (FS) bacteria that could be identified statistically over time. Laboratory-scale mesocosms were created in triplicate for three manure sources: cattle, poultry, and swine. The average rate of decay for fecal bacteria in surface water ranged from $-0.085\ hr^{-1}$ for FC in cattle manure to $-0.140\ hr^{-1}$ for FS in swine manure. Discriminant analysis of the FC and FS species composition data indicated that distinct populations of fecal bacteria exist for each manure source examined. The rates of correct classification (RCCs) were highest in the FS species compositions with all three sources having RCCs of 100%. The RCCs for FC species composition were lower than those for FS, with RCCs of 83.3%, 71.4%, and 100% for cattle, poultry, and swine, respectively. Distinct populations of both FC and FS appear to exist and can be classified correctly in surface water even in the presence of native bacterial populations. Based on the

findings of this study, research into the development of an MST methodology based on fecal bacterial species composition is warranted.

4.2. INTRODUCTION

As early as 1914, coliform bacteria have been used as an indicator of fecal contamination of drinking water (Gerba, 2000). Fecal coliform (FC) and fecal streptococci (FS) bacteria are commonly used as indicators of fecal contamination both in drinking water and surface water because they are more clearly associated with contamination by fecal matter. It has even been suggested that the FC/FS ratio can indicate the source of the fecal contamination because mammalian species have unique FC/FS ratios, although recent research has downplayed the usefulness of this ratio due to its instability (Gerba, 2000).

Research is currently being conducted in the area of microbial source tracking (MST) as an alternative to traditional uses of fecal bacteria. MST methods typically use genetic or biochemical assays to identify species of bacteria or physiological characteristics that are specific to the source of contamination. Methods currently being researched include antibiotic resistance analysis, ribotyping, and fecal bacterial species composition (Wiggins et al., 1999; Hagedorn et al., 1999; Bernhard and Field, 2000; Carson et al., 2001; Parveen et al., 1999; Evenson and Strevett, unpublished data).

The authors are currently developing an MST methodology involving the use of fecal bacterial species composition, and a critical question not addressed in current literature is the stability and distinctness of fecal bacterial populations once they have entered surface water. Therefore, this laboratory-based study was designed to

determine if cattle, swine, and poultry manure had distinct fecal bacterial populations that could be identified statistically over time.

4.3. MATERIALS AND METHODS

To examine the decay rates and patterns of fecal bacteria in surface water, laboratory-scale mesocosms were created in triplicate for three manure sources: cattle, poultry, and swine. Mesocosms were set up in sterile 2L polypropylene containers. The containers were filled with 1.5L of surface water collected from a nearby creek (Clear Creek, Norman, OK). Aseptic technique was used when handling the surface water samples to minimize indigenous microbial community disturbance. This was done in order to determine if specific fecal bacterial compositions existed for each manure source that could be detected even in the presence of natural bacterial populations. Within 6 hours of surface water sample collection, the mesocosms were inoculated with 100mL of manure slurry consisting of tap water and aged manure from a single source.

The manure slurries had a total solids (TS) concentration of 11 g TS/L, as measured using *Standard Methods* 2540 B (APHA, 1998). The creation of the manure slurries was designed to mimic the creation of a waste lagoon in which manure is flushed from the animal housing units using tap water on site. Once inoculated with manure slurry, the mesocosms were mixed thoroughly, and the zero hour samples were taken. Mesocosms were stirred continuously throughout the length of the experiment to limit the settling of solids or bacteria.

Initial FC and FS concentrations were determined for both the surface water and the mesocosms using selective media, mFC agar for FC and mEnterococcus agar

for FS (Difco Laboratories), using the membrane filtration technique as described by *Standard Methods* 9222D and 9230C, respectively (APHA, 1998). The mesocosms were sampled every 6 hours after inoculation until concentrations were below detectable limits.

Decay rates were calculated for each manure source using the following equation:

$$X=X_0e^{-kt} \qquad \text{(Equation 4.1)}$$

Only the linear portion of each curve was used for this calculation. The decay rates for each manure source were then analyzed statistically using the PROC GLM function of SAS version 8 (SAS Institute Inc., Cary, NC).

To determine the dominant species composition in each mesocosm over time, five colonies from both the FC and FS membrane filtration plates were randomly selected and isolated on selective media. Colonies were isolated from FC and FS plates containing between 20 and 80 colony-forming units for each 6-hour interval. After the colony had been isolated and a pure plate obtained, an isolated colony from the pure plate was transferred to Biolog Universal Growth Agar containing 5% defibrinated sheep's blood. The maximum number of transfers performed was limited to five to prevent unnecessary stress and limit metabolic changes, as specified by identification procedures set by Biolog, Inc. (Hayward, CA). The growth from the blood agar plate was then diluted and used to inoculate GN2 or GP2 Microplates™ according to the procedures developed by Biolog, Inc. (Hayward, CA) for the identification of gram-negative and gram-positive bacteria, respectively. Each microplate contains 95 different sole carbon sources, which produce a metabolic

fingerprint that is specific to species and strains of microorganisms. After incubation, the pattern of positive, negative, and borderline responses were read manually and input into the MicroLog Microbial Identification System (Biolog, Inc.). The results obtained for each colony identified were then compiled and used to calculate fecal bacterial species composition as an average percentage basis from the three replicates.

The species composition data for each source over time was analyzed statistically using Statistical Analysis Software (SAS) version 8. The PROC DISCRIM function was used to classify the fecal bacterial species composition data into source categories (cattle, poultry, or swine).

4.4. RESULTS AND DISCUSSION

The difference in fecal bacterial decay for each manure source in surface water is illustrated by the changes in FC (Figure 4.1) and FS (Figure 4.2) concentrations over time. All three FC decay curves were sigmoidal with lag periods of 6 hours (Figure 4.1). After this lag period, FC concentrations in the surface water mesocosms containing cattle and swine manure began to decrease exponentially while FC concentrations in poultry manure mesocosms increased from a log concentration of 7.0 to 7.7 over the next 12 hours. A Student's t-test of the 0 hour and 18 hour concentrations indicated a trend toward statistical significance ($t = 0.0972$, $0.05 < p > 0.10$). This period of regrowth in the poultry mesocosms was followed by exponential decay, similar to that observed in the cattle and swine mesocosms. Although the reasons the regrowth of FC occurred in only the poultry mesocosms were not determined in this study, these results do correspond with results from other studies

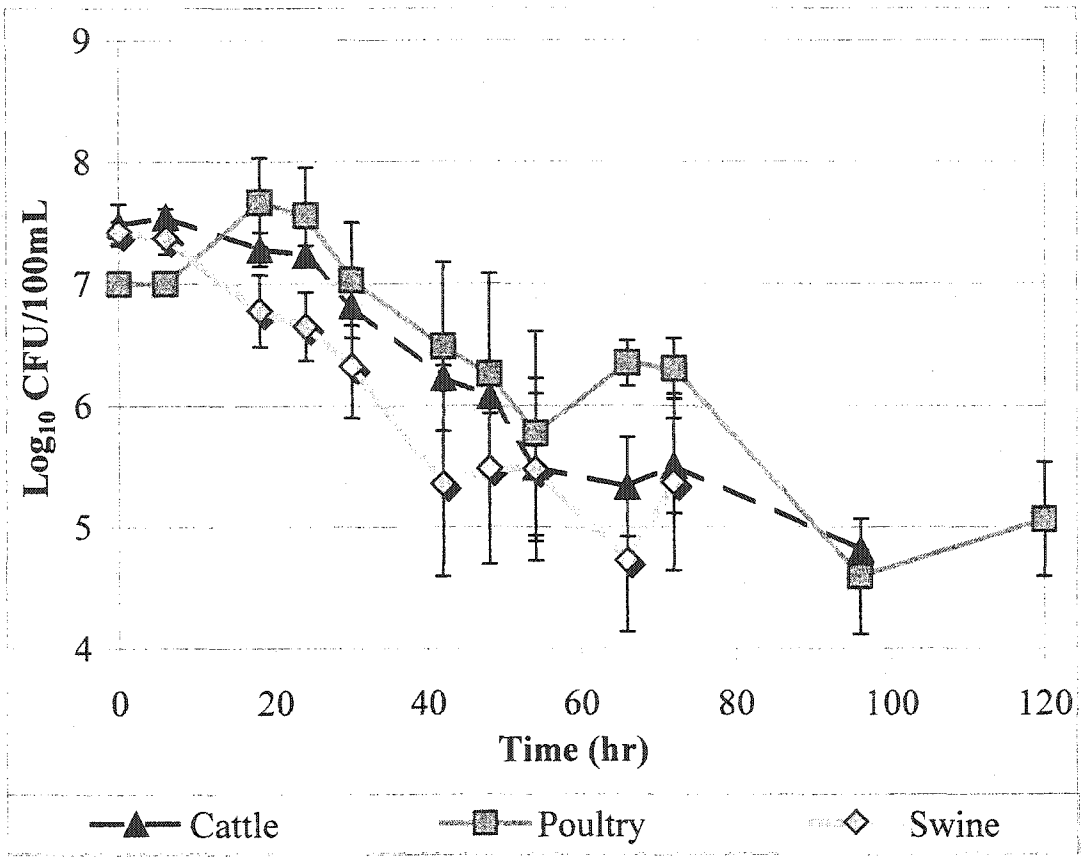

Figure 4.1: Fecal coliform decay patterns. Error bars represent one standard deviation of the mean of three replicates. Data points without error bars have small standard deviations with error bars masked by the symbol.

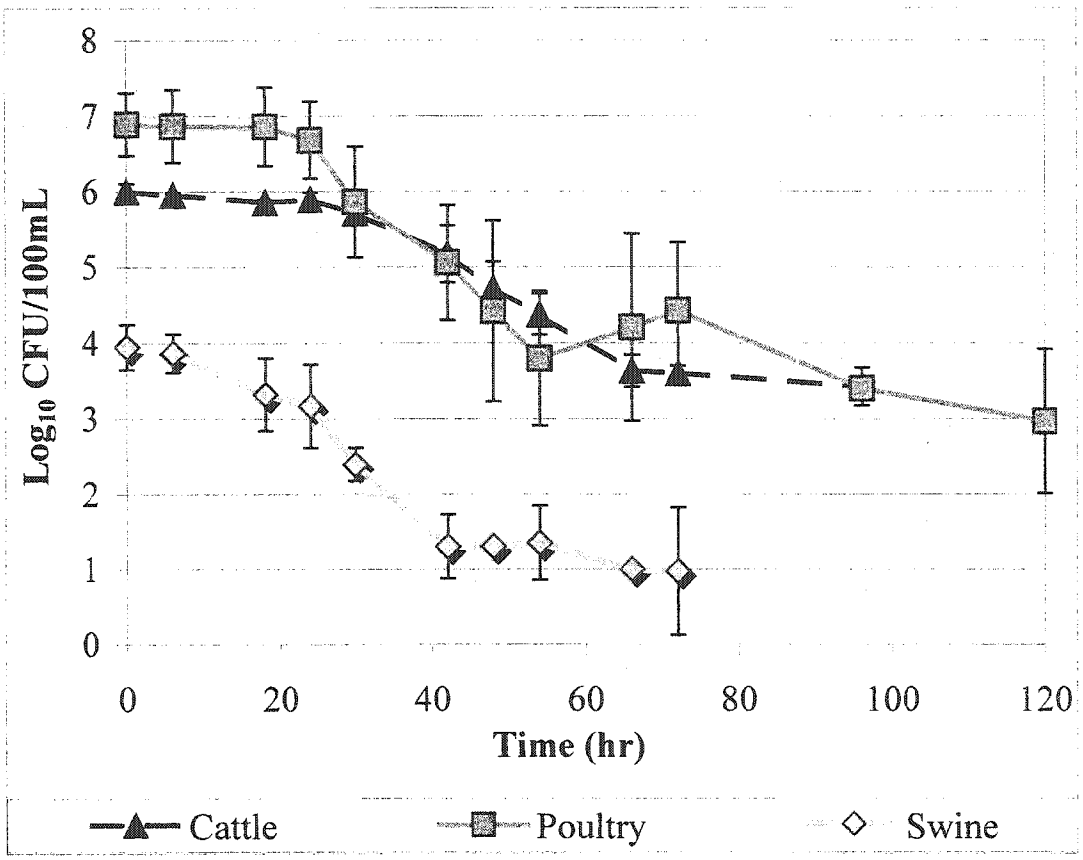

Figure 4.2: Fecal streptococci decay patterns. Error bars represent one standard deviation of the mean of three replicates. Data points without error bars have small standard deviations with error bars masked by the symbol.

that have indicated FC regrowth as a disadvantage to using FC as the sole indication of contamination of surface water (Bitton, 1994; Howell et al., 1996).

The decay curves for FS were not as tightly clustered as were the FC decay curves. Analysis of variance (ANOVA) for FS concentrations in swine mesocosms indicated that the concentrations were significantly less than those in the cattle and poultry mesocosms, with FS concentrations 2 to 3 orders of magnitude lower than cattle or poultry ($p=0.05$). The FS concentrations in the cattle and poultry mesocosms were not statistically different ($p=0.05$). However, like the decay curves for FC, all three curves were sigmoidal and showed lag periods between 6 and 24 hours. Regrowth after initial inoculation was not observed for FS in any of the mesocosms.

The average rate of decay for fecal bacteria in surface water ranged from $-0.085\ hr^{-1}$ for FC in cattle manure to $-0.140\ hr^{-1}$ for FS in swine manure (Table 4.1). ANOVA results indicated that the decay rate observed for FC in cattle manure was significantly different from the decay rates observed for FS in both cattle and swine manure ($p=0.05$) (Table 4.2). The decay rate for FS in swine manure was also significantly different from the rate calculated for FC in poultry manure ($p=0.05$).

Table 4.1: Fecal Bacterial Decay Rates

Manure Source	Fecal Coliform		Fecal Streptococci	
	Average (hr^{-1})	Std Dev	Average (hr^{-1})	Std Dev
Cattle	-0.084	0.009	-0.133	0.015
Poultry	-0.085	0.006	-0.126	0.018
Swine	-0.107	0.031	-0.140	0.021

Table 4.2: Analysis of Variance for Fecal Bacterial Decay Rates*

Manure Source		N	MEAN	Duncan Grouping**	
Cattle	FC	3	-0.085	A A	
Poultry	FC	2	-0.086	A A	
Swine	FC	3	-0.107	A A	B B
Poultry	FS	3	-0.126	A A	B B
Cattle	FS	3	-0.133	A	B B
Swine	FS	3	-0.140		B

* Results obtained using the SAS version 8, PROC GLM, and Duncan's Multiple Range Test for x.

** Means with the same letter are not significantly different.

It is important to note that the differences in decay rates occur between, not within, FC and FS groups. This indicates that an MST method based on either FC or FS species composition could be used because the bacteria within each manure source decay at roughly the same rate. This is supported by a study performed by Harwood et al. (2000) in which a MST method based on antibiotic resistance patterns showed that FC and FS databases classified isolates from known sources with similar accuracy.

Discriminant analysis of the FC and FS species composition data indicated that distinct populations of fecal bacteria exist for each manure source examined (Table 4.3 and 4.4). The average rate of correct classification (ARCC) for FC species composition (ARCC = 84.9%) was lower than the ARCC for FS species composition (ARCC = 100%). For FS species composition data, all three manure sources had rates of correct classification (RCCs) of 100%. This indicates that FS species composition data for each manure source were correctly classified throughout the entire length of the study. This is important because these data indicate that FS species composition for each manure source can be correctly classified as long as these bacteria are present in detectable numbers in surface water. An MST method based on FS species composition can be useful in identifying major contributors to nonpoint source pollution because each manure source has a distinct composition of dominant species that can be detected in the presence of native bacteria.

Table 4.3: Discriminant Analysis Results for Fecal Coliform

Manure Source	Cattle	Poultry	Swine	Total
Cattle	5 83.3	1 16.7	0 0.0	6 100.0
Poultry	1 14.3	5 71.4	1 14.3	7 100.0
Swine	0 0.0	0 0.0	5 100.0	5 100.0
Total	6 33.3	6 33.3	6 33.3	18 100.0

* Discriminant analysis was performed SAS v8 using PROC DISCRIM.

** The number of observations and percent classified into source are listed in each cell.

Table 4.4: Discriminant Analysis Results for Fecal Streptococci

Manure Source	Cattle	Poultry	Swine	Total
Cattle	6 100.0	0 0.0	0 0.0	6 100.0
Poultry	0 0.0	7 100.0	0 0.0	7 100.0
Swine	0 0.0	0 0.0	5 100.0	5 100.0
Total	6 33.3	6 33.3	6 33.3	18 100.0

*Discriminant analysis was performed SAS v8 using PROC DISCRIM.

** The number of observations and percent classified into source are listed in each cell.

The RCCs for FC species composition were lower than those for FS in all manure sources except swine (RCC= 100%). Poultry FC species composition had the lowest RCC at 71.4%. This was followed by cattle with an RCC of 83.3%. When the species composition data for the inoculum used for each mesocosm were removed from the SAS dataset and reanalyzed, the RCCs for both cattle and swine increased. The cattle mesocosms had an RCC of 100%, and the poultry mesocosms had an RCC of 83.3%. The RCC for poultry mesocosms was increased to 100% when the species composition data for the 0-hour sample was removed from the SAS dataset and reanalyzed using PROC DISCRIM. The regrowth of FC in the poultry mesocosms may have contributed to the misclassification of the inoculum and 0-hour samples. The regrowth of FC may have changed the percentages of dominant FC species found in each manure source and led to the incorrect classification of the inoculum and 0-hour samples. This is supported by a study performed by McFeters et al. (1974), which also indicated that FS, as a group, showed more agreement between the die-off rates for individual species than FC. An MST method based on FS species composition may be more useful in identifying the major contributor to non-point source pollution because the dominant FS species in each waste source persist longer in the environment and remain in a constant ratio to one another unlike the dominant FC species.

4.5. CONCLUSIONS

Based on the findings of this study, research into the development of an MST methodology based on fecal bacterial species composition is warranted. Distinct populations of both FC and FS appear to exist and can be classified correctly in

surface water even in the presence of native bacterial populations. However, caution should be used when interpreting FC species composition results because FC regrowth may hinder correct classification in recent contamination.

4.6. ACKNOWLEDGMENTS

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CHAPTER 5

A PHENOTYPIC APPROACH TO SOURCE IDENTIFICATION FOR SURFACE WATER CONTAMINATION

5.1. ABSTRACT

With swine production increasing and becoming centralized on large farms, there is a need for a better, more conclusive method to pinpoint the source of pollution in environmental samples. Traditional methods involving the examination of samples for nutrient enrichment have proven to be inconclusive to name a particular agricultural source as the cause of nutrient enrichment. Therefore, this study was conducted to determine whether a phenotypic microbial source tracking method could be used to provide a more conclusive method for determining the source of pollution in an agriculturally impacted watershed. During the summer of 2000, samples were collected from 11 stream sites and 12 concentrated animal feeding operation lagoons within the North Canadian River watershed in northwest Oklahoma and were analyzed for fecal coliform and fecal streptococci species. After tier testing was performed, four organisms were identified as potential indicator organisms: *L. lactis* ss lactis, *Ent. casseliflavus*, *Ent. gallinarum*, and *C. braakii.* Based on these preliminary results, it is likely that a microbial source tracking methodology can be created based on phenotypic identification of fecal bacterial species in surface water.

5.2. INTRODUCTION

Over the past decade, the number of swine in Oklahoma has increased by 1,053%, while the total number of farms reported to be producing swine has decreased from a high of 8,000 to 2,800 (Williams and Luce, 2000; Willoughby et al., 1998). This indicates that swine production is tending toward larger farms with high swine production (termed concentrated animal feeding operations (CAFOs)). Because of the large number of swine produced on these farms, a large amount of waste is generated. Waste management strategies vary depending on climate, topography, and hydrology and involve solid or liquid waste management systems (NPPC, 1997). Most operations favor a liquid waste management system in which wastes are pumped, flushed, or gravity-fed to clay-lined earthen basins, or lagoons (NPPC, 1997; Liu et al., 1997; Ham and DeSutter, 1999), and excess effluent must be removed periodically to prevent the lagoons from overflowing. This effluent is typically land applied to cropland or pastureland and serves as a low-cost fertilizer.

There are several negative environmental impacts to groundwater and surface water quality associated with effluent infiltration or runoff. One way in which lagoon effluent can impact groundwater and surface water quality occurs with over-application. When lagoon effluent is over-applied to a small area, nutrient concentrations exceed crop uptake and soil retention levels, with the excess being transported to ground and surface waters. Accidental leaks and spills are another way in which lagoon effluent can negatively impact surface and ground waters. Because of the many ways in which lagoon effluent can reach surface and ground waters, it is necessary to have a tool to indicate when CAFO effluent has reached a body of water.

One way in which CAFO pollution has traditionally been monitored is through analysis of nutrient content in surface water and groundwater (Huffman and Revels, 1998; Gilliam et al., 1996; Jokela, 1992). An increase in nutrient concentrations over background or historical levels generally indicates pollution, but because many different agricultural, industrial, and municipal wastes contain high concentrations of nutrients, the pollution cannot be conclusively linked to any particular source. Therefore, a more conclusive method for pinpointing the source of pollution is needed.

The use of microorganisms as indicators of fecal contamination is a common practice. Although fecal coliform (FC) and fecal streptococci (FS) bacteria may be useful for indicating fecal contamination, these groups of organisms are still not specific enough to conclusively identify CAFOs as the source of pollution. Microbial source tracking (MST) can, however, be used to determine the source of undesirable microorganisms in water supplies and food processing facilities (Lyhs et al., 1999; Wong et al., 1999; Hielm et al., 1998; Zanetti et al., 1999; Ralyea et al., 1998, Wiggins et al., 1999, Parveen et al., 1997, Hagedorn et al., 2000). Wiggins et al. (1999) found that measurable differences in the antibiotic resistance patterns of FS isolated from several different nonpoint sources could be used to identify and classify these sources. In an earlier study also involving antibiotic resistance analysis, Parveen et al. (1997) found that multiple-antibiotic-resistance profiles could be used to identify the source of *Escherichia coli* isolates. Ralyea et al. (1998) used ribotyping to identify the source of contamination in a dairy plant. They found that the contaminant, *Pseudomonas fluorescens*, was coming from raw milk and being

passed on to the pasteurized product via contaminated filler nozzles. Hurd et al. (2001) used pulsed-field gel electrophoresis and ribotyping to trace the source of a multistate listeriosis outbreak back to meat produced at a specific turkey processing facility. The source of an outbreak of melioidiosis in Western Australia was also identified using MST (Inglis et al., 1998). In this case, polymerase chain reaction (PCR) ribotyping was used to trace the causal agent, *Burkholderia pseudomallei*, to its source, the domestic water supply.

The objective of this study was to develop a rapid strain tracing method based on phenotypic species identification. The methodology is based on a tier testing approach using microbial identifications obtained through metabolic fingerprinting. A description of the methodology and the results of its application to a watershed in northwest Oklahoma are discussed.

5.3. METHODOLOGY DEVELOPMENT

The first step in this methodology involves the isolation and identification of FC and FS organisms in both potential contaminant source samples and surface water samples to the species or strain. After the bacteria are isolated, a suitable method for identification needs to be determined. This method should be inexpensive, relatively simple to perform, and allow a large number of samples to be analyzed accurately in a short amount of time. Potential methods include both genotypic and phenotypic assays.

One genotypic method for identifying bacterial species or strains is polymerase chain reaction (PCR) ribotyping. Ribotyping uses 16S rDNA sequences that are unique to each microorganism to identify a bacterial isolate (Marlowe et al.,

2000). Although ribotyping gives unambiguous results and can tell if two isolates are different, there are several disadvantages to ribotyping. These include the inability to determine if isolates are the same species when the fingerprints are the same, the large amount of time it takes to complete ribotyping, and the additional metabolic and antibiotic sensitivity tests required to identify a particular strain of bacteria. A final disadvantage to this method is the cost of performing this type of identification. PCR ribotyping is cost prohibitive for most environmental studies due to the large number of colonies necessary to identify to provide an accurate measure of the microbial community in a single sample. Thus, the combination of time and cost factors made PCR ribotyping an unrealistic option for this methodology.

A preferred alternative is a rapid strain tracing method that yields accurate strain identifications with limited time and transfer requirements to prevent changes in strain characteristics while remaining cost effective. This can currently be accomplished through the use of a series of biochemical assays in combination with Gram staining and phenotypic classification.

The biochemical assay used in this study employs a 96 well microtitre plate with a different carbon source in each well. Wells in which the carbon source can be oxidized by the microorganism turn purple, indicating a positive reaction, due to the concomitant reduction of a tetrazolium dye present in each well (Bochner, 1989). Wells in which the carbon source cannot be utilized by the microorganism remain clear. Therefore, the plates can be read manually with relative ease. The pattern of positive and negative reactions produces a metabolic "fingerprint" that is unique to each species or strain of microorganism. Sample plate results are compared to a

database of over 1400 species using a software package. Identification of the sample is made based on the similarity of the pattern to a database organism's pattern. The large amount of carbon sources tested, the relative ease of set up and inoculation of the Microplates™, and the ability to create a database containing microorganisms not currently in the database made this a rapid method for identifying microorganisms that was very useful for this study.

For these reasons, this study performed MST using the biochemical assay described above to identify an unknown isolate from an environmental sample to the strain or species level and then used tier testing to eliminate organisms that were too common in the environment to be reliably used as indicator organisms. Once the isolates are identified, the species identifications for the surface water samples are compared to the species identifications for the potential contaminant sources. This involves a set of three decision criteria in a tier testing methodology (Figure 5.1). The first tier of decision criteria applies to potential indicator organisms based on identifications that were strain-specific. The second tier of decision criteria applies to potential indicator organisms based on identifications that were species-specific. If a strain or species satisfies all decision criteria in tier testing, then it is a potential indicator of contamination from a specific source.

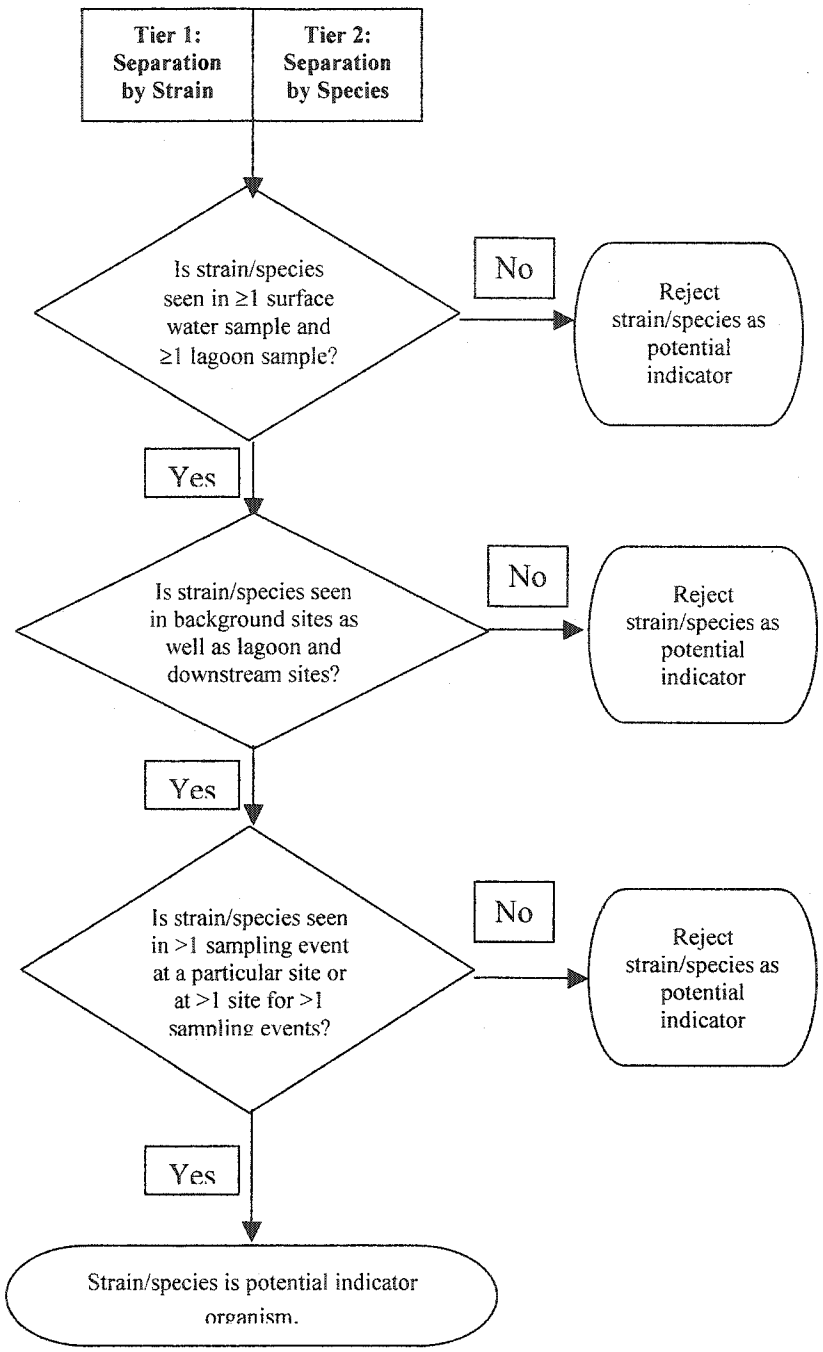

Figure 5.1: Tier testing methodology and decision criteria for determining potential indicator organisms.

5.4. MATERIALS AND METHODS

5.4.1. Sample Collection and Microbial Analysis

To determine if the above methodology could be used successfully to determine potential indicator organisms in an area where swine CAFOs may contribute to water quality degradation, water samples were taken from 11 stream sites and 12 CAFO lagoons in the North Canadian River watershed in northwest Oklahoma. The surface water samples were collected monthly from June to September 2000. These samples were collected from 9 sites that are potentially impacted by CAFO pollution and from 2 background sites. Samples were only collected once from the CAFO lagoons. All samples were preserved as necessary, stored on ice during transportation, and analyzed within 24 hours of collection (APHA, 1992).

Samples were analyzed for FC and FS using the membrane filtration technique (Standard Method 9225 and 9230, respectively) (APHA, 1992). After 24 to 48 hours of incubation, five colonies from each of the FC and FS plates were selected at random and isolated on mFC agar or mEnterococcus agar, respectively. Colonies were reisolated as necessary until a pure culture was obtained; no more than three transfers were performed to limit the potential for changes in metabolic characteristics. All pure culture plates (FC and FS) were stored at 4°C if biochemical assay analysis could not begin immediately.

5.4.2. Biochemical Assay

Each pure culture was reisolated on Biolog Universal Growth agar with 5% sheep's blood and incubated at 35°C for 16-24 hours. Cell mass from these plates

was then transferred to inoculating fluid at a transmittance of 63% for Gram-negative bacteria and 22% for Gram- positive bacteria (Solit, 1999). This culture was then used to inoculate microtitre plates containing 95 carbon sources. These MicroPlates™ provide a metabolic "fingerprint" for the microorganism based on its ability to utilize the 95 carbon sources and are specifically designed for use with either Gram-positive or Gram-negative bacteria. A purple color in any well indicated a positive reaction, while a clear color in any well indicated a negative reaction. After a 16-24 hour incubation period, the positive and negative results were manually determined and input into the MicroLog™ Microbial Identification system (MicroLog™). This system compared the pattern of the sample plate with patterns in the MicroLog™ database of known microorganisms and issued a genus, species, or strain identification based on the results of this comparison. Identification results were then compiled and analyzed through tier testing to determine if an indicator species for CAFO pollution existed.

For every set of 30 microplates analyzed, a set of 5 known microorganisms was also analyzed using the above procedure. The following is a list of the Gram negative and Gram-positive microorganisms that were used: Gram-negative bacteria – *Alcaligenes xylosoxydans*, *Cedecea neteri*, and *Providencia stuartii*; Gram-positive bacteria – *Corynebacterium minutissimum, Rhodococcus equi*, and *Staphylococcus aureus*. Greater than 95% of all quality control organisms were correctly identified.

5.5. RESULTS AND DISCUSSION

5.5.1. Species Identification

The first step in the proposed methodology was to identify the species present in the lagoons and the surface water samples. Twelve lagoons were sampled and analyzed for FC and FS bacteria. Of the total number of FS bacteria identified in the lagoon samples, 87% were *Enterococcus* spp.. Another 4% were identified as *Lactococcus* sp. while the remaining 9% could not be identified. The following distribution was observed for the FC bacteria identified: 44% *Escherichia* spp., 16% *Enterobacter* spp., 5% *Citrobacter* spp., 2% *Klebsiella* spp., 1% *Serratia* spp., and 32% unidentified. Once the species present in the lagoons had been identified, this provided a database of organisms from which potential indicator organisms could be obtained.

The next step was to identify the FC and FS bacteria present in the surface water samples. Overall, 89% of the total FS bacteria identified belonged to the genus *Enterococcus* while 6% could not be identified. The remaining FS bacteria were identified as the following genera: *Carnobacterium* (2%), *Lactococcus* (3%), and *Staphylococcus* (1%). *Escherichia* spp. accounted for 89% of the total number of FC bacteria identified in the surface water samples. Other FC genera identified in the surface water included *Citrobacter* (6%) and *Klebsiella* (3%). Only 3% of the FC bacteria could not be identified. Each species identified was then compared to the database of organisms identified in the lagoon samples. This comparison was used to eliminate organisms that were not potential indicator organisms. Potential indicator organisms are defined as microorganisms that are present in both the treatment

lagoons and impacted surface water samples (but not in background surface water samples).

A total of four microbial strains were present in both lagoon and surface water samples. These strains included *Aeromonas veronii* DNA Group 10, *Pseudomonas putida* Biotype A, *Lactococcus lactis* ss lactis, and *Escherichia coli* (USP5-7085). Nine species were detected in both lagoon and surface water samples: *Aeromonas encheleia*, *Enterococcus faecalis*, *Enterococcus mundtii*, *Escherichia coli*, *Enterococcus avium*, *Pseudomonas alcaligenes*, *Citrobacter braakii*, *Enterococcus casseliflavus*, and *Enterococcus gallinarum*. The species- and strain-specific identifications were then subjected to three decision criteria. These decision criteria were designed to narrow the pool of potential indicator organisms by eliminating microorganisms that are common in the environment or identifications were not reproducible.

5.5.2. Tier Testing

The decision criteria for tier testing are presented in Figure 5.1. *A. veronii* DNA Group 10 was seen in lagoon and downstream surface water samples, however it was also found in the background samples and was, therefore, rejected as a possible indicator species. *P. putida* Biotype A and *E. coli* (USP5-7085) were not seen at more than one sample site and were also rejected. Therefore, from Tier 1, only *L. lactis* ss lactis remained as a potential indicator organism (Table 5.1).

Tier 2 separated microorganisms based on identifications at the species-specific level in both lagoon and surface water samples. *A. encheleia*, *Ent. faecalis*, *Ent. mundtii*, and *E. coli* were eliminated as potential indicator species because of

their presence in the background surface water samples despite being present in the downstream surface water samples. *Ent. avium* and *P. alcaligenes* were not seen in more than one sample for the same sampling event nor were they seen in more than one sampling event at a one site; therefore, they were also rejected as potential indicator organisms. Only *C. braakii*, *Ent. casseliflavus*, and *Ent. gallinarum* remained as potential indicator species from Tier 2 (Table 5.1).

After tier testing was completed, the original pool of 12 potential indicator organisms was decreased to three FS organisms and one FC organism. These organisms were *L. lactis* ss lactis, *Ent. casseliflavus*, *Ent. gallinarum*, and *C. braakii.* Although the three Tier 2 microorganisms are useful indicator organisms because they are specific to fecal matter of mammals, there may be several strains of each of these microorganisms present in the environment. In other words, the species may not be specific enough to conclusively pinpoint the source of pollution. The strain identification is much more useful because it is more specific, and this strain is much less likely to be found in other environments not impacted by CAFO waste.

Table 5.1: Tier Testing Results

	Species Name
Tier 1	*Lactococcus lactis* ss lactis
Tier 2	*Citrobacter braakii*
	Enterococcus casseliflavus
	Enterococcus gallinarum

Ent. gallinarum, *Ent. casseliflavus*, and *L. lactis* ss lactis may be more conclusive indicators of the remaining organisms because they are FS bacteria as opposed to *C. braakii*, which is a FC bacteria. FS are generally rare in uncontaminated surface water and can persist longer in the environment than FC (McFeters, 1974; Bitton, 1999). FS are also more resistant to environmental stress than coliform bacteria (Gerba, 2000). These factors make *Ent. gallinarum*, *Ent. casseliflavus*, and *L. lactis* ss lactis more conclusive indicators of swine waste contamination than *C. braakii*.

5.6. CONCLUSION

When using the source identification methodology proposed in this study, four bacterial species were determined to be the most useful indicators of swine waste contamination: *L. lactis* ss lactis, *Ent. casseliflavus*, *Ent. gallinarum*, and *C. braakii*. Of these, *Ent. gallinarum*, *Ent. casseliflavus*, and *L. lactis* ss lactis are the most promising indicator organisms.

This study shows that a MST method based on phenotypic species identification holds promise for future use. In particular, MST methods that focus on identifying FS organisms as indicators of pollution source may hold more promise because of the advantages these organisms have over FC bacteria, such as rarity in uncontaminated surface water, rates of survival, and resistance to environmental stress.

5.7. ACKNOWLEDGMENTS

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5.8. METHOD REFINEMENT

This chapter was submitted to *Water Environment Research* for publication as a short note. During the review process, a critical self-review of the methodology was performed. Through this self-review, significant omissions of analysis were identified and led to the modification of the methodology. Several of the key questions suggested by the reviewer were also identified during the in-house review. As a result, the decision was made not to resubmit the article with revisions, but rather to address the changes made to the method and how these changes answer key questions brought up by the reviewer about the method's design.

One concern raised during the in-house review of the methodology dealt with the presence of the four organisms discussed in this chapter in the gut fauna of animals other than swine. To address this concern, the method has been revised to give less significance to the presence of a single fecal bacterial species. In the revised method, the composition of FC and FS populations is used to distinguish between waste sources. A known source database has been established with species composition data from four waste sources: human, cattle, swine, and poultry. Species composition data from surface water sources with unknown waste sources are then compared to the database statistically using discriminant analysis, which allows

for classification of data based on qualitative criterion variables (i.e. human, cattle, swine, or poultry). Using a known source database and the composition of the fecal bacterial populations gives less significance to individual species that can be found in numerous waste sources and aids in the discrimination between waste sources. The original methodology called for a tier assessment system. In the revised method, a tier system is no longer used to identify single fecal bacterial species of interest for the indication of swine contamination. From the information obtained through this study, the authors learned that use of a single indicator species was not an adequate method of source tracking.

During review of the methodology, two scenarios appeared necessary to test its effectiveness: one where only fecal bacteria from wildlife are present and one where livestock other than swine are present. This has been addressed in method revision by the inclusion of a known source database that will be validated with samples from a single known waste source. It has also been addressed in this and other future work by the inclusion of background samples with no known source of contamination other than wildlife. This combined with the use of discriminant analysis should strengthen the method.

This chapter was an important first step in the development of this phenotypic microbial source tracking method. Despite the fact that the method has been revised, it is important to present this work to show the progression of the method from its very first stages to the latest, more polished version. Also through this chapter, the importance of FS identifications and the limitations of FC identifications were realized.

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CHAPTER 6

DISCRIMINANT ANALYSIS OF FECAL BACTERIAL SPECIES COMPOSITION FOR USE AS A PHENOTYPIC MICROBIAL SOURCE TRACKING METHOD

6.1. ABSTRACT

A rapidly growing method to identify origins of nonpoint source (NPS) pollution is microbial source tracking (MST). Current MST research utilizes either an organism's genetic or physiological traits to establish source identification. To determine if an MST method based on fecal bacterial species composition can be used to determine sources of NPS pollution, samples from known NPS contributors (human, cattle, poultry, and swine) were collected and analyzed for fecal coliform (FC) and fecal streptococci (FS). Five colonies from each bacterial type were randomly selected, isolated, and identified using phenotypic profiles. The species composition was calculated from these data and analyzed statistically via discriminant analysis. The rates of correct classification (RCC) for FC species composition patterns were 64%, 71%, 47%, and 70% for cattle, human, poultry, and swine, respectively. The RCC for FS species composition patterns were 87%, 86%, 74%, and 83% for cattle, human, poultry, and swine, respectively. The average rate of correct classification for samples from all known sources was significantly higher ($p=0.05$) for FS species composition data (82%) than for FC (63%). The average rate of correct classification was increased when the FC and FS species composition data were combined (93%). The results from this study indicate that a phenotypic MST

methodology based on species composition of dominant fecal bacteria may be useful in determining major contributors to NPS pollution. Based on the average rates of correct classification, the use of FS species composition patterns appears to be more useful in identifying source than the use of FC patterns.

6.2. INTRODUCTION

Traditionally, increases in nutrient concentrations over background levels have been used to assess the environmental impacts of concentrated animal feeding operations (CAFOs) (Huffman and Revels, 1998; Gilliam et al., 1996; Jokela, 1992). However, many sources of pollution, including agricultural, industrial, and urban sources, can increase a surface water body's nutrient content. For instance, nitrate pollution can be caused by such varied sources as crop production, CAFOs, municipalities, industries, and geologic formations (Thompson, 1996). Therefore, pinpointing the specific source of ground or surface water contamination in a watershed with multiple sources of pollution can be difficult.

An alternative to nutrient enrichment studies involves the use of microorganisms for identifying nonpoint pollution sources. One method that has been around for decades involves the use of fecal bacteria, such as fecal coliform (FC) and fecal streptococci (FS), as indicators of fecal contamination of surface and drinking water supplies (Gerba, 2000). Other methods involve the use of microorganisms to identify the source of undesirable microorganisms in food products or to identify the source of disease outbreaks (Ralyea et al., 1998; Hurd et al., 2001). Microbial source tracking (MST) has developed in response to the limitations of the methods.

Recent advances in genetic and biochemical methods of species identification have improved the ease with which environmental samples can be analyzed for specific strains or species of microorganisms. These advances make the use of microorganisms to pinpoint the source of environmental contamination more promising and increase the interest in MST as a method of identifying contributors to NPS for regulatory purposes.

MST methods currently under development can be divided into two main categories: genotypic and phenotypic. Some of the genotypic MST methods being researched include ribotyping, pulsed-field gel electrophoresis, and polymerase chain reaction (PCR) of repetitive intergenic sequences (Carson et al., 2001; Parveen et al., 1999, Simmons et al., 1995; Dombek et al., 2000). These genotypic methods use DNA sequences that are unique to each microorganism to identify the source of the bacterial isolate (Marlowe et al., 2000). Carson et al. (2001) have reported average rates of correct classification (ARCC) of 73.56% for riboprints of *Escherichia coli* from eight separate known sources. Simmons et al. (1995) also reported results using pulsed-field gel electrophoresis (ARCC=51.6%). Dombek et al. (2000) used rep-PCR DNA fingerprinting to successfully differentiate *E. coli* isolates from human and animal sources (ARCC=87.5%).

Phenotypic MST methods under development include antibiotic resistance analysis (ARA) and fecal bacterial species composition. ARA uses the antibiotic resistance patterns of FC or FS bacteria as phenotypic fingerprints to determine the source of fecal bacterial pollution (Wiggins, 1996; Wiggins et al., 2000; Hagedorn et al., 1999; Harwood et al., 2000). Wiggins (1996) was one of the first to report the

usefulness of discriminant analysis in differentiating between human and animal sources of fecal pollution. In this study, Wiggins (1996) reported an ARCC of 74% for six animal sources and 92% for human isolates when antibiotic resistance patterns of FS were analyzed using discriminant analysis. Hagedorn et al. (1999) also used antibiotic resistance patterns of FS isolates to determine sources of fecal pollution, which included beef and dairy cattle, deer, chickens, humans, and waterfowl. ARCC for this study was 87%. When animal sources were pooled, the ARCC for separations between animal and human sources increased to 95%. Harwood et al. (2000) used FC and FS antibiotic resistance patterns to identify sources of fecal pollution in subtropical waters. The sources analyzed in this study included wild birds, cattle, chickens, dogs, pigs, and raccoons. The ARCC were 62.3% and 63.9% for FS and FC, respectively. Each study indicated that ARA could be used to successfully identify the source of fecal pollution in surface water.

Although both the genotypic and phenotypic methods described above have been used successfully to identify nonpoint sources, many of these methods are complex, labor-intensive, and costly. Genotypic MST methods typically concentrate on FC, *E. coli* in particular. This reliance on a single organism may cause problems; especially since studies have shown that *E. coli* have been found in pristine environments (Toranzos and McFeters, 1997). Also, detection and isolation of FC in some composted animal wastes and poultry litter is more difficult than is that of FS (Hagedorn, 1998). In this study, a phenotypic MST method based on fecal bacterial species composition is described. This method was developed to differentiate

between the most common sources of contamination in rural, agricultural settings: human, cattle, poultry, and swine waste sources.

6.3. MATERIALS AND METHODS

To develop an MST method based on fecal bacterial species composition, samples were taken from four known sources: human, cattle, poultry, and swine. Each known source sample was analyzed for FC and FS bacteria using selective media, mFC agar for FC and mEnterococcus agar for FS (Difco Laboratories), and the membrane filtration technique as described by *Standard Methods* 9222C and 9230C, respectively (APHA, 1998) for liquid samples and the swab inoculation technique for solid samples. From each of the resulting FC and FS agar plates, five colonies were randomly selected and isolated on selective media. Five colonies were chosen based on a calculation used for choosing a sampling design for environmental data collection (USEPA, 2002) and existing data for FC and FS concentrations in environmental samples.

After an FC or FS colony had been isolated and a pure plate obtained, an isolated colony from that pure plate was transferred to Biolog Universal Growth Agar containing 5% defibrinated sheep's blood. The maximum number of transfers performed was limited to five to prevent unnecessary stress and limit metabolic changes, as specified by identification procedures set by Biolog, Inc. (Hayward, CA). The growth from the blood agar plate was then diluted and used to inoculate GN2 or GP2 Microplates™ according to the procedures developed by Biolog, Inc. for the identification of gram-negative and gram-positive bacteria, respectively. GN2 Microplates™ required an inoculum with a 63% transmission (%T) for gram-

negative, enteric bacteria while GP2 Microplates™ required an inoculum with a 20%T for gram-positive cocci. For every set of 30 Microplates™ analyzed, a set of 5 known microorganisms and duplicates was also analyzed using the above procedure. The following is a list of the gram-negative and gram-positive microorganisms that were used: gram-negative bacteria – *Escherichia coli (*ATCC 11775) and *Providencia stuartii* (ATCC 33672); gram-positive bacteria – *Corynebacterium minutissimum* (ATCC 23348), *Enterococcus faecalis* (ATCC 19433), and *Staphylococcus aureus ss aureus (*ATCC 12600). Greater than 95% of all quality control organisms were correctly identified.

Each microplate contains 95 different sole carbon sources, which produce a metabolic fingerprint that is specific to species and strains of microorganisms. After incubation, the patterns of positive, negative, and borderline responses were read manually and input into the MicroLog™ Microbial Identification System (Biolog, Inc.; Hayward, CA). The results obtained for each of the five colonies identified were compiled and used to calculate fecal bacterial species composition as an average percentage basis. FC, FS, and fecal bacterial species composition data were obtained for a single known source sample. The fecal bacterial species composition data were obtained by combining the identification results for FC and FS to create a single dataset. The species composition data for all known source samples tested were compiled to create a known source database. Three known source databases were created to determine which database could provide the best separation between source categories.

The species composition data for each known source (human, cattle, poultry, and swine) were analyzed statistically using Statistical Analysis Software (SAS) version 8 (SAS Institute, Inc.; Cary, NC). The PROC DISCRIM function was used to classify the fecal bacterial species composition data into source categories (prior probabilities, equal; covariance matrix, pooled). This function produces a source-by-source matrix with the number and percentage of correctly classified samples for each source located on the diagonal. Average rate of correct classification (ARCC) for each fecal bacterial type was calculated by averaging the percentages of correctly classified samples on the diagonal as reported previously by Wiggins (1996). Ten samples from each known source were used to check the accuracy of the classification results obtained from the known source database. These samples were analyzed statistically using PROC DISCRIM and classified into sources based on the similarities between the known source dataset and the sample.

Spatial plots of the species composition patterns for each database (FC, FS, and fecal bacteria) were generated using canonical discriminant analysis (PROC CANDISC, SAS software). Canonical discriminant analysis derive canonical variables that are a linear combination of variables with the highest multiple correlation with the groups. Plotting the first two canonical variables on an x-y scatter plot displays the accuracy of source identification. A large separation between source groups indicates highly accurate source identification.

6.4. RESULTS

A total of 365 species composition patterns were generated from four known sources (cattle, human, poultry, and swine) and analyzed statistically using

discriminant analysis. The rates of correct classification (RCC) for FC, FS, and combined FC and FS (or fecal bacteria) species composition pattern databases for all four known sources are listed in Tables 6.1-6.3, respectively. The number of observations (top number) and percent classified into source (bottom number) are listed in each cell. RCCs for each source are located along the left-to-right diagonal of each table. ARCCs were calculated for each database by averaging the RCC for each source. Comparison of ARCC for the FS species composition database and the FC species composition database showed a higher RCC for the FS database (82%) than for the FC database (63%). The ARCC increased when the FC and FS species composition data was combined (93%) to create the fecal bacteria species composition patterns.

In order to test the accuracy of the classifications and provide a measure of quality control for each database, ten samples for each known source were reanalyzed against each species composition pattern database (Tables 6.4-6.6). The ARCCs for each species composition pattern database were 68%, 83%, and 95% for the FC, FS, and fecal bacterial databases respectively. These ARCCs were all similar to the ARCCs for the known sources. This indicated that the correct classifications for each known source were not simply due to chance but that true differences between the species composition patterns for each source exist.

Plots of the first two canonical variables (Can1 and Can2) are presented in Figures 6.1 and 6.2. The canonical variables are the linear combinations of the species composition variables that have the highest possible multiple correlations with the source categories. They also summarize between-source category variation.

Table 6.1: Discriminant Analysis Results[1] for Fecal Coliform Species Composition

Manure Source	Cattle	Human	Poultry	Swine
Cattle	35 63.6	0 0.0	11 20.0	9 16.4
Human	0 0.0	10 71.4	0 0.0	4 28.6
Poultry	14 32.6	0 0.0	20 46.5	9 20.9
Swine	11 29.7	0 0.0	0 0.0	26 70.3

Table 6.2: Discriminant Analysis Results[1] for Fecal Streptococci Species Composition

Manure Source	Cattle	Human	Poultry	Swine
Cattle	39 86.7	3 6.7	2 4.4	1 2.2
Human	2 14.3	12 85.7	0 0.0	0 0.0
Poultry	3 15.8	1 5.3	14 73.7	1 5.3
Swine	1 3.3	3 10.0	1 3.3	25 83.3

[1]The number of observations and percent classified into source are listed in each cell.

Table 6.3: Discriminant Analysis Results[1] for Fecal Bacterial Species Composition

Manure Source	Cattle	Human	Poultry	Swine
Cattle	44 97.8	0 0.0	0 0.0	1 2.2
Human	1 7.1	13 92.9	0 0.0	0 0.0
Poultry	3 15.8	0 0.0	16 84.2	0 0.0
Swine	0 0.0	1 3.3	0 0.0	29 96.7

Table 6.4: Discriminant Analysis Results[1] for Check Samples using Fecal Coliform Species Composition

Manure Source	Cattle	Human	Poultry	Swine
Cattle	9 90.0	0 0.0	1 10.0	0 0.0
Human	4 40.0	6 60.0	0 0.0	0 0.0
Poultry	2 20.0	0 0.0	5 50.0	3 30.0
Swine	3 30.0	0 0.0	0 0.0	7 70.0

[1]The number of observations and percent classified into source are listed in each cell.

Table 6.5: Discriminant Analysis Results[1] for Check Samples using Fecal Streptococci Species Composition

Manure Source	Cattle	Human	Poultry	Swine
Cattle	10 100.0	0 0.0	0 0.0	0 0.0
Human	2 20.0	8 80.0	0 0.0	0 0.0
Poultry	1 10.0	1 10.0	8 80.0	0 0.0
Swine	1 10.0	2 20.0	0 0.0	7 70.0

Table 6.6: Discriminant Analysis Results[1] for Check Samples using Fecal Bacteria Species Composition

Manure Source	Cattle	Human	Poultry	Swine
Cattle	9 90.0	0 0.0	0 0.0	1 10.0
Human	0 0.0	10 100.0	0 0.0	0 0.0
Poultry	1 10.0	0 0.0	9 90.0	0 0.0
Swine	0 0.0	0 0.0	0 0.0	10 100.0

[1]The number of observations and percent classified into source are listed in each cell.

Canonical discriminant analysis finds linear combinations of the quantitative values for species composition data that provide the maximum separation between the known sources and displays these linear combinations as canonical variables on spatial plots. These plots show Can1 on the x-axis and Can2 on the y-axis. The spatial displays for all three species composition pattern databases (FC, FS, and fecal bacteria) and all four known sources are displayed in Figure 6.1. In this figure, human species composition patterns in the FS and fecal bacterial databases were clearly distinguishable from the animal sources but did not show as clear a separation for the FC database. The animal sources showed some overlap for all three sources in this figure, and the distinct clustering of these sources was not clearly distinguishable because the human source stretches the x- and y-axes and does not allow for adequate visualization of the separation between the animal sources. In Figure 6.2, the human species composition patterns were removed, and the comparison of the three animal sources resulted in distinct separation of each source. The least distinct separation occurred for the FC database (Figure 6.2a), in which a large amount of overlap occurred between all three animal sources in the center of the spatial plot. In Figure 6.2b, distinct separation between the swine source and the other two animal sources occurred. However, some overlap toward the center of the graph still occurred between the cattle and poultry sources. The most distinct separation of animal sources occurred in the fecal bacteria database, where only a few of the poultry species composition patterns were located near the cattle species composition patterns (Figure 6.2c).

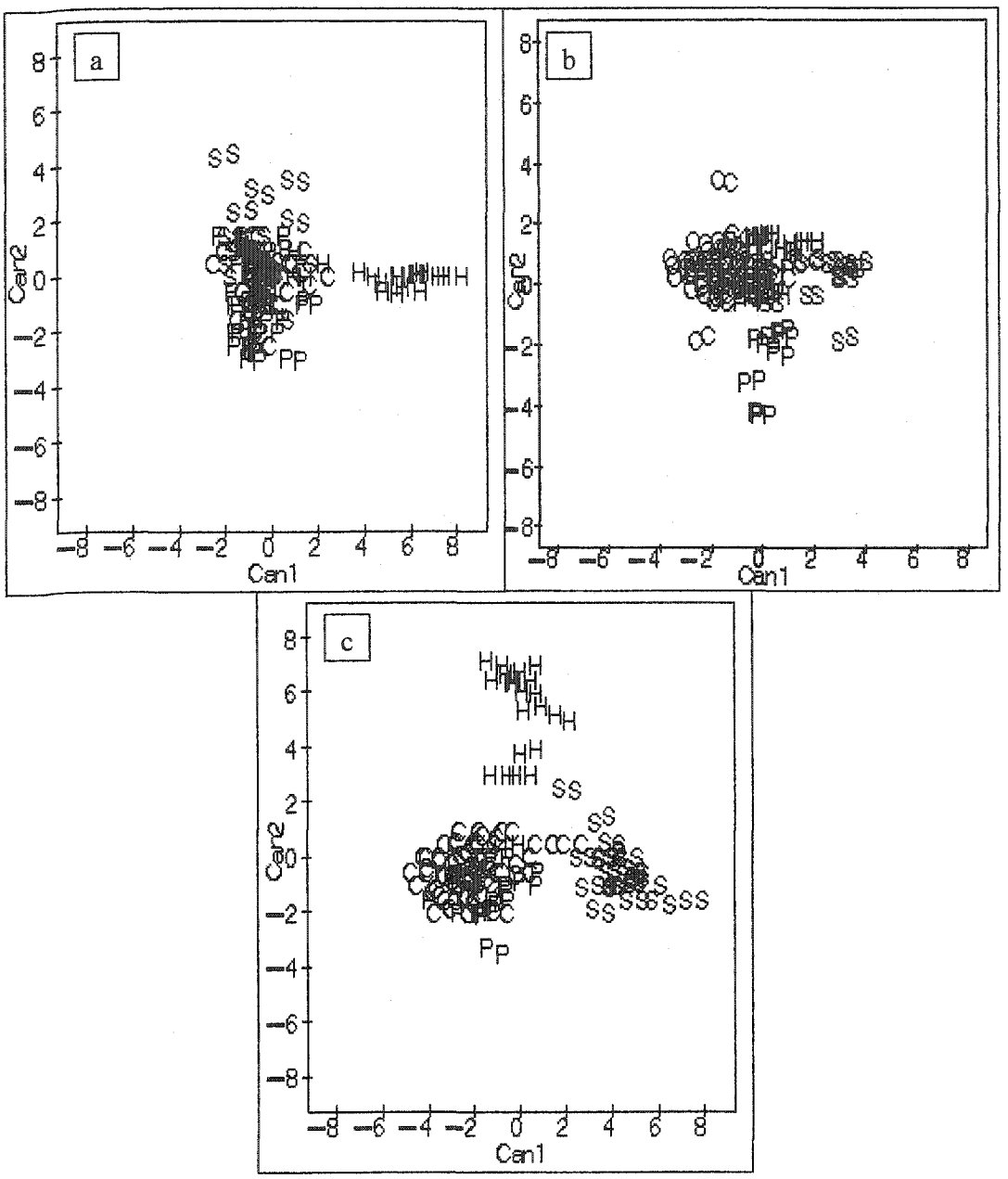

Figure 6.1: Spatial plots of species composition patterns for FC (a), FS (b), and fecal bacteria (c). Position of patterns is indicated by the following symbols: cattle (C), human (H), poultry (P), and swine (S). Can1 is on the x-axis, and Can2 is on the y-axis.

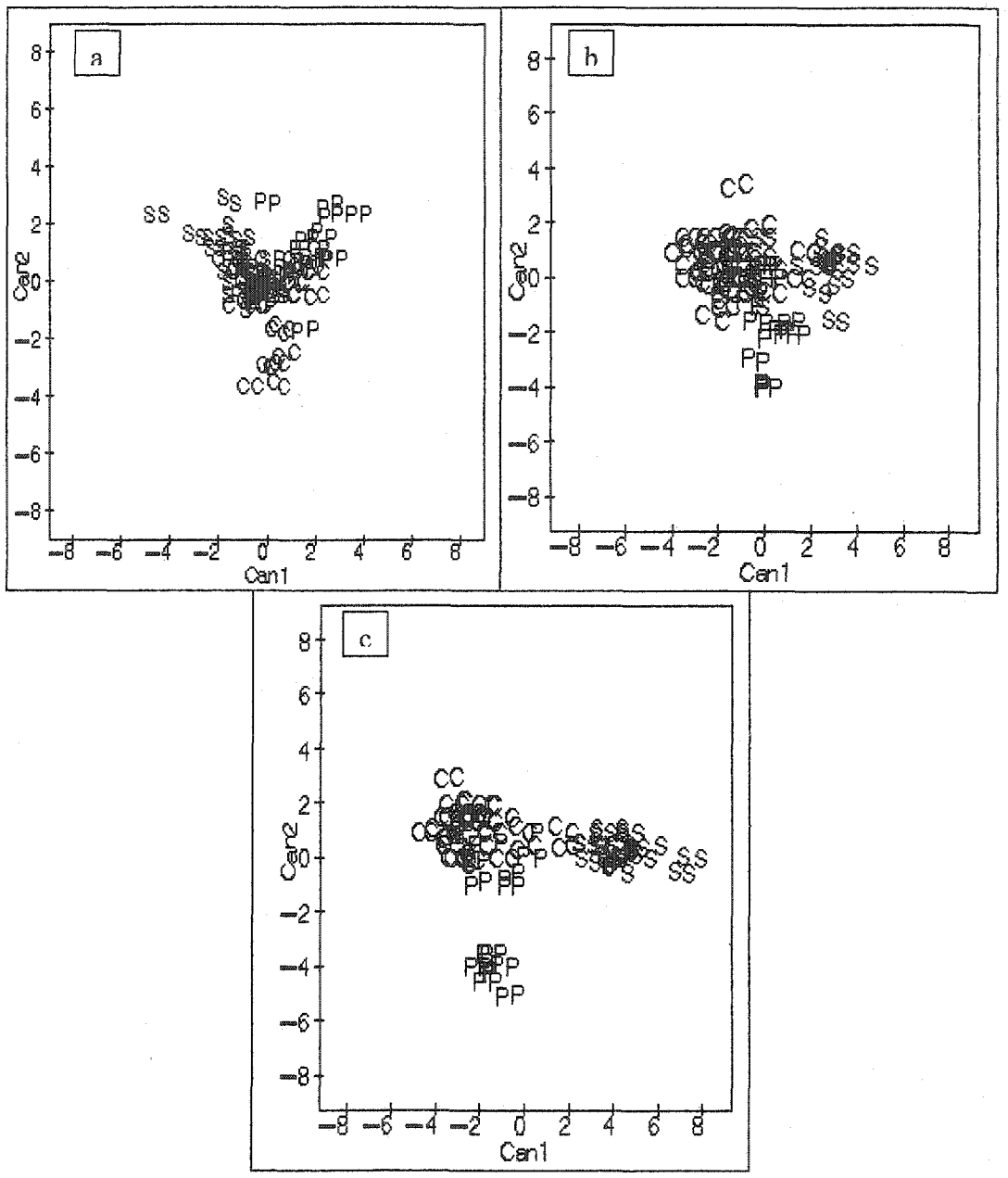

Figure 6.2: Spatial plots of species composition patterns for FC (a), FS (b), and fecal bacteria (c). Position of patterns is indicated by the following symbols: cattle (C), poultry (P), and swine (S). Can1 is on the x-axis, and Can2 is on the y-axis.

6.5. DISCUSSION

The results from this study show that a phenotypic MST methodology based on species composition of dominant fecal bacteria is useful in determining major contributors to NPS pollution. All four sources had distinct species composition patterns that could be correctly classified, with ARCCs ranging from 67.5% to 95%. These ARCCs are similar to those of other genotypic and phenotypic methods, such as ribotyping and antibiotic resistance analysis. Carson et al. (2001) reported an ARCC of 73.56% for riboprints of *E. coli* from eight separate known sources while Dombek et al. (2000), using rep-PCR, reported an ARCC of 87.5% for the differentiation of *E. coli* isolates from human and animal sources. The ARCCs in this study were also similar to those reported by researchers working on antibiotic resistance analysis. For example, using discriminant analysis of FS antibiotic resistance patterns, Wiggins (1996) reported an ARCC of 74% for six animal sources and 92% for human isolates and between 64 and 78% when using four pooled known sources to identify the source of pollution. Hagedorn et al. (1999) also used antibiotic resistance patterns of FS isolates to determine sources of fecal pollution with an ARCC of 87%. Harwood et al. (2000) used FC and FS antibiotic resistance patterns to identify sources of fecal pollution in subtropical waters. The sources analyzed in this study included wild birds, cattle, chickens, dogs, pigs, and raccoons. The ARCC were 62.3% and 63.9% for FS and FC, respectively.

When the FS and FC species composition data were combined to produce the fecal bacteria species composition database, the known sources were the most distinct and have the highest RCC, ranging between 84.2% and 97.8%. The species

composition patterns in this database also showed the most separation in spatial plots (Figures 6.1 and 6.2). The fecal bacteria species composition patterns provide greater separation between known source categories because there are more variables in this database to give each known source more distinct patterns. Most of the current research being conducted in MST deals with *E. coli*, FC, or FS databases, individually (Carson et al., 2001; Wiggins, 1996; Hagedorn et al., 1999). This method would be unique in MST research because it uses both FC and FS in a combined database to improve the classification rates for known sources.

The FC species composition database provided the least separation between the four known sources due primarily to the dominance of *E. coli* in all four known sources; *E. coli* accounted for 36%, 43.7%, 45.2%, and 51.1% of the total FC species identified for cattle, human, poultry, and swine known source samples, respectively. If a human source of contamination were not suspected in a watershed, the human known source species composition patterns could be removed from each database to improve the separation between the animal sources if the FC database is to be used (Figure 6.2). Analysis of water samples with only the animal sources could differentiate between the three most common agricultural sources of fecal pollution. This is supported by a study conducted by Wiggins (1996) that reported improved classification rates, and thus more distinct separation, when the human source was removed and only the animal sources (cattle and poultry in this case) were compared.

The highest percentages of correctly classified isolates were found for the cattle and human sources. Cattle sources were correctly classified most frequently in the FC database while human sources were correctly classified most frequently in the

FS and fecal bacteria databases. The greatest number of samples per source was obtained for the cattle source (55 in the FC database). In contrast, the human source dataset had the fewest number of samples (n=14). Although the different numbers of samples making up each known source dataset is not ideal and will be addressed in future work, it was not a factor that affected classification results. If the number of samples were a crucial factor in the RCC, one would expect the cattle source to have the lowest RCC in all three databases because it represents the source with the most diversity. Instead, the lowest RCC for all three databases occurred in the poultry source, which had 43 samples analyzed in the FC database and 19 samples analyzed in the FS and fecal bacteria databases. Also, when the data were truncated to equalize the number of samples in each known source, the RCCs were not significantly different than those obtained with the unequal sample dataset (data not shown).

Each known source species composition pattern consisted of five FC, five FS, or a combination of the five FC and five FS species identifications. Although this may not be a large number of isolates per sample, it is not meant to provide the complete fecal bacterial community within each sample, which would be an extremely costly and labor-intensive process. It was also determined to be an adequate number according to a method used by the USEPA to calculate sample sizes (USEPA, 2002). The purpose of the five species identifications is to provide only the dominant fecal bacterial species in each sample. This method has shown that five species identifications are sufficient to provide good separation between known sources while balancing the need for cost and labor efficiency. By basing the method on the dominant fecal bacterial species composition patterns instead of the genetic

patterns of a single organism or the antibiotic resistance pattern of a single isolate, it is also believed that some of the changes that may occur due to genetic instability or changes in antibiotic use can be avoided.

The use of this method takes several days to obtain results due to the need for isolation, purification, and identification procedures. The minimum length of time to obtain results is one week and is unlikely to be reduced any farther. Although this may limit the use of this method for water quality testing that requires rapid decision-making, such as beach closures, it will still be useful for regulatory purposes in watersheds with chronic fecal pollution problems or that have recently experienced a spill from an unknown source. The purpose of this method is to determine the source of fecal pollution in impaired watersheds. This will allow regulatory agencies to target best management practices in areas that will do the most to limit the input of fecal bacteria into these watersheds and improve water quality.

The ultimate goal of this methodology is to be able to identify the major contributors to nonpoint source pollution in surface water and groundwater. The results of this study indicate that distinct populations of dominant fecal bacteria, both FC and FS, exist that can be correctly classified into the four major sources of interest with regards to fecal contamination in rural watersheds. Seasonal, temporal, and geographic variations in fecal bacteria may occur that could affect the use of this method, but these issues were not addressed in the present study. With the push for the creation of total maximum daily loads for impaired watersheds across the nation, this method may prove to be useful to regulatory agencies interested in determining the major contributors to fecal pollution.

6.6. CONCLUSIONS

The results from this study indicate that a phenotypic MST methodology based on species composition of dominant fecal bacteria can be useful in determining major contributors to nonpoint source pollution. Based on the average rates of correct classification, the use of fecal bacteria or FS species composition patterns appears to provide more separation between known source species composition patterns than does the use of FC species composition patterns.

6.7. ACKNOWLEDGMENTS

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CHAPTER 7

APPLICATION OF TWO MICROBIAL SOURCE TRACKING METHODS IN A RURAL WATERSHED IN NORTHWEST OKLAHOMA

7.1. ABSTRACT

Several microbial source tracking (MST) methods have been used to identify contributors to nonpoint source pollution, but few studies have compared the results of multiple MST methods used in a single watershed during a single study. In this study, a phenotypic MST method based on FC, FS, and fecal bacterial species composition patterns and a genotypic MST method using ribotyping are used to identify the major contributor to fecal pollution in the Turkey Creek (TC) watershed in north central Oklahoma from August, 2002 to June, 2003 at sample sites TC1, TC2, TC4, and TC5. The phenotypic MST method, fecal bacteria species composition, identified cattle as the major source of pollution in 55.6% of the total samples taken when analyzed via discriminant analysis. The genotypic MST method, ribotyping, identified cattle as the source of 36.9% of the total unknown *Escherichia coli* isolates. Fecal bacteria species composition MST classified 66.7%, 50.0%, 58.3%, and 41.7% of the total species composition patterns as belonging to a cattle source for TC1, TC2, TC4, and TC5, respectively. Ribotyping MST classified 44.6%, 45.1%, and 35.6% of the total isolates as belonging to cattle sources for TC2, TC4, and TC5, respectively, while 30.5% of the total isolates identified for TC1 were classified as human sources. Both methods identified the major source of fecal

pollution in the Turkey Creek watershed as cattle manure and served to cross-validate the results of each method.

7.2. INTRODUCTION

Recent advances in genetic and biochemical methods of species identification have improved the ease with which environmental samples can be analyzed for specific strains or species of microorganisms (Stahl, 1997; White et al., 1997). This has made it feasible to use microorganisms to pinpoint the source of environmental contamination and has increased the interest in microbial source tracking (MST) as a method of identifying contributors to nonpoint source pollution for regulatory purposes.

MST methods currently under development can be divided into two main categories: genotypic and phenotypic. Some of the genotypic MST methods being researched include ribotyping, pulsed-field gel electrophoresis, and PCR of repetitive intergenic sequences (Carson et al., 2001; Parveen et al., 1999, Simmons et al., 1995; Dombek et al., 2000). These genotypic methods use DNA sequences that are unique to each microorganism to identify the source of the bacterial isolate (Marlowe et al., 2000). Carson et al. (2001) have reported average rates of correct classification rates (ARCC) of 73.56% for riboprints of *Escherichia coli* from eight separate known sources. Simmons et al. (1995) reported similar results using pulsed-field gel electrophoresis (ARCC=51.6%). Dombek et al. (2000) used rep-PCR DNA fingerprinting to successfully differentiate *E. coli* isolates from human and animal sources.

Phenotypic MST methods under development include antibiotic resistance analysis (ARA) and fecal bacterial species composition. ARA uses the antibiotic resistance patterns of FC or FS bacteria as phenotypic fingerprints to determine the source of fecal bacterial pollution (Wiggins, 1996; Wiggins et al., 2000; Hagedorn, 1998; Hagedorn et al., 1999; Harwood et al., 2000). Wiggins (1996) was one of the first to report the usefulness of discriminant analysis in differentiating between human and animal sources of fecal pollution. In this study, Wiggins (1996) reported an ARCC of 74% for six animal sources and 92% for human isolates when antibiotic resistance patterns of FS were analyzed using discriminant analysis. Hagedorn et al. (1999) also used antibiotic resistance patterns of FS isolates to determine sources of fecal pollution. The ARCC for this study was 87%. When animal sources were pooled, the ARCC for separations between animal and human sources increased to 95%. Harwood et al. (2000) used FC and FS antibiotic resistance patterns to identify sources of fecal pollution in subtropical waters. The ARCC were 62.3% and 63.9% for FS and FC, respectively. Each study indicated that ARA could be used to successfully identify the source of fecal pollution in surface water.

Although both the genotypic and phenotypic methods described above have been used successfully to identify nonpoint sources, very few studies have examined the use of multiple MST methods to identify the major contributors to fecal pollution in a single watershed. In this study, a phenotypic MST method based on fecal bacterial species composition and a genotypic MST method using ribotyping were used to identify the major contributor to fecal pollution in the Turkey Creek watershed in north central Oklahoma.

7.3. MATERIALS AND METHODS

7.3.1. Background on Turkey Creek Watershed

Turkey Creek is located in north central Oklahoma and is a tributary of the Cimarron River. It originates in southeast Alfalfa County, travels northwest to southeast through Garfield and Major Counties, and connects with the Cimarron River near the city of Dover, OK in Kingfisher County (Figure 7.1). It is located in an area of wheat and cattle production. As a result of the agricultural activities occurring in this watershed, Turkey Creek is listed as a priority-one watershed under section 303D of the Clean Water Act (Becker, 2001). Pollutants of concern in this watershed include nutrients, suspended solids, and fecal bacteria (Becker, 2001; OCC, 1997). In order to determine where to target best management practices in this watershed, a study was conducted by the United States Geological Survey (USGS) to identify the source of fecal pollution through ribotyping MST. The authors were given permission by the USGS project manager to perform the recently developed species composition MST method in conjunction with ribotyping as a way to cross-validate the results of each MST method. The USGS project manager provided ribotyping results and methods.

7.3.2. Sampling Methods

To determine the major contributor to fecal pollution in the Turkey Creek watershed (Figure 7.1), grab samples were taken from four locations (TC1, TC2,

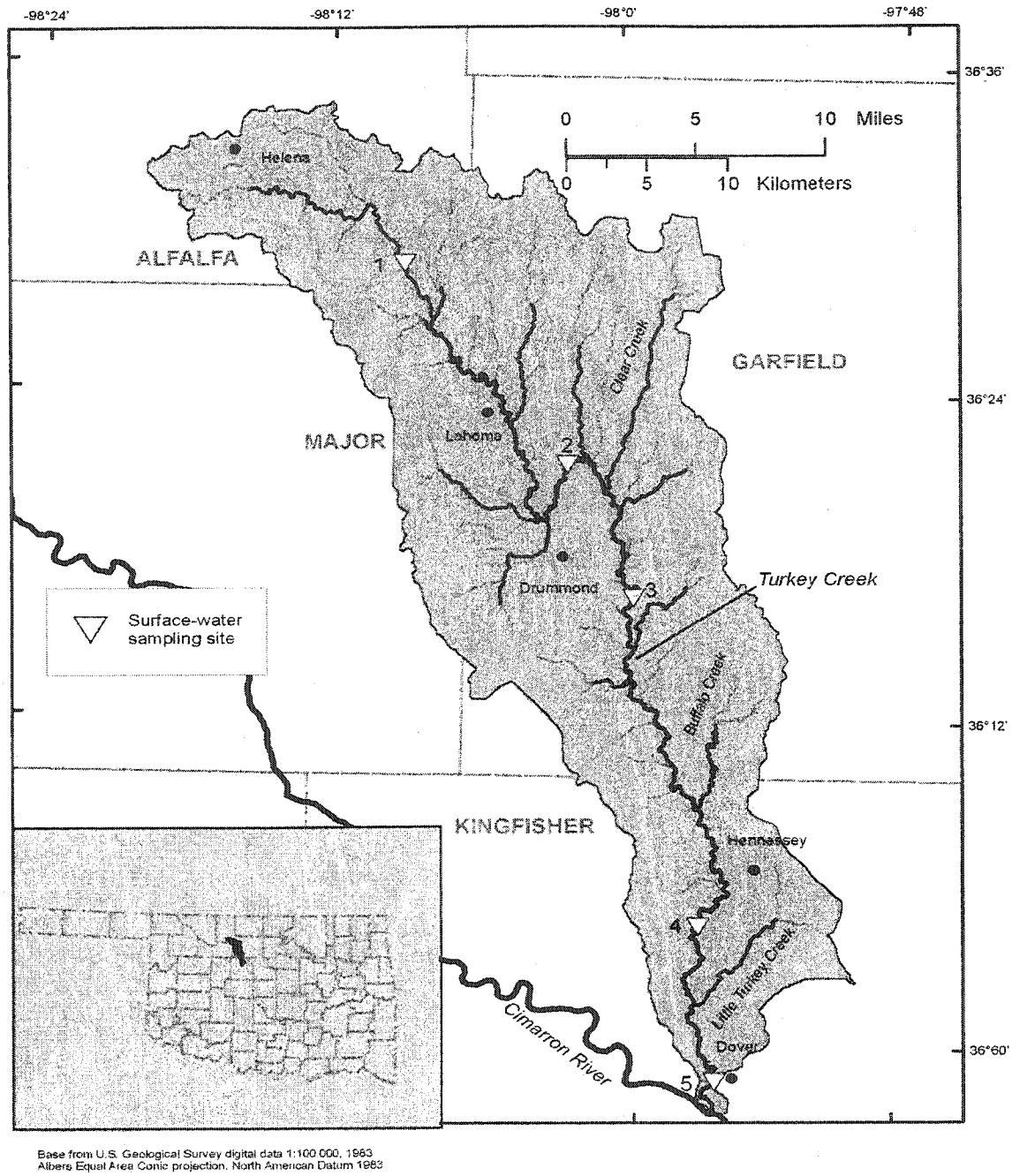

Figure 7.1: Sampling locations on Turkey Creek in the Turkey Creek watershed in North Central Oklahoma.

Sampling locations are labeled with numbered, inverted triangles. Site 3 was not sampled for this study. Map courtesy of Carol J. Becker, United States Geological Survey, Oklahoma City, Oklahoma.

TC4, and TC5) on four separate occasions. Samples for species composition MST were collected in sterile 500 mL polypropylene bottles and stored on ice until returned to the laboratory. Samples for ribotyping MST were collected in sterile 1L polypropylene bottles and stored at 4˚C until the samples could be analyzed.

7.3.3. Species Composition Microbial Source Tracking

A species composition MST method was developed based on fecal bacterial species composition patterns from four known sources: human, cattle, poultry, and swine (Evenson and Strevett, in press). Each known source sample was analyzed for fecal coliform (FC) and fecal streptococci (FS) bacteria using selective media, mFC agar for FC and mEnterococcus agar for FS (Difco Laboratories), and the membrane filtration technique as described by *Standard Methods* 9222D and 9230C, respectively (APHA, 1998). From each of the resulting FC and FS agar plates, five colonies were randomly selected, isolated, and identified for each sample. Five colonies were chosen based on a calculation used for choosing a sampling design for environmental data collection (USEPA, 2002) and existing data for FC and FS concentrations in environmental samples. The identification results were compiled to create FC, FS, or combined FC and FS (fecal bacteria) species composition patterns. A total of 365 species composition patterns were generated from four known sources and analyzed statistically using discriminant analysis to create the known source databases. The ARCC for FC, FS, and fecal bacteria species composition pattern databases for all four known sources were 63%, 82%, and 93%, respectively for FC, FS, and fecal bacteria (FB) species composition databases.

The Turkey Creek samples were analyzed for FC and FS bacteria within 8 hours of collection using selective media, mFC agar for FC and mEnterococcus agar for FS (Difco Laboratories), and the membrane filtration technique as described by *Standard Methods* 9222D and 9230C, respectively (APHA, 1998) in the Center for Restoration of Ecosystems and Watersheds. From each of the resulting FC and FS agar plates, five colonies were randomly selected and isolated on selective media. After an FC or FS colony had been isolated and a pure plate obtained, an isolated colony from that pure plate was transferred to Biolog Universal Growth Agar containing 5% defibrinated sheep's blood. The maximum number of transfers performed was limited to five to prevent unnecessary stress and limit metabolic changes, as specified by identification procedures set by Biolog, Inc. (Hayward, CA). The growth from the blood agar plate was then diluted and used to inoculate GN2 or GP2 Microplates™ according to the procedures developed by Biolog, Inc. for the identification of gram-negative and gram-positive bacteria, respectively. GN2 Microplates™ required an inoculum with a 63%T for gram-negative, enteric bacteria while GP2 Microplates™ required an inoculum with a 20%T for gram-positive cocci. For every set of 30 Microplates™ analyzed, a set of 5 known microorganisms and duplicates was also analyzed using the above procedure. The following is a list of the gram-negative and gram-positive microorganisms that were used: gram-negative bacteria – *Escherichia coli (*ATCC 11775) and *Providencia stuartii* (ATCC 33672); gram-positive bacteria – *Corynebacterium minutissimum* (ATCC 23348), *Enterococcus faecalis* (ATCC 19433), and *Staphylococcus aureus ss aureus (*ATCC 12600). Greater than 95% of all quality control organisms were correctly identified.

Each microplate contains 95 different sole carbon sources, which produce a metabolic fingerprint that is specific to a single species or strain of microorganism. After incubation, the patterns of positive, negative, and borderline responses were read manually and input into the MicroLog™ Microbial Identification System (Biolog, Inc.; Hayward, CA). The results obtained for each of the five colonies identified were compiled and used to calculate FC, FS, or FB species composition patterns for each sample as a percentage. The FB species composition patterns were obtained by combining the identification results for FC and FS to create a single dataset. The species composition data for all known source samples tested were compiled to create a known source database.

The species composition data for each sample were analyzed statistically using Statistical Analysis Software (SAS) version 8 (SAS Institute, Inc.; Cary, NC). The PROC DISCRIM function was used to classify the fecal bacterial species composition patterns into source categories based on pattern similarity between known source and sample. This function produces a source-by-source matrix with the number and percentage of samples classified into each source for each sample.

7.3.4. Ribotyping Microbial Source Tracking

According to the USGS project manager, known source samples were collected from within the Turkey Creek watershed and were analyzed for *Escherichia coli* within 24 hours of collection using Tergitol 7 agar with TTC for samples on swabs as specified by the *Manual of Clinical Microbiology* (Murray et al., 1999) or membrane filtration and mFC agar for liquid samples as specified by *Standard Methods* (APHA, 1998) at the Oklahoma State University Animal Disease Diagnostic

Laboratory (Stillwater, Oklahoma). The isolates were confirmed by inoculation on Triple Sugar Iron Agar, Indole, Citrate, Urea, motility, Lysine Iron Agar, Methyl Red, and Vogas-Proskauer reactions. Isolates that were confirmed to be *E. coli* were sent to Purdue University for ribotyping analysis using the RiboPrinter® Microbial Characterization System (DuPont Qualicon; Wilmington, DE) using methods described previously by Bruce et al. (1997). The riboprints for these known source samples were compiled to create a watershed-specific library.

Surface water samples from Turkey Creek were also analyzed for *E. coli* isolates by the Oklahoma State University Animal Disease Diagnostic Laboratory (Stillwater, Oklahoma) as specified above. *E. coli* isolates were confirmed by inoculation on EC-MUG agar and sent to Purdue University Calumet for ribotyping analysis. Ribotyping analyses were performed at the Department of Biological Sciences at Purdue University Calumet in Hammond, Indiana under the supervision of Dr. Charles Tseng and Dr. Evert Ting. A total of 225 isolates were tested against the watershed-specific library. The isolates that were not identified using that library were reanalyzed using the Purdue University Calumet library.

7.4. RESULTS

In a previously reported study (Evenson and Strevett, in press), an MST method involving the use of fecal bacteria species composition patterns to identify major contributors to fecal pollution was described. Three databases, each with four known sources (cattle, human, poultry, and swine), were developed for FC, FS, and FB species composition patterns with ARCC of 63%, 82%, and 93%, respectively (Evenson and Strevett, in press). In order to test the accuracy of the classifications

and provide a measure of quality control for each database, ten samples for each known source were reanalyzed against each species composition pattern database, with ARCC of 68%, 83%, and 95% for the FC, FS, and FB databases, respectively. These ARCCs were all similar to the ARCCs for the known sources. This indicated that the correct classifications for each known source were not simply due to chance but that true differences between the species composition patterns for each source exist. Results from this study indicated that species composition patterns could be used to accurately identify the source of fecal pollution.

In this study, the ability of the known source databases to correctly identify the source of fecal pollution in a watershed was examined and compared to a well-established MST method that has received considerable research (i.e. ribotyping). Several researchers have used ribotyping to identify the source of fecal pollution in known and environmental samples (Carson et al., 2001; Parveen et al., 1999, Simmons et al., 1995; Dombek et al., 2000). The use of these two MST methods in a single watershed served as a way to cross-validate the results of both methods.

7.4.1. Species Composition Microbial Source Tracking

FC, FS, and FB species composition patterns for the Turkey Creek (TC) samples were obtained and compared to the corresponding known source database via discriminant analysis. Tables 7.1-7.3 present the classification results for the FC, FS, and FB known source databases, respectively. The number of observations (top number) and percent classified into source (bottom number) are listed in each cell. When the FC species composition patterns for each of the samples from the TC watershed were compared to the FC known source database, 87.5% species

Table 7.1: Sample Classification Results[1] using the Fecal Coliform Species Composition Pattern Database

Manure Source	Cattle	Human	Poultry	Swine
TC1[2]	4 100.0	0 0.0	0 0.0	0 0.0
TC2	3 75.0	0 0.0	1 25.0	0 0.0
TC4	4 100.0	0 0.0	0 0.0	0 0.0
TC5	3 75.0	1 25.0	0 0.0	0 0.0

Table 7.2: Sample Classification Results[1] using the Fecal Streptococci Species Composition Pattern Database

Manure Source	Cattle	Human	Poultry	Swine
TC1[2]	1 25.0	0 0.0	2 50.0	1 25.0
TC2	1 25.0	1 25.0	1 25.0	1 25.0
TC4	1 25.0	1 25.0	1 25.0	1 25.0
TC5	1 25.0	2 50.0	0 0.0	1 25.0

[1] The number of observations and percent classified into source are listed in each cell.

[2] TC: Turkey Creek

Table 7.3: Sample Classification Results[1] using the Fecal Bacteria Species Composition Pattern Database

Manure Source	Cattle	Human	Poultry	Swine
TC1[2]	3 75.0	1 25.0	0 0.0	0 0.0
TC2	2 50.0	0 0.0	1 25.0	1 25.0
TC4	2 50.0	2 50.0	0 0.0	0 0.0
TC5	1 25.0	2 50.0	1 25.0	0 0.0

[1] The number of observations and percent classified into source are listed in each cell.

[2] TC: Turkey Creek

composition patterns were classified into the cattle source. The remaining FC species composition patterns were classified into the human and poultry source (6.3% each).

A lower percentage of the FB species composition patterns were classified into the cattle source. Using this database to identify the source of fecal pollution, the source of contamination was identified as cattle for 50% of the species composition patterns (Table 7.3). The source of contamination for another 31.3% of the FB species composition patterns was identified as human. Another 12.5% of the species composition patterns were classified as coming from a poultry source. Finally, 6.2% of the FB species composition pattern was identified as belonging to a swine source. The FS species composition patterns had the most diversity in its sample classification results (Table 7.2). Cattle were identified as the source of fecal pollution in only 25% of the FS species composition patterns. The source of fecal

pollution was evenly distributed at 25% between all four known sources (cattle, human, poultry, and swine).

To determine the major contributor to nonpoint source pollution in the Turkey Creek watershed, the source identifications for all four sampling locations were combined. Cattle manure was identified as the major source of pollution in 30 of the 54 samples via discriminant analysis, which corresponded to 55.6% of the total samples, using the species composition MST method (Table 7.6). When the species composition patterns were averaged for each site and the source identified based on the average species composition pattern, both the FC and FS databases identified cattle manure as the major source of pollution for all four sampling sites. The FB known source database identified cattle manure as the primary source of fecal pollution for all but TC5, which was identified as coming from a human source.

7.4.2. Ribotyping MST

Riboprints for 225 unknown *E. coli* isolates from the four sampling events for TC1, TC2, TC4, and TC5 were compared to riboprints of *E. coli* isolates from a watershed-specific database and a known source database established by Purdue University Calumet for use in ribotyping MST at two different cut-off values (p=0.10 and p=0.05). The classification results for each sampling location are presented in Tables 7.4 and 7.5. Using the less stringent cut-off value (p=0.10), 83 of the 225 unknown *E. coli* isolates were classified as belonging to a cattle source of fecal pollution, corresponding to 36.9% of the total isolates identified (p=0.10) (Table 7.6). When separated into individual sampling locations, 44.6%, 45.1%, and 35.6% of the total isolates isolated for TC2, TC4, and TC5, respectively, were identified as coming

from a cattle source. The greatest percentage of isolates for TC1 was identified as coming from a human source (30.5%). The second most frequently identified source was human fecal pollution (p=0.10). Sixty-five of the 225 unknown isolates from Turkey Creek samples were classified into the human source, corresponding to 28.9%. Deer were also identified as a minor contributor with 13.8% of the total isolates identified as coming from this source. All other sources, including the unidentifiable isolates, were identified as the source of less than 10% of the total isolates.

When riboprints for the unknown isolates were compared to the known source database at the p=0.05 cut-off level, cattle and human sources were identified as the major sources of fecal pollution in the Turkey Creek samples. Fifty-eight of the 225 unknown isolates were classified into the cattle and human sources, corresponding to 25.8% (Table 7.6). The number of unidentifiable isolates increased at this cut-off level, from 5.4% to 28.4% of the total unknown isolates. When separated into individual sampling locations, 28.6%, and 29.4% of the total isolates isolated for TC2 and TC4, respectively, were identified as coming from a cattle source (Table 7.5). The greatest percentage of isolates for TC1 and TC5 were identified as coming from a human source at 25.4% and 32.2% of the unknown isolates, respectively. Deer were also identified as a minor contributor with 12.0% of the total isolates identified as coming from this source. All other sources were identified as the source of less than 5% of the total isolates. For each site, the percentage of unidentifiable isolates ranged from 45.1% and 22.0%.

Table 7.4: Classification Results[1] for *E. coli* Isolates from Each Sample Location using the USGS/Purdue Ribotyping Database (p=0.10)

Manure Source	Cattle	Human	Deer	Horse	Raccoon	Seagull	Sheep	Unknown
TC1[2]	14	21	13	7	1	1	1	1
	23.7	35.6	22.0	11.9	1.7	1.7	1.7	1.7
TC2	25	12	10	4	0	2	0	3
	44.6	20.4	17.9	7.1	0.0	3.6	0.0	5.4
TC4	23	13	2	5	1	1	0	6
	45.1	25.5	3.9	9.8	2.0	2.0	0.0	11.8
TC5	21	19	6	4	1	0	0	7
	35.6	32.2	10.2	6.8	1.7	0.0	0.0	11.9

Table 7.5: Classification Results[1] for *E. coli* Isolates from Each Sample Location using the USGS/Purdue Ribotyping Database (p=0.05)

Manure Source	Cattle	Human	Deer	Horse	Goose	Raccoon	Seagull	Sheep	Unknown
TC1[2]	10	15	13	3	3	1	0	1	13
	17.0	25.4	22.0	5.1	5.1	1.7	0.0	1.7	22.0
TC2	16	13	8	1	1	0	1	1	15
	28.6	23.2	14.3	1.8	1.8	0.0	1.8	1.8	26.8
TC4	15	11	0	1	0	0	1	0	23
	29.4	21.5	0.0	2.0	0.0	0.0	2.0	0.0	45.1
TC5	17	19	6	3	0	1	0	0	13
	28.8	32.2	10.2	5.1	0.0	1.7	0.0	0.0	22.0

[1] The number of observations and percent classified into source are listed in each cell.

[2] TC: Turkey Creek

Table 7.6: Average Classification Results[1] for the USGS/Purdue Ribotyping Database and the Fecal Bacteria Species Composition Databases

Manure Source	Cattle	Human	Poultry	Swine	Deer	Horse	Goose	Raccoon	Seagull	Sheep	No ID
Species Composition Databases	30 55.6	11 20.3	8 14.8	5 9.3	NA	NA	NA	NA	NA	NA	NA
USGS/Purdue Database (p=0.10)	83 36.9	65 28.9	NA[2]	NA	31 13.8	20 8.9	1 0.4	3 1.3	4 1.8	1 0.4	3 5.4
USGS/Purdue Database (p=0.05)	58 25.8	58 25.8	NA	NA	27 12.0	8 3.6	4 1.8	2 0.9	2 0.9	2 0.9	64 28.4

[1] The number of observations and percent classified into source are listed in each cell.

[2] NA: Not Applicable. These sources were not used as known sources for each database.

7.5 DISCUSSION

7.5.1. Source Identification

The Turkey Creek watershed contains approximately 15,200 people and 9,300 cattle (C.J. Becker, personal communication). TC1 was the northern-most sample taken in this watershed (Figure 7.1). Its surrounding land use consists primarily of pasture/hay and row crops. Samples taken from this site and analyzed with species composition MST were classified as having species composition patterns consistent with a 66.7% cattle source of fecal pollution. Ribotyping identified the source of fecal pollution as human for 35.6% of the *E. coli* isolates and cattle for 23.7% of the isolates at the p=0.10 cut-off value. At the p=0.05 cut-off value, 25.4% of the *E. coli* isolates were classified as associated with a human source of fecal pollution while 16.9% of the *E. coli* isolates were classified as associated with a cattle source. At this site, cattle are the primary source of fecal pollution given the land use that immediately surrounds the site as opposed to a human source (Figure 7.2). The nearest major source of human fecal pollution to TC1 is located upstream of this site and comes from the city of Helena and a correctional facility located near Helena. The city of Helena provides sewage utilities for residents and businesses within city limits via primary sewage treatment in sewage lagoons and is permitted for land application of the sewage solids on alluvial deposits along Turkey Creek (C.J. Becker, personal communication). The sewage lagoons operated by the city of Helena are located south of the city, and solids from these lagoons are applied on 80 acres in the upper reaches of the Turkey Creek watershed. Households and businesses outside

of city limits use private septic tank systems for wastewater disposal. These local septic tanks may also contribute fecal bacteria to Turkey Creek at this site.

A cattle source of fecal pollution was identified for TC2 using both the species composition and ribotyping MST methods. Fifty percent of the species composition patterns were classified as associated with a cattle source while 44.6% ($p=0.10$) and 28.6% ($p=0.05$) of the riboprints for E. coli isolates at this site were identified as belonging to a cattle source. These identifications are supported by the land use immediately surrounding the site (Figure 7.2), which is primarily row crop/grains and pasture/hay. During sampling cattle were seen to be grazing near this sample site and have access directly to Turkey Creek and its tributaries in many locations throughout the watershed.

TC4 was located on the southern portion of Turkey Creek in Kingfisher County. Fifty-eight percent of the species composition patterns obtained for this site was identified as associated with a cattle source of fecal pollution. Ribotyping also identified cattle as the source of fecal pollution at TC4 at both the 0.10 and 0.05 cut-off values (45.1% and 29.4%, respectively). As with TC2, this site is located near pasture/hay, grassland, and row crop/small grains land use areas. This land use supports the identification of cattle as the major contributor to fecal pollution at this site.

Unlike the other sampling locations, TC5 did not clearly identify a single source of fecal pollution in either MST method. Both MST methods identified both cattle and human as major contributors to nonpoint source pollution. Using the species composition MST method, 41.7% species composition patterns were

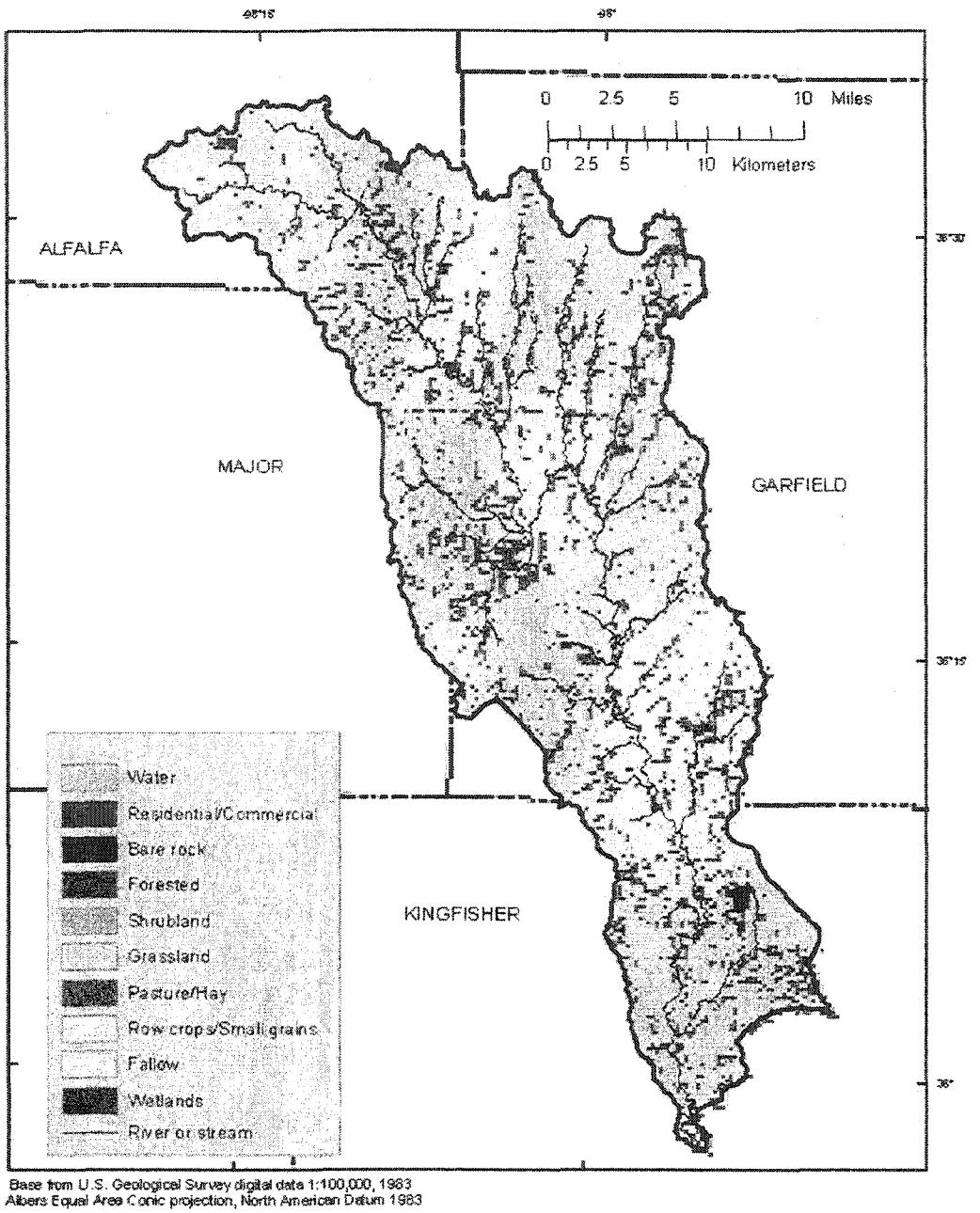

Figure 7.2: Land use in the Turkey Creek watershed, 1992.

Land use breaks down into the following percentages: row crop/small grains – 75.63%, pasture/hay – 10.95%, grassland – 9.35%, forested – 1.78%, residential/commercial – 0.78%, water - 0.68%, shrubland – 0.60%, wetlands – 0.19%, urban grasses – 0.02%, and bare rock – 0.01%. (US Environmental Protection Agency, 2000). Map courtesy of Carol J. Becker, United States Geological Survey, Oklahoma City, Oklahoma.

classified into both the cattle source and the human source. Ribotyping identified 32.2% *E. coli* isolates at this site as associated with a human source at both the 0.05 and 0.10 cut-off values. The next most frequently classified source for the ribotyping MST method was cattle at 28.8% and 35.6% for the 0.05 and 0.10 cut-off value, respectively. The mixed classification results at this site can be explained by the land use surrounding this site. In addition to the pasture/hay and row crop/small grains land use categories found at the other three Turkey Creek sampling locations, TC5 was also located downstream of the city of Dover. This site may be receiving fecal loads from both cattle grazing near this site as well as from the municipal wastewater treatment facility. The city of Dover uses primary sewage treatment for residents and businesses within city limits. Sewage is pumped into one or more lagoons where solids settle out and organic material decomposes. While the sewage lagoons are located outside of the Turkey Creek watershed, Dover is permitted for land application of the sewage solids on alluvial deposits along Turkey Creek (C.J. Becker, personal communication). Households and businesses outside of city limits also use private septic tank systems for wastewater disposal, which may also be contributing fecal bacteria to the watershed.

Both MST methods identified cattle as the major contributor to fecal pollution in the Turkey Creek watershed with human sources of fecal pollution as a secondary contributor to the watershed near the residential area of Dover, OK. This source identification is supported by the land use and agricultural production occurring in the watershed. The primary land use in this watershed is row crops/small grains followed by pasture/hay, which is an indirect indicator of the

presence of cattle in the watershed. Direct comparison of all of the results from the two MST methods was complicated by the lack of poultry and swine known source categories in ribotyping known source database and the lack of a wild known source category in the species composition databases.

7.5.2. Recommendations for Species Composition MST

In a previous study (Evenson and Strevett, in press), it was recommended that either the FS known source database or the fecal bacteria database be used to identify fecal pollution sources. This was recommended because these two databases provided the most separation between the known sources. The FC database did not provide as much separation as between the animal sources as the other two databases because of the dominance of *E. coli* in each known source sample and the inability of the MicroLog™ Microbial Identification System to distinguish between environmental strains of *E. coli*. When samples were analyzed using the FS database, no clear source of contamination was identified despite the fact that the watershed contains no swine or poultry concentrated animal feeding operations. The fecal bacteria species composition patterns gives less weight to the presence of *E. coli* in known source samples while still providing adequate separation between known sources. Based on the results of this study, it is recommended that all future work be focused on the fecal bacteria species composition database.

One advantage the fecal bacteria species composition MST method has over the ribotyping MST method is its use of fecal bacteria population composition rather than a reliance on a specific organism. In an article by Harwood (2002), three directions were identified with regards to research in MST. The first was the search

for a single indicator organism that is specific to a single species. The second approach involved the use of subtypes that are exclusive to certain sources. The final research direction focused on the composition of populations of bacteria in sources instead of the use of individual species. Harwood (2002) concluded that this third approach has the greatest potential to identify the sources of fecal pollution in natural waters. The species composition MST method uses these compositions and has been shown to adequately identify the source of fecal pollution in a watershed as well as providing comparable results to a well-researched method such as ribotyping. Based on the results of this study, further research into the species composition MST method is warranted.

7.6. CONCLUSIONS

Both methods identified the major source of fecal pollution in the Turkey Creek watershed as cattle manure and served to cross-validate the results of each method. The species composition MST method warrants further research to expand the database and research its ability to identify sources of fecal pollution in other watersheds. Future research on the species composition MST method should be focused on the fecal bacteria species composition database instead of the individual FC or FS databases. Best management practices that control cattle access to Turkey Creek and its tributaries will be the most effective ways to improve the water quality in this watershed.

7.7. ACKNOWLEDGMENTS

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CONCLUDING REMARKS

The goal of this project was to investigate the possible uses of microorganisms in the assessment of the environmental impact of animal wastes on water quality. It was achieved through the examination of three main topic areas: microbial community analysis of CAFO lagoons, fecal bacterial survivability, and microbial source tracking (MST) in surface water. The results of each study described in this dissertation showed that fecal bacteria could be used through a fecal bacteria species composition microbial source tracking (MST) method to identify the major contributors to fecal pollution.

The results from Chapter One indicate that an MST method based on fecal bacteria species composition patterns could be used to distinguish between breeding, nursery, and finishing lagoons as sources of fecal pollution attributed to swine in a watershed. Further work needs to be done to determine if the differences between species composition patterns could be determined in surface water with other contributing sources.

Chapter Two showed that CLPP could be used to determine differences between production stage-associated lagoons. Each production stage had unique carbon source utilization patterns that could be identified statistically via principal component analysis and canonical discriminant analysis. Based on the results of this study, an MST method based carbon source utilization patterns could be used to distinguish between breeding, nursery, and finishing lagoons as sources of fecal pollution attributed to swine in a watershed. Carbon source utilization patterns may

prove to be a quick, inexpensive, and accurate method for identifying nonpoint source pollution attributed to swine to the production stage level. Further work needs to be done to determine if the differences between carbon source utilization patterns could be determined in surface water with other contributing sources.

The stability and distinctness of fecal bacteria species composition patterns in lagoon mesocosms was examined in Chapter Three. FC and FS bacteria were shown to be capable of survival under the conditions encountered in the initial days after deposition and flushing into lagoons. The secondary addition of wastes to the mesocosms did not significantly alter the concentrations in each mesocosm. Dominant species composition patterns were correctly classified for each known source throughout the first 14 days of incubation under lagoon conditions. This indicated that a microbial source tracking method based on species composition patterns could be used successfully to identify major contributors to nonpoint source pollution even if the source of the pollution was aged under lagoon conditions.

Chapter Four examined the survival rates and patterns of fecal bacteria in surface water mesocosms. The results from this study showed that research into the development of an MST methodology based on fecal bacterial species composition was warranted. Distinct populations of both FC and FS appear to exist and can be classified correctly in surface water even in the presence of native bacterial populations. However, caution should be used when interpreting FC species composition results because FC regrowth may hinder correct classification in recent contamination.

Chapters Five and Six presented work done to develop a phenotypic microbial source tracking method using fecal bacteria species composition patterns. Chapter Five presented the initial work on such a method and was an important first step in the development of this phenotypic microbial source tracking method. It showed the progression of the method from its very first stages to the latest, more polished version, which was presented in Chapter Six. The results from Chapter Six indicated that a phenotypic MST methodology based on species composition of dominant fecal bacteria could be useful in determining major contributors to nonpoint source pollution. Based on the average rates of correct classification, the use of fecal bacteria or FS species composition patterns appears to provide more separation between known source species composition patterns than does the use of FC species composition patterns. The importance of fecal streptococci identifications and the limitations of fecal coliform identifications were identified primarily in these two chapters.

In Chapter Seven, the species composition MST method developed in Chapter Six and a ribotyping MST method were used in the Turkey Creek watershed in north-central Oklahoma. Both methods identified the major source of fecal pollution in the Turkey Creek watershed as cattle manure and served to cross-validate the results of each method. The species composition MST method warrants further research to expand the database and test its ability to identify sources of fecal pollution in other watersheds. Future research on the species composition MST method should be focused on the fecal bacteria species composition database instead of the individual FC or FS databases. Best management practices that control cattle access to Turkey

Creek and its tributaries will be the most effective ways to improve the water quality in this watershed.

When used by regulatory agencies, the MST method developed in this dissertation could be use to target best management practices and improve water quality in impaired watersheds. In order to be useful to regulatory agencies and increase the robustness of the method, several additional projects need to be completed. The first thing that needs to be done to increase the strength of this method in the identification of sources of fecal pollution is the addition of known source species composition patterns to each database. Although results from Chapter Six indicated that the unequal number of known source species composition patterns did not adversely affect the methods ability to correctly classify these patterns, increasing and equalizing the number of patterns for each known source would improve the statistical reliability of the method. Determining an adequate known source database size also needs to be examined.

A second area of research that merits consideration is the effect of geography on species composition patterns from known sources. It is not yet known how these patterns will vary between herds or flocks from one part of the country to the next. A study would need to be designed to examine the geographical stability of known source species composition patterns across the country and perhaps worldwide. The geographical stability of these patterns will determine whether a known source database created in Oklahoma would be applicable in North Carolina or other parts of the world or if a known source database would need to be created for each watershed,

state, or country, thus increasing the cost of using the method for any regulatory purpose.

The temporal stability of the MST method presented in this dissertation should also be examined. An interesting question that should be addressed is if the species composition patterns of each known source are stable over time. While this method deals with the dominant fecal bacteria in known source samples and should prevent changes in known source species composition patterns as a result of minor changes in diet or environmental conditions, it has yet to be determined how stable these dominant species composition patterns are over the long term, say over a period of months or years, within a single organism.

Finally, I believe it is necessary for other researchers to use this method in order to determine its repeatability. Ideally, this would be accomplished through a national study in which several laboratories would analyze the same samples using a set of standard methods for this MST method. It is important to have multiple investigators obtain the same results for the same samples if this method is to prove to be useful for regulatory purposes.

Overall, the results presented in this dissertation show that an MST method based on fecal bacteria species composition can be used to correctly classify species composition patterns. It has also been used successfully to identify sources of fecal pollution in a watershed and provides results comparable to ribotyping, an established method for MST. One advantage the MST method developed here has over its genotypic counterparts is that its results can be communicated relatively easily to

public and managerial audiences without the need for extensive knowledge of complex genetic techniques.

APPENDICES

APPENDIX 1: IN SITU PARAMETER MEASUREMENTS

Site #	Site Visit	Water Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
SRS1	1	16.6	8.10	1.13	6560
	2	11.4	NA	1.28	6690
	3	7.4	8.11	0.46	7210
	4	13.6	7.77	0.92	8130
	5	21.3	7.90	NA	9630
	6	24.3	7.90	0.11	8980
SRS2	1	17.4	8.07	1.09	5140
	2	12.8	NA	1.02	5300
	3	6.6	7.95	0.56	5830
	4	13.3	8.00	0.43	7220
	5	21.1	7.86	NA	8370
	6	25.4	7.85	0.12	7810
SRS3	1	16.9	8.23	1.07	8010
	2	11.0	NA	0.24	9990
	3	6.8	8.10	1.44	9930
	4	12.4	7.78	0.33	9310
	5	21.9	7.75	NA	9560
	6	25.8	6.73	0.28	8320
SRS4	1	17.9	7.79	0.11	9400
	2	12.8	NA	0.51	9140
	3	5.7	7.93	0.89	10000
	4	12.0	7.67	0.19	9880
	5	21.2	7.74	NA	9310
	6	27.4	7.75	0.13	7860
SRS5	1	18.2	8.34	0.43	11260
	2	9.7	NA	0.26	11810
	3	9.7	8.29	0.69	11540
	4	14.5	8.24	0.20	11570
	5	22.3	7.97	NA	12090
	6	28.6	8.04	0.27	11150
SRS6	1	18.9	8.30	0.14	13640
	2	10.7	NA	0.13	13990
	3	7.0	8.34	0.17	13950
	4	12.4	8.24	0.09	14170
	5	22.4	7.97	NA	13310
	6	29.2	7.95	0.09	12410

APPENDIX 2: WEATHER PARAMETER MEASUREMENTS

Site #	Site Visit	Wind Speed (m/s)	Wind Direction	Ambient Temperature (°C)	Solar Radiation (W/m²)
SRS1	1	6.7	NNW	13	3.6E+05
	2	8.5	NNW	16	3.3E+05
	3	5.4	SSE	-7	8.2E+04
	4	7.2	NNE	10	4.5E+05
	5	4.9	NNE	19	5.0E+05
	6	7.2	SW	31	5.0E+05
SRS2	1	6.7	NNW	14	4.1E+05
	2	6.7	NNW	18	3.7E+05
	3	4.9	SSE	-8	5.1E+04
	4	7.6	NNE	9	2.5E+05
	5	4.5	N	18	1.1E+05
	6	7.6	SW	29	4.3E+05
SRS3	1	6.7	NNW	14	4.3E+05
	2	7.6	NNW	18	3.8E+05
	3	5.8	SSE	-5	1.7E+05
	4	7.6	NNE	12	5.3E+05
	5	2.7	NE	19	5.4E+05
	6	6.7	SW	32	5.8E+05
SRS4	1	5.8	NNW	15	4.7E+05
	2	5.8	NNW	19	3.3E+05
	3	5.8	SE	-7	1.3E+05
	4	7.2	NNE	13	5.3E+05
	5	4.0	NNW	19	4.1E+05
	6	6.7	SSW	32	5.5E+05
SRS5	1	5.8	NNW	16	2.3E+05
	2	7.2	NNW	19	2.7E+05
	3	6.3	SSE	-4	1.5E+05
	4	7.6	NNE	13	4.8E+05
	5	4.9	NE	21	4.7E+05
	6	6.7	SSW	33	6.1E+05
SRS6	1	6.3	NNE	17	2.9E+05
	2	6.3	NNE	18	1.8E+05
	3	7.6	SSE	-4	1.8E+05
	4	6.3	NNE	14	6.2E+05
	5	2.7	NNE	20	5.1E+05
	6	7.6	S	33	6.3E+05

APPENDIX 3: SITE AVERAGE ANALYSIS OF VARIANCE FOR AMBIENT TEMPERATURE

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparison-wise error rate, not the experiment-wise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	150.8029

Number of Means	2	3	4	5	6
Critical Range	14.48	15.22	15.69	16.04	16.29

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	16.467	6	SRS6
A			
A	16.133	6	SRS5
A			
A	15.183	6	SRS4
A			
A	15.083	6	SRS3
A			
A	13.617	6	SRS1
A			
A	13.250	6	SRS2

APPENDIX 4: SITE AVERAGE ANALYSIS OF VARIANCE FOR WATER TEMPERATURE

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	52.85778

Number of Means	2	3	4	5	6
Critical Range	8.573	9.009	9.292	9.494	9.646

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	17.167	6	SRS5
A			
A	16.767	6	SRS6
A			
A	16.167	6	SRS4
A			
A	16.100	6	SRS2
A			
A	15.800	6	SRS3
A			
A	15.767	6	SRS1

APPENDIX 5: SITE AVERAGE ANALYSIS OF VARIANCE FOR WIND SPEED

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	2.043556

Number of Means	2	3	4	5	6
Critical Range	1.686	1.771	1.827	1.867	1.897

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	6.6500	6	SRS1
A			
A	6.4167	6	SRS5
A			
A	6.3333	6	SRS2
A			
A	6.1833	6	SRS3
A			
A	6.1333	6	SRS6
A			
A	5.8833	6	SRS4

APPENDIX 6: SITE AVERAGE ANALYSIS OF VARIANCE FOR SOLAR RADIATION

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	2.901E10

Number of Means	2	3	4	5	6
Critical Range	200840	211062	217688	222418	225990

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	438333	6	SRS3
A			
A	403333	6	SRS4
A			
A	401667	6	SRS6
A			
A	370333	6	SRS1
A			
A	368333	6	SRS5
A			
A	270167	6	SRS2

APPENDIX 7: SITE AVERAGE ANALYSIS OF VARIANCE FOR DISSOLVED OXYGEN

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	0.142743

Number of Means	2	3	4	5	6
Critical Range	0.4932	0.5180	0.5339	0.5452	0.5536

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	ID
	A	0.7800	5	SRS1
	A			
	A	0.6720	5	SRS3
	A			
B	A	0.6440	5	SRS2
B	A			
B	A	0.3700	5	SRS5
B	A			
B	A	0.3660	5	SRS4
B				
B		0.1240	5	SRS6

APPENDIX 8: SITE AVERAGE ANALYSIS OF VARIANCE FOR pH

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	0.074582

Number of Means	2	3	4	5	6
Critical Range	.3565	.3744	.3859	.3941	.4001

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	8.1760	5	SRS5
A			
B A	8.1600	5	SRS6
B A			
B A C	7.9560	5	SRS1
B A C			
B A C	7.9460	5	SRS2
B C			
B C	7.7760	5	SRS4
C			
C	7.7180	5	SRS3

APPENDIX 9: SITE AVERAGE ANALYSIS OF VARIANCE FOR CONDUCTIVITY

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	881759.4

Number of Means	2	3	4	5	6
Critical Range	1107	1164	1200	1226	1246

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	13578.3	6	SRS6
B	11570.0	6	SRS5
C	9265.0	6	SRS4
C			
C	9186.7	6	SRS3
D	7866.7	6	SRS1
E	6611.7	6	SRS2

APPENDIX 10: SAMPLING EVENT AVERAGE ANALYSIS OF VARIANCE FOR AMBIENT TEMPERATURE

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	2.238889

Number of Means	2	3	4	5	6
Critical Range	1.764	1.854	1.912	1.954	1.985

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	31.6667	6	July
B	19.3333	6	May
B			
B	18.0000	6	Nov
C	14.8333	6	Sept
D	11.8333	6	March
E	-5.8333	6	Jan

APPENDIX 11: SAMPLING EVENT AVERAGE ANALYSIS OF VARIANCE FOR WATER TEMPERATURE

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	1.495222

Number of Means	2	3	4	5	6
Critical Range	1.442	1.515	1.563	1.597	1.622

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	26.7833	6	July
B	21.7000	6	May
C	17.6500	6	Sept
D	13.0333	6	March
E	11.4000	6	Nov
F	7.2000	6	Jan

APPENDIX 12: SAMPLING EVENT AVERAGE ANALYSIS OF VARIANCE FOR DISSOLVED OXYGEN

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	25
Error Mean Square	0.149329

Number of Means	2	3	4	5
Critical Range	0.4595	0.4827	0.4976	0.5081

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	ID
	A	0.7017	6	Jan
	A			
B	A	0.6617	6	Sept
B	A			
B	A	0.5733	6	Nov
B	A			
B	A	0.3600	6	March
B				
B		0.1667	6	July

APPENDIX 13: SAMPLING EVENT AVERAGE ANALYSIS OF VARIANCE FOR CONDUCTIVITY

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	7021195

Number of Means	2	3	4	5	6
Critical Range	3124	3283	3386	3460	3516

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	10378	6	May
A			
A	10047	6	March
A			
A	9743	6	Jan
A			
A	9487	6	Nov
A			
A	9422	6	July
A			
A	9002	6	Sept

APPENDIX 14: SAMPLING EVENT AVERAGE ANALYSIS OF VARIANCE FOR pH

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	25
Error Mean Square	0.075679

Number of Means	2	3	4	5
Critical Range	0.3271	0.3436	0.3542	0.3617

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	8.1383	6	Sept
A			
A	8.1200	6	Jan
A			
B A	7.9500	6	March
B A			
B A	7.8650	6	May
B			
B	7.7033	6	July

APPENDIX 15: SAMPLING EVENT AVERAGE ANALYSIS OF VARIANCE FOR WIND SPEED

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	0.581111

Number of Means	2	3	4	5	6
Critical Range	0.899	0.945	0.974	0.995	1.011

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	7.2500	6	March
A			
A	7.0833	6	July
A			
A	7.0167	6	Nov
A			
B A	6.3333	6	Sept
B			
B	5.9667	6	Jan
C	3.9500	6	May

APPENDIX 16: SAMPLING EVENT AVERAGE ANALYSIS OF VARIANCE FOR SOLAR RADIATION

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for Concentration

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	1.052E10

Number of Means	2	3	4	5	6
Critical Range	120941	127096	131086	133934	136085

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	ID
A	550000	6	July
A			
B A	476667	6	March
B A			
B A C	423333	6	May
B C			
B C	365000	6	Sept
C			
C	310000	6	Nov
D	127167	6	Jan